

Original Article

TRIM21 causes abnormal expression of IL-6 in oral lichen planus via the TRIB2-MAPK signal axis

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Abstract: Objective: Oral lichen planus (OLP) is a common chronic inflammatory disease in the oral cavity, and has the risk of developing into oral squamous cell carcinoma (OSCC). It is necessary to discover the role of TRIM21 in the pathogenesis of OLP and its underlying mechanism. Methods: Western blot and qPCR assays were used to detect the effects of TRIM21 on cellular levels of ERK, p-ERK, AP-1, IL-6, TRIB2, IRF3, and IRF7, while co-immunoprecipitation was performed to verify the interaction between Trim21 and TRIB2 protein. The TRIM21 effect on TH1/TH2 balance in T cells was also evaluated using ELISA. Results: The results of western blot showed that TRIM21 overexpression significantly increased p-ERK, c-fos, c-jun, IL-6 and TRIB2 levels in H9 cells ($P < 0.01$ and $P < 0.001$), however, inhibited the IRF3 and IRF7 levels ($P < 0.05$). On the other hand, TRIM21 did not regulate the phosphorylation of ERK and the mRNA expression of AP-1 and TRIB2. In addition, TRIM21 was in relation to the proteasome degradation in TRIB2-ERK. TRIM21 also regulated the level of TRIB2 not only by inhibiting the ubiquitination of TRIB2, but also by affecting IL-6 through the ERK pathway. Conclusion: TRIM21 caused abnormal expression of IL-6 in OLP via regulating TRIB2-MAPK signal axis, leading to the disrupted Th1/Th2 balance in T lymphocytes.

Keywords: Oral lichen planus, tripartite motif-containing protein 21, tribbles homolog 2, mitogen-activated protein kinase, interleukin-6

Introduction

Oral lichen planus (OLP) is a common chronic inflammatory disease of the oral mucosa with approximately 0.5% to 4% incidence [1]. The World Health Organization (WHO) lists OLP as a potential malignant lesion in the oral cavity due to the possibility of developing into oral squamous cell carcinoma (OSCC). It is known that patients with OLP have 10 times higher chance of getting oral cancer than healthy people [2]. The etiology and pathogenesis of OLP may be involved in the immune system, genetic factors, sociopsychological factors, and infection factors [3]. OLP has currently been confirmed as T lymphocyte-mediated immune disease, and

therefore it is of great significance to investigate the pathogenesis of OLP in the aspects of diagnosis, treatment and prognosis.

Tripartite motif (TRIM) protein family, contains more than 77 members, are implicated in a wide range of physiologic processes, including immune regulation, cell proliferation, anti-viral processes, oncogenesis, and tumor metastasis [4]. Many TRIM proteins function as the E3 ubiquitin ligases due to the presence of RING domain [5]. The tripartite motif-containing protein 21 (TRIM21) is an important member of the TRIM protein family which was first discovered in 1988. TRIM21 is usually present in Hela cells of the patients' serum, and considered as an

auto-antigen that causes Sjogren's syndrome and systemic lupus erythematosus [6]. In addition, TRIM21 is widely expressed in a variety of cells and plays an important role in regulating immune system. The E3 ligase activity of TRIM21 links ubiquitin molecules to its substrates, and thereby mediating protein degradation through the ubiquitin-proteasome pathway. TRIM21 can also play different biological roles by interacting with different substrates [7]. For example, TRIM21 can stimulate the activation of transcription factors, such as nuclear factor κ B (NF- κ B), protein 1 (AP) -1, interferon regulatory factor 3 (IRF3), IRF5, IRF7 and IRF8, through its K63-linked ubiquitin ligase activity [8]. The activation of these transcription factors trigger the production of multiple pro-inflammatory cytokines and chemokines, such as interleukin-6 (IL-6), IL-12p40, tumor necrosis factor- α (TNF- α), type I interferon (IFN), CXCL10, CCL2 and CCL4 in mouse embryonic fibroblasts (MEFs) and macrophages [9].

A previous study has shown that compared with healthy subjects, IL-6 level in the serum of OLP patients was significantly higher [10]. Yet, whether this difference was regulated by TRIM21 has not been reported before. This study aimed to investigate the role and mechanism of TRIM21 in the IL-6 expression of patients with OLP through *in vitro* cell experiments.

Methods

Cell culture

The human CD3⁺CD4⁺ T cells from healthy volunteers and H9 cell lines were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin (complete medium) and maintained at 37°C in a humidified atmosphere of 5% CO₂.

Cell proliferation assay

Cell proliferation was analyzed using the Cell Counting Kit-8 (CCK-8; cat. no. E606335-0500; BBI Life Sciences). The CD3⁺CD4⁺ T cells and H9 cell lines in the logarithmic growth phase were seeded into a 96-well plate at a density of

