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Trim32 deficiency enhances Th2 immunity and predisposes to features of atopic dermatitis

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Abstract

Altered innate immunity is a feature of certain skin inflammatory diseases such as psoriasis and atopic dermatitis (AD). In this study, we provide evidence that deficiency in Trim32 (a tripartite motif (TRIM) protein with innate antiviral activity) contributes to a Th2 biased response and predisposes to features of AD in mice. Upon treatment with the TLR7 agonist imquimod (IMQ), *Trim32* knockout (KO) mice displayed compromised psoriasiform phenotypes and defective Th17 response. Instead, IMQ treatment of *Trim32* KO mice induced AD-like phenotypes with enhanced skin infiltration of eosinophils and mast cells, elevation of Th2 cytokines/chemokines expression, and reduced expression of filaggrin protein expression. Furthermore, while the induction of phosphorylated Stat3 and RelA were compromised following IMQ treatment in the KO mice, phosphorylated Stat6 was elevated. CCL20 induction by TNFα and IL-17A was reduced in Trim32 deficient keratinocytes whereas CCL5 induction by TNFα and IL-4 was enhanced. In addition, Trim32 protein levels were elevated in mice treated with IMQ. Unlike Trim32 overexpression in psoriasis, TRIM32 levels were low in AD patients. Based on Trim32 induction by IMQ, the lower levels of TRIM32 in AD skin compared to healthy control and psoriatic skin suggest a defective TRIM32 pathway in AD pathogenesis.

Keywords

Trim32; innate immunity; atopic dermatitis; psoriasis; Th2

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CONFLICT OF INTEREST

There are no conflicts of interest to declare.

INTRODUCTION

Innate immunity is the first line of defense against environmental insults and is crucial for initiation of the adaptive immune response. Aberrant innate immunity has been implicated in many skin inflammatory diseases, including psoriasis and atopic dermatitis (AD). Enhanced innate immune response is associated with psoriasis, whereas defective innate immunity contributes to AD pathogenesis, as indicated by reduced expression of antimicrobial peptides and susceptibility to bacterial and viral infection (De Benedetto et al. 2009). Single nucleotide polymorphisms in genes in the innate immune signaling pathways, such as TLR1/2/6/9 and NOD1/2, have been associated with AD (Ahmad-Nejad et al. 2004; Weidinger et al. 2005; Novak et al. 2007; EArly Genetics and Lifecourse Epidemiology (EAGLE) Eczema Consortium et al. 2015). However, the causal relationship between defective innate immunity and AD pathogenesis in animal models remains to be defined.

Trim32 is a member of Trim E3-ubiquitin ligase family. The Trim proteins in this family are mainly involved in innate antiviral immunity. They inhibit viral infection through different mechanisms, such as blocking viral entry, transcription, assembly, and release (Rajsbaum et al. 2014) . Trim32 was initially identified as an HIV tat associated protein (Fridell et al. 1995). Trim32 restricts the replication of influenza A virus by targeting viral RNA polymerase for degradation (Fu et al. 2015). Like many other TRIM proteins, it regulates the signaling pathways triggered by innate immunity pattern recognition receptors. Trim32 activates STING, which is a sensor for cytoplasmic DNA essential for innate antiviral response (Zhang et al. 2012) . We previously showed that Trim32 can activate NF-κB through the degradation of Piasy, a negative regulator of NF-κB (Albor et al. 2006). *TRIM32* gene and protein mutations are associated with human diseases, including limb-girdle muscular dystrophy (Shieh et al. 2011) and Bardet-Biedl syndrome (Chiang et al. 2006), as well as attention deficit/hyperactivity disorder (Lionel et al. 2011), a condition which has been associated with AD (Schmitt et al. 2013; Eichenfield et al. 2014).

In the current study, we discovered that *Trim32* knockout (KO) mice displayed an AD-like inflammatory skin condition in response to imiquimod (IMQ) treatment, including 1) elevated IL-4, IL-5, and CCL5 and reduced IL-17A, CCL20, and S100A9 expression; 2) increased presence of eosinophils and mast cells; and 3) reduced expression of filaggrin protein. Compared to patient-matched non-lesional skin and skin of healthy controls, TRIM32 protein levels were high in psoriasis and low in AD lesional skin. Taken together, our results suggest that Trim32 is required for normal Th17 response, while Trim32 deficiency favors features of a Th2 atopic response.

