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Transcriptional Regulator CTR9 Inhibits Th17 Differentiation via Repression of IL-17 Expression

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PAF complex is an evolutionarily conserved transcriptional complex that associates with RNA polymerase II in the coding region of actively transcribing genes. Although its transcriptional activity is closely related to diverse cellular processes, such as cell-cycle progression or development in mammals, its role in immune responses has not been addressed yet. In this study, we show that CTR9, a component of PAF complex, functions as a repressor of Th17 differentiation. Both mRNA and protein levels of CTR9 were significantly decreased during the differentiation processes of naive T into Th17 effector cells. When CTR9 was depleted, IL-17 expression was induced and differentiation into Th17 cells enhanced. In naive T cells, CTR9 occupied the coding region of Il17a, but dissociated under Th17 in vitro-polarizing conditions. In contrast, both CDC73 and PAF1 were recruited to the Il17a locus under Th17-differentiation conditions. In the IL-6-stimulated splenocytes, expression of CTR9 was decreased, and chromatin-bound CTR9 disappeared in the coding region of Il17a. IL-6 also directly repressed expression of CTR9 gene, as promoter activity of CTR9 was similarly repressed by IL-6 treatment. Moreover, in mice with collagen-induced arthritis, lentivirus-mediated CTR9 overexpression in the joints ameliorated arthritis severity, decreasing the frequency of CD4IL-17+ T cells in lymph nodes. In conclusion, our data propose a novel feed-forward loop of IL-17 transcriptional regulatory circuit, via IL-6-mediated repression of CTR9 which is a transcriptional repressor of IL-17. The Journal of Immunology, 2014, 192: 1440–1448.

The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; CIL, type II collagen; H2Bub, histone H2B; Lenti-CTR9, CTR9-expressing lentiviral particle; Lenti-shRNA, CTR9–short hairpin RNA–expressing lentiviral particle; m, murine; mCr-v-LUC, luciferase reporter containing CTR9 promoter fragments; PAF, PAF complex; qRT-PCR, quantitative real-time PCR; RORγt, retinoic acid–related orphan receptor γt; shRNA, short hairpin RNA.

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T he adaptive immune response is mediated by interplay between immune cells such as APCs, B cells, and T cells. Among them, CD4-positive effector T cells play essential roles in the regulation of immune responses through cell-to-cell interactions and inflammatory cytokine secretion. Effector Th cells, such as Th1, Th2, and Th17, and regulatory T cells help B cells to produce Abs, enhance activation of CD8-positive T cells, or remain as memory cells to fight effectively against pathogens during the subsequent infections (1). Th17 is a newly identified subset of effector T cells that mainly secret inflammatory cytokines IL-17 and IL-21 upon activation. Neutralization of IL-6 in the supernatant of LPS-activated dendritic cells or genetic ablation of TGF-β failed to induce Th17 differentiation, indicating that IL-6– and TGF-β–mediated signals are important to drive the differentiation of naive T cells into Th17 lineage (2–4). Th17 is clinically important, as it is involved in the onset of autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis (5). IL-17, a key cytokine of Th17, plays critical roles in the recruitment of neutrophils to the site of inflammation (6). Overexpression of IL-17 is a key feature of the autoimmune diseases in human patients, and IL-17A knockout impaired the induction of experimental autoimmune encephalomyelitis in mice, demonstrating the importance of IL-17 in the onset of autoimmune disease (7–9).

Transcription factors activated by IL-6 and TGF-β are known to play critical roles in the differentiation of Th17 cells. Specifically, STAT3 functions as a central transcription factor orchestrating the transcriptional regulatory program of Th17 differentiation. Both IL-6 and IL-21 trigger STAT3 phosphorylation, which in turn induces the expression of retinoic acid–related orphan receptor γt (RORγt), a cell fate–determining factor of Th17 differentiation (10–12). STAT3-deficient T cells fail to produce IL-17A, thus blocking the differentiation of naive T cells into Th17 lineage (10). Both STAT3 and RORγt bind to the promoter region of Il17a locus and collaboratively induce IL-17 production (12–14). Due to the clinical significance of Th17 cells, there have been active efforts to identify transcription factors that control IL-17 expression or Th17 differentiation. Recently, basic leucine zipper transcription factor 3, EOMES, IFN regulatory factor 4, and Kruppel-like factor 4 have been identified as novel transcriptional regulators that control Th17 differentiation (15–18). In addition to the lineage-specific transcriptional control by transcription factors, epigenetic regulation of chromatin also plays essential roles for maintaining the lineage commitment and inheritng expression profiles to progenitor cells. In Th17 cells, chromatins surrounding Il17a locus are enriched with acetylated histone 3 and histone 3 trimethylated at lysine 4 residue (H3K4me3), but lacks histone H trimethylated at lysine 3 residue (H3K4me3).
lysin 27 (H3K27me3), whereas the opposite pattern of chromatin modifications are observed in Th1 and Th2 cells (19, 20).

