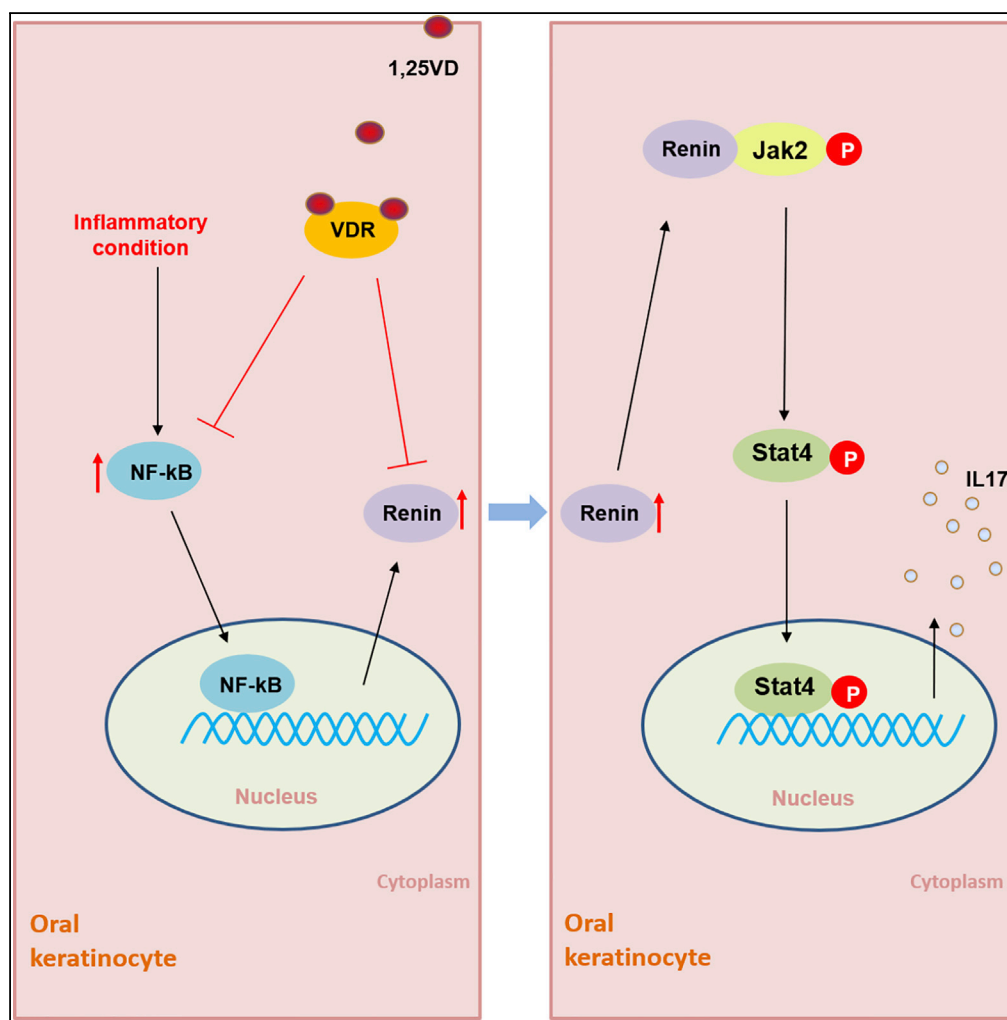


Article

Renin Promotes STAT4 Phosphorylation to Induce IL-17 Production in Keratinocytes of Oral Lichen Planus



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HIGHLIGHTS

NF-κB pathway induces renin expression in oral lichen planus

Renin upregulation promotes IL-17 expression in oral keratinocytes

Renin regulates IL-17 by promoting STAT4 phosphorylation in oral keratinocytes



Article

Renin Promotes STAT4 Phosphorylation to Induce IL-17 Production in Keratinocytes of Oral Lichen Planus

Xuejun Ge,^{1,7} Hanting Xie,^{2,7} Tivoli Nguyen,^{3,7} Bin Zhao,^{4,5,6} Jing Xu,² and Jie Du^{4,6,8,*}**SUMMARY**

Interleukin-17 (IL-17) is highly expressed in the epithelial layer of oral lichen planus (OLP), but the underlying mechanism for IL-17 overexpression remains unknown. Here, we identify renin that is induced by NF- κ B pathway contributes to the increase of IL-17 in human oral keratinocytes (HOKs). We describe that the release of cellular renin leads to the phosphorylation of Janus kinase 2 (JAK2) protein. The phosphorylated JAK2 recruits and activates the signal transducer and activator of transcription 4 (STAT4) by phosphorylating STAT4's tyrosine residue 693 (Tyr693). The now-activated STAT4 translocates into nucleus and binds to the promoter region of IL-17 gene in HOKs. Genetic interference of renin restores IL-17 levels in OLP cell models. Collectively, our results reveal that renin upregulates IL-17 expression by enhancing STAT4 phosphorylation. This discovery unveils an underpinning by which IL-17 is increased in oral keratinocytes and provides potential targeted therapies for OLP patients.

INTRODUCTION

Oral lichen planus (OLP) is a chronic relapsing inflammatory disorder affecting the mucous membranes of gums, buccal mucosa, palate, and tongue (Cheng et al., 2016). Crucial in the initiation and development of this disease is T-lymphocyte infiltration, an event that triggers inflammatory responses of both the lamina propria and the epithelial layer and leads to keratinocyte apoptosis in oral epithelium (Lavanya et al., 2011). Although numerous studies have suggested that OLP is an autoimmune response-driven disease in a common consensus, the exact pathogenesis remains unclear. To date, multiple pathogenic factors, such as autoimmune response, mental stress, infection, and hypersensitivity, have been found to contribute to OLP onset (Cheng et al., 2016). There are six recognized subtypes of clinical expression for OLP: erosive, plaque, papular, reticular, atrophic, and bullous (Cheng et al., 2016; Gorouhi et al., 2014). Patients who are smokers and alcoholics take more risks for malignant transformation (Aghbari et al., 2017). Because OLP is refractory in clinic, much attention has been given to symptomatic improvement (Cheng et al., 2016). At present, topical and systemic immunosuppressants are typically administered to relieve clinical symptoms, but these leave significant side effects upon long-term management (Cheng et al., 2016; Crincoli et al., 2011; De Rossi and Ciarrocca, 2014). Thus, explorations regarding the pathogenies of OLP and curative treatments of it are required.

T-helper (Th) 17, a CD4 T cell subset, has been described to play a prominent role in allergic reactions, autoimmune development, and host defense by generating IL-17, a type of pro-inflammatory cytokine (Bettelli et al., 2006). IL-17 can activate diverse cells such as epithelial cell, fibroblast, and chondrocyte to produce powerful inflammatory molecules (Gaffen, 2008; Onishi and Gaffen, 2010). Exogenous IL-17 in oral keratinocytes has been reported to induce CCL-20, IL-8, and TNF- α production (Lu et al., 2014). Previous studies have stated that, compared with unaffected controls, increased levels of IL-17 mRNA and protein are detected in OLP (Lu et al., 2014), indicating IL-17 may have a critical role in OLP disease. Interestingly, serum IL-17 concentrations in erosive OLP patients are higher than those in the non-erosive subtype (Pouralibaba et al., 2013), suggesting a positive correlation between IL-17 levels and OLP severity.

Renin is a key component of the renin-angiotensin system (RAS), which is broadly recognized to mediate blood pressure and vascular resistance (Re, 2004). After being synthesized in kidney, renin is secreted into the circulation in which angiotensinogen is cleaved to angiotensin I, which is converted to angiotensin II further by angiotensin-converting enzyme to exert biological and physiological functions (He et al., 2019; Sparks et al., 2014). In addition to playing a known role in circulatory regulation, RAS has been emphasized

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to result in autoimmune disorders recently by some studies that suggest RAS facilitates colitis through activating Th17 cells, and renin expression is significantly increased in inflamed colonic tissues (He et al., 2019). Moreover, RAS suppression limits IL-17 production in lung diseases (Weber et al., 2012), implying renin may be involved in regulating IL-17 expression. Given the important roles of IL-17 in OLP and the relationship between RAS and IL-17, in this study, we explored renin as well as IL-17 in the context of OLP and found that the overexpression of renin enhances IL-17 production by phosphorylating signal transducer of activated transcription 4 (STAT4) in oral keratinocytes of OLP.

RESULTS

Renin Levels Are Upregulated in the Field of OLP

Because renin is reported to be involved in autoimmune diseases (He et al., 2019), we collected oral biopsies from patients with OLP and healthy individuals, sampled from clinically lesion and unaffected mucosa, to test renin expression in these tissues. As shown in Figure 1, mRNA and protein levels of renin showed increases of more than 3-fold in the epithelial layer of specimens derived from OLP in comparison to healthy controls (Figures 1A–1C). Our immunohistochemistry staining data confirmed the upregulation of renin in OLP and indicated that the overexpressed renin is localized in the cytoplasm of oral keratinocyte rather than in nucleus (Figure 1D). Furthermore, the enhanced expression of renin in the lamina propria of OLP was detected as well (Figures S1A and S1B). To gain a better understanding of the molecular mechanism of OLP development, we employed LPS and the culture medium of activated CD4⁺ T cells to establish OLP cell models as described in previous investigation (Zhao et al., 2019). Consistent with our hypothesis, renin mRNA and protein levels were elevated by LPS or activated CD4⁺ T cell stimulation in a time-dependent manner (Figures 1E–1H). These results demonstrate that renin is induced under OLP circumstances.

