ARTICLE

REGy controls Th17 cell differentiation and autoimmune inflammation by regulating dendritic cells

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Interleukin-17A (IL-17A)-producing helper T (Th17) cells are a subset of CD4⁺ T cells that play important pathological roles in autoimmune diseases. Although the intrinsic pathways of Th17 cell differentiation have been well described, how instructive signals derived from the innate immune system trigger the Th17 response and inflammation remains poorly understood. Here, we report that mice deficient in REGy, a proteasome activator belonging to the 11S family, exhibit significantly deteriorated autoimmune neuroinflammation in an experimental autoimmune encephalomyelitis (EAE) model with augmented Th17 cell polarization in vivo. The results of the adoptive transfer of CD4⁺ T cells or dendritic cells (DCs) suggest that this phenotype is driven by DCs rather than T cells. Furthermore, REGy deficiency promotes the expression of integrin $\alpha\beta 8$ on DCs, which activates the maturation of TGF- $\beta 1$ to enhance Th17 cell development. Mechanistically, this process is mediated by the REGy-proteasome-dependent degradation of IRF8, a transcription factor for $\alpha\beta 8$. Collectively, our findings delineate a previously unknown mechanism by which REGy-mediated protein degradation in DCs controls the differentiation of Th17 cells and the onset of an experimental autoimmune disease.

Key words: REGy; EAE; DC; TGF- β ; Th17

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INTRODUCTION

The role of Th17 cells, defined as a distinct group of CD4⁺ effector T cells that secrete IL-17A, IL-17F, IL-21, and IL-22, in the pathogenesis of autoimmune disorders such as experimental autoimmune encephalomyelitis (EAE) has been well documented.^{1–4} It is essential to understand the in vivo mechanisms underlying the development and maintenance of Th17 cells. Previous studies have reported that the differentiation of Th17 cells is induced by IL-6 and IL-1 β or IL-21 in the presence of transforming growth factor- $\beta 1$ (TGF- $\beta 1$) through the activation of retinoic-acid-receptor-related orphan receptors α and γ (ROR α and ROR γ , respectively) and signal transducer and activator of transcription 3 (STAT3).^{5–13} Once developed, IL-23 has been implicated in maintaining and expanding differentiated Th17 cells. More importantly, IL-23 directly endows Th17 cells with pathogenic effector functions since Th17 cells are nonpathogenic without further exposure to IL-23. In addition to inducing the expression of ROR γ and favoring Th17 cell differentiation, TGF- $\beta 1$ stimulates the expression of Foxp3, a transcription factor required for the generation of regulatory T cells (Tregs) and the maintenance of Treg functions.^{5,14,15} Additionally, IL-6 is required for the stabilization of ROR γ and repression of Foxp3.^{5,16,17} In contrast to T cell-intrinsic mechanisms, how instrumental signals derived from the innate immune systems drive Th17 cell

development and organ-specific autoimmune inflammation remains poorly understood.

Dendritic cells (DCs) orchestrate adaptive immune responses by triggering the activation and differentiation of naïve T cells. Through pattern-recognition receptors expressed on DCs, microbial pathogens initiate the activation of DCs, followed by the induction of adaptive immunity. Accordingly, recent studies have revealed that DCs contribute to the pathogenesis of most autoimmune diseases.^{18–20} Nevertheless, how DCs sense innate immune signals and further shape T cell responses, especially Th17 cell development and autoimmune pathogenesis, remains to be illuminated.

REGy, also known as PA28 γ , Ki antigen, and PSME3, is a member of the 11S family of proteasome activators that binds to the 20S core proteasome and mediates the degradation of multiple intact proteins in an ATP- and ubiquitin-independent manner.^{21–23} Previous studies have demonstrated that REGy plays important roles in innate immune responses during fungal and viral infection, acute colitis, and associated cancers.^{24–26} Moreover, REGy deficiency does not affect the development of T cells and B cells but increases the immunoglobulin class switch through mediating the degradation of activation-induced cytidine deaminase (AID) in B cells.^{27,28} Nevertheless, the impact of REGy on the functions of helper T cells and adaptive autoimmune immunity has not been defined.

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In this study, we demonstrate how the proteasome activator REGy functions in DCs to regulate Th17 cell differentiation and autoimmune inflammation. Mice deficient in REGy exhibited exacerbated EAE progression with enhanced Th17 cell proliferation, for which DCs are indispensable. REGy was found to regulate the expression of integrin $\alpha\text{v}\beta 8$ by degrading IRF8 in DCs to control Th17 cell differentiation. Thus, our studies elucidate an essential mechanism by which DC-T cell crosstalk supports Th17 cell differentiation and autoimmune inflammation.

RESULTS

Depletion of REGy aggravates the autoimmune disease EAE Having defined the contribution of REGy to the innate immune response and viral infection,^{26,29} we investigated the requirement of REGy in adaptive immunity and autoimmune disease. Wild-type (WT) and REGy-deficient (REGy^{-/-}; KO) mice were immunized with myelin oligodendrocyte glycoprotein peptide (MOG₃₅₋₅₅) to induce EAE pathogenesis, establishing a mouse model characterized by inflammatory cell infiltration and demyelination reminiscent of multiple sclerosis (MS) in humans. Compared with immunized WT mice, REGy^{-/-} mice developed more severe phenotypes reflected by significantly higher clinical scores and the earlier onset of disease (Fig. 1a). Moreover, histological analysis revealed significantly increased inflammation (hematoxylin and eosin staining) and demyelination (Luxol fast blue staining) in the REGy^{-/-} mouse spinal cord (Fig. 1b). As shown by flow cytometry and quantitative analysis, we observed a remarkable increase in the numbers of CD4⁺ T cells and CD11b⁺CD45^{hi} macrophages in the REGy^{-/-} mouse spinal cord but no differences in the numbers of CD8⁺ T cells, CD19⁺ B cells, and CD11b⁺CD45^{lo} microglia between WT and KO mice (Fig. 1c, d). Furthermore, ex vivo analysis of the MOG-specific proliferation of T cells indicated increased proliferative capacity in T cells from REGy^{-/-} mice than in T cells from WT mice in response to MOG₃₅₋₅₅ (Fig. 1e). Collectively, these data indicate that REGy deficiency exacerbates autoimmune disease.

