Regulation of the Psoriatic Chemokine CCL20 by E3 Ligases Trim32 and Piasy in Keratinocytes

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Psoriasis is an inflammatory skin disorder with aberrant regulation of keratinocytes and immunocytes. Although it is well known that uncontrolled keratinocyte proliferation is largely driven by proinflammatory cytokines from the immunocytes, the functional role of keratinocytes in the regulation of immunocytes is poorly understood. Recently, we found that tripartite motif-containing protein 32 (Trim32), an E3-ubiquitin ligase, is elevated in the epidermal lesions of human psoriasis. We previously showed that Trim32 binds to the protein inhibitor of activated STAT-Y (Piasy) and mediates its degradation through ubiquitination. Interestingly, the Piasy gene is localized in the *PSORS6* susceptibility locus on chromosome 19p13, and Piasy negatively regulates the activities of several transcription factors, including NF-κB, STAT, and SMADs, that are implicated in the pathogenesis of psoriasis. In this study, we show that Trim32 activates, and Piasy inhibits, keratinocyte production of CC chemokine ligand 20 (CCL20), a psoriatic chemokine essential for recruitment of DCs and T helper (Th)17 cells to the skin. Further, Trim32/Piasy regulation of CCL20 is mediated through Piasy interaction with the RelA/p65 subunit of NF-κB. As CCL20 is activated by Th17 cytokines, the upregulation of CCL20 production by Trim32 provides a positive feedback loop of CCL20 and Th17 activation in the self-perpetuating cycle of psoriasis.

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INTRODUCTION

CC chemokine ligand 20 (CCL20) is an inflammatory chemokine responsible for recruitment of leukocytes to sites of injury and inflammation. It is mainly expressed in surface epithelial cells such as keratinocytes (Homey *et al.*, 2000), bronchial epithelial (Reibman *et al.*, 2003), and intestinal epithelial cells (Fujiie *et al.*, 2001). CCL20 expression from keratinocytes is induced by proinflammatory cytokines such as tumor necrosis factor (TNFα), IL-1, and IL-17 (Homey *et al.*, 2000; Nograles *et al.*, 2008; Harper *et al.*, 2009). In contrast to the promiscuous nature of many other chemokines and their receptors, CC chemokine receptor6 (CCR6) is the only known receptor for CCL20. CCR6 is expressed in a variety of immunocytes, including neutrophils, memory B-cells, Treg, immature dendritic cells (DCs), and T helper (Th)17 cells. CCL20/CCR6 play important roles in the chemostasis of immunocytes, particularly for those in the Th17 pathway. CCR6 is induced by the nuclear receptor RORgammat (Manel *et al.*, 2008), and found in virtually all Th17 cells (Singh *et al.*, 2008). Moreover, CCR6 is essential for the pathogenesis of encephalomyelitis and psoriasis in animal models (Hedrick *et al.*, 2009; Reboldi *et al.*, 2009).

Th17 activation is a defense mechanism against extracellular infection in tissues (Ghilardi and Ouyang, 2007). The receptors for Th17 cytokines are expressed in a variety of epithelial cells, including keratinocytes. Th17 cytokines contribute to tissue immunity through (1) production of antimicrobial peptides, (2) recruitment of immunocytes through induction of chemokines such as CCL20, and (3) tissue repair by enhancing epithelial proliferation (Zheng *et al.*, 2007). The Th17-signaling pathway is tightly controlled to ensure tissue homeostasis, and is terminated after infection is ablated and tissue repair is complete. However, Th17 signaling is persistently activated in psoriasis (Blauvelt, 2008). Recruitment of Th17 cells by CCL20 and induction of CCL20 from keratinocytes by Th17 cytokines in psoriasis highlights the critical role of CCL20 in Th17 activation and psoriasis pathogenesis. Yet, regulation of CCL20 induction in keratinocytes is largely unknown.

