nature portfolio

Peer Review File

Single-cell transcriptome reveals keratinocyte subclusters contributing to altered differentiation and inflammatory responses in atopic dermatitis

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors present a large dataset of AD skin and primarily focus on keratinocyte populations. Keratinocytes have previously been identified as key players in AD, given the defective epidermal barrier in these patients. This barrier defect might facilitate AD development via an outside-in mechanism, e.g. allergic sensitisation to environmental allergens and irritants and enhanced detection of those via innate or adaptive immune mechanisms. It is very well established that this barrier defect is primarily established by keratinocytes, the most abundant cell type in the epidermis.

Major

The authors might want to align their findings to the abundance of already available single-cell datasets published over the last few years from various centres globally. Given the heterogeneity of the disease, this might help us to better understand how their findings correlate with results from other research groups that have published their single-cell datasets of lesional and nonlesional AD skin biopsies.

Why the focus on keratinocytes in this paper? Was this also technically driven? Isolating high quality cells from skin can sometimes be a challenge – Were the authors concerned that their dermal cells as well as immune infiltrates less reliably detected by their isolation method? Again, aligning their finding with published datasets might add to the robustness of their dataset to exclude potential technical bias.

The authors might want to include a QC table listing the number of cells isolated per biopsy, and the genes per cell per biopsy, both before and after QC.

TWEAK is not part of a newly discovered pathway. This mediator has been previously shown to be highly up-regulated in AD (PMID 29038621, 38072390). Also, AD models showed the dependence of AD-like inflammation on TWEAK (PMID 28530223, 31515807). Other authors already demonstrated that “The activity of TWEAK in keratinocytes contributes to AD development, and neutralising TWEAK represents a future potential therapeutic option in human AD similar to targeting IL-13.“ (PMID 37650473).

Functional experiments on stimulated human immortalised keratinocytes are controversial, as they might not sufficiently reflect the situation in vivo. Organoids, in vitro cultivated skin or reconstructed epidermis might be a more appropriate approach, better reflecting actual in vivo human skin situations.

While this is a very large dataset of skin biopsies from human disease, it mostly corroborates markers previously published by other authors (including the effect of IL13 on keratinocytes). Novelty of this manuscript thus remains unclear.

Reviewer #2

(Remarks to the Author)

Atopic dermatitis is driven by a pathogenic crosstalk of immune cells and keratinocytes. This study investigates an impressive number of patient biopsies at single-cell level using both single-cell RNA sequencing and spatial transcriptomics to describe this crosstalk at an unprecedented depth. The authors show that in lesional AD, keratinocyte differentiation is altered in the microenvironment of type 2 inflammation in particular in a subset of keratinized keratinocytes with high metabolic activity and oxidative stress. They further show that besides the well-known cytokine IL-13, a criticial mediator of T cell-keratinocyte interaction and with that disturbed epidermal differentiation is the molecule TWEAK. TWEAK has been associated with AD before and also proposed in the context of novel therapeutic strategies (e.g. PMID: 37650473). The study is highly relevant also as a resource due to the very large amount of data and state of the art techniques. I have a few more specific comments:1
1) Term „keratinocyte subset“: are the clusters observed really stable over time, or do they represent distinct cellular states induced by type 2 immunity? In other words, can it be reverted blocking type 2 inflammation?
2) Lower amount of endithelial cells and more keratinocytes in non-lesional AD – could that be related to the size of the biopsy or the anatomical region where the biopsies were obtained?
3) Would the authors discuss why sebocytes and eccrine cells where regarded keratinocyte subsets?
4) The authors describe apoptosis to be a key result of TWEAK/FN14 binding. Apoptosis is not typically observed in AD skin, in contrast to drug reactions or interface dermatitis diseases. In that context, it may be interesting that IL-13 is also upregulated in lichen planus in some reports – is the mechanism shown here exclusive to AD or classical type 2 diseases?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
the author have responded to all the reviewers comments.

Reviewer #2

(Remarks to the Author)
The manuscript clearly improved and all comments of the reviewer´s have been carefully considered. Especially adding mechanistic cell culture models increase the validity of the study. The one commen that is remaining and could be addressed further is from my perspective one comment of reviewer 1: it is clear that the much higher number of high-quality cells would result in more differentially expressed genes in this dataset, highlighting how important this study also is as a resource. However, I would like to understand whether the proposed subtypes of keratinocytes were also found in the published, substantially smaller datasets – could individual cells be identified that have a similar gene expression profile as the distinct keratinocyte subsets of the original dataset also in the published sources? And were there eventually keratinocytes that were totally different to the subsets identified in the current manuscript? This cannot be answered by a global DEG analysis.

Reviewer #1 (Remarks to the Author):

We thank the reviewer for their thoughtful and constructive comments and for recognizing the importance of keratinocytes in atopic dermatitis (AD) pathogenesis.

The authors present a large dataset of AD skin and primarily focus on keratinocyte populations. Keratinocytes have previously been identified as key players in AD, given the defective epidermal barrier in these patients. This barrier defect might facilitate AD development via an outside-in mechanism, e.g. allergic sensitization to environmental allergens and irritants and enhanced detection of those via innate or adaptive immune mechanisms. It is very well established that this barrier defect is primarily established by keratinocytes, the most abundant cell type in the epidermis.

Response to reviewers:

We agree that keratinocytes and epidermal dysfunction are central to AD and have been extensively studied. However, relatively few studies have comprehensively examined the heterogeneity within keratinocyte compartments and how each distinct keratinocyte states interact with immune cells across lesional and non-lesional skin. In this study, we leverage a large cohort to enable high-resolution, subtype-specific analysis of keratinocytes. This allows us to identify keratinocytes compartment-specific immune interactions and transcriptional programs, providing a more refined view of epidermal involvement in AD than previously reported.

Major

The authors might want to align their findings to the abundance of already available single-cell datasets published over the last few years from various centers globally. Given the heterogeneity of the disease, this might help us to better understand how their findings correlate with results from other research groups that have published their single-cell datasets of lesional and nonlesional AD skin biopsies.

Response to reviewers:

We appreciate this important suggestion. To address this, we compared our dataset (PERG) with two independent scRNA-seq studies (Ma et al. [1]: 6 lesional vs. 5 non-lesional AD samples and Wu et al. [2]: 5 lesional vs. 5 non-lesional AD samples).

