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Loss of Sprouty4 in T cells ameliorates experimental autoimmune encephalomyelitis in mice by negatively regulating IL-1β receptor expression

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ABSTRACT

Th17 cells, which have been implicated in autoimmune diseases, require IL-6 and TGF-β for early differentiation. To gain pathogenicity, however, Th17 cells require IL-1β and IL-23. The underlying mechanism by which these confer pathogenicity is not well understood. Here we show that Sprouty4, an inhibitor of the PLCγ-ERK pathway, critically regulates inflammatory Th17 (iTh17) cell differentiation. Sprouty4-deficient mice, as well as mice adoptively transferred with Sprouty4-deficient T cells, were resistant to experimental autoimmune encephalitis (EAE) and showed decreased Th17 cell generation *in vivo*. *In vitro*, Sprouty4 deficiency did not severely affect TGF-β/IL-6-induced Th17 cell generation but strongly impaired Th17 differentiation induced by IL-1/IL-6/IL-23. Analysis of Th17-related gene expression revealed that Sprouty4-deficient Th17 cells expressed lower levels of IL-1R1 and IL-23R, while RORγt levels were similar. Consistently, overexpression of Sprouty4 or pharmacological inhibition of ERK upregulated IL-1R1 expression in primary T cells. Thus, Sprouty4 and ERK play a critical role in developing iTh17 cells in Th17 cell-driven autoimmune diseases.

1. Introduction

$CD4^+$ T helper (Th) cells play a central role in the immune response. Upon activation by antigens, Th cells follow distinct developmental pathways, such as Th1, Th2 and Th17, with specialized properties and effector functions. These subsets are characterized by the production of their signature cytokines, IFN-γ, IL-4 and IL-17A, respectively. Th17 cells produce IL-17F and IL-22 in addition to IL-17A, and function primarily for host defense against fungal and bacterial pathogens. However, Th17 cells have emerged as crucial mediators of various inflammatory diseases such as multiple sclerosis (MS), psoriasis and rheumatoid arthritis (RA). The differentiation of Th17 cells from naïve T cells requires TGF-β and IL-6, both *in vitro* and *in vivo*, which induces the nuclear orphan receptors RORγt and RORα. In contrast, TGF-β and IL-2 induce Foxp3, the master regulator of Tregs, which inhibits RORγt by direct binding [1].

TGF-β and IL-6 have been shown to drive early Th17 cell differentiation, while IL-23 and IL-1β are necessary for maintenance and pathogenic maturation of Th17 cells [2]. Moreover, IL-1, IL-6 and IL-23 (IL-1/6/23) have been shown to induce Th17 differentiation in murine T cells even in the absence of exogenous TGF-β [2,3]. It still remains controversial whether IL-1/6/23 action is dependent on TGF-β signaling, since TGF-β3 has been shown to be produced in T cells by IL-1β or IL-23 [4]. Nonetheless, Th17 cells induced by IL-1/6/23 were found to express higher levels of IL-23R than IL-6/TGF-β-induced Th17 cells and those cells were thought to be highly pathogenic and called inflammatory Th17 (iTh17) cells [3]. In contrast, Th17 cells induced by IL-6/TGF-β seem to acquire a regulatory phenotype [5]. These observations suggest that Th17 is not a uniform population and their inflammatory and anti-inflammatory or regulatory features may be induced in response to different stimulations and environmental factors. Whatever the case, the mechanisms that regulate iTh17 and regulatory Th17 remain to be clarified.

Sprouty was originally identified in *Drosophila* as a negative regulator of fibroblast growth factor (FGF) signaling during tracheal development [6] and then came to be regarded as a general inhibitor of the growth factor-induced receptor tyrosine kinase (RTK)-dependent ERK signaling pathways involved in

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Drosophila development and organogenesis. In mammals, four Sprouty orthologues (Sprouty1–4) have been identified. In addition, three Sprouty-related genes, known as Spreds (*Sp*routy-*r*elated *E*na/VASP homology 1 *d*omain-containing proteins), have been identified [7]. Mammalian Sproutys and Spreds inhibit growth factor-induced cellular responses by inhibiting the RTK-dependent ERK signaling pathway [8]. Several mechanisms for the Sprouty-mediated inhibition of the ERK pathway have been proposed. *XtSprouty* inhibits FGF-induced PLCγ-mediated Ca^{2+} mobilization and PKC signaling with normal ERK activation during the early stages of gastrulation, while *XtSpred* inhibits ERK activation with little effect on Ca^{2+} flux and PKC signaling [9,10]. Similarly, we and others have demonstrated that Sprouty4 inhibits VEGF-A-induced PLCγ-PKC-mediated ERK activation, by inhibiting PIP_2 hydrolysis, resulting in the suppression of Ca^{2+}-mobilization and the various PKC downstream pathways [11].

