Activated β-catenin in Foxp3+ regulatory T cells links inflammatory environments to autoimmunity

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Foxp3+ regulatory T cells (Treg cells) are the central component of peripheral immune tolerance. Whereas a dysregulated Treg cytokine signature has been observed in autoimmune diseases, the regulatory mechanisms underlying pro- and anti-inflammatory cytokine production are elusive. Here, we identify an imbalance between the cytokines IFN-γ and IL-10 as a shared Treg signature present in patients with multiple sclerosis and under high-salt conditions. RNA-sequencing analysis on human Treg subpopulations revealed β-catenin as a key regulator of IFN-γ and IL-10 expression. The activated β-catenin signature was enriched in human IFN-γ+ Treg cells, as confirmed in vivo with Treg-specific β-catenin-stabilized mice exhibiting lethal autoimmunity with a dysfunctional Treg phenotype. Moreover, we identified prostaglandin E receptor 2 (PTGER2) as a regulator of IFN-γ and IL-10 production under a high-salt environment, with skewed activation of the β-catenin–SGK1–Foxo axis. Our findings reveal a novel PTGER2–β-catenin loop in Treg cells linking environmental high-salt conditions to autoimmunity.

The homeostatic maintenance of T cells is finely tuned by Treg cells. Treg cells play a distinct role from the other CD4+ T cells in dampening prolonged inflammation and preventing aberrant autoimmunity1. Although Treg cells are potent suppressors of immunological function, the number of Treg cells is often normal in a variety of autoimmune diseases, including multiple sclerosis (MS)14–16. These observations suggest that not only a quantitative but also a functional dysregulation of Treg cells contributes to the development of autoimmunity.

Treg cells display their suppressive ability through both contact-dependent and cytokine-mediated mechanisms. Treg cells show substantial heterogeneity, and the balance between pro- and anti-inflammatory populations is finely regulated to maintain immunological homeostasis14. IFN-γ marks dysfunctional Treg cells in patients with autoimmunity (in MS17 and type 1 diabetes18) and cancer (in glioblastoma). Additionally, Treg cells producing the anti-inflammatory cytokine IL-10 play prominent roles in suppression of the immune response at environmental interfaces and in the development of mature memory CD8+ T cells, thereby preventing autoimmune and chronic infection in mice19. These studies suggest that the balance between IFN-γ and IL-10 production in Treg cells is central in the maintenance of immune homeostasis; however, the molecular mechanisms underlying this regulatory balance are not known.

Human autoimmune disease results from an interplay between genetic factors and environmental triggers. In this regard, MS is an autoimmune disease that results from the complex interaction of predominantly common genetic variants and environmental factors14, and 233 common risk haplotypes have been identified to date19,20. Several environmental factors are associated with an increased risk of MS, including vitamin D insufficiency, smoking, obesity, and a high-salt diet (HSD)21. Previous studies have shown that an HSD exacerbates neuroinflammation in an experimental autoimmune encephalomyelitis model of MS, and that higher salt concentrations within the physiological range skew naïve CD4+ T cells to proinflammatory type 17 helper T (T H17) cells and impair Treg-suppressive function through the induction of IFN-γ expression14,15,22. Studies using mouse models of autoimmune disease increasingly support this possibility23,24, and recent magnetic resonance imaging studies have revealed higher sodium intensity in acute MS lesions than chronic lesions, thus suggesting more sodium accumulation within the pathogenic microenvironment in the MS brain25. However, whether an HSD directly affects MS clinical activity remains unknown26.

β-catenin, an essential component of the canonical Wnt signaling pathway, is involved in a variety of biological processes including carcinogenesis, stem cell maintenance, organogenesis, and aging27,28. Although β-catenin and canonical Wnt signaling have been studied in the immune system, the specific mechanisms through which β-catenin affects Treg function and the roles of these signaling pathways in modulating cytokine production by Treg cells, particularly in the context of human autoimmune disease, is poorly understood.

Here, we show that the imbalance between IFN-γ and IL-10 is a shared Treg signature observed in patients with MS and in a high-salt environment. By performing unbiased RNA-seq analysis on human Treg subpopulations, we identified β-catenin as a central player in maintaining Treg function and regulating the production of both IFN-γ and IL-10 cytokines. Moreover, we clarified β-catenin’s role in mediating the high-salt-induced proinflammatory signature: β-catenin participates in a feed-forward loop with PTGER2, which is uniquely upregulated under high-salt conditions. Our findings suggest that the β-catenin–PTGER2 axis serves as a bridge between environmental factors and autoimmune disease by modulating...
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that mTreg cells isolated from patients with MS (MS-Treg), compared and IL-10 production. mTreg cells isolated from the periphery and inflammatory (IL-10) cytokines by circulating human Treg cells from healthy subjects and patients with MS through flow cytometry. On the basis of our observation that CD25+CD4+CD45RO− Treg cells (memory Treg (mTreg) cells) are the major source of effector cytokine expression in human Treg cells (Supplementary Fig. 1), we focused on mTreg cells to avoid the potential bias caused by the variable ratio of naive Treg cells to mTreg cells among subjects. We found that mTreg cells isolated from patients with MS (MS-Treg), compared with healthy donors, produced more IFN-γ and less IL-10, and the ratio of IFN-γ to IL-10-producing Treg cells further highlighted this imbalance (Fig. 1a,b). Furthermore, we examined the mRNA expression of IFNG and IL10 in mTreg cells without PMA plus ionomycin stimulation, thus better reflecting the situation in vivo, and identified a trend similar to that seen in protein expression (Fig. 1c).

We have recently demonstrated that Treg cells exposed to high salt concentrations exhibit a dysfunctional phenotype with a pro-inflammatory-cytokine signature skewed toward IFN-γ (ref. 13). We sought to determine whether high salt could also impair the IFN-γ/IL-10 balance and found that the high-salt environment caused an increase in IFN-γ production and a decrease in IL-10 production in human mTreg cells after 96h in culture (Fig. 1d,e). We determined the gene expression kinetics of IFNG and IL10 on the basis of qPCR-identified early (8h) and late (96h) waves of gene expression. High-salt stimulation suppressed the early wave of IFNG and IL10, and enhanced the late wave of IFNG but not IL10 (Fig. 1f). These findings suggest that the imbalance in IFN-γ/IL-10 induced by continuous exposure to high-salt conditions, which was not observed at the phase of acute response to high-salt stress, might capture the dysfunctional Treg properties in the setting of autoimmunity.

**β-catenin regulates relative production of IFN-γ and IL-10 in human Treg cells.** The molecular mechanisms underpinning the balance between IFN-γ and IL-10 in Treg cells were largely unknown. To address this lack of knowledge, we performed RNA-seq-based genome-wide transcriptome analysis on human Treg subsets defined by IFN-γ and IL-10 production. mTreg cells isolated from the peripheral mononuclear cells of healthy subjects were stimulated with PMA plus ionomycin for 4h ex vivo. After using cytokine capture kits for IFN-γ and IL-10, we sorted four different subpopulations (IFN-γ single positive (IFN-γSP), IL-10 single positive (IL10SP), IFN-γ and IL-10 double positive (DP), and IFN-γ and IL-10 double negative (DN)), and we performed RNA-seq on each subpopulation (Fig. 2a). We identified 672 differentially expressed genes between IFN-γ SP and IL10SP, and the four populations could be distinguished on the basis of their gene expression profiles (Fig. 2b). Notably, the IFN-γ-producing populations were highly distinct from the IFN-γ-negative populations, thus suggesting that IFN-γ-secreting Treg cells have a more dominant signature than IL-10-secreting Treg cells. We also identified ten clusters of coexpressed genes (C1–C10) across the populations. IFN-γ- and IL-10-associated genes were enriched in C9 and C10 (for example, CXCR3, CD226, and NKG7), and C1 and C2 (for example, MAI, SOCS3, and NOTCH2), respectively.

