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RIPK4 activates an IRF6-mediated proinflammatory cytokine response in keratinocytes

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Running Title: RIPK4-elicited keratinocyte inflammation

Abbreviations: RIPK4, receptor-interacting protein kinase 4; IRF6, interferon regulatory factor 6; PKC, protein kinase C.

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ABSTRACT

Keratinocytes of the oral mucosa and epidermis play key roles in host defense. In addition to functioning as a physical barrier, they also produce cytokines to elicit inflammation in response to infection or injury. We recently established that receptor-interacting protein kinase 4 (RIPK4) and interferon regulatory factor 6 (IRF6) function as a cell-intrinsic signaling axis to regulate keratinocyte differentiation. In this study, we have demonstrated a functional relationship between RIPK4 and IRF6 in the control of proinflammatory cytokine expression in keratinocytes. The overexpression of RIPK4 by oral keratinocytes induced the strong expression of CCL5 and CXCL11. In contrast, the expression of other cytokines (e.g. IL8 and TNF) was largely unaffected, thus demonstrating specificity in the induction of proinflammatory cytokine expression by RIPK4. CCL5 and CXCL11 expression were also induced in response to the activation of the PKC pathway, and gene silencing experiments indicated that their inducible expression was dependent on RIPK4 and IRF6. Moreover, gene reporter assays suggested that RIPK4 induces CCL5 and CXCL11 expression by stimulating the transactivation of their promoters by IRF6. Accordingly, our findings suggest that the RIPK4-IRF6 signaling axis plays a multifaceted role in barrier epithelial homeostasis through its regulation of both keratinocyte inflammation and differentiation.

Keywords: Keratinocyte; CCL5; CXCL11; RIPK4; IRF6.

1. Introduction

The oral mucosa and epidermis function as protective barriers against mechanical and chemical damage and biological insults (e.g. microbial pathogens) [1]. The homeostatic maintenance of these barriers requires a delicate balance between proliferation and differentiation where epithelial stem cells in the basal layer periodically withdraw from the cell cycle and differentiate into keratinocytes as they migrate towards the surface [2]. When the integrity of these barriers is compromised, for instance following injury, a wound healing response is triggered. The re-epithelialization phase of the response involves the proliferation and migration of keratinocytes to the site of injury, and differentiation to restore barrier integrity [3].

Keratinocytes also play active roles in providing protection against infection [4]. They express various pattern recognition receptors, including Toll-like receptors (TLRs), which positions them to carry out immunosurveillance [5]. Keratinocytes respond to pathogens by producing cytokines, including interleukin-8 (IL8) and chemokine (C-C motif) ligand 5 (CCL5), which elicit local inflammation through the activation and recruitment of immune cells. IL8 is a key mediator of the activation and recruitment of neutrophils to sites of infection [6], and is critical for periodontal homeostasis due to the presence of a polymicrobial biofilm that accretes to the surface of the teeth [7]. CCL5, whose expression is likewise upregulated in activated keratinocytes [8], is chemotactic for various immune cell populations, including T cells, monocytes, eosinophils, and mast cells [9]. Despite being important mediators of host protection, the overproduction of cytokines by keratinocytes also contributes to the pathogenesis of several chronic inflammatory diseases (e.g. periodontitis, psoriasis, and atopic dermatitis) [7, 10, 11].

Receptor-interacting protein kinase 4 (RIPK4) is a key regulator of keratinocyte differentiation, as indicated by the disorganized and expanded epidermis of Ripk4-deficient mice [12]. Moreover, mutations in RIPK4 cause Bartsocas-Papas syndrome, a congenital

syndrome that is characterized by severe epidermal abnormalities, and typically causes death early in life [13, 14]. We recently demonstrated that RIPK4, which functions in the protein kinase C (PKC) pathway [15, 16], regulates keratinocyte differentiation by directly stimulating the transactivator function of interferon regulatory factor 6 (IRF6) [17]. IRF6 is a key transcriptional regulator of keratinocyte differentiation and regulates the switch from proliferation to differentiation by transactivating differentiation-associated genes (e.g. Grainyhead-Like 3) [17-20]. RIPK4 has also been shown to stimulate the activation of NF-κB [13, 16, 17, 21, 22], which is also a key transcriptional regulator of epithelial homeostasis [23].

Significantly, RIPK4 function may also extend to the regulation of inflammation. Mice with epidermal-specific expression of a Ripk4 transgene developed spontaneous inflammation that was exacerbated further by the topical application of the well-established skin irritant and PKC agonist, phorbol myristate acetate [24]. Given that RIPK4 and IRF6 function as a signaling axis downstream of PKC activation to promote keratinocyte differentiation [17], we investigated if the pathway also regulates proinflammatory cytokine expression in keratinocytes. The overexpression of RIPK4 in keratinocytes was sufficient to induce the strong expression of CCL5 and chemokine (C-X-C motif) ligand 11 (CXCL11). Moreover, gene silencing and gene reporter experiments indicated that RIPK4 and IRF6 function together in the PKC pathway to stimulate CCL5 and CXCL11 expression. Therefore, our findings potentially extend the RIPK4-IRF6 signaling axis to the regulation of inflammation of the oral mucosa and epidermis through its control of cytokine expression in keratinocytes.

