Control of peripheral tolerance by regulatory T cell-intrinsic Notch signaling

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Abstract

Notch receptors direct the differentiation of T helper (T<sub>H</sub>) cell subsets, but their influence on regulatory T (T<sub>reg</sub>) cell responses is obscure. We here report that lineage-specific deletion of components of the Notch pathway enhanced T<sub>reg</sub> cell-mediated suppression of T<sub>H</sub>1 responses, and protected against their T<sub>H</sub>1 skewing and apoptosis. Expression in T<sub>reg</sub> cells of gain of function transgene encoding Notch1 intracellular domain resulted in lymphoproliferation, exacerbated T<sub>H</sub>1 responses and autoimmunity. Cell-intrinsic canonical Notch signaling impaired T<sub>reg</sub> cell fitness, promoted the acquisition by T<sub>reg</sub> cells of a T<sub>H</sub>1 cell-like phenotype, whereas Rictor-dependent non-canonical Notch signaling activated the AKT-Foxo1 axis and impaired Foxp3 epigenetic stability. These findings establish a critical role for Notch signaling in controlling peripheral T<sub>reg</sub> cell functions.

Notch signaling serves pleiotropic roles in the immune system by influencing multiple lineage decisions of developing lymphoid and myeloid cells. In mammals, the Notch family is composed by 4 Notch receptors (Notch1–4) and 5 ligands (Delta-like1, 3, and 4 and Jagged1 and 2). After ligand-receptor interaction, the intracellular domain of the Notch receptor is cleaved, traffics to the nucleus and forms complexes with the DNA binding factor RBPJ and the transcriptional co-activators MAML1-3, promoting expression of target genes. In addition to this canonical pathway, cleaved intracellular domains of Notch receptors engage non-canonical signaling components, including the metabolic checkpoint kinase complex mTORC2 and its associated adaptor Rictor. Notch intracellular domain also interacts with components of the NF-κB, TGF-β and the hypoxia response pathways.
Notch signaling is activated at various stages of commitment and development of T cell lineages, such as commitment to the T cell versus the B cell lineage, αβ versus γδ T cell differentiation and CD4 T versus CD8 single-positive T cell differentiation, and during T cell-mediated immune responses, such as peripheral cytotoxic and helper T (T_H) cell differentiation and function. Pathogen-associated molecular patterns are known to promote expression of Notch ligand at the surface of antigen-presenting cells. Activation of naive CD8+ T cells requires binding of Delta-like1 on antigen-presenting cells by Notch1 or Notch2 leading to expression of Eomes, Gzmb, Ifng and Pfr1. In naive CD4+ T cells, Delta-like1 and 4 activate Notch signaling and Tbx21 transcription, encoding the T_H1 transcriptional regulator T-bet. During T_H2 differentiation, activation of Notch1 and 2 by Jagged1 and Jagged2 favor the expression of Gata3 and Il4.

Notch1 signaling has been described to be important in the differentiation of T_H17 and T_H9 subsets of helper T cells by promoting Rorc and Il9 expression, respectively. The role of Notch signaling in the regulatory T (T_reg) cell compartment remains controversial. In vitro, Jagged ligands and Notch 1 and Notch3 signaling seem to promote T_reg cell differentiation and survival. In contrast, several in vivo studies have demonstrated that blockade of the Notch pathway, in particular Notch1 and Notch2, promotes tolerance in murine models of graft versus host disease, in association with the expansion of T_reg cells. Studies have shown tolerogenic functions for antibodies to Notch1 in a humanized mouse model of vasculitis and in a murine model of aplastic anemia. In this study, we have employed T_reg cell lineage-specific genetic and functional approaches to identify a key role for the Notch pathway in destabilizing T_reg cells, promoting their apoptosis and inhibiting their function in the context of inflammation.

Results

Notch negatively regulates T_reg cell functions and homeostasis

To elucidate the role of the Notch pathway in peripheral tolerance, we examined the functional consequences of interrupting Notch receptor signaling in a T_reg cell-specific manner. To this end, we derived mice with a bacterial artificial chromosome (BAC) expressing an enhanced green fluorescent protein fused with the Cre recombinase under the control of Foxp3 promoter together with loxP-flanked Pofut1, encoding the enzyme protein O-fucosyltransferase 1 (called Foxp3EGFPCrePofut1Δ/Δ here; Supplementary Fig. 1a, b). The latter mediates fucosylation of Notch receptors, which is essential for receptor ligand interaction; its deficiency abrogates Notch signaling. T_reg cell-specific Pofut1 deficiency resulted in a decrease in peripheral CD3+ T cells and CD4+ T cell numbers by about 25% compared to Foxp3EGFPCre mice (Fig. 1a). It also resulted in a reciprocal increase in T_reg cell frequency, with decreased CD4+CD62LloCD44hi T effector memory and a relative increase in CD62LloCD44lo naïve T cells as compared to Foxp3EGFPCre mice (Fig. 1b-e).

Expression of IFN-γ in splenic CD4+ T cells was markedly decreased in Foxp3EGFPCrePofut1Δ/Δ as compared to Foxp3EGFPCre mice, whereas expression of IL-17 was unaffected (Fig. 1f, g). Similar results were obtained for the IFN-γ production by CD8+ T cells (Fig. 1h, i). Expression of several T_reg cell markers, including Foxp3, CD25, CTLA-4, Helios and neuropilin 1 (Nrp1) was increased in Pofut1-deficient compared to...
Foxp3EGFPCre T_reg cells (Fig. 1j). We examined the role of the canonical Notch signaling in T_reg cells by lineage-specific deletion of loxP-flanked Rbpj (Foxp3EGFPCreRbpjΔ/Δ, Supplementary Fig. 1a, b) 27. The key phenotypes of Foxp3EGFPCrePofut1Δ/Δ mice were recapitulated in Foxp3EGFPCreRbpjΔ/Δ mice (Fig. 1a–j), indicating that the canonical pathway is the primary mediator of Notch signaling in T_reg cells. Of the four Notch receptors, Notch1 was the most highly expressed in T_reg cells, followed by Notch 2, whereas Notch3 and Notch4 expression was negligible (Supplementary Fig. 1c, d). The phenotype of mice with Notch1-deficient T_reg cells, achieved by lineage-specific deletion of loxP-flanked Notch1 (Foxp3EGFPCreNotch1Δ/Δ, Supplementary Fig. 1a, b), approximated those of mice with Pofut- or RBPJ-deficient T_reg cells, indicating that Notch1 was the main receptor through which Notch signaling was triggered in T_reg cells (Fig. 1a–j) 28.

We also assessed the effect of loss of function mutations in genes encoding members of the Notch pathway on the generation of thymus-derived T_reg cells. We observed similar frequencies and numbers of Foxp3+ T_reg cells among the mature CD4 single positive (CD4SP) thymocyte compartment in Foxp3EGFPCre, Foxp3EGFPCrePofut1Δ/Δ, Foxp3EGFPCreRbpjΔ/Δ and Foxp3EGFPCreNotch1Δ/Δ mice (Supplementary Fig. 2a, b). We employed Foxp3YFPCre mice, which express a fusion of yellow fluorescent protein (YFP) and Cre recombinase in Treg cells under control of the endogenous Foxp3 locus 29. We found that the in vitro differentiation of naive CD4+ T cells from Foxp3YFPCreRbpjΔ/Δ or Foxp3EGFPCreNotch1Δ/Δ into induced T_reg (iT_reg) cells was similar to that of Foxp3YFPCre control cells (Supplementary Fig. 2c, d). These results indicate normal thymic development and peripheral differentiation of T_reg cell populations.

To further elucidate the cell-intrinsic impact of loss of function Notch mutations on T_reg cells, we took advantage of random X chromosome inactivation in females to analyze both central thymic and peripheral splenic T_reg cells in heterozygous Foxp3YFPcre/+RbpjΔ/Δ female mice. Compared to heterozygote Foxp3YFPcre/+ littermate control female mice, in which approximately 50% of T_reg cells within the thymus and spleen are YFP+, a higher proportion of YFP+ T_reg cells was observed in the periphery of Foxp3YFPcre/+RbpjΔ/Δ females (Supplementary Fig. 2e, f). YFP+ T_reg cells had higher expression of several T_reg cell markers, including CD25, Helios and Nrp1 compared to YFP− T_reg cells (Supplementary Fig. 2g, h). Overall, loss of function Notch signaling mutations exerted a positive, cell intrinsic effect on T_reg cell fitness and function both at steady state and in the context of inflammation.

