Trim32 Deficiency Enhances Th2 Immunity and Predisposes to Features of Atopic Dermatitis

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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 T helper type 2 biased response and predisposes to features of AD in mice. On treatment with the toll-like receptor 7 agonist imiquimod (IMQ), Trim32 knockout mice displayed compromised psoriasiform phenotypes and defective T helper type 17 response. Instead, IMQ treatment of Trim32 knockout mice induced AD-like phenotypes with enhanced skin infiltration of eosinophils and mast cells, elevation of T helper type 2 cytokines/chemokines expression, and reduced expression of filaggrin protein expression. Furthermore, although the induction of phosphorylated Stat3 and RelA was compromised after IMQ treatment in the knockout mice, phosphorylated Stat6 was elevated. CC chemokine ligand 20 induction by tumor necrosis factor-α and IL-17A was reduced in Trim32-deficient keratinocytes, whereas CC chemokine ligand 5 induction by tumor necrosis factor-α 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 patients with AD. Based on Trim32 induction by IMQ, the lower levels of TRIM32 in AD skin compared with healthy control and psoriatic skin suggest a defective TRIM32 pathway in AD pathogenesis.

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, such as psoriasis and atopic dermatitis (AD). Enhanced innate immune response is associated with psoriasis, whereas defective innate immune 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 toll-like receptor (TLR)1/2/6/9 and NOD1/2, have been associated with AD (Ahmad-Nejad et al., 2004; Early Genetics and LifeCourse Epidemiology Eczema Consortium and 3Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Manitoba, Canada

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Abbreviations: AD, atopic dermatitis; CCL, CC chemokine ligand; IMQ, imiquimod; KO, knockout; Th, T helper; TLR, toll-like receptor; TNF, tumor necrosis factor; TRIM32, tripartite motif-containing 32; WT, wild type

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increased presence of eosinophils and mast cells; and (3) reduced expression of filaggrin protein. Compared with patient-matched nonlesional 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 T helper type 17 (Th17) response, whereas Trim32 deficiency favors features of a Th2 atopic response.

RESULTS
IMQ-induced psoriasis-like phenotypes are compromised by Trim32 deficiency
Based on 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, histologic, and molecular endpoints associated with IMQ-induced psoriasis pathogenesis in Trim32 KO mice and their control wild-type (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 and c, respectively. Th2 cytokines, IL-5 and IL-4, were upregulated in skin in response to IMQ in Trim32 KO mice (Figure 2d and e), corroborated by increased serum IL-4 in Trim32 KO mice (Supplementary Figure S2a online). 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 (Supplementary Figure S3a–f online). 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 (Supplementary Figure S4 online). The expression of IL-17A and CCL20 is mediated through Stat3 (Hau et al., 2014; Li et al., 2015; Mack et al., 2012), whereas the expression of IL-4 and IL-5 is mediated through Stat6 (Chan et al., 2001; Nelms et al., 1999). 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 was 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, Supplementary Figure S5 online). 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 2b). In keeping with this upregulation, Trim32 KO mice displayed significantly increased infiltration of eosinophils (Figure 3d and e) and mast cells (Figure 3f and 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 (Supplementary Figure S6 online, Figure 4a). Compared with WT mice, the levels of total filaggrin protein, filaggrin multimer, and filaggrin fragments were reduced in Trim32 KO mice (Figure 4b and c, respectively).
mice. Filaggrin mRNA levels were not affected by Trim32 deficiency (data not shown), suggesting that Trim32 effects are posttranscriptional. Further analysis of filaggrin by immunostaining revealed that cellular filaggrin expression in the epidermis was significantly reduced in Trim32 KO mice (Figure 4b and c).

**Trim32 deficiency enhances AD-like phenotypes in an 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). Histologic analysis of the treated skin revealed that Trim32 KO mice displayed a significantly thicker epidermis in response to MC903 treatment (Supplementary Figure S7 a and b online). Furthermore, eosinophil infiltration was significantly elevated in MC903-treated KO mice compared with their WT control littermates (Supplementary Figure S7c and d). IL-4 was elevated in Trim32 KO mice and further increased in response to MC903 treatment (Supplementary Figure S7e). Similar to IL-4, thymic stromal lymphopoietin, another key cytokine essential for Th2-associated inflammation in AD, was increased in Trim32 KO mice in response to MC903. Thymic stromal lymphopoietin basal levels were higher in Trim32 KO mice and further induced by MC903 than in WT mice (Supplementary 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**

Because changes in the chemokines in whole skin samples (shown in Figure 2 and Supplementary Figure 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 tumor necrosis factor-α (TNF-α) and IL-17A and that CCL5 expression was enhanced by TNF-α or TNF-α plus IL-4 in Trim32 KO keratinocyte cultures (Figure 5a and b). Further analysis of the induction pattern revealed that Trim32 primarily affected the induction by TNF-α 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 TNF-α 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 TNF-α and IL-17A was compromised in Trim32-deficient keratinocytes (Figure 5d).

