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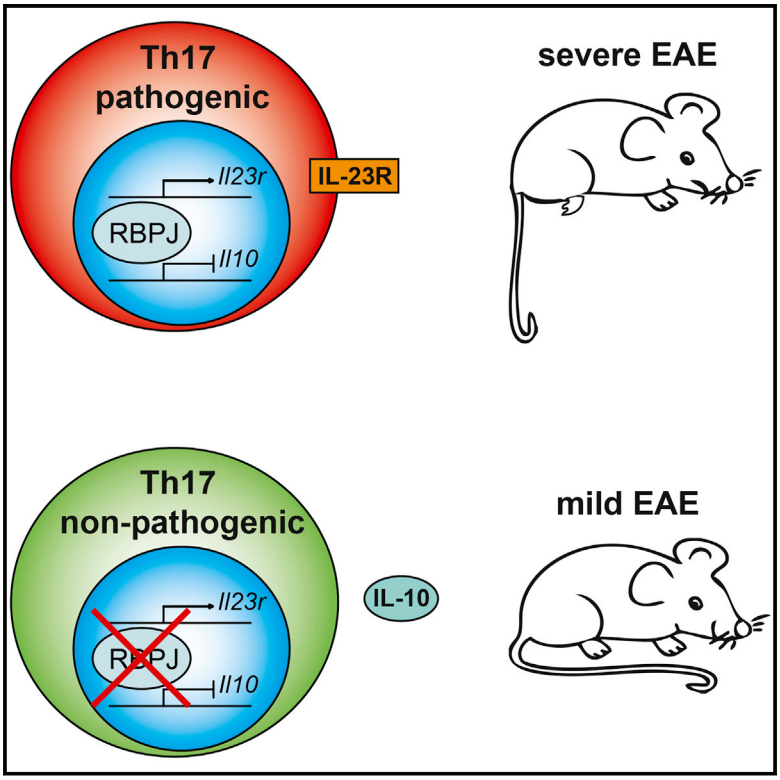
RBPJ Controls Development of Pathogenic Th17 Cells by Regulating IL-23 Receptor Expression

Graphical Abstract



Authors

Gerd Meyer zu Horste, Chuan Wu, Chao Wang, ..., Sheng Xiao, Aviv Regev, Vijay K. Kuchroo

Correspondence

vkuchroo@evergrande.hms.harvard.edu

In Brief

Meyer zu Horste et al. find that RBPJ promotes the pathogenicity of Th17 cells by directly enhancing expression of the interleukin-23 receptor and repressing interleukin-10 production.

Highlights

- RBPJ promotes experimental CNS autoimmunity via the IL-23R
- RBPJ-deficient Th17 cells fail to express IL-23R and related transcripts
- RBPJ binds and *trans*-activates the *Il23r* promoter together with RORγt
- RBPJ represses expression of IL-10 in Th17 cells

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RBPJ Controls Development of Pathogenic Th17 Cells by Regulating IL-23 Receptor Expression

Gerd Meyer zu Horste,[1,2] Chuan Wu,[1,2] Chao Wang,[1,2] Le Cong,[3] Mathias Pawlak,[1,2] Youjin Lee,[1,2] Wassim Elyaman,[2] Sheng Xiao,[1,2] Aviv Regev,[3] and Vijay K. Kuchroo[1,2,3,*]

[1]Evergrande Center for Immunologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, MA 02215, USA
[2]Ann Romney Center for Neurologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, MA 02215, USA
[3]The Broad Institute of the Massachusetts Institute of Technology and Harvard, Cambridge, MA 02142, USA
*Correspondence: vkuchroo@evergrande.hms.harvard.edu
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SUMMARY

Interleukin-17 (IL-17)-producing helper T cells (Th17 cells) play an important role in autoimmune diseases. However, not all Th17 cells induce tissue inflammation or autoimmunity. Th17 cells require IL-23 receptor (IL-23R) signaling to become pathogenic. The transcriptional mechanisms controlling the pathogenicity of Th17 cells and IL-23R expression are unknown. Here, we demonstrate that the canonical Notch signaling mediator RBPJ is a key driver of IL-23R expression. In the absence of RBPJ, Th17 cells fail to upregulate IL-23R, lack stability, and do not induce autoimmune tissue inflammation in vivo, whereas overexpression of IL-23R rescues this defect and promotes pathogenicity of RBPJ-deficient Th17 cells. RBPJ binds and *trans*-activates the *Il23r* promoter and induces IL-23R expression and represses anti-inflammatory IL-10 production in Th17 cells. We thus find that Notch signaling influences the development of pathogenic and non-pathogenic Th17 cells by reciprocally regulating IL-23R and IL-10 expression.

INTRODUCTION

Interleukin-17 (IL-17)-producing helper T cells (Th17 cells) have been identified as a distinct subset of effector $CD4^+$ T cells and are considered critical drivers of autoimmune tissue inflammation (Bettelli and Kuchroo, 2005; Korn et al., 2009). Differentiation of naive $CD4^+$ T cells into Th17 cells is achieved with the cytokines transforming growth factor (TGF)-β1 and IL-6 (Bettelli et al., 2006). This cytokine combination, however, generates Th17 cells, which co-produce IL-10 together with IL-17 and do not induce autoimmunity (Lee et al., 2012; McGeachy et al., 2007) and have therefore been called non-pathogenic Th17 cells. To acquire the ability to induce autoimmunity in vivo, IL-17-producing T cells need to either be re-stimulated with IL-23 (McGeachy et al., 2009) or be generated with alternative cytokine combinations triggering IL-23 receptor (IL-23R) expression and signaling, such as IL-1β, IL-6, and IL-23 (Ghoreschi et al., 2010) or TGF-β3 and IL-6 (Lee et al., 2012). IL-23R controls the production of a proinflammatory transcriptional module in Th17 cells (Lee et al., 2012), including many essential effector cytokines (Codarri et al., 2011; El-Behi et al., 2011). IL-23R is thus a key determinant of the pathogenicity of Th17 cells and of autoimmunity in general (Cua et al., 2003). Understanding the mechanism by which IL-23R regulates the functional phenotype of pathogenic and non-pathogenic Th17 cells and how this balance is transcriptionally regulated is therefore critical for the selective inhibition of pathogenic Th17 cells in human autoimmune diseases.

Differentiation of Th17 cells is transcriptionally controlled by the lineage-defining transcription factor RORγt (Ivanov et al., 2006; Xiao et al., 2014), and the transcriptional networks controlling Th17 cell differentiation have recently been identified in large-scale transcriptomic analyses (Ciofani et al., 2012; Yosef et al., 2013). Our study predicted that Notch signaling and RBPJ, a downstream regulator of Notch signaling, were two of the 22 major nodes that positively regulated the development of Th17 cells (Yosef et al., 2013). This is consistent with previous studies that demonstrated that pharmacological and antibody-mediated inhibition of Notch ameliorated Th17-dependent autoimmune disease models (Bassil et al., 2011; Jurynczyk et al., 2008; Keerthivasan et al., 2011; Reynolds et al., 2011). Despite these detailed transcriptomic data, the molecular mechanism by which Notch regulates Th17 development has not been identified. In addition, which subtype of Th17 cells (pathogenic or non-pathogenic) is regulated by Notch signaling has not been addressed.

Here we demonstrate that the canonical Notch signaling molecule RBPJ in Th17 cells regulates the development of pathogenic and non-pathogenic Th17 cells. We show that RBPJ directly promotes the expression of IL-23R by binding and *trans*-activating the *Il23r* promoter and repressing anti-inflammatory IL-10 production in Th17 cells. Consistent with this observation is that RBPJ-deficient Th17 cells show a non-pathogenic Th17 transcriptional profile, that RBPJ deficiency in Th17 cells protects mice from the development of experimental autoimmune encephalomyelitis (EAE), and that IL-23R overexpression rescues this defect. We have therefore identified a transcription factor that controls the generation of pathogenic and non-pathogenic Th17 cells by directly driving IL-23R expression and repressing production of the anti-inflammatory cytokine IL-10.

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RESULTS

RBPJ Is Required for the Pathogenicity of Th17 Cells

IL-23R is essential for the pathogenicity of Th17 cells, but its transcriptional control is unknown. We previously identified Notch1 and RBPJ, which form the Notch signaling pathway, as predicted positive regulators of Th17 cell differentiation (Yosef et al., 2013). However, the exact role of Notch signaling in Th17 cells was not analyzed. In a time course expression analysis, RBPJ had high expression and was continuously upregulated in Th17 cells (Figures S1A–S1C). We therefore generated $CD4^{Cre}RBPJ^{fl/fl}$ mice and found that RBPJ deficiency in T cells did not affect Th17 differentiation in the presence of TGF-β1 and IL-6, a condition that induces non-pathogenic Th17 cells independent from IL-23 (Lee et al., 2012; Figure 1A). Under these non-pathogenic conditions, $CD4^{Cre}RBPJ^{fl/fl}$ Th17 cells instead showed a dramatic increase in IL-10 production (Figures 1A and 1B). When differentiated with IL-1β + IL-6 + IL-23, which generates pathogenic Th17 cells dependent on IL-23 (Ghoreschi et al., 2011), naive $CD4^{Cre}RBPJ^{fl/fl}$ cells showed a significant decrease in IL-17 expression (Figures 1C and 1D). In addition, such pathogenic Th17 cells from $CD4^{Cre}RBPJ^{fl/fl}$ mice began to produce IL-10 (Figure 1D), which is normally not observed under these conditions. Also, memory T cells from $CD4^{Cre}RBPJ^{fl/fl}$ mice that were stimulated with IL-23 showed an increase in IL-10 (Figure 1E), although IL-23 suppresses IL-10 production from wild-type Th17 cells (Lee et al., 2012; McGeachy et al., 2009). Lack of RBPJ thus affected the ability of Th17 cells to adequately respond to IL-23.