Across shared keratinocyte populations (basal, differentiated and keratinized keratinocytes), our dataset identified a substantially larger number of differentially expressed genes (DEGs) (9-fold and 14-fold more DEGs than the Ma et al. and Wu et al datasets, respectively),

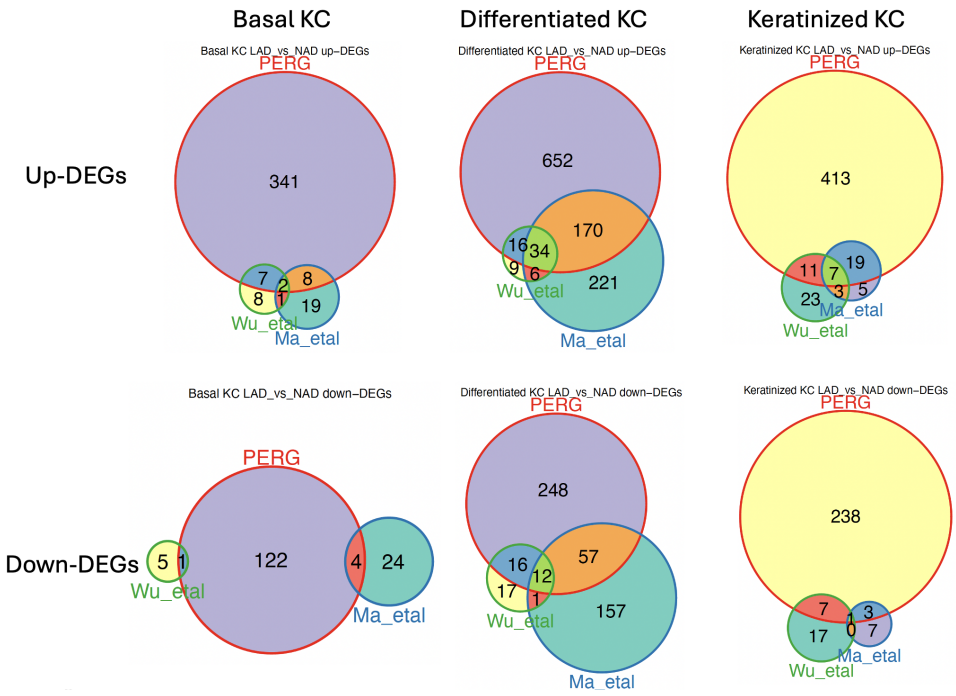
reflecting increased statistical power due to a large cohort size. On average, 74% of the union set of DEGs across the three datasets were identified exclusively in PERG dataset, whereas only 16% were detected in the two independent datasets (**Response to Reviewers Figure 1**). Notably, upregulation of *TNFRSF12A* (i.e. TWEAK receptor, *FN14*) in differentiated and keratinized keratinocytes was uniquely identified in our dataset. Conversely, 40% of the DEGs in the Ma et al. dataset 46% of those in the Wu et al. datasets were also identified in our dataset, with a maximum overlap of 77% observed in keratinized keratinocytes. Fewer than 25 DEGs were uniquely identified in either independent dataset across most cell types, with one exception: differentiated KC in Ma et al., which showed 221 upregulated and 157 downregulated DEGs unique to that dataset (**Response to Reviewers Figure 1**). Moreover, minimal overlap was observed between the two independent datasets (0 to 40 DEGs across cell types), suggesting limited reproducibility between them.

Functional enrichment analysis further supported the robustness of our findings. Among unique DEGs of differentiated KC in our dataset, 134 upregulated and 22 downregulated Gene Ontology Biological Process (GOBP) terms were significantly enriched. In contrast, only seven downregulated GOBP terms were enriched among DEGs unique to Ma et al., and no enriched terms were found among its 221 upregulated DEGs (**Response to Reviewers Supplementary Table S1**). These results suggest that the differential signals identified in the Ma et al. dataset may exhibit weaker robustness and limited functional coherence, potentially reflecting limited statistical power or reduced reproducibility. Notably, multiple immune-related terms, including interferon responses and cytokine production, were strongly upregulated among unique DEGs in our dataset (**Response to Reviewers Figure 2**). By comparison, Ma_etal-specific DEGs were enriched primarily for muscle cell differentiation-related processes, which were also detected within the unique downregulated DEGs in our dataset.

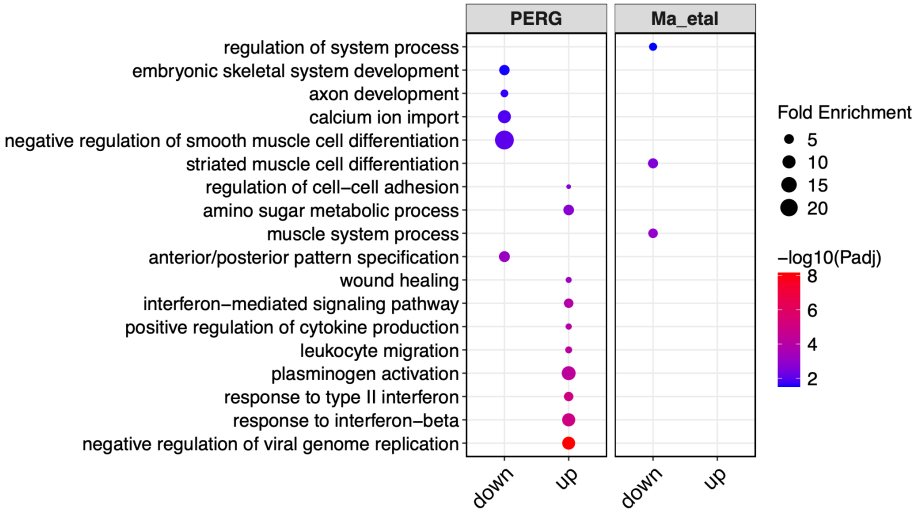
Overall, **these findings highlight the markedly enhanced statistical power of our dataset, enabling the detection of additional biologically meaningful signals in lesional AD, including the key observation of upregulated TWEAK signaling in keratinocytes.** This is facilitated by the large sample size, which allows for pseudobulk analysis – a robust approach in single-cell RNA-seq that reduces false positives while maintaining statistical power.

In addition, the unprecedented scale of our dataset enabled high-resolution characterization of keratinocytes differentiation states, identifying fine-grained subpopulations within differentiated KCs (DK1-DK7) and keratinized KCs (KK1 and KK2). This led to the discovery of a potentially pathogenic subtype (DK6) and evidence of altered differentiation trajectories between KK1 and KK2 in lesional AD skin. These observations

are consistent with the prior report of disrupted keratinocyte differentiation in AD by Fiskin et al. (section “Altered keratinocyte differentiation and cornified layer in lesional AD skin”) [3].



Response to Reviewers Figure 1. Lesional vs non-lesional AD DEG comparison between our dataset (PERG) and additional two independent datasets (Ma_etal and Wu_etal) in individual keratinocyte subtypes (basal, differentiated and keratinized KC). Up and down-regulated pseudobulk DEGs (adjusted p value < 0.05 and absolute fold change > 2) were shown separately (top: up-DEGs, bottom: down-DEGs).