Only a few studies have explored the function of Sproutys in T cells [12]. In this study, we show that Sprouty4 gene (*Spry4*) deletion in $CD4^+$ T cells impairs IL-1/6/23-induced iTh17 differentiation. Under IL-1/6/23 conditions, RORγt expression was not significantly affected in the absence of Sprouty4, however, Sprouty4 deficiency strongly reduced IL-1R and IL-23R expression. From the effect of pharmacological ERK inhibitors, we propose that ERK activation suppressed IL-1 receptor expression, which may explain why Sprouty4-deficienct mice were resistant to the typical Th17-mediated disease model, experimental autoimmune encephalomyelitis (EAE). Our findings have thus uncovered a novel and critical role of the ERK pathway in the RORγt-mediated signaling program for pathogenic Th17 gene expression.

2. Materials and methods

2.1. Mice and EAE

Sprouty4-KO mice have been described previously [13,14]. *Sprouty4*-KO mice were generated with a 129/C57BL/6J-mixed background, and then backcrossed into C57BL/6J at least 7 times. C57BL6/J mice were purchased from Nihon Jikken Doubutsu (Tokyo, Japan). Experimental autoimmune encephalomyelitis (EAE) and passive EAE were induced as described previously [15,16]. Animals were maintained under specific pathogen-free conditions in animal facilities certified by the Animal Care Committees of the Keio University School of Medicine. All experiments using these mice were approved by the Animal Ethics Committee of the Keio University School of Medicine, and performed according to their guidelines.

2.2. T cell preparation and differentiation

Naïve $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ T cells (4×10^4) were isolated as previously described [17]. Naïve $CD4^+$ T cell were stimulated with 3 μg/ml of plate-bound anti-CD3 (clone 145-2C11) and 0.5 μg/ml of soluble anti-CD28 in 96-well flat-bottomed plates. For Th0 cell differentiation, the cells were stimulated with anti-CD3/CD28 (TCR) antibodies. For conventional Th17 cell differentiation in the presence of TGF-β1, the cells were treated with 1 ng/ml human TGF-β1, 30 ng/ml human IL-6, 5 μg/ml anti-IL-4 (11B11) and 5 μg/ml anti-IFN-γ (R4-6A2) [18]. For Th17 cell differentiation in the absence of TGF-β1, naïve $CD4^+$ T cells were cultured with anti-TCR antibodies in the presence of 30 ng/ml IL-6, 30 ng/ml IL-1β, 50 ng/ml IL-23, 5 μg/ml anti-IL-4 and 5 μg/ml anti-IFN-γ. For iTreg differentiation, naïve $CD4^+$ T cells were cultured with anti-TCR antibodies in the presence of 5 ng/ml TGF-β1, 5 μg/ml anti-IL-4 and 5 μg/ml anti-IFN-γ antibodies. DCs were prepared as described [19]. Flow cytometry was performed as described [20]. Anti-IL-1R1 antibody was purchased from Biolegend.

2.3. Retroviral transduction

Sprouty4 cDNA was subcloned into the pMX-IRES-EGFP vector and the retrovirus was prepared as described previously [21]. Naïve $CD4^+$ T cells were plated and subjected to the Th17 cell differentiation conditions described above, starting on day 0. On day 1, fresh retrovirus supernatant was added and the cells were centrifuged at 2500 rpm for 2 hr at 35 °C. After spin infection, the cells were cultured in the Th17 cell differentiation media and harvested on day 4 for intracellular cytokine staining and quantitative RT-PCR analysis (Q-PCR). Cell sorting was performed with a FACSAria II cell sorter to obtain EGFP-positive cells.

2.4. Q-PCR

Total RNA was extracted using the RNA-iso (Takara Bio, Shiga, Japan) according to the manufacturer's protocols, and cDNA was then synthesized with a High Capacity cDNA reverse transcription kit (Applied BioSystems). Gene expression was examined using a CFX96 Q-PCR detection system (Bio-Rad) and a Kapa SYBR FAST qPCR kit (Kapa Biosystems). The results were normalized to GAPDH levels. The primers were described previously [22,23].

3. Results

3.1. *Spry4*$^{-/-}$ mice were resistant to EAE with decreased Th17 cells generation in vivo

A previous study found that Sprouty family members are highly expressed in primary T cells [12]. We confirmed this by Q-PCR. We were particularly interested in Sprouty4 because Sprouty4 expression is maintained at high levels in Th17 differentiation conditions, while other members were not (Fig. 1A). There was no increase in Sprouty1, 2 or 3 expression caused by Sprouty4-deficiency, suggesting that expression of each Sprouty is independently regulated.

To examine the role of Sprouty4 in Th17, we first examined EAE by immunizing wild type (WT) and *Spry4*$^{-/-}$ mice with MOG peptide. As shown in Fig. 1B, *Spry4*$^{-/-}$ mice exhibited delayed onset and less severe EAE symptoms compared with WT mice. We isolated the splenoctyes from these mice on day 9 after immunization and stimulated them with MOG peptide. We observed a slight reduction of IL-17A production in splenoctyes from *Spry4*$^{-/-}$ mice compared with those from WT mice (Fig. 1C). However, infiltration of IL-17A$^+$ and IFN-γ$^+$ as well as double positive (IL-17A$^+$ IFN-γ$^+$) T cells were dramatically reduced in the CNS (brain and spinal cord) on day 12 (Fig. 1D), whereas Foxp3 expression in cells from *Spry4*$^{-/-}$ mice was increased compared with that from WT mice (Fig. 1E). These data suggest that differentiation of pathogenic Th17 cells and their infiltration into the CNS were impaired in Sprouty4-deficient mice.

