


RKIP mediates autoimmune inflammation by positively regulating IL-17R signaling

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Abstract

Th17 cells contribute to the development of autoimmune diseases by secreting interleukin-17 (IL-17), which activates its receptor (IL-17R) that is expressed on epithelial cells, macrophages, microglia, and resident neuroectodermal cells. However, the mechanisms through which IL-17R-mediated signaling contributes to the development of autoimmune disease have not been completely elucidated. Here, we demonstrate that Raf-1 kinase inhibitor protein (RKIP) deficiency in mice ameliorates the symptoms of experimental autoimmune encephalomyelitis (EAE). Adoptive T-cell-transfer experiments demonstrate that RKIP plays a predominant role in Th17-mediated, but not in Th1-mediated immune responses. RKIP deficiency has no effect on Th17-cell differentiation *ex vivo*, nor does it affect Th17-cell differentiation in EAE mice. However, RKIP significantly promotes IL-17R-induced proinflammatory cytokine and chemokine production. Mechanistically, RKIP directly interacts with IL-17RA and Act1 to promote the formation of an IL-17R-Act1 complex, resulting in enhanced MAPK- and P65-mediated NF- κ B activation and downstream cytokine production. Together, these findings indicate that RKIP functions as an essential modulator of the IL-17R-Act1 axis in IL-17R signaling, which promotes IL-17-induced inflammation and autoimmune neuroinflammation.

Keywords Act1; EAE; IL-17; RKIP

Subject Categories Immunology; Molecular Biology of Disease; Neuroscience

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Introduction

Multiple sclerosis (MS) is a type of human demyelinating disease that causes central nervous system (CNS; i.e., the brain and spinal

cord) inflammation, neuronal demyelination, and axonal damage, resulting in both physical and mental health impairment. MS is the most common autoimmune disorder affecting the central nervous system [1]. Experimental autoimmune encephalomyelitis (EAE) is a well-established MS-like animal disease model induced by immunizing mice with myelin oligodendrocyte glycoprotein peptide (MOG₃₅₋₅₅) and pertussis toxin (PTX) [2]. It has been demonstrated that myelin-specific T cells, particularly interleukin-17 (IL-17)-producing Th17 cells, play a crucial pathogenic role in the mouse EAE model [3,4]. Specifically, Th17 cells participate in the development of EAE by secreting IL-17, which comprises IL-17A to IL-17F [5,6]. IL-17 levels have been shown to be increased in patients with MS and ulcerative colitis (UC) [7,8]. The binding of IL-17 to a type I cell surface receptor known as the IL-17 receptor (IL-17R) activates IL-17R signaling and ultimately triggers the production of proinflammatory cytokines (TNF- α , IL-1, IL-6, IL-8, G-CSF, and GM-CSF) and chemokines (CXCL1, CXCL2, CXCL5, CCL2, CCL7, and CCL20) [9]. These cytokines subsequently contribute to the activation of resident CNS cells, including epithelial cells, macrophages, microglia, and astrocytes, resulting in the secretion of additional chemokines and the recruitment of additional leukocytes into the CNS, which leads to disseminated CNS inflammation, neuronal demyelination, and the development of EAE symptoms [3,6,10]. Consistent with these findings, the findings of additional studies indicate that mice deficient in IL-17 or IL-17R display less severe EAE than wild-type (WT) mice because they lack the cellular machinery responsible for facilitating IL-17-mediated IL-17R signal transduction [11,12].

IL-17R signaling involves the recruitment of the key adaptor protein Act1 (also known as CIKS) and the E3 ubiquitin ligase TRAF6 to the cytoplasmic region of IL-17R, where they bind with its SEFIR domain. Specifically, the SEFIR domain of IL-17R interacts with the adaptor protein Act1 in response to IL-17 stimulation [13,14]. Act1 subsequently recruits TRAF6 to IL-17R, then triggers activation of the MAPK and NF- κ B pathways, and induces the production of proinflammatory cytokines and chemokines [15,16].

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In addition to activating TRAF6, Act1 also recruits TRAF2 and TRAF5 to prolong the half-life of the mRNA of some cytokines and chemokines, such as IL-6 and CXCL1 [17,18]. Previous studies have demonstrated the regulatory mechanisms through which multiple signaling molecules downstream of the IL-17R-Act1 complex exert their effects. For example, Xiao *et al* [10] reported that the serine/threonine kinase TPL2 functions as a pivotal mediator of TAK1 activation in the IL-17R signaling pathway. However, the mechanism through which the IL-17R-Act1 signalosome is modulated remains poorly understood [14].

Raf-1 kinase inhibitor protein (RKIP) is a member of the phosphatidylethanolamine-binding protein (PEBP) family and is widely expressed in both prokaryotic and eukaryotic organisms. RKIP modulates and controls crucial intracellular signaling networks, including the Raf/MEK/ERK, NF- κ B, glycogen synthase kinase-3 β (GSK3 β), and G protein-coupled receptor (GPCR) signaling cascades [19–22]. Therefore, RKIP is involved in regulating a variety of physiological processes, such as cell differentiation, cell cycle progression, and apoptosis [23]. RKIP has been shown to inhibit cancer cell invasion and metastasis in several cancers and functions as an important tumor suppressor [24,25]. We recently determined that RKIP also plays a crucial role in mediating human and mouse colitis by promoting intestinal epithelial cell apoptosis [26]. Furthermore, we determined that RKIP promotes anti-viral innate immune responses by promoting TBK1 auto-phosphorylation [27]. Therefore, RKIP functions in more processes than tumor suppression. In the present study, we first obtained genetic evidence that RKIP deficiency in non-hematopoietic cells renders affected mice refractory to EAE induction. Adoptive T-cell-transfer experiments demonstrated that RKIP promotes Th17-mediated EAE development. We further demonstrated that RKIP deficiency in astrocytes significantly inhibits IL-17-induced proinflammatory cytokine and chemokine expression. More importantly, we found that RKIP serves as a pivotal mediator of the interactions between IL-17R-Act1 in the IL-17R signaling pathway, resulting in enhanced IL-17-induced NF- κ B and the MAPK activation. RKIP deficiency in mice significantly impairs IL-17-induced pulmonary inflammation and peritonitis. IL-17R signaling is involved in various inflammatory diseases, and these findings not only provided novel insights into the regulatory mechanisms through which IL-17R signaling occurs, but also showed that

targeting IL-17R signaling may be useful for the treatment of IL-17-mediated autoimmune diseases.

Results

RKIP is a crucial mediator of the pathogenesis of EAE

We previously determined that RKIP is involved in colitis and anti-viral immune responses [26,27]. We thus attempted to determine whether RKIP can regulate autoimmune diseases. The well-known mouse MS model, that is, the EAE model, was firstly established to determine the role of RKIP in the pathogenesis of EAE. We subcutaneously injected RKIP WT mice and their knockout (KO) littermates with MOG₃₅₋₅₅ and then intravenously injected them with pertussis toxin (PTX) to induce the EAE model. As shown in Fig 1A and B, *RKIP-KO* mice suffered from relatively mild EAE disease symptoms compared to WT mice and had lower EAE clinical scores than their counterparts. Moreover, WT mice displayed diminished inflammatory cell infiltration (Fig 1C) and demyelination (Fig 1D), as demonstrated by hematoxylin–eosin (H&E) and luxol fast blue (LFB) staining, respectively, compared to their counterparts. Consistent with these results, flow cytometry analysis of mouse CNS tissues (brains and spinal cords) demonstrated that both the percentages and the absolute numbers of CNS-infiltrating CD11b⁺Gr-1⁺ neutrophils were significantly reduced in *RKIP-KO* mice compared to WT mice (Fig 1E). Within the CNS-infiltrating CD4⁺ T-cell population, the percentages and absolute numbers of IL-17⁺ Th17 cells and Foxp3⁺ Treg cells in *RKIP-KO* mice were comparable to those in WT mice (Fig 1F), while the numbers of Th1 (IFN γ ⁺ CD4⁺ T) cells in *RKIP-KO* mice were slightly decreased compared to those in WT mice (Fig 1F). We also assessed the composition of the immune cell population in the spleen during EAE. The results of the assessment showed that RKIP deficiency inhibited CD11b⁺Gr-1⁺ neutrophil infiltration into the spleen (Fig EV1A–C) without affecting the absolute numbers or percentages of other immune cells, namely CD4⁺ T cells, CD8⁺ T cells, and CD11b⁺Gr-1[–] monocytes in the spleen. RKIP deficiency either did not alter T-helper-cell differentiation (namely Th1-, Th17-, and Treg-cell differentiation; Fig EV1D and E). We

Figure 1. RKIP is a crucial mediator of the pathogenesis of EAE.

- A The mean clinical scores for *RKIP-KO* (KO, $n = 5$) mice and their wild-type littermates (WT, $n = 7$) induced by being immunized with MOG₃₅₋₅₅ were assessed from day 0 to day 31 after immunization.
- B Linear regression curves of (A) dashed lines indicate the 95% confidence intervals of the regression lines.
- C, D Histology of the spinal cord from WT and KO mice on day 31 after MOG₃₅₋₅₅ immunization was analyzed by hematoxylin–eosin (H&E) (C) and luxol fast blue (LFB) (D) staining. Scale bars (a whole spinal cord, 100X), 100 μ m; scale bars (a portion of the spinal cord, 200X), 50 μ m.
- E In a separated experiment, summary graph of the percentages of cells (left) and the absolute numbers of cells (right) in the CNS (brains and spinal cords). CNS-infiltrating cells isolated from mice treated as in (A) on day 31 were stained with mouse anti-CD4, anti-CD8, anti-CD11b, and anti-Gr-1 antibodies and analyzed by flow cytometry (FACS; WT $n = 5$, KO $n = 5$).
- F T-helper cells isolated from the CNS of mice treated as in (A) on day 31 were fixed and permeabilized, and the CD4⁺ T cells were analyzed by flow cytometry to measure intracellular IFN- γ , IL-17, and Foxp3 expression. The data are presented in summary graphs of percentages (left) and absolute cell numbers (right; WT $n = 5$, KO $n = 5$).
- G The expression levels of proinflammatory cytokines and chemokines in the CNS tissues of WT and KO EAE mice, as determined by real-time PCR (WT $n = 5$, KO $n = 5$).