2. Materials and methods

2.1 Reagents

Cell culture medium (Keratinocyte serum-free medium, Dulbecco's-modified Eagle's medium (DMEM), and Opti-MEM I reduced serum medium) and supplements (human EGF, bovine

pituitary extract, penicillin/streptomycin, and GlutaMax-1), fetal bovine serum (FBS), Lipofectamine 2000 and Lipofectamine RNAiMAX transfection reagents, and the Silencer Select RIPK4 siRNA were from Life Technologies. KGM-Gold keratinocyte growth medium was from Lonza. The ON-TARGETplus IRF6 siRNA was from GE Healthcare. FuGENE 6 transfection reagent was from Promega. Phorbol myristate acetate, and the HRP-conjugated anti-FLAG (M2) antibody were from Sigma-Aldrich. The anti-HSP90 antibody was from BD Biosciences, and the human IL8 and CCL5 ELISA kits were from Life Research.

2.2 Expression plasmids

The expression plasmids encoding FLAG-tagged mouse Ripk4 [15] and HA-tagged human IRF6 [25] have been described.

2.3 Cell culture

The human oral keratinocyte cell line, OKF6/TERT-2, was cultured in Keratinocyte serum-free medium supplemented with 0.2 ng/ml human EGF, 25 µg/ml bovine pituitary extract, 0.4 mM $CaCl_2$, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM GlutaMax-1. Normal human epidermal keratinocytes were cultured in KGM-Gold medium according to the protocol provided by the supplier (Lonza). HEK293T cells were cultured in DMEM medium supplemented with 10% FBS, 100 units/ml Penicillin, 100 µg/ml Streptomycin, and 2 mM GlutaMax-1. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

2.4 Overexpression and siRNA-mediated gene silencing

A reverse-transfection protocol was used for the transfection of OKF6/TERT-2 cells and primary human epidermal keratinocytes. For overexpression, the Ripk4 plasmid was diluted in 100 µl Opti-MEM I reduced serum medium, mixed with 100 µl Opti-MEM medium containing 1 µl Lipofectamine 2000 transfection reagent, and incubated at room temperature

for 15-20 min. For gene silencing, the siRNA was diluted to 120 nM with 100 µl Opti-MEM I medium, mixed with 100 µl Opti-MEM I medium containing 1 µl Lipofectamine RNAiMAX transfection reagent, and incubated at room temperature for 15-20 min. Keratinocytes (5×10^5 cells for overexpression; 2×10^5 cells for gene silencing) in 1 ml of antibiotic-free growth medium were seeded into 12-well plates and the transfection cocktail added. For gene overexpression experiments, the cells were analyzed 24 h post-transfection. For gene silencing experiments, the medium was replaced after 16 h, and the cells treated with phorbol myristate acetate 48 h (OKF6/TERT-2 cells) or 72 h (normal human epidermal keratinocytes) post-transfection.

2.5 Phorbol myristate acetate stimulation

OKF6/TERT-2 cells and normal human epidermal keratinocytes were allowed to adhere overnight. Thereafter, the cells were stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) in dimethylsulfoxide (DMSO), while time-matched control cells were treated with 0.1% DMSO.

2.6 RNA purification and reverse-transcription

Total RNA was purified using the ReliaPrep RNA Cell miniprep system (Promega), which includes an on-column DNase-treatment step. RNA was reverse-transcribed into cDNA using GoScript Reverse Transcriptase (Promega) and random primers.

2.7 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed in triplicate using GoTaq Probe qPCR Master Mix (Promega) and pre-developed TaqMan assays (Life Technologies) for the following genes: CCL5 (Hs00174575_m1), CXCL11 (Hs00171138_m1), IL8 (Hs00174103_m1), IRF6 (Hs00196213_m1), RIPK4 (Hs01062501_m1), Ripk4

147 (Mm00458366_m1), and TNF (Hs0113624_g1). Real-time PCR was performed on an ABI
148 PRISM 7900HT (Applied Biosystems) or QuantStudio 7 Flex (Life Technologies), and the
149 data normalized against HPRT gene expression [26].
150
151 *2.8 Cell lysis and Western blotting*
152 Cells were washed twice with ice-cold PBS, and then lysed (20 mM Tris-HCl (pH 7.4), 150
153 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, 10 mM NaF, 10 mM β-glycerol
154 phosphate, and Complete™ protease inhibitors) on ice for 60 min. Thereafter, the lysates
155 were clarified by centrifugation (13,000 x *g* for 10 min at 4°C) and protein concentrations
156 measured using a protein assay kit (Bio-Rad). Lysates were subjected to electrophoresis on
157 10% Nu-PAGE gels, followed by Western blotting according to standard protocols.
158 Immunoreactive bands were visualized using ECL reagents (Millipore) and a LAS-3000
159 Imager (Fujifilm).
160
161 *2.9 Enzyme-linked immunosorbent assays (ELISA)*
162 ELISA assays were performed by incubating diluted culture supernatants and standards in 96-
163 well microplates for 2 h. The wells were washed prior to the addition of biotinylated anti-IL8
164 and anti-CCL5 antibodies according to the manufacturer's instructions. After incubation at
165 room temperature for 2 h, the plates were washed and then incubated with a streptavidin-HRP
166 conjugate for 45 min. The plates were again washed, 3,3',5,5'-tetramethylbenzidine substrate
167 added, and color development measured at 450 nm using a microplate reader (Model 680,
168 Bio-Rad).
169
170 *2.10 Gene promoter reporter assays*
171 HEK293T cells were seeded in 12-well tissue culture plates (3×10^5 cells per well) and
172 transfected in duplicate the next day using FuGENE 6 transfection reagent. The total amount

of plasmid in each transfection was kept constant using empty plasmid. The cells were lysed 24 h post-transfection with Passive Lysis Buffer (Promega), and assayed for firefly and *Renilla* luciferase activity using the Dual-Glo luciferase assay system and a GloMax 96 microplate luminometer (Promega, USA). *Renilla* luciferase activity was used to normalize transfection efficiencies. The luciferase-based CCL5, CXCL11, and IL8 gene promoter reporter plasmids were as previously reported [27-29]. The pRL-TK luciferase reporter plasmid was from Promega.