2 $\times$ 10³ cells/well and placed in a cell culture incubator. At 2-4 h before termination of culture, CCK-8 solution (10 μ L) was added into each well, and the mixture was incubated for 1 h. An enzyme-labeled instrument was carried out to measure each well with the absorbance values (OD value) at 450 nm. The results were obtained from three independent experiments in triplicate. The OD value was considered to be directly proportional to the number of viable cells in the culture.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-6, IL-10, TGF- β 1, IFN- γ and IL-4 in saliva were detected using ELISA kit in accordance with the manufacturer's instructions (IL-6: EK106/2-48; IL-10: EK110/2-48; TGF- β 1: EK981-48; IFN- γ : EK180-01; IL-4: EK104-01; MultiSciences Biotech Co., Ltd., China). The ELISA plate was treated and incubated with 50 μ L of bovine serum albumin (BSA) blocking solution (10 mg/ml), 50 μ L of serum and 50 μ L of biotin-conjugated detector mAb at 37°C for 1 hour, and probed with avidin-HRP solution (1:100). After final rinsing with PBST, 100 μ L of tetramethylbenzidine (TMB) substrate solution (10 mg/ml) was added for color reaction with Ag-Ab complex. The relative absorbance was measured at 450 nm using an automated ELISA reader (Bio-Rad model 550, Irvine, CA). The concentration was determined by comparing the optical density with standard curve.

Transfection

The siRNAs targeting TRIM21 knockdown: TGAGAAGTTGGAA GTGGAAATACC, TRIM21 primers: GAGTTGGCTGAG AAGTTGGAA and scrambled controls: CCTAAGGTTAAG TCGCCCTCG were purchased from GenePharma (Shanghai, China). Cells were transfected with appropriate miRNA, siRNA oligonucleotides and plasmids using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. The medium was replenished 6 h after transfection.

Western blot

The cells from different groups were collected to extract and quantify proteins. Protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein bands were then transferred to

Table 1. Real-Time PCR primer sequences

Gene	Primer sequence (5'→3')
c-fos-F	GGGGCAAGGTGGAACAGTTAT
c-fos-R	CCGCTTGGAGTGTATCAGTCA
c-jun-F	TCCAAGTGCCGAAAAAGGAAG
c-jun-R	CGAGTTCTGAGCTTTCAAGGT
ERK-F	TCACACAGGGTTCCTGACAGA
ERK-R	ATGCAGCCTACAGACCAATATC
TRIB2-F	CTTTTGCCTGTCTGCTCATAGT
TRIB2-R	ATAGCTTCGCTCAAAGAACACA
IRF3-F	AGAGGCTCGTGATGGTCAAG
IRF3-R	AGGTCCACAGTATTCTCCAGG
IRF7-F	GCTGGACGTGACCATCATGTA
IRF7-R	GGGCCGTATAGGAACGTGC
GAPDH-F	GGAGCGAGATCCCTCCAAAAT
GAPDH-R	GGCTGTTGTCATACTTCTCATGG

a nitrocellulose membrane and blocked with 5% skim milk at room temperature for 30 min. The blots were incubated overnight at 4°C with primary antibody and HRP-labeled secondary antibody. The primary antibodies included TRIM21 (1:1000, 92043, CST), ERK (1:1000, 4695, CST), p-ERK (1:1000, 4370, CST), c-fos (1:1000, 4384, CST), c-JUN (1:1000, ab314-19, Abcam), IL-6 (1:1000, ab208113, Abcam), TRIB2 (1:1000, 13533, CST), IRF3 (1:1000, 11904, CST), IRF7 (1:1000, 4920, CST) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:20000, 5174, CST). After that, the membranes were rinsed with TBST for 3 times (5 min per each). An enhanced chemiluminescence (ECL) was applied to visualize the bands, while the gel image was taken by Tanon-5200 gel imager (Tanon, Shanghai, China). The absorbance analysis was conducted by Quantity-One software. The expression of the target protein was represented as the ratio of the target protein absorbance to the corresponding GAPDH absorbance. The experiment was repeated three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from H9 cell lines using Trizol Reagent (15596-018, Invitrogen). RNA was reverse transcribed to cDNA after DNA elimination. The prepared cDNA was then amplified using SYBR Green Master Mixture (Takara, Otsu, Japan), of which the results were calculated by ABI Stepone plus™ Real-Time

PCR System (illumina eco). The thermocycling conditions were started with 5 min of DNA regeneration at 95°C, 40 cycles at 95°C for 30 sec, followed by primer annealing at 60°C for 30 sec, and primer extension at 72°C for 5 min. The $2^{-\Delta\Delta C_q}$ method was applied to normalize the relative expression of genes to GAPDH. The used primers are listed in **Table 1**. The experiment was also repeated three times.

Co-immunoprecipitation

Cells were lysed in protease inhibitors-supplemented Tris lysis buffer (50 mM Tris buffer, 150 mM NaCl, 0.5% IGEPAL® CA-630, 5% glycerol and 1 mM EDTA) after 24 h transfection. Prior to immunoprecipitation (IP), protein lysates were pre-cleared by incubation with Protein G Agarose beads (Millipore, Cork, Ireland) at 4°C for 30 min. Pre-cleared lysates were then incubated with the antibody and Protein G Agarose beads overnight in a rotary tube mixer at 4°C. Immunoprecipitated proteins were washed from beads by boiling at 95°C for 5 min in Laemmli buffer and analyzed by Western blotting.

Statistical analysis

Data were analyzed using SPSS software (Chicago, IL, USA). Normally distributed data are presented as the mean ± standard deviation (SD), while the non-normally distributed data are presented as the mean ± interquartile range or as the median. One-way and two-way ANOVA were used for comparison between groups, and the Bonferroni test was carried out for multiple comparisons. $P < 0.05$ was considered as statistically significant.