Tripartite motif-containing protein 32 (Trim32) is a scaffold protein with E3-ubiquitin ligase activity (Ozato *et al.*, 2008). We found that Trim32 contributes to survival of keratinocytes under conditions that induce terminal differentiation and apoptosis (Horn *et al.*, 2004). Furthermore, we identified

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Abbreviations: CCL20, CC chemokine ligand 20; CCR, CC chemokine receptor; DC, dendritic cell; LPS, lipopolysaccharide; Piasy, protein inhibitor of activated STAT-Y; Th, T helper; TNF, tumor necrosis factor; Trim32, tripartite motif-containing protein 32

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protein inhibitor of activated STAT-Y (Piasy) as a Trim32 physiological substrate (Albor *et al.*, 2006), where Trim32 mediates Piasy ubiquitylation and degradation in keratinocytes. Piasy is a transcriptional repressor for a number of transcription factors with roles in inflammation and keratinocyte proliferation, such as STATs, SMADs, and NF-κB (Shuai and Liu, 2005). The *PIASY* gene is localized in a psoriasis-susceptibility locus (*PSORS6*) on chromosome 19p13 (Lee *et al.*, 2000); however, the genetic alterations responsible for the pathogenesis of psoriasis in this locus remain to be identified. The fact that Piasy regulates a number of factors whose activation is associated with psoriasis-like phenotypes in mouse models, including constitutive NF-κB activation by IκBα deletion (Klement *et al.*, 1996), epidermal overexpression of STAT3 (Sano *et al.*, 2005), and transforming growth factor-β, which activates Smads (Li *et al.*, 2004), highlights a central role for Trim32 and Piasy in psoriasis pathogenesis.

In this study, we found that Trim32 is elevated in psoriatic epidermis. Furthermore, we identified CCL20 as a downstream target of Trim32. Trim32 activates, and Piasy inhibits, keratinocyte production of CCL20 in mouse and in human keratinocytes. Further molecular dissection of this pathway revealed Trim32/Piasy regulation of CCL20 through NF-κB, mediated by Piasy interaction with the RelA/p65 subunit of NF-κB. As CCL20 is activated by Th17 cytokines, the upregulation of CCL20 production by Trim32 provides a novel positive feedback loop and direct keratinocyte role of CCL20 and Th17 activation in the self-perpetuating cycle of psoriasis.

RESULTS

Trim32 is upregulated in psoriatic epidermis

Trim32 is an E3-ubiquitin ligase that inhibits apoptosis in normal keratinocytes in response to UV light and TNFα (Horn *et al.*, 2004). On the basis of reports of resistance to apoptosis as one of the common features of psoriatic keratinocytes (Wrone-Smith *et al.*, 1997), we tested Trim32 expression in lesional specimens from human psoriasis patients, as compared with that in uninvolved control epidermis (Figure 1a). We found Trim32 overexpression in 20 out of 33 psoriasis specimens by immunohistochemical staining, with elevated Trim32 in the basal and spinous epidermal cell layers. Indirect immunofluorescence analysis revealed that Trim32 expression is predominantly cytoplasmic in the psoriatic keratinocytes (Figure 1b), as expected on the basis of previous studies (Reymond *et al.*, 2001; Albor *et al.*, 2006).

Regulation of psoriatic chemokine CCL20 by Trim32/Piasy

To explore how Trim32 might potentially contribute to the pathogenesis of psoriasis, we analyzed the expression of a panel of psoriatic cytokines and chemokines in keratinocytes infected with Trim32 or Piasy adenoviruses. We found that CCL20 expression was significantly increased in keratinocytes with Trim32 adenovirus and decreased in keratinocytes with Piasy adenovirus, as compared with those infected with GFP adenovirus (Figure 2a). In addition, CCL20 expression is reduced in primary keratinocytes isolated from Trim32-null mice as compared with those from their wild-type littermates (Figure 2b).