PAF complex (PAFc) is a transcriptional regulatory complex composed of Ctr9, Paf1, Leo1, Cdc73, and Rtf1 in yeast. It interacts with RNA polymerase II and controls transcription at multiple stages: transcriptional initiation, elongation, and RNA processing of eukaryotic transcription (21). Consistent with their genomic distribution along the coding region of genes, PAFc has genetic and physical interactions with elongation factors, such as Sp4–Spt5 (DRB sensitivity-inducing factor) and Spt16-Pob3 (facilitates chromatin transcription) complexes (22). Recruitment of both Set1 and Set2 histone methyltransferase to actively transcribing target gene loci depends on PAFc (23, 24), as deletion mutants of Rtf1 and Paf1 were defective in H3K4 and H3K79 trimethylation (25). In addition, loss of Paf1 and Ctr9 result in complete loss of H3K36 trimethylation (26). PAFc is also required for Rad6/Bre1-dependent ubiquitination of histone H2B (H2Bub) of the actively transcribing genes, which is prerequisite for trimethylation of H3K4 and H3K79 (25, 27). Although H2Bub and H3K4me3 are closely linked with active genes, they are also reported to repress gene expressions possibly through recruitment of histone deacetylases (28).

Human PAFc also interacts with signal-specific transcription factors at promoter regions. CDC73 associates with β-catenin to induce Wnt target gene expression (29), and CTR9 affects recruitment of STAT3 to the target gene promoter through physical interaction to activate transcription of IL-6–inducible genes (30). It suggests that PAFc might link signal-specific gene expression and epigenetic chromatin regulation via its protein interactions with multiple transcription regulators. Although most of PAFc studies published thus far have focused on its role in the cell-cycle progression and development (21), recent data suggest that PAFc might also be involved in the immune-related processes (31). Regulatory potential of PAFc in the IL-6–induced gene expression prompted us to investigate its possible role in the transcriptional regulatory network of Th17 differentiation. Using an in vitro differentiation system of Th17, in this study, we demonstrate that CTR9 inhibits Th17 differentiation via its direct regulation of IL-17 expression.

Materials and Methods

In vitro differentiation of mouse T cells

Naive CD4+ T cells (CD4+CD62Lhi) were obtained from spleens of C57BL/6 wild-type mice. CD4+ T lymphocytes were separated by negative selection using the MACS CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD62Lhi cells were then separated by positive sorting using anti-CD62L–conjugated microbeads. The purity of isolated T cell populations was >99%. For Th17 differentiation, naive T cells were stimulated with Abs against CD3 (5 μg/ml) and CD28 (10 μg/ml) in the presence of human TGF-β (5 ng/ml) and murine (m)IL-6 (20 ng/ml). Neutralizing Abs against mIL-17A (10 μg/ml) and mL-4 (10 μg/ml) were also included. For Th1 differentiation, naive T cells were stimulated with Abs against CD3 (5 μg/ml) and CD28 (10 μg/ml) in the presence of mIL-12 (10 ng/ml) and mL-2 (10 ng/ml). Neutralizing Abs against mL-4 (10 μg/ml) were also included. For Th0 conditions, naive T cells were stimulated with Abs against CD3 (5 μg/ml) and CD28 (10 μg/ml). Anti-CD3 and anti-IL-4 Abs were purchased from eBioscience (San Diego, CA). Anti-CD28, anti–IFN-γ Abs, and all cytokines were purchased from R&D Systems (Minneapolis, MN). For Th2 differentiation, naive T cells were stimulated with Abs against CD3 (5 μg/ml) and CD28 (2 μg/ml) in the presence of mL-2 (20 ng/ml), mL-4 (10 ng/ml), and anti–IFN-γ Abs (10 μg/ml) for 3 d. After 3 d, cells were harvested, washed, and rested with T cell culture media for 1 d. And then, cells were recultured for an additional 3 d two times with a set of the above Th2–polarizing reagents with CD3/CD28 stimulation.

Cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions. Briefly, cells were incubated with isotype control Ab in PBS solution (1% FBS) for 30 min at 4°C for blocking. After washing with PBS solution (1% FBS) at 1000 × g, cells were permeabilized with BD Cytoperm solution (BD Biosciences) for 15 min at 4°C. After washing with Perm/Wash buffer (BD Biosciences), cells were stained with anti-mouse IL-17A–PE and anti–IFN-γ–APC Abs in Perm/Wash Buffer (BD Biosciences) for 30 min at 4°C. After washing with Perm/Wash buffer (BD Biosciences), cells were resuspended in Perm/Wash Buffer, and images were collected on a BD FACSCalibur (BD Biosciences). Abs were purchased from eBioscience (San Diego, CA). All acquisitions were performed with a BD FACSCalibur cytometer equipped with BD CellQuest software (BD Biosciences).

Quantitative real-time PCR analysis

Total RNA from T cells was extracted with RNAiso (Takara). A total of 100–500 ng RNA was reverse-transcribed into cDNA with the Improm-II Reverse Transcription System (Promega, Madison, WI). cDNA were quantified by real-time PCR with a SYBR (Takara) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative mRNA levels were determined with the ΔΔCT threshold cycle method. Values were expressed relative to β-actin. Primer information is described in Supplemental Table I.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChiP) was performed as previously described (30). Briefly, I × 106 cells was cross-linked with 1% formaldehyde solution and sonicated to produce soluble chromatin with an average size of 400 bp. The soluble chromatin was immunoprecipitated with CTR9, CD73, or PAF1 Abs at 4°C overnight. Ab complex was then bound to protein A/G beads (Calbiochem, San Diego, CA) for 2 h. DNA was eluted from the immunoprecipitated complex by heating at 65°C overnight and bound to a DNA-binding column (Bioneer). Quantitative real-time PCR (qRT-PCR) was performed using primers designed for specific loci. PAFc Abs were purchased from Bethyl Laboratories (Montgomery, TX). The sequences of the oligonucleotides used are described in Supplemental Table I.

Reporter constructs

Reporter constructs were obtained by inserting mouse CTR9 promoter fragments amplified by high-fidelity PCR into the plg3 basic vector (Promega). Primer information for PCR is described in Supplemental Table I.

Lentivirus generation and transduction

Short hairpin RNA (shRNA) specific for mouse Ctr9 (5'-CGTGTTGCGCT-CCAAAACCTTT-3') was cloned into pLL3.7 vector under U6 promoter. Mouse CTR9 coding sequence was cloned into pLL3.7 vector under CMV promoter. A total of 10 μg lentiviral vector and 7 and 3 μg each packaging vector (Δ8.9 and vesicular stomatitis virus-G, respectively) were cotransfected into 293T cells on 100-mm dishes by calcium phosphate transfection method (32). Supernatants were collected 48 h after transfection and filtered through a 0.45-μm filter. The viral supernatant was centrifuged at 25,000 rpm in a Beckman SW28 rotor (Beckman Coulter) for 1.5 h. The viral pellet was resuspended in PBS and stored at −80°C. For lentiviral transduction of CD4+ T cells, naive T cells were cultured under corresponding polarizing conditions for 24 h. Cells were centrifuged with concentrated lentiviral particles at a multiplicity of infection of 5 in culture media containing 4 μg/ml polybrene at 700 × g for 2 h at 37°C. Multiplicity of infection indicates the number of infectious particles per cell. To measure the number of infectious particles, we infected 293FT cells with lentivirus and measured GFP+ population by flow cytometry. Then infectious particle numbers per unit volume were calculated. After centrifugation, viral supernatants were replaced with T cell culture medium containing appropriate cytokines and Abs. For titration, we infected HEK293 cells with lentiviral particles and measured GFP+ cells population by FACS analysis.

Transient transfections and luciferase assays

HEK293 cells in a 24-well plate were transfected with luciferase reporters containing CTR9 promoter fragments (mCtr9-LUC) vector (100 ng/well) along with Renilla luciferase plasmid (pRL-TK) (2 ng/well) that encodes the Renilla luciferase for normalization of transfection efficiency by calcium phosphate transfection method (32). At 48 h posttransfection, cells were treated with IL-6 plus IL-6R (40 ng/ml each) for 12 h, and a dual luciferase assay was performed according to the manufacturer’s instructions (Promega).
ELISA
FACS-sorted naive T cells (CD4+CD25−CD44lowCD62Lhi) were cultured for 5 d in RPMI 1640 (containing 10% FCS, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, 1 mM sodium pyruvate, 55 µM 2-ME, 1 mM NEAA, and 2 mM l-glutamine) after transfection of lentivirus. For Th17, naive T cells were stimulated with precoated anti-CD3ε (5 µg/ml) and anti-CD28 (2 µg/ml) in the presence of rTGF-β (5 ng/ml), IL-6 (20 ng/ml), anti–IFN-γ (5 µg/ml), anti–IL-4 (5 µg/ml), and anti–IL-2 (2.5 µg/ml). Culture supernatants were collected, and IL-17A was detected with a Ready-SET-Go kit provide by eBioscience in accordance with the manufacturer’s protocol.