Results

Effects of TRIM21 on cell activity and cytokine secretion in primary CD3⁺CD4⁺ T cells and H9 cell lines

The CCK8 result showed that in primary CD3⁺CD4⁺ T cells, the overexpression of transfected TRIM21 plasmid significantly enhanced cell viability compared with negative control (NC) group ($P < 0.01$). In contrast, the TRIM21 silencing significantly inhibited cell viability ($P < 0.05$, **Figure 1A**). Similarly, the overexpression of transfected TRIM21 plasmid significantly increased cell activity in H9 cell lines

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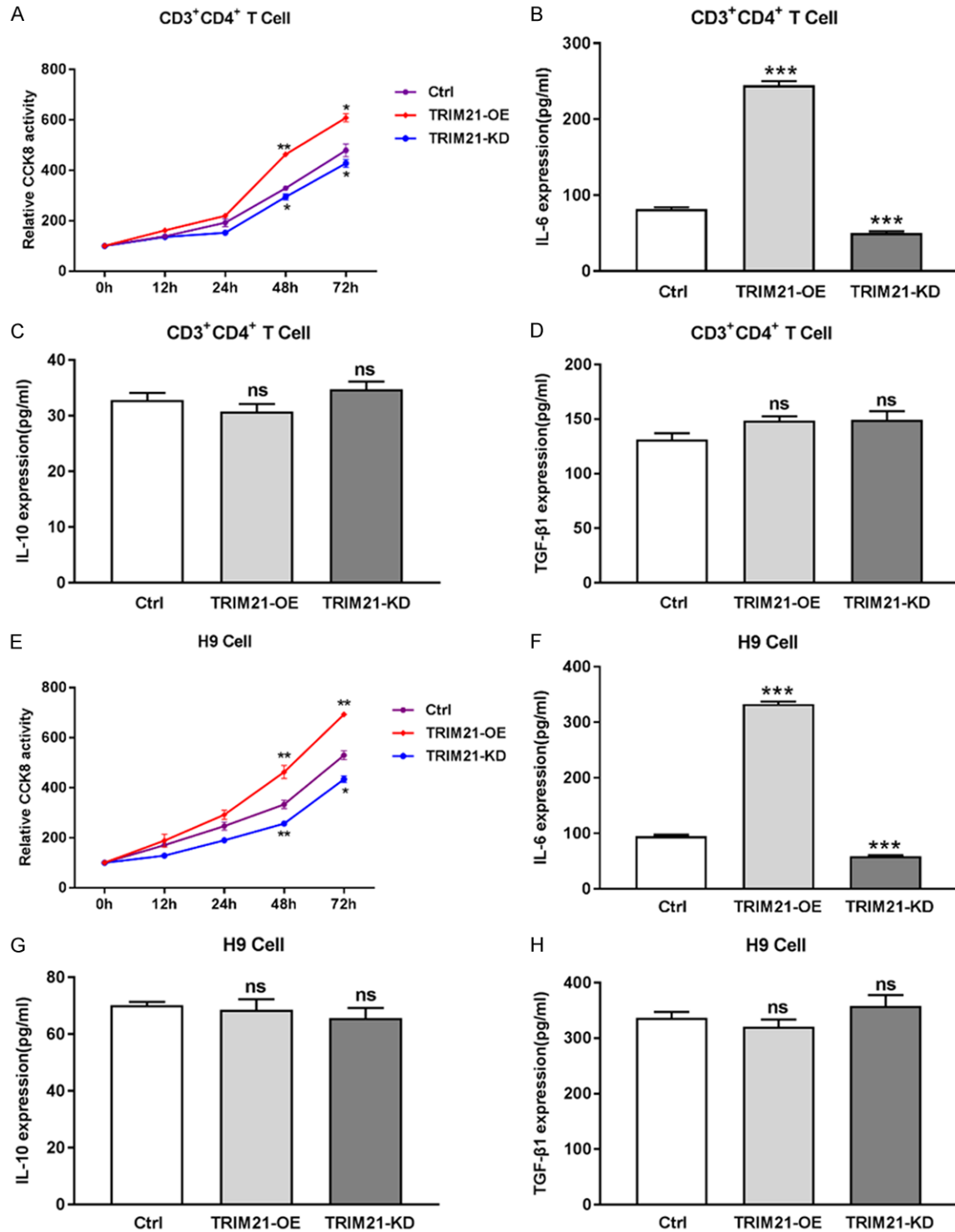


Figure 1. Effect of TRIM21 on cell activity and cytokine secretion by primary CD3⁺CD4⁺ T cells and H9 cell lines. In primary CD3⁺CD4⁺ T cells, (A) cell viability, the level of (B) IL-6, (C) IL-10 and (D) TGF-β1; In H9 cells, (E) cell viability, the level of (F) IL-6, (G) IL-10 and (H) TGF-β1. *P<0.05, **P<0.01, ***P<0.001, compared with the Ctrl group. ns, not significant.

(P<0.01), whereas the knockdown of TRIM21 remarkably suppressed cell activity (P<0.01, Figure 1E).