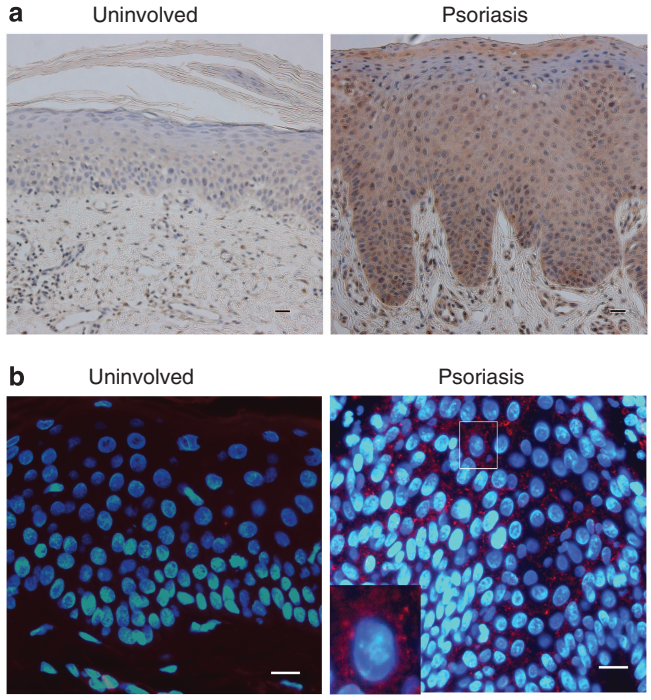


Figure 1. Elevated Trim32 expression in human psoriasis. (**a**) Immunohistochemical analysis of Trim32 expression in psoriasis specimens. Trim32 was visualized in paraffin sections using 0.2 μg ml^{-1} chicken anti-Trim32 antibody and stained by the ABC technique. Trim32 level was elevated in the epidermis of psoriatic lesions relative to that in uninvolved skin (×200 magnification). (**b**) Indirect immunofluorescence analysis of Trim32 expression in psoriasis specimens. Trim32 was visualized with chicken anti-Trim32 antibody followed by Texas red secondary antibody (×600 magnification). The nucleus was stained with Hoechst 33342. The micrographs are representative of 20 Trim32-positive specimens out of 33 psoriatic skin specimens. Bar = 50 μm.

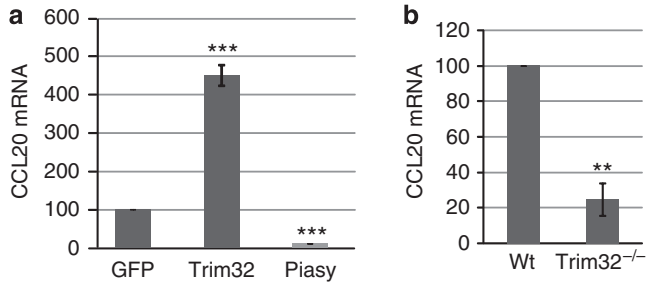


Figure 2. Regulation of CCL20 expression in keratinocytes by Trim32 and Piasy. (**a**) CCL20 mRNA expression in the mouse keratinocyte line 291 infected with adenovirus expressing GFP, Trim32, or Piasy. (**b**) Analysis of CCL20 mRNA in primary mouse keratinocytes isolated from Trim32-null mice (*Trim32*$^{-/-}$) and wild-type littermates (Wt). CCL20 mRNA was normalized to GAPDH. The data are representative of three independent experiments for Figure 1a and two independent experiments for Figure 1b (**$P < 0.01$; ***$P < 0.001$).

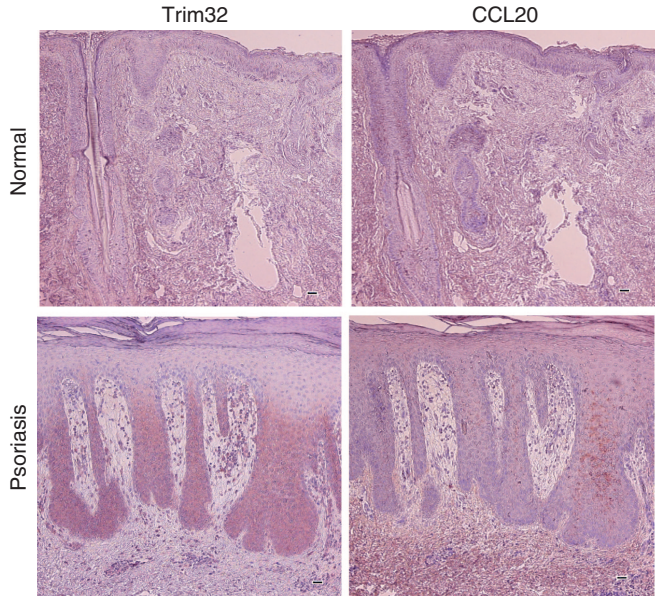
Trim32 correlates with CCL20 expression in psoriasis