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, no significant difference (unpaired, two-tailed Student's *t*-test). Data are representative of three independent experiments with similar results. Data are means \pm SEM values.

subsequently examined the expression levels of several proinflammatory cytokines and chemokines in the CNS tissues of WT and *RKIP-KO* EAE mice. As shown in Fig 1G, the gene expression

levels of the proinflammatory cytokine IL-6 and several chemokines known to mediate immune cell recruitment, such as CXCL1 (KC) and CXCL2, were significantly decreased in *RKIP-KO* EAE

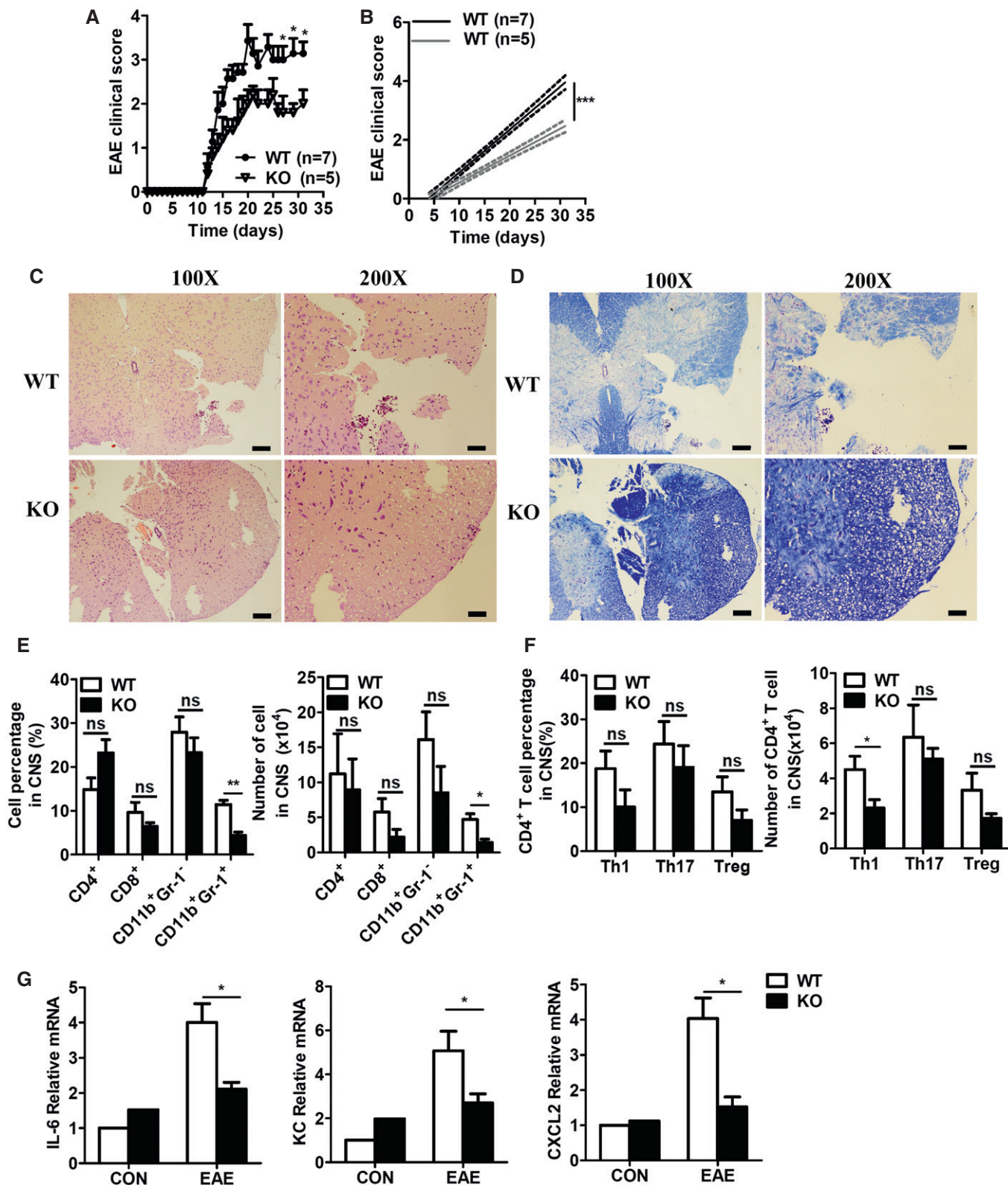


Figure 1.

mice versus WT EAE mice. These results suggest that RKIP deficiency restricts EAE development by reducing the gene expression levels of several proinflammatory cytokines and chemokines.

RKIP deficiency in non-hematopoietic cells impairs the pathogenesis of EAE

We subsequently attempted to identify the cellular compartment in which RKIP mediates the pathogenesis of EAE. We generated bone marrow chimeric mice by transferring WT or *RKIP-KO* bone marrow cells to lethal dose-irradiated (9.5 Gy) WT or *RKIP-KO* recipient mice. After 8 weeks of reconstitution, the chimeric mice were immunized with MOG₃₅₋₅₅ and injected with PTX to induce EAE. WT mice reconstituted with WT bone marrow cells (WT→WT group) or *RKIP-KO* bone marrow cells (KO→WT group) displayed comparable EAE clinical scores, while *RKIP-KO* mice reconstituted with WT bone marrow cells (WT→KO group) displayed lower EAE clinical scores than their counterparts (Fig 2A and B). H&E and LFB staining showed that inflammatory cell infiltration and neuronal demyelination were ameliorated in the WT→KO group compared to the KO→WT and WT→WT groups (Fig 2C and D). Consistently, the percentage of CNS-infiltrating CD11b⁺Gr-1⁺ neutrophils was significantly reduced in the WT→KO group compared to that in the KO→WT and WT→WT groups (Fig 2E and F). However, neuronal demyelination and infiltration of inflammatory cells were comparable between the spinal cords of KO→WT chimeras and that of WT→WT (Fig 2C and D). Moreover, the percentages and absolute numbers of IL-17⁺ Th17 cells, IFN- γ ⁺ Th1 cells, and Foxp3⁺ Treg cells in the CNS-infiltrating CD4⁺ T-cell population were comparable among the three groups (Fig 2G). These results indicate that RKIP in non-hematopoietic cells regulates the development of EAE.

Homozygous *RKIP-KO* mice were viable and did not display any abnormalities with respect to growth or survival, suggesting that RKIP is not essential for mouse growth and development. There are no underlying deficits in T-cell, B-cell, NK-cell, or CD11b⁺ APC populations in homozygous *RKIP-KO* mice [28]. The compositions of the T-cell populations in various organs (including the thymus, spleen, and lymph nodes) in 2- to 3-month-old *RKIP-KO* mice were similar to those in their WT littermates (Fig EV1F and G), indicating that RKIP deficiency in mice does not affect the development of various types of immune cells. CD44^{lo}CD62L^{hi} naive CD4⁺ T cells were sorted by FACS, and *in vitro* polarization assays were performed under different T-cell polarization conditions, including Th1, Th2, Th17, and Treg polarization conditions. As shown in Fig EV1H and I, knocking out RKIP had no significant effect on Th1-, Th2-, Th17-, and Treg-cell production. Taken together, these results indicate that RKIP deficiency does not affect T-cell activation and differentiation *in vivo* and *in vitro*.

RKIP positively regulates the pathogenesis of EAE via IL-17R-mediated inflammation

To elucidate the mechanisms through which RKIP contributes to EAE development, we transferred pre-activated MOG₃₅₋₅₅-specific Th17 cells or Th1 cells into γ -irradiated (5Gy) WT or *RKIP-KO* recipient mice to induce EAE. After 6–8 days of MOG₃₅₋₅₅-specific Th17-cell transfer, both WT and *RKIP-KO* recipient mice (Th17→WT

and Th17→KO) exhibited severe EAE. However, *RKIP-KO* recipient mice developed relatively mild EAE compared to WT recipient mice and exhibited lower clinical scores than their counterparts (Fig 3A and B). Moreover, CNS-infiltrating immune cell recruitment, particularly CD11b⁺Gr-1⁻ monocytes, and CD11b⁺Gr-1⁺ neutrophils recruitment, was significantly decreased in *RKIP-KO* recipient mice compared to WT recipient mice (Fig 3C and D). Diminished inflammation and demyelination were also observed in *RKIP-KO* recipient mice compared to WT recipient mice (Fig 3E). The gene expression levels of chemokines, such as CXCL2 and CCL20, in the CNS tissues of Th17→KO EAE mice were significantly lower than those in the CNS tissues of Th17→WT EAE mice (Fig 3F). We also transferred WT MOG₃₅₋₅₅-specific Th1 cells into γ -irradiated (5Gy) WT or *RKIP-KO* recipient mice and then assessed the EAE clinical scores of these mice. As shown in Fig EV2A and B, EAE clinical scores were comparable between the two groups (Th1→WT and Th1→KO). Recruitment of CD4⁺ T cells, CD8⁺ T cells, CD11b⁺Gr-1⁻ monocytes, and CD11b⁺Gr-1⁺ neutrophils was also comparable between *RKIP-KO* recipient mice and WT recipient mice (Fig EV2C and D). Moreover, inflammation, demyelination, and chemokine gene expression levels in the CNS tissues (Th1→WT and Th1→KO) were almost identical between the two groups (Fig EV2E and F). Taken together, these results indicate that RKIP plays a predominant role in the Th17-mediated immune response but not in the Th1-mediated immune response.

Th17 cells secrete IL-17A which initiates the IL-17R signaling and plays an important role in the EAE pathogenesis [6]. We next investigated whether the reduced EAE pathogenesis in RKIP deficiency mice was dependent on IL-17R-mediated signaling and its inflammation, an anti- α -IL-17A-specific blocking antibody was continually *i.p.* injected into RKIP WT and KO mice during the induction of EAE. As shown in Fig 3G and H, the injection of anti- α -IL-17A antibody resulted in a significant inhibited EAE symptom, including clinical scores, inflammation, and demyelination, in RKIP WT and KO mice compared to the anti-isotype control antibody-injected RKIP WT group. The RKIP-deficient mice exhibited much reduced EAE severity compared to WT mice, which was obliterated after the injection of IL-17A-blocking antibody. Consistently, the induction of IL-17A-induced genes, such as IL-6, CXCL2, and TNF- α , in CNS tissues was substantially attenuated in *RKIP-KO* mice compared to WT mice. And the expression of these genes in WT mice decreased to comparable levels in KO mice after treatment with IL-17A-blocking antibody (Fig 3I). Collectively, these data suggest RKIP regulates EAE pathogenesis via IL-17R-mediated signaling-associated inflammation.

RKIP positively regulates IL-17-induced gene expression and protein production

Myelin-specific Th17 cells secrete IL-17A, which bind IL-17R-expressing epithelial cells, macrophages, microglia, and resident neuroectodermal cells (neurons, astrocytes, and oligodendrocytes) and induce proinflammatory cytokine and chemokine gene expression in these resident CNS cells, particularly in astrocytes, a well-known cell type that has been reported to be crucial for IL-17-induced EAE [10,29]. Given that RKIP regulates the pathogenesis of EAE via IL-17R signaling, we assessed the involvement of RKIP in IL-17-induced inflammation in astrocytes by stimulating primary