REGy deficiency promotes polarization of Th17 cells in vivo Given that CD4⁺ T cells, including Th1 cells, Th17 cells, and Tregs, play critical roles in EAE development,^{1,15,30} we analyzed the global effects of REGy on cytokine expression in CD4⁺ T cells purified from draining lymph nodes at the peak of disease. In comparison to WT mice, REGy^{-/-} mice displayed significantly higher levels of *Il17a* (IL-17A) and *Il17f* (IL-17F), whereas *Il23r* (IL-23R), *Il21* (IL-21), *Il22* (IL-22), *Rorc* (ROR γ t), *Stat3* (STAT3), *Ifng* (IFN- γ), and *Foxp3* (Foxp3) levels were no different (Fig. 2a). As shown by intracellular staining and flow cytometry, the REGy-deficient EAE mouse model displayed significantly increased numbers of Th17 cells in the spleen, draining lymph nodes and spinal cord, whereas the numbers of Th1 cells and Tregs were comparable to those in WT mice (Supplementary Fig. 1A, Fig. 2b, c). Among the panel of pro- and anti-inflammatory cytokines evaluated, IL-17A was significantly elevated in splenocytes from REGy^{-/-} mice restimulated with MOG peptide (Fig. 2d). Granulocyte-macrophage colony-stimulating factor (GM-CSF) has emerged as a proinflammatory cytokine with nonredundant functions in autoimmune diseases including EAE, and the neutralization of GM-CSF is currently being tested in clinical trials for a number of diseases including MS.^{31,32} We then analyzed CD4⁺ T cell-intrinsic GM-CSF expression by flow cytometry and found that REGy^{-/-} mice exhibited levels of GM-CSF similar to those in WT controls (Supplementary Fig. 1B). To investigate the reactivity of effector T cells to specific antigens, draining lymph node cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and challenged with MOG₃₅₋₅₅. Upon stimulation, REGy^{-/-} mouse-derived Th17 cells proliferated much faster than those from WT controls, whereas the proliferation of Th1 cells was unchanged

(Fig. 2e), leaving only minor differences between the WT and KO mouse CD4⁺ T cell populations (Supplementary Fig. 1C). CD40 ligand (CD40L) is rapidly expressed after activation and can therefore serve as a marker for MOG-specific effector T cells;³³ therefore, we examined MOG-specific Th17 or Th1 cells by gating on CD40L^{high} effector T cells. Consistently, the frequency of MOG-specific Th17 cells was significantly higher in REGy^{-/-} mice relative to WT controls, whereas the frequency of Th1 cells was no different (Supplementary Fig. 1D). To determine whether REGy suppresses polarization of Th17 cells exclusive to MOG-specific responses, we immunized WT and REGy^{-/-} mice with the antigen keyhole limpet hemocyanin (KLH). As shown by intracellular staining, a much higher frequency of Th17 cells was observed in draining lymph node cells from REGy^{-/-} mice, while the frequencies of Th1 and Tregs in REGy^{-/-} and WT mice were comparable (Fig. 2f). Taken together, our data demonstrate that REGy attenuates the in vivo development of Th17 cells following specific antigen stimulation.

DCs are the key determinant of REGy-dependent regulation of Th17 cell development and autoimmune disease

We next sought to determine whether REGy affects the process of intrinsic Th17 cell differentiation from naïve CD4⁺ T cells. We performed the ex vivo differentiation of isolated naïve CD4⁺ T cells under Th1-, Th17- or inducible Treg-polarizing conditions. To our surprise, the absence of REGy did not have cell-autonomous effects on Th1, Th17, or Treg differentiation (Supplementary Fig. 2A). To substantiate our findings in vivo, we transferred WT or REGy^{-/-} CD4⁺ T cells into *Rag1*^{-/-} mice and immunized them with MOG₃₅₋₅₅. Mice to whom WT CD4⁺ T cells were transferred exhibited clinical scores similar to those of mice to whom REGy^{-/-} CD4⁺ T cells were transferred (Fig. 3a). We conclude that REGy^{-/-} CD4⁺ T cells are not intrinsically defective in their ability to differentiate into Th1 cells, Th17 cells, and Tregs.

DCs orchestrate innate and adaptive immunity by priming naïve T cells. To investigate whether REGy has an impact on DC function, we employed transgenic CD11c-DTR mice, in which the administration of diphtheria toxin (DT) ablates the CD11c⁺ population.³⁴ We confirmed that the application of DT successfully and effectively ablated the CD11c⁺ cell population in CD11c-DTR mice within 48 h (Supplementary Fig. 2B). We then transferred WT or REGy^{-/-} splenic DCs into CD11c-DTR mice in which endogenous CD11c⁺ cells had been depleted and immunized the recipients with MOG₃₅₋₅₅. In contrast to WT-DTR recipients, REGy^{-/-}-DTR recipients developed more severe EAE (Fig. 3b), which was accompanied by the increased infiltration of inflammatory cells and demyelination (Fig. 3c). In addition, flow cytometry analysis revealed an increased frequency of Th17 cells in draining lymph nodes from REGy^{-/-}-DTR recipients, whereas the frequencies of Th1 cells and Tregs were comparable to those in WT-DTR recipients (Fig. 3d). These findings demonstrate the essential role of DCs in promoting EAE in REGy^{-/-} mice.

