i) Short informative title

Single-cell RNA sequencing reveals 2D cytokine assay can model atopic dermatitis more accurately than immune-competent 3D setup (125 characters)

ii) Short working title

Single-cell transcriptomics rates eczema models (47 characters)

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AUTHOR CONTRIBUTIONS

BA and JAS designed the experiments. ST and SF recruited the patients. BA performed the experiments. ODB supervised the scRNA-seq workflow and the sequencing. BA and NH analysed the data. BA wrote the manuscript. JAS, TW and HR supervised the project. All authors provided critical feedback to support all steps of the projects.

vi) Abstract and keywords

Background: Modelling atopic dermatitis (AD) *in vitro* is paramount to understand the disease pathophysiology and identify novel treatments. Previous studies have shown that the Th2 cytokines IL-4 and IL-13 induce AD-like features in keratinocytes *in vitro*. However, it has not been systematically researched whether the addition of Th2 cells, their supernatants or a 3D structure are superior to model AD compared to simple 2D cell culture with cytokines.

Methods: For the first time, we investigated what *in vitro* option most closely resembles the disease *in vivo* based on single-cell RNA sequencing data (scRNA-seq) obtained from skin biopsies in a clinical study and published datasets of healthy and AD donors. *In vitro* models were generated with primary fibroblasts and keratinocytes, subjected to cytokine treatment or Th2 cell cocultures in 2D/3D. Gene expression changes were assessed using qPCR and Multiplex Immunoassays.

Results: Of all cytokines tested, incubation of keratinocytes and fibroblasts with IL-4 and IL-13 induced the closest *in vivo*-like AD phenotype which was observed in the scRNA-seq data. Addition of Th2 cells to fibroblasts failed to model AD due to the downregulation of ECM-associated genes such as POSTN. While keratinocytes cultured in 3D showed better stratification than in 2D, changes induced with AD triggers did not better resemble AD keratinocyte subtypes observed *in vivo*.

Conclusions: Taken together, our comprehensive study shows that the simple model using IL-4 or IL-13 in 2D most accurately models AD in fibroblasts and keratinocytes *in vitro*, which may aid the discovery of novel treatment options.

Key words:

single-cell RNA sequencing – Th2 cells – *in vitro* models – human skin equivalents – atopic dermatitis

vii) Main text

1. INTRODUCTION

Atopic dermatitis (AD) is a common non-infectious inflammatory skin disease that is characterized by a Th2/Th22 dominated immune reaction, a dysfunctional epidermal barrier and pronounced itch. The skin barrier changes involve keratinocytes downregulating differentiation and cell adhesion genes such as FLG or CLDN1[1]. Keratinocytes typically also upregulate genes encoding anti-microbial peptides such as S100A7 and PI3, and genes associated with proliferation (KRT16), neuronal survival and activity (NELL2, TSLP) and inflammation (e.g. CCL2, CCL20, CCL27)[2-7]. In the dermis, fibroblasts upregulate genes associated with type 2 inflammation such as specific cytokines (e.g. CCL26, CCL2, CCL19) and drivers of fibrosis (e.g. COL6A6, COL6A5, TNC, POSTN)[4-8].

Most AD research has focussed on modelling the disease *in vivo* and *in vitro* to find new AD treatments. Fully human *in vitro* models are particularly sought after for ethical and translational concerns regarding animal models. The Th2 cytokines IL-4 and IL-13 have been found to be especially useful in inducing key hallmarks of AD in 2D and 3D cultured skin cells, driving the epidermal barrier dysfunction, fibrosis and inflammation described above[9-19] (also reviewed in [20-22]). However, many of the genes typically tested are not AD-exclusive but are also changed in other inflammatory skin diseases such as in Th1/Th17 cell-driven psoriasis. Only some genes such as CCL27 in keratinocytes and TNC and POSTN in fibroblasts are differently regulated between AD and psoriasis[23-26]. Distinguishing different skin diseases and specifically modelling AD therefore require carefully selected marker gene sets. Since prior research on AD *in vitro* models typically tested for only few marker genes mainly utilizing a single modelling approach, a comprehensive and AD-specific comparison between different modelling approaches is still lacking. Additionally, there is currently no direct comparison of *in vitro* approaches with *in vivo* data. The recent advent of scRNA-seq allows us to assess pathological conditions *in vivo* on cell-subtype levels and can therefore serve as an ideal reference for mRNA-readouts from *in vitro* experiments. Moreover, unlike T cells or Th1 cells for psoriasis[27-29], the effect of Th2 cells, one of the main Th2 cytokine producers has not been assessed so far on keratinocytes and fibroblasts in human skin equivalents (HSEs). To address these issues, we aimed at comparing different *in vitro* models in order to identify the one that most closely resembles the active disease state in the patients, using scRNA-seq data as reference.

