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IRAK2 Has a Critical Role in Promoting Feed-Forward Amplification of Epidermal Inflammatory Responses

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AUTHOR CONTRIBUTIONS

Conceptualization: SS, JEG; Data Curation, SS, JC, RU, ACB, XX, CZ, MKS, RW, YJ, JK, JS, OP; Formal Analysis: LCT, WRS, FM, MP, AM; Investigation, SS, JC, RU, ACB, XX, CZ, MKS, RW, YJ, JK, JS, OP; Methodology: SS, JC, RU, ACB, XX, CZ, MKS, RW, YJ, JK, JS, OP; Writing - Original Draft Preparation: SS, JEG; Writing - Review and Editing: SS, JEG, GW, PWH, JJV, NLW, MP, AM, BEPW, RLM, BA, EM, SW, JMK

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 https://doi.org/10.1016/j.jid.2021.03.019.

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Abstract

Many inflammatory skin diseases are characterized by altered epidermal differentiation. Whether this altered differentiation promotes inflammatory responses has been unknown. Here, we show that IRAK2, a member of the signaling complex downstream of IL-1 and IL-36, correlates positively with disease severity in both atopic dermatitis and psoriasis. Inhibition of epidermal IRAK2 normalizes differentiation and inflammation in two mouse models of psoriasis- and atopic dermatitis-like inflammation. Specifically, we demonstrate that IRAK2 ties together proinflammatory and differentiation-dependent responses and show that this function of IRAK2 is specific to keratinocytes and acts through the differentiation-associated transcription factor ZNF750. Taken together, our findings suggest that IRAK2 has a critical role in promoting feed-forward amplification of inflammatory responses in skin through modulation of differentiation pathways and inflammatory responses.

INTRODUCTION

The epidermis provides both a physical and immunologic barrier to a variety of external threats (Elias, 2007). Keratinocytes (KCs) are the major cellular constituent of the epidermis and exhibit excessive proliferation and aberrant differentiation when reacting to a wide range of environmental and internal stimuli, evident in diseases such as atopic dermatitis (AD) and psoriasis (Boehncke and Schön, 2015; Dainichi et al., 2018; de Vries et al., 1998; Guttman-Yassky et al., 2011a; Kim et al., 2018; Moos et al., 2019). The role of KCs in contributing to inflammation is well-known and occurs through secretion of cytokines, chemokines, and antimicrobial peptides (Dainichi et al., 2018; Nestle et al., 2009; Veldhoen, 2017; Ye et al., 2014). In addition, during inflammation, proliferation of KCs frequently leads to epidermal thickening or acanthosis (Mansbridge and Knapp, 1987). Whether this altered differentiation plays a passive or active role in promoting inflammatory responses in the epidermis, and whether there is a shared mechanism linking the two, has remained unclear.

IRAKs, including IRAK1, IRAK2, IRAK3 (IRAK-M), and IRAK4, are a family of serine-threonine kinases that mediate IL-1 and toll-like receptor (TLR) signaling in immune cells (Flannery and Bowie, 2010; Jain et al., 2014). Despite the role of IL-1R and TLR signaling in skin inflammation (Cataisson et al., 2012; Vu et al., 2010), little is known about the function and significance of IRAK2 signaling in the epidermis under healthy or inflammatory states.

Here, we show that IRAK2 expression correlates with disease severity in two chronic inflammatory diseases of the skin, AD and psoriasis, and knockdown of epidermal

IRAK2, through topical application of *IRAK2* small interfering RNA (siRNA), alleviates the epidermal thickness and inflammation in both psoriasis- and AD-like mouse models. We further demonstrate that IRAK2 function is primarily in KCs but not in fibroblasts, where it regulates an alternative differentiation pathway in the epidermis through its effects on the epidermal differentiation-associated transcription factor (TF), ZNF750. In turn, this facilitates and amplifies epidermal immune responses, promoting a feed-forward amplification in a more active proinflammatory state.

RESULTS

IRAK2 is increased and correlates with disease severity in psoriasis and AD

mRNA expression of *IRAK* members in AD and psoriasis (Tsoi et al., 2019) was assessed, with *IRAK2* being most significantly upregulated in both psoriasis (fold change [FC] = 4.9-fold, $P = 2 \times 10^{-25}$) and AD (FC = 2.2-fold, $P = 2 \times 10^{-11}$) compared with healthy control skin (Figure 1a and Supplementary Figure S1a). Notably, *IRAK2* mRNA expression was positively correlated with disease severity, as measured by PASI and SCORing Atopic Dermatitis) in psoriasis (Spearman correlation = 0.45, $P = 1.5 \times 10^{-2}$) and AD (Spearman correlatin = 0.51, $P = 2.7 \times 10^{-3}$), respectively (Figure 1b). Consistent with this observation, IRAK2 protein was abundantly expressed in the epidermis of both AD and psoriasis (Figure 1c).

To identify IRAK2 inducers, we stimulated primary human KCs with a panel of cytokines involved in the pathogenesis of psoriasis and AD. RNA-sequencing (RNA-seq) analysis showed that TNF, a combination of TNF and IL-17A, or IL-36γ significantly induced *IRAK2* mRNA expression in primary KCs (FC = 1.8-fold, false discovery rate [FDR] = 8.7×10^{-16}; FC = 1.7-fold, FDR = 7.6×10^{-18}; FC = 1.5-fold, FDR = 2.3×10^{-23}, respectively; n = 38) (Figure 1d). Quantitative real-time PCR validated TNF-mediated upregulation of *IRAK2* mRNA in primary human KCs (n = 10) in a dose- and time-dependent manner (Supplementary Figure S1b and c). Western blot validated TNF-induced expression and activation of IRAK2 (Supplementary Figure S1d).

IRAK1, IRAK2, and IRAK4 have been reported to form a complex mediating signaling downstream of TLR, IL-1, and IL-36 receptors (Lin et al., 2010). We therefore analyzed the mRNA expression of *IRAK1* and *IRAK4* in primary KCs stimulated with the above panel of cytokines based on RNA-seq data. In contrast to IRAK2, none of these stimuli except IFN-α increased the expression of *IRAK1* or *IRAK4* mRNA (Supplementary Figure S1e), suggesting that the regulation of IRAK2 is more dynamic in KCs than that of other IRAK members. Consistent with this idea, mRNA expression of *IRAK2* normalized rapidly after anti-TNF therapy in psoriasis by week 12 (n = 37, $P = 2.5 \times 10^{-13}$; Supplementary Figure S1f).

IRAK2 knockdown alleviates psoriasis- and AD-like inflammation in vivo

To validate the role of IRAK2 in vivo, we used the imiquimod (IMQ)-induced psoriasis-like and the MC903-induced AD-like models. *Irak2*-specific siRNA was topically applied on mouse ears, and efficacy of *Irak2* suppression was assessed by quantitative real-time PCR,

western blotting, and immunofluorescence (IF) staining (Supplementary Figure S2a-c). In the IMQ-induced mouse model, topical *Irak2* silencing over a 5-day period resulted in decreased ear thickness, epidermal proliferation, acanthosis, and decreased inflammatory infiltrate (Figure 2a-c). quantitative real-time PCR analysis of inflamed skin from IMQ mice revealed significantly lower expressions of *Il17a*, *Tnf*, *Il1b*, *Il36g*, *Il36a*, *Defb4*, *S100a7*, *Ccl20*, keratin (K) gene *K16*, *Lor*, *Znf750*, and *Grhl3* in the *Irak2* siRNA-treated group compared with control siRNA (Figure 2d and Supplementary Figure S2d). Moreover, in the MC903-induced AD-like mouse model, ear thickness, epidermal thickness, and inflammatory cell infiltration were attenuated by *Irak2* siRNA application (Figure 2e-g). The expression of *Tslp*, *Il4*, *Il13*, *Il6*, *K16*, *Lor*, *Znf750*, and *Grhl3* was reversed by *Irak2* siRNA in MC903-induced mice (Figure 2h), illustrating a dual role of IRAK2 in regulating epidermal differentiation and inflammatory responses in vivo.