To further assess how REGy regulates DC-T cell crosstalk during the activation and differentiation of naïve T cells in vivo, we isolated naïve CD4⁺ T cells (CD45.1⁺) from OT-II mice (which transgenically express a TCR specific for ovalbumin peptide 323–339) and transferred them into WT and REGy^{-/-} mice, followed by immunization with OVA. REGy^{-/-} recipients exhibited a higher frequency of Th17 cells than WT mice, whereas the Th1 cell frequency was unaltered (Fig. 3e). Additionally, we cocultured naïve OT-II⁺ CD4⁺ T cells with LPS/IFN- γ -pretreated bone marrow-derived dendritic cells (BMDCs) in the presence of OVA to mimic the interaction between DCs and T cells. Consistent with the in vivo data, primed BMDCs from REGy^{-/-} mice stimulated a significant increase in the frequency of Th17 cells compared to BMDCs from WT mice; however, the Th1 cell frequency was unchanged (Fig. 3f). Collectively, these data suggest that increased

Fig. 1 REG γ deficiency deteriorates EAE progression. **a** Clinical scores of age-matched female WT and REG $\gamma^{-/-}$ mice (n = 8 per group) after the induction of EAE with MOG₃₅₋₅₅. **b** Spinal cord sections obtained from the mice at day 16 after immunization were analyzed for inflammation or demyelination by hematoxylin and eosin (H&E) staining or Luxol fast blue (LFB) staining, respectively. Differences are highlighted by red arrows. Original magnification, $\times 10$. Flow cytometry analysis (**c**) and the absolute number (**d**) of immune cells infiltrated into the spinal cord at day 16 after immunization. **e** Proliferation of cells from draining lymph nodes in response to MOG₃₅₋₅₅ at the indicated concentrations, as measured by [3 H]thymidine incorporation. Data are representative of two (**b-e**) or three (**a**) independent experiments with three (**b**) or five (**c-e**) mice per group. Data are the means $\pm$ SEMs. * P < 0.05; ** P < 0.01 (Student's t -test)

Th17 cell polarization and aggravated EAE phenotypes in REG $\gamma^{-/-}$ mice are determined by DCs and not T cell-intrinsic effects.

REG γ modulates Th17 cells by DCs partially via IL-6. To determine the cellular mechanism by which REG γ acts on DCs to modulate Th17 cell differentiation, we analyzed the development of subclasses of DCs. Surprisingly, no significant differences in the development of CD4 $^{+}$ DC, CD8 $^{+}$ DC, CD11b $^{+}$ DC, and B220 $^{+}$ DC in both the spleens and draining lymph nodes of REG $\gamma^{-/-}$ and WT mice were observed (Supplementary Fig. 3A, B). In addition, the expression levels of CD40, CD80, CD86, and MHC II

in REG $\gamma^{-/-}$ and WT mice were comparable (Supplementary Fig. 3C). To further understand the ability of REG $\gamma^{-/-}$ DCs to prime the CD4 $^{+}$ T cell response, we performed an in vivo antigen-presentation assay and found that OT-II $^{+}$ CD4 $^{+}$ T cells proliferated to a similar extent as their REG $\gamma^{-/-}$ and WT counterparts, suggesting that REG γ in DCs is dispensable for priming CD4 $^{+}$ T cells (Supplementary Fig. 3D).

We next investigated the profiles of inflammatory genes in isolated DCs at EAE onset. The expression of *Il6* was modestly increased in REG $\gamma^{-/-}$ splenic DCs compared to WT DCs, while *Il1b*, *Tnfa*, and *Il10* expression levels were no different (Supplementary

Fig. 2 Deletion of REGγ promotes the generation of Th17 cells in vivo. **a** Quantitative PCR analysis of CD4⁺ T cells isolated from draining lymph nodes at day 16 after immunization. **b** Flow cytometry analysis of Th1 cells, Th17 cells, and Tregs from draining lymph nodes (**b**) and the spinal cord (**c**) at day 16 after immunization. **d** Cytokine production by draining lymph nodes was measured after ex vivo MOG₃₅₋₅₅ stimulation for 72 h. **e** Draining lymph node cells were labeled with CFSE and stimulated with MOG₃₅₋₅₅ for 72 h, followed by examination of Th1 cells and Th17 cells in CFSE^{low} populations of CD4⁺ T cells. **f** Flow cytometry analysis of Th1 cells, Th17 cells, and Tregs from draining lymph nodes at day 10 after keyhole limpet hemocyanin immunization. Data are representative of two (**a**, **e**, and **f**) or three (**b–d**) independent experiments with four (**b–e**), five (**a**), or six (**f**) mice per group. Data are the means ± SEMs. *P < 0.05; **P < 0.01 (Student's *t*-test)

Fig. 4A). In the presence of lipopolysaccharide (LPS) and IFN-γ, REGγ-deficient BMDCs exhibited slightly more secreted IL-6 than WT controls (Supplementary Fig. 4B). To determine whether IL-6 is indeed the critical determinant that promotes Th17 cell development in REGγ-deficient DCs, we cocultured DC-T cells with or without exogenous recombinant IL-6 protein. Interestingly, exogenous IL-6 boosted the generation of Th17 cells from WT DCs to a level comparable to that in the untreated REGγ-deficient group; however, significantly increased Th17 cell polarization was still observed in the REGγ-deficient class following treatment with exogenous IL-6, indicating the existence of other critical mechanisms in DCs that orchestrate Th17 cell differentiation (Supplementary Fig. 4C).