Western blot
A total of 1.0 × 106 cells was harvested at differentiation days 0, 1, and 2. Cells were lysed in lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 0.1 mg/ml PMSE, 1 mM DTI, 1 mM sodium orthovanadate, 5 µg/ml aprotonin, 2 µg/ml pepstatin, 5 µg/ml leupeptin, and 1 mM benzamidine). Total cell lysates (20 µg/sample) were resolved using 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membrane and blotted with Abs. Abs against CTR9, PAF1, and LEO1 were purchased from Bethyl Laboratories.

CFSE labeling
Lentivirally transduced T cells were polarized under Th17 conditions for 3 d. Then cells were labeled with 5 µM CFSE for 5 min at room temperature in PBS. After washing with PBS, CFSE-labeled or unlabeled cells were plated in 96-well round-bottom plates and cultured under Th17-differentiation conditions for an additional 3 d. Cells were analyzed with a BD FACSCalibur (BD Biosciences) cytometer equipped with CellQuest software (BD Biosciences).

Induction of collagen-induced arthritis
Male DBA/1 mice (The Jackson Laboratory, Bar Harbor, ME) were immunized with bovine type II collagen (CII; Chondrex, Redmond, WA) at 7 to 8 wk of age, as described previously (33, 34). Briefly, CII was dissolved in 0.05 N-acetic acid (2.5 mg/ml) at 4˚C and emulsified with CFA (ratio 1:1). The mice received 0.1 ml emulsion (containing 100 µg CII) in the base of the tail as a primary immunization. Booster injections were given 14 d after primary immunization into the footpad with 50 µg CII dissolved and emulsified as before. The incidence and severity of arthritis were determined by visual inspection. The mice were observed three times a week for the onset, duration, and severity of joint inflammation after the primary immunization. Each limb was assessed on a 0–4-point scale, as described earlier (35). The hindfoot that received the booster immunization was excluded from the evaluation.

Results

Both mRNA and protein levels of CTR9 were decreased during Th17 differentiation

Based on the ability of CTR9 to modulate IL-6–inducible acute-phase protein gene expression (30), we examined whether CTR9 controls Th17 differentiation. For this purpose, we first examined the expression of PAFc components during the course of Th17 differentiation. Naive CD4+ T cells were isolated from the spleen of mice using the MACS system. Anti-CD3/anti-CD28 stimulation with IL-6 and TGF-β treatment successfully induced naive CD4+ T cells to differentiate into Th17 populations (Fig. 1A). To examine the PAFc expression patterns during Th17 differentiation, we harvested cells from days 0, 1, 3, and 5 during Th17 polarization and analyzed IL-17A and PAFc mRNA levels by qRT-PCR (Fig. 1B). After 5 d of culture, >600-fold induction of IL-17A was observed. In the same set of cultured cells, CTR9 mRNA levels were significantly decreased, whereas LEO1 mRNA levels were increased with >10-fold induction at day 5 (Fig. 1B). In contrast, mRNA levels of CDC73, PAF1, and SKI8 were either not changed or slightly increased during Th17 differentiation. To confirm the patterns of PAFc expression during Th17 differentiation, we separately harvested cells from days 0, 1, and 2 of Th17-polarizing in vitro culture and examined protein levels by Western blot analysis (Fig. 1C). The expression of CTR9 protein was detected in naive T cells (day 0). However, the expression of either PAF1 or LEO1 protein was undetectable at day 0. In agreement with mRNA expression patterns, CTR9 protein levels were decreased, whereas PAF1 and LEO1 levels were increased after 2 d of Th17-polarizing in vitro culture. This result was somewhat surprising, as PAFc is known to function as a complex (21). Both the basal expression of the individual PAFc components in the naive T cells and the induction patterns during Th17 differentiation suggested that PAFc in T cells might behave differently from PAFc in other cell types.

