A mechanism for expansion of regulatory T-cell repertoire and its role in self-tolerance

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T-cell receptor (TCR) signalling has a key role in determining T-cell fate. Precursor cells expressing TCRs within a certain low-affinity range for complexes of self-peptide and major histocompatibility complex (MHC) undergo positive selection and differentiate into naive T cells expressing a highly diverse self-MHC-restricted TCR repertoire. In contrast, precursors displaying TCRs with a high affinity for ‘self’ are either eliminated through TCR-agonist-induced apoptosis (negative selection)3 or restrained by regulatory T (Treg) cells, whose differentiation and function are controlled by the X-chromosome-encoded transcription factor Foxp3 (reviewed in ref. 2). Foxp3 is expressed in a fraction of self-reactive T cells that escape negative selection in response to agonist-driven TCR signals combined with interleukin 2 (IL-2) receptor signalling. In addition to Treg cells, TCR-agonist-driven selection results in the generation of several other specialized T-cell lineages such as natural killer T cells and innate mucosal-associated invariant T cells5. Although the latter exhibit a restricted TCR repertoire, Treg cells display a highly diverse collection of TCRs6. Here we explore in mice whether a specialized mechanism enables agonist-driven selection of Treg cells with a diverse TCR repertoire, and the importance this holds for self-tolerance. We show that the intronic Foxp3 enhancer conserved noncoding sequence 3 (CNS3) acts as an epigenetic switch that confers a poised state to the Foxp3 promoter in precursor cells to make Treg cell lineage commitment responsive to a broad range of TCR stimuli, particularly to suboptimal ones. CNS3-dependent expansion of the TCR repertoire enables Treg cells to control self-reactive T cells effectively, especially when thymic negative selection is genetically impaired. Our findings highlight the complementary roles of these two main mechanisms of self-tolerance.

TCR signalling plays an essential role in Treg cell differentiation and function7–10. Previous studies have shown that a broad range of self-reactivity can promote Treg cell differentiation in the thymus, consistent with the highly diverse TCR repertoire of these cells4,6. We reasoned that a dedicated mechanism, linked to the regulation of Foxp3 gene expression, might enable selection of Treg cells with a diverse TCR repertoire. Previously, we showed that an intronic element of the Foxp3 gene, CNS3, increases the efficiency of Treg cell generation, raising the possibility that it might affect the composition of the Treg repertoire. To account for the potential effects of a mixed 129/B6 genetic background in our previous study, we backcrossed the CNS3 knock-out Foxp3ΔCNS3/− strain onto a B6 genetic background and generated male Foxp3ΔCNS3/− and Foxp3+/+ littermates carrying identical amino-terminal enhanced green fluorescent protein (GFP) reporters11,12. Consistent with our previous observation11, we found an ~40% reduction in Foxp3+CD4+ thymocytes in CNS3-deficient mice, compared to CNS3-sufficient littermate controls (2.05 ± 0.38% and 3.38 ± 0.70% (mean ± s.d.) of CD4 single-positive (SP) thymocytes, respectively). The size of other thymocyte subsets was unaffected (Extended Data Fig. 1a, b). In contrast, peripheral Treg cells were present at comparable frequencies, probably owing to homeostatic expansion7,13–15 (Extended Data Fig. 1a). Interestingly, loss of CNS3 had no effect on Foxp3 expression in differentiated Treg cells (Extended Data Fig. 1c). Our previous study suggested that CNS3 is epigenetically marked in precursor cells, raising the question of which stage of T-cell differentiation CNS3 acts to facilitate Treg cell development. We found that ablation of a conditional CNS3 allele in double positive (DP) or double negative (DN) thymocytes using Cd4cre or Lckcre drivers, respectively, resulted in similarly defective thymic Treg cell generation (Extended Data Fig. 1d, e). To assess the requirement for CNS3 immediately preceding Foxp3 induction, we acutely ablated CNS3 using tamoxifen-inducible Cre and observed decreased Foxp3 induction upon activation of naive CD4+ T cells in the presence of TGFβ and IL-2 (Extended Data Fig. 1f). Notably, in mature Treg cells, CNS3 was fully dispensable for the maintenance of Foxp3 expression during cell division in the presence of pro-inflammatory cytokines (Extended Data Fig. 1g, h), and for their suppressor function in vivo (Extended Data Fig. 2).

These findings raised the question of how, mechanistically, CNS3 could selectively facilitate the initiation but not the maintenance of Foxp3 expression. To address this problem, we identified the stage of thymocyte differentiation at which the CNS3 region first acquires the characteristic features of a poised enhancer. We previously found that CNS3 is marked by lysine 4 mono-methylation of histone H3 (H3K4me1) in DP thymocytes11. Unexpectedly, we found increased H3K4me1 levels at CNS3 at the DN1 stage and in haematopoietic stem cells, comparable to the levels observed in DP thymocytes, CD4 SP thymocytes and naive CD4+ and CD8+ T cells (Fig. 1a–c and unpublished data). In contrast, CNS3 chromatin was not enriched for H3K4me1 in embryonic stem cells, macrophages or dendritic cells (Fig. 1b, c). These results indicate that the poised state of CNS3 is established at a very early stage of haematopoiesis, but is lost in ‘non-T-cell’ lineages. As CNS3 appeared to be the earliest epigenetically modified region in the Foxp3 locus, it might exert its function by facilitating chromatin remodelling at the Foxp3 promoter.

While depostition of the ‘active’ histone modifications H3K4me3 and H3K27ac at the Foxp3 promoter occurred exclusively in Treg cells (Extended Data Fig. 3a, b), we found an enrichment of H3K4me1 in mature CD4 SP thymocytes and naive CD4+ T cells (Fig. 1d). In the absence of CNS3, both mature CD4 SP thymocytes and naive CD4+ T cells showed impaired H3K4me1 accumulation at the Foxp3 promoter (Fig. 1e, f), suggesting that CNS3 facilitates epigenetic remodelling of the Foxp3 promoter in Treg cell precursors. Notably, differentiated CNS3-deficient Treg cells showed normal levels of H3K4me3 and...
induction between CNS3-sufficient and -deficient CD4+ T cells was markedly decreased in the presence of higher amounts of CD3 antibody (Fig. 2a and Extended Data Fig. 4a), suggesting that increased TCR signal strength can partially compensate for the lack of Foxp3 promoter poisoning in the absence of CNS3, and that differentiation of Treg precursors receiving lower TCR stimulation might be disproportionately impeded by CNS3 deficiency. These results suggest that the mature Treg cells differentiated from CNS3-deficient precursors are enriched for TCRs at the higher end of the self-reactivity spectrum, and depleted of those with lower self-reactivity.