RESULTS

Imiquimod (IMQ) induced psoriasis-like phenotypes are compromised by Trim32 deficiency

Based upon our previous report that TRIM32 protein is elevated in the epidermal lesions of human psoriasis and activates keratinocyte production of CCL20 (Liu et al. 2010), we investigated whether Trim32 is required for the development of psoriasis-like phenotypes in

mice. Using an established IMQ-treated mouse psoriasis model (van der Fits et al. 2009), we evaluated gross, histological, and molecular endpoints associated with IMQ-induced psoriasis pathogenesis in FVB *Trim32* KO mice and their control WT littermates. The gross appearance of the back skin of the WT mice treated with IMQ displayed more scaling than that of KO mice (Figure 1A). Consistent with less skin scaling in *Trim32* KO mice, hyperkeratosis and parakeratosis were less pronounced in *Trim32* KO mice (Figure 1B & Figure S1). Taken together, these results indicate that the development of psoriasis-like phenotypes is compromised by Trim32 deficiency.

Reduced Th17 and increased Th2 cytokines by Trim32 deficiency

To determine the molecular features underlying these phenotypic changes, we evaluated the expression profiles of cytokines and chemokines in response to IMQ in *Trim32* WT versus KO mice. As expected, IL-17A was induced by IMQ treatment in the WT mice; however, its induction was significantly reduced in the KO mice under the same conditions (Figure 2A). In keeping with defective Th17 immunity, the induction of CCL20 and antimicrobial peptide S100A9 were significantly reduced in the *Trim32* KO mice (Figure 2B & C, respectively). Th2 cytokines, IL-5 and IL-4, were up-regulated in skin in response to IMQ in *Trim32* KO mice (Figure 2D & E), corroborated by increased serum IL-4 in *Trim32* KO mice (Figure S2A). Similar chemokine/cytokine profiling was observed in *Trim32* KO mice in another genetic background (129XC57BL/6J), indicating that the essential findings are reproducible and not strictly mouse strain dependent (Figure S3A–F). Consistent with Trim32 deficiency conferring defective Th17 response, overexpressing Trim32 in epidermis predictably enhanced psoriasis-like phenotypes with increased expression of IL-23, IL-17f, CCL20, and neutrophil chemokine CXCL5 (Figure S4). The expression of IL-17A and CCL20 is mediated through Stat3 (Mack et al. 2012; Hau et al. 2014; Li et al. 2015), whereas the expression of IL-4 and IL-5 is mediated through Stat6 (Nelms et al. 1999; Chan et al. 2001). Examination of the IMQ-treated mouse skin revealed induction of phosphorylated Stat3 (Y705) in the WT mice and, conversely, induction of phosphorylated Stat6 (Y641) in the *Trim32* KO mice (Figure 2G). Collectively, these results indicate that Trim32 contributes to IMQ mediated Th17 activation and that Trim32 deficiency impairs Th17 response and favors Th2 activation.

IMQ differentially induces the infiltration of T helper, mast cells and eosinophils in Trim32 deficient mice

To test the cell types that contribute to altered cytokine expression by Trim32 deficiency, the presence of Th2 and Th17 cells in IMQ treated skin were evaluated using multiplex sequential immunohistochemistry. Consistent with reduced IL-17A expression in *Trim32* KO mice (Figure 2A), Th17 cells (IL23R+/CD4+) were significantly reduced, and the presence of Th2 cells (Gata3+/CD4+) was marginally increased in *Trim32* KO mice (Figure 3A–C, Figure S5). Besides Th2 cells, mast cells and eosinophils are other major cell types that express IL-4 (Gessner et al. 2005; McLeod et al. 2015). We found that CCL5, a chemokine for eosinophils and mast cells (Beck et al. 1997; Juremalm et al. 2002), was significantly increased in the skin of *Trim32* KO mice (Figure 2F). In keeping with this up-regulation, *Trim32* KO mice displayed significantly increased infiltration of eosinophils (Figure 3D & E) and mast cells (Figure 3F & G) in response to IMQ treatment. Furthermore,

serum IgE levels were significantly elevated in the *Trim32* KO mice (Figure S2B). These lines of evidence indicate that Trim32 deficiency contributes to the development of AD-like phenotypes in response to IMQ.

Trim32 deficiency compromises filaggrin expression in response to IMQ

AD is an inflammatory skin disease featured by Th2 polarization, barrier defects, and susceptibility to infection. To further define the relevance of Trim32 deficiency in AD, we evaluated the expression of filaggrin, a barrier protein mutated or downregulated in AD (O'Regan et al. 2008). Filaggrin protein was barely detectable in the whole skin tissue lysate but was prominently induced by IMQ (Figure S6 and Figure 4A). Compared to WT mice, the level of total filaggrin protein, filaggrin multimer, and filaggrin fragments were reduced in *Trim32* KO mice. Filaggrin mRNA levels were not affected by Trim32 deficiency (data not shown), suggesting that Trim32 effects are post-transcriptional. Further analysis of filaggrin by immunostaining revealed that cellular filaggrin expression in the epidermis was significantly reduced in *Trim32* KO mice (Figure 4B & 4C).