To demonstrate the intrinsic effects of Sprouty4 on T cells, we performed a passive EAE model, which is induced by an adoptive transfer of activated T cells from MOG-immunized mice. The mice that received MOG-specific T cells from WT mice developed EAE, whereas the mice that received MOG-specific T cells from *Spry4*$^{-/-}$ mice developed much milder symptoms (Fig. 1F). These data suggest that reduction of pathogenic Th17 cells in *Spry4*$^{-/-}$ mice results in amelioration of experimentally induced EAE.

3.2. Differentiation of iTh17 cells, but not immature Th17 cells, was impaired in *Spry4*$^{-/-}$ T cells

Next, we examined T cell activation and differentiation *in vitro*. WT or *Spry4*$^{-/-}$ T cells were co-cultured with WT splenic DCs in the presence of soluble anti-CD3, and LPS with TGF-β1. We observed similar IFN-γ-producing Th1 differentiation, while reduced Th17

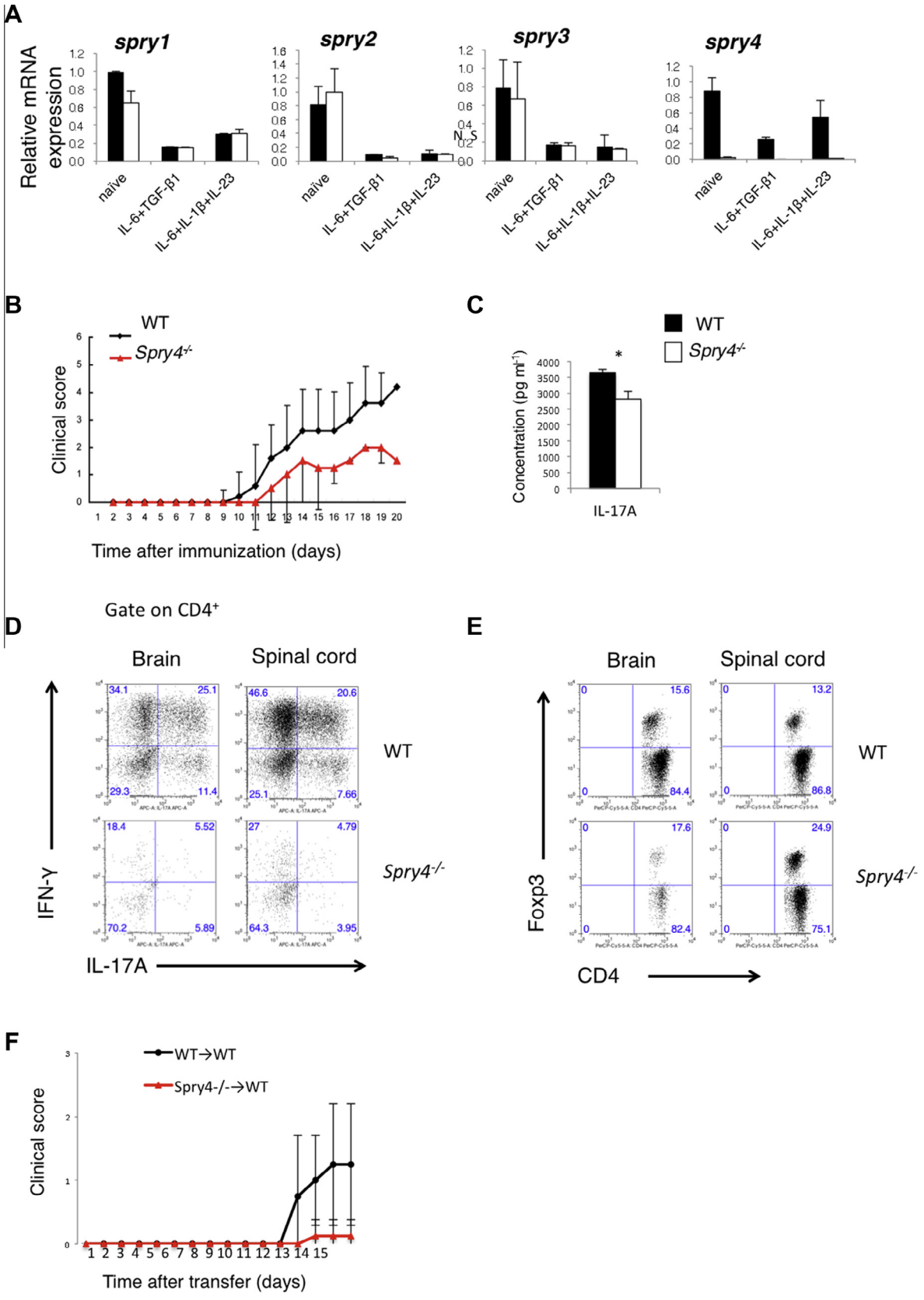

Fig. 1. Reduced EAE severity in *Spry4*$^{-/-}$ mice. (A) Expression of Sprouty family genes under Th17 conditions. WT or *Spry4*$^{-/-}$ naïve CD4^{+} T cells were activated under the indicated Th17 conditions for 4 days. The mRNA expression of Sprouty1, Sprouty2, Sprouty3, and Sprouty4 were analyzed by quantitative real-time PCR and normalized to GAPDH. Data are shown as mean ± SD. (B) WT and *Spry4*$^{-/-}$ mice were immunized with MOG peptide. Mean clinical EAE scores are shown. $N = 5$. (C) Nine days after EAE induction, the concentration of IL-17A in the supernatants from splenocytes restimulated with MOG peptide was measured by ELISA. $^{*}P < 0.05$. Data are shown as mean ± SD. (D and E) MNCs (mononuclear cells) were isolated from brain and spinal cord of MOG-immunized mice on day 12. IL-17A and IFN-γ productions (D) or Foxp3 expression (E) in CD4^{+} T cells was examined by flow cytometry. Data are represented by a dot plot and numbers for all FACS plots indicate the percentage of cells in each gate. (F) Passive EAE was induced by adoptive transfer of T cells isolated from mice immunized with MOG peptide. Mean clinical EAE scores are shown. Data are shown as mean ± SD. $N = 4$.

differentiation in *Spry4*$^{-/-}$ T cells (Fig. 2A). Foxp3^{+}iTreg differentiation was not different between WT and *Spry4*$^{-/-}$ T cells (Fig. 2B). This suggests that Sprouty4 plays a specific role in promoting Th17 differentiation in T cells.

