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Pregnancy Enables Expansion of Disease-Specific Regulatory T Cells in an Animal Model of Multiple Sclerosis

Jan Broder Engler,*,[1] Nina F. Heckmann,*,[1] Jan Jäger,* Stefan M. Gold,*,†,‡ and Manuel A. Friese*

Disease activity of autoimmune disorders such as multiple sclerosis and its mouse model experimental autoimmune encephalomyelitis (EAE) is temporarily suppressed by pregnancy. However, whether disease amelioration is due to nonspecific immunomodulation or mediated by Ag-specific regulation of disease-causing conventional T cells (Tcon) and immunosuppressive regulatory T cells (Tregs) remains elusive. In the current study, we systematically analyzed changes of the TCRβ repertoire driven by EAE and pregnancy using TCR sequencing. We demonstrate that EAE, but not pregnancy, robustly increased TCR repertoire clonality in both peripheral Tcon and Treg. Notably, pregnancy was required for the expansion of Treg harboring the dominant EAE-associated TRBV13-2 chain and increased the frequency of EAE-associated clonotypes within the Treg compartment. Our findings indicate that pregnancy supports the expansion of Treg clonotypes that are equipped to recognize EAE-associated Ags. These Treg are thereby particularly suited to control corresponding encephalitogenic Tcon responses and likely contribute to pregnancy-associated protection in autoimmunity. ***The Journal of Immunology*****, 2019, 203: 000–000.**

Multiple sclerosis (MS) is the most frequent inflammatory disease of the CNS, with around 2.5 million affected individuals worldwide. MS pathogenesis is attributed to a breakdown of immune tolerance toward CNS autoantigens, leading to infiltration of autoreactive conventional T cells (Tcon), which trigger demyelination and neuroaxonal degeneration (1). Intriguingly, pregnancy is one of the strongest suppressors of disease activity in MS (2) and its animal model experimental autoimmune encephalomyelitis (EAE) (3, 4), with an ∼80% reduction of MS relapse rates in third trimester pregnancy (5). Importantly, in both humans and mice, this protective effect is limited to the period of pregnancy with rebounding disease activity after delivery. Despite the potential therapeutic implications of this phenomenon, the identity and relative contribution of involved protective mechanisms is still incompletely understood (6).

Regulatory T cells (Treg) are potent suppressors of autoreactive Tcon (7) but are also required to control maternal immune responses against the semiallogeneic fetus during gestation in placental mammals (8–11). Protection of the fetus is achieved by local expansion of Treg in the reproductive tract (10, 12) and draining lymph tissue (13), which is partly mediated by selection of hormone-resistant Treg over hormone-sensitive Tcon (4, 14). However, Treg expansion during pregnancy has also been shown to be fetal Ag specific (8–11, 15), raising the question of to what extent is it driven by Ag-specific versus nonspecific cues? Similarly, it remains enigmatic whether protection from EAE during pregnancy is predominantly driven by Treg and whether it happens in an Ag-specific manner.

Whereas Ag specificity in Tcon is clearly a prerequisite for targeted immune responses, the necessity for Treg to recognize the same Ag to properly control the corresponding Tcon response is less obvious. For example, TCR analysis revealed only a partial repertoire overlap between Treg and non-Treg (16–19), contributing to the discussion whether suppressive Treg effects are mainly mediated through Ag-specific activity (20, 21) or bystander suppression (22). In EAE, which is induced by active immunization with myelin oligodendrocyte glycoprotein peptide 35–55 ($MOG_{35–55}$), the MHC class II restriction of inbred C57BL/6 mice typically leads to the preferred usage of the TRBV13-2 segments in both MOG-reactive Tcon and Treg (23, 24). Yet, although C57BL/6 mice are genetically identical, each mouse starts with a unique TCR repertoire (25) and develops a singular composition of expanded MOG-specific clones (23, 24). Moreover, autoreactive immune responses tend to broaden their antigenic scope toward unrelated copresented Ags, a phenomenon referred to as "epitope spreading" (26). As a consequence, expanded EAE-associated clonotypes are not necessarily MOG specific, which further complicates their interindividual tracking.

In this study, we set out to systematically analyze the TCR repertoire of both Tcon and Treg in EAE and pregnancy to better

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J.B.E. and N.F.H. contributed equally to this work. J.B.E., N.F.H., and M.A.F. designed the experiments for the study. N.F.H. conducted animal breeding, EAE, and cell sorting for sample collection. J.J. performed DNA isolation. J.B.E. performed bioinformatic processing of TCR sequencing data. J.B.E. and N.F.H. analyzed and visualized the data. S.M.G. made intellectual contributions to design and discussion. J.B.E., N.F.H., and M.A.F. wrote the manuscript. M.A.F. conceived and supervised the study.

The sequences presented in this article have been submitted to the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE122894.

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Abbreviations used in this article: E, gestational day; EAE, experimental autoimmune encephalomyelitis; $MOG_{35–55}$, myelin oligodendrocyte glycoprotein peptide 35–55; MS, multiple sclerosis; Tcon, conventional T cell; Treg, regulatory T cell.

understand whether Ag specificity is a relevant driving force of pregnancy-associated protection from autoimmunity. We provide evidence that the EAE-related TRBV13-2 chain as well as EAE-associated CDR3 clonotypes are selectively expanded in Treg of pregnant EAE animals but not in EAE animals without pregnancy. These findings support the notion that pregnancy might foster a CNS autoantigen-specific Treg response that is suited to counteract autoimmune inflammation.

Materials and Methods

Mice

Previously described DEREG female mice (Jax no. 32050; The Jackson Laboratory) (27) and BALB/c males (Jax no. 000651) were kept under specific pathogen-free conditions in the Central Animal Facility at the University Medical Center Hamburg-Eppendorf. Age-matched adult mice were used in all experiments.

Allogenic mating of mice

Age-matched female DEREG mice were mated with fertile BALB/c males for three consecutive nights. Successfully mated females, where identified by the presence of a vaginal plug, were separated and weighted on consecutive days to confirm pregnancy. The day of plug was considered gestational day 0.5 (E0.5).

EAE induction

Mice were immunized s.c. with 200 μg of $MOG_{35–55}$ peptide (peptides and elephants) in CFA (Becton Dickinson) containing 4 mg/ml *Mycobacterium tuberculosis* (Becton Dickinson). In addition, 200 ng of pertussis toxin (Merck Chemicals) was injected i.v. on the day of immunization and 48 h later. Animals were scored daily for clinical signs by the following system: 0, no clinical deficits; 1, tail weakness; 2, hind limb paresis; 3, partial hind limb paralysis; 3.5, full hind limb paralysis; 4, full hind limb paralysis and forelimb paresis; and 5, premorbid or dead. Animals reaching a clinical score ≥4 had to be killed according to the regulations of the local Animal Welfare Act. EAE induction in pregnant animals was performed on E7.5. Samples for TCR repertoire analysis were typically taken on E18.5.

Immune cell isolation from lymphoid organs and CNS

Mice were anesthetized and killed by inhalation of CO_2, and paraaortal, inguinal, axillary, brachial, and superficial cervical lymph nodes and spleen were harvested with sterile instruments and collected in ice-cold PBS. Pooled single-cell suspensions were prepared by homogenization through a 40-μm cell strainer, cells were pelleted by centrifugation (300 × *g*, 10 min, 4°C), and lysis of splenic erythrocytes was initiated by RBC lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH = 7.4) for 2.5 min at 4°C and stopped with MACS Buffer (PBS, 0.5% BSA, 2 mM EDTA). Cells were washed with PBS and used in follow-up applications. For fluorescence-activated cell sorting, cell suspensions were stained with CD4–Pacific Blue (clone RM4-5; BioLegend) and Fc-Block (True Stain anti-mouse CD16/32, clone 93; BioLegend) for 30 min at 4°C in the dark, washed with PBS, and filtered and resuspended in PBS with EDTA (1:10,000). For isolation of CNS-infiltrating leukocytes, mice were intracardially perfused with ice-cold PBS immediately after killing to remove blood from intracranial vessels. Brain and spinal cord were prepared with sterile instruments and incubated with agitation in RPMI medium 1640 (PAN-Biotech) containing 1 mg/ml collagenase A (Roche) and 0.1 mg/ml DNaseI (Merck) for 60 min at 37°C. Tissue was triturated through a 40-μm cell strainer and washed with PBS (300 × *g*, 10 min, 4°C). Homogenized tissue was resuspended in 30% isotonic Percoll (GE Healthcare) and underlaid with 78% isotonic Percoll. After gradient centrifugation (1500 × *g*, 30 min, 4°C), CNS-infiltrating immune cells were recovered from the gradient interphase and washed twice in ice-cold PBS. Isolated cells were depleted for $CD8^+$ T cells using CD8a (Ly-2) MACS Microbeads and MS columns (both Miltenyi Biotec). Cells were washed with PBS and used in follow-up applications.

