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CDK inhibitors suppress Th17 and promote iTreg differentiation, and ameliorate experimental autoimmune encephalomyelitis in mice

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ABSTRACT

Th17 cells, which have been implicated in autoimmune diseases, require IL-6 and TGF-β for early differentiation. Several Smad-independent pathways including the JNK and the RhoA-ROCK pathways have been implicated in the induction of RORγt, the master regulator of Th17, however, molecular mechanisms underlying Smad-independent pathway remain largely unknown. To identify novel pathways involved in Th17 differentiation, we screened 285 chemical inhibitors for known signaling pathways. Among them, we found that Kenpaullone, a GSK3-β and CDK inhibitor, efficiently suppressed TGF-β-mediated RORγt induction and enhanced Foxp3 induction in primary T cells. Another CDK inhibitor, Roscovitine, but not other GSK3-β inhibitors, suppressed Th17 differentiation and enhanced iTreg development. Kenpaullone and Roscovitine suppressed experimental autoimmune encephalomyelitis (EAE), a typical Th17-mediated autoimmune disease model. These two compounds enhanced STAT5 phosphorylation and restored IL-2 production in the presence of TGF-β. These data suggest that CDK inhibitors modulate TGF-β-signaling pathways, which restore TGF-β-mediated suppression of IL-2 production, thereby modifying the Th17/iTreg balance.

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, through which they develop specialized properties and effector functions. CD4$^+$ T cells were traditionally thought to differentiate into two subsets: Th1 cells and Th2 cells [1]. Recently, an additional subset of Th cells has been identified; these are the Th17 cells and iTregs (induced regulatory T cells). Th17 cells, characterized by the production of IL-17A play an important role in autoimmune diseases, elimination of extracellular bacteria, and cancer. The differentiation of Th17 cells from naïve T cells requires TGF-β and IL-6, both *in vitro* and *in vivo* [2–5]. TGF-β and IL-6 induce the nuclear orphan receptors RORγt and RORα, which have also been shown to be required for the differentiation of Th17 cells [6,7]. In contrast, Foxp3, the master regulator of Tregs is induced by TGF-β and IL-2. We and others have also reported that Foxp3 inhibits *IL-17A* expression through interaction with RORγt [8,9], and that growth factor-independent 1 (Gfi1) negatively regulates Th17 differentiation by inhibiting RORγt activity [10,11]. Thus, Th17 and iTregs reciprocally inhibit their differentiation.

The main TGF-β signaling pathway is the Smad pathway. However, we and others have demonstrated that *Rorc* induction by TGF-β is independent of the Smad signaling pathway [12]. Smad-independent TGF-β has now been discovered, including the Ras-extracellular signal-regulated kinase (Erk), TGF-β-activated kinase-mitogen-activated protein kinase (MAPK) kinase, 4-c-Jun N-terminal kinase (TAK-MKK4-JNK), TAK-MKK3/6-p38, Rho-Rac-cdc42 MAPK, and phosphatidylinositol 3-kinase (PI3K)-Akt pathways [2]. The pathway for RORγt induction, however, remained to be clarified prior to the present study. Recently, we found that the expression of Eomesodermin (Eomes) was substantially down-regulated via a Smad-independent mechanism activated by TGF-β. We established that the TGF-β-JNK-c-Jun signaling pathway strongly repressed expression of Eomes. Repression of Eomes was necessary for Th17 induction and suppression of JNK could inhibit the Th17-mediated disease model,

