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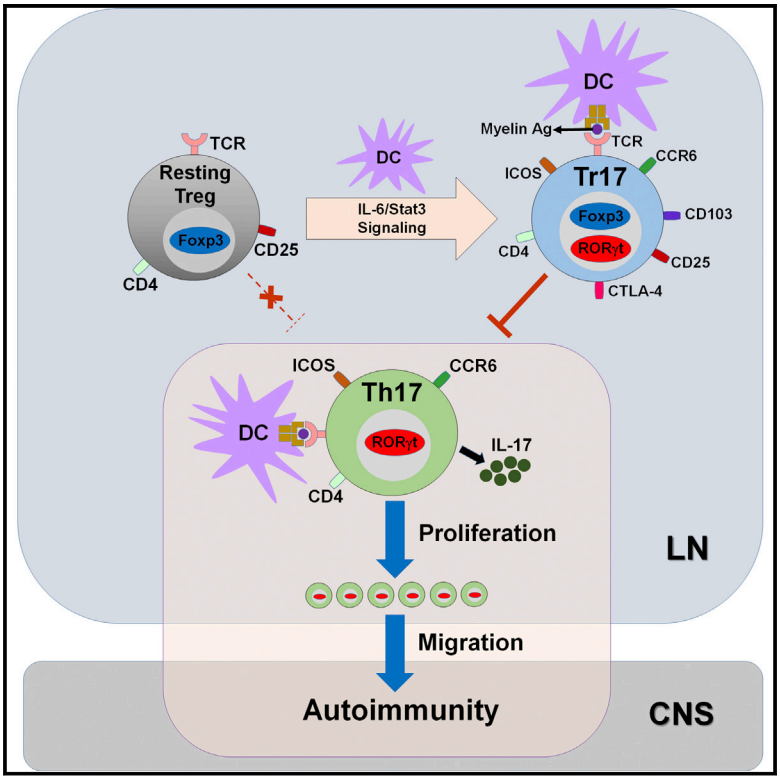
Generation of RORγt$^+$ Antigen-Specific T Regulatory 17 Cells from Foxp3$^+$ Precursors in Autoimmunity

Graphical Abstract



Highlights

- RORγt$^+$Foxp3$^+$CD4$^+$ Tr17 cells are found in lymph nodes after immunization
- Tr17 cells upregulate Treg cell-associated effector molecules and CCR6
- Tr17 cells originate from resting Treg cells via Stat3 signaling
- Tr17 cells potentially modulate Th17-cell-driven CNS autoimmunity

Authors

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In Brief

Kim et al. find that RORγt$^+$Foxp3$^+$ T regulatory 17 (Tr17) cells are induced in lymph nodes after immunization. Tr17 cells are generated from thymic Treg cells in an antigen-specific manner through Stat3 signaling. Their data suggest that Tr17 cells represent antigen-specific effector Treg cells that can regulate Th17-cell-dependent autoimmunity.

Data and Software Availability

GSE103319

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Cell Reports
Article

Generation of RORγt$^+$ Antigen-Specific T Regulatory 17 Cells from Foxp3$^+$ Precursors in Autoimmunity

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SUMMARY

Th17 cells are potent mediators in autoimmune diseases, and RORγt is required for their development. Recent studies have shown that RORγt$^+$ Treg cells in the gut regulate intestinal inflammation by inhibiting effector T cell function. In the current study, we report that RORγt$^+$ Treg cells were also found in lymph nodes following immunization. Not only distinct from intestinal RORγt$^+$ Treg cells in their transcriptomes, peripheral RORγt$^+$ Treg cells were derived from Foxp3$^+$ thymic Treg cells in an antigen-specific manner. Development of these RORγt$^+$ Treg cells, coined T regulatory 17 (Tr17) cells, depended on IL-6/Stat3 signaling. Tr17 cells showed suppressive activity against antigen-specific effector T cells in vitro. In addition, Tr17 cells efficiently inhibited myelin-specific Th17-cell-mediated CNS auto-inflammation in a passive EAE model. Collectively, our study demonstrates that Tr17 cells are effector Treg cells that potentially restrict autoimmunity.

INTRODUCTION

Interleukin-17 (IL-17)-producing T helper cells (Th17 cells) have been associated with the progression of autoimmune diseases (Dong, 2008). An orphan nuclear hormone receptor, RORγt, is required for the development of Th17 cells; RORγt-deficient T cells are impaired in Th17 differentiation (Ivanov et al., 2006). Accordingly, in an experimental autoimmune encephalomyelitis (EAE) model, the severity of autoimmunity in the CNS is significantly decreased in RORγt-deficient mice compared with control mice, along with the decreased Th17 cells in the CNS, indicating that RORγt-dependent Th17 generation is critical for the development of CNS autoimmunity (Ivanov et al., 2006).

Foxp3$^+$ regulatory T cells (Treg cells) are essential for preventing autoimmunity against self-antigens and preventing tissue destruction resulting from excessive immune responses. Recent studies have shown that Treg cells differentiate into distinct subsets to inhibit distinct T helper cell subsets (Campbell and Koch, 2011; Sakaguchi et al., 2013). For example, T-bet$^+$CXCR3$^+$ Treg cells are required for the inhibition of Th1 cell-mediated inflammation, whereas *Irf4* expression in Foxp3$^+$ Treg cells is necessary to prevent Th2 cell-mediated spontaneous immunopathology (Koch et al., 2009; Zheng et al., 2009). In addition, CXCR5$^+$ follicular regulatory T cells (Tfr cells), whose development is dependent on Bcl6, are critical for regulating germinal center reactions mediated by CXCR5$^+$Bcl6$^+$ follicular helper T cells (Tfh cells) (Chung et al., 2011; Linterman et al., 2011). Moreover, selective Stat3 or IL-10R deficiency in Treg cells led to dysregulation of Th17 cell responses and subsequent development of inflammation in Th17-cell-rich mucosal tissues such as the lung, skin, and intestine, suggesting that IL-10-mediated Stat3 activation in Treg cells is critical for Th17 regulation (Chaudhry et al., 2009, 2011).

RORγt$^+$Foxp3$^+$CD4$^+$ T cells or IL-17-producing Foxp3$^+$ Treg cells have been demonstrated both in mice and humans (Du et al., 2014). However, whether RORγt$^+$Foxp3$^+$CD4$^+$ T cells represent a subset of Treg cells, a precursor of Th17 cells, or an intermediate differentiation stage with a bipolar potential to develop into either Treg cells or Th17 cells has been a matter of debate. Human studies showed that CD4$^+$CD25^{hi} CD45RA$^-$HLA-DR$^-$ Treg cells or CD4$^+$CD25^{hi}CCR6$^+$ Treg cells produce IL-17, but their suppressive activity against effector cells is maintained unless the stimulation is too strong (Beriou et al., 2009; Voo et al., 2009). A mouse study also found that RORγt$^+$Foxp3^{int} cells that highly express membrane-bound transforming growth factor β (TGF-β) can regulate autoimmune diabetes (Tartar et al., 2010). In contrast, several mouse studies

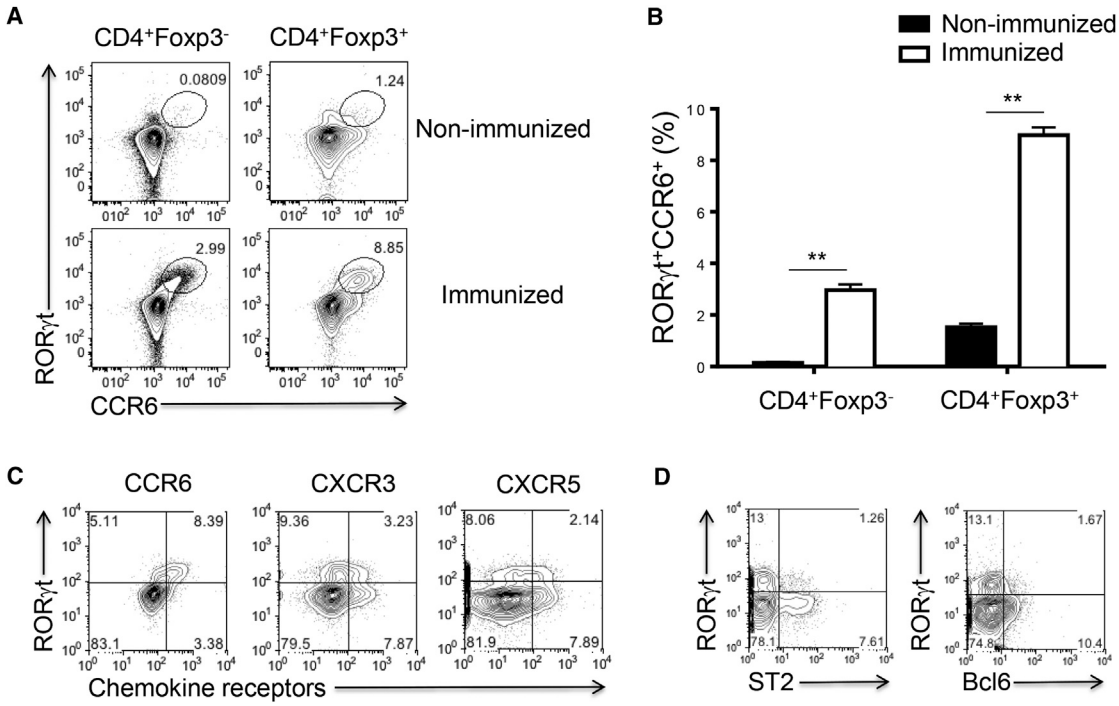

Figure 1. Induction of RORγt$^+$CCR6$^+$ Treg Cells after Immunization

C57BL/6 mice were s.c. immunized with MOG/CFA. Seven days later, dLNs were isolated for analysis.

(A and B) RORγt and CCR6 expression in CD4$^+$Foxp3$^+$ cells or CD4$^+$Foxp3$^-$ cells was analyzed by flow cytometry (A) and shown as graph (B). Non-immunized mice were included as a control.

(C) Expression of CCR6, CXCR3, or CXCR5 was compared with RORγt expression in CD4$^+$Foxp3$^+$ cells.

(D) Expression of ST2 or Bcl6 was compared with RORγt expression in CD4$^+$Foxp3$^+$ cells.