Trim32 deficiency enhances AD-like phenotypes in MC903 induced AD mouse model

The role of Trim32 deficiency in AD pathogenesis was further evaluated in an AD mouse model induced by MC903 (Li et al. 2006, 2008). Histological analysis of the treated skin revealed that *Trim32* KO mice displayed a significantly thicker epidermis in response to MC903 treatment (Figure S7A & B). Furthermore, eosinophil infiltration was significantly elevated in MC903-treated KO mice compared to their WT control littermates (Figure S7C & D). IL-4 was elevated in *Trim32* KO mice and further increased in response to MC903 treatment (Figure S7E). Similar to IL-4, TSLP, another key cytokine essential for Th2-associated inflammation in AD, was increased in *Trim32* KO mice in response to MC903. TSLP basal levels were higher in *Trim32* KO mice and further induced by MC903 than in WT mice (Figure S7F). Thus, Trim32 deficiency associated with AD-like phenotypes was validated in an AD mouse model.

Trim32 deficiency reduces CCL20 and enhances CCL5 expression in keratinocytes

Since changes in the chemokines in whole skin samples (shown in Figure 2 and S3 for the IMQ treated mice) are mainly attributed to keratinocytes, we further characterized cultured keratinocytes from WT and *Trim32* KO mice. Analysis of chemokine expression revealed that CCL20 expression was reduced in response to TNFα and IL-17A and that CCL5 expression was enhanced by TNFα or TNFα plus IL-4 in *Trim32* KO keratinocyte cultures (Figure 5A & B). Further analysis of the induction pattern revealed that Trim32 primarily affected the induction by TNFα but not IL-4 or IL-17A. This suggests that the converse regulation of CCL20 and CCL5 expression by Trim32 is mediated at least in part through TNFα signaling. Consistent with NF-κB activation by Trim32 overexpression in our previous studies (Albor et al. 2006), NF-κB activation was compromised in the skin of *Trim32* KO mice as indicated by reduced RelA phosphorylation and reduced expression of NF-κB downstream gene A20 (Figure 5C). Similarly, NF-κB activation by TNFα and IL-17A was compromised in Trim32 deficient keratinocytes (Figure 5D).

Trim32 induction is defective in atopic dermatitis

To determine whether Trim32 deficiency is relevant to human AD, we compared TRIM32 levels in AD and psoriasis biopsy samples to healthy controls. TRIM32 levels in the non-lesional skin from psoriasis were similar to that in the skin from control individuals. Compared to non-lesional skin, the TRIM32 levels were significantly higher in the lesional psoriatic epidermis; further, the TRIM32 protein levels were significantly lower in both lesional skin and non-lesional skin from AD patients compared to that from psoriatic epidermis and healthy skin (Figure 6A & B). This is consistent with reduced *TRIM32* mRNA expression reported in AD patients (Guttman-Yassky et al. 2009). As an innate anti-viral protein, the expression of many TRIM proteins can be induced by viral infection and CpG stimulation (Rajsbaum et al. 2008). Analysis of Trim32 expression in the IMQ model revealed that Trim32 was induced by IMQ at the protein level (Figure 6C). Considering that AD skin is under constant challenge from pathogens (Hauser et al. 1985; Park et al. 2013), the low levels of TRIM32 in AD lesional skin suggest defective TRIM32 induction in AD.

DISCUSSION

In this study, we provide evidence to support the contribution of defective innate immunity in AD pathogenesis with Trim32 knockout mice as a model. Trim32 belongs to a family of proteins with members involved in innate immunity (Kawai and Akira 2010; Versteeg et al. 2014). Specifically, TRIM32 contributes to innate immunity by 1) restricting viral replication (Uchil et al. 2008; Fu et al. 2015), 2) inhibiting viral protein activity (Fridell et al. 1995; Fatima et al. 2015), and 3) activating innate immune signaling pathways (Albor et al. 2006; Zhang et al. 2012; Uchil et al. 2013). Consistent with our previous report that TRIM32 protein is elevated in the epidermal lesions of human psoriasis and that TRIM32 can activate keratinocyte production of CCL20 (Liu et al. 2010), we verified that *Trim32* KO mice were deficient in mounting features of a Th17 response to TLR activation in the IMQ model of psoriasis-like disease in mice. Interestingly, *Trim32* KO mice developed AD-like phenotypes characterized with dermal infiltration of eosinophils and mast cells, overexpression of Th2 cytokines, and enhanced Stat6 phosphorylation. In conjunction with reduced Trim32 expression in AD, the development of AD-like phenotypes in *Trim32* KO mice provides *in vivo* evidence that defects in innate immunity contribute to Th2 polarization and AD pathogenesis.

