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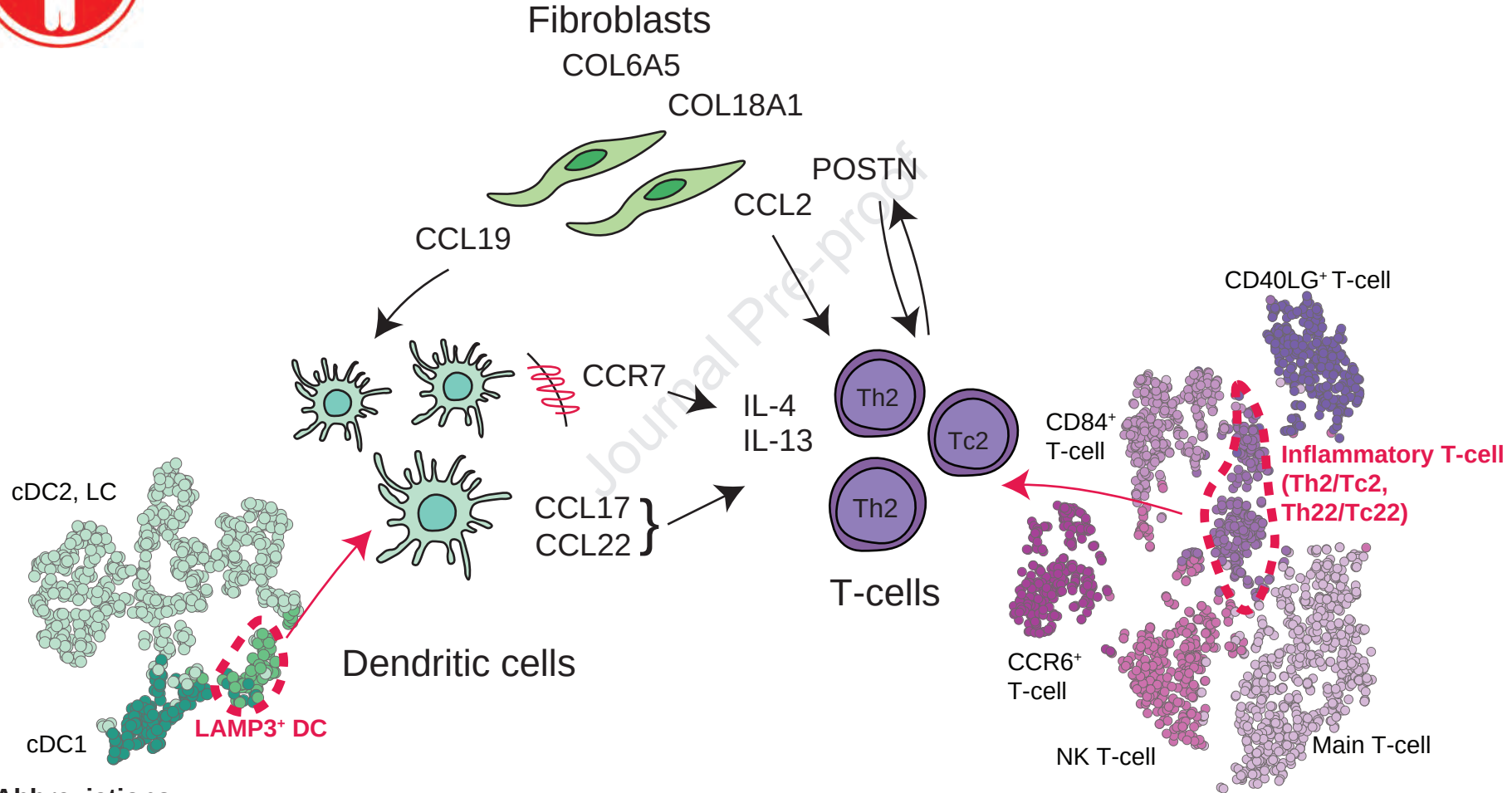
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Novel inflammatory fibroblasts cross-talk with DCs and T-cells, and may orchestrate T-cell migration and Th2/Tc2 polarization in AD



Abbreviations

cDC: Conventional dendritic cell; IL: Interleukin; LC: Langerhans cell; NK: Natural killer; Th: T-helper; Tc: T-cytotoxic

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Abstract**Background**

Atopic dermatitis (AD) is a prevalent inflammatory skin disease with a complex pathogenesis, involving immune cell and epidermal abnormalities. Despite whole tissue biopsy studies that have advanced the mechanistic understanding of AD, single-cell-based molecular alterations are largely unknown.

Objective

To construct a detailed, high-resolution atlas of cell populations, and to assess variability in cell composition and cell-specific gene expression in the skin of AD patients versus controls

Methods

We performed single-cell RNA-sequencing on skin biopsies from 5 patients with AD (4 lesional samples, 5 non-lesional samples) and 7 healthy control subjects, using 10x Genomics.

Results

We created transcriptomic profiles for 39,042 AD (lesional and non-lesional) and healthy skin cells. Fibroblasts demonstrated a novel *COL6A5*⁺*COL18A1*⁺ subpopulation that was unique to lesional AD, and expressed *CCL2* and *CCL19* cytokines. A corresponding *LAMP3*⁺ dendritic cell (DC) population that expressed the *CCL19* receptor *CCR7* was also unique to AD lesions, illustrating a potential role for fibroblast signaling to immune cells. Lesional AD samples were characterized by expansion of inflammatory DCs (*CD1A*⁺*FCER1A*⁺) and tissue resident memory T-cells (*CD69*⁺*CD103*⁺). The frequencies of type 2 (*IL13*⁺)/type 22 (*IL22*⁺) T-cells were higher than type 1 (*IFNG*⁺) in lesional AD, while this ratio was diminished slightly in non-lesional AD and further in controls.

Conclusion

70 AD lesions were characterized by expanded type 2/type 22 T-cells and inflammatory DCs, and a
71 unique inflammatory fibroblast that may interact with immune cells to regulate lymphoid cell
72 organization and type 2 inflammation.

Clinical Implications

This is the first single-cell transcriptome defining constitutive cytokine activation by fibroblasts and polarized T-cells in AD skin, suggesting these inflammatory products as potential therapeutic targets. Single-cell profiling may be used to evaluate therapeutic responses in clinical trials for inflammatory skin diseases.

Capsule Summary

Single-cell transcriptomic profiling of AD reveals distinct compositional and cell-specific gene expression changes, and a novel inflammatory fibroblast subpopulation that may orchestrate T-cell migration/trafficking and type 2 immune responses.