To test for the in vivo relevance of these findings, we induced the autoimmune disease model EAE in $CD4^{Cre}RBPJ^{fl/fl}$ mice using myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide. *$CD4^{Cre}RBPJ^{fl/fl}$* mice developed reduced peak severity and had faster recovery from EAE, but disease onset was unchanged (Figures 1F and 1G), consistent with RBPJ deficiency affecting the generation of pathogenic Th17 cells. We isolated CNS-infiltrating mononuclear cells from mice with EAE (Figure S1D) and found a decrease in IL-17A-producing and IL-17A/interferon γ (IFN-γ) double-producing $CD4^+$ T cells in the CNSs of $CD4^{Cre}RBPJ^{fl/fl}$ mice at the peak of EAE (Figure 1H), and IL-$17A^+IFN\text{-}\gamma^+$ T cells are generally considered to be pathogenic in EAE (Abromson-Leeman et al., 2009). There was no effect on IFN-γ-producing single-positive T cells and on the production of other cytokines in the CNS at early time points (Figure S1D). At the time of recovery, production of anti-inflammatory IL-10 by $CD4^+$ T cells in the CNS in $CD4^{Cre}RBPJ^{fl/fl}$ mice was increased (Figures 1I and 1J), whereas IL-23-dependent early generation of Th17 cells in draining lymph nodes in $CD4^{Cre}RBPJ^{fl/fl}$ mice was reduced (Figure S1E). We thus hypothesized that RBPJ promotes the pathogenicity of Th17 cells by altering their ability to respond to pro-inflammatory IL-23 and/or by repressing the production of anti-inflammatory IL-10.

RBPJ is known to have a role in the differentiation of other T helper cells (Amsen et al., 2009), and, accordingly, we observed an increased differentiation of Th1 cells (Figure S1F) and decreased generation of induced regulatory T cells (iTregs) (Figure S1G) in $CD4^{Cre}RBPJ^{fl/fl}$ mice. To circumvent these non-Th17-derived effects, we crossed $RBPJ^{fl/fl}$ mice to $IL17A^{Cre}$ mice to delete RBPJ only in IL-17A-producing cells. The differentiation of Th1 (Figure S2A) and iTreg (Figure S2B) cells was unchanged in $IL17A^{Cre}RBPJ^{fl/fl}$ cells, supporting that the loss of RBPJ is indeed restricted to IL-17-producing T cells in this mouse line.

When differentiating $IL17A^{Cre}RBPJ^{fl/fl}$ cells under non-pathogenic Th17 conditions (TGF-β1 + IL-6), RBPJ was indeed deleted (Figure S2C), and we observed an increase in IL-10 production but little effect on IL-17 production (Figures 2A and 2B). In contrast, under pathogenic differentiation conditions (IL-1β, IL-6, and IL-23), which require the effect of IL-23R signaling, IL-17A production was significantly reduced in $IL17A^{Cre}RBPJ^{fl/fl}$ cells (Figures 2C and 2D), whereas IL-10 protein was not detectable (data not shown). After immunization with $MOG_{35–55}$, $IL17A^{Cre}RBPJ^{fl/fl}$ mice developed less severe EAE than wild-type controls (Figures 2E and 2F), with a significant reduction in peak EAE severity but no change in disease onset. At the peak of EAE, fewer CNS-infiltrating $CD4^+$ cells produced IL-17 and IL-17/IFN-γ concurrently (Figure 2G) in $IL17A^{Cre}RBPJ^{fl/fl}$ mice, and there was a trend toward more IL-10^+ $CD4^+$ T cells at recovery that did not reach statistical significance (Figure 2H). Production of other cytokines was unchanged (Figure S2D). Moreover, the IL-23-dependent generation of Th17 cells was affected in the periphery (Figure S2E), and $IL17A^{Cre}RBPJ^{fl/fl}$ cells produced more IL-10 at recovery (Figure 2I). We next co-cultured $IL17A^{Cre}RBPJ^{fl/fl}$ cells with congenically marked wild-type cells and found a marked reduction in pathogenic Th17 cell differentiation only in $CD45.2^+$ $IL17A^{Cre}RBPJ^{fl/fl}$ cells in comparison with the cocultured $CD45.1^+$ wild-type cells, arguing for a cell-intrinsic effect of RBPJ in Th17 cells (Figure S2F). Thus, Th17 cell-restricted RBPJ deficiency also impairs their response to pro-inflammatory IL-23 and their pathogenicity.

IL-17A is also expressed by γδ T cells and innate lymphoid cells. To exclude the contribution of such cell types and to address the role of RBPJ specifically in pathogenic Th17 cells in vivo, we crossed $CD4^{Cre}RBPJ^{fl/fl}$ mice to 2D2 mice, which express a $MOG_{35–55}$-specific T cell receptor (TCR) transgene (Bettelli et al., 2003). Th17 cells differentiated in vitro under pathogenic Th17 conditions from $CD4^{Cre}RBPJ^{fl/fl}$ 2D2 mice and were unable to induce EAE upon adoptive transfer, in contrast to wild-type 2D2 Th17 cells (Figure 2J), although their IL-17 production before transfer was unchanged (Figure S2G). Our data obtained with this targeted deletion of RBPJ thus indicate that RBPJ is required specifically for the differentiation of pathogenic Th17 cells generated with IL-23 and that loss of RBPJ results in an increase in anti-inflammatory IL-10 and loss of pathogenicity in vivo. RBPJ deficiency thus causes the generation of non-pathogenic Th17 cells even under pathogenic Th17 differentiation conditions.

Notch signaling controls various aspects of $CD4^+$ T cell function, including chemokine receptor expression (Reynolds et al., 2011) and viability of memory T cells (Helbig et al., 2012; Maekawa et al., 2015). We did not find evidence for an altered expression of *Ccr6* (Figure S3A) or an altered survival of RBPJ-deficient Th17 cells in vitro (Figure S3C) or in vivo (Figures S3D and S3E), arguing for a specific effect of RBPJ on the pathogenicity of Th17 cells under IL-23-dependent Th17 differentiation conditions. We next investigated the mechanism of how RBPJ enhanced pathogenicity of Th17 cells.

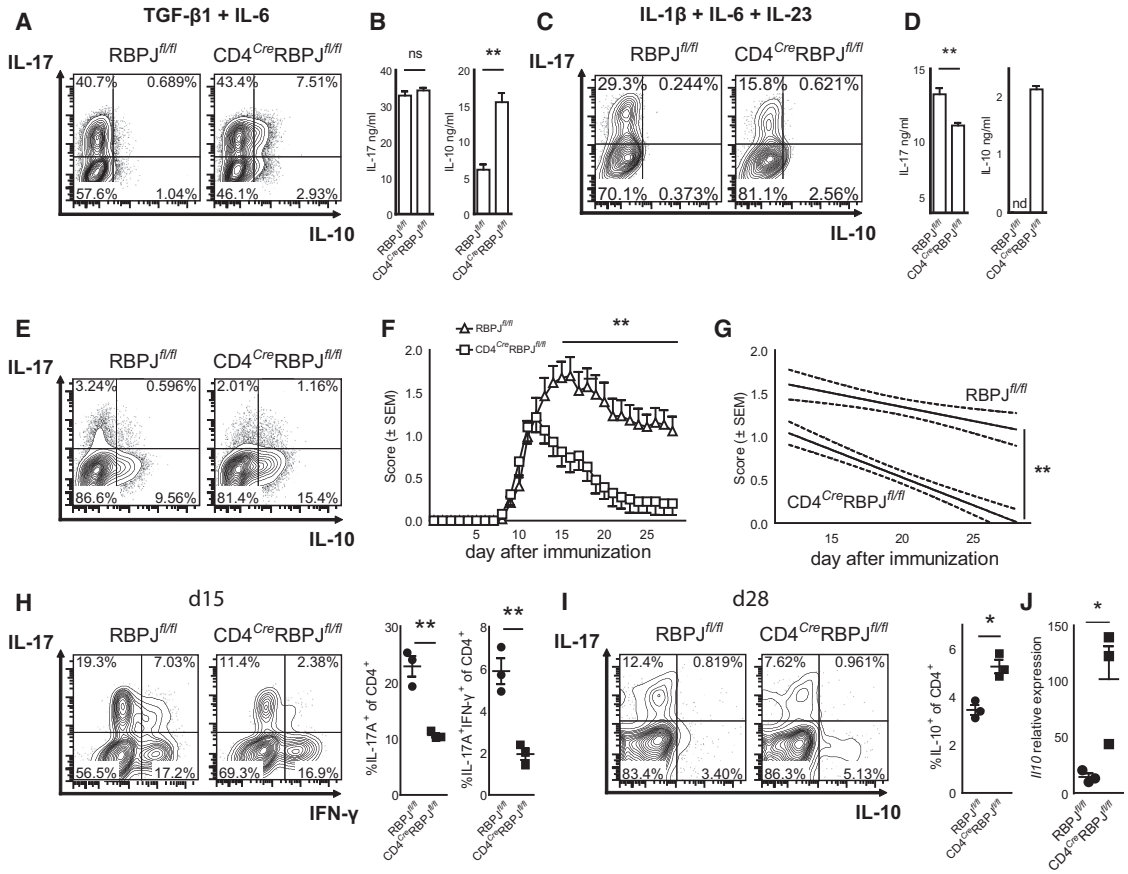

Figure 1. RBPJ in T Cells Maintains the Pathogenicity of Th17 Cells

(A) Naive $CD4^+CD62L^{high}CD44^{low}CD25^-$ T cells were sorted from $RBPJ^{flox/flox}$ and $CD4^{Cre}RBPJ^{flox/flox}$ mice, differentiated with TGF-β1 and IL-6, and analyzed by intracellular cytokine staining after 4 days.