Response to Reviewers Figure 2. Enriched Gene Ontology Biological Process (GOBP) terms among the lesional vs non-lesional DEGs in differentiated keratinocytes that are unique in PERG (our dataset) or Ma_etal dataset (adjusted p value < 0.05). Top 10 enriched terms after removing highly relevant ones (semantic similarity > 0.5) were shown.

Why the focus on keratinocytes in this paper? Was this also technically driven? Isolating high quality cells from skin can sometimes be a challenge – Were the authors concerned that their dermal cells as well as immune infiltrates less reliably detected by their isolation method? Again, aligning their finding with published datasets might add to the robustness of their dataset to exclude potential technical bias.

Response to reviewers:

Our focus on keratinocytes is driven by disease biology rather than technical considerations. Although many single-cell studies of AD have provided a broad overview of the cellular landscape in AD skin, none have comprehensively examined the molecular alterations within keratinocytes, which are central to disease pathogenesis. We also refer to our recent genetic study published in *Nature Communications* [4], which highlighted the contribution of multiple genetic defects affecting epidermal function and further supports the need for an in-depth investigation of epidermal biology and keratinocyte subtypes/subclusters in disease mechanisms.

To characterize skin cell populations in an unbiased manner, we performed scRNA-seq on whole skin biopsies containing both epidermal and dermal compartments in an approximate 1:3 ratio. After rigorous quality control (QC), we obtained more than 800,000 high-quality cells and identified 10 major cell types (**Figure 1A**) and 43 subtypes, encompassing most known epidermal and dermal cell populations, including endothelial cells, fibroblasts, pericyte, Schwann cells, melanocytes, and infiltrating immune cells such as T cells, myeloid cells, mast cells. The composition of cell types across sample groups is shown in Figure 1B, and post-QC cell counts for each cell types are provided in **Response to Reviewers Table 1**. The distribution of sample-level QC metrics is shown in **Response to Reviewers Figure 3,** including the number of cells, number of reads, number of genes and average number of reads across cells before and after QC. In addition, we have included **Supplementary Table S11** with detailed sample-level quality metrics in the Discussion section of the revised manuscript.

We have provided the comparisons with independent scRNA-seq studies on AD corresponding to Response to reviewers point #1, which demonstrated the robustness our results.

Response to Reviewers Table 1. Number of post-QC cells in individual cell types stratified by sample types.

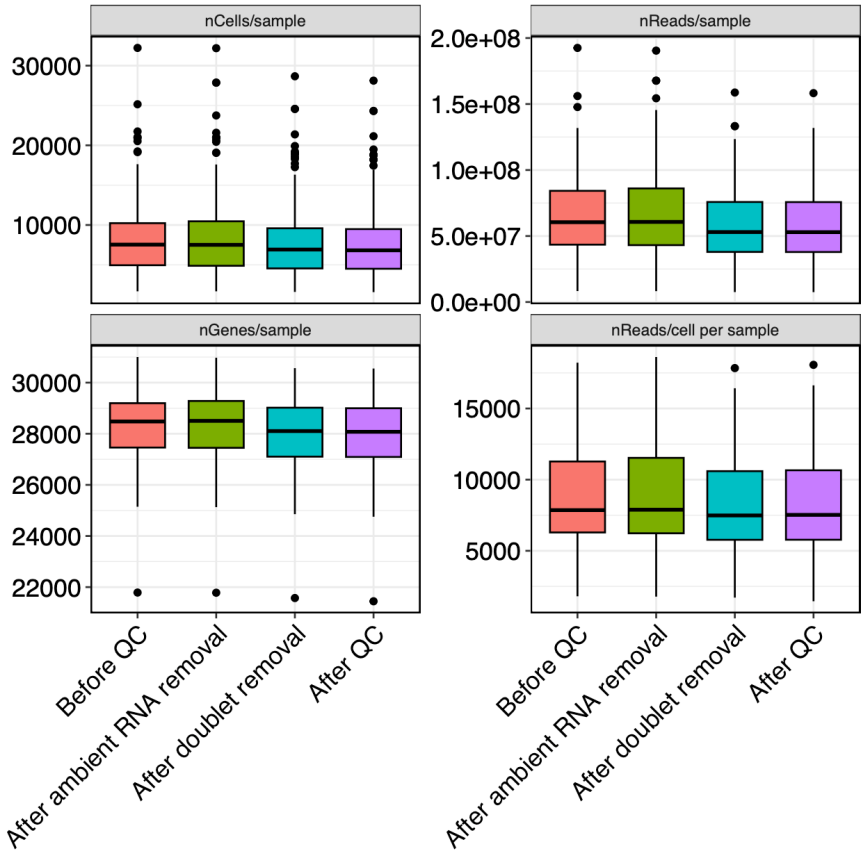
Cell type	Healthy	Non-lesional	Lesional
Eccrines	3,931	1,842	11,978
Endothelial cells	13,829	14,916	26,235
Fibroblasts	36,515	47,444	71,206
Keratinocytes	71,171	118,967	216,888
Lymphatic endothelial cellsECs	4,092	3,934	8,801
Mast Cells	1,965	2,552	3,917
Myeloid Cells	7,287	8,894	22,584
Pericytes	16,231	19,118	29,755
Schwann/Melanocytes	2,389	4,234	7,798
T Cells	12,081	13,123	35,858

The authors might want to include a QC table listing the number of cells isolated per biopsy, and the genes per cell per biopsy, both before and after QC.

Response to reviewers:

We thank the reviewer for this suggestion. We have included the per-sample QC metrics in **Supplementary Table S11** and clarified that "our focus on keratinocytes is driven by disease biology rather than technical considerations in the Discussion section (1st paragraph) of the revised manuscript. The distributions of the number of cells per sample, number of reads per sample, number of genes per sample and average number of reads in cells per sample were shown in **Response to Reviewers Figure 3**, which were consistent across the QC filtering steps.

In our dataset, each sample yielded an average of 8,085 high-quality cells after QC filtering, with a mean sequencing depth of ~8,432 UMIs per cell (**Response to Reviewers Table 2**). This cell number falls within the typical range targeted in droplet-based single-cell RNA-seq experiments (3,000-10,000 cells per sample), and the sequencing depth is sufficient for cell-type identification and clustering analyses. Across samples, we detected ~28,000 genes per library, indicating high transcriptome complexity. This is consistent with typical droplet-based single-cell RNA-seq datasets, which commonly detect ~20,000–30,000 genes across thousands of cells [5, 6].



Response to Reviewers Figure 3. QC metrics per sample before ("Before QC") and after each step of filtering ("After ambient RNA removal", "After doublet removal" and "After QC"). The metrics includes number of cells per sample, number of reads per sample, number of genes per sample, and the average number of reads in cells per sample. In the "After QC" step, the cells with <200 or >5000 genes, and/or >25% mitochondrial genes were filtered out.