To predict the key transcriptional regulators that account for IFN-γ and IL-10 production, we performed an upstream regulator analysis in Ingenuity Pathway Analysis, using differentially expressed genes from each population (Supplementary Table 1). We identified β-catenin (CTNNB1) as one of the top upstream regulators in the Treg populations producing IFN-γ and/or IL-10, as compared with DN. Intriguingly, β-catenin was the top-ranked upstream regulator in the comparison between IFN-γSP and IL10SP. These results suggest that β-catenin plays a critical role in driving the production of both IFN-γ and IL-10 in Treg cells, especially that of IFN-γ. We also identified several upstream regulators that have been demonstrated to have critical roles in maintaining Treg function, including MYB, SATB1, NFAFC2, and KLF2, thus suggesting that our upstream regulator analysis provides a reliable readout.

In agreement with these findings, gene-set enrichment analysis (GSEA) identified significant enrichment of the Wnt-β-catenin signaling pathway in IFN-γ-producing Treg subsets (Fig. 2c). IFN-γ SP exhibited the highest enrichment score for the Wnt-β-catenin signaling pathway. Further GSEA analysis with different gene sets also yielded similar results (Supplementary Fig. 2a). Together, these findings indicate that Wnt-β-catenin signaling is more activated in IFN-γ-secreting Treg cells than in other human Treg subpopulations.

**β-catenin is stabilized in the IFN-γ-secreting Treg population.** We first confirmed that β-catenin was stabilized and transcriptionally active in IFN-γSP compared with DN in ex vivo Treg cells by examining the level of active β-catenin, the dephosphorylated form of β-catenin with established active transcriptional activity (Fig. 3a). Notably, the DP and IL10SP subsets also exhibited higher active β-catenin expression than did the DN subset ex vivo, thus suggesting that β-catenin signaling is important for production of not only IFN-γ but also IL-10 in Treg cells, in agreement with the results from our upstream regulator and enrichment analyses. To exclude the possibility that PMA plus ionomycin stimulation might have affected β-catenin stability, we measured active β-catenin levels in CXCR3+ type 1 helper T (T(H)1)-like Treg cells, which comprise most of the IFN-γ-producing Treg cells without PMA plus ionomycin stimulation; these analyses confirmed that expression of active β-catenin was significantly elevated in the CXCR3+ T(H)1-like Treg population (Fig. 3b). In agreement with these data, the downstream β-catenin-target genes AXIN2 and TCF7, and the protein TCF7-1 (encoded by TCF7), were upregulated in IFN-γSP compared with DN ex vivo (Fig. 3c and Supplementary Fig. 2b). This result was consistent with previously published microarray data for IFN-γ-positive and IFN-γ-negative Treg cells (Supplementary Fig. 2c).

To examine whether the in vitro model was able to recapitulate the ex vivo results, we assayed levels of active β-catenin in each of the Treg subsets after 4 d of culture with anti-CD3 and anti-CD28 stimulation. IFN-γ-producing Treg populations (IFN-γSP and DP) showed higher active β-catenin expression than did IL10SP and DN (Fig. 3d), thus indicating that stabilization of β-catenin was more enhanced in IFN-γSP than IL10SP under T cell–receptor stimulation. IL-12 is an essential cytokine for the induction of IFN-γ-producing pathogenic Treg cells under T cell–receptor stimulation. IL-12 is an essential cytokine for the induction of IFN-γ-producing pathogenic Treg cells under T cell–receptor stimulation. IL-12 is an essential cytokine for the induction of IFN-γ-producing pathogenic Treg cells under T cell–receptor stimulation.

We found that upregulation of β-catenin was also present in IL-12-induced T(H)1-like Treg cells, especially in the IFN-γ-producing population (Fig. 3e). To determine whether Wnt-β-catenin signaling was necessary for IFN-γ production in T(H)1-like Treg cells, we blocked β-catenin signaling with the inhibitor PKF115-584 (PKF). Treg cells treated with PKF exhibited significantly decreased production of IFN-γ (Fig. 3f,g). IL-10 production was also suppressed by PKF treatment, albeit less dramatically than IFN-γ. Knocking down the CTNNB1 gene in Treg cells with short hairpin RNA (shRNA) (Supplementary Fig. 2d) ameliorated IL-12-induced IFN-γ and IL-10 production (Fig. 3h,i). These data suggest that β-catenin plays a critical role in IFN-γ and IL-10 induction in human Treg cells, but does so more profoundly for IFN-γ production under T cell–receptor stimulation.

**Results**

**Treg cytokine imbalance in multiple sclerosis and a high-salt environment.** Previous studies have identified a proinflammatory Treg population characterized by the secretion of IFN-γ. This population is dysfunctional both in vitro and in vivo, and a high frequency of this population is associated with autoimmune disease1–6. However, the balance between pro- and anti-inflammatory Treg populations has not been defined. To address this lack of knowledge, we evaluated the production of proinflammatory (IFN-γ) and anti-inflammatory (IL-10) cytokines by circulating human Treg cells from healthy subjects and patients with MS through flow cytometry. Previous studies have identified a proinflammatory-cytokine signature skewed toward IFN-γ (ref. 13). We sought to determine whether high salt could also impair the IFN-γ/IL-10 balance and found that the high-salt environment caused an increase in IFN-γ production and a decrease in IL-10 production in human mTreg cells after 96h in culture (Fig. 1d,e). We determined the gene expression kinetics of IFNG and IL10 on the basis of qPCR-identified early (8h) and late (96h) waves of gene expression. High-salt stimulation suppressed the early wave of IFNG and IL10, and enhanced the late wave of IFNG but not IL10 (Fig. 1f). These findings suggest that the imbalance in IFN-γ/IL-10 induced by continuous exposure to high-salt conditions, which was not observed at the phase of acute response to high-salt stress, might capture the dysfunctional Treg properties in the setting of autoimmunity.
Fig. 1 | IFN-γ/IL-10 balance of human T<sub>reg</sub> cells in MS and under high-salt treatment. a, Representative flow cytometric analysis of ex vivo human T<sub>reg</sub> cells (CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>–negCD45RO<sup>+</sup>) isolated from healthy donors and patients with MS. Flow cytometry–isolated T<sub>reg</sub> cells were stimulated with PMA plus ionomycin for 4 h, then subjected to intracellular staining for IFN-γ and IL-10. Data are representative of 12 experiments. b, IFN-γ and IL-10 cytokine profiles of ex vivo human T<sub>reg</sub> cells. Left, percentages of IFN-γ- and/or IL-10-producing T<sub>reg</sub> cells. Right, ratio between IFN-γ-positive and IL-10-positive T<sub>reg</sub> cells (healthy control (HC), n = 12; MS, n = 14 subjects). *P < 0.05, **P < 0.01 (by two-way analysis of variance (ANOVA) with Sidak’s multiple comparisons test (left), or two-sided Student’s t test (right)). c, IFNG and IL10 gene expression, determined on ex vivo T<sub>reg</sub> cells by qPCR. Right, ratio between IFNG and IL10 expression (HC, n = 36; MS, n = 27 subjects). P values were calculated by two-sided Student’s t test. d, Representative flow cytometric analysis of IFN-γ and IL-10 production in human T<sub>reg</sub> cells stimulated with anti-CD3 and anti-CD28 in the normal medium (control) or medium supplemented with an additional 40 mM NaCl (NaCl) for 96 h. Data are representative of 8 experiments. The percentage of IFN-γ- and/or IL-10-producing T<sub>reg</sub> cells is shown at bottom (n = 21 subjects). P values were calculated by two-sided Student’s t test. e, IFNG and IL10 mRNA expression, assessed 96 h after stimulation as in d (n = 41 subjects). Right, ratio between IFNG and IL10 expression. Each symbol represents an individual subject. P values were calculated by two-sided Student’s t test. f, mRNA expression kinetics of IFNG and IL10 from nine different time points (n = 6 subjects). *P < 0.05, **P < 0.01, ***P < 0.001 (two-way ANOVA with Sidak’s multiple comparisons test). Data are represented as mean (b–e) or mean ± s.e.m. (f).
Constitutive activation of β-catenin in $\Delta_{\text{ctn}}$ cells induces Scurfy-like autoimmunity. To ascertain the physiological relevance of β-catenin signaling in $\Delta_{\text{ctn}}$ cells, we generated $\Delta_{\text{ctn}}$-specific β-catenin-stabilized mice by crossing Foxp3-IREs-Cre mice (Foxp3$^{\text{Cre}}$) with Ctnnb1$^{\Delta\beta\text{catenin}}$ mice (Supplementary Fig. 3a), in which the active form of β-catenin was specifically induced in $\Delta_{\text{ctn}}$ cells. In these Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ mice, β-catenin was highly stabilized in Foxp3$^{\text{Cre}}$ $\Delta_{\text{ctn}}$ cells but not Foxp3$^{\text{Cre}}$ non-$\Delta_{\text{ctn}}$ cells (Fig. 4a and Supplementary Fig. 3b). Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ mice spontaneously developed a hunched posture and crusting of the ears, eyelids, and tail, and they showed thymic atrophy, splenomegaly, and lymphadenopathy (Fig. 4b). Histological analysis demonstrated lymphocyte infiltration into several tissues, such as the lung, pancreas, liver, and intestine, thus indicating systemic inflammation in Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ mice (Fig. 4c). This Scurfy-like fulminant autoimmunity led to premature death within 40 d of birth with 100% penetrance (Fig. 4d).

