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# A20 and ABIN1 Suppression of a Keratinocyte Inflammatory Program with a Shared Single-Cell Expression Signature in Diverse Human Rashes



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Genetic variation in the NF- $\kappa$ B inhibitors, ABIN1 and A20, increase risk for psoriasis. While critical for hematopoietic immune cell function, these genes are believed to additionally inhibit psoriasis by dampening inflammatory signaling in keratinocytes. We dissected ABIN1 and A20's regulatory role in human keratinocyte inflammation using an RNA sequencing-based comparative genomic approach. Here we show subsets of the IL-17 and tumor necrosis factor- $\alpha$  signaling pathways are robustly restricted by A20 overexpression. In contrast, ABIN1 overexpression inhibits these genes more modestly for IL-17, and weakly for tumor necrosis factor- $\alpha$ . Our genome-scale analysis also indicates that inflammatory program suppression appears to be the major transcriptional influence of A20/ABIN1 overexpression, without obvious influence on keratinocyte viability genes. Our findings thus enable dissection of the differing anti-inflammatory mechanisms of two distinct psoriasis modifiers, which may be directly exploited for therapeutic purposes. Importantly, we report that IL-17-induced targets of A20 show similar aberrant epidermal layer-specific transcriptional upregulation in keratinocytes from diseases as diverse as psoriasis, atopic dermatitis, and erythrokeratoderma variabilis, suggesting a contributory role for epidermal inflammation in a broad spectrum of rashes.

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## INTRODUCTION

Specific blockade of immune cell signaling pathways has sharply expanded the efficacy and diversity of dermatologic therapies in the past 2 decades. Systemic biologic treatments that block tumor necrosis factor (TNF)- $\alpha$  and IL-17 function (e.g., infliximab and secukinumab, respectively) now represent some of the most effective psoriasis therapies (Langley et al., 2014; Reich et al., 2005). The cytokine TNF- $\alpha$ ,

produced mainly by activated macrophages, is increased in psoriatic skin and believed to accelerate epidermal immune cell infiltration and induce dendritic cell activation (Uchi et al., 2000). IL-17, a cytokine produced by T helper type 17 and innate lymphoid cells (Cua and Tato, 2010), potentiates inflammatory states by stimulating keratinocyte secretion of proinflammatory mediators and recruiting neutrophils and other inflammatory cells (Ogawa et al., 2018). In inflammatory skin diseases such as psoriasis, TNF- $\alpha$  and IL-17 synergistically augment dysregulated immune response (Krueger and Brunner, 2018).

These infiltrating immune cells clearly play a fundamental role in cutaneous inflammation. However, recent attention has turned to the immunologic role of target tissues in inflammatory disease. In psoriasis, keratinocytes themselves influence and establish the inflammatory milieu by producing an array of inflammatory cytokines, such as TNF- $\alpha$ , ILs, and IFNs (Rupec et al., 2010), while also undergoing abnormal differentiation and hyperproliferation. Molecular abnormalities occurring primarily in the epidermis cause other rashes, including pityriasis rubra pilaris, ichthyoses, and erythroderma keratoderma variabilis. These conditions, which share the clinical phenotype of inflammatory scaly plaques, all show histopathologically scant immune cell infiltrates, underscoring the importance of keratinocyte-driven inflammation (Hanifin, 2009).

NF- $\kappa$ B activation, a common pathway downstream of both TNF- $\alpha$  and IL-17, has been implicated recurrently in keratinocyte-driven rashes. Gain-of-function mutations in *CARD14*, an NF- $\kappa$ B activator, underlie familial forms of

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Abbreviations: CPM, counts per million; FC, fold-change; FDR, false discovery rate; KGM, keratinocyte growth media; NHEK, normal human epidermal keratinocyte; scRNA-seq, single-cell RNA sequencing; TNF, tumor necrosis factor

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pityriasis rubra pilaris (Fuchs-Telem et al., 2012). The rare inherited ichthyoses, often caused by genes important for epidermal barrier establishment, typically manifest early in childhood with cutaneous inflammation, diffuse scaling, and barrier defects. It has recently been noted that ichthyotic skin displays a strong IL-17 dominant immune profile, also suggestive of a link between epidermal-based dysfunction and NF- $\kappa$ B-mediated inflammation (Paller et al., 2017). Erythrokeratoderma variabilis is a rare genetic disorder characterized by migratory erythematous scaly plaques. The majority of cases are due to loss-of-function connexin gap junction mutations (Scott et al., 2012), which appear to activate keratinocyte-autonomous inflammatory programs, although NF- $\kappa$ B has not been established as the central pathway.

NF- $\kappa$ B signaling is critical for both innate and adaptive immunity, as well as epidermal homeostasis. In its baseline inactive state, the NF- $\kappa$ B transcription factor complex is bound to the inhibitory protein I $\kappa$ B $\alpha$  and sequestered in the cytoplasm (Mitchell et al., 2016). Canonical NF- $\kappa$ B activation occurs through phosphorylation of I $\kappa$ B $\alpha$  by the I $\kappa$ B kinase complex (Mercurio et al., 1997). I $\kappa$ B $\alpha$  is then ubiquitinated and degraded by the proteasome, releasing NF- $\kappa$ B for nuclear translocation and subsequent transcriptional activation. Multiple human linkage studies and mouse models have demonstrated a role for NF- $\kappa$ B signaling pathway alterations in autoimmune disease and inflammatory skin disease (Sun et al., 2013). For example, genome-wide association studies in psoriasis, rheumatoid arthritis, and systemic lupus erythematosus have identified the NF- $\kappa$ B signaling pathway genes *ABIN1* (G'Sell et al., 2015; Nair et al., 2009), *A20* (Catrysse et al., 2014), and *CARD14* (Jordan et al., 2012) as disease susceptibility loci.

The NF- $\kappa$ B inhibitors A20 (encoded by *TNFAIP3*) and ABIN1 (encoded by *TNIP1*) are well established as regulators of hematopoietic immune cell activity (Zhou et al., 2011). The adapter protein ABIN1 physically links the A20 deubiquitinase to the I $\kappa$ B kinase complex, where A20 deubiquitinates and inactivates I $\kappa$ B kinase to prevent phosphorylation and inactivation of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ . Immune cell-specific deletions of A20 or ABIN1 in mice produce a wide variety of systemic hyperinflammatory phenotypes, reminiscent of systemic lupus erythematosus and rheumatoid arthritis (Catrysse et al., 2014; G'Sell et al., 2015). Recently, however, experimental manipulation has also revealed a role for A20 and ABIN1 in keratinocyte-mediated inflammation. In response to inflammatory stimuli (imiquimod and IL-17), ABIN1 epidermal depletion in mice enhances chemokine expression (Ippagunta et al., 2016). Correspondingly, HaCaT human keratinocyte cultures exposed to the double-stranded RNA mimic Poly (I:C) markedly increase inflammatory gene expression when ABIN1 is depleted (Rudraiah et al., 2018). While a link between A20 depletion and epidermal inflammation has not been shown yet, its overexpression in HaCaT cells represses a small set of profiled cytokines when exposed to Poly (I:C) (Sohn et al., 2016).