(B) Cytokine ELISA of cultures described in (A). ns, not significant.

(C) Naive T cells were differentiated with IL-1β, IL-6, and IL-23.

(D) Cytokine ELISA of cultures described in (C). nd, not detected.

(E) $CD4^+CD62L^{low}CD44^{high}CD25^-$ memory T cells were sorted from $RBPJ^{flox/flox}$ and $CD4^{Cre}RBPJ^{flox/flox}$ mice, cultured with IL-23 for 5 days, and stained for intracellular cytokines.

One representative of five independent experiments is shown in (A)–(E).

(F) Active EAE was induced in $RBPJ^{flox/flox}$ (n = 26) and $CD4^{Cre}RBPJ^{flox/flox}$ (n = 26) mice by subcutaneous immunization with 100 μg of $MOG_{35–55}$ peptide in complete Freund's adjuvant together with intraperitoneal injection of pertussis toxin (200 ng) on days 0 and 2.

(G) Regression analysis of the clinical scores observed between days 12 and 28.

(H) CNS-infiltrating mononuclear cells were extracted and stained for intracellular cytokines on day 15 after immunization (d15). The percentage of CNS-infiltrating, cytokine-producing $CD4^+$ cells was calculated.

(I) The cytokine production by CNS-infiltrating $CD4^+$ cells was assessed on d28, and the proportion of IL-10-producing $CD4^+$ T cells was calculated.

(J) CNS-infiltrating $CD4^+$ T cells were sorted from $RBPJ^{flox/flox}$ (n = 3) and $CD4^{Cre}RBPJ^{flox/flox}$ (n = 3) mice, and relative *Il10* expression was measured by qPCR using GAPDH as a housekeeping gene.

Results are summed from three independent experiments in (F–I), with three individual mice analyzed per group in (H) and (I). *$p < 0.05$, **$p < 0.01$. See also Figure S1.

RBPJ Controls the Expression of IL-23R in Th17 Cells

We performed a multiplex gene expression analysis using nanostring technology, applying a codeset of 200 predefined Th17-related transcripts (Yosef et al., 2013) and comparing the results with a previously defined pathogenic/non-pathogenic Th17 gene signature (Lee et al., 2012). Non-pathogenic Th17 cells (TGF-β1 + IL-6) differentiated from $IL17A^{Cre}RBPJ^{fl/fl}$ mice showed a reduced expression of *Il23r* and increased expression of *Il10* (Figure 3A) that is part of a regulatory module in non-pathogenic Th17 cells. Conversely, the expression of *Il23r*, *Il22*, and *Casp1* was reduced in pathogenic Th17 cells (IL-1β + IL-6 + IL-23) differentiated from $IL17A^{Cre}RBPJ^{fl/fl}$ mice (Figure 3B). These transcripts were previously identified as part of a pro-inflammatory module in pathogenic Th17 cells (Lee et al., 2012). In addition, *Il17a*, which is the effector cytokine of pathogenic Th17 cells, was downregulated under both conditions in $IL17A^{Cre}RBPJ^{fl/fl}$ cells. RBPJ thus partly controls the expression of both pathogenic and non-pathogenic signature genes in Th17 cells.

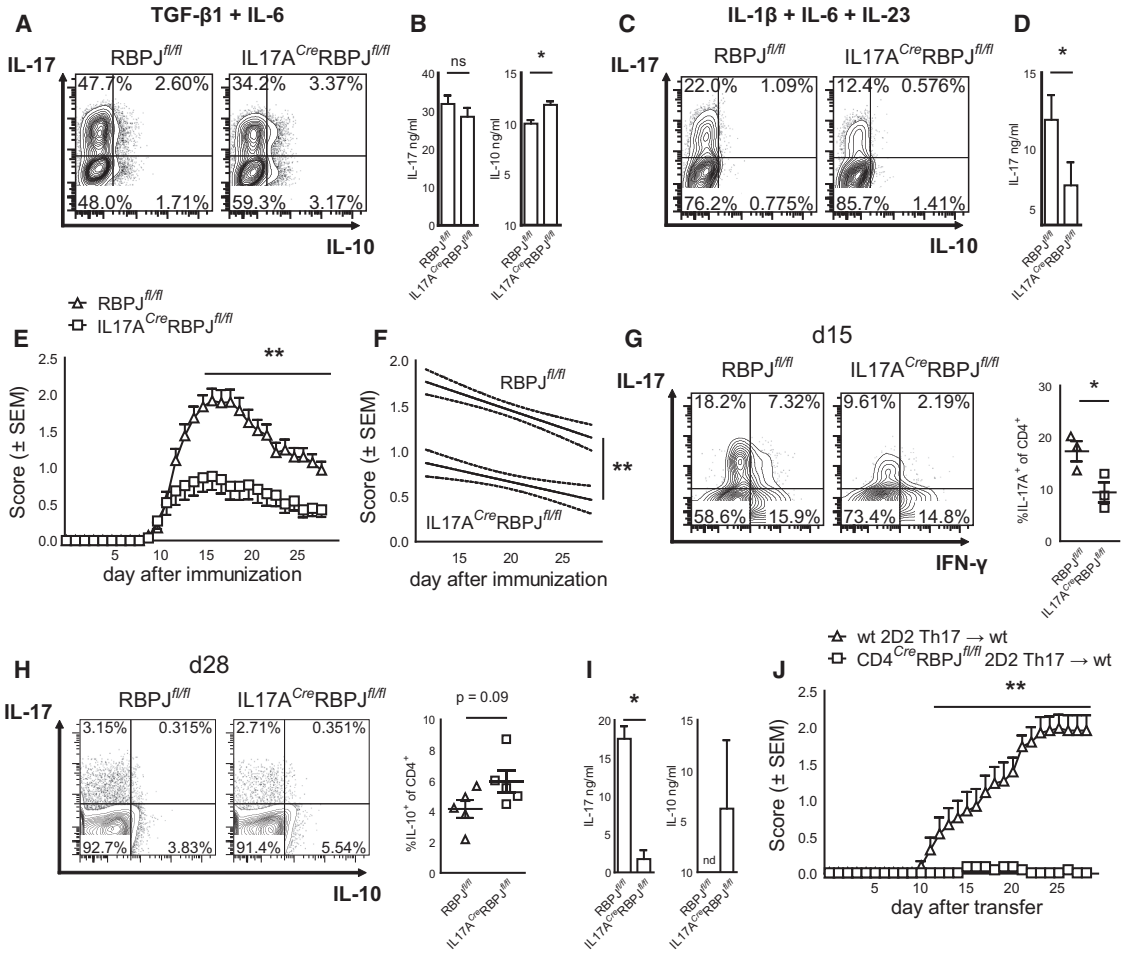

Figure 2. RBPJ Is Required in Th17 Cells to Maintain Their Pathogenicity

(A) Naive CD4$^+$CD62L^{high}CD44^{low}CD25$^-$ T cells were sorted from RBPJ$^{flox/flox}$ and IL17A^{Cre}RBPJ$^{flox/flox}$ mice, differentiated with TGF-β1 and IL-6, and stained for intracellular cytokines after 4 days.

(B) Cytokine ELISA of cultures described in (A).

(C) Naive T cells were differentiated in vitro with IL-1β, IL-6, and IL-23 and analyzed as in (A).

(D) Cytokine ELISA of cultures described in (C).

One representative of five independent experiments is shown in (A)–(D).

(E) RBPJ$^{flox/flox}$ (n = 43) and IL17A^{Cre}RBPJ$^{flox/flox}$ (n = 28) mice were immunized subcutaneously with MOG$_{35–55}$ peptide emulsified in complete Freund's adjuvant with intraperitoneal injections of pertussis toxin.

(F) Regression analysis of the clinical scores observed between days 12 and 28.

The data in (E) and (F) are summed from five independent experiments.

(G) CNS-infiltrating cells were extracted and analyzed by intracellular cytokine staining on day 15 after immunization.

(H) CNS-infiltrating lymphocytes were stained for intracellular cytokines on d28, and the percentage of IL-10$^+$ CD4$^+$ cells was calculated.

(I) Draining lymph node cells of mice on d15 were restimulated in vitro with 10 μg/ml MOG$_{35–55}$ peptide and IL-23 for 5 days. Cytokine concentrations were measured by ELISA.

(J) Naive CD4$^+$CD62L^{high}CD44^{low}CD25$^-$ T cells were sorted from wild-type 2D2 and CD4^{Cre}RBPJ$^{fl/fl}$ 2D2 mice and differentiated in vitro with TGF-β1, IL-6, and IL-23. Sfter 7 days of culture, 5 × 10^6 cytokine-producing T cells were intravenously injected into C57BL/6 wild-type recipients.

The data in (G–I) are summed from three independent experiments with 3–5 mice/group. *p < 0.05, **p < 0.01. See also Figure S2.