CCL20 is a psoriatic chemokine overexpressed in psoriatic epidermis (Homey *et al.*, 2000). To test whether Trim32 expression correlated with CCL20 expression, we performed immunostaining of tissue sections for each of these antigens in human psoriatic skin specimens *versus* uninvolved skin

controls (Figure 3). CCL20 was not detectable in uninvolved skin. However, CCL20 was detected along with Trim32 overexpression in a majority of psoriatic skin specimens. CCL20 was detected in 23 out of 33 psoriasis specimens obtained from 33 different patients, correlating with Trim32 positivity in 19 cases. Lack of Trim32 correlated with lack of CCL20 expression in nine additional cases. Thus CCL20 expression in psoriatic lesions correlated with Trim32 status in 87% of the psoriatic specimens (Figure 3).

Trim32 sensitizes keratinocytes to TNFα- and IL-17A-induced CCL20 expression

To define the role of CCL20 in T-cell response, we evaluated CCL20 expression in keratinocytes treated with Th1 (IFN-γ, IL-2, IL-12), Th2 (IL-4, IL-13), and Th17 (IL-17A, TNFα) cytokines. TNFα and IL-17A showed potent effects on CCL20 expression in keratinocytes (Figure 4a), while neither Th1 nor



	Trim32+	Trim32–
CCL20+	19	4
CCL20–	1	9

Figure 3. Trim32 and CCL20 expression in human psoriasis. Paraffin-embedded psoriatic lesional tissues from 33 patients were sectioned serially and stained with chicken anti-Trim32 antibody and goat anti-CCL20 antibody (×100 magnification). The results are summarized in the table. Correlation of Trim32 overexpression with CCL20 expression was statistically significant (*two-tailed Fisher's exact test, *P*-value = 0.00015). Bar = 50 μm.