IMQ is a potent inducer for Th1/Th17 activation and commonly used to induce psoriasis-like phenotypes in mice (van der Fits et al. 2009). The development of AD-like phenotypes and enhanced Th2 activity in response to IMQ suggest that Trim32 contributes to the determination of T helper cell response. Pathogen-associated molecular patterns (PAMPs)-mediated innate immunity is essential for the polarization of Th1 and Th17 to combat viral and bacterial infection. Th2 polarization is generally induced when innate immunity is compromised as demonstrated by the evidence from MyD88 deficient mice (Schnare et al. 2001; Sun et al. 2005) and vaccination without TLR agonist (Korsholm et al. 2010). The demonstration that *Trim32* KO mice display compromised Th17 response and enhanced Th2 response in response to IMQ supports the view that PAMP-mediated innate immunity determines the polarization of T helper cells.

Atopic dermatitis is an inflammatory skin disease with defective innate immunity (Kuo et al. 2013). AD patients are susceptible to bacterial and viral infection. Despite the highly inflamed nature and the presence of pathogens in AD skin, antimicrobial peptide expression is compromised in AD (Ong et al. 2002). Furthermore, genetic polymorphisms of genes in innate signaling pathways have been associated with AD, such as TLR2/9 and NOD1/2. Although the role of defective innate immunity in AD is appreciated, its contribution in Th2 activation and AD pathogenesis remains to be defined. The causal role of defective innate immunity was evaluated to date only in *Tlr2* KO mice (Kuo et al. 2013a) and *Tlr4* KO mice (Brandt et al. 2013) showing barrier defects but not Th2 activation and the infiltration of eosinophils and mast cells. In the IMQ mouse model, *Trim32* KO mice displayed many AD features including epidermal thickening, enhanced Th2 cytokine expression, infiltration of Th2, mast cells and eosinophils, reduced filaggrin expression, and increased serum IgE level. Thus, we provide evidence that Trim32 deficiency can result in a Th2, AD type of skin disorder in response to TLR activation.

NF-κB activation is essential for innate immune response and T cell activation (Hatada et al. 2000). Consistent with NF-κB activation by Trim32 overexpression (Albor et al. 2006), we showed that NF-κB activation is compromised by Trim32 deficiency (Figure 5). As NF-κB signaling is essential for Th17 differentiation (Brüstle et al. 2012; Molinero et al. 2012) , compromised NF-κB activation may attribute to reduced Th17 activation in *Trim32* KO mice. Similar to *Trim32* KO mice, NF-κB inhibition in transgenic mice with constitutive active IκBa mounted enhanced allergic inflammation with increased IL-4 expression and serum IgE (Aronica et al. 1999) . Furthermore, *RelB* KO mice developed AD like phenotypes with increased Th2 cytokine expression and impaired viral clearance in response to vaccinia viral infection (Freyschmidt et al. 2007) . These lines of evidence suggest that Trim32 mediated NF-κB activation is critical in determining T helper cell differentiation in response to innate immune activation. Coupled with the low level of TRIM32 in AD lesional skin, these results provide a pathological basis for defective innate immunity and Th2 activation in AD patients.

In summary, our data provide *in vivo* evidence that genetic manipulation of Trim32 regulates Th17 vs Th2 immunity in response to TLR activation, supporting findings that TRIM32 protein expression is defective in AD lesional skin. Thus, we provide evidence at the molecular level to support deficiency in innate immunity as leading to Th2 polarization and AD pathogenesis. Future examination of the contribution of Trim32 in other genetically modified mouse models that recapitulate features of human psoriasis or AD, using a wider array of stimuli, will be critical in further defining the role of Trim32 in inflammatory diseases and conditions of compromised innate immunity. Expanding knowledge of TRIM32 regulation in skin response to pathogen promises to have clinical relevance in diagnosis, prognosis of severity, and sub-classification of patients for clinical trials of novel treatments for human inflammatory diseases such as atopic dermatitis and psoriasis.

