The H3K9 demethylase plant homeodomain finger protein 2 regulates interleukin 4 production in CD4$^+$ T cells

Yuya Arakawa[a,c], Yuzuki Tano[a], Moe Fujii[a], Yuuki Imai[b], Yoshiaki Norimatsu[a], Masaki Yasukawa[a], Mikio Watanabe[c], Takeshi Yamada[a,*]

[a] Department of Medical Technology, Faculty of Health Sciences, Ehime Prefectural University of Health Sciences, Iyo-gun, Ehime, Japan.

[b] Department of Pathophysiology, Graduate School of Medicine, Ehime University, Toon, Ehime, Japan.

[c] Department of Clinical Laboratory and Biomedical Sciences, Osaka University Graduate School of Medicine, Osaka, Japan

[*]**Corresponding author:** Takeshi Yamada, Department of Medical Technology, Faculty of Health Sciences, Ehime Prefectural University of Health Sciences, 543, Takoda, Tobe-cho, Iyo-gun, Ehime 791-2101, Japan

E-mail: tyamada@epu.ac.jp;

TEL: +81-89-958-2111, FAX: +81-89-958-2177

Abstract

$CD4^+$ T cells play a key role in the immune response via their differentiation into various helper T cell subsets that produce characteristic cytokines. Epigenetic changes in $CD4^+$ T cells are responsible for cytokine production in these subsets, although the exact molecular mechanisms remain unclear. Therefore, we investigated the effects of plant homeodomain finger protein 2 (PHF2), a histone H3K9 demethylase, on cytokine production in $CD4^+$ T cells using T cell-specific *Phf2*-conditional knockout (cKO) mice in this study. we showed that interleukin 4 (*Il4)* expression was significantly decreased in *Phf2*-cKO $CD4^+$ T cells compared to that in wild-type cells. To further elucidate the role of PHF2 *in vivo*, we assessed immune responses in a mouse model of ovalbumin (OVA)-induced atopic dermatitis. *Phf2*-cKO mice exhibited lower serum levels of OVA-specific IgE than those in wild-type mice. These findings suggest that PHF2 plays a role in promoting T helper 2 cell (Th2) function and may contribute to the pathogenesis of Th2-related allergies such as atopic dermatitis. This study demonstrated the impact of PHF2 on cytokine production in $CD4^+$ T cells for the first time. Further studies on the PHF2-mediated epigenetic mechanisms may lead to the development of treatments for a variety of immune diseases.

Keywords

Plant homeodomain finger protein 2, $CD4^+$ T cells, Histone methylation, T helper 2 cells, Interleukin 4, Interferon-gamma

1. INTRODUCTION

$CD4^+$ T cells play a key role in the immune system by responding to antigen presentation and differentiating into various helper T cell subsets. For example, T helper 1 (Th1) cells contribute to the development of antiviral and antimicrobial immunity by producing cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNFα) [1]. Th1 cells activate cytotoxic T cells and macrophages to enhance the immune response against intracellular pathogens [1]. In contrast, T helper 2 (Th2) cells produce cytokines such as interleukin (IL)-4, IL-5, and IL-13, which activate B cells to enhance the humoral immune response against extracellular pathogens such as parasites [1, 2]. IFN-γ and IL-4 inhibit the production of each other, thereby maintaining the Th1/Th2 balance [1]. In addition, T helper 17 (Th17) cells, particularly those in the mucous membrane, enhance immune responses against bacterial infections by secreting IL-17 and IL-21 [3]. These cytokines promote inflammation and activate T and natural killer cells [3]. Although

$CD4^+$ T cells contribute to immune defense, they are also involved in the development of allergies and autoimmune diseases owing to dysregulation of the immune system [4].

Activation of $CD4^+$ T cells leads to epigenetic changes, such as histone methylation via intracellular signaling, and changes to the expression of transcription factors, cytokines, receptors, and other genes [5, 6]. Histone methylation, which is catalyzed by various enzymes to amino acids on histone N-tails, has been reported to affect T cell differentiation and function. Dot1L, an H3 lysine 79 methyltransferase, and the menin/trithorax group complex, an H3K4 methyltransferase, regulate Th1/Th2 differentiation, whereas UTX, an H3K27 demethyltransferase, regulates Th17 differentiation [2, 7]. In addition, the methylation state of H3K9 in cytokine genes shifts significantly before and after $CD4^+$ T cell differentiation, thereby suggesting that H3K9 methylation is involved in $CD4^+$ T cell differentiation and function [8]. G9a, an H3K9 methyltransferase, promotes Th2 cytokine expression in the absence of Th2 transcription factors [8]. Suv39h1, another H3K9 methyltransferase, does not affect Th1/Th2 differentiation but suppresses the plasticity of Th2 to switch to Th1 phenotypes [9]. However, the mechanisms underlying H3K9 demethylation during $CD4^+$ T cell differentiation and function remain unclear.