2. RESULTS

2.1 Different scRNA-seq datasets show similar changes for specific AD genes

To evaluate different AD modelling approaches, we generated a list of genes described to be specifically altered in AD and associated with fibroblasts or keratinocytes[3,4,7,8]. To distinguish between cutaneous inflammation in general and changes specific to AD, we also included genes described to have opposite regulation in psoriasis, such as POSTN, TNC and CCL27[6,24-26,30,31].

To verify this AD gene selection, we analysed 6 healthy and 4 AD lesional samples (Table S1) in a scRNA-seq study. We identified keratinocytes and fibroblasts as the largest cell groups (Fig. 1A). We also compared and integrated our own data with two other published scRNA-seq AD datasets by He et al[8] and Reynolds et al[32] (Table S2), to reduce data bias.

Similar distinct differences between AD and healthy control samples were observed among the fibroblast genes in all three datasets (Fig. 1B). For example, extracellular and matricellular proteins such as COL6A6, COL6A5, TNC and POSTN were all upregulated (Fig. 1B). IL13RA2 and IL6 regulation was similar in our and the He et al datasets. In keratinocytes most AD genes were also regulated similarly (such as S100A7, KRT16, NELL2, CA2 and FLG) with few exceptions (CCL27, CCL20, CCL2, TSLP) (Fig. 1C). We used the average gene expression of all three studies to generate an *in vivo* benchmark for all further analyses.

To further evaluate the AD gene panel, we compared changes between AD and healthy samples to data from psoriasis lesions published by Reynolds et al[32]. Many of the AD defining genes such as CCL2, CCL26, and POSTN were downregulated in psoriasis fibroblasts (Fig. 1D). In contrast, most keratinocyte genes increased in AD were also increased in psoriasis, except for TIMP1, CCL27 and CLDN1 (Fig. 1E).

2.2 IL-4 and IL-13 can induce changes to fibroblasts and keratinocytes *in vitro* that resemble cells from AD patients

We next tested key gene expression in response to the cytokines IL-17A, IFNγ, IL-4 or IL-13 and a type 2 cytokine mix (IL-4, IL-13, IL-25, IL-33 and TNFα) on fibroblasts and keratinocytes in conventional 2D cultures. Relative changes in gene expression to the untreated control were compared to changes found in the *in vivo* scRNA-seq datasets.

For both cell types, gene expression in response to Th2 cytokine treatment resembled strongly *in vivo* AD data, whereas IL-17A induced psoriasis-like features (Fig. 1D, E). Clustering probability to AD *in vivo* was highest for IL-4 and IL-13 treatment in fibroblasts (Fig. S1A), with induction of all expected AD-associated genes in fibroblasts except for ICAM1. In keratinocytes these Th2

cytokines also induced expression of most AD genes except for TIMP1 and CLDN1. Interestingly, no differences between IL-4 and IL-13 treatment were observed in any cell type, clustering together with very high probability (Fig. S1A, S1B). The AD cytokine mix did not induce significantly different changes in keratinocytes compared to IL-4 or IL-13 treatment alone but more strongly promoted AD genes such as IL13RA2, CCL5 and IL-6 in fibroblasts, deviating from the scRNA-seq data. IFNγ or IL-17 treatment resulted in different expression patterns in both cell types compared to Th2 cytokine treatment, for instance due to the lack of POSTN and TNC expression in fibroblasts but increased CXCL8 and CCL20 expression in keratinocytes. Since IL-13 but not IL-4 was upregulated in lymphoid cells from lesional compared to healthy samples (Fig. S2A), we decided to use IL-13 as *in vitro* AD reference for subsequent experiments.

2.3 IL-13 treated fibroblasts resemble AD more closely than fibroblasts cultured with Th2 cells

Van den Bogaard et al[27] previously showed that Th1 cells, one of the main contributors to psoriasis pathology, were able to induce psoriasis-like characteristics HSEs. We therefore investigated whether complex models with one of the main producers of Th2 cytokines, i.e. activated Th2 cells, or their supernatants could model AD *in vitro* even better than simply cytokine treatment.

To this end, we isolated mature CD4+CRTh2+ Th2 cells from blood (Fig. S2B, S2C). As these cells only make up approximately 2% of peripheral blood cells, we also generated induced Th2 cells (iTh2) from naïve blood T cells for greater cell yield. Both Th2 cell types produced high amounts of IL-13 and low amounts of IFNγ, confirming their general Th2 characteristics (Fig. 2A). However, mature Th2 cells (mTh2) showed much higher ability to produce Th2 cytokines than iTh2 cells, particularly IL-4 and IL-31.