Key Words: Atopic dermatitis, single-cell RNA-sequencing, fibroblasts, dendritic cells, T-cells, cytokines

Abbreviations

AD: Atopic dermatitis

BSA: Body surface area

CCL: CC chemokine ligand

CXCL: C-X-C motif ligand

DC: Dendritic cell

FB: Fibroblast

IDEC: Inflammatory dendritic epidermal cell

Ig: Immunoglobulin

IL: Interleukin

- 96 IRS: Inner root sheath
- 97 KC: Keratinocyte
- 98 LC: Langerhans cell
- 99 LEC: Lymphatic endothelial cell
- 100 MAC: Macrophage
- 101 MEL: Melanocyte
- 102 NC: Neuronal cell
- 103 ORS: Outer root sheath
- 104 PC: Pericyte
- 105 PNS: Peripheral nervous system
- 106 POSTN: Periostin
- 107 SC: Schwann cell
- 108 scRNA-seq: Single-cell RNA-sequencing
- 109 SGC: Sweat gland cell
- 110 TC: T-cell
- 111 TEWL: Transepidermal water loss
- 112 Th: T-helper
- 113 TPM: Transcripts per kilobase million
- 114 T_{RM}: Tissue resident memory T-cell
- 115 VEC: Vascular endothelial cell
- 116 vSMC: Vascular smooth muscle cell

Introduction

Atopic dermatitis (AD) is a common inflammatory skin disease, affecting 7% of adults and 15% of children in the United States.^{1, 2} AD involves skin immune and barrier abnormalities, including T-cell and dendritic cell (DC) infiltration,^{3, 4} epidermal hyperplasia, and disrupted keratinocyte differentiation.^{4, 5} AD is primarily characterized by a type 2 immune response,³ but may also involve type 1, 17, and 22 pathways to varying degrees.⁶⁻⁸ In moderate-to-severe AD patients, defined as involving >10% body surface area, these abnormalities extend beyond the visibly affected skin to also involve non-lesional skin.⁹

Until recently, skin profiling was solely based on characterization of bulk tissue skin biopsies, using transcriptomic profiling approaches such as RNA-sequencing and microarrays to provide insights into AD pathogenesis.^{10, 11} While whole skin biopsies provided important information on the cytokine profile, they did not readily define the originating cell types. Single-cell RNA-sequencing (scRNA-seq) offers a novel approach to define the cellular composition in the skin, including novel or rare cell populations, and is able to resolve cell-specific changes in gene expression in AD versus controls. Current scRNA-seq studies in healthy skin are highly specific, focusing either on epidermal cells,¹² fibroblast subpopulations,^{13, 14} or the hair follicle.¹⁵ Single-cell analysis of skin and kidney biopsies from lupus patients revealed important insights in resolving cell type-specific interferon-stimulated gene expression and fibrotic events that may have prognostic value.^{16, 17} To our knowledge, deep single-cell profiling using the latest emulsion-capture technology with all epidermal and dermal cells in healthy skin is unavailable, and single-cell analysis of AD skin was never reported.

Here, we report scRNA-seq analysis for 39,042 cells obtained from three conditions: lesional and non-lesional skin from patients with AD and skin from healthy individuals. We used

140 established cell lineage markers to construct a comprehensive map of all cell types in healthy,
141 non-lesional, and lesional AD skin. We evaluated both composition and gene expression of cells
142 for each condition, including detailed characterization of specific immune cell subsets. Finally,
143 we identified abundantly expressed receptors and ligands for each cell type to highlight putative
144 intercellular communication in the skin microenvironment.

Methods

Patient and sample information

Lesional and non-lesional skin biopsies were taken from the extremities of 5 moderate-to-severe AD patients (mean SCORAD 68.7, EASI 20.7, IGA 3, BSA 44.4, Eosinophils 500/ μ L) and 7 matching controls. All subjects provided IRB-approved consent. AD patients had chronic disease (age of onset <5 years old), no topical steroid/immunomodulator use in the last week, no systemic immunosuppressants or phototherapy in the last four weeks, and no moisturizer use within 12hrs.

Single-cell capture, sequencing, and data processing

Biopsies were cryopreserved, dissociated, and processed by 10x Genomics. The library was sequenced on the Illumina HiSeq 2500 platform to get ~150-200 million reads. The 10x raw data were processed using the standard Drop-seq pipeline (<http://mccarrolllab.com/dropseq/>), with modifications.

Clustering analysis using Seurat and t-SNE visualization

Clustering analysis using the *Seurat* package (version 2.3.4) was implemented for both first-level analysis across all cells, and for second-level higher-resolution analysis within cell types. ScRNA-seq datasets were combined into a single Seurat object and aligned by canonical correlation analysis (CCA) to remove batch effects (Fig E1A). Cells with <100 genes, >5,000 genes, or >25% mitochondria content were excluded. Ubiquitously expressed ribosomal protein-coding (RPS and RPL) and MALAT noncoding RNA, miRNA, and snoRNA genes were also excluded. Wilcoxon rank sum test was used to identify differentially expressed genes (DEGs) in

each cluster to define broad cell types, and then repeated for each resultant cell type to further characterize subpopulations. Read counts for each gene were normalized to 10,000 to generate TPM-like values. After CCA alignment, each cluster was represented by all samples (Fig E1B-I).

Composition, gene expression, and receptor-ligand interaction analyses

Pairwise comparisons were performed for all conditions, using unpaired t-test for lesional or non-lesional versus healthy samples, and paired t-test for lesional versus non-lesional samples. *SingleR* was used to annotate T-cells with fine-tuning, using the Human Primary Cell Atlas as the reference dataset (http://biogps.org/dataset/BDS_00013/primary-cell-atlas/).¹⁸ DEG analysis was performed using *DESeq2*, using the criteria absolute value of log (fold-change)>1 and false discovery rate<0.05. To identify potential intercellular interactions, we used a list of ligand-receptor pairs from the Database of Interacting Proteins (<http://dip.doe-mbi.ucla.edu>) and the IUPHAR/BPS guide to pharmacology (www.guidetopharmacology.org), as described earlier.¹⁹ The list of interactions was intersected with the list of DEGs and with TPM-normalized gene levels (Table E1). See the Online Repository for details.

RESULTS

Strategy for scRNA-seq and data analysis

Cell suspensions, prepared upon dissociation of cryostored skin biopsies from 5 AD patients (4 lesional, 5 non-lesional) and 7 controls, were subjected to droplet-based encapsulation (using 10x Genomics)²⁰ followed by library preparation and sequencing (Fig 1A-B). Bioinformatics analysis identified 22,220, 10,169, and 6,653 high quality scRNA-seq profiles from healthy, lesional AD, and non-lesional AD samples, respectively, which were aggregated and analyzed by graph-based clustering (Fig 1C-D). Average expression profiles for each sample are listed in Table E1.

Cell type composition of healthy, non-lesional, and lesional skin

DEG analysis (Wilcoxon rank sum test) segregated 39,042 combined (healthy, lesional, non-lesional) cells into 10 broad cell types: keratinocytes (KC1-3), fibroblasts (FB1-3), pericytes and vascular smooth muscle cells (PC-vSMC), melanocytes (MEL), neuronal and Schwann cells (NC-SC), sweat gland cells (SGC), vascular endothelial cells (VEC1-2), lymphatic endothelial cells (LEC), macrophages and DCs (MAC-DC), and T-cells (TC) (Fig 1C-D, Table E2). We determined the relative proportion of each cell type in all samples, showing increased immune cells in lesional AD versus controls (Fig E2).

KCs separated into 3 subpopulations by first-level tSNE analysis (Fig 1C-D), but second-level analysis further separated them into 7 subpopulations, namely basal, proliferating, suprabasal, inner root sheath (IRS)-sebaceous, outer root sheath (ORS), channel-ATPase, and channel-gap (Fig 2A). Basal KCs highly expressed *KRT5*, *KRT14* and *KRT15*, while suprabasal KCs specifically expressed early differentiation keratins (*KRT1*, *KRT10*), and also highly expressed hyper-proliferative (*KRT6A*, *KRT16*) and S100 (*S100A7*, *S100A8*) genes (Fig E3A).