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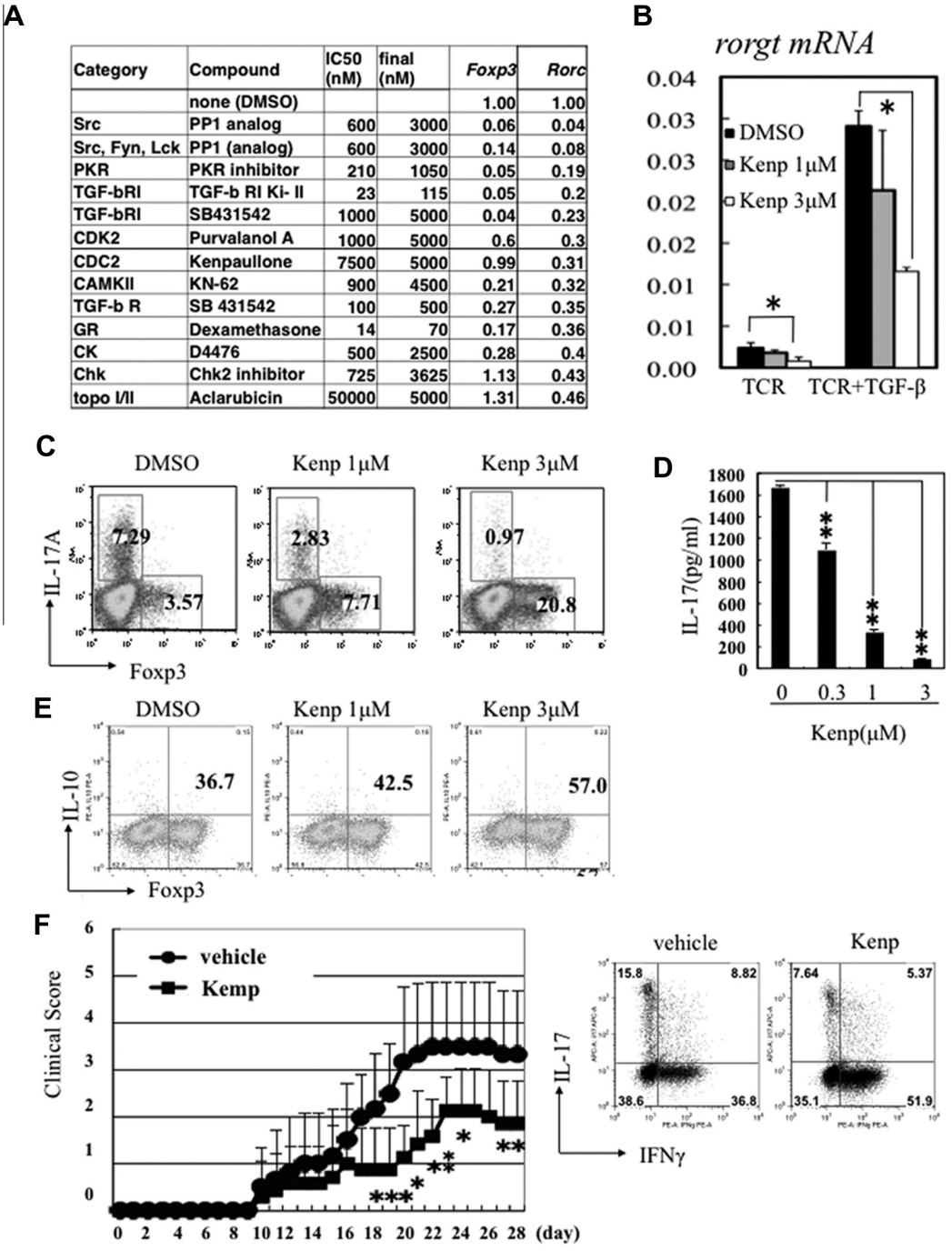
experimental autoimmune encephalomyelitis (EAE). A recent paper also suggests that TGF-β-RhoA-ROCK2 pathway activation regulates Th17 differentiation [13]. However, the entire TGF-β pathway for Th17 differentiation was previously unknown.

To identify a novel mechanism that regulates TGF-β-mediated Th17 differentiation, we screened a chemical library containing known inhibitors for more than 80 signaling pathways. By this screening, we found that CDK inhibitors strongly suppressed



Category	Compound	IC50 (nM)	final (nM)	*Foxp3*	*Rorc*
	none (DMSO)			1.00	1.00
Src	PP1 analog	600	3000	0.06	0.04
Src, Fyn, Lck	PP1 (analog)	600	3000	0.14	0.08
PKR	PKR inhibitor	210	1050	0.05	0.19
TGF-bRI	TGF-b RI Ki- II	23	115	0.05	0.2
TGF-bRI	SB431542	1000	5000	0.04	0.23
CDK2	Purvalanol A	1000	5000	0.6	0.3
CDC2	Kenpaullone	7500	5000	0.99	0.31
CAMKII	KN-62	900	4500	0.21	0.32
TGF-b R	SB 431542	100	500	0.27	0.35
GR	Dexamethasone	14	70	0.17	0.36
CK	D4476	500	2500	0.28	0.4
Chk	Chk2 inhibitor	725	3625	1.13	0.43
topo I/II	Aclarubicin	50000	5000	1.31	0.46