**T_reg cell-specific loss of Notch function protects mice from GVHD**

We examined the suppressive capacities of Notch signaling-deficient T_reg cells in the context of a strong inflammatory response, using the model of major histocompatibility complex (MHC) class I&II disparate graft versus host disease (GVHD). Adoptive transfer of T cell-depleted bone marrow from Foxp3EGFPCre, Foxp3EGFPCreRbpjΔ/Δ or Foxp3EGFPCreNotch1Δ/Δ C57Bl/6 mice into BALB/c mice lead to recovery from the lethal irradiation (Fig. 2a). Co-transfer of total spleen cells from Foxp3EGFPCre mice induced lethal GVHD, associated with expansion of donor-derived (H-2Kb) IFN-γ-producing CD4+ and CD8+ T cells (Fig. 2a–f), while co-transfer of Foxp3EGFPCreRbpjΔ/Δ or

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Foxp3^EGFP^Cre^Notch1^Δ/Δ^ total spleen cells resulted in protection from lethal GVHD in recipient mice, with attenuated GVHD severity scores and decreased IFN-γ production by donor T cells (Fig. 2a–f). Moreover, transfer of total spleen cells from Foxp3^EGFP^Cre^RBPJ^Δ/Δ^ or Foxp3^EGFP^Cre^Notch1^Δ/Δ^ mice lead to increased frequency and number of donor T^reg^ cells, associated with decreased apoptosis, as assessed by Annexin V (AnnV) staining, and decreased IFN-γ production (Fig. 2c–d, g–j). In the context of exaggerated inflammation induced by GVHD, donor Foxp3^EGFP^Cre^T^reg^ cells exhibited higher expression of the cleaved intracellular domain of Notch1 (N1c), as compared to their CD4^+^Foxp3^−^ T^conv^ cell counterparts (Fig. 2h–i). Together, these results reveal a direct role for cell-intrinsic Notch signaling in destabilizing T^reg^ cells in the context of inflammation by promoting their apoptosis and IFN-γ production.

**T^reg^ cell-specific gain of Notch function disrupts peripheral tolerance**

To investigate the role of Notch1 signaling in T^reg^ cells, we generated Foxp3^EGFP^Cre^Rosa26^N1c/N1c^ mice, which constitutively express N1c in their T^reg^ cells (Supplementary Fig. 1e). We found increased expression of N1c in Foxp3^EGFP^Cre^Rosa26^N1c/N1c^ T^reg^ cells, associated with heightened expression of several Notch signaling target genes, including Hes1, Hey1, Heyl and Dtx1 (Supplementary Fig. 1f, g). In contrast to the mutations that resulted in loss of Notch function, constitutive expression of N1c in T^reg^ cells resulted in an autoimmune lymphoproliferative disease, whose manifestations included large vessel vasculitis and lymphocytic end organ infiltration in the Foxp3^EGFP^Cre^Rosa26^N1c/N1c^ mice (Fig. 3a, b). The CD4^+^CD62L^lo^CD44^hi^ T effector memory cell pool was expanded and the numbers of CD4^+^ and CD8^+^ T cells expressing IFN-γ were increased by 50%, while the frequency of T^reg^ cells was decreased by 45% (Fig. 3c, d). There was also dysregulation of the B cell compartment with significant increase of several circulating autoantibodies (to 18 out of 98 screened endogenous antigens) in Foxp3^EGFP^Cre^Rosa26^N1c/N1c^ mice as compared to Foxp3^EGFP^Cre^ mice (Fig. 3e).

Overexpression of N1c in T^reg^ cells dramatically worsened the incidence and severity of type I diabetes in the genetically susceptible NOD mice, particularly in normally resistant NOD males (Fig. 3f–h). Whereas N1c expression in T cell precursors precipitates the occurrence of T cell acute lymphocytic leukemia, no evidence of leukemia was found in Foxp3^EGFP^Cre^Rosa26^N1c/N1c^ mice up to 6 months of age (data not shown).

Analysis of Foxp3^EGFP^Cre^Rosa26^N1c/N1c^ mice revealed an age-dependent increase in the frequency of T^reg^ cells that do not express the Foxp3 BAC-driven EGFP-Cre transgene (data not shown). Accumulation of EGFP^−^ T^reg^ cells was observed during thymic development and reached up to 30% of the peripheral T^reg^ cell pool at 2 month of age (Supplementary Fig. 3a, b). EGFP^−^ T^reg^ cells were observed at very low frequencies in Foxp3^EGFP^Cre^, Pofut1, RBPJ or Notch1 mice (data not shown). Whereas the GFP^+^ Foxp3^EGFP^Cre^Rosa26^N1c/N1c^ T^reg^ cells expressed high amounts of N1c, consistent with the expression of the transgene from the Rosa26 locus, N1c expression in GFP^−^ Foxp3^EGFP^Cre^Rosa26^N1c/N1c^ T^reg^ cells was similar to that of control Foxp3^EGFP^Cre^ T^reg^ cells (Supplementary Fig. 3c), suggesting that the GFP^−^ T^reg^ cells never expressed the Foxp3^EGFP^Cre^ BAC gene and that transgenic expression of N1c induced a profound competitive disadvantage in T^reg^ cells. To overcome this 'escape' phenotype, we used...
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**Foxp3**YFP**Cre** mice that expressed a YFP-Cre fusion protein under control of the endogenous **Foxp3** locus. Unlike **Foxp3**EGFPCreRosa26 Nic/N1c mice, 95% of **Foxp3**YFP**Cre**Rosa26 Nic/N1c Treg cells expressed the **Foxp3**-driven Cre recombinase (Supplementary Fig. 3d, e). Analysis of heterozygous **Foxp3**YFP**Cre**WTRosa26 Nic/N1c females revealed marked skewing of X-chromosome utilization by Treg cells in the periphery in favor of expressing the **Foxp3**WT allele (Supplementary Fig. 3f, g). YFP+ **Foxp3**YFP**Cre**WT R)$^\text{Rosa26 Nic/N1c}$ Treg cells expressed significantly lower amounts of **Foxp3** compared to their YFP- Treg cell counterparts (Supplementary Fig. 3h, i). Double mutant **Foxp3**YFP**Cre**Rosa26 Nic/N1c male mice developed a more aggressive and accelerated autoimmune lymphoproliferative disease than **Foxp3**EGFPCreRosa26 Nic/N1c males, as revealed by tissue histology and immunological analyses (Supplementary Fig. 4).

Altogether, these data indicate a profoundly deleterious impact of N1c expression on Treg cell fitness.