Data are representatives of at least two independent experiments. **$p < 0.005$.

identified RORγt$^+$Foxp3$^+$ cells as one of the intermediate stages during Th17 cell development both in vitro and in vivo, although their function has not been addressed (Ichiyama et al., 2008; Yang et al., 2008; Zhou et al., 2008). In addition, others found that RORγt expression in Foxp3$^+$ cells represents an unstable subpopulation of Treg cells that can convert to IL-17-producing cells or pathogenic Treg cells to promote the development of autoimmune diseases or cancer (Blatner et al., 2012; Komatsu et al., 2014). Two recent reports demonstrated the enrichment of RORγt-expressing Foxp3$^+$ Treg cells in the mouse colon (Ohnmacht et al., 2015; Sefik et al., 2015). These gut RORγt$^+$ Treg cells originated from naive CD4$^+$ T cell precursors, dependent on intestinal microbiota. Although gut RORγt$^+$ Treg cells are required to inhibit intestinal inflammation mediated by Th1/Th17 cells (Sefik et al., 2015) or Th2 cells (Ohnmacht et al., 2015), whether RORγt$^+$ Treg cells are also present outside of the gut and regulate peripheral T helper cell immune responses is unknown.

In this study, we identified RORγt-expressing Foxp3$^+$ Treg cells that were induced in lymphoid tissues after immunization. These RORγt$^+$ Treg cells selectively co-expressed the chemokine receptor CCR6 and represented activated Treg cells with high proliferative potential. We found that RORγt$^+$CCR6$^+$ Treg cells shared similar molecular regulation with Th17 cells for their development, and most of them were derived from thymic Treg cells. Moreover, RORγt$^+$CCR6$^+$ Treg cells potently inhibited Th17-cell-mediated CNS auto-inflammation in a Th17 transfer passive EAE model. Thus, immunization-induced lymph node (LN) RORγt$^+$ Treg cells, which we named T regulatory 17 cells (Tr17 cells), represent a type of antigen (Ag)-specific effector Treg cells with inhibitory function against autoreactive effector T cells.

RESULTS

RORγt$^+$CCR6$^+$Foxp3$^+$ T Cells Are a Distinct Treg Cell Subset

Although RORγt$^+$Foxp3$^+$ Treg cells have been shown in the gut constitutively, less than 2% of Treg cells in the peripheral lymph nodes were RORγt$^+$ in unimmunized C57BL/6 mice; upon subcutaneous (s.c.) immunization with myelin oligodendrocyte glycoprotein (MOG) peptide emulsified in complete Freund's adjuvant (CFA), about 10% of CD4$^+$Foxp3$^+$ Treg cells expressed RORγt in draining lymph nodes (dLNs) (Figures 1A and 1B). RORγt expression was highly correlated with cell surface expression of CCR6, a chemokine receptor preferentially expressed by Th17 cells (Yamazaki et al., 2008), in both Treg cells and non-Treg cells (Figure 1A). In addition, most of the RORγt$^+$ Treg cells were negative for CXCR3 and CXCR5, markers for T-bet$^+$ and Bcl6$^+$ Treg cells, respectively (Figure 1C).

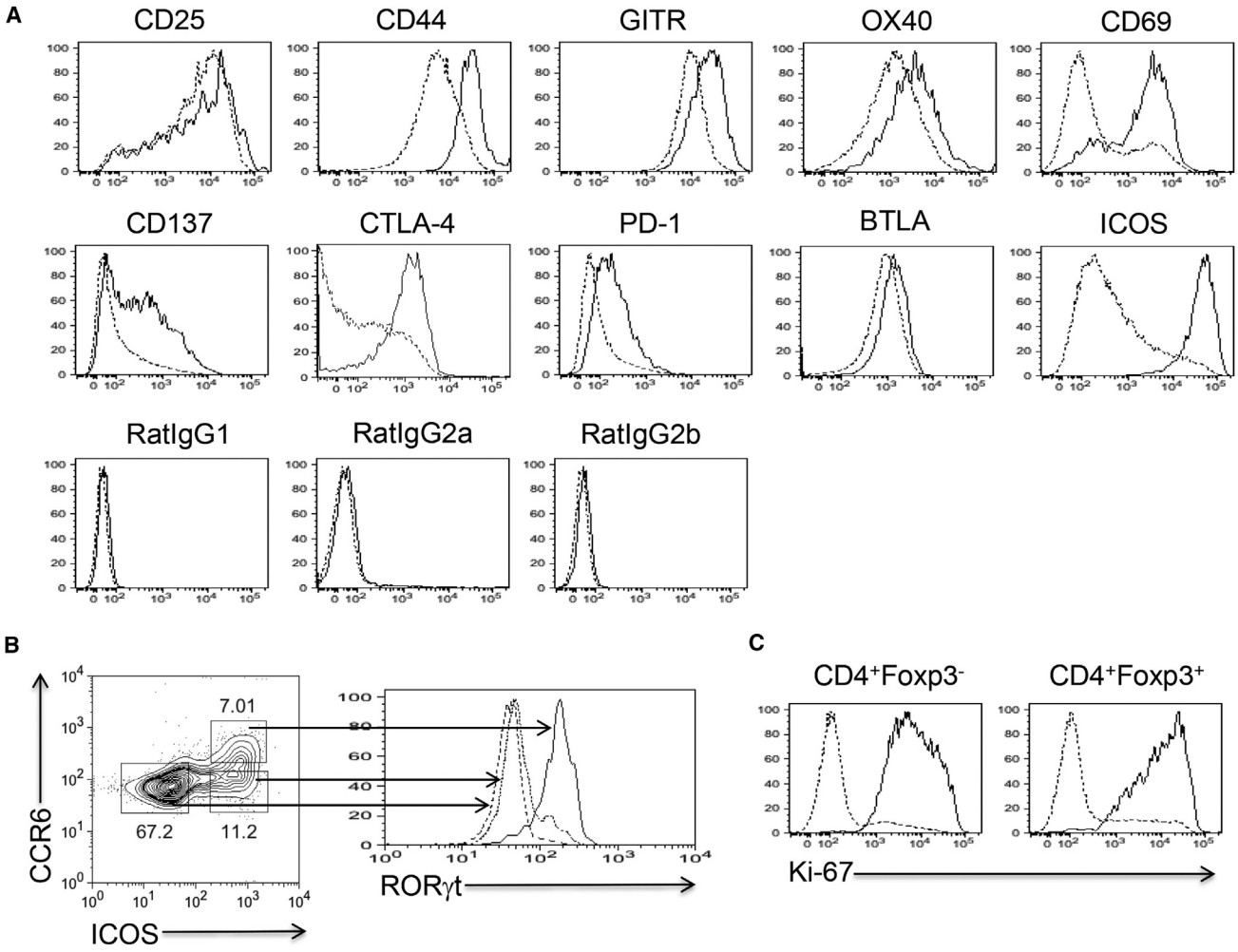

Figure 2. Treg Cell-Specific Cell Surface Marker Expression in RORγt$^+$CCR6$^+$ Treg Cells

(A) Seven days after MOG/CFA s.c. immunization, dLNs were isolated from C57BL/6 mice. Cell surface marker expression in RORγt$^+$CCR6$^+$CD4$^+$Foxp3$^+$ cells (solid) was compared with that in RORγt$^-$CCR6$^-$CD4$^+$Foxp3$^+$ cells (dashed).

(B) Seven days after MOG/CFA immunization, RORγt expression in each CD4$^+$Foxp3$^+$ subsets in dLNs was analyzed after gating based on ICOS and CCR6 expression.

(C) Ki-67 expression in RORγt$^+$CCR6$^+$ (solid) or RORγt$^-$CCR6$^-$ (dashed) cells was analyzed. Data are representatives of at least two independent experiments. See also Figure S1.

Interestingly, RORγt$^+$ Treg cells were mutually exclusive from Bcl6$^+$ Treg cells as well as ST2$^+$ Treg cells, a subpopulation of Treg cells recently identified in mucosal tissues (Figure 1D; Arpaia et al., 2015; Molofsky et al., 2015; Schiering et al., 2014).

To characterize RORγt$^+$ Treg cells, we analyzed the expression of Treg cell-associated cell surface molecules. Compared with RORγt$^-$CCR6$^-$ Treg cells, RORγt$^+$CCR6$^+$ Treg cells highly expressed cell activation markers, including CD25, CD44, GITR, OX40, and CD69 (Figure 2A). In addition, the expression levels of CD137, CTLA-4, PD-1, and BTLA, which have been attributed to the functional aspects of Treg cells, were also higher in RORγt$^+$CCR6$^+$ Treg cells than in RORγt$^-$CCR6$^-$ Treg cells. Of note, most of the RORγt$^+$CCR6$^+$ Treg cells were selectively ICOS^{hi}, resulting in exclusive expression of RORγt in ICOS^{hi} CCR6$^+$ Treg cells compared with ICOS^{hi}CCR6$^-$ Treg cells or ICOS^{lo}CCR6$^-$ Treg cells (Figures 2A and 2B). The cell surface marker expression pattern of RORγt$^+$CCR6$^+$ non-Treg cells was very similar to that of RORγt$^+$CCR6$^+$ Treg cells except for the low expression of CD137 (Figure S1). Consistent with their activated phenotype, most of the RORγt$^+$CCR6$^+$ Treg cells as well as RORγt$^+$CCR6$^+$ non-Treg cells were actively proliferating cells with high levels of Ki-67 expression (Figure 2C). Collectively, these results suggest that RORγt$^+$CCR6$^+$ Treg cells induced following immunization are activated cells.

