# Type I Immune Response Induces Keratinocyte Necroptosis and Is Associated with Interface Dermatitis

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Interface dermatitis is a characteristic histological pattern that occurs in autoimmune and chronic inflammatory skin diseases. It is unknown whether a common mechanism orchestrates this distinct type of skin inflammation. Here we investigated the overlap of two different interface dermatitis positive skin diseases, lichen planus and lupus erythematosus. The shared transcriptome signature pointed toward a strong type I immune response, and biopsy-derived T cells were dominated by IFN-γ and tumor necrosis factor alpha (TNF-α) positive cells. The transcriptome of keratinocytes stimulated with IFN-γ and TNF-α correlated significantly with the shared gene regulations of lichen planus and lupus erythematosus. IFN-γ, TNF-α, or mixed supernatant of lesional T cells induced signs of keratinocyte cell death in three-dimensional skin equivalents. We detected a significantly enhanced epidermal expression of receptor-interacting-protein-kinase 3, a key regulator of necroptosis, in interface dermatitis. Phosphorylation of receptor-interacting-protein-kinase 3 and mixed lineage kinase domain like pseudokinase was induced in keratinocytes on stimulation with T-cell supernatant—an effect that was dependent on the presence of either IFN-γ or TNF-α in the T-cell supernatant. Small hairpin RNA knockdown of receptor-interacting-protein-kinase 3 prevented cell death of keratinocytes on stimulation with IFN-γ or TNF-α. In conclusion, type I immunity is associated with lichen planus and lupus erythematosus and induces keratinocyte necroptosis. These two mechanisms are potentially involved in interface dermatitis.

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## INTRODUCTION

Inflammatory skin diseases (ISD) are a heterogeneous group of complex, immune-mediated, and noninfectious skin disorders leading to disability, systemic inflammation, social stigmatization, and a poor quality of life (Boehncke and Schon, 2015; Brunner et al., 2017; Eyerich et al., 2015). Over the last few years, an increasing understanding of underlying immune mechanisms led to the development of specific therapeutic compounds, such as monoclonal antibodies targeting key cytokines (Noda et al., 2015). So far, research has focused on single diseases aiming at elucidating master regulators of disease progression and, thereby, identifying new drug targets (Lauffer and Ring, 2016). Interestingly, most of the new compounds are not exclusively effective in the treatment of one ISD, but also show beneficial effects in the treatment of ISD with a similar pathogenic mechanism. Namely, monoclonal antibodies against tumor necrosis factor alpha (TNF-α) were shown to be effective in the treatment of psoriasis, hidradenitis suppurativa (acne inversa), and pityriasis rubra pilaris (Chaudhari et al., 2001; Kimball et al., 2016; Petrof et al., 2013). To exploit the full potential of available compounds, a new translational scientific approach investigating general immune response patterns of the skin in a disease-independent manner is needed (Eyerich and Eyerich, 2017).

Interface dermatitis (ID), also called lichenoid tissue reaction, is a unique histological pattern that can be detected in several inflammatory and autoimmune skin diseases, such as lichen planus (LP), lupus erythematosus (LE), dermatomyositis, erythema multiforme, fixed drug eruption, and many others. According to common definitions, ID is composed of two characteristic parts: an immune cell infiltrate close to the basal membrane of the epidermis and cell swelling and death of the undermost keratinocytes (Sontheimer, 2009). Although the initial stimulus might be different among certain skin diseases, a dominance of cytotoxic T cells and involvement of plasmacytoid dendritic cells was described in several ID positive skin diseases (Wenzel and Tuting, 2008). However, the mechanism of epidermal reaction remains poorly understood. This is reflected by a nonuniform terminology describing keratinocyte swelling as oncosis, apoptosis, necrotic degeneration, or single cell necrosis. The lack of

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*Abbreviations: ID, interface dermatitis; ISD, inflammatory skin disease; LE, lupus erythematosus; LP, lichen planus; MLKL, mixed lineage kinase domain like pseudokinase; RIP3, receptor-interacting-protein-kinase 3; ShRNA, small hairpin RNA; TCS, T-cell supernatant; Th, T helper; TNF, tumor necrosis factor*

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understanding ID pathogenesis is paralleled by an unmet medical need for new therapeutic targets to treat ID positive skin diseases. Thus, ID is a reasonable issue to investigate general mechanisms of skin inflammation.

The aim of this study was to investigate the underlying molecular mechanism of ID in a disease-independent manner focusing on the overlap of LP and LE in terms of histological architecture, genetic regulations, and cellular immune response.

## RESULTS

### The molecular signature of ID reflects immune cell infiltration as well as epidermal reaction

Skin biopsies of LP and LE were investigated as both diseases show an ID despite their different clinical presentations (Figure 1a). To investigate ID-specific gene regulations, whole genome expression analysis was performed in human skin biopsies of LP ($n = 11$) and LE ($n = 5$) in comparison with autologous healthy skin. A total of 5,675 genes were regulated exclusively in LP, and 4,354 genes were regulated exclusively in LE. A total of 3,888 genes showed differential regulation in both LE and LP when compared with healthy skin (Figure 1b). Only these genes were regarded as independent of the specific diseases, LP and LE, but shared among ID diseases and included in the further analysis. Pathway enrichment of shared genes showed an activation of interferon, chemokine, and T-cell-related pathways (Figure 1c). To get a deeper understanding of gene interactions leading to ID, we performed induced modules network analysis of the top 500 regulated genes in the overlap of LP and LE (gene list in Supplementary Table S1 online). Highly connected nodes (hub genes) could be attributed to T-cell immunology (TBX21, IL12R), interferon signaling (IFN-γ, IRF1, IRF2, IRF3), and response to interferon stimulation (signal transducer and activator of transcription 1, NF-κB, RELA proto-oncogene, interferon-stimulated gene factor 3 [ISGF3]) (Figure 1d). Considering characteristics of ID, we hypothesized that hub genes indicate a type I immune dominance of infiltrating immune cells, whereas response to interferon signaling might correspond to the epidermal reaction pattern.

