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Inverse correlation of TRIM32 and PKCζ in Th2 biased inflammation

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Abstract

Atopic dermatitis (AD) is a Th2 biased disease with elevated expression of Th2 cytokines that responds to Th2 signaling blockade. Tripartite motif-containing protein 32 (TRIM32) is an E3 ubiquitin ligase with innate antiviral activity. In our previous studies, we showed that *Trim32* null mice developed Th2 biased skin inflammation in response to imiquimod and associated low level of TRIM32 with AD. In this study, we provide evidence that TRIM32 deficiency contributes to enhanced Th2 cell differentiation *in vitro*. Analysis of TRIM32-associated proteins from public databases identified PKCζ as a TRIM32-associated protein that contributes to the regulation of Th2 signaling. We demonstrated that PKCζ was specifically ubiquitinated by TRIM32, and further, that PKCζ stability tended to be increased in Th2 cells with a *Trim32* null background. Furthermore, *Prkcz* null mice showed compromised AD-like phenotypes in the MC903 AD model. Consistently, a high PKCζ and low TRIM32 ratio was associated with $CD4^+$ cells in AD human skin compared with healthy controls. Taken together, these findings suggest that TRIM32 functions as a regulator of PKCζ that controls the differentiation of Th2 cells important for AD pathogenesis.

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Author contributions
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Conflict of interest
The authors have declared that no conflict of interest exists.

Introduction

Atopic dermatitis (AD) is a chronic skin inflammatory disease characterized by skin barrier defects and the sustained activation of Th2-related inflammation (Hanifin 2018). Th2 immune pathways mediate immune responses against helminths and allergic reactions with the production of Th2 cytokines (Zhu 2018). Th2 immunity plays a critical role in the pathogenesis of AD as demonstrated by overexpression of Th2 cytokines in AD (Chan et al. 1996), development of AD-like phenotypes in mice with overexpression of Th2 cytokines and constitutively active STAT6 (Chan et al. 2001; Sehra et al. 2010), the association of genetic variants in genes in the Th2 signaling pathway (Bin and Leung 2016), and more importantly, the alleviation of AD clinical conditions by antibodies against Th2 cytokines (Simpson et al. 2016). Despite the importance of Th2 activation in AD pathogenesis, the mechanism of Th2 activation in AD remains largely elusive.

Defective innate immunity is another key feature of AD, manifested by patient susceptibility to bacterial and viral infection such as *S. aureus* colonization, herpes and vaccinia viral infection. Despite the highly inflamed nature and the presence of pathogens in AD skin, antimicrobial peptide expression is compromised in AD. We have previously added to the evidence supporting the role of defective innate immunity in AD pathogenesis, showing that *Trim32*$^{-/-}$ mice develop AD-like phenotypes in response to imiquimod, a TLR7 agonist that induces psoriasis-like phenotypes in wild type mice (Liu et al. 2017). TRIM32 is a member of TRIM E3-ubiquitin ligase family with innate anti-viral activity (Rajsbaum et al. 2014). TRIM32 was initially identified as an HIV Tat-associated protein (Fridell et al. 1995). It induces interferon-beta expression and represses influenza A viral infection (Fu et al. 2015; Versteeg et al. 2013). Trim32 is also an activator of STING (Zhang et al. 2012), a sensor for cytoplasmic DNA essential for protection against DNA viral infection (Chan and Gack 2016).

In the current study, we demonstrate that differentiation of CD4 naïve T cells to Th2 cells *in vitro* was enhanced by TRIM32 deficiency. Biochemically, TRIM32 was associated with protein kinase C zeta (PKCζ), an atypical protein kinase C isoform implicated in the activation of IL-4/STAT6 signaling. Moreover, TRIM32 ubiquitinated PKCζ and modulated its stability. Mice lacking PKCζ displayed reduced AD phenotypes in response to MC903 treatment. Consistently, CD4^{+} cells with high PKCζ and low TRIM32 were increased in AD human skin. These findings suggest a reciprocal role of TRIM32 and PKCζ in Th2 regulation.

Results

Th2 cell infiltration is enhanced by TRIM32 deficiency in the MC903 AD mouse model.

To explore the contribution of TRIM32 in Th2 regulation, we evaluated the status of Th2 cell infiltration in MC903-treated *Trim32* deficient mice. Th2 cells were measured by co-staining of CD4 and GATA3, a Th2 lineage specific transcription factor (Zheng and Flavell 1997). Consistent with enhanced AD-like phenotype in *Trim32*$^{-/-}$ mice, the infiltration of GATA3 positive T cells was significantly enhanced in *Trim32*$^{-/-}$ mice compared with

Trim32$^{+/+}$ mice treated with MC903 (Figure 1a&b). These findings suggest that *Trim32* deficiency contributes to Th2 biased inflammation in the MC903-induced AD model.

Th2 cell differentiation *in vitro* is enhanced by *Trim32* deficiency.

The increased Th2 infiltration in *Trim32*$^{-/-}$ mice could result from increased recruitment of Th2 cells or increased Th2 cell differentiation. The former was not supported by any significant changes in Th2 chemokines CCL1 and CCL17 (data not shown). However, analysis of Th2 cell differentiation *in vitro* revealed enhanced Th2 differentiation from naïve T cells from *Trim32*$^{-/-}$ mice (Figure 1c&d). Consistently, quantitative RT-PCR showed increased expression of IL-4 in Th2 cells with a *Trim32*$^{-/-}$ background (Figure 1e). These data indicate that Th2 cell differentiation is enhanced by Trim32 deficiency.