MATERIALS & METHODS

Reagents and antibodies

Reagents were purchased as follows: 5% imiquimod cream manufactured by Perrigo (Yeruham, Israel), calcipotriol (MC903) from Cayman Chemical (Ann Arbor, MI), sirius red (Direct Red 80) from Sigma-Aldrich (St. Louis, MO), anti-phospho-RelA(S536), anti-TNFAIP3 (A20), anti-GAPDH, anti-phospho-Stat3 (Y705), and anti-phospho-Stat6 (Y641) from Cell Signaling (Danvers, MA), anti-filaggrin antibody from Santa Cruz Biotech (Dallas, TX), anti-keratin 5 antibody from LifeSpan Biosciences (Seattle, WA), anti-GATA3 and anti-IL23R from Abcam (Cambridge, MA), anti-CD4 from eBioscience (San Diego, CA), and NovaUltra™ Toluidine Blue Stain from Fisher Scientific (Pittsburgh, PA). Rabbit anti-Trim32 antibody for immunoblotting and chicken anti-Trim32 antibody for immunostaining were generated in our laboratory (Albor et al. 2006).

Mouse skin inflammation models

Psoriasis-like disease was induced by imiquimod (IMQ) in mice as described previously (van der Fits et al. 2009). Specifically, the back hair of WT and KO mice was removed using an electric razor. Mice received a daily topical dose of 62.5mg IMQ per mouse or Cetaphil® cream vehicle alone as control. Mice deficient in Trim32 and their WT littermates (provided by Dr. Hao Ding, University of Manitoba, Canada) were mated into pure FVB genetic background and mixed background (129XC57BL/6J) and used for the IMQ experiments. FVB mice were used for MC903 studies as described previously (Zhang et al. 2009). All animals were bred under specific pathogen-free conditions and used for experiments at 8–11 weeks of age. Animals were sex-matched for experiments. All animal experiments were conducted according to animal protocol (IS00001640) approved by OHSU.

Mouse primary keratinocyte cell culture and cytokine treatment

Primary mouse keratinocytes were isolated from the epidermis of neonatal *Trim32* KO and their WT littermates. These cells were maintained in “low calcium” medium (final concentration of 0.03–0.05 mM Ca^{2+}) as described (Kulesz-Martin et al. 1988; Dlugosz et al. 1995). Once the keratinocyte culture reached 100% confluence, the keratinocytes were treated for 24 hours with species-specific cytokines: TNFα (20 ng/ml), IL-4 (50 ng/ml), IL-17A (100ng/ml), purchased from Peprotech (Rocky Hill, NJ).

Human studies

The human subject research component was approved by the OHSU Institutional Review Board (IRB, 2568). After informed consent, 4mm punch biopsies were obtained from both psoriasis and AD patients and diagnosis was confirmed after histology was reviewed by a dermatopathologist. The uninvolved skin biopsies were taken 2–4 cm from the affected skin. Skin biopsies from healthy individuals with no history of psoriasis, AD, or inflammatory disease served as controls.

Histological analysis, mast cell, eosinophil staining, indirect immunofluorescence and quantification

Sections (4–6 μm) from mouse back skin were stained with toluidine blue for mast cells per manufacturer's instruction (NovaUltra™ Cat# IW-3013) or Harris hematoxylin and sirius alkaline red for eosinophil (Llewellyn 1970) . Indirect immunofluorescence of TRIM32 in human tissues and filaggrin in mouse tissues were performed as described (Liu et al. 2010). For quantification of mast cells and eosinophils, four pictures of the stained skin sections were taken for each animal using a 10x objective and 40x objective, respectively, capturing representation of all the layers of the skin. The number of mast cells and eosinophils were quantified using ImageJ software and averaged for each animal.

Quantitative RT-PCR

Total RNAs extracted from back skins or ears in Ambion® RNAlater solution (Fisher Scientific, Pittsburgh PA) were converted into total cDNAs using the RNA-cDNA kit (Applied Biosystems, Foster City, CA). The primers used are listed in Supplemental Table 1. Quantitative PCR was set up in triplicates using Power SYBR® Green mix (Applied Biosystems, Foster City, CA) on a real-time PCR system (ViiA™ 7 Real-Time PCR System).

Immunoblotting analysis, Serum IgE and IL-4 detection

Skin tissues were lysed with tissue extraction buffer (100 mM Tris, Ph7.4, 150mM NaCl, 1mM EGTA, 1mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate) supplemented with both protease inhibitor tablet and phosphatase inhibitor tablet (Roche Diagnostics, Indianapolis, IN). Mouse serum IgE was measured using ELISA kits from eBioscience (San Diego, CA) and mouse serum IL-4 was measured using ELISA kits from Abcam (Cambridge, MA).