The Increase of Renin Is Regulated by NF- κ B Pathway in Oral Keratinocytes

The mechanism of renin's upregulation under inflammatory condition is still not well explained. To explore this, we examined human *renin* gene promoter and found a putative κ B motif (Figure 2A). The NF- κ B pathway, which plays a critical mediatory role in OLP, was found to be robustly activated in diseased tissues and cell models (Figures 2B, 2C, S2A, and S2B). We next designed primers flanking the κ B-binding site and performed ChIP assays in HOKs transfected with IKK β plasmid reportedly being an activator for NF- κ B pathway (Chen et al., 2013). As shown in Figure 2, the binding of p65 to the κ B site was accelerated by IKK β plasmid transfection (Figure 2D), stating NF- κ B pathway most likely regulates renin transcription. Next, we performed ChIP assays in both human biopsies and OLP cell models and these results coincide with previous findings (Figures S2C–S2E). To determine whether the activated NF- κ B pathway strengthens renin expression in HOKs, we transfected IKK β plasmids into this cell line and found that renin levels were increased in the presence of IKK β overexpression in a dose dependent-manner (Figures 2E and 2F). In the loss-of-function assays, we silenced the p65 gene using siRNAs in order to suppress NF- κ B pathway as described previously (Wu et al., 2017) (Figure 2G). As shown, inhibition of NF- κ B pathway blocked the enhanced renin expression induced by LPS or activated CD4⁺ T cells (Figures 2H–2K). Bay 11-7082 compound, an NF- κ B pathway inhibitor, also decreased renin levels in OLP cell models (Figures S2F and S2G).

Renin Mediates IL-17 Expression in HOKs

Some studies have reported renin to be closely related with IL-17 expression (He et al., 2019). To explore this, we first examined IL-17 status in the context of OLP. As expected, compared with healthy controls, mRNA and protein levels of IL-17 in oral epithelia and secretion of IL-17 in serum were increased for OLP patients (Figures 3A–3C). Renin and IL-17 levels of participants showed good positive correlations (Figures 3D and 3E). Accordingly, IL-17 expression was elevated in the OLP cell models in a time-course-dependent manner (Figures S3A–S3D). Does renin regulate IL-17 expression in HOKs? To answer this question, we transfected human renin plasmids into HOKs (Figure 3F). As manifested, IL-17 was induced in a dose-dependent fashion after transfection (Figures 3G and 3H). IL-17 expression was also enhanced after recombinant renin treatment in HOKs (Figure S3E). In agreement, the expression of IL-17 in oral epithelia from renin transgene mice was considerably increased (Figures 3I–3K). IKK β overexpression was also shown to stimulate IL-17 transcription and production in a dose-dependent way (Figures S3F and S3G).

For *renin* gene knockdown, we transfected renin-siRNAs into HOKs (Figure 4A). Our data suggested that LPS or activated CD4⁺ T cells failed to induce IL-17 expression in the knockdown of *renin* in HOKs (Figures

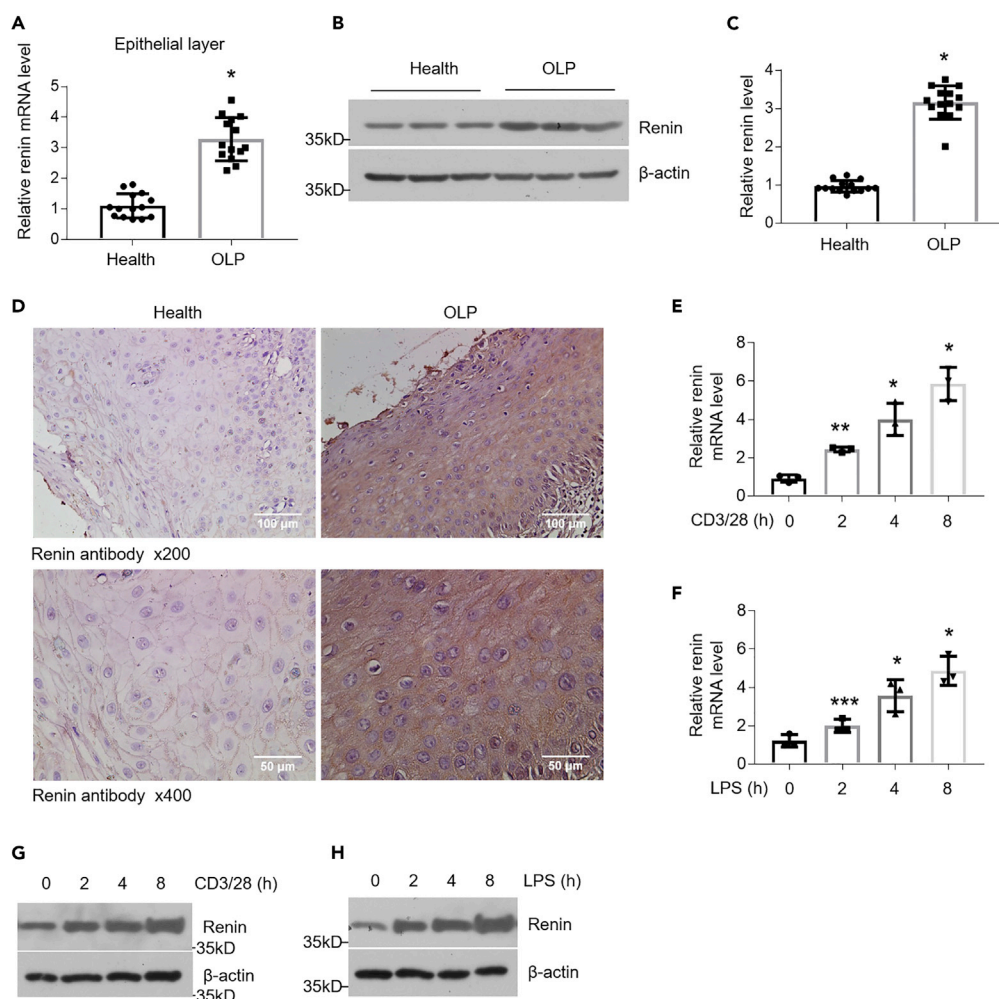


Figure 1. Renin Expression Is Upregulated in the Keratinocytes of OLP

(A) Real-time PCR quantification of renin in the oral mucosal epithelial layer, $n = 14$ each group.

(B and C) Western blot analysis (B) and densitometric quantitation (C) of renin in epithelial layer of oral mucosa, $n = 14$ each group.

(D) Renin immunostaining in the oral tissues of healthy controls and OLP patients.

(E and F) Real-time PCR quantification of renin in HOKs challenged by anti-CD3/28-activated $CD4^+$ T cells (E) or 100 ng/mL LPS (F) for 0, 2, 4, 8 h, $n = 3$.

(G and H) Western blot determination of renin in HOKs challenged by anti-CD3/28-activated $CD4^+$ T cells (G) or 100 ng/mL LPS (H) for different time points as shown, $n = 3$.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus corresponding control. Data were shown as means \pm SD. Two-tailed Student's t test was used.

4B–4E). Importantly, p65-siRNA transfection and Bay 11-7082 treatment both compromised IL-17 increases in OLP cell models (Figures 4F–4I and S4A–S4D), indicating that inhibition of the upstream signaling of renin may regulate IL-17 productions.

Renin Upregulates IL-17 Expression via Phosphorylating STAT4

In searching for the mechanism by which renin regulates IL-17 productions, we analyzed the human *IL-17* gene promoter and found STAT4 motif (Figure S5A). ChIP data showed that renin plasmid transfection enhanced the binding action between STAT4 and its motif in the *IL-17* promoter region (Figure S5B), implying renin mediates IL-17 transcription through activating STAT4. How does renin influence STAT4 activity? To answer it, we carried out a Co-IP assay using renin and STAT4 plasmids but observed no protein interaction (Figure S5C). The Janus kinase (JAK)/STAT signaling pathway is well known to transduce the

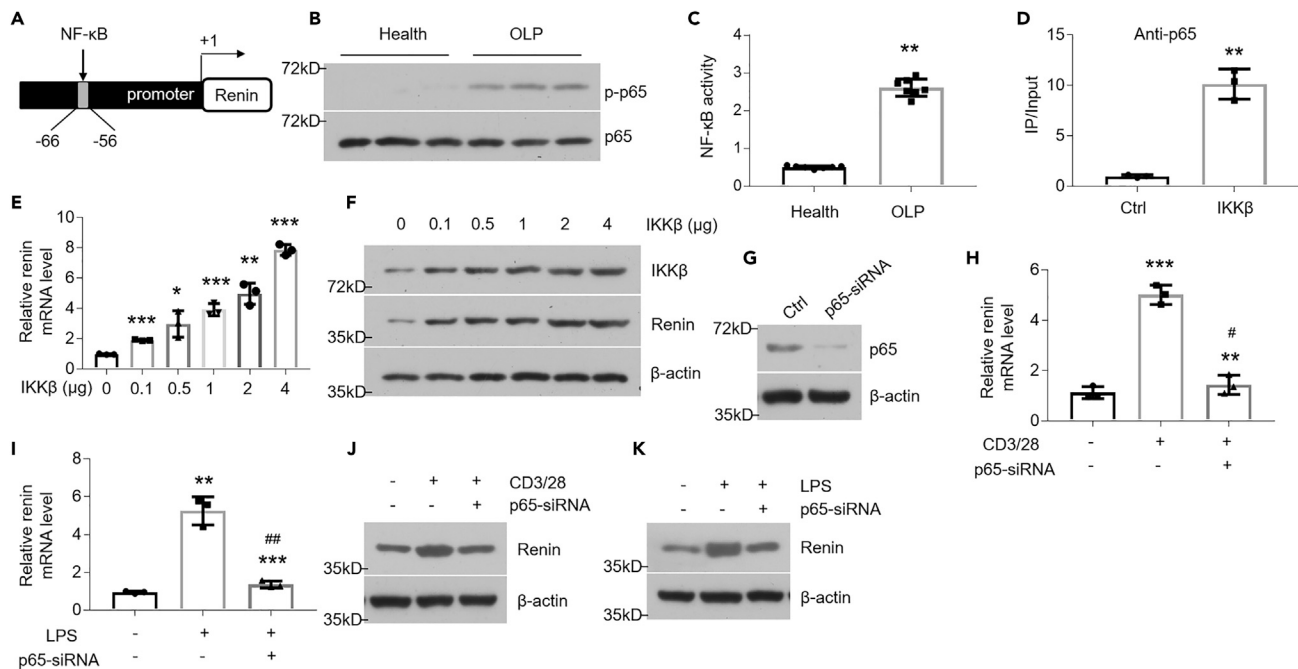


Figure 2. Renin Is Induced by NF- κ B Pathway

(A) Schematic illustration of κ B motif in the promoter of *renin* gene.