2.11 Statistical analysis

Data combined from at least three independent experiments are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism software version 6.01 (GraphPad Software). Differences between two groups were evaluated using the Student's *t* test. For multiple comparisons, statistical analysis was performed using a one-way analysis of variance (ANOVA). A p value <0.05 was considered to be statistically significant.

3. Results

3.1 Overexpression of RIPK4 induces proinflammatory cytokine expression in keratinocytes

Although RIPK4 is a key regulator of keratinocyte differentiation [12, 17], its transgenic expression in the epidermis of mice causes spontaneous inflammation [24]. Therefore, we investigated if the overexpression of RIPK4 in keratinocytes was sufficient to induce the expression of proinflammatory cytokines. Briefly, OKF6/TERT-2 human oral keratinocytes (hereafter referred to as OKF6 cells) were transfected with a plasmid expressing FLAG-tagged mouse Ripk4. The overexpression of FLAG-Ripk4 was confirmed by qPCR (**Fig. 1A**) and Western blotting with an anti-FLAG antibody (**Fig. 1B**). The overexpression of Ripk4 in OKF6 cells resulted in a 3-fold increase in IL8 mRNA levels (**Fig. 1C**). IL8 protein levels in the culture medium were also moderately increased (**Fig 1D**). Tumor necrosis factor (TNF)

mRNA levels were also weakly affected by the overexpression of Ripk4 (**Fig. 1E**). In contrast to IL8 and TNF, however, CCL5 gene expression was strongly induced (>150-fold) in response to the overexpression of Ripk4 (**Fig. 1F**); CCL5 protein levels in the culture medium were also greatly increased (**Fig. 1G**). Similarly, the overexpression of Ripk4 resulted in the strong induction (>900-fold) of CXCL11 gene expression (**Fig. 1H**). These data strongly suggest that RIPK4 is a key regulator of CCL5 and CXCL11 gene expression in keratinocytes.

3.2 Activation of the PKC pathway induces CCL5 and CXCL11 expression in keratinocytes

RIPK4 has been shown to function downstream of PKC [15, 16], for example to promote keratinocyte differentiation [17]. Given our findings above, we tested the ability of PKC to regulate CCL5 and CXCL11 gene expression. The stimulation of OKF6 cells with the PKC agonist phorbol myristate acetate (PMA) resulted in the strong upregulation of CCL5 gene expression (**Fig. 2A**). The expression levels of CXCL11 were also found to be strongly upregulated in PMA-treated cells (**Fig. 2B**). In contrast to the overexpression of Ripk4 (**Fig. 1**), the stimulation of OKF6 cells with PMA induced strong IL8 (**Fig. 2C**) and TNF (**Fig. 2D**) gene expression. We also examined the effects of PMA stimulation on RIPK4 gene expression. Although declining moderately initially, RIPK4 mRNA levels returned to pre-stimulus levels by 6 h post-PMA stimulation, and had moderately increased after 24 h of PMA stimulation (**Fig. 2E**). Collectively, these findings indicate that the PKC pathway regulates the expression of CCL5 and CXCL11, along with IL8 and TNF, in keratinocytes.

3.3 RIPK4 regulates the induction of proinflammatory cytokine expression by the PKC pathway in keratinocytes

To determine if RIPK4 was necessary for the upregulation of CCL5, CXCL11, IL8 and TNF gene expression by the PKC pathway, RIPK4 expression was silenced prior to stimulating the

cells with PMA. The transfection of OKF6 cells with a RIPK4 siRNA, which reduced RIPK4 mRNA levels by >80% (**Fig. 3A**), strongly inhibited (>65%) the stimulation of CCL5 gene expression by PMA (**Fig. 3B**). Similarly, the induction of CXCL11 expression was also greatly inhibited in cells transfected with the RIPK4 siRNA (**Fig. 3C**). The silencing of RIPK4 also inhibited the PMA-inducible expression of IL8 (**Fig. 3D**) and TNF (**Fig. 3E**). The same gene silencing approach was taken to establish if RIPK4 likewise regulates the PMA-inducible expression of these cytokines in epidermal keratinocytes. The silencing of RIPK4 gene expression in normal human epidermal keratinocytes consistently reduced PMA-inducible CCL5 expression (**Fig. 3F**). As was the case for oral keratinocytes (e.g. OKF6 cells), the silencing of RIPK4 in epidermal keratinocytes also inhibited the PMA-inducible expression of CXCL11 (**Fig. 3G**) and IL8 (**Fig. 3H**). Together these results indicate that RIPK4 functions downstream of PKC to regulate the expression of proinflammatory cytokines in both oral and epidermal keratinocytes.

3.4 IRF6 regulates the induction of proinflammatory cytokine expression by the PKC pathway in keratinocytes

We recently established that RIPK4 promotes keratinocyte differentiation by directly stimulating the transactivator function of IRF6 [17]. IRF6 also functions in the TLR2 pathway to stimulate proinflammatory cytokine expression in oral keratinocytes [25]. Therefore, a role for IRF6 in mediating the expression of proinflammatory cytokines in response to PKC activation was investigated. The transfection of OKF6 cells with an IRF6 siRNA, which reduced IRF6 expression by >75% (**Fig. 4A**), strongly inhibited (>80%) the stimulation of CCL5 gene expression by PMA (**Fig. 4B**). Likewise, the silencing of IRF6 also inhibited the PMA-inducible expression of CXCL11 (**Fig. 4C**) and IL8 (**Fig. 4D**). In contrast, the stimulation of TNF gene expression was not significantly affected (data not shown). Comparable findings were obtained when IRF6 expression in normal human epidermal

keratinocytes was silenced. Specifically, the silencing of IRF6 significantly inhibited the PMA-inducible expression of CCL5 (**Fig. 4E**), CXCL11 (**Fig. 4F**), and IL8 (**Fig. 4G**). These findings are consistent with IRF6 functioning together with RIPK4 to regulate the expression of specific proinflammatory cytokines (e.g. CCL5 and CXCL11) in response to the activation of the PKC pathway in keratinocytes.