Plant homeodomain finger protein 2 (PHF2) belong to the Jumonji-C

superfamily. It consists of an N-terminal plant homeodomain (PHD) and a Jumonji-C (JmjC) domain [10]. The PHD domain recognizes dimethylated lysine residues, and the JmjC domain catalyzes the demethylation of lysine residues using Fe^{2+} and α-ketoglutarate as cofactors [10]. *PHF2* is downregulated in malignant tumors, such as squamous cell carcinoma, and it suppresses cancer progression by targeting the *p53* gene, which is a tumor suppressor gene [11]. PHF2 is also essential for the proliferation of neural progenitor cells [12] and affects adipogenesis by targeting CCAAT/enhancer-binding protein alpha [13]. However, the role of PHF2 in immune cells remains unclear. Therefore, we aimed to determine the effect of PHF2 on cytokine expression in $CD4^+$ T cells.

2. MATERIAL AND METHODS

2.1. Experimental animals

C57BL/6 $Thy1.2^+$ mice and *Cd4*-Cre transgenic mice obtained from the Jackson laboratory (Bar Harbor, ME, USA) were used in the study. *Phf2*$^{flox/flox}$ transgenic mice [13] were provided by Dr. Y. Imai of Ehime University. We generated T cell-specific *Phf2*-conditional knockout mice (*Phf2*-cKO; *Cd4*-Cre *Phf2*$^{flox/flox}$) by crossing *Phf2*$^{flox/flox}$ and *Cd4*-Cre mice. All animal experiments were performed in accordance with the guidelines

of the Animal Care and Use Committee of the Ehime Prefectural University of Health Sciences.

2.2. $CD4^+$ T cell isolation and western blotting

Spleens were harvested from the mice, and $CD4^+$ T cells were isolated from the spleens using the MojoSort Isolation Kit (BioLegend, San Diego, CA, USA) following the manufacturer's instructions. $CD4^+$ T cells were activated in culture for 3 d. Activated $CD4^+$ T cells were lysed in EzApply (ATTO, Tokyo, Japan) at 95°C for 5 min, sonicated, centrifuged at 15,000 ×g for 10 min, and the supernatants were then collected. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (ATTO). After blocking with EzBlock Chemi (ATTO) for 30 min at room temperature (23–25° C), the blots were incubated with antibodies against PHF2 (Cell Signaling Technology, Danvers, MA, USA) and β-actin (BioLegend). The immunocomplexes were visualized using Ez-Capture Mg (ATTO) with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG antibody; BioLegend) and ImmunoStar Zeta (FUJIFILM WAKO Pure Chemical Industries, Osaka, Japan).

2.3. Cell culture and RNA analysis

Naive $CD4^+$ T cells were purified from the spleens of wild type (WT; n = 4) and *Phf2*-cKO (n = 4) mice and then stimulated with immobilized anti-CD3ε monoclonal antibodies (10 μg/ml, clone 145-2C11; Biolegend) and anti-CD28 mAb (1 μg/ml, clone 37.51; BioLegend). Cells were cultured in RPMI 1640 (FUJIFILM WAKO Pure Chemical Industries) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), IL-2 (10 ng/ml; FUJIFILM WAKO Pure Chemical Industries), 2 mM L-glutamine (FUJIFILM WAKO Pure Chemical Industries), 1 mM sodium pyruvate (FUJIFILM WAKO Pure Chemical Industries), 1% minimum essential medium nonessential amino acid solution (FUJIFILM WAKO Pure Chemical Industries), 10 mM HEPES (FUJIFILM WAKO Pure Chemical Industries), 55 μM 2-mercaptoethanol (Thermo Fisher Scientific), and 1% penicillin–streptomycin (FUJIFILM WAKO Pure Chemical Industries). Total RNA was extracted from activated $CD4^+$ T cells after 2 d of culturing using RNAiso Plus (Takara Bio Inc., Shiga, Japan), and cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative polymerase chain reaction (qPCR) was performed using TaqMan Gene Expression Assays (Thermo Fisher Scientific), Thunderbird Probe qPCR Mix (TOYOBO, Osaka, Japan) and Quant Studio 1 (Thermo Fisher Scientific). Probes for