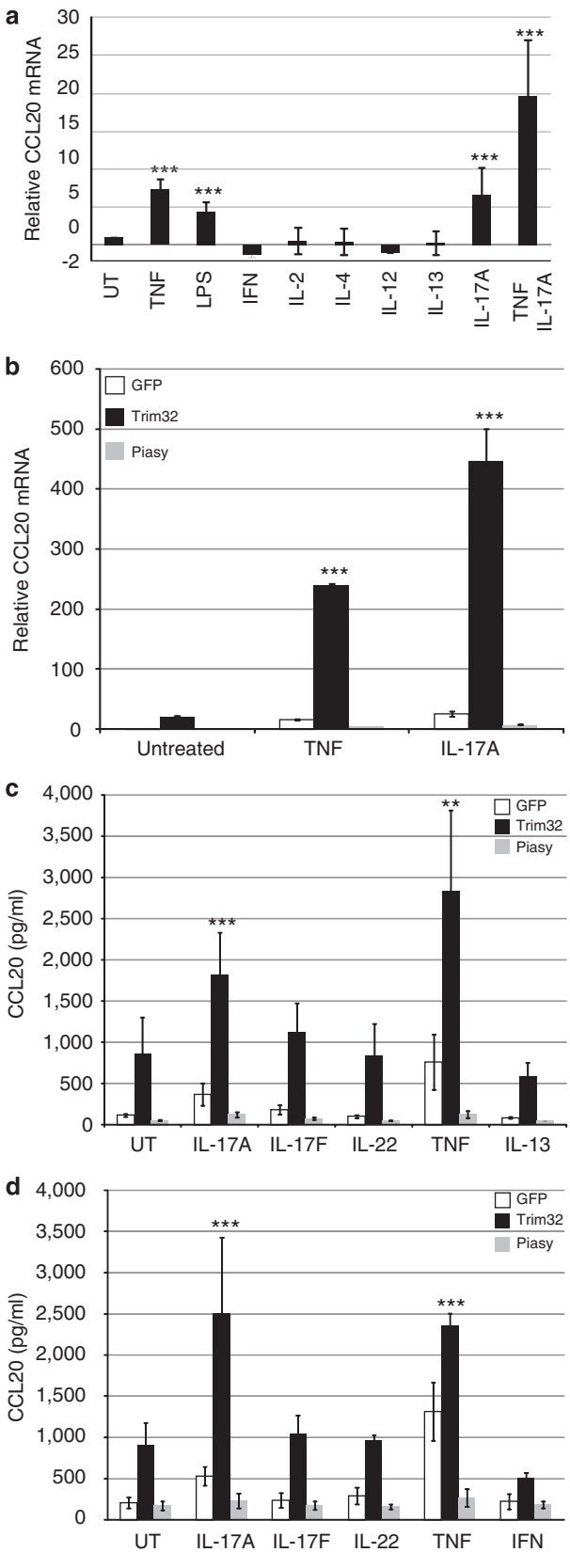


Figure 4. Regulation of Th17 induced CCL20 by Trim32 and Piasy. (**a**) CCL20 mRNA was measured by quantitative real-time PCR in mouse 291 keratinocytes treated with the indicated cytokines and LPS. (**b**) CCL20 mRNA was measured by quantitative real-time PCR in mouse 291 keratinocytes infected overnight with adenovirus expressing GFP, Trim32, or Piasy, then treated with indicated cytokines for 4 hours. CCL20 mRNA was normalized to GAPDH. (**c**) CCL20 protein was measured by ELISA in mouse 291 keratinocytes infected overnight with adenovirus expressing GFP, Trim32, or Piasy, then treated with indicated cytokines for 6 hours. (**d**) The human keratinocyte line HEKnV was infected with adenovirus expressing GFP, Trim32, or Piasy, and then treated with the indicated cytokines for 6 hours. CCL20 protein was measured from the culture supernatant by ELISA. Data are representative of three independent experiments. Statistical analysis was conducted by one-way analysis of variance followed by Bonferroni post-test (**$P < 0.01$; ***$P < 0.001$).

Th2 cytokines showed any significant effect on CCL20 expression. As activated Th17 cells produce both IL-17A and TNFα, we evaluated their combined effects on CCL20 induction. Although TNFα is also produced from other Th cells, the enhanced induction of CCL20 by the combined treatment of IL-17A and TNFα is consistent with CCL20 as a Th17 chemokine regulated by Th17 cytokines.

To test whether Trim32/Piasy regulates the expression of CCL20 in response to TNFα and IL-17A, we measured CCL20 expression in keratinocytes infected with Trim32- or Piasy-expressing adenoviruses, followed by treatment with TNFα or IL-17A. Trim32 significantly increased, whereas Piasy repressed, TNFα and IL-17A induction of CCL20 expression (Figure 4b). These effects of Trim32 and Piasy on the induction of CCL20 by TNFα or IL-17A were confirmed by ELISA analysis of CCL20 protein expression in both mouse (Figure 4c) and human (Figure 4d) keratinocytes. Trim32 showed enhanced induction of CCL20 by TNFα or IL-17A, but not by IL-17F, IL-13, or IFN-γ. The cooperation of Trim32 expression with TNFα or IL-17 signaling to induce CCL20 expression in keratinocytes suggests that Trim32 is a positive regulator that targets the rate-limiting steps in the TNFα- and IL-17-signaling pathways.

CCL20 regulation by Piasy is mediated through the p65/RelA subunit of NF-κB

The regulation of TNFα and IL-17 induced CCL20 by Trim32/Piasy suggests Trim32/Piasy acts at a common pathway between TNFα and IL-17. In fact, NF-κB is a common downstream effector of TNFα, IL-17, IL-1, and lipopolysaccharide (LPS). As our previous results showed that NF-κB is regulated by Trim32/Piasy (Albor *et al.*, 2006), we evaluated the effect of Trim32 and Piasy on NF-κB dependent CCL20 expression. We found that CCL20 is induced upon transfection of a constitutively active p65/RelA subunit of NF-κB (Figure 5a), but that p65-mediated CCL20 expression was only marginally increased by co-transfection of Trim32. This suggests that p65 acts downstream of Trim32, and therefore bypasses the regulation of CCL20 expression by Trim32. Note that the overall induction of CCL20 by Trim32 plasmid transfection (9.6-fold) was less than CCL20 induction by Trim32 adenovirus (20-fold in Figure 4), consistent with plasmid transfection being less efficient than adenoviral infection. p65-dependent CCL20 expression was significantly repressed by co-transfection of Piasy, suggesting that p65 is a target of Piasy. This is supported by Piasy's association with p65, as detected by co-immunoprecipitation of endogenous p65 with Flag-tagged Piasy in 291 keratinocytes (Figure 5b), suggesting that CCL20 regulation by Trim32/Piasy is mediated through NF-κB.