To test this possibility we analysed Foxp3+ and Foxp3− CD4+ T cells for the expression of orphan nuclear receptor Nur77 (also known as Nr4a1), the product of a prominent TCR target gene in Treg and conventional T cells that accurately reports the strength of TCR signalling. In agreement with the in vitro Foxp3 induction studies, we found that both thymic and peripheral CNS3-deficient Treg cells, but not Foxp3− CD4+ T cells, were markedly enriched for cells expressing higher levels of Nur77 in comparison to their CNS3-sufficient counterparts. This trend was observed in non-competitive settings of male Foxp3+ and Foxp3ΔCNS3-gfp littersmates, as well as in competitive settings, on ablation of CNS3 in bone marrow chimera and heterozygous female Foxp3ΔCNS3+ and Foxp3ΔCNS3-gfp+ littersmates (Fig. 2b, Extended Data Fig. 4b–d and unpublished data). Notably, Nur77 levels were increased in both resting (CD4+CD25+CD69−) and activated (CD4+CD25+CD69+) CNS3-deficient Treg cells (Extended Data Fig. 4e–g). In contrast, Nur77 expression in Foxp3− thymocytes and peripheral T cells was unaffected in the absence of CNS3 (Extended Data Fig. 4d). Consistently, CNS3-deficient Treg cells expressed increased amounts of CTLA4, a major negative feedback regulator of TCR signalling, and cell proliferation marker Ki-67 (Extended Data Fig. 4h, i). Finally, we found that CNS3-deficient and -sufficient Foxp3+ CD4 SP thymocytes and resting peripheral Treg cells exhibited distinct gene expression profiles, in contrast to Foxp3− subsets (Extended Data Fig. 5a). Specifically, the expression of TCR-dependent genes and genes characteristic of activated Treg cells was significantly increased in CNS3-deficient Foxp3+ thymocytes and resting Treg cells in comparison to their CNS3-sufficient counterparts (Fig. 2c, d and Extended Data Fig. 5b, d). In contrast, transcriptional profiles of CNS3-sufficient and -deficient activated Treg cells and Foxp3+ CD4 SP thymocytes were similar (Extended Data Fig. 5a, c, e and unpublished data). These results further support the notion that loss of CNS3 results in enrichment of thymic Treg cells with heightened TCR signal strength.

To assess the auto-reactivity of CNS3-deficient versus -sufficient Treg cells in vivo, we examined their capacity for MHC class II (MHC-II)-dependent homeostatic expansion under lymphopenic conditions, known to be proportional to TCR affinity for self5. CNS3-deficient Treg cells expanded markedly compared to CNS3-sufficient counterparts after co-transfer with congenically labelled effector T cells into lymphopenic hosts (Tcrb−/− Tcrd−/−), probably driven by the recognition of self-antigens presented by MHC-II molecules because antibody-mediated blockade of MHC-II prevented expansion of Treg cells and erased the advantage of CNS3-deficient Treg cells over their CNS3-sufficient counterparts (Fig. 2e, f and Extended Data Fig. 5f). Accordingly, the frequency of CNS3-deficient Treg cells was noticeably increased in the periphery of Foxp3ΔCNS3-gfp+ compared to Foxp3ΔCNS3−/+ heterozygous female mice (Extended Data Fig. 5g). Thus, Treg cells developed from precursors lacking CNS3 resulted in a skewed TCR repertoire.

We next examined the TCR repertoires of Treg cells and naive and activated CD4+ T cells in Foxp3ΔCNS3-gfp or Foxp3ΔCNS3-Tcrb−/− mice expressing the DO11.10 TCR3 chain transgene, through which TCR diversity is limited to a single functional TCRα chain locus 8. Barcoded TCRα libraries were generated using an optimized protocol, and high-throughput sequencing data were analysed using the MIGEC software package 22. Cluster analysis using VDJtools 23 showed

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Figure 1 | CNS3 acts as an epigenetic switch for the Foxp3 promoter poising. a, Chromatin immunoprecipitation and quantitative PCR (ChIP-qPCR) of H3K4me1 at the Foxp3 locus and control loci (Hspa2, Rpl30 and Gm5069) in B cells, DP thymocytes, naive CD4+ T (Tn) and Treg cells.

H3K27ac deposition at the Foxp3 regulatory regions (Extended Data Fig. 3c–e), consistent with the dispensable role of CNS3 in differentiated Treg cells (Extended Data Fig. 1c, g, h).

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To address whether the CNS3-dependent poised state of the Foxp3 promoter assists deposition of additional permissive marks and further chromatin remodelling that facilitates the initiation of Foxp3 expression, we cultured naive CD4+ T cells from male Foxp3+ and Foxp3ΔCNS3-gfp littersmates under Treg cell differentiation conditions, and isolated Foxp3− cells that had been exposed to Foxp3-inducing conditions but had not yet acquired Foxp3 expression. We observed a CNS3-dependent increase in H3K27ac at the Foxp3 promoter preceding Foxp3 expression (Extended Data Fig. 3f), consistent with the defect in Treg cell differentiation in the absence of CNS3 (Extended Data Fig. 1f). Furthermore, blocking the recruitment of chromodomains-containing histone acetyltransferases using the inhibitor iBET sharply reduced Treg cell induction efficiency in a dose-dependent manner 16 (Extended Data Fig. 3g). Conversely, blocking histone deacetylase activity using butyrate increased H3K27ac at the Foxp3 promoter in agreement with recent reports 17–19 (Fig. 1g). Notably, provision of butyrate rescued impaired in vitro Treg cell differentiation associated with loss of CNS3 (Fig. 1h). These observations suggest that a CNS3-dependent poised state at the Foxp3 promoter, probably via looping, in precursor cells may enhance their sensitivity to Foxp3-inducing signals (Extended Data Fig. 3h).

While CNS3-dependent poising of the Foxp3 promoter could facilitate Foxp3 induction in a probabilistic manner, it might also enable lower strength TCR signals to promote Treg cell differentiation. To address this possibility, we tested whether impaired Foxp3 induction in CNS3-deficient naive CD4+ T cells could be rescued by increasing amounts of CD3 antibody under in vitro Treg cell differentiation conditions. We found that the relative difference in the efficiency of Foxp3
that TCRα repertoires of thymic and peripheral CNS3-deficient and -sufficient Treg cells, but not naive or activated effector CD4+ T cells, were distinct (Fig. 2g). Further analysis showed a significantly reduced TCRα diversity of CNS3-deficient Treg cells, but not naive or effector CD4+ T cells, in comparison to their CNS3-sufficient counterparts (Fig. 2h). As the TCR complementarity-determining region 3 (CDR3) largely determines TCR specificity for peptide–MHC (pMHC) complexes, we assessed the frequencies of strongly interacting amino acid residues in the TCRα chain CDR3 by leveraging a mathematical model linking the features of amino acid residues in the CDR3 to TCR affinity for pMHC.24 Interestingly, the TCRα CDR3s were significantly enriched for strongly interacting amino acid residues (Extended Data Fig. 5h, i), and for more randomly added nucleotides (Supplementary Table 1) in CNS3-deficient versus -sufficient Treg cells, but not naive or activated effector CD4+ T cells. These results implied higher affinities of CNS3-deficient Treg TCRs for self-antigens and further supported the notion that CNS3 shapes Treg TCR repertoire by increasing its diversity, probably by enabling Treg differentiation in response to a broad range of self-reactivity.