We next evaluated the expression of CTR9 in other subsets of T cells in which IL-17 is suppressed. For this purpose, naive CD4+ T cells were activated with anti-CD3/anti-CD28 Abs in the presence of IL-12 and IL-2 to induce Th1 differentiation and IL-4 to induce Th2 differentiation (Fig. 1D, Supplemental Fig. 1A). Over
80% of CD4$^+$ T cells were IFN-γ$^+$IL-17$^-$ in Th1 differentiation (Fig. 1D), and 20% were IL-4$^+$IL-17$^-$ in Th2 differentiation (Supplemental Fig. 1A), respectively. CTR9 mRNA level was not significantly changed in Th0, Th1, or Th2 cells, but was in Th17 cells (Fig. 1E, Supplemental Fig. 1A).

Because the Th17-polarizing culture condition is mainly obtained by costimulation with IL-6 and TGF-β, we asked whether expression of CTR9 can be directly controlled by IL-6 signal. For this, we prepared splenocytes from a mouse spleen and measured the mRNA levels of IL-17A and CTR9 after IL-6 stimulation. IL-6 treatment alone was sufficient to induce IL-17A expression, and at the same time, the mRNA level of CTR9 was dramatically reduced (Fig. 1F). We next tested IL-21, which is also known to drive Th17 differentiation (36). However, IL-21 treatment to splenocytes exhibited a very weak effect on IL-17 expression, and CTR9 expression was not significantly affected (Fig. 1G).

**Th17 differentiation was enhanced by CTR9 knockdown**

Reduction in CTR9 expression during the differentiation into Th17 cells can be interpreted as follows: CTR9 blocks Th17 differentiation in naive T cells and must be absent to allow Th17 differentiation. If this is true, alterations in CTR9 levels during the Th17-polarizing in vitro culture will change the percentage of differentiated Th17 cells. To test the repressive potential of CTR9, we produced a lentivirus harboring CTR9-shRNA under the U6 promoter and a CMV-EGFP reporter cassette. At 24 h after culture under Th17 conditions, T cells were transduced with control (Lenti) or CTR9-shRNA–expressing (Lenti-shCTR9) lentiviral particles and cultured 3 more d in the Th17-polarizing conditions. The silencing efficiency of CTR9-shRNA was separately confirmed in the mouse embryonic fibroblast cells (Supplemental Fig. 1B). Both Lenti and Lenti-shCTR9 infected T cells with similar efficiency, confirmed by GFP signal, without significant changes to general properties of T cells following the infection (Fig. 2A). The effect of CTR9 deficiency in the progression of Th17 differentiation was then monitored by FACS analysis. Among the T cells transduced with control virus (Lenti), only 10.4% were IL-17A$^+$. In contrast, 17.1% of the T cells were IL-17A$^+$ in the cells transduced with CTR9-shRNA virus, confirming that CTR9 inhibits differentiation into Th17 cells (Fig. 2A). In separate experiments with enhanced Th17 differentiation efficiency, more IL-17A$^+$ cells were observed in the CTR9-shRNA virus–transduced GFP–positive cells (Supplemental Fig. 2). In addition, increased IL-17A production was detected in the CTR9-shRNA virus–transduced cells compared with Lenti (Fig. 2B). Finally, we examined the effect of CTR9 overexpression on Th17 differentiation. When CD4$^+$ T cells were transduced with CTR9-expressing lentiviral particles (Lenti-CTR9), less cells differentiated into Th17 population, compared with cells transduced with Lenti (Fig. 2C). On the contrary, Th1 differentiation was not affected by CTR9 knockdown (Supplemental Fig. 1C). These data altogether indicate that CTR9 specifically functions in the Th17 differentiation.

**CTR9 specifically controls transcription of IL-17**

To understand how CTR9 affects Th17 cell differentiation, we monitored the expression of key regulatory molecules involved in the differentiation, amplification, and stabilization of Th17 cells in the CTR9-deficient condition. For this purpose, T cells were transduced with Lenti or Lenti-shCTR9 and cultured under the Th17-polarizing conditions. At day 5, lentivirally transduced T cells were sorted by GFP signal using a cell sorter, and the mRNA expression profiles were analyzed by qRT-PCR. General silencing efficiency was ~50%, determined by endogenous CTR9 mRNA expression in the transduced cells (Fig. 3A). We first assessed the expression of inflammatory cytokines IL-17A/F, IL-21, and IL-22. The expression of IL-17A/F, key cytokines produced by Th17 cells, was significantly enhanced by CTR9 silencing (Fig. 3B). In contrast, expression levels of IL-21, which acts in an autocrine manner and is important for the amplification of Th17 cells (36), and IL-22, another cytokine secreted by Th17 (37), were not significantly altered in the CTR9-deficient cells. The expression of IL-22, although not statistically significant, appears to decrease following CTR9 silencing. Therefore, it seems that IL-17 is the main cytokine affected by CTR9 silencing.