In addition, the effects of TRIM21 on cytokine secretion by primary CD3⁺CD4⁺ T cells and H9 cells were detected by ELISA. The results dem-

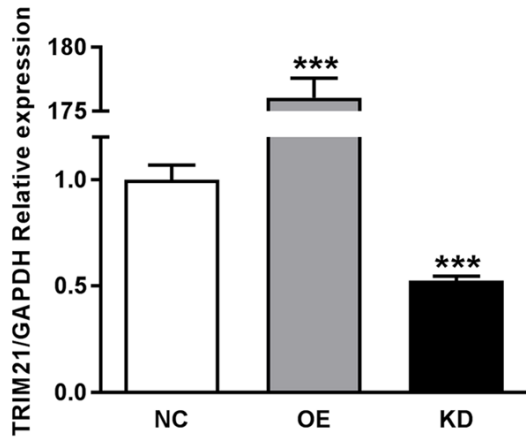


Figure 2. The efficiency of TRIM21 overexpression and knockdown was evaluated by Western blot. *** $P < 0.001$, compared with the Ctrl group.

onstrated that compared with the NC group, the primary $CD3^+CD4^+$ T cells had relatively higher expression level of IL-6 in TRIM21-overexpressed cells ($P < 0.001$), while IL-6 level in TRIM21-silenced cells was dramatically dropped ($P < 0.001$, **Figure 1B**). The similar results were also obtained in H9 cell lines (**Figure 1F**). The expression of TRIM21 had no obvious effect on the secretion of IL-10 and TGF- $\beta 1$ in both $CD3^+CD4^+$ T cells and H9 cells (**Figure 1C, 1D, 1G, 1H**).

The above results revealed that the expression of TRIM21 had similar effects on both H9 cell lines and in primary $CD3^+CD4^+$ T cells, and thereby H9 cell lines were selected for the subsequent experiments in this study.

The mechanism of TRIM21 affecting IL-6 expression

In order to understand the mechanism of TRIM21 in the IL-6 expression, TRIM21 overexpression plasmid or sh-TRIM21 plasmid were transfected into H9 cells. The results of western blot demonstrated the successful transfection of overexpression or silence of TRIM21 into H9 cells ($P < 0.001$) (**Figure 2**).

The activations of TRIM21, ERK, p-ERK, AP-1, IL-6, TRIB2, IRF3, and IRF7 were detected using western blot. The results clarified that TRIM21 overexpression significantly elevated the p-ERK, c-fos, c-jun, IL-6 and TRIB2 levels in H9 cells ($P < 0.01$, $P < 0.001$), however, remarkably inhibited the levels of IRF3 and IRF7 ($P < 0.05$).

The suppression of TRIM21 expression notably reduced the levels of p-ERK, c-fos, c-jun, IL-6, and TRIB2 ($P < 0.05$, $P < 0.01$), and increased the levels of IRF3 and IRF7 ($P < 0.05$, $P < 0.01$, **Figure 3**).

Furthermore, the mRNA expression of the above factors were detected by RT-PCR, which indicated the increase in mRNA levels of IL-6, IRF3 and IRF7 with the overexpression of TRIM21 ($P < 0.05$, $P < 0.001$ respectively), whereas the changes of TRIM21 could not affect the phosphorylation of ERK and the mRNA expression of AP-1 and TRIB2 ($P > 0.05$, **Figure 4**).

TRIM21 affects TH1/TH2 balance in H9 cell line

Interferon-gamma (IFN- γ) is a cytokine produced by TH1 cells, while interleukin 4 (IL-4) is produced by TH2 cells. The changes of IFN- γ and IL-4 concentrations and IFN- γ /IL-4 ratio were detected using ELISA, which showed the increase in the IL-4 concentration with the TRIM21 overexpression (**Figure 5A**), and the decrease in IFN- γ concentration (**Figure 5B**). Compared with the Ctrl group, the ratio of IFN- γ /IL-4 was significantly reduced in the OE group ($P < 0.001$), while this ratio was relatively raised in the KD group ($P < 0.001$, **Figure 5C**).

TRIM21 interacts with TRIB2

In order to confirm the effect of TRIM21 on TRIB2 protein, actidione (CHX) and proteasome inhibitor (MG132) were used to inhibit protein synthesis and proteasome degradation respectively. The western blot was applied to detect the expressions of TRIB2, p-ERK, and ERK protein. In the CHX treatment group, the expression of TRIB2 was significantly elevated with the TRIM21 overexpression ($P < 0.001$), while the knockdown of TRIM21 resulting in the decreased expression of p-ERK protein ($P < 0.001$). After treatment with MG132, there were no significant differences in the expressions of TRIB2 and p-ERK protein were found ($P > 0.05$, **Figure 6A, 6B**).

Both co-immunoprecipitation and western blot analysis were carried out to detect the interaction between TRIM21 and TRIB2 in H9 cells. The interaction between TRIM21 and IgG provides a false positive result. As indicated in **Figure 6C**, the results of FLAG (TRIM21)/IgG