3.5 RIPK4 stimulates the IRF6-mediated transactivation of the CCL5 and CXCL11 promoters

The regulation of CCL5, CXCL11 and IL8 gene expression by RIPK4 and IRF6 was investigated further by performing gene promoter reporter assays in HEK293T cells. As shown in **Fig. 5A**, the co-expression of Ripk4 and IRF6 resulted in the strong (>50-fold) transactivation of the CCL5 promoter. Ripk4 activated only weakly (<10-fold) the gene reporter in the absence of co-expressed IRF6, and IRF6 was unable to activate the reporter in the absence of co-transfected Ripk4 (**Fig. 5A**). The co-expression of Ripk4 resulted in the strong (>30-fold) transactivation of the CXCL11 promoter by IRF6 (**Fig. 5B**). Again, Ripk4 activated only weakly the CXCL11 gene reporter in the absence of co-transfected IRF6 (**Fig. 5B**). In contrast to the CCL5 and CXCL11 gene reporters, Ripk4 strongly activated (>150-fold) the IL8 gene reporter in the absence of co-expressed IRF6 (**Fig. 5C**), and co-transfection of IRF6 resulted in a small, but significant, additional increase in reporter activity (**Fig. 5C**). These findings suggest that RIPK4 regulates CCL5 and CXCL11 gene expression by stimulating the transactivation of their promoters by IRF6.

4. Discussion

In addition to being important for maintaining the barrier functions of the oral mucosa and epidermis, keratinocytes also play active roles in host defense by producing cytokines that elicit inflammation in response to infection or injury [4, 5]. RIPK4 has previously been shown

to be a key regulator of keratinocyte differentiation [12, 17]. In the current study, we identified an additional role for RIPK4 in keratinocytes, namely in regulating the expression of proinflammatory cytokines. Therefore, RIPK4 likely plays a multifaceted role in the maintenance of epithelial homeostasis, and thus host defense, through its regulation of both keratinocyte inflammation and differentiation.

A role for RIPK4 in regulating the inflammatory properties of keratinocytes was suggested by the finding that the overexpression of RIPK4 in oral keratinocytes (e.g. OKF6 cells) was sufficient to induce the strong expression of CCL5 and CXCL11. Notably, the expression of other proinflammatory cytokines (e.g. IL8 and TNF) was largely unaffected, indicating that the induction of CCL5 and CXCL11 gene expression was directly and specifically correlated with increased RIPK4 levels. Therefore, RIPK4 activation in response to infection or injury is likely to be important in mediating the expression of CCL5 and CXCL11 by keratinocytes.

We recently demonstrated that RIPK4 mediates keratinocyte differentiation downstream of PKC activation by forming a signaling axis with IRF6 to induce the expression of differentiation-associated genes (e.g. GRHL3) [17]. Our findings here suggest that RIPK4 likewise cooperates with IRF6 to mediate the stimulation of proinflammatory cytokine expression by the PKC pathway in keratinocytes. Specifically, RIPK4 and IRF6 were shown to be necessary for the stimulation of CCL5 and CXCL11 gene expression by PMA. In line with their expression being directly regulated by the RIPK4-IRF6 pathway, the co-expression of RIPK4 and IRF6 induced the synergistic transactivation of the CCL5 and CXCL11 gene promoters. RIPK4 also stimulates the transactivator functions NF-κB and AP-1 downstream of PKC activation [16, 17, 21]. We have shown previously that IRF6 functions in concert with NF-κB to induce CCL5 gene expression downstream of TLR2 activation in oral keratinocytes (e.g. OKF6 cells) [25]. Similarly, IRF1 functionally cooperates with NF-κB to regulate respiratory syncytial virus-inducible CCL5 expression in lung epithelial cells [30], and

interferon-inducible CXCL11 expression in B-lymphoblast cells [31]. Therefore, RIPK4 likely induces the expression of CCL5 and CXCL11 in keratinocytes by activating the transactivator functions of both NF-κB and IRF6.

RIPK4 and IRF6 were also shown to be important for the PMA-inducible expression of IL8 in keratinocytes. In addition to NF-κB and AP-1 [29, 32, 33], IRFs have also been demonstrated to play a role in regulating IL8 gene expression in epithelial cells [34-36]. Similar to CCL5 and CXCL11, maximal stimulation of IL8 gene expression by the PKC pathway in keratinocytes is likely to require functional cooperation between NF-κB, AP-1 and IRF6 downstream of RIPK4 activation. Interestingly, RIPK4 was shown by gene reporter assay in HEK293T cells to activate the IL-8 promoter in the absence of IRF6. However, the co-transfection of IRF6 potentiated the activation of the IL8 gene reporter by RIPK4, and therefore HEK293T cells may express IRF family members that can functionally compensate for IRF6 to mediate the transactivation of the IL8 promoter.

The IRF6-dependent stimulation of IL8 expression by PKC in OKF6 cells contrasted with its IRF6-independent regulation by TLR2 [25]. The differences in the dependence on IRF6 for the activation of IL8 transcription may provide a mechanism for imparting signaling specificity to the stimulation of IL8 production by the PKC and TLR2 pathways in keratinocytes. This may in turn be important in dictating the nature of the inflammatory responses elicited by different stimuli. However, further studies are required to fully understand the mechanisms of IRF6 action in regulating IL8 expression.