IL-23 promotes the pathogenicity and stability of Th17 cells. We next addressed whether RBPJ affects the stability of the Th17 cell lineage by crossing IL17A^{Cre}RBPJ$^{fl/fl}$ mice with *R26*^{RFP} mice, generating IL-17A fate reporter mice as described previously (Hirota et al., 2011; Figure S3F). Th17 cells differentiated in vitro from IL17A^{Cre}RBPJ$^{fl/fl}$R26^{RFP} mice had unchanged red fluorescent protein (RFP) expression (Figure 3C), indicating an unchanged initial commitment to the Th17 lineage. Instead, we observed a reduced production of IL-17 by CD4$^+$RFP$^+$ (i.e., lineage-committed Th17 cells) generated under pathogenic differentiation conditions (IL-1β + IL-6 + IL-23) but not under non-pathogenic conditions (TGF-β1 + IL-6) from IL17A^{Cre}RBPJ^{fllf} R26^{RFP} mice (Figure S3G). This indicates that RBPJ deficiency affects Th17 stability only under IL-23-dependent differentiation

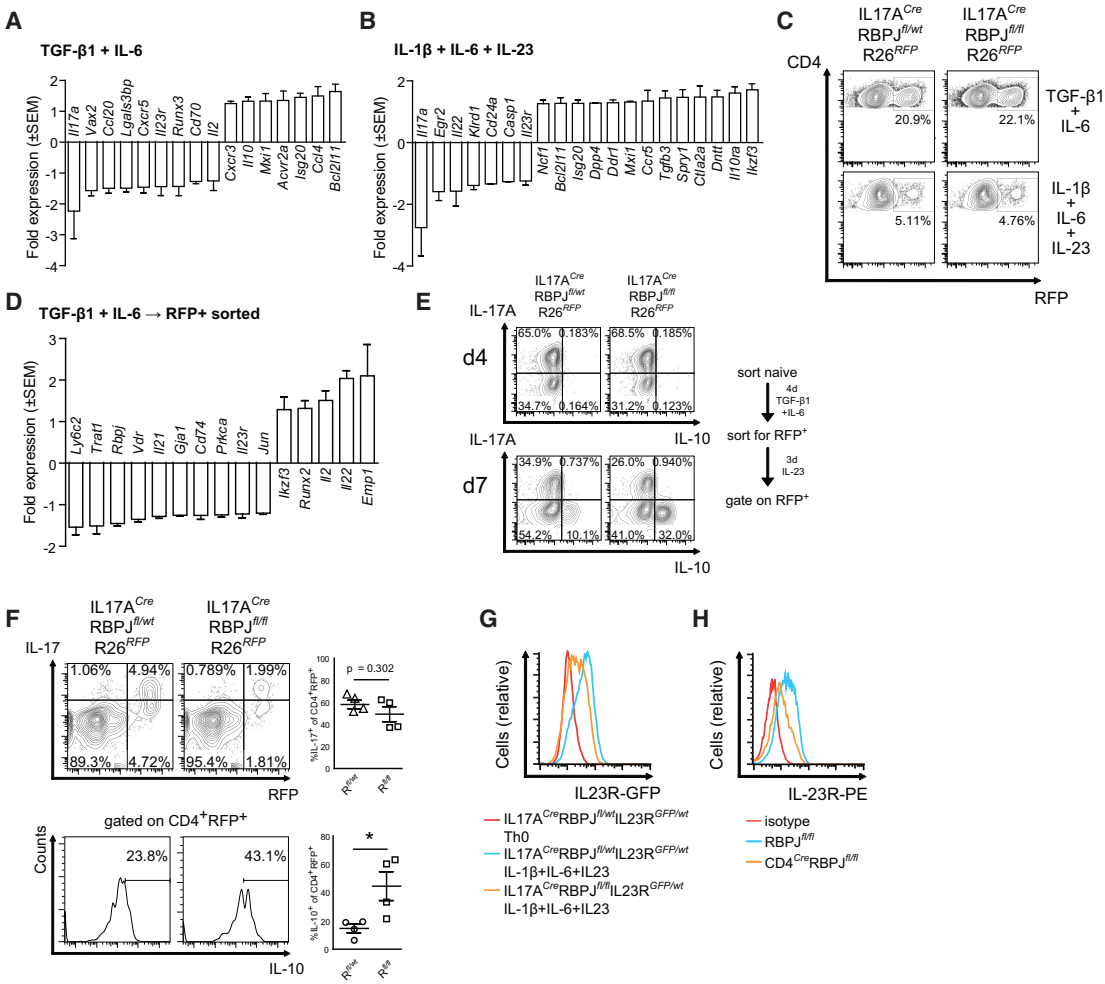

Figure 3. RBPJ-Deficient Th17 Cells Show Non-pathogenic Gene Expression and Are Unstable In Vitro Because of a Lack of IL-23R Expression

(A) Naive $CD4^+CD62L^{high}CD44^{low}CD25^-$ T cells were sorted from $IL17A^{Cre}RBPJ^{flox/wt}$ and $IL17A^{Cre}RBPJ^{flox/flox}$ mice and differentiated with TGF-β1 and IL-6. Expression of a pre-defined set of Th17-relevant genes was quantified using Nanostring nCounter after 4 days of culture.

(B)An identical experiment as in (A) using naive $CD4^+$ cells differentiated with IL-1β, IL-6, and IL-23.

Average values from three independent experiments with technical duplicates per sample are depicted in (A and B) as fold change of $IL17A^{Cre}RBPJ^{flox/flox}$ versus $IL17A^{Cre}RBPJ^{flox/wt}$ cells. Please note that *Il10* is upregulated 1.47-fold (data not shown) but does not reach the defined cutoff for minimum read counts; i.e., transcript abundance. The cutoff for differentially regulated genes was ≥1.25-fold regulation.

(C) Naive T cells from $IL17A^{Cre}RBPJ^{flox/wt}R26^{RFP}$ and $IL17A^{Cre}RBPJ^{flox/flox}$*R26*^{RFP} mice were differentiated in vitro with TGF-β1 and IL-6 or IL-1β, IL-6, and IL-23 for 4 days, and the proportion of $CD4^+RFP^+$ cells was assessed by flow cytometry.

(D) $CD4^+RFP^+$ cells were sorted from cultures differentiated with TGF-β1 and IL-6 described in (C) and analyzed for gene expression using Nanostring nCounter. Expression is depicted as fold-change of $IL17A^{Cre}RBPJ^{flox/flox}R26^{RFP}$ versus $IL17A^{Cre}RBPJ^{flox/wt}R26^{RFP}$ cells. The cutoff for differentially regulated genes was ≥1.25-fold, and changes were averaged from three independent experiments.

(E) Naive T cells were differentiated with TGF-β1 and IL-6 from $IL17A^{Cre}RBPJ^{flox/wt}R26^{RFP}$ and $IL17A^{Cre}RBPJ^{flox/flox}R26^{RFP}$ mice as in (D). Live $CD4^+RFP^+$ cells were sorted from the cultures and restimulated in vitro for 3 days with IL-23 only. Cytokine production was assessed before (d4) and after (d7) restimulation after gating on live RFP^+ cells.

(F) $IL17A^{Cre}RBPJ^{flox/wt}R26^{RFP}$ ($R^{fl/wt}$) and $IL17A^{Cre}RBPJ^{flox/flox}R26^{RFP}$ ($R^{fl/fl}$) mice were immunized with $MOG_{35–55}$, and, at the peak of EAE, draining lymph nodes cells were cultured in the presence of 1 μg/ml MOG and IL-23 for 5 days. Intracellular cytokine production was assessed after gating on $CD4^+$ (top) and $CD4^+RFP^+$ (bottom) cells. Data are summarized from four independent mice in three independent experiments.

(G) *Il23r* promoter-driven GFP fluorescence was quantified in naive T cells differentiated for 4 days without cytokines from $IL17A^{Cre}RBPJ^{flox/wt}IL23R^{GFP/wt}$ mice (red histogram) or with IL-1β, IL-6, and IL-23 from $IL17A^{Cre}RBPJ^{flox/wt}IL23R^{GFP/wt}$ mice (blue histogram) and $IL17A^{Cre}RBPJ^{flox/flox}IL23R^{GFP/wt}$ mice (orange histogram).

(H) Naive $CD4^+$ T cells were sorted from $RBPJ^{flox/flox}$ and $CD4^{Cre}RBPJ^{flox/flox}$ mice and differentiated with IL-1β, IL-6, and IL-23 for 4 days. IL-23R was detected by cell surface staining for IL-23R.

The data in (C), (E), (G), and (H) are representative of three independent experiments. *$p < 0.05$. See also Figure S3.