Inhibition of target tissue inflammation may improve a broad range of rashes and could potentially lead to localized treatments that avoid the serious side effects that can accompany systemic immunosuppression. While localized

repression of NF- $\kappa$ B signaling may represent one such strategy, a full accounting of the ability for A20 and ABIN1 to suppress keratinocyte inflammation has not been reported, nor has their potential non-inflammatory effects. Here we report a global transcriptional analysis of the inflammation repressing effects of the NF- $\kappa$ B inhibitory binding partners, A20 and ABIN1, in human keratinocytes. We show that overexpressed A20 is a more potent inhibitor of keratinocyte inflammation than ABIN1, in the context of IL-17A or TNF- $\alpha$  cytokine stimulation. Single cell RNA sequencing of epidermis from diverse rashes revealed a common inflammatory gene expression signature, the genes of which were repressed by A20 overexpression in primary keratinocyte culture.

## RESULTS

We utilized lentivirus to overexpress A20 and ABIN1 in primary normal human epidermal keratinocyte (NHEK) culture, leading to a  $\sim$ 1,300 and 500% increase in protein expression, respectively (Supplementary Figure S1 online). RNA sequencing was performed on mock-infected (GFP), A20, or ABIN1 overexpressing NHEK cells to assess global expression patterns. The edgeR software package (Robinson et al., 2010), which uses an overdispersed Poisson model to address biological and technical variability, was used to identify genes differentially expressed between treatment conditions. We first examined downregulation of inflammation-related genes, given the known roles of A20 and ABIN1 in suppressing inflammation. Under standard (no cytokine stimulation) culture conditions, expression of relatively few inflammatory genes, or genes overall, were repressed by A20 or ABIN1 overexpression in NHEK cells (Figure 1). A20 overexpression downregulated 53 genes ( $\log_2$  fold-change [FC]  $<$   $-0.5$ , false discovery rate [FDR]  $<$  0.05) (Supplementary Table S1a online), among these were several inflammatory genes, such as *CXCL1*, *C1R*, *IL24*, *IL32*, *MMP9*, and *SAA1*. ABIN1 repressed fewer genes (31 overall genes with  $\log_2$  FC  $<$   $-0.5$ , FDR  $<$  0.05) (Supplementary Table S1b), including a subset of the A20 repressed inflammatory genes (*C1R*, *IL32*, *MMP9*, and *SAA1*), with similar or slightly greater effect than A20.

Given the relatively modest gene regulatory effects exerted by overexpression of these two genes under standard culture conditions, we next sought to understand their regulatory power when challenged with inflammatory stimuli. We utilized the pro-inflammatory cytokines IL-17A and TNF- $\alpha$ , given their known roles and specific blockade by biological therapeutics in cutaneous inflammatory disease. To better understand the specific roles for A20 and ABIN1 in each of these cytokines' signaling pathways, we stimulated keratinocytes with single cytokine exposure. RNA sequencing was performed on mock-infected (GFP), A20, and ABIN1 overexpressing NHEK cells that were cytokine stimulated with either IL-17A or TNF- $\alpha$  for 1 or 24 hours.

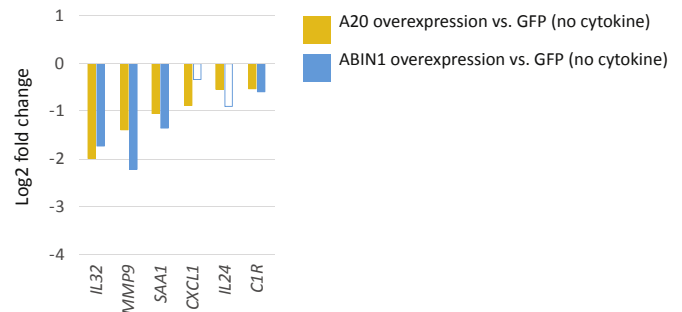
We used expression data from mock-infected (GFP expressing) keratinocytes as a proxy for cytokine stimulation of phenotypically normal keratinocytes. Incubation of these keratinocytes with IL-17A for 24 hours led to greater transcriptional upregulation of genes than at 1 hour (131 to 36 genes, respectively ( $\log_2$  FC  $>$  0.5, FDR  $<$  0.05)

(Supplementary Tables S1d, S1e). We thus focused our attention on the 24-hour IL-17A cytokine stimulation time point. Consistent with previous reports, 24-hour IL-17A exposure upregulated genes such as antimicrobial peptides (*DEFB4A*, *LCN2*, and *S100A7*), CXC chemokines (*CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, *CXCL6*, and *CXCL8*), and interleukins (*IL1A*, *IL1B*, *IL23A*, *IL24*, *IL33*, and *IL36G*) (Figure 2) (Chiricozzi et al., 2011; Nograles et al., 2008).

While overexpression of A20 in NHEK culture robustly inhibited IL-17A (24-hour) induced inflammatory genes, ABIN1 overexpression produced more modest effects (Figure 2, Supplementary Tables S1e, S1f). With IL-17A exposure, most of the few inflammatory genes repressed by A20 overexpression under unstimulated conditions continued to be repressed by a similar magnitude; however, repression of an extended set of inflammatory genes became evident, for example, *DEFB4A*, *S100A7*, *S100A8*, *CCXLI*, *CXCL3*, *CXCL5*, *CXCL6*, *CXCL8*, *IL24*, and *IL36G* (Figure 2). Most of this set of genes were lowly expressed under standard culture conditions before IL-17A stimulation. For ABIN1 overexpressing keratinocytes exposed to IL-17A (24-hour) stimulation, the small set of inflammatory genes downregulated under baseline conditions also continue to be repressed, along with repression of a similar extended set of inflammatory genes as with A20 overexpression, albeit with lesser effect (Figure 2). With 1 hour of IL-17A cytokine stimulation, a similar pattern emerged where A20 overexpression exhibited stronger inflammatory gene repressive activity (both in terms of number of genes and degree of repression) than ABIN1 (Supplementary Figure S2 online, Supplementary Tables S1d, S1g, and S1h).

We next tested the effects of A20 and ABIN1 overexpression on NHEK cells stimulated with either 1 or 24 hours of TNF- $\alpha$  exposure. We noted that the greatest modulation by A20 or ABIN1 of inflammatory gene expression occurred with 1 hour of TNF- $\alpha$  exposure, so we focused on this time point. In mock-infected keratinocytes, 90 genes were upregulated with 1 hour of TNF- $\alpha$  exposure (Figure 3,  $\log_2$  FC > 0.5, FDR < 0.05) (Supplementary Table S1i). Similar to previous findings, upregulated genes include neutrophil chemoattractants (e.g., *CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, and *CXCL8*), ILs (*IL16*, *IL1A*, *IL1B*, *IL20*, *IL23A*, *IL36G*, and *IL6*), *CCL20*, *ICAM1*, *CSF1/2*, and *TNF* itself (Banno et al., 2004).