The balance between $\Delta_{\text{ctn}}$ effector T cells is critical to maintaining T cell homeostasis in both central and peripheral lymphoid tissue. The percentage of $\Delta_{\text{ctn}}$ cells within the thymic CD4$^{\text{+}}$ T cells of Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ mice remained at the same level as that in Foxp3$^{\text{Cre}}$ mice by the age of 3 weeks and even increased at the age of 5 weeks; however, the number of $\Delta_{\text{ctn}}$ cells in the thymus began to decline at the age of 3 weeks in Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ mice (Fig. 4e). In contrast, Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ mice displayed an elevated number of CD4$^{\text{+}}$ and CD8$^{\text{+}}$ conventional T cells in secondary lymphoid organs (Fig. 4f) and high expression of effector cytokine genes such as Ifng, Il14, and Il10, but not Il17a (Supplementary Fig. 3c). Downregulation of Rorc in both $\Delta_{\text{ctn}}$ cells and conventional CD4$^{\text{+}}$ T cells is opposite for Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ mice (Supplementary Fig. 3b) compared with Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ mice, thus highlighting the difference between $\Delta_{\text{ctn}}$ cells from these genotypes. We examined Helios expression to characterize the functional stability of $\Delta_{\text{ctn}}$ cells and found that Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ $\Delta_{\text{ctn}}$ cells lost Helios expression, a result supporting the unstable and dysfunctional features of Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ $\Delta_{\text{ctn}}$ cells (Supplementary Fig. 3d). These results suggest that forced expression of a stabilized form of β-catenin in $\Delta_{\text{ctn}}$ cells influences their functional stability in the periphery more than in the central compartment.

In vitro suppression assays revealed that Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ $\Delta_{\text{ctn}}$ cells showed less suppressive activity than did Foxp3$^{\text{Cre}}$ $\Delta_{\text{ctn}}$ cells (Fig. 5a). Given that the direct interaction of β-catenin with Foxo1 has been reported, we noted that the morphological and pathophysiological phenotype of Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ $\Delta_{\text{ctn}}$ cells was similar to that of mice with Foxo1-deleted $\Delta_{\text{ctn}}$ cells (Fig. 5b). Further assessment with GSEA revealed similar transcriptional profiles between Ctnnb1$^{\Delta\beta\text{catenin}}$; Foxp3$^{\text{Cre}}$ $\Delta_{\text{ctn}}$ cells and Foxo1-depleted $\Delta_{\text{ctn}}$ cells (Supplementary Fig. 3e). In agreement with this...
observation, the levels of phosphorylated (p-) Foxo1 and Foxo3a were higher in \textit{Ctnnb1}\textsuperscript{Als1}; \textit{Foxp3}\textsuperscript{Cre}\textsubscript{CCR} T\textsubscript{reg} cells than in \textit{Foxp3}\textsuperscript{Cre}\textsubscript{CCR} T\textsubscript{reg} cells (Fig. 5c). To determine whether β-catenin and Foxo1 directly interact with each other, we performed an in situ proximity ligation assay (PLA) on human T\textsubscript{reg} cells and detected the PLA signal in human T\textsubscript{reg} cells (Fig. 5d). Collectively, our results indicated that β-catenin regulates the proinflammatory T\textsubscript{h}1-skewing program in T\textsubscript{reg} in concert with the Foxo pathway.