### ID is dominated by a type I immune response

Considering the strong activation of T-cell pathways in our network analysis, T cells were isolated from lesional skin biopsies for in-depth analysis. Intracellular cytokine staining revealed a high frequency of IFN-γ and TNF-α positive cells in both LE and LP. The frequency of $CD4^+$/IFN-$\gamma^+$/TNF-$\alpha^+$ was significantly higher than in psoriasis (percentage of $CD4^+$ cells: LP 64.5 ± 24.1, LE 63.1 ± 18.6, psoriasis 27.9 ± 11.2, $P = 0.0346$ LP vs. psoriasis, $P = 0.0466$ LE vs. psoriasis), an ISD without ID (Figure 2a and b). In contrast, there were no significant differences in the levels of IL-4, TNF-α, and IL-17A (Figure 2c). To verify a type I immune dominance in situ, immunohistochemical stainings for T-bet were performed. In line with the T-cell immunophenotyping, a significantly higher number of T-bet-positive cells were observed in LE and LP than in psoriasis tissue samples (10.3 ± 4.6 vs. 2.2 ± 1.2; $P = 0.0002$) (Figure 2d).

### The molecular signature of ID resembles keratinocytes stimulated with IFN-γ and TNFα

We next addressed the question: which T-cell stimulus induces gene expression signatures in keratinocytes similar to the molecular signature of ID? We therefore stimulated primary human keratinocytes with the cytokine milieu produced by the T helper (Th) cell subsets Th1, Th2, Th17, and Th22 cells. Transcriptome analysis followed by induced network analysis revealed distinct molecular response patterns integrated into networks for each of the four cytokine combinations (Figure 3a). The network for keratinocytes stimulated with IFN-γ and TNF-α, lead cytokines of Th1 cells, showed the highest similarity to the network detected in human punch biopsies as both shared highly connected nodes such as IRF1, NF-κB, ISGF3, and signal transducer and activator of transcription 1 (Figures 1d and 3a). When comparing the shared expression profile of ID with the top 100 most differentially regulated genes of keratinocytes, we detected a significant correlation with keratinocytes stimulated with IFN-γ and TNF-α, IL-17, and IL-22 as well as with IL-22 (Figure 3b). Of note, the most significant correlation was evident on IFN-γ and TNF-α stimulation, indicating that keratinocytes in ID are exposed to a type I immune response microenvironment. Finally, we stimulated three-dimensional skin models, mimicking keratinocyte differentiation of the human epidermis, with different T-cell cytokine combinations. Although this in vitro model is limited due to the lack of immune cells, we observed that only keratinocytes stimulated with IFN-γ and TNF-α were swollen and showed pyknotic nuclei as signs of cell death (Figure 3c).

### Necroptosis and apoptosis pathways are activated in ID

Given the clear type I dominance in the cellular compartment and keratinocyte death that can be induced by type I cytokines, we next aimed at understanding the underlying mechanisms. Gene network analysis of the ID shared gene signature revealed signals of apoptosis (FASL), direct cytotoxicity (granzyme B), and necroptosis (ISGF3 complex) for ID (Figures 1d and 4a). In line with this observation, regulation of genes belonging to the apoptosis and necroptosis pathway was detectable in the ID signature (Supplementary Figure S1 online). Although the number of cleaved caspase 3 positive cells was higher in ID than in psoriasis, the majority of keratinocytes in LP and LE were negative for this marker of apoptosis, as detected by immunohistochemistry (Figure 4b, upper panel). However, LP and LE tissue sections showed an epidermal expression of receptor-interacting-protein-kinase 3 (RIP3), a key protein of necroptosis (Figure 4b, lower panel), which was significantly stronger than in psoriasis samples. In vitro stimulation of keratinocytes with IFN-γ and TNF-α (10 ng/ml) resulted in induction of RIP3 like in ID, but not cleaved caspase 3 (Figure 4c). IFN-α (10 ng/ml) neither induced RIP3 nor cleaved caspase 3 in keratinocytes (Figure 4c). Next, we stimulated keratinocytes with mixed T-cell supernatant (TCS) derived from LE and LP lesional T cells. Here, we observed that high doses of TCS induced both RIP3 and cleaved caspase 3, whereas lower concentrations led to predominant induction of RIP3 and only a faint band of cleaved caspase 3 (Figure 4d). Phosphorylated mixed lineage kinase domain like pseudokinase