(B) Western blot analysis of phosphorylated NF- κ B p65 in the epithelial layer of human samples, $n = 14$.

(C) NF- κ B activity in the epithelial layer of human samples, $n = 7$.

(D) IKK β plasmids transfection in HOKs helps NF- κ B bind to the promoter of endogenous *renin*. Chromatin immunoprecipitation (ChIP) was performed using anti-p65 antibody.

(E and F) Real-time PCR (E) and Western blot (F) analyses of renin in HOKs transfected with IKK β plasmids.

(G) Efficacy of siRNA-mediated knockdown on p65 expression was detected by Western blot in HOKs.

(H–K) Quantitative PCR (H and I) and Western blot (J and K) analyses of renin levels in HOKs, which were treated with activated CD4⁺ T cells or 100 ng/mL LPS for 8 h, following 36-h scramble- or p65-siRNA transfection.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus corresponding control; # $p < 0.05$, ## $p < 0.01$ versus LPS or CD3/28 group, $n = 3$. Ctrl, control. Data were shown as means \pm SD. Two-tailed Student's t test and one-way analysis of variance were used.

activity of cellular signals. Upon binding with signals, JAK proteins are phosphorylated on their tyrosine residues, resulting in recruitment and tyrosine-phosphorylation of STAT proteins (Aaronsen and Horvath, 2002; Brooks et al., 2014). To explore this phenomenon, we tested the activities of three types of JAK proteins (JAK1–3) in renin-transfected HOKs. Among these, we found JAK2 to be the only protein phosphorylated by renin (Figure 5A). STAT4 is phosphorylated on tyrosine residue 693 (Tyler et al., 2007). Consistently, STAT4 phosphorylation on Tyr693 was significantly increased in HOKs overexpressing renin (Figure 5B). The roles of renin in JAK2 and STAT4 were also validated using a dose-dependent experiment (Figure S5D). Co-IP assays confirmed the interaction between renin and JAK2 (Figure 5C), uncovering the underpinning whereby renin regulates JAK2 phosphorylation.

We then transfected STAT4 plasmids into HOKs and found STAT4 overexpression has no effect on IL-17 transcription and expression (Figures S5E–S5G), indicating that the overexpression, itself, of STAT4 is not sufficient to induce IL-17 expression and that phosphorylation may be a key event that allows for STAT4's induction of IL-17 expression. To validate it, we confirmed the translocation of phosphorylated STAT4 from cytoplasm into nucleus following renin plasmid transfection (Figure 5D) and detected the increase of phosphorylated STAT4 in the nucleus of oral keratinocytes of diseased biopsies and in the oral epithelial layer of renin-Tg mice (Figures S5H and S5I). In addition, wild-type STAT4 was able to bind efficiently to the biotin-labeled STAT4-binding element (SBE) with renin overexpression, but not Y693F mutant (Figure 5E). Next, we deleted the endogenous *STAT4* gene in HOKs using CRISPR/Cas 9 system (Figure S5J), followed by establishment of cell lines stably expressing wild-type STAT4 or its Y693F mutant (Figure S5K). These results demonstrated that renin phosphorylated Tyr693 on STAT4, which facilitated occupancy of STAT4 on the promoter of *IL-17* in HOKs that express wild-type STAT4 rather than mutant Y693F

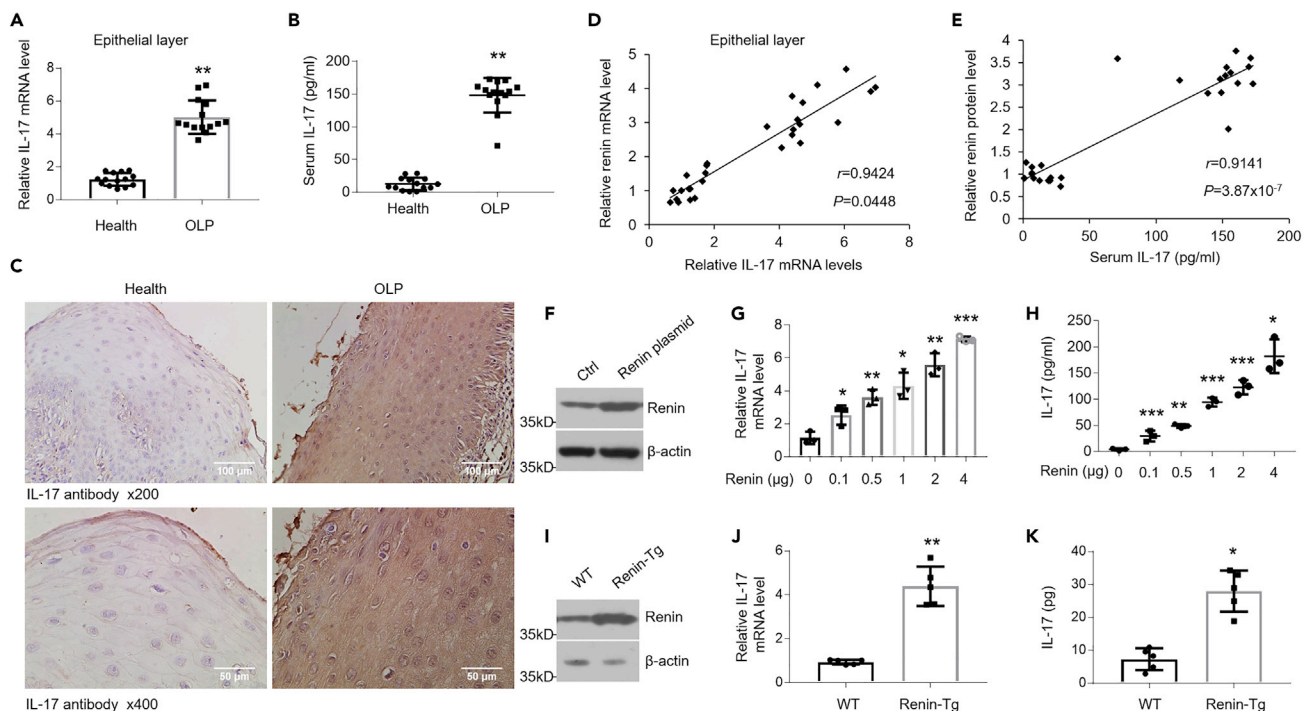


Figure 3. Renin Upregulates IL-17 Expression in Oral Keratinocytes

(A) Real-time PCR quantification of IL-17 mRNA levels in oral keratinocytes from human specimens, $n = 14$.
 (B) ELISA measurement of IL-17 concentrations in serum from healthy individuals or OLP patients, $n = 14$.
 (C) IL-17 immunostaining in the oral tissues of healthy controls and OLP patients.
 (D) Correlation of fold change between renin mRNA levels and IL-17 mRNA status in oral epitheliums of human biopsies.
 (E) Correlation between renin protein levels in the human oral epithelial layer and IL-17 concentrations in serum of participants.
 (F) Western blot analysis of renin protein levels in HOKs transfected with empty or renin plasmids, $n = 3$.
 (G) Real-time PCR quantification of IL-17 mRNA levels in HOKs after renin transfection with different doses as shown, $n = 3$.
 (H) ELISA detection of IL-17 productions in the culture medium of HOKs after renin transfection with different doses as shown, $n = 3$.
 (I and J) Western blot (I) of renin expression or quantitative PCR test (J) of IL-17 mRNA levels in oral keratinocytes obtained from wild-type or renin-transgene mice, $n = 5$.
 (K) ELISA examination of IL-17 expression in oral keratinocytes obtained from wild-type or renin-transgene mice, $n = 5$.
 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus corresponding control. Ctrl, control; WT, wild type. Data were shown as means \pm SD. Two-tailed Student's t test was used.

(Figures 5F and 5G). Tyrosine Kinase Inhibitor (TKI) acting as a tyrosine phosphorylation suppressor blocked renin-induced IL-17 increases (Figures 5H and 5I). Together, these findings support the hypothesis that renin phosphorylates STAT4 on Tyr693 to improve IL-17 transcripts.