REGγ potentiates the activation of latent TGF-β1 for Th17 cell differentiation
TGF-β1 is a critical factor for Th17 cell development.^{5,9,11} To understand whether REGγ deficiency affects the production of TGF-β1 in DCs, we examined *Tgfb1* expression by quantitative PCR. However, deletion of REGγ did not alter *Tgfb1* transcription in MOG-immunized splenic DCs (Supplementary Fig. 5A). Previous studies suggested that active TGF-β1 is released from a complex comprising latent TGF-β1 and its binding protein, LAP (latency associated peptide).³⁵ The expression of integrin αv, especially integrin αvβ8, on DCs enables the switch of latent TGF-β1 to biologically active TGF-β1, which in turn triggers the differentiation of Th17 cells and Tregs.^{36,37} To test the hypothesis that REGγ is

Fig. 3 Aggravated EAE progression in REGy^{-/-} mice is driven by dendritic cells and not a T cell-intrinsic effect. **a** Clinical EAE scores in *Rag1*^{-/-} mice reconstituted with CD4⁺ T cells from WT or REGy^{-/-} mouse (*n* = 6 per group). **b** Clinical EAE scores in CD11c-DTR recipient mice in which splenic DCs from WT or REGy^{-/-} mice were transferred after the elimination of intrinsic DCs (*n* = 7 per group). **c** Pathological analysis of the spinal cords of recipient mice in **b**; differences are highlighted by red arrows. **d** Flow cytometry analysis of Th1 cells, Th17 cells, and Tregs in the recipient mice in **b**. **e** Flow cytometry analysis of Th1 cells and Th17 cells from donor CD45.1⁺ OT-II T cells in the draining lymph nodes of OVA-immunized recipients. **f** Flow cytometry analysis of Th1 cells and Th17 cells from OT-II T cells after their coculture with activated and OVA-pulsed WT or REGy^{-/-} BMDCs for 4 days ex vivo. Data are representative of two (**a–e**) or three (**f**) independent experiments with four (**c, d** and **f**) or five (**e**) mice per group. Data are the means ± SEMs. **P* < 0.05; ***P* < 0.01 (Student's *t*-test)

involved in the activation of TGF-β1 in DCs to prime naïve CD4⁺ T cells, we analyzed the development of Th17 cells in vitro following the coculture of MOG-immunized WT or REGy^{-/-} splenic DCs with naïve CD4⁺ T cells in the presence of TGF-β1 or latent TGF-β1 under Th17 cell-polarizing conditions. Although REGy^{-/-} DCs and WT controls in their ability to generate Th17 cells with or without recombinant TGF-β1, the REGy^{-/-} group exhibited the significantly augmented production of Th17 cells upon treatment with latent TGF-β1, indicating more efficient activation of TGF-β1 by REGy^{-/-} DCs than WT DCs (Fig. 4a). Consistent with this finding, the secretion of IL-17A was much higher in the REGy^{-/-} group in the presence of latent TGF-β1 (Fig. 4b). In contrast, no significant difference was observed in the production of IFN-γ between WT and REGy^{-/-} mice under any conditions (Supplementary Fig. 5B). In ex vivo recall experiments with the MOG peptide, we found much more active TGF-β1 in the supernatants of REGy^{-/-}

splenocytes, whereas total TGF-β1 levels were no different in comparison to those in WT controls (Fig. 4c, d). To determine whether integrin αvβ8 liberates active TGF-β1 in REGy^{-/-} DCs, we examined the transcripts of *Itgb8*, which encodes integrin αvβ8. Quantitative PCR analysis revealed the elevated expression of *Itgb8* in activated splenic DCs and BMDCs from REGy^{-/-} mice (Fig. 4e, f). To determine the mechanism by which the REGy-mediated regulation of integrin αvβ8 impacts Th17 cell differentiation via the release of active TGF-β1 from DCs, we analyzed latent TGF-β1-mediated Th17 cell polarization in the presence of an anti-αvβ8 neutralizing antibody or an isotype control. Notably, neutralization of αvβ8 effectively inhibited Th17 cell generation, and the frequency of Th17 cells was comparable between the WT and REGy^{-/-} groups (Fig. 4g). These findings suggest that REGy deficiency regulates Th17 cell differentiation via the upregulation of integrin αvβ8 on DCs to facilitate the release of TGF-β1.

Fig. 4 REGy modulates Th17 cell polarization by controlling active TGF-β1 production. Naïve CD4⁺ T cells were cultured with MOG-immunized splenic DCs for 4 days under Th17 polarization conditions in the absence or presence of active TGF-β1 or latent TGF-β1. Cells expressing IL-17A were detected by flow cytometry (**a**), and culture supernatants were collected for ELISA (**b**). ELISA analysis of active TGF-β1 (**c**) and total TGF-β1 (**d**) protein levels in the supernatants of draining lymph node cells from MOG-immunized mice MOG rechallenge for 3 days ex vivo. Transcriptional levels of *Itgb8* in MOG-immunized splenic DCs (**e**) and BMDCs with or without 6 h of stimulation (**f**). **g** Naïve CD4⁺ T cells were cultured with MOG-immunized splenic DCs for 4 days under latent TGF-β1-mediated Th17 development conditions in the absence or presence of anti-αvβ8 neutralization antibody. Th17 cells were examined by flow cytometry. Data are representative of two (**c**, **d** and **g**) or three (**a**, **b**, **e** and **f**) independent experiments with four (**a**–**d**, **g**) or five (**e** and **f**) mice per group. Data are the means ± SEMs. **P* < 0.05; ***P* < 0.01 (Student's *t*-test or two-way ANOVA)