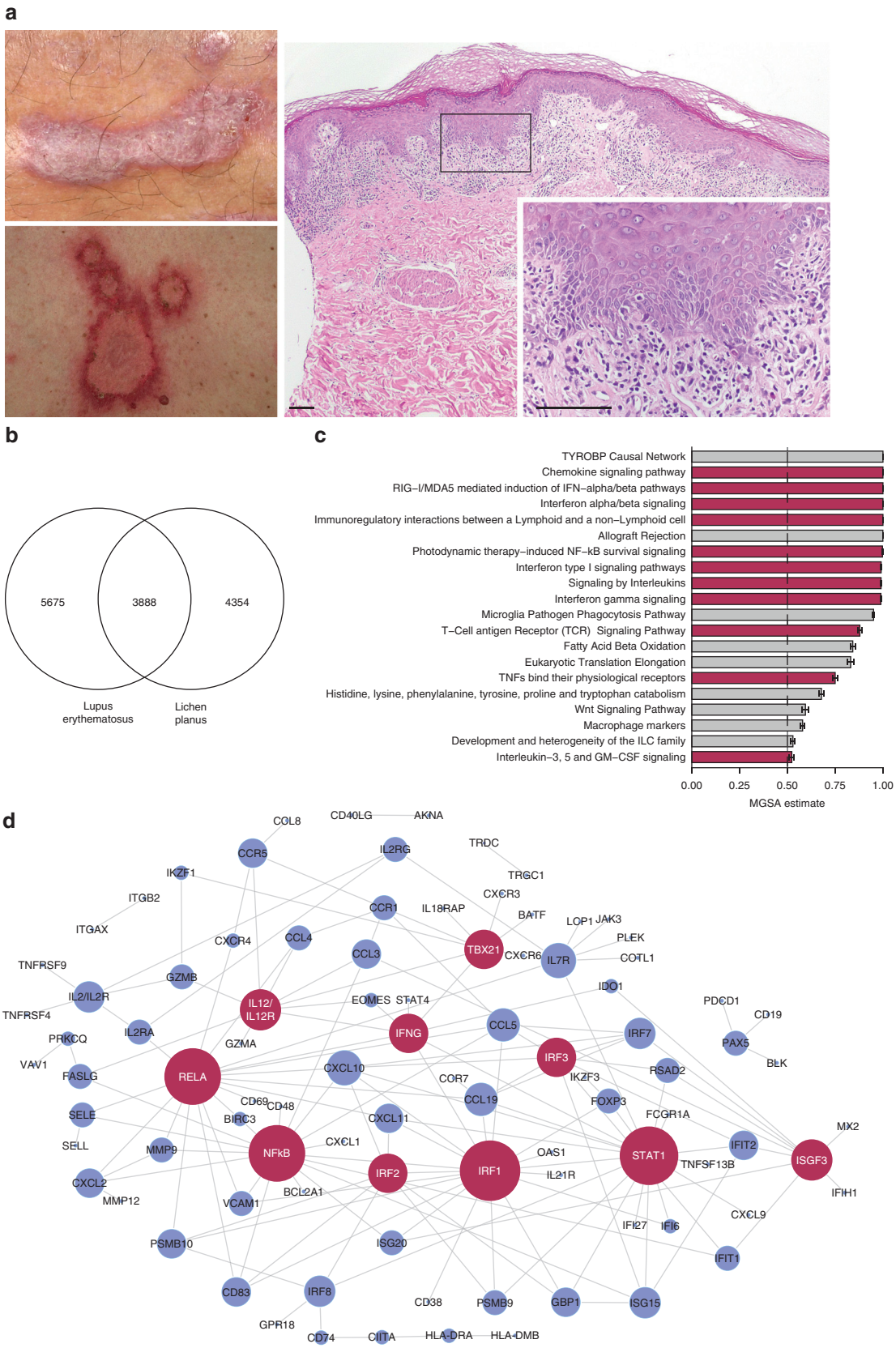


**Figure 1. The molecular signature of interface dermatitis.** (**a**) Clinical picture of lichen planus (LP, upper-left picture) and lupus erythematosus (LE, lower-left picture). Histological picture of a representative LP demonstrating a dense lichenoid infiltration of lymphocytes and vacuolated basal keratinocytes. Scale bars indicate 50 μm. (**b**) Transcriptome analysis of LP (n = 11) and LE (n = 5). Depicted is the number of regulated genes shared by both LP and LE. Only the shared genes were taken into account for further analysis. (**c**) Active pathways (estimate larger than 0.5) of shared interface dermatitis genes. Pathways related to type I immune responses are marked in red. (**d**) Induced modules network analysis of the top 500 regulated genes shared by LP and LE. Node size correlates with the number of connected nodes and edges. Nodes with ≥ 8 connections are marked in red.

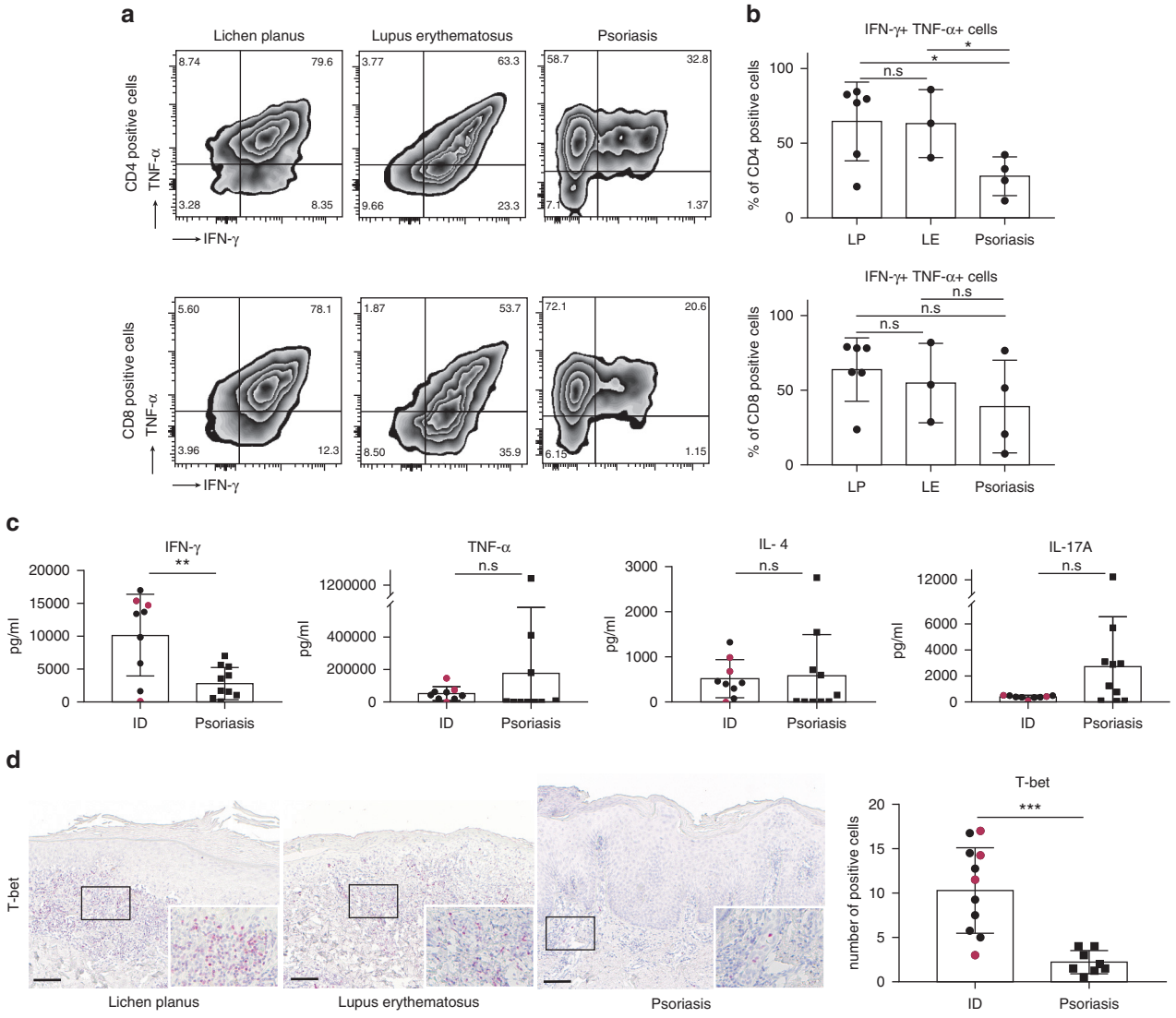


**Figure 2. Interface dermatitis is mediated by a strong type I immune response.** (**a**) Representative flow cytometry plots of lichen planus (LP), lupus erythematosus (LE), and psoriasis-derived T cells. (**b**) Frequency of IFN-γ, tumor necrosis factor-α (TNF-α) double positive cells gated on CD4-positive cells, and CD8-positive cells of LP (n = 6), LE (n = 3), and psoriasis (n = 4). (**c**) IFN-γ, TNF-α, IL-4, and IL-17 secretion into supernatant of biopsy-derived T cells of LP, LE, and psoriasis. Lesional T cells of LP and LE are displayed in one bar (interface dermatitis: ID). Red points indicate LE samples. (**d**) Representative immunohistochemical stainings for T-bet and number of T-bet positive cells per high power field (×400). Red points indicate LE samples. Scale bars indicate 50 μm. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