Flow cytometric analysis of peripheral Treg cells in the **Foxp3**EGFPCreRosa26 Nic/N1c and **Foxp3**YFP**Cre**Rosa26 Nic/N1c mice revealed decreased expression of Treg cell markers, including Foxp3, CD25, CTLA-4, OX40, Nrp1 and Eos (Fig. 4a and Supplementary Fig. 5g, h), suggesting an inhibitory function of N1c in both thymus and periphery-derived Treg cells. Naive CD4+ cells among CD4 single positive thymocytes were found in **Foxp3**EGFPCre and **Foxp3**YFP**Cre**Rosa26 Nic/N1c mice (Supplementary Fig. 5c–d). In addition, the demethylation status of the conserved non-coding sequence 2 (CNS2) CpG elements in the **Foxp3** promoter was similar in thymic Treg cells of **Foxp3**EGFPCre and **Foxp3**YFP**Cre**Rosa26 Nic/N1c mice. In vitro, the differentiation of naive CD4+ Foxp3YFP**Cre**Rosa26 Nic/N1c T cells into induced Treg (iTreg) cells following stimulation with anti-CD3+CD28 mAb and TGF-β was decreased compared to those of **Foxp3**YFP**Cre** mice (Supplementary Fig. 5g, h), suggesting an inhibitory function of N1c in both thymus and periphery-derived Treg cells. Next, we investigated the effect of N1c on the ability of adoptively transferred Treg cells to suppress colitis in Rag1-deficient mice. Naïve CD45Rb+CD4+ Tconv cells were isolated from CD45.1+ **Foxp3**EGFPCre donor mice and transferred alone or in combination with Treg cells from CD45.2+ **Foxp3**EGFPCre or CD45.2+ **Foxp3**EGFPCreRosa26 Nic/N1c mice into T cell–deficient Rag1−/− host mice. We found that such co-transfer of Treg cells from CD45.2+ **Foxp3**EGFPCreRosa26 Nic/N1c mice failed to suppress the colitis induced by the transfer of naive CD45.1+ CD45Rb+CD4+ Tconv cells: the recipient Rag1−/− mice exhibited substantial weight loss and tissue inflammation, as well as shorter colons and excess generation of IFN-γ+ and IL-17+ CD45.1+ effector T cells (Fig. 4e–i). Analysis of the CD45.2 cell compartment in recipient mice at d35 showed increased EGFP loss in **Foxp3**EGFPCreRosa26 Nic/N1c (60%) as compared to control **Foxp3**EGFPCre cells (45%) (Fig. 4j), indicative of the heightened instability of N1c-expressing Treg cells.

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To delineate the intrinsic impact of N1c on the functional phenotype of T<sub>reg</sub> cells, as opposed to a cell-extrinsic effect mediated by systemic inflammation, we analyzed heterozygous Foxp3<sup>YFP<sub>cre</sub>/WT</sup>Rosa26<sup>N1c/N1c</sup> female mice for lymphoproliferation, immune cell activation and T<sub>reg</sub> cell phenotype. In contrast to homozygous Foxp3<sup>YFP<sub>cre</sub>/YFP<sub>cre</sub></sup>Rosa26<sup>N1c/N1c</sup> females, Foxp3<sup>YFP<sub>cre</sub>/WT</sup>Rosa26<sup>N1c/N1c</sup> females exhibited no signs of lymphoproliferation or T<sub>H1</sub> skewing of CD4<sup>+</sup> T<sub>conv</sub> cells at steady state (Supplementary Fig. 6a–d). However, YFP<sup>+</sup> T<sub>reg</sub> cells from Foxp3<sup>YFP<sub>cre</sub>/WT</sup>Rosa26<sup>N1c/N1c</sup> females showed decreased expression of T<sub>reg</sub> cell markers such as Foxp3, CD25, CTLA4, OX40, Helios and Nrp1, as compared to YFP<sup>−</sup> T<sub>reg</sub> cells from the same mice (Supplementary Fig. 6e, f). These results indicate that N1c overexpression destabilizes the phenotype and impairs the function of T<sub>reg</sub> cells by a cell-intrinsic mechanism, leading to immune cell activation, lymphoproliferation and autoimmunity.

The canonical Notch pathway mediates T<sub>reg</sub> cell destabilization

To determine the overall contribution of the canonical Notch pathway to the inflammation and autoimmunity observed in Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c</sup></sup> mice, we concurrently deleted Rbpj in the T<sub>reg</sub> cells of these mice. Most major phenotypes of Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c</sup></sup> mice, including lymphoproliferation, T<sub>H1</sub> and T<sub>C1</sub> skewing, autoantibody production and alterations in T<sub>reg</sub> cell markers and suppressor function were reversed in Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c</sup>Rbpj<sup>Δ/Δ</sup></sup> mice (Fig. 5a–i). Thus, activation of canonical Notch signaling has a predominant role in the immune dysregulation observed in the Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c</sup></sup> mice.

Notch activates RBPJ-dependent and independent transcription in T<sub>reg</sub> cells

T<sub>reg</sub> cells have a transcriptional signature reflective of their regulatory function. We compared the transcriptional profiles of splenic T<sub>reg</sub> cells isolated from Foxp3<sup>EGFP<sub>cre</sub></sup>, Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c</sup></sup>, Foxp3<sup>EGFP<sub>cre</sub>/Rbpj<sup>Δ/Δ</sup></sup> and Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c/Rbpj<sup>Δ/Δ</sup></sup></sup> mice. We compared the transcriptional profiles of splenic Treg cells isolated from Foxp3<sup>EGFP<sub>cre</sub></sup>, Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c</sup></sup>, Foxp3<sup>EGFP<sub>cre</sub>/Rbpj<sup>Δ/Δ</sup></sup> and Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c/Rbpj<sup>Δ/Δ</sup></sup></sup> mice. Comparison of the transcriptional profiles of Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c</sup></sup> T<sub>reg</sub> cells and Foxp3<sup>EGFP<sub>cre</sub></sup> T<sub>reg</sub> cells revealed a limited set of genes whose transcription was affected by N1c expression (mean expression value, >120; false-discovery rate, <0.1; coefficient variation, <0.5; Fig. 6a). Plotting of the transcriptional profiles as the difference in expression in Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c</sup></sup> T<sub>reg</sub> cells versus Foxp3<sup>EGFP<sub>cre</sub></sup> T<sub>reg</sub> cells against the difference in expression Foxp3<sup>EGFP<sub>cre</sub>/Rosa26<sup>N1c/N1c/Rbpj<sup>Δ/Δ</sup></sup></sup> T<sub>reg</sub> cells versus Foxp3<sup>EGFP<sub>cre</sub></sup> T<sub>reg</sub> cells revealed both RBPJ-dependent alterations in gene expression and RBPJ-independent alterations in gene expression (Fig. 6b). Hierarchical clustering analysis of genes whose expression was significantly altered by N1c expression revealed a subgroup of genes that were either upregulated (Dtx1, Ifng, Gemb, Pde3b) or down-regulated (Nrp1, Socs2, Il11r1, Ikkf2, Ikkf4) in an RBPJ-dependent manner (Fig. 6c). A second subgroup included genes that were modulated by N1c in an RBPJ-independent manner, consistent with their regulation by non-canonical pathway(s) (Fig. 6d). This subgroup included genes downstream of Foxo1 signaling, such as Bmp7, Gzma, Cd55 and others. These results indicated a
profound impact of Notch signaling on the T\textsubscript{reg} cell transcriptome by both canonical and non-canonical pathways.

We further analyzed the binding of RBPJ at two Foxp3-regulated genes that were affected by N1c expression. These included \textit{Pde3b}, whose expression is normally suppressed by Foxp3, but was upregulated by N1c expression in T\textsubscript{reg} cells, and \textit{Ikzf2}, whose expression is normally upregulated by Foxp3, but was decreased by N1c. Chromatin-immunoprecipitation analysis indicated that Foxp3 binding to the \textit{Pde3b} and \textit{Ikzf2} promoters was decreased, and that of RBPJ was increased, in Foxp3\textsuperscript{EGFP}\textsubscript{Cre}Rosa26\textsuperscript{N1c/N1c} as compared to Foxp3\textsuperscript{EGFP}\textsubscript{Cre} T\textsubscript{reg} cells (Supplementary Fig. 7a, b). Analysis of epigenetic histone-methylation markings at the \textit{Pde3b} promoter revealed more H3K4me3 and less H3K27me2 in Foxp3\textsuperscript{EGFP}\textsubscript{Cre}Rosa26\textsuperscript{N1c/N1c} T\textsubscript{reg} cells than in Foxp3\textsuperscript{EGFP}\textsubscript{Cre} T\textsubscript{reg} cells (Supplementary Fig. 7a), a pattern associated with decreased Foxp3-dependent suppression of gene expression\textsuperscript{37}. Reciprocally, H3K27me2 was increased at the \textit{Ikzf2} promoter (Supplementary Fig. 7b). These results indicate that canonical Notch signaling directly targeted a subset of Foxp3-regulated genes, antagonized Foxp3 binding and altered Foxp3-induced epigenetic markings at those loci.