Response to Reviewers Table 2. Average per-sample QC metrics before and after QC

Metric	Before QC	After QC	% retained
nCells/sample	8,350	8,085	97%
nReads/sample	65,514,055	59,415,238	91%
nGenes/sample	28,266	28,009	99%
nReads/cell per sample	8,844	8,432	95%

TWEAK is not part of a newly discovered pathway. This mediator has been previously shown to be highly up-regulated in AD (PMID 29038621, 38072390). Also, AD models showed the dependence of AD-like inflammation on TWEAK (PMID 28530223, 31515807). Other authors already demonstrated that "The activity of TWEAK in keratinocytes contributes to AD development, and neutralising TWEAK represents a future potential therapeutic option in human AD similar to targeting IL-13." (PMID 37650473).

Response to reviewers:

We thank the reviewers for highlighting previous studies supporting the connection between TWEAK/Fn14 signaling and inflammatory mechanisms in AD, as well as its close correlation with IL-13 and IL-22 targets, suggesting it as a potential therapeutic pathway. However, none of these prior studies were based on AD patient cohorts, and most findings were derived from mouse models [7-9] or *in vitro* experiments [10]. Liu et al reviewed the central role of TWEAK/Fn14 pathway in multiple inflammatory skin diseases by integrating results from mouse models, *ex vivo* human patient samples, *in vitro* experiments on primary cells and cell lines, as well as structural and biochemical studies[11].

To our knowledge, no previous single-cell RNA-seq study has directly demonstrated the upregulation of this pathway in a cell type-specific manner, particularly in the context of different keratinocyte states. In our study, we identified the Fn14 gene upregulation specifically in AD basal and differentiated keratinocytes, and, for the first time, demonstrated the spatial proximity and interactions of IL13+ Th2 cells and Fn14+ keratinocytes. This provides novel insight into the TWEAK/Fn14-mediated inflammatory axis in AD pathogenesis. Moreover, the TWEAK and Fn14 interaction was identified as the only lesional AD-specific upregulated ligand-receptor pair by both CellChat and CellPhoneDB approaches, representing an unbiased finding. Therapeutic targeting of this axis has not been evaluated in AD, despite favorable safety profiles observed in Phase I trials in other indications. Therefore, our results advance our understanding of TWEAK/Fn14 in AD pathology with *in vivo* and *in situ* data, supporting the potential of using TWEAK/Fn14 as a therapeutic target in AD.

Besides the upregulation of Fn14 in AD keratinocytes, we also identified several intermediate signals potentially involved in AD pathogenesis, including the marked downregulation of *TGFBI*. The strong anti-correlation between *Fn14* and *TGFBI* expression in differentiated keratinocytes was observed uniquely in lesional AD, suggesting a link between the TGF-β and TWEAK/Fn14 pathways. Furthermore, knockdown of *APOD* and *LYZ* in NTERT cells

significantly reversed the downregulation of *TGFBI*. These findings highlight additional therapeutic targets that may act indirectly through the TWEAK/Fn14 axis.

Functional experiments on stimulated human immortalised keratinocytes are controversial, as they might not sufficiently reflect the situation in vivo. Organoids, in vitro cultivated skin or reconstructed epidermis might be a more appropriate approach, better reflecting actual in vivo human skin situations.

Response to reviewers:

We thank the reviewer for raising this important concern. Although human immortalized keratinocytes (e.g. NTERT) are widely used as a primary-like human keratinocyte model in conventional 2D culture for inflammatory and AD studies [12-14], we agree that their relatively uniform differentiation states may not fully recapitulate the complex, highly stratified architecture of human epidermis in vivo.

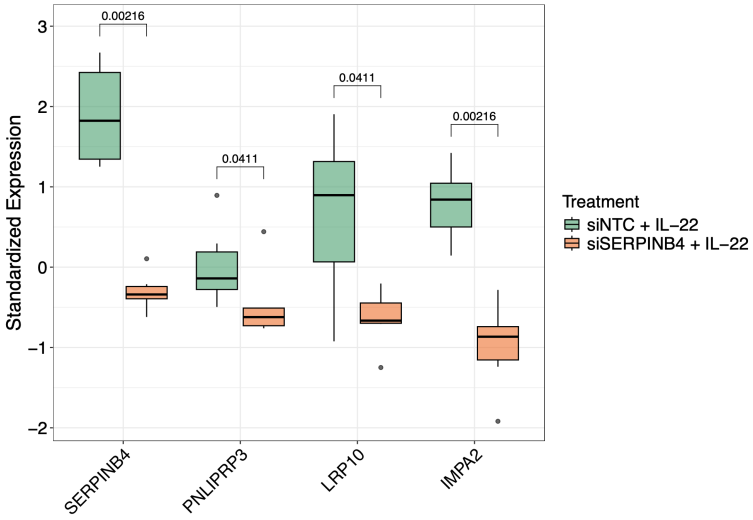
To address this limitation and validate our findings in a more physiologically relevant system, we first examined a public bulk RNA-seq dataset (GSE29284) generated from bio-stabilized human skin stimulated with IL-4, IL-13 and/or IL-22 [15]. In this dataset, *Fn14* was significantly upregulated following IL-13 stimulation compared to control (log2FC = 1.1, FDR = 0.02), as well as in IL-13+IL-4-treated samples (log2FC = 0.87, FDR = 0.047). In addition, *SERPINB4*, which we identified as highly IL-13 responsive in our PERG keratinocyte data, was also strongly induced upon IL-13 stimulation (log2FC = 2.5, FDR = 0.0001). Notably, the tumor necrosis factor (TNF) production pathway was among the top enriched in IL-4-upregulated genes, supporting enhanced TNF signals in this ex vivo inflammatory skin model and consistent with our observations.

In addition, we established a 3D organotypic epidermal model to further validate our findings in a system that better recapitulates *in vivo* skin structure. These reconstructed epidermal cultures were treated with cytokine stimulation and siRNA targeting either a control (siNTC) or *SERPINB4* (siSERPINB4) (see Methods). RNA was extracted and qRT-PCR was performed to assess the expression of genes identified as repressed by siSERPINB4 in the NTERT scRNA-seq dataset (Figure 3E), including *PNLIPRP3, LRP10* and *IMPA2*. In this 3D model, *SERPINB4* was robustly induced by IL-22 stimulation (**Response to Reviewers Figure 4**), consistent with results from our dataset (**Figure 4D**) and NTERT scRNA-seq results. Knockdown of *SERPINB4* under IL-22 treatment effectively reduced its expression (p value = 2.16×10^{-3}), and recapitulated the NTERT findings, with all three downstream target genes showing consistent repression. Specifically, *PNLIPRP3*, which was upregulated in lesional

AD skin in our dataset and specifically reduced by siSERPINB4 in the NTERT scRNA-seq dataset, was also significantly decreased in the 3D model under siSERPINB4 and IL-22 condition (p = 0.04). *LRP10* and *IMPA2*, which were upregulated specifically in DK6/DK7 keratinocyte compartments in lesional AD skin but lost significance upon *SERPBIN4* knockdown in the NTERT dataset, were likewise consistently repressed in the 3D model (p = 0.04 and 0.002, respectively). We also evaluated PBS control and IL-13 conditions, however, *SERPINB4* expression was extremely low under these conditions, limiting the ability to assess the knockdown efficiency. Therefore, downstream gene expression changes in these settings are not readily interpretable.