Vitamin D/VDR Signaling Blunts Renin and IL-17 Increases in the OLP Cell Models

Vitamin D/VDR signaling, which is reported to inhibit cytokine productions and apoptosis of oral keratinocytes, is demonstrated to play a suppressive role in renin expression and RAS (Ge et al., 2019; Li, 2003; Yuan et al., 2007; Zhao et al., 2019). To explain the effects of vitamin D/VDR signaling on renin and IL-17 productions in the context of OLP, we administered different doses of vitamin D and VDR plasmids to HOKs. As shown, both vitamin D and VDR ameliorated renin-induced IL-17 overexpression in HOKs (Figures 6A, 6B, S6A, and S6B). Moreover, the vitamin D-VDR signaling is capable of impeding renin and IL-17 increases in OLP cell models (Figures 6C–6F and S6C–S6J). Together, these data indicate that vitamin D/VDR signaling suppresses renin and IL-17 expression in OLP.

DISCUSSION

IL-17 has been reported to play potential pathogenic roles in autoimmune disease development, including oral lichen planus (He et al., 2019; Lu et al., 2014). Increased levels of IL-17 have been observed in the biopsies and serum from OLP patients (Lu et al., 2014; Pouralibaba et al., 2013). Some recent studies suggest that renin is

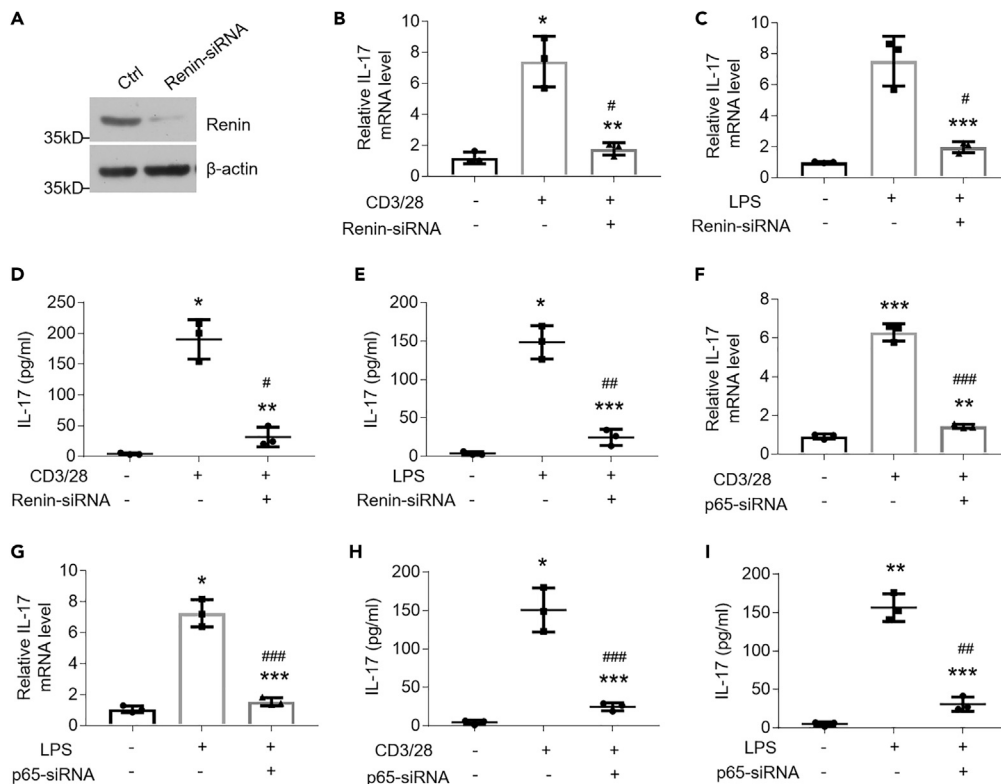


Figure 4. Interference of Renin Prohibits IL-17 Productions in OLP Cell Models

(A) Efficacy of siRNA-mediated knockdown on renin expression was detected by Western blot in HOKs.

(B and C) Real-time PCR quantification of IL-17 mRNA levels in HOKs stimulated with activated CD4⁺ T cells (B) or 100 ng/mL LPS (C) for 8 h, following 36-h scramble- or renin-siRNA pretreatment.

(D and E) ELISA detection of IL-17 secretions in the culture medium from HOKs stimulated with activated CD4⁺ T cells (D) or 100 ng/mL LPS (E) for 8 h, following 36-h scramble- or renin-siRNA pretreatment.

(F–I) HOKs were transfected with scramble- or p65-siRNA for 36 h, followed by 8-h activated CD4⁺ T cells or 100 ng/mL LPS treatment. Real-time PCR (F and G) and ELISA (H and I) tests were performed.

p* < 0.05, *p* < 0.01, ****p* < 0.001 vs. corresponding control; #*p* < 0.05, ###*p* < 0.01, ####*p* < 0.001 versus LPS or CD3/28 group, *n* = 3. Ctrl, control. Data were shown as means ± SD. Two-tailed Student's *t* test and one-way analysis of variance were used.

robustly elevated in the microenvironment of inflamed tissues, and RAS plays a role in activating Th17 cells and increasing IL-17 production (He et al., 2019; Platten et al., 2009). We examined the roles of renin and IL-17 in OLP and explained the mechanism by which renin upregulates IL-17 in oral keratinocytes.

Renin, an important component of the RAS, has crucial effects on multiple pathophysiological processes (Re, 2004). It is well known that renin exerts its regulatory actions in cardiovascular and renal systems, but the role of local renin in oral keratinocytes remains poorly understood. In this report, we present evidence that renin is dramatically enhanced in the epithelial layer and lamina propria of OLP tissues, a result consistent with other investigations demonstrating renin is induced in colonic inflamed biopsies (He et al., 2019). This finding is confirmed in OLP cell models, where renin is induced by LPS or activated CD4⁺ T cells in a dose-dependent manner. Although previous studies have reported that renin may be secreted from the lymphocytes in the lamina propria, our cell model data suggest that the oral keratinocytes are capable of producing renin in response to stimulation. Because there are no well-established animal models for mimicking OLP, animal studies are not involved here.

Previous studies have suggested that cyclic AMP response induces renin production (Yuan et al., 2007), but cyclic AMP signaling is not activated in OLP (Data not shown). Our finding of the κB motif in the promoter of *renin* gene led us to hypothesize that NF-κB pathway may induce renin production in OLP. Consistent with data from our previous studies (Zhao et al., 2018a, 2018b), we observed that the activated NF-κB pathway in keratinocytes of