RORγt$^+$CCR6$^+$ Treg Cells Are Derived from Foxp3$^+$ Thymic Treg Cells

Next we examined the origin and antigen specificity of RORγt$^+$ Treg cells. To determine whether RORγt$^+$ Treg cells are preferentially derived from thymic Foxp3$^+$ Treg (tTreg) cells or naive

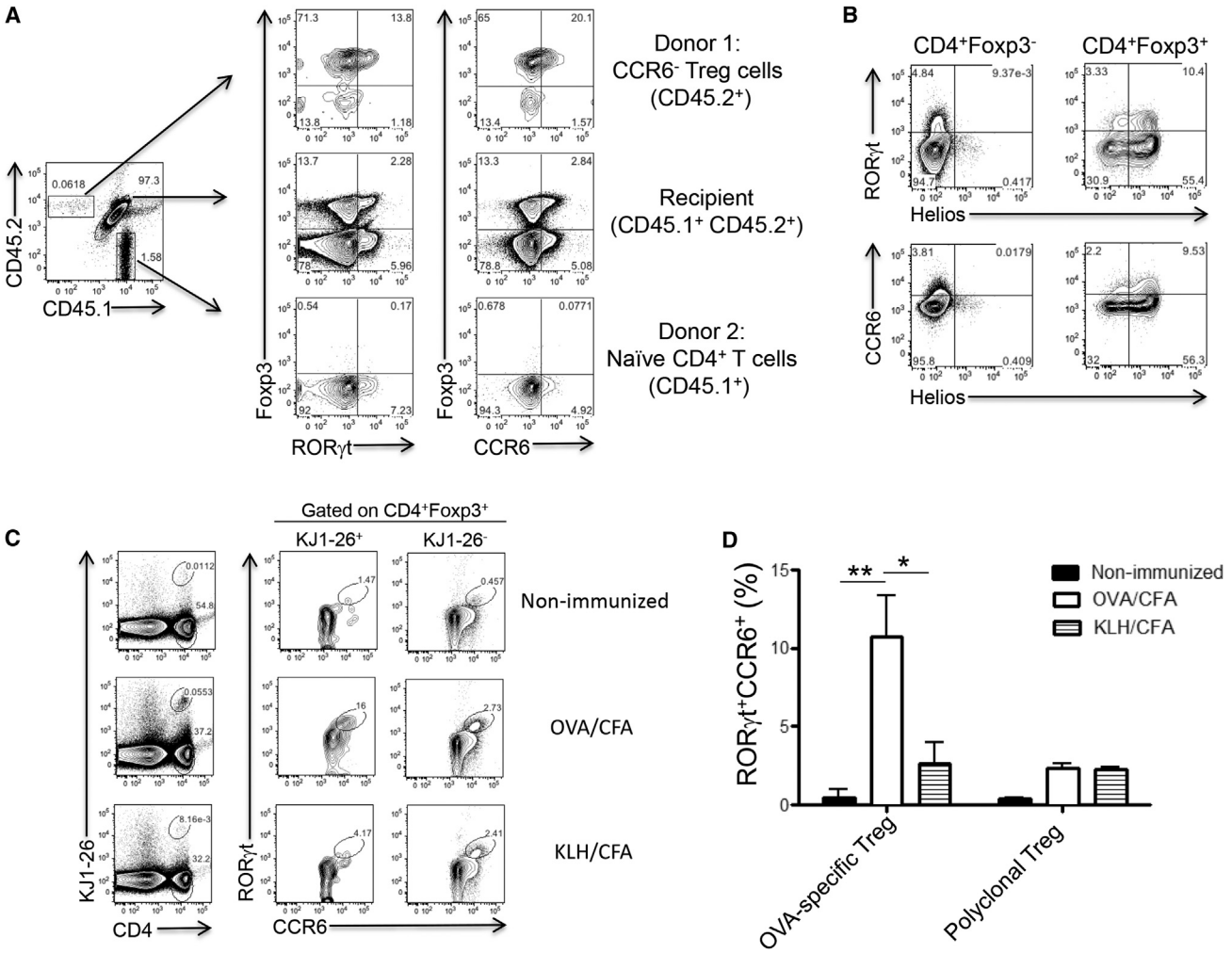

Figure 3. Origin of RORγt$^+$CCR6$^+$ Treg Cells

(A) CD4$^+$YFP$^+$CCR6$^-$ Treg cells isolated from Foxp3$^{YFP\text{-}Cre}$ reporter mice (CD45.2$^+$) were mixed with CD4$^+$CD25$^-$CD44^{lo}CD62L$^+$ naive T cells isolated from B6.SJL mice (CD45.1$^+$) at a 1:9 ratio and then adoptively transferred to CD45.1$^+$CD45.2$^+$ recipient mice. One day after the transfer, recipient mice were s.c. immunized with MOG/CFA. Seven days later, CD4$^+$ T cells in dLNs were analyzed for Foxp3, RORγt, and CCR6 expression after gating populations based on CD45.1 and CD45.2 expression.

(B) Helios expression in CD4$^+$Foxp3$^+$ or CD4$^+$Foxp3$^-$ cells in dLNs was compared with RORγt or CCR6 expression.

(C and D) OVA-specific CD4$^+$KJ1-26$^+$GFP$^+$CCR6$^-$ Treg cells isolated from DO11.10xFoxp3^{GFP} reporter mice were adoptively transferred to BALB/c mice. One day after the transfer, mice were s.c. immunized with OVA/CFA or KLH/CFA or left untreated. Seven days after immunization, KJ1-26$^+$ or KJ1-26$^-$ CD4$^+$Foxp3$^+$ Treg cells in dLNs were analyzed for RORγt and CCR6 expression by flow cytometry (C) and shown as graph (D).

Data are representatives of two independent experiments. *$p < 0.05$. **$p < 0.005$. See also Figure S2.

CD4$^+$ T cell precursors, we co-transferred CD4$^+$CCR6$^-$ yellow fluorescent protein (YFP)$^+$ Treg cells isolated from Foxp3$^{YFP\text{-}Cre}$ reporter mice (CD45.2$^+$) with naive CD4$^+$ T cells isolated from congenic mice (CD45.1$^+$) into CD45.1$^+$CD45.2$^+$ recipient mice. Seven days after immunization with MOG/CFA, about 10% of host Treg cells were RORγt$^+$CCR6$^+$. Of interest, the majority of the transferred CCR6$^-$ tTreg cells maintained their Foxp3 expression, and 15%–20% of them were RORγt$^+$CCR6$^+$ (Figure 3A). In contrast, few, if any, Foxp3$^+$ cells were induced from naive CD4$^+$ T cells. These results suggest that RORγt$^+$ Treg cells are preferentially derived from tTreg cells after immunization. In support of this notion, unlike their intestinal counterparts (Ohnmacht et al., 2015; Sefik et al., 2015), more than 75% of the RORγt$^+$CCR6$^+$ Treg cells induced by MOG/CFA immunization expressed Helios, one of the markers preferentially expressed by tTreg cells (Figure 3B; Thornton et al., 2010). To directly confirm their thymic origin, we sorted Treg cells from thymocytes of Foxp3^{GFP} reporter mice and co-transferred them with naive CD4$^+$ T cells into TCRβ$^{-/-}$ mice. Seven days after MOG/CFA immunization, we confirmed that more than 95% of the RORγt$^+$CCR6$^+$ Treg cells originated from thymic Treg cells (Figure S2).

To assess whether RORγt$^+$ Treg cell induction is antigen-specific, ovalbumin (OVA)-specific CCR6$^-$ Treg cells isolated from

DO11.10xFoxp3^{GFP} mice were adoptively transferred to naive BALB/c recipient mice followed by s.c. immunization with either OVA/CFA or keyhole limpet hemocyanin (KLH)/CFA. Without immunization, very few CD4$^+$KJ1-26$^+$ cells were recovered, and less than 0.5% of KJ1-26$^-$ polyclonal Treg cells were RORγt$^+$CCR6$^+$ (Figures 3C and 3D). Interestingly, however, the numbers of OVA-specific KJ1-26$^+$ Treg cells were remarkably increased by OVA antigen immunization, whereas 10%–16% of the OVA-specific Treg cells became RORγt$^+$CCR6$^+$. In contrast, we could not observe any increase in the frequencies of OVA-specific CD4$^+$KJ1-26$^+$ Treg cells after KLH immunization, and most of the donor Treg cells remained RORγt$^-$CCR6$^-$. The similar induction efficiency of RORγt$^+$CCR6$^+$ cells among KJ1-26$^-$ Treg cells between OVA and KLH immunization rules out the possibility of intrinsic differences in the immunization protocols (Figures 3C and 3D). Altogether, these results indicate that, unlike intestinal RORγt$^+$ Treg cells, those in the peripheral lymphoid tissues mainly originated from tTreg cells in an antigen-specific manner after immunization.

Immunization-Induced Peripheral RORγt$^+$Foxp3$^+$ Cells Are Distinct from Conventional Treg Cells or Colonic RORγt$^+$ Treg Cells

To further characterize RORγt$^+$ Treg cells in their transcriptome, we isolated RORγt$^+$CCR6$^+$ and RORγt$^-$CCR6$^-$ Treg cells from dLNs of MOG/CFA-immunized Foxp3$^{YFP\text{-}Cre}$ reporter mice by flow cytometry, based on their ICOS and CCR6 expression patterns (Figures S3A and S3B). We confirmed that CD4$^+$YFP$^+$ ICOS^{hi}CCR6$^+$ cells and CD4$^+$YFP$^+$ICOS^{lo}CCR6$^-$ cells represent RORγt$^+$ Treg cells and RORγt$^-$ Treg cells, respectively, at the mRNA level by RT-PCR (Figure S3C). Then we compared the gene expression profiles by performing RNA sequencing (RNA-seq) analysis. Based on transcriptomic profiling by RNA-seq analysis, ICOS^{hi}CCR6$^+$ Treg cells were found to be distinct from ICOS^{lo}CCR6$^-$ Treg cells, which were relatively similar to CCR6$^-$CXCR3$^-$CXCR5$^-$ naive Treg cells isolated from unimmunized mice (Figures 4A–4C). A total of 3,111 genes were differentially regulated in ICOS^{hi}CCR6$^+$ Treg cells compared with ICOS^{lo}CCR6$^-$ Treg cells (Figure 4D).

To sort out RORγt-dependent genes among them, we generated RORγ-floxed mice and then crossed them to Foxp3$^{YFP\text{-}Cre}$ mice to specifically delete RORγt in Treg cells. We then performed RNA-seq analysis for CD4$^+$YFP$^+$ cells isolated either from ROR$\gamma^{f/f}$Foxp3$^{YFP\text{-}Cre}$ mice (RORγt$^{-/-}$ Treg cells) or from ROR$\gamma^{+/+}$Foxp3$^{YFP\text{-}Cre}$ mice (wild-type [WT] Treg cells) 7 days after MOG/CFA immunization. When we compared differentially regulated genes between ICOS^{hi}CCR6$^+$ Treg and ICOS^{lo}CCR6$^-$ Treg cells with those between WT Treg and RORγt$^{-/-}$ Treg cells, 102 common genes were selected (Figure 4D). Then we further identified 29 RORγt-bound loci among them based on chromatin immunoprecipitation (ChIP) sequencing analysis of in vitro-generated Th17 cells (Figure 4E; Ciofani et al., 2012). Intriguingly, a number of Th17-cell-related genes, such as *Rorc*, *Il22*, *Il23r*, *Il1r1*, *Il17a*, and *Il17f*, were highly expressed by ICOS^{hi}CCR6$^+$ Treg cells, suggesting that ICOS^{hi}CCR6$^+$RORγt$^+$ Treg cells share some of the features of Th17 cells (Figure 4E). We confirmed the high expression of the selected genes (*Il17a*, *Il17f*, *Il22*, *Il1r1*, *Il23r*, and *Il10*) by ICOS^{hi}CCR6$^+$ Treg cells at the mRNA level, whereas the expression of irrelevant Th1-specific genes (*Tbx21* and *Ifng*) was not increased (Figure S3C).