Proliferating KCs were characterized by expression of mitotic markers like *UBE2C* and *TOP2A*. Sebaceous glands are lipid producing epithelial cell structures that drain sebum to the infundibulum through a duct at the junction of infundibulum and isthmus.²¹ A combined subpopulation of IRS and sebaceous gland cells (IRS-sebaceous) expressed *KRT79* and genes involved in lipid synthesis (*FASN*, *THRSP*, *ELOVL5*), metabolism (*FADS2*, *ACSBG1*), and transport (*APOC1*). A cluster of *KRT6B*⁺*KRT17*⁺ KCs corresponded to ORS cells. Finally, two clusters expressed genes that regulate ion channels. ‘Channel-ATPase’ expressed components of ATPases (*ATP1B1*, *ATP1A1*) and its regulators (*DEFB1*, *SAT1*), while ‘channel-gap’ consisted of gap junctions, specifically expressing *GJB6*, *GJB2*, and *GJA1*.

Fibroblasts (FB1-3) expressed *COL1A1* and *DCN*, and separated into four subgroups on second-level analysis (Fig 2B, Table E2). The two major FB populations both expressed lipid transport/homeostasis (*APOD*, *APOE*, *ABCA6*) and complement genes (*C3*), but were distinguished by *FBN1* and *MFAP5* expression (Fig 2B, E3B). A third minor FB population expressed fibrocartilage- (*COL11A1*) and myofibroblast- (*POSTN*, *EDNRA*) related genes, suggesting that they may be in a differentiating state. Finally, a previously unrecognized FB subtype expressed *COL6A5*, *COL6A6*, and *COL18A1*, along with chemokines *CCL2* and *CCL19*.

First level analysis showed that PC-vSMCs expressed *TAGLN* and *ACTA2* (Fig 1C-D), which further separated into pericyte (PC1-2) and vSMC clusters distinguished by expression of *RGS5*, *PDGFRB*, *NOTCH3*, and *REGL*, *CNN1*, respectively (Fig 2C, E3C). The PC-2 population had high *CD36* and *FABP4*, suggesting their involvement in fatty acid transport across blood vessels.²²

Melanocytes (MELs), neuronal cells (NCs), and Schwann cells (SCs), which initially clustered together, formed separate clusters and were identified by established lineage markers

MLANA, *NRXN1*, and *GLDN*, respectively (Fig 2D, Fig E3D). Interestingly, MELs expressed lipid transport (*APOE*) and cholesterol biosynthesis (*FDFT1*) genes, and follicular stem cell marker *PHLDA1*. NCs expressed genes corresponding to sodium channels subunits (*SCN7A*), axons (*ANK3*, *SEMA3B*), and cell adhesion (*CADM1*, *CADM3*). SCs expressed the tight junction gene *TJP1*, Na^+/K^+ ATPase regulator *FXYD6*, and creatine synthesis protein *GATM*.

Sweat gland cells (SGCs) that form the secretory coil are broadly of two secretory cell types: clear cells that secrete sodium chloride-rich sweat and dark cells that secrete glycoproteins and *DCD*/dermicidin).²³ Sub-clustering analysis differentiated clear from dark cells, respectively expressing *CA2* and *MUCL1* (Fig 2E, E3E). Although all SGCs expressed secretoglobins such as *SCGB1D2* and *SCGB2A2*, only dark cells expressed *SCGB1B2P*.

All VECs expressed canonical markers *PECAM1* and *VWF* (Fig 1D). Further analysis separated VECs into venous/venular, capillary and arterial/arteriolar origins based on their respective expression of *ACKR1*, *GPIHBP1*, and *HEY1* lineage markers (Fig 2F). Capillary VECs abundantly expressed genes involved in fatty acid transport (*CD36*, *FABP4*) (Fig E3F). Arterial/arteriolar VECs expressed regulators of lymphatic vessel migration and formation (*SEMA3G* and *CXCL12*) and ion exchangers (*SLC9A3R2*, *SLC12A2*, *SLC6A6*, *ATP13A3*). An additional cluster of venous/venular VECs expressed *NR2F2*, *SELP*, and *CCL23*, likely reflecting a state of vascular inflammation. LECs did not further sub-cluster.

MAC-DCs separated into one macrophage (MAC) and two DC (DC-A-B) populations (Fig 2G, E3G). MACs were identified by expression of *RNASE1*, *CD68*, and *SEPP1*. Although both DC clusters expressed *CD1C*, DC-A expressed higher *CD1A*, a LC marker, and *MRC1/CD206*, a marker of immature and inflammatory DCs.²⁴

TCs split into 6 clusters, namely TC-main, TC-inflam, *CD40LG*⁺, *CD84*⁺, *CCR6*⁺, and NK T-cells (NKT) (Fig 2H, E3H). TC-inflam was distinguished by high expression of AD-related cytokine *CSF2*,²⁵ but also abundantly expressed type 2 cytokine *IL13*, type 22 cytokine *IL22*, *TNFRSF4/OX40*, and *TNFSF14/LIGHT*, likely representing activated and polarized TCs. The *CD40LG*⁺, *CD84*⁺, and *CCR6*⁺ subgroups likely represent TCs with specialized roles in mediating B-cell activation/class-switching, immune regulation via formation of hemophilic dimers,²⁶ and recruitment of Th17 and regulatory T-cells, respectively. Finally, NKTs expressed *CD3D*, but also *NKG7*, *GZMA*, and *GZMK*, highlighting their cytotoxic nature.

To assess the quality of cells from cryopreserved versus fresh samples, an additional fresh biopsy was taken from a healthy volunteer, and subject to the same analysis, revealing a cell type landscape that overwhelmingly consisted of KCs (92% of all cells) (Fig E4). In the fresh healthy sample, 145 FBs and 42 VECs were captured from a total of 4,161 cells. In contrast, averaging together all cryopreserved healthy samples, 744 FBs and 821 VECs were captured out of a comparable total of 3,174 cells.

Novel inflammatory *COL6A5*⁺*COL18A1*⁺ fibroblast subpopulation in atopic dermatitis lesions

Most clusters, including fibroblasts, were distributed among all conditions (Fig E5). However, *COL6A5*⁺*COL18A1*⁺ FBs primarily comprised of lesional AD cells (Fig 3A). These FBs expressed inflammatory cytokines (*CCL2*, *CCL19*, *IL32*) and ECM products that are induced by type 2 inflammation (*POSTN*, *TNC*, *VCAM1*) (Fig 3B, Table E2).²⁷⁻³⁰ All lesional AD samples showed expansion of *COL6A5*⁺*COL18A1*⁺ FBs (Fig 3C), which were not reported by previous

studies on healthy fibroblasts (Fig E6).^{13, 14} *MFAP5⁺FBN1⁺* FBs were expanded in both lesional and non-lesional AD compared to healthy skin (Fig 3C).

DEG analysis of FBs demonstrated that *POSTN*, S100 genes (*S100A10*, *S100A11*) and *CRIP1*, a zinc finger suggested to induce type 2 cytokine production in model systems,³¹ were up-regulated, while *PTGDS*, a negative regulator of cell migration/invasion and platelet aggregation, was down-regulated, in lesional and/or non-lesional AD versus controls (Fig 3D, Table E3).³² *CCL26*, perhaps the best marker of response to type 2 targeting by dupilumab,³³ was highly expressed by lesional AD FBs, primarily in *COL6A5⁺COL18A1⁺* and *MFAP5⁺FBN1⁺* FBs (Fig 3D-E). Like FBs, lesional PCs highly expressed *CCL2*, *CCL19*, and *CCL26* (Fig 3F).