(pMLKL) as a downstream target of RIP3 was most prominently expressed in the absence of cleaved caspase 3, indicating a dose-dependent, dichotomous regulation of different cell death mechanisms. To test whether IFN-γ, TNF-α, or the combination of both cytokines leads to the activation of necroptosis in keratinocytes, we depleted these cytokines in TCS using biotinylated antibodies (Supplementary Table S2 online). Stimulation of keratinocytes with IFN-γ-only-depleted TCS as well as stimulation with TNF-α-only-depleted TCS resulted in induction of RIP3 and pMLKL, whereas depletion of both cytokines prevented the activation of both RIP3 and pMLKL (Figure 4e). As observed with recombinant cytokines (Figure 3c), TCS induced cell swelling in three-dimensional skin equivalents. Similar to the induction of RIP3 and pMLKL, this effect was induced by IFN-γ-depleted TCS as well as by TNF-α-depleted TCS, but not by IFN-γ- and TNF-α-depleted TCS (Figure 4f).

### RIP3 is involved in keratinocyte cell death on stimulation with TNF-α and IFN-γ

To test the impact of RIP3 for keratinocyte cell death, we performed a shRNA knockdown of RIP3 in primary human keratinocytes. Compared with control shRNA, knockdown of RIP3 using shRNA1 and shRNA3 led to a decreased phosphorylation of MLKL on stimulation with IFN-γ and TNF-α (Figure 5a). Keratinocytes stimulated with IFN-γ and TNF-α (both 10 ng/ml) showed significantly lower frequencies of dead cells, when shRNA knockdown of RIP3 (using a 50:50 mix of shRNA1 and shRNA3) was performed compared with keratinocytes transduced with control shRNA (Figure 5b and c).

## DISCUSSION

A better understanding of general immune reaction patterns of the skin is a prerequisite to identify new therapeutic options for large groups of ISD. Beyond apotosis, we here