We next analyzed regulation of Th2 signaling by Trim32 through analysis of STAT6, a Th2 lineage-specific transcription factor (Kaplan et al. 1996; Zhu et al. 2001), and its downstream targets, GATA3 and GFI-1 (Kurata et al. 1999; Zhu et al. 2006). Consistent with enhanced Th2 cell differentiation, STAT6 protein phosphorylation (Figure 2a & 2b) as well as mRNA levels of *Gata3* (Figure 2c) and *Gfi-1* (Figure 2d) were significantly increased in Th2 cells with *Trim32*$^{-/-}$ background, suggesting TRIM32 acts upstream of STAT6 in the Th2 pathway.

PKCζ is ubiquitinated by TRIM32, and PKCζ stability in Th2 cells is enhanced by *Trim32* deficiency.

To define a mechanism by which *Trim32* deficiency enhances Th2 activation, we reviewed TRIM32 associated proteins from public databases and cross-referenced them to phenotypes produced by their respective knockout mice. PKCζ emerged as a TRIM32-associated protein that contributes to the regulation of Th2 immunity. PKCζ was initially identified as a TRIM32-associated protein in regulating neural stem cell maintenance and differentiation (Hillje et al. 2011). Comparison of the phenotypes of *Trim32*$^{-/-}$ mice and *Prkcz*$^{-/-}$ mice showed inversed Th2 immune profiles (Supplemental Table S1). PKCζ deficiency impaired Th2 differentiation and dramatically inhibited ovalbumin-induced allergic airway disease (Martin et al. 2005). PKCζ expression is induced in Th2 cells but not in Th0 or Th1 cells, and its contribution to Th2 activation is mediated through activation of IL-4 signaling (Durán et al. 2004; Martin et al. 2005).

In addition to PKCζ, other PKC family members, such as PKCθ and PKCι, have also been implicated in T helper cell differentiation under different conditions (Marsland et al. 2004; Yang et al. 2009). Co-immunoprecipitation with PKC isoform(s) revealed that TRIM32 associated with PKCζ but not PKCθ or PKCι (Figure 3a), indicating that TRIM32 binds specifically to PKCζ. To define the domains involved in the interaction between TRIM32 and PKCζ, we evaluated various TRIM32 deletional mutants for their interaction with PKCζ. The TRIM32 mutants containing the NHL domain bound well to PKCζ, whereas the one without the NHL domain (ΔNHL) showed minimal binding indicating that the NHL domain contributes to PKCζ association (Figure 3b). NHL is the domain involved in binding to substrates of TRIM32, thus suggesting that PKCζ is a substrate for TRIM32. Analysis of TRIM32 binding to PKCζ deletional mutants revealed that TRIM32 associated with the full length PKCζ only, not with the N-terminal or C-terminal PKCζ mutants (Figure 3c), indicating that TRIM32 binding requires the hinge region between the N-terminal or C-terminal domains. The major sequence difference between PKCζ and PKCι lies in the hinge region, thus providing a basis for TRIM32 specific binding to PKCζ but not to PKCι.

PKCζ ubiquitination by TRIM32 has been suggested previously, by immunoprecipitation with PKCζ antibody followed by immunoblotting with ubiquitin antibody (Hillje et al. 2011). However, due to non-denaturing conditions used in these experiments, it was not clear whether the ubiquitinated proteins detected were PKCζ or other proteins in an immune-complex with PKCζ. To validate the specificity of PKCζ ubiquitination by TRIM32, PKCζ ubiquitination by TRIM32 was compared with other PKC isoforms by an *in vivo* ubiquitination assay that measures ubiquitinated PKCζ protein under denaturing conditions. PKCζ was ubiqutinated by TRIM32 but not the RING domain deletional mutant of TRIM32 (dRING) that is defective in E3 ligase activity (Figure 4a). Consistent with TRIM32 binding studies above (Figure 3a), PKCθ and PKCι were not ubiquitinated by TRIM32 (Figure 4a). Taken together, these results demonstrated that PKCζ is ubiquitinated by TRIM32.

To test whether TRIM32 contributes to PKCζ degradation, we evaluated the half-life of PKCζ in HEK293T cells stably overexpressing PKCζ. Compared with cells expressing wild type TRIM32, the half-life of PKCζ was increased in cells expressing a TRIM32 mutant with a RING domain deletion (Figure 4b). Furthermore, we evaluated endogenous PKCζ turnover in T cells with different *Trim32* background. The turnover of endogenous PKCζ was similar between the undifferentiated T cells (Th0) with different *Trim32* genotypes (Figure 4c). Interestingly, the half-life of PKCζ was increased in Th2 cells compared with Th0 cells (Figure 4c&d). Moreover, there was a trend of a greater increase of half-life of PKCζ in *Trim32*$^{-/-}$ Th2 cells compared with *Trim32*$^{+/+}$ Th2 cells (Figure 4d), suggesting that TRIM32 contributes to PKCζ degradation. Such altered stability was not observed in PKCθ (data not shown). Moreover, the half-life of PKCλ showed opposite results: 1) the half-life of PKCλ was extended in Th0 cells compared with Th2 cells; 2) a trend of extended half-life of PKCλ in *Trim32*$^{+/+}$ Th2 cells compared with Trim32$^{-/-}$ Th2 cells (Supplemental Figure S1). Cell viability showed no difference in T cells with different Trim32 backgrounds during cycloheximide treatment (Supplemental Figure S2). Taken together, the negative regulation of PKCζ by TRIM32 is consistent with the opposing effects of TRIM32 and PKCζ on Th2 regulation.

AD-like phenotypes are compromised in *Prkcz*$^{-/-}$ mice treated with MC903.

Although PKCζ is important in controlling Th2 cell function and PKCζ deficiency limits ovalbumin-induced allergic airway disease (Martin et al., 2005), the contribution of PKCζ to AD pathogenesis has not been reported yet. To address the role of PKCζ in AD pathogenesis, we evaluated Th2 responses and AD-like phenotypes in *Prkcz*$^{-/-}$ mice. *Prkcz*$^{-/-}$ mice showed reduced skin inflammation induced by MC903 (Figure 5a&b). The ear skin of *Prkcz*$^{-/-}$ mice treated with MC903 displayed less scaling and epidermal thickening than that of the wild type mice (Figure 5c). The expression of *Tslp*, a direct target of MC903, was reduced in *Prkcz*$^{-/-}$ mice compared with their wild type littermates (Figure 5d).