Fig. 1. Identification of Kenpaullone (Kenp) as a Th17-inhibitor. (A) Naive $CD4^+$ T cells were stimulated with anti-CD3/CD28 (TCR) antibodies in the presence or absence of 5 ng/ml TGF-β for 24 h with various compounds from the SCADS inhibitor kits at a concentration five times higher than their IC_{50}. After isolation of total RNA, mRNA levels of *Foxp3* and *Rorc* (RORγt gene) were measured using quantitative real-time PCR. The top 13 compounds that inhibited *Rorc* expression were listed. (B) Effects of various Kenpaullone concentrations on *Rorc* mRNA levels in $CD4^+$T cells stimulated with TCR ± TGF-β for 24 h were determined. $^*p < 0.05$ (C) IL-17A and Foxp3 profiles were determined by intracellular staining. Naïve $CD4^+$T cells were cultured under Th17 differentiation conditions (see Materials and Methods) with the indicated Kenpaullone (Kenp) concentrations. T cells were recovered on day 4, restimulated with PMA, ionomycin and brefeldin A for 5 h, and analyzed using FACS. One representative data set from at least three independent experiments is shown. (D) IL-17A levels, determined using ELISA. $CD4^+$ T cells were cultured for 3 days under Th17 conditions in the presence of the indicated Kenpaullone concentrations, then 1×10^5 cells were stimulated with anti-CD3 antibody for 24 h.$^*p < 0.05$ $^{**}p < 0.01$ (E) IL-10 and Foxp3 profiles were determined by intracellular staining. Naïve $CD4^+$T cells were cultured under iTreg differentiation conditions in the presence of the indicated Kenpaullone concentrations. (F) EAE disease course in vehicle-treated and Kenpaullone-treated mice ($n = 6$ mice per group). Vehicle or Kenpaullone (25 mg /kg/day) was administered intraperitoneally every second day from day 0 to day 8 $^*p < 0.05$, $^{**}p < 0.01$. Right panels; cytometric data for IL-17A and IFN-γ production by $CD4^+$ T cells in the central nerve system from EAE mice treated with or without Kenpaullone on day 20 after EAE induction. One representative data set from at least three independent experiments is shown.

IL-17 and RORγt expression in T cells. We found that CDK inhibition suppressed Th17 and promoted iTreg differentiation, suggesting that CDK modulates TGF-β signaling pathways, and could be a therapeutic target for Th17-related diseases such as rheumatoid arthritis, psoriasis and intestinal bowel disease.

2. Materials and methods

2.1. Mice

C57BL6/J mice were purchased from Nihon Jikken Doubutsu (Tokyo, Japan). Animals were maintained under specific pathogen-free