**Fig. 3 | β-catenin is stabilized in the IFN-γ-secreting T\textsubscript{reg} subpopulation.** a, Relative expression of active β-catenin on ex vivo T\textsubscript{reg} subpopulations analyzed by flow cytometry (n = 11 subjects). Fold change in geometric mean fluorescence intensity (gMFI) over DN is shown. *P < 0.05, **P < 0.01 (one-way ANOVA with Tukey’s multiple comparisons test). b, Expression of active β-catenin between CXCR3$^+$ and CXCR3$^-$ ex vivo T\textsubscript{reg} cells from healthy controls. Left, representative histogram; right, summary of results (n = 7 subjects). P values were calculated by two-sided Student’s t test. c, Expression of target genes of Wnt–β-catenin signaling (AXIN2 and TCF7), assessed by RNA-seq (n = 8 subjects). *P < 0.05, **P < 0.01 (one-way ANOVA with Tukey’s multiple comparisons test). d, Relative expression of active β-catenin on T\textsubscript{reg} cells stimulated with anti-CD3 and anti-CD28 for 4 d, then with PMA plus ionomycin for 4 h, and subjected to intracellular cytokine staining for IFN-γ and IL-10 (n = 12 subjects). Fold change in gMFI over DN is depicted. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA with Tukey’s multiple comparisons test). e, Expression of β-catenin on T\textsubscript{reg} cells stimulated with anti-CD3 and anti-CD28 in the presence (T\textsubscript{h}1) or absence (T\textsubscript{0}) of IL-12 for 4 d. Left, β-catenin levels on IFN-γ-positive/negative T\textsubscript{reg} populations, determined after 4 h of stimulation with PMA plus ionomycin (n = 4 subjects). Right, representative histogram of β-catenin expression. P values were calculated by two-sided Student’s t test. f, g, Frequencies of IFN-γ- and IL-10-positive cell numbers (f) and expression of IFNG and IL10, determined by qPCR (g), T\textsubscript{reg} cells were stimulated with anti-CD3 and anti-CD28 in the presence of the Wnt-β-catenin-signaling inhibitor PKF, IL-12 (T\textsubscript{h}1), or IL-12 plus PKF (T\textsubscript{h}1+PKF) (n = 4 subjects). NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA with Tukey’s multiple comparisons test). h, i, Relative frequencies of IFN-γ- and IL-10-positive cell numbers (fold over scrambled shRNA/control condition) (h) and expression of IFNG and IL10, determined by qPCR (i). T\textsubscript{reg} cells were transduced with a scrambled shRNA or a \textit{Ctnnb1} shRNA (\textit{shCtnnb1}) and cultured in T\textsubscript{0} or T\textsubscript{h}1 conditions for 5 d (h, n = 7 subjects; i, n = 5 subjects). *P < 0.05, **P < 0.01 (one-way ANOVA with Tukey’s multiple comparisons test). Data are representative of two experiments (e, f, or are from more than three experiments. Data are represented as mean ± s.d.
**Fig. 4** | Treg-specific activation of β-catenin induces Scurfy-like autoimmunity. **a**, Flow cytometric analysis of β-catenin on peripheral lymph node T<sub>reg</sub> cells and Foxp3<sup>+</sup> T cells (CD4<sup>+</sup>T cells) from Foxp3<sup>Cre</sup> and Ctnnb<sup>ΔEx3;Cre</sup> mice. Data are representative of four experiments. **b**, Left, images of 4-week-old Foxp3<sup>Cre</sup> and Ctnnb<sup>ΔEx3;Cre</sup> mice. Right, representative pictures of thymus, peripheral lymph nodes, and spleens isolated from 4-week-old Foxp3<sup>Cre</sup> or Ctnnb<sup>ΔEx3;Cre</sup> mice. **c**, Hematoxylin and eosin staining of thymus, spleen, liver, intestine, pancreas, and lung sections from 4-week-old Foxp3<sup>Cre</sup> or Ctnnb<sup>ΔEx3;Cre</sup> mice. Scale bars, 300 μm for lower magnification and 150 μm for higher magnification. Results in **b** and **c** are representative of six experiments. **d**, Survival of Foxp3<sup>Cre</sup> and Ctnnb<sup>ΔEx3;Cre</sup> mice. **e**, Percentage of T<sub>reg</sub> cells within CD4<sup>+</sup> T cells and cell numbers of T<sub>reg</sub> cells in the spleen (top) and thymus (bottom) from Foxp3<sup>Cre</sup> and Ctnnb<sup>ΔEx3;Cre</sup> mice at 3 weeks and 5 weeks of age (n=2-4 mice; each plotted point represents one mouse). **f**, Flow cytometric analysis of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in peripheral lymph nodes and spleens from Foxp3<sup>Cre</sup> and Ctnnb<sup>ΔEx3;Cre</sup> mice at 3 weeks of age. Cell counts and percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells among CD3<sup>+</sup> T cells from the spleens are shown at the bottom (n=3 or 4 mice; each plotted point represents one mouse). **P < 0.01, ****P < 0.0001 (two-way ANOVA with Sidak’s multiple comparisons test). Data are represented as mean ± s.d. Data are representative of three experiments.
A high-salt environment activates the β-catenin–SGK1–Foxo axis and produces IFN-γ/IL-10 imbalance. The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)–AKT1–Foxo axis has previously been shown to play a pivotal role in inducing IFN-γ-producing dysfunctional Treg cells\(^1\). Furthermore, we observed that p-Foxo1/3a and SGK1 were upregulated in Ctnnb1\(^{Δ\text{Ctnnb1}}\), Foxp3\(^{\text{Ctnnb1}}\) Treg cells. To assess whether the SGK1–Foxo axis might be activated in human Treg subpopulations, we examined p-SGK1 and p-Foxo1 levels through flow cytometry and found that both were highly expressed in the IFN-γ-producing Treg population ex vivo (Supplementary Fig. 4a). Additionally, we demonstrated the direct interaction between β-catenin and Foxo1 in IFN-γ-producing human Treg cells by using PLA (Supplementary Fig. 4b). These findings prompted us to hypothesize that activation of β-catenin might be involved in high-salt-induced IFN-γ production as an upstream regulator of the SGK1–Foxo axis. Higher expression of active β-catenin, p-SGK1, and p-Foxo1 was observed specifically in the IFN-γ-producing human Treg subset under high-salt conditions (Fig. 6a) but not in the IL-10-producing subset (Supplementary Fig. 4c). β-catenin–target genes (AXIN2 and TCF7) and TCF-1 protein were also upregulated in human Treg cells treated with increased salt concentrations (Fig. 6b and Supplementary Fig. 4d). Additional salt treatment skewed the IL-12-induced, T\(_\gamma\)1-like Treg cells to produce more IFN-γ and less IL-10, thus suggesting that the high-salt environment might exacerbate the IFN-γ-skewing pathogenic Treg signature that resembles the MS-Treg phenotype (Fig. 6c and Supplementary Fig. 4e). To determine whether β-catenin activation is necessary for IFN-γ induction under high-salt conditions, we pharmacologically blocked β-catenin signaling with two different Wnt–β-catenin signaling inhibitors: PKF and IWR-1. Each inhibitor significantly downregulated high-salt-induced IFN-γ expression in human Treg cells (Fig. 6d and Supplementary Fig. 4f). Similar results were also observed after genetic knockdown of CTNNB1 by shRNA (Fig. 6e–g). Because SGK1 is a target of β-catenin...
**Fig. 6 | A high-salt environment induces β-catenin-signaling activation and IFN-γ/IL-10 cytokine imbalance.** **a,** Flow cytometric analysis of active β-catenin, p-SGK1 (Thr256), and p-Foxo1 (Ser256) expression in human IFN-γ-producing T<sub>reg</sub> cells. T<sub>reg</sub> cells were stimulated with anti-CD3 and anti-CD28 in the presence (NaCl) or absence (control) of an additional 40 mM NaCl for 96 h, then subjected to 4 h PMA plus iomomycin stimulation (active β-catenin, n = 18 subjects; p-SGK1, n = 13 subjects; p-Foxo1, n = 10 subjects). P values were calculated by two-sided Student’s t test. **b,** mRNA expression kinetics for Wnt-β-catenin-target genes (AXIN2 and TCF7) from nine time points; each plotted point represents the average of four different experiments. *P < 0.05, **P < 0.001 (two-way ANOVA with Sidak’s multiple comparisons test). Data are represented as mean ± s.e.m. **c,** IFNG mRNA expression in human T<sub>reg</sub> cells cultured in T<sub>0</sub> or T<sub>1</sub> conditions in the presence (NaCl) or absence (control) of an additional 40 mM NaCl for 96 h (n = 19 subjects). *P < 0.05, **P < 0.01, ***P < 0.001 (two-way ANOVA with Tukey’s multiple comparisons test). **d,** IFNG mRNA expression in human T<sub>reg</sub> cells stimulated in the presence (NaCl) or absence (control) of an additional 40 mM NaCl with or without Wnt-β-catenin-signaling inhibitor (PKF) or IWR-1 (IWR) for 96 h (n = 7–10 subjects; each plotted point represents one subject). *P < 0.05 (one-way ANOVA with Tukey’s multiple comparisons test). **e,** Representative flow cytometric analysis of IFN-γ and IL-10 production in human T<sub>reg</sub> cells transduced with a nontargeting shRNA or a CTNNB1 shRNA and cultured in normal medium (control) or medium supplemented with an additional 40 mM NaCl (NaCl) for 96 h. Data are representative of three experiments. **f,** IFNG and IL10 mRNA expression on T<sub>reg</sub> cells (f) and frequency of IFN-γ- and IL-10-producing T<sub>reg</sub> cells relative to control/scrambled shRNA conditions (g). T<sub>reg</sub> cells were treated as in e (f, n = 9 subjects; g, n = 8 subjects). NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA with Tukey’s multiple comparisons test). Data are represented as mean ± s.d.
Next, we measured the production of IFN-γ Ser473 was increased in IFN-γ-expressing Jurkat T cells (Supplementary Fig. 5c). These data, along with the β-catenin-knockout CRISPR–Cas9 technology and found that high-salt-induced SGK1 production, in agreement with our Treg data (Supplementary Fig. 5b). In addition, we generated β-catenin-depleted Jurkat T cells by using CRISPR–Cas9 technology and found that high-salt-induced SGK1 and Foxo1 phosphorylation were attenuated in β-catenin-knockout Jurkat T cells (Supplementary Fig. 5c). These data, along with the evidence from nonimmune cells, support our hypothesis that the β-catenin–SGK1–Foxo1 axis is activated by high-salt stimulation.