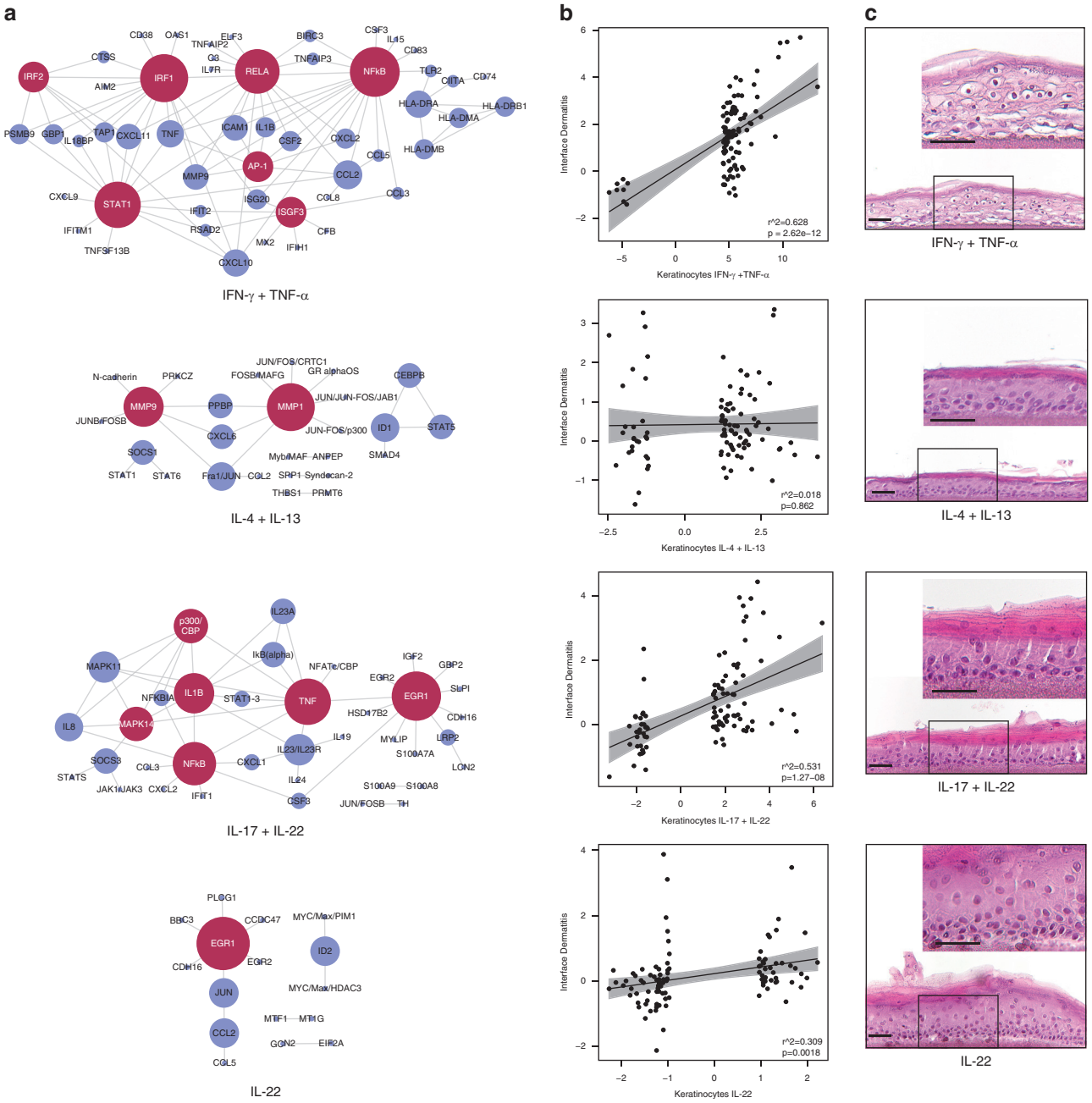


**Figure 3. The molecular signature of interface dermatitis resembles keratinocytes stimulated with IFN-γ and tumor necrosis factor-α (TNFα).** (**a**) Primary human keratinocytes were stimulated with the indicated cytokine combinations (each 50 ng/ml). Induced module networks of top 100 regulated genes as compared with unstimulated control are displayed. Node size correlates with the number of connected nodes and edges. Nodes with ≥5 connections are marked in red. (**b**) Comparison of top 100 differentially regulated genes in stimulated keratinocytes and the shared genes of lichen planus (LP) and lupus erythematosus (LE) (referred to as "interface dermatitis") based on fold changes. (**c**) Stimulation of three-dimensional skin models with indicated cytokine combinations (each 50 ng/ml) for 72 hours. Scale bars indicate 50 μm.

define two additional mechanisms associated with ID: a type I dominant cellular immune response with the key cytokine IFN-γ and an activation of the necroptosis pathway mediated by the phosphorylation of RIP3 in keratinocytes.

We hypothesized that a common mechanism would orchestrate the inflammatory pattern of ID in a disease-independent manner. To test this hypothesis, we chose LP and LE as model diseases as they share ID, but are clinically distinct. However, when comparing the current knowledge of LP and LE pathogeneses, certain analogies are apparent, albeit both diseases are not understood entirely. Apart from case reports about patients with LP-LE-overlap syndromes (Komori et al., 2017; Lospinoso et al., 2013), there are several studies pointing toward a similar immune polarization. Observational studies described high numbers of cytotoxic T cells in LE and LP lesions (de Carvalho et al., 2016; Wenzel and Tuting, 2008). A dominance of type I immune cells could be detected by transcriptome analysis of LE skin lesions (Jabbari et al., 2014), and a high IFN-γ expression was measured in oral and cutaneous LP (Weber et al., 2017).