Fluorescence-activated cell sorting

Cell suspensions were sorted into Treg ($CD4^+$, $Foxp3^+$) and Tcon ($CD4^+$, $Foxp3^–$) populations with a FACSAria device (Becton Dickinson). Collection tubes were coated with FCS and filled with complete RPMI 1640 medium (1% penicillin, streptomycin, 0.1% 2-ME) with 20% FCS. Purity of sorted populations was routinely above 95%. Then cells were pelleted by centrifugation at 300 × *g* for 10 min at 4°C, dry frozen in liquid nitrogen, and stored at –80°C until DNA isolation.

DNA isolation and TCR sequencing

Dry-frozen pellets were thawed on ice, and DNA was isolated according to the AllPrep DNA/RNA Micro kit (Qiagen). Purified DNA was quantified on a Qubit 3 Fluorometer (Thermo Fisher Scientific) and stored at –80°C until library preparation. Amplification of TCRβ gene rearrangements was performed by HS Diagnomics (Berlin) using their established multiplex PCR approach, which includes 21 forward and 15 reverse primers that anneal to the alleles of 23 International Immunogenetics Information System-listed functional TCRβ V segments and 13 J segments, respectively. The primer sequences were as follows: MV1, 5′-GGAGCTGAGGCTGCAAGTGGCC-3′; MV2, 5′-CATTTAGACCTTCAGATCACAGCTCT-3′; MV3, 5′-TATTTCACTCTGAAAATCCAACCCACA-3′; MV4, 5′-CTCATTTGAATCTTCGAATCAAGTCTG-3′; MV5, 5′-CCAGACAGCTCCAAGCTACTTTTAC-3′; MV12-1/-2, 5′-CACTCTGAAATGAACATGAGTGCCTT-3′; MV13-1, 5′-TCTTCCTCCTGCTGGAATTGGCT-3′; MV13-2/-3, 5′-TCTCCCTCATTCTGGAGTTGGCT-3′; MV14, 5′-CTCCACTCTCAAGATCCAGTCTGCA-3′; MV15, 5′-ACTCTGAAGATTCAACCTACAGAAC-3′; MV16, 5′-CAACTCTGAAGATCCAGAGCACGCA-3′; MV17, 5′-CTGCTCTCTCTACATTGGCTCTGCA-3′; MV19, 5′-TCTTTTTCTCTCACTGTGACATCTGC-3′; MV20, 5′-CCAACTTATCCTTTTCATCTATGACAGTT-3′; MV21, 5′-CATGTACCATAGAGATCCAGTCCAGC-3′; MV23, 5′-TGCAGCCTGGGAATCAGAACGTGC-3′; MV24, 5′-CATCCTGGAAATCCTATCCTCTG-3′; MV26, 5′-GCAGCCTAGAAATTCAGTCCTCTG-3′; MV29, 5′-CATTTCTCCCTGATTCTGGATTCTGC-3′; MV30, 5′-ATTCTCAACGTTGACAGTGAACAATG-3′; MV31, 5′-TTCATCCTAAGCACGGAGAAGCTG-3′; MJ1-1*01, 5′-TTACCTACAACTGTGAGTCTGGTTC-3′; MJ1-1*02, 5′-TTACCTACAACTGTGAGTGTGGTTC-3′; MJ1-2, 5′-CTTACCTATTACCAAAAGCCTGGT-3′; MJ1-3, 5′-TACCTACAACAATGAGCCGGCTTCC-3′; MJ1-4*01/*02, 5′-TACCCAAGACAGACAGCTTGGTT-3′; MJ1-5, 5′-AGTTTACCTAGAACAGAGAGTCGAGT-3′; MJ1-6, 5′-CCTGTCACAGTGAGCCGGGTGC-3′; MJ1-7, 5′-GTCTTATCTTATACCTAAGTTCCTTTCCAA-3′; MJ2-1, 5′-TACCTAGGACGGTGAGTCGTGTC-3′; MJ2-2, 5′-ACCCAGCACTGTCAGCTTTGAGCC-3′; MJ2-3, 5′-TACCGAGAACAGTCAGTCTGGTTC-3′; MJ2-4, 5′-CCTAGCACCGATAGTCGGGTGC-3′; MJ2-5, 5′-CCTAACACGAGGAGCCGAGTGC-3′; MJ2-7*01, 5′-TACCTAAAACCGTGAGCCTGGTGC-3′; and MJ2-7*02, 5′-TACCTAAAACCGTGAGCCTAGTGC-3′. HS Diagnomics performed extensive calibration experiments with the multiplex PCR primer sets to establish homogeneous amplification rates by means of a synthetic template set as a reference containing all relevant primer sites and an artificial CDR3 region. In a second PCR step, universal Illumina adapter sequences including additional barcodes were added to the generated TCRβ amplicons. Amplicon sequencing was performed at the Transcriptome and Genome Analysis Laboratory (University Medical Center Goettingen) on the MiSeq platform, generating 0.5–1 million paired-end reads (2 × 150 bp) per sample.

TCR sequencing analysis

Productive TCRβ rearrangements were extracted from fastq files using mixcr 2.1.10 (28) with standard parameters. Down-stream analysis was performed with VDJtools 1.1.8 (29) and custom R scripts. As a metric for the skewing of the TCR repertoire by clonal expansion, we used "clonality," defined as 1 − normalized Shannon–Wiener index. Clonality values approaching 0 indicate a very even distribution of clonotype abundances, whereas values approaching 1 indicate an increasingly asymmetric distribution in which a few clonotypes are present at high frequencies. Usage of TRBV and TRBJ segments was calculated by using the VDJtools function "CalcSegmentUsage," and pairing of V and J segments in representative samples was visualized in circus plots via "PlotFancyVJUsage." Data set–wide similarity of CDR3 amino acid sequence usage was assessed by calculating the pairwise overlap between samples by "CalcPairwiseDistances" with standard parameters. Pairwise distances were projected into a two-dimensional plane by multidimensional scaling (MASS package) and color-coded by experimental condition. Overlap between peripheral and CNS samples was calculated and visualized using the VDJtools function "OverlapPair" with standard parameters; however, color-coding was extended to the top 100 clonotypes (default is 20). EAE clonotypes (Supplemental Table I) were required to be present in at least two of three analyzed CNS samples (see Fig. 5B). As negative control for EAE clonotypes, 1000 peripheral clonotypes were randomly selected. Tracking of EAE and random clonotypes in independent data sets was performed on the level of CDR3 amino acid sequence and quantified as cumulative abundance within each sample. All plots were either generated by VDJtools, Prism 7 (GraphPad), or the R package ggplot2.

Statistics

Statistical analysis was performed with Prism 7 (GraphPad). Data are shown as mean values + SEM. The *n* values refer to the number of biological replicates (mice). Differences between two groups were determined by unpaired Student *t* tests. Comparisons between three or more groups were performed by two-way ANOVA with Sidak multiple comparison post hoc test. Post hoc tests were only applied when a significant group or interaction effect was observed in the initial two-way ANOVA. Significant results are indicated by asterisks: $*p < 0.05$, $**p < 0.01$.

Data availability

Data generated for this study are available through the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE122894.

Study approval

All animal care and experimental procedures were performed according to institutional guidelines and conformed to requirements of the German Animal Welfare Act. Ethical approvals were obtained from the State Authority of Hamburg, Germany (approval no. G45/17).

Results

EAE but not pregnancy increases TCR repertoire clonality

To explore Ag-specific responses in Tcon and Treg during EAE and their modification by pregnancy, we sampled spleens and lymph nodes from four experimental groups, including naive and EAE-immunized mice that were either pregnant or nonpregnant (Fig. 1A). Importantly, pregnant EAE mice were fully protected from disease activity at the time point of sampling (day 10–12 postimmunization), whereas nonpregnant animals showed acute disease activity (Fig. 1B). We used DEREG mice (27) to isolate Tcon and Treg from pooled spleen and lymph nodes by sorting Foxp3-eGFP–negative and Foxp3-eGFP–positive $CD4^+$ T cells, respectively (Fig. 1C); extracted genomic DNA; and performed TCRβ sequencing (Fig. 1A). Per sample, we recovered ∼0.5 million reads mapping to the TCR locus (Supplemental Fig. 1A), of which ∼75% encoded productive CDR3 amino acid sequences (Supplemental Fig. 1B).