The overexpression of RIPK4 in OKF6 cells was not sufficient to induce TNF gene expression, despite the fact that TNF expression is largely regulated by NF-κB and AP-1 [37, 38]. However, RIPK4 was necessary for the PMA-inducible expression of TNF. This indicates that PKC likely stimulates TNF expression via both RIPK4-dependent and RIPK4-independent pathways. Notably, IRF6 was not required for the simulation of TNF expression by PKC. IRF5 has been shown to functionally cooperate with NF-κB in regulating TNF gene

expression by the TLR4 pathway in myeloid cells [39]. Therefore, IRFs may contribute to the differential regulation of TNF expression in a cell-type or stimulus dependent manner.

CCL5 stimulates the recruitment of various immune cell populations, including T cells, monocytes, eosinophils, and mast cells [40, 41]. CXCL11 acts via chemokine (C-X-C motif) receptor 3 (CXCR3) to stimulate the recruitment of inflammatory T cells (e.g. Th1 and Th17 cells) [42], whereas IL8 is a key regulator of the trafficking and homing of neutrophils [6]. Mouse models of infection have demonstrated that CCL5, CXCL11 and IL8 are important mediators of host defense [43-49]. Accordingly, our findings potentially position RIPK4 as a key regulator of the inflammatory responses of the oral mucosa and epidermis to microbial pathogens through its control of IRF6-mediated inflammatory cytokine expression in keratinocytes. Such a conclusion is consistent with the finding that the transgenic expression of *Ripk4* in the epidermis of mice caused spontaneous inflammation [24], and exacerbated the epidermal inflammation induced by the topical application of the well-established skin irritant PMA.

We recently demonstrated a role for IRF6 in mediating the inflammatory cytokine responses of oral keratinocytes to *Porphyromonas gingivalis* [50], a keystone pathogen in periodontitis. In this context, IRF6 transactivator function was regulated at least in part by IL-1R-associated kinase 1. However, the findings presented in this study raise the possibility that RIPK4 may also play a role in promoting oral inflammation to *P. gingivalis*, and potentially other pathogens, through its activation of IRF6.

In addition to producing proinflammatory cytokines, activated keratinocytes are also migratory and hyperproliferative, and facilitate re-epithelialization following damage to surface epithelia (e.g. oral mucosa and epidermis). Once re-epithelialization is complete, the activated keratinocytes revert to differentiation to restore the functional integrity of the epithelium [51]. In addition to T cells, CXCL11 can also stimulate keratinocyte migration [52, 53], and CXCL11 has been shown to be produced by keratinocytes in an excisional wound

healing model [54]. Significantly, wound closure and re-epithelialization was delayed in Cxcl11-deficient [52] and Cxcr3-deficient mice [54]. However, given that T cells can produce growth factors which stimulate keratinocyte and fibroblast proliferation [55, 56], CXCL11 may promote wound healing by acting in both an autocrine and paracrine manner. Therefore, in addition to its regulation of keratinocyte differentiation, RIPK4 may also promote wound healing by stimulating wound closure and re-epithelialization through its regulation of CXCL11 expression.

In conclusion, our findings extend the RIPK4-IRF6 signaling axis to the regulation of proinflammatory cytokine expression, particularly in oral keratinocytes. As the oral epithelium is pivotal as both a physical barrier and an immune tissue, this potentially places RIPK4 in the signaling framework as an important regulator of the inflammatory response of the oral mucosa following infection or injury. Moreover, by governing both keratinocyte inflammation and differentiation, RIPK4 may function as a key regulatory nodal point in the maintenance of epithelial homeostasis. For the same reason, because the dysregulation of this signaling framework may lead to chronic inflammation, future studies aimed at exploring the role of the RIPK4-IRF6 axis in the context of chronic inflammatory diseases offer the potential for therapeutic exploitation.

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Conflict of Interest

The authors declare no conflicts of interest.

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Figure Legends

Fig. 1. Overexpression of RIPK4 in human keratinocytes induces proinflammatory cytokine expression and secretion. OKF6 cells were transfected with a FLAG-Ripk4 expression plasmid or empty plasmid. Twenty-four h post-transfection, (A) Ripk4, (C) IL8, (E) TNF, (F) CCL5, and (H) CXCL11 mRNA levels were measured by qPCR. Ripk4 mRNA levels are shown relative to HPRT, and IL8, TNF, CCL5 and CXCL11 mRNA levels as the –fold increase over cells transfected with empty plasmid. (B) Cell lysates were subjected to Western blotting with anti-FLAG and anti-HSP90 (loading control) antibodies. (D) IL8 and (G) CCL5 protein levels in the cell culture medium were measured by ELISA. All data are combined from three independent experiments and presented as the mean ± SEM (** $p<0.01$,* $p<0.05$, ND = Not detected).

Fig. 2. Stimulation of proinflammatory cytokine expression in human keratinocytes by PMA. OKF6 cells were treated with PMA for the times indicated. (A) CCL5, (B) CXCL11, (C) IL8, (D) TNF, and (E) RIPK4 mRNA levels were measured by qPCR and are shown as the –fold increase over control cells. All data are combined from at least three independent experiments and presented as the mean ± SEM (** $p<0.01$, * $p<0.05$).