Activated Th2 cells were able to survive in direct contact with fibroblasts but not with keratinocytes (Fig. 2B). Consequently, we only tested the effects of Th2 cells on fibroblasts in direct 2D cocultures. When added to fibroblasts, iTh2 cells required continuous activation during coculture to still produce cytokines and induce strong gene expression changes (Fig. S2D, S2E). When cocultured with Th2 cells, fibroblasts strongly upregulated key AD genes such as CCL26, COL6A5 and IL6 (Fig. 2C). However, both mTh2 and iTh2 cocultures did not cluster as closely with the *in vivo* AD scRNA-seq data as the IL-13 treatment due to the significant downregulation of the ECM proteins POSTN ($P = 0.043$), COL6A1 ($P = 0.013$) and COL3A1 ($P = 0.013$) compared to IL-13 treated controls (Fig. 2C). MTh2 coculture more closely resembled the *in vivo* data than +iTh2. While the iTh2 supernatant induced similar changes as the direct T cell cocultures in fibroblasts, the mTh2 supernatant more closely resembled IL-13 treated fibroblasts. In any of these cases

however, clustering probability was not very high (Fig. S1C). Some genes could also be evaluated on protein level in the supernatant, confirming a similar profile for type VI collagens and COL6A5 in fibroblasts after incubation with IL-13 and mTh2 supernatant (Fig. S3A).

Since these experiments were conducted with T cells and fibroblasts from allogenic donors, we additionally determined the effect of MHC mismatches. Interestingly, fibroblast origin itself was more relevant for gene expression than T cell matching (Fig. S3B).

Since T cells were not able to survive in direct coculture with keratinocytes in 2D, we tested Th2 cells in 3D skin models in the additional presence of fibroblasts. After seeding iTh2 cells into HSEs, we could still detect T cells 8 days later (Fig. 2D). When comparing the effects of Th2 cells on keratinocytes in 3D culture compared to IL-13 treated 3D models, the general gene expression profile did not strongly differ (Fig. 2E).

2.4 The response to IL-13 is not better modelled in 2D than in 3D

Since keratinocytes stratify *in vivo* and we expected that 3D cultured keratinocytes show a higher range of differentiation, we next determined whether 3D keratinocytes modelled AD more accurately than in 2D.

First, we compared profiles between more and less differentiated keratinocytes *in vivo*. We subclustered the keratinocyte scRNA-seq data and identified six different groups: basal, proliferating, spinous, granular, cornified and gland-associated keratinocytes (Fig. 3A). Expression profiles in different healthy keratinocyte subsets revealed that S100A7, KRT16, PI3 and FLG were mostly expressed in differentiated, i.e. granular and cornified, keratinocytes. In contrast, CCL2 and KRT15 were more highly expressed in basal keratinocytes (Fig. 3B).

We then compared changes of these AD genes in untreated keratinocytes cultured in 3D versus 2D. As depicted in Fig. 3C, most AD genes were strongly upregulated in 3D compared to 2D cultured keratinocytes in general, particularly differentiation genes including S100A7, PI3 and FLG. Genes connected with basal keratinocytes such as CCL2 and KRT15 were also upregulated in 3D. Interestingly, TSLP was the only gene reproducibly downregulated in 3D. We confirmed the expression of selected genes at protein level using immunohistochemistry on HSEs, showing clear filaggrin expression in the stratum corneum and keratin15 expression in the stratum basale (Fig. 3D).

We next assessed if the 3D model would also resemble *in vivo* AD more closely when undergoing IL-13 treatment compared to 2D. We again first looked at changes in lesional compared to healthy skin *in vivo* (Fig. 3E). Some of the genes in the AD gene panel were differentially expressed by all

keratinocyte subtypes in lesional compared to healthy skin, such as KRT16 and CCL27. However, TIMP1, CCL2 and KRT15 were mainly changed in undifferentiated keratinocytes (basal and proliferating), NELL2, HAS3, CA2 and KRT1 in intermediate keratinocytes (spinous and granular) and S100A7, PI3 and FLG in highly differentiated, cornified keratinocytes. When comparing subsets from *in vivo* lesional skin with IL-13-treated keratinocytes *in vitro* in 2D and 3D, changes in 2D culture most closely resembled changes in spinous and granular AD keratinocytes (Fig. 3F). Compared to 2D, IL-13-treated 3D keratinocyte gene expression was less like basal keratinocytes. Surprisingly, they also failed to model the response to IL-13 of more differentiated AD keratinocyte subtypes.

3. DISCUSSION

Based on a set of AD-associated genes, we showed that 2D cell cultures treated with IL-13 induced AD-like features as good as or better than more complex models including 3D or co-culture.

We used skin scRNA-seq data obtained from patients with AD and healthy individuals as benchmark for the comparison of different culture conditions of fibroblasts and keratinocytes. Since sample and data processing typically vary between scRNA-seq studies and can therefore lead to inconsistent results, we compared our own scRNA-seq results with two other scRNA-seq studies, involving healthy and lesional AD skin and used the mean as reference for the *in vitro* experiments. Minor differences in the fibroblast dataset by Reynolds *et al* might be due to longer and different enzyme treatment of the dermis (Table S2). Differences in keratinocytes in our scRNA-seq data could result from the presence of more differentiated keratinocytes, possibly since we refrained from dead cell removal steps.