To understand the functional significance of CNS3-dependent regulation of the Treg cell repertoire, we first assessed the immune status of male Foxp3ΔCNS3-gfp and their wild-type Foxp3gfp littermates. CNS3 deficiency had no observed effect on the numbers of activated or memory CD4+ or CD8+T cells, or on cytokine production by T cells in the secondary lymphoid organs of 8–12-week-old mice (Fig. 3a–c and unpublished data). Although CNS3-deficient Treg cells were capable of preventing systemic autoimmunity (Fig. 3a–c and Extended Data Fig. 1a), it remained possible that the skewed Treg TCR repertoire might have ‘holes’. Thus, we reasoned that a select few non-lymphoid organs may exhibit focused immune activation in CNS3-deficient versus CNS3-sufficient mice, whereas others might be similarly or even more protected against autoimmunity by the over-represented highly autoreactive Treg cells. Indeed, we found increased numbers of activated effector T cells and elevated IL-13 and IFN-γ production by T cells in the lungs of Foxp3ΔCNS3-gfp mice (Fig. 3a–c). We also observed markedly increased titres of circulating autoantibodies against several self-antigens in the sera of CNS3-deficient mice versus their wild-type Foxp3gfp littermates, whereas the absolute amounts of immunoglobulin (Ig) isotypes were comparable (Fig. 3d, Extended Data Fig. 6a and unpublished data). This notion was further supported by the observed modest, but consistent, decrease in the severity of experimental autoimmune encephalomyelitis in Foxp3ΔCNS3-gfp versus Foxp3gfp littermates (Extended Data Fig. 6b–f). To compare the suppressive capacity of Treg cells developed in CNS3-deficient and -sufficient mice, we transferred these Ly5.2+ Treg cells together with Ly5.1+ Foxp3-null (ΔFoxp3) effector T cells into T-cell-deficient recipients (Fig. 3e). Despite comparable expansion of CNS3-sufficient and -deficient Treg cells in the recipients, we observed more pronounced weight loss and increased pro-inflammatory cytokine production by effector ΔFoxp3 T cells in the presence of CNS3-deficient Treg cells in comparison to the control (Fig. 3f–h and Extended Data Fig. 6g, h). These results indicate that Treg cells developed from CNS3-deficient precursors were selectively impaired in their capacity to suppress self-reactive effector T cells.

Both negative selection and Treg cell generation are driven by self-antigen recognition in the thymus and probably have complementary roles in self-tolerance.1,2,25,26 We reasoned that the relatively mild impairment in suppressive capacity of Treg cells from CNS3-deficient mice on a B6 genetic background, resistant to autoimmunity, may not fully reveal the biological significance of CNS3-dependent broadening of the Treg cell repertoire because of efficient negative selection. Therefore, we assessed the consequences of combined deficiency in CNS3 and Aire (autoimmune regulator), a nuclear factor required for thymic negative selection and optimal Treg cell generation. Loss of Aire leads to diminished expression of a subset of tissue-restricted antigens in the thymus and, consequently, an enlarged self-reactive T-cell pool and diminished Treg repertoire.25,27–29 In contrast to late-onset and mild autoimmunity observed in Aire-knockout (KO)
mice on a B6 genetic background, deficiency of both CNS3 and Aire resulted in fatal early-onset aggressive autoimmune lesions in multiple tissues as early as 3–4 weeks of age, whereas detectable autoimmune inflammation was lacking in littermates with a single deficiency in Aire or CNS3 (Fig. 4a and Extended Data Fig. 7a). We noticed a 100% (n = 35) penetrance with a stochastic gender-independent variation in manifestations expected from perturbations in randomly generated repertoires of self-reactive T cells as well as the probabilistic nature of negative selection (Extended Data Fig. 7a and unpublished data). This was accompanied by significant increases in CD4+ T-cell activation, IFNγ* production (Fig. 4b, c), serum Ig levels (Extended Data Fig. 7b) and autoantibody production (Fig. 4d). Combined Aire and CNS3 deficiency resulted in a further reduction in thymic Treg cell frequency in comparison to the single-deficient mice (1.66 ± 0.28% and 0.91 ± 0.35% in Foxp3*CD4*CD62L*KO mice and Foxp3*CD4*CD62L*KO mice, respectively) (Fig. 4e). However, peripheral Treg cells reached normal levels in young Foxp3*CD4*CD62L*KO mice before development of clinical signs of disease, probably owing to homeostatic proliferation (Fig. 4f). Despite their normal quantities and Foxp3 expression, these Treg cells were unable to suppress pathogenic self-reactive T cells resulting from impaired negative selection in the absence of Aire (Fig. 4b–d and Extended Data Fig. 7c). As diminished thymic Treg cell numbers and their skewed TCR repertoire probably contributed to disease severity in Foxp3*CD4*CD62L*KO mice, we directly assessed the ability of CNS3-deficient and -deficient Treg cells developed in the presence of Aire to control Foxp3*CD4*CD62L*KO effector T cells when adoptively transferred into T-cell-deficient hosts. Although the negative effect of CNS3 deficiency on the TCR repertoire was probably mitigated by Treg cell expansion in lymphopenic settings, CNS3-deficient Treg cells still exhibited compromised ability to suppress the responses of transferred Foxp3*CD4*CD62L*KO effector T-cell recipients and resident B cells in comparison to the controls (Extended Data Fig. 7d–i). These results suggest that control of broad self-reactive T cells requires a diverse CNS3-dependent repertoire of Treg cells.

Our studies suggest that CNS3, an intronic Foxp3 regulatory element, establishes a poised state of the Foxp3 promoter in precursor cells and increases the probability of Foxp3 induction in response to TCR stimulation, particularly within a lower range of signal strength (Extended Data Fig. 8). Similar mechanisms of promoter poising may operate in other cell types and enable them to respond to a wider spectrum of growth factor or morphogen concentrations through receptor-triggered analogue signalling. CNS3-mediated Foxp3 promoter poising expands
Figure 4 | CNS3-deficient Tng cells fail to maintain self-tolerance in the absence of Aire. a. Analysis of tissue inflammation in CNS3 Aire double knockout (DKO) mice. n = 3 per group except for Foxp3-CNS3-gfp Aire−/− (n = 4). Two-tailed unpaired Mann–Whitney test. b. Analysis of activated CD4+ Tng cells in DKO mice. Foxp3-CNS3-gfp Aire−/− (n = 10); Foxp3-CNS3-gfp Aire−/WT (n = 6); Foxp3-CNS3-gfp Aire−/WT (n = 11); Foxp3-CNS3-gfp Aire−WT (n = 9). Data are representative of ≥2 experiments. Two-tailed unpaired Mann–Whitney test. c. Analysis of IFNγ production by CD4+ Tng cells in DKO mice. Foxp3-CNS3-gfp Aire−/− (n = 5); Foxp3-CNS3-gfp Aire−WT (n = 3); Foxp3-CNS3-gfp Aire−/− (n = 7); Foxp3-CNS3-gfp Aire−/WT (n = 9). Data are representative of ≥2 experiments. Two-tailed unpaired Mann–Whitney test. d. Analysis of tissue-specific autoantibodies in the serum of DKO mice. Sections of skin, intestine and eye from gender-matched Rag1−/− mice were stained with serum IgG (>8 mice per group). DAPI, 4',6-diamidino-2-phenylindole. e, f. Analysis of Foxp3+ CD4 SP thymocytes (e) and peripheral Tng cells (f) in Foxp3-CNS3-gfp Aire−/− mice. Foxp3-CNS3-gfp Aire−/− (n = 10); Foxp3-CNS3-gfp Aire−/WT (n = 6); Foxp3-CNS3-gfp Aire−/WT (n = 11); Foxp3-CNS3-gfp Aire−WT (n = 11). Two-tailed unpaired Mann–Whitney test.