To deepen our understanding of the role of T-cell intrinsic Sprouty4 in Th17 differentiation, we induced differentiation of naïve T cells into Th17 *in vitro* by TCR stimulation and cytokines. It has been proposed that IL-6/TGF-β induces immature Th17 cell differentiation, while IL-1β, IL-6 and IL-23 (IL-1/6/23) condition promotes iTh17 development. We found that there was no significant difference in the differentiation of immature Th17 cells induced by IL-6/TGF-β between WT and *Spry4*$^{-/-}$ naïve CD4^{+} T cells when we performed intracellular cytokine staining (Fig. 3A). However, pathogenic Th17 cell development by IL-1/6/23 was markedly reduced in *Spry4*$^{-/-}$ CD4^{+} T cells (Fig. 3A). This was confirmed by ELISA assay at various concentrations of anti-CD3 antibody (Fig. 3B, left). However, IL-2 production under the same conditions was not affected by Sprouty4 deficiency (Fig. 3B, right).

To characterize *Spry4*$^{-/-}$ Th17 cells in more detail, we analyzed additional Th17 signature genes by Q-PCR. Every gene, including *Il17a*, in IL-6/TGF-β-driven Th17 differentiation seemed comparable in expression between WT and *Spry4*$^{-/-}$ cells (Fig. 3C and data not shown). On the contrary, under IL-1/6/23-induced Th17 conditions, not only *Il17a* but also *Il17f*, *Il22*, *Il1r* and *Il23r* expression was reduced. Interestingly, we found the expression of the lineage-specific transcription factor *Rorc* in *Spry4*$^{-/-}$ mice Th17 was comparable to that in WT cells under IL-1/6/23 conditions (Fig. 3C), suggesting that reduced expression of signature cytokines in *Spry4*$^{-/-}$ Th17 cells is not due to reduced RORγt expression.

We also studied whether Sprouty4 deletion has any influence on other Th cell lineages by performing Th1, Th2 and iTreg polarization *in vitro*. Flow cytometry and Q-PCR analysis revealed no defect in any signature gene expression among these Th subsets (data not shown). Thus, Sprouty4 seems to play a specific, critical role only in IL-1/6/23-induced Th17 differentiation.

3.3. Sprouty4 positively regulates IL-1R1 expression

Since IL-1 has been shown to play essential roles in the pathogenicity of Th17 cells [2], we investigated the mechanism of