To see whether peripheral RORγt$^+$ Treg cells are similar to colonic RORγt$^+$ Treg cells, we compared the transcriptome of these two RORγt$^+$ Treg populations (Sefik et al., 2015). A fold change/fold change comparison between colonic RORγt$^+$ Treg cells and LN RORγt$^+$ Treg cells, after calculating the fold change based on their respective RORγt$^-$ populations, revealed that several Th17-cell-related genes (*Rorc*, *Il23r*, *Il1r1*, and *Il17a*) were highly expressed by both colonic RORγt$^+$ Treg cells and peripheral RORγt$^+$ Treg cells (Figure 4F). Genes selectively upregulated in RORγt$^+$ Treg cells in peripheral LNs compared with colonic ones include those encoding Th17-cell-associated effector cytokines and IL-17 receptor family members (*Il22*, *Il17f*, and *Il17re*), Treg cell-associated effector cytokines (*Gzmb* and *Il10*), a G protein-coupled receptor (*Gpr15*), integrin (*Itgae*), chemokine receptors (*Ccr2*, *Cxcr6*, *Ccr4*, and *Ccr6*), a costimulatory molecule (*Tnfrsf8*), and Helios (*Ikzf2*). In contrast, the gut homing integrin and receptor (*Itaga4* and *Ccr9*), IFN-γ receptors (*Ifngr1* and *Ifngr2*), and the transcription factor *Bcl6* were selectively downregulated in LN Treg cells (Figure 4F). We further compared RORγt-dependent, differentially regulated genes in LN Treg cells with those in colonic Treg cells. Accordingly, several Th17-cell-associated effector cytokines (*Il17a*, *Il17f*, and *Il22*) and cell surface receptors (*Il1r1* and *Il17re*) were selectively elevated in LN Treg cells in an RORγt-dependent manner, whereas *Il23r* and the chemokine *Cxcl3* were commonly upregulated by both populations. In contrast, expression of Th1 (*Ifng*) and Th2 (*Il4* and *Il13*) effector cytokines was significantly inhibited by RORγt in both colon and LN Treg cells (Figure 4G). We confirmed the relatively high expression of CCR6, CD103, Helios, and IL-17A in peripheral RORγt$^+$ Treg cells compared with colonic RORγt$^+$ Treg cells at the protein level, whereas the granzyme B expression level was not consistent with the transcriptome analysis result (Figure 4H; Figures S3D and S3E). In contrast, the Tim-3 (encoded by *Havcr2*) expression level was higher in colonic RORγt$^+$ Treg cells than in peripheral RORγt$^+$ Treg cells at the protein level, despite the common upregulation of the molecule by both populations in a transcriptome analysis (Figures 4F and 4H). Of note, about 10%–25% of peripheral RORγt$^+$ Treg cells expressed IL-17A, whereas colonic RORγt$^+$ Treg cells did not produce IL-17A (Figure 4I; Figure S3E). Based on this finding, we named peripheral RORγt$^+$ Treg cells Tr17 cells. Altogether, these results indicate that immunization-induced peripheral RORγt$^+$ Tr17 cells are distinct from conventional Treg cells as well as gut RORγt$^+$ Treg cells.

Factors Involved in the Generation of RORγt$^+$CCR6$^+$ Tr17 Cells

Increased expression of Th17-cell-associated genes in Tr17 cells prompted us to address whether these cells share developmental cues with Th17 cells. To test whether RORγt is required for the generation of Tr17 cells following immunization, ROR$\gamma^{f/f}$CD4^{Cre} mice were immunized with MOG/CFA. As expected, the induction of RORγt$^+$CCR6$^+$ Tr17 cells was completely abolished in ROR$\gamma^{f/f}$CD4^{Cre} mice (Figure 5A). Intriguingly, however, we could still detect some CCR6^{int}ICOS^{hi} Treg

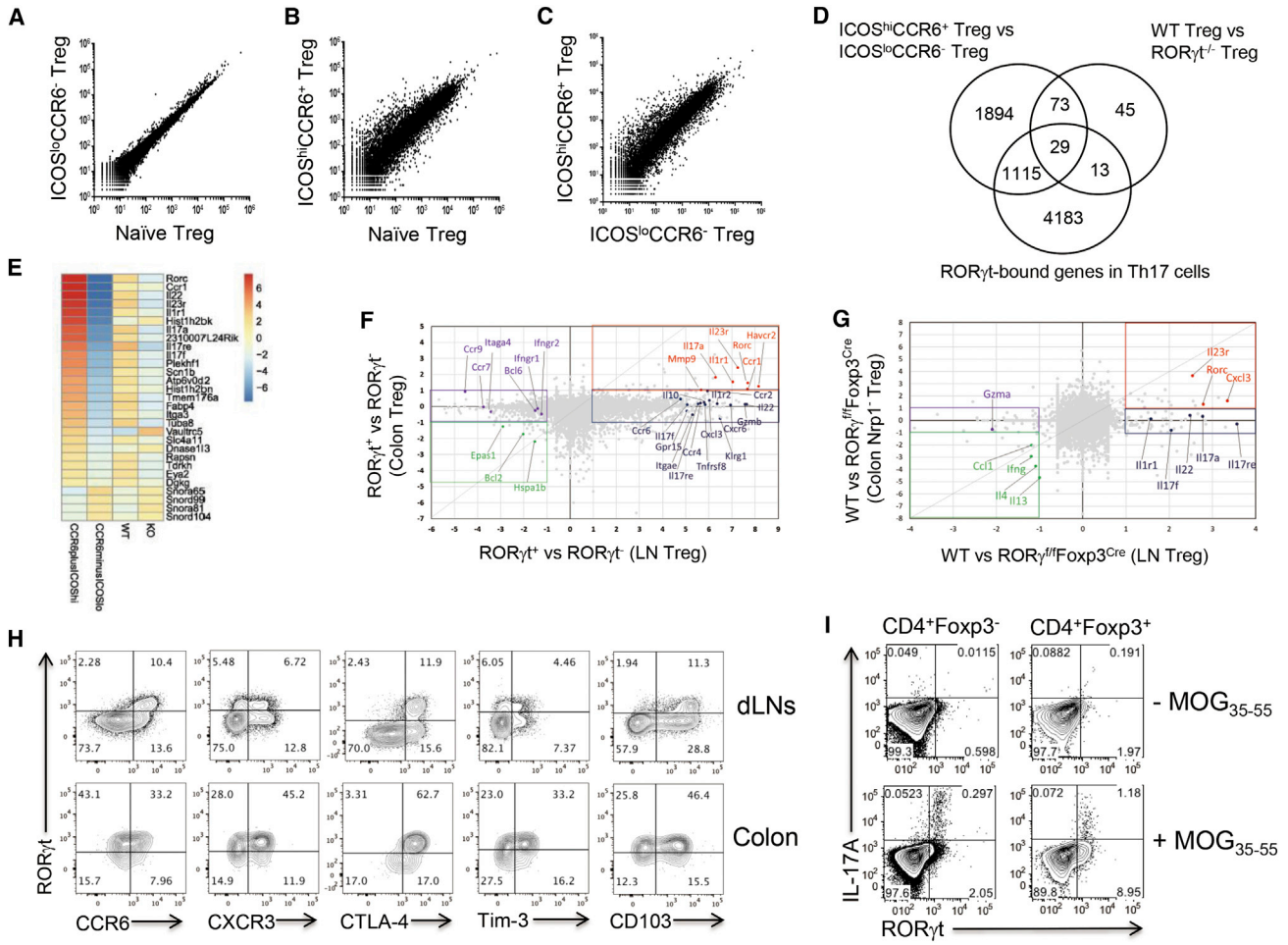

Figure 4. Transcriptional Profiling of RORγt$^+$CCR6$^+$ Treg Cells

(A–C) Pairwise comparison between Naïve Treg versus ICOS^{lo}CCR6$^-$ Treg (A), Naïve Treg versus ICOS^{hi}CCR6$^+$ Treg (B), and ICOS^{lo}CCR6$^-$ Treg versus ICOS^{hi}CCR6$^+$ Treg (C). Values indicate normalized counts.

(D) Common differentially regulated genes between "ICOS^{hi}CCR6$^+$ Treg versus ICOS^{lo}CCR6$^-$ Treg" and "WT Treg (WT) versus RORγt$^{-/-}$ Treg (knockout [KO])" were compared and are shown in a Venn diagram. RORγt-bound gene loci identified from ChIP-sequencing data of in vitro-generated Th17 cells were further compared.

(E) Heatmap showing 29 common differentially regulated genes selected from (D).

(F) Relatively upregulated or downregulated genes in LN ICOS^{hi}CCR6$^+$ Treg cells (RORγt$^+$ Treg) over ICOS^{lo}CCR6$^-$ Treg cells (RORγt$^-$ Treg) were compared with those in colonic RORγt$^+$ Treg cells over colonic RORγt$^-$ Treg cells. Values indicate $\log_2$ (fold change).

(G) Comparison of RORγt-dependent genes (WT versus RORγ$^{f/f}$Foxp3^{Cre} mice) between colon Nrp1$^-$ Treg cells and LN Treg cells isolated from s.c. immunized mice. Values indicate $\log_2$ (fold change).

(H) Expression of the indicated molecules in CD4$^+$Foxp3$^+$ cells in dLNs or the colon were analyzed by flow cytometry 7 days after MOG/CFA s.c. immunization.

(I) Antigen-specific expression of IL-17A and RORγt in CD4$^+$Foxp3$^+$ cells or CD4$^+$Foxp3$^-$ cells was analyzed 24 hr after MOG peptide restimulation. Brefeldin A and monensin were added for the last 6 hr of culture.