We next explored the molecular mechanisms underlying high-salt-induced β-catenin activation. First, we examined whether Wnt ligands might play a role in this aberrant activation of β-catenin signaling. We used the fragment crystallizable region fused to the cysteine-rich domain of Frizzled-8 protein (Fzd8-FC), a known scavenger of Wnt ligands, to inhibit the effects of Wnt ligands on Treg cells. Activation of β-catenin, as assessed by levels of active β-catenin or production of IFN-γ and IL-10, was not affected by Fzd8-FC treatment in control or high-salt conditions, thus suggesting a dispensable role of Wnt ligands in high-salt-induced activation of β-catenin (Supplementary Fig. 6a,b). Although a salinity stress sensor has not been fully described in mammalian cells, a number of pathways contributing to the salt-stress response have been identified. Within these pathways, we focused on AKT kinase, because it is well known to regulate β-catenin signaling via direct phosphorylation of β-catenin or indirectly through GSK3β, a negative regulator of β-catenin. Indeed, the PI3K–AKT pathway was highly enriched in the IFN-γ-producing Treg subset, and AKT phosphorylation at Ser473 was increased in IFN-γ-producing Treg cells (Supplementary Fig. 6c,d). We then investigated whether β-catenin could be directly activated by AKT by examining AKT-specific phosphorylation of β-catenin (Ser522), which stabilizes β-catenin. The phosphorylation of β-catenin (Ser522) was increased in a high-salt environment, and this effect was reversed by the AKT inhibitor MK2206, thus indicating that activation of AKT is responsible for stabilizing β-catenin during high-salt stimulation (Supplementary Fig. 6e). Furthermore, we demonstrated that phosphorylation of GSK3β at Ser9, an important site of phosphorylation by AKT, was increased by high-salt stimulation, whereas the amounts of p-AKT and p-GSK3β were not affected by silencing β-catenin (Supplementary Fig. 6f), suggesting that both of these proteins act upstream of β-catenin. These data indicate that AKT regulates β-catenin activation through both direct and indirect mechanisms under high-salt conditions.

A high-salt-induced PTGER2–β-catenin loop leads to imbalance between IFN-γ and IL-10. Both IFN-γ and IL-10 were upregulated in IL-12-induced Treg cells in a β-catenin-dependent manner (Fig. 3f–i). However, IL-10 expression was significantly suppressed by high-salt treatment, in contrast to IFN-γ expression. In fact, the β-catenin–SGK1–Foxo axis was not activated in IL-10SP after high-salt treatment (Supplementary Fig. 4c). Additionally, the effect of high salt on IL-10 production could not be explained by activated β-catenin signaling (Fig. 6c,f and Supplementary Fig. 4f). Then, we hypothesized the existence of a factor that was uniquely induced in the high-salt environment but not in IL-12-driven Treg conditions, thus resulting in IL-10 inhibition. We compared the gene expression profiles of Treg cells between control medium and IL-12-supplemented medium (Treg), and also between control medium and NaCl-supplemented medium. Among the group of differentially expressed genes in each comparison, we identified six genes that were upregulated in high-salt conditions but downregulated in Treg conditions, and four genes that were regulated in the opposite direction, which may potentially account for the high-salt-induced IFN-γ/IL-10 imbalance (Fig. 7a).

PTGER2 regulates the production of cytokines in a context-dependent manner35–37, and the action of PTGER2 on cytokine production, especially on IFN-γ production in T cells, is affected by the strength of PI3K–AKT signaling44. Because we have observed a role of PTGER2 in promoting the pathogenic phenotype by modulating IFN-γ/IL-10 balance in T17 cells38, and high-salt treatment induces a pathogenic Treg signature, we hypothesized that PTGER2 might regulate the IFN-γ/IL-10 balance in salt-stimulated Treg cells. Indeed, PTGER2 was upregulated after high-salt treatment in human Treg cells and T17 cells (Fig. 7b) and was more highly expressed in IFN-γSP than IL10SP (Supplementary Fig. 7a).

Given the reported evidence of a positive relationship between β-catenin signaling and PTGER2 (refs 38,43), we investigated whether β-catenin and PTGER2 might form an autoregulatory loop during chronic high-salt exposure. We used Jurkat T cells and demonstrated that high-salt-induced PTGER2 was suppressed by genetic deletion of CTNNB1 (Supplementary Fig. 7b) and that PTGER2 knockout partially ameliorated high-salt-induced β-catenin activation (Supplementary Fig. 7c). These results suggest the presence of a β-catenin–PTGER2 feed-forward loop under high-salt conditions. PTGER2 silencing abolished the high-salt-induced IFN-γ/IL-10 imbalance in human Treg cells and eliminated the high-salt-induced activation of β-catenin in IFN-γSP, but it did not affect the level of active β-catenin in IL10SP (Fig. 7c–e), thus suggesting that high-salt-induced IFN-γ depends on the PTGER2–β-catenin loop, but IL-10 suppression by high salt is dependent on PTGER2 but not β-catenin.

We further investigated whether the high-salt-induced PTGER2–β-catenin loop could be amplified in cells in which AKT is activated, such as in IFN-γ-producing Treg cells, but not in cells with lower AKT activity, such as IL-10-producing Treg cells. We then tested the effect of modulating AKT signaling on the production of IFN-γ and IL-10 in Treg cells under high-salt conditions via increasing CD28 co-stimulation. High-salt-induced IFN-γ production was boosted by strengthening AKT signaling with higher CD28 co-stimulation (Supplementary Fig. 7d). In contrast, high-salt-induced IL-10 inhibition was not altered. Together, these data indicate that high salt induces a positive feedback loop between β-catenin and PTGER2 in conjunction with activated AKT status, thereby resulting in amplification of IFN-γ production in Treg cells.

Stabilized β-catenin is observed in Treg cells from mice fed a high-salt diet and in patients with MS. To examine whether β-catenin is stabilized under high-salt conditions in vivo, we fed wild-type mice either a normal-salt diet, containing 0.4% of NaCl, or an HSD, containing 4% NaCl, and assayed β-catenin expression on Treg cells. We found that β-catenin and phosphorylated Foxo1/3a/4, as assayed with a monoclonal antibody for detecting phosphorylation sites on Foxo1 (Thr24), Foxo3a (Thr32), and Foxo4 (Thr28), were elevated in Treg cells from HSD mice (Fig. 8a). Next, we determined whether β-catenin might be more stabilized in MS-Treg cells than in Treg cells from healthy subjects. The level of active β-catenin in IFN-γ-producing Treg cells from healthy subjects was significantly lower than that in Treg cells from mice fed a high-salt diet.
or IFNG expression in MS-Treg cells, we assessed the expression of these factors in Treg cells from healthy subjects and patients with MS. Notably, higher expression of PTGER2 and active β-catenin level correlated with IFNG expression in MS-Treg cells but not in Treg cells from healthy subjects (Fig. 8d,e). These findings provide in vivo evidence supporting our hypothesis that the PTGER2-β-catenin loop plays an important role in the salt-induced malfunction of Treg cells and links this salt signature to the pathogenic profile of MS-Treg cells (Supplementary Fig. 8).