We then used the *in vivo* scRNA-seq data to test how different AD *in vitro* models perform. We confirmed that Th2 cytokines induced AD-like characteristics in both fibroblasts and keratinocytes *in vitro*, particularly regarding ECM-related genes in fibroblasts. Many of the upregulated genes in fibroblasts such as COL6A5, COL6A6, CCL2, and CCL19 corresponded to the AD specific (COL6A5+COL18A1+) subtype of fibroblasts described by He et al[8]. We detected no significant differences between IL-4 and IL-13 treatment, implying a less prominent role for IL13RA2, which was suggested as an IL-13 sink[33], since IL-4 binds to IL13RA1 but not to IL13RA2. Interestingly, complex additives such as the AD cytokine mix or Th2 cell supernatants did not provide clear benefits over just IL-4 or IL-13 treatment, emphasizing their role in AD pathophysiology.

More complex cultures involving Th2 cells did not result in gene expression profiles closer to AD scRNA-seq data. While Th2 cells increased the expression of tested pro-inflammatory cytokines and chemokines such as CCL5, IL-6 and CCL2 in keratinocytes and fibroblasts, they strongly downregulated ECM genes such as POSTN and COL6A1 in fibroblasts. T cells have been shown to downregulate collagens in direct coculture with fibroblasts[34,35], potentially mediated via membrane-bound TNF-α[35]. TNF-α was produced by the Th2 cells in our study and we additionally found a downregulation of TGF-β in supernatants of fibroblast/T cell coculture (data not shown), another well-known regulator of ECM production[36]. We also revealed that these effects are more pronounced in iTh2 cells compared to mTh2 cells, most likely since mTh2 cells produced higher amounts of IL-4 and IL-13, which might counteract ECM downregulation. The limited potential to induce AD-like features in direct contact with fibroblasts suggests a role for other type 2 cytokine sources, such as granulocytes and ILC2s. For instance, Jiao et al[37] showed the pro-inflammatory effect of basophils and eosinophils in an *in vitro* coculture with fibroblasts in presence of NOD2 and TLR2 ligands. In a recent scRNA-seq study ILC2s have been confirmed to infiltrate human AD skin and express high levels of Th2 cytokines[38]. Since T cells cluster with other lymphoid cells *in vivo*, the contact ratio of T cells to fibroblasts might be much lower than 1:2 used in the *in vitro* experiments and therefore might mutually alter gene expression. Interestingly, we did not detect significant gene expression changes in fibroblasts after allogenic and autologous coculture with iTh2 cells, alleviating the potential downside of allogenic cocultures for AD modelling.

To our knowledge, no results on HSEs incorporating functional Th2 cells have been published so far. Prior 3D skin models either only involved total/bulk blood-derived T cells[28,39] or cells that did not appear to produce Th2 cytokines[27]. We could only test direct coculture of Th2 cells with fibroblasts but not with keratinocytes in 2D. This ability of fibroblasts to prevent apoptosis in T cells and the potential mediators have been described in the literature[40-42].

In HSEs, addition of T cells induced gene expression changes in keratinocytes that closely resembled the *in vivo* reference similarly to IL-13 treatment. While three-dimensionality did increase stratification of untreated keratinocytes and resulted in a higher expression of most genes, IL-13 treatment did not generate more AD-like changes compared to 2D culture. High expression variability of HSEs might contribute to that. ScRNA-seq would need to be performed to obtain subset-specific gene expression in 3D cultured keratinocytes upon IL-13 treatment. Due to low benefit and high effort to construct HSEs, we recommend using the simplest IL-4 or IL-13 induced AD model in 2D. For more specific questions however, which for instance consider spatial gene expression differences, 3D models might still be indispensable.

To our knowledge, this study is one of the most comprehensive efforts to model AD *in vitro* and the first to directly compare larger gene sets to *in vivo* patterns identified within individual patient cell populations using scRNA-seq. However, we are aware that a larger set of genes might produce even more accurate results. While some of the top upregulated genes in AD are included in this study, it would be interesting to expand the list for other highly upregulated genes such as PLA2G2A and SERPINB4. However, in summary we believe it is good news that a simpler model (IL-13 treatment of 2D cultures) is more accurate in recapitulating disease features *in vitro* since it will simplify and accelerate the identification of new treatments for AD.

4. MATERIALS AND METHODS

Patient recruitment and samples

Clinical studies were approved by the ethics committee of the MHH (Nr. 8775_B0_S_2019 and Nr. 9464_B0_K_2020). All participants provided written consent prior to sample taking. For *in vivo* AD reference data, one half of a 5 mm skin biopsy was used for scRNA-seq from each participant (Table S1). Skin fibroblasts for autologous coculture were collected from skin cancer local wide excisions. Peripheral blood was collected from the same donors (Table S3). Other fibroblasts and keratinocytes were isolated from excess abdominal skin from cosmetic surgery and provided by Alphenyx® (female, age 18 – 46 years).