To validate and determine the anatomical location of these *COL6A5⁺COL18A1⁺* FBs, we performed immunohistochemistry staining with cell counts for representative markers (Fig 3G, Fig E7A). *COL6A5⁺* cells were significantly increased in both lesional and non-lesional AD versus controls, most prominently in the upper dermis near the dermo-epidermal junction. AD lesions showed increased infiltration of *CCL2⁺* cells compared to non-lesional skin and controls, with similar trends for *CCL19*, and *POSTN*. Immunofluorescence staining further confirmed *COL6A5⁺CCL19⁺* and *COL6A5⁺POSTN⁺* double staining in the upper dermis of AD lesions, likely by fibroblast cells (Fig 3H). *COL6A5⁺* and *CCL19⁺* cells were juxtaposed to *CD3⁺* T-cells, further supporting a role for these inflammatory FBs in T-cell recruitment and organization (Fig E7B).

Compositional differences and differentially expressed genes in keratinocytes and other non-immune cell types in atopic dermatitis compared to controls

Select KC subpopulations were further sub-clustered for more detailed profiling of rare subsets. The $TOP2A^+UBE2C^+$ proliferating KC cluster separated into KC.proliferating.1 (expressing *MGST1*, *APOE*, *KRT17*) and KC.proliferating.2 (*KRT1*, *CCL27*) (Fig 4A). KC.proliferating.1, likely rapidly dividing cells in the hair follicle, contained mostly healthy cells, while KC.proliferating.2 overwhelming comprised of lesional AD cells. Suprabasal KCs resolved into two $KRT1^+KRT10^+$ subgroups, one of which was predominated by lesional AD cells and expressed keratins enriched in hyper-proliferative and wound healing states (*KRT6*, *KRT6A*, *KRT16*),³⁴ consistent with epidermal hyperplasia (Fig 4B). Accordingly, lesional AD samples demonstrated increased proportions of $KRT1^+KRT10^+$ and $KRT6A^+KRT16^+$ KCs compared to controls (Fig 4C).

This marked epidermal hyperplasia in lesional AD KCs coincided with increased expression of genes associated with epidermal proliferation, including S100 (*S100A7*, *S100A8*, *S100A9*) and protease inhibitor (*SERPINB4*) genes (Fig 4D, Table E3), consistent with previous bulk RNA-sequencing.¹⁰ The type 2 chemokine *CCL2* was significantly up-regulated in lesional AD versus controls. *CCL27*, required for T-cell recruitment and shown to cause allergen-specific skin inflammation,³⁵ and *AQP3*, a water channel component associated with KC proliferation and trans-epidermal water loss,³⁶ were increased in lesional and moderately in non-lesional AD KCs. While lesional AD increases in *CCL27* were seen in both basal and suprabasal KCs, *CCL2* and also pro-inflammatory cytokine *IL32* were only increased in basal KCs, while *KRT16*, *AQP3*, and S100s (*S100A2*, *S100A10*) were only increased in suprabasal KCs (Fig 4E-F, Table E3). Pathway enrichment analysis using the DAVID functional annotation tool³⁷ showed lesional AD KCs with enrichment of terms like epidermis development, immune response, and response

to wound healing, whereas healthy KCs were enriched in negative regulation of cell differentiation, regulation of growth, and regulation of lipid metabolic process (Fig 4G).

Identification and expansion of $LAMP3^+CCR7^+$ and other dendritic cell subsets in atopic dermatitis

The major DC subsets in healthy human skin include epidermal Langerhans cells (LC) and dermal conventional DCs (cDCs), further subdivided into type 1 (cDC1) and type 2 (cDC2) subsets.³⁸ DC.A and DC.B were combined and further resolved into three clusters (Fig 5A). The largest subgroup had highest *CD1C* and also uniquely expressed *CD11C/ITGAX* and *CD207/langerin*, likely corresponding to a combined cDC2 and LC cluster (cDC2-LC). The second cluster highly expressed the cDC1 markers *CLEC9A* and *IRF8*. Finally, a small but important population expressing *LAMP3*, a marker of mature DCs, consisting primarily of lesional AD cells, was distinguished by robust expression of the type 2 chemokines *CCL17* and *CCL22*, and the *CCL19* receptor *CCR7* (Fig 5A-B). cDC2 ($CD11C^+$) and $LAMP3^+CCR7^+$ DCs, and also $CD68^+$ MACs were expanded in lesional AD versus controls (Fig 5C). Proportional frequencies of other DC subsets that were previously found in the skin³⁹⁻⁴² but were not resolvable by clustering, likely due to their rarity, were calculated based on characteristic markers (Fig 5D). Inflammatory dendritic epidermal cells (IDECs), which are $CD1A^+FCER1A^+$ and highly specific to AD,⁴¹ were expanded in lesional AD relative to other DC subsets. $BDCA3^+$ DCs, which are tolerogenic and promote regulatory T-cell differentiation,³⁹ and $BDCA2^+$ plasmacytoid DCs, were also slightly higher in lesional AD than controls (Fig 5D). Lesional AD MAC-DCs showed increases in type 2 chemokines *CCL13* and *CCL18*, which are expressed by MACs (Fig 5E-F).

Expansion of CD8⁺ tissue resident memory T-cells, Th2/Tc2, and Th22 cells in AD lesions

Because mitotic cells clustered together based on characteristic markers (e.g. *UBE2C*, *TOP2A*), the proliferating KC subpopulation also featured a proliferating TC cluster, dominated by lesional AD cells (Fig 4A). Resident memory T-cells (T_{RM}), which express *CD69* and/or *CD103*,^{43, 44} were significantly expanded in lesional AD compared to healthy and non-lesional AD skin, only within the CD8⁺ subset (Fig 6A). Immunofluorescence co-staining also showed relative expansion of *CD69*⁺*CD103*⁺ cells, especially in the epidermis, for lesional AD (45 cells; 24 in epidermis, 21 in dermis) compared to non-lesional AD (17 cells; 13 in epidermis, 4 in dermis) and controls (1 cell; 0 in epidermis, 1 in dermis) (Fig 6B). The frequency of *CD4*⁺/*CD8*⁺ type 2 (*IL13*⁺) and type 17 (*IL26*⁺) cells was significantly increased in lesional AD compared to both healthy and non-lesional AD samples (Fig 6A). Type 22 (*IL22*⁺) TCs were also expanded in lesional AD, more in the *CD4*⁺ subset, while type 1 (*IFNG*⁺) TCs were comparable in AD and controls.

The proportion of TCs that were type 2, type 22, or type 17 was markedly higher (2-98 to 3.87-fold) compared to type 1 in lesional AD (type 2: 22.9%, type 22: 20.1%, type 17: 17.6%, type 1: 5.9%), with a similar trend for non-lesional AD (type 2: 11.9%, type 22: 12.7%, type 17: 11.9, type 1: 6.0%), while healthy controls showed the reverse trend, with type 1 cells outnumbering both type 2 and type 22 (type 2: 2.7%, type 22: 0.95%, type 17: 4.4%, type 1: 5.1%) (Fig 6C). Similar trends were observed within *CD4*⁺ and *CD8*⁺ subsets. A higher percentage of proliferating *UBE2C*⁺*TOP2A*⁺ lesional AD TCs were identified to be *IL13*⁺ (33.9%), *IL22*⁺ (20.7%), or *IL26*⁺ (38.0%) compared to *IFNG*⁺ (8.3%), suggesting active expansion of memory/effector type 2, 22, and 17 cells in AD lesions.