hypoxanthine-guanine phosphoribosyl transferase (*Hprt*), *Ifng*, *Tnf*, *Il4*, and *Il17a* were obtained from Applied Biosystems (Catalog No. Mm01324427_m1, Mm01168134_m1, Mm00443258_m1, Mm00445259_m1, and Mm00439618_m1, respectively). Gene expressions was normalized to the expression level of *Hprt*.

2.4. Induction of atopic dermatitis and immune response assessment

Atopic dermatitis was induced in 6- to 8-week-old WT and *Phf2*-cKO mice (n = 20/genotype, female) via epicutaneous exposure to ovalbumin (OVA) as described previously (Figure 2A) [14]. The mice were anesthetized via isoflurane inhalation and their back skin was shaved. The skin was tape-stripped four times to create a standardized skin injury. Gauze patches (1 × 1 cm^2) soaked in different concentrations of OVA (0.05%, 0.1%, and 0.2%) or saline were placed on the back skin (n = 5) and covered using a Tegaderm Transparent Film Roll (3M, Saint Paul, MN, USA). The experiment comprised three one-week exposures, with a two-week interval between each exposure week. Sera were collected after immunization, and OVA-specific IgE levels were quantified via enzyme-linked immunosorbent assays (ELISAs) using the LBIS® mouse anti-OVA-IgE ELISA kit (FUJIFILM WAKO Pure Chemical Industries).

2.5. Statistical analysis

The Student's t-tests were used to evaluate the significance of differences in mRNA expression levels and OVA-specific IgE levels. *P*-values for mRNA expression levels were adjusted using the Bonferroni correction for multiple comparisons. Data were analyzed using JMP17 software (SAS Institute Inc., Tokyo, Japan). Statistical significance was defined as p-values < 0.05.

3. RESULTS

3.1. Deletion of *Phf2* in $CD4^+$ T cells

We generated *Phf2*-cKO mice and performed western blotting to confirm the absence of PHF2 expression in $CD4^+$ T cells. Western blotting showed the absence of PHF2 protein expression in $CD4^+$ T cells obtained from *Phf2*-cKO mice, whereas expression was detected in WT mice (Figure 1A).

Figure 1.

(A) Western blot analysis confirming PHF2 expression. PHF2 protein expression was detected at approximately 150 kDa, while the β-actin protein was detected at approximately 42 kDa. (B) mRNA expression of *Ifng*, *Tnf*, *Il4*, and *Il17a* in $CD4^+$ T cells

from WT (white bars) and *Phf2*-cKO (gray bars) mice. Error bars indicate standard deviation. *** indicates $p < 0.001$

PHF2, plant homeodomain finger protein 2; WT, $CD4^+$ T cell extracts from wild-type mice; cKO, *Phf2*-conditional knockout $CD4^+$ T cell extracts; *Ifng*, interferon-gamma; *Tnf*, tumor necrosis factor alpha; *Il4*, interleukin 4; *Il17a,* interleukin 17 alpha.

3.2. Regulatory role of PHF2 in cytokine production

To investigate the impact of PHF2 on cytokine gene expression, we cultured $CD4^+$ T cells extracted from *Phf2*-cKO and WT mice and measured the mRNA expression of these cells. mRNA expression analysis revealed that the expression level of *Il4* in *Phf2*-cKO $CD4^+$ T cells was significantly lower than that in WT $CD4^+$ T cells ($p < 0.001$). However, mRNA expression levels of *Ifng*, *Tnf*, and *Il17a* did not differ significantly between *Phf2*-cKO and WT $CD4^+$ T cells (Figure 1B).

3.3. Allergic immune responses

To investigate the effect of PHF2 to immune response *in vivo*, we generated a mouse model of atopic dermatitis in *Phf2*-cKO and WT mice (Figure 2A) and measured serum levels of OVA-specific IgE in these mice. Serum levels of OVA-specific IgE were

significantly lower in *Phf2*-cKO mice immunized with 0.2% OVA than those in WT mice (p = 0.008; Figure 2B). No significant differences were observed in IgE levels of mice immunized with OVA at concentrations <0.2%.