IRAK2 has a greater biological effect in KCs than in fibroblasts

To determine the respective role of IRAK2, we silenced *IRAK2* using siRNA in KCs and fibroblasts stimulated with and without TNF (24 hours, n = 3) because it robustly induced IRAK2 expression (Figure 1d and Supplementary Figure S3a), followed by RNA-seq. The efficiency of *IRAK2* knockdown in fibroblasts (Supplementary Figure S3b) was similar to that achieved in KCs (Supplementary Figure S3c). Principal component analyses showed clear separation between all four groups (*IRAK2*-silenced [*siIRAK2*] ± TNF, *NC*-silenced ± TNF) in KCs but not in fibroblasts (Figure 3a). *IRAK2* silencing had a greater effect on KCs than fibroblasts. Thus, 2,845 differentially expressed genes were affected by *siIRAK2* in unstimulated KCs versus only nine in fibroblasts (with three genes overlapping). Similarly, 2,634 differentially expressed genes were affected by *siIRAK2* in TNF-stimulated KCs, in contrast to 83 in TNF-stimulated fibroblasts (27 overlapping) (FC > 1.5 or < 0.5, respectively; FDR ≤ 0.1) (Figure 3b). Gene Ontology term analyses revealed that IRAK2-regulated genes in unstimulated KCs were involved in biological categories such as cornification, epidermis development, skin barrier function, and antimicrobial responses, whereas IRAK2-regulated genes in unstimulated fibroblasts showed enrichment for mesenchyme migration and regulation of cellular process (Figure 3c). Expression of *IRAK2* mRNA was approximately 30% higher on average in KCs than in fibroblasts, and the expressions of differentiation-associated and proliferation genes, including *ZNF750* and *GRHL3*, and immune genes (*IL1B* and *S100As*) were regulated by IRAK2 in KCs but not in fibroblasts (Figure 3d). These findings were further confirmed using quantitative real-time PCR (Supplementary Figure S3d and e). These data suggest that IRAK2 plays an important and specific role in KC homeostasis and differentiation.

In KCs, *IRAK2* silencing suppressed induction of *IL36G*, *S100A7*, and *S100A8* and expression of KC proliferation and differentiation-associated genes, including *K6*, *K16*, *DSC2*, and desmoglein gene *DSG1* (Figure 3e), suggesting that IRAK2 plays a role in promoting the altered differentiation that is characteristic of AD and psoriasis (Mansbridge and Knapp, 1987). Consistent with this, RNA-seq analysis demonstrated that IRAK2-regulated genes overlap with differentially expressed genes in both psoriasis and AD (Supplementary Figure S4a). Thus, of the 1,394 IRAK2-regulated genes in KCs, 549 were also differentially expressed in psoriasis lesions compared with nonlesional psoriatic skin,

and 322 were differentially expressed in human AD lesions compared with nonlesional AD skin (Figure 3f, upper panel). Furthermore, 215 psoriasis-related genes and 130 AD-related genes were altered in the direction predicted by the *IRAK2* knockdown experiment, supporting the existence of an IRAK2-regulated gene expression pathway in psoriasis and AD (Figure 3f, lower panel). Biological processes shared between IRAK2-regulated genes and psoriasis or AD were enriched for Gene Ontology categories involving both differentiation and inflammatory responses, including KC differentiation, keratinization, epidermis development, and positive regulation of NF-κB TF activity (Supplementary Figure S4b).

Moreover, KCs overexpressing *IRAK2* (Supplementary Figure S5a) had increased mRNA expression of proinflammatory genes, including *IL1B*, *IL36G*, and *DEFB4*, and decreased induction of KC differentiation markers, including *IVL* and *FLG*, during TNF stimulation (Supplementary Figure S5b), supporting a role for IRAK2 in modulating both epidermal differentiation and immune responses.

NF-κB signaling pathway mediates epidermal regulation by IRAK2

To assess the underlying mechanism of IRAK2 in regulation of proinflammatory and epidermal genes, we analyzed gene expression across 118 transcriptomic datasets from skin (Swindell et al., 2014) to identify *IRAK2*-coexpressed genes. A total of 13,007 genes were identified with detectable expression ($P < 0.05$, signed rank test) in at least 5% (≥6 of 118) of the samples. Of the 13,007 genes, the expression pattern of 5,423 was positively correlated with *IRAK2* ($r_S > 0$). A graphical approach was used to identify a critical point in the decay of correlations (Figure 4a) to define a set of 108 genes having *IRAK2*-correlated expression ($r_S \geq 0.45$) (Figure 4b). Genes most strongly correlated with *IRAK2* expression included *CCL20*, *NFKB1*, and *IL36G* ($r_S \geq 0.62$) (Figure 4c). As a group, the 108 *IRAK2*-correlated genes were enriched with respect to the Gene Ontology biological process terms negative regulation of synthesis, negative regulation of EGFR signaling, positive regulation of immune system process, and cytokine metabolism (Figure 4d). Semi-parametric generalized additive logistic models were next used to identify DNA motifs enriched in regions upstream of the 108 *IRAK2*-correlated genes (Swindell et al., 2013). With respect to 1 kilo base upstream regions, an NF-κB motif was the most significantly enriched among *IRAK2*-correlated genes ($P = 1.09 \times 10^{-05}$; FDR = 0.029; Figure 4e). A second NF-κB motif was also significantly enriched in 1 kilo base upstream regions ($P = 0.00014$; FDR = 0.074; Figure 4e). Among these *IRAK2*-correlated genes, the top-ranked NF-κB motif was most frequently identified within a region of 500 base pair upstream from the transcription start site (Figure 4f). Predicted targets of the first motif included genes such as *CCL20*, *NFKBIZ*, *RELB*, *CXCL8*, *GNA15*, *IL36G*, and *NFKB1*. Examples of genes positively correlated with IRAK2 expression are shown (Figure 4g). To confirm IRAK2-mediated activation of NF-κB, we evaluated phosphorylated levels of NF-κB in *IRAK2* siRNA–transfected KCs and in the IMQ mouse model. Phosphorylation and activation of IκB kinaseand p65 was impaired in *IRAK2*-silenced, TNF-stimulated KCs (Figure 4h) and in skin lesions of IMQ- or MC903-treated mice (Supplementary Figure S6a). Other signaling pathways, including MAPK, were not obviously affected by *siIRAK2*

(Supplementary Figure S6b). Importantly, IκB kinase and p65 were activated by IRAK2 overexpression without additional TNF stimulation (Supplementary Figure S6c).