Lesional TCs showed elevated expression of T-cell activation and monocyte chemotactic chemokine *CCL1*, type 2/type 17 cytokines *IL13* and *IL26*, and annexin genes (*ANXA1*, *ANXA2*) that have various roles in regulating inflammation, wound healing, and apoptosis (Fig 6D, Table E3). Taking only *CD4*⁺ cells, lesional AD showed additional up-regulation of *TNFRSF4/OX40*, which promotes type 2 responses and may be a therapeutic target in AD,⁴⁵ *TNFSF10/TRAIL*, a TC activation and pro-apoptotic marker, *TNFRSF18*, which may function in self-tolerance,⁴⁶ and *IFITM2*, an interferon-induced gene (Fig 6E). Pathway enrichment analysis revealed enrichment of immune response, antigen processing and presentation, and regulation of apoptosis in lesional AD TCs (Fig 6F).

Cell-cell communication in atopic dermatitis

To investigate potential intercellular communications among cell types, we visualized average expression levels of the most abundant (>3 TPM) ligands and their cognate receptors (>0.4 TPM) that were differentially expressed in lesional AD versus controls (Fig 7). In AD lesions, the interaction between *CCL19* produced by inflammatory *COL6A5*⁺*COL18A1*⁺ FB subpopulation, and *CCR7* on TCs and MAC-DCs, particularly *LAMP3*⁺ DCs, is critical for regulating lymphoid cell organization and trafficking. The type 2 chemokine *CCL2* was abundantly expressed by inflammatory FBs and KCs, while its receptors *CCR1* and *CCR2* were expressed on MAC-DCs. Lesional TCs highly expressed the key type 2 cytokine *IL13*, signaling via *IL4R* and *IL13RA1* on FBs and PC-vSMCs. *CCL27* was up-regulated in lesional KCs, suggestive of signaling via *CCR10* on TCs, highlighting communication between primarily “non-immune” (e.g. FBs, KCs) and immune cells.

DISCUSSION

We present, to our knowledge, the first comprehensive, high-resolution, single-cell transcriptomic analysis of all cell types and expression programs from patients with AD compared to healthy controls. Our atlas of combined AD and healthy skin comprises 10 broad cell types. Through high-resolution sub-clustering, we identified rare subpopulations unreported in previous scRNA-seq skin studies.¹²⁻¹⁵ Examples include clear and dark cells in the secretory coil of sweat glands, and gap junctions and ATPase channels in KCs, which concur with but add further resolution to ‘channel KCs’ identified by Cheng et al.¹²

Previous bulk RNA-sequencing and microarray transcriptomic studies on AD skin revealed dysregulation of inflammatory (*IL13*, *CCL2*) and barrier (*KRT16*, *S100A8*, *S100A9*) genes,^{10, 47-49} but many markers, including those representing polar T-helper cytokines, often remain masked in whole-skin transcriptomic analyses.^{10, 48, 49} Here, we provide cell type level resolution of primary cytokines associated with AD (e.g. *IL13*, *IL22*), and identified novel *COL6A5*⁺*COL18A1*⁺ FBs that produce key molecules driving AD inflammation (e.g. *CCL2*, *CCL19*, *POSTN*, *TNC*).

This FB cluster, unique to AD lesions, harbors a distinct collagen expression pattern, with high levels of *COL6A5* and *COL18A1*. While we localized *COL6A5* to the papillary dermis, and particularly near the dermo-epidermal junction, consistent with previous reports,^{13, 50, 51} we now also demonstrate marked *COL6A5* expansion in AD lesions. *COL6A5* has been identified as an AD susceptibility gene,⁵⁰ though these studies were inconsistent⁵²⁻⁵⁶ and without a clear mechanism. The pathogenic role of *COL6A5* in AD may be due to the formation of unstable heterotrimers leading to aberrant fibroblast adhesion, collagen synthesis and metabolism, and barrier disruption.⁵¹ *COL6A5* is also found in other sites of allergic disorders (e.g. skin, lung,

small intestine, colon), and may promote allergen sensitization and inflammation by regulating LCs and T-cells.⁵⁰ *COL18A1*/endostatin potently inhibits angiogenesis and binds to several other ECM components,⁵⁷ and may similarly contribute to ECM disorganization and instability in AD.

Along with promoting barrier and structural instability, these FBs also have an inflammatory role. They highly express *CCL2*, implicating a function in type 2 cell polarization,⁵⁸ and *CCL19*, which is important for lymphocyte recirculation, homing, and migration to secondary lymphoid organs.⁵⁹ The cross talk between fibroblasts and DCs in AD is supported by the lesional skin enrichment of mature (*LAMP3*⁺) DCs expressing *CCR7*, the receptor for *CCL19*, and type 2 chemokines *CCL17* and *CCL22*, suggesting that FBs interact with macrophages and DCs to facilitate T-cell trafficking, lymphoid tissue organization, and type 2 cell recruitment. These FBs also express *POSTN* and *TNC*, which encode ECM proteins that are induced by the defining and pro-fibrotic type 2 cytokines *IL4* and *IL13*,^{60, 61} up-regulated in AD,^{28, 62} and may further propagate type 2 inflammation and fibrotic remodeling in a vicious inflammatory cycle. These FBs may also play a role in fibrotic skin conditions that also have an inflammatory (type 2) component (e.g. scleroderma, keloids).^{63, 64} Recent studies describing stromal cells in lung and other tissues that promote type 2 responses (e.g. *IL33*, *TSLP*, *CCL18*), and are inducible by *IL4/IL13*, further support a role for fibroblasts in mediating type 2 immunity and atopy across organ systems.^{63, 65-67}

These inflammatory *COL6A5*⁺*COL18A1*⁺ FBs were unrecognized by prior single-cell studies on healthy dermal fibroblasts.^{13, 14} While our overall FB composition, including *COL11A1*⁺ FBs, agreed with these studies, we report different gene expression patterns in the major clusters, likely reflecting the plasticity within fibroblasts and/or transcription responses to increased inflammatory cytokines in AD. For example, while Tabib et al. found that *FMO1*⁺ FBs

constituted a major population, we found very low levels of *FMO1* in FBs.¹⁴ Interestingly, Philippeos et al. did report a FB cluster that expressed *COL6A5*, but lacked other pro-inflammatory properties, and instead attributed this cluster to general papillary fibroblasts.¹³

ScRNA-seq provides a direct, reliable, and more detailed characterization of immune subsets in AD, compared to indirect cell origin inference by open chromatin analysis used in bulk transcriptomic studies.⁴⁷ For example, actively proliferating *UBE2C*⁺*TOP2A*⁺ type 2/type 22 TCs were expanded in lesional AD, and were either absent or less abundant in non-lesional and healthy samples. T_{RM} cells, which are crucial to cutaneous homeostasis in healthy skin but become aberrant in certain autoimmune and inflammatory diseases like psoriasis,^{43, 44, 68} and while not previously reported in AD, were expanded in AD lesions. It is unclear whether T_{RM} expansion constitutes a homeostatic response to inflammation or an active contributor to AD pathogenesis. In future studies, to fully understand the role of T_{RM} persistence in disease recurrence, as is seen in psoriasis,^{30,31,47} it would be beneficial to evaluate T_{RM} expression in resolved AD lesions after successful treatment.

While flow cytometry studies showed increases in type 2/type 22 populations in AD lesions,^{69, 70} the current study shows preferential activation of Th2/Tc2 and Th22 TCs, as compared to controls, where type 1 TCs are preferentially expanded. This study is able to define transcriptionally activated cells that are producing polar T-cell cytokines, without the artificial activation stimulus required for flow cytometric studies. Respective interactions between *CCL2* and *CCL27* in KCs and *CCR2* and *CCR10* on TCs likely promote type 2/type 22 differentiation, suggesting that KCs are part of a “feed forward” inflammatory response by producing chemokines that promote AD-related immune abnormalities. In turn, type 22 TCs mediate many of the compositional or gene expression changes observed in KCs, including S100 gene

production and epidermal hyperplasia,⁷⁰ as reflected by enrichment of hyper-proliferative (*KRT6A*, *KRT16*) and early differentiation (*KRT1*, *KRT10*) keratins.