Discussion

Loss of Foxp3+ Treg function is associated with a number of autoimmune diseases, and previous studies have linked environmental factors to autoimmunity through affecting Treg cell homeostasis. Here, using both mouse and human systems, we extended this concept and also described a novel molecular mechanism. Our results demonstrate a novel role of β-catenin as a regulatory molecule for Treg functional plasticity and also provide molecular mechanisms that link a high-salt environment to autoimmune disease.

Our transcriptional profiling of human Treg subsets on the basis of IFNG and IL-10 production provides new insights into Treg heterogeneity. We identified β-catenin as a key regulator and demonstrated its role in skewing Treg into a dysfunctional state in human Treg cells and in mouse models. Although several studies have demonstrated the role of β-catenin in Treg cells, how β-catenin contributes to Treg function and the effector-cytokine signature remains unknown. One study has demonstrated that β-catenin is an anti-inflammatory factor in the context of generating long-lived suppressive Treg cells via antiapoptotic-gene induction, and two previous studies have shown that activation of β-catenin provokes Treg dysfunction, thereby leading to exaggerated colitis in a mouse model.

We showed that Treg-specific stabilization of β-catenin resulted in a loss of suppressive properties of
The incidence of autoimmune diseases has been increasing in the past half century, a phenomenon that cannot be explained by genetic adaptation. Thus, there is great interest in studying the interplay between genetic risk and environmental triggers. Among several environmental triggers, a high-salt diet might increase the incidence of autoimmune diseases, although this possibility requires further epidemiological investigation. Previous studies have shown that higher salt concentrations affect T<sub>reg</sub> cell development and T<sub>reg</sub> stability, as manifested by aberrant IFN-γ production. Notably, our observation of an IFN-γ/IL-10 imbalance not only in MS-T<sub>reg</sub> cells but also under high-salt conditions suggested that the salt-induced signature may overlap with the MS pathogenic profile. The importance of IFN-γ/IL-10 balance in the context of salt-induced immune alteration is supported by a previous study showing that elevated sodium content in the colon tissue in HSD mice results in excessive inflammation in models of inflammatory bowel disease. Interestingly, β-catenin signaling was activated in both T<sub>reg</sub> and T<sub>17</sub> cells under stimulation with high salt (data not shown). Furthermore, we demonstrated that PTGER2 accounted for the high-salt-induced IFN-γ/IL-10 imbalance in T<sub>reg</sub> cells by creating a positive feed-forward loop with β-catenin. Given that T<sub>reg</sub> cells can produce PGE2 (ref. 50) and that PGE2 is enriched in experimental autoimmune encephalomyelitis lesions, PTGER2–PTGER2 signaling may be amplified in T<sub>reg</sub> cells under high-salt conditions and also in MS lesions. However, the role of PGE2 in experimental autoimmune encephalomyelitis and MS remains unclear, and further investigation is needed.

In summary, we determined genome-wide transcriptomic profiles of human ex vivo T<sub>reg</sub> subpopulations, which unveil the heterogeneity of T<sub>reg</sub> cells in terms of IFN-γ and IL-10 production. Aberrant β-catenin activation modulates T<sub>reg</sub> cytokine plasticity and integrity in both human and mouse T<sub>reg</sub> cells. Under a high-salt environment, this effect occurs in conjunction with upregulation of PTGER2, thereby establishing a feed-forward loop between PTGER2 and β-catenin. Notably, T<sub>reg</sub> cells from patients with MS display positive correlations among IFN-γ production, PTGER2 expression, and active β-catenin levels, which are not observed in T<sub>reg</sub> cells from healthy subjects. Together, our results in humans with autoimmune disease, as confirmed in mouse models, indicate that the PTGER2–β-catenin axis serves as a bridge between environmental factors and autoimmune disease by modulating T<sub>reg</sub> properties.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41590-018-0236-6.

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Author contributions

T.S., D.M.R., and C.M.U. performed in vitro experiments with the help of M.R.L. and M.D.-V.; T.S. and A.T.N. provided supervision in overall study. M.R.L., M.D.-V., and M.D.-M. provided supervision in the overall study.

Competing interests

D.A.H. has consulted for the following companies: Bayer Pharmaceuticals, Biohaven Pharmaceuticals, Bristol Myers Squibb, Compass Therapeutics, Eisai Pharmaceuticals, EMD Serono, Genentech, Juno Therapeutics, McKinsey & Co., MedImmune/AstraZeneca, Mylan Pharmaceuticals, Neurophage Pharmaceuticals, NKT Therapeutics, Novartis Pharmaceuticals, Procella Biosciences, Questcor Pharmaceuticals, Roche, Sage Therapeutics, Sanofi Genzyme, Torny Industries, and Versant Venture.

Additional information

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Methods

Study subjects. Peripheral blood was drawn from healthy individuals and patients with MS after informed consent was obtained and approval was granted by the Institutional Review Board at Yale University. The patients were diagnosed with either clinically isolated syndrome or relapsing-remitting MS according 2010 MacDonald Criteria and were not treated with any immunomodulatory therapy at the time of the blood draw. All experiments conformed to the principles of the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. The clinical characteristics of evaluated patients are listed in Supplementary Table 2.

Mice. C57BL/6j mice were purchased from the Jackson Laboratory or CLEA Japan. Fic mice and Cnmh1(+/-) mice were as previously described, and mice backcrossed into the C57BL/6j strain were used. Cnmh1(+/-), Foxp3-cre mice were studied at 3–5 weeks of age. For HSD experiments, six-week-old male wild-type mice with a CD4(+/+)B2M(−/−) transgene were used. CD4(+/+)B2M(−/−)T cells were isolated with a CD4 T cell isolation kit (Stemcell), and CD4+CD25+CD127−/−/CD45R0+ Treg cells were sorted on a FACSaria (BD Biosciences) cell sorter. Treg cells were cultured in RPMI 1640 medium supplemented with 5% human serum, 2 mM L-glutamine, 5 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 mM sodium pyruvate, 0.05 mM nonessential amino acids, and 5% human AB serum (Gemini Bio-Products). 96-well round-bottom plates (Corning) were precoated with anti-human CD3 (UCHT1) (1–2 μg/ml) (BD Bioscience) and used for Treg in vitro culture with soluble anti-human CD28 (28.2) (1–5 μg/ml) (BD Bioscience) and human IL-2 (50 U/ml). Human IL-2 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). T1+ Treg cells were induced with human recombinant IL-2 (10 ng/ml) and the Lenti/β-catenin inhibitor PKEF151-584 (Tocris) was used at 200 nM, and 1W-1 (Tocris) was used at 20 μM. The SGK1 inhibitor GSK650394 (Tocris) was used at 10 μM. The AKT inhibitor MK2206 (Tocris) was used at 5 μM. Fizzled 8 FC chimerica protein (R&D) was used at 500 ng/ml.

Suppression assays. CD4+CD25− T responder cells and CD4+CD25+ T responder cells were isolated from the spleen and lymph nodes of Foxp3+ mice or Cnmh1(+/-) mice with a CD4 T cell isolation kit (Stemcell), and CD4+CD25+CD127−/−/CD45R0+ Treg cells were sorted on a FACSaria (BD Biosciences) cell sorter. T responder cells were cultured with CD3e and then cocultured with Treg cells (5 x 10^5) at a 1:1 ratio in RPMI 1640 medium supplemented with 10% FBS (HyClone), 50 μM 2-mercaptoethanol (Sigma-Aldrich), 1 x GlutaMAX, 50 U/ml penicillin, and 100 μg/ml streptomycin with Dynabeads Mouse T-Activator CD3/CD28 at 2:1 bead/cell ratio. The proliferation of T responder cells was determined at day 4 through flow cytometry on a FACSVerse instrument (BD Bioscience).