Figure 2.

(A) Atopic dermatitis induction protocol. (B) The serum levels of OVA-specific IgE are shown for OVA sensitization concentrations (0%, 0.05%, 0.1%, and 0.2%) in WT (white bars) and *Phf2*-cKO (gray bars) mice. Error bars indicate standard errors. ** indicates p < 0.01

OVA, ovalbumin; *Phf2*, plant homeodomain finger protein 2; WT, $CD4^+$ wild type; cKO, *Phf2*-conditional knockout.

4. DISCUSSION

In this study, we showed that *Phf2*-deficient activated $CD4^+$ T cells exhibited decreased *Il4* expression, which suggests that PHF2 can promote Th2 function ($p > 0.001$, Figure 1B). Changes in *Ifng* expression were not significant; however, it was higher in *Phf2*-deficient activated $CD4^+$ T cells ($p = 0.065$; Figure 1B). This suggests the possibility that *Il4* upregulation might have contributed to the suppression of IFN-γ. The impact of

PHF2 on Th1 differentiation may be limited because there was no difference in *Tnf* expression, despite an increase in *Ifng* expression. However, PHF2 may not affect Th17 function, because there was no significant change in *Il17* expression in *Phf2*-deficient activated $CD4^+$ T cells (Figure 1B). Thus, PHF2 may be directly or indirectly involved in the hypomethylation of *Il4*. Previous studies have shown that H3K9 methyltransferases (G9a and Suv39h1) are involved in Th1/Th2 function [8, 9]. G9a promotes Th2 cytokine production as a transcription factor independent of H3K9 methylation [8]. While Suv39h1 is not involved in Th1/Th2 differentiation, it plays a key role in inhibiting the conversion of Th2 cells to Th1 cells [9]. Thus, the molecular mechanisms involved in H3K9 methylation are diverse, and further investigation of the PHF2 targets is necessary to elucidate the mechanism underlying the regulation of *Il4* expression by PHF2.

IL-4 is a Th2 cytokine that exacerbates allergic diseases by promoting IgE production [2]. Mouse models of atopic dermatitis induced via OVA sensitization exhibit acute inflammation due to Th2 responses accompanied by an increase in OVA-specific IgE levels [15]. Therefore, we evaluated OVA-specific IgE levels in mice as an indicator of pathological conditions. We found that OVA-specific IgE expression was reduced in *Phf2*-cKO atopic dermatitis-induced mice compared to that in WT mice (Figure 2B), indicating that PHF2 enhances IgE production by promoting IL-4 production in atopic

dermatitis. Our findings also suggest that targeting PHF2 can be used to treat Th2-driven allergies such as atopic dermatitis.

In this study, cell culture experiments revealed that PHF2 regulated *Il4* expression in $CD4^+$ T cells. However, because T cell-specific *Phf2*-cKO mice were used in mouse experiments, the results may have reflected the effect of PHF2 in not only $CD4^+$ but also other T cells. Therefore, it will be necessary to generate $CD4^+$ T cell-specific *Phf2*-cKO mice to exclusively investigate the function of PHF2 in $CD4^+$ T cells *in vivo*.

In conclusion, this study revealed for the first time the potential role of PHF2 in the regulation of IL-4 production in $CD4^+$ T cells. This study is also the first report to explore the function of PHF2 in lymphocytes and suggests that PHF2 is an important regulator in Th1/Th2 differentiation mechanism through H3K9 methylation regulation. Moreover, our findings suggest that PHF2 may function as an exacerbating factor in Th2-mediated allergic diseases.

CRediT authorship contribution statement

Yuya Arakawa: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review and editing, Visualization, Project administration, Funding acquisition. **Yuzuki Tano:** Formal analysis and Investigation.

Moe Fujii: Formal analysis and Investigation. **Yuuki Imai:** Methodology, Resources, Writing – review and editing. **Yoshiaki Norimatsu:** Writing – review and editing, Supervision. **Masaki Yasukawa:** Writing – review and editing, Supervision. **Mikio Watanabe:** Writing – review and editing, Supervision. **Takeshi Yamada:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – review and editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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