Sequential immunohistochemistry

Multiplex sequential immunohistochemistry was performed with FFPE (5μm) tissue sections as we previously reported (Gunderson et al. 2016). Primary rat or rabbit antibodies were then serially stained for 1 hour at room temperature using rat anti-CD4 (4SM95, 1:50, eBioscience), Goat anti-IL23R (1:100, Abcam), and rabbit anti-GATA3 (EPR16651, 1:500, Abcam). Histofine Simple Stain MAX PO HRP conjugated polymer (Nichirei Biosciences Inc.) was utilized for detection followed by AEC for peroxidase detection. Multiplex images were coregistered using CellProfiler software (Broad Institute), deconvoluted using Image J, pseudocolored, and merged in ImageScope (Aperio, Leica). High magnification images were created with a 4x zoom from a 20x original magnification. Total positive cells were manually counted for each cross section, and the results were normalized to total tissue area.

Statistical analysis

Data values shown are mean +/− SD. Statistical significance was determined by an unpaired Student *t* test (**) and two-way ANOVA followed by Bonferroni post-test for multiple comparisons (*) using GraphPad Prism (La Jolla, CA). The *p* value is denoted for each analysis and $p<0.05$ was considered statistically significant unless otherwise indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AD	atopic dermatitis
KO	knockout
WT	wild type
qPCR	quantitative real time polymerase chain reaction
TRIM32	tripartite motif-containing 32
IMQ	imiquimod
STAT	the signal transducer and activator of transcription
CCL	CC chemokine ligand
IL	interleukin
Th	T helper
TNF	tumor necrosis factor
TSLP	thymic stromal lymphopoietin
Ig	immunoglobulin
OHSU	Oregon Health & Science University

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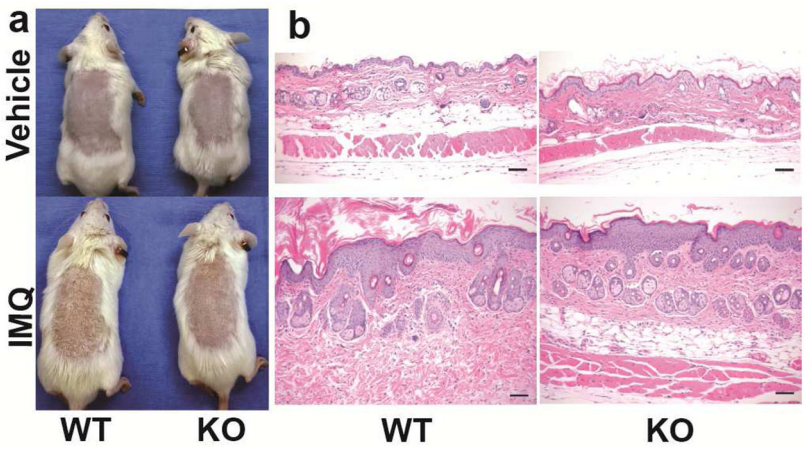

Figure 1. Skin morphological abnormalities induced by IMQ in WT and KO mice

(**A**) Representative pictures of gross appearance of back skin from WT and KO mice in response to vehicle or IMQ treatment at day 6. (**B**) Representative H&E staining of tissue sections of back skin treated with vehicle or IMQ at day 6. Scale Bar=200 μm. n=3–5 per group.

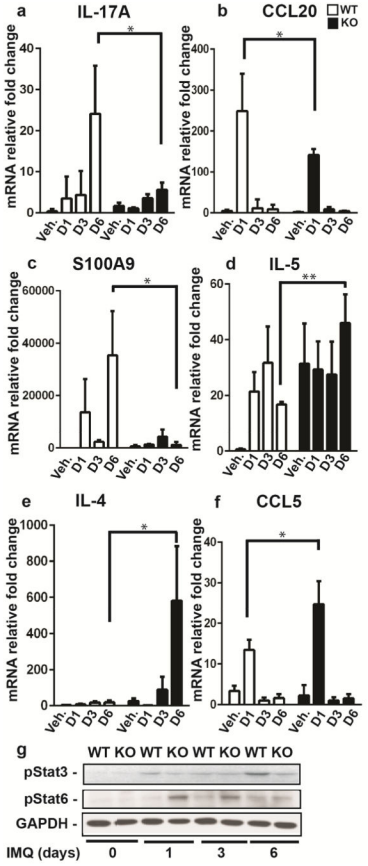

Figure 2. IMQ treatment of *Trim32* KO mice enhanced Th2 while repressing Th17 cytokine profile

(A–F) Relative mRNA fold change for vehicle or IMQ-treated mice by qRT-PCR. Data were normalized by GAPDH mRNA expression followed by normalization to the WT vehicle-exposed mice (*p<0.05, two-way ANOVA followed by Bonferroni post-test, **p<0.05, an unpaired Student t test). **(G)** Western blot analysis of phospho-STAT3 (Y705) and phospho-Stat6 (Y641) from the skin of *Trim32* WT and KO mice treated with IMQ. GAPDH was used as loading control.