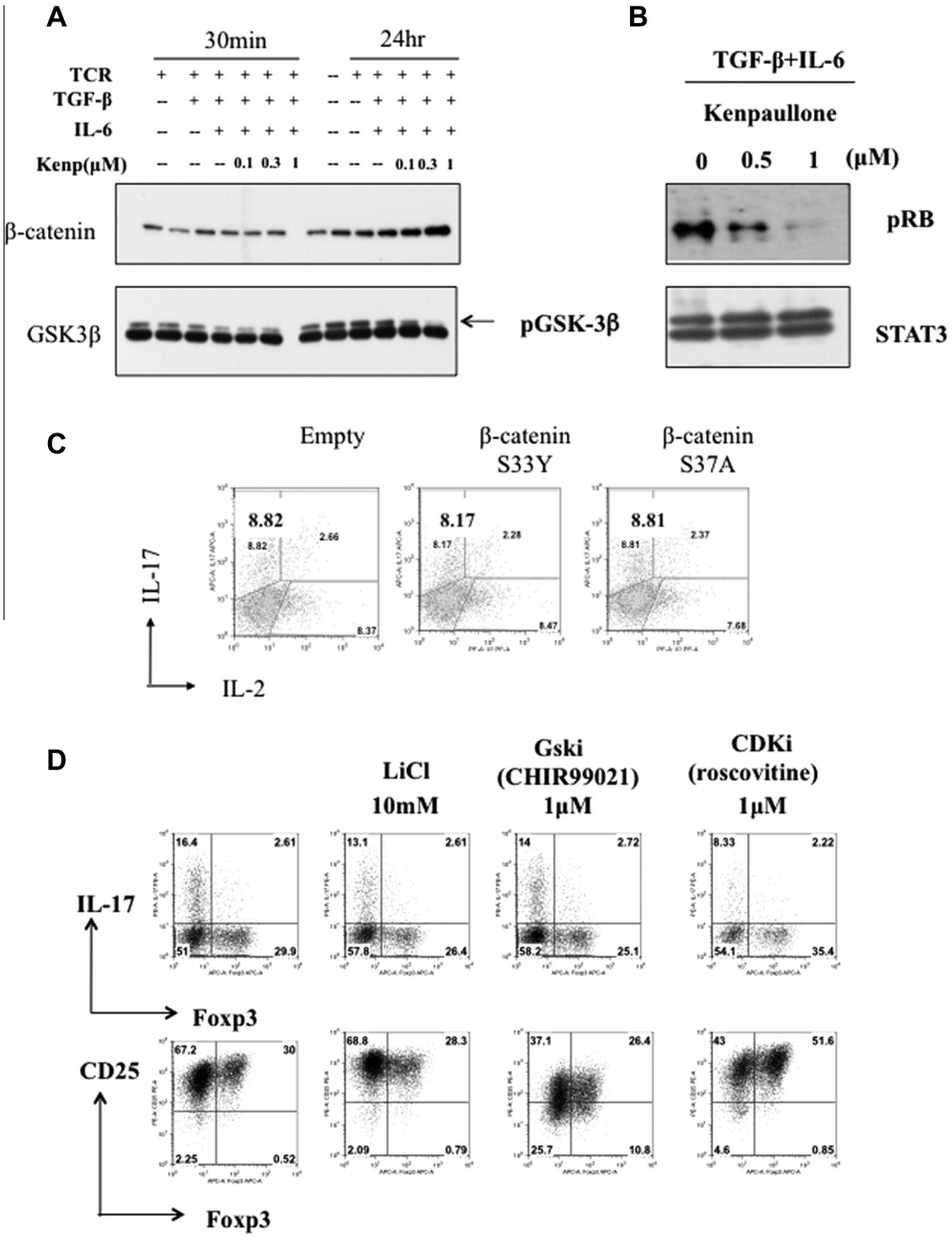


Fig. 2. Effect of Kenpaullone on GSK-3β and CDKs. (A and B) Naïve CD4$^+$ T cells were stimulated with anti-CD3/CD28 (TCR), TGF-β (5 ng/ml) and IL-6 (20 ng/ml) in the presence of the indicated Kenpaullone (Kenp) concentrations for 30 min (A) or 24 h (A and B). After lysing, the cell extracts were subjected to SDS–PAGE and Western blotting using the indicated antibodies. Positions for phosphorylated GSK-3β (pGSK-3β) are indicated. (C) One day after activation under Th17-differentiation conditions, naïve CD4$^+$ T cells were transduced with retroviral vectors encoding IRES-GFP (empty) and β-catenin (S33Y or S37A)-IRES-GFP. The cells were analyzed after a total of 4 days in Th17-skewed culture. The panels were gated on GFP-positive cells. The production of IL-17A and IL-2 by the transduced (GFP-positive) cells were examined using intracellular staining. One representative experiment out of three independent experiments is shown. (D) Naïve CD4$^+$ T cells were cultured under Th17 conditions (upper panels) or iTreg conditions (lower panels) in the presence of the indicated LiCl, CHIR99021, or Roscovitine concentrations. T cells were recovered on day 4, restimulated with PMA, ionomycin and brefeldin A for 5 h, and analyzed using FACS. The quadrant percentiles of cells staining positively for the indicated cytokines are shown. One representative data set from three independent experiments is shown.

conditions in animal facilities certified by the Animal Care Committee of the Keio University School of Medicine. Animal protocols were approved by the same Animal Care Committee.

2.2. Chemicals

Chemicals. The SCADS (Screening Committee of Anticancer Drugs) inhibitor kits [14] (http://gantoku-shien.jfcr.or.jp/) containing 285 compounds were kindly provided by a Grant-in-Aid for Scientific Research in the Priority Area "Cancer" from the Ministry of Education, Culture, Sports, Science and Technology (Tokyo, Japan). Kenpaullone was purchased from Selleck Chemicals, LLC (Houston, TX, USA), and Roscovitine was purchased from ShangHai Biochempartner Co., Ltd., (Shanghai, China). CHIR99021 was purchased from Wako Chemicals Co., (Japan).

2.3. T cell preparation and differentiation

Naïve $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ T cells were isolated as previously described [15]. Naïve T cell were stimulated with 3μ g/ml of plate-bound anti-CD3 (clone 145–2C11) and 0.5 μg/ml of soluble anti-CD28 (eBioscience). For Th0 cell differentiation, the cells were stimulated with anti-CD3/CD28 (TCR) antibodies. For Th17 cell differentiation, the cells were treated with 5 ng/ml human TGF-β (R&D Systems), and 20 ng/ml human IL-6 (R&D Systems) [16]. For iTreg differentiation, naïve $CD4^+$T cells were culture with anti-TCR antibodies in the presence of 5 ng/ml TGF-β. Intracellular cytokine staining was perofmed as previously described [17,18]. Phosphorylation was analyzed using SDS–PAGE and Western blotting as described previously [19].