the TCR repertoire of Tng cells, which is essential for controlling pathogenic self-reactive T cells that escape negative selection.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions Y.F. and A.R. conceived and designed the study. Y.F. performed animal and in vivo studies, flow cytometric, TCR sequencing and gene expression analyses. J.v.d.V. analysed the epigenetic modifications of CNS3 and how they affect Foxp3 transcriptional regulation. M.S., E.V.P. and D.M.C. analysed TCR sequencing data. H.U.O. and C.S.L. analysed RNA sequencing data. B.E.H. performed serum Ig isotype analysis. S.D. and S.H. participated in phenotypic analysis of mice. S.H. generated the Cre retroviral vectors. Y.F. and A.R. conceived and designed the study. Y.F. and A.R. wrote the manuscript.

Author Information All RNA and TCR sequencing data have been deposited in the Gene Expression Omnibus under accession numbers GSE71309 and GSE90790, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.Y.R. (rudenska@mskcc.org).
METHODS

No statistical methods were used to determine sample size. Mice. Foxp3-CNS3-fl-gfp mice were generated using ES cell line C24.1 (C56BL/6) as previously described. CD4GFP, LacZ, UbGFP-CRE, and ROSA26-stop-YFP (R26Y) mice were obtained from the Jackson Laboratories. DO11.10 TCR3 transgenic and Aire-knockout mice were provided by P. Marrack, and D. Mathis and C. Benoist, respectively. Heterozygous females carrying Foxp3-CNS3-fl-gfp and Foxp3+2 were crossed with B6 males to generate hemizygous Foxp3-CNS3-fl-gfp and wild-type Foxp3+2 littersmate Foxp3+2, Foxp3-null, Rag1−/−, CD45.1− Foxp3+2 and TcrbΔ− Tcrd+ T cell–mice were maintained in our animal facility. To study the genetic interactions between CNS3 and Aire, heterozygous females of Foxp3-CNS3-fl-gfp were first crossed with AireCre+/−, and F1 harbouring AireCre+ and Foxp3ΔCNS3-fl-gfp or Foxp3+2 were then intercrossed to generate AireKk0 or AireKk0 mice carrying Foxp3-CNS3-fl-gfp or Foxp3+2. To examine TCR diversity with restricted repertoire, Foxp3-CNS3-fl-gfp heterozygous females were crossed to the DO11.10 TCR3 transgenic and Tcr−/− males. F1 males of Foxp3-CNS3-fl-gfp or Foxp3+2 mice carrying the DO11.10 TCR3 transgene and Tcr−/− were used for T-cell isolation and TCR sequencing. To induce deletion of CNS3 in vivo, tamoxifen solution (40 mg/ml in olive oil) was administered by gavage to Ubc-Cre+2F3R26R2Y6 mice more than 3 days before lymphocyte isolation.

All mice were maintained in the MSKCC animal facility under SPF conditions, and the experiments were approved by the Institutional Review Board (IACUC 08-10-023). The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Statistical analysis. Statistical tests were performed with Prism (GraphPad), Excel (Microsoft) or R statistical environment. Box-and-whisker plots show minimum, maximum, first and third quartiles and median.

Cell culture. For in vitro Treg cell differentiation, naive CD4+ T cells (GFP−CD25−CD44−CD62Lhi), or mature CD4+ CD8− SP (TCRβGFP−CD25−CD62LhiCD69hi) T cells were sorted from Foxp3+2, Foxp3ΔCNS3-fl-gfp or Foxp3−/− mice after the enrichment of CD4+ T cells or depletion of CD8+ T cells using Dynabeads FlowComp Mouse CD4 or CD8 kits, respectively (Life Technologies), and then cultured with lethally irradiated (20 Gy) antigen-presenting cells (splenocytes depleted of T cells with Dynabeads FlowComp (Life Technologies), and then cultured with lethally irradiated (20 Gy) antigen-presenting cells (splenocytes depleted of T cells with Dynabeads FlowComp Mouse CD4 kit, Life Technologies) or depleted of CD8− T cells (Dynabeads FlowComp Mouse CD8 kit, Life Technologies), respectively, and Treg cells (CD4+Foxp3+). Mature Foxp3+3 Foxp3−/− thymocytes (CD4+CD8−），mature Foxp3+3 Foxp3−/− SP thymocytes (CD4+CD8−），mature Foxp3+3 Foxp3−/− SP thymocytes (CD4+CD8−），mature Foxp3+3 Foxp3−/− SP thymocytes (CD4+CD8−），mature Foxp3+3 Foxp3−/− SP thymocytes (CD4+CD8−）, peripheral naive (CD4−CD8−), and effector (CD4−CD8−) Treg cells were isolated using a FACSaria II sorter (BD) gated on TCR-Vβ8. Extracted from total RNA from TRizol-preserved cell lysates was performed according to the manufacturer's instructions (Life Technologies). mRNA was purified from total RNA with Dynabeads mRNA DIRECT Kit (Life Technologies) and used for reverse transcription.

cDNA synthesis. To maximize the priming efficiency of reverse transcription, a mixture of oligo(dT)18 and eight DNA oligonucleotides corresponding to the tissue-specific constant region was used. The oligonucleotides used in this study were synthesized by Integrated DNA Technologies, Inc. Primer R1, RT-1': 5'-GGGACTCCGAGACGATCAG-3', TRAC_RT1: 5'-TTCTGAACCTTTAATGAATG-3', TRAC_RT2: 5'-CTCTCAGCTATGACCAGGATGAA-3', TRAC_RT3: 5'-GTATATTTTGTCCTCAGCAATT-3', TRAC_RT4: 5'-TTCTGAACCTTTAATGAATG-3', TRAC_RT5: 5'-AGGATCCTATGACCAGGATGAA-3', TRAC_RT6: 5'-AGGATCCTATGACCAGGATGAA-3', TRAC_RT7: 5'-TCTATGACCAGGATGAA-3', TRAC_RT8: 5'-GTATATTTTGTCCTCAGCAATT-3', TRAC_RT9: 5'-TCTATGACCAGGATGAA-3'. To label the 5′ end of TRcRc mRNA, a DNA–RNA hybrid oligonucleotide with 12 random nucleotides serving as barcodes to tag individual mRNA molecules was synthesized as previously reported.