Next, we examined the expression of key transcription factors that determine Th17 differentiation. STAT3, a transcription factor that transmits signals from activated IL-6R, is expressed in two
different isoforms, STAT3α and STAT3β. STAT3α usually activates signal-specific gene expression, whereas STAT3β acts as a transcriptional repressor (38, 39). Therefore, we examined the expression of both isoforms of STAT3 using primers that specifically distinguish them. In the CTR9-deficient cells, both STAT3α and STAT3β were similarly expressed compared with control cells (Fig. 3C). RORα and RORγt, key transcription factors for Th17 differentiation, were not changed significantly or slightly increased by CTR9 knockdown (Fig. 3D). To examine whether CTR9 alters proliferative potential of Th17 cells, without affecting the expression of IL-17, we used the CFSE labeling method to measure the proliferation rate of cells transduced with either control or CTR9-shRNA lentivirus (Fig. 3E). The data clearly suggested that CTR9 does not affect the Th17 cell proliferation rate, indicating that IL-17 expression might be directly controlled by CTR9 at the transcription level.

**CTR9 association to the coding region of Il17a is altered during Th17 differentiation**

PAFCs regulate gene expression through physical association with chromatin. To determine whether CTR9 or PAFCs is directly involved in the transcriptional regulation of IL-17 expression, genomic occupancy patterns of PAFCs in the Il17a locus were explored during the Th17-polarizing in vitro culture condition (Fig. 4A). Naive CD4+ T cells cultured in the Th17-polarizing condition were harvested at differentiation days 0, 1, and 2. ChIP assay was performed with Abs directed against the PAFC components. At day 0, CTR9 was rarely found in the promoter region of Il17a. Instead, the highest CTR9 occupancy was observed in the coding regions of Il17a. At differentiation days 1 and 2, quite opposite patterns of CTR9 occupancy were observed; upon the treatment with IL-6 and TGF-β for Th17 differentiation, CTR9 was dissociated from the coding region, and at the same time, CTR9 was recruited to the promoter region of Il17a (Fig. 4B). The dissociation of CTR9 was not observed in cells activated with TGF-β alone or cells activated without cytokine (Fig. 4C). In contrast to CTR9, both CDC73 and PAF1 occupancy levels were increased as cells differentiate in the Il17a locus (Fig. 4D, 4E).

We also separately confirmed IL-6-mediated recruitment of STAT3 and CTR9 to the Il17a locus. As observed during the Th17-polarizing in vitro culture condition, both STAT3 and CTR9 were recruited to the promoter region of Il17a after IL-6 stimulation in splenocytes (Fig. 4F). Simultaneously, highly occupied CTR9 in the coding region of Il17a was dissociated upon IL-6 stimulation (Fig. 4F). These results indicate that both chromatin association and complex formation of PAFCs dynamically change during the whole processes of transcription as T cells differentiate.

Finally, to determine whether IL-6 represses CTR9 expression directly, the promoter activity of CTR9 gene was measured. For this purpose, mCrt9-LUC was constructed (Fig. 4G). HEK293 cells were cotransfected with three different mCrt9-LUC vectors and pRL-TK vector. Luciferase activity of each construct was measured upon IL-6 stimulation. Luciferase activity of the CTR9 reporter constructs correlated with the decreased transcript level of CTR9 mRNA after IL-6 treatment, indicating that IL-6 directly controls the transcription of CTR9 (Fig. 4G).

**CTR9 regulates Th17 differentiation in vivo**

Finally, we examined whether CTR9 regulates Th17 differentiation in vivo. To this end, we injected Lenti-shCTR9 intra-articularly into the ankle joints of mice with collagen-induced arthritis twice a week for 3 wk from 15 d after the primary immunization. We found that the onset of arthritis was more rapid in mice injected with shRNA for CTR9 than in mice injected with Lenti control (Fig. 5A, 5B). The severity of arthritis was also significantly higher in mice with CTR9 shRNA (Fig. 5B). Reduced expression of CTR9 in mice injected with CTR9 shRNA was confirmed in sorted CD4+ T cells of draining lymph nodes at 3 d after the injection of CTR9 shRNA into the joints (Fig. 5C). In parallel, the number of CD4+IL-17+ T cells in draining lymph node cells was increased by downregulation of CTR9 transcripts in the joints (Fig. 5D, 5E). On the contrary, intra-articular injection of Lenti–CTR9 ameliorated the onset and severity of arthritis (Fig. 5A, 5B) and decreased the frequency of CD4+IL-17+ T cells in lymph node cells (Fig. 5D, 5E), indicating that CTR9 overexpression suppresses the Th17 response. Taken together, these results are consistent with our in vitro findings that CTR9 regulates CD4+IL-17+ T cell differentiation.