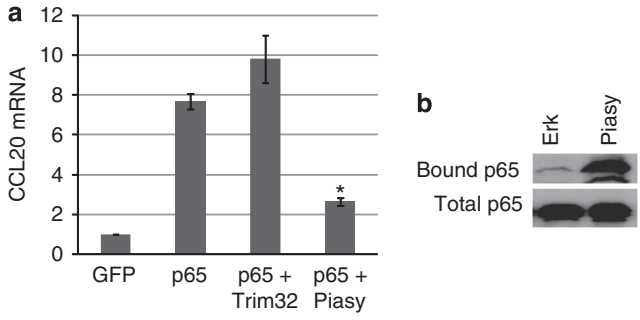


Figure 5. Piasy regulates CCL20 through p65/RelA subunit of NF-κB. (**a**) CCL20 mRNA was measured in 291 keratinocytes transfected with plasmids expressing GFP, p65, Trim32 + p65, or Piasy + p65. Data are representative of two independent experiments. Statistical analysis of Trim32 and Piasy effect was conducted relative to p65 alone control (*$P<0.05$). (**b**) Piasy interacts with endogenous p65 in keratinocytes. Flag-tagged Piasy and Flag-tagged Erk (negative control) were transfected into 291 keratinocytes. Bound p65 was immunoprecipitated with an anti-Flag antibody and visualized by immunoblotting with an anti-p65 antibody.

DISCUSSION

Psoriasis is a chronic inflammatory skin disorder characterized by epidermal hyperproliferation and immunocyte infiltration (Lowes *et al.*, 2007; Nickoloff *et al.*, 2007). T-cell-derived cytokines have been viewed prevalently as the driving force for aberrant keratinocyte proliferation and differentiation, whereas keratinocytes are generally viewed as the downstream effectors of T-cell activation. Yet, unlike many other autoimmune diseases such as systemic lupus erythematosus and pemphigus vulgaris, psoriasis is triggered and exacerbated by external insults such as microbial infections and physical injury (Koebner phenomenon), suggesting that defects in the immune system alone are not sufficient for psoriasis pathogenesis. The perturbation of keratinocyte response to external insults may contribute to the pathogenesis of psoriasis through chemokine production to recruit immunocytes. CCL20 is a chemokine abundant in psoriatic plaques (Homey *et al.*, 2000; Kryczek *et al.*, 2008; Harper *et al.*, 2009), and is induced by proinflammatory cytokines. In addition, CCL20 can be induced by physical stimuli, such as tape stripping of skin (Schmuth *et al.*, 2002) and dynamic compression of chondrocytes (Haudenschild *et al.*, 2008). Induction of CCL20 can also be achieved through infections by bacteria such as *Staphylococcus aureus* (Fahy *et al.*, 2004) and *Streptococcus mutans* (Takahashi *et al.*, 2008), with different toll-like receptors in keratinocytes specific to different types of microbes. As keratinocytes constitute the first line of defense to protect the body from external insults, CCL20 production from keratinocytes could be an initial trigger for Th17 activation, and aberrant CCL20 induction in keratinocytes may contribute to the pathogenesis of psoriasis.

In this study, we showed upregulation of Trim32, an E3-ubiquitin ligase, in psoriatic epidermis and identified Trim32 and its substrate Piasy as regulators of CCL20. Trim32 increases CCL20 induction by TNFα and IL-17 in keratinocytes. Systematic analysis of CCL20 induction in response to cytokines revealed that CCL20 is induced by Th17 but not Th1 or Th2 cytokines. This indicates that CCL20 is a chemokine specific to Th17 activation. These results are consistent with the results of Harper *et al.* (2009) demonstrating that CCL20 is induced by TNFα and IL-17A. In conjunction with CCL20 induction by Th17 cytokines, the involvement of CCL20 in Th17 activation is also supported by (1) CCL20 recruitment of CCR6-positive $CD11c^+$ DCs that produce IL-23 for Th17-cell activation (Lee *et al.*, 2004); (2)

high CCR6 expression in virtually all Th17 cells but not in Th1 and Th2 cells (Singh *et al.*, 2008; Yamazaki *et al.*, 2008); and (3) CCL20 overexpression in psoriatic epidermis (Homey *et al.*, 2000). Therefore, CCL20 expression in keratinocytes could be a key component in maintaining the self-perpetuating cycle of Th17 activation in psoriasis.