Hybrid oligonucleotide: GAGAGAGGTGATCAGACGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
reverse primer 8 (5'-TTTTGTGACTGGAACCGTT-3'), 0.2 mM each dNTP, 1.5 mM MgCl₂ and 0.02 U/lUC-1 KOD Hot Start DNA Polymerase (EMD Millipore). PCR parameters were as follows: initial denaturation at 95°C for 2 min; 10 cycles of 95°C for 20 s, 70°C for 10 s with an increment of 1°C per cycle, and 70°C for 30 s; 15 cycles of 95°C for 20 s, 60°C for 10 s and 70°C for 30 s; and final cycle at 70°C for 3.5 min. Amplified DNA was purified with Agencourt AMPure XP magnetic beads for the subsequent reaction. The second PCR reaction used the same reaction conditions except that the reverse primer was replaced by a nested primer. Nested PCR products were purified with Agencourt AMPure XP magnetic beads and used for the fourth PCR amplification with primers P1 (5'-AAGGATCCACCGACCATCG-3') and P2 (5'-AAGGATCCACCGACCATCG-3'). The cycling parameters were: 95°C for 2 min; 6 cycles of 95°C for 20 s, 60°C for 10 s and 70°C for 30 s; and a final cycle at 70°C for 3.5 min. DNA from individual samples was extracted with Agencourt AMPure XP magnetic beads and used for the fourth PCR amplification with primers P1 (5'-AAGGATCCACCGACCATCG-3') and P2 (5'-AAGGATCCACCGACCATCG-3'). The cycling parameters were: 95°C for 2 min; 6 cycles of 95°C for 20 s, 55°C for 10 s and 70°C for 30 s; and a final cycle at 70°C for 3.5 min. The PCR products were purified with Agencourt AMPure XP magnetic beads and used for the fourth PCR amplification with primers P1 (5'-AAGGATCCACCGACCATCG-3') and P2 (5'-AAGGATCCACCGACCATCG-3'). The cycling parameters were: 95°C for 2 min; 5 cycles at 95°C for 20 s, 57°C for 10 s and 70°C for 30 s; and a final cycle at 70°C for 3.5 min. The final PCR products were separated by agarose gel electrophoresis and a single band around 600 base-pairs was cut and extracted with Gel Extraction and PCR Clean-Up kits (Takara).

High-throughput sequencing. Samples were quantified with Kapa Library Quantification kits (Kapa Biosystems) and sequenced on a MiSeq sequencer (Illumina) using 200 cycles of read 1, 6 cycles of index read and 200 cycles of read 2 with the following customized primers: read 1: 5'-CTTCCGCTTGTGAATGC-3', index read: 5'-TCTGTCAGTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'; and read 2: 5'-AGTCATCGACCCGACGATCCACAGA-3'.

Data analysis. Barcoded sequencing data were analysed with MIGEC software. Briefly, unique molecular identifier sequences were isolated from raw sequencing data (read 1) with MIGEC/Checkout routine. Reads (≥5) bearing the same unique molecular identifier were grouped and assembled to generate consensus sequences with MIGEC/Assembly. Variable (V) and joining (J) segment mapping, CDR3 extraction, and error correction were performed with MIGEC/CdrBlast as previously described, which eliminates PCR and sequencing errors, as well as normalizes the output data as CDNA counts that represent the TCR clonotypes in a population.

Comparison of TCRα repertoires between CNS3-deficient and -sufficient mice at protein level was evaluated using VDJtools post-analysis framework (https://github.com/mikeshe/vdjtools). Pearson correlation of clonotype frequencies for the shared TCR clones was used for the calculation of the denrogram. Clonal diversities of TCRα repertoires were evaluated using inverse Simpson index computed separately for individual samples after downsampling to the repertoire size of the smallest sample from the same organ. Similar downsampling strategy, not weighted by clonotype frequencies, was used to compute the average size of added nucleotides in CDR3. A mathematical model was used to assess the strength of CDR3 amino acid interactions with pMHC complexes. Numbers of strongly interacting amino acid residues (LFMVMWCY) were calculated for the V-segment part of TCRα CDR3 and V-J segment junction. Those numbers were then weighted by the corresponding clonotype frequencies and the resulting sums were used for the comparisons between samples.

RNA sequencing and data analysis. Mature Foxp3+ CD4+ CD8- SP (TCRα-β) CD45RA- (CD69) thymocytes, Foxp3+ CD4 SP thymocytes (thyroidic, Treg Cells), peripheral resting (CD44loCD62Lhi) and activated (CD44hiCD62Llo) Treg Cells were FACS-sorted from 6–8-week-old male Foxp3GFP and Foxp3GFP mice resuspended in 250 μl nuclear lysis buffer containing 1% SDS. Chromatin input samples were prepared by sonication of cross-linked nuclear lysates. For histone Chdps, nuclear lysates were subjected to micrococcal nuclease (MNase) digestion before sonication. Nuclear lysates were resuspended in 100 μl MNase (New England Biolabs) at 12,000 U ml⁻¹ for 1 min at 37°C. The reaction was stopped by addition of 10 μl of 0.5 M EDTA. Chromatin input samples were incubated overnight at 4°C with antibodies against H3K4me1 (Abcam), H3K4me3 (Millipore) or H3K27ac (Abcam), and precipitated for 90 min at 4°C using protein A Dynabeads (Life Technologies). After thorough washing, bead-bound chromatin was subjected to proteinase K digestion and decrosslinking overnight at 65°C. DNA PCR was isolated using the Qiagen PCR purification kit. Relative abundance of precipitated DNA fragments was analysed by qPCR using Power SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used for qPCR: Gm5069: forward: 5'-AAAGGCTATCATCTCCTGCTCG-3'; reverse: 5'-TAAGCTGATGTTCTGACGAGG-3'; Fbo3: forward: 5'-TCGGTGAGATGTTGGAGGAGGACG-3'; reverse: 5'-AGCTTGAAGCAGCAGAGG-3'; Hspa2: forward: 5'-TCAAGCAGAAGACGGCATACGA-3', reverse: 5'-TTACTCTGACCCGAGGAAGCGCTAGTA-3', reverse: 5'-TTCGGGAGCCTCTTTGAGTACC-3'; Rpc3: forward: 5'-TGGGCTTACCTCATCCGCTTCTTCTT-3', reverse: 5'-TGCTCCTGCTGTATGCTAGGTGG-3'; Foxp3 promoter: forward: 5'-TATATGGGCGACCTTCACCAAGG-3', reverse: 5'-AAACCTCTCTCGGTACCTTGGCAGAC-3'; CNS1: forward: 5'-AGACTGCTTGGAACACACTACGCT-3', reverse: 5'-TGAGGAGACGAGAAGGACGCT-3'; CNS2: forward: 5'-ATCTGGGAGAAGGGTGAAGATGAC-3', reverse: 5'-GGGCGTTCTCGTTGAGCGTGTCTCT-3'; CNS3: forward: 5'-TCTCGAGGGTCCAGATCCAGAAGG-3', reverse: 5'-GCAATGCACTGGATAGAGGCTGAG-3'.