Data are representatives of at least two independent experiments. See also Figure S3.

cells in RORγ$^{f/f}$CD4^{Cre} mice, suggesting RORγt-independent CCR6 expression in Treg cells despite the suboptimal level of expression (Figure 5A). A previous report has shown that Stat3 in Treg cells is essential for the regulation of Th17 cells, at least in part, by inducing the expression of CCR6 (Chaudhry et al., 2009). Thus, we determined whether Stat3 is required for Tr17 cell generation using Stat3$^{f/f}$CD4^{Cre} mice. After immunization with MOG/CFA, induction of RORγt$^+$CCR6$^+$ Tr17 cells was completely abrogated in the absence of Stat3 in T cells, demonstrating that Stat3 is indispensable for the generation of Tr17 cells (Figures 5B and 5C). We and others found previously that ICOS-ICOSL (B7h) signaling is critical for Th17 cell development in vivo (Bauquet et al., 2009; Park et al., 2005). Increased expression of ICOS in RORγt$^+$CCR6$^+$ Treg cells prompted us to define whether ICOS-ICOSL signaling is also involved in the generation of Tr17 cells. When we immunized B7h-deficient mice with MOG/CFA, we found a significant reduction in RORγt$^+$CCR6$^+$ Tr17 cell generation compared with control mice (Figures 5D and 5E). Taken

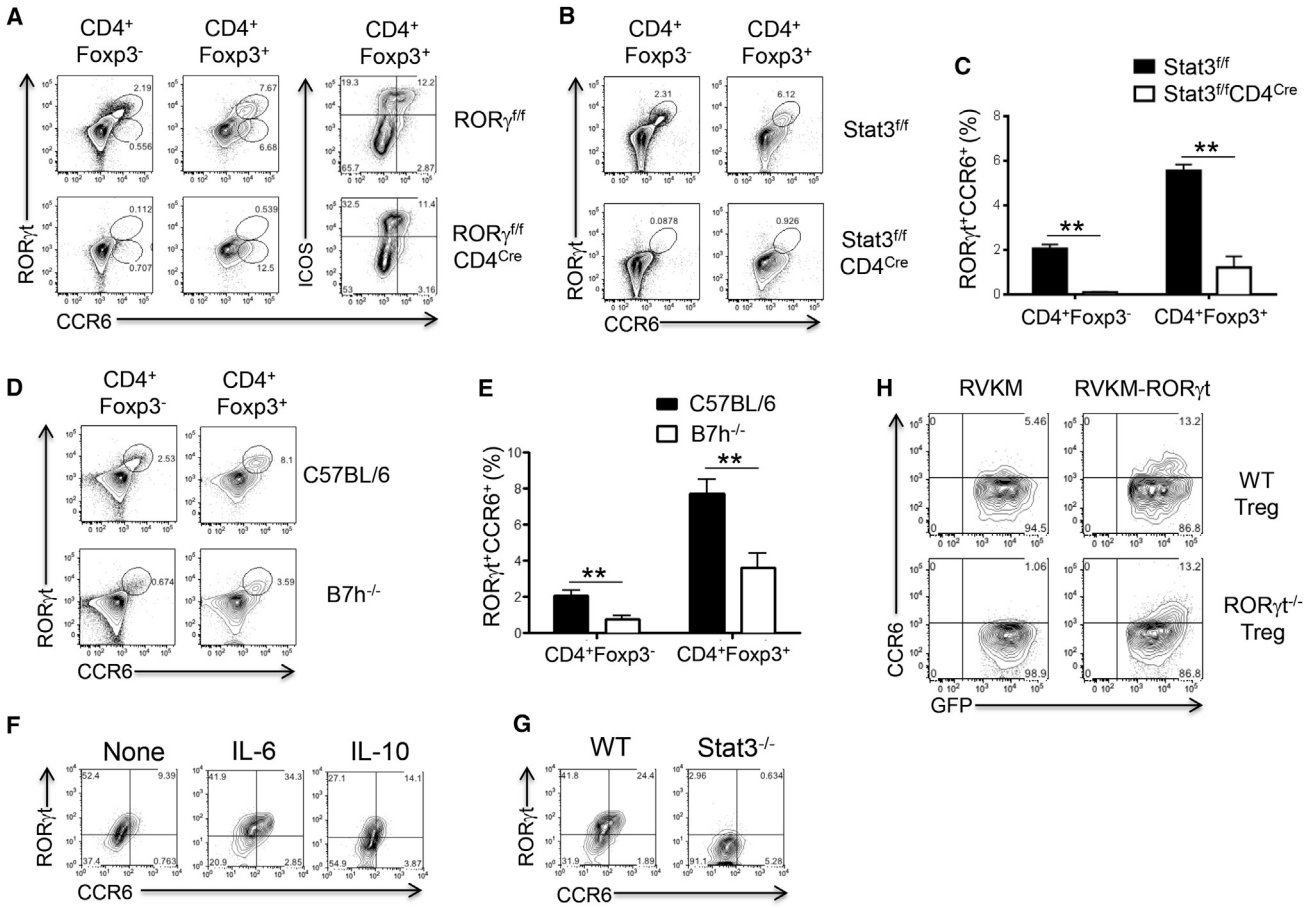

Figure 5. Requirements for the Induction of RORγt$^+$CCR6$^+$ Treg Cells

(A) Seven days after MOG/CFA s.c. immunization of RORγ$^{f/f}$ CD4^{Cre} mice and littermate control RORγ$^{f/f}$ mice, dLNs were isolated for analysis of RORγt$^+$CCR6$^+$ cell induction in CD4$^+$Foxp3$^+$ cells or CD4$^+$Foxp3$^-$ cells.

(B) Stat3$^{f/f}$ CD4^{Cre} mice and littermate control Stat3$^{f/f}$ mice were immunized with MOG/CFA s.c. and analyzed by flow cytometry as in (A).

(C) The result summary of (B) is shown.

(D) B7h$^{-/-}$ mice and control C57BL/6 mice were immunized with MOG/CFA s.c. and analyzed by flow cytometry as in (A).

(E) The result summary of (D) is shown.

(F) CD4$^+$CCR6$^-$GFP$^+$ Treg cells isolated from Foxp3^{GFP} mice were stimulated with anti-CD3 and anti-CD28 antibodies (Abs) for 4 days in the presence of IL-6 (20 ng/mL) or IL-10 (100 ng/mL). RORγt and CCR6 expression in gated CD4$^+$Foxp3$^+$ cells was analyzed by flow cytometry.

(G) CD4$^+$CD25^{hi}CCR6$^-$ Treg cells isolated from Stat3$^{f/f}$CD4^{Cre} mice or littermate control mice were stimulated with anti-CD3 and anti-CD28 Abs in the presence of IL-6 (20 ng/mL). RORγt and CCR6 expression in gated CD4$^+$Foxp3$^+$ cells was analyzed.

(H) CD4$^+$CD25^{hi}CCR6$^-$ Treg cells isolated from RORγ$^{f/f}$CD4^{Cre} mice (RORγt$^{-/-}$ Treg) or littermate control mice (WT Treg) were transduced with a retroviral vector expressing RORγt (RVKM− RORγt) or an empty vector (RVKM). CCR6 and GFP expression was analyzed after gating on GFP$^+$ cells.

Data are representatives of at least two independent experiments. **$p < 0.005$.

together, these results suggest that Tr17 cells utilize similar molecular machinery for their development as Th17 cells.

RORγt Induced by IL-6-Stat3 in Treg Cells Regulates CCR6 Expression

It has been demonstrated that IL-10-IL-10R signaling in Treg cells is mandatory to induce Stat3 activation and subsequent regulation of Th17 cells by Treg cells (Chaudhry et al., 2011). Thus, we hypothesized that IL-10-mediated activation of Stat3 induces RORγt and CCR6 expression in Treg cells. To address this hypothesis, CCR6$^-$ Treg cells isolated from Foxp3 reporter mice were T cell receptor (TCR)-stimulated in the presence of IL-6 or IL-10. TCR activation alone induced few, if any, RORγt$^+$CCR6$^+$Foxp3$^+$ cells. Interestingly, exogenous IL-6 induced a considerable proportion of the RORγt$^+$CCR6$^+$ population among Foxp3$^+$ cells, whereas the effect of exogenous IL-10 was minimal (Figure 5F). As expected, Stat3 expression in Treg cells was essential for IL-6-mediated induction of RORγt$^+$CCR6$^+$Foxp3$^+$ cells (Figure 5G).

Next we addressed whether RORγt transduction is sufficient to induce CCR6 expression in Treg cells. To this end, we isolated CCR6$^-$ Treg cells from RORγ$^{f/f}$ CD4^{Cre} mice or littermate control mice and then transduced them with a retroviral vector expressing RORγt-GFP or an empty vector expressing GFP alone.

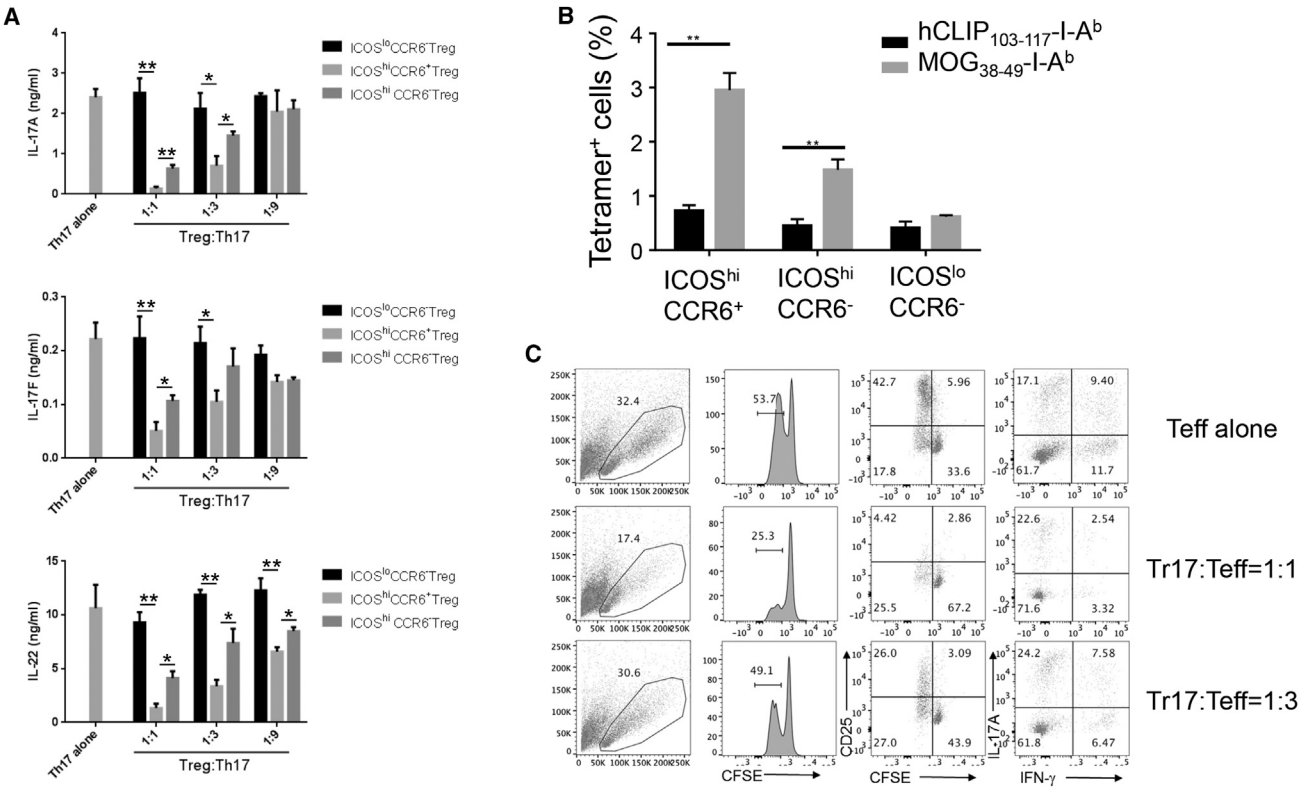

Figure 6. ICOS^{hi}CCR6^{+}RORγt^{+} Treg Cells Are Ag-Specific Regulatory T Cells with Potent Suppressive Activity In Vitro