2.4. Experimental autoimmune encephalomyelitis (EAE)

For the induction of EAE, mice were immunized s.c. on day 0 with 100 μg/mouse myelin oligodendrocyte glycoprotein (MOG) peptide, as described previously [20]. Kenpaullone or Roscovitine (50 mg/kg) was injected i.p. every other day from day 0 to day 8.

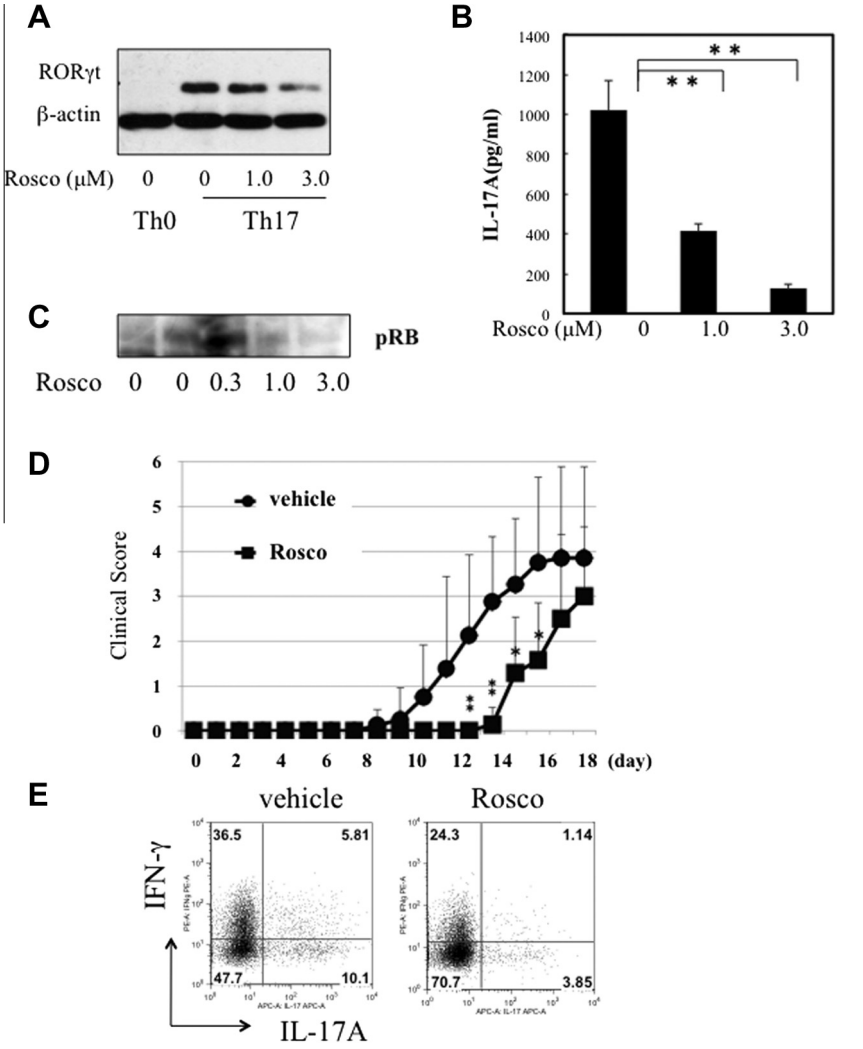


Fig. 3. Effect of Roscovitine (Rosco) on Th17 development and disease. (A and B) Naive $CD4^+$ T cells (1×10^6) were stimulated with Th0 or Th17 conditions for 48 h in the presence of various Roscovitine compounds. After lysing, the cell lysate was subjected to Western blotting with anti-RORγt or β-actin antibodies (A). IL-17A in the culture supernatant was measured using ELISA (B) $^{**}p < 0.01$. (C) Naïve $CD4^+$ T cells were cultured under Th17 conditions for 24 h in the presence of the indicated Roscovitine (Rosco) concentrations. After lysing, the cell extracts were subjected to SDS–PAGE and Western blotting with anti-CDK substrate antibody. Phosphorylated Rb (pRb) is shown. (D) EAE disease course in vehicle-treated and Roscovitine-treated mice ($n = 6$ mice per group). Vehicle or Roscovitine (50 mg /kg/day) were administered intraperitoneally every second day from day 0 to day 8. $^*p < 0.05$, $^{**}p < 0.01$ (E) Cytometric data for IL-17A and IFN-γ production by $CD4^+$ T cells in the central nerve system from EAE mice treated with or without Kenpaullone on day 20 after EAE induction. One representative data set from at least three independent experiments is shown.