A role for IRAK2 in IL-1R–mediated immune responses is well-established (Flannery and Bowie, 2010) and mediated through interaction of IRAKs with MYD88. This results in downstream activation of NF-κB signaling (Lin et al., 2010). To determine if IL-1R signaling contributes to IRAK2-associated responses in KCs, we used siRNA targeting *IL1R* or *MYD88* or blocked IL-1R signaling with a neutralizing anti–IL-1R antibody. Both approaches resulted in reduction of *IRAK2* mRNA expression in KCs, as shown by quantitative real-time PCR (Supplementary Figure S7a), and decreased mRNA expression of *IL36G* in response to TNF treatment (Supplementary Figure S7b). Consistent with the contribution of IL-1/IL-36 signaling to IRAK2 expression, IF showed widespread expression of MyD88 protein in psoriatic epidermis colocalized with IRAK2 (Supplementary Figure S7c). These results suggest that IL-1 and IL-36 contribute to epidermal TNF responses through an IRAK2-dependent mechanism.

IRAK2 contributes to altered differentiation in inflammatory states

To further elucidate the role of IRAK2 in differentiated KCs under inflammatory states, we used three-dimensional human skin equivalents (Arnette et al., 2016) and examined the impact of TNF stimulation with or without *siIRAK2*. TNF treatment induced signs of accelerated differentiation as reflected in thickening of the epidermis (acanthosis), with increased prominence of the granular cell layer of the epidermis. Strikingly, *IRAK2* silencing in epidermal rafts reversed the effects of TNF (Figure 4i, left panel). IF staining confirmed downregulation of IRAK2 and validated the effect of IRAK2 on expression of differentiation-associated molecules, such as LOR (Figure 4i, right panel). In addition, silencing *IRAK2* reduced the expression of immune response–related genes, including *IL36G*, *CCL20*, and *DEFB4*, and induction of TFs *ZNF750* and *GRHL3* (Figure 4j).

To assess the clustering of these genes in vivo in healthy, psoriatic, and AD lesional skin, we used gene coexpression networks based on the RNA-seq data from AD and psoriasis (Merleev et al., 2018). Similar to before, *IRAK2* was dissociated from epidermal differentiation markers and inflammatory genes in normal skin (Figure 5a and Supplementary Figure S8a), but in AD or psoriatic skin (Figure 5a and b), *IRAK2* clustered with inflammatory response genes, including *IL36G* and *DEFB4*, and epidermal differentiation regulators, such as *ZNF750* or *GRHL3*. No correlation was seen with the epidermal stem marker *TP63* (Figure 5a and b). Tissue IF confirmed that IRAK2 was mostly absent in normal control skin but colocalized with IL-36γ, hDB2 (DEFB4), IVL, and K16 in psoriatic skin (Supplementary Figure S8b). Using single-cell RNA-seq data, gene expression in $IRAK2^+$ versus $IRAK2^-$ KCs were compared from both lesional and uninvolved psoriatic skin. Although the $IRAK2^+$ KC population was much smaller in uninvolved psoriatic skin than that in lesional psoriatic skin (data not shown), this again demonstrated that $IRAK2^+$ KCs were specifically enriched for genes involved in both epidermal cell differentiation and inflammatory responses (Figure 5c and d).

IRAK2 acts through ZNF750 to modulate proinflammatory epidermal responses

ZNF750 is a TF involved in epidermal differentiation (Sen et al., 2012). Genetic variants close to ZNF750 have been suggested as a putative risk factor for psoriasis (Birnbaum et al., 2011; Dębniak et al., 2014; Yang et al., 2008). GRHL3 is essential for epidermal development and skin homeostasis (Gordon et al., 2014). Expression of both *ZNF750* and *GRHL3* were decreased by *siIRAK2* in our RNA-seq data (Figure 6a and b). To address the interaction between *IRAK2* and *ZNF750* and *GRHL3*, we demonstrated that IRAK2 (cytoplasmic) was colocalized with ZNF750 and GRHL3 in psoriatic epidermis but to a minimal extent in healthy skin (Figure 6c). Moreover, single-cell RNA-seq analyses (Supplementary Figure S9a and b) showed that *IRAK2* and *ZNF750/GRHL3* expression was divergent in healthy skin and nonlesional psoriatic skin, whereas in psoriatic skin, their expression was correlated, along with increased expression of *IL36G* and *CCL20*. Similar to *IRAK2* silencing, siRNA targeting of *ZNF750* led to a robust decrease in mRNA expression of multiple host defense genes (*IL1B*, *IL36G*, *CCL20*, *DEFB4*, and *S100A7*) and differentiation genes (*IVL*, *FLG*, and *LOR*) (Figure 6d). Moreover, *IL36G*, *DEFB4*, and *S100A7*, which increased in *IRAK2*-overexpressing KCs, were reduced by *ZNF750* knockdown (Supplementary Figure S10). These data suggest that ZNF750 is a downstream mediator of IRAK2 signaling that links inflammatory responses with epidermal differentiation pathways.

DISCUSSION

The mechanisms involved in epidermal responses to inflammatory stimuli and amplification of feed-forward immune responses remain unclear. Here, we show that IRAK2, a member of the signaling complex downstream of IL-1 and IL-36 and/or TLRs, plays an important role in promoting abnormal epidermal differentiation and amplifying epidermal inflammatory responses that are characteristic of diseases such as AD and psoriasis (Guttman-Yassky et al., 2011a). Specifically, we identify IRAK2 as a critical component in the feed-forward amplification of skin inflammation, whose function likely applies to other chronic inflammatory diseases involving the epidermis.

The role of IRAK2 in regulating inflammatory responses has been well-established by multiple research groups. IRAK2, along with MYD88 and IRAK4, is a component of the Myddosome complex and a critical signaling mediator of the TLR and/or IL-1R superfamily (Lin et al., 2010). IRAK2 kinase activity has been shown to be critical for signaling through this complex (Liu et al., 2018) and promotes early NF-κB activation (Conner et al., 2009). In addition, IRAK2 may also facilitate nuclear export of inflammatory-related mRNAs as has been shown in murine macrophages (Zhou et al., 2017). IRAK2-deficient mice produce reduced levels of proinflammatory cytokine mRNAs after stimulation with a TLR2 agonist (Kawagoe et al., 2008), and IRAK2-deficient mice are more resistant to septic shock than WT mice (Kawagoe et al., 2008; Wan et al., 2009). Notably, IRAK2 function appears to be more critical than that of IRAK1 because IRAK2-deficient mice have a more drastic reduction in secretion of proinflammatory cytokines compared with IRAK1-deficient mice (Kawagoe et al., 2008). No studies, however, have been done to address the potential role of IRAK2 in inflammatory skin diseases despite prominent involvement of IL-1 and

IL-36 in driving inflammatory responses in KCs (Foster et al., 2014; Mahil et al., 2017), and their upregulation suggested critical roles in psoriasis (Johnston et al., 2017) and AD (Suárez-Fariñas et al., 2015).

AD and psoriasis are each considered to be T cell–mediated inflammatory diseases. Although T cell–derived proinflammatory mediators are central to their disease pathogenesis, these diseases also have prominent amplification of the inflammatory response in the epidermis (Guttman-Yassky et al., 2011b). In psoriasis, this has been called a feed-forward amplification, where epidermal KCs activated by T cell–derived cytokines secrete various antimicrobial peptides, proinflammatory cytokines, and chemokines that in turn bring in more leukocytes and heighten the inflammatory state. Our observation that IRAK2, but not the other members of the IRAK family, correlates with disease severity of AD and psoriasis suggests that IRAK2 uniquely contributes to this heightened feed-forward inflammatory state, which in turn is reflected in greater disease severity.