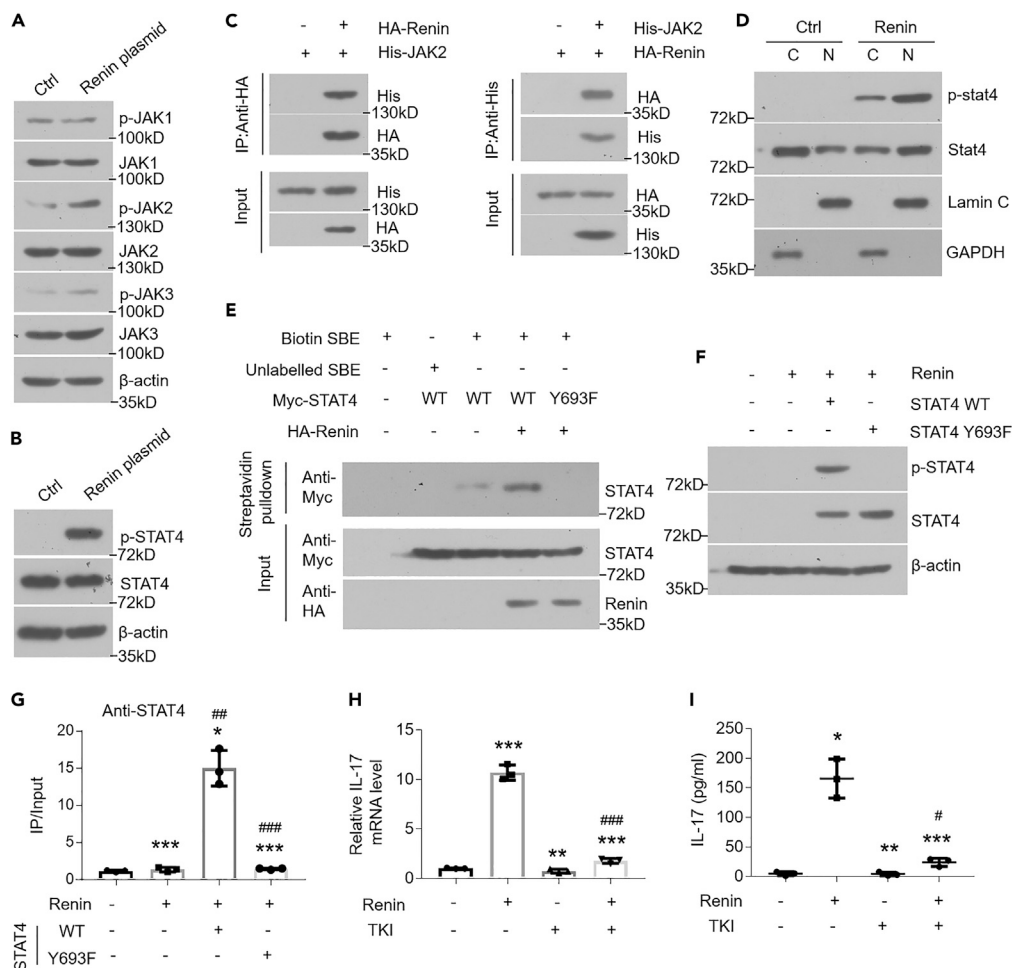


Figure 5. Renin Phosphorylates STAT4 to Induce IL-17 Expression

(A) Western blot analysis of HOKs transfected with empty or renin plasmids, using antibodies as indicated. (B) Western blot analysis of STAT4 and phospho-STAT4 in HOKs transfected with empty or renin plasmids. (C) Co-IP and Western blot analyses of cell lysates from HOKs transfected with the indicated plasmids. (D) HOKs were transfected with empty or renin plasmids. Cytoplasmic (C) and nuclear (N) fractions were extracted and analyzed by Western blotting using indicated antibodies. Lamin C is selected to be a nuclear marker, and GAPDH serves as a cytoplasmic marker. (E) Y693F mutation eliminated DNA binding of STAT4 in a pull-down assay. Biotin-labeled SBE probes were added into the transfected HOKs lysates to bind proteins and then pulled down by streptavidin beads. Retrieved proteins were detected by Western blot. Unlabeled SBE probe is chosen for a negative control. (F–I) The endogenous STAT4 in HOKs was deleted by CRISPR/Cas9 system, and different plasmids were transfected into the STAT4-knockout cell line. Western blot was carried out to determine the p-STAT4 and STAT4 levels in transfected HOKs (F). Chromatin immunoprecipitation (ChIP) was performed using anti-STAT4 antibody and showed that Y693F mutation impeded STAT4 binding to the promoter region of *IL-17* gene (G). Real-time PCR (H) or ELISA (I) detection of *IL-17* in HOKs transfected with renin plasmids in the presence or absence of TKI. *p < 0.05, **p < 0.01, ***p < 0.001 versus corresponding control; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. renin group, n = 3. Ctrl, control; WT, wild type; Y693F, Tyr693 mutant; SBE, STAT4 binding element; TKI, tyrosine kinase inhibitor. Data were shown as means \pm SD. Two-tailed Student's t test and one-way analysis of variance were used.

OLP considerably enhances renin production. Similarly, genetic or chemical interference of NF- κ B pathway was associated with decreased renin production. The active NF- κ B pathway is described to be induced by proinflammatory cytokines (such as TNF- α) (Chen et al., 2013). Therefore, in the setting of OLP, bacteria or secretions of CD4⁺ T-cell-induced cytokines may activate NF- κ B signaling pathway. One limitation is that our results do not consider the possibility that renin may be induced directly by other signaling pathways. However, we explained, at least in part, the cause of upregulated renin in OLP in this study.

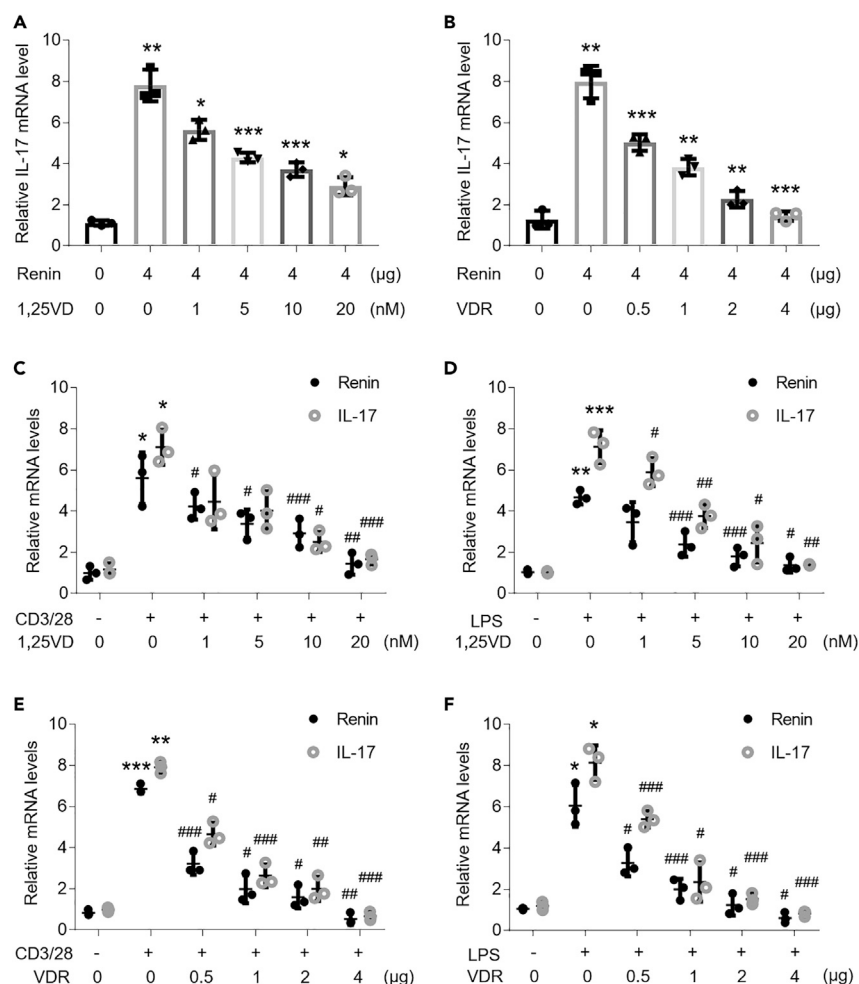


Figure 6. Vitamin D/VDR Signaling Inhibits Renin and IL-17 Transcripts in OLP Cell Models

(A) HOKs were pretreated with different doses of 1,25VD for 12 h, and then challenged by renin plasmids transfection for 36 h

(B) HOKs were co-transfected with VDR and renin plasmids for 36 h.

(C and D) HOKs were pretreated with different doses of 1,25VD for 12 h and then challenged by activated CD4⁺ T cells (C) or 100 ng/mL LPS (D) for 8 h.

(E and F) HOKs were transfected with different doses of VDR plasmids for 36 h, followed by 8-h activated CD4⁺ T cells (E) or 100 ng/mL LPS (F) treatment. Renin and IL-17 transcripts were quantified by real-time PCR.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus corresponding control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus LPS or CD3/28 group, $n = 3$. 1,25VD, 1,25(OH)₂D₃. Data were shown as means \pm SD. Two-tailed Student's *t* test and one-way analysis of variance were used.

We found IL-17 production is dramatically increased in the diseased tissues and serum from patients with OLP, in agreement with other studies (Lu et al., 2014; Pouralibaba et al., 2013). In immune cells, RAS is reported to elevate IL-17 secretions by stimulating mucosal Th17 immunity (He et al., 2019). Inhibition of RAS plays a suppressive role in IL-17 expression in airway diseases (Weber et al., 2012). Similarly, we found renin affects IL-17 productions in the gain-of-function or loss-of-function assays in HOKs. Unlike with other studies (Weber et al., 2012), we did not block RAS with angiotensin II receptor blockers (ARBs) or angiotensin-converting enzyme inhibitors (ACEIs) because we aimed to explore the function of renin in HOKs rather than the whole RAS.

To exploit the mechanism by which renin regulates IL-17, we found SBE in the promoter of *IL-17* gene and confirmed renin takes effect on IL-17 transcripts via STAT4 activation. Furthermore, our data indicate renin interacts with JAK2 to phosphorylate STAT4, which is consistent with the notion that RAS

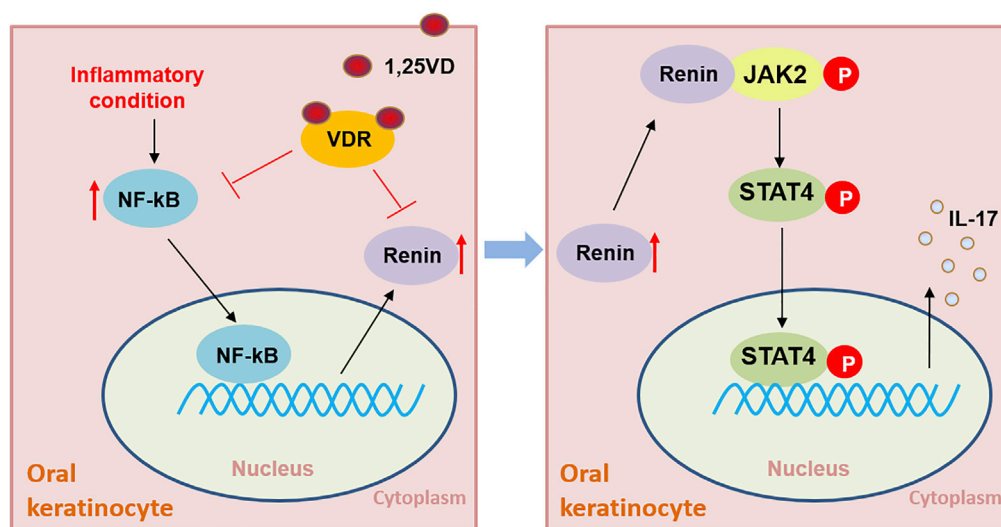


Figure 7. Schematic of Renin's Effects on IL-17 Expression in Oral Keratinocytes

performs its functions in immune cells via JAK2/STAT pathway (He et al., 2019). JAKs phosphorylate STATs on tyrosine residues to transduce cellular signaling (Aaronson and Horvath, 2002). To this end, we assessed STAT4 phosphorylation and found renin phosphorylates tyrosine residue 693 of STAT4 protein. This event helps STAT4 translocate into nucleus to bind to the *IL-17* promoter. It appears that serine residue 721 is another potential site for STAT4 phosphorylation (Morinobu et al., 2002). Therefore, we tested Ser721 phosphorylation by Western blot analysis, wherein we observed overexpression of renin did not lead to the phosphorylation of Ser721 (Data not shown). Moreover, we mutated tyrosine residue 693 and showed STAT4 mutant's regulatory effects on IL-17 transcripts are abolished completely in HOKs with renin plasmids transfection, indicating Tyr693 as the only modification site.