2.5. Statistical analysis

All data were analyzed by Student's *t* test. A *p*-value of <0.05 was considered to be significant. All error bars shown in this article represent standard deviations.

3. Results

3.1. Screening of a chemical library for inhibitors of TGF-β-mediated RORγt induction

We have shown that Smad-independent TGF-β signals can induce RORγt under Th17 conditions [21]. We found that TGF-β alone can rapidly induce RORγt mRNA but the IL-6 signal is synergistically required for stable expression of RORγt (data not shown). Thus, to elucidate the signal for TGF-β, we have screened a chemical library composed of 285 compounds that inhibit various kinases and signaling molecules. As shown in Fig 1A, several kinase inhibitors were selected as potent inhibitors of TGF-β-induced RORγt induction. Of these inhibitors, Kenpaullone suppressed RORγt induction but did not affect Foxp3 induction. Thus, we focused on Kenpaullone. Kenpaullone is a potent inhibitor of CDK1/cyclin B (IC_{50} = 400 nM), CDK2/cyclin A (IC_{50} = 680 nM), CDK5 (IC_{50} = 850nM) and also, to a lesser extent, other kinases [22]. More recently, Kenpaullone has been found to be a strong GSK-3β inhibitor (IC_{50} = 23 nM) [23,24].

3.2. Characterization of Kenpaullone as a Th17 inhibitor

First we characterized Kenpaullone as a Th17 inhibitor. As shown in Fig. 1B, Kenpaullone dose-dependently inhibited TGF-β-induced RORγt induction. Next, we examined the effect of Kenpaullone on Th17 and iTreg development under Th17 conditions (TCR + IL-6 + TGF-β). As expected, Kenpaullone suppressed Th17 differentiation (Fig. 1C), which was also confirmed using ELISA; 1 μM Kenpaullone was sufficient to inhibit IL-17A production (Fig. 1D), while 1 μM Kenpaullone did not much affected cell proliferation (~20% reduction. data not shown). Interestingly,