In this context, it is of interest that the IRAK2 promoter has enriched RXR binding sites, suggesting that the effect of retinoids, which have been used to treat both psoriasis (Harrison et al., 1987) and AD (Mihály et al., 2011) and still have an unknown mechanism of action (Khalil et al., 2017), could be mediated, at least partially, through an IRAK2-dependent mechanism. Finally, these data suggest that IRAK2 could be targeted pharmacologically to disrupt the feed-forward mechanism in skin inflammation. As our mouse experiments demonstrate, silencing IRAK2 expression alleviates skin inflammation in both psoriasis- and AD-like mouse models. Inhibitors of IRAK2 have been generated (Li et al., 2018) that can competitively interfere in the protein–protein interaction of the α-helical domain of IRAK2 with IRAK4 and inhibit NF-κB activity in asthma-like models (Li et al., 2018), but to our knowledge, these agents have not been used to treat inflammatory skin diseases. It has been shown that NF-κB signaling may contribute to epidermal hyperplasia and production of proinflammatory genes in KCs, including *IL1B*, *IL36G*, and antimicrobial proteins (Wang et al., 2018; Yan et al., 2015). This is consistent with IRAK2 overexpression leading to induction of proinflammatory gene expressions through activation of downstream NF-κB signaling.

Our data suggest that IRAK2 modulates epidermal differentiation responses through its effects on epidermal TFs, such as ZNF750 and GRHL3, which are increased in psoriatic skin (Gordon et al., 2014; Li et al., 2014), and to a lesser extent, GATA3 and KLF4, which are decreased in psoriatic skin (Kim et al., 2014; Zeitvogel et al., 2017), but all four TFs were affected in the same direction by IRAK2 silencing in vitro (Figure 6). This coordinated process between IRAK2 induction and effect on epidermal TFs and inflammatory responses suggests that inflammatory responses in the epidermis reflect a finely tuned and highly coordinated process.

Although IRAK2 affects expression of both ZNF750 and GRHL3, we have not identified the mechanisms by which IRAK2 influences their expression. ZNF750 and GRHL3 do not have NF-κB binding sites, suggesting that alternative signaling mechanisms downstream of IRAK2 affect their expression. In addition, how IRAK2 engages with these factors during inflammatory states is unclear, as well as the nature of the cross-talk between GRHL3,

ZNF750, and other TFs, but several TFs involved in epidermal immune responses have been predicted to regulate ZNF750 (PROMO database), including GR-β, Pax-5, p53, peroxisome proliferator-activated receptor-α/RXR-α, ETF, GR-α, and signal transducer and activator of transcription 4 (data not shown). The epidermal master regulator, p63, a member of the p53 family, is known to bind the ZNF750 promoter and is necessary for its induction (Sen et al., 2012) and notably is also a known genetic risk factor for development of psoriasis (Yin et al., 2015). We also note that although epidermal thickening is characteristic in both AD and psoriasis, there are also distinct histologic differences. For example, neutrophils are present in the epidermis of psoriasis but not AD, and the alterations in keratinization are distinct in the two diseases. This suggests that although IRAK2 contributes to epidermal thickening in both AD and psoriasis, other factors must contribute to the distinct clinical disease features.

In conclusion, our results identify IRAK2 as a critical mediator in promoting altered epidermal maturation that is more responsive to proinflammatory stimuli, thereby promoting and amplifying epidermal inflammatory responses through NF-κB activation. This work reveals that IRAK2 can be a novel potential therapeutic target for inflammatory skin diseases, including AD and psoriasis.

MATERIALS AND METHODS

RNA-seq and analysis

The RNA-seq experiment was carried out on RNA extracted from skin biopsy samples obtained from lesional (n = 28) and nonlesional (n = 27) psoriatic skin, chronic (n = 28) and acute (n = 11) lesional and nonlesional (n = 27) AD skin, and healthy controls (n = 38). The RNA-seq experiments and analysis for cells were obtained from KCs or fibroblasts treated with a variety of cytokines or knocked down with siRNAs. Data processing and analyses are detailed in Supplementary Materials and Methods.

Primary KC isolation and culture

Normal human KCs were established from healthy adults as previously described (Swindell et al., 2017). Informed written consent was obtained from human subjects under a protocol approved by the Institutional Review Board of the University of Michigan Medical School. Cultures were maintained with serum-free medium (Medium 154, Invitrogen/Cascade Biologics, Portland, OR) and used at passage 2 or 3 with a calcium concentration of 0.1 mM. Cells were starved of GFs for 24 hours before treatment with stimulus, which is detailed in Supplementary Materials and Methods.

Cell culture, stimulation, and RNA interference

N/TERTs, an immortalized KC cell line (Dickson et al., 2000), was grown in Keratinocyte-serum-free medium (17005-042, Thermo Fisher Scientific, Waltham, MA) supplemented with 30 μg/ml bovine pituitary extract, 0.2 ng/ml EGF, and 0.3 mM calcium chloride. Cells at proper confluency were subsequently treated with recombinant human IL-1β (10 ng/ml, R&D Systems, Minneapolis, MN), IL-36γ (10 ng/ml, R&D Systems), and TNF (10 ng/ml, R&D Systems) for the indicated time before RNA or protein extraction.

siRNA targeting *IRAKs*, including *IRAK2* (Accell Human IRAK2 siRNA, E-004761-00-0010), *IRAK1* (E-004760-00-0010), and *IRAK4* (E-003302-00-0010); *ZNF750* (E-014417-00-0010); *IL1R* (E-005188-00-0005); *MYD88* (E-004769-00-0005); and control siRNA (D-001910-01-20) were purchased from Dharmacon company (Lafayette, CO) and introduced into cells by Delivery medium (B-005000-100, Dharmacon) according to the manufacturer's instructions.

Three-dimensional human skin equivalents

Three-dimensional human skin equivalent were established as previously described using primary neonatal foreskin-derived KCs (Arnette et al., 2016). Cells were treated with *IRAK2* siRNA at the time of plating onto the collagen hydrogel as previously described (Simpson et al., 2010). Cultures were then treated with TNF (10 ng/ml, R&D Systems) on days 3, 4, and 5 and harvested on day 6.

The skin models were fixed with 10% neutral-buffered formalin (Sigma-Aldrich, St. Louis, MO) overnight and then paraffin wax–embedded and cut to 4-μm thickness. The sections were subsequently stained with H&E to assess general morphology and inflammatory states.

The methods for single-cell RNA sequencing and data analysis, mouse experiments, cell culture, generation of IRAK2 overexpressing N/TERTs, quantitative real-time PCR, western blot analysis, and tissue IF are provided in Supplementary Materials and Methods.

Statistics

Calculations were made using GraphPad Prism Version 8 (GraphPad Software, San Diego, CA). Appropriate control groups were used in all experiments, and details of sample number and independent replicates are provided in the respective figure legends. All data are mean ± SEM unless otherwise indicated. Statistical significance was determined using unpaired two-tailed Student's *t*-test or ANOVA as indicated in the legends, unless otherwise noted. For RNA-seq analysis, FDR was used to control for multiple testing. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ were considered significant and are referred to as such in the text.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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Data availability statement

The authors confirm that the data supporting the findings of this study are available in the article and its Supplementary Materials. Datasets related to this article can be found at GSE121212, hosted at Gene Expression Omnibus database.