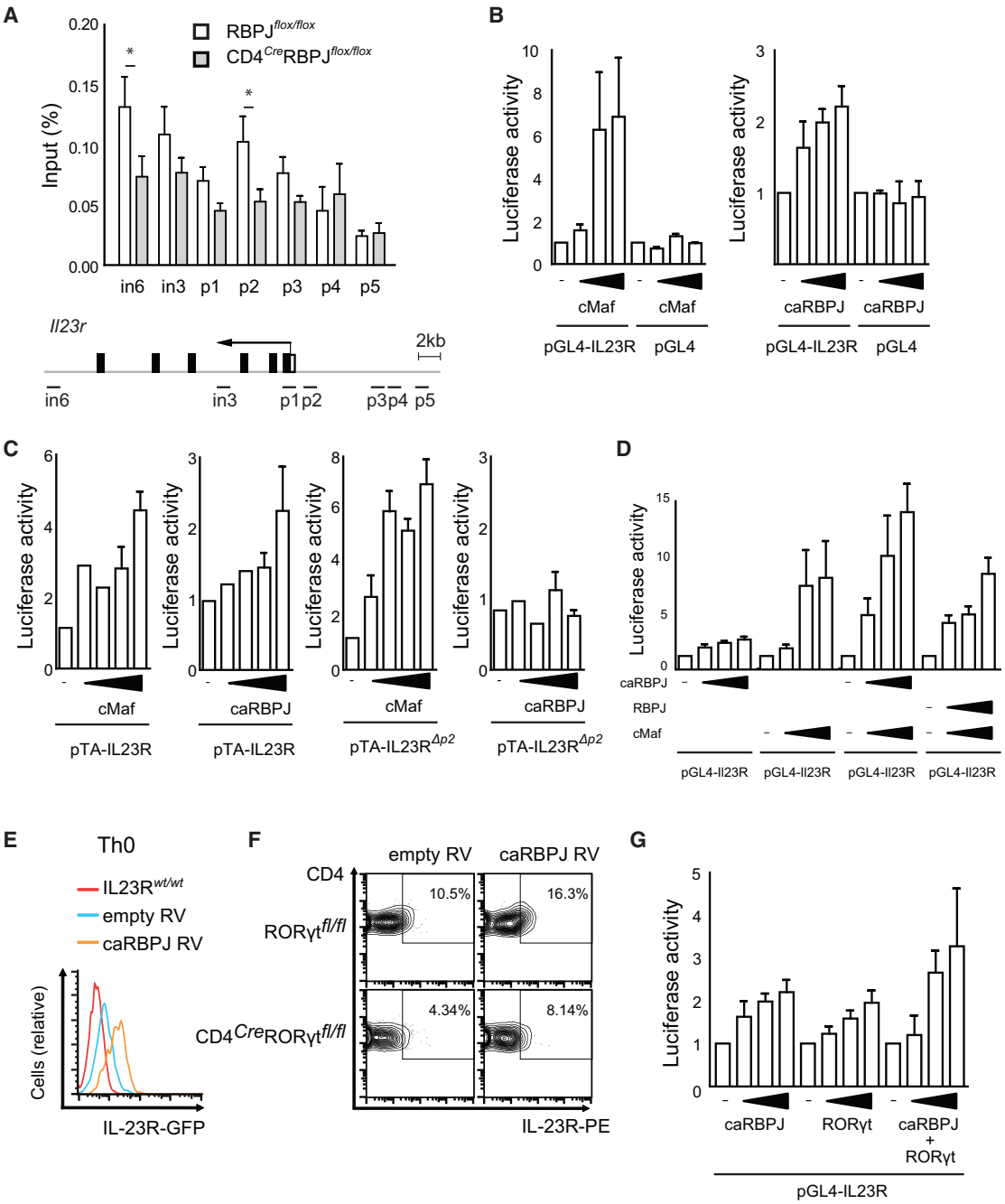

Figure 4. RBPJ Binds and *trans*-Activates the Il23r Promoter and Induces IL-23R Expression in Cooperation with RORγt

(A) Naive CD4$^+$ T cells were sorted from RBPJ$^{flox/flox}$ and CD4^{Cre}RBPJ$^{flox/flox}$ mice and differentiated with TGF-β1 and IL-6 for 4 days, followed by IL-23 for 3 days. Cross-linked protein-DNA complexes were immunoprecipitated using an anti-RBPJ antibody. DNA was purified and used for qPCR with primers spanning parts of the *Il23r* promoter predicted to contain RBPJ binding sites, indicated as horizontal bars in the schematic of the *Il23r* locus. in, intron; p, promoter. DNA binding was calculated as percentage of input DNA. One representative of three independent experiments with three replicate samples per immunoprecipitation (IP) is shown.

(B) *Il23r* promoter activity was measured in HEK293T cells transfected with a luciferase vector driven by 2.7 kb of the IL23R promoter (pGL4-IL23R) and increasing amounts of constructs encoding c-Maf and (ca)RBPJ (RBPJ-VP16).

(C) IL23r promoter activity was measured after transfection with a luciferase vector driven by 1.5 kb of the IL23R promoter (pTA-IL23R) and constructs encoding c-Maf and caRBPJ (RBPJ-VP16). Luciferase activity induced by c-Maf and RBPJ-VP16 was measured using a pTA-IL23R promoter construct with mutated RBPJ binding site p2 (pTA-IL23R^{Δp2}).

(D) pGL4-IL23R luciferase activity was measured after transfection with combinations of RBPJ, caRBPJ, and c-Maf constructs.

(legend continued on next page)

conditions. We also tested how efficiently the RBPJ$^{fl/fl}$ allele was deleted by the IL17A^{Cre} allele and found a marked reduction but no absence of RBPJ in CD4^{+}RFP^{+} cells (Figure S3H). Even the reduction of RBPJ expression in the IL17A^{Cre}RBPJ^{flox} mouse line is thus sufficient to impair an adequate response to IL-23 in Th17 cells.

We next sorted CD4^{+}RFP^{+} cells, which constitute lineage-committed Th17 cells, following in vitro differentiation with TGF-β1 + IL-6 and subjected them to nanostring analysis. Of note, expression of *Il23r* and *Il21* was lower in RBPJ-deficient CD4^{+}RFP^{+} cells compared with RBPJ-competent cells (Figure 3D). When we cultured these sorted CD4+RFP+ cells in the presence of IL-23 to test their stability, IL17A^{Cre}RBPJ^{fllflf}R26^{RFP} cells lost IL-17 and gained IL-10 production to a greater extent than controls (Figure 3E). We also used the IL17A^{Cre} RBPJ^{fllflf}R26^{RFP} mouse to address the lineage ontogeny of the CD4^{+}IL-10^{+} population we had observed in draining lymph nodes in the absence of RBPJ (Figure 2I). We found that a greater proportion of RFP^{+} cells was IL-10^{+} in IL17A^{Cre}RBPJ^{fllflf}R26^{RFP} mice (Figure 3F), indicating that the increased IL-10^{+} cells in the absence of RBPJ derive from Th17 cells. Together, these data indicate that RBPJ-deficient T cells are unaffected in their initial commitment to the Th17 lineage but lose stability and expression of members of the pathogenic transcriptional module, including IL-23R, while gaining expression of members of the regulatory module. We thus speculated that RBPJ helps to maintain the stability of the Th17 lineage by directly regulating the expression of IL-23R.

To address this, we examined the protein expression of IL-23R in pathogenic Th17 cells, which are known to express high levels of IL-23R (Lee et al., 2012). We crossed IL17A^{Cre}RBPJ$^{fl/fl}$ mice with IL23R^{GFP} reporter mice (Awasthi et al., 2009) and found that pathogenic Th17 cells from IL17A^{Cre}RBPJ$^{fl/fl}$IL23R^{GFP} mice showed reduced GFP expression compared with heterozygous IL17A^{Cre}RBPJ$^{fl/wt}$IL23R^{GFP} controls (Figure 3G). IL-23R surface levels were also reduced in pathogenic Th17 cells from CD4^{Cre}RBPJ$^{fl/fl}$ mice stained with an anti-IL-23R antibody (Figure 3H), confirming that RBPJ is, in fact, required to induce IL-23R protein expression in Th17 cells. A reduction in IL-23R expression was also observed in RBPJ-deficient non-pathogenic Th17 cells, albeit at lower levels because of the low expression of IL-23R under these conditions (data not shown). Furthermore, activation of the Notch pathway by overexpression of the intracellular domain of Notch receptors 1–3 (NICD) also induced surface expression of IL-23R in Th17 cells (Figure S4A). RBPJ and the Notch signaling pathway thus promote expression of the IL-23R, and RBPJ is consequently required for the stability and pathogenicity of Th17 cells.

RBPJ Binds and *trans*-Activates the Il23r Promoter in Cooperation with RORγt

We next addressed how RBPJ controls the expression of the IL-23R on a transcriptional level. We identified canonical RBPJ binding sites (Wang et al., 2011) in the proximal and distal promoter region (Figure 4A) and in both enhancer regions in intron 3 and intron 6 of the *Il23r* gene (Figure 4A), where Th17-related transcription factors such as RORγt bind (Xiao et al., 2014). To experimentally test for binding of RBPJ, we performed chromatin immunoprecipitation (ChIP) PCR of the predicted RBPJ binding sites. *Il23r* promoter regions—preferentially a region ~1,100 bp upstream of the transcriptional start site termed *p2*—showed enriched binding (Figure 4A), indicating that RBPJ indeed binds the *Il23r* promoter. Of note, the site with most enrichment (p2) is distinct from sites bound by other Th17 cell-related transcription factors (Ciofani et al., 2012; Xiao et al., 2014; Yosef et al., 2013).

Using a previously described IL23R promoter luciferase construct spanning 1.2 kb of the *Il23r* promoter (pTA-Il23r), constitutively active (ca) RBPJ (RBPJ-VP16) *trans*-activated the *Il23r* promoter (Figure 4B). c-Maf, which is known to activate *Il23r* expression (Sato et al., 2011), was used as a positive control. Mutating site *p2* in this construct abrogated its activation by caRBPJ but not by c-Maf (Figure 4C). This indicates that RBPJ directly controls transcription from the *Il23r* promoter dominantly through the p2 binding site, which is distinct and independent from other Th17 cell-related transcription factors. We next tested whether non-modified RBPJ exerted repressive effects on the *Il23r* promoter. Non-modified RBPJ had no effect on c-Maf-induced *Il23r* promoter activation, whereas caRBPJ had additive effects together with c-Maf on the *Il23r* luciferase construct (Figure 4D). Thus, the Notch pathway drives IL-23R expression through direct transcriptional control.