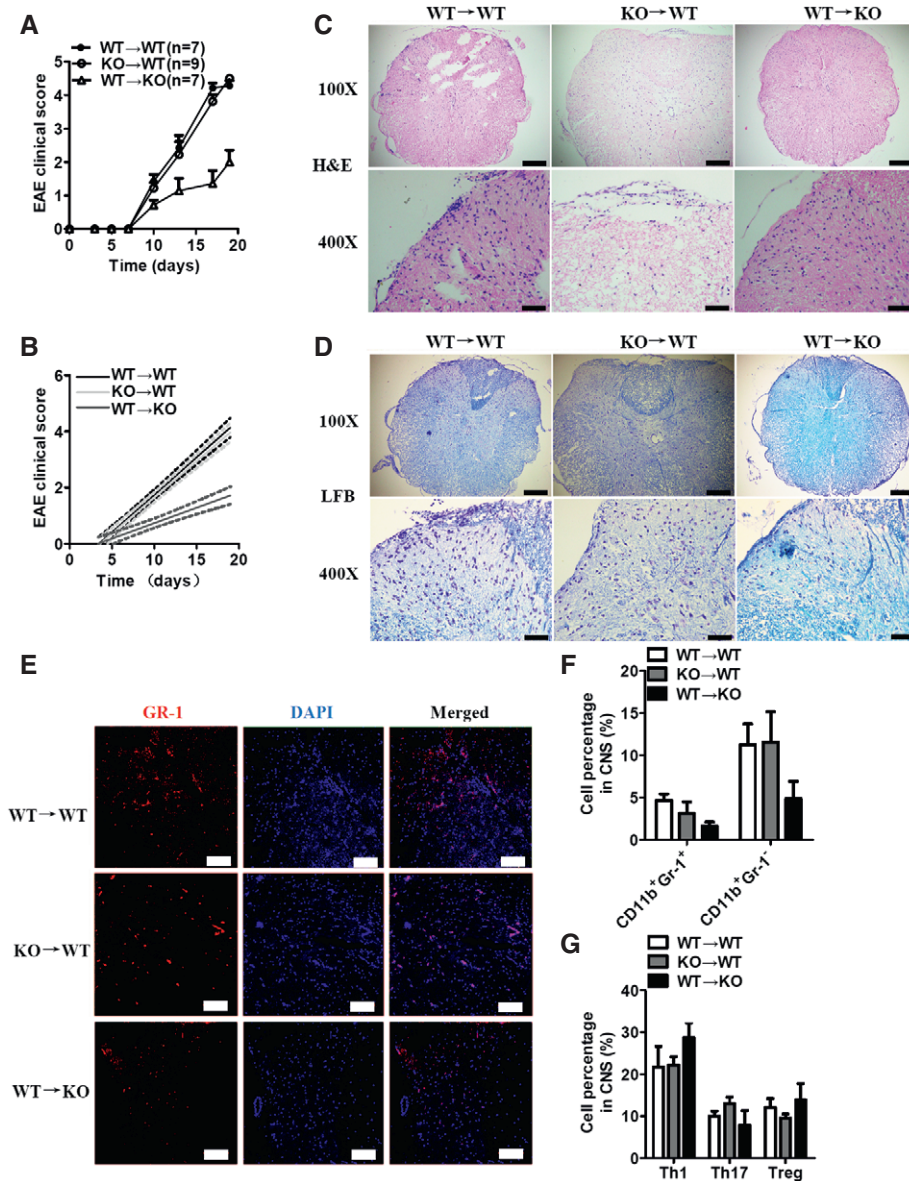


Figure 2. RKIP deficiency in non-hematopoietic cells mediates the pathogenesis of EAE.

A Mice with reconstituted bone marrow cells (WT→WT $n = 7$, WT→KO $n = 7$, and KO→WT $n = 9$) were immunized with MOG₃₅₋₅₅ to induce EAE. The mean clinical scores were calculated every other day.

B Linear regression curves of (A) dashed lines indicate the 95% confidence intervals of the regression lines.

C, D Histology of spinal cord sections from MOG₃₅₋₅₅-immunized mice was analyzed by H&E (C) and LFB (D) staining. Scale bars (a whole spinal cord, 100X), 200 μ m; scale bars (a portion of the spinal cord, 400X), 50 μ m.

E Gr-1 immunofluorescence staining of spinal cord sections from MOG₃₅₋₅₅-immunized mice treated as in (A). The nuclei were counterstained with DAPI (blue). Scale bars, 100 μ m.

F In a separated experiment, CNS cells isolated from chimeric mice on day 18 after MOG₃₅₋₅₅-immunization were analyzed as in Fig 1D (WT $n = 5$, KO $n = 5$).

G CNS T-helper cells isolated from chimeric mice on day 18 after MOG₃₅₋₅₅ immunization were analyzed as in Fig 1E (WT $n = 5$, KO $n = 5$).

Data information: Data are representative of two independent experiments with similar results. Data are means \pm SEM values.

astrocytes isolated from WT and *RKIP-KO* neonatal mouse brains with a recombinant murine IL-17A protein. As shown in Fig 4A and B, both the gene (Fig 4A) and protein expression levels (Fig 4B) of IL-6, CXCL2 and CCL20 were significantly induced by IL-17A stimulation. However, the inductions were significantly lower in *RKIP-KO* mice than those in WT mice (Fig 4A and B). Since the function of

IL-17F is similar to that of IL-17A, we also investigated the gene expression levels of IL-17F-induced proinflammatory cytokines and chemokines in astrocytes. As shown in Fig 4C and D, *RKIP* deficiency significantly inhibited IL-17F-induced IL-6, CXCL2, and CCL20 mRNA expression and protein production in astrocytes. U-87MG [30], a kind of human glioblastoma-like epithelial cells,

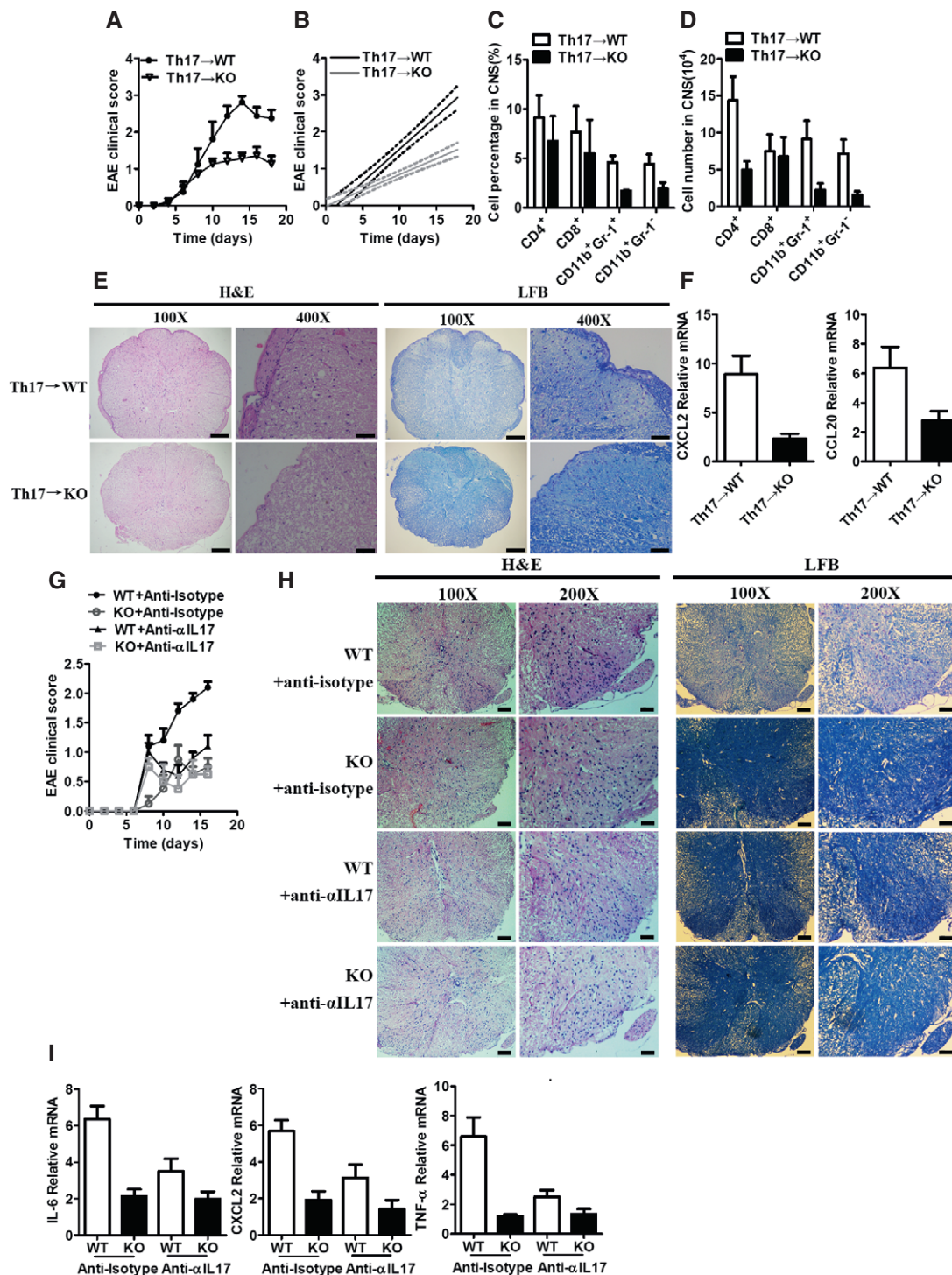


Figure 3.

were transfected with RKIP-specific small interfering RNA (siRKIP) to investigate whether RKIP has effect on IL-17-triggered signaling in human cells. As shown in Fig 4E, RKIP knockdown in U-87MG cells significantly inhibited the expression level of IL-6 and CCL20 induced by IL-17A. Taken together, these data suggest that RKIP promotes IL-17-induced proinflammatory gene expression in resident CNS cells.

HeLa cells are epithelial cells and have been widely used to investigate IL-17R signaling pathways [13,30,31]. We have transfected RKIP-specific small interfering RNA into HeLa cells to inhibit endogenous RKIP expression. RKIP knockdown significantly inhibited IL-17A- or IL-17F-induced IL-6, CXCL2, and CCL20 gene and protein expression (Fig EV3A–D), while RKIP overexpression increased IL-17A-induced IL-6, CXCL2, and CCL20 mRNA and

Figure 3. RKIP deficiency inhibits Th17 cell passive transfer-induced EAE.

- A The mean EAE clinical scores for γ -irradiated (5Gy) WT and *RKIP-KO* mice (WT $n = 8$, KO $n = 7$) reconstituted with WT MOG₋₃₅₋₅₅-pre-sensitized Th17 cells were determined from day 0 to day 18 after Th17-cell transfer.
- B Linear regression curves of (A) dashed lines indicate the 95% confidence intervals of the regression lines.
- C, D Summary graph of the percentages of cells (C) and absolute numbers of cells (D) in the CNS. CNS-infiltrating cells isolated from mice treated as in (A) (WT $n = 8$, KO $n = 7$) were stained with the appropriate antibodies.
- E Histology of the spinal cord from mice reconstituted with Th17 cells was analyzed by H&E (left) and LFB (right) staining. Scale bars (a whole spinal cord, 100X), 200 μ m; scale bars (a portion of the spinal cord, 400X), 50 μ m.
- F Real-time PCR analysis of *CXCL2* and *CCL20* mRNA expression in the CNS tissues of WT and *RKIP-KO* mice treated as in (A) (WT $n = 8$, KO $n = 7$).
- G The mice (WT $n = 5$, KO $n = 4$) were treated with intraperitoneal (*i.p.*) injection of an anti-IL-17A antibody (100 μ g per mouse each time) or appropriate isotype controls on days 7, 9, 11, and 13 after the second MOG₋₃₅₋₅₅ immunization. Mean clinical scores were calculated every other day according to the standards described in the Materials and Methods section.
- H Histology of the spinal cord was analyzed by H&E (left) or LFB (right) staining on day 14 after the second MOG₋₃₅₋₅₅ immunization. Scale (a whole spinal cord, 100X), 100 μ m; scale (a portion of the spinal cord, 200X), 50 μ m.
- I IL-6, *CXCL2*, and TNF- α mRNA in the CNS tissues (including brain and spinal cords) were measured by real-time PCR on day 14 after the second MOG₋₃₅₋₅₅ immunization, (WT $n = 5$, KO $n = 4$).