Relative enrichment was calculated by normalizing to background binding to the control region (Gm5069). Ig isotype ELISA and immunofluorescence staining. Quantification of serum Ig isotypes was performed by ELISA as previously described. Tissue sections from gender matched Rag1-/- mice were used to detect mouse autoantibodies. Briefly, organs from the Rag1-/- mice were dissected, fixed with neutral buffered formalin, embedded with paraffin and sectioned. After deparaffinization with EZ Prep buffer (Ventana Medical Systems) and antigen retrieval with cell conditioning solution (Ventana Medical Systems) the sections were blocked for 30 min with Background Buster solution (Innoven), followed by avidin/biotin blocking for 8 min, mouse serum (1:50 dilution) incubation for 5 h and biotinylated horse anti-mouse IgG (Vector Labs) incubation for 1 h. The detection was performed with streptavidin–horseradish peroxidase (Ventana Medical Systems) followed by incubation with Tyramide Alexa Fluor 488 (Invitrogen). The slides were then counterstained with DAPI (Sigma Aldrich) for 10 min, mounted, scanned with a Mirax scanner and visualized with Pannoramic Viewer (3DHISTECH). Scanned images were scored and representative snapshots were processed with Photoshop (Adobe) to switch the green and red channels for presentation purpose.

Generation of mixed bone marrow chimaeras. Mixed bone marrow chimaeras were generated as previously described. Briefly, recipient mice were irradiated (9.5 Gy) 24 h before intravenous injection of 10⁶ bone marrow cells from CD45.1+ Foxp3GFP and CD45.2+ Foxp3GFP mice mixed at a 1:1 ratio. After bone marrow transfer, the recipient mice were administered with 2 mg ml⁻¹ neomycin in drinking water for 3 weeks and analysed 8–10 weeks later.

Histological analysis. Tissue samples were fixed in 10% neutral buffered formalin and processed for haematoxylin and eosin staining. Stained slides were scored for tissue inflammation as previously described.

Experimental autoimmune encephalomyelitis induction. Experimental autoimmune encephalomyelitis was induced by immunization with myelin oligodendrocyte glycoprotein peptide 35-55 (MOG35-55, GenScript) in complete Freund’s adjuvant (CFA, Sigma) and mice were monitored for disease as previously described.
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Extended Data Figure 1 | CNS3 is required in precursor cells for optimal T<sub>reg</sub> cell differentiation. a, Diminished numbers of thymic T<sub>reg</sub> cells in 6–8-week-old CNS3-deficient mice. Two-tailed Mann–Whitney test. The data show individual mice and median, and represent 1 of >2 independent experiments. Foxp3<sup>ΔCNS3</sup> (n = 9); Foxp3<sup>CNS3-fl-gfp</sup> (n = 11). b, Flow cytometric analysis of CD4 and CD8 SP thymocyte subsets, including thymic T<sub>reg</sub> precursor (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>−</sup>) cells (b) and Foxp3 expression (c) in 6–8-week-old Foxp3<sup>CNS3-fl-gfp</sup> mice (n = 11) and Foxp3<sup>ΔCNS3</sup> (n = 9) littermates. Unpaired Mann–Whitney test. c, CNS3-dependent T<sub>reg</sub> cell differentiation in heterozygous Foxp3<sup>CNS3-fl-gfp</sup> and Cd4<sup>Cre</sup> Foxp3<sup>CNS3-fl-gfp</sup> (d), or Foxp3<sup>CNS3-fl-gfp</sup> and Lck<sup>Cre</sup> Foxp3<sup>CNS3-fl-gfp</sup> females (e). GFP<sup>+</sup> and GFP<sup>−</sup> T<sub>reg</sub> cells in these mice express Foxp3<sup>CNS3-fl-gfp</sup> or wild-type Foxp3<sup>+</sup> alleles, respectively. The data represent 1 of >2 independent experiments (n ≥ 3 mice per group). f, Acute ablation of CNS3 impairs T<sub>reg</sub> induction in vitro. Yellow fluorescent protein (YFP)<sup>+</sup> (tamoxifen treated) or YFP<sup>−</sup> (vector control) naive CD4<sup>+</sup> T cells from UbcCre-ERT2 Foxp3<sup>CNS3-fl-gfp</sup>R26Y males were cultured under in vitro T<sub>reg</sub> induction conditions. The data show mean ± s.e.m. of triplicate cultures and represent 1 of 2 independent experiments. Two-tailed unpaired t-test. g, h, Acute ablation of CNS3 in differentiated T<sub>reg</sub> cells does not affect Foxp3 expression level on a per cell basis or the stability of mature T<sub>reg</sub> cells. g, Expression of Foxp3, CD25 and CD44 in T<sub>reg</sub> cells on day 4 after tamoxifen treatment. h, YFP<sup>+</sup> and YFP<sup>−</sup> T<sub>reg</sub> cells from tamoxifen-treated UbcCre-ERT2 Foxp3<sup>CNS3-fl-gfp</sup>R26Y males were cultured in the presence of IL-2, IFNγ, IL-4, IL-6 and IL-12 for 4 days. The data represent 2 independent experiments.
Extended Data Figure 2 | CNS3 is dispensable for the suppressor function of differentiated Treg cells in vivo. a–f, In vivo assessment of the suppressor function of Treg cells upon acute ablation of CNS3. Treg cells (CD4+GFP+) isolated from Foxp3GFP or Foxp3CNS3-fl-gfp mice were activated with CD3 and CD28 antibody-coated beads in vitro for five days and then transduced with retroviruses expressing Cre recombinase and a Thy1.1 reporter. Three days later, Thy1.1+CD4+GFP+ cells were sorted by FACS for the suppressor assay. a, CD4+Foxp3− and CD8+ effector T cells (Teff) sorted from Foxp3DTR reporter mice seven days after diphtheria toxin (DT) injection (1 μg intraperitoneal per mouse) were transferred alone or with equal amounts of Thy1.1+Cre-transduced Foxp3GFP or Foxp3CNS3-fl-gfp Treg cells into Tcrb−/−Tcrd−/− recipients. b, Mice were weighed before and after T-cell transfer, and relative weight changes were assessed at weeks 3 and 4 post-transfer. c–f, Four weeks after adoptive transfer, cells were recovered and analysed for Treg frequencies and Foxp3 expression (c), CD4+TCRβ+Foxp3− and CD8+TCRβ− cell numbers (d), IFNγ (e) and IL-13 (f) production. Unpaired Mann–Whitney test (n = 5 per group).
Extended Data Figure 3 | Epigenetic modifications at the Foxp3 locus during T\(_{\text{reg}}\) differentiation. a, b, ChIP–qPCR analysis of H3K4me3 (a) and H3K27ac (b) at the Foxp3 locus and control loci (Hspa2, Rpl30 and Gm5069) in B cells, DP thymocytes, naïve CD4\(^+\) T and T\(_{\text{reg}}\) cells. FAC-sorted cells from wild-type male Foxp3 DTR mice were used for ChIP–qPCR. Relative enrichment was calculated by normalizing to background binding to control region (Gm5069).