As in the IL-17A stimulation experiments, A20 overexpression strongly inhibited expression of inflammatory genes induced by 1 hour of TNF- $\alpha$  exposure, repressing ~79% of all induced genes (Figure 3,  $\log_2$  FC < -0.5, FDR < 0.05; Supplementary Table S1j). Similar to IL-17A exposure, genes downregulated under baseline conditions continue to be downregulated by A20 overexpression, despite an overall increase in their absolute expression levels with TNF- $\alpha$  stimulation. A20 repressed inflammatory genes that were evident only upon TNF- $\alpha$  stimulation including *CSF1/2*, *ICAM1*, *TNF*, chemokines (*CCL20*, *CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, and *CXCL8*), and ILs/IL receptors (*IL16*, *IL1A*, *IL1B*, *IL6*, *IL24*, *IL20*, *IL23A*, *IL36G*, and *IL7R*). In contrast, ABIN1 overexpression produced only a small effect, repressing 4/89 TNF- $\alpha$ -stimulated genes, (e.g., *IL20*, *CCL20*, and *C3*) and continued to repress genes that were already repressed at

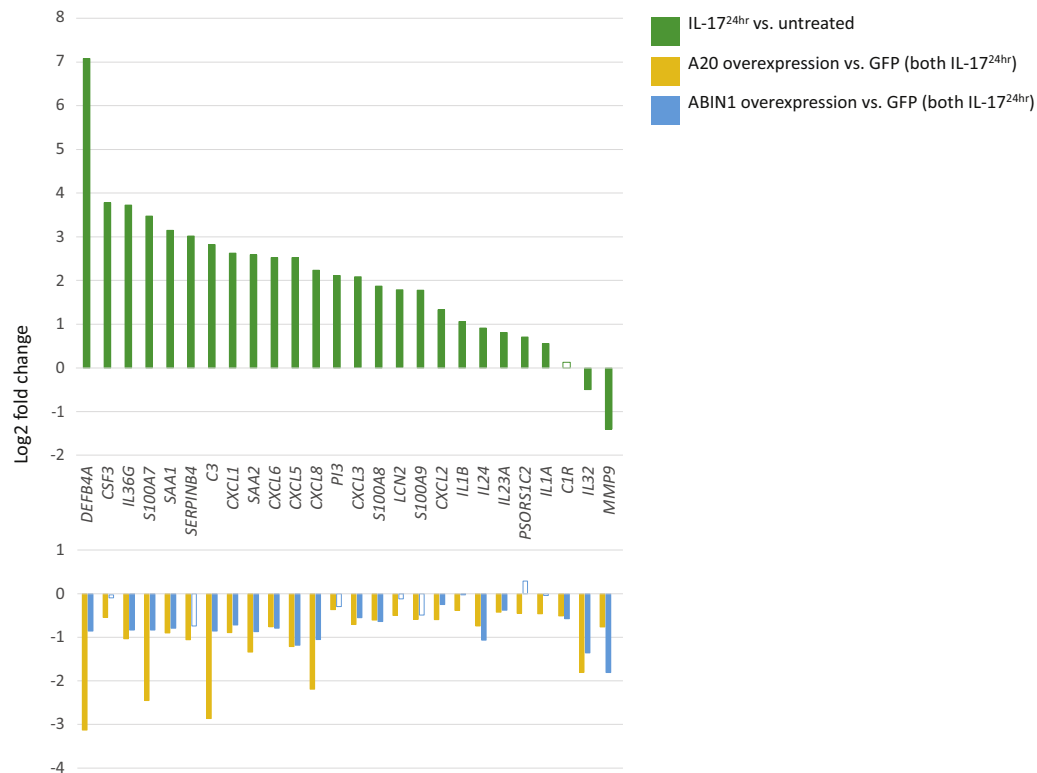


**Figure 1. A20 and ABIN1 repress transcription of relatively few inflammatory genes under unstimulated conditions.** Relative expression ( $\log_2$  fold-change) of inflammatory genes in A20 or ABIN1 versus mock-infected (GFP) overexpressing keratinocytes. Data shown represent edgeR RNA-sequencing differential expression results for six replicates for each permutation (false discovery rate < 0.05 except for non-shaded bars).

baseline (e.g., *C1R*, *IL32*, *MMP9*, and *SAA1*, Figure 3,  $\log_2$  FC < -0.5, FDR < 0.05) (Supplementary Table S1k). Twenty-four-hour TNF- $\alpha$  exposure for ABIN1 and A20 overexpressing keratinocytes showed similar trends with A20 clearly repressing inflammatory genes, such as chemokines and ILs, although to a slightly lesser extent than with 1 hour of TNF- $\alpha$  exposure. The ABIN1 overexpression effect was still weak, although slightly more appreciable, with extended TNF- $\alpha$  stimulation (Supplementary Figure S3 online, Supplementary Table S1l, S1m, and S1n).

Given a potential association between keratinocyte inflammation and differentiation (Schröder et al., 2006), we next focused on the effects of A20 and ABIN1 overexpression on keratinocyte differentiation. We noted that A20 repressed late differentiation genes, such as small proline-rich proteins (*SPRR*; *SPRR2A*, *SPRR2G*, and *SPRR3*), LCE genes (*LCE1B*, *LCE3D*, and *LCE3E*), as well as the early differentiation marker *KRT1*. ABIN1 did not consistently repress this set of differentiation genes (Figure 4,  $\log_2$  FC < -0.5, FDR < 0.05) (Supplementary Tables S1a, S1b).

We were curious if our focus on inflammation had caused us to overlook other transcriptional programs controlled by A20 and ABIN1 that might complicate clinical treatment strategies. We performed unsupervised hierarchical clustering on 861 differentially expressed genes when comparing the A20, ABIN1, or GFP (control) keratinocyte overexpression, with and without 24 hours of IL-17A or TNF- $\alpha$  stimulation permutations (FDR <  $1 \times 10^{-5}$  for each condition, six replicates each). These data are depicted as a heat map in Figure 5. As expected, we noted inflammatory gene clusters, with three groupings that were enriched for Gene Ontology terms, such as immune response, immune system response, and immune system process. One cluster represents genes strongly upregulated by IL-17A and downregulated by A20 and ABIN1 overexpression, characterized by genes such as *DEFB4A*, *CXCL6*, *SAA2*, and *CSF3*. A second cluster generally encompasses genes upregulated by both TNF- $\alpha$  and IL-17A, and repressed by A20 and ABIN1 (e.g., *C3*, *CXCL5*, *CSF2*, and *CXCL3*). The third cluster represents a third set of inflammatory genes, containing genes, such as *IL32*, *C1R*, *MMP9*, and *TNF*, that are more strongly upregulated by TNF- $\alpha$  and downregulated by A20 and/or ABIN1 overexpression.