Although integrin αvβ8-mediated TGF-β1 activation also induces Treg cell differentiation, we did not observe any differences in Treg cell polarization between the WT or KO groups during DC-T cell coculture assays in the presence of either TGF-β1 or latent TGF-β1 (Supplementary Fig. 5C). We postulate that the overproduction of IL-6 in REGy^{-/-} DCs (Supplementary Fig. 4A, B) suppressed Treg cell polarization in the REGy^{-/-} group. We tested our hypothesis in DC-T cell coculture assays in which Treg cell differentiation is mediated in the presence of an anti-IL-6

neutralizing antibody or an isotype control. Interestingly, neutralization of DC-derived IL-6 was sufficient to promote Treg cell polarization, and a more dramatic increase in the number of Tregs in the REGy^{-/-} group compared to those in the WT group was observed (Supplementary Fig. 5D). A previous study suggested that CD11b⁺Sirpα⁺ DCs can trans-present IL-6 through a complex containing DC-expressed IL-6Rα bound to IL-6 that is able to interact with gp130 expressed on T cells. This “IL-6 cluster signaling” is indispensable for generating pathogenic Th17 cells

Fig. 5 REGγ negatively regulates the protein level of IRF8. **a** Immunoblot analysis of IRF family members in MOG-immunized splenic DCs. **b** Transcriptional analysis of *Irf8* in MOG-immunized splenic DCs. **c** Immunoblot analysis of IRF family members in BMDCs in the presence of IFN-γ (20 ng/ml). **d** Endogenous stability of IRF8 was detected in BMDCs after cycloheximide (100 mg/ml) treatment for the indicated time with the calculated rate of decrease. **e** Exogenous stability of HA-IRF8 was detected in 293 cells expressing WT and N151Y REGγ (dominant-negative mutation) after cycloheximide (100 mg/ml) treatment for the indicated time. Data are representative of two (**a**, **c**, **d** and **e**) or three (**b**) independent experiments. Data are the means ± SEMs. *P < 0.05; **P < 0.01 (Student's *t*-test)

in vivo¹⁹. To determine whether REGγ deficiency affects the composition of CD11b⁺Sirpa⁺ DCs and CD11b⁺IL6Rα⁺ DCs, we analyzed these DC subsets by flow cytometry and observed comparable profiles between both populations in WT and REGγ^{-/-} mice (Supplementary Fig. 5E, F).

REGγ regulates DC functions through negatively regulating IRF8 at the protein level

IRF8 (Interferon Regulatory Factor 8) activates integrin αvβ8-mediated TGF-β signaling and facilitates Th17 cell-dependent neuroinflammation.³⁸ In our efforts to elucidate the molecular

mechanisms of these functions, we found a selective increase in the IRF8 level, but not the *Irf8* transcription level (Fig. 5b), in REGγ^{-/-} splenic DCs from MOG-immunized mice (Fig. 5a), whereas levels of the other IRF family members IRF4 and IRF5 were unaltered (Fig. 5a). Consistently, REGγ^{-/-} BMDCs exhibited notable enhancement of the IRF8 protein in the presence of IFN-γ in comparison to WT BMDCs (Fig. 5c). In degradation kinetics studies, the half-life of IRF8 in WT BMDCs was obviously shorter than that in REGγ^{-/-} BMDCs in the presence of cycloheximide, suggesting a REGγ-dependent degradation mechanism (Fig. 5d). Furthermore, in a well-established, REGγ-inducible cell system in

Fig. 6 REG γ mediates IRF8 turnover. **a** Flag-tagged REG γ was transfected into 293T cells with empty vector or HA-tagged IRF8. Cells were harvested and lysed after treatment with 10 μ M MG132 for 6 h. Immunoprecipitated products were analyzed by immunoblotting. **b** The endogenous interaction between IRF8 and REG γ was detected by coimmunoprecipitation in BMDCs. **c** REG γ mediated the proteolytic degradation of IRF8 in a cell-free system. Purified REG γ and in vitro-translated IRF8, p21, or I κ B α were incubated along with the 20S proteasome for 2 h at 30 $^{\circ}$ C, followed by immunoblotting. Data are representative of two (**b**, **c**) or three (**a**) independent experiments

which functional REG γ or dominant-negative mutant (N151Y) REG γ is expressed upon the addition of doxycycline, we demonstrated that cells expressing only intact REG γ could repress IRF8 expression (Fig. 5e).

To understand whether IRF8 is a direct substrate of REG γ , we validated the physical interaction between REG γ and IRF8 by reciprocal coimmunoprecipitation assays (Fig. 6a, b). We next determined whether the REG γ -proteasome can degrade IRF8 in a cell-free system. Incubation of in vitro-translated IRF8 with purified REG γ and the 20S proteasome significantly degraded IRF8, whereas the 20S proteasome or REG γ alone was unable to promote the destruction of IRF8 (Fig. 6c). Taken together, these data reveal that REG γ exerts opposing effects by negatively regulating the stability of IRF8 in DCs, thereby orchestrating a program of DC-dependent Th17 cell differentiation.