We next addressed whether RBPJ acted independently or in cooperation with the Th17 cell lineage-defining transcription factor RORγt, which was previously shown to bind the *Il23r* promoter at moderate levels (Ciofani et al., 2012; Xiao et al., 2014). Retroviral overexpression of caRBPJ induced IL23R^{GFP} reporter expression even in Th0 cells, which do not express RORγt (Figure 4E). Also, caRBPJ induced surface expression of IL-23R in the absence of RORγt in CD4^{Cre}RORγt$^{flox/flox}$ cells cultured in the presence of IL-1β + IL-6 + IL-23 (Figure 4F). In accordance, both caRBPJ and RORγt individually activated the *Il23r* promoter luciferase construct and, when combined, exhibited an additive effect in inducing IL-23R expression (Figure 4G). RBPJ did not interact directly with RORγt on the protein level (Figure S4B). Taken together, our data indicate that the Th17 transcriptional program is not required for RBPJ to promote the expression of IL-23R but that RBPJ and RORγt may

The data in (B–D) are representative of five independent experiments.

(E) Naive T cells were sorted from IL23R$^{GFP/wt}$ reporter mice and transduced with either an empty retrovirus (RV) (murine stem cell virus [MSCV]-Thy1.1) or a RV encoding caRBPJ (MSCV-RBPJ-VP16-Thy1.1) and cultured without cytokines. GFP expression was detected after gating on live CD4^{+}Thy1.1^{+} cells after 4 days of culture.

(F) Naive T cells were sorted from Rorγt$^{flox/flox}$ and CD4^{Cre}Rorγt$^{flox/flox}$ mice and transduced with either an empty RV or caRBPJ RV. Cell surface expression of IL-23R was analyzed after 4 days in culture after gating on live CD4^{+}Thy1.1^{+} cells.

(G) IL23r promoter activity was measured in HEK293T cells transfected with the pGL4-IL23R vector and increasing amounts of caRBPJ and Rorγt constructs or both.

Luciferase activities were calculated as fold empty vector in (B–D) and (G). One representative of three independent experiments is shown in (E)–(G). *$p < 0.05$.

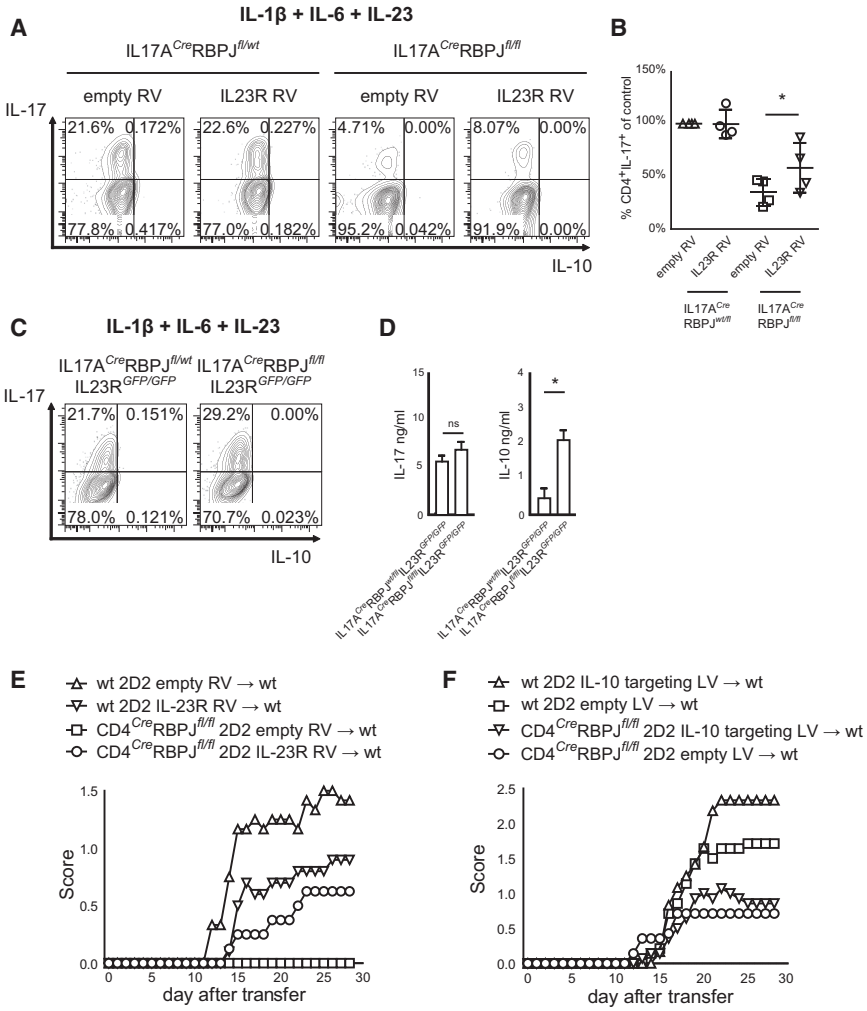

Figure 5. RBPJ Is Required to Promote IL-23R Expression Rather Than IL-17 in Th17 Cells

(A) Naive T cells from IL17A^{Cre}RBPJ$^{flox/wt}$ and IL17A^{Cre}RBPJ$^{flox/flox}$ mice were transduced with either an empty RV or with an RV encoding mouse IL-23R and stained for intracellular cytokines after differentiation with IL-1β, IL-6, and IL-23 for 4 days. Gating was on live CD4$^+$GFP$^+$ cells.

(B) The percentage of IL-17$^+$ cells was calculated, defining empty RV in IL17A^{Cre}RBPJ$^{flox/wt}$ cells as 100% in each experiment. Significance was calculated using Student's t test for paired samples.

(C) Naive T cells were sorted from IL17A^{Cre}RBPJ$^{flox/wt}$IL23R$^{GFP/GFP}$ and IL17A^{Cre}RBPJ$^{flox/flox}$IL23R$^{GFP/GFP}$ mice and differentiated with IL-1β, IL-6, and IL-23. Intracellular cytokine staining was performed after 4 days.

(D) Cytokine ELISA of cell culture supernatants described in (C).

One representative of three independent experiments is shown in (C) and (D).

(E) Naive T cells were sorted from wild-type 2D2 and CD4^{Cre}RBPJ$^{fl/fl}$ 2D2 mice and, during Th17 differentiation, were transduced with either an empty or IL-23R-encoding RV. After 7 days of culture, 6 × 10^6 cytokine-producing T cells were intravenously injected into C57BL/6 wild-type recipients. The clinical score was assessed daily.

(F) Naive T cells were sorted from wild-type 2D2 and CD4^{Cre}RBPJ$^{fl/fl}$ 2D2 mice and, during Th17 differentiation in vitro, transduced with a lentivirus (LV) encoding both Cas9 and an IL-10-targeting CRISPR guide RNA or Cas9 only (empty). After 7 days of culture, 8 × 10^6 cytokine-producing T cells were intravenously injected into C57BL/6 wild-type recipients.

The data in (A) are representative of and in (B) are summarized from four independent experiments. *$p < 0.05$.

work in a combinatorial manner to induce maximum expression of IL-23R but do not form a protein-protein complex.

The Dominant Function of RBPJ in Th17 Cells Is to Maintain IL-23R Expression

Our data indicate that RBPJ controls the generation and stability of pathogenic Th17 cells by directly driving IL-23R transcription. Previous studies, however, had reported that Notch controls transcription of IL-17A and RORγt itself, although not testing for changes in IL-23R expression (Keerthivasan et al., 2011; Mukherjee et al., 2009), and we replicated these findings (Figures S5A–S5C). We thus wanted to address whether RBPJ primarily controls Th17 cell differentiation through regulating IL-17A expression or through regulating IL-23R expression.

To this end, we retrovirally overexpressed IL-23R in IL17A^{Cre}RBPJ$^{fl/fl}$ Th17 cells during differentiation under pathogenic Th17 conditions (IL-1β + IL-6 + IL-23), where IL-23R is required to stabilize IL-17 production and is maximally expressed in wild-type cells. Overexpression of IL-23R partly rescued the loss of IL-17A production in IL17A^{Cre}RBPJ$^{fl/fl}$ cells (Figures 5A and 5B), although the effect was small. To further dissect whether RBPJ mainly controls expression of IL-17 or IL-23R, we generated IL-23R/RBPJ double-deficient mice by crossing IL17A^{Cre}RBPJ$^{fl/fl}$ mice with IL23R$^{GFP/GFP}$ mice that lack functional IL-23R at homozygosity (Awasthi et al., 2009). Pathogenic Th17 cells (IL-1β + IL-6 + IL-23) differentiated from IL-23R/RBPJ double-deficient IL17A^{Cre}RBPJ$^{fl/fl}$IL23R$^{GFP/GFP}$ mice showed no significant change in IL-17 production compared with IL-23R deficient/RBPJ-competent IL17A^{Cre}RBPJ$^{fl/wt}$IL23R$^{GFP/GFP}$ mice (Figures 5C and 5D). This was in contrast to the reduced differentiation of pathogenic Th17 cells we had observed in IL17A^{Cre}RBPJ$^{fl/fl}$ mice (Figures 2C and 2D) and indicates that RBPJ deficiency impairs Th17 cell differentiation only in the presence of IL-23R. Similar effects were observed in CD4^{Cre}RBPJ$^{fl/fl}$IL23R$^{GFP/GFP}$ mice (data not shown).