Furthermore, the induction of *Il-4* and *Gata3* by MC903 was ablated in *Prkcz*$^{-/-}$ mice, suggestive of a defective Th2 pathway in *Prkcz*$^{-/-}$ mice (Figure 5d). Additionally, there were fewer GATA3/CD4 double positive cells in the dermis of *Prkcz*$^{-/-}$ mice (Figure 5e&f). Taken together, these results strongly suggest that PKCζ positively contributes to Th2 activation and AD pathogenesis.

CD4+ T cells with high PKCζ and low TRIM32 are increased in AD skin.

PKCζ degradation by TRIM32 and their opposing effects on the Th2 immune profile (Supplemental Table S1) suggests there is a reciprocal relationship between TRIM32 and PKCζ in AD. Analysis of PKCζ and TRIM32 expression in AD patient skin by indirect immunofluorescence staining revealed that TRIM32 epidermal expression in AD skin was lower than in skin from healthy controls (Figure 6a), consistent with our previous observation (Liu et al. 2017). However, PKCζ expression in AD epidermis was not increased overall (Figure 6a). The PKCζ antibody (H1) used in this study recognizes an epitope that is shared by PKCζ and PKCι. However, immunoblotting of primary keratinocytes showed that PKCζ/ι immunoreactivity measured with this antibody was not reduced in *Prkcz*$^{-/-}$ keratinocytes (Supplemental Figure S3). This indicates that PKCι but not PKCζ is the major atypical PKC in keratinocytes. In human T cells, however, atypical PKC immunoreactivity was detected only with a PKCζ specific antibody (Supplemental Figure S4). This indicates that PKCζ is the major form in human T cells. Therefore, immunostaining analysis with PKCζ/ι antibody (H1) reflects the status of PKCι in epidermal keratinocytes and PKCζ in CD4^{+} T cells. Analysis of overall signal intensity of TRIM32 and PKCζ in CD4+ cells in the dermis showed no significant differences between AD and control skin (data not shown). However, the percentage of CD4^{+} cells with high PKCζ and low TRIM32 was significantly increased in AD skin (Figure 6b&c). While it remains to be determined the nature of these CD4^{+} cells with high PKCζ and low TRIM32, these data support roles for PKCζ and TRIM32 in atopic dermatitis.

Discussion

In this study, we provide evidence that Th2 cell differentiation is enhanced by TRIM32 deficiency and its association with PKCζ in Th2 biased inflammation. This finding provides a basis for Th2 biased phenotypes found in *Trim32*$^{-/-}$ mice (Liu et al. 2017). PKCζ is an atypical protein kinase involved in a variety of cellular activities, including Th2 regulation (Moscat et al. 2006). PKCζ deficiency contributes to compromised Th2 immunity with low level of serum IgE, impaired Th2 differentiation, and inhibition of ovalbumin-induced allergic airway disease. These are inversely correlated with indications of Th2 biased inflammation observed in *Trim32*$^{-/-}$ mice (Supplemental Table S1). The importance of PKCζ in Th2 regulation was highlighted by our *in vivo* evidence that Th2 signaling pathway was defective in *Pkcζ*$^{-/-}$ mice treated with MC903 (Figure 5). Consistent with an increased PKCζ protein level in Th2 cells (21), we found the half-life of PKCζ was increased in Th2 cells compared with Th0 cells (Figure 4c&d). More importantly, there was a trend of further increase of half-life of PKCζ in *Trim32*$^{-/-}$ Th2 cells compared with *Trim32*$^{+/+}$ Th2 cells (Figure 4d). This indicates that the enhanced Th2 differentiation by TRIM32 deficiency is mediated through PKCζ. Interestingly, we noticed opposite effect on PKCλ in T cells with different Trim32 backgrounds (Supplemental Figure S1) suggesting a compensatory effect between atypical PKC members.

Th2 signaling is largely mediated through STAT6 phosphorylation to activate the expression of the Th2 specific transcription factor GATA3 and Th2 cytokines (Kaplan et al. 1996; Zheng and Flavell 1997). In addition, IL-4/STAT6 signaling integrates with PI3K/mTOR signaling through IRS2, an insulin receptor substrate phosphorylated by the IL-4 receptor (Heller et al. 2008). IRS2 activates PI3K/mTOR signaling which plays an indispensable role in Th2 cell differentiation (Delgoffe et al. 2011; Yang et al. 2013). PKCζ is an atypical protein kinase C activated by PDK1 (phosphoinositide-dependent protein kinase-1) (Balendran et al. 2000), an essential kinase in the PI3K/mTOR signaling pathway. The impaired activation of JAK1 and STAT6 in response to IL-4 in Th2 cells with a *Prkcz* null background (Martin et al. 2005) suggests that PKCζ acts as a co-activator in the IL-4/STAT6 signaling pathway for Th2 regulation. Negative regulation of PKCζ by TRIM32 thus provides a potential mechanism in regulating Th2 signaling.

Low levels of PKCζ in T cells from human cord blood have been associated with high risk of allergic diseases including AD (D’Vaz et al. 2012; Prescott et al. 2007). However, it is unclear how to reconcile this association with compromised asthma-like phenotypes (Martin et al. 2005) and AD-like phenotypes in *Prkcz*$^{-/-}$ mice (Figure 5) and increased CD4^{+} T cells with high PKCζ and low TRIM32 in AD skin (Figure 6). It is likely that PKCζ levels in fetal T cells is regulated differently in the allergen free environment in utero or regulated through maternal Th1/Th2 dichotomy (Dealtry et al. 2000). Nevertheless, a low level of PKCζ protein was associated with low IL-4 production in T cells from cord blood in the same study (D’Vaz et al. 2012). Although such association contradicts the association of low PKCζ in infants with allergic diseases, it is consistent with the role of PKCζ as a positive regulator of Th2 signaling in this study and those of others (Durán et al. 2004; Martin et al. 2005).