Droplet based scRNA-seq

Fresh biopsies (Table S1) were immediately processed with the Whole Skin Dissociation Kit (Miltenyi-Biotec) and prepared for loading on the Chromium Controller (10X Genomics; Single Cell 3′ Library & Gel Bead Kit v3.1) according to the manufacturers' instructions. The samples were sequenced on an Illumina NovaSeq 6000 sequencer as described in the Supplement.

Isolation of primary skin cells and blood T cells

Epidermis was separated from dermis using 2.4 U/ml dispase II. Fibroblasts were isolated from the dermis using the Whole Skin Dissociation Kit (Miltenyi-Biotec) and keratinocytes from the epidermis using a 0.025% trypsin-EDTA solution.

iTh2 were generated by isolating naïve CD4+ T cells from blood PBMCs (EasySep™ Human Naïve T Cell Isolation Kit and Human CD4+ T Cell Isolation Kit, STEMCELL) and polarized for one week using anti-CD2/CD3/CD28 MACSiBeads Particles (Miltenyi-Biotec), IL-4 (50 ng/ml, STEMCELL) and anti-IFNγ antibodies (5 µg/ml, BioLegend). mTh2 cells i.e. CD4+CRTh2+ T cells, were isolated from PBMCs using the EasySep™ Human CD4+ T cell isolation kit and subsequent FACS.

2D- and 3D cocultures

For cytokine treatments, fibroblasts or keratinocytes were treated for 3 days with IL17 (100 ng/ml), IFNγ (200 U/ml), IL-4 (100 ng/ml), IL-13 (100 ng/ml) or an AD cytokine mix (20 ng/ml IL-4, 25 ng/ml IL-13, 25 ng/ml IL-25, 15 ng/ml IL-31, 20ng/ml TNFα).

For 2D culture T cells were (re-)activated for 24 hours before being added in a ratio 1:2 to precultured fibroblasts or keratinocytes. For continuous activation either MACSiBeads Particles or Concanavalin A (= ConA; 10µg/ml) were added. After three days of coculture, T cells and

fibroblasts or keratinocytes were collected separately and analysed for purity and viability using flow cytometry. Additionally, cell pellets and supernatants were frozen for RNA and protein analysis.

Control HSEs were generated as described previously[43]. For immune-competent skin models Th2 cells were added to fibroblasts in a ratio 1:1 when fibroblasts were seeded into 3 mg/ml bovine type I collagen (PureCol®, Advanced Biomatrix) and cultured with ConA after adding keratinocytes. After airlift and one week of culture, the epidermis was collected for RNA analysis or the whole skin model was frozen in O.C.T for immunohistochemistry. For cytokine treatment 100 ng/ml IL-13 was added 3 days before the collection of cells.

Flow cytometry and FACS

To assess viability and purity via flow cytometry or sort T cells with FACS, cells were stained (Table S4) and analyzed using a BD LSRFortessa™ X-20 Analyzer (BD Biosciences) or sorted using a BD FACSMelody Cell Sorter (BD Biosciences) and interpreted using FlowJo 7.6.5.

Real-time PCR

RNA was extracted from fibroblasts and keratinocytes using the RNeasy Mini Kit (Qiagen). After RNA quality assessment cDNA was synthesized and quantified using TaqMan MasterMix Reagent Kits and the 7900HT Fast Real-Time PCR System (AppliedBiosystems). Gene expression was normalized to GAPDH and YWHAZ and quantified using the $\Delta\Delta$Ct analysis. Ratios of treated and untreated controls were used to compare the similarity of different culture conditions in heatmaps, which were created with R using the Manhattan distance function.

Protein analysis

T cell supernatants were analysed using ELISAs (IFNγ, IL-4, IL-13, IL-31, TNFα, IL-5; R&D Systems). Additionally, periostin (R&D Systems) and type VI collagen levels (Assay Genie) were assessed from fibroblast supernatants cultured under different conditions using ELISAs, and IL-6, CCL2, CCL20 and CCL26 were measured using the Bio-Plex 200 System (Bio-Rad), to validate mRNA differences on a protein level.

Immunohistochemistry

Cryosections (7 µm) of frozen HSEs were fixated with 4% PFA for 20 min, incubated in fish serum blocking buffer (Thermo Scientific) for 1 h to prevent unspecific binding, and incubated with primary antibodies overnight at 8°C (Table S4). On the next day, samples were incubated for 1 h

with fluorescent-dye-conjugated secondary antibodies and analysed using a Zeiss Axio Observer (ZEISS).

Data and Statistical analyses

Downstream analysis of the scRNA-seq data was performed in R (v. 4.0.3). Background contamination from lysed cells was removed using SoupX (v. 1.5.2) and doublets identified and filtered using scDblFinder (v. 1.4.2). Further processing and analysis of the data was performed using Seurat (v. 4.0.2) and is described in detail in the Supplement.