Quantitative PCR. Total RNA was extracted with an RNeasy Micro Kit (Qiagen) or ZR 96 Quick RNA kit (Zymo Research), according to the manufacturer’s instructions. RNA was treated with DNase and reverse transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems) or SuperScript IV VILO Master Mix (Invitrogen). cDNAs were amplified with TaqMan probes (TaqMan Gene Expression Arrays) and TaqMan Fast Advanced Master Mix on a StepOne Real-Time PCR System (Applied Biosystems). cDNAs were generated directly from reverse-transcribed cDNA. Barcoded libraries were generated with a SMART-Seq v4 Ultra Low Input RNA Kit for sequencing (Takara/Clontech). Barcoded libraries were generated with a Nextera XT DNA Library Preparation kit (Illumina) and sequenced with a 2 x 100 bp paired-end protocol on the HiSeq 2000 Sequencing System (Illumina).

RNA-seq data analysis. RNA-seq data was generated from healthy individuals and patients with MS after informed consent was obtained and approval was granted by the Institutional Review Board at Yale University. The patients were diagnosed with either clinically isolated syndrome or relapsing-remitting MS according 2010 MacDonald Criteria and were not treated with any immunomodulatory therapy at the time of the blood draw. All experiments conformed to the principles of the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. The clinical characteristics of evaluated patients are listed in Supplementary Table 2.

Human T cell isolation and culture. Peripheral blood mononuclear cells were isolated from donors with Ficol-Paque PLUS (GE Healthcare) or Lymphoprep (Stemcell) gradient centrifugation. Total CD4+ T cells were isolated through negative magnetic selection with a CD4 T cell isolation kit (Stemcell), and CD4+CD25+CD127−/−/CD45R0+ Treg cells were sorted on a FACSaria (BD Biosciences) cell sorter. Treg cells were cultured in RPMI 1640 medium supplemented with 5% human serum, 2 mM L-glutamine, 5 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 mM sodium pyruvate, 0.05 mM nonessential amino acids, and 5% human AB serum (Gemini Bio-Products). 96-well round-bottom plates (Corning) were precoated with anti-human CD3 (UCHT1) (1–2 μg/ml) (BD Bioscience) and used for Treg in vitro culture with soluble anti-human CD28 (28.2) (1–5 μg/ml) (BD Bioscience) and human IL-2 (50 U/ml). Human IL-2 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). T1+ Treg cells were induced with human recombinant IL-2 (12 ng/ml) and the Lenti/β-catenin inhibitor PKEF151-584 (Tocris) was used at 200 nM, and 1W-1 (Tocris) was used at 20 μM. The SGK1 inhibitor GSK650394 (Tocris) was used at 10 μM. The AKT inhibitor MK2206 (Tocris) was used at 5 μM. Fizzled 8 FC chimera protein (R&D) was used at 500 ng/ml.

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For in vitro T1+ Treg and high-salt T1+ T responder cells, differently expressed genes (fold change > 2, P value < 0.05) were used.

Microarray analysis. For the oligo DNA microarray analysis, total RNA samples were isolated from sorted CD4+CFSE+Foxp3+ cells of Cnmh1(+/-) mice. CD4+CFSE+ mice. Microarray analysis was performed with a 3D-Genome Oligo chip 24k (Toray Industries). Total RNA was labeled with Cy5 with an Amino Alhy MessageAMP II aRNA Amplification Kit (Applied Biosystems). The Cy5-labeled aRNA pools were mixed with hybridization buffer and hybridized for 16h. The hybridization signals were obtained with a 3D-Genome Scanner and processed in 3D-Genome Extraction software (Ray Industries). Detected signals for each gene were normalized with the global normalization method (the median of the detected signal intensity was adjusted to 25).

Histology. Mouse tissues were fixed in Ufix (Sakura Finetek Japan) and embedded in paraffin. 6-μm tissue sections were stained with hematoxylin and eosin.

Lentiviral transduction for shRNA gene silencing and CRISPR-Cas9-mediated gene deletion. Lentiviral plasmids encoding shRNAs were obtained from Sigma-Aldrich, and all-in-one vectors carrying CTNBN1 shRNA/Cas9 with GFP reporter were obtained from Applied Biological Materials. Each plasmid was transformed into One Shot Stbl3 chemically competent cells (Invitrogen) and purified with a ZymoPURE plasmid Maxiprep kit (Zymo research). Lentiviral pseudovirions were obtained after plasmid transfection of 293FT cells with LentiFectamine 2000 (Invitrogen). The lentivirus-containing medium was harvested 48 or 72h after transfection and concentration 50-50 times with Lenti-X concentrator (Takara/Clontech). Sorted T cells were cultured with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml) for 24h and transduced with lentiviral particles by spinfection (1,000g for 90 min at 32°C) in the presence of polybrein (5μg/ml) on plates coated with retronectin (50μg/ml) (Takara/Clontech) and anti-CD3 (1–2 μg/ml). Human Jurkat T cells were directly transduced with lentiviral particles by spinfection. Five days after transduction, cells were sorted on the basis of...
expression of GFP. GFP-expressing human Jurkat T cells were further purified by flow cytometry at least three times before being used for experiments.

Proximity ligation assay (PLA). PLA was performed with Duolink In situ Detection Reagents Orange (Sigma) according to the manufacturer’s recommendations with minor modifications. T<sub>reg</sub> cells were cultured for 4 d and harvested, and cells were fixed with 2% paraformaldehyde for 10 min at RT. Fixed cells were incubated in Foxp3 Fix/Perm buffer set for 30 min at 4°C, then stained with mouse anti-β-catenin (14/β-catenin) (BD Bioscience) and rabbit anti-Foxo1 (C29H4) (CST) for 1 h at room temperature in Foxp3 staining buffer. Cells were washed and stained in Foxp3 staining buffer with the secondary mouse PLUS and rabbit MINUS antibodies for 30 min at RT. Cells were washed in TBS (0.01 M Tris and 0.15 M NaCl) with 0.5% BSA, and the ligation reaction was performed at 37°C for 30 min and was followed by the amplification reaction at 37°C for 100 min. Cells were washed in TBS (0.2 M Tris and 0.1 M NaCl) with 0.5% BSA and stained with anti-Foxp3 (PCH101) (eBioscience) or anti-IFN-γ (B27) (BD Bioscience) for 30 min at 4°C. Cells were analyzed with a 60× or 100× objective on a Leica DM6000 CS confocal microscope.