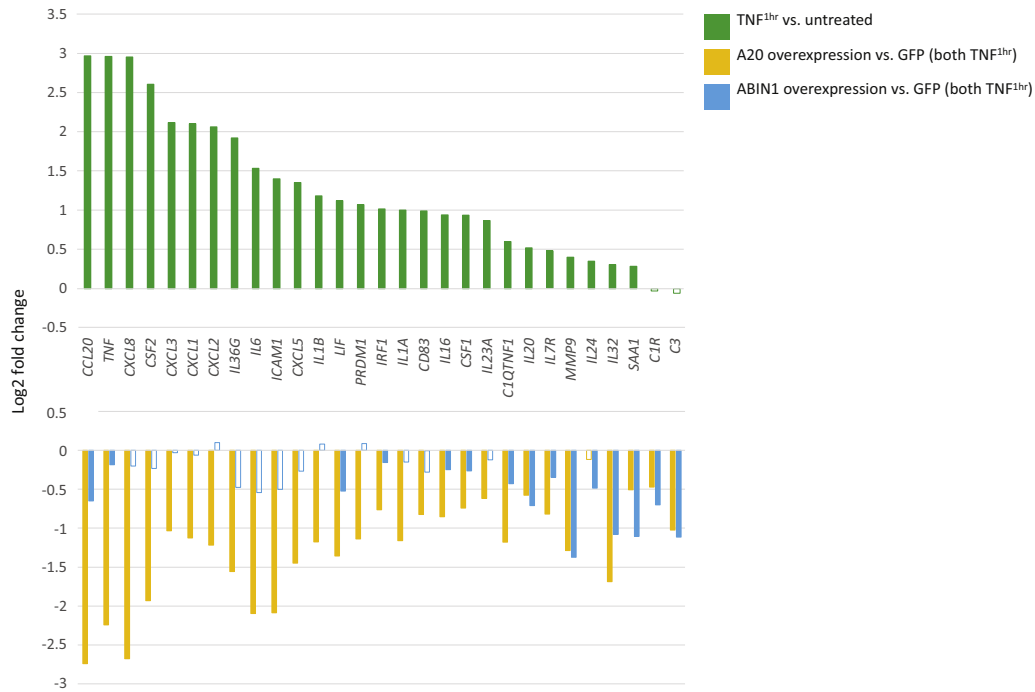
**Figure 2. A20 robustly and ABIN1 modestly represses IL-17A induced inflammatory transcriptional programs.** Top panel depicts relative expression ( $\log_2$  fold-change) of mock-infected (GFP) keratinocytes incubated with IL-17A for 24 hours versus untreated mock-infected keratinocytes. Bottom panel depicts relative expression ( $\log_2$  fold change) for either A20 or ABIN1 overexpressing keratinocytes compared to mock-infected (GFP) keratinocytes, all samples treated with IL-17A for 24 hours. Data shown represents edgeR RNA-sequencing differential expression results for six replicates for each permutation (false discovery rate  $< 0.05$  except for non-shaded bars).

We also noted non-inflammatory gene clusters that showed regulation by TNF- $\alpha$  and/or A20/ABIN1 overexpression. A small cluster is enriched for a set of cornification and keratinization genes, such as small proline-rich proteins (*SPRR2E*, *SPRR2B*, and *SPRR2G*) that are modestly downregulated by A20 and weakly by ABIN1. Another cluster includes genes strongly downregulated by TNF- $\alpha$  treatment and is enriched for Gene Ontology terms, such as chromatin (e.g., *HIST1H1B* and *HIST1H2AC*) and cell proliferation (e.g., *CCNA2* and *CDC20*). This cluster of genes likely corresponds with genes affected by TNF-mediated reduction in cell proliferation (Banno et al., 2004; Detmar and Orfanos 1990). Lastly, we noted a cluster enriched for cell adhesion genes (e.g., *LAMA3*, *LAMB3*, and *ITGA5*), which is strongly upregulated by TNF- $\alpha$  without much effect from A20 or ABIN1 overexpression. There was no clearly assignable function or biological process associated with the remaining gene clusters.

Given the greater repression of inflammation by A20 compared to ABIN1, we sought to understand whether keratinocytes in psoriasis and other rashes display upregulation of the A20 repressed gene sets discovered in our NHEK experiments. Such a finding would suggest a potential inflammation-suppressive effect of A20 in certain human rashes that could be therapeutically targeted. We generated single-cell RNA sequencing (scRNA-seq) expression data from epidermis freshly isolated from three normal, three psoriasis, one atopic dermatitis, and one erythrodermia

variabilis skin samples, each of which was validated by a board-certified dermatopathologist. To assess for whole epidermis-level expression alteration, scRNA-seq data for keratinocytes from each sample type were aggregated in bulk and differential expression analysis between normal skin samples and each of the disease types was performed using limma-trend (Law et al., 2014a). Expression for 67 genes was strongly increased in psoriatic epidermis ( $\log_2FC > 0.9$ , adjusted FDR  $< 0.05$ ) (Supplementary Table S2 online). In our NHEK experiments, 49 genes were upregulated by 24 hours of IL-17A exposure ( $\log_2FC > 0.9$ , FDR  $< 0.05$ ) (Supplementary Table S1c). Ten genes overlapped between these sets (*S100A7*, *S100A8*, *S100A9*, *SPRR2A*, *PDZK11*, *PI3*, *SAA1*, *CRABP2*, *SERPINB3*, and *SERPINB4*). Six of these 10 genes also overlapped with upregulated transcripts in the erythrodermia variabilis and atopic dermatitis samples ( $\log_2FC > 0.9$ , FDR  $< 0.05$ ) (Supplementary Table S2). Of the 10 genes that were upregulated in both psoriatic epidermis and IL-17A stimulated NHEK cells, all but two were repressed in our A20 overexpression 24-hour IL-17A exposure NHEK experiments, suggestive of in vivo relevance for A20-mediated gene repression in inflammatory skin disease (FDR  $< 0.05$ ) (Supplementary Table S1e).

If this set of A20 repressible inflammatory genes shared a regulatory mechanism, indicative of a potentially targetable common pathway, we might expect to see shared expression dynamics corresponding with keratinocyte differentiation (e.g., differing expression levels between basal and late



**Figure 3. A20 robustly and ABIN1 weakly represses tumor necrosis factor- $\alpha$ -induced inflammatory transcriptional programs.** Top panel depicts relative expression ( $\log_2$  fold-change) of mock-infected (GFP) keratinocytes incubated with tumor necrosis factor- $\alpha$  for 1 hour versus untreated mock-infected keratinocytes. Bottom panel depicts relative expression ( $\log_2$  fold change) for either A20 or ABIN1 overexpressing keratinocytes compared to mock-infected (GFP) keratinocytes, all samples treated with tumor necrosis factor- $\alpha$  for 1 hour. Data shown represents edgeR RNA-sequencing differential expression results for 6 replicates for each permutation (false discovery rate < 0.05 except for non-shaded bars). TNF, tumor necrosis factor.

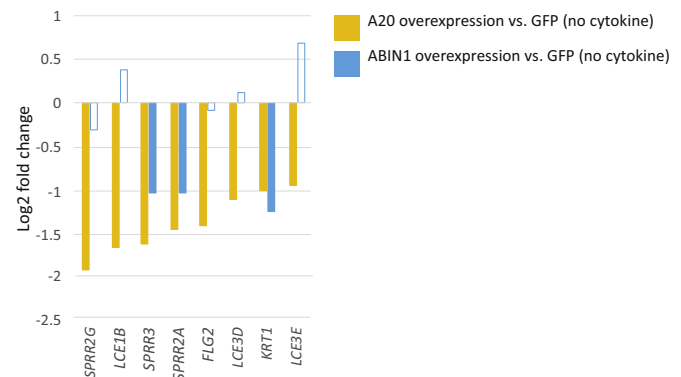
differentiated keratinocytes). We examined our scRNA-seq data by displaying transcript abundance for these genes and *KRT10* (which we used as a marker of keratinocyte differentiation state) on a single-cell level (Figure 6). In normal skin, these eight genes all showed decreased expression in the most differentiated keratinocytes (as represented by highest *KRT10* expression). However, seven of these genes showed coordinated aberrant upregulation in the most differentiated keratinocytes in the psoriatic samples, suggesting an epidermal layer-specific expression signature and common therapeutically targetable mechanism. Many of these genes also showed similar differentiation-related transcript upregulation in the erythrokeratoderma variabilis and atopic dermatitis scRNA-seq data, suggesting that these A20 regulated inflammatory transcripts are more generally aberrantly expressed in diverse types of skin disease (Figure 6).