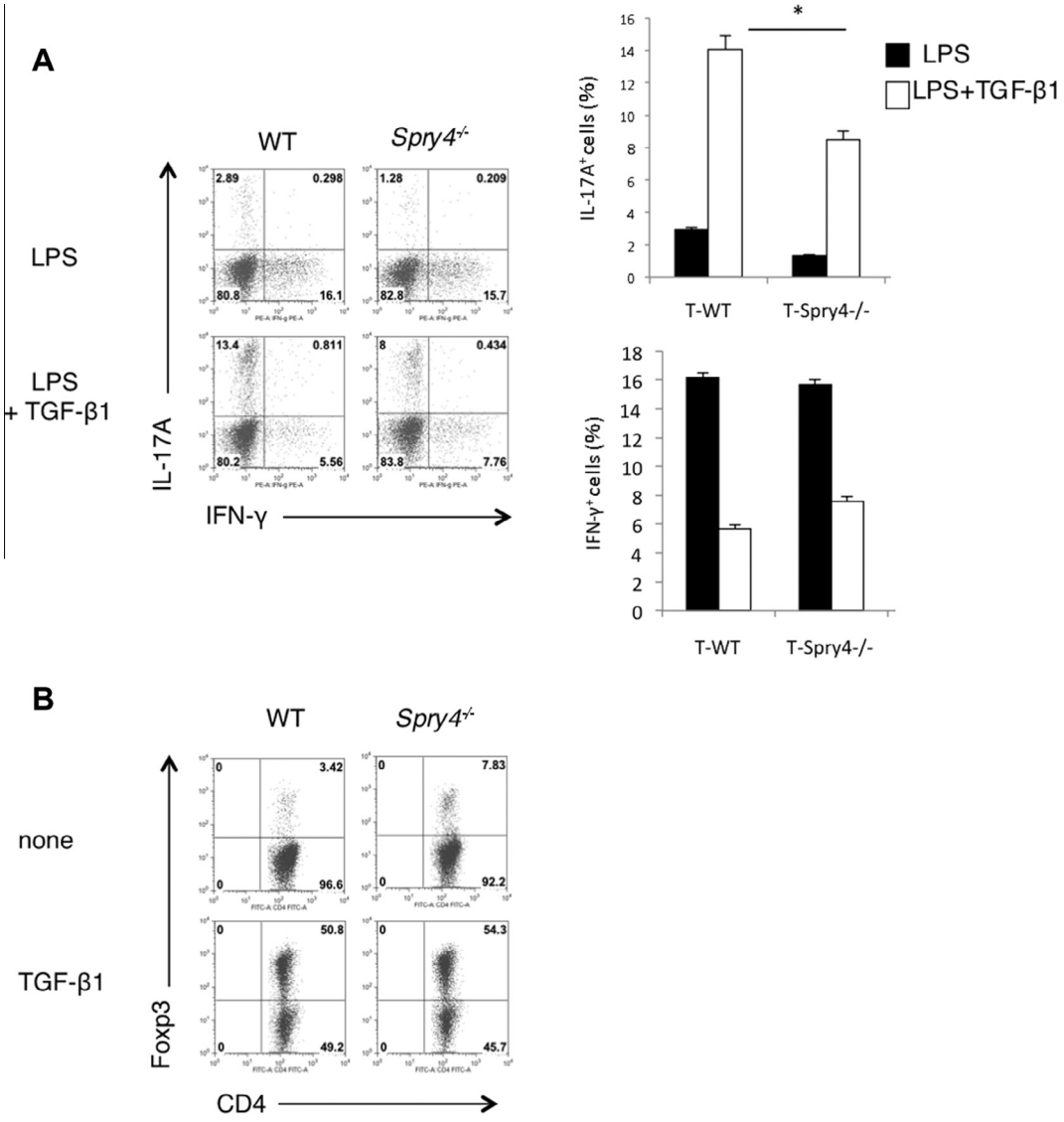

Fig. 2. *In vitro* induction of Th17 with DCs. (A and B) IL-17A and IFN-γ production (A) or Foxp3 expression (B) in CD4^{+} T cells obtained from WT or *Spry4*$^{-/-}$ mice in co-culture with DCs from WT mice. Co-cultures were performed in the presence of soluble anti-CD3, anti-IFN-γ, anti-IL-4, TGF-β1 (B), and/or LPS (100 ng/ml) (A) for 3 days. Data are represented by a dot plot and numbers for all FACS plots indicate the percentage of cells in each gate. One representative experiment out of three independent experiments is shown. The bar graph represents the average of the IL-17A^{+} or IFN-γ^{+} or Foxp3^{+} population in the three independent experiments. $^{*}P < 0.05$; Data are shown as mean ± SD.