As a tissue immune defensive mechanism, the Th17-signaling pathway is activated upon exposure to pathogens or tissue injury, and is tightly controlled to ensure tissue homeostasis (Ghilardi and Ouyang, 2007). Th17 signaling is terminated after infection is ablated and tissue repair is complete. The persistent Th17 activation in psoriasis suggests that defects exist in Th17 signal regulation in psoriasis. Persistent Th17 activation in psoriatic skin is characterized by (1) infiltration of IL-23-producing DCs and Th17 cells, and (2) epidermal overexpression of Th17 chemokines. As recruitment of Th17 cells and IL-23-producing DCs is an essential step for Th17 activation, uncontrolled CCL20 production by keratinocytes could be central to persistent Th17 activation in psoriasis. In this study, we have demonstrated that CCL20 induction by TNFα and IL-17A is enhanced by Trim32 and attenuated by Piasy (Figure 4). Thus, Trim32 overexpression in human psoriatic lesions observed in this study provides a plausible mechanism for how keratinocytes in epidermis could contribute to excessive CCL20 production in psoriasis. As Piasy is a substrate of Trim32, Piasy alterations could be primary causes of aberrant CCL20 induction in psoriasis cases without elevated Trim32. In this regard it will be interesting to determine whether the *PIASY* gene is functionally involved in the psoriasis susceptibility associated with the *PSORS6* locus on chromosome 19p13 (Lee *et al.*, 2000). As Piasy is also a negative regulator for NF-κB, Smad, and Stat transcription factors that have been implicated in the development of psoriasis-like phenotypes in animal models, taking advantage of animal models and genetic approaches will help to further define the role of the Trim32/Piasy axis in Th17 activation and psoriasis pathogenesis.

So far, the mechanism of CCL20 regulation by the Trim32/Piasy axis is largely unknown. The magnitude of CCL20 induction enhanced by Trim32 suggests that Trim32 acts at the rate-limiting step in CCL20 regulation. As NF-κB is the common downstream effector of TNFα, IL-1, IL-17, and LPS, we focused our study on the NF-κB pathway. Although CCL20 induction by TNFα and IL-17 was significantly sensitized by Trim32 (Figure 4), CCL20 induction by the p65/RelA subunit of NF-κB was only marginally enhanced by Trim32 (Figure 5a). Bypassing the action of Trim32 by the constitutively active p65/RelA suggests that p65/RelA is the downstream effector of Trim32. Our evidence that p65/RelA is a Piasy-interacting protein, and that p65/RelA-mediated CCL20 induction was repressed by Piasy (Figure 5a), suggests that p65/RelA is a target regulated by the Trim32/Piasy axis in CCL20 expression.

Taken together, our results show that Trim32 and Piasy can regulate CCL20 in mouse and human keratinocytes; that Trim32 and CCL20 expression positively correlate in psoriasis lesional samples; and that Trim32 antigen activity is elevated in psoriatic epidermis. These results provide evidence for a novel pathway by which CCL20 is regulated in keratinocytes, and by which keratinocytes may contribute to Th17's role in psoriasis. Further study to define the mechanism of CCL20 regulation by the Trim32/Piasy axis in keratinocytes will be essential to develop novel therapeutic approaches that target these E3 ligases to disrupt the self-perpetuating cycle of Th17 activation in psoriasis.

MATERIALS AND METHODS

Keratinocyte cell cultures

The primary mouse keratinocyte cell strain 291 was derived from neonatal mouse skin and displays normal regulation of proliferation and differentiation by extracellular Ca^{2+} characteristic of primary epidermal cultures (Kulesz-Martin *et al.*, 1985). Primary mouse keratinocytes were isolated from neonatal Trim32-knockout mice epidermis and their wild-type littermates. These cells were maintained in low-calcium Eagle's medium (LCEM, final concentration of 0.03–0.05 mM Ca^{2+}) as described by Kulesz-Martin *et al.* (1988) and Dlugosz *et al.* (1995). The human keratinocyte HEKnV was cultured in EpiLife keratinocyte medium supplemented with a semi-defined human keratinocyte growth supplement (Cascade Biologics, Portland, OR) as described by Iordanov *et al.* (2002).