Statistical analyses of the heatmaps were performed using the pvclust function in R. For comparisons of single genes Mann-Whitney U tests were used and differences were considered significant if $P < 0.05$ after Bonferroni-correction.

Biorender was used for designing selected images.

REFERENCES

1. Batista DIS, Perez L, Orfali RL, et al. Profile of skin barrier proteins (filaggrin, claudins 1 and 4) and Th1/Th2/Th17 cytokines in adults with atopic dermatitis. *Journal of the European Academy of Dermatology and Venereology.* 2015;29(6):1091-1095.
2. Sugiura H, Ebise H, Tazawa T, et al. Large-scale DNA microarray analysis of atopic skin lesions shows overexpression of an epidermal differentiation gene cluster in the alternative pathway and lack of protective gene expression in the cornified envelope. *British Journal of Dermatology.* 2005;152(1):146-149.
3. Rojahn TB, Vorstandlechner V, Krausgruber T, et al. Single-cell transcriptomics combined with interstitial fluid proteomics defines cell type–specific immune regulation in atopic dermatitis. *Journal of Allergy and Clinical Immunology.* 2020;146(5):1056-1069.
4. Suárez-Fariñas M, Ungar B, Correa da Rosa J, et al. RNA sequencing atopic dermatitis transcriptome profiling provides insights into novel disease mechanisms with potential therapeutic implications. *Journal of Allergy and Clinical Immunology.* 2015;135(5):1218-1227.
5. Ewald DA, Malajian D, Krueger JG, et al. Meta-analysis derived atopic dermatitis (MADAD) transcriptome defines a robust AD signature highlighting the involvement of atherosclerosis and lipid metabolism pathways. *BMC Medical Genomics.* 2015;8(1):60.
6. Nomura I, Gao B, Boguniewicz M, Darst MA, Travers JB, Leung DYM. Distinct patterns of gene expression in the skin lesions of atopic dermatitis and psoriasis: A gene microarray analysis. *Journal of Allergy and Clinical Immunology.* 2003;112(6):1195-1202.
7. Zhu J, Wang Z, Chen F. Association of Key Genes and Pathways with Atopic Dermatitis by Bioinformatics Analysis. *Medical science monitor : international medical journal of experimental and clinical research.* 2019;25:4353-4361.
8. He H, Suryawanshi H, Morozov P, et al. Single-cell transcriptome analysis of human skin identifies novel fibroblast subpopulation and enrichment of immune subsets in atopic dermatitis. *Journal of Allergy and Clinical Immunology.* 2020;145(6):1615-1628.
9. Danso MO, van Drongelen V, Mulder A, et al. TNF-alpha and Th2 cytokines induce atopic dermatitis-like features on epidermal differentiation proteins and stratum corneum lipids in human skin equivalents. *The Journal of investigative dermatology.* 2014;134(7):1941-1950.
10. Kim K, Kim H, Sung GY. An Interleukin-4 and Interleukin-13 Induced Atopic Dermatitis Human Skin Equivalent Model by a Skin-On-A-Chip. *International journal of molecular sciences.* 2022;23(4):2116.
11. Lee SH, Bae IH, Choi H, et al. Ameliorating effect of dipotassium glycyrrhizinate on an IL-4- and IL-13-induced atopic dermatitis-like skin-equivalent model. *Archives of dermatological research.* 2019;311(2):131-140.
12. Kamsteeg M, Bergers M, de Boer R, et al. Type 2 helper T-cell cytokines induce morphologic and molecular characteristics of atopic dermatitis in human skin equivalent. *The American journal of pathology.* 2011;178(5):2091-2099.
13. do Nascimento Pedrosa T, De Vuyst E, Mound A, Lambert de Rouvroit C, Maria-Engler SS, Poumay Y. Methyl-β-cyclodextrin treatment combined to incubation with interleukin-4 reproduces major features of atopic dermatitis in a 3D-culture model. *Archives of dermatological research.* 2017;309(1):63-69.
14. Rouaud-Tinguely P, Boudier D, Marchand L, et al. From the morphological to the transcriptomic characterization of a compromised three-dimensional in vitro model mimicking atopic dermatitis. *British Journal of Dermatology.* 2015;173(4):1006-1014.
15. Sriram G, Bigliardi PL, Bigliardi-Qi M. Full-Thickness Human Skin Equivalent Models of Atopic Dermatitis. *Methods Mol Biol.* 2019;1879:367-383.