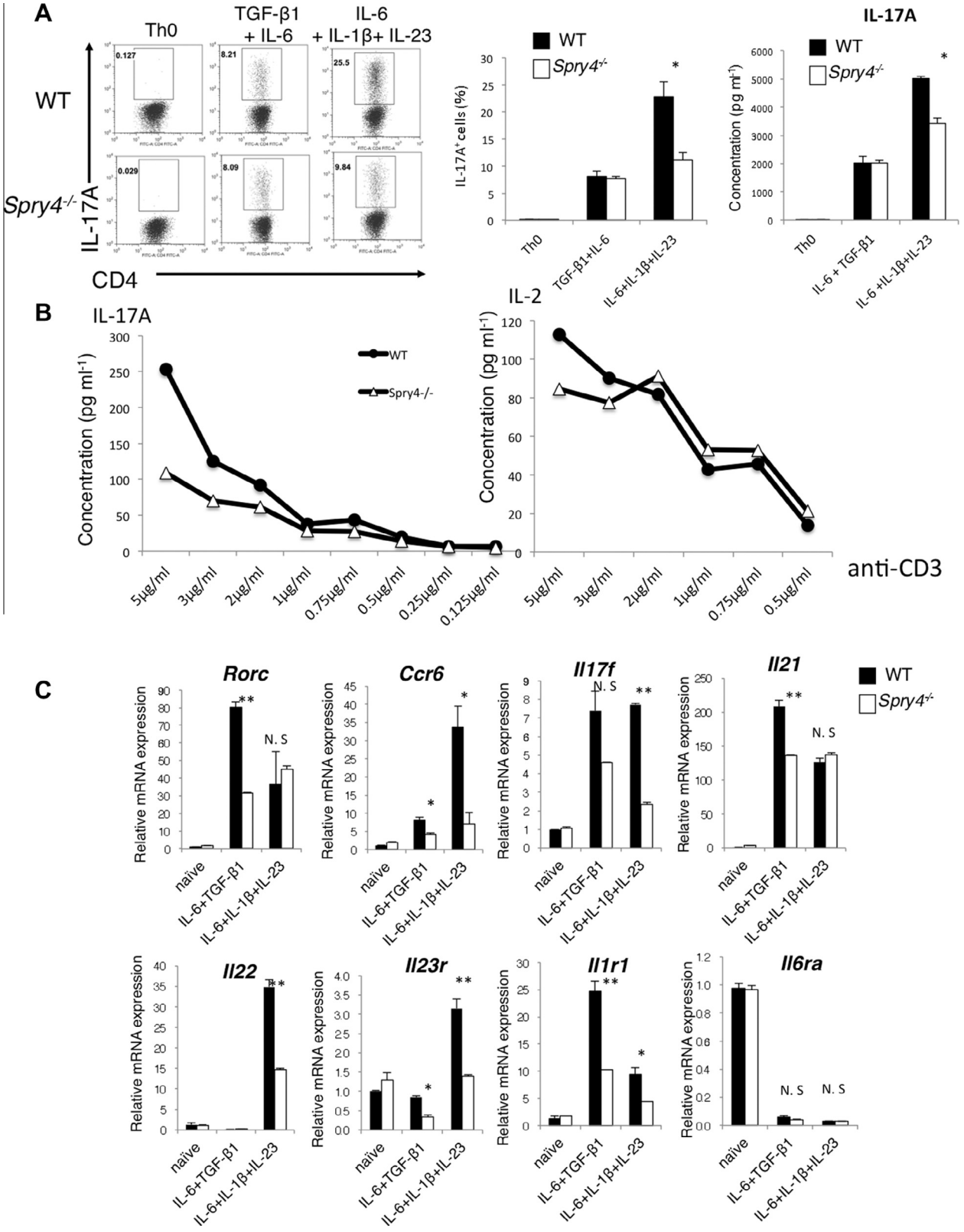

Fig. 3. Sprouty4 is related in Th17 differentiation *in vitro*. (A) WT or *Spry4*$^{-/-}$ naïve CD4^{+} T cells were activated under indicated Th17 conditions for 4 days. Data are represented by a dot plot and numbers for all FACS plots indicate the percentage of cells in each gate. One representative experiment out of three independent experiments is shown. The bar graph represents the average of the IL-17A^{+} population in the three independent experiments. Cytokine levels in the culture supernatants were measured by ELISA. (B) WT or *Spry4*$^{-/-}$ naïve CD4^{+} T cells were activated with indicated concentrations of anti-CD3 antibody in the presence of IL-1, IL-6 and IL-23 as indicated in (A). IL-17A and IL-2 levels in the culture supernatant were measured by ELISA. (C) The mRNA expression of the indicated genes were analyzed by Q-PCR and normalized to GAPDH. In this figure, $^{*}P < 0.05$; $^{**}P < 0.01$; N. S., not significant. Data are shown as mean ± SD.