This study has some limitations. We were unable to recover certain KC populations like stratum corneum cells expressing late differentiation markers (e.g. *FLG*), possibly attributed to cell damage induced by cryopreservation and/or cell type sensitivity to enzymatic digestion. However, we still characterized several keratinocyte subpopulations corresponding to multiple anatomic compartments of the epidermis, aligning with previously published scRNA-seq on keratinocyte populations in healthy fresh tissue.¹² Our data suggest that cryopreservation may even improve recovery of dermal cell populations like FBs and VECs. Secondly, our study involved a limited number of samples, restricting our ability to use graph-based clustering to resolve certain cell types, like DCs or *CD4*⁺ versus *CD8*⁺ TCs, an issue shared by other scRNA-seq skin studies,¹² requiring us to identify these groups by other means like automatic cell annotation tools. Our findings require validation with a larger cohort.

This is the first detailed single-cell transcriptomic profiling of lesional and non-lesional skin from AD patients compared to healthy controls, providing new insights into the complex interplay of cell types and gene expression changes involved in AD pathogenesis. Our study demonstrates the utility of scRNA-seq on small cryopreserved skin biopsies to detect disease-related compositional and gene expression characteristics in AD and other inflammatory skin diseases at steady states or in response to therapeutics during clinical trials. We are providing a detailed molecular map of various cell types and potential interactions involved in orchestrating AD-specific inflammation and barrier alterations. This previously unrecognized fibroblast cluster with inflammatory properties is of particular interest, because while immune and barrier abnormalities are established in AD, the role of ECM cells and genes in these processes are

478 largely unknown. These fibroblasts may signal to immune cells to mediate ECM breakdown,
479 fibrotic remodeling, and T-cell recruitment and polarization, requiring validation in future
480 studies. These cells and their respective products are potential future therapeutic targets in AD
481 and other inflammatory or fibrotic skin diseases.

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Main Figure Legends

Figure 1. scRNA-seq atlas of cell populations in atopic dermatitis and healthy skin. (A)

Cross-sectional schematic illustrating diverse cell populations in skin. (B) Workflow for single-cell profiling of skin, including biopsy dissociation, droplet-based scRNA-seq, and graph-based clustering. (C) tSNE-plot for 39,042 skin cells, derived from 5 atopic dermatitis patients (4 lesional, 5 non-lesional samples) and 7 healthy subjects. (D) Distinct gene signatures (top 10 differentially expressed genes, Wilcoxon rank sum test.) of skin cell populations. DC, dendritic cell; FB, fibroblast; KC, keratinocyte; LEC, lymphatic endothelial cell; MAC, macrophage; MEL, melanocyte; NC, neuron cell; NKT, natural killer T-cell; PC, pericyte; SC, Schwann cell; SGC, sweat gland cell; TC, T-cell; VEC, vascular endothelial cell; vSMC, vascular smooth muscle cell

Figure 2. Heterogeneity of cell subpopulations in atopic dermatitis and healthy skin. tSNE

plots and accompanying violin plots of representative genes for (A) keratinocytes, (B) fibroblasts, (C) pericytes and vascular smooth muscle cells, (D) melanocytes, neuron cells, and Schwann cells, (E) sweat gland cells, (F) vascular endothelial cells, (G) macrophages and dendritic cells, (H) T-cells. IRS, inner root sheath; NK, natural killer; ORS, outer root sheath; vSMC, vascular smooth muscle cell

Figure 3. Novel fibroblast subpopulation in lesional atopic dermatitis. (A) tSNE plot of

combined fibroblast cells, color-coded by tissue type/condition (healthy, non-lesional AD, and lesional AD), with the $COL6A5^+COL18A1^+$ and $FBN1^+MFAP5^+$ fibroblast clusters delineated by a dotted black line. (B) Feature plots displaying expression of specific markers among the set of all fibroblasts: *DCN*, *COL6A5*, *COL18A1*, *CCL2*, *CCL19*, *TNC*. (C) Frequencies of $COL6A5^+COL18A1^+$ and $MFAP5^+FBN1^+$ fibroblasts, as a proportion of all fibroblasts, with

pairwise comparisons (with accompanying P -values for $P < 0.1$) among disease conditions. **(D)** Unsupervised clustering heatmap showing relative expression (z-score) levels of the top 50 most abundantly and differentially expressed genes in fibroblasts using criteria log fold-change > 1 and FDR < 0.05 , calculated on samples with > 50 fibroblasts. **(E)** Violin plots showing *CCL26* mRNA expression, written as log transcripts per kilobase million, within fibroblast subpopulations. **(F)** Heatmap showing relative expression (z-score) levels of *CCL19*, *CCL2*, *CCL26* in pericytes, calculated on samples with > 10 pericytes. **(G)** Cell counts for *COL6A5*, *CCL2*, *CCL19*, and *POSTN* in healthy, non-lesional atopic dermatitis, and lesional atopic dermatitis samples with pairwise comparisons and accompanying P -values for $P < 0.1$. **(H)** Immunofluorescence staining of *COL6A5* (green)/*CCL19* (red), *COL6A5* (green)/*POSTN* (red), and their respective overlays, in healthy, lesional atopic dermatitis, and non-lesional atopic dermatitis sample, with dotted line outlining the dermal-epidermal junction. Zoomed-in insert of *COL6A5*/*POSTN* co-staining in individual cells. LS, lesional; NL, non-lesional; tpm, transcripts per kilobase million.

Figure 4. Keratinocyte compositional and gene expression changes in atopic dermatitis. (A) tSNE plot of proliferating keratinocytes and T-cells, color-coded by cell subpopulation and by disease condition, with an accompanying heatmap of distinct gene signatures (top 10 differentially expressed genes, Wilcoxon rank sum test). **(B)** tSNE plot of suprabasal keratinocytes, color-coded by cell subpopulation and by disease condition, with an accompanying heatmap of distinct gene signatures (top 10 differentially expressed genes, Wilcoxon rank sum test). **(C)** Frequencies of *KRT1*⁺*KRT10*⁺ and *KRT16*⁺*KRT6A*⁺ keratinocytes, as percentage of all keratinocytes, with pairwise comparisons (and accompanying P -values for $P < 0.1$) among disease conditions (healthy, non-lesional atopic dermatitis, lesional atopic dermatitis). **(D)** Unsupervised clustering heatmap showing relative expression (z-score) levels of

the top 50 most abundantly and differentially expressed genes in keratinocytes using criteria log fold-change >1 , FDR <0.05 , calculated on samples with >50 keratinocytes. **(E)** Unsupervised clustering heatmap showing relative expression (z-score) levels of differentially expressed genes in basal keratinocytes using criteria log fold-change >1 , FDR <0.05 , calculated on samples with >10 basal keratinocytes. **(F)** Unsupervised clustering heatmap showing relative expression (z-score) levels of differentially expressed genes in suprabasal keratinocytes using criteria log fold-change >1 , FDR <0.05 , calculated on samples with >10 suprabasal keratinocytes. **(G)** Gene ontology (GO) terms enriched in lesional versus healthy keratinocytes, using DAVID. The top 5 most up-regulated and down-regulated gene sets in lesional AD versus controls, with lowest P -value that are composed of >3 genes, are depicted as bar plots displaying $-\log_{10}(P\text{-value})$. **(H)** Interactions between differentially expressed ligands and receptors in lesional atopic dermatitis versus healthy subjects. Arrows are pointing to the receptors, with the size of the arrow stem proportionate to expression levels of ligands in log-scale. Cutoffs were >3 transcripts per kilobase million for ligands and >0.4 transcripts per kilobase million for receptors. Cell-types were only included if they comprised >100 cells in over 50% of samples. LS, lesional; NL, non-lesional; tpm, transcripts per kilobase million