To address the importance of RBPJ in driving IL-23R expression versus IL-17 expression in vivo, we retrovirally overexpressed IL-23R in pathogenic 2D2 (expressing a MOG$_{35–55}$-specific TCR) Th17 cells before transferring them into wild-type hosts. We found that overexpression of IL-23R partially rescued the protection from EAE conferred by RBPJ deficiency (Figure 5E). Conversely, knockout of IL-10 in 2D2 Th17 cells using

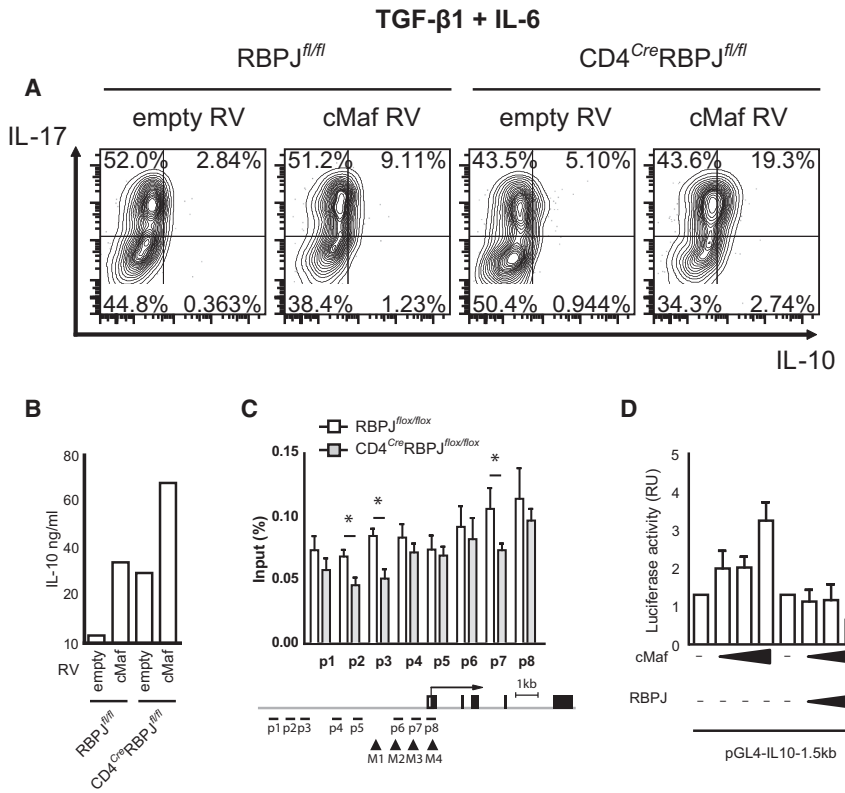

Figure 6. RBPJ Represses c-Maf-Induced IL-10 Production in Th17 Cells

(A) Sorted naive RBPJ$^{flox/flox}$ and CD4^{Cre}RBPJ$^{flox/flox}$ CD4^{+} T cells were transduced with either empty MSCV-GFP or MSCV-cMaf-GFP RV, cultured with TGF-β1 and IL-6 for 4 days, and analyzed by intracellular cytokine staining.

(B) Cell culture supernatants from cultures described in (A) were analyzed by ELISA.

(C) Naive CD4^{+} T cells were sorted from RBPJ$^{flox/flox}$ and CD4^{Cre}RBPJ$^{flox/flox}$ mice and differentiated in vitro with TGF-β1 and IL-6 for 4 days, followed by IL-23 for 3 days. Cross-linked protein-DNA complexes were immunoprecipitated with an anti-RBPJ antibody. DNA was purified and used for qPCR spanning predicted RBPJ binding sites in the IL-10 promoter. DNA binding was calculated as percentage of input DNA. Predicted RBPJ-binding sites are indicated as p1–p8, and previously identified MARE sites (Apetoh et al., 2010) are indicated as M1–M4. One representative of four independent experiments is shown, with three replicate IPs per sample.

(D) Il10 promoter activity was measured in HEK293T cells transfected with the pGL4-IL10-1.5kb luciferase vector and constructs encoding c-Maf and RBPJ. Luciferase activities were calculated as fold change compared with the empty vector.

One representative of three independent experiments is shown in (A), (B), and (D). *p < 0.05.

a clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) lentiviral system did not rescue the protection from EAE conferred by RBPJ deficiency (Figure 5F), and neutralization of IL-10 did not revert protection from actively induced EAE in CD4^{Cre}RBPJ$^{fl/fl}$ mice (Figure S5D). Together, these data indicate that driving IL-23R expression, not driving IL-17 or repressing IL-10, is the dominant function of RBPJ in Th17 cells in vivo.

Of note, RBPJ-deficient IL17A^{Cre}RBPJ$^{fl/fl}$IL23R$^{GFP/GFP}$ pathogenic (IL-1β + IL-6 + IL-23) Th17 cells also produced higher amounts of IL-10 detected by ELISA, although not by intracellular cytokine staining, than RBPJ-competent IL17A^{Cre}RBPJ$^{fl/wt}$ IL23R$^{GFP/GFP}$ Th17 cells (Figure 5D). Thus, RBPJ deficiency enhances production of the non-pathogenic cytokine IL-10 even in the absence of IL-23R. This indicated that RBPJ may also serve as a repressor of IL-10 production in Th17 cells in vitro independently from its effect on IL-23R expression and although this is not its dominant function in vivo (Figures 5E and 5F).

RBPJ Serves as a Repressor of c-Maf-induced IL-10 Production in Th17 Cells

The transcription factor c-Maf is a known inducer of IL-10 in type 1 regulatory (Tr1) cells induced by IL-27 (Apetoh et al., 2010) and in Th17 cells (Bauquet et al., 2009; Xu et al., 2009), and we thus speculated that RBPJ may act as a repressor of the regulatory module, including IL-10 controlled by c-Maf in Th17 cells. We overexpressed c-Maf in Th17 cells generated in the presence of TGF-β1 + IL-6 and found that, in CD4^{Cre}RBPJ$^{fl/fl}$ cells, c-Maf overexpression induced more IL-10 than in wild-type cells (Figures 6A and 6B). We conclude that RBPJ may serve as a transcriptional repressor of c-Maf-induced IL-10 expression in Th17 cells. The IL-10 genomic locus contains multiple predicted RBPJ binding sites, and ChIP-PCR confirmed binding of RBPJ to the IL-10 locus in Th17 cells (Figure 6C). Of note, two previously identified c-Maf response elements (MAREs) in the *Il10* promoter (Apetoh et al., 2010) were located within a 150-bp range of predicted RBPJ binding sites (Figure 6C). We did not observe protein-protein interaction between RBPJ and c-Maf (Figure S6). Using a luciferase assay, we observed that non-modified RBPJ inhibited c-Maf-induced activation of the IL-10 promoter (Figure 6D), indicating that RBPJ can indeed repress IL-10 production induced by c-Maf. In conclusion, we identify RBPJ as an essential transcriptional regulator controlling the generation of pathogenic and non-pathogenic Th17 cells by promoting the expression of IL-23R and repressing the production of IL-10. However, the effects of RBPJ on IL-23R are dominant in promoting the pathogenic phenotype of Th17 cells in vivo.

DISCUSSION

Our previously generated transcriptional network of Th17 cells implicated RBPJ, the canonical Notch signaling molecule, as a positive regulator of Th17 differentiation (Yosef et al., 2013). Although the previous study had relied on analysis and perturbation on the mRNA level, here we examined RBPJ in Th17 cells with technically more stringent approaches by testing for the

in vitro differentiation and the in vivo function of Th17 cells in the absence of RBPJ and identifying the mechanisms by which RBPJ controls Th17 cell differentiation. Using different genetic approaches, we observed that RBPJ deficiency does not affect the early stage of Th17 cell differentiation but, rather, affects the stabilization stage when Th17 cells are rendered pathogenic through the effects of IL-23. We further demonstrate that RBPJ is required to maintain a pathogenic state of Th17 cells by driving expression of IL-23R and by repressing production of the anti-inflammatory cytokine IL-10. RBPJ thus serves a highly specific purpose in Th17 cells: to promote the pathogenicity of Th17 cells by promoting the expression of members of the pro-inflammatory module and repressing expression of the regulatory module. Therefore, this observation expands the array of functions of the Notch signaling pathway in the differentiation of various T cell subsets (reviewed in Amsen et al. (2009).

Consistent with our observations, pharmacological inhibition of Notch cleavage or Notch ligand binding was previously shown to ameliorate EAE (Bassil et al., 2011; Jurynczyk et al., 2008; Keerthivasan et al., 2011; Maekawa et al., 2015; Reynolds et al., 2011), but the mechanisms were not elucidated. Here we did not rely on pharmacological inhibitors but, rather, used genetic approaches to ablate the canonical Notch signaling molecule RBPJ in a cell-type-specific manner in Th17 cells. Driving Cre expression from the endogenous *Il17a* promoter or from an *Il17f* bacterial artificial chromosome/clone (BAC) transgenic line (Croxford et al., 2009; Hirota et al., 2011) allows deletion of RBPJ in all IL-17-expressing cells. RBPJ deletion by *Il17a* promoter-driven Cre expression was well detectable but did not reach 100% deletion, indicating that RBPJ mRNA may persist after recombination of the *Rbpj* genomic locus, providing a potential explanation for the more subtle in vitro phenotype in $IL17A^{Cre}RBPJ^{fl/fl}$ than in $CD4^{Cre}RBPJ^{fl/fl}$ cells. Therefore, combining our data derived from the $CD4^{Cre}$ and $IL17A^{Cre}$ lines and using the 2D2 adoptive transfer model, in which RBPJ-deletion was specifically targeted to CNS-specific pathogenic Th17 cells, and by overexpressing IL-23R, we demonstrate that RBPJ is required for the pathogenicity of Th17 cells by directly controlling IL-23R expression. IL-23 did not induce the expression of RBPJ, indicating that RBPJ is an important upstream controller of IL-23R expression but does not form a positive feedforward loop with IL-23R signaling.