Abbreviations:

AD	atopic dermatitis
FC	fold change
FDR	false discovery rate
IF	immunofluorescence
IMQ	imiquimod
K	keratin
KC	keratinocyte
RNA-seq	RNA-sequencing
siIRAK2	IRAK2-silenced
siRNA	small interfering RNA
TF	transcription factor
TLR	toll-like receptor

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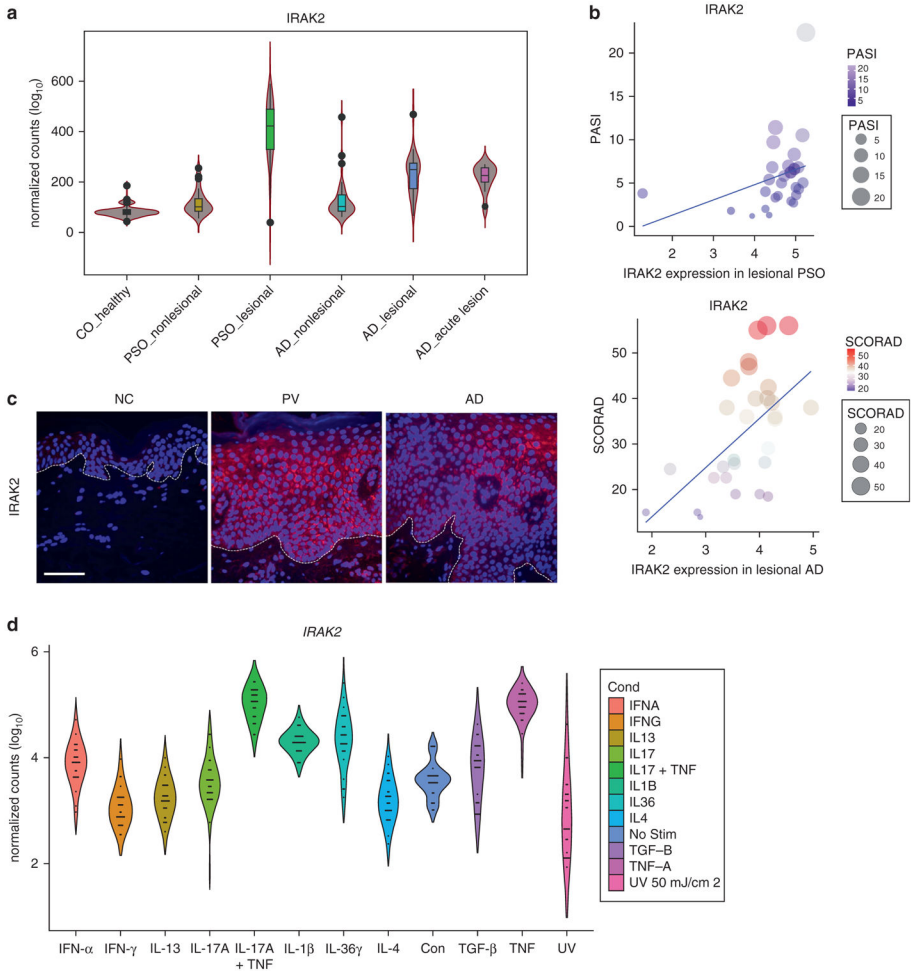


Figure 1. IRAK2 is highly expressed in skin lesions of patients with AD and PSO.

(**a**) Violin plot showing the expression level of IRAK2 as detected by RNA-seq analysis in lesional skin of patients with PSO (n = 28, FC = 4.9-fold, $P = 2 \times 10^{-25}$), nonlesional skin of patients with PSO (n = 27), chronic (n = 28, FC = 2.2-fold, $P = 2 \times 10^{-11}$) and acute lesional (n = 11, FC = 1.8-fold, $P = 2 \times 10^{-10}$) skin of patients with AD, nonlesional skin of patients with AD (n = 27), and healthy CO (n = 38). (**b**) Positive correlation of IRAK2 expression level in psoriatic lesions with PASI (upper panel) and in AD lesions with SCORAD (lower panel). (**c**) Representative immunofluorescent staining of IRAK2 (red) in lesional AD and PSO skin. White dashed line indicates the epidermal and dermal junction (n = 3, biological replicates). Bar = 100 μm. (**d**) Violin plots highlighting the expression level of *IRAK2* mRNA in primary keratinocytes (n = 38) stimulated with various cytokines, such as TNF (FC = 1.8-fold, FDR = 8.7×10^{-16}), combination of TNF + IL-17A (FC = 1.7-fold, FDR = 7.6×10^{-18}), or IL-36γ (FC = 1.5-fold, FDR = 2.3×10^{-23}) for 24 hours. One-way ANOVA. Error bars represent SEM. AD, atopic dermatitis; CO, control; FC, fold change; FDR, false discovery rate; NC, normal control; PSO, psoriasis; PV, psoriasis vulgaris; RNA-seq, RNA-sequencing.

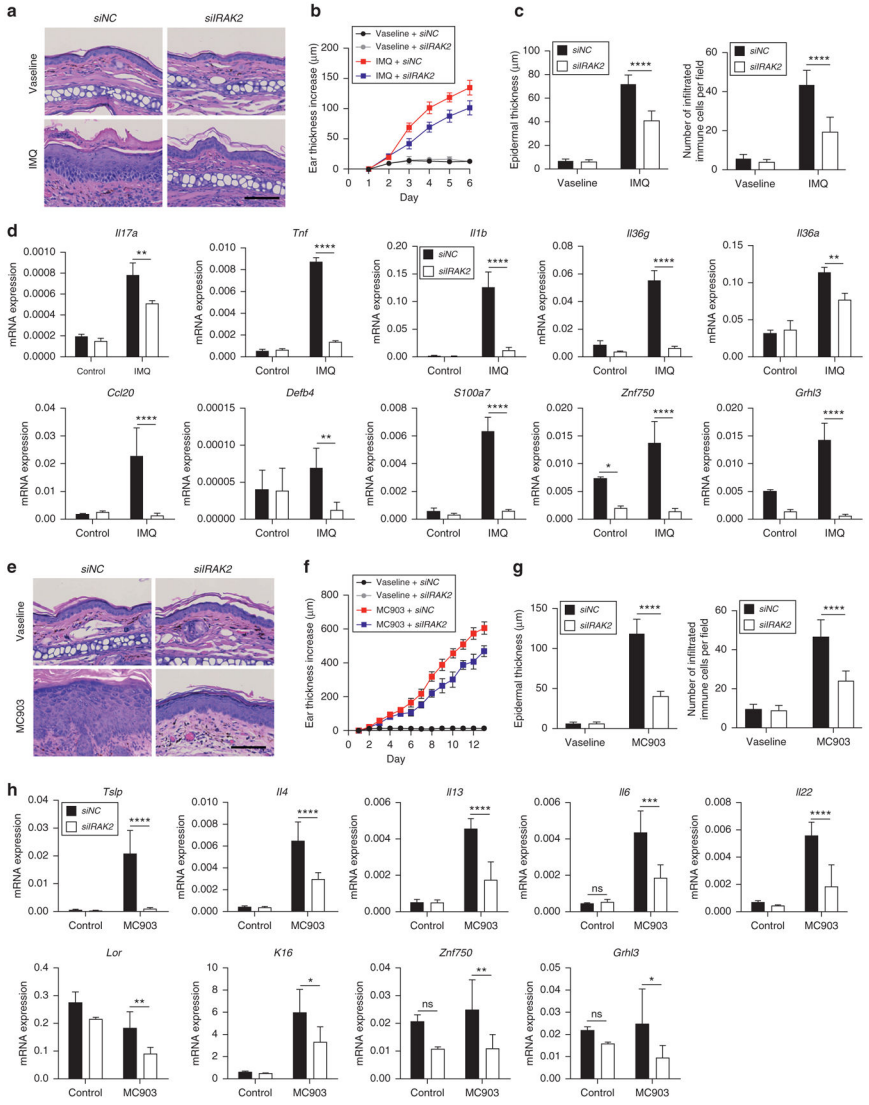


Figure 2. *IRAK2* knockdown improves inflammatory responses in both psoriasis and AD mouse models.