Statistical analysis. All statistical analyses were performed in GraphPad Prism 6 (GraphPad Software). Detailed information about statistical analysis, including tests and values used, is provided in the figure legends. Values of $P < 0.05$ were considered significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
RNA-seq data are available in the GEO repository under accession code GSE116283. The remaining data that support the findings of this study are available from the corresponding authors upon reasonable request.
Corresponding author(s): Tomokazu Sumida

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- n/a
- Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | Flow cytometry data were collected using BD FACSDiva software (v8). |
|-----------------|------------------------------------------------------------------|
| Data analysis   | Partek flow (v6.0), GraphPad Prizm 6, Image J (1.51), FlowJo (v10), STAR (2.5.0e) |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Provide your data availability statement here.
Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical methods were used to predetermine sample size estimates. Sample size was determined based on the experimental results that we obtained from preliminary experiments and published papers. |
|-------------|------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded from the analysis. |
| Replication | All findings shown have been reproduced in at least two independent experiments. |
| Randomization | Age-, sex-, and body weight- matched C57BL/6J wild-type mice were randomly allocated to high salt diet or normal diet. Age- and sex-matched Foxp3-IRES-Cre/wild-type mice (Foxp3Cre) and Foxp3-IRES-Cre/Ctnnb1ΔEx3 (Ctnnb1ΔEx3/Foxp3Cre) mice were analyzed. |
| Blinding | Blinding was not relevant to the study. |

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| Involved in the study | Involved in the study |
| ☑ ☐ Unique biological materials | ☑ ☐ ChIP-seq |
| ☑ ☐ Antibodies | ☐ ☐ Flow cytometry |
| ☑ ☐ Eukaryotic cell lines | ☑ MRI-based neuroimaging |
| ☑ Palaeontology | ☐ |
| ☑ ☐ Animals and other organisms | ☐ |
| ☑ Human research participants | ☐ |

Antibodies

| Antibodies used | All of the antibodies are commercially available. The commercial source and the clone for each specific antibody are listed in the Supplementary Table 3. |
| Validation | All of the antibodies have been validated by the vendors or other researchers. |

Eukaryotic cell lines

| Policy information about cell lines |
|-----------------------------------|
| Cell line source(s) | Human Jurkat T cells were purchased from ATCC. |
| Authentication | No further authentication has been performed. |
| Mycoplasma contamination | Not tested. |
| Commonly misidentified lines (See ICLAC register) | No commonly misidentified cell lines were used. |
Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

CS7BL/6J mice were purchased from the Jackson Laboratory or CLEA Japan. Foxp3-IRES-Cre (Foxp3Cre) mice were obtained from Dr. Shimon Sakaguchi (Osaka University). Ctnnb1ΔEx3 mice were crossed to Foxp3Cre mice to generate Ctnnb1ΔEx3/Foxp3Cre mice. All experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments and strictly adhered to the guidelines for animal experiments of the University of Tokyo.

All mice were on C57BL/6J background and all of experiments were done in male mice. All mice were bred under specific-pathogen-free (SPF) conditions with a 12-hour light/dark cycle in a temperature-controlled and humidity-controlled environment, and, allowed ad libitum access to normal laboratory chow. For high salt diet (HSD) experiments, six-week-old male wild type mice were fed with normal chow (control group) or sodium-rich chow containing 4% NaCl (Research Diets; HSD group) with normal tap water for 3 weeks.

Wild animals

Not applicable. This study didn’t involve wild animals.

Field-collected samples

Not applicable. This study didn’t involve samples collected from the field.

Human research participants

Policy information about studies involving human research participants

Population characteristics

MS patients’ characteristics are listed as below and also in Supplementary table 2.

| Patient # | Age | Sex | Ethnicity | Duration of Disease | Treatment | EDSS score |
|-----------|-----|-----|-----------|---------------------|-----------|------------|
| #1        | 57  | M   | African american | 16 yrs | untreated | 4.5        |
| #2        | 46  | F   | Caucasian/Non-Hispanic | 7 yrs | untreated | 2.5        |
| #3        | 36  | F   | Caucasian/Hispanic | 1 mo | untreated | 3.5        |
| #4        | 29  | F   | Caucasian/Non-Hispanic | 3 yrs | untreated | 2          |
| #5        | 48  | F   | Caucasian/Non-Hispanic | 18 yrs | untreated | 6.5        |
| #6        | 41  | M   | Caucasian/Non-Hispanic | 10 yrs | untreated | 1.5        |
| #7        | 31  | M   | Caucasian/Non-Hispanic | 2 mo | untreated | 6          |
| #8        | 41  | F   | Caucasian/Non-Hispanic | 3 mo | untreated | 3          |
| #9        | 42  | M   | Caucasian/Non-Hispanic | <1 mo | untreated | 1.5        |
| #10       | 34  | F   | Caucasian/Non-Hispanic | 4 mo | untreated | 4          |
| #11       | 38  | M   | Caucasian/Non-Hispanic | <1 mo | untreated | 0          |
| #12       | 43  | F   | African american | 1 yr untreated | 1 |
| #13       | 53  | F   | Caucasian/Non-Hispanic | 28 yrs | untreated | 4.5        |
| #14       | 32  | F   | Caucasian/Non-Hispanic | 1 mo | untreated | 1.5        |
| #15       | 32  | F   | Caucasian/Non-Hispanic | 3 yrs | untreated | not assessed |
| #16       | 28  | F   | Caucasian/Non-Hispanic | 2 yrs | untreated | 1          |
| #17       | 50  | F   | Caucasian/Non-Hispanic | <1 mo | untreated | 3.5        |
| #18       | 43  | F   | Caucasian/Non-Hispanic | 5 mo | untreated | 2.5        |
| #19       | 23  | F   | Caucasian/Non-Hispanic | <1 mo | untreated | 1          |
| #20       | 58  | F   | Caucasian/Non-Hispanic | 13 yrs | untreated | 6          |
| #21       | 34  | F   | Caucasian/Non-Hispanic | 1 mo | untreated | 1.5        |
| #22       | 30  | F   | Caucasian/Non-Hispanic | 12 yrs | untreated | 2          |
| #23       | 27  | F   | Caucasian/Non-Hispanic | <1 mo | untreated | 0          |
| #24       | 61  | F   | Caucasian/Non-Hispanic | 20 yrs | untreated | 4          |
| #25       | 35  | F   | Caucasian/Hispanic | 1 yr | untreated | 2          |
| #26       | 33  | F   | Caucasian/Non-Hispanic | 1 mo | untreated | 1.5        |
| #27       | 44  | F   | Caucasian/Non-Hispanic | 8 yrs | untreated | 2.5        |

Recruitment

The patients recruited to this study were diagnosed either CIS or RRMS by 2010 MacDonald Criteria and were not treated with any immunomodulatory medicine at the blood draw.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Single-cell suspensions were prepared from human PBMCs or mouse tissues. For human Treg isolation, total CD4+ T cells were isolated by negative selection using CD4 T cell isolation kit (Stemcell). Cells were stained with fixable viability dye in PBS for 10
min at RT, followed by staining with surface antibodies in 2% FBS + 1mM EDTA containing PBS for 30 min at 4°C. For intracellular staining, cells were fixed and permeabilized with the Foxp3 Fix/Perm buffer set (eBioscience) for 1 hr at 4°C, followed by staining with intracellular antibodies. For cytokine staining, cells were stimulated with PMA/ionomycin in the presence of GolgiPlug (BD Bioscience) for 4 hr at 37°C, followed by intracellular staining method.

| Instrument                  | BD FACSVerse or LSR Fortessa flow cytometer (BD Bioscience) |
|-----------------------------|-------------------------------------------------------------|
| Software                    | FlowJo software (Treestar)                                  |
| Cell population abundance   | The purity of each population were confirmed by flow cytometry and in each case was above 85% purity. |
| Gating strategy             | At first, cells were gated on FSC-A/SSC-A on the location known to contain T cells. Doublet cells were excluded by gating on FSC-H/FSC-W and SSC-H/SSC-W. Fixable viability dye negative gate was used to select live cells. CD4+CD25hiCD127low-negCD45RO+ cells were sorted as human memory Tregs, CD4+CD25negCD127+CD45RO+ cells were sorted as human memory T effector cells (Teff), and CD3+CD4+CD25hi cells were sorted as mice Tregs. Flow cytometry analysis for cytokines and intracellular molecules were done on Foxp3 positive Tregs. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.