So far the significance of PKCζ regulation by TRIM32 in Th2 differentiation is largely unknown. TRIM32 is an E3 ubiquitin ligase with innate anti-viral activity (Rajsbaum et al. 2014), and the regulation of PKCζ by TRIM32 in Th2 cell suggests the integration of innate immune signaling in Th2 cell differentiation. TRIM32 is an activator of stimulator of interferon genes protein (STING) (Zhang et al. 2012), a sensor for cytoplasmic DNA essential for protection cell against DNA viral infection (Chan and Gack 2016). The activation of STING inhibits the activities of mTOR and STAT5 (Imanishi et al. 2019), which play critical roles in Th2 cell differentiation (Delgoffe et al. 2011; Yang et al. 2013; Zhu et al. 2003). The integration of TRIM32 signal in Th2 cell differentiation provides a potential molecular association between defective innate immunity and Th2 polarization in AD.

Taken together, our findings indicate that TRIM32 deficiency contributes to Th2 cell differentiation, providing potential connection between defective innate immunity and Th2 polarization in AD. Furthermore, we identified PKCζ as a TRIM32-associated protein that contributes to the regulation of Th2 signaling, thus providing evidence at the molecular level to integrate TRIM32 innate immune signaling and PKCζ signaling in Th2 regulation. Future studies to define the interaction of TRIM32 and PKCζ pathways in Th2 regulation will shed light on our understanding of the interplay between innate immunity and Th2 activation in Th2 biased inflammation.

Materials and Methods

Reagents, antibodies, and plasmids

Cytokines and antibodies are summarized in Supplemental Table S2. The V5-TRIM32 and its deletional mutants were constructed by cloning *Trim32* cDNA (Albor et al. 2006) and its deletional segments into BamHI and EcoRI digested pcDNA-V5 vector (Invitrogen). Flag-tagged PKCζ was obtained from Alex Toker (Addgene plasmid #10799). The Flag-tagged N-terminal domain of PKCζ (1–183 a.a.) and C-terminal domain of PKCζ (184–592a.a.) were subcloned into pCMV-Tag2B (Stratagene). PKCζ recombinant lentivirus was constructed by cloning full-length PKCζ cDNA into pSL35 lentiviral vector for lentiviral production as described (Liu et al. 2007).

Mouse skin inflammation models

AD-like disease was induced by MC903 (Li et al. 2006) in *Trim32*$^{-/-}$ mice in pure FVB genetic background (Liu et al. 2017) and *Prkcz*$^{-/-}$ mice (Lee et al. 2013) on a mixed genetic background (C57BL/6 X 129S6_SvEvTac). The mutant mice and their wild type littermates from the same breeding generation were used in the experiments.

All animals were bred under specific pathogen-free conditions and used for experiments at 8–11 weeks of age.

In vitro differentiation of CD4 T cells and flow cytometry

Naïve CD4 T cells were isolated from the spleen of 7–9 weeks-old mice using the EasySep™ CD4+ T Cell Isolation Kit (STEMCELL, Cambridge, MA). Naive CD4 T cells were cultured and induced into Th2 helper cells as described (Sekiya and Yoshimura 2016). Cultured CD4 T cells were stimulated with 50ng/ml PMA,1μg/ml ionomycin and 2uM Brefeldin-A for 5 hours before being labeled with anti-CD4 (PerCP-Cy5.5, RM4–5)and then fixed and labeled with anti-IL-4 (AlexaFluro647, 11B11) for flow cytometry analysis with a Becton-Dickinson LSR II.

Histological analysis, staining and quantification

Paraffin embedded skin tissue sections were stained with hematoxylin-eosin (H&E). The number of CD4 and GATA3 double positve cells were stained and quantified using ImageJ software.

Quantitative RT-PCR

Quantitative PCR was set up in triplicate using Power SYBR® Green mix (Applied Biosystems, Foster City, CA) on a real-time PCR system (ViiA™ 7 Real-Time PCR System). The primers used to amplify the fragments of the indicated genes are summarized in Supplemental Table S3.

Immunoprecipitation

HEK293T cells were transfected with Flag-tagged PKCζ and V5-tagged TRIM32 plasmids. The transfected cells were lysed in RIPA buffer as described (Liu et al. 2012). The lysates were precipitated with agarose-coupled anti-Flag antibody (Sigma). The precipitated Trim32 proteins were blotted with anti-V5 antibody (Sigma). The total lysate was blotted as input reference.

In vivo Ubiquitination Assay

In vivo ubiquitination assays were conducted in cells using a Ni2+-NTA pulldown method as described (Liu et al. 2012). Briefly, HEK293T cells transfected with His-tagged ubiquitin and indicated plasmids were treated with 25 μM MG132 for 6 hours before harvesting. The cell lysates were subjected to Ni2+-NTA pulldown under denaturing conditions. The bead bound proteins were analyzed by immunoblotting.

Cycloheximide chase analysis and immunoblotting

HEK293T cells with stable expression of exogenous PKCζ were generated by infection of PKCζ recombinant lentivirus. The PKCζ transduced HEK293T cells were transfected with TRIM32 or its RING domain deletional mutant for 16–18 hours followed by treating with 50 μM of cycloheximide for indicated times. To measure the half-life of endogenous PKCζ, naïve $CD4^+$ T cells were cultured under Th0 or Th2 conditions as described previously (Sekiya and Yoshimura 2016). Cells were incubated with 20 μg/ml of cycloheximide for various times. Cell lysates were collected for PKCζ immunoblotting with a ChemiDoc Touch Imaging System. PKCζ protein was quantified by ImageJ and scaled to the values of the loading controls.