DISCUSSION

In this study, we demonstrate that REG γ integrates extrinsic signals in DCs to drive the differentiation of Th17 cells during autoimmune inflammation, shedding new light on the regulation of DC-T cell crosstalk and Th17 cell polarization. The DC-intrinsic role of REG γ causes mice lacking REG γ to be more susceptible than WT mice to EAE progression, which is characterized by the

increased polarization of Th17 cells. In addition to the increased production of IL-6, REG γ deficiency triggers the differentiation of Th17 cells via stabilizing IRF8 and upregulating integrin $\alpha\beta$ 8 on DCs, which facilitates the release of active TGF- β 1. The regulation of integrin $\alpha\beta$ 8 in DCs is molecularly mediated by the REG γ -dependent degradation of IRF8 (Fig. 7).

DCs bridge innate and adaptive immunity by efficiently processing and presenting pathogen-derived peptides, resulting in the activation of functional T cells. Increasing evidence suggests that DCs transduce and integrate innate signals for Th17 cell differentiation, tissue homeostasis, and inflammation via distinct mechanisms.^{19,20,39} The classic ubiquitin proteasome system (UPS), including the UPS components PDLIM2, SIAH1/2, and Itch, has been well documented to modulate T cell functions.⁴⁰⁻⁴² We are the first to identify the function of a noncanonical, ubiquitin- and ATP-independent proteasome pathway in DCs to control Th17 cell polarization and regulate autoimmune inflammation via IRF8 that plays essential roles in DC development. Surprisingly, we did not observe differences in the number of DC subsets between WT and REG γ -deficient mice (Supplementary Fig. 3A, B). Previously, we reported that REG γ activity is increased during inflammation.²⁶ We believe that REG γ mediates an increased rate of IRF8 turnover under inflammatory conditions.

Fig. 7 A schematic depicting REGγ-mediated Th17 cell differentiation. Incursive antigens are sampled by dendritic cells (DCs), and antigen-loaded DCs then migrate to draining lymph nodes, where they activate naïve CD4⁺ T cells, resulting in the differentiation of Th17 cells. DC-intrinsic REGγ controls the expression of integrin αvβ8 by regulating the protein degradation of IRF8, thereby modulating the release of active TGF-β1 production associated with the modest regulation of IL-6 to manipulate Th17 cell development

A diverse range of environmental signals endow Th17 cells with dichotomous plasticity that supports the nonpathogenic production of IL-17 and the pathogenic roles of Th17 cells in many inflammatory diseases.^{43–47} In fact, Th17 cells generated by TGF-β1 and IL-6 alone are nonpathogenic since they are not ready to induce autoimmune diseases without further exposure to IL-23. Thus, IL-23, which is synthesized with additional signaling, is a determinant of the pathogenicity of Th17 cells, leading to the stabilization of Th17 cells, suppression of IL-10 production, promotion of GM-CSF, and maintenance of TGF-β3.^{31,45,48–50} Our observation that REGγ deficiency promotes DC-mediated polarization of Th17 cells in vitro and in vivo prompts us to speculate that the progression of EAE in REGγ-deficient mice is initiated by the generation of more Th17 cells at the differentiation stage, followed by exposure to IL-23, resulting in the accumulation of pathogenic Th17 cells and a deteriorated disease status in vivo. This notion may explain why the differences in disease scores between WT and KO mice are most dramatic at the peak of EAE.

A meta-analysis of genome-wide association scans for multiple sclerosis (MS) susceptibility mapped single nucleotide polymorphisms (SNPs) to the 3' noncoding region of the *Irf8* gene, indicating that anomalous regulation of IRF8 accounts for MS susceptibility.⁵¹ The degradation of IRF8 can occur after ubiquitination by Cbl, a member of the RING finger E3 ligase family.⁵² In this study, we identified the REGγ-proteasome as an additional regulatory pathway to control the protein stability of IRF8 in a ubiquitin- and ATP-independent manner. As with many other REGγ substrate proteins that can be degraded by both the UPS and REGγ-proteasome system, IRF8 provides an additional example of the regulation of protein stability by dual systems. However, we know little about how these two systems differentially regulate the IRF8 protein in vivo. We speculate that under physiological conditions, the relatively low activity of REGγ may exert a negligible effect on

the regulation of IRF8, justifying why we did not observe a significant difference in the development of DC subsets between WT and REGγ-deficient mice (Supplementary Fig. 3A, B). However, upon pathological challenge, increased REGγ activity may tip the balance between ubiquitin-dependent and ubiquitin-independent degradation pathways. Thus, future studies are needed to understand the contribution of these two proteasome degradation systems in the regulation of IRF8 stability under different environmental conditions.

In summary, our findings delineate REGγ as an essential regulatory factor in Th17 cell differentiation and autoimmune inflammation. REGγ modulates the function of DCs, thereby regulating the initiation of Th17 cells and the progression of EAE via crosstalk between DCs and T cells, which highlights REGγ as a potential therapeutic target for the treatment of autoimmune diseases.

MATERIALS AND METHODS

Mice

Wild-type and REGγ^{-/-} mice with a C57BL/6 genetic background were provided by Dr. John J. Monaco at the University of Cincinnati. CD11c-DTR mice (which express the diphtheria toxin receptor under the control of CD11c regulatory elements) were provided by Dr. Nan Shen at the Shanghai Jiaotong University School of Medicine. OT-II mice (which transgenically express a T cell antigen receptor specific for chicken ovalbumin amino acids 323–339) were obtained from Dr. Hongyan Wang at the Chinese Academy of Sciences. *Rag1*^{-/-} and CD45.1⁺ mice were purchased from the Jackson Laboratory. OT-II mice were bred with CD45.1⁺ mice to generate CD45.1⁺ OT-II mice. All experiments were performed with age- and sex-matched mice from 8 to 12 weeks of age housed under specific pathogen-free conditions and handled

according to high ethical and scientific standards by the Animal Center at the institute.