Together, these results from *ex vivo* human skin and a reconstructed 3D epidermal system provide orthogonal validations of our findings and support the relevance of SERPINB4-mediated regulatory effects beyond the NTERT model. Given the time and technical complexity associated with 3D epidermal reconstruction, we focused on SERPINB4 as a representative, strongly cytokine-responsive gene with potential therapeutic relevance in AD.

In the revised manuscript, we have included the 3D organotypic epidermal model in the method section and the qRT-PCR results in **Supplementary Figure S7E**.



Response to Reviewers Figure 4. Gene expression in 3D organotypic epidermal culture treated with IL-22 and siRNA targeting either a non-targeting control (siNTC) or SERPINB4 (siSERPINB4). Standardized relative expression of four genes (*SERPINB4, PNLIPRP3, LRP10 and IMPA2*), assessed by qRT-PCR, is shown. A total of six samples were analyzed from two

experiments, each with three replicate per condition. Wilcoxon Rank-Sum test p values < 0.05 are indicated.

While this is a very large dataset of skin biopsies from human disease, it mostly corroborates markers previously published by other authors (including the effect of IL13 on keratinocytes). Novelty of this manuscript thus remains unclear.

Response to reviewers:

We thank the reviewer's comment. In the revised manuscript, we have now clarified in the Discussion the novelty and significance of our study, substantially beyond corroborating previously reported markers:

1. **Reversed keratinocyte differentiation trajectory.**
   We identified molecular signatures, such *APOD* and *LYZ*, that may contribute to a reversed cell-fate trajectory between two keratinized KC compartments in AD, suggesting aberrant epidermal maturation.

2. **Identification of a potentially pathogenic KC subcluster (DK6).**
   We discovered a distinct differentiated KC compartment (DK6) characterized by exceptionally strong signatures related to mitochondrial and endoplasmic reticulum (ER) functions, processed known to be implicated in AD pathogenesis, suggesting DK6 as a potential disease-relevant KC subpopulation.

3. **Comprehensive cytokine response profiling across KC subtypes.**
   No prior study has systematically profiled cytokine response burden across KC subtypes. Leveraging 404,932 keratinocytes across 14 KC subtypes, we demonstrated that each KC subtype exhibit distinct response to different cytokine stimuli. Notably, we identified cycling T cells as an additional IL-13-producing population that may cooperate with Th2 cells, and we found that SERPINB family genes (e.g. *SERPINB4*) were among the most IL-13-responsive genes in keratinocytes.

4. **Cell-type-specific TWEAK/FN14-mediated immune-keratinocyte interactions unique to lesional AD.**
   We delineated the specific immune and KC subtypes involved in type2 immune responses and uncovered lesional AD-specific TWEAK/FN14 interactions between defined immune cells and KC subtypes. To our knowledge, this is the first in situ demonstration of these interactions at single-cell resolution using spatial

transcriptomic data. In addition, *TGFBI* was found to be markedly downregulated in AD and strongly anti-correlated with *FN14* and *TGFB1* expression, suggesting a potential intermediate role linking TGF-β and TWEAK/FN14 pathways.

5. **Proposed integrative molecular model of AD pathogenesis.**
   We proposed a mechanistic framework involving specific interactions between infiltrating immune cells and keratinocyte compartments. The model highlights:
   - Cycling T- and Th2-derived TWEAK/Fn14 activation in basal and differentiated KCs
   - Enhanced Fn14 expression in these cell compartments
   - Dysregulated mitochondrial/ER functions in the DK6 compartment
   - Potential crosstalk between TGF-β and TWEAK/Fn14 signaling mediated by *TGFBI*.

   Beyond TWEAK and FN14, this model identifies additional candidate genes that may contribute to AD pathogenesis and represent potential therapeutic targets, including *TGFBI, APOD, LYZ* and *SERPINB4*.

6. **A large, high-quality community resource.**
   Finally, we provide one of the largest high-quality single-cell datasets of human AD skin to date, which we anticipate will serve as a valuable resource for the research community.

Reviewer #2 (Remarks to the Author):

Atopic dermatitis is driven by a pathogenic crosstalk of immune cells and keratinocytes. This study investigates an impressive number of patient biopsies at single-cell level using both single-cell RNA sequencing and spatial transcriptomics to describe this crosstalk at an unprecedented depth. The authors show that in lesional AD, keratinocyte differentiation is altered in the microenvironment of type 2 inflammation in particular in a subset of keratinized keratinocytes with high metabolic activity and oxidative stress. They further show that besides the well-known cytokine IL-13, a criticial mediator of T cell-keratinocyte interaction and with that disturbed epidermal differentiation is the molecule TWEAK. TWEAK has been associated with AD before and also proposed in the context of novel therapeutic strategies (e.g. PMID: 37650473). The study is highly relevant also as a resource due to the very large amount of data and state of the art techniques. I have a few more specific comments:1

We thank the reviewer for their positive evaluation and thoughtful questions.

1) Term „keratinocyte subset“: are the clusters observed really stable over time, or do they represent distinct cellular states induced by type 2 immunity? In other words, can it be reverted blocking type 2 inflammation?