Quantification of TRIM32 and PKCζ in CD4+ cells

Paraffin embedded skin sections from AD control skin were stained with Anti-CD4 antibody (Fluor-647) (Abcam, Cambridge, MA), Anti-PKCζ/ι antibody (Fluor-555) (Santa Cruz, Texas), and anti-TRIM32 antibody (Fluor-790) (Liu et al. 2010). The images were captured using a Yokogawa CSU-X1 on a Zeiss Cell Observer microscope. The images were tiled to form an image of the entire tissue section for quantification. The signal intensity of TRIM32 and PKCζ in CD4+ cells was quantified individually by ZEN 2.6 (Blue edition) software. The mean intensity of PKCζ (555), CD4 (647), and TRIM32 (790) in each cell in the entire dermis was quantified. Background intensity for each channel was obtained and was used to normalize the mean intensity values across samples. Of the CD4+cells, mean intensity pixel values were plotted (x-axis: TRIM32, y-axis: PKCζ) and sorted into quadrants based on a threshold of 100 pixels above the background for each of the axes. The percentage of cells in the quadrant of high PKCζ (>100pixel on y-axis) and low TRIM32 (<100 pixel on x-axis) was calculated.

Statistical analysis

Statistical significance was determined using Student's *t* test for paired samples, two-way ANOVA followed by Tukey's multiple comparison test for multiple sample analysis.

Study approval

The human subject research component was approved by the OHSU Institutional Review Board (IRB-2568). Written informed consent was obtained from all patients and healthy controls. Skin punch biopsies of 4mm were obtained from AD patients and diagnosis was confirmed after histology was reviewed by a dermatopathologist. Skin biopsies from individuals with no history of psoriasis, AD, or inflammatory disease served as healthy controls. All animal experiments were conducted according to animal protocol (IS00001640) approved by OHSU IACUC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data availability statement

No datasets were generated or analyzed during the current study. Primary data are available upon request.

Abbreviations:

AD	atopic dermatitis
TRIM32	tripartite motif-containing 32
PKCζ	protein kinase C zeta
PKCι	protein kinase C iota
PKCθ	protein kinase C theta
Prkcz	protein kinase C zeta coding gene
Th	T helper
CD4	cluster of differentiation 4
STAT	the signal transducer and activator of transcription
IL	interleukin
CCL	CC chemokine ligand
TSLP	thymic stromal lymphopoietin
STING	stimulator of interferon genes
GATA3	GATA binding protein 3

IgE	immunoglobulin E
PI3K	phosphoinositide 3-kinases
mTOR	the mammalian target of rapamycin
PDK1	phosphoinositide-dependent kinase-1

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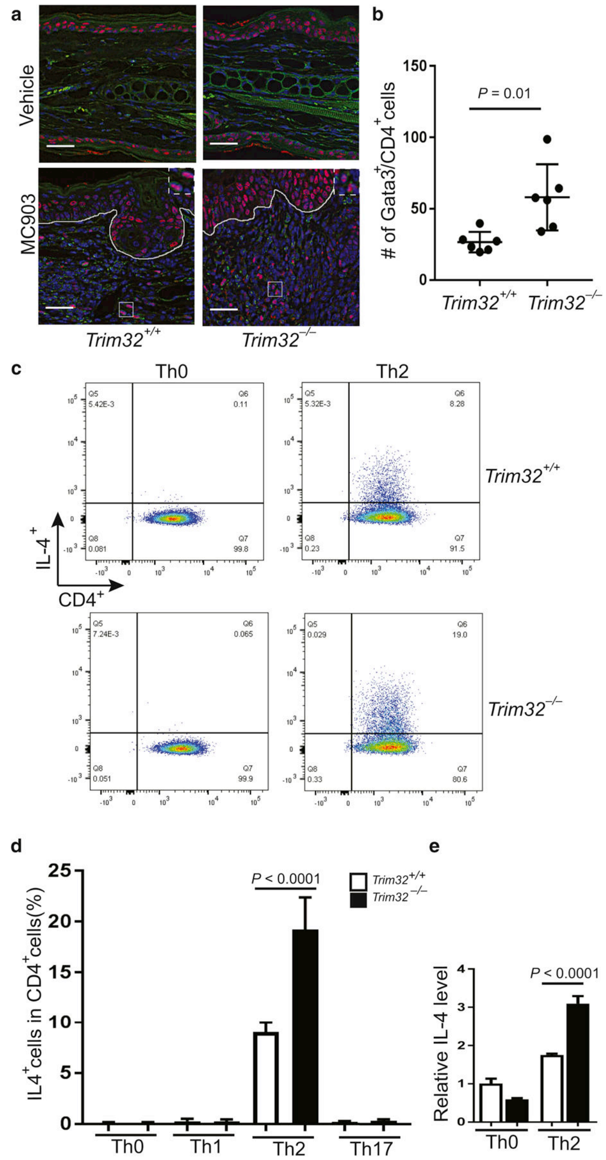

Figure 1. Th2 polarization by Trim32 deficiency.

(**a**) Representative images of CD4+/GATA3+ cells in the skin of *Trim32* wild type (*Trim32*$^{+/+}$) and null (*Trim32*$^{-/-}$) mice treated with MC903 for 9 days (n=6 for MC-903-treated *Trim32*$^{+/+}$ and *Trim32*$^{-/-}$ mice). (**b**) Quantification of dermal CD4$^+$/GATA3$^+$ cells (Student *t* test). (**c**) Flow cytometry analysis of Th2 cells (IL-4$^+$/CD4$^+$) differentiated from naïve CD4$^+$ T cells *in vitro* and undifferentiated CD4$^+$ T cells (Th0). (**d**) Quantification of IL-4$^+$/CD4$^+$ T cells (n=4 for each group). (Two-way ANOVA with Tukey's post-test: $F(3, 24)=37.31$, $P<0.0001$). (**e**) RT-PCR quantification of IL-4 expression in both Th0 and Th2 cells (Two-way ANOVA with Tukey's post-test: $F(1, 8)=162$, $P<0.0001$). Scale bar=50μM.