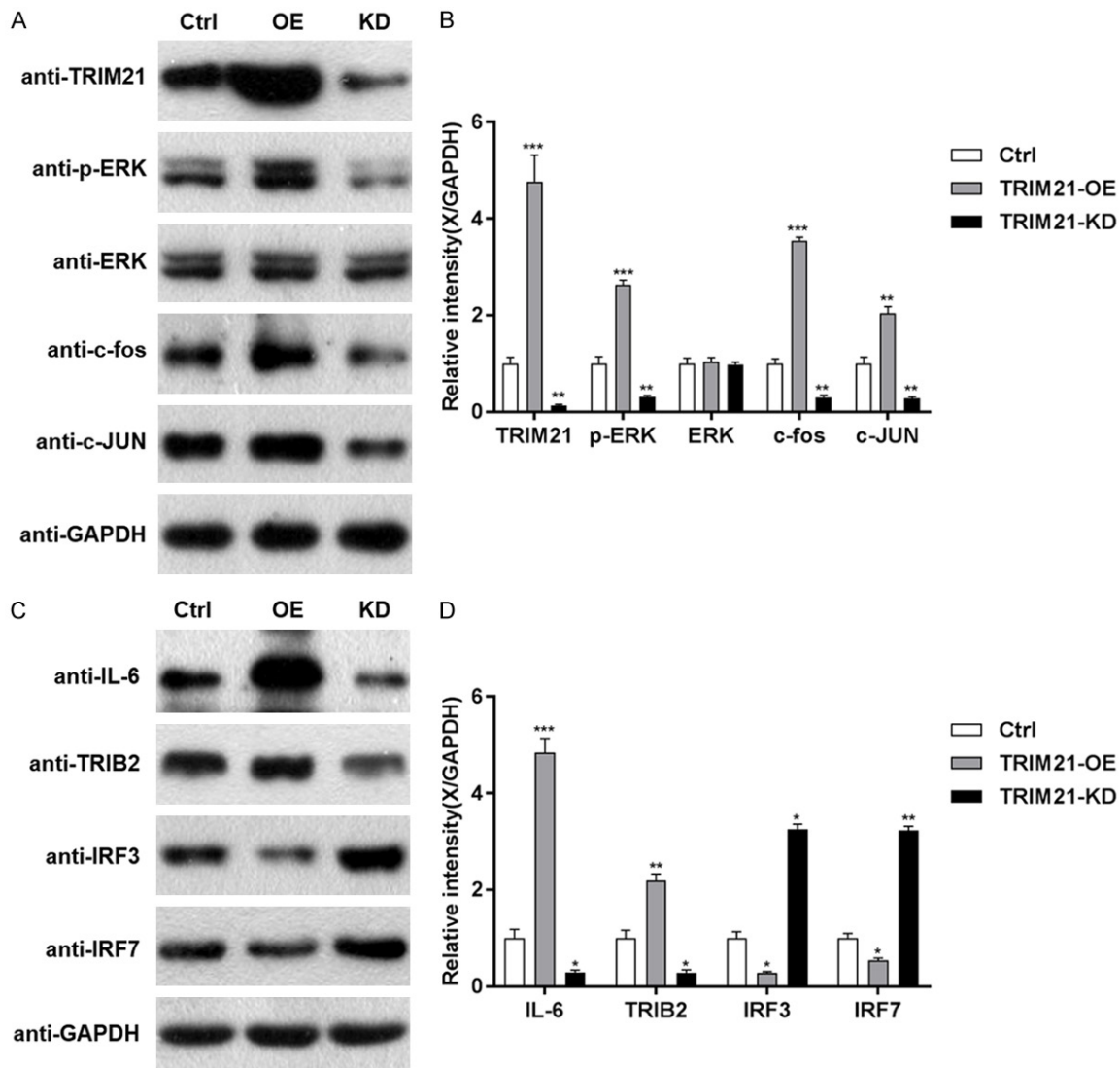


Figure 3. The activation of (A, B) TRIM21, p-ERK, ERK, c-fos, c-jun, (C, D) IL-6, TRIB2, IRF3, IRF7 was detected by Western blot. *P<0.05, **P<0.01, ***P<0.001, compared with the Ctrl group.

antibody immunoprecipitation showed the interaction between TRIM21 and TRIB2.

Furthermore, TRIB2-IP grade antibodies were used for immunoprecipitation, and HA antibodies were used to detect the ubiquitination of the samples after IP. The results of western blot showed that TRIM21 not only inhibited the ubiquitination of TRIB2 and regulated the level of TRIB2 in the cell, but also affected IL-6 through the ERK pathway (Figure 7).

Discussion

In the current study, the *in vitro* cell experiments proved that TRIM21 caused the abnor-

mal expression of IL-6 in OLP through regulating TRIB2-MAPK signal axis, suggesting the imbalance of Th1/Th2 in T lymphocytes. The interaction and mechanism between TRIM21 and TRIB2 proteins were also studied.

It is suggested to prevent the development of OSCC by early detection of OLP. TRIM21 is a cytoplasmic antibody receptor that catalyzes the formation of free K63 ubiquitin chains through its RING domain, followed by activate the AP-1, NF- κ B and IRF3/5/7 signaling pathways, allowing the increased production of pro-inflammatory cytokines in patients with OLP [8, 11]. IL-6 is a multifunctional cytokine which is capable of stimulating inflammatory responses