EAE induction

Mice were immunized subcutaneously with 200 µg of MOG_{35–55} in complete Freund's adjuvant (Sigma-Aldrich) containing 500 µg of killed *M. tuberculosis* strain H37Ra (Difco) and received an intraperitoneal injection of 200 ng of pertussis toxin (List Biological Laboratories) at the time of immunization and 48 h later. Mice were monitored daily for clinical signs and assigned scores on a 5-point scale as follows: 0, no disease; 1, tail paralysis; 2, weakness of hind limbs; 3, paralysis of hind limbs; 4, paraplegia or quadriparalysis; 5, moribund or death.

Histopathology

Mice were euthanized at the peak of disease, and their spinal cords were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or Luxol fast blue.

MOG-specific T cell proliferation and cytokine measurement

For ex vivo recall experiments, 10 days after MOG immunization, draining lymph node cells (2×10^5 per well) were labeled with or without CFSE and stimulated with MOG peptide (20 µg/ml) for 72 h. T cell responses were examined by intracellular staining or [³H]thymidine incorporation during the last 18 h of culture with a βcounter (Perkin Elmer). For cytokine measurements, 5×10^5 cells from draining lymph nodes were treated with MOG (20 µg/ml) for 72 h, and cytokines in supernatants were measured with mouse ELISA kits (R&D Systems or eBioscience) according to the manufacturer's instructions.

Cell purification and isolation

We used FACS (FACS Aria, BD Biosciences) to sort naïve CD4⁺ T cells (CD4⁺ CD25[−]CD44^{lo}CD62L^{hi}). CD4⁺ T cells were purified from the spleen and draining lymph nodes by anti-CD4 microbeads (L3T4, Miltenyi Biotec). CD11c⁺ DCs were isolated from spleens using anti-CD11c⁺ microbeads (N418, Miltenyi Biotec) according to the manufacturer's protocol. To isolate CNS leukocytes, the spinal cord was dissected, cut into small pieces, and digested in Hank's balanced salt solution containing collagenase D (5 mg/ml; Roche) for 45 min at 37°C. At the end of the digestion, the solutions were mixed thoroughly and passed through a 70-µm cell strainer, washed once in PBS, suspended in a 37% Percoll solution (GE Healthcare) and pelleted for 20 min at 800 × g. Pellets were suspended in media for subsequent analysis.

In vitro T helper cell differentiation

Purified naïve CD4⁺ T cells were stimulated with antibodies against CD3 (3 µg/ml) and CD28 (1 µg/ml) for 72 h under Th17 cell differentiation conditions as follows: rm IL-6 (10 ng/ml; R&D Systems), rm IL-23 (10 ng/ml; R&D Systems), rh TGF-β1 (1 ng/ml; R&D Systems), anti-IL-4 and anti-IFN-γ (10 µg/ml; BD Biosciences). The Th1 cell differentiation conditions were as follows: rm IL-12 (10 ng/ml; R&D Systems) and anti-IL-4 (10 µg/ml; BD Biosciences). The Treg cell differentiation conditions were as follows: rh TGF-β1 (5 ng/ml; R&D Systems).

Culture and activation of bone marrow-derived DCs

Bone marrow was collected by aspiration from mouse femurs and tibias and processed to generate erythrocyte-free cell suspensions. Cells were cultured with recombinant mouse GM-CSF (10 ng/ml; R&D Systems) and IL-4 (1 ng/ml; R&D Systems), and the media was removed and replaced with fresh differentiation media every other day. After 6 days of culture, loosely adherent cells were collected for subsequent experiments. BMDCs were challenged with 100 ng/ml LPS (Sigma-Aldrich) and IFN-γ (20 ng/ml) for activation.

Flow cytometry

For intracellular staining, T cells were stimulated with PMA (phorbol 12-myristate 13-acetate, 50 ng/ml, Sigma-Aldrich) and ionomycin (750 ng/ml; Sigma-Aldrich) for 5 h in the presence of Golgi-Plug (555029; BD Biosciences). T cells were subjected to intracellular staining for IL-17A (eBio17B7; eBioscience) and IFN-γ (XMG1.2; eBioscience) with a staining kit (555028; BD Biosciences) according to the manufacturer's instructions. T cells were subjected to intracellular staining for Foxp3 (FJK-16S; eBioscience) with a staining kit (00-5523-00; eBioscience) according to the manufacturer's instructions. For surface marker staining, we utilized antibodies against CD4 (RM4-5, GK1.5), CD25 (PC61.5), CD44 (IM7), CD62L (MEL-14), CD11c (N418), CD11b (M1/70), CD8a (53-6.7), CD40 (1C10), MHC II (M5/114.15.2), CD45.2 (104) and CD19 (eBio1D3), all of which were from eBioscience. Antibodies against CD45 (30-F11; BioLegend), CD45.1 (A20; BioLegend), CD80 (16-10A1; BD Biosciences), IL-6Rα (D7715A7), Sirpα (P84), GM-CSF (MP1-22E9) and CD86 (GL-1, BD Biosciences) were used. Samples were analyzed on a FACSCalibur or LSRII flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

Adoptive transfer and antigen challenge

CD45.1⁺ CD4⁺ T cells (2×10^6) were sorted from OT-II mice, labeled with CFSE and transferred intravenously into CD45.2⁺ WT or REGy^{−/−} recipient mice. Twenty-four hours later, the mice were immunized by the subcutaneously intraperitoneal injection of 0, 10, or 100 µg of endotoxin-free OVA (Sigma-Aldrich) emulsified in incomplete Freund's adjuvant. Three days after immunization, the mice were sacrificed, and their draining lymph node cells were collected, incubated with dilute dye and examined by flow cytometry.