First, we analyzed the distribution of clonotype abundances, which showed that EAE samples were more dominated by one or few expanded clones (Fig. 1D). Correspondingly, using sample clonality as a global measure of TCR repertoire skewing, we observed a strong increase of clonality in EAE samples in comparison with naive samples ($p < 0.0001$; Fig. 1E). This EAE effect was consistent across Tcon and Treg subsets of virgin as well as pregnant mice (Tcon, nonpregnant, naive mice versus EAE: $p = 0.0497$; Tcon, pregnant, naive mice versus EAE: $p = 0.0028$; Treg, nonpregnant, naive mice versus EAE: $p = 0.0120$; Treg, pregnant, naive mice versus EAE: $p = 0.0032$; Fig. 1F). Pregnancy alone or in addition to immunization did not induce significant clonality shifts (Tcon, naive, nonpregnant mice versus pregnant mice: $p = 0.9724$; Treg, naive, nonpregnant mice versus pregnant mice: $p = 0.8841$; Tcon, EAE, nonpregnant mice versus pregnant mice: $p = 0.2421$; Treg, EAE, nonpregnant mice versus pregnant mice: $p = 0.5092$). Thus, clonality of the TCR repertoire appeared to be mainly driven by immunization in our data set.

Treg show increased usage of EAE-associated TRBV13-2 in pregnancy-protected EAE mice

Because TCRβ (TRB) segment usage can deliver first indications of Ag specificity, we next investigated the relative contribution of canonical TRBV and TRBJ segments across experimental conditions. Previously reported EAE-associated TRBV segments include 13-2, 19, 20, and 31, all of which have originally been identified in CNS infiltrates of EAE animals (23, 24, 30). However, it was unclear whether significant changes in TRBV segment usage could also be identified in the peripheral immune system. Indeed, we did not observe significant changes in TRBV (Fig. 2) and TRBJ (Supplemental

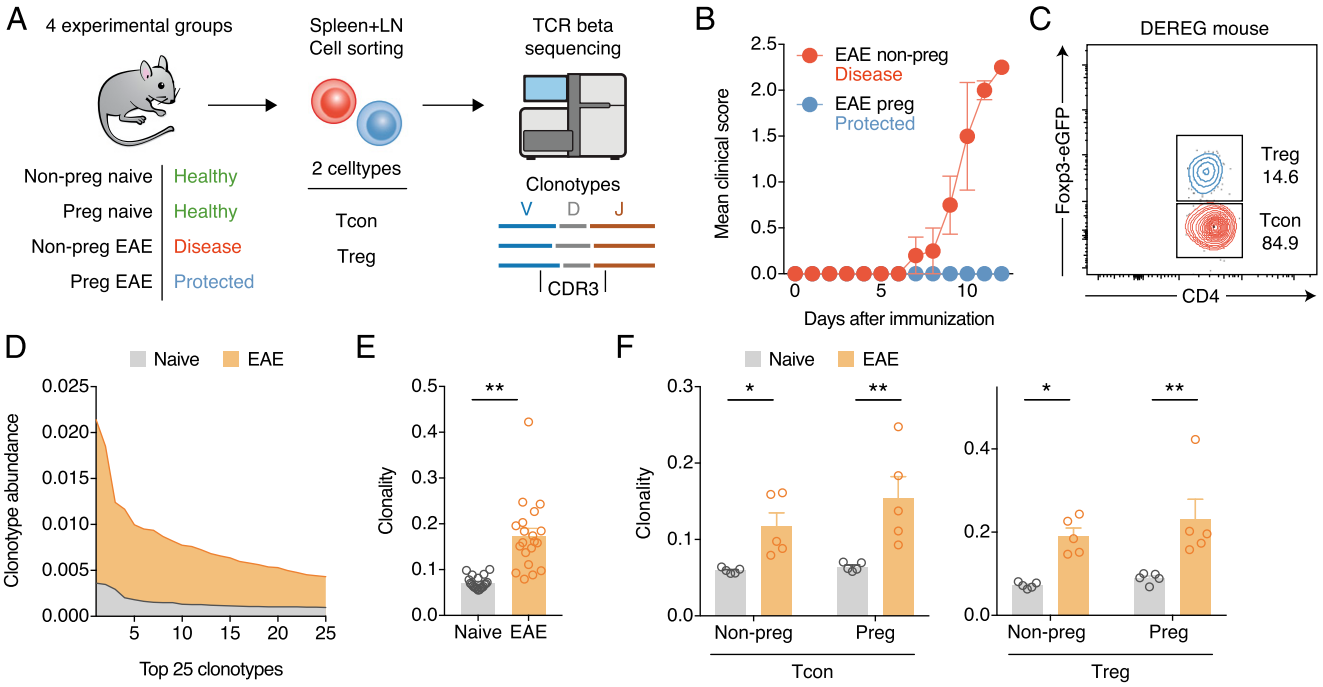

FIGURE 1. EAE but not pregnancy increases TCR repertoire clonality. (**A**) Scheme of experimental setup ($n = 5$ biological replicates per group). (**B**) Representative EAE disease courses of nonpregnant (non-preg; $n = 5$) and pregnant (preg) ($n = 7$) C57BL/6 DEREG mice. Animals were sampled for TCR sequencing on day 10–11 after immunization, corresponding to E18.5. (**C**) Representative gating for FACS sorting of pooled spleen and lymph nodes from $CD4^+$Foxp3-eGFP$^-$ Tcon and $CD4^+$Foxp3-eGFP$^+$ Treg from C57BL/6 DEREG mice. (**D**) Clonotype abundance curves (top 25 clonotypes) of representative naive and EAE samples. (**E**) Clonality analysis of naive versus EAE samples ($n = 20$ per group). (**F**) Clonality analysis of naive versus EAE samples, grouped by T cell subtype (Tcon versus Treg) and gestational state (non-preg versus preg), with $n = 5$ per group. Data are presented as mean values + SEM. Statistical analysis was performed by unpaired Student *t* test in (E) and two-way ANOVA with subsequent Sidak multiple comparison post hoc test in (F). $*p < 0.05$, $**p < 0.01$.