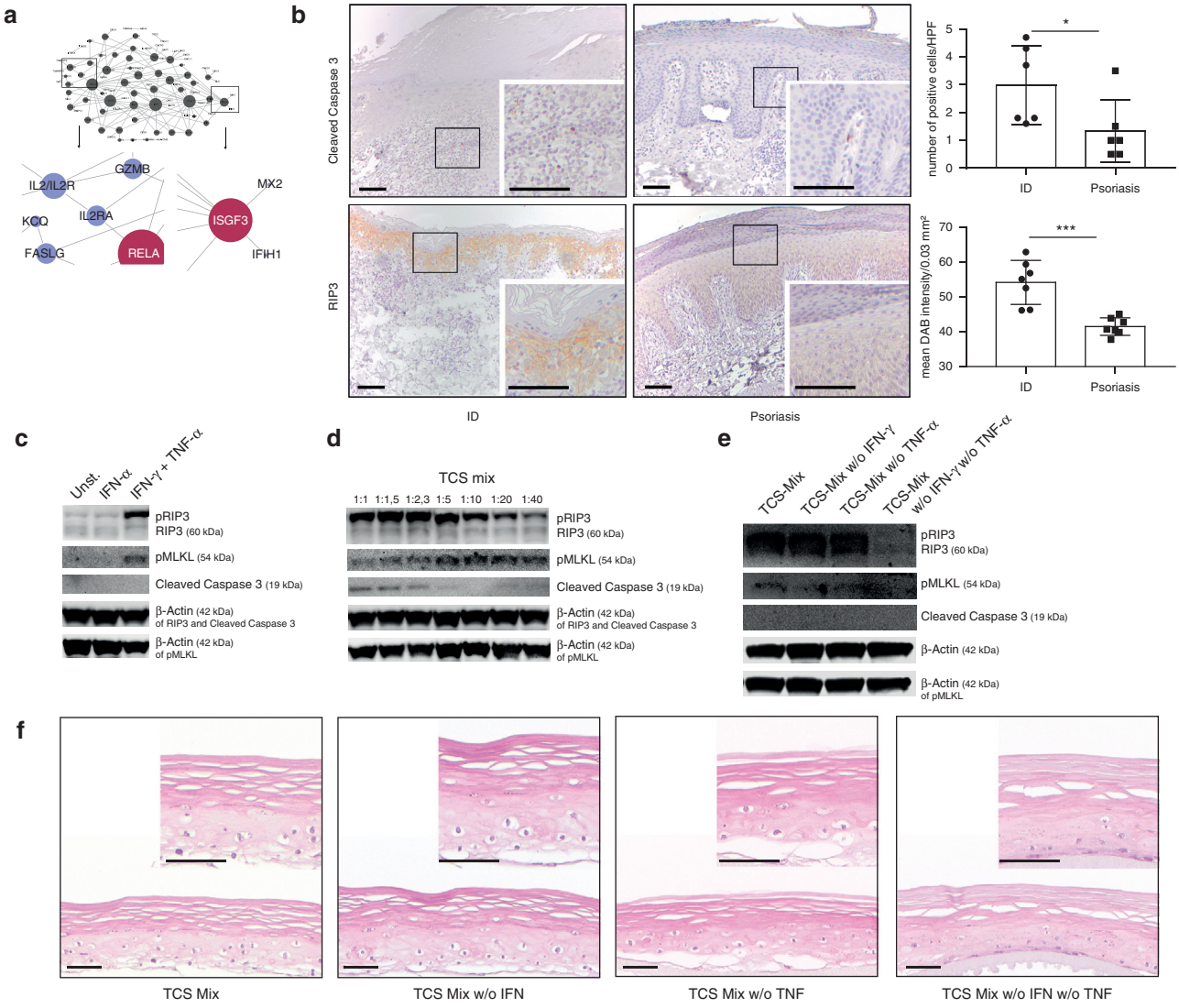


**Figure 4. Different cell death mechanisms are activated in interface dermatitis.** (**a**) Gene network analysis revealed upregulation of apoptosis (FASLG: Fas ligand), direct cytotoxicity (GZMB: granzyme B), and necroptosis (ISGF3: interferon stimulated gene factor 3 complex). Outtake of the network is shown in Figure 1d. (**b**) Representative immunohistochemical stainings of interface dermatitis (LE) and psoriasis biopsies for cleaved caspase 3 and RIP3. Number of cleaved caspase 3 positive cells per high power field (×400) and mean diaminobenzidine (DAB) intensity per 0.03 $mm^2$ in interface dermatitis (LE: n = 3, LP: n = 3) or psoriasis (n = 6). Scale bars indicate 100 μm. (**c**) Primary human keratinocytes were stimulated with IFN-α (10 ng/ml) or IFN-γ + tumor necrosis factor-α (TNF-α) (each 10 ng/ml). Western blot for receptor-interacting-protein-kinase 3 (RIP3), phosphorylated mixed lineage kinase domain like pseudokinase (pMLKL), and cleaved caspase 3 compared with unstimulated (US). (**d**) Primary human keratinocytes were stimulated with mixed lesional T-cell supernatant (TCS mix) derived from LP (n = 6) and LE (n = 3) (as shown in Figure 2). TCS was diluted with cell culture medium as indicated. Western blot for RIP3, pMLKL, and cleaved caspase 3. (**e**) Primary human keratinocytes were stimulated with TCS mix (1:10 diluted in cell culture medium), IFN-γ-depleted TCS mix (1:10), TNF-α-depleted TCS mix (1:10), and IFN-γ- and TNF-α-depleted TCS mix. Western blot for RIP3, pMLKL, and cleaved caspase 3. (**f**) Stimulation of three-dimensional skin models with TCS mix (1:10 diluted in cell culture medium), IFN-γ-depleted TCS mix (1:10), TNF-α-depleted TCS mix (1:10), and IFN-γ- and TNF-α-depleted TCS mix. Scale bars indicate 50 μm.

Furthermore, genetic polymorphisms of IFN-γ are linked to LP susceptibility (Al-Mohaya et al., 2016). Some authors also claimed an important role for plasmacytoid dendritic cells and type I interferons in the pathogenesis of LE and LP (Wenzel et al., 2006, 2009). Of note, these data were mainly obtained in studies of LE, whereas the knowledge about LP pathogenesis is limited to small case series (Saadeh et al., 2016). Animal studies demonstrated that a lack of IFN-α inhibiting interferon-regulatory-factor-2 leads to skin inflammation, which is either described as psoriasis-like or lichenoid (Arakura et al., 2007; Dutz, 2009; Hida et al., 2000). Importantly, in these models, the epidermal reaction is dependent on the presence of cytotoxic T cells, indicating that T-cell-derived IFN-γ and not IFN-α is indispensable for ID. In line with these findings, we observed a high production of IFN-γ by lesional T cells and an enhanced number of T-bet-positive cells in both LP and LE. In our study, only stimulation with IFN-γ or TNF-α, but not stimulation with IFN-α, activated cell death cascades in keratinocytes, thus indicating that IFN-α might be an upstream regulator of T-cell response, but not directly inducing epidermal cell death.

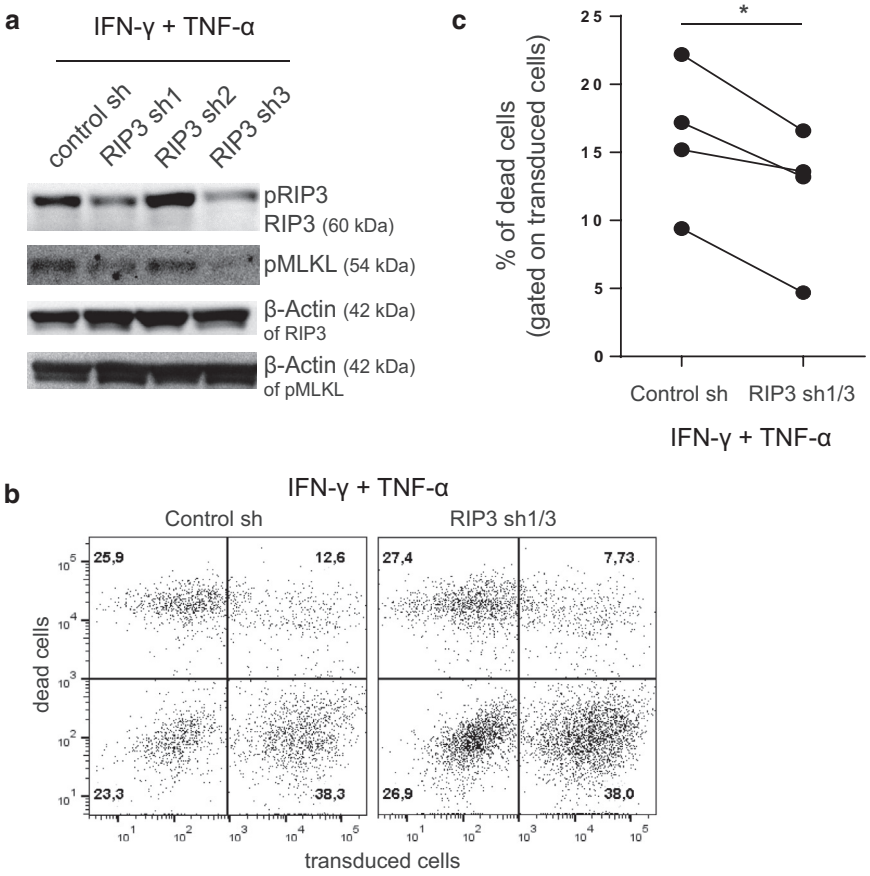


**Figure 5. Receptor-interacting-protein-kinase 3 (RIP3) is involved in keratinocyte death on stimulation with IFN-γ and tumor necrosis factor-α (TNF-α).** (**a**) Western blot for RIP3, pMLKL, and cleaved caspase 3 of RIP3 shRNA (three different target sequences) and control shRNA transduced primary human keratinocytes followed by stimulation with 50 ng/ml IFN-γ and TNF-α. (**b**) Representative FACS staining of keratinocytes transduced with RIP3 shRNA1 and shRNA3 (ratio 50:50) or control shRNA and stimulated with IFN-γ and TNF-α (each 10 ng/ml). Dead cells were stained with DAPI. (**c**) Proportion of DAPI-positive (dead) keratinocytes within the group of RIP3 shRNA or control shRNA transduced cells. Each pair of dots represents an independent experiment. Difference in means was tested using the paired *t*-test. $*P < 0.05$. pMLKL, phosphorylated mixed lineage kinase domain like pseudokinase; shRNA, small hairpin RNA.