Data information: Data are representative of two independent experiments with similar results. Data are means \pm SEM values.

protein expression compared to mock control treatment (Fig EV3E and F). Simultaneously, we investigated the function of RKIP on TNF- α -, IL-1 β -, and LPS-induced inflammation in HeLa cells. As shown in Fig EV4A–C, RKIP silencing has no significant effect on the TNF- α -, IL-1 β -, or LPS-induced gene expression of proinflammatory cytokines and chemokines, such as IL-6, *CXCL2*, and *CCL20* in HeLa cells. The efficiency of RKIP knockout, knockdown, and overexpression was confirmed by Western blotting (Fig EV3G).

RKIP mediates IL-17R signaling by interacting with Act1 and IL-17RA

Upon the binding of IL-17A or IL-17F to the homodimer or heterodimer IL-17R (IL-17RA or IL-17RA/F), IL-17R recruits the key adaptor protein Act1 (also known as CIKS, an E3 ubiquitinase) to the cytoplasmic tail of IL-17R and activates both MAPK and NF- κ B signaling to induce proinflammatory cytokine and chemokine production [10,15]. RKIP deficiency inhibited IL-17A/F-induced TAK1, NF- κ B P65 subunit, JNK1/2, ERK1/2, and P38 phosphorylation in astrocytes (Fig 5A and B). Reduced IL-17A/F-induced phosphorylation of these molecules was also observed in RKIP-silenced HeLa cells and U-87MG cells compared to negative control small interfering RNA (siNC)-transfected cells (Fig 5C–E). Conversely, overexpressing RKIP in HeLa cells promoted IL-17A-induced NF- κ B P65 subunit, ERK1/2, and P38 phosphorylation (Fig EV4D). However, knockdown of RKIP in HeLa cells did not affect the TNF- α -, IL-1 β -, or LPS-induced signaling pathways activation (Fig EV4E–G). We next performed co-immunoprecipitation assays in HEK293T cells to examine the interactions between RKIP and key molecules involved in IL-17R signaling, such as Act1, TRAF2, TRAF3, TRAF5, and TRAF6. As shown in Fig 5F, Myc-tagged RKIP specifically interacted with Flag-tagged Act1 but not with TRAF2, TRAF3, and TRAF5 [17,18]. Slighter interactions between RKIP and TRAF6, which have been reported in some cancer cell lines, were also observed (Fig 5F) [32]. We also observed interactions between RKIP and IL-17RA in HEK293T cells (Fig 5G).

RKIP promotes interactions between Act1 and IL-17RA to promote IL-17R signaling

We subsequently examined the endogenous interactions between RKIP and IL-17R and Act1 in HeLa cells. We stimulated HeLa cells

or Flag-tagged RKIP-expressing HeLa cells with IL-17A for the indicated time and then incubated the whole-cell lysate (WCL) with anti-RKIP, anti-Flag, or anti-Act1 antibodies to immunoprecipitate endogenous RKIP or Flag-tagged RKIP or endogenous Act1, respectively. As shown in Fig 6A and B, endogenous Act1 and IL-17R were co-immunoprecipitated by endogenous RKIP and Flag-tagged RKIP, and the interactions between the two molecules peaked at 10 min after IL-17A stimulation. Endogenous Act1 also dynamically bound with Flag-tagged RKIP or IL-17R, and this interaction also peaked at 10 min after IL-17A stimulation (Fig 6B and C). Since the commercially available anti-IL-17R antibody could not efficiently immunoprecipitate the endogenous IL-17R, we stimulated HA-tagged IL-17RA-overexpressing HeLa cells with IL-17A for the indicated time and then immunoprecipitated the HA-tagged IL-17RA with the anti-HA antibody. Endogenous RKIP and Act1 bound with HA-IL-17RA, and RKIP dynamically interacted with the Act1-IL-17RA complex, an interaction that also peaked at 10 min after IL-17A treatment (Fig 6D). We next investigated which domain of Act1 or IL-17RA binds with RKIP. It has been reported that the SEFIR domain of Act1 is critical for the binding of Act1 to the SEFIR domain of IL-17RA and TRAF6 [15,33]. Therefore, we mutated WT Flag-tagged Act1 to Flag-tagged Act1-SEFIR or Flag-tagged Act1- Δ SEFIR, as shown in Fig EV5A. We found that Myc-tagged RKIP bound with both WT Flag-tagged Act1 and Flag-tagged Act1-SEFIR but not with the Flag-tagged Act1- Δ SEFIR, indicating that RKIP bound with Act1 in a SEFIR domain-dependent manner. WT HA-tagged IL-17RA and its truncated counterparts were screened via co-immunoprecipitation assay in HEK293T cells to identify the IL-17RA domain that interacts with RKIP. As shown in Fig EV5B, Myc-tagged RKIP bound with all the HA-tagged IL-17RA mutants but not with HA-tagged IL-17RA Δ SEFIR. We then examined whether RKIP can directly bind with Act1 or IL-17RA. For this experiment, GST-tagged RKIP proteins were purified from *Escherichia coli* strain *BL21*, and Flag-tagged EGFP, Flag-tagged Act1 protein, or Flag-tagged Act1- Δ SEFIR were purified from Flag-tagged EGFP, Flag-tagged Act1, or Flag-tagged Act1- Δ SEFIR-transfected HEK293T cells. Flag-tagged Act1 but not Flag-tagged EGFP control protein or Flag-tagged Act1- Δ SEFIR pulled down GST-RKIP (Fig 6E). HA-tagged IL-17RA, which was purified from HA-tagged IL-17RA-transfected HEK293T cells, could pull down GST-tagged RKIP protein, but not HA-tagged EGFP or HA-tagged IL-17RA- Δ SEFIR protein (Fig 6F). Taken together,

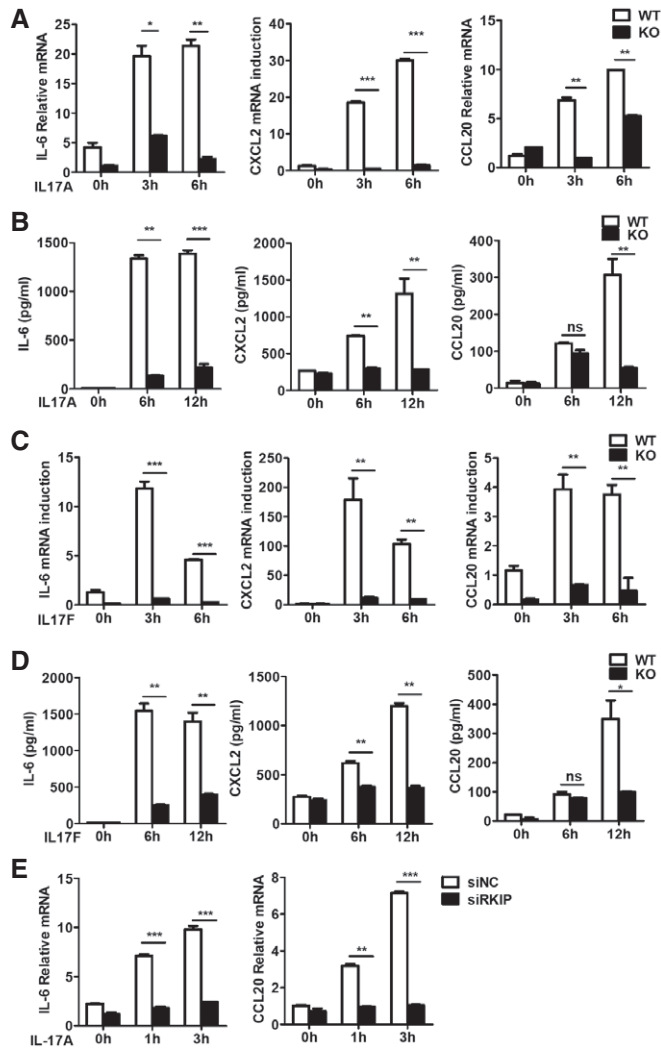


Figure 4. RKIP deficiency inhibits IL-17A-induced proinflammatory cytokine and chemokine expression in primary astrocytes.

A, B Real-time PCR analysis (A) and ELISA (B) of IL-6, CXCL2, and CCL20 mRNA expression and protein production in WT and *RKIP-KO* astrocytes stimulated with IL-17A (100 ng ml⁻¹) for the indicated time.
 C, D Real-time PCR analysis (C) and ELISA (D) of IL-6, CXCL2, and CCL20 mRNA expression and protein production in WT and *RKIP-KO* astrocytes stimulated with IL-17F (100 ng ml⁻¹) for the indicated time.
 E Real-time PCR analysis of IL-6, and CCL20 mRNA expression in RKIP-specific siRNA (siRKIP) or negative control siRNA (siNC) transfected human glioblastoma-like epithelial cells, U-87MG cells, stimulated with IL-17A (100 ng ml⁻¹) for the indicated time.

Data information: **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, no significant difference (unpaired, two-tailed Student's *t*-test). Data are representative of three independent experiments with similar results. Data are means ± SEM values.

these data suggest that RKIP directly interacts with both Act1 and IL-17RA in a SEFIR domain-dependent manner.

Since RKIP binds to the structurally homologous SEFIR domains of Act1 and IL-17RA, we next assessed whether RKIP can promote interactions between Act1 and IL-17RA under IL-17A stimulation to positively regulate the IL-17R signaling pathway. HA-IL-17RA-overexpressing HeLa cells were transfected with RKIP siRNA

or RKIP-expressing plasmids and then incubated with anti-HA or anti-Act1 antibodies to immunoprecipitate HA-IL-17RA or Act1, respectively. As shown in Fig 6G and H, RKIP knockdown prevented interactions between Act1 and HA-IL-17RA, while RKIP overexpression promoted interactions between Act1 and HA-IL-17RA (Fig 6I and J). HA-tagged IL-17RA and Flag-tagged Act1 were co-expressed in HEK 293T cells in which RKIP was overexpressed or silenced. Flag-tagged Act1 was immunoprecipitated using anti-Flag (M2) beads. As shown in Fig EV5C, overexpressing RKIP in HEK293T cells promoted interactions between Flag-tagged Act1 and HA-tagged IL-17RA, while knocking down RKIP inhibited this interaction (Fig EV5D). These data indicate that RKIP promotes interactions between IL-17RA and Act1. We subsequently investigated whether RKIP modulates IL-17A-mediated stabilization of CXCL1 mRNA. We stimulated RKIP-silenced HeLa cells with TNF- α and then treated them with actinomycin D (ActD) with or without IL-17A. As shown in Fig EV5E, the half-life of CXCL1 was similar in siNC- and siRKIP-transfected HeLa cells treated with actinomycin D alone; however, treatment with IL-17A plus actinomycin D promoted greater degradation of CXCL1 mRNA in RKIP-silenced HeLa cells than in siNC-transfected HeLa cells. Collectively, these data indicate that RKIP functions upstream of IL-17R signaling and serves as a crucial adaptor to promote the formation of the IL-17RA-Act1 complex, which controls multiple downstream signal transduction pathways and the expression of downstream cytokines or chemokines.