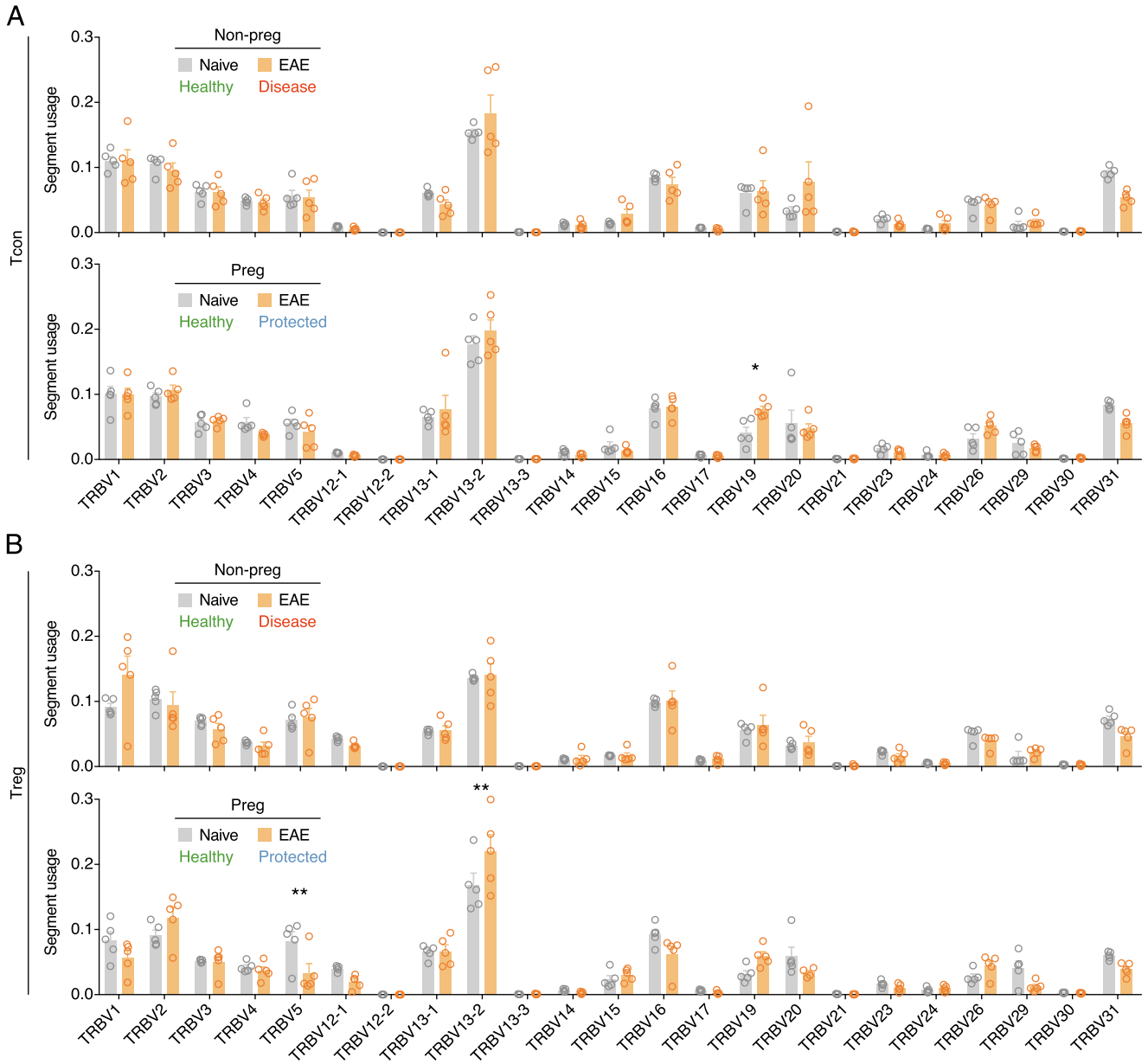

FIGURE 2. Treg show increased usage of EAE-associated TRBV13-2 in pregnancy-protected EAE mice. (**A** and **B**) TRBV segment usage of Tcon (A) and Treg (B) isolated from naive versus EAE mice, grouped by gestational state (nonpregnant [non-preg] versus pregnant [preg]), with $n = 5$ per group. Data are presented as mean values + SEM. Statistical analysis was performed by two-way ANOVA with subsequent Sidak multiple comparison post hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$.

Fig. 2) segment usage in virgin EAE mice. In contrast, we found a significant induction of TRBV19 in Tcon (TRBV19: $p = 0.0171$; Fig. 2A) and TRBV13-2 in Treg ($p = 0.0003$; Fig. 2B) of pregnant EAE mice. Also, the direct comparison of pregnant versus non-pregnant EAE mice yielded a significant induction of TRBV13-2 in Treg ($p < 0.0001$). TRBJ usage, in contrast, was unchanged (Supplemental Fig. 2). Thus, expansion of the TRBV13-2 segment that was repeatedly shown to harbor MOG-specific clones (23, 24, 30–32) was restricted to Treg of pregnant EAE mice. In summary, pregnancy appeared to expand Treg clonotypes that use a potentially EAE-associated TCR and could thereby contribute to controlling encephalitogenic T cell responses of the same Ag specificity.

Usage of CDR3 amino acid sequences is driven by immunization and T cell subtype

Next, we zoomed in on the actual TCR clonotype sequences. To assess the overall similarity of CDR3 amino acid sequence usage in our data set, we calculated the pairwise distances between samples and projected the results into a two-dimensional plane by multi-dimensional scaling (Fig. 3). Color-coding of immunization status and T cell subtype resulted in a clear separation of naive versus EAE and Tcon versus Treg samples, respectively (Fig. 3A, 3B). This indicated that the overall variance in CDR3 amino acid usage was prominently driven by these two parameters. In contrast, color-coding of gestational state (nonpregnant versus pregnant) led to an intermingled picture (Fig. 3C). Thus, pregnancy presents as a much weaker determinant of shared CDR3 amino acid usage than either disease state or T cell subset. Of note, we observed a consistent clustering of Treg from pregnant EAE mice across all three parameters (Fig. 3A–C, dashed circle), indicating that these samples were tied together by strong CDR3 amino acid similarity. Taken together, our findings identify disease status and T cell subtype as strong determinants of CDR3 amino acid usage. Remarkably, even EAE immunization did not blur but rather intensified the CDR3 amino acid similarity of Tcon versus Treg. The tight clustering of Treg from pregnant EAE animals suggests a

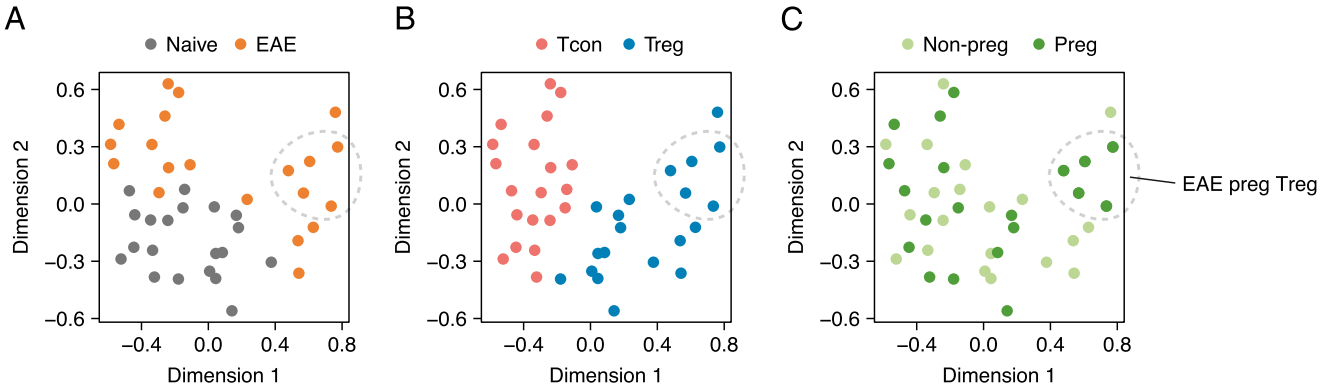

FIGURE 3. Usage of CDR3 amino acid sequences is driven by immunization and T cell subtype. (**A**–**C**) Multidimensional scaling plots of CDR3 amino acid similarity across samples. Color-coding of naive versus EAE (A), Tcon versus Treg (B), and nonpregnant (non-preg) versus pregnant (preg) (C) samples ($n = 5$ per group). Dashed line highlights Treg samples of pregnant EAE animals.

selective pressure onto Treg under this specific condition that coincides with the protective effect of pregnancy in EAE.

CNS-infiltrating T cells show preferred usage of TRBV13-2 and TRBV20

Despite being genetically identical, each C57BL/6 mouse possesses a singular TCR repertoire. To identify public EAE clonotypes that are associated with encephalitogenic activity in EAE and shared between individual mice, we sampled peripheral (spleen and lymph nodes) and CNS-infiltrating CD4$^+$ T cells from an independent cohort of three EAE mice (Fig. 4A). Thus, all samples contained a mixture of Tcon and Treg. Thereby, we aimed to identify EAE-driving public clonotypes that could be used to assess their change during EAE and pregnancy in our peripheral primary data set. Expectedly, we observed that CNS samples showed a pronounced increase of clonality in comparison with peripheral samples from the same mouse ($p = 0.0026$; Fig. 4B). This indicated a local accumulation or expansion of encephalitogenic TCRs in CNS tissue. When looking at the usage of TRBV and TRBJ, we observed a significant enrichment of TRBV13-2 ($p < 0.0001$) and TRBV20 ($p = 0.0054$; Fig. 4C, 4D, Supplemental Fig. 3), as previously described for EAE infiltrates (23, 24, 30). Together, the acquired CNS samples presented as a suitable source for the identification of EAE-associated clonotypes.

CNS samples enable identification of EAE clonotypes

To identify public EAE-associated C57BL/6 TCRs in our experimental setup, we explored the overlap of CDR3 nucleotide sequences