Here, we propose a coherent feed-forward regulatory loop that regulates IL-17 expression via signal specific recruitment of STAT3 and repression of transcription repressor CTR9 (Fig. 6).
Discussion

Due to its tight link to autoimmune disease, the transcriptional regulatory program of Th17 differentiation, which is mainly controlled by sequence specific transcription factors and epigenetic chromatin modifiers, has received much attention lately. PAFc is a transcriptional regulator that associates with RNA polymerase II and controls recruitment of histone-modifying enzymes to target gene locus. It is usually found associated with the coding regions of the actively transcribing genes (40, 41) and hence suggested as an activator of transcription. However, there are also a handful of reports proposing a negative role of PAFc in transcription (42–46). Both Paf1 and Ctr9 repress the SER3 gene by reducing nucleosome occupancy and recruiting of Spt16 (43). Rtf1-dependent ubiquitination of H2Bub and methylation of H3K4 represses ARG1 expression in yeast (28). In this study, we also showed that CTR9 represses IL-6–mediated transcription of IL-17 in Th17 cells.

PAFc-mediated transcriptional regulation is closely associated with the cellular differentiation program of stem cells (47). PAFc is downregulated during embryoid body formation, and CTR9 knockdown induces extensive differentiation of embryonic stem cells. Similarly, we also observed that CTR9 is downregulated during Th17 differentiation, and loss of CTR9 leads to augmented differentiation of Th17 cells. Furthermore, CTR9 was associated with the IL-17a locus in the undifferentiated T cell condition and removed after Th17 differentiation. These data collectively suggest that PAFc or CTR9 might be important for maintaining undifferentiated state of cells, and its downregulation is required to initiate cellular differentiation.

Interestingly, CTR9 was recruited to the promoter region of IL-17a in response to IL-6. CTR9 is known to stabilize STAT3 interaction with chromatin at the promoter region of acute-phase responsive genes (30). IL-17a also is a direct target gene of STAT3, as it contains a STAT3 binding site in its promoter region (13). Therefore, we hypothesized that CTR9 plays dual function at the promoter and coding region of IL-17a. In undifferentiated T cells, CTR9 at the coding region represses IL-17 expression. Under Th17 conditions, repressive CTR9 at coding region is removed to facilitate RNA polymerase II–mediated transcription, and another CTR9 is recruited to the promoter region to stabilize the interaction between STAT3 and IL-17a promoter. It is not clear whether the CTR9 dissociated from the coding region relocates to the promoter region, or CTR9-containing complex is newly recruited to the promoter region. Although transcriptional activation mechanism

**FIGURE 4.** CTR9 is dissociated from the coding region of IL-17a during Th17 differentiation. (A) Schematic illustration of IL-17a locus. Locations of primers used in ChIP analysis are marked with double-sided arrows. (B) At differentiation days 0, 1, and 2, ChIP assay was performed using anti-CTR9 Ab. ChIP was coupled with qRT-PCR using primers specific for promoter (Pro), transcription start site (TSS), and coding regions (Mid1, Mid2, Mid3, and Mid4) of IL-17a. (C) Naive T cells were activated with or without TGF-β for a total of 2 d. ChIP assay was performed as described in (A). (D and E) Naive T cells were polarized under Th17 conditions for a total of 2 d. Cells were harvested at differentiation days 0, 1, and 2, and ChIP assay was performed using anti-CDC73 (D) and anti-PAF1 (E) Abs. ChIP was coupled with qRT-PCR using primers specific for indicated loci. *p < 0.1, **p < 0.05, ***p < 0.02, Student t test. Error bars represent SD (n = 3). (F) Mouse splenocytes were stimulated with IL-6 (20 ng/ml) for 1 h, and the occupancies of STAT3 and CTR9 on IL-17a promoter (Pro) or coding region (Mid2) were measured by ChIP analysis. (G) mCtr9-LUC and pRL-TK were cotransfected into HEK293 cells. At 48 h later, IL-6 (40 ng/ml) was treated for 12 h. Firefly relative to Renilla luciferase activity was measured. Error bars represent SD (n = 3). ***p < 0.01 by Student t test. N.D., Below detection range.
of CTR9 at the promoter has been previously proposed, molecular mechanism of transcriptional repression in the coding region of target gene has not been thoroughly investigated yet. We propose that CTR9 might interact with negative regulators of transcriptional elongation or histone remodelers to inhibit transcription of IL-17 in basal conditions. However, detailed molecular mechanisms of regulation need to be further investigated.