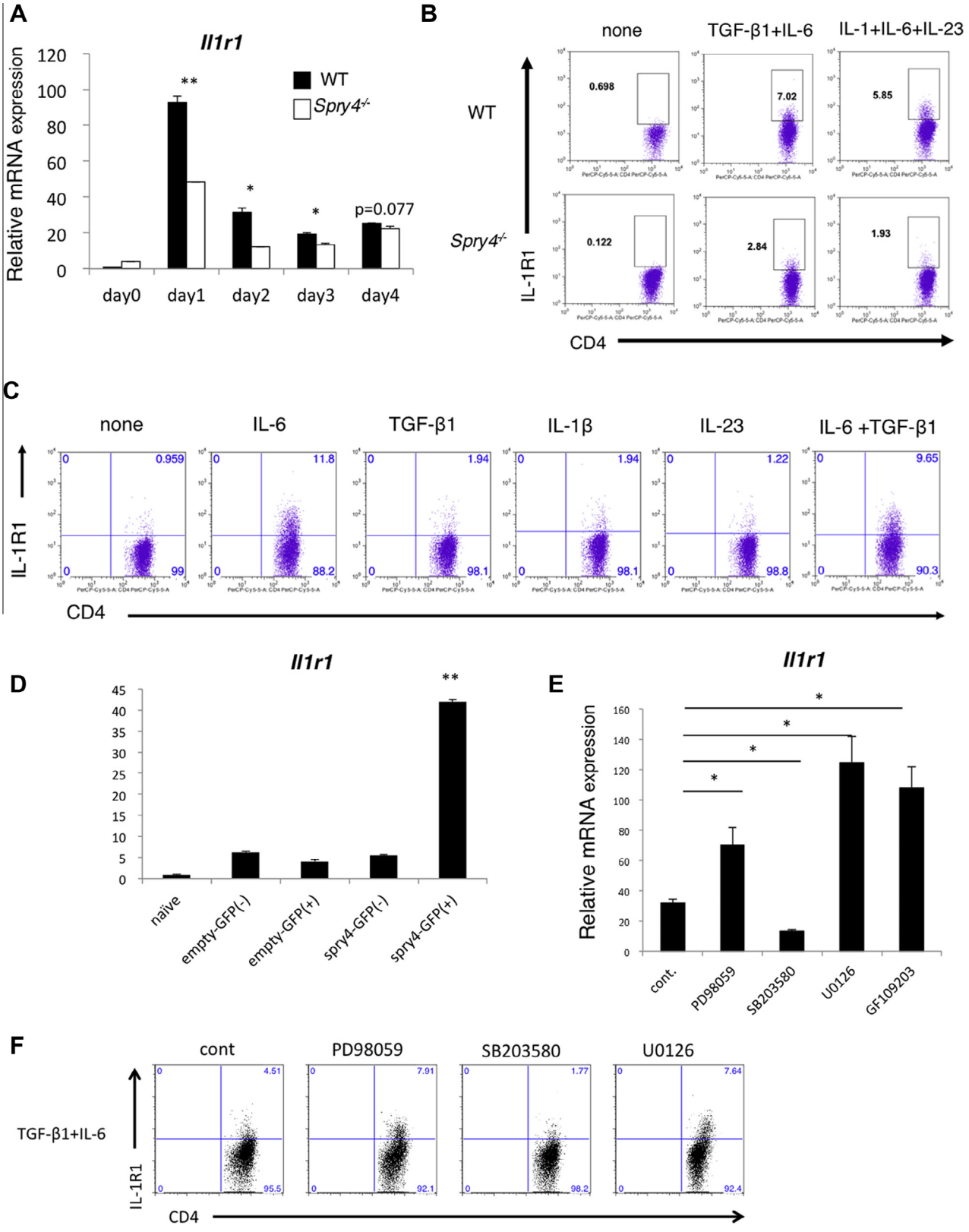

Fig. 4. Sprouty4 and TGF-β1 enhanced IL-1R1 expression. (A) WT or *Spry4*$^{-/-}$ naïve CD4$^+$ T cells were cultured in the presence of IL-6 for the indicated number of days. Total RNA was extracted from the CD4$^+$ T cells and mRNA expression of *Il1r1* was analyzed by Q-PCR and normalized to GAPDH. $^*P < 0.05$; $^{**}P < 0.01$. Data are shown as mean ± SD. (B) WT or *Spry4*$^{-/-}$ naïve CD4$^+$ T cells were activated under the indicated Th17 conditions for one day. IL-1R1 expression was analyzed by flow cytometry. Data are represented by a dot plot and numbers for all FACS plots indicate the percentage of cells in each gate. One representative experiment out of three independent experiments is shown. (C) WT naïve CD4$^+$ T cells were cultured in the presence of indicated cytokines for one day, and IL-1R expression on the cell surface was measured by flow cytometry. (D)WT naïve CD4$^+$ T cells were transduced with retroviral vector encoding for empty-IRES-EGFP or Sprouty4-IRES-EGFP and cultured in the presence of IL-6 and TGF-β1 for further 3 days. Total RNA was extracted from the FACS-sorted GFP(−) cells or GFP(+) cells. The mRNA expression of *Il1r1* was analyzed by Q-PCR and normalized to GAPDH. $^{**}P <$ 0.01.(E and F) WT naïve CD4$^+$ T cells were cultured in the presence of IL-6 and TGF-β1 supplemented with 5 μM indicated compounds for one day, and then total RNA was isolated. The mRNA expression of *Il1r1* was analyzed by Q-PCR and normalized to GAPDH (E). IL-1R expression on the cell surface was measured by flow cytometry (F). One representative experiment out of three independent experiments is shown. In this figure, $^*P < 0.05$ Data are shown as mean ± SD.