The Notch canonical pathway mediates \textit{T}H1 reprogramming of T\textsubscript{reg} cells

Aborted \textit{T}H1 responses\textsuperscript{38}, T\textsubscript{reg} cells respond to IFN-\gamma treatment by upregulating \textit{Tbx21} expression in a STAT1-dependent manner\textsuperscript{38}. T-bet expression in T\textsubscript{reg} cells induces a partial Th1 program, including expression of CXCR3, but fails to upregulate expression of IL-12R\beta2, necessary to complete STAT4-dependent \textit{T}H1 differentiation\textsuperscript{39}. N1c over-expression in Foxp3\textsuperscript{EGFP}\textsubscript{Cre}Rosa26\textsuperscript{N1c/N1c} T\textsubscript{reg} cells upregulated the transcription of several genes associated with \textit{T}H1 cells, including \textit{Il12rb2} and \textit{Ifng} as well as a number of genes downstream of IL-12-STAT4 signaling, including \textit{Ffar1}, \textit{Id2} and \textit{Nkg7} (Fig. 7a and Supplementary Table 1). Treatment of Foxp3\textsuperscript{EGFP}\textsubscript{Cre}Rosa26\textsuperscript{N1c/N1c} T\textsubscript{reg} cells with IL-12 lead to expression of IFN-\gamma, which was lost in Foxp3\textsuperscript{EGFP}\textsubscript{Cre}Rosa26\textsuperscript{N1c/N1c}Rbpj\textsuperscript{Δ/Δ} T\textsubscript{reg} cells (Fig. 7b, c). Treatment with IL-12 resulted in the phosphorylation of STAT4 in a subset of N1c-expressing T\textsubscript{reg} cells in an RBPJ-dependent manner (Fig. 7d, e). Foxp3\textsuperscript{EGFP}\textsubscript{Cre}Rosa26\textsuperscript{N1c/N1c} T\textsubscript{reg} cells exhibited increased RBPJ and N1c binding to the \textit{Ifng} CNS22 element, which mediates transcriptional activation of \textit{Ifng} by Notch signaling in cooperation with T-bet (Fig. 7f, g)\textsuperscript{40}. IL-12-induced STAT4 phosphorylation was only observed in YFP\textsuperscript{+}, but not YFP\textsuperscript{−} T\textsubscript{reg} cells from heterozygous Foxp3\textsuperscript{YFP}\textsubscript{cre/+}Rosa26\textsuperscript{N1c/N1c} females (Supplementary Fig. 6g, h), indicating that expression of \textit{Il12rb2} in Foxp3\textsuperscript{YFP}\textsubscript{cre/WT}Rosa26\textsuperscript{N1c/N1c} T\textsubscript{reg} cells was due to intrinsic N1c-overexpressing and not to systemic inflammation. These results indicated that N1c promoted \textit{T}H1 programming of T\textsubscript{reg} cells in a cell-intrinsic and canonical pathway-dependent manner.

Notch regulates \textit{Foxp3} epigenetic stability via Rictor

Notch signaling activates the mammalian target of Rapamycin (mTOR) kinase complex 2 (mTORC2) and its downstream kinase AKT independent of RBPJ\textsuperscript{3, 4}. AKT phosphorylates the transcription factor Foxo1, resulting in its retention in the cytosol and its ubiquitination and degradation\textsuperscript{42, 43}. In turn, Foxo1 negatively regulates \textit{Th}1 differentiation.
of T\textsubscript{reg} cells by suppressing \textit{Ifig} transcription, as well as other type 1 genes\textsuperscript{35, 44}. Because N\textsubscript{1c} overexpression increased the transcription of Foxo1-suppressed genes in \textit{Foxp3\textsubscript{EGFPCre}\textit{Rosa26}N\textsubscript{1c}N\textsubscript{1c} T\textsubscript{reg} cells, we examined mTORC2-Akt activation in these cells. Anti-CD3+CD28 mAb treatment resulted in increased phosphorylation of S473 in AKT, which is a target of mTORC2, in \textit{Foxp3\textsubscript{EGFPCre}\textit{Rosa26}N\textsubscript{1c}N\textsubscript{1c} T\textsubscript{reg} cells, but not in control \textit{Foxp3\textsubscript{EGFPCre} T\textsubscript{reg} cells. Anti-CD3 mAb treatment did not induce the phosphorylation of T308 in AKT, which is a target of phospho-inositide 3-kinase (PI3K) (Fig. 8a, b and data not shown). AKT S473 phosphorylation was detected in \textit{Foxp3\textsubscript{EGFPCre}\textit{Rosa26}N\textsubscript{1c}N\textsubscript{1c}\textit{Rbpj}\Delta/\Delta T\textsubscript{reg} cells as well, suggesting it was independent of RPBJ (Fig. 8a, b). AKT S473 phosphorylation was not observed in \textit{Foxp3\textsubscript{EGFPCre}\textit{Rosa26}N\textsubscript{1c}N\textsubscript{1c}\textit{Rictor}\Delta/\Delta T\textsubscript{reg} cells, bearing a Foxp3-driven deletion of Rictor, an essential component of the mTORC2 complex following anti-CD3+CD28 mAb stimulation (Supplementary Fig. 1a, b). In contrast, AKT S473 phosphorylation proceeded unaffected in stimulated CD4\textsuperscript{+} T\textsubscript{conv} cells of all genotypes (Fig. 8a, b). There was increased Foxo1 translocation to the cytosol upon CD3 activation in \textit{Foxp3\textsubscript{EGFPCre}\textit{Rosa26}N\textsubscript{1c}N\textsubscript{1c} T\textsubscript{reg} cells, which was partially reversed by Rictor but not by RBPJ deficiency in these cells (Fig. 8c, d).

Analysis of \textit{Foxp3\textsubscript{EGFPCre}\textit{Rosa26}N\textsubscript{1c}N\textsubscript{1c}\textit{Rictor}\Delta/\Delta} mice revealed that the frequencies and absolute numbers of splenic T\textsubscript{reg} and IFN-\textgamma\textsuperscript{-}CD4\textsuperscript{+} T\textsubscript{conv} cells were decreased and the lymphoproliferative disease was reduced as compared to \textit{Foxp3\textsubscript{EGFPCre} T\textsubscript{reg} cells, (Supplementary Fig. 8a,b). However, in the absence of N\textsubscript{1c} overexpression, Rictor deficiency did not impact the frequencies or the total numbers of IFN-\textgamma\textsuperscript{-} CD4\textsuperscript{+} T\textsubscript{reg} cells, memory CD4\textsuperscript{+} T cells or T\textsubscript{reg} cells in \textit{Foxp3\textsubscript{EGFPCre} T\textsubscript{reg} cells as compared to \textit{Foxp3\textsubscript{EGFPCre} T\textsubscript{reg} cells, which was partially reversed by Rictor but not by RBPJ deficiency in these cells (Fig. 8c, d). Epigenetic demethylation of the Foxp3 CNS2 region has been linked to sustained Foxp3 expression and overall T\textsubscript{reg} cell stability\textsuperscript{45, 46}. The demethylation of the Foxp3 CNS2 region was decreased in \textit{Foxp3\textsubscript{EGFPCre} T\textsubscript{reg} cells, which was largely reversed by concurrent Rictor but not RBPJ deficiency (Fig. 8g, h). These findings indicated that N\textsubscript{1c} destabilized T\textsubscript{reg} cells in part by altering their Foxp3 CNS2 epigenetic demethylation signature in a Rictor-dependent manner.