So far, it has been assumed that apoptosis was the mechanism leading to characteristic epidermal changes of ID. Many studies have detected proapoptotic and antiapoptotic signals in single keratinocytes of ID (Bascones-Ilundain et al., 2006; Skiljevic et al., 2017; Yoneda et al., 2008). In line with these observations, we detected significantly higher numbers of cleaved caspase 3 positive cells in ID than in psoriasis samples. However, regarding the typical cell morphology of apoptotic cells, there is a discrepancy to histological observations: in ID, keratinocytes are vacuolated and not shrunk as it would be expected for apoptotic cells (Belizario et al., 2015; Elmore, 2007; Kroemer et al., 2009). Nevertheless, the consistent picture of ID in several ISD indicates that a regulated mechanism and not accidental necrosis orchestrates the epidermal reaction.

We could observe that IFN-γ, TNF-α, and a mix of lesional TCS led to cell swelling and signs of keratinocyte death in three-dimensional skin models. As expected for an in vitro model, these histological changes do not fully resemble the natural picture of ID in humans. In humans, degeneration and vacuolar changes of keratinocytes can usually be observed in the basal layer of the epidermis and keratinocytes do not show swelling as we detected in our model. Three-dimensional skin models are limited because of the lack of immune cells, and stimulation with TCS or recombinant cytokines cannot be as fine-tuned as the cell-cell interaction in vivo. We used this model, however, to study the impact of single cytokines, demonstrating that IFN-γ as well as TNF-α alone induces morphological changes in three-dimensional skin equivalents. Of note, in a mouse model, it has been demonstrated that desmoglein-specific CD4-positive IFN-γ-producing T cells are indispensable for the generation of ID (Takahashi et al., 2011). Interestingly, skin lesions that develop as a side effect of novel check-point inhibitor therapies also show an ID (Schaberg et al., 2016), thus representing an in vivo analogy of our observation. Check-point inhibitors induce a strong production of IFN-γ-producing T cells and are approved for the treatment of advanced-stage melanoma (Liakou et al., 2008).

Challenging current pathology concepts, in addition to apoptosis, we detected regulation of necroptosis pathway and high epidermal expression of RIP3 in ID. Necroptosis is a regulated form of cell death with a cascade comprising phosphorylated RIP3 and MLKL that eventually builds pores of the inner and outer cell membranes (Cai et al., 2014). Influx of extracellular liquids and destruction of the osmotic barrier are paralleled by consecutive cell swelling and cell death, respectively (Linkermann and Green, 2014). IFN-γ and TNF-α are known inducers of necroptosis (Jorgensen et al., 2017), and RIP3 is central for generation of inflammatory reactions induced by viral infections (Xu et al., 2017).

In our study, we detected RIP3 broadly in the epidermis of ID. This observation conflicts with the fact that in LP or LE not all keratinocytes die. More and more reports support the hypothesis that there is an inflammatory status of necroptosis before cell death. For instance, activation of necroptosis leads to increased aerobic respiration (Yang et al., 2018) and RIP3 deficiency is associated with an induction of DNA repair pathways (Sun et al., 2018). Once activated, necroptosis can

be reversed by prosurvival signals, such as the Fas-associated death domain protein-caspase8-cellular FLICE inhibitory protein complex (Dillon et al., 2012). As contra-regulations might eventually prevent cell death in LP and LE, the broad expression of RIP3 indicates that necroptosis is activated in ID, even if not all affected cells finally die. In our study, RIP3 was induced in primary human keratinocytes stimulated with the supernatant of lesional T cells. Depletion of IFN-γ or TNF-α revealed that both cytokines are able to induce RIP3 in human keratinocytes. RIP3 knockdown and consecutive reduction of MLKL phosphorylation led to a significant reduction of dead cells on stimulation with IFN-γ and TNF-α. These findings are concordant with the observation that necroptosis is a key event in toxic epidermal necrolysis, a blistering skin disease characterized by cell death of the majority of keratinocytes. In fact, toxic epidermal necrolysis might be regarded as a disease with maximal ID and a failure of contra-regulations (Kim et al., 2015).

Understanding general immune response patterns of the skin is crucial to detect new drug targets. Recently, the discovery of necroptosis inhibitors, which interact with either RIP3 or MLKL, has been reported (Fauster et al., 2015; Yan et al., 2017). Furthermore, AMG811, a monoclonal antibody depleting IFN-γ, was tested in a phase I trial in LE. IFN-γ-associated biomarkers decreased, even if there was no clinical effect on LE skin lesions after administration of one single dose (Werth et al., 2017). Phase II studies with different dosage regimes ought to clarify if there is a beneficial effect. Based on our findings, inhibition of necroptosis and targeting the IFN-γ axis are both promising therapeutic approaches for ID positive skin reactions. This concept is supported by the fact that B-cell-targeting therapies are not effective for the treatment of cutaneous LE (Vital et al., 2015) and that inhibition of T-cell immunology using a Janus kinase inhibitor (Klaeschen et al., 2017) or inhibition of NF-κB by fumaric acid esters has proven efficacy in first studies (Kuhn et al., 2017).

Overall, our data highlight the importance of a type I cellular immune response and suggest a role of necroptosis in the pathogenesis of ID. Given the possibility of precisely targeting the aforementioned cascades, our data warrant future translational approaches using new compounds for ID positive ISD.

## MATERIAL AND METHODS

### Patient characteristics

A total of 34 patients with either LP (n = 14), LE (n = 11), or psoriasis (n = 9) were enrolled in this study after obtaining written informed consent. Exclusion criteria were systemic or topical immune suppressive treatment within the last 3 months (systemic) or 1 week (topical), respectively. Patient characteristics are listed in Supplementary Table S3 online. The study was approved by the local ethics committee and conducted according to ethical principles laid down in the Declaration of Helsinki.