siRNA targeting IRAK2 was topically applied to IMQ-induced psoriasis-like mouse or MC903-induced AD-like mouse ears to silence IRAK2 expression. (**a**) H&E staining of ear sections from IMQ-induced mice at day 6. Bar = 50 μm. (**b**) Dynamic changes in ear thickness at the indicated time points. (**c**) Epidermal thickness and inflammatory infiltrates were evaluated based on (**a**). (**d**) Quantitative real-time PCR results showing mRNA expression of various cytokines in the ears of IMQ mice on day 6. (**e**) H&E staining of ear sections from MC903-induced mice at day 15. Bar = 50 μm. (**f**) Dynamic changes in ear thickness at the indicated time points. (**g**) Epidermal thickness and inflammatory infiltrates were evaluated based on (**e**). (**h**) Quantitative real-time PCR results showing mRNA expression of various cytokines in the ears of MC903 mice on day 15. Two-way ANOVA. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. AD, atopic dermatitis; IMQ, imiquimod; ns, nonsignificant; K, keratin; NC, normal control; *siIRAK2*, *IRAK2*-silenced; si-NC, NC-silenced; siRNA, small interfering RNA.

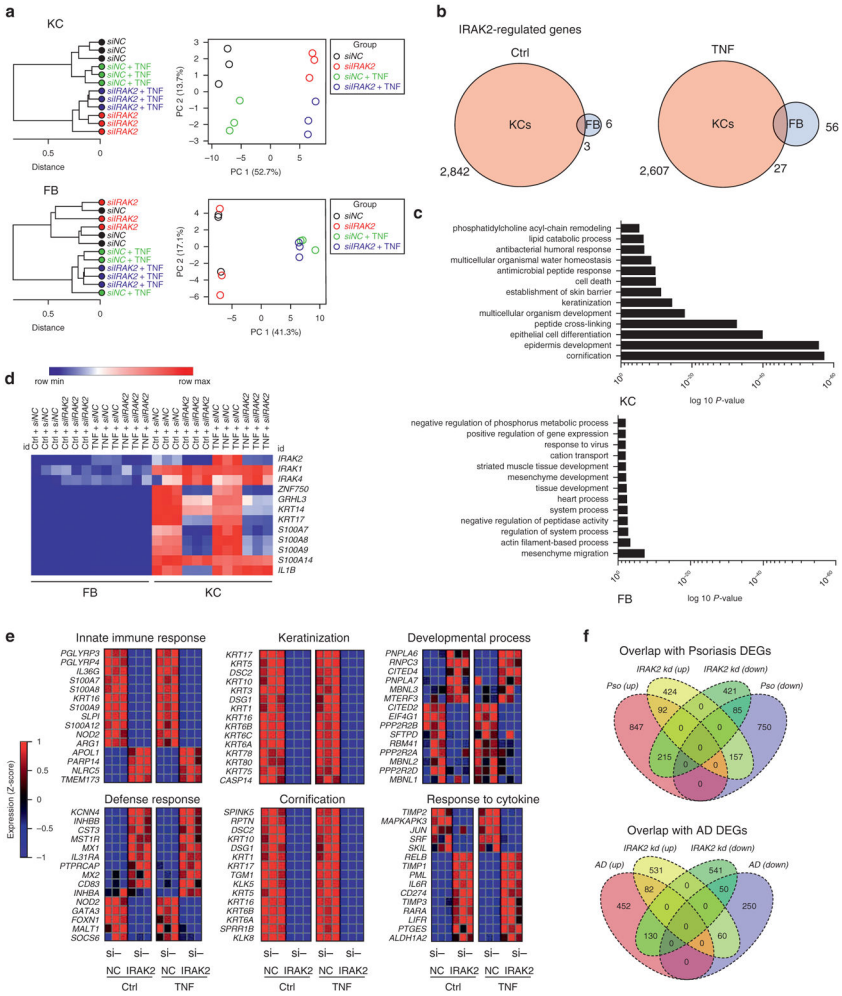


Figure 3. IRAK2′s role is primarily in KCs instead of FBs.

(**a**) KCs and FBs were transfected with *IRAK2* siRNA or ctrl siRNA and stimulated with TNF (10 ng/ml) for 24 hours (n = 3 samples per group). Cluster analysis of four experimental groups in KCs (upper panel) and FBs (lower panel) are shown based on the expression changes detected by RNA-seq. (**b**) Venn diagrams showing the number of DEGs similarly altered by *IRAK2* siRNA in KCs and FBs with or without TNF stimulation (10 ng/ml). (**c**) Functional categories enriched in IRAK2-regulated genes in KCs and FBs. (**d**) Heatmap showing expression level of selected IRAK2-regulated genes enriched in differentiation and innate immune responses in FBs and KCs, respectively. (**e**) Heatmap showing expression levels of IRAK2-regulated genes in different GO categories with or without TNF stimulation. (**f**) Overlap between IRAK2-regulated genes (upregulated and downregulated) and DEGs in AD and psoriasis skin lesions. AD, atopic dermatitis; ctrl, control; DEG, differentially expressed gene; FB, fibroblast; GO, Gene Ontology; KC, keratinocyte; max, maximum; min, minimum; NC, normal control; PC, principal component; RNA-seq, RNA-sequencing; *siIRAK2*, *IRAK2*-silenced; *siNC*, *NC*-silenced; siRNA, small interfering RNA.