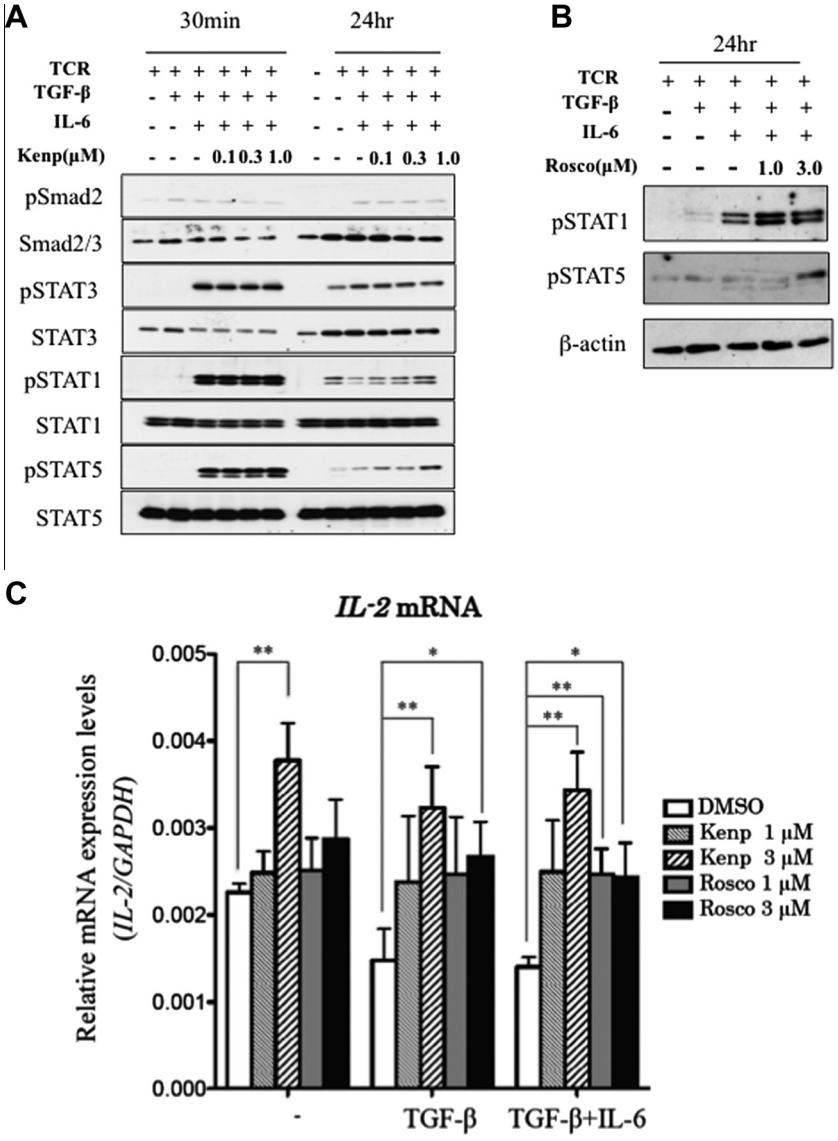


Fig. 4. CDK inhibitors enhanced STAT5 activation and IL-2 production. (A, B) Naïve $CD4^+$T cells were cultured with the indicated cytokine for 30 min or 24 h in the presence of the indicated compound concentrations. Cells were then lysed and blotted with the indicated Abs. (C) Effect of Kenpaullone and Roscovitine on *IL-2* mRNA levels in $CD4^+$T cells stimulated with TCR ± TGF-β and IL-6 for 24 h. Naïve $CD4^+$ T cells were stimulated with anti-TCR antibodies alone or in the presence of TGF-β ± IL-6. Total RNA was recovered from T cells and the IL-2 mRNA levels were measured using quantitative real-time RT-PCR. $^*p < 0.05$ $^{**}p < 0.01$.

Kenpaullone strongly promoted iTreg development under Th17 conditions (Fig. 1C). Kenpaullone also promoted Foxp3 protein levels, but not IL-10 under iTreg conditions (TCR + TGF-β) (Fig. 1E), which was not recognized by an initial mRNA screening. Finally, we examined the effect of Kenpaullone on an *in vivo* Th17 disease model, EAE. As shown in Fig. 1F, Kenpaullone significantly ameliorated the severity of EAE and reduced infiltration of Th17 cells into the central nerve system (CNS). Taken together, Kenpaullone inhibited Th17 differentiation *in vitro* and *in vivo*, thereby suppressing EAE in the model.

3.3. Suppression of CDK and GSK-3β by Kenpaullone

Kenpaullone has been shown to inhibit both CDKs and GSK-3β. Thus, we examined whether Kenpaullone actually inhibited GSK-3β and CDKs under Th17 conditions. As shown in Fig. 2A, Kenpaullone at 1 μM slightly reduced GSK-3β phosphorylation and enhanced β-catenin accumulation. However, phosphorylation of the Rb protein, a well known cellular CDK substrate, was strongly inhibited by 1 μM Kenpaullone (Fig. 2B). These data suggest that Kenpaullone inhibited CDKs more strongly than GSK-3β under Th17 conditions.

To verify whether CDK or GSK-3β is responsible for the effect of Kenpaullone on IL-17 production, we examined other CDK and GSK-3β inhibitors. First we introduced the constitutively active form of β-catenin by retrovirus into primary T cells. As shown in Fig. 2C, the active form of β-catenin did not suppress Th17 development. Then we examined the effect of the GSK-3β inhibitors, LiCl and CHIR99021. These two independent GSK-3β inhibitors did not affect Th17 differentiation (Fig. 2D).

Next, we examined a novel CDK inhibitor, Roscovitine (Fig. 2D, right). Roscovitine is an olomoucine-related purine flavopiridol, and is a highly potent inhibitor of CDK1, CDK2, CDK5, and CDK7 kinase activity ($IC_{50} \sim 0.5$ to 0.2 μM) but it is a weak inhibitor of CDK 4 and CDK 6 ($IC_{50} > 100$ μM) [25,26]. As shown in (Fig. 2D right). Roscovitine suppressed IL-17A production and enhanced Foxp3 expression, similar to Kenpaullone. These data suggest that CDK, rather than GSK-3β, is involved in Th17 and iTreg differentiation.

3.4. Roscovitine is a potent Th17 inhibitor

We characterized Roscovitine as a Th17 inhibitor; we observed dose-dependent Roscovitine-induced suppression of RORγt and IL-17 expression under Th17 conditions *in vitro* (Fig. 3A and B). As expected, Roscovitine inhibited phosphorylation of Rb protein (Fig. 3C). Similar to Kenpaullone, Roscovitine efficiently suppressed EAE and infiltration of Th17 cells into CNS (Fig. 3D). Roscovitine required a higher dose for Th17 and EAE suppression than Kenpaullone, which may reflect the higher Roscovitine IC_{50} for CDK suppression compared to Kenpaullone.