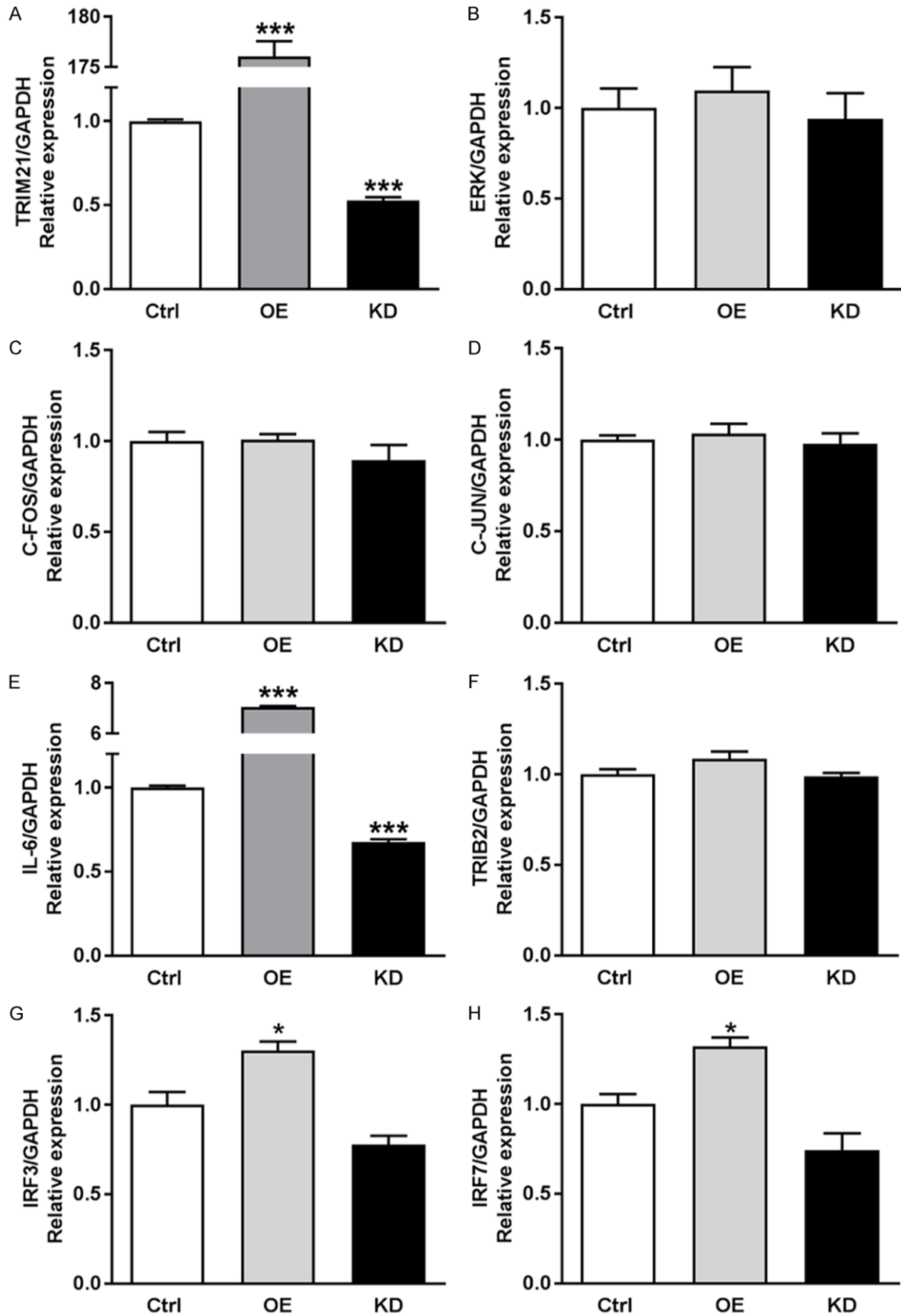


Figure 4. RT-PCR detection of (A) TRIM21, (B) ERK, (C) c-fos, (D) c-jun, (E) IL-6, (F) TRIB2, (G) IRF3, (H) IRF7 mRNA expression levels. *P<0.05, compared with the Ctrl group.

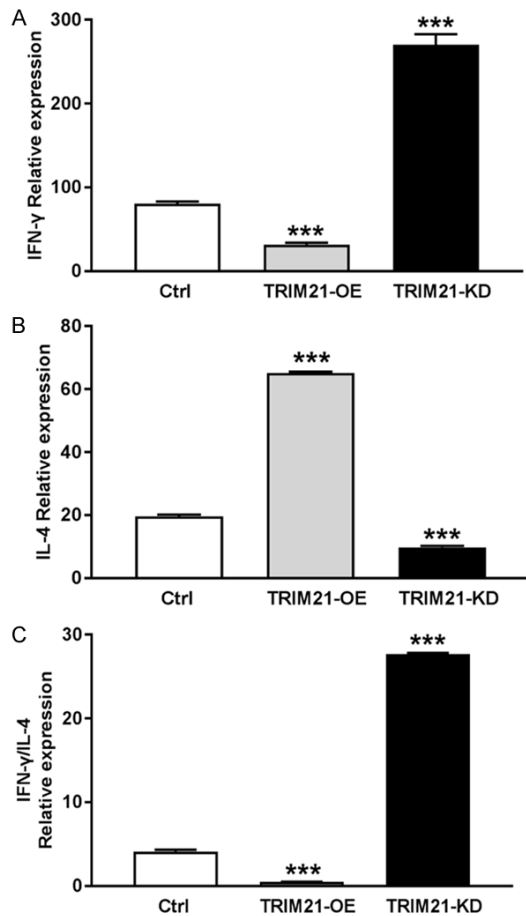


Figure 5. The levels of (A) IFN- γ , (B) IL-4 and (C) IFN- γ /IL-4 in the supernatant of H9 cells were detected by ELISA.

and various other biological processes. Several studies have reported that IL-6 is highly expressed in patients with OLP [10, 12, 13], which is consistent with our ELISA results. Moreover, TRIM21 is an important regulator for the production of pro-inflammatory cytokines. A significant increase in IL-6 ($P < 0.001$) after the overexpression of TRIM21 in T cells suggested that TRIM21 may be the main pro-inflammatory molecule. The major cause of the inflammatory responses in OLP epithelial cells is the dysregulated immune network formed by the epithelial cell membrane surface and intracellular protein signaling pathways [14]. However, the major signaling pathways that mediate the cellular behaviors in OLP patients, especially those key transcription factors, have not been fully identified yet. As a potential malignant lesion of the oral cavity, the pathogenesis of OLP has certain implications for the relationship between chronic inflammation and tumorigenesis.