Response to reviewers:

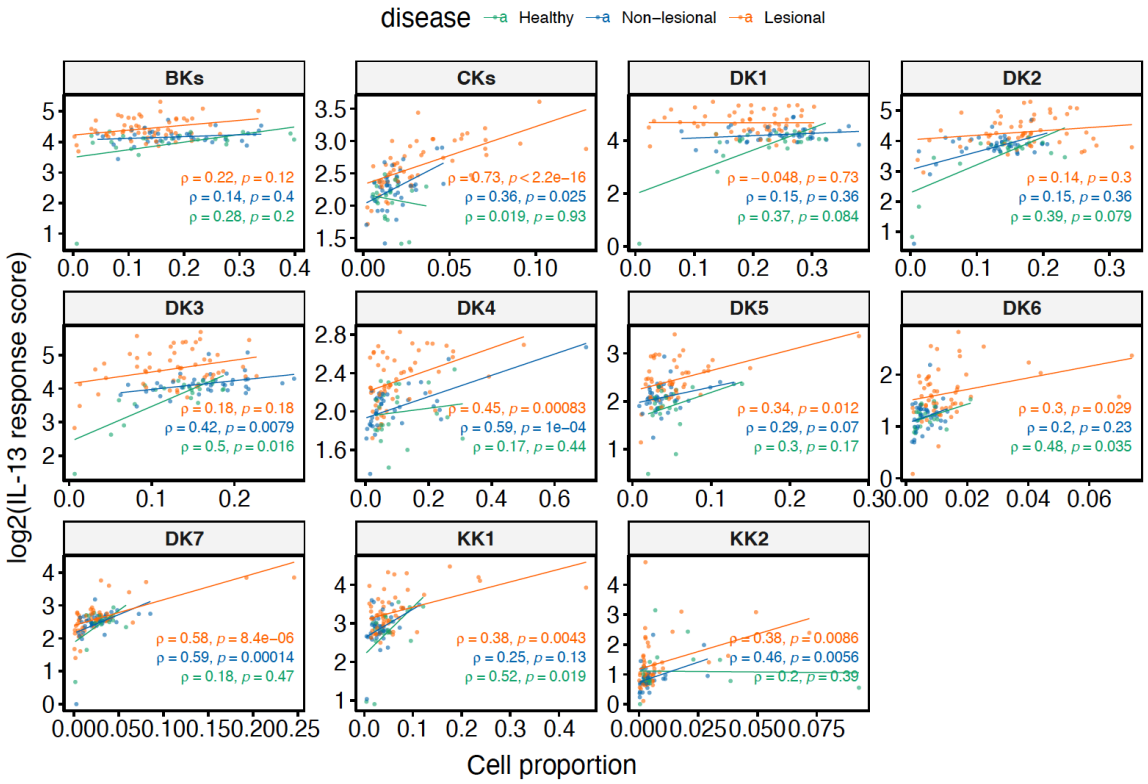
We thank the reviewer for this insightful question regarding the identified keratinocyte subsets (subclusters) represent stable subtypes or inflammation-induced states.

We defined the 14 keratinocyte subsets (or subclusters/subtypes) across all disease conditions (healthy control, non-lesional and lesional AD) by leveraging marker gene signatures and cell differentiation trajectory information (see **Methods**). These subsets align along the expected differentiation continuum from basal keratinocytes (BKs) through differentiated keratinocytes (DK1 – DK7) and finally keratinized keratinocytes (KK1/KK2), suggesting that they primarily represent differentiation-associated transcriptional states.

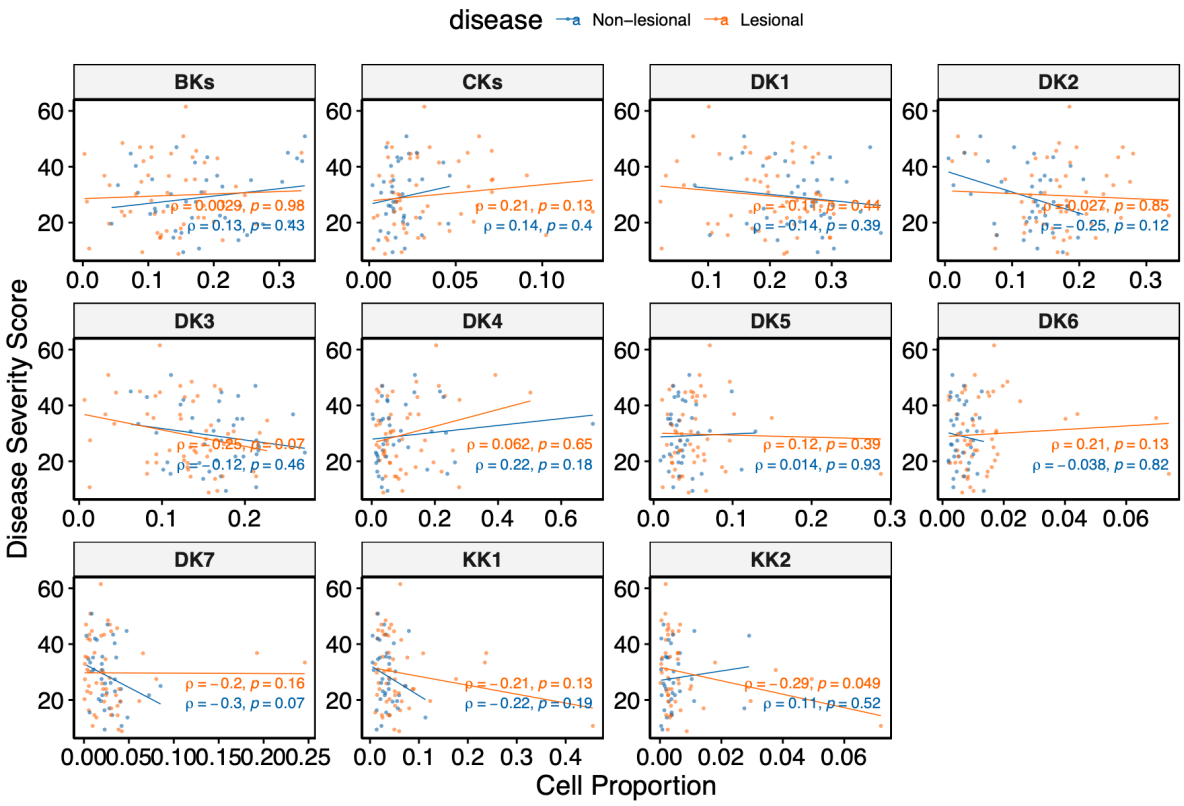
Importantly, no subset was exclusively dominated by either healthy or lesional samples (Figure 2A). All subsets contained cells from healthy, non-lesional and lesional samples, although their relative abundances differed. For example, the putative pathogenetic subset K6—the subset most enriched in lesional skin—still included cells from healthy individuals. This distribution argues against the emergence of entirely disease-specific populations Consistent with this interpretation, IL-13 response signatures were detectable across all keratinocyte subsets, with higher responses observed in lesional samples but comparable baseline activity between healthy and non-lesional skin (Figure 4B). This indicates that type 2 inflammation acts broadly across pre-existing differentiation states rather than defining a single disease-restricted population.

To further address whether inflammation selectively drives expansion of specific subset, we examined correlation between IL-13 response scores and the subset proportions across samples. Among healthy samples, most subsets showed no significant association. In contrast, lesional and non-lesional samples demonstrated moderate correlations for several subsets, including cycling keratinocytes (CKs), several differentiated keratinocytes (DK4-DK7) and keratinized keratinocytes (KK1/KK2) **(Response to Reviewers Figure 5**), suggesting inflammation-dependent modulation of abundance rather than generation of new cell types. Notably, subset proportions were not significantly associated with clinical disease severity (**Response to Reviewers Figure 6**), further indicating that these populations are not solely defined by disease status.