**Discussion**

In this study, we provide evidence that cell-intrinsic Notch signaling regulates the T\textsubscript{reg} cell compartment in the periphery and controls T\textsubscript{reg} cell programming towards a T\textsubscript{H}1 phenotype, as well as T\textsubscript{H}1 cell responses. Blockade of different steps of the Notch signaling pathway in T\textsubscript{reg} cells, by means of lineage-specific targeted gene inactivation of \textit{Pofut1}, \textit{Rbpj} or \textit{Notch1}, resulted in increased T\textsubscript{reg} cell frequency, decreased CD4\textsuperscript{+} T cell compartment size, and decreased IFN-\textgamma production by CD4 and CD8 cells. T\textsubscript{reg} cell-specific deletion of Notch
signaling components protected against full MHC-mismatched GVHD in association with decreased T<sub>reg</sub> cell apoptosis, T<sub>reg</sub> cell programming towards a T<sub>H1</sub> phenotype and alloreactive T<sub>H1</sub> and T<sub>C1</sub> responses. Reciprocally, over-expression of N1c in T<sub>reg</sub> cells resulted in lymphoproliferation, increased T<sub>reg</sub> cell apoptosis, T<sub>H1</sub> programming of T<sub>reg</sub> cells, dysregulated T<sub>H1</sub> and T<sub>C1</sub> responses and autoimmunity. N1c expression down-regulated the expression of several critical components of the T<sub>reg</sub> cell transcriptome and weakened the T<sub>reg</sub> cell epigenetic imprint. Thus, homeostatic Notch signaling in T<sub>reg</sub> cells defines the size and function of the T<sub>reg</sub> cell compartment and controls the magnitude of T<sub>H1</sub> and T<sub>C1</sub> cell responses in vivo.

The effects of Notch signaling on T<sub>reg</sub> cells were predominantly exerted by the canonical pathway. Interruption of the canonical Notch pathway by means of T<sub>reg</sub> cell-specific Rbpj deletion rescued the key manifestations associated with enforced N1c expression in T<sub>reg</sub> cells, including lymphoproliferation and autoimmunity. Several T<sub>reg</sub> cell phenotypic, transcriptional and functional alterations induced by enforced N1c expression were reversed upon RBPJ deletion, indicating their dependence on canonical Notch signaling. RBPJ binds to a subset of Foxp3 target gene, such as Pde3b and Iklf2, antagonized the binding of Foxp3 and reversed the Foxp3-associated histone methylation profile at those loci, indicative of a direct effect of the canonical pathway on at least a subset of Foxp3-regulated genes. Additional regulation may also be exerted by downstream gene targets of Notch signaling, such as Hes1 and Dtx1, which may engage in secondary transcriptional circuits that contribute to gene expression changes induced by Notch in T<sub>reg</sub> cells.

In contrast to the above, a number of T<sub>reg</sub> cell transcripts appeared to respond to Notch signaling in a canonical pathway-independent manner. Several of these have been previously associated with Foxo1 dependent regulation, consistent with their induction by the mTOR-AKT-Foxo1 pathway. N1c-driven impairment of epigenetic demethylation of Foxp3 CNS2 was also independent of the canonical pathway, suggesting a putative role for non-canonical signaling in Notch-mediated T<sub>reg</sub> cell destabilization. This conclusion was reinforced by the demonstration that N1c-induced increase in CpG methylation of Foxp3 CNS2 was largely reversed in N1c overexpressing but Rictor deficient T<sub>reg</sub> cells.

These studies highlight the important role played by canonical Notch signaling in T<sub>reg</sub> cells in enabling T<sub>H1</sub> and T<sub>C1</sub> responses. In the course of controlling a T<sub>H1</sub> response, T<sub>reg</sub> cells normally undergo an aborted program of T<sub>H1</sub> differentiation that limits their capacity to express IL-12Rβ2. Activation of the canonical Notch signaling bypassed this blockade, leading to STAT4 activation and IFN-γ production in response to IL-12 in T<sub>reg</sub> cells. RBPJ and N1c associated with CNS22 in the Ifng locus in N1c-overexpressing T<sub>reg</sub> cells, consistent with previous data showing direct activation of Ifng expression by Notch signaling in synergy with T-bet. N1c also enabled more effective activation of mTORC2-AKT by TCR engagement and Foxo1 cytosolic sequestration, a mechanism previously implicated (by means of CD4<sup>+</sup> T cell- or T<sub>reg</sub> cell-specific Foxo1 deletion) in promoting T<sub>H1</sub> differentiation of T<sub>reg</sub> cells. The two mechanisms, canonical and non-canonical, may thus act cooperatively to induce T<sub>H1</sub> skewing.
Notch signaling in T<sub>reg</sub> cells played a particularly deleterious role in the context of severe inflammation in the full MHC-mismatched GVHD. Whereas control (Foxp3<sup>EGFP</sup>Cre) T<sub>reg</sub> cells were susceptible to apoptosis and T<sub>H1</sub> programming, Notch1 or RBPJ-deficient T<sub>reg</sub> cells were resistant to both processes, leading to the accumulation of T<sub>reg</sub> cells, decreased skewing towards the T<sub>H1</sub> phenotype and increased survival. Notch1 inactivation in T cells protects against GVHD<sup>22</sup>, and our studies indicated that T<sub>reg</sub> cells were the key cellular effectors of this effect. In the context of overwhelming T<sub>H1</sub> immune responses, such as experimental <i>T. gondii</i> infection, T<sub>reg</sub> cells undergo T<sub>H1</sub> programming and apoptosis, recapitulating the fate of T<sub>reg</sub> cells in GVHD<sup>47</sup>. The exaggerated T<sub>H1</sub> response in <i>T. gondii</i>-infected mice was associated with decreased IL-2 production, which contributed to T<sub>reg</sub> cell apoptosis. In addition, interference with Notch signaling in T<sub>reg</sub> cells attenuated the T<sub>H1</sub> and T<sub>C1</sub> response and increased IL-2 production, consistent with a role for IL-2 in enhanced survival of Notch-deficient T<sub>reg</sub> cells in GVHD.

The inhibitory effects of Notch signaling on T<sub>reg</sub> cell function offer mechanistic insights into how blockade of Notch receptors may induce tolerance, as has been reported in some experimental mouse models of graft versus host disease. Intervention strategies targeting the Notch pathway may thus offer innovative therapeutic approaches in transplant and autoimmune diseases.

### Online Methods

**Mice**

Foxp3<sup>EGFP</sup>Cre, Foxp3<sup>YFP</sup>Cre, Notch1<sup>fl/fl</sup>, Rosa26<sup>N1c/N1c</sup>, Rag1<sup>−/−</sup>, CD45.1, Foxp3<sup>EGFP</sup> and BALB/c mice were obtained from the Jackson Laboratory<sup>28, 30, 48</sup>. Rictor<sup>fl/fl</sup> mice were obtained from the Mutant Mouse Regional Resource Center. Pofut1<sup>fl/fl</sup> and Rbpj<sup>fl/fl</sup> were kind gifts of Pamela Stanley and Tasuku Honjo, respectively, and were generated as described<sup>26, 27</sup>. All Foxp3 mutant mouse strains and their respective crosses were backcrossed 8–10 generations on C57BL/6 or NOD background where specified. Excepted when it was specified, 8 weeks old mice were used in this study. The mice were housed under specific pathogen-free conditions and used according to the guidelines of the institutional Animal Research Committees at the Boston Children’s Hospital.

**Real-time PCR**

Total RNA was isolated from sorted cells with RNeasy kit (Qiagen). Reverse transcription was performed with the SuperScript III RT-PCR system (Invitrogen) and quantitative real-time reverse transcription (RT)-PCR with Taqman<sup>®</sup> Fast Universal PCR master mix, internal house keeping gene mouse (GAPD VIC-MGB dye) and specific target gene primers (FAM Dye) (Applied Biosystems) on Step-One- Plus machine. Relative expression was normalized to GAPD for Notch1-4 receptor and calculated as fold change compared to wild-type CD4<sup>+</sup>GFP<sup>−</sup> conventional T cells for Pofut1, Notch1, RBPJ and Rictor regarding the regulatory T cell-specific deficiency and fold change normalized to wild-type Treg cells for IFNg and IL12rb2.
Flow cytometry