A total of 2×10^6 naïve CD45.1⁺ CD4⁺ T cells were sorted from OT-II mice and transferred intravenously into CD45.2⁺ WT or REGy^{−/−} recipient mice. Twenty-four hours later, the recipients were immunized subcutaneously with OVA in the presence of LPS (1 µg per mouse; Sigma-Aldrich), emulsified in incomplete Freund's adjuvant. Seven days after immunization, the mice were sacrificed, and draining lymph node cells were analyzed following intracellular staining.

A total of 2×10^6 CD4⁺ T cells were purified from WT or REGy^{−/−} mice and transferred intravenously into recipient *Rag1*^{−/−} mice. One day later, EAE was induced in the recipient mice.

CD11c-DTR mice were given an intraperitoneal injection of diphtheria toxin (8 ng per gram of body weight) on day 0. Twenty-four hours later, 1×10^6 splenic DCs derived from WT or REGy^{−/−} mice were transferred intravenously into DT-treated CD11c-DTR mice. On day 2, EAE was induced in the recipient mice, and on days 3 and day 6, the mice were given an injection of a low dose of diphtheria toxin (4 ng per gram of body weight).

In vitro DC-T cell coculture

A total of 4×10^4 BMDCs were challenged with 100 ng/ml LPS (Sigma-Aldrich) and IFN-γ (20 ng/ml) for 6 h, pulsed with OVA for 2 h, washed, and mixed with 2×10^5 naïve CD4⁺ T cells. After 4 days of coculture, the cells were stimulated with PMA, ionomycin, and Golgi-Plug for 5 h for intracellular staining. For cytokine treatment, coculture media were supplemented with IL-6 (10 ng/ml; R&D Systems).

For latent TGF-β1-induced Th17 or Treg polarization, 1×10^5 splenic DCs were isolated from WT or REGy^{−/−} mice at EAE onset and cocultured with 2×10^5 naïve CD4 T cells in Th17 cell or Treg differentiation conditions with latent TGF-β1 (10 ng/ml or 30 ng/ml; R&D Systems) substituted for TGF-β1. Four days later, the cells were collected for intracellular staining. For neutralization antibody treatment, coculture media were supplemented with anti-IL6 (10 µg/ml; R&D Systems) or anti-αvβ8 (5 µg/ml, a kind gift from Professor Stephen L. Nishimura, UCSF).

Coimmunoprecipitation and immunoblot analysis

Cells were harvested with cold PBS and lysed with lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% NP-40, 150 mM NaCl, 10% glycerol, and a mixture of protease inhibitors for 30 min on ice. Lysate supernatants were collected after centrifugation. For endogenous colP, lysates were incubated with 5 µg of anti-REGy antibody (generated by our lab) overnight at 4°C, and 20 µl of protein A/G agarose (sc-2003; Santa Cruz Biotechnology) was added and incubated for an additional 2 h. For exogenous colP, lysates were incubated with 5 µl of anti-HA antibody conjugated with agarose (M20013; Abmart) for 2 h at 4°C. After incubation, the agarose was washed 3–5 times with wash buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% NP-40, 150 mM NaCl, 10% glycerol and a mixture of protease inhibitors). The agarose was then suspended in SDS sample buffer, and proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with primary antibodies. Antibodies against IRF8 (#5628), IRF5 (#4950), IRF4 (#4299), Flag (#2368), and HA (#3724) were purchased from Cell Signaling Technology. Anti-β-actin antibody (A3854) was purchased from Sigma-Aldrich. Anti-IRF8 antibody (ab3428) was purchased from Abcam. Horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG (H + L), 115-035-166; goat anti-rabbit IgG (H + L), 111-035-144) were obtained from Jackson ImmunoResearch. Immunoblots were analyzed by chemiluminescence.

In vitro proteolytic assay

The REGy protein was expressed in *E. coli* by the pPAL7-REGy plasmid and purified by Profinity eXact affinity chromatography with a fast protein liquid chromatography system. Substrates of IRF8 or p21 were generated by a cell-free protein expression system (L1170, Promega) according to the manufacturer's instructions. Proteolytic assays were performed by incubating substrate, REGy heptamers and the 20S proteasome (Boston Biochem) for 2 h in a 50 µl reaction system at 30°C, followed by immunoblotting analysis.

Study approval

All animal experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 8023, revised 1978). The studies were reviewed and approved by IACUCs of East China Normal University.

Statistical analysis

All the experiments in this manuscript were repeated at least three times. Statistical analysis was performed using Prism software. Statistical significance was assessed by two-tailed Student's *t*-test or two-way ANOVA, with a value of *P* < 0.05 indicating statistical significance.

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AUTHOR CONTRIBUTIONS

L.Z., X.L. and B.Z. designed the research. L.Z., L.Y. and Q.Z. performed most molecular, cell biology, immunological, and animal experiments. W.X., X.W., J.X., Q.L., Q.L., Y.X., H.Z., L.J., L.W., Weicang Wang, Weichao Wang, and T.S. helped with the experiments. L.F., B.Z., S.L. and L.L. provided scientific advice and valuable expertise. X.T.L., B.Z. and L.Z. wrote the manuscript with input from all the authors.

ADDITIONAL INFORMATION

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