Fig. 3. RIPK4-dependent stimulation of proinflammatory cytokine expression in human keratinocytes by PMA. (A-E) OKF6 cells and (F-H) normal human epidermal keratinocytes were transfected with a RIPK4 (+) or control (-) siRNA. Forty-eight h post-transfection, (A) RIPK4 mRNA levels were measured by qPCR. RIPK4 mRNA levels in cells transfected with the control siRNA were given an arbitrary value of 100%. (B-H) The cells were treated with PMA for 2 h (TNF) or 6 h (CCL5, CXCL11 and IL8). (B and F) CCL5, (C and G) CXCL11, (D and H) IL8, and (E) TNF mRNA

563 levels were measured by qPCR and are shown as the –fold increase over control cells.
564 All data are combined from three independent experiments and presented as the mean ±
565 SEM (** $p<0.01$, * $p<0.05$).
566
567 **Fig. 4.** IRF6-dependent stimulation of proinflammatory cytokine expression in human
568 keratinocytes by PMA. (A-D) OKF6 cells and (E-G) normal human epidermal keratinocytes
569 were transfected with an IRF6 (+) or control (-) siRNA. Forty-eight h post-transfection, (A)
570 IRF6 mRNA levels were measured by qPCR. IRF6 mRNA levels in cells transfected with the
571 control siRNA were given an arbitrary value of 100%. (B-G) The cells were treated with
572 PMA for 2 h (TNF) or 6 h (CCL5, CXCL11 and IL8). (B and E) CCL5, (C and F) CXCL11,
573 and (D and G) IL8 mRNA levels were measured by qPCR and are shown as the –fold increase
574 over control cells. All data are combined from three independent experiments and presented
575 as the mean ± SEM (** $p<0.01$, * $p<0.05$).
576
577 **Fig. 5.** RIPK4 stimulates the transactivation of the CCL5 and CXCL11 gene promoters by
578 IRF6. HEK293T cells were transfected with an (A) CCL5, (B) CXCL11, and (C) IL8 gene
579 promoter reporter plasmid together with plasmids expressing Ripk4 and IRF6. Gene reporter
580 activity was measured 24 h post-transfection and shown as the –fold increase over cells
581 transfected with empty plasmid. All data are combined from three independent experiments
582 and presented as the mean ± SEM (** $p<0.01$, * $p<0.05$).

Figure 1

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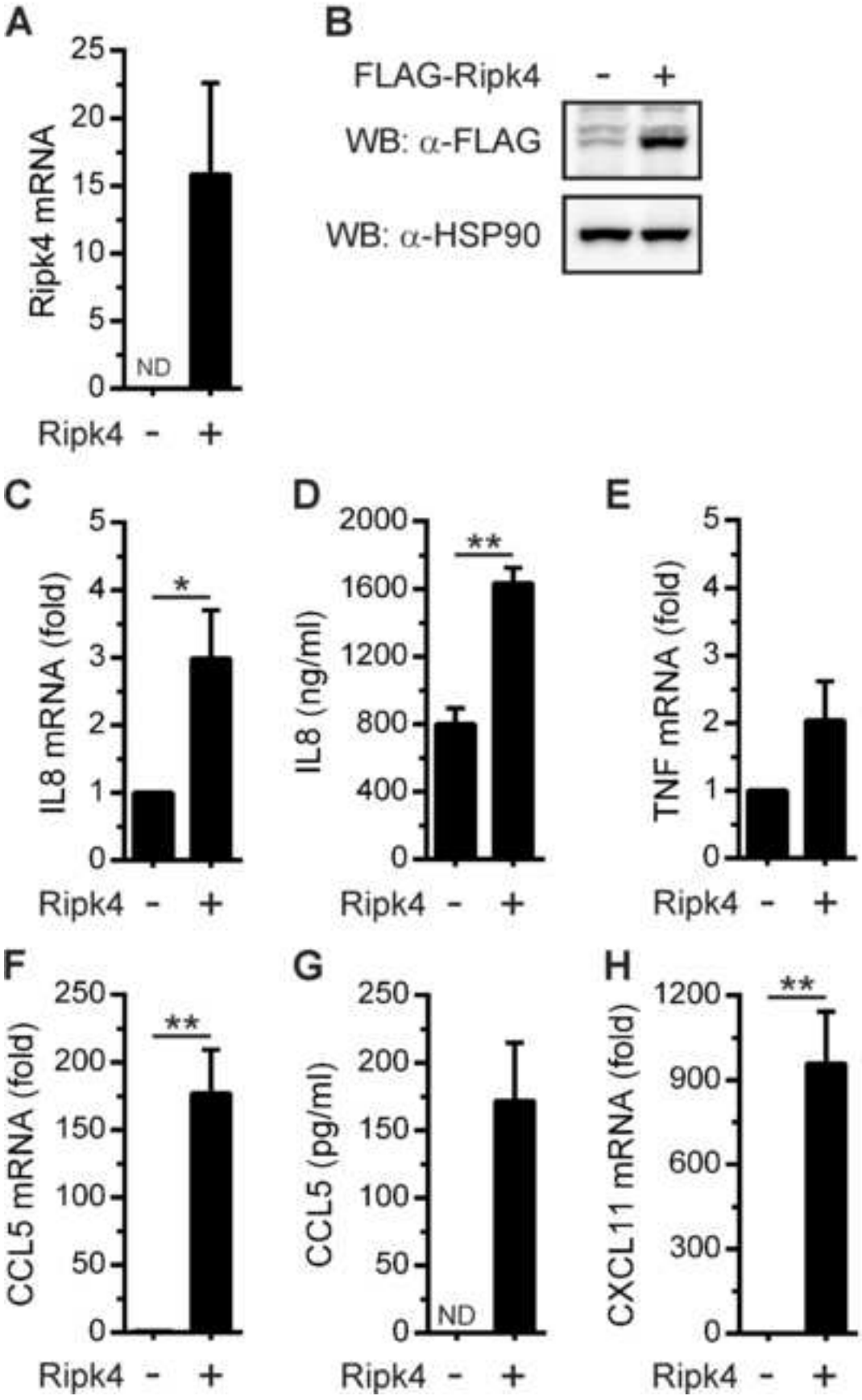