16. Oriente A, Fedarko NS, Pacocha SE, Huang S-K, Lichtenstein LM, Essayan DM. Interleukin-13 Modulates Collagen Homeostasis in Human Skin and Keloid Fibroblasts. *Journal of Pharmacology and Experimental Therapeutics.* 2000;292(3):988-994.
17. Leonardi A, Cortivo R, Fregona I, Plebani M, Secchi AG, Abatangelo G. Effects of Th2 Cytokines on Expression of Collagen, MMP-1, and TIMP-1 in Conjunctival Fibroblasts. *Investigative ophthalmology & visual science.* 2003;44(1):183-189.
18. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nature reviews Immunology.* 2004;4(8):583-594.
19. Hubaux R, Bastin C, Salmon M. On the relevance of an in vitro reconstructed human epidermis model for drug screening in atopic dermatitis. *Experimental dermatology.* 2018;27(12):1403-1407.
20. De Vuyst E, Salmon M, Evrard C, Lambert de Rouvroit C, Poumay Y. Atopic Dermatitis Studies through In Vitro Models. *Frontiers in medicine.* 2017;4:119-119.
21. Moon S, Kim DH, Shin JU. In Vitro Models Mimicking Immune Response in the Skin. *Yonsei Med J.* 2021;62(11):969-980.
22. Sarama R, Matharu PK, Abduldaiem Y, Corrêa MP, Gil CD, Greco KV. In Vitro Disease Models for Understanding Psoriasis and Atopic Dermatitis. *Front Bioeng Biotechnol.* 2022;10:803218.
23. Riis JL, Johansen C, Vestergaard C, Bech R, Kragballe K, Iversen L. Kinetics and differential expression of the skin-related chemokines CCL27 and CCL17 in psoriasis, atopic dermatitis and allergic contact dermatitis. *Experimental dermatology.* 2011;20(10):789-794.
24. Garzorz N, Eyerich K. NOS2 and CCL27: clinical implications for psoriasis and eczema diagnosis and management. *Expert Review of Clinical Immunology.* 2015;11(2):167-169.
25. Ogawa K, Ito M, Takeuchi K, et al. Tenascin-C is upregulated in the skin lesions of patients with atopic dermatitis. *Journal of Dermatological Science.* 2005;40(1):35-41.
26. Zhang B, Roesner LM, Traidl S, et al. Single-cell profiles reveal distinctive immune response in atopic dermatitis in contrast to psoriasis. *Allergy.* 2022.
27. van den Bogaard EH, Tjabringa GS, Joosten I, et al. Crosstalk between Keratinocytes and T Cells in a 3D Microenvironment: A Model to Study Inflammatory Skin Diseases. *Journal of Investigative Dermatology.* 2014;134(3):719-727.
28. Lorthois I, Simard M, Morin S, Pouliot R. Infiltration of T Cells into a Three-Dimensional Psoriatic Skin Model Mimics Pathological Key Features. *International journal of molecular sciences.* 2019;20(7):1670.
29. Rioux G, Simard M, Morin S, Lorthois I, Guérin SL, Pouliot R. Development of a 3D psoriatic skin model optimized for infiltration of IL-17A producing T cells: Focus on the crosstalk between T cells and psoriatic keratinocytes. *Acta Biomaterialia.* 2021;136:210-222.
30. Tsoi LC, Rodriguez E, Degenhardt F, et al. Atopic Dermatitis Is an IL-13–Dominant Disease with Greater Molecular Heterogeneity Compared to Psoriasis. *Journal of Investigative Dermatology.* 2019;139(7):1480-1489.
31. He H, Bissonnette R, Wu J, et al. Tape strips detect distinct immune and barrier profiles in atopic dermatitis and psoriasis. *Journal of Allergy and Clinical Immunology.* 2021;147(1):199-212.
32. Reynolds G, Vegh P, Fletcher J, et al. Developmental cell programs are co-opted in inflammatory skin disease. *Science (New York, NY).* 2021;371(6527):eaba6500.
33. Kasaian MT, Raible D, Marquette K, et al. IL-13 antibodies influence IL-13 clearance in humans by modulating scavenger activity of IL-13Rα2. *Journal of immunology (Baltimore, Md : 1950).* 2011;187(1):561-569.
34. Rezzonico R, Burger D, Dayer JM. Direct contact between T lymphocytes and human dermal fibroblasts or synoviocytes down-regulates types I and III collagen production via