In this study, we demonstrated that both mRNA expression and promoter activity of CTR9 were reduced by IL-6 stimulation. This explains how CTR9 expression level decreased under the Th17-differentiation conditions. Contrary to the negative regulation of CTR9 by IL-6, STAT3 is activated by IL-6 stimulation and positively controls differentiation of Th17 by orchestrating the expression of regulatory factors and key cytokines. As a result, STAT3 and CTR9 form a coherent feed-forward regulatory loop for the regulation of IL-17 expression under IL-6–stimulation conditions. IL-17 is induced by IL-6–mediated activation and recruitment of STAT3. At the same time, IL-6 repressed expression of CTR9, a repressor of IL-17 transcription, and IL-6 also induced CTR9 dissociation from the \( \text{Il17a} \) locus (Fig. 6). Because the coherent feed-forward loop often ensures the delayed-type response (48), it is possible that CTR9 controls the kinetics of IL-17 production and Th17 differentiation through the epigenetic change of histone modifications during Th17 differentiation. Our work provides a novel insight into the repressive role of PAFc in the regulation of immune cell differentiation.

![Figure 5](http://www.jimmunol.org/)

**Figure 5.** Lentivirus-mediated CTR9 gene silencing augments arthritis severity and increases Th17 response. Arthritis incidence (A) and severity (B) in mice with Lenti (n = 14), Lenti-shCTR9 (n = 14), and Lenti-CTR9 (n = 9). DBA/1 mice were immunized with CII plus CFA to induce collagen-induced arthritis. Fifteen days after the primary immunization, \( 1 \times 10^6 \) infectious viral particles (20 μl) was injected into the ankle joints of fore and hind foot twice a week for 3 wk. Clinical arthritis scores were regularly assessed until day 39 postimmunization. The mean ± SDs are shown. \( *p < 0.05, **p < 0.01, ***p < 0.001 \) versus control mice treated with Lenti only by Student \( t \) test. (C) Downregulation of CTR9 transcripts by the intra-articular injection of Lenti-shCTR9. Lenti (n = 3) or Lenti-shCTR9 (n = 4) was injected into the ankle joints 3 wk after primary immunization. Draining (inguinal) lymph nodes (LN) were isolated from the mice at 3 d after the lentivirus injection. CTR9 mRNA expression was determined in sorted CD4+ T cells of lymph nodes by real-time PCR analysis. \( *p < 0.05 \) versus control mice treated with Lenti by Student \( t \) test. (D and E) FACS analysis of cells in inguinal lymph node cells of arthritic mice injected with Lenti, Lenti-shCTR9, and Lenti-CTR9, which was determined at 3 wk after primary immunization (n = 3 for each group). Data are the representative of three independent experiments. \( *p < 0.05, ***p < 0.001 \) versus control mice by Student \( t \) test.

![Figure 6](http://www.jimmunol.org/)

**Figure 6.** Negative regulation of IL-17 transcription by CTR9. CTR9 association with the coding region of \( \text{Il17a} \) in undifferentiated T cells. Under Th17 conditions, IL-6 signal activates IL-17 transcription via recruitment of STAT3 and dissociation of CTR9 from the \( \text{Il17a} \) locus.
IL-17 has been demonstrated to be effective for suppressing arthritis (50). In the current study, the onset of arthritis was more rapid in mice injected with Lenti-shCTR9 than in mice injected with Lenti control. The severity of arthritis was also significantly higher in mice with Lenti-shCTR9. Conversely, lentivirus-mediated CTR9 overexpression in the joints ameliorated arthritis severity, decreasing the frequency of CD4+IL-17+ T cells in lymph nodes. These results, together with the previous results (49, 50), suggest that CTR9 regulates inflammatory arthritis by inhibiting Th17 cell generation.

In conclusion, our work provides a novel insight into the repressive role of PAFc in the regulation of immune cell chronic inflammation. In addition, our findings open a new possibility of developing CTR9 modulator(s) as a therapeutic agent for treating chronic inflammatory arthritis.

Disclosures

The authors have no financial conflicts of interest.

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