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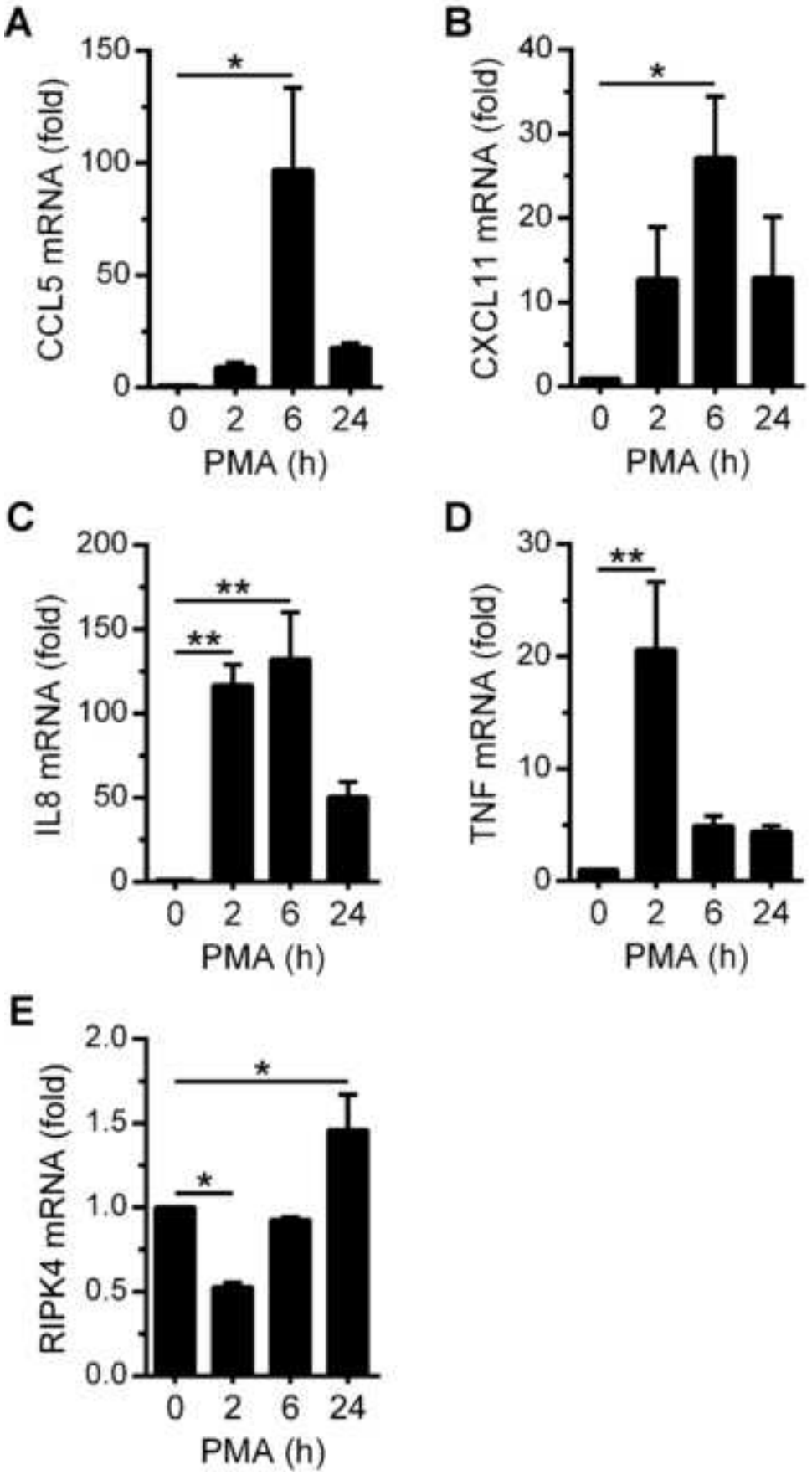