3.5. CDK inhibitors suppress Th17 by inhibiting TGF-β-mediated IL-2 suppression

Lastly, we attempted to define the mechanism of Th17 suppression and iTreg promotion by CDK inhibitors. First, we examined the effect of CDK inhibitors on STAT phosphorylation under Th17 conditions. There was no effect of Kenpaullone and on STAT1, STAT3 or Smad2 phosphorylation (Fig. 4A). However, STAT5 phosphorylation was enhanced by Kenpaullone after 24 h stimulation but not after 30 min stimulation (Fig. 4A). A similar increase in STAT5 phosphorylation was observed by Roscovitine (Fig. 4B). Since STAT5 is a strong inhibitor of Th17 and promoter of iTreg differentiation [27,28], modulation of STAT5 activity explains the effect of Kenpaullone and Roscovitine.

Since STAT5 is mostly activated by IL-2 in T cells, we examined the effect of Kenpaullone on IL-2 levels. As reported previously, TGF-β suppresses IL-2 production, which is dependent on Smad2 and Smad3 [29]. We found that Kenpaullone as well as Roscovitine restored the IL-2 expression levels (Fig. 4C). These data suggest that CDK inhibitors indirectly suppress Th17 differentiation by reversing Smad2/3-mediated IL-2 suppression.

4. Discussion

In this study, we showed that CDK inhibitors are a potent suppressor of Th17 differentiation, which can be applied to Th17-related diseases, such as EAE. CDK inhibitors have also been used in rodent arthritis models such as collagen-induced arthritis (CIA), another Th17-dependent disease model. Overexpression of protein CDK inhibitors and chemical CDK inhibitors showed an inhibitory effect in experimental arthritis models [30–32]. Several mechanisms of CIA amelioration by the CDK inhibitors have been proposed, including inhibiting proliferation of synovial fibroblasts and exerting anti-inflammatory effects in CDK activity-dependent and -independent manners [32,33]. Another study showed that the CDK inhibitor p16(INK4) also suppressed LPS-induced production of IL-6 but not TNF-α from macrophages [34]. p16(INK4a) promoted ubiquitin-dependent IRAK1 degradation, impaired AP-1 activation, and suppressed IL-6 production. However, we did not observe strong suppressive effect of Kenpaullone and Roscovitine at 1∼3 μM on IL-6 production from Raw 264.7 macrophage cell line in response to LPS (data not shown), suggesting that CDK inhibitors did not suppress the NF-kB pathway at the concentration which inhibited IL-17A production.

TCR stimulation strongly activates CDKs, which induce phosphorylation of Rb (see Fig. 2B). Chunder et al. reported that CDK2, the major target of p27kip1, is highly active in T cells that infiltrate and reject cardiac allografts, and CDK2 deficiency in recipients led to long-term allograft survival [35]. Antagonism of CDK2 resulted in decreased production of IFN-γ without affecting proliferation, and CDK2-deficient mice exhibited increased infiltration of Foxp3 + Tregs *in vivo*. These data are consistent with our data, which shows that CDK inhibitors suppressed T cells pathogenicity and increased Tregs proliferation, although the other study did not examine Th17. However, the mechanism of CDK inhibitor-induced tolerance remains to be clarified.

Recently, CDK was shown to antagonize TGF-β action by phosphorylating Smad2 and Smad3 [36,37]. CDK-dependent phosphorylation of Smad2 on Thr-8 (T8) was linked to impaired Smad activity and T8, T178, and Ser-212 of Samd3 were reported to be phosphorylated by CDK4 and CDK2 [36]. Thus, it is possible that CDKs reduce Smad2/3 activity, thereby weakening TGF-β-mediated IL-2 suppression. Therefore, CDK inhibitors suppress Th17 by restoring IL-2 expression as shown in Fig. 4C. However, this hypothesis cannot account for upregulation of Foxp3, which was also dependent on Smad2/3 [12]. The relationship between Smads and CDK or CDK inhibitors for gene-specific transcriptional regulation remains to be clarified.

It is believed that Th17 plays a key role in certain types of autoimmune diseases, including psoriasis and inflammatory bowel disease. Th17-specific inhibitors such as RORγt inhibitors have been developed, and have been shown to be effective in EAE models. Although the mechanism has not been completely elucidated, several compounds have been shown to be a Th17-selective inhibitor. Our study suggests that CDK inhibitors could lead to novel and effective therapies for autoimmune diseases.

Acknowledgments

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