RKIP deficiency inhibits IL-17A-induced inflammation *in vivo*

To confirm the role of RKIP in IL-17A-induced inflammation responses *in vivo*, we intraperitoneally injected WT and *RKIP-KO* mice with PBS or IL-17A and examined IL-17A-induced IL-6, CXCL1, CXCL2, and CCL20 mRNA expression in peritoneal mesothelial cells. As shown in Fig 7A, RKIP deficiency significantly inhibited IL-17A-induced IL-6, TNF- α , CXCL1 (KC), CXCL2, and CCL20 mRNA expression in peritoneal mesothelial cells. IL-17A-induced chemokines amplify inflammatory responses by recruiting neutrophils to local tissues, as observed in an inflammation model in which administration of aerosolized IL-17A caused considerable pulmonary inflammation [31]. To determine whether RKIP can affect IL-17A-induced pulmonary inflammation by recruiting neutrophils to lung tissues, we treated WT and *RKIP-KO* mice with intratracheal injections of PBS or IL-17A and quantified the total numbers of cells and neutrophils (CD11b⁺Gr1⁺) in mouse bronchoalveolar lavage fluid (BALF) by FACS analysis. As shown in Fig 7B, *RKIP-KO* mice displayed significantly reduced IL-17A-induced total cell and neutrophil recruitment in BALF compared to WT mice. Moreover, *RKIP-KO* mice showed less inflammation and tissue damage in lung tissues than WT mice (Fig 7C). Consistently, the mRNA level of IL-6, TNF- α , and KC in lung tissues and IL-6 and TNF- α production in BALF were much lower in *RKIP-KO* mice than that in WT mice (Fig 7D and E). Taken together, these data demonstrate that RKIP positively regulates IL-17A-induced inflammation *in vivo*.

Discussion

Th17 cells and IL-17/IL-17R signaling have been extensively studied, as they play important roles in various autoimmune diseases

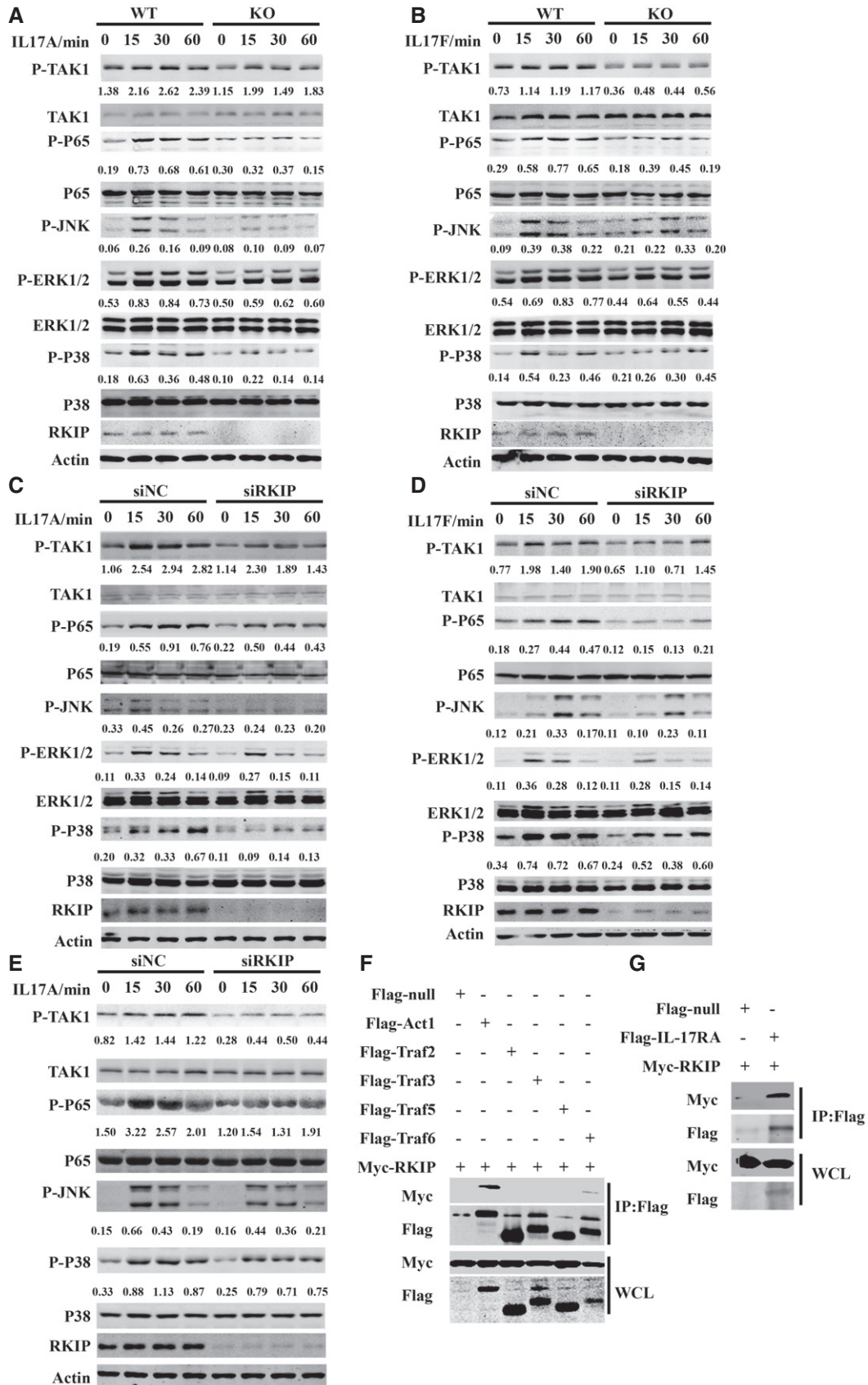


Figure 5.

Figure 5. RKIP positively regulates IL-17R signaling.

- A, B WT or *RKIP-KO* astrocytes were treated with 100 ng ml⁻¹ IL-17A (A) or IL-17F (B) for the indicated time, and then, the whole-cell lysates (WCLs) were immunoblotted with the indicated antibodies.
- C, D HeLa cells were transfected with RKIP-specific siRNA (siRKIP) or negative control siRNA (siNC) and treated with 50 ng ml⁻¹ IL-17 A (C) or IL-17F (D) for the indicated time, and then, the WCLs were immunoblotted with the indicated antibodies.
- E U-87MG cells were transfected with RKIP-specific siRNA (siRKIP) or negative control siRNA (siNC) and then treated with 100 ng ml⁻¹ IL-17A for the indicated time. The WCLs were subsequently immunoblotted with the indicated antibodies.
- F Flag-null, Flag-tagged Act1, or Flag-tagged TRAF2/3/5/6 were co-expressed with Myc-tagged RKIP in HEK293T cells and then immunoprecipitated with anti-Flag beads (M2 beads). The WCLs were subsequently immunoblotted with the indicated antibodies.
- G Flag-null or the Flag-tagged IL-17RA was co-expressed with Myc-tagged RKIP in HEK293T cells and then immunoprecipitated with anti-Flag beads (M2 beads). The WCLs were subsequently immunoblotted with the indicated antibodies. Numbers between two blots indicate densitometry of phosphorylated proteins relative to that of total proteins, respectively. Densitometry of phosphorylated JNK was relative to actin.

Data information: Data are representative of three independent experiments with similar results.

Source data are available online for this figure.

and inflammatory processes [4,14,16]. In the current study, we determined that the Raf-1 kinase inhibitor protein (RKIP) participates in the pathogenesis of IL-17-mediated autoimmune diseases and inflammation. Specifically, we found that RKIP deficiency in mice ameliorated MOG₃₅₋₅₅-induced EAE disease symptoms without affecting CD4⁺ T-cell development and polarization *in vivo* and *in vitro*. CD4⁺ T-cell-transfer experiments demonstrated that RKIP was involved in the pathogenesis of Th17 cell-mediated EAE but not Th1 cell-mediated EAE. Further mechanism studies revealed that RKIP regulated IL-17-induced signal transduction and inflammation by directly binding with IL-17RA and Act1 and promoting interactions between IL-17RA and Act1.

Raf-1 kinase inhibitor protein belongs to the phosphatidylethanolamine-binding protein (PEBP) family and is widely involved in functionally regulating many types of cancer cells [34,35]. However, RKIP is much more than a well-defined cancer inhibitory protein. We recently demonstrated that RKIP contributed to the development of inflammatory bowel disease (IBD) and antiviral immune responses [26,27]; however, its functional role in autoimmune diseases remains unclear. Using genetic approaches, we found that RKIP contributes to the development of MOG₃₅₋₅₅-induced EAE disease symptoms and is responsible for increases in EAE clinical scores and increases in inflammatory cell infiltration and neuronal demyelination. Bone marrow cell-transfer experiments demonstrated that RKIP positively regulates the pathogenesis of

EAE in non-hematopoietic cells, findings supported by the observation that the EAE phenotype was significantly attenuated in the WT→KO group compared to KO→WT and WT→WT groups. The myelin-specific Th17 cell or Th1 cell passive transfer-induced EAE experiments confirmed the hypothesis that RKIP mediated the pathogenesis of Th17-mediated EAE but not Th1-mediated EAE. We also found that RKIP deficiency does not affect Th17-cell differentiation *in vitro* and *in vivo*. Taken together, these data confirmed that RKIP plays a crucial role in positively regulating the pathogenesis of Th17 cell-mediated EAE.

It has been reported that IL-17-mediated activation of IL-17R signal transduction and cytokine production in astrocytes is involved in the pathogenesis of EAE [10,36]. By investigating IL-17R signaling-mediated inflammation in RKIP WT and *RKIP-KO* astrocytes, we found that RKIP promoted IL-17-induced proinflammatory cytokine and chemokine expression. Moreover, our signaling pathway studies demonstrated that RKIP serves as an indispensable regulatory protein that mediates IL-17-induced MAPK (including P38, JNK and ERK) and NF-κB (including TAK1 and NF-κB) signaling. All of these data suggest that RKIP mediates the pathogenesis of EAE by positively regulating IL-17-induced signaling and pathological cytokine and chemokine production in astrocytes in the CNS. Consistently, RKIP knockdown inhibits IL-17-induced signaling and downstream cytokine production, while overexpressing RKIP promotes IL-17-induced signaling in HeLa cells.

Figure 6. RKIP directly binds to Act1 and IL-17RA and promotes formation of the IL-17RA-Act1 complex.

- A The lysates from HeLa cells stimulated with IL-17A (50 ng ml⁻¹) were immunoprecipitated with anti-RKIP antibody and then subjected to immunoblot analysis with the indicated antibodies.
- B, C The lysates from Flag-RKIP-stable expressing HeLa cells stimulated with IL-17A (50 ng ml⁻¹) were immunoprecipitated with anti-Flag beads (B) or anti-Act1 antibody (C) and then subjected to immunoblot analysis with the indicated antibodies.
- D The lysates from HA-IL-17RA-stable expressing HeLa cells stimulated with IL-17A (50 ng ml⁻¹) were immunoprecipitated with anti-HA beads and then subjected to immunoblot analysis with the indicated antibodies.
- E Purified Flag-tagged EGFP, Flag-tagged Act1, or Flag-tagged Act1-ΔSEFIR proteins were mixed with recombinant GST-tagged RKIP proteins, and then, the proteins were pulled down with anti-Flag beads. Asterisk (*) indicates the target band.
- F Purified HA-tagged EGFP, HA-tagged IL-17RA, or HA-tagged IL-17RA-ΔSEFIR proteins were mixed with recombinant GST-tagged RKIP proteins, and then, the proteins were pulled down with anti-HA beads. Asterisk (*) indicates the target band.
- G, H HA-IL-17RA-overexpressing HeLa cells were transfected with siRKIP or siNC and then stimulated with IL-17A (50 ng ml⁻¹), followed by immunoprecipitation (IP) with antibodies to HA (G) or Act1 (H) and subjected to immunoblot analysis with the indicated antibodies.
- I, J HA-IL-17RA-overexpressing HeLa cells were transfected with Flag-tagged RKIP or control (Flag-tagged null) plasmids and then stimulated with IL-17A (50 ng ml⁻¹), followed by immunoprecipitation (IP) with antibodies to HA (I) or Act1 (J) and subjected to immunoblot analysis with the indicated antibodies.