c–e, ChIP–qPCR analysis of H3K4me1 (c), H3K4me3 (d) and H3K27ac (e) in the Foxp3 locus in mature T\(_{\text{reg}}\) cells isolated from wild-type Foxp3\(^{\text{gfp}}\) and Foxp3\(^{\Delta\text{CNS3}-\text{gfp}}\) male mice normalized to the background binding to the Gm5069 locus. FACS.

f, CNS3-dependent deposition of H3K27ac at the Foxp3 promoter in Foxp3\(^{-}\)CD4\(^+\) T cells during in vitro T\(_{\text{reg}}\) cell induction. Foxp3\(^{\text{gfp}}\) or Foxp3\(^{\Delta\text{CNS3}-\text{gfp}}\) naive CD4\(^+\) T cells were cultured under in vitro T\(_{\text{reg}}\) cell differentiation conditions. After three days of culture, GFP\(^{-}\) and GFP\(^{+}\) cells were sorted for ChIP–qPCR analysis. Two-tailed unpaired t-test.

g, Inhibition of T\(_{\text{reg}}\) induction in vitro by bromodomain protein inhibitor iBET. Naïve CD4\(^+\) T cells from wild-type Foxp3\(^{\text{gfp}}\) males were used for Foxp3 in vitro induction in the presence of indicated concentrations of iBET or vehicle.

h, Schematic of the chromatin dynamics at CNS3 and the Foxp3 promoter during T\(_{\text{reg}}\) cell differentiation. The data are shown as means ± s.e.m. of triplicates and represent 1 of 2 independent experiments.
Extended Data Figure 4 | CNS3 facilitates Foxp3 induction and shapes Treg cell repertoire. a, Differential effect of CNS3 on Treg cell in vitro development of mature non-Treg CD4 SP T cells. CD4 SP thymocytes (CD4^+CD8^-TCRβ^+GFP^- CD25^-CD62L^+CD69^+) were pooled and sorted from male Foxp3^GFP^ and Foxp3^ΔCNS3-GFP^ littermates (n = 7 each group) for in vitro Foxp3 expression in CNS3-deficient and -sufficient groups. Data depict means ± s.e.m. of five replicate cultures and represent 1 of 3 independent experiments. Foxp3 expression was analysed four days later and the relative changes in the ratios of Foxp3-expressing cells in the absence of CNS3 were calculated by comparing to CNS3-sufficient groups. Data depict means ± s.e.m. of five replicate cultures and represent 1 of 3 independent experiments. b, Flow-cytometric analysis of Nur77 protein expression in CNS3-deficient and -sufficient Treg cells (n = 5 for each group). Two-tailed unpaired Mann–Whitney test. The data represent 1 of 2 independent experiments. c, Increased Nur77 protein levels in CNS3-deficient Treg cells developed after conditional ablation of CNS3 upon tamoxifen-induced activation of Ubc^Cre-ERT2. Bone marrow of CD45.1+ Foxp3^GFP^ and CD45.2+ Ubc^Cre-ERT2 Foxp3^ΔCNS3-GFP^ R26Y mice were collected from donor mice treated with tamoxifen, mixed at a 1:1 ratio and transferred into lethally irradiated Tcrb^−/−Tcrd^−/− recipients. CD45.1+ CD4^-GFP^, CD45.2+ YFP^-GFP^- and CD45.2+ YFP^-GFP^ cells were sorted for flow cytometric analysis of Nur77 protein levels 10 weeks after bone marrow transfer (n = 5). Unpaired Mann–Whitney tests were used to compare CD45.2+ YFP^-GFP^- and CD45.2+ YFP^-GFP^ or CD45.2+ YFP^-GFP^- and control (CD45.1+ CD4^-GFP^-) groups. The data show medians of individual mice and represent >3 independent experiments. d, Nur77 expression levels in thymic Treg precursors (CD25^-Foxp3^-, immature (CD62L^hiCD69^lo) and mature (CD62L^-CD69^-) CD4 SP thymocytes, and peripheral Foxp3^-CD4^+ and CD8^- T cells in 6–7-week-old Foxp3^{ΔCNS3} (n = 5) and Foxp3^{ΔCNS3-GFP} (n = 4) littermates. Unpaired Mann–Whitney test. The data show medians of individual mice and represent >3 independent experiments. e, Differential Nur77 expression in peripheral CD4^-Spleen Thymus (CD4^-CD62L^hi) and mature (CD62L^-CD69^-) CD4 SP thymocytes, and peripheral Foxp3^-CD4^+ and CD8^- T cells in 6–7-week-old Foxp3^{ΔCNS3} (n = 5) and Foxp3^{ΔCNS3-GFP} (n = 4) littermates. Unpaired Mann–Whitney test. The data represent 1 of 3 independent experiments. f, g, Upregulation of Nur77 expression in resting (CD4^-CD62L^hi) (f) and activated (CD4^-CD62L^lo) (g) CNS3-deficient Treg cells in 6–7-week-old Foxp3^{ΔCNS3} (n = 5) and Foxp3^{ΔCNS3-GFP} (n = 4) littermates. Unpaired Mann–Whitney test. The data represent >3 experiments. h, i, CTLA4 (h) and Ki-67 (i) expression by CNS3-deficient and -sufficient Treg cells in Foxp3^{ΔCNS3} (n = 9) and Foxp3^{ΔCNS3-GFP} (n = 11) mice (h), Foxp3^{ΔCNS3} (n = 5), Foxp3^{ΔCNS3-GFP} (n = 4) (i). Two-tailed unpaired Mann–Whitney test. The data represent 1 of >3 independent experiments.
Extended Data Figure 5 | Influence of CNS3 on Treg cell repertoire.

**a.** Principal component analysis of mRNA expression in CNS3-deficient and -sufficient mature Foxp3\(^{-}\) and Foxp3\(^{+}\) CD4 SP thymocytes, and peripheral resting and activated Treg cells. RNA-seq was performed with three and four biological replicates for cells sorted from male Foxp3\(^{gfp}\) and Foxp3\(^{ΔCNS3-gfp}\) littermates, respectively. Dots represent samples from individual mice.