reduced expression of IL-1 receptor (IL1R) in *Spry4*$^{-/-}$ Th17 cells. First, we examined the time course of IL-1R gene (*Il1r1*) expression during Th17 differentiation. As shown in Fig. 4A, *Il1r1* expression was rapidly induced on day 1 after stimulation with TCR and IL-1/6/23 and the levels in *Spry4*$^{-/-}$ T cells were lower than in WT T cells. Then, we confirmed the reduced levels of IL1-R on the cell surface in *Spry4*$^{-/-}$ T cells by flow cytometry (Fig. 4B). Again, IL-1R expression was reduced by Sprouty4 deficiency both in TGF-β/IL-6 and IL-1/6/23 conditions. Among cytokines for Th17 differentiation, we found that IL-6 has the greatest effect on IL-1R expression and TGF-β somewhat reduced IL-1R expression by using flow cytometer analysis (Fig. 4C). Importantly, Sprouty4 overexpression by the retrovirus enhanced the expression of *Il1r1* under IL-1/6/23 conditions (Fig. 4D). These data indicate that Sprouty4 positively regulates IL-1R expression in T cells, which can account for the reduced iTh17 responses in *Spry4*$^{-/-}$ T cells.

Then, we characterized the signals that induce IL-1R expression. Sprouty4 has been shown to inhibit the PLCγ-PKC-ERK pathway in non-hematopoietic cells, thus, we examined effect of various signal transduction inhibitors. Among them, as expected, MEK inhibitors (PD98059, U0126) strongly upregulated *Il1r1* expression. In addition, a PKC inhibitor (GF109203) also enhanced *Il1r1* expression. However, a p38 inhibitor, SB203580, reduced *Il1r1* levels. These data indicate that the PLC-PKC-ERK pathway is involved in the suppression of IL-1R expression, which was negatively regulated by Sprouty4 (Fig. 4E). These data were confirmed by cell surface expression (Fig. 4F) and they indicate that the PKC-ERK pathway negatively regulates IL-1R expression in T cells, which is suppressed by Sprouty4.

4. Discussion

The mechanism by which RORγt regulates inflammatory or regulatory Th17 cell differentiation has not been completely studied. Although various stimulations induce different traits of IL-17A-producing Th17 cell generation, the intrinsic mechanisms underlying these differential cell fate decisions remain unknown. In this paper, we report that Sprouty4 is required for RORγt-mediated iTh17 gene expression when T cells are activated, especially under the IL-1/6/23 condition rather than the conventional IL-6/TGF-β condition. Sprouty4 deletion in T cells leads to the abrogation of iTh17 generation in the IL-1/6/23-induced condition in spite of normal expression of RORγt. Furthermore, *Spry4*$^{-/-}$ mice exhibited a significant resistance to the induction and development of EAE. By Th17 signature gene expression analysis, we found that Sprouty4 regulates the genes necessary for the inflammatory properties in Th17, including *Il1r1*.

We have shown that Sprouty4-overexpression suppressed PKC-mediated ERK activation [24]. Our study suggests that the ERK pathway negatively regulates pathogenic iTh17 differentiation. This is consistent with previous finding that MEK inhibitors enhance Th17 differentiation. Tan et al. reported that ERK signaling is found to potentiate the production of IL-2, thereby suppressing Th17 development [25]. We have proposed similar mechanism for the suppression of Th17 by CDK inhibitors [26]. However, we found that *Spry4*$^{-/-}$ T cells produce similar amounts of IL-2 compared with WT T cells (Fig. 3B). Therefore, it is unlikely Sprouty4 regulates the IL-2-STAT5 pathway. On the other hand, Liu et al. reported that blockade of ERK activation inhibited Th17-cell development while upregulated Treg cells under Th17 polarization conditions [27]. They also reported that inhibition of ERK enhanced TGF-β induction of Foxp3. However, as shown in Fig. 2B, we observed no significant differences in Foxp3 induction between WT and *Spry4*$^{-/-}$ T cells. Therefore, Sprouty4 probably regulates other ERK-target genes that are directly important for iTh17 development.

We confirmed that Sprouty4 positively regulates *Il1r1* expression under iTh17 conditions. Among the cytokines we examined, IL-6 was the most potent cytokine that induced IL-1R. Therefore, the IL-6 signaling pathway, especially STAT3, could be a target of Sprouty4. Since IL-6 activates STAT3 in addition to ERK [28], STAT3 may play an important role in *Il1r* induction. We confirmed that Sprouty4-overexpression suppressed IL-6-mediated ERK activation in a cultured T cell line (Okada et al. unpublished data). ERK has been shown to downregulate STAT3 transcriptional activity by inducing phosphorylation of Ser727 of STAT3 [29]. Another possibility is a direct or indirect phosphorylation of RORγt by ERK, since *Il1r1* expression has been shown to be dependent on RORγt [2]. Alternatively, the PKC-ERK pathway could be downstream of TCR and directly regulate IL-1R expression. Further study is necessary to define the mechanism of regulation of *Il1r1* expression by ERK/Sprouty4. Although the specific molecular mechanism remains to be clarified, our study suggests that Sproutys could be a therapeutic target for Th17-mediated diseases.

Acknowledgments

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