(A) ICOS^{hi}CCR6^{+}GFP^{-}CD4^{+} Th17 cells were isolated 7 days after MOG/CFA s.c. immunization of CD45.2^{+}Foxp3^{GFP} reporter mice. In parallel, ICOS^{hi}CCR6^{+}GFP^{+}CD4^{+} Treg cells, ICOS^{lo}CCR6^{-}GFP^{+}CD4^{+} Treg cells, and ICOS^{hi}CCR6^{-}GFP^{+}CD4^{+} Treg cells were isolated from MOG/CFA-immunized CD45.1^{+}CD45.2^{+} Foxp3^{GFP} reporter mice. Th17 cells were stimulated with MOG peptide (50 μg/mL) in the presence of irradiated T cell-depleted splenocytes (TdS, CD45.1^{+}). 3-fold serially diluted number of each Treg cells were added to the culture to test the suppressive activity. Three days later, IL-17A, IL-17F, or IL-22 production in the culture supernatant was analyzed by ELISA.

(B) Foxp3^{GFP} reporter mice were s.c. immunized with MOG/CFA. Seven days later, MOG-specific cells in the indicated populations of dLNs were identified by tetramer staining.

(C) ICOS^{hi}GFP^{-}CD4^{+}CD45.1^{+} T effector cells (Teffs) were labeled with CFSE (2.0 μM) and then stimulated with splenic CD11c^{+} dendritic cells (DCs) in the presence or absence of ICOS^{hi}CCR6^{+}GFP^{+}CD4^{+}CD45.2^{+} Tr17 cells in the indicated Tr17-to-Teff cell ratio. Three days later, forward scatter (FSC) value, CFSE dilution, CD25 expression, and cytokine production of CD45.1^{+} Teff cells were analyzed by flow cytometry after phorbol-12-myristate-13-acetate (PMA)/ionomycin restimulation.

Data are representatives of at least two independent experiments. *$p < 0.05$. **$p < 0.005$. See also Figure S4.

Although WT Treg cells expressed a marginal level of CCR6 even without RORγt transduction, RORγt$^{-/-}$ Treg cells were totally deficient in CCR6 expression without RORγt transduction. In addition, CCR6 expression was dramatically increased in GFP^{+} cells of WT Treg cells by RORγt transduction in a GFP intensity-dependent manner. Of note, RORγt transduction was adequate to restore CCR6 expression in RORγt$^{-/-}$ Treg cells at a level comparable with that in WT Treg cells (Figure 5H). These results suggest that RORγt expression in Treg cells is dependent on IL-6-mediated Stat3 signaling and, in turn, that RORγt expression is essential and sufficient for CCR6 expression in Treg cells.

RORγt^{+}CCR6^{+} Tr17 Cells Have an Inhibitory Function against Ag-Specific Th17 and Th1 Cells In Vitro

To address whether Tr17 cells can regulate Ag-specific Th17 cell responses, CD4^{+}ICOS^{hi}CCR6^{+}GFP^{-} Th17 cells were isolated from dLNs of MOG/CFA-immunized Foxp3^{GFP} reporter mice. Three days after MOG peptide stimulation, copious amounts of IL-17A, IL-17F, and IL-22 were detected in the culture supernatant. Of note, ICOS^{hi}CCR6^{+}RORγt^{+} Tr17 cells significantly suppressed cytokine production by Th17 cells in a Treg cell-to-Th17 cell ratio-dependent fashion. In contrast, ICOS^{lo}CCR6^{-} resting Treg cells failed to do so, even at the highest Treg-to-Th17 ratio (1:1) (Figure 6A). Of interest, ICOS^{hi}CCR6^{+} Tr17 cells were almost three times more potent at suppressing Ag-specific Th17 cells than ICOS^{hi}CCR6^{-} activated Treg cells (Figure 6A). We also observed that ICOS^{hi}CCR6^{+} Treg cells were superior at suppressing IFN-γ production as well as IL-17A production by total ICOS^{hi} effector CD4^{+} T cells compared with ICOS^{hi}CCR6^{-} Treg cells and ICOS^{lo}CCR6^{-} Treg cells (Figure S4A). These results indicate that Tr17 cells are Ag-specific Treg cells with potent regulatory activity against Ag-specific Th17 cells and Th1 cells in vitro.

RORγt$^+$CCR6$^+$ Tr17 Cells Inhibit Proliferation and Activation of Effector Cells

Because RORγt$^+$ Tr17 cells exerted a more potent suppressive function than resting Treg cells, we hypothesized that Ag-specific Treg cells were enriched within the RORγt$^+$ subpopulation. To test this hypothesis, we benefitted from the $MOG_{38–49}$-I-A^b tetramer, which enabled us to detect MOG-reactive CD4$^+$ T cells by flow cytometry. Of interest, we observed that ICOS^{hi}CCR6$^+$ Tr17 cells contained more MOG-specific cells than ICOS^{hi}CCR6$^-$ or ICOS^{lo}CCR6$^-$ Treg cells, suggesting that enrichment of Ag-specific Treg cells contributed to the enhanced suppression meditated by Tr17 cells (Figure 6B; Figure S4B).

We then determined the mode of action by which Tr17 cells inhibited effector T cells. After labeling CD4$^+$GFP(Foxp3)$^-$ICOS^{hi} effector T cells with carboxyfluorescein succinimidyl ester (CFSE) dye to monitor cell proliferation, we restimulated the cells with MOG peptide. Three days after restimulation, about 50% of effector cells diluted CFSE and highly expressed the cell activation marker CD25, suggesting proliferation and activation of MOG-specific effector T cells. When we added titrated numbers of ICOS^{hi}CCR6$^+$ Tr17 cells to the culture, effector cell proliferation and activation were significantly reduced in a Treg-to-T effector cell (Teff cell) ratio-dependent manner (Figure 6C). Of note was that the frequency of Th1 cells was considerably reduced by Tr17 cells whereas that of Th17 cells was maintained (Figure 6C). These results suggest that the suppressive mode of action exerted by Tr17 cells might be different depending on the type of effector T cells. We observed a similar pattern of suppression at the single-cell level in the presence of polyclonal restimulation (Figure S4C).

Because our transcriptome analysis revealed that Tr17 cells upregulated the *Il10* transcript, and it was confirmed by RT-PCR (Figure 4F; Figure S3C), we tested whether IL-10 is required for the suppression mediated by Tr17 cells. As shown in Figure S4D, Tr17 cell-mediated suppression of IL-17A and IFN-γ production by effector T cells was significantly impaired by the anti-IL-10R blocking antibody, implying that Tr17 cell-produced IL-10 is, at least in part, involved in their suppressive function.

RORγt$^+$CCR6$^+$ Tr17 Cells Can Dampen Th17-Cell-Driven Autoimmunity

Having shown that RORγt$^+$CCR6$^+$ Tr17 cells are activated cells with potent suppressive activity against Th17 cells in vitro, we set out to assess the suppressive function of Tr17 cells under inflammatory conditions in vivo. To assess whether RORγt$^+$Foxp3$^+$ Tr17 cells were required for the regulation of Ag-specific Th17 cells in vivo, we compared EAE progression between RORγ$^{f/f}$ Foxp3^{Cre} mice and littermate control mice. Despite the potent suppressive activity of Tr17 cells in vitro, EAE progression was not exacerbated in Tr17 cell-deficient RORγ$^{f/f}$Foxp3^{Cre} mice compared with control mice (data not shown). We also found that the frequency and total number of Treg cells in the CNS were not significantly different between RORγ$^{f/f}$Foxp3^{Cre} mice and control mice, suggesting that RORγt expression in Treg cells was not essential for CNS homing of Treg cells (data not shown).