**DISCUSSION**

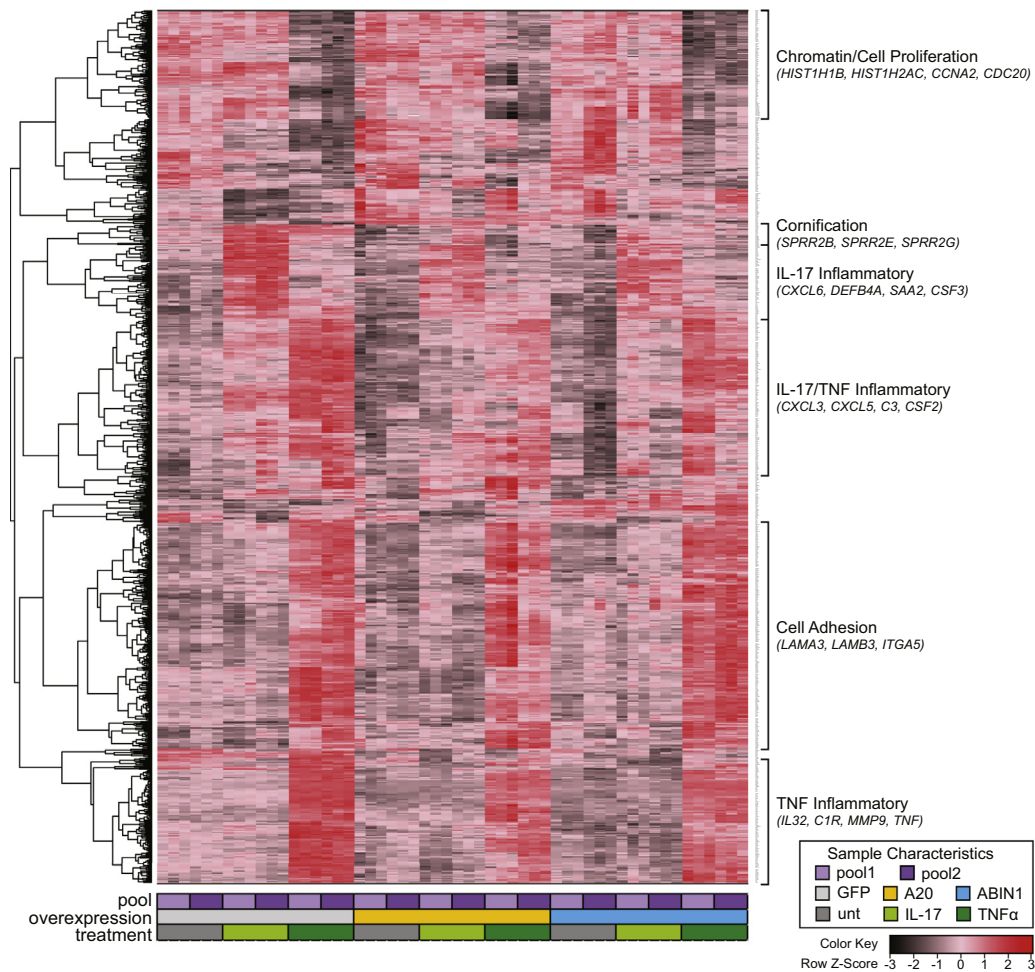
Numerous lines of evidence, from human disease linkage analyses to mouse experimental models, attest to critical roles for the NF- $\kappa$ B inhibiting partner proteins A20 and ABIN1 in systemic and cutaneous inflammatory disease. Given the increasing awareness of the role keratinocytes play in potentiating inflammatory cutaneous disease, we focused on the function of these two genes in keratinocyte inflammatory response. The combination of A20 and ABIN1 comparative analyses, RNA-seq, and cytokine stimulation treatments enabled us to substantially expand upon previous reports that focused on the effects of ABIN1 overexpression in unstimulated HaCaT keratinocytes (Ramirez et al., 2015) or on a small defined inflammatory gene set with A20

overexpression in Poly (I:C)-stimulated NHEK cells (Sohn et al., 2016). Global transcriptional analysis of unstimulated ABIN1 overexpressing HaCaT cells had previously identified enrichment in repressed genes for the “immunological disease” biological process, without further exploration of specific inflammatory genes (Ramirez et al., 2015). We found that under baseline unstimulated conditions, A20 and ABIN1 both repress a small set of inflammatory genes (e.g., *C1R*, *IL32*, *MMP9*, and *SAA1*) (Figure 1).

More importantly, we discovered a robust and broader role for A20 compared to ABIN1 in repressing inflammation



**Figure 4. A20 represses late differentiation gene transcription.** Relative expression ( $\log_2$  fold-change) of either A20 or ABIN1 overexpressing keratinocytes versus mock-infected (GFP) keratinocytes. Data shown represents edgeR RNA-sequencing differential expression results for six replicates for each permutation (false discovery rate < 0.05 except for non-shaded bars).