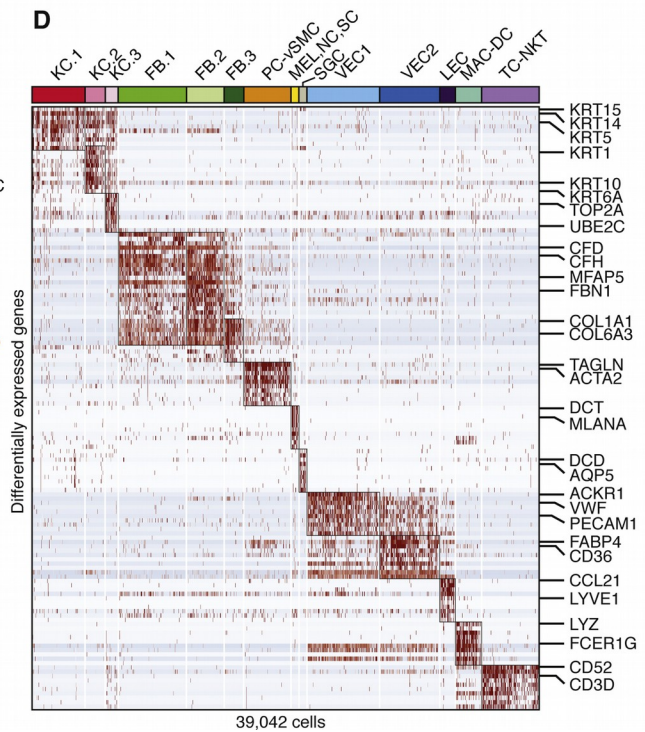
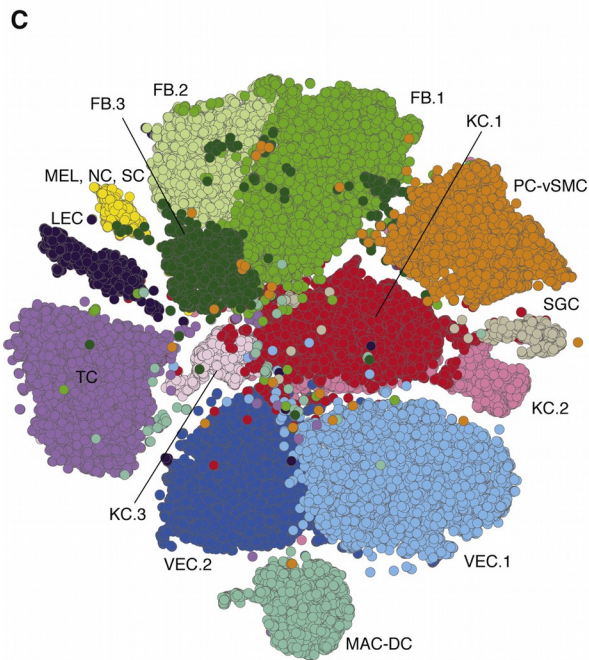
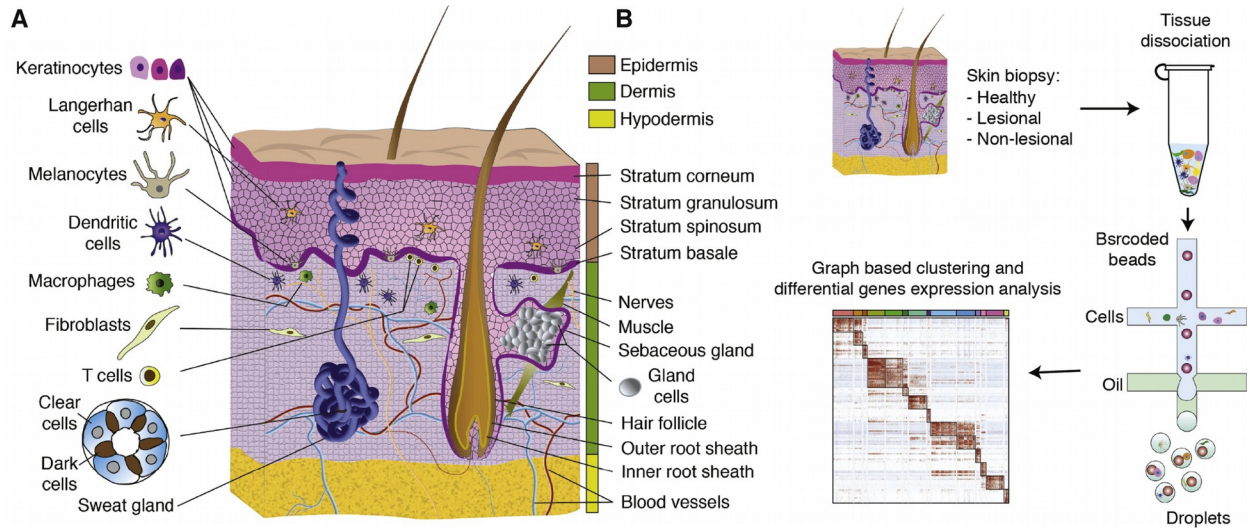
Figure 5. Compositional and gene expression changes among macrophages and dendritic cells in atopic dermatitis. **(A)** tSNE plot of dendritic cells, color-coded by cell subpopulation and by disease condition, with an accompanying heatmap of distinct gene signatures (top 10 differentially expressed genes, Wilcoxon rank sum test). **(B)** Violin plot showing *CCL17* mRNA expression, written as log tpm value, within dendritic cell subpopulations. **(C-D)** Frequencies of dendritic cell and macrophage subsets as a percentage of all macrophages and dendritic cells in the sample, with pairwise comparisons among disease conditions (healthy, non-lesional atopic

dermatitis, lesional atopic dermatitis), as obtained from (C) sub-clustering and from (D) previously defined lineage markers. (E) Unsupervised clustering heatmap showing relative expression (z-score) levels of differentially expressed genes in macrophages and dendritic cells using criteria log fold-change >1, FDR <0.05, calculated on samples with >10 macrophages and dendritic cells. (F) Violin plot showing *CCL13* and *CCL18* mRNA expression, written as log tpm, transcripts per kilobase million, within macrophage/dendritic cell subpopulations. cDC, conventional dendritic cell; IDEC, inflammatory dendritic epidermal cell; LS, lesional; MAC, NL, non-lesional; tpm, transcripts per kilobase million

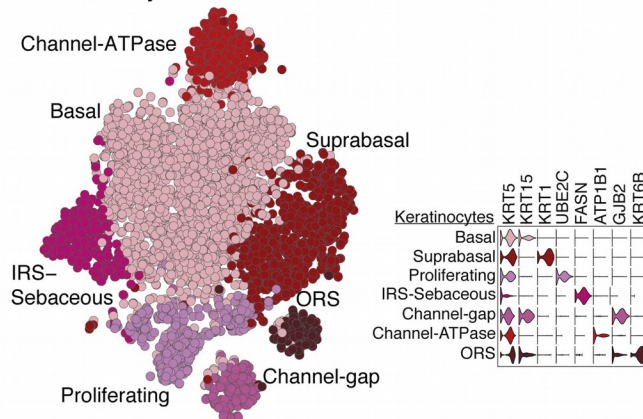
Figure 6. Compositional and gene expression changes among T-cells in atopic dermatitis.