Vitamin D/VDR signaling is proved to suppress both renin and IL-17 levels in OLP in this work, consistent with the point that vitamin D inhibits *renin* gene transcription (Yuan et al., 2007). Because vitamin D/VDR signaling is able to inactivate NF-κB pathway (Chen et al., 2013), they may regulate renin expression via blocking NF-κB pathway in HOKs.

In this report, we present evidence that renin increases STAT4 phosphorylation on Tyr693 to increased IL-17 productions in keratinocytes of OLP (Figure 7). Some studies suggest that recombinant human IL-17 treatment robustly increases matrix metalloproteinases 9 (MMP9) expression, which results in the deterioration of the mucosal extracellular matrix (Wang et al., 2018). Although the role of IL-17 in apoptosis of oral keratinocytes is still unclear, it is reported to induce apoptosis in hepatocyte (Zhao et al., 2018a, 2018b), indicating IL-17 may be related with oral keratinocytes apoptosis. Thus, overexpression of renin and IL-17 in OLP may affect the onset and pathogenesis of this disease by damaging the extracellular matrix and inducing apoptosis of oral keratinocytes. Our results provide insights into the understanding of pathogenesis with respect to OLP and possible new therapeutic targets for clinic management of this disease. However, more investigations are required to guarantee the efficiency of this kind of new treatment.

Limitations of the Study

Because much of the data in this study still rely on overexpression or knockdown in HOK lines, it would be great to perform these experiments in a primary cell system. Moreover, animal models mimicking OLP are required to better explore this kind of disease.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.100983>.

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AUTHOR CONTRIBUTIONS

Du J conceived and designed the research. Ge XJ and Xie HT performed the experiments. Nguyen T analyzed data. Zhao B and Xu J supervised the project. Du J wrote the manuscript. Nguyen T proofread the manuscript. Du J and Ge XJ acquired funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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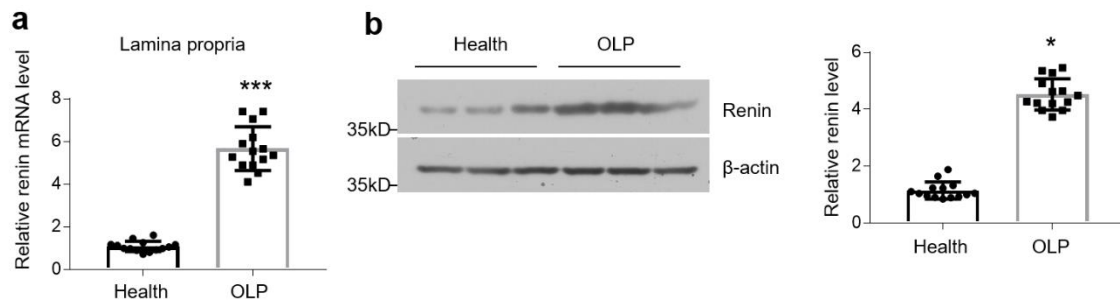
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Supplemental Information

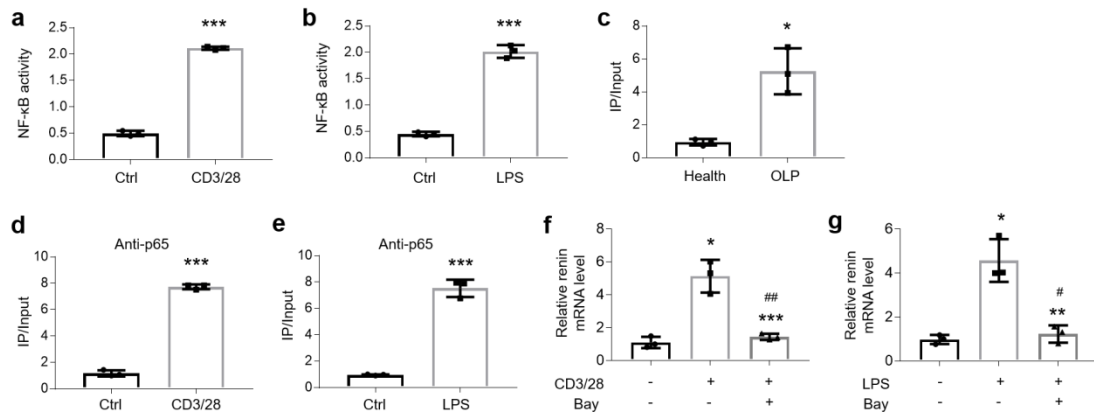
**Renin Promotes STAT4 Phosphorylation
to Induce IL-17 Production
in Keratinocytes of Oral Lichen Planus**

Xuejun Ge, Hanting Xie, Tivoli Nguyen, Bin Zhao, Jing Xu, and Jie Du

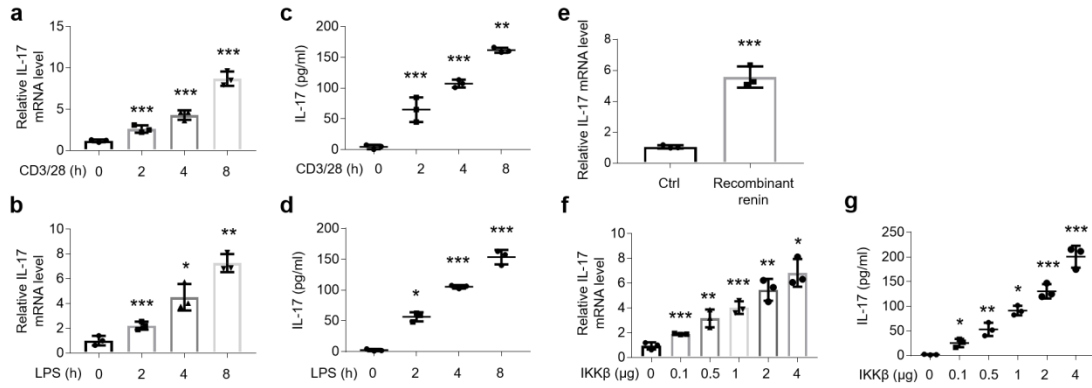
Supplemental Figures



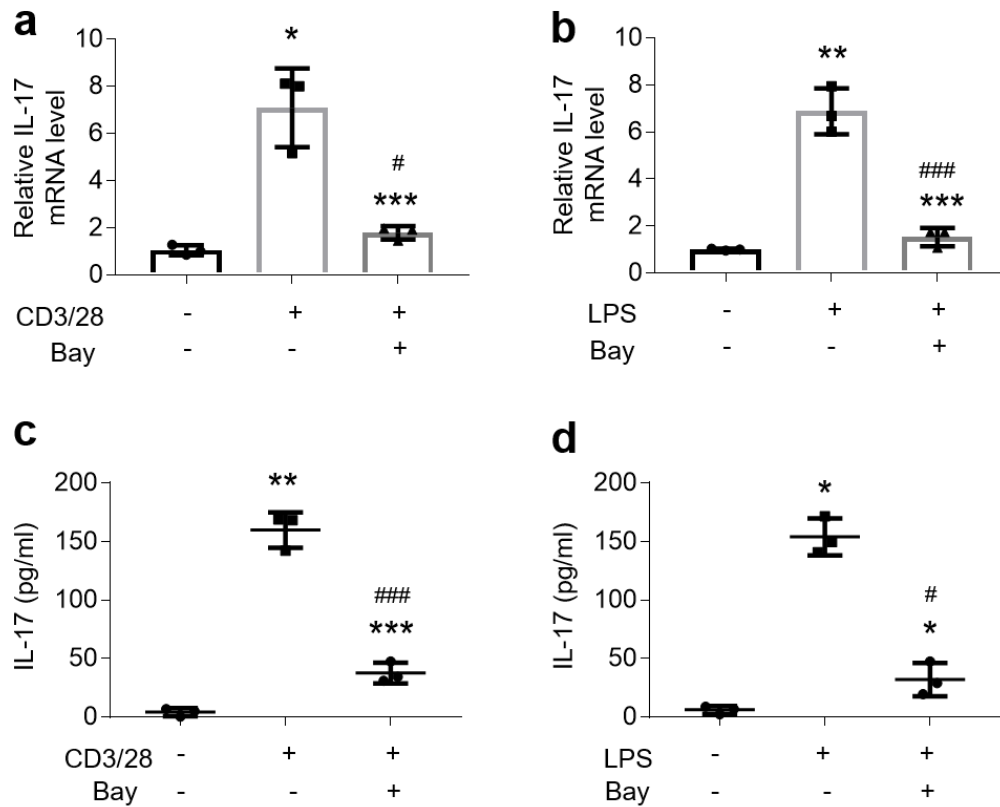
Supplemental figure 1. Renin expression is elevated in the lamina propria of OLP, Related to Figure 1. (a) Real-time PCR quantification of renin in the lamina propria of oral mucosal membranes. (b) Western blot analysis and densitometric quantitation of renin in the lamina propria of oral samples. * $P < 0.05$, *** $P < 0.001$ vs. corresponding healthy controls, $n=14$ each group. Data were shown as means \pm SD. 2-tailed Student's t test was used.