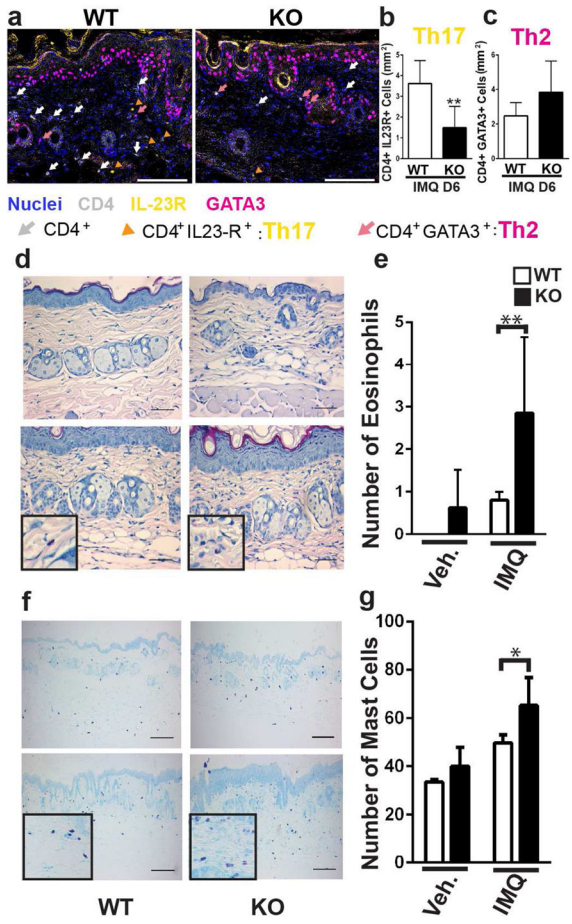

Figure 3. IMQ treatment results in lower levels of Th17 cells and elevated mast cell and eosinophil infiltration in the *Trim32* KO mice

(A) Representative images of CD4+, CD4+/IL23R+, and CD4+/GATA3+ cells in the skin of WT and *Trim32* KO mice treated with IMQ for 6 days (n=4). (**B**) Quantification of the number of CD4+/IL23R+ cells (Th17) averaged for each animal (**p<0.05, an unpaired Student t test) (**C**) Quantification of the number of CD4+/GATA3+ cells (Th2) averaged for each animal.(**D**) Representative images of eosinophil staining of vehicle or IMQ-treated mice (n 4). (**E**) Quantification of the number of eosinophils averaged for each animal (**p<0.05, an unpaired Student *t* test). (**F**) Representative images of the mast cell staining of vehicle or IMQ-treated mice (n 4). (**G**) Quantification of the number of mast cells averaged for each animal (*p<0.05, two-way ANOVA followed by Bonferroni post-test). Scale Bar= 100 μm (A) and 200 μm (D & E)

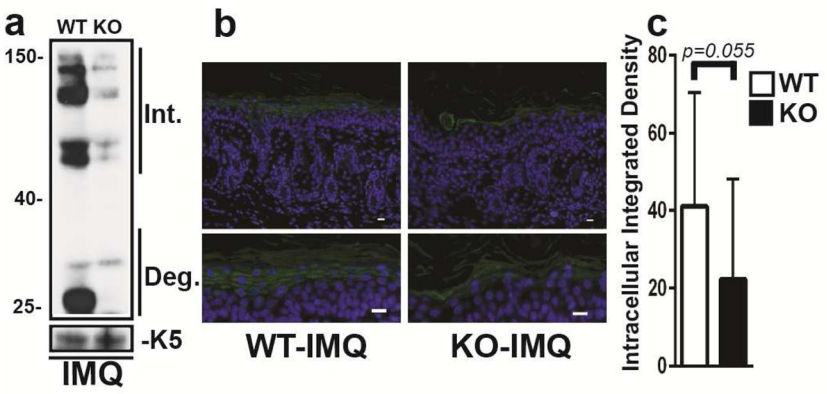

Figure 4. Filaggrin is down-regulated in *Trim32* KO mice in response to IMQ treatment

(A) Western blot analysis of filaggrin expression from the skin of *Trim32* WT and KO mice treated with IMQ for 6 days. Keratin 5 was used as loading control (n=4). **(B)** Representative images of indirect immunofluorescence of filaggrin antibody reactivity in IMQ treated mice and zoomed in images (lower panels). Scale Bar=50 μm. **(C)** Quantification of integrated fluorescent density of epidermal filaggrin staining. (FIJI ImageJ Software, p=0.055).