Data information: Data are representative of three independent experiments with similar results.

Source data are available online for this figure.

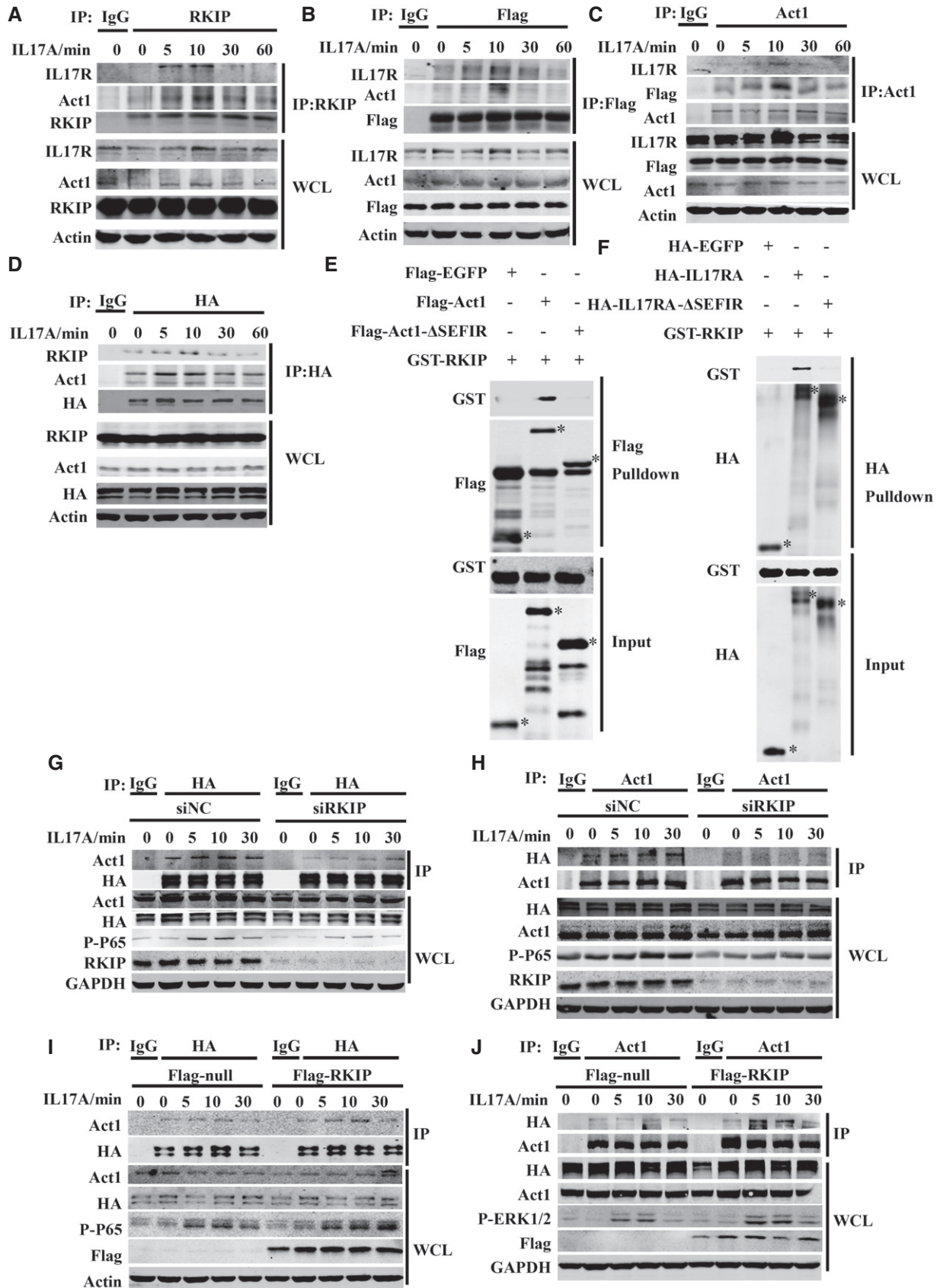


Figure 6.

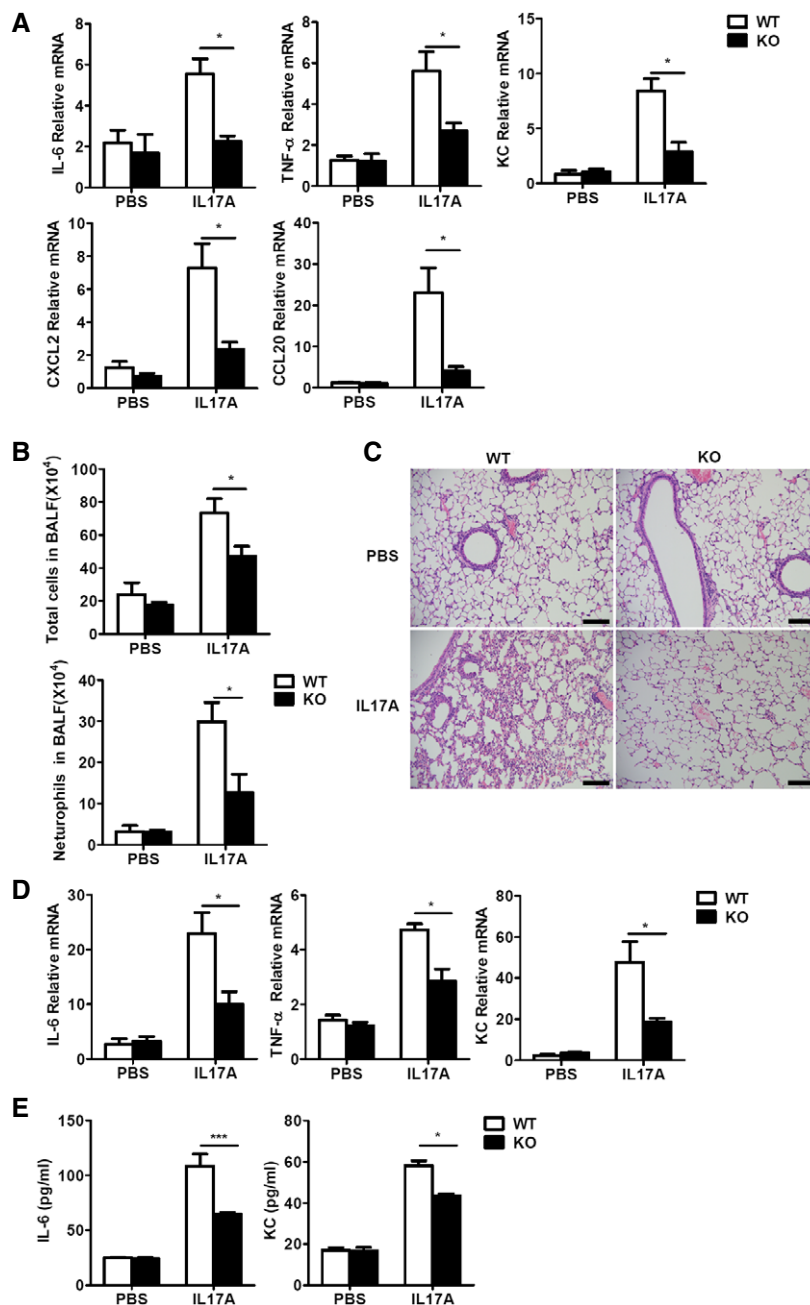


Figure 7. RKIP promotes IL-17A-induced inflammation *in vivo*.

A Real-time PCR analysis of *IL-6*, *TNF- α* , *KC*(*CXCL1*), *CXCL2*, and *CCL20* mRNA expression in peritoneal mesothelial cells isolated from WT and *RKIP-KO* mice treated with intraperitoneal injection of PBS ($n = 3$) or IL-17A ($n = 7$; 0.5 μ g in 200 μ l PBS).

B Total cells and Gr-1⁺ neutrophil infiltration in BALF from mice treated with intranasal injections of PBS (25 μ l, $n = 3$) or IL-17 (2 μ g in 25 μ l of PBS, $n = 5$) was assessed 24 h after injection.

C Histology of lung tissues from mice treated as in (B) was analyzed by H&E staining of. Scale bars, 50 μ m.

D Real-time PCR analysis of *IL-6*, *TNF- α* , and *KC* mRNA expression in the lung tissues of WT and *RKIP-KO* mice treated as in (B).

E ELISA of IL-6 and TNF- α production in the BALF of mice treated as in (B).

Data information: * $P < 0.05$, *** $P < 0.001$; ns, no significant difference (unpaired, two-tailed Student's *t*-test). Data are representative of three independent experiments with similar results. Data are means \pm SEM values.

IL-17 receptors are structurally analogous to the toll-like receptors (TLRs), the IL-1 β receptor and the TNF receptors [37]. The IL-17R signaling pathway shares some of the downstream regulatory

molecules with the other three signaling pathways but has different upstream mediators. Upon IL-17 stimulation, IL-17R first recruits the adaptor protein and E3 ubiquitinase Act1, which utilizes the E3

ubiquitinase TRAF6 to activate the TAK1/IKK complex to activate multiple downstream signaling pathways, including the NF- κ B, MAPK, JNK, and P38 signaling pathways [17,38]. Researchers have made a concerted effort to construct a detailed map of IL-17R-mediated signaling events [13,33,39,40]; however, the upstream mediators of IL-17R-mediated signaling remain unclear. Using biochemical approaches, we determined that RKIP positively regulates IL-17R-mediated signaling by promoting interactions between Act1 and IL-17RA. RKIP physically associates with Act1 and IL-17RA in HeLa cells, and IL-17A stimulation enhances this association. *In vitro* pull-down assays demonstrated that RKIP directly binds with both Act1 and IL-17RA, and domain mapping studies of Act1 and IL-17RA showed that RKIP interacts with Act1 and IL-17RA through their SEFIR domains. Moreover, RKIP knockdown significantly inhibited Act1-IL-17RA interaction, while RKIP overexpression strongly promoted this interaction. Collectively, our data indicate that RKIP serves as a crucial upstream adaptor protein by directly promoting interaction between Act1 and IL-17RA in the IL-17R signaling pathway. We further demonstrated that RKIP is indispensable for IL-17A-mediated inflammation *in vivo*, including IL-17A-induced pulmonary inflammation and peritonitis. RKIP deficiency in mice significantly impaired IL-17A-induced cytokine and chemokine expression in peritoneal mesothelial cells. In addition, fewer neutrophils were recruited to the lung tissues of *RKIP-KO* mice than to the lung tissues of WT mice after administration of aerosolized IL-17A.