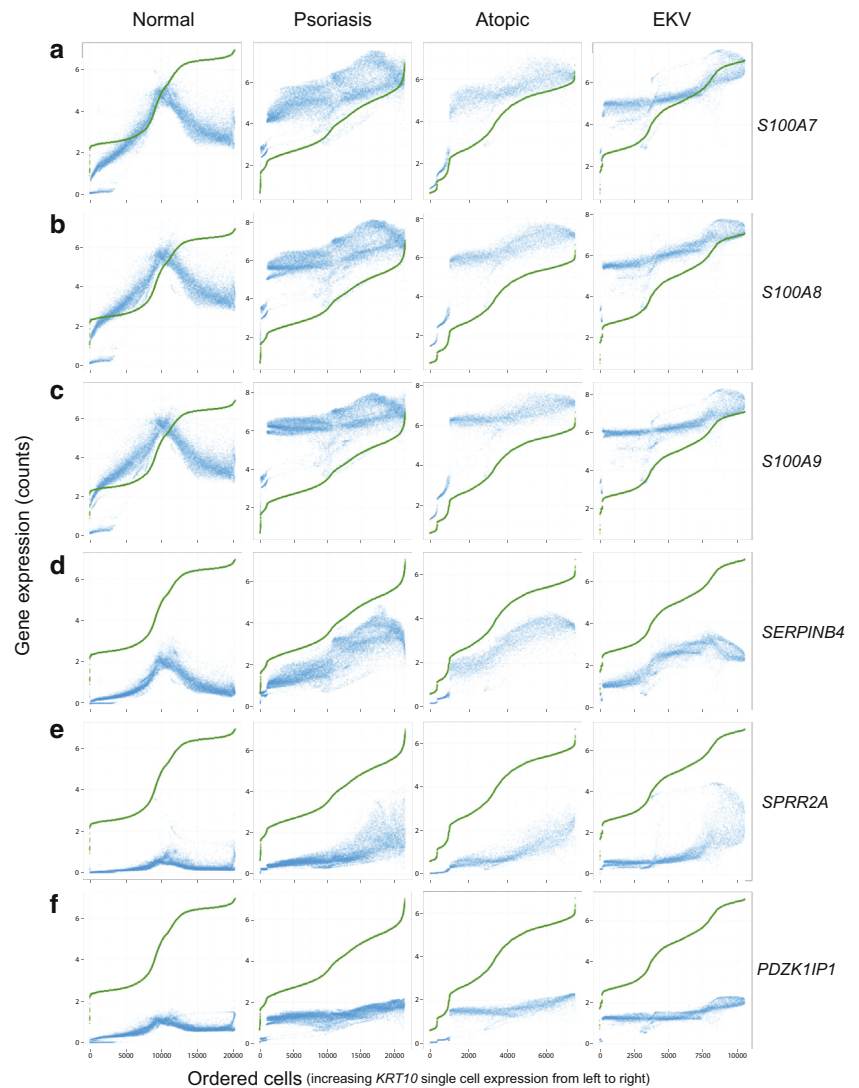
**Figure 5. Inflammation predominates the response to A20 and ABIN1 overexpression in the context of cytokine stimulation.** Unsupervised hierarchical clustering dendrogram and heatmap of gene RNA expression values. Each column represents RNA-sequencing counts per million values for one of six replicates (three replicates each for two pooled keratinocyte isolates) for nine different treatment conditions (GFP-expressing [negative control], A20-overexpressing, or ABIN1-overexpressing keratinocytes, with and without 24 hours of IL-17A or tumor necrosis factor- $\alpha$  stimulation). Each row represents 1 of the 861 differentially expressed genes with false discovery rate  $< 1 \times 10^{-5}$ . The color key represents for each row, deviation from the mean in standard deviations. TNF, tumor necrosis factor.

induced by the critical pro-inflammatory cytokines, IL-17A or TNF- $\alpha$ . This suggests that modulating A20 activity may be a more promising therapeutic target than ABIN1, given its greater inflammation-repressing role. With IL-17A and TNF- $\alpha$  exposure, both ABIN1 and A20 generally continued to downregulate the few baseline repressed genes to a similar extent, despite a greater overall expression level for cytokine responsive genes. However, upon cytokine stimulation, differences in A20 and ABIN1's repressive effect on inflammatory genes become more evident. With 24-hour IL-17A stimulation, a large set of inflammatory genes were repressed by both A20 or ABIN1 overexpression: *DEFB4A*, *C3*, *S100A7*, *CXCL8*, *SAA2*, *CXCL5*, *CSF2*, *IL36G*, *SAA1*, *CXCL1*, *CXCL6*, *IL24*, *IL74*, *CXCL3*, *S100A8*, and *CXCL22* ( $\log_2$  FC  $< -0.5$ , FDR  $< 0.05$ ), although generally to a greater extent by A20. However, there were a few genes uniquely repressed by A20, including *SERPINB4*, *S100A9*, and *LCN2*. For TNF- $\alpha$  exposure, common repressed genes by A20 or ABIN1 overexpression include *CCL20*, *IL32*, *C3*, *SAA1*, *IL32*, *IL20*, and *C1R*, but there were many genes uniquely repressed by A20, including *CXCL8*, *IL6*, *IL36G*, *CXCL5*, *IL1B*, *IL1A*, *CXCL1*,

*CXCL3*, *PI3*, and *IL23A* ( $\log_2$  FC  $< -0.5$ , FDR  $< 0.05$ ). While A20 repressed a large shared set of genes for both IL-17A or TNF- $\alpha$  stimulation, genes that were repressed only with IL-17A stimulation (e.g., *DEFB4A*, *CSF3*, *SERPINB4*, *SAA2*, *CXCL6*, and *LCN2*) or only with TNF- $\alpha$  stimulation (e.g., *TNF*, *CCL20*, and *ICAM1*) generally had low baseline expression levels before strong upregulation with the respective cytokine. These results substantially expand upon the five repressed inflammatory genes seen with A20 overexpression in Poly (I:C)-stimulated NHEK cells (Sohn et al., 2016). For ABIN1, given its greater effect in the context of IL-17A stimulation, genes such as *CXCL5*, *CXCL8*, *DEFB4A*, *IL36G*, *CXCL1*, and *CXCL3*, were repressed only with IL-17A stimulation and *CCL20* was one of the few inflammatory genes uniquely repressed in the context of TNF- $\alpha$  stimulation.

The differential repressive potency and gene targets of A20 and ABIN1 overexpression with IL-17A and TNF- $\alpha$  exposure suggest unique roles for these two genes in modulating keratinocyte inflammatory response. In the context of TNF- $\alpha$  signaling, ABIN1 is believed to primarily function as a facilitative adapter protein with A20 to inhibit NF- $\kappa$ B

**Figure 6. Shared single-cell RNA expression differentiation-related patterns in psoriatic, erythrokeratoderma variabilis, and atopic dermatitis keratinocytes, for selected IL-17A induced and A20 repressed genes.** Each row of panels represents normalized/imputed single cell RNA expression data for a different gene (a) *S100A7*, (b) *S100A8*, (c) *S100A9*, (d) *SERPINB4*, (e) *SPRR2A*, or (f) *PDZK1IP1*; see Materials and Methods). Each column of panels represents a distinct inflammatory state (normal, psoriasis, erythrokeratoderma variabilis, or atopic dermatitis-affected epidermis). In each panel, the dots represent single cells. For each panel, cells are ordered along the x-axis by *KRT10* expression from low to high starting with the lowest-expressing single cell on the far left and the highest-expressing single cell on the far right (i.e., increasing differentiation status of single cells from left to right on the x-axis). The scale of the x-axis differs for each inflammatory state, reflective of the differing number of profiled single cells. On the y-axis of each panel, transcript abundance (in log<sub>2</sub> counts per 10,000) for each single cell is displayed for a given gene (blue dot) or *KRT10* (green dot). As there are small incremental differences in *KRT10* expression between adjacent points and the large number of plotted cells, the green dots that represent *KRT10* single-cell expression merge into a contiguous line. In the normal skin samples, there is a decrease in gene expression in the highest *KRT10* expressing single cells; however, in the inflamed disease samples, there is an aberrant coordinated increase in gene expression in the highest *KRT10*-expressing single cells.



signaling, by preventing phosphorylation and inactivation of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  (G'Sell et al., 2015). A20 also employs additional inhibitory mechanisms, such as restricting TNF receptor initiation of NF- $\kappa$ B and JNK signaling through TRAF2 and RIP1 (Catrysse et al., 2014). We speculate that overexpressed ABIN1 may readily saturate endogenous levels of A20 so that increased expression in keratinocytes only modestly correlates with functional activity when exposed to TNF- $\alpha$ .