**b, c.** Relative gene expression levels (cumulative fraction of genes) in CNS3-sufficient and -deficient peripheral resting (**b**) or activated (**c**) Treg cells in comparison to those up- and downregulated in activated versus resting Treg cells isolated from Foxp3\(^{gfp}\) mice. The numbers of genes in each comparison group are indicated in parentheses.

**d, e.** Relative gene expression levels in CNS3-sufficient and -deficient peripheral rTreg (**d**) or aTreg (**e**) cells in comparison to those downregulated in activated Treg cells subjected to acute TCR ablation versus mock treatment. The numbers of genes in each comparison group are indicated in parentheses. One-tailed Kolmogorov–Smirnov test.

**f.** Flow cytometric analysis of Foxp3 expression level (median fluorescence intensity (MFI)) in CNS3-sufficient and -deficient Treg cells after expansion in lymphopenic recipients. Treg cells were sorted from mixed bone marrow chimaeras of CD45.1\(^{+}\) Foxp3\(^{gfp}\) and CD45.2\(^{+}\) Foxp3\(^{ΔCNS3-gfp}\) mice and mixed at a 1:1 ratio, and co-transferred with wild-type naive Foxp3\(^{-}\)CD4\(^{+}\) T cells into Tcrb\(^{-}\) Tcrd\(^{-}\) recipients treated with MHC-II-blocking antibody or isotype-control IgG before and after the transfer (n = 5 per group).

**g.** Comparison of CNS3-sufficient and -deficient Treg cells in competitive environment of heterozygous Foxp3\(^{gfp/+}\) and Foxp3\(^{ΔCNS3-gfp/+}\) females (6–8 weeks of age). In contrast to CNS3-sufficient Treg cells, CNS3-deficient cells are relatively enriched in the periphery in comparison with the thymus. Ratios of GFP\(^{-}\) to GFP\(^{+}\) Treg cells are inversely proportional to the relative abundance of Foxp3\(^{gfp}\) or Foxp3\(^{ΔCNS3-gfp}\) Treg cells in the Treg pool. Wilcoxon matched-pairs signed rank test; Foxp3\(^{gfp/+}\) (n = 5), Foxp3\(^{ΔCNS3-gfp/+}\) (n = 8). Linked circles represent samples from the same mice. Data represent 1 of 2 independent experiments.

**h, i.** Numbers of strongly interacting amino acid residues (LFIMVWCY) were calculated for the V-segment of TCR α CDR3 (binned to germline) and V–J segment junction, and weighted by the corresponding clonotype frequencies. Sums of the weighted scores were used for the comparisons between CNS3-deficient and -sufficient groups (unpaired t-test). The data represent the analysis of pooled TCR sequences derived from the indicated thymic (**h**) and peripheral (**i**) CD4\(^{+}\) naive (Tn), activated effector (Teff) and Treg cell subsets isolated from individual Foxp3\(^{gfp}\) (n = 5) and Foxp3\(^{ΔCNS3-gfp}\) (n = 3) mice. Box-and-whisker plots show minimum, maximum, first and third quartiles and median.
Extended Data Figure 6 | Selective modulation of autoimmune responses in mice lacking CNS3. 

**a**, CNS3 deficiency does not affect antibody production against a subset of autoantigens. Foxp3ΔCNS3-gfp and Foxp3gfp littermates (n = 4 per group). Box-and-whisker plots show minimum, maximum, first and third quartiles and median. Data represent 1 of 2 independent experiments. 

**b**–**f**, CNS3 deficiency decreases experimental autoimmune encephalomyelitis severity. On immunization with MOG peptide in CFA, mice of indicated genotypes were assessed for the severity of limb paralysis (**b**), effector T-cell numbers (**c**), Treg cell frequency (**d**), Foxp3 expression levels (**e**) and inflammatory cytokine production (**f**). Foxp3gfp (n = 8); Foxp3ΔCNS3-gfp (n = 11). Unpaired t-test (**b**) or Mann–Whitney test (**c**–**f**). Mean and s.e.m. are presented (**b**). *P < 0.05, **P < 0.01. The data represent 2 independent experiments. 

**g**, Analysis of the proportion of Treg cells in CD4+ TCRβ+ cell population (**g**) and level of Foxp3 expression (MFI) (**h**) in an in vivo suppressor assay of CNS3-deficient or -sufficient Treg cells (Fig. 3e–h). Two-tailed unpaired Mann–Whitney test.
Extended Data Figure 7 | Compromised suppressive function of CNS3-deficient Treg cells. 

a. Autoimmune diseases in Foxp3ΔCNS3-gfp AireKO/KO (DKO) mice. Arrow indicates the inflammatory lesions in the tail of a 3-week-old mouse with an early onset of autoimmunity ($n > 11$) (i).

De-pigmentation in a 6-week-old mouse with delayed onset of autoimmunity ($n > 16$) (ii).

b. Analysis of serum Ig isotypes in Foxp3ΔCNS3-gfp AireKO/KO and littermate control mice using ELISA ($n = 8$ per group). Error bars, mean ± s.e.m. Two-way ANOVA.

c. Flow cytometric analysis of Foxp3 expression by Treg cells. The data show one of at least three mice per group and represent >3 independent experiments.

d–i. Analysis of the ability of CNS3-deficient and -sufficient Treg cells to control CNS3 Aire DKO effector T cells on adoptive transfer into T-cell-deficient recipients. Flow cytometric analysis of non-Treg CD4+ T-cell numbers (d), Treg cell numbers (e), Foxp3 expression levels (f), IFNγ production (g), IL-17 production (h), and serum IgG1 and IgG2b levels (i) in recipient mice transferred with CNS3 and Aire DKO effector T cells (Foxp3− CD4+ and CD8+) at a 10:1 ratio with Treg cells from Aire-sufficient Foxp3ΔK or Foxp3ΔCNS3-gfp mice. Two-tailed unpaired Mann–Whitney tests (d–h) or unpaired t-test (i). Error bars, mean ± s.e.m. (i). The recipient mice were analysed 7 weeks after adoptive T-cell transfer ($n = 5$ per group).
Extended Data Figure 8 | Theoretical impact of CNS3 on Treg TCR repertoire. **a**, Hypothetical distribution of TCRs expressed by T<sub>reg</sub> and non-T<sub>reg</sub> CD4<sup>+</sup> T cells according to their affinities for self-antigens. Precursor cells expressing TCRs within a certain low-affinity window are positively selected and become 'conventional' CD4<sup>+</sup> T cells, and those with higher affinities for self-antigens differentiate into T<sub>reg</sub> cells. CNS3 promotes the differentiation of T<sub>reg</sub> cells and broadens their TCR repertoire by facilitating Foxp3 expression predominantly in response to lower strength ('suboptimal') inducing TCR signals. **b**, After expansion in the periphery, CNS3-deficient T<sub>reg</sub> cells reach similar numbers as their wild-type counterparts, with some TCRs underrepresented (A), some minimally affected (B), and some overrepresented (C). T<sub>conv</sub>, conventional T cells.