According to a recent study, it is possible that depletion of RORγt$^+$ Treg cells is needed to examine their function rather than deletion of RORγt expression in Treg cells (Levine et al., 2017). However, the availability of an experimental system for selective depletion of RORγt$^+$ Treg cells in vivo is limited at the moment. To directly examine whether Tr17 cells can regulate Ag-specific Th17 cells in vivo, we isolated CD4$^+$YFP^{hi} Th17 fate-mapped (Th17fm) cells from dLNs of MOG/CFA-immunized IL-17F^{Cre}xRosa26^{YFP} fate-mapping mice and confirmed that about 50% of the CD4$^+$YFP^{hi} cells produced IL-17A after MOG restimulation (Figure S5A). We then adoptively transferred Th17fm cells alone or together with either ICOS^{hi}CCR6$^+$YFP$^+$ Tr17 cells or ICOS^{lo}CCR6$^-$YFP$^+$ resting Treg cells (rTreg cells) into RAG1$^{-/-}$ mice at a 4:1 Treg to Th17fm ratio (Figure S5B). After immunization with MOG peptide emulsified in incomplete Freund's adjuvant (IFA) followed by pertussis toxin (PTX) injections, mice receiving Th17fm cells alone developed sequential clinical signs of regular EAE. Notably, disease progression by Th17fm cells was completely abrogated by co-transfer of Tr17 cells (Figure 7A). Interestingly, however, about 60% of rTreg cell co-transfer recipient mice developed the disease with a similar onset and severity as Th17fm cell-only transfer mice, whereas the remainder of the mice did not develop the disease (Figure 7A). Of note, CD4$^+$ T cells were barely detectable in mice receiving Tr17 co-transfer, whereas a significant infiltration of CD4$^+$ T cells was found in those receiving Th17fm alone or rTreg cell co-transfer (Figures 7B and 7C). Because Th17fm cells permanently express YFP, and its intensity is much higher than that in Treg cells in this experimental system, we could differentiate YFP^{hi} Th17fm cells from YFP^{int} Treg cells after induction of EAE. Analysis of YFP expression in total CD4$^+$ T cells in the CNS confirmed that a majority of the CNS-infiltrated CD4$^+$ T cells were Th17fm cells in both Th17-only mice and diseased rTreg cell co-transfer mice (Figure 7C). We found that most of the YFP^{hi} Th17fm cells had been converted to IFN-γ$^+$GM-CSF$^+$ cells, whereas some cells still produced IL-17A (Figure 7D). When we analyzed YFP expression in total CD4$^+$ T cells in the spleen, the ratio of YFP^{int} Treg cells to YFP^{hi} Th17fm cells in Tr17 cell co-transfer mice was 2.56 ± 0.72, which was about eight times higher than that in rTreg cell co-transfer mice (0.32 ± 0.08) (Figures 7E and 7F). We also confirmed that the absolute number of YFP^{hi} Th17fm cells was significantly reduced by Tr17 cell co-transfer (Figure 7G). These results suggest that Tr17 cells efficiently regulated expansion of Ag-specific Th17 cells in the periphery. Intracellular cytokine staining after MOG peptide restimulation revealed that most of the MOG-specific Th17fm cells in the spleen already converted to IFN-γ-producing Th1 cells after EAE induction under lymphopenic conditions. The absolute number and frequency of IFN-γ-producing exTh17 cells were significantly suppressed by Tr17 cells, whereas rTreg cells marginally suppressed IFN-γ production at the single-cell level (Figures 7G and 7H). After 3 days of culture with MOG peptide, splenocytes isolated from Th17fm-only mice produced a significant amount of IFN-γ with little production of IL-17A. Interestingly, IFN-γ production by splenocytes was significantly reduced by co-transfer of Tr17 cells, whereas IL-17A production was marginally increased compared with Th17fm alone (Figures 7I and 7J). In contrast, co-transfer of rTreg cells did not significantly suppress IFNγ production by splenocytes. Taken together, these results indicate that thymic Treg-derived RORγt$^+$ Tr17

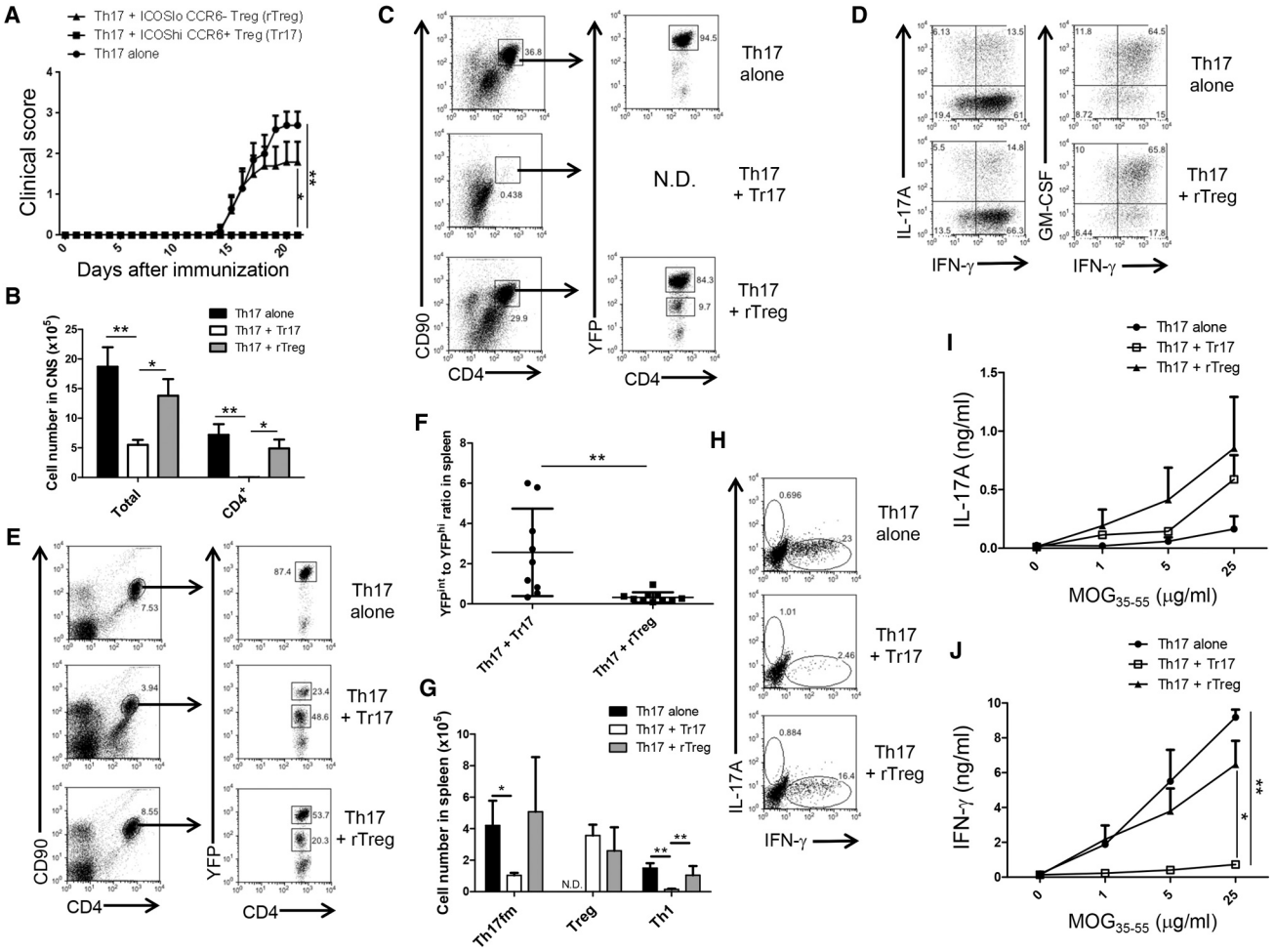

Figure 7. ICOS^{hi}CCR6^{+}RORγt^{+} Treg Cells Can Regulate Th17-Cell-Mediated CNS Autoimmunity

CD4^{+}YFP^{hi} Th17 cells isolated from MOG/CFA-immunized IL-17F^{Cre}xRosa26^{YFP} fate-mapping mice were adoptively transferred into RAG1$^{-/-}$ mice. CD4^{+}YFP^{+} ICOS^{hi}CCR6^{+} cells (Tr17) or CD4^{+}YFP^{+}ICOS^{lo}CCR6^{-} cells (rTreg) isolated from MOG/CFA-immunized Foxp3$^{YFP\text{-}Cre}$ mice were co-transferred with Th17 cells in some recipient mice. All recipient mice were immunized with MOG/IFA 1 day after adoptive transfer. Pertussis toxin was injected on the day of immunization and 2 days later.

(A) The EAE score was monitored daily: Th17 alone (n = 10), Th17 + Tr17 (n = 9), and Th17 + rTreg (n = 10).

(B) The numbers of total cells and CD4^{+} T cells in the CNS were analyzed.

(C) Representative dot plot of YFP expression in gated CD4^{+}CD90^{+} T cells in the CNS.

(D) Cytokine production in CNS CD4^{+}YFP^{hi} T cells was analyzed after PMA/ionomycin stimulation.

(E) YFP expression in splenic CD4^{+} T cells.

(F) The YFP^{int} Treg cell-to-YFP^{hi} Th17 fate-mapped cell ratio in the spleen was analyzed.

(G) Absolute numbers of CD4^{+}YFP^{hi} Th17 fate-mapped cells (Th17fm), CD4^{+}YFP^{int} Treg cells (Treg), and MOG-specific CD4^{+}IFN-γ^{+} Th1 cells in the spleen.

(H) MOG-specific cytokine production by splenic CD4^{+} T cells was analyzed after MOG peptide (50 μg/mL) restimulation.

(I and J) IL-17 (I) and IFN-γ (J) production by splenocytes was analyzed by ELISA 3 days after restimulation with titrated doses of MOG peptide.

*p < 0.05. **p < 0.005. N.D., not detected. Data are combined results (A and F) or representatives (B–E and G–J) of two independent experiments. See also Figure S5.

cells can suppress Th17-cell-mediated CNS inflammation by repressing peripheral expansion and CNS migration of Th17 cells.

DISCUSSION

Recent studies have identified intestinal RORγt^{+} Treg cells and found that these microbiota-induced Treg cells are required for the regulation of specific T helper cells in the gut (Ohnmacht et al., 2015; Sefik et al., 2015). The immunization-induced RORγt^{+} Tr17 cells in the current study share some features with intestinal RORγt^{+} Treg cells, including a CD44^{hi}, ICOS^{hi}, CTLA4^{hi}, CD103^{hi} activated phenotype; CCR6 expression; Stat3-dependent development; distinction from ST2^{+} Treg cells; and elevated expression of genes encoding *Il1r1*, *Il23r*, *Havrc2*, and *Il10*. However, peripheral RORγt^{+} Tr17 cells are distinct from intestinal RORγt^{+} Treg cells. First, we found that peripheral

RORγt$^+$ Tr17 cells are mostly Helios$^+$Nrp-1$^+$ tTreg cells, whereas intestinal RORγt$^+$ Treg cells were totally Helios$^-$Nrp-1$^-$, indicating a different origin of peripherally induced Treg cells (pTreg cells). Second, unlike intestinal RORγt$^+$ Treg cells, about 10% of peripheral RORγt$^+$ Tr17 cells produced IL-17A upon restimulation. Third, Stat3 was essential for the development of peripheral RORγt$^+$ Tr17 cells, whereas Stat3 was not absolutely required for RORγt expression in intestinal Treg cells. Finally, gene expression profiling data obtained from two independent transcriptome analyses revealed that these two Treg cell populations have some similarities but are distinct from each other.