Both ABIN1 and A20 appear to independently inhibit IL-17 signaling. A20 inhibits TRAF6, a molecule required for IL-17–induced NF- $\kappa$ B and MAPK signaling (Garg et al., 2013). A20 also directly binds to and inhibits the IL-17 receptor, an additional mechanism for inhibiting IL-17–induced NF- $\kappa$ B and MAPK activation (Garg et al., 2013). The role of ABIN1 in IL-17 signaling (e.g., whether and how ABIN1 and A20 cooperatively interact to inhibit NF-

$\kappa$ B activation as in TNF signaling) is less well defined; however, ABIN1 can inhibit IL-17 signaling independent of A20 (Cruz et al., 2017). Whether ABIN1 also directly interacts with the IL-17 receptor like A20 also remains an open question. ABIN1's greater inflammation-repressing effects in the context of IL-17A stimulation (compared to TNF- $\alpha$ ), likely arise from its A20-independent role in IL-17 repression. ABIN1's lower overall activity compared to A20 may stem from its inability to bind the IL-17 receptor or inhibit other A20-specific functions. Alternatively, the greater levels of A20 protein overexpression compared to ABIN1 may account for its greater overall inflammatory repression. However, this appears unlikely, given that expression levels for both proteins are increased by at least fivefold.

Our results also punctuate the importance of keratinocyte-mediated inflammation and thus its potential as a target for clinical intervention. By utilizing single-cell RNA-seq on



rash-affected epidermis, we identified a set of epidermal layer-specific, IL-17A-inducible inflammatory transcripts in diverse skin diseases, such as psoriasis, eczema, and erythrodermia variabilis, that are repressed by A20 overexpression. While systemic blockade of cytokine signaling pathways, such as IL-17A and TNF- $\alpha$ , have had immense therapeutic benefit in patients, the ability to specifically block their effect in a readily accessible target tissue, such as skin, would be highly desirable in minimizing the systemic side effects of these potent immunosuppressants. Based on our findings, we hypothesize that *in vivo* upregulation of cutaneous A20 activity may represent a therapeutic path to dampen target tissue inflammation in diverse inflammatory skin diseases.

## MATERIALS AND METHODS

### Keratinocyte isolation and primary culture

Primary human keratinocyte cultures were isolated from neonatal foreskins as described previously (Lowdon et al., 2014). Written informed consent for surgical tissue discards was obtained using protocols approved by the University of California, San Francisco Institutional Review Board. Briefly, skin was incubated overnight at 4°C in 25 U/ml dispase solution (Corning Life Sciences, Corning, NY), followed by mechanical separation of epidermis from dermis. Epidermis was incubated in 0.05% trypsin for 15 minutes at 37°C. Dissociated epidermal cells were filtered using a 100- $\mu$ m nylon cell strainer (Corning Life Sciences) and cultured in keratinocyte growth media (KGM; Medium 154CF supplemented with 0.07 mM CaCl<sub>2</sub> and Human Keratinocyte Growth Supplement; Life Technologies, Waltham, MA).

### ABIN1 and A20 lentiviral overexpression and cytokine treatment

ABIN1, A20, and GFP open reading frame expression vectors were purchased from GeneCopoeia (Rockville, MD) (Supplementary Table S3 online). Lentivirus particles were prepared by the University of California, San Francisco ViraCore. Briefly, HEK293T cells were seeded at  $7 \times 10^4$  cells/cm<sup>2</sup> in 15-cm tissue culture dishes in 20 ml media (DMEM, 10% FBS; Life Technologies). Twenty-four hours after plating, 12  $\mu$ g lentiviral transfer vector was transfected alongside 7  $\mu$ g psPAX2 (Addgene #12260) and 3  $\mu$ g pMD2.G (Addgene #12259) with 50  $\mu$ l jetPRIME transfection reagent (Polyplus, New York, NY) according to manufacturer's protocol. Seventy-two hours post-transfection, lentiviral supernatant was collected and passed through a 0.45- $\mu$ m filter (EMD Millipore, Burlington, MA). Lentiviral titer was determined using the p24 ELISA kit from Takara (Mountain View, CA). Pooled primary cultured keratinocytes from three different individuals were transduced at a multiplicity of infection of 10–100 as described previously (Kuhn et al., 2002). One day prior to transduction, keratinocytes were plated at  $1 \times 10^4$  cells/cm<sup>2</sup>. Lentivirus supernatants were diluted using KGM and 4  $\mu$ g/ml Polybrene (EMD Millipore). Keratinocytes were incubated with lentivirus for 16 hours, after which they were allowed to recover for 24 hours in fresh KGM. Lentivirus infection was selected for using 2  $\mu$ g/ml puromycin (Life Technologies) for 48 hours. At ~80% confluency, cells were incubated in fresh KGM or KGM containing cytokine (10 ng/ml TNF- $\alpha$  or 200 ng/ml IL-17A; PeproTech, Rocky Hill, NJ) for 1 or 24 hours. Samples from triplicate experiments performed upon two distinct pooled primary cultured keratinocytes from three different individuals (six total replicates) were harvested for protein and RNA extraction.

### Western blot

Whole-cell lysate was extracted using radioimmunoprecipitation assay buffer with freshly dissolved protease/phosphatase inhibitor as per manufacturer's protocol (Life Technologies). Protein concentration was measured using DC Protein Assay (BioRad Laboratories, Hercules, CA). Equal amounts of protein were separated using a NuPAGE 4–12% Bis-Tris Protein Gel and transferred to polyvinylidene difluoride membrane (Life Technologies). Membranes were blocked with Odyssey PBS Blocking Buffer (Li-Cor, Lincoln, NE) for 30 minutes at room temperature and incubated with primary antibodies against ABIN1(TNIP1) (Proteintech, Rosemont, IL; 1:1000, rabbit) and A20 (TNFAIP3) (Cell Signaling, Danvers, MA; 1:1000, rabbit). Glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling; 1:10000, mouse) was used as loading control. Anti-rabbit and anti-mouse secondary antibody conjugated to an infrared dye (IRDye800CW and IRDye 680RD, respectively; Li-Cor) or anti-rabbit and anti-mouse secondary antibody conjugated to horseradish peroxidase were used (Cell Signaling) and the images were acquired on an Odyssey FC imaging instrument (Li-Cor) or peroxidase activity was detected using Pierce ECL Western Blotting Substrate (Life Technologies).

### Statistical analysis for Western blot band intensity analysis

When applicable, the results are presented as mean  $\pm$  standard deviation. Statistical analysis was conducted using GraphPad Prism, version 5.0f (La Jolla, CA). Student *t* test was used to compare two separate sets of independent and identically distributed samples with a *P*-value < 0.05 considered as significant.

### RNA isolation and RNA-seq

Total RNA was extracted using TRIzol reagent (Life Technologies) as per manufacturer's protocol. RNA-seq libraries were prepared with 300–1,000 ng of total RNA using KAPA Biosystems Stranded RNA-Seq Kits with RiboErase HMR (Roche, Pleasanton, CA). Technical duplicate sequencing libraries were generated for each RNA sample to minimize batch effect. Total RNA samples were depleted for ribosomal RNA through hybridization of cDNA oligonucleotides, followed by treatment with RNase H and DNase to remove ribosomal RNA duplexed to DNA and original DNA oligonucleotides, respectively. The resulting ribosomal-depleted RNA then underwent RNA fragmentation using heat and magnesium. First-strand cDNA synthesis was performed using random primers, followed by second-strand synthesis. To the 3' ends of the double-stranded cDNA library fragments, deoxyadenosine monophosphate was added (A-tailing). Double-stranded DNA adapters with 3' deoxythymidine monophosphate was ligated to the A-tailed library fragments. Library fragments with appropriate adapter sequences were amplified via ligation-mediated PCR. Post-amplified cDNA libraries were quantitated with either Quant-iT double-stranded DNA or Qubit double-stranded DNA high-sensitivity assay kits (Life Technologies). Quality assessment was performed using the LabChip GX Touch HT microfluidics platform (Perkin Elmer, Waltham, MA). A 2  $\times$  150-bp sequencing on a NovaSeq 6000 instrument was performed on libraries with a PhiX Control, version 3 (Illumina, San Diego, CA).