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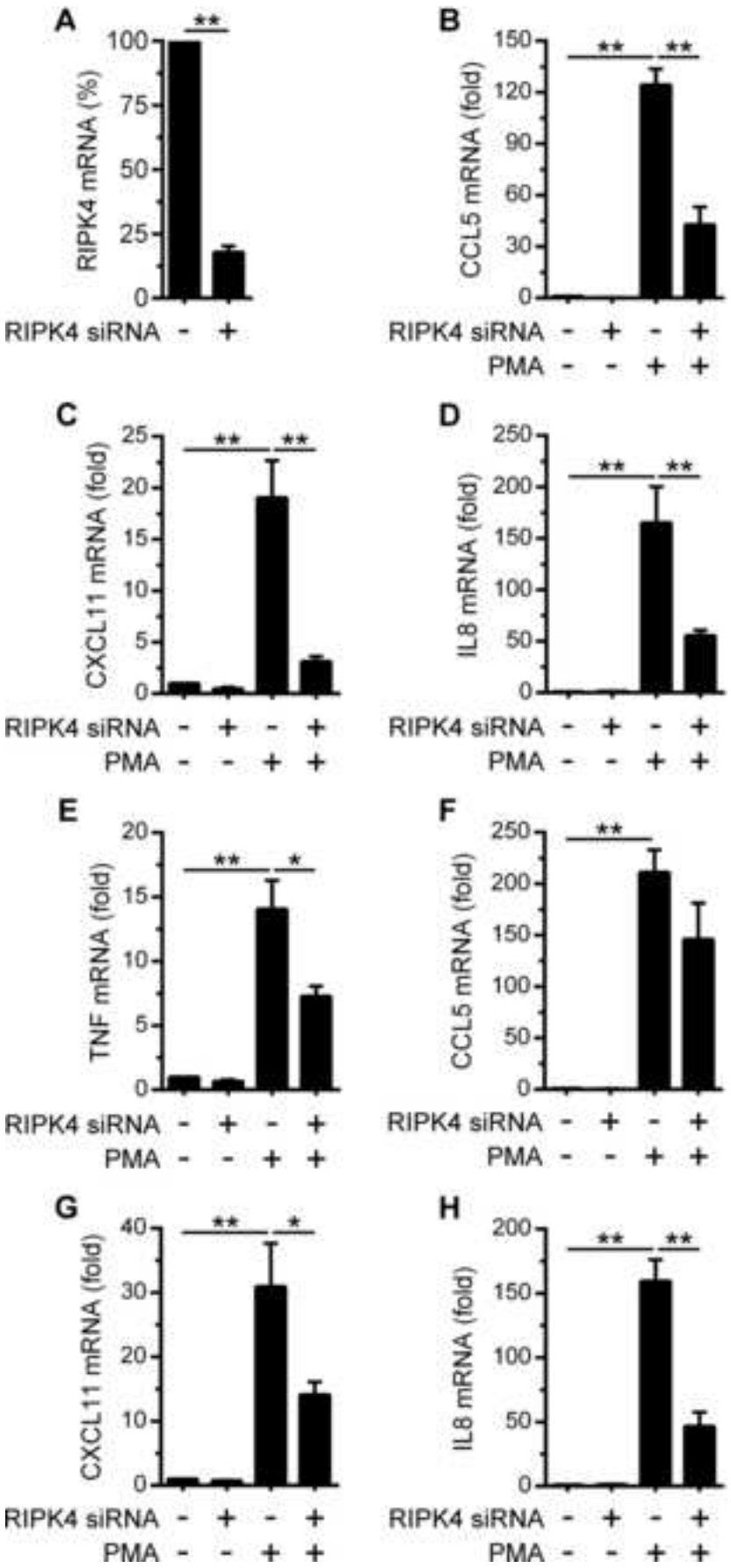

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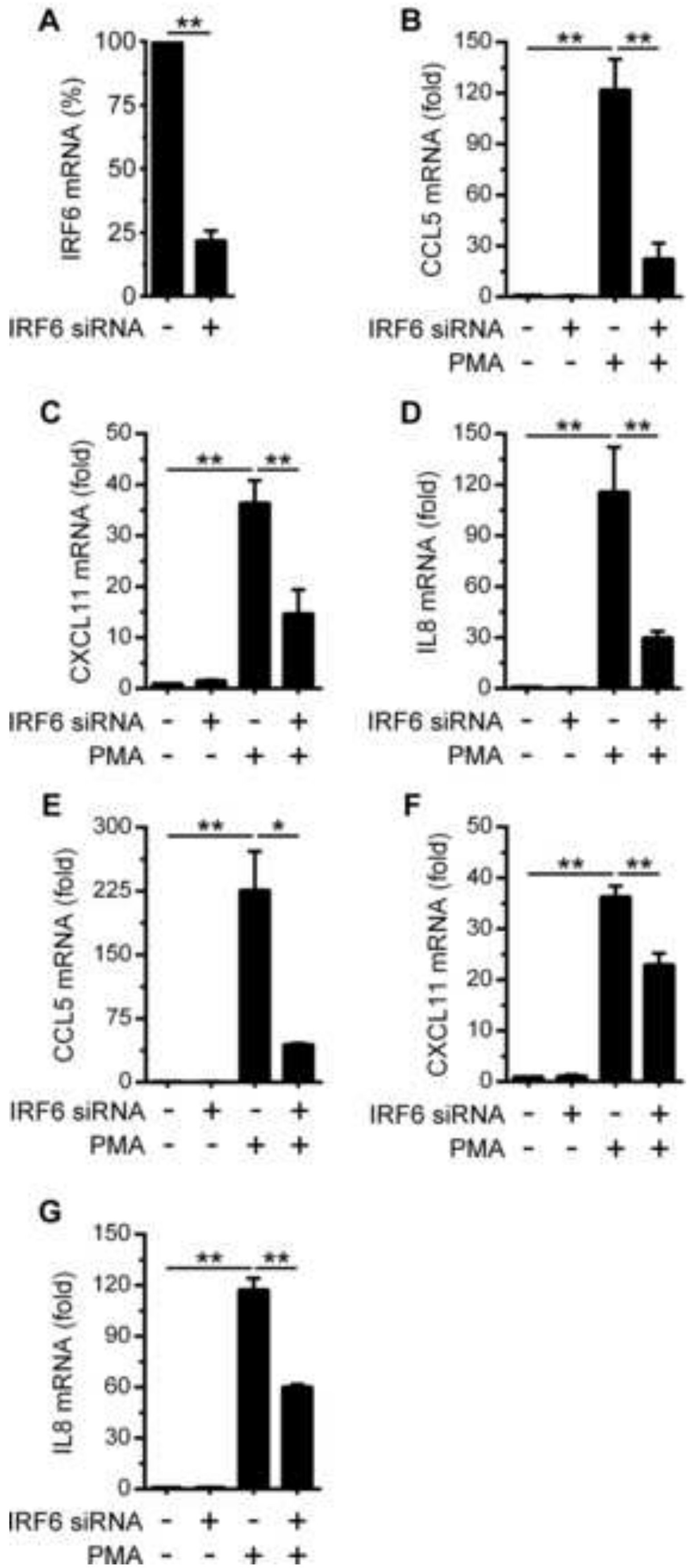

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