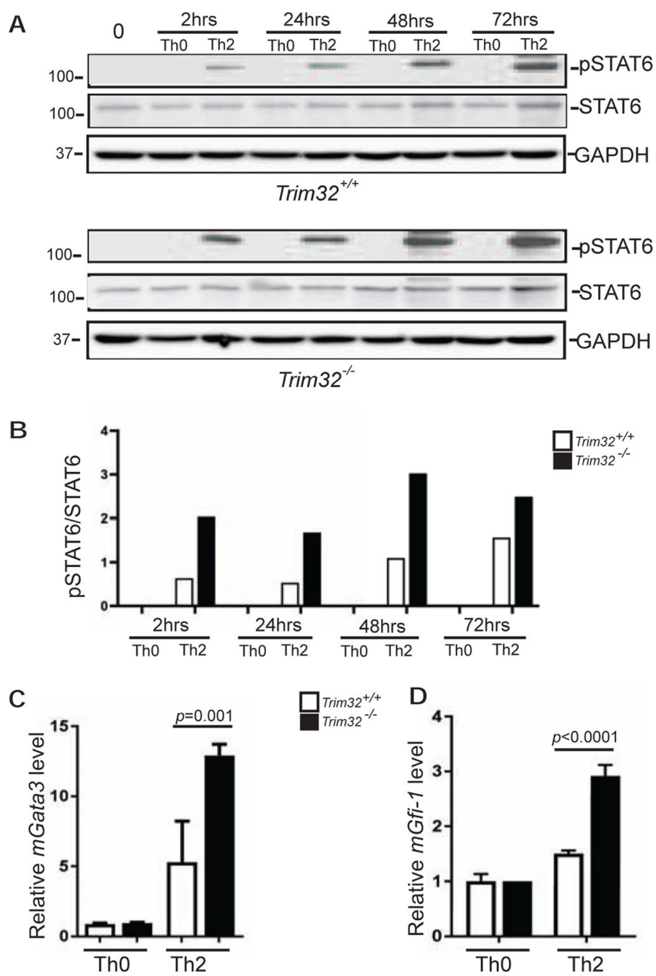

Figure 2. IL-4 signaling pathway enhanced by TRIM32 deficiency in Th2 cells.

Naïve T cells (Th0) and Th2 cells (Th2) from *Trim32*$^{+/+}$ and *Trim32*$^{-/-}$ mice were analyzed by (**a**) Immunoblotting of phospho-STAT6 level and (**b**) quantification of pSTAT6/STAT6; (**c**) RT-PCR quantification of *Gata3* mRNA (Two-way ANOVA with Tukey's post-test: F(1,8)=18.28 P=0.0027); (**d**) RT-PCR quantification of *Gfi-1* mRNA. (Two-way ANOVA with Tukey's post-test: F(1,8)=98.02 P=0.0001).

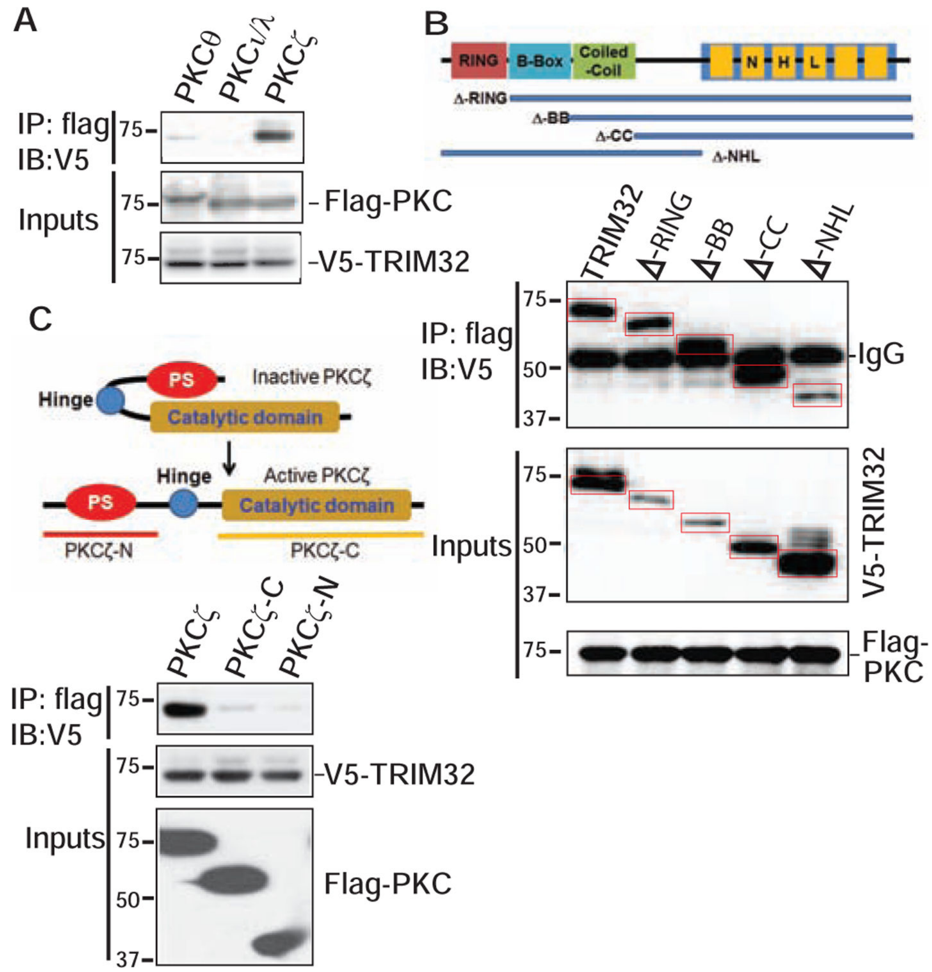

Figure 3. Specificity of TRIM32 association with PKCζ.