**Trim32 induction is defective in AD**

To determine whether Trim32 deficiency is relevant to human AD, we compared TRIM32 levels in AD and psoriasis biopsy samples with healthy controls. TRIM32 levels in the nonlesional skin from psoriasis were similar to those in the skin from control individuals. Compared with nonlesional skin, the TRIM32 levels were significantly higher in both lesional skin and nonlesional...
skin from patients with AD compared with that from psoriatic epidermis and healthy skin (Figure 6a and b). This is consistent with reduced TRIM32 mRNA expression reported in patients with AD (Guttman-Yassky et al., 2009). As an innate antiviral 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 (Fu et al., 2015; Uchil et al., 2008), (2) inhibiting viral protein activity (Fatima et al., 2016; Fridell et al., 1995), and (3) activating innate immune signaling pathways (Albor et al., 2006; Uchil et al., 2013; Zhang et al., 2012). Consistent with our previous report that TRIM32 protein is elevated

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 analysis of variance followed by the Bonferroni post-test). Scale bar = 100 μm (a) and 200 μm (d and f). IMQ, imiquimod; KO, knockout; Th, T helper; WT, wild type.
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 Th cell response. Pathogen-associated molecular pattern-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 pathogen-associated molecular pattern-mediated innate immunity determines the polarization of Th cells.

AD is an inflammatory skin disease with defective innate immunity (Kuo et al., 2013). Patients with AD 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., 2013) 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 IkBα 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 Th cell differentiation in response to innate immune activation. Coupled with the low level of TRIM32 in AD lesional skin, these results provide a
pathologic basis for defective innate immunity and Th2 activation in patients with AD.

In summary, our data provide in vivo evidence that genetic manipulation of Trim32 regulates Th17 versus 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 the pathologic basis for defective innate immunity and Th2 activation in patients with AD.

MATERIALS AND METHODS

Reagents and antibodies
Reagents were purchased as follows: 5% IMQ 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-TNFαIP3 (A20), anti-glyceroldehyde-3-phosphate dehydrogenase (GAPDH), anti-phospho-Stat3 (Y705), and anti-phospho-Stat6 (Y641) from Cell Signaling (Danvers, MA); anti-filaggrin antibody from Santa Cruz (Cambridge, MA); 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 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 62.5 mg 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/6) 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 Oregon Health and Science University.

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 Ca2+) as described (Dlugosz et al., 1995; Kulesz-Martin et al., 1988). Once the keratinocyte culture reached 100% confluence, the keratinocytes were treated for 24 hours with species-specific cytokines: TNF-α (20 ng/ml), IL-4 (50 ng/ml), and IL-17A (100 ng/ml), purchased from PeproTech (Rocky Hill, NJ).

Human studies
The human subject research component was approved by the Oregon Health and Science University Institutional Review Board (2568). After informed consent, 4-mm punch biopsies were obtained from both patients with psoriasis and those with AD 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.

Histologic 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 eosinophils, four pictures of the stained skin sections were taken for each animal using a ×10 objective and ×40 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) were converted into total cDNAs using the RNA-cDNA kit (Applied Biosystems, Foster City, CA). The primers used are listed in Supplemental Table S1 online. Quantitative PCR was
set up in triplicates using Power SYBR Green mix (Applied Biosystems) 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, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM 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, and mouse serum IL-4 was measured using ELISA kits from Abcam.

**Sequential immunohistochemistry**

Multiplex sequential immunohistochemistry was performed with formalin-fixed, paraffin-embedded (5 μm) 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 (45M95, 1:50, Ebioscience), Goat anti-IL23R (1:100, Abcam), and rabbit anti-GATA3 (EPR16651, 1:500, Abcam). Histone Simple Stain MAX PO horseradish peroxidase-conjugated polymer (Nichirei Biosciences) was used for detection followed by 3-amino-9-ethylcarbazole (AEC) for peroxidase detection. Conjugated polymer (Nichirei Biosciences) was used for detection followed by 3-amino-9-ethylcarbazole (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 ×4 zoom from a ×20 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 ± standard deviation. Statistical significance was determined by an unpaired Student t test (***) and two-way analysis of variance followed by the 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.

**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 www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.09.020.

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Y Liu et al.

Trim32 Deficiency Enhances Th2 Atopic Response
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