In summary, this study provided us with additional information regarding the functional role of RKIP in IL-17R signaling and IL-17-induced gene expression and showed that RKIP regulates IL-17R-related autoimmune diseases (Fig EV6). Thus, RKIP serves as a multi-functional protein in inflammation and autoimmunity and, based on our findings, may be a target for the treatment of IL-17R-related inflammation and autoimmune disorders.

Materials and Methods

Mice

RKIP knockout (*RKIP*^{-/-}, *RKIP-KO*) mice were provided by Professor John Sedivy of Brown University, and 5–6-week-old *RKIP-KO* mice and their littermates with a C57BL/6 background were used for the EAE induction, and 6–8-week-old mice were used for the induction of pulmonary inflammation and peritonitis. Mice were genotyped by PCR analysis of isolated tail DNA using the following primers: *RKIP-KO* sense (5'-GAGCCCTGGCCGGTCTCCCTGTCCC AAACCTT-3'), *RKIP* WT antisense (5'-CACAAAACCAATCTTAAAGAGCCA-3'), *RKIP-KO* antisense (5'-CCAAAAGGGTCTTTGAGCAC CAGAGGACATCCG-3'). The mice were maintained and bred in specific pathogen free (SPF) conditions. Animal care and experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals with approval from the Scientific Investigation Board of Zhejiang University, Hangzhou.

Induction and assessment of EAE

Acute EAE was induced and assessed as previously described [29,30]. Briefly, acute EAE was induced by a subcutaneous

immunization with 200 μ g of the MOG₃₅₋₅₅ peptide (Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys, Sangon Biotech Co.) in CFA containing 5 mg ml⁻¹ heat-killed H37Ra strain of *Mycobacterium tuberculosis* (Chondrex, Inc) in the back region and both sides of the vertebrae. And the immunized mice were *i.v.* injected with pertussis toxin (List Biological Laboratories, Inc.) at a dose of 200 ng per mouse in PBS on the day of immunization and once more 48 h after first injection. Passive EAE was also induced as previously described [10]. Briefly, to prepare MOG₃₅₋₅₅-specific polarized T cells, WT donor mice were subcutaneously immunized with MOG₃₅₋₅₅ plus twice injection *i.v.* of PTX. Draining lymph node cells were prepared from donor mice 10 days after immunization. Cells were cultured with MOG₃₅₋₅₅ at a concentration of 25 μ g ml⁻¹ under Th17-polarizing conditions (20 ng ml⁻¹ rmIL-23, R&D) or Th1-polarizing conditions (20 ng ml⁻¹ rmIL-12, R&D, and 2 μ g ml⁻¹ anti-mIL23p19) for 5 days. And then, the 5–6-week-old *RKIP* wild-type or *RKIP-KO* recipient mice were injected with 3.0×10^7 polarized MOG₃₅₋₅₅-specific Th17 cells or Th1 cells 4 h after 5Glyc γ -irradiation. The clinical score was performed in double-blinded manner. Mice were examined every 2–3 days for disease symptoms and were double-blinded scored for disease severity using the EAE scoring rulers: 0, no clinical signs; 1, limp tail; 2, paraparesis (weakness, incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with fore limb weakness or paralysis; and 5, moribund state or death.

Isolation and analysis of CNS inflammatory cells

Central nervous system tissues, including spinal cords and brains, were homogenized in ice-cold tissue grinders, filtered through a 70- μ m cell strainer (Falcon), and the cells were collected by centrifugation at 160 g for 5 min at 4°C. Cells were re-suspended in 10 ml of 35% Percoll (GE) and centrifuged onto a 5 ml 70% Percoll cushion in 15-ml tubes at 280 g for 25 min with a low accelerating or breaking speed. Cells at the 35–70% interface were collected and subjected to flow cytometry. Fluorescence-conjugated monoclonal antibodies to APC-Cy7-CD4, Pacific Blue-CD8, FITC-CD11b, APC-F4/80, and PE-Gr-1 were stained together for the cell face marker analysis. Antibodies to PE-IL-17A, APC-IFN- γ , and Pacific Blue-Foxp3 were stained together for the intracellular analysis.

Reagents and plasmids

MOG₃₅₋₅₅ peptide was purchased from Shengong Biomedicals (purity > 99.0%); CFI, and M2 (anti-Flag) beads were purchased from Sigma-Aldrich. CFA with 10 mg/ml heat-killed H37Ra strain of *Mycobacterium tuberculosis* was purchased from Chondrex, Inc. Human recombinant IL-17A/F, murine IL-17A, and human CXCL2 ELISA kit were purchased from PeproTech. Mouse IL-6 and human IL-6 ELISA kit were purchased from eBioscience. Mouse CXCL2 and CCL20 ELISA kit were purchased from R&D. Antibodies for P-P65 (3033s), P-P38(4511S), P-ERK1/2(4370S), P-JNK1/2(4668S), P65 (8242), P38(8690), and ERK1/2(4695) were purchased from Cell Signaling Technology. Antibodies P-TAK1 (S439) (ab109404) and TAK1 (ab109526) were purchased from Abcam. Antibodies for RKIP (sc-28837), Act1 (sc-11444), IL-17R (sc-376600), and protein A/G agarose (sc-2003) were purchased from Santa Cruz. Antibodies for

β -actin (M20011), GAPDH (M130718), Flag (M20008), Myc (M20002), HA (M20003), and HA beads (M20013S) were purchased from Abmart. Antibodies for Flag (M1403-2), Myc (R1208-1), HA (0906-1), and GST (ET1611-47) were purchased from HuaBio. DAPI was purchased from Life Technology. GST beads were purchased from Thermo Scientific. Fluorescence-conjugated monoclonal antibodies to APC-Cy7-CD4 (100413), Pacific Blue-CD8 (100728), FITC-CD11b (101211), Alexa Fluor[®]594-Gr-1(108448), and Pacific Blue-Foxp3 (126409) were purchased from BioLegend. Recombinant mouse IL-6 (575702), TGF- β (763102), IL-4(715004), anti-CD3 (B202621), anti-CD28 (B209363), anti-IL-4 (B213843), anti-IFN- γ (B193599), and anti-IL-12/IL-23P40 (B174522) were purchased from BioLegend. Antibodies for PE-IL-17A (12-7177-81) and APC-IFN- γ (17-7319-82) were obtained from eBioscience. Anti- α -IL-17A neutralization antibody (16-7173-95) and relative isotype IgG were purchased from eBioscience. Mouse naïve CD4⁺ T Cell Isolation Kit (15L66873) was purchased from STEMCELL Technologies. PEI was purchased from Polyscience, and INTERFERin@ was purchased from Polyplus Transfection. Scramble siRNA, and RKIP-targeted siRNA were purchased from Genepharma (Shanghai, China). Myc-RKIP plasmid was cloned from the HeLa cell line cDNA and constructed in the pcDNA3.1-Myc-His vector, and GST-His-RKIP were subcloned into pGEX-4T1-10His vector, respectively. Flag-Act1 plasmid and HA-IL-17RA-expressed stable HeLa cells were kind gifts from Qian's Laboratory (Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). Flag-TRAF2 and TRAF5 were kind gifts from Xia's Laboratory (Life Sciences Institute of ZJU, LSI). Flag-Act1 deletion mutants were subcloned into the pcDNA3.1-Flag-His vector. HA-IL-17RA plasmid was purchased from Addgene, and its deletion mutants were subcloned into pCMV4-HA-Neo vector.

Histological analysis

Paraffin-embedded sections (4 mm thick) were subjected to either hematoxylin and eosin (H&E) or luxol fast blue (LFB) to evaluate inflammation and demyelination, respectively. H&E and LFB were performed by the Histomorphology Platform, Zhejiang University, with the standard protocol performed according to the manufacturer's instructions.

Culture of primary astrocytes

Astrocytes were prepared from 1-day-old neonatal mice as previously described [10,41]. Briefly, brains freed of meninges were dissociated with 1-ml pipettes. Debris was removed by filtration with a 70- μ m cell strainer (Falcon). Cells were cultured in DMEM plus 10% fetal bovine serum (vol/vol) supplemented with 50 μ g ml⁻¹ penicillin and 50 μ g ml⁻¹ streptomycin. After 7 day, astrocytes were stained with antibody to GFAP (Sigma, G3893, 1:500) and purity was > 95%.

Bone marrow chimeras

Bone marrow chimeras were performed as reported before [26,30]. Briefly, recipient mice underwent sublethal dose of γ -ray irradiation (9.5Gy, which was divided into two times of irradiation at a dose of 5Gy or 4.5Gy, respectively) to kill the bone marrow cells, and 4 h

post-irradiation WT or *RKIP-KO* recipients were received 100 μ l fresh WT or *RKIP-KO* bone marrow cells at the concentration of 1×10^8 ml⁻¹, respectively, which are WT \rightarrow WT, WT \rightarrow KO, and KO \rightarrow WT groups. Eight weeks after bone marrow transplantation, mice's blood was collected and determined with a RKIP genotyping analysis to exclude failure mice and then immunized with MOG₃₅₋₅₅ for indicated time to induce EAE.

In vitro CD4⁺ T-cell differentiation

In vitro CD4⁺ T-cell differentiation was performed as previously described with little modification [10]. Purified naïve CD4⁺ T cells (CD44^{lo}CD62L^{hi}) were activated with 1 μ g ml⁻¹ of plate-bound anti-CD3 and 3 μ g ml⁻¹ anti-CD28 under Th0 (10 μ g ml⁻¹ anti-IL-4 and 10 μ g ml⁻¹ anti-IFN- γ), Th1 (10 μ g ml⁻¹ anti-IL-4 and 10 ng ml⁻¹ IL-12), Th2 (50 ng ml⁻¹ IL-4 and 10 μ g ml⁻¹ anti-IFN- γ), Th17 (10 μ g ml⁻¹ anti-IL-4, 10 μ g ml⁻¹ anti-IFN- γ , 20 ng ml⁻¹ IL-6, and 5 ng ml⁻¹ TGF- β), or Treg cells (10 μ g ml⁻¹ anti-IL-4, 10 μ g ml⁻¹ anti-IFN- γ , and 5 ng ml⁻¹ TGF- β) conditions. After 4 day of activation, the differentiated T cells were stained with Foxp3 to quantify the frequency of Treg cells or re-stimulated for 4 h with PMA and ionomycin in the presence of the protein transport inhibitor monensin (BFA), followed by intracellular staining of IFN- γ , IL-4, and IL-17 to quantify the frequency of Th1, Th2, and Th17 cells.

Cell culture, plasmid transfection, and siRNA silencing

HeLa and HEK293T cells were obtained from American Type Culture Collection (ATCC), and primary astrocytes were isolated from *RKIP*^{+/-} mice and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Bioindustry). The HeLa and HEK293T cells were transfected with PEI according to the manufacturer's protocol. RKIP-specific siRNA (siRKIP) or negative control siRNA (siNC) were transfected in HeLa cells using INTERFERin@ according to the manufacturer's protocol. The following siRNA oligonucleotide sequences were used: RKIP siRNA (5-TGGTCAACATGAAGGGTAA-3).

Immunoprecipitation and Western blot analysis

Western blots were performed as described previously [26,27]. Stably expressed HeLa cell or HEK293T cell lysates were immunoprecipitated using anti-Flag, anti-HA, or anti-Act1 antibodies plus protein A/G agarose. The proteins were then separated using SDS-PAGE and subjected to Western blot analysis with anti-Act1, anti-HA, anti-Flag, anti-IL-17R, or anti-RKIP antibodies.