### RNA-seq analysis

The RNA-seq by Expectation Maximization algorithm was used to quantify gene expected counts used for differential expression analysis and counts per million (CPMs) used for heat-map clustering. Differential expression analysis was performed using edgeR (version 3.22.3 [Robinson et al., 2010]; R, version 3.5.1). For each comparison, very-

low-expressing genes with a CPM  $\leq 1$  in 6 (the number of replicate per treatment condition) or more samples were removed. An additive model formula was then used to adjust for batch-effect differences between the two batches of keratinocyte pools within edgeR's glmQLFit framework (i.e., genewise negative binomial generalized linear models with quasi-likelihood tests, which fits a quasi-likelihood negative binomial generalized log-linear model to count data to conduct genewise statistical tests for a given coefficient or contrast) (Lund et al., 2012), and a quasi-likelihood F-test (glmQLFTest) was used to identify those genes significantly different (FDR  $P < 0.05$  after correcting for multiple hypothesis testing using the Benjamini-Hochberg procedure) between each pairwise comparison. Gene Ontology analysis was performed using Bioconductor's goseq package (version 1.32.0) (Young et al., 2010).

To generate the heat map, variation in gene expression across samples was visualized using R's (version 3.4.4) gplots package (version 30.1). Pearson's correlation distance was calculated between Z scores for each gene, and complete linkage clustering was performed to group genes by common patterns across samples. The resulting dendrogram was then cut to yield 14 clusters. Gene Ontology was performed on genes in each cluster as compared to a background of all expressed genes using R's goseq package (version 1.28.0). The R scripts for these analyses as well as their resulting output are available on GitHub: [https://github.com/SRHilz/ModOfKerInf\\_RNAseqAnalysis](https://github.com/SRHilz/ModOfKerInf_RNAseqAnalysis).

### Single-cell RNA-seq

Human epidermal cells were isolated from normal surgical tissue discards or lesional skin from psoriasis, eczema, or erythrodermia variabilis patients. Written informed consent for skin samples was obtained using protocols approved by the University of California, San Francisco Institutional Review Board. Skin was incubated for 2 hours at 37°C in 25 U/ml dispase solution followed by mechanical separation of epidermis from dermis. Epidermis was incubated in 0.05% trypsin for 15 minutes at 37°C. Dissociated epidermal cells were washed with KGM and filtered using a 40- $\mu$ m nylon cell strainer (Corning Life Sciences). FACS was performed on dissociated cells to exclude debris, doublets, and DAPI-positive cells. The sorted cells were resuspended in 0.04% BSA in phosphate buffered saline (Life Technologies) prior to Chromium Single cell 3' Solution V2 (10x Genomics, Pleasanton, CA) library preparation, performed by the University of California, San Francisco Institute for Human Genetics Core as per manufacturer's protocol. Four scRNA-seq sample data sets were originally published in Cheng et al. (2018) and were re-analyzed along with the new samples as described below.

### scRNA-seq data processing and quality control filtering

Cellranger (10x genomics, version 2.0.2) was used to de-multiplex the raw Illumina sequencing data, from scRNA-seq libraries, quantify unique molecular identifiers (using the GRCh38, version 1.2.0, reference transcriptome), and aggregate data for the eight samples. We managed and filtered the resulting data from 59,502 cells with Seurat (version 2.2.0 [Macosko et al., 2015]). To control for damaged cells, we filtered out cells in the top 5th percentile of proportion of mitochondrial unique molecular identifiers, which corresponded to cells with >14% of total unique molecular identifiers accounted for by mitochondrial transcripts. We used multiplet rate estimates provided by 10X genomics ([https://assets.ctfassets.net/an68im79xiti/UhAMGmlaEMmYMaA4A4Uwa/274a813b81e42cba81345d49380432d7/CG00052\\_SingleCell3\\_ReagentKitv2UserGuide\\_RevD.pdf](https://assets.ctfassets.net/an68im79xiti/UhAMGmlaEMmYMaA4A4Uwa/274a813b81e42cba81345d49380432d7/CG00052_SingleCell3_ReagentKitv2UserGuide_RevD.pdf)) to fit a linear model to estimate the percentage of multipllets in each

sample based on the number of loaded cells. In order to avoid including partial cells and multipllets in our analysis, we filtered on the number of detected genes in each cell. On a per-sample basis, we removed cells using a low threshold of 0.5% and high threshold of 100 - 2\* multipllet rate on the percentiles of number of genes detected. These filters left 42,105 cells to include in our primary analyses.

### scRNA-seq dimensional reduction and data imputation

Using the pipeline described in Cheng et al. (2018), we used ZINB-WaVE (version 1.0.0) (Risso et al., 2018) to remove confounding signal due to the percent mitochondrial expression, the total number of unique molecular identifiers detected (log scaled) and the sample membership from the raw count data of highly expressed (at least 500 CPM in 0.1% of cells) genes in each cell. We used the 20-dimensional projection of the data produced by ZINB-WaVE to construct an affinity matrix (with adaptive distance parameters  $k_a = 10$  and  $k = 30$ ) to input to the MAGIC (Dijk et al., 2017) imputation algorithm. For the imputation step, we set the parameter  $t$  to 10.

### scRNA-seq differential expression analysis

We used limma-trend (Law et al., 2014b) to compare the single-cell expression profiles from each disease to that in the normal skin samples. For this analysis, unique molecular identifier count data were converted to library-size normalized CPM and log<sub>2</sub>-scaled with an offset of 1. We performed this analysis on 10,194 moderately expressed genes in our cohort of cells (at least 300 CPM in 0.1% of cells). We removed melanocytes by dropping cells with *PMEL* expression >500 CPM. To remove immune cells, we used an *HLA-DRA* expression cutoff of >2000 CPM. Limma-trend calculates the difference in mean log-CPM for each group of cells, and to evaluate statistical significance, moderated t-statistics are calculated based on an empirical Bayes approach. We use the FDR of the *P*-values associated with these statistics to correct for multiple hypothesis testing.

### Data access

Sequence data have been deposited at the European Genome-Phenome Archive, which is hosted by the European Bioinformatics Institute and the Centre for Genomic Regulation, under accession number EGAS00001002981. Further information about European Genome-Phenome Archive can be found on <https://ega-archive.org>.

### CONFLICTS OF INTEREST

AJS, CJV, and SCB are employees of Nantomics, LLC. CJV and SCB are equity holders of Nantomics, LLC. The remaining authors disclose no conflicts.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at [www.jidonline.org](http://www.jidonline.org), and at <https://doi.org/10.1016/j.jid.2018.10.046>.

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