To ensure a clear diagnosis of ID, standardized histological assessment using 24 objective and subjective criteria (Supplementary Tabel S4) of 25 skin biopsies (LP: n = 14; LE: n = 11) was performed by two independent expert pathologists. Clustering analysis revealed a correlation of subjective ID criteria (e.g., ID subtype, ID strength) with objective criteria (the number of dyskeratotic epidermal cells, lymphocytic exocytosis; Supplementary Figure S2 online). Given the clear relationship between the number of dyskeratotic epidermal cells and subjective rating of ID, only skin biopsies with ≥1 dyskeratotic epidermal cells were included in gene expression analysis that left 11 LP and 5 LE samples.

### Punch biopsy specimens, histology, and immunohistochemistry

Six-millimeter punch biopsies of lesional and autologous nonlesional skin were obtained under local anesthesia. Skin samples were fixed in 10% formalin and embedded in paraffin, and 2.5-μm sections were cut and dewaxed. After rehydration, sections were stained with hematoxylin and eosin using standard methods. For immunohistochemistry, heat-induced epitope retrieval was performed in citrate buffer pH 6 (Leica Biosystems, Germany). Sections were incubated with the primary antibodies mouse anti-T-bet (Abcam, Cambridge, UK; 1:100), rabbit anti-cleaved-caspase3 (Cell Signaling Technology, Danvers, MA; 1:100), or mouse anti-RIP3 (R&D Systems, Minneapolis, Minnesota; 1:1,000) overnight at 4°C. A secondary polymeric alkaline-phosphatase-linked antirabbit/mouse antibody or a horseradish-peroxidase-linked antirabbit antibody (Zytomed Systems Germany) was applied, and the complex was visualized by the substrate chromogen Fast Red or 3,3′-diaminobenzidine. Eventually, slides were counterstained with hematoxylin. As a negative control, primary antibodies were omitted or replaced with an irrelevant isotype-matched monoclonal antibody. Information about quantification of immunohistological stainings can be found in the Supplementary Materials and Methods online.

### Isolation of total RNA from skin biopsy and gene expression microarray

Total RNA was isolated from biopsies stored in RNA-later with the miRNeasy Mini Kit (Qiagen, Netherlands) according to the manufacturer's protocol. The RNA yield and quality was determined with a Nanodrop ND1000 UV-vis spectrophotomer. Moreover, the RNA integrity numbers were measured using the 2100 Bioanalyzer (Agilent Santa Clara, California) according to the manufacturer's protocol (Agilent RNA 6000 Nano Kit). RNA samples with a RNA integrity number ≥ 6 were Cy3 labeled and amplified using the low input quick Amp labeling kit and hybridized on SurePrint G3 Human GE 8x60K BeadChips (Agilent Technologies). Fluorescence detection with the iScan microarray scanner and signal extraction with the Agilent Feature Extraction Software were used to determine specific gene expression. All microarrays were preprocessed together using the limma package in R (details in the Supplementary Materials and Methods).

### Modeling gene expression data

A detailed description about the modeling approach can be found in the Supplementary Materials and Methods. In short, two models were used for analyzing the human biopsy samples. One model included LP, LE, and psoriasis samples and their corresponding healthy skin samples. The second model combined LP and LE in one predictor and compared it with autologous healthy skin and psoriasis. In both models, a linear mixed-effects approach was used to adjust for interindividual variability. This results in an intercept calculated for each individual patient (=random effect) and an overall adjusted fold change (=fixed effect) for each predictor (LE, LP, psoriasis) compared with healthy. *P*-values were adjusted for multiple testing using Benjamini Hochberg correction.

### Statistical analysis

Data were visualized using the GraphPad Prism 7.00 software, and the unpaired or paired *t*-test was used to test for difference in the means. The significance level was defined as $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

### Induced network modules

Induced network modules of the 500 (LP and LE) or 150 (stimulated keratinocytes) top genes were calculated and displayed using *ConsensusPathDB* and *Cytoscape version 3.4.0* software (Herwig et al., 2016; Shannon et al., 2003). Top genes are defined as significant (Benjamini Hochberg adjusted $P < 0.05$) and having the highest absolute fold change over healthy and unstimulated samples, respectively.

### Pathway analysis

We performed pathway analysis by applying the model-based gene set enrichment method *mgsa* that takes into account the hierarchical structure of pathways (Bauer, 2016). *Wikipathways* was used as the pathway resource (Kutmon et al., 2016). Pathways with an mgsa estimate larger than 0.5 are considered active.

### Isolation and stimulation of lesional T cells

A detailed description about the isolation of lesional T cells can be found in the Supplementary Materials and Methods. For generation of TCS, expanded lesional T cells were stimulated again with α-CD3 and α-CD28 for 72 hours. IFN-γ and TNF-α were depleted using biotinylated antibodies against IFN-γ and TNF-α (R&D Systems) and streptavidin beads. Concentrations of IFN-γ and TNF-α before and after depletion were determined by ELISA (R&D Systems) and are listed in Supplementary Table S3. For intracellular cytokine staining, T cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 5 hours in the presence of golgi inhibitors.

### Lentiviral transduction of primary human epidermal keratinocytes

Second-passage primary human epidermal keratinocytes were cultured in six-well plates and transduced with freshly concentrated lentiviral supernatant on two consecutive days. A detailed description can be found in the Supplementary Materials and Methods.

### Three-dimensional skin models

Three-dimensional skin models were generated as described before (Poumay et al., 2004). A detailed description can be found in the Supplementary Materials and Methods.

### Supplementary material and methods

Information on preprocessing of microarray data, modeling the gene expression data, cell culture, lentiviral vector construction, lentiviral vector production, lentiviral transduction of primary human epidermal keratinocytes, isolation of lesional T cells, three-dimensional skin models, western blot, and quantification of immunohistochemical stainings can be found in the Supplementary Materials and Methods.

## CONFLICT OF INTEREST

TB gave advice to or got an honorarium for talks or research grant from the following companies: Alk-Abelló, Astellas, Bencard, Biogen, Janssen, Leo, Meda, MSD, Novartis, Phadia, and Thermo Fisher. The rest of the authors state no conflict of interest.

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## AUTHOR CONTRIBUTIONS

FL contributed to designing the project, performed experiments, and wrote the manuscript; MJ, NG-S, and SE performed experiments; LK performed statistical analysis and contributed to designing the project; RF, SR, and AB performed histological examination; NSM and FJT supervised statistical analysis; CBS-W and TB supervised the study design; and KE designed and supervised the project. All authors have read and approved the final version of the manuscript.

## 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.2018.02.034.

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