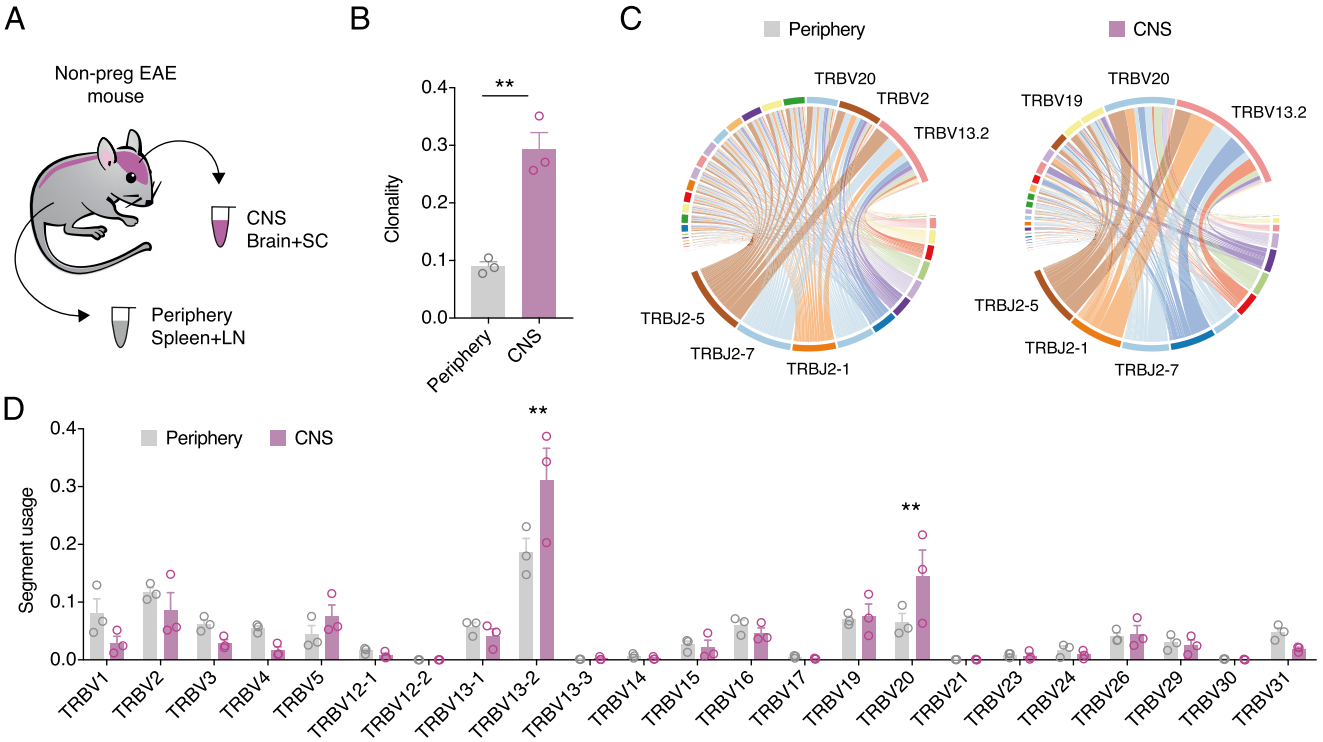

FIGURE 4. CNS-infiltrating T cells show preferred usage of TRBV13-2 and TRBV 20. (**A**) Scheme for sampling of CNS-infiltrating (brain and spinal cord) versus peripheral (spleen and lymph nodes) CD4$^+$ T cells ($n = 3$ nonpregnant EAE mice at day 14 after immunization). (**B**) Clonality analysis of peripheral versus CNS samples ($n = 3$ per group). (**C**) Circus plots showing relative abundance and pairing of TRBV and TRBJ segments in peripheral versus CNS samples of one representative animal. (**D**) TRBV segment usage in peripheral versus CNS samples ($n = 3$ per group). Data are presented as mean values + SEM. Statistical analysis was performed by unpaired Student t test in (B) and two-way ANOVA with subsequent Sidak multiple comparison post hoc test in (D). $^*p < 0.05$, $^{**}p < 0.01$.

within and across individual mice and sample locations. First, we identified and tracked clonotypes that were shared between EAE mice in the peripheral lymphatic tissue (Fig. 5A, top row). This analysis yielded a limited clonotype overlap of ~4%, emphasizing the interindividual heterogeneity of peripheral TCR repertoires even after immunization with the same peptide Ag.

Next, we compared periphery and CNS within the same mouse, revealing a consistent expansion of shared clonotypes from the periphery (~5%) toward the CNS (~40%) (Fig. 5A, middle row). These data are in line with an Ag-specific infiltration and/or local proliferation of EAE clonotypes within the CNS. Finally, we contrasted CNS samples across different mice and observed a remarkable overlap despite the fact that we were conducting interindividual comparisons (Fig. 5A, bottom row). For example, clonotypes shared between CNS1 and CNS2 were present with a cumulative abundance of ~45% in CNS1 and ~75% in CNS2. Clonotypes shared between CNS1 and CNS3 accounted for as much as ~90% of productive TCR reads in CNS3 (Fig. 5A, bottom row). Hence, despite the high peripheral diversity, CNS-infiltrating clonotypes showed a striking overlap between mice.

We further explored this interindividual overlap between CNS samples by intersecting the identified shared clonotypes in a Venn diagram (Fig. 5B). As a result, we obtained 918 CDR3 nucleotide clonotypes that were shared in at least two CNS samples, including 91 clonotypes that were present in all three CNS samples. We reasoned that these 918 identified EAE clonotypes could be useful to track EAE-specific responses in our main data set.

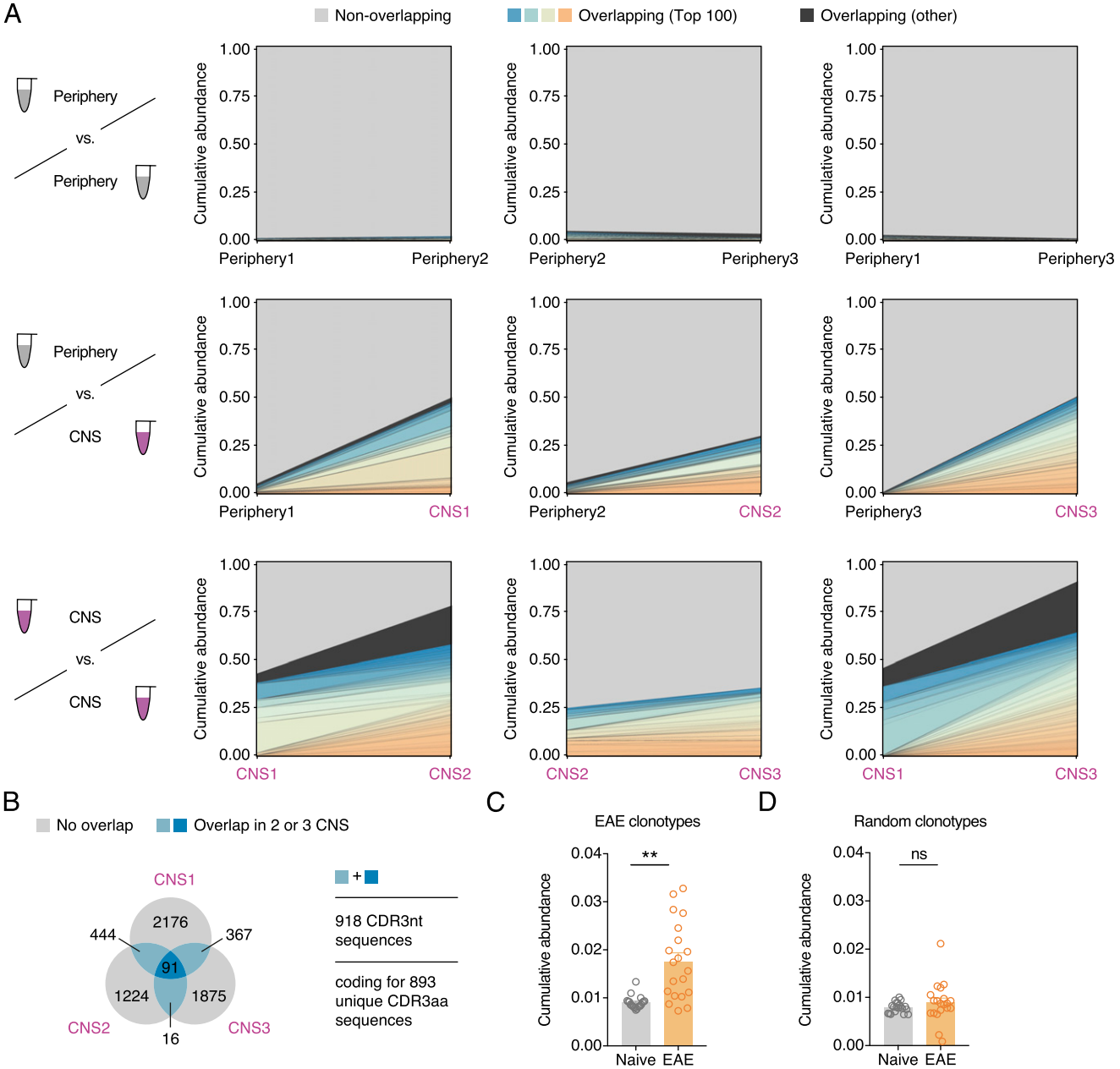

FIGURE 5. CNS samples enable identification of EAE clonotypes. (**A**) Cumulative abundance of clonotypes shared between peripheral samples across mice (top row), between peripheral and CNS samples of the same mouse (middle row), and between CNS samples across mice (bottom row). Non-overlapping clones are depicted in light gray, top 100 overlapping clones are depicted by colors (light blue to orange), and remaining overlapping clones that were not part of the top 100 are depicted in dark gray. (**B**) Venn diagram of overlap between CNS samples. Clonotypes present in at least two of three CNS samples were considered EAE clonotypes. (**C** and **D**) Cumulative abundance of EAE clonotypes (C) and 1000 random clonotypes (D) in naive versus EAE samples ($n = 20$ per group) of the main data set (see Fig. 1A). Statistical analysis was performed by unpaired Student t test in (C) and (D). $^{**}p < 0.01$.