In vitro Flag-tagged pull-down and HA-tagged pull-down assay

The fusion proteins of GST-His-RKIP protein were expressed in *E. coli* B21 strain and purified according to standard protocols. For Flag-tagged pull-down assay, Flag-tagged Act1, Flag-tagged Act1- Δ SEFIR, or Flag-tagged EGFP proteins were purified from Flag-Act1, Flag-Act1- Δ SEFIR, or Flag-EGFP-transfected HEK293T cells, \sim 3 μ g GST fusion proteins were mixed with Flag-tagged proteins, respectively, and incubated with M2 beads at 4°C for 4 h with gentle rotation. The beads were washed three times with cell lysis buffer, and the bound proteins were eluted with SDS sample buffer and

analyzed with Western blotting. For HA-tagged pull-down assay, HA-tagged IL-17RA, HA-tagged IL-17RA-ΔSEFIR, or HA-tagged EGFP proteins were purified from HA-IL-17RA, HA-IL-17RA-ΔSEFIR, or HA-EGFP-transfected HEK293T cells and performed pull-down assay similarly as that in *in vitro* Flag pull-down assay.

Quantitative real-time PCR

Quantitative real-time PCR was performed using SYBR Green fluorescence as previously described [26]. The primer sequences are listed in Table EV1.

Statistical analysis

All data are expressed as the mean ± SEM. Results presented as fold induction were relative to those of the unstimulated cells or control group, and the baseline values were set as “1” in each panel. Statistical significance between two experimental groups was assessed using unpaired two-tailed Student’s *t*-test.

Expanded View for this article is available online.

Acknowledgements

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Author contributions

WL, NW, KZ, FS, and CM performed the experiments. JS and HL assisted in the conduction of experiments. YJ, HL, KW, and YQ contributed the reagents; XW and WL designed experiments and assisted in analyzing the data and edited the manuscripts.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Compston A, Coles A (2008) Multiple sclerosis. *Lancet* 372: 1502–1517
- Simmons SB, Pierson ER, Lee SY, Goverman JM (2013) Modeling the heterogeneity of multiple sclerosis in animals. *Trends Immunol* 34: 410–422
- Goverman J (2009) Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol* 9: 393–407
- Iwakura Y, Nakae S, Saijo S, Ishigame H (2008) The roles of IL-17A in inflammatory immune responses and host defense against pathogens. *Immunol Rev* 226: 57–79
- Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M, Giuliani F, Arbour N, Becher B, Prat A (2007) Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med* 13: 1173–1175
- Iwakura Y, Ishigame H, Saijo S, Nakae S (2011) Functional specialization of interleukin-17 family members. *Immunity* 34: 149–162
- Kolls JK, Linden A (2004) Interleukin-17 family members and inflammation. *Immunity* 21: 467–476
- Yang J, Sundrud MS, Skepner J, Yamagata T (2014) Targeting Th17 cells in autoimmune diseases. *Trends Pharmacol Sci* 35: 493–500
- Isailovic N, Daigo K, Mantovani A, Selmi C (2015) Interleukin-17 and innate immunity in infections and chronic inflammation. *J Autoimmun* 60: 1–11
- Xiao Y, Jin J, Chang M, Nakaya M, Hu H, Zou Q, Zhou X, Brittain GC, Cheng X, Sun SC (2014) TPL2 mediates autoimmune inflammation through activation of the TAK1 axis of IL-17 signaling. *J Exp Med* 211: 1689–1702
- Gonzalez-Garcia I, Zhao Y, Ju S, Gu Q, Liu L, Kolls JK, Lu B (2009) IL-17 signaling-independent central nervous system autoimmunity is negatively regulated by TGF-beta. *J Immunol* 182: 2665–2671
- Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y (2006) IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 177: 566–573
- Qian Y, Liu C, Hartupee J, Altuntas CZ, Gulen MF, Jane-Wit D, Xiao J, Lu Y, Giltiay N, Liu J et al (2007) The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. *Nat Immunol* 8: 247–256
- Song X, Qian Y (2013) The activation and regulation of IL-17 receptor mediated signaling. *Cytokine* 62: 175–182
- Liu C, Qian W, Qian Y, Giltiay NV, Lu Y, Swaidani S, Misra S, Deng L, Chen ZJ, Li X (2009) Act1, a U-box E3 ubiquitin ligase for IL-17 signaling. *Sci Signal* 2: ra63
- Zhu S, Qian Y (2012) IL-17/IL-17 receptor system in autoimmune disease: mechanisms and therapeutic potential. *Clin Sci* 122: 487–511
- Bulek K, Liu C, Swaidani S, Wang L, Page RC, Gulen MF, Herjan T, Abbadi A, Qian W, Sun D et al (2011) The inducible kinase IKKi is required for IL-17-dependent signaling associated with neutrophilia and pulmonary inflammation. *Nat Immunol* 12: 844–852
- Sun D, Novotny M, Bulek K, Liu C, Li X, Hamilton T (2011) Treatment with IL-17 prolongs the half-life of chemokine CXCL1 mRNA via the adaptor TRAF5 and the splicing-regulatory factor SF2 (ASF). *Nat Immunol* 12: 853–860
- Yeung K, Seitz T, Li S, Janosch P, McFerran B, Kaiser C, Fee F, Katsanakis KD, Rose DW, Mischak H et al (1999) Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. *Nature* 401: 173–177
- Al-Mulla F, Bitar MS, Al-Maghrebi M, Behbehani AI, Al-Ali W, Rath O, Doyle B, Tan KY, Pitt A, Kolch W (2011) Raf kinase inhibitor protein RKIP enhances signaling by glycogen synthase kinase-3beta. *Can Res* 71: 1334–1343
- Yeung KC, Rose DW, Dhillon AS, Yaros D, Gustafsson M, Chatterjee D, McFerran B, Wyche J, Kolch W, Sedivy JM (2001) Raf kinase inhibitor protein interacts with NF-kappaB-inducing kinase and TAK1 and inhibits NF-kappaB activation. *Mol Cell Biol* 21: 7207–7217
- Van Tongel A, Karelse A, Berghs B, Van Isacker T, De Wilde L (2014) Diagnostic value of active protraction and retraction for sternoclavicular joint pain. *BMC Musculoskelet Disord* 15: 421
- Lorenz K, Schmid E, Deiss K (2014) RKIP: a governor of intracellular signaling. *Crit Rev Oncog* 19: 489–496
- Frankenberger C, Rabe D, Bainer R, Sankarasharma D, Chada K, Krausz T, Gilad Y, Becker L, Rosner MR (2015) Metastasis suppressors regulate the tumor microenvironment by blocking recruitment of prometastatic tumor-associated macrophages. *Cancer Res* 75: 4063–4073
- Yesilkanal AE, Rosner MR (2014) Raf kinase inhibitory protein (RKIP) as a metastasis suppressor: regulation of signaling networks in cancer. *Crit Rev Oncog* 19: 447–454

26. Lin W, Ma C, Su F, Jiang Y, Lai R, Zhang T, Sun K, Fan L, Cai Z, Li Z et al (2016) Raf kinase inhibitor protein mediates intestinal epithelial cell apoptosis and promotes IBDs in humans and mice. *Gut* 66: 597–610
27. Gu M, Liu Z, Lai R, Liu S, Lin W, Ouyang C, Ye S, Huang H, Wang X (2016) RKIP and TBK1 form a positive feedback loop to promote type I interferon production in innate immunity. *EMBO J* 35: 2553–2565
28. Wright KT, Vella AT (2013) RKIP contributes to IFN-gamma synthesis by CD8+ T cells after serial TCR triggering in systemic inflammatory response syndrome. *J Immunol* 191: 708–716
29. Kang Z, Altuntas CZ, Gulen MF, Liu C, Giltiay N, Qin H, Liu L, Qian W, Ransohoff RM, Bergmann C et al (2010) Astrocyte-restricted ablation of interleukin-17-induced Act1-mediated signaling ameliorates autoimmune encephalomyelitis. *Immunity* 32: 414–425
30. Zhu S, Pan W, Shi P, Gao H, Zhao F, Song X, Liu Y, Zhao L, Li X, Shi Y et al (2010) Modulation of experimental autoimmune encephalomyelitis through TRAF3-mediated suppression of interleukin 17 receptor signaling. *J Exp Med* 207: 2647–2662
31. Zhong B, Liu X, Wang X, Chang SH, Liu X, Wang A, Reynolds JM, Dong C (2012) Negative regulation of IL-17-mediated signaling and inflammation by the ubiquitin-specific protease USP25. *Nat Immunol* 13: 1110–1117
32. Tang H, Park S, Sun SC, Trumbly R, Ren G, Tsung E, Yeung KC (2010) RKIP inhibits NF-kappaB in cancer cells by regulating upstream signaling components of the IkkappaB kinase complex. *FEBS Lett* 584: 662–668
33. Onishi RM, Park SJ, Hanel W, Ho AW, Maitra A, Gaffen SL (2010) SEF/IL-17R (SEFIR) is not enough: an extended SEFIR domain is required for il-17RA-mediated signal transduction. *J Biol Chem* 285: 32751–32759
34. Das SK, Bhutia SK, Sokhi UK, Azab B, Su ZZ, Boukerche H, Anwar T, Moen EL, Chatterjee D, Pellicchia M et al (2012) Raf kinase inhibitor RKIP inhibits MDA-9/syntenin-mediated metastasis in melanoma. *Can Res* 72: 6217–6226
35. Park S, Yeung ML, Beach S, Shields JM, Yeung KC (2005) RKIP downregulates B-Raf kinase activity in melanoma cancer cells. *Oncogene* 24: 3535–3540
36. Ma C, Lin W, Liu Z, Tang W, Gautam R, Li H, Qian Y, Huang H, Wang X (2017) NDR1 protein kinase promotes IL-17- and TNF-alpha-mediated inflammation by competitively binding TRAF3. *EMBO Rep* 18: 586–602
37. Maitra A, Shen F, Hanel W, Mossman K, Tocker J, Swart D, Gaffen SL (2007) Distinct functional motifs within the IL-17 receptor regulate signal transduction and target gene expression. *Proc Natl Acad Sci USA* 104: 7506–7511
38. Qu F, Gao H, Zhu S, Shi P, Zhang Y, Liu Y, Jallal B, Yao Y, Shi Y, Qian Y (2012) TRAF6-dependent Act1 phosphorylation by the IkkappaB kinase-related kinases suppresses interleukin-17-induced NF-kappaB activation. *Mol Cell Biol* 32: 3925–3937
39. Garg AV, Amatya N, Chen K, Cruz JA, Grover P, Whibley N, Conti HR, Hernandez Mir G, Sirakova T, Childs EC et al (2015) MCP1P1 endoribonuclease activity negatively regulates interleukin-17-mediated signaling and inflammation. *Immunity* 43: 475–487
40. Garg AV, Ahmed M, Vallejo AN, Ma A, Gaffen SL (2013) The deubiquitinase A20 mediates feedback inhibition of interleukin-17 receptor signaling. *Sci Signal* 6: ra44
41. Kang Z, Wang C, Zepp J, Wu L, Sun K, Zhao J, Chandrasekharan U, DiCorleto PE, Trapp BD, Ransohoff RM et al (2013) Act1 mediates IL-17-induced EAE pathogenesis selectively in NG2+ glial cells. *Nat Neurosci* 16: 1401–1408