Annexin V and antibodies against CD4, CD8, CD16/32, CD90.2, CTLA4, ICOS, H-2Kd (biolegend), CD3ε, CD25, CD44, CD45.1, CD45.2, CD45Rb, CD62L, Eos, Foxp3, Helios, IFN-γ, IL-17A, IL-2, Ki67, OX40, CXCR3, H-2Kb (eBioscience), N1c, P-STAT4 (BD biosciences), P-AKT (S473) (Cell signaling) and Nrp1 (R&D system) were used. Cell suspensions were incubated for 10 min with CD16/32 then stained for surface markers for 20 min on PBS/1%FCS. Foxp3, Helios, Eos, CTLA4, Ki67 staining was performed by using eBioscience Fixation/Permeabilization kit. For cytokine detection, cell suspensions were pre-incubated with 50 ng/mL PMA, 500 ng/mL ionomycin and 10 μg/mL brefeldin A for 4h in complete medium before CD16/32 blocking followed by surface staining, permeabilization and intracellular Foxp3, IFN-γ, IL-2 and IL-17 staining. For phospho-AKT experiments, spleen cells were stimulated for 30 min with soluble anti-CD3 (1μg/mL) and anti-CD28 (5μg/mL). In some experiments, spleen cells were pre-treated with Rapamycin (250nM, Tocris) for 0, 1 or 24h. After stimulation, cells are fixed with PBS/2%PFA for 20min, permeabilized in 90% methanol for 30 min on ice and stained for CD4, Foxp3 and P5473-AKT. For phospho-STAT4 experiments, splenic CD4+ T cells were isolated and stimulated for 30min with 25ng/mL of recombinant mouse IL-12 (Biolegend). Cells were fixed with PBS/2%PFA for 20min, permeabilized in 90% methanol for 30 min on ice and stained for CXCR3, CD4, Foxp3 and P5693-STAT4. For ex-vivo Treg cell stimulation, isolated Treg cells were cultured for 3 days with IL-2 at 200U/mL ± IL-12 at 25 ng/mL (Biolegend) and PMA/ionomycin/BrefeldinA the last 4 hours before staining for IFN-γ and Foxp3. All flow cytometry acquisitions were performed on a Fortessa cytometer using DIVA software (BD Biosystems) and analyzed using FlowJo (Tree Star).

Graft versus host disease

Eight weeks old Balb/c mice were lethally irradiated (8.5–9 Gy) 4 hours prior reconstitution with 5.10^6 T cell depleted bone marrow cells alone or in presence of 10^7 spleen cells from C57BL/6 Foxp3EGFPcre, Foxp3EGFPcreRbpjΔΔ or Foxp3EGFPcreNotch1ΔΔ mice. T cell deplete bone marrow was prepared using CD90.2 microbeads (Miltenyi Biotech). Clinical GVHD score was evaluated every 2 to 3 days by assessment of five clinical parameters as followed: Weight loss (<10%, grade 0; >10% to <20%, grade 1; >20%, grade 2), posture (Normal, grade 0; Hunching noted only at rest, grade 1; Severe hunching and/or impairs movement, grade 2), activity (Normal, grade 0; Mild to moderately decreased, grade 1; Stationary unless stimulated, grade 2), fur texture (Normal, grade 0; Mild to moderate ruffling, grade 1; Severe ruffling/poor grooming, grade 2) and skin integrity (Normal, grade 0; Scaling of paws/tail, grade1; Obvious areas of denuded skin, grade 2). In selected experiments, mice were sacrificed 5 days post transplantation and donor H-2Kd−H-2Kb+ T cells were evaluated for apoptosis, cytokine production and Foxp3 expression.

Adoptive transfer induced Colitis

Naïve (CD4+CD45RBhighGFP−) and Treg (CD4+GFP+) cells are respectively isolated from the spleen of CD45.1 and CD45.2 Foxp3EGFPcre or Foxp3EGFPcreRosa26N1c/N1c mice. Colitis was induced in RAG1-deficient males by i.p. injection of 5.10^5 CD45.1 naïve ± 2.10^5 Treg cells. Mice were weighed and monitored for signs of disease twice weekly.
**Suppression assays**

CD4+ T cells were isolated using a CD4 negative isolation kit (Miltenyi), then labeled with CellTrace™ Violet Cell Proliferation dye (Life technologies) according to the manufacturer’s instructions and used as responder cells. Treg (CD4+GFP+) cells were isolated on FACSARia and used as suppressor cells. Responders were used at a fixed concentration of 10^5 cells per well and stimulated for 3 days with 2 μg/mL of soluble anti-CD3 antibody and 5 μg/mL of soluble anti-CD28 antibody in the presence of 4.10^5 feeder Rag1−/− spleen cells in 96-well, round-bottom plates in triplicate.

**Gene-expression profiling**

Spleen T_{reg} (CD3+CD4+GFP+) cells were double-sorted from 6 week old male Foxp3^{EGFPcre}, Foxp3^{EGFPcreRbpjΔ/Δ}, Foxp3^{EGFPcreRosa26^{N1c/N1c}} and Foxp3^{EGFPcreRosa26^{N1c/N1cRbpjΔ/Δ}} mice (n=3–4 per group). Cells were collected directly into TRIzol. RNA was purified and used for probe synthesis for hybridization to Affymetrix Mouse Gene M1.0 ST microarrays. Raw data were background-corrected and normalized with the RMA algorithm in the GenePattern software package.

**ChIP assays**

Chromatin immunoprecipitation on purified T_{reg} cells was performed with Agarose ChIP Kit (Pierce) and anti-RBPJ (Cell signaling), anti-Notch1 (biolegend), anti- Foxp3 (MBL), anti-Histone H3 trimethylated on Lysine 4 (H3K4me3) (abcam), anti-dimethylated on Lysine 27 (H3K27me2) (Millipore) and respective isotype control antibodies. Purified DNA was subjected to real-time PCR with primers flanking RBPJ binding site at Ifng CNS-22 or with primers flanking Foxp3 binding sites on Pde3b and Iκzf2 as previously described.

**Histology**

Pancreatic inflammation was scored based on the degree of inflammatory cellular infiltrations present in the islets of Langerhans: 0, no inflammation; 1, mild inflammatory infiltrates; 2, perisulitis; 3, moderate and diffuse or severe and focal insulitis; 4, severe and diffuse insulitis; 5, severe insulitis and destruction of the tissue. For colitis experiments, colon sections were stained by H&E and scored as followed: 0, no inflammation; 1, mild, scattered infiltrates; 2, moderate infiltrates without loss of epithelium integrity; 3, moderate and diffuse or severe inflammation; 4, Severe inflammation associated with loss of the epithelial barrier integrity.

**Autoantibody arrays**

Screening for a broad panel of IgG autoantibodies was performed with autoantibody arrays (University of Texas Southwestern Medical Center, Genomic and Microarray Core Facility) as described.

**Methylation analysis**

The methylation status of Foxp3 T_{reg}-specific demethylation region (TSDR or CNS2) in thymic and splenic Treg cells of 8 weeks old male mice was assessed by bisulfite sequence analysis, as described. The TSDR of converted DNA was amplified by methylation-
specific primer sequences: *Foxp3 CNS2* Forward 5′-TATTTTTTTTGGGTTTTGGATATTA-3′ (forward) and *Foxp3 CNS2* Reverse 5′-AACCAACCAACTTCTACACTCTAT-3′. The PCR product was subcloned and sequenced. Blast analyses were done by comparing the resulting sequences with converted *Foxp3* gene sequences.

**Confocal microscopy**

*T*<sub>reg</sub> cells were purified and incubated on pre-coated coverslip (poly-L-lysine 50μg/mL, ± anti-CD3 0.1 or 1 μg/mL for low and high dose respectively) at 37°C for 30 min in RPMI/10%FCS. After fixation with PBS/4% paraformaldehyde, cells were permeabilized with PBS/0.1% saponin and blocked on PBS/4% bovine serum albumin (BSA). Cells were incubated with 1:100 diluted rabbit anti-Foxo1 (C29H4, Cell Signaling) followed by 1:500 diluted Alexa fluor 555-anti rabbit secondary antibody (Life technologies) in PBS/1%BSA. Slides were mounted with gold anti-fade reagent with DAPI (Invitrogen). Images were acquired with a Zeiss LSM700 confocal microscopy and ZEN imaging software. Five to 10 fields were selected randomly and total cells in the field were analyzed for percentage of Foxo1 nuclear localization using ImageJ software. Percentage of nuclear Foxo1 localization was obtained by the formula: 100 × corrected nuclear fluorescence/corrected total cell fluorescence and corrected fluorescence was obtained by the formula: Integrated Density – (Area of selected cell or nucleus × Mean fluorescence of background).