Taken together, these findings support a model in which AD inflammation reshapes the distribution and transcriptional programs of pre-existing keratinocyte differentiation states rather than inducing entirely novel keratinocyte populations. We note that our study is cross-sectional and therefore cannot directly assess temporal stability or reversibility following therapeutic blockade of type 2 inflammation. However, the presence of all keratinocyte subsets in healthy skin suggests that disease-associated features likely reflect dynamic, microenvironment-driven reprogramming of conserved epidermal states.



Response to Reviewers Figure 5. Cross-sample correlation between cell proportion and IL-13 response score in individual keratinocyte subsets. Each dot represents a sample, colored by disease status: lesional AD (LAD, orange), non-lesional AD (NAD, blue), and healthy controls (HC, green). Lines indicate group-specific linear regression fits, and the corresponding spearman correlation (ρ) and p value were noted.



Response to Reviewers Figure 6. Cross-sample correlation between cell proportion and disease severity score in individual keratinocyte subsets. Each dot represents a sample, colored by disease status: lesional AD (LAD, orange) and non-lesional AD (NAD, blue). Lines indicate group-specific linear regression fits, and the corresponding spearman correlation (ρ) and p values were noted.

2) Lower amount of endothelial cells and more keratinocytes in non-lesional AD – could that be related to the size of the biopsy or the anatomical region where the biopsies were obtained?

Response to reviewers:

We thank the review for raising this critical question that might induce a biased observation in single-cell data. To control for the bias in cell composition potentially introduced by biopsy approach, we took 6mm skin biopsies for all samples and used the mixture of epidermal and dermal cells in approximately 1:3 ratio for the single-cell sequencing. All healthy control and non-lesional skin biopsies were taken from normal-appearing skin on the buttock, and lesional biopsies were taken from well-developed AD lesions, primarily on the trunk and extremities. In response to this comment, we have incorporated further clarification about

the anatomic sites of the biopsies in the methods. As for explanation of the differences mentioned in this comment, expansion of keratinocytes in non-lesional AD skin agrees with prior findings[16] and leads to an apparent reduction in other cell types including EC and LEC. In lesional skin, variation in body site and disease process (including variable expansion of immune cells) may obscure these differences. As absolute representation of cell types is difficult to infer from scRNA-seq, however, we do not expand upon this in the main text.

3) Would the authors discuss why sebocytes and eccrine cells where regarded keratinocyte subsets?

Response to reviewers:

We thank the reviewer for this important point. In the initial global clustering, eccrine gland cells (ECRs) were positioned adjacent to the major keratinocyte cluster, reflecting their shared epithelial origin and close developmental relationship with epidermal appendages (Figure 1A). Subsequent sub-clustering of the epithelial compartment enabled us to resolve these populations as distinct appendage-derived epithelial cell types, including sebocytes originating from hair follicles and eccrine gland cells associated with sweat glands (Figure 1D). Because these cells emerged from the broader epithelial cluster containing keratinocytes during the first round of clustering, they were initially included within the keratinocyte-lineage compartment. However, we acknowledge that sebocytes and eccrine cells represent specialized epithelial cell types rather than canonical interfollicular keratinocytes. Importantly, the downstream analyses presented in this study were keratinocyte-focused (basal, cycling, differentiated and keratinized keratinocytes), and thus their inclusion in the initial clustering does not affect the identification or interpretation of keratinocyte subsets or the conclusions regarding keratinocyte biology in AD.

In the revised manuscript Results section (2nd paragraph), we have clarified that “For the downstream analysis, we focused on basal, cycling, differentiated and keratinized keratinocytes as keratinocyte subsets.”

4) The authors describe apoptosis to be a key result of TWEAK/FN14 binding. Apoptosis is not typically observed in AD skin, in contrast to drug reactions or interface dermatitis diseases. In that context, it may be interesting that IL-13 is also upregulated in lichen planus in some reports – is the mechanism shown here exclusive to AD or classical type 2 diseases?

Response to reviewers:

We thank the reviewer for this thoughtful comment. We agree that widespread keratinocyte apoptosis is not a classical histopathological hallmark of AD, in contrast to interface dermatitis or severe drug reactions. In our study, we did not observe evidence for extensive cell death at the tissue level. Rather, our transcriptomic analyses indicate activation of apoptosis-related pathways, particularly in specific keratinocyte subsets.

Pathway enrichment analysis revealed significant upregulation of apoptosis and apoptosis-regulation signatures in lesional AD keratinocytes, most prominently in the putative pathogenic DK6 subset and basal keratinocytes when compared with non-lesional or healthy skin (**Response to Reviewers Supplementary Table S2**). These signatures included key regulators of intrinsic and extrinsic apoptotic pathways (e.g., BAX, BAK1, BID, CYCS, TNFSF10, TNFRSF10A/B, TRAF2), as well as multiple proteasome components (e.g., PSMA/PSMB/PSMC/PSMD families) and stress-response factors (e.g., STAT3, TRAF2, BAX/BAK1, 14-3-3 proteins). Importantly, such transcriptional activation does not necessarily imply ongoing widespread apoptosis but may reflect heightened cellular stress, priming for apoptosis, or engagement of non-lethal TNF-family signaling pathways.

Consistent with this interpretation, several studies have reported increased keratinocyte susceptibility to apoptosis or enhanced apoptotic signaling in AD skin, particularly under inflammatory or barrier-disruptive conditions, although overt apoptotic cell death is typically limited [17-19]. Barrier disruption, oxidative stress, and cytokine exposure can sensitize keratinocytes to apoptosis without causing extensive epidermal loss. TWEAK/Fn14 signaling has been shown to induce diverse cellular outcomes depending on context, including apoptosis, proliferation, inflammation, and tissue remodeling, and may therefore contribute to keratinocyte dysfunction without causing extensive cell loss [11, 20]. Zimmermann, M., et al. specifically demonstrated that TWEAK cooperates with TNF-α in the induction of keratinocyte apoptosis in AD skin [21].

Regarding disease specificity, we agree that it will be worthwhile to assess whether the mechanism described here is exclusive to AD or classical type 2 diseases. IL-13 upregulation has also been reported in other inflammatory dermatoses, including lichen planus, suggesting that cytokine-driven epithelial stress responses may occur across multiple conditions. However, our data indicate that in AD, the combination of strong type 2 cytokine signaling and TWEAK/Fn14 activation in keratinocytes creates a distinct transcriptional program characterized by altered differentiation, metabolic stress, and apoptosis-related signaling. Whether similar pathways operate in interface dermatitis or other inflammatory diseases remains an important question for future investigation.