The identity of RORγt$^+$Foxp3$^+$ cells has been debatable. We hypothesized that there are at least three different possibilities regarding the fate of immunization-induced peripheral RORγt$^+$ Foxp3$^+$ cells: transient precursor cells during Th17 cell development from naive CD4$^+$ T cells, unstable Foxp3$^+$ Treg cells that can convert to IL-17-producing pathogenic cells under inflammatory conditions, and an activated Treg cell subpopulation generated under a Th17-prone microenvironment. To address whether a considerable proportion of Th17 cells undergo the RORγt$^+$ Foxp3$^+$ intermediate stage as demonstrated before (Ichiyama et al., 2008; Yang et al., 2008; Zhou et al., 2008), we generated Foxp3^{Cre}xRosa26^{YFP} mice to track Th17 cells that expressed Foxp3 at any stage throughout their developmental process. Unexpectedly, however, we found few, if any, YFP$^+$IL-17A$^+$ or YFP$^+$IFN-γ$^+$ cells in the CNS of Foxp3^{Cre}xRosa26^{YFP} mice after EAE induction (data not shown). This result indicates that the contribution of RORγt$^+$Foxp3$^+$ intermediate precursors to the total Th17 cell pool is minor, at least in our EAE model. In addition, a low expression level of IL-17A compared with that of Th17 cells and no evidence for pathogenic potential in our cell-adoptive transfer EAE experiment indicate that peripheral RORγt$^+$Foxp3$^+$ cells are not a distinct IL-17-producing pathogenic cell population. Finally, high expression of Treg cell-associated cell surface molecules, stable expression of Foxp3, increased proliferative potential, Stat3-dependent development, and strong suppressive activity against effector cells suggest that peripheral RORγt$^+$ Foxp3$^+$ cells are activated Treg cells with potent suppressive activity induced under a Th17-prone microenvironment in a Stat3-dependent manner, as suggested previously (Yang et al., 2015).

Although we have observed that RORγt$^+$ Tr17 cells had strong suppressive activity against MOG-specific Th17 cells in vitro and in vivo, we did not see any difference in onset and severity of the EAE between RORγt$^+$ Treg cell-deficient RORγ$^{f/f}$Foxp3^{Cre} mice and WT mice. It is more plausible that RORγt and CCR6 expression is rather a phenotypic marker of the Ag-specific activated Treg cells generated under the Th17-cell-promoting microenvironment than a functional component necessary for Treg cell activity. Considering that the total ICOS^{hi} Treg cell frequency was not changed after immunization, even in the absence of RORγt (Figure 4A), ICOS^{hi}CCR6$^-$ Treg cells might have compensated for the absence of ICOS^{hi}RORγt$^+$CCR6$^+$ Treg cells in RORγ$^{f/f}$ Foxp3^{Cre} mice. A recent study demonstrated that depletion of T-bet$^+$ Treg cells rather than deletion of the *Tbx21* gene in Treg cells resulted in Th1-dependent immunopathology (Levine et al., 2017). In this regard, selective depletion of RORγt$^+$CCR6$^+$ Treg cells after their development is required to address the functional relevance of the population in EAE, as suggested previously (Kim et al., 2007). Another possibility is that the numbers of Tr17 cells generated in EAE were not sufficient to suppress autoimmunity during an excessive inflammation reaction.

The role of CCR6 in EAE has been controversial (Elhofy et al., 2009; Kleinewietfeld et al., 2005; Liston et al., 2009; Moriguchi et al., 2013; Reboldi et al., 2009; Villares et al., 2009; Yamazaki et al., 2008). We and others have demonstrated that CCR6 expression is required for Th17 cells to enter into the CNS to initiate CNS inflammation by showing that EAE development is attenuated in CCR6-deficient mice (Liston et al., 2009; Reboldi et al., 2009; Yamazaki et al., 2008). In contrast, others showed that CCR6 deficiency exacerbated EAE progression by regulating different immune cells, such as Treg cells or dendritic cells (Elhofy et al., 2009; Villares et al., 2009). In the current study, we found that most of the adoptively transferred RORγt$^+$CCR6$^+$ Tr17 cells reside in the periphery without migrating to the CNS, whereas they suppress MOG-specific Th17-mediated CNS inflammation in a Th17 cell transfer EAE model (Figures 7C and 7E). In addition, Treg cell-specific deletion of RORγt in RORγ$^{f/f}$ Foxp3^{Cre} mice did not critically affect the CNS homing of Treg cells after EAE induction (data not shown). These results indicate that RORγt-dependent CCR6 expression in Treg cells is neither sufficient nor necessary for CNS homing of Treg cells. As previously suggested by others, it is conceivable that CCR6 expression is not required for Treg cells to migrate to the CNS when CNS inflammation is initiated by Th17 cells (Reboldi et al., 2009). Also, we do not rule out RORγt-independent CCR6 expression in Treg cells because we detected CCR6^{int} Treg cells even in the absence of RORγt. The role of CCR6 in EAE progression warrants a more detailed analysis with cell-type-specific deletion of CCR6 in different immune cells.

The role of Treg cells in human multiple sclerosis has been unclear. Although the frequency of CD4$^+$CD25^{hi} Treg cells in peripheral blood was not significantly different between multiple sclerosis (MS) patients and healthy subjects, a decrease in Foxp3 expression and a defect in Treg function have been found in MS patients (Kleinewietfeld and Hafler, 2014; Venken et al., 2008; Viglietta et al., 2004). In contrast, a recent study did not support that Treg function is defective in MS patients (Michel et al., 2008). Because most of these studies focused on conventional Treg cells, it is important to dissect Treg cell subsets based on additional cellular markers. In this regard, it has been reported that CD103$^+$ Treg cells are increased in relapsing-remitting MS patients, especially after IFN-β treatment (Venken et al., 2008). In addition, CD39$^+$ Treg cells that are selectively required for Th17 cell regulation have been identified in MS patients (Borsellino et al., 2007). Whether a human counterpart for RORγt$^+$ Tr17 cells can be found in MS patients and whether there is any relationship between the frequency of RORγt$^+$CCR6$^+$ Treg cells and the progression of MS will be important questions to be elucidated in the future.

Overall, we found that RORγt$^+$CCR6$^+$Foxp3$^+$ Tr17 cells induced by s.c. immunization represent activated Ag-specific effector regulatory T cells. RORγt$^+$ Tr17 can regulate Th17-mediated CNS autoimmunity by inhibiting the peripheral expansion and CNS migration of myelin-specific Th17 cells. This study will provide a guide to the development of an effective Treg cell immunotherapy against multiple sclerosis and other autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice

Mice were bred and maintained in the specific pathogen-free (SPF) animal facility at the University of Texas (UT) MD Anderson Cancer Center in accordance with institutional guidelines. We generated $ROR\gamma^{f/f}$ mice in which exons 2 and 3 of the *Rorc* gene are flanked by *LoxP* sites. The resultant $ROR\gamma^{f/f}$ mice were bred to FLPeR mice to remove the Neomycin resistance cassette and then backcrossed to C57BL/6 mice for six generations. $Foxp3^{YFP\text{-}Cre}$ knockin mice and $Foxp3^{GFP}$ reporter mice were kindly provided by Dr. Alexander Rudensky (Memorial Sloan Kettering Cancer Center). $Foxp3^{GFP\text{-}Cre}$ bacterial artificial chromosome (BAC) transgenic mice were kindly provided by Drs. Jeffrey Bluestone (University of California, San Francisco [UCSF]) and Shao-Cong Sun (UT MD Anderson Cancer Center). $Stat3^{f/f}CD4^{Cre}$ and $B7h^{-/-}$ mice were described previously (Nurieva et al., 2003; Yang et al., 2007). C57BL/6 and BALB/c mice were purchased from Jackson Laboratories. $Foxp3^{GFP}$ reporter mice on the BALB/c background and DO11.10 mice were obtained from Jackson Laboratories and crossed in our animal facility. Female and male mice at 8–12 weeks of age were used for experiments.

Antigen-Specific Induction of $ROR\gamma t^+CCR6^+$ Treg Cells In Vivo

$CD4^+KJ1\text{-}26^+CCR6^-GFP^+$ Treg cells isolated from DO11.10x$Foxp3^{GFP/Y}$ male mice were adoptively transferred into BALB/c recipient mice. One day after the transfer, recipient mice were left untreated or immunized with either OVA (grade V, Sigma) or KLH (Sigma), both of which were emulsified in IFA supplemented with *M. tuberculosis* extract. Seven days after immunization, $CD4^+KJ1\text{-}26^+$ T cells in draining inguinal lymph nodes were analyzed for RORγt, CCR6, and Foxp3 expression.

Induction of EAE

For active EAE induction, mice were immunized s.c. with MOG_{35-55}/CFA at the tail base on day 0. Pertussis toxin (List Biological Laboratories) was injected intraperitoneally (i.p.) on day 0 and day 2. For Th17 transfer passive EAE, $CD4^+YFP^{hi}$ Th17 cells isolated from MOG_{35-55}/CFA-immunized $IL\text{-}17F^{Cre}xRosa26^{YFP}$ fate-mapping mice were adoptively transferred into $RAG1^{-/-}$ mice (2.0×10^4 Th17 cells/mouse). To test the suppressive activity of Treg cells, $CD4^+ICOS^{hi}CCR6^+YFP^{int}$ Tr17 cells or $CD4^+ICOS^{lo}CCR6^-YFP^{int}$ resting Treg cells isolated from MOG_{35-55}/CFA-immunized $Foxp3^{YFP\text{-}Cre}$ mice were adoptively transferred into $RAG1^{-/-}$ mice together with Th17 cells (8.0×10^4 Treg cells/mouse). The recipient mice were immunized with MOG_{35-55}/IFA followed by two PTX injections on the day of immunization and 2 days later. EAE disease progression was monitored daily, and the disease score was assigned on a scale as follows: 0, no disease; 1, loss of tail tonicity; 2, wobbly gait; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, moribund.

Statistical Analysis

Statistical analysis was performed by two-tailed t test. A p value of less than 0.05 was considered statistically significant: *, $p < 0.05$. **, $p < 0.005$. All error bars were drawn based on SE.

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA-seq data reported in this paper is GEO: GSE103319.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.09.021.

AUTHOR CONTRIBUTIONS

C.D., S.H.C., Y.C., and B.-S.K. designed the research and analyzed the data. B.-S.K. performed most of the experiments, and K.I., X.C., K.T., Y.-h.L., and S.H.C. participated in specific experiments. X.Y. and Y.-B.Z. generated $ROR\gamma^{f/f}$ mice. R.N. provided $B7h^{-/-}$ mice. B.-S.K., H.L., N.A.M., L.Z., and W.J. analyzed the RNA sequencing data. B.-S.K., S.H.C., and C.D. prepared the manuscript.

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