A large number of studies have confirmed that mitogen-activated protein kinase (MAPK) is highly activated in some malignancies, and the activation of MAPK may be involved in the carcinogenic process [15-17]. The MAPK signaling pathway is a conserved signal transduction system that activates transcription factors through a three-stage enzymatic cascade to regulate specific gene expression, and thereby cause inflammatory responses and cytokine productions. Hence, we speculated that the MAPK signaling pathway may be involved in modulating the inflammatory responses induced by OLP and the process OSCC conversion. Extracellular signal-regulated kinase (ERK1/2), also called as mitogen-activated MAPK pathway, can promote cell proliferation and differentiation after activation [18]. Theocharis et al. have found that over-activation of ERK signaling is closely related to the malignant lesions of OLP [19]. TRIB2 plays a role in the degradation of key proteins and the regulation of MAPK signaling pathways, as well as the survival and proliferation of hematopoietic cells [20]. In our study, the overexpression of TRIM21 resulted in the remarkably increased level of p-ERK, TRIB2 ($P < 0.01$, $P < 0.001$), and two proto-oncogenes: c-fos and c-jun ($P < 0.01$, $P < 0.001$), indicating that TRIM21 overexpression in OLP can activate ERK and AP-1 signaling. In order to further verify the effect of TRIM21 on the TRIB2 protein, CHX and MG132 were used to inhibit protein synthesis and proteasome degradation, respectively. The results of western blot demonstrated that the effect of TRIM21 on TRIB2-ERK was only related to proteasome degradation. Moreover, there was an interaction between TRIM21 and TRIB2, where TRIM21 regulated the level of TRIB2 by inhibiting the ubiquitination of TRIB2 and affected IL-6 through the ERK pathway, suggesting that TRIM21 triggered the inflammatory responses in OLP, especially the expression of IL-6 via modulating the TRIB2-MAPK signal axis.

The dynamic balance of Th1 and Th2 cells is extremely important for maintain the normal immune system. Numerous studies have revealed that there is an imbalance of Th1 and Th2 in lesions, saliva, or tissue exudate of OLP patients [21], suggesting the relationship between OLP pathogenesis and Th cell imbalance. In this study, after TRIM21 overexpression, the level of Th1 type cytokine IFN- γ was significantly lower than in the Ctrl group ($P < 0.001$), whereas the Th2 type cytokine IL-4