**In vitro induced T*<sub>reg</sub>* cell generation**

Naive CD4<sup>+</sup>CD62L<sup>hi</sup>CD44<sup>lo</sup> T cells were isolated from spleen using FACS Aria (purity>98%, data not shown). Cells are stimulated in vitro with coated anti-CD3 (2μg/mL) and anti-CD28 (5μg/mL) for 5 days in the presence of different concentrations of rhTGF-β1 (0, 1, 2 or 5 ng/mL).<sup>52</sup>

**Statistical analysis**

Data were analyzed by paired and unpaired two-tailed Student’s *t*-test, one and two way ANOVA with post test analyses and log-rank test, as indicated. Differences in mean values were considered significant at a *p* < 0.05.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Interruption of Notch signaling in T$_{\text{reg}}$ cells results in a super-regulatory phenotype
(a) Frequencies and numbers of CD3 and CD4 T cells from the spleen of 8 weeks old Foxp3$^{\text{EGFPcre}}$, Foxp3$^{\text{EGFPcrePofut}^{\Delta/\Delta}}$, Foxp3$^{\text{EGFPcreRbpj}^{\Delta/\Delta}}$ and Foxp3$^{\text{EGFPcreNotch1}^{\Delta/\Delta}}$ mice. (b) Flow cytometric analyses of CD4 and Foxp3 markers on CD3$^+$ T cells are shown. (c) Frequencies and numbers of T$_{\text{reg}}$ cells for each group of panel (a). (d) Flow cytometric analysis of CD62L and CD44 markers on CD4$^+$ T cells are shown. (e) Frequencies and numbers of memory CD4$^+$ T cells for each group. (f) Flow cytometric analyses of IFN-γ and IL-17 on CD4$^+$ T cells are shown. (g) Frequencies and numbers of IFN-γ producing CD4$^+$ T cells for each group. (h) Flow cytometric analyses of IFN-γ and IL-17 expression in CD8$^+$ T cells. (i) Frequencies and numbers of IFN-γ producing CD8$^+$ T cells shown in panel (H). (j) Expression of Foxp3, CD25, CTLA4, Helios and Nrp1 markers were evaluated of splenic T$_{\text{reg}}$ cells and expressed as mean fluorescence intensity (MFI). Results are representative of at least 3 experiments per panel. * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 by one way ANOVA with post test analysis.
Fig. 2. T_{reg} cell-specific loss of function Notch signaling mutations protect mice from lethal graft versus host disease
(a) Survival and (b) severity score of lethally irradiated (8.5/9Gy) BALB/c mice infused with C57Bl/6 Foxp3^EGFP^Cre^,^ Foxp3^EGFP^Cre^Rbpj^Δ/Δ or Foxp3^EGFP^Cre^Notch1^Δ/Δ T cell-depleted bone marrow, either alone (BM only) or together with spleen cells of the respective genotypes. For T cell subpopulation analyses were carried out on spleen cells at day 5 post adoptive transfer. The BM only groups were not included in panel b. (c) Flow cytometric analyses of IFN-γ and Foxp3 markers on CD4^+^ T cells are shown. (d) Frequencies of IFN-γ producing CD4^+^ T_{conv} (Foxp3^−^) and T_{reg} (Foxp3^+^) cells for each group. (e) Flow cytometric analyses of IFN-γ and IL-2 markers on CD8^+^ T cells. (f) Frequencies and numbers of IFN-γ producing CD8^+^ T cells for each group. (g) Flow cytometric analyses of Foxp3 marker on CD4^+^ T cells are shown. (h) Frequencies and numbers T_{reg} cells for each group. (i) Viability dye and Annexin V (AnnV) staining of T_{reg} cells are shown (j) Frequencies of apoptotic (AnnV^+) T_{conv} and T_{reg} cells for each group. (k) Overlay of representative N1c expression on T_{conv} and T_{reg} cells are shown. (l) MFI of N1c expression in T_{conv} and T_{reg} cells for each group. Results are representative of at least 2 experiments per panel. * p<0.05, ** p<0.01,
*** p<0.001 and **** p<0.0001 by log-rank test, one way ANOVA and two way ANOVA with post test analysis.
**Fig. 3. Exacerbated Notch signaling in T\textsubscript{reg} cells results in immune dysregulation and autoimmunity**

(a) A representative picture of spleens and peripheral lymph nodes from 6 months old \textit{Foxp3\textsuperscript{EGFP}Cre} and \textit{Foxp3\textsuperscript{EGFP}Cre\textsubscript{Rosa26\textsuperscript{N1c/N1c}}} mice. (b) Representative pictures of H&E staining of lung, spinal vessels, spinal cord and pancreas from 6 month old \textit{Foxp3\textsuperscript{EGFP}Cre\textsubscript{Rosa26\textsuperscript{N1c/N1c}}} mice. Frequencies (c) and numbers (d) of CD3, CD4, CD8 T cells, T\textsubscript{reg} cells, IFN-\gamma producing CD4 and CD8 T cells and memory CD62L\textsuperscript{lo}CD44\textsuperscript{hi} CD4 T cells from the spleen of 8 weeks old \textit{Foxp3\textsuperscript{EGFP}Cre} (white circles) and \textit{Foxp3\textsuperscript{EGFP}Cre\textsubscript{Rosa26\textsuperscript{N1c/N1c}}} (black circles) mice. (e) Heatmap summarizing the expression of circulating autoantibodies significantly increased in 8 weeks old \textit{Foxp3\textsuperscript{EGFP}Cre\textsubscript{Rosa26\textsuperscript{N1c/N1c}}} compared to \textit{Foxp3\textsuperscript{EGFP}Cre} mice (serum from \textit{Foxp3\textsuperscript{K276X}} was used as a positive control). (f) Diabetes incidence of littermate control and \textit{Foxp3\textsuperscript{EGFP}Cre\textsubscript{Rosa26\textsuperscript{N1c/N1c}}} female (n=18 and n=13 respectively) and male (n=16 and n=8 respectively) mice on NOD background. (g, h) Representative pictures of H&E staining of pancreas and histological score of 15 weeks old control females (white circles) or males (white squares) and \textit{Foxp3\textsuperscript{EGFP}Cre\textsubscript{Rosa26\textsuperscript{N1c/N1c}}} females (Black circles) or males (black squares) on NOD background. * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 by unpaired two-tailed Student’s t-test and log-rank test.
Fig. 4. Notch signaling compromises T<sub>reg</sub> cell phenotype and suppressive functions

(a) Expression of Foxp3, CD25, CTLA4, OX40, Helios, Nrp1 and Eos markers were evaluated in splenic T<sub>reg</sub> cells of Foxp3<sup>EGFP<sup>Cre</sup></sup> (Dashes lines and white circles) and Foxp3<sup>EGFP<sup>Cre</sup></sup>Rosa26<sup>N1c/N1c</sup> (Solid lines and black circles) mice. Gray plains represent expression of those markers in T<sub>conv</sub> of Foxp3<sup>EGFP<sup>Cre</sup></sup> mice. Cell turn-over was assessed in T<sub>reg</sub> cells of Foxp3<sup>EGFP<sup>Cre</sup></sup> (Dashes lines and white circles), Foxp3<sup>EGFP<sup>Cre</sup></sup>Rosa26<sup>N1c/N1c</sup> (Solid black lines and black circles) and Foxp3<sup>EGFP<sup>Cre</sup></sup>Rbpj<sup>Δ/Δ</sup> (Solid gray lines and Gray triangles) by Ki67 staining (b) for active cell cycle phases and by AnnV staining (c) for apoptosis. (d) In vitro suppression of responder CD4<sup>+</sup> T cell proliferation (T<sub>Eff</sub>) was assessed by evaluation of proliferation dye dilution upon anti-CD3/CD28 stimulation in the