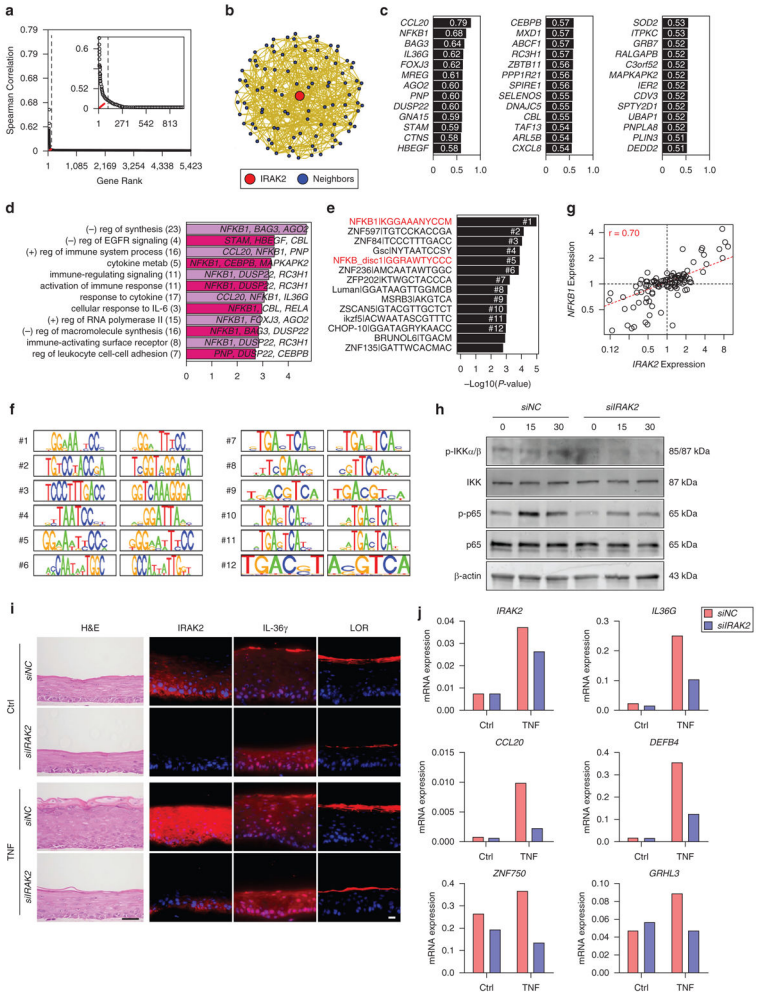
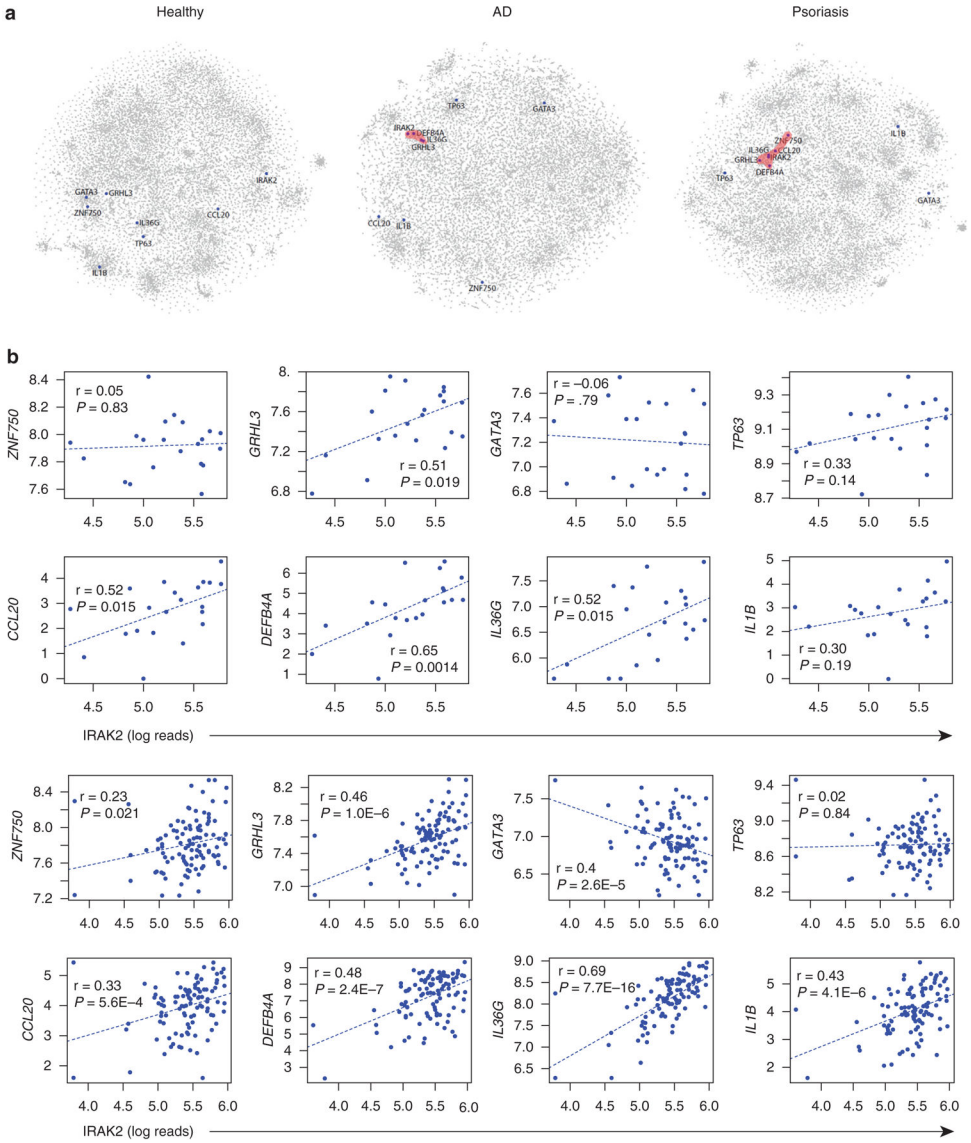


Figure 4. IRAK2 regulatory network and effect on epidermal differentiation during inflammatory state.

(**a**) Correlation coefficients relative to the ranked list of 5,423 genes detected by RNA-seq. The vertical axis is exponentially scaled (r7) to emphasize higher correlations. The red line represents the minimal distance between the lower-left origin and correlation curve, which defines a set of 108 genes having IRAK2-correlated expression (rs ≥ 0.45). (**b**) IRAK2-correlated genes are plotted in a spherical network with IRAK2 at center. The top three intranetwork correlations were used for each gene to draw connections. (**c**) Network genes most strongly correlated with IRAK2. (**d**) GO biological process terms most strongly enriched with respect to the 108 IRAK2-correlated genes. The number of genes associated with each term are shown (parentheses, left margin) and exemplar genes are listed within the figure. (**e, f**) DNA motifs (**e**) most heavily enriched in 5-kb regions upstream of IRAK2-correlated genes, with (**f**) the 12 most significant motifs shown (see corresponding names in **e**). (**g**) The correlation of *IRAK2* with *NFKB1* expression. (**h**) Representative western blot showing the activation of IKK and NF-κB p65 signaling in siIRAK2 versus Ctrl keratinocytes in response to TNF stimulation. (**i**) Representative H&E staining (left panel) of HSEs showing the effect of *IRAK2* silencing with or without TNF stimulation, and immunofluorescent staining (right panel) of IRAK2, IL-36γ, and LOR (all red) at

day 6. The experiment was repeated twice. Bar = 100 μm. (**j**) Representative quantitative real-time PCR analysis of proinflammatory and differentiation genes in *siIRAK2*-treated 3D HSE with or without TNF stimulation. 3D, three-dimensional; Ctrl, control; GO, Gene Ontology; HSE, human skin equivalent; IKK, IκB kinase; kb, kilo base; NC, normal control; p-IKKα/β, phosphorylated IκB kinaseα/β; p-P65, phosphorylated p65; reg, region; RNA-seq, RNA-sequencing; *siIRAK2*, *IRAK2*-silenced; *siNC*, *NC*-silenced.