In summary, our findings suggest that TWEAK/Fn14 signaling in AD is associated with activation of apoptosis-related pathways at the transcriptional level, reflecting keratinocyte

stress and dysregulation rather than extensive tissue-level apoptosis, and may represent a context-dependent mechanism that is not restricted exclusively to AD.

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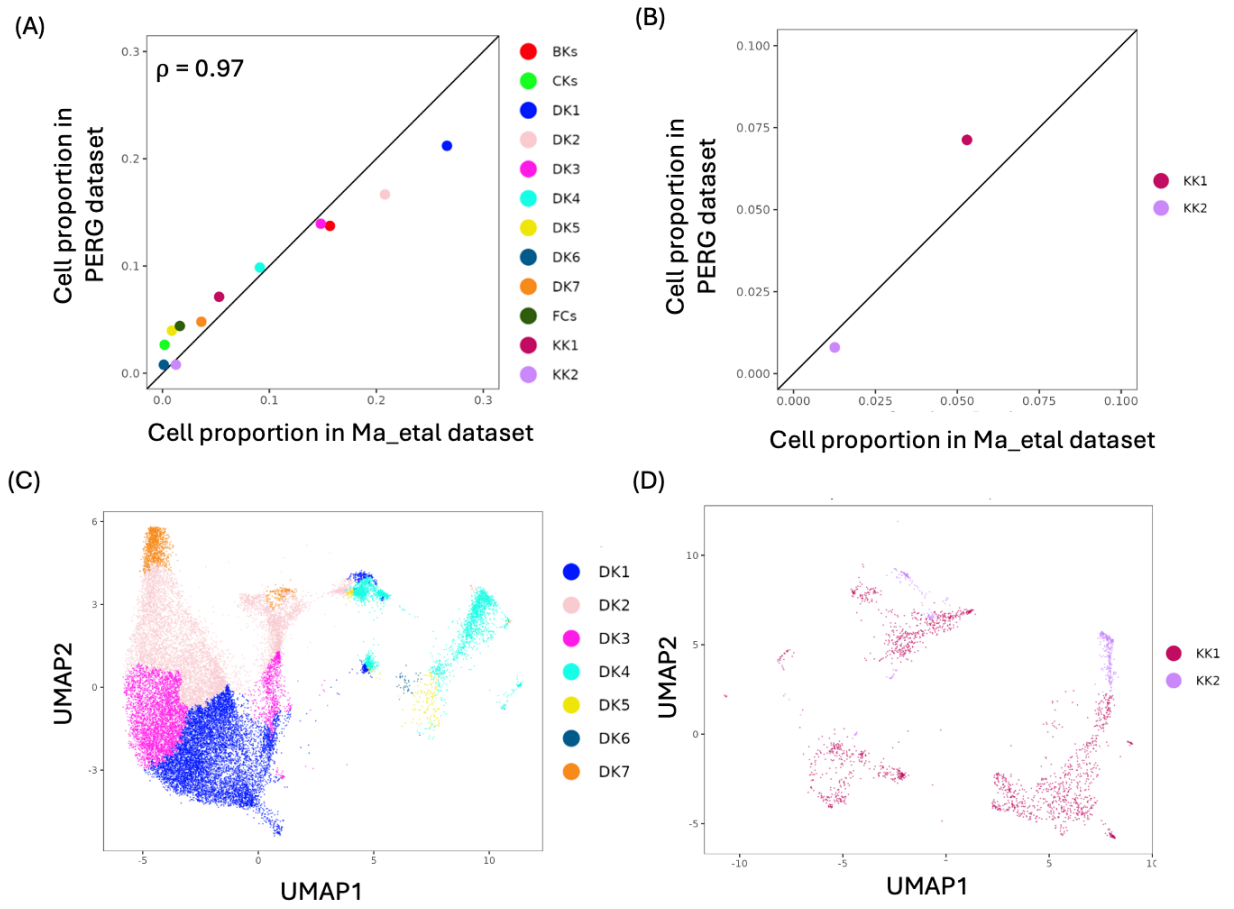
Reviewer 2: The manuscript clearly improved and all comments of the reviewer´s have been carefully considered. Especially adding mechanistic cell culture models increase the validity of the study. The one commen that is remaining and could be addressed further is from my perspective one comment of reviewer 1: it is clear that the much higher number of high-quality cells would result in more differentially expressed genes in this dataset, highlighting how important this study also is as a resource. However, I would like to understand whether the proposed subtypes of keratinocytes were also found in the published, substantially smaller datasets – could individual cells be identified that have a similar gene expression profile as the distinct keratinocyte subsets of the original dataset also in the published sources? And were there eventually keratinocytes that were totally different to the subsets identified in the current manuscript? This cannot be answered by a global DEG analysis.

Response to reviewer 2:

We thank the reviewer for the insightful comment. To access whether the keratinocyte subclusters identified in our study can be reproduced in independent datasets, we analyzed the keratinocytes from the published Ma et al. dataset (Ma et al. [1]: 6 lesional vs. 5 non-lesional AD samples). We projected keratinocytes from the published data onto the reference embedding precomputed from our dataset (PERG) using Symphony framework [2]. We found that the keratinocyte subclusters defined in our dataset were successfully recapitulated in the Ma et al. dataset, with highly concordant cell-type proportions (Spearman rank correlation $\rho = 0.97$, $p < 10^{-16}$; **Response to Reviewers Figure 1A-B**). In particular, the seven differentiated keratinocyte subclusters (DK1-DK7) and two keratinized keratinocytes (KK1-KK2) were clearly segregated in the UMAP embedding (**Response to Reviewers Figure 1C-D**). We note, however, that several subclusters, particularly DK4 - DK7 and KK1, were underrepresented in Ma et al. dataset, likely reflecting the smaller cohort size and reduced cellular depth of the published dataset. Notably, DK6 and DK7, which were identified as the key disease-associated populations in our dataset, were under-represent in the Ma, et al. dataset, suggesting their study would have lack of power to reveal more granular keratinocyte subpopulation and their molecular profiles. Overall, these results demonstrate that the keratinocyte subclusters identified in our study are reproducible in an independent dataset, while also highlighting that rarer disease-associated cell populations may be less well captured in smaller scRNA-seq cohorts.



Response to Reviewers Figure 1. Recapitulation of the identified keratinocyte subclusters in an independent keratinocyte scRNA-seq dataset (Ma_etal). (A-B) Correlation of subcluster proportions between Ma_etal and our dataset (PERG) across all subclusters (A) and the two keratinized keratinocyte subclusters (B). Spearman rank correlation coefficiant is shown. (C-D) UMAP visualization of differentiated keratinocyte subclusters (C) and keratinized keratinocyte sublucters (D) identified in Ma_etal dataset.

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