The immunoprecipitation was conducted in lysates of HEK293T cells co-transfected with V5-tagged TRIM32 and Flag-tagged PKC plasmids as indicated. (**a**) TRIM32interacts with PKCζ but not PKCθ or PKCι/λ as measured by immunoprecipitation (IP) followed by immunoblotting (IB). (**b**) PKCζ binds to Trim32 NHL domain. (**c**) TRIM32 binds to full length PKCζ but not the N-terminal or C-terminal domains of PKCζ.

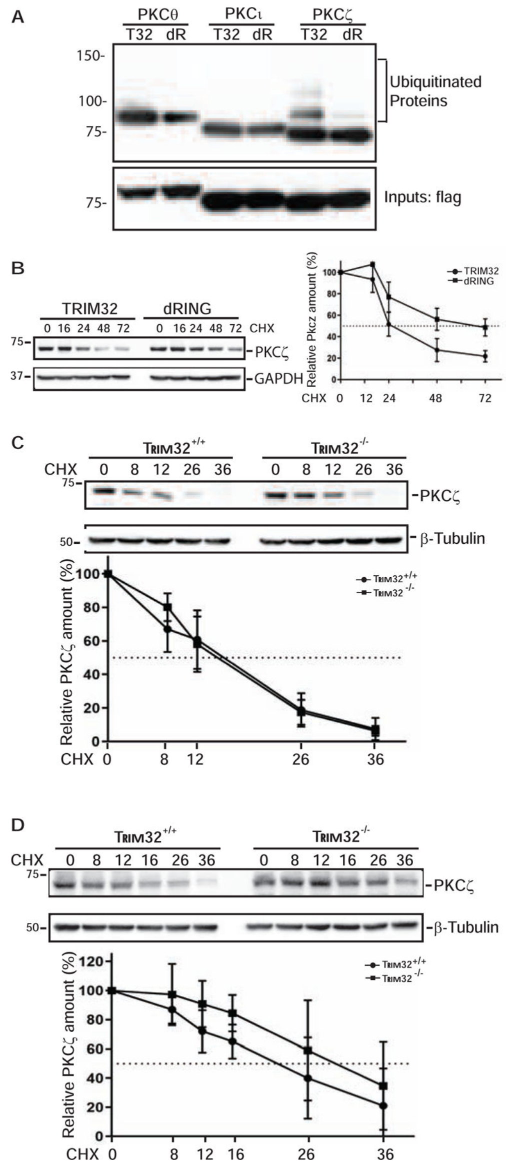

Figure 4. Analysis of TRIM32mediated PKCζ ubiquitination and stability.

(**a**) PKCζ but not PKCθ or PKCι is ubiquitinated by TRIM32 (T32) but not by TRIM32with RING domain deletion (dR). Ubiquitinated PKC proteins were measured by Ni2+-NTA bead pull down followed by immunoblotting. (**b**) PKCζ degradation is enhanced by TRIM32 but not its RING domain deletion mutant (dRING). HEK293T cells with stable expression of exogenous PKCζ were generated by infection of PKCζ recombinant lentivirus. PKCζ protein in HEK293T cells transfected with V5-TRIM32 or V5-dRING was measured at indicated time points after cycloheximide treatment (CHX). The data were quantified with three independent experiments. (**c**) Analysis of endogenous PKCζ stability in undifferentiated T cells (Th0) from *Trim32*$^{+/+}$ and *Trim32*$^{-/-}$ mice. (**d**) Analysis of PKCζ stability in Th2 cells from *Trim32*$^{+/+}$ and *Trim32*$^{-/-}$ mice. The levels of PKCζ at different time points after cycloheximide treatment were quantified and normalized with tubulin loading controls. The data were quantified with four independent experiments.