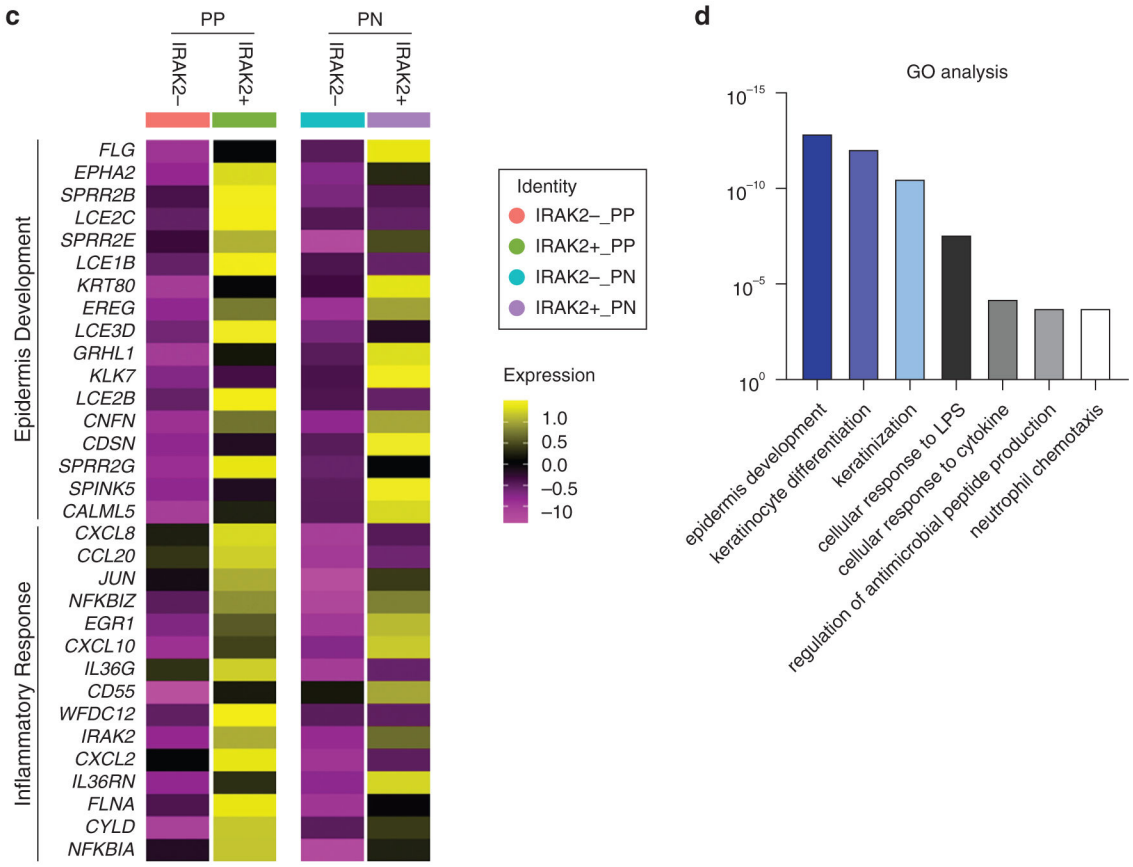


Figure 5. IRAK2-associated genes in keratinocytes involve inflammatory response genes and markers of epidermal development.

(**a**) 2DCoexpression networks of gene expression (RNA-seq) of healthy skin, psoriatic, and AD skin lesions with labeled IRAK2 and key epidermal differentiation genes showing grouping and tight clustering of these genes in psoriatic skin but not normal skin. (**b**) The correlation analysis of IRAK2 with selected inflammatory and epidermal differentiated genes in skin lesions of AD (upper panel) and psoriasis (lower panel) based on the RNA-seq data. (**c**) Heatmap and (**d**) GO categories of epidermis development and inflammatory response of IRAK2^{+} keratinocytes based on single-cell RNA-seq of psoriasis skin lesions. 2D, two-dimensional; AD, atopic dermatitis; GO, Gene Ontology; LPS, lipopolysaccharide; NC, normal control; PN, psoriatic non-lesional skin; PP, psoriatic lesional skin; RNA-seq, RNA-sequencing.

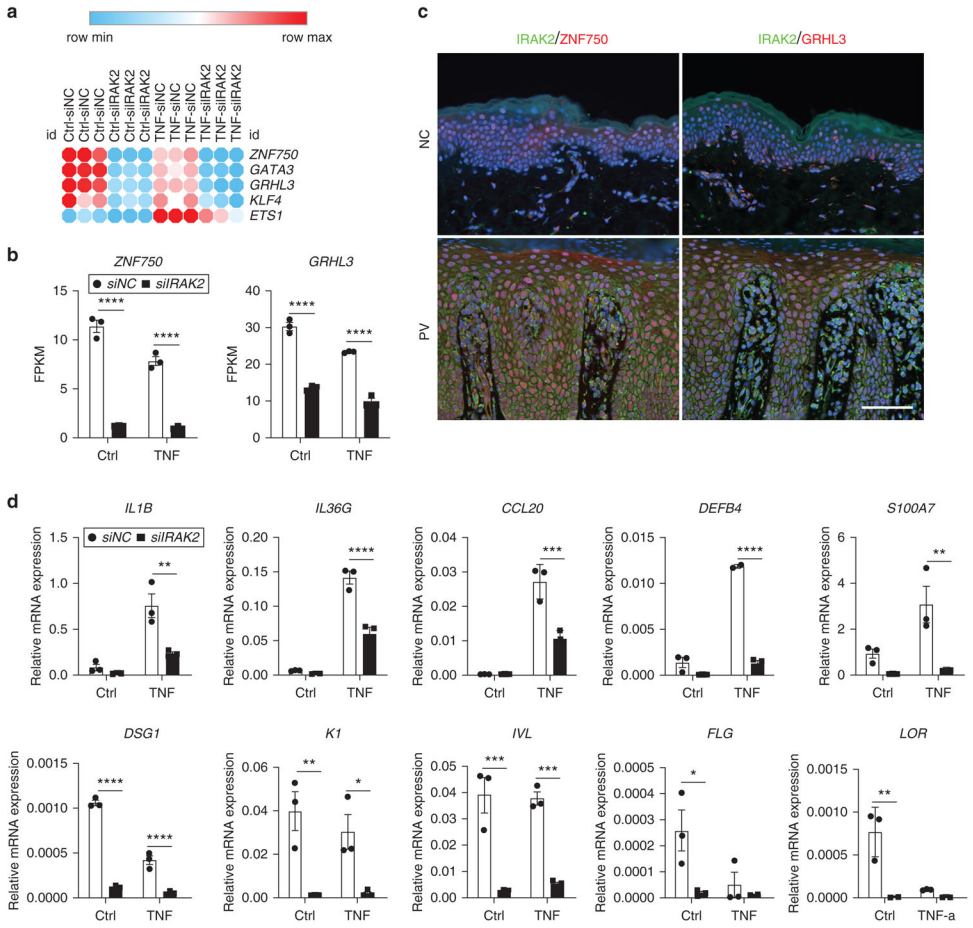


Figure 6. IRAK2 affects ZNF750 expression to modulate epidermal differentiation and proinflammatory responses.

(**a**) Heatmap analysis showing the critical differentiation genes affected by siIRAK2 in keratinocytes. (**b**) RNA-seq validation of *ZNF750* and *GRHL3* mRNA expression by siIRAK2 in keratinocytes (n = 3). (**c**) Immunofluorescent costaining of IRAK2 (green) against ZNF750 and GRHL3 (all red) in normal and lesional psoriatic skin. Bar = 100 μm. (**d**) Quantitative real-time PCR for mRNA expression of differentiation and proinflammatory genes in ZNF750-silenced keratinocytes with or without TNF stimulation (n = 3). Two-way ANOVA. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Ctrl, control; DSG, desmoglein; FPKM, Fragments Per Kilobase of transcript per Million mapped reads; id, identification; K, keratin; max, maximum; min, minimum; NC, normal control; ns, no significance; PV, psoriasis vulgaris; RNA-seq, RNA-sequencing; *siIRAK2*, *IRAK2*-silenced; *siNC*, *NC*-silenced.