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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.

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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

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