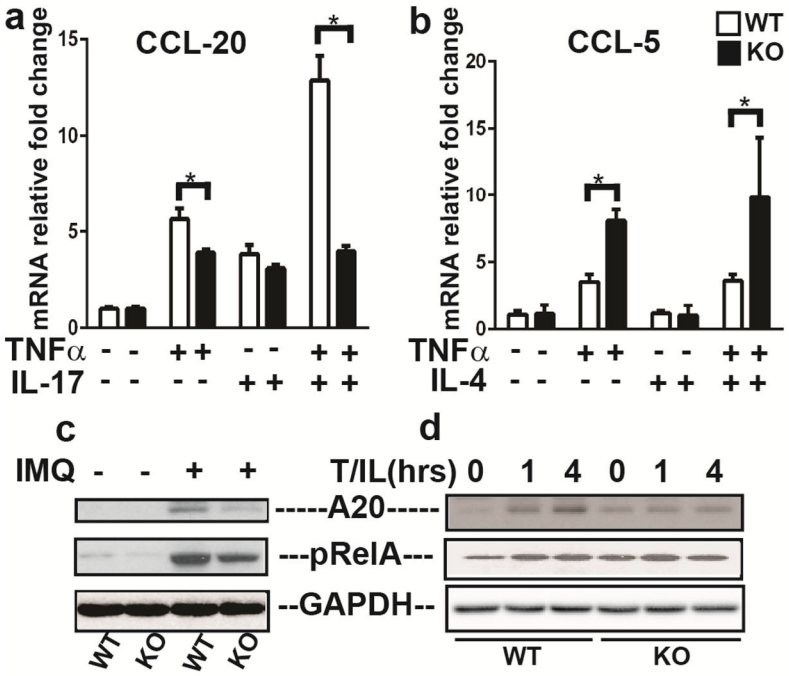

Figure 5. Altered chemokine expression from neonatal keratinocytes derived from WT and KO mice and compromised NF-κB activation in Trim32 KO keratinocytes

(A & B) mRNAs were isolated from primary neonatal keratinocytes following cytokine treatment as indicated. Expression levels were determined by qPCR and normalized by GAPDH. (*p<0.05, two-way ANOVA followed by Bonferroni post-test). Data are representative of three independent experiments. (C) Western blot analysis of phosphorylation of RelA and its downstream targets A20 expression from the skin of *Trim32* WT and KO mice treated with IMQ for 6 days. (D) Western blot analysis of RelA phosphorylation and A20 expression in response to TNFα/IL-17 (T/IL) in cultured keratinocytes. GAPDH was used as loading control.

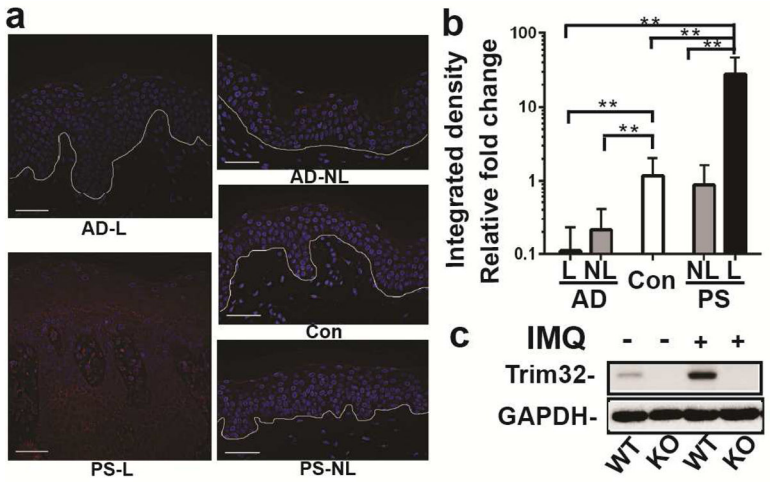

Figure 6. Evaluation of TRIM32 levels in skin from AD, psoriasis, and healthy individuals and induction of Trim32 in mouse skin by TLR ligand IMQ

(A) Representative images of indirect immunofluorescence of Trim32 antibody reactivity at 20X magnification. White line defines boundary between epidermis (top) and dermis. Scale Bar= 50 μm. (**B**) Quantification of integrated density of epidermal TRIM32 staining (AD lesional (AD-L) n=15; AD non-lesional (AD-NL) n=8; psoriasis lesional (PS-L) n=7; psoriasis non-lesional (PS-NL) n=8; control n=9). Fluorescence was quantified using FIJI ImageJ Software. Data were normalized to control group and displayed as a log scale (**p<0.0001, an unpaired Student t test). (C) Western blot analysis of Trim32 expression from the skin of *Trim32* WT and KO mice treated with IMQ for 6 days. Total cell lysates were extracted from the back skin of IMQ-treated mice. GAPDH was used as loading control.