cell-associated cytokines. *The Journal of biological chemistry.* 1998;273(30):18720-18728.
35. Chizzolini C, Parel Y, De Luca C, et al. Systemic sclerosis Th2 cells inhibit collagen production by dermal fibroblasts via membrane-associated tumor necrosis factor α. *Arthritis & Rheumatism.* 2003;48(9):2593-2604.
36. Akhmetshina A, Palumbo K, Dees C, et al. Activation of canonical Wnt signalling is required for TGF-β-mediated fibrosis. *Nat Commun.* 2012;3:735.
37. Jiao D, Wong C-K, Qiu H-N, et al. NOD2 and TLR2 ligands trigger the activation of basophils and eosinophils by interacting with dermal fibroblasts in atopic dermatitis-like skin inflammation. *Cellular & Molecular Immunology.* 2016;13(4):535-550.
38. Alkon N, Bauer WM, Krausgruber T, et al. Single-cell analysis reveals innate lymphoid cell lineage infidelity in atopic dermatitis. *Journal of Allergy and Clinical Immunology.* 2022;149(2):624-639.
39. Engelhart K, El Hindi T, Biesalski H-K, Pfitzner I. In vitro reproduction of clinical hallmarks of eczematous dermatitis in organotypic skin models. *Archives of dermatological research.* 2005;297(1):1-9.
40. Pilling D, Akbar AN, Girdlestone J, et al. Interferon-β mediates stromal cell rescue of T cells from apoptosis. *European journal of immunology.* 1999;29(3):1041-1050.
41. Buckley CD, Amft N, Bradfield PF, et al. Persistent Induction of the Chemokine Receptor CXCR4 by TGF-β1 on Synovial T Cells Contributes to Their Accumulation Within the Rheumatoid Synovium. *The Journal of Immunology.* 2000;165(6):3423-3429.
42. Van Linthout S, Miteva K, Tschöpe C. Crosstalk between fibroblasts and inflammatory cells. *Cardiovascular Research.* 2014;102(2):258-269.
43. Mildner M, Ballaun C, Stichenwirth M, et al. Gene silencing in a human organotypic skin model. *Biochemical and biophysical research communications.* 2006;348(1):76-82.

viii) Tables

Only Tables in Supplementary Material

ix) Figure legends

Figure 1: Relative AD marker gene expression in *in vivo* skin samples and comparison to cytokine-treated fibroblasts and keratinocytes *in vitro*.

(A) UMAP of integrated scRNA-seq data from healthy and lesional AD full thickness skin biopsies (our scRNA-seq data only).

(B), (C): Comparison of scRNA-seq data of fibroblasts (B) or keratinocytes (C) from three different studies showing the changes in AD lesional skin compared to healthy skin (log10 fold changes displayed).

(D), (E): Comparison of RNA differences in fibroblasts (D) or keratinocytes (E) induced with cytokine treatment in *in vi*tro 2D culture (relative to untreated cells; using RT-qPCR) to differences in *in vivo* AD or psoriasis (pso) skin samples (compared to healthy controls; using scRNA-seq). Means of at least 3 different independent experiments for the *in vitro* data.

AD cyt mix (20 ng/ml IL-4, 25 ng/ml IL-13, 25 ng/ml IL-25, 15 ng/ml IL-31, 20 ng/ml TNFα), IL-17 (100 ng/ml), IFNγ (200 U/ml), IL-4 (100 ng/ml), IL-13 (100 ng/ml).

Figure 2: Effect of Th2 cell coculture on fibroblasts and keratinocytes *in vitro* compared to *in vivo*.

(A) Cytokine profiling of supernatants from iTh2 or mTh2 cells after 3d of (re-)activation using ELISA.

(B) Viability of iTh2 cells after 2D coculture with keratinocytes and fibroblasts using flow cytometry.

(C) Heatmap showing relative changes of AD RNA in fibroblast cultured in 2D after incubation with IL-13 or coculture with iTh2 or mTh2 cells or after incubation with their supernatants (sup) compared to untreated controls (log10) using RT-qPCR compared to changes in AD skin using scRNA-seq.

(D) Representative immunostaining of fibroblasts (vimentin) and T cells (CD45) in a 3D skin model containing keratinocytes, fibroblasts and iTh2 cells, which were continuously activated using ConcanavalinA.

(E) Heatmap containing relative changes of AD RNA in keratinocytes extracted from epidermis in 3D skin models after incubation with IL-13 or with iTh2 cells compared to untreated controls (log10 values) using RT-qPCR compared to changes in AD skin using scRNA-seq.

All data summarized at least three independent experiments. Mann-Whitney U test, **P<0.01

Figure 3: Relative AD marker gene expressions in subsets of healthy and lesional kerationcytes compared to keratinocytes cultured in 2D or 3D *in vitro*.

(A) UMAP plots of keratinocyte subsets from lesional and healthy full thickness skin biopsies using our scRNA-seq data.

(B) Dot plot showing the expression strength for all keratinocyte subtypes from healthy skin samples using scRNA-seq data from this study.

(C) Log10 fold changes of AD related genes in keratinocytes cultured in 3D (HSEs) compared to 2D using RT-qPCR. Means of 3 independent experiments.

(D) Representative immunostaining filaggrin (green) and keratin15 (red) in epidermis of human skin equivalents.

(E) Dot plot showing expression differences in lesional and healthy samples using scRNA-seq (this study only), with upregulation being colored in red and downregulation in blue, percentage of expressing cells being encoded by circle size and significance being depicted by filled circles.

(F) Correlogram depicting Pearson correlation coefficient of gene expression changes among different keratinocyte subsets cultured in 2D and 3D *in vitro* (RT-qPCR) compared to AD keratinocytes *in vivo* (scRNA-seq), with positive correlation colored in red and negative correlation in blue.