Indeed, the cumulative abundance of EAE clonotypes showed a significant increase in EAE-immunized animals in comparison with naive animals ($p < 0.0001$; Fig. 5C). We also tracked 1000 randomly selected peripheral clonotypes, which were unchanged by EAE immunization (Fig. 5D). This highlights the relevance of our identified EAE clonotypes. Although not having formally shown that these clonotypes recognize the MOG Ag, we would anticipate that they include MOG-reactive T cells as well as T cells cross-reacting to secondary epitopes generated in the course of epitope spreading. Importantly, the cohort we used for the discovery of EAE clonotypes was completely independent of the cohort we sampled for the analysis of Tcon and Treg during EAE and pregnancy. Thus, our approach enabled us to compile a comprehensive list of clonotypes (Supplemental Table I) that can be used to track EAE responses in any cohort of MOG-immunized C57BL/6 mice.

Pregnancy is required to expand EAE clonotypes within Treg

After having identified EAE clonotypes that could be tracked in the peripheral samples of our main data set, we plotted each EAE clonotype as a separate row in an abundance heat map (Fig. 6A). In total, we identified 918 CDR3 nucleotide clonotypes, coding for 893 unique CDR3 amino acid sequences (Fig. 5B). Of those, we

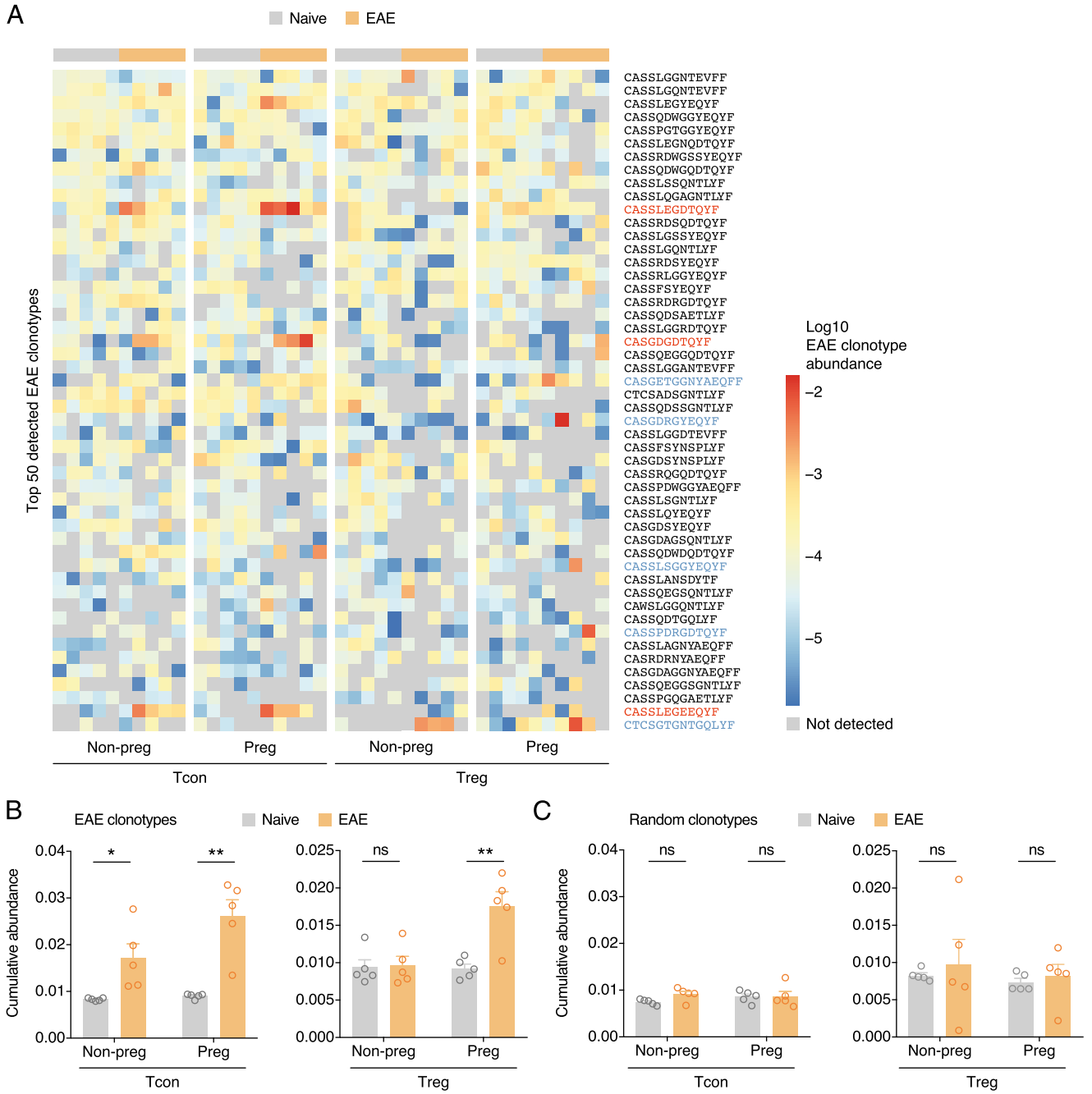

FIGURE 6. Pregnancy is required to expand EAE clonotypes within Treg. (**A**) Abundance heatmap of top 50 EAE clonotypes across samples. Light gray color in heatmap indicates that clonotype was not detected in the respective sample. Sequences of clonotypes contributing to Tcon and Treg responses in EAE are marked in red and blue, respectively. (**B** and **C**) Cumulative abundance of EAE clonotypes (B) and 1000 random clonotypes (C) in naive versus EAE samples, grouped by T cell subtype (Tcon versus Treg) and gestational state (nonpregnant [non-preg] versus pregnant [preg]). Data ($n = 5$ per group) are presented as mean values + SEM. Statistical analysis was performed by two-way ANOVA with subsequent Sidak multiple comparison post hoc test in (B) and (C). *$p < 0.05$, **$p < 0.01$.

found 489 clonotypes (~55%) in at least one sample of our main data set and ordered them by the number of samples they were detected in. Focusing on the top 50 clonotypes (Fig. 6A), we observed that those clonotypes contributing to Tcon responses in EAE (marked in red) were not the same as those that contributed to Treg responses (marked in blue). Additionally, Treg EAE clonotypes appeared more sporadic in individual mice, whereas EAE clonotype usage by Tcon was more consistent across animals of the same experimental group (Fig. 6A). This suggests that Tcon responses to EAE are more dominantly driven by "public" clonotypes than Treg responses to EAE.

To quantitatively assess changes during EAE induction and protection by pregnancy, we analyzed the cumulative abundance of all EAE clonotypes across experimental groups. Tcon showed a robust increase of public EAE clonotype abundance in immunized versus naive animals, regardless of whether they were pregnant or not (nonpregnant, naive versus EAE: $p = 0.0327$; pregnant, naive versus EAE: $p = 0.0002$; Fig. 6B). In Treg, however, EAE immunization alone did not lead to an expansion of public EAE clonotypes ($p = 0.9840$). Instead, only in the additional presence of pregnancy, Treg responded with an increased usage of public EAE clonotypes ($p = 0.0007$; Fig. 6B). Also, the direct comparison of pregnant versus nonpregnant EAE mice yielded a significant induction of EAE clonotypes in Treg ($p = 0.0011$). Importantly, 1000 peripheral random clonotypes did not show any significant changes, supporting that the observed induction of EAE clonotype abundance was indeed Ag specific (Fig. 6C). Assuming that the specificity for EAE-related Ag enhances the power of these Treg to control encephalitogenic Tcon, this finding mirrors the suppression of disease activity in pregnant EAE mice (Fig. 1B).

Together, these observations show that pregnancy was required to generate detectable EAE clonotype expansion in Treg of EAE mice. Moreover, Tcon and Treg showed a district usage of EAE-associated CDR3 amino acid sequences, consistent with a distinct clonal origin of both cell types.

Discussion

In the current study, we investigated shifts of the TCR repertoire as a potential driver of pregnancy-associated suppression of autoimmunity. We employed in-depth characterization in key T cell subsets on the clonal level during active EAE as well as EAE amelioration during pregnancy. These data suggest clonotype-specific, rather than global, immunoregulation during pregnancy that contributes to amelioration of EAE.

As a first global measure of TCR repertoire shifts, we employed clonality analysis, which indicated a consistent clonal expansion in EAE samples in both Tcon and Treg. In contrast, pregnancy alone or in addition to immunization did not increase clonality. Thus, we did not pick up clonal expansion induced by BALB/c-associated Ags, which were expressed by semiallogeneic fetuses in the pregnant dams. Similarly, CDR3 amino acid usage was prominently driven by MOG peptide immunization, whereas the effect of pregnant versus nonpregnant was marginal. These findings might be explained by the fact that active peptide immunization was specifically designed to yield supraphysiologic immune responses, whereas expansion of fetus-specific clones, although verifiable, is much subtler (11). Of note, we also did not observe a difference in Tcon clonality in protected versus diseased EAE animals, suggesting that protection during pregnancy cannot be explained by a complete blockage of clonal expansion of MOG-reactive Tcon clones. However, pregnancy still reliably led to a suppression of disease activity, which we previously found to be associated with reduced CNS infiltration (4).