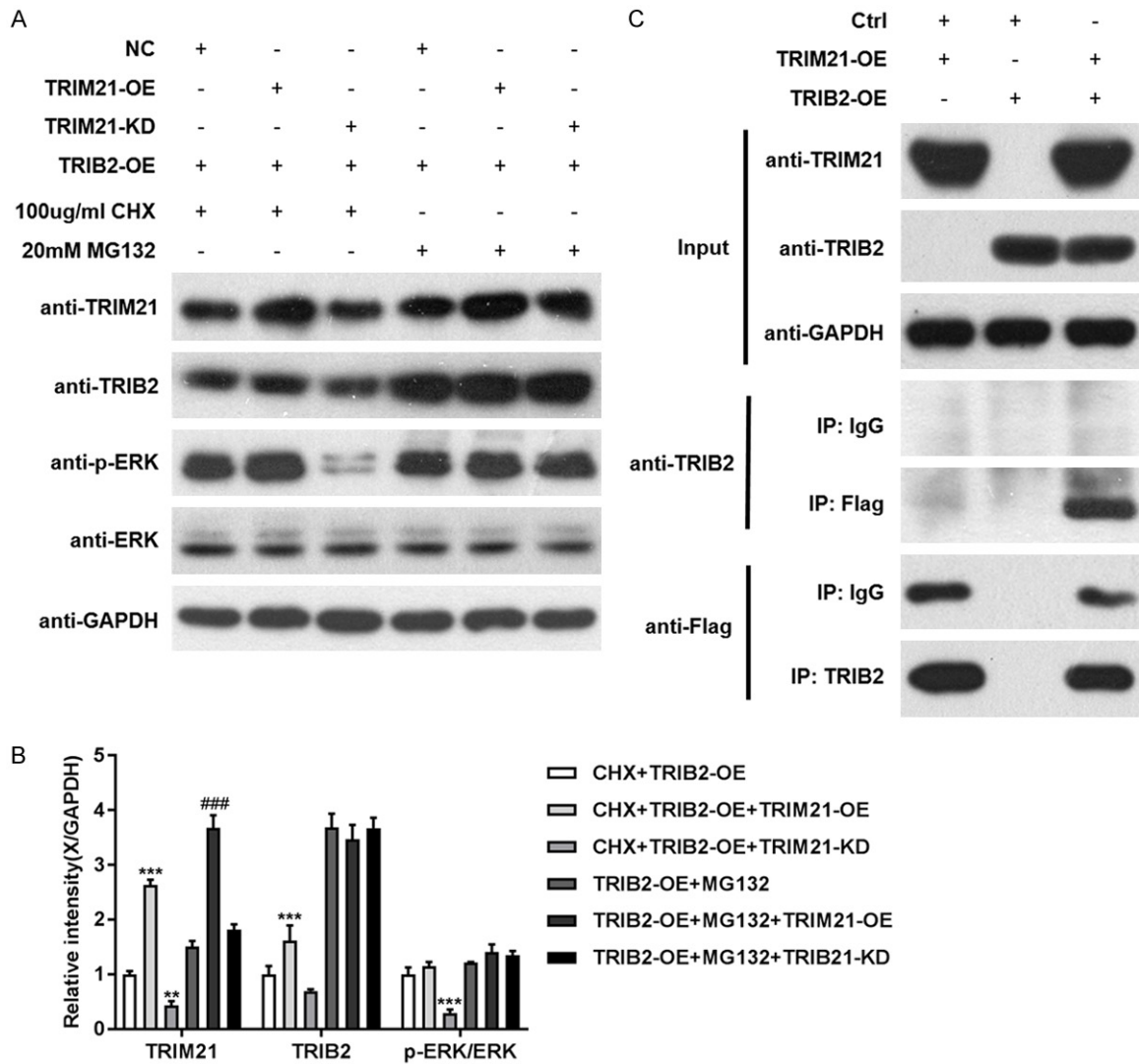


Figure 6. A, B. Western blot was used to detect the change of TRIB2, p-ERK, and ERK protein expression. C. Co-immunoprecipitation and western blot analysis validated TRIM21 and TRIB2 protein interactions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the Ctrl group.

was relatively higher ($P < 0.001$). Furthermore, the ratio of IFN- γ /IL-4 was significantly reduced ($P < 0.001$), which means there is a Th1/Th2 imbalance in OLP patients. Both IFN- γ and IL-4 are the co-immune etiological factors of different types of OLP.

Conclusions

In conclusion, our research revealed the key role of TRIM21 in patients with OLP. To our knowledge, this is the first study investigating the underlying mechanism of TRIM21 in the inflammatory response of OLP pathogenesis. Despite the occurrence and development of OLP involve in a variety of immune-active cells,

inflammatory cytokines and interferon conversion factors, the specific mechanism still needs to be further studied.

Acknowledgements

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Disclosure of conflict of interest

None.

TRIM21 affects IL-6 expression in oral lichen planus

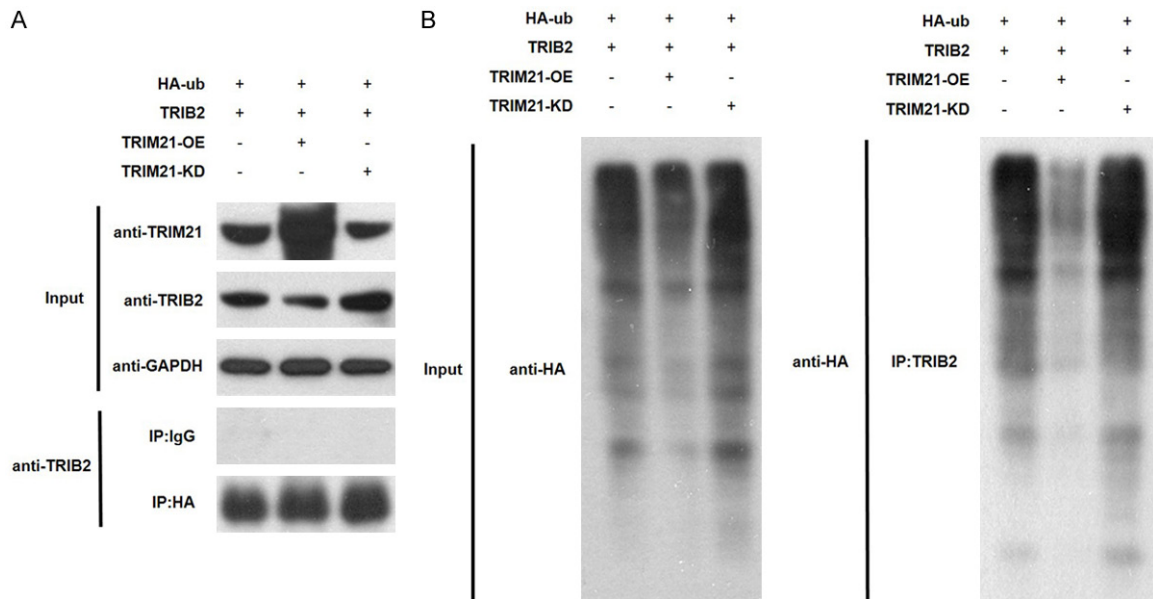


Figure 7. TRIM21 regulated the level of TRIB2 by inhibited the ubiquitination of TRIB2. A. Cell lysates were immuno-precipitated with anti-HA to detect the TRIM21 and TRIB2 proteins expression. B. TRIB2 was immunoprecipitated using an anti-HA antibody and the TRIB2 ubiquitination were detected by western blot.

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