(A) Frequencies of tissue resident memory T-cells ($CD69^+CD103^+$), Th2/Tc2 cells ($IL13^+$), Th22/Tc22 cells ($IL22^+$), Th1/Tc1 cells ($IFNG^+$), and Th17/Tc17 cells ($IL26^+$), as percentage of all T-cells, within both $CD4^+$ and $CD8^+$ subsets, with pairwise comparisons (and accompanying P -values for $P<0.1$) among disease conditions (healthy, non-lesional atopic dermatitis, lesional atopic dermatitis). (B) Immunofluorescence staining of tissue resident memory (T_{RM}) T-cell markers $CD69$ (red), $CD103$ (green), and $CD69/CD103$ overlay in healthy, non-lesional atopic dermatitis, and lesional atopic dermatitis sample, with dotted line outlining the dermal-epidermal junction. (C) Table of percentages of $IL13^+$, $IL22^+$, $IL26^+$, and $IFNG^+$ cells, as a percentage of all T-cells, all $CD4^+$ cells, and all $CD8^+$ cells, as well as $IL13^+/IFNG^+$, $IL22^+/IFNG^+$, and $IL26^+/IFNG^+$ ratios for each condition (healthy, non-lesional atopic dermatitis, lesional atopic dermatitis). (D) Unsupervised clustering heatmap showing relative expression (z-score) levels of the top 50 most abundantly and differentially expressed genes in T-cells using criteria log fold-change >1, FDR <0.05, calculated on samples with >50 T-cells. (E) Unsupervised clustering heatmap showing relative expression (z-score) levels of differentially expressed genes in $CD4^+$

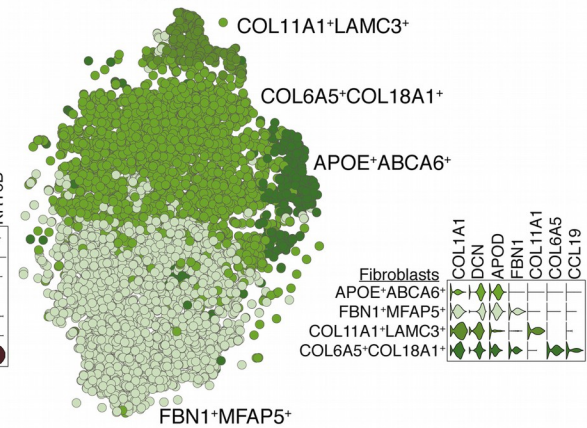
790 T-cells using criteria log fold-change >1 , FDR <0.05 . **(F)** Gene ontology (GO) terms enriched in
791 lesional AD versus healthy T-cells using DAVID. The 5 gene sets with lowest P -value that are
792 composed of >3 genes are depicted as bar plots displaying $-\log_{10}(P\text{-value})$.



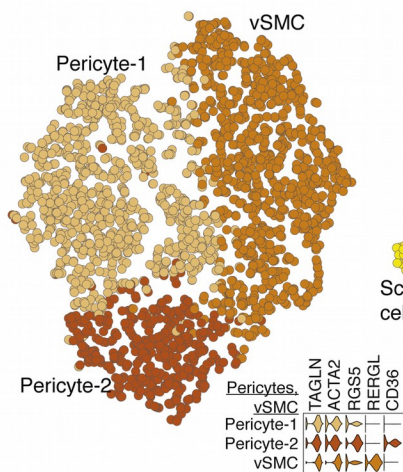
A. Keratinocytes



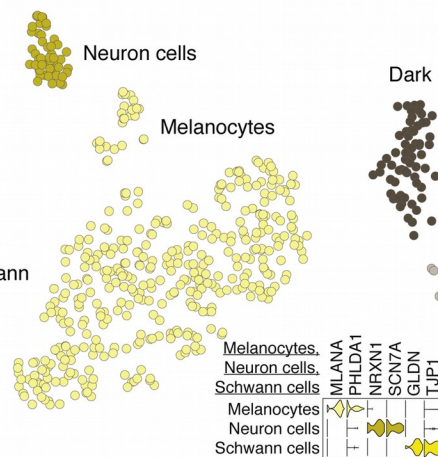
B. Fibroblasts



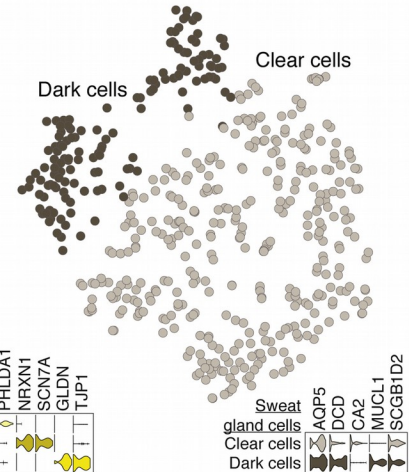
C. Pericytes, Vascular smooth muscle cells



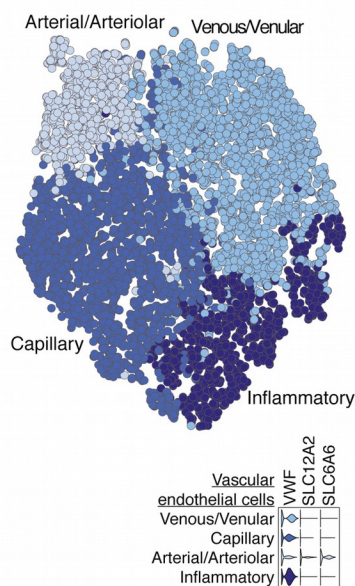
D. Melanocytes, Neuron and Schwann cells



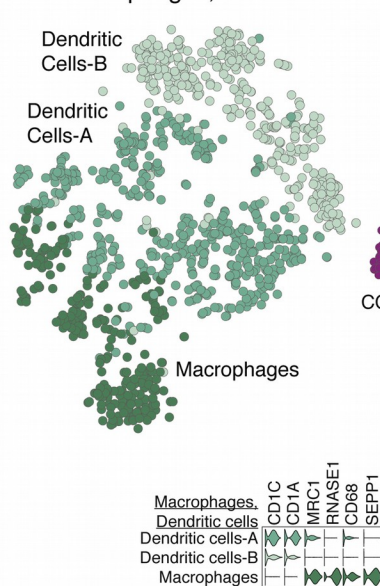
E. Sweat gland cells



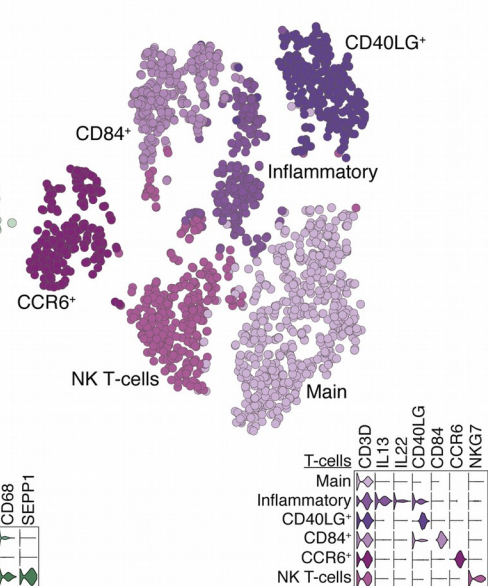
F. Vascular endothelial cells