When analyzing TRBV and TRBJ segment usage, reported EAE-associated segments were not generally induced by immunization in the periphery. The prototypic EAE-associated TRBV13-2 segment (23, 24, 30, 31), which we also found enriched in CNS infiltrates, was only increased in Treg of pregnancy-protected EAE animals. Although constituting indirect evidence, these findings are indicative of an expansion of EAE-related clonotypes within the immunoregulatory Treg compartment that was dependent on the presence of pregnancy. Additionally, we observed a tight clustering of Treg from pregnant EAE animals, which suggests a pregnancy-driven relatedness of their CDR3 amino acid sequences. To further substantiate the notion of an expanded EAE-related Treg pool in pregnancy, we explored strategies to identify and track EAE-related clonotypes. Therefore, we sampled an independent cohort of EAE mice and identified 918 EAE clonotypes from CNS-infiltrating $CD4^+$ T cells.

This additional data set enabled us to explore the overlapping clonotype usage in the periphery versus CNS within individuals and across individual mice. Although peripheral overlap was limited, shared clonotypes increased in abundance toward the CNS. Remarkably, interindividual overlap between CNS samples was profound. This allowed for the identification of public EAE clonotypes that could be tracked in our main experimental cohort. Clearly, MOG specificity was not formally established for these clonotypes. However, we think that because of their targeted CNS enrichment observed across mice, the identified clonotypes capture the disease-relevant EAE-associated T cell response. Hence, we compiled a comprehensive list (Supplemental Table I) that can be used as a resource for tracking EAE clonotypes in any cohort of $MOG_{35–55}$–immunized C57BL/6 mice. A similar approach might also be applicable to human studies in autoimmunity and pregnancy, in which cerebrospinal fluid or peptide restimulation could be employed for MS clonotype identification.

Importantly, this data-driven approach confirmed pregnancy as a necessary prerequisite for the expansion of EAE-related clonotypes within the immunosuppressive Treg compartment. As a negative control, we used 1000 peripheral random clonotypes, which showed no dynamic across immunization, cell type, and gestation, highlighting the use and relevance of the identified EAE clonotypes. This complements our findings indicating that pregnancy can shift the EAE-specific immune response toward tolerance. Because Treg are locally expanded in the fetomaternal interface and draining lymph nodes (10, 12, 13), one possible explanation would be the transportation and local presentation of MOG Ag in these tolerogenic microenvironments (33, 34). However, to what degree APCs deliver the $MOG_{35–55}$ epitope to these specific locations after s.c. administration remains to be investigated. Other possible mechanisms could involve systemic levels of pregnancy hormones, which have been extensively studied in the context of pregnancy (35–38) and might steer the EAE Ag-specific response toward tolerogenic Treg (4, 14, 39, 40).

Another striking observation from our study is the level of determination of CDR3 amino acid usage that is imposed by belonging to either Tcon or Treg. This can be appreciated in the clear clustering by cell type in CDR3 amino acid–based multidimensional scaling and the heatmap of EAE clonotypes, which identifies distinct clonotype usage in Tcon and Treg. These observations are in line with previous work, showing that Tcon and Treg strongly differ in clonal composition (23, 41), indicating that they are recruited from different clonal pools during thymic development (42) and that *trans*-differentiation between both subtypes is limited (23).

Together, we deliver a comprehensive analysis of the TCR repertoire of Tcon and Treg in EAE and its suppression by pregnancy. We provide evidence that pregnancy is a prerequisite for the expansion of Treg that target EAE-related Ags. Because autoantigen-specific Treg can be particularly potent suppressors of