Adenoviral production and infection

The recombinant Trim32 and Piasy adenoviruses were generated as described using Adeno-X (Clontech, Mountain View, CA) (Liu *et al.*, 2004). The recombinant Trim32 adenovirus was characterized using an anti-Trim32 antibody to detect Trim32 expression in 291 keratinocytes infected with the virus. The viral particles were titered by immunostaining of Trim32 in 291 keratinocytes infected with limiting dilutions of virus. The minimum number of viral particles required for 100% infection of 291 keratinocytes was calibrated for experiments.

Cytokine treatment

Once the keratinocyte culture reached 100% confluence, the medium was replaced with LCEM without the fibroblast-conditioned media. The mouse and human keratinocyte cultures were treated with species-specific cytokines, TNFα (20 ng ml^{-1}), 1 μg ml^{-1} LPS (Sigma, St Louis, MO), IFNγ (20 ng ml^{-1}), IL-2 (20 ng ml^{-1}), IL-4 (50 ng ml^{-1}), IL-13 (50 ng ml^{-1}), IL-17A (100 ng ml^{-1}), IL-17F (100 ng ml^{-1}), or IL-22 (100 ng ml^{-1}; purchased from Peprotech, Rocky Hill, NJ).

Quantitative real-time PCR for CCL20

Triplicate qPCR reactions (15 μl) for each sample and primer set were performed using a 384-well plate (Applied Biosystems, Foster City, CA), using the *Ccl20*-gene-specific primers (6 μM each, validated for linearity and target specificity) Fwd, TGCTCTTCCTTGCTTTGGCATGGGTA and Rev, TCTGTGCAGTGATGTGCAGGTGAAGC, in the presence of UDP-*N*-glycosylase (Invitrogen, Carlsbad, CA) and SYBR-Green-I dye (Applied Biosystems). Gene expression data were collected using a 7900HT thermocycler and SYBR-Green-I fluorescence was analyzed by the $\Delta\Delta C_t$ method (Applied Biosystems). The relative *Ccl20* mRNA was normalized with GAPDH.

Keratinocyte transfection and immunoprecipitation

291 keratinocytes were transfected with a Flag-tagged Piasy vector (Albor *et al.*, 2006) using the Mirus transfection reagent (Mirus Bio

LLC, Madison, WI) when the culture reached 70% confluence. The cell lysates were collected in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0) and subjected to immunoprecipitation with agarose beads conjugated to anti-Flag antibody, clone M2 (Sigma). The bound proteins were eluted for immunoblotting with anti-p65/RelA antibody, clone C20 (Santa Cruz Biotechnology, Santa Cruz, CA).

Indirect immunofluorescence and immunohistochemistry

Archival human psoriasis lesional skin specimens biopsied and diagnosed in the OHSU Department of Dermatology were accessed with approved OHSU IRB protocol. Uninvolved human skin controls were obtained from non-psoriasis donors undergoing surgery at the Department of Dermatology or Otolaryngology. Non-specific binding of antibody was blocked with PAB (PBS, 0.1% sodium azide, 0.5% BSA) containing 10% normal goat serum for CCL20 or 10% blokhen (AVES Lab, Portland, OR) for Trim32. The sections were then incubated with an affinity-purified chicken anti-Trim32 antibody generated in our laboratory (Albor *et al.*, 2006) or with a goat anti-CCL20 antibody, clone AF360 (R&D Systems, Minneapolis, MN) overnight at 4 °C. The specificity of the chicken anti-Trim32 antibody was verified by immunoblotting and immunostaining as described by Albor *et al.* (2006) and detailed in Supplementary Figure S1. The staining signal was visualized by the ABC approach (Vector Laboratories, Burlingame, CA) and counterstaining was performed with hematoxylin. Immunofluorescence staining of Trim32 was performed with the Texas red secondary antibody. The nucleus was stained with 1 $\mu g\,ml^{-1}$ Hoechst 33342.

Statistics

The data were expressed as mean ± SD. The statistical significance of CCL20 regulation by Trim32 and Piasy was determined by two-tailed Student's *t* test (Figures 2 and 5) and one-way analysis of variance followed by Bonferroni post-test (Figure 4). Statistical significance of correlated expression of Trim32 and CCL20 (Figure 3) was determined by two-tailed Fisher's exact test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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