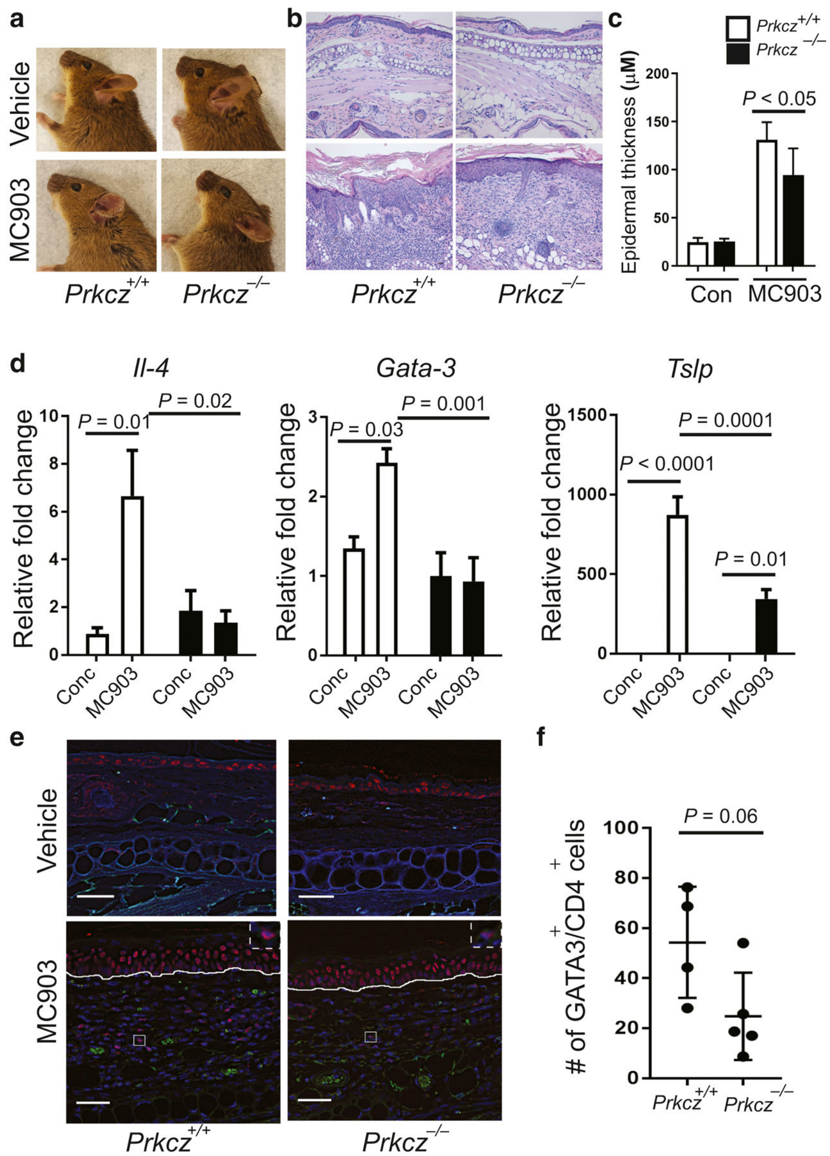

Figure 5. Compromised AD-like phenotypes by PKCζ deficiency.

(**a**) Representative pictures of gross appearance of ear from *Prkcz*$^{+/+}$ and *Prkcz*$^{-/-}$ mice in response to topical MC903 treatment. (**b**) Representative images of H&E staining of tissue sections of ears treated with MC903. (**c**) Quantification of epidermal thickening (n=3–5). (**d**) qRT-PCR analysis of the mRNA levels of *IL-4* (Two-way ANOVA with Tukey's post-test: F(1,18)=7.302 P=0.0146), *Gata3* (Two-way ANOVA with Tukey's post-test: F(1,18)=5.58, P=0.0296), and *Tslp* (Two-way ANOVA with Tukey's post-test: F(1,18)=14.26, P=0.0014). (**e**) Representative images of dermal CD4^{+}/GATA3^{+} cells in the skin treated with MC903. (**f**) Quantification of dermal CD4^{+}/GATA3^{+} cells in the skin treated with MC903 (n=4 for *Prkcz* $^{+/+}$ mice and n=5 for *Prkcz*$^{-/}$ mice, Student *t* test). Scale bar=50μM.

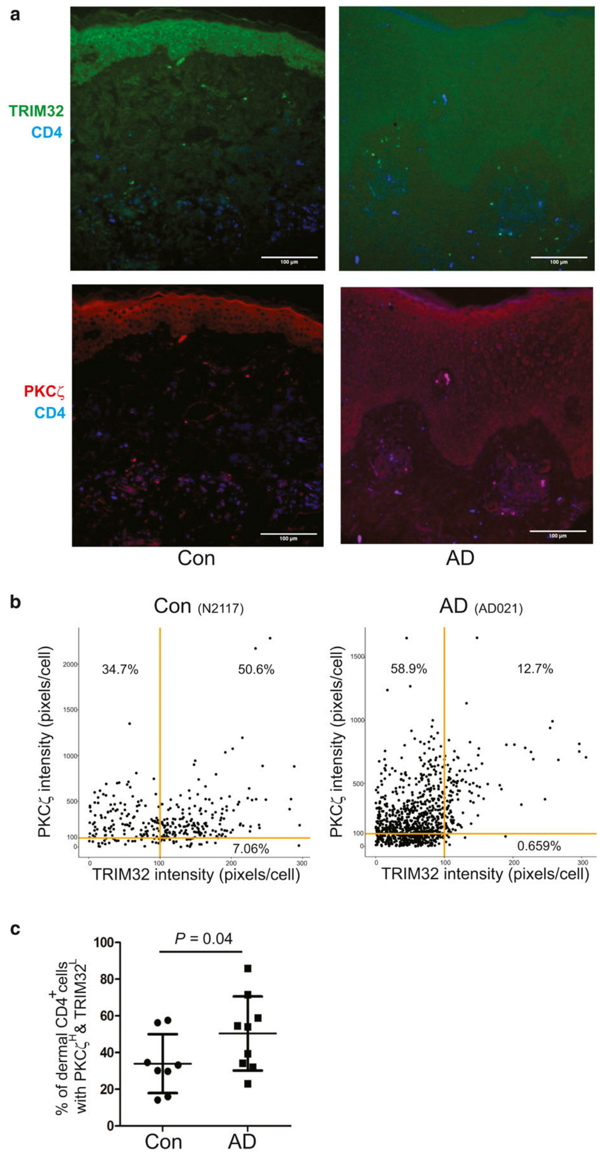

Figure 6. Evaluation of PKCζ and TRIM32 levels in skin from AD patients.

Paraffin embedded skin sections from AD (n=9) and control skin (n=8) were stained with anti-CD4 antibody, anti-PKCζ/ι antibody, and anti-TRIM32 antibody. (**a**) Representative images of indirect immunofluorescence of TRIM32 (green), PKCζ (red), and CD4 (blue) antibody reactivity. Scale Bar=100 μm. (**b**) Quantification of the signal intensity of PKCζ and TRIM32 in $CD4^+$ cells. The mean intensity pixel values of PKCζ and TRIM32 in each $CD4^+$ cell were calculated and plotted into quadrants based on a threshold of 100 pixel above the background for each of the axes. (**c**) The percentages of $CD4^+$ cells with high PKCζ (>100 pixel/per cell on y-axis) and low TRIM32 (<100 pixel/per cell on x-axis) were calculated and plotted (Student *t* test).