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presence of various number of T\textsubscript{reg} cells from each genotype. In vivo suppressive capacity of
T\textsubscript{reg} cells from Foxp3\textsuperscript{EGFP\textsubscript{Cre} (white circles), Foxp3\textsuperscript{EGFP\textsubscript{Cre} Rosa26\textsuperscript{N1c/N1c (black circles))}
was assessed in the CD4 T cell transfer-induced colitis model. (e) Changes in body weight
over time are shown (n=4–7). One representative experiment of 3 is shown. (f–h) Disease
severity was evaluated by H&E staining of colon sections and end point colon length. (i)
Absolute number of total, IFN-\& and IL-17-producing CD45.1\textsuperscript{+}CD4\textsuperscript{+} T cells from the naïve
T cell input was quantified within the entire colon. (j) Stability of T\textsubscript{reg} cell lineage was
shown by percent of EGFP expression of the CD45.2 T cell compartment. * p<0.05, **
p<0.01, *** p<0.001 and **** p<0.0001 by unpaired two-tailed Student’s t-test, One way
ANOVA with post test analysis and two way ANOVA.
Fig. 5. T<sub>reg</sub> cell failure and disease manifestations in Foxp<sup>3</sup>EGFP<sub>Cre</sub><sub>Rosa26</sub><sup>N1c/N1c</sup> mice proceed by a canonical pathway dependent mechanism

(a) Frequencies and numbers of CD3, CD4 and T<sub>reg</sub> cells from the spleen of 8 weeks old Foxp<sup>3</sup>EGFP<sub>Cre</sub>, Foxp<sup>3</sup>EGFP<sub>Cre</sub>Rosa26<sup>N1c/N1c</sup>, Foxp<sup>3</sup>EGFP<sub>Cre</sub>Rbpj<sup>Δ/Δ</sup> and Foxp<sup>3</sup>EGFP<sub>Cre</sub>Rosa26<sup>N1c/N1c</sup>Rbpj<sup>Δ/Δ</sup> mice. (b) Flow cytometric analysis of CD62L and CD44 markers on CD4<sup>+</sup> T cells. (c) Scatter plots represent frequencies and numbers of memory CD4<sup>+</sup> T cells for each group. (d) Flow cytometric analysis of IFN-γ and IL-17 secretion by CD4<sup>+</sup> T cells are shown. (e) Scatter plots represent frequencies and numbers of IFN-γ producing CD4<sup>+</sup> T cells for each group. (f, g) Expression of Foxp3, CD25, Helios, Nrp1 markers were evaluated in splenic T<sub>reg</sub> cells of each group. n=4–5 per group. (h) In vitro suppression of responder CD4<sup>+</sup> T cell proliferation (T<sub>Eff</sub>) was assessed by evaluation of proliferation dye dilution upon anti-CD3/C28 stimulation in the presence of various number of T<sub>reg</sub> cells from each genotype. (i) Heatmap summarizing the expression of significantly modulated circulating autoantibodies in 8 weeks old Foxp<sup>3</sup>EGFP<sub>Cre</sub>, Foxp<sup>3</sup>EGFP<sub>Cre</sub>Rosa26<sup>N1c/N1c</sup> and Foxp<sup>3</sup>EGFP<sub>Cre</sub>Rbpj<sup>Δ/Δ</sup> and Foxp<sup>3</sup>EGFP<sub>Cre</sub>Rosa26<sup>N1c/N1c</sup>Rbpj<sup>Δ/Δ</sup> mice (serum from Foxp<sup>3</sup>K276X was used as positive control). One representative experiment of 2 or 3 is shown for panels (a-h). * p<0.05 and ** p<0.01 by One way ANOVA with post test analysis and two way ANOVA.
Fig. 6. Impact of Notch signaling on the T_{reg} cell transcriptome

(a) Microarray analysis of gene expression (mean) of Foxp3^{EGFPCre}Rosa26^{N1c/N1c} (n = 4) (X axis) versus Foxp3^{EGFPCre} T_{reg} cells (n=4). Numbers in plots indicate total genes downregulated (red) or upregulated (blue) in N1c overexpressing T_{reg} cells relative to their expression in Foxp3^{EGFPCre} T_{reg} cells (cutoff of a two fold change). (b) Comparison of changes in gene expression induced by Notch1 signaling (Foxp3^{EGFPCre}Rosa26^{N1c/N1c} vs Foxp3^{EGFPCre}; horizontal axis) and those induced by Notch gain of function in absence of canonical pathway (Foxp3^{EGFPCre}Rosa26^{N1c/N1c}Rbpj^{Δ/Δ} vs Foxp3^{EGFPCre}; vertical axis) within T_{reg} cells. Blue lines mark a fold change of 2. (c, d) Genes whose expressions are significantly modulated (p<0.05 by one way ANOVA) were segregated in 2 clusters based on the pattern of modulation (canonical and non-canonical) and representative heat maps for each cluster are shown.
Fig. 7. N1c mediates T_{H}1 reprogramming of T_{reg} cells
(a) qPCR analysis of Il12rb2 and Ifng transcripts in T_{reg} cells isolated from Foxp3^{EGFPcre}, Foxp3^{EGFPcre}Rosa26^{N1c/N1c} and Foxp3^{EGFPcre}Rosa26^{N1c/N1c}Rbpj^{Δ/Δ} mice (n=4–6 per group). Results represent mean fold change + S.E.M. compared to mean of Foxp3^{EGFPcre} T_{reg} cells. Representative flow cytometry dot plots (b) and histograms (c) of IFN-γ production by sorted T_{reg} cells after ex-vivo IL-12 stimulation (n=3 per group). Representative histograms (d) and dot plots (e) of P-STAT4 on gated Foxp3^{−}CXCR3^{+} and CD4^{+}Foxp3^{+}CXCR3^{+} cells after CD4 enrichment and IL-12 stimulation (n=3–8 per group). ChIP graphs represent quantitative PCR analysis of the ratio of enriched Ifng CNS22 binding site immunoprecipitated with anti-RBPJ (f) and anti-N1c (g) to the input DNA on T_{reg} cells isolated from Foxp3^{EGFPcre}, Foxp3^{EGFPcre}Rosa26^{N1c/N1c} and Foxp3^{EGFPcre}Rosa26^{N1c/N1c}Rbpj^{Δ/Δ} mice (n=3 per group). * p<0.05, ** p<0.01 and *** p<0.001 by unpaired two-tailed Student’s t-test and One Way ANOVA with post test analysis.
Fig. 8. Rictor-dependent non-canonical signaling dysregulates AKT-Foxo1 axis in Foxp3EGFPCreRosa26N1c/N1c Treg cells
(a) Flow cytometric analysis and (b) scatter plot analysis of MFI of unstimulated and anti-CD3/anti-CD28 stimulated Treg cells from Foxp3EGFPCre, Foxp3EGFPCreRosa26N1c/N1c, Foxp3EGFPCreRosa26N1c/N1cRictorΔ/Δ and Foxp3EGFPCreRosa26N1c/N1cRbpjΔ/Δ mice. (c) N1c augments anti-CD3 mAb-induced translocation of nuclear Foxo1 to the cytosol in a Rictor dependent manner. Unstimulated and anti-CD3 mAb stimulated Treg cells were stained for nuclear DNA (DAPI) and Foxo1 and examined by confocal microscopy for Foxo1 distribution in the nucleus vs. cytosol. (d) Quantitation of percent Foxo1 nuclear expression. Each point represents one cell. (e) Flow cytometric analyses of Foxp3 and EGFP expression in peripheral Treg cells from Foxp3EGFPCreRosa26N1c/N1c, Foxp3EGFPCreRosa26N1c/N1cRictorΔ/Δ and Foxp3EGFPCreRosa26N1c/N1cRbpjΔ/Δ mice. (f) Fractions of EGFP+ cells among Foxp3+ Treg cells from panel (e). (g) Methylation status of individual CpG motifs within the TSDR of CNS2 in Foxp3. Individual CpG motifs are numbered with reference to the transcription initiation site of Foxp3. (h) Global methylation status of the TSDR of CNS2 in Foxp3. * p<0.05, ** p<0.01 and *** p<0.001 by unpaired two-tailed Student’s t-test and One Way ANOVA with post test analysis.