Canonical Notch signaling serves a multitude of functions in T cells. It controls thymic development of T cells (Tanigaki and Honjo, 2007), the differentiation of several T helper cell lineages (Amsen et al., 2009; Bailis et al., 2013), the longevity of helper cells in vitro (Helbig et al., 2012), the expression of chemokine receptors in vitro (Reynolds et al., 2011), and the survival of memory T cells in a re-stimulation paradigm in vivo (Maekawa et al., 2015). However, by using Th17 cell-specific deletion of RBPJ, we did not find evidence for survival-related effects in our experimental setting (Figures S5A–S5E), and, by overexpressing IL-23R (Figure 5), we demonstrate that RBPJ regulates Th17 pathogenicity specifically by controlling IL-23R expression. By generating RBPJ/IL-23R double-deficient mice and by overexpressing IL-23R in Th17 cells transferred in vivo, we also demonstrate that the *dominant* function of RBPJ in Th17 cells in vivo is to promote the expression of IL-23R and the pathogenic functional state of Th17 cells.

Th17 cells do not all induce autoimmune disease. It is becoming increasingly clear that Th17 cells mediate tissue homeostasis at mucosal surfaces. In fact, IL-17 production by T cells may serve a protective function in the gut without inducing tissue inflammation (O'Connor et al., 2009), and gut-resident Th17 cells partly resemble non-pathogenic Th17 cells that co-produce IL-17 and IL-10 (Esplugues et al., 2011; Gagliani et al., 2015). These different functional states of Th17 cells may have evolutionarily arisen to defend against specific pathogens, as shown in humans (Zielinski et al., 2012). Identifying the factors controlling the balance between such pathogenic and non-pathogenic Th17 cells may provide the key to understanding how to inhibit pathogenic Th17 cells that induce tissue inflammation and autoimmune diseases but spare those that maintain gut barrier functions and promote tissue homeostasis. Our data indicate that the Notch signaling pathway may be one of the key checkpoints in determining the balance between the pathogenic and non-pathogenic states of Th17 cells. The biological importance is further strengthened by genetic linkage of the RBPJ genomic locus with rheumatoid arthritis (Stahl et al., 2010), in which Th17 cells are important (Miossec and Kolls, 2012).

In the canonical Notch signaling pathway, RBPJ is bound to DNA and acts as a transcriptional repressor in the absence of NICD (Amsen et al., 2009). Only after ligand binding will NICD *trans*-locate to the nucleus and convert RBPJ to a transcriptional co-activator (Amsen et al., 2009). RBPJ can thus exert dual functions as either a transcriptional repressor or inducer depending on the nuclear availability of NICD and, thus, on the activity of the Notch pathway. This dual function of RBPJ may account for the induction of IL-10 by NICD in Th1 cells (Kassner et al., 2010; Rutz et al., 2008) but inhibition of IL-10 by RBPJ, which we describe here. In fact, overexpression of NICD also induces IL-10 in Th17 cells (data not shown) in accordance with the dual role of RBPJ as transcriptional activator or repressor. We describe here that the *Il10* and *Il23r* loci respond differently to RBPJ-mediated repression in the absence of Notch pathway activation. RBPJ does not alter cMaf-induced transcription of the *Il23r* locus but inhibits cMaf-induced transcription of the *Il10* locus (Figure 6). The activity of the Notch signaling pathway may thus serve as a transcriptional switch to determine how c-Maf and potentially other transcription factors control the transcription of IL-10 and IL-23R expression in Th17 cells.

We describe here a dual function of RBPJ in Th17 cells as both promoting pathogenicity by inducing IL-23R expression and inhibiting the production of anti-inflammatory IL-10. We therefore identify RBPJ as a transcriptional switch in Th17 cells and provide a mechanistic explanation of how RBPJ can promote the generation of pathogenic Th17 cells and inhibit the generation of non-pathogenic Th17 cells. Our identification of RBPJ as a regulator that can differentially affect pathogenic and non-pathogenic Th17 cells will offer targets to inhibit pathogenic Th17 cells but spare beneficial tissue-protective Th17 cells.

EXPERIMENTAL PROCEDURES

Animals and EAE

$CD4^{Cre}$ mice were from Taconic. B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ mice (named CD45.1) and B6;129S6-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J mice (Madisen et al., 2010) (named $R26^{RFP}$), were from The Jackson Laboratory. $IL17A^{Cre}$ (Hirota et al., 2011), $RBPJ^{flox}$ (Tanigaki et al., 2002), $IL23R^{GFP}$ (Awasthi et al., 2009), and 2D2 (Bettelli et al., 2003) mice were described previously. All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School. Age- and sex-matched mice (7–9 weeks old) were immunized for EAE as described previously (Xiao et al., 2014). IL-10 neutralization in vivo was performed with 500 μg of low endotoxin azide-free rat immunoglobulin G_1 (IgG_1) or anti-IL-10 antibody (clone JES5-16E3, BioLegend) intraperitoneally on days 0, 2, and 7 as described previously (McGeachy et al., 2007). Adoptive transfer of EAE using Th17 differentiated 2D2 cells was performed as described previously (Jäger et al., 2009). For transduction before adoptive transfer, 2D2 cells were spin-infected (600 × *g* for 45 min at 25°C) with retroviral supernatants on day 1 after plating with Polybrene (8 μg/ml) or with lentiviral supernatants. Cells were not sorted or selected for transduction before transfer.

$CD4^+$ T Cell Differentiation and Retroviral Transduction

$CD4^+CD44^{low}CD62L^{high}CD25^-$ naive CD4+ T cells were purified by flow cytometry following magnetic activated cell sorting (MACS) bead isolation of $CD4^+$ cells. Naive T cells were activated with plate-bound anti-CD3 (2 μg/ml, 145-2C11) and anti-CD28 (2 μg/ml, PV-1) antibody in 96-well or 24-well plates. In vitro T cell differentiation was performed for 96 hr. For non-pathogenic Th17 cell differentiation, cultures were supplemented with IL-6 (20 ng/ml) and TGF-β1 (2 ng/ml). For pathogenic Th17 cell differentiation, cultures were supplemented with IL-1β (20 ng/ml), IL-6 (20 ng/ml), and IL-23 (10 ng/ml). Retrovirus-containing supernatants were produced by transiently transfecting HEK293T producer cells with retroviral packaging constructs (PCL-Eco, gag/pol) together with retroviral expression plasmids using Fugene HD (Roche). Culture supernatants were harvested after 72 hr, supplemented with Polybrene (8 mg/ml), and added to sorted naive T cells previously stimulated for 24 hr (1×10^5 cells/well, 96-well plate, plate-bound anti-CD3/CD28, both 2 μg/ml, and indicated cytokines). Cultures were centrifuged at 600 × *g* for 45 min at 25°C. After an additional 72 hr of culturing, intracellular cytokine staining was performed. Gating was on $CD4^+7AAD^-Thy1.1^+$ cells. The CRISPR/Cas9 was described previously (Parnas et al., 2015). Cas9 was driven by a modified embryonal fyn-associated substrate (EF-S) promoter, and single guide RNA (sgRNA) was driven by a mammalian U6 promoter. Packaging was with a vesicular stomatitis virus G protein (VSV-G) envelope.

Generation of Constructs and Luciferase Assays

The pTA-IL23R plasmid was described previously (Sato et al., 2011). The pTA-$IL23R^{\Delta p2}$ plasmid was generated by PCR site-directed mutagenesis modifying the GTGGGAA sequence at −1,151 bp from the *Il23r* transcriptional start site (TSS) to GTAAAAA. The pGL4-IL23R construct was generated by PCR, amplifying a 2.7-kb part of the *Il23r* promoter (−2.714 to 0 bp from TSS) from a mouse BAC clone (RPCI-23-34P19, Bacterial artificial chromosome [BAC]/P1-derived artificial chromosome [PAC] Children's Hospital Oakland Research Institute [CHORI] repository) and cloning the product into the pGL4.10 vector (Promega) using NheI/EcoRV sites. The coding part of all constructs was verified by sequencing. Luciferase assays were performed in HEK293T cells using the Dual-Luciferase reporter assay system (Promega). The firefly luciferase activity was normalized to *Renilla* luciferase activity and is expressed as relative values compared with the empty vector control.

Statistics

The clinical score of EAE was analyzed by Fisher's exact test. All other results were analyzed by Student's t test. $p < 0.05$ was considered significant (*$p < 0.05$; **$p < 0.01$). Statistical analysis was performed using GraphPad Prism 5.0.

For a detailed description of all experimental procedures, see the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.088.

AUTHOR CONTRIBUTIONS

G.M.z.H., C. Wu, C. Wang, L.C., M.P., Y.L., and S.X. performed the experiments. W.E., S.X., and A.R. provided reagents. G.M.z.H., C. Wu, and V.K.K. conceived the study. G.M.z.H. and V.K.K. wrote the manuscript.

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