autoreactive responses (20, 21, 43), this finding might contribute to the marked protection from EAE during pregnancy. Moreover, the existence of cell-specific or possibly Ag-specific mechanisms that contribute to amelioration of autoimmunity during pregnancy might help to explain why it has been difficult to fully replicate the protective effect of pregnancy in MS by using global immunomodulators, such as pregnancy levels of estrogens (44, 45). However, a central limitation of this study should be noted. Although we provide correlational evidence for a suppressive function of pregnancy-induced autoantigen-restricted Treg in EAE, the applied methodology does not allow to infer causation. Thus, additional interventional studies are required to corroborate our findings. Nevertheless, this work delivers valuable insights into the interaction of autoimmunity and reproduction and their effect on the TCR repertoire in Tcon and Treg. Targeted expansion of Treg, as eminent during pregnancy, has the potential to be explored as a treatment strategy in MS and other autoimmune diseases.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Dendrou, C. A., L. Fugger, and M. A. Friese. 2015. Immunopathology of multiple sclerosis. *Nat. Rev. Immunol.* 15: 545–558.
2. Confavreux, C., M. Hutchinson, M. M. Hours, P. Cortinovis-Tourniaire, and T. Moreau, Pregnancy in Multiple Sclerosis Group. 1998. Rate of pregnancy-related relapse in multiple sclerosis. *N. Engl. J. Med.* 339: 285–291.
3. Gatson, N. N., J. L. Williams, N. D. Powell, M. A. McClain, T. R. Hennon, P. D. Robbins, and C. C. Whitacre. 2011. Induction of pregnancy during established EAE halts progression of CNS autoimmune injury via pregnancy-specific serum factors. *J. Neuroimmunol.* 230: 105–113.
4. Engler, J. B., N. Kursawe, M. E. Solano, K. Patas, S. Wehrmann, N. Heckmann, F. Lühder, H. M. Reichardt, P. C. Arck, S. M. Gold, and M. A. Friese. 2017. Glucocorticoid receptor in T cells mediates protection from autoimmunity in pregnancy. *Proc. Natl. Acad. Sci. USA* 114: E181–E190.
5. Finkelsztejn, A., J. B. Brooks, F. M. Paschoal, Jr., and Y. D. Fragoso. 2011. What can we really tell women with multiple sclerosis regarding pregnancy? A systematic review and meta-analysis of the literature. *BJOG* 118: 790–797.
6. Patas, K., J. B. Engler, M. A. Friese, and S. M. Gold. 2013. Pregnancy and multiple sclerosis: feto-maternal immune cross talk and its implications for disease activity. *J. Reprod. Immunol.* 97: 140–146.
7. Josefowicz, S. Z., L. F. Lu, and A. Y. Rudensky. 2012. Regulatory T cells: mechanisms of differentiation and function. *Annu. Rev. Immunol.* 30: 531–564.
8. Schumacher, A., P. O. Wafula, A. Z. Bertoja, A. Sollwedel, C. Thuere, I. Wollenberg, H. Yagita, H. D. Volk, and A. C. Zenclussen. 2007. Mechanisms of action of regulatory T cells specific for paternal antigens during pregnancy. *Obstet. Gynecol.* 110: 1137–1145.
9. Zenclussen, A. C., K. Gerlof, M. L. Zenclussen, S. Ritschel, A. Zambon Bertoja, S. Fest, S. Hontsu, S. Ueha, K. Matsushima, J. Leber, and H. D. Volk. 2006. Regulatory T cells induce a privileged tolerant microenvironment at the fetal-maternal interface. *Eur. J. Immunol.* 36: 82–94.
10. Tilburgs, T., S. A. Scherjon, B. J. van der Mast, G. W. Haasnoot, M. Versteeg-V D Voort-Maarschalk, D. L. Roelen, J. J. van Rood, and F. H. Claas. 2009. Fetal-maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. *J. Reprod. Immunol.* 82: 148–157.
11. Rowe, J. H., J. M. Ertelt, L. Xin, and S. S. Way. 2012. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. *Nature* 490: 102–106.
12. Tsuda, S., X. Zhang, H. Hamana, T. Shima, A. Ushijima, K. Tsuda, A. Muraguchi, H. Kishi, and S. Saito. 2018. Clonally expanded decidual effector regulatory T cells increase in late gestation of normal pregnancy, but not in preeclampsia, in humans. *Front. Immunol.* 9: 1934.
13. Aluvihare, V. R., M. Kallikourdis, and A. G. Betz. 2004. Regulatory T cells mediate maternal tolerance to the fetus. *Nat. Immunol.* 5: 266–271.
14. Mao, G., J. Wang, Y. Kang, P. Tai, J. Wen, Q. Zou, G. Li, H. Ouyang, G. Xia, and B. Wang. 2010. Progesterone increases systemic and local uterine proportions of CD4+CD25+ Treg cells during midterm pregnancy in mice. *Endocrinology* 151: 5477–5488.
15. Kahn, D. A., and D. Baltimore. 2010. Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. *Proc. Natl. Acad. Sci. USA* 107: 9299–9304.
16. Hsieh, C. S., Y. Liang, A. J. Tyznik, S. G. Self, D. Liggitt, and A. Y. Rudensky. 2004. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity* 21: 267–277.
17. Pacholczyk, R., H. Ignatowicz, P. Kraj, and L. Ignatowicz. 2006. Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells. *Immunity* 25: 249–259.
18. Pacholczyk, R., J. Kern, N. Singh, M. Iwashima, P. Kraj, and L. Ignatowicz. 2007. Nonself-antigens are the cognate specificities of Foxp3+ regulatory T cells. *Immunity* 27: 493–504.
19. Wong, J., R. Obst, M. Correia-Neves, G. Losyev, D. Mathis, and C. Benoist. 2007. Adaptation of TCR repertoires to self-peptides in regulatory and nonregulatory CD4+ T cells. *J. Immunol.* 178: 7032–7041.
20. Spence, A., W. Purtha, J. Tam, S. Dong, Y. Kim, C. H. Ju, T. Sterling, M. Nakayama, W. H. Robinson, J. A. Bluestone, et al. 2018. Revealing the specificity of regulatory T cells in murine autoimmune diabetes. [Published erratum appears in 2018 *Proc. Natl. Acad. Sci. USA* 115: E5634.] *Proc. Natl. Acad. Sci. USA* 115: 5265–5270.
21. LaMothe, R. A., P. N. Kolte, T. Vo, J. D. Ferrari, T. C. Gelsinger, J. Wong, V. T. Chan, S. Ahmed, A. Srinivasan, P. Deitemeyer, et al. 2018. Tolerogenic nanoparticles induce antigen-specific regulatory T cells and provide therapeutic efficacy and transferrable tolerance against experimental autoimmune encephalomyelitis. *Front. Immunol.* 9: 281.
22. Kim, Y. C., A. H. Zhang, J. Yoon, W. E. Culp, J. R. Lees, K. W. Wucherpfennig, and D. W. Scott. 2018. Engineered MBP-specific human Tregs ameliorate MOG-induced EAE through IL-2-triggered inhibition of effector T cells. *J. Autoimmun.* 92: 77–86.
23. Nguyen, P., W. Liu, J. Ma, J. N. Manirarora, X. Liu, C. Cheng, and T. L. Geiger. 2010. Discrete TCR repertoires and CDR3 features distinguish effector and Foxp3+ regulatory T lymphocytes in myelin oligodendrocyte glycoprotein-induced experimental allergic encephalomyelitis. *J. Immunol.* 185: 3895–3904.
24. Zhao, Y., P. Nguyen, J. Ma, T. Wu, L. L. Jones, D. Pei, C. Cheng, and T. L. Geiger. 2016. Preferential use of public TCR during autoimmune encephalomyelitis. *J. Immunol.* 196: 4905–4914.
25. Sun, Y., K. Best, M. Cinelli, J. M. Heather, S. Reich-Zeliger, E. Shifrut, N. Friedman, J. Shawe-Taylor, and B. Chain. 2017. Specificity, privacy, and degeneracy in the CD4 T cell receptor repertoire following immunization. *Front. Immunol.* 8: 430.
26. Vanderlugt, C. L., and S. D. Miller. 2002. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat. Rev. Immunol.* 2: 85–95.
27. Lahl, K., C. Loddenkemper, C. Drouin, J. Freyer, J. Arnason, G. Eberl, A. Hamann, H. Wagner, J. Huehn, and T. Sparwasser. 2007. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* 204: 57–63.
28. Bolotin, D. A., S. Poslavsky, I. Mitrophanov, M. Shugay, I. Z. Mamedov, E. V. Putintseva, and D. M. Chudakov. 2015. MiXCR: software for comprehensive adaptive immunity profiling. *Nat. Methods* 12: 380–381.
29. Shugay, M., D. V. Bagaev, M. A. Turchaninova, D. A. Bolotin, O. V. Britanova, E. V. Putintseva, M. V. Pogorelyy, V. I. Nazarov, I. V. Zvyagin, V. I. Kirgizova, et al. 2015. VDJtools: unifying post-analysis of T cell receptor repertoires. *PLoS Comput. Biol.* 11: e1004503.
30. Mendel, I., N. Kerlero de Rosbo, and A. Ben-Nun. 1995. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells. *Eur. J. Immunol.* 25: 1951–1959.
31. O'Connor, R. A., K. H. Malpass, and S. M. Anderton. 2007. The inflamed central nervous system drives the activation and rapid proliferation of Foxp3+ regulatory T cells. *J. Immunol.* 179: 958–966.
32. Mendel Kerlero de Rosbo, N., and A. Ben-Nun. 1996. Delineation of the minimal encephalitogenic epitope within the immunodominant region of myelin oligodendrocyte glycoprotein: diverse V beta gene usage by T cells recognizing the core epitope encephalitogenic for T cell receptor V beta b and T cell receptor V beta a H-2b mice. *Eur. J. Immunol.* 26: 2470–2479.
33. Arck, P. C., and K. Hecher. 2013. Fetomaternal immune cross-talk and its consequences for maternal and offspring's health. *Nat. Med.* 19: 548–556.
34. Erlebacher, A. 2013. Mechanisms of T cell tolerance towards the allogeneic fetus. *Nat. Rev. Immunol.* 13: 23–33.
35. Lydon, J. P., F. J. DeMayo, C. R. Funk, S. K. Mani, A. R. Hughes, C. A. Montgomery, Jr., G. Shyamala, O. M. Conneely, and B. W. O'Malley. 1995. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev.* 9: 2266–2278.
36. Solano, M. E., M. K. Kowal, G. E. O'Rourke, A. K. Horst, K. Modest, T. Plösch, R. Barikbin, C. C. Remus, R. G. Berger, C. Jago, et al. 2015. Progesterone and HMOX-1 promote fetal growth by CD8+ T cell modulation. *J. Clin. Invest.* 125: 1726–1738.
37. Kim, M. R., D. W. Park, J. H. Lee, D. S. Choi, K. J. Hwang, H. S. Ryu, and C. K. Min. 2005. Progesterone-dependent release of transforming growth factor-beta1 from epithelial cells enhances the endometrial decidualization by turning on the Smad signalling in stromal cells. *Mol. Hum. Reprod.* 11: 801–808.
38. Norwitz, E. R., D. J. Schust, and S. J. Fisher. 2001. Implantation and the survival of early pregnancy. *N. Engl. J. Med.* 345: 1400–1408.
39. Lee, J. H., B. Ulrich, J. Cho, J. Park, and C. H. Kim. 2011. Progesterone promotes differentiation of human cord blood fetal T cells into T regulatory cells but suppresses their differentiation into Th17 cells. *J. Immunol.* 187: 1778–1787.
40. Papenfuss, T. L., N. D. Powell, M. A. McClain, A. Bedarf, A. Singh, I. E. Gienapp, T. Shawler, and C. C. Whitacre. 2011. Estriol generates tolerogenic dendritic cells in vivo that protect against autoimmunity. *J. Immunol.* 186: 3346–3355.

41. Duurland, C. L., C. C. Brown, R. F. O'Shaughnessy, and L. R. Wedderburn. 2017. CD161^{+} tconv and CD161^{+} Treg share a transcriptional and functional phenotype despite limited overlap in TCRβ repertoire. *Front. Immunol.* 8: 103.
42. Li, M. O., and A. Y. Rudensky. 2016. T cell receptor signalling in the control of regulatory T cell differentiation and function. *Nat. Rev. Immunol.* 16: 220–233.
43. Munoz-Suano, A., M. Kallikourdis, M. Sarris, and A. G. Betz. 2012. Regulatory T cells protect from autoimmune arthritis during pregnancy. *J. Autoimmun.* 38: J103–J108.
44. Voskuhl, R. R., H. Wang, T. C. Wu, N. L. Sicotte, K. Nakamura, F. Kurth, N. Itoh, J. Bardens, J. T. Bernard, J. R. Corboy, et al. 2016. Estriol combined with glatiramer acetate for women with relapsing-remitting multiple sclerosis: a randomised, placebo-controlled, phase 2 trial. *Lancet Neurol.* 15: 35–46.
45. Pozzilli, C., L. De Giglio, V. T. Barletta, F. Marinelli, F. D. Angelis, V. Gallo, V. A. Pagano, S. Marini, M. C. Piattella, V. Tomassini, and P. Pantano. 2015. Oral contraceptives combined with interferon β in multiple sclerosis. *Neurol. Neuroimmunol. Neuroinflamm.* 2: e120.