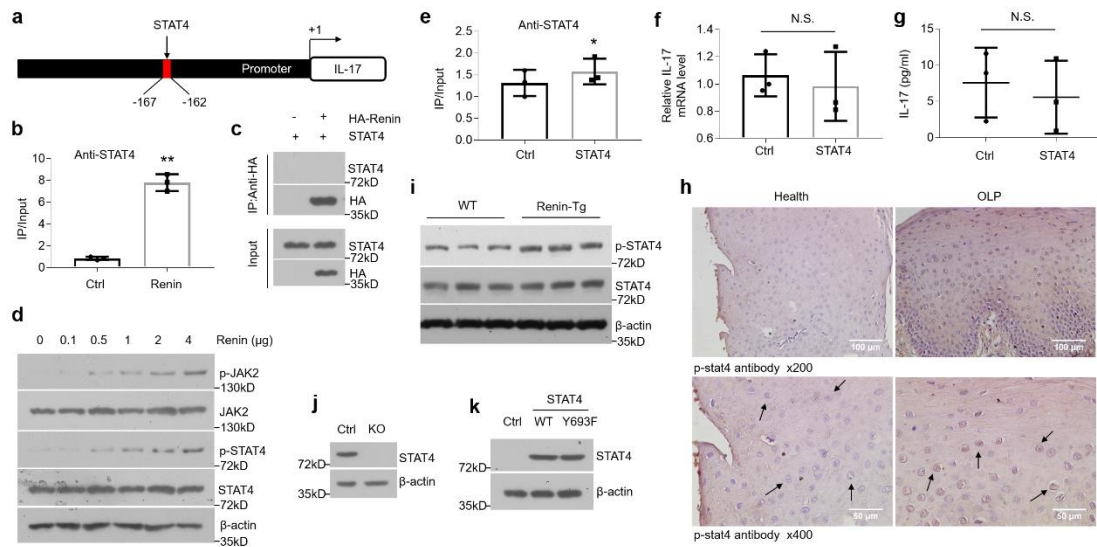
Supplemental figure 2. Effects of NF- κ B pathway on renin expression, Related to Figure 2. (a and b) NF- κ B activity in HOKs after activated CD4⁺ T cells (a) or LPS (b) treatment. (c-e) Binding of NF- κ B to the promoter of endogenous *renin* was increased in the epithelial layer of OLP biopsies (c), and in HOKs treated with activated CD4⁺ T cells (d) or 100 ng/ml LPS (e) for 8 hours. Chromatin immunoprecipitation (ChIP) was performed using anti-p65 antibody. (f and g) Real-time PCR quantification of renin mRNA levels in HOKs challenged by activated CD4⁺ T cells (f) or 100 ng/ml LPS (g) for 8 hours with or without 12-hour Bay 11-7082 (20 nM) pretreatment. *P < 0.05, **P < 0.01, ***P < 0.001 vs. corresponding control; #P < 0.05, ##P < 0.01 vs. LPS or CD3/28 group, n = 3. Ctrl, control; Bay, Bay 11-7082. Data were shown as means \pm SD. 2-tailed Student's *t* test and one-way analysis of variance were used.



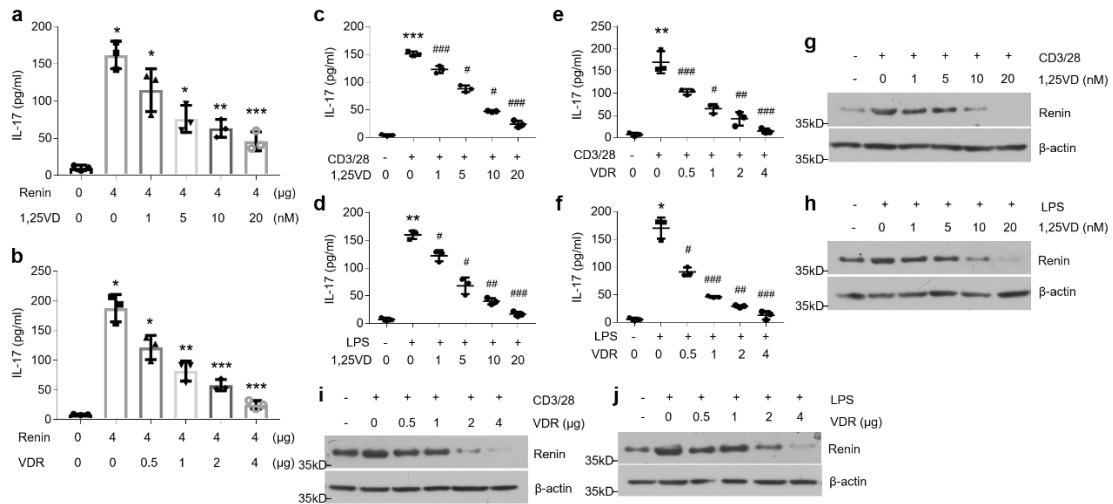
Supplemental figure 3. Expression of IL-17 in OLP or HOKs with IKK β overexpression, Related to Figure 3. (a and b) Real-time PCR analysis of IL-17 in HOKs with activated CD4⁺ T cells (a) or 100 ng/ml LPS (b) treatment. (c and d) The amounts of IL-17 productions in culture medium from activated CD4⁺ T cells- (c) or 100 ng/ml LPS- (d) treated HOKs were assessed by Elisa. (e) Real-time PCR analysis of IL-17 in HOKs treated with or without recombinant renin (50 ng/ml, Enzo Life Sciences, Cat: ENZ-PRT193-0010). (f and g) Real-time PCR (f) or Elisa (g) analysis of IL-17 in HOKs transfected with IKK β plasmids or culture medium from these cells. n = 3 each group of cell line studies. *P < 0.05, **P < 0.01, ***P < 0.001 vs. corresponding control. Data were shown as means \pm SD. 2-tailed Student's *t* test was used.



Supplemental figure 4. Inhibition of NF- κ B pathway blunts IL-17 increases in OLP cell models, Related to Figure 4. (a and b) Real-time PCR quantification of IL-17 mRNA levels in HOKs treated with activated CD4⁺ T cells (a) or 100 ng/ml LPS (b) for 8 hours, with or without 12-hour Bay compound pretreatment. (c and d) Elisa assessment of IL-17 secretions in the culture medium from HOKs stimulated with activated CD4⁺ T cells (c) or 100 ng/ml LPS (d) for 8 hours, following 12-hour Bay compound pretreatment. *P < 0.05, **P < 0.01, ***P < 0.001 vs. corresponding control; #P < 0.05, ###P < 0.001 vs. LPS or CD3/28 group, n = 3. Data were shown as means \pm SD. 2-tailed Student's *t* test and one-way analysis of variance were used.



Supplemental figure 5. The occupancy of STAT4 on the promoter of *IL-17* gene, Related to Figure 5. (a) Schematic illustration of STAT4 motif in the promoter of *IL-17* gene. (b) Renin plasmids transfection in HOKs helps STAT4 bind to the promoter of endogenous *IL-17*. Chromatin immunoprecipitation was performed using anti-STAT4 antibody. (c) Co-IP and western blot analyses of cell lysates from HOKs transfected with the indicated plasmids. (d) Western blot analysis of proteins in renin-transfected HOKs lysates. (e) Chromatin immunoprecipitation analysis of empty or STAT4 plasmids-transfected HOKs using anti-STAT4 antibody. (f and g) Real-time PCR (f) and Elisa (g) measurements of IL-17 expression in HOKs transfected with empty or STAT4 plasmids. (h) phospho-STAT4 immunostaining in the oral tissues of healthy controls and OLP patients. (i) Western blot analysis of proteins in mice oral epitheliums lysates. (j) Endogenous STAT4 in HOKs was deleted using CRISPR/Cas9 system and demonstrated by western blot. (k) Wild-type STAT4 and Y693F mutant were transfected into HOKs in which the endogenous STAT4 was knocked out. The expression of STAT4 was detected by western blot. n = 5 each group of mice samples, n = 3 each group of cell line studies. *P < 0.05, **P < 0.01 vs. corresponding control. Ctrl, control. Data were shown as means \pm SD. 2-tailed Student's *t* test was used.



Supplemental figure 6. Vitamin D/VDR signaling inhibits renin and IL-17 protein expression in OLP cell models, Related to Figure 6. (a) Elisa test of culture medium of HOKs which were pretreated with different doses of 1,25VD for 12 hours, and then challenged by renin plasmids transfection for 36 hours. (b) Elisa detection of culture medium of HOKs which were co-transfected with VDR and renin plasmids for 36 hours. (c-j) HOKs were pretreated with different doses of 1,25VD (12 hours) or of VDR plasmids (36 hours), and then challenged by activated CD4⁺ T cells or 100 ng/ml LPS. Real-time PCR quantification of IL-17 in HOKs (c and d), Elisa detection of IL-17 secretions in the culture medium (e and f), western blot analysis of renin (g and j). *P < 0.05, **P < 0.01, ***P < 0.001 vs. corresponding control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. LPS or CD3/28 group, n = 3. 1,25VD, 1,25(OH)₂D₃. Data were shown as means ± SD. 2-tailed Student's *t* test and one-way analysis of variance were used.

Supplemental Tables

Supplemental table 1. Primer sequences involved in this work, Related to Figure 1 and Figure 5.

Primer name	Forward(5'-3')	Reverse(5'-3')
hRenin	ACCTTTGGTCTCCCGACAGA	CACCTCGTTCCTTCAGGCTTT
hIL-17	TCCCACGAAATCCAGGATGC	GGATGTTTCAGGTTGACCATCAC
hGADPH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
mIL-17	TCCCTCTGTGATCTGGGAAG	AGCATCTTCTCGACCCTGAA
mGAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA
Renin (ChIP)	GGTAATAAATCAGGGCAGAGCA	GGTAATAAATCAGGGCAGAGCA
IL-17 (ChIP)	TGCCCTTCCCATTTTCCTTCA	TGGTCACTTACGTGGCGTG

Supplemental table 2. Antibodies information, Related to Figure 1-5.

Antibody	Source	Identifier
Anti-Renin	Santa Cruz	sc-27318
Anti- β -actin	Santa Cruz	sc-47778
Anti-NF- κ B p65	Cell Signaling	8242
Anti-Phospho-NF- κ B p65	Cell Signaling	3031
Anti-IKK β	Cell Signaling	8943
Anti-p-JAK1	Invitrogen	PA5-37617
Anti-JAK1	BD Bioscience	610231
Anti-p-JAK2	Cell Signaling	3771
Anti-JAK2	Cell Signaling	3230
Anti-p-JAK3	Cell Signaling	5031
Anti-JAK3	Cell Signaling	8863
Anti-Phospho-STAT4 (Tyr693)	Cell Signaling	5267
Anti-STAT4	Cell Signaling	2653
Anti-HA	Cell Signaling	3724
Anti-His	Cell Signaling	12698
Anti-Myc	Cell Signaling	2276
Anti-Lamin C	Abcam	Ab125679
Anti-GAPDH	Sigma	G8795
Anti-IL-17	Abcam	ab79056

Transparent Methods

Human samples collection. Human oral mucosal tissues and blood samples were collected from participants at the Stomatological Hospital of Shanxi Medical University. Unaffected control samples were got from volunteers who underwent wisdom teeth extraction. Modified World Health Organization (WHO) diagnostic criteria were used for selecting for OLP patients. The human sample studies were approved by the Institutional Ethical Committee of Shanxi Medical University. Written informed consent was got from each participant involved in this investigation. Detailed information regarding OLP patients was in detail elaborated here (Zhao et al., 2019). There were 6 males and 8 females in OLP patients. Most of patients were middle-aged and all of them showed the reticular subtype.

Cell culture. Human oral keratinocytes (HOKs, ScienCell, Catalog #2610) were cultured in oral keratinocyte medium (ScienCell, Catalog #2611) with 10% FBS. There are two methods for mimicking OLP *in vitro*. In one, HOKs were challenged with 100 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich, O111:B4 *E. coli*) for 8 hours. In the other, HOKs were treated for 8 hours with the culture medium from CD4⁺ T cells, which were activated with or without anti-CD3/CD28. The supernatant of CD4⁺ T cell cultures was added to HOKs at a 30% final volumetric concentration (Zhao et al., 2019). In another assay, HOKs were treated with 1,25 VD or Bay 11-7082 compound for 12 hours or transfected with plasmids or siRNAs for 36 hours prior to 8-hour LPS or activated CD4⁺ T cells challenge.

Isolation and stimulation of CD4⁺ T cells. Peripheral blood from OLP patients was subjected to Ficoll-Hypaque density gradient centrifugation. Anti-CD4 magnetic particles (BD Biosciences) were used to purify CD4⁺ T cells. After suspension in RPMI 1640 medium, CD4⁺ T cells were stimulated by anti-CD3 and anti-CD28 antibodies (BD Biosciences Pharmingen).

Animal studies. Transgenic mouse line RenTgMK 108 (RenTg) used in this study carries a single copy of mouse renin transgene which is driven by liver-specific albumin 109 promoter/enhancer (He et al., 2019). 8-week-old mice were chosen for

this study, they were sex matched and cohoused. All animal studies were approved by the Institutional Ethical Committee affiliated with Shanxi Medical University.

Oral mucosal epithelium isolation. Oral epithelial layer separation assays were performed according to ZB et al (Zhao et al., 2019). Briefly, whole oral buccal tissues from individuals or mice were digested for 12 hours by 0.25% dispase II, followed by direct separation with muscle forceps. After epithelial layer separation, the rest of tissues are lamina propria.

CRISPR/Cas9-regulated knockout of *STAT4*. sgRNA sequence targeting the *STAT4* gene (5'- TGACACGTTGTACCAAATGA-3') was inserted into lentiCRISPRv2 vector (Addgene, catalog 52961) by the BsmBI restriction enzyme. Lenti-vector and packaging plasmids (pMD2.G and psPAX2) were cotransfected into HEK293T cells. Virus was isolated from the culture medium of HEK293T cells after 48-hour transfection. Lentivirus particles were added into HOKs with polybrenes (4 µg/ml) supplement. Puromycin (0.5 µg/ml) was used to select transduced cells.

RT-PCR. Total RNAs were isolated from cells or tissues with TRIzol reagent (Invitrogen). mRNAs were converted to first-stranded cDNAs by a PrimeScript reagent kit (TaKaRa). Real-time PCR was performed with a SYBR Premix kit (TaKaRa). GAPDH was chosen as an internal control. Relative quantification of transcripts was analyzed using the $2^{-\Delta\Delta Ct}$ formula. Primers sequences were provided in supplemental table 1.

Western blot. Western blot analyses were carried out according to previous studies(Zhao et al., 2019). Briefly, cells were dissolved into lysis buffer containing protease inhibitors (Roche) and heated at 95°C for 5 min. The same amount of protein was separated by SDS-PAGE and electrotransferred onto PVDF membranes (Millipore). The membranes were incubated at cold room overnight using primary antibodies, following one-hour blocking buffer treatment. On day 2, HRP-conjugated secondary antibodies were selected to incubate membranes at room temperature for 1 hour. Bands were detected by ECL kit (Thermo Fisher Scientific) and visualized with x-ray films at dark room. More details on primary antibodies are provided in supplemental table 2.

Immunohistochemical staining. Immunohistochemical assays were conducted according to ZB et al (Zhao et al., 2019). Oral tissues were fixed in 10% formalin and embedded with paraffin. Blocks were cut into 4 μ m slides using a microtome (Leica). After deparaffinization and dehydration, slides were treated with citrate buffer for antigen retrieval and hydrogen peroxide for endogenous peroxidase activity blockage. Primary antibodies (anti-Renin, anti-p-STAT4 and anti-IL-17) were used to incubate slides at cold room overnight, followed by one-hour secondary incubation. After a series of washes, antibody-connected tissues were treated with 3,3'-Diaminobenzidine (DAB) and hematoxylin. Slides were observed under a light microscope. More details on primary antibodies are provided in supplemental table 2.

Chromatin immunoprecipitation (ChIP). Cells were washed with PBS for 3 times, followed by 10-min crosslinking with 1% formaldehyde. ChIP assays were carried out using a commercial kit (Pierce) according to the manufacturer's instructions. Anti-p65 and anti-STAT4 antibodies were involved in this assay.

DNA pulldown assay. DNA pulldown assay was performed according to ZQ et al (Zhang et al., 2019). Briefly, HOKs transfected with plasmids were lysed in a DNA binding buffer, followed by 30-min incubation with 30 pmol biotinylated STAT4-binding element (SBE) oligonucleotides (CATTGGGGGCGGAAATTTTAACCAA). DNA-protein complexes were precipitated via streptavidin beads, and then subjected to western blot analysis.

Co-immunoprecipitation (Co-IP). Co-IP assays were performed according to ZG et al (Zhao et al., 2019). Briefly, transfected cells were lysed and supernatants were collected before incubation with antibodies and protein A/G beads (Thermo Fisher Scientific). After 4-hour incubation, complexes were washed with NETN buffer 4 times and then subjected to SDS-PAGE for analyses.

Elisa. Blood samples obtained from participants were stored at cold room overnight for serum collection. IL-17 levels in human serum samples, HOKs culture medium and the lysates of mice oral epithelial layer were detected using a commercial Elisa kit (eBioscience) according to the instructions. The OD values were monitored by a microplate reader.

Transfection assay. Plasmids and siRNAs were transfected into HOKs with Lipofectamin 3000 (Invitrogen). HA-Renin plasmids were obtained from Sino Biological (Cat: HG10969-CY), His-JAK2 plasmids from Biological (Cat: HG11198-CH), and Myc-STAT4 plasmids from Origene (Cat: RC206892). Y693F mutant of STAT4 was performed with the Site-Directed Mutagenesis Kit (Clontech). Oligonucleotides (5'-GACAAGGGTTTCGTCCCTTCTGTTTTTATCCC-3') were used to change tyrosine to phenylalanine at codon 693 (Morinobu et al., 2002; Visconti et al., 2000). Renin-siRNAs (Cat: sc-41644) and NF- κ B p65-siRNAs (sc-29410) were got from Santa Cruz.

NF- κ B activity assay. NF- κ B activity assay was carried out by using NF- κ B luciferase reporter kit (BPS Bioscience). Primary oral keratinocytes from human samples were isolated and cultured in Petri dishes. Both HOKs and primary oral keratinocytes were transfected with NF- κ B luciferase reporter using Lipofectamine 3000. The luciferase activity was detected by Dual Luciferase Assay System.

Statistical analysis. Data were shown as means \pm SD. Statistical analyses were performed by 2-tailed Student's *t* test or one-way analysis of variance for 2 or multiple groups, respectively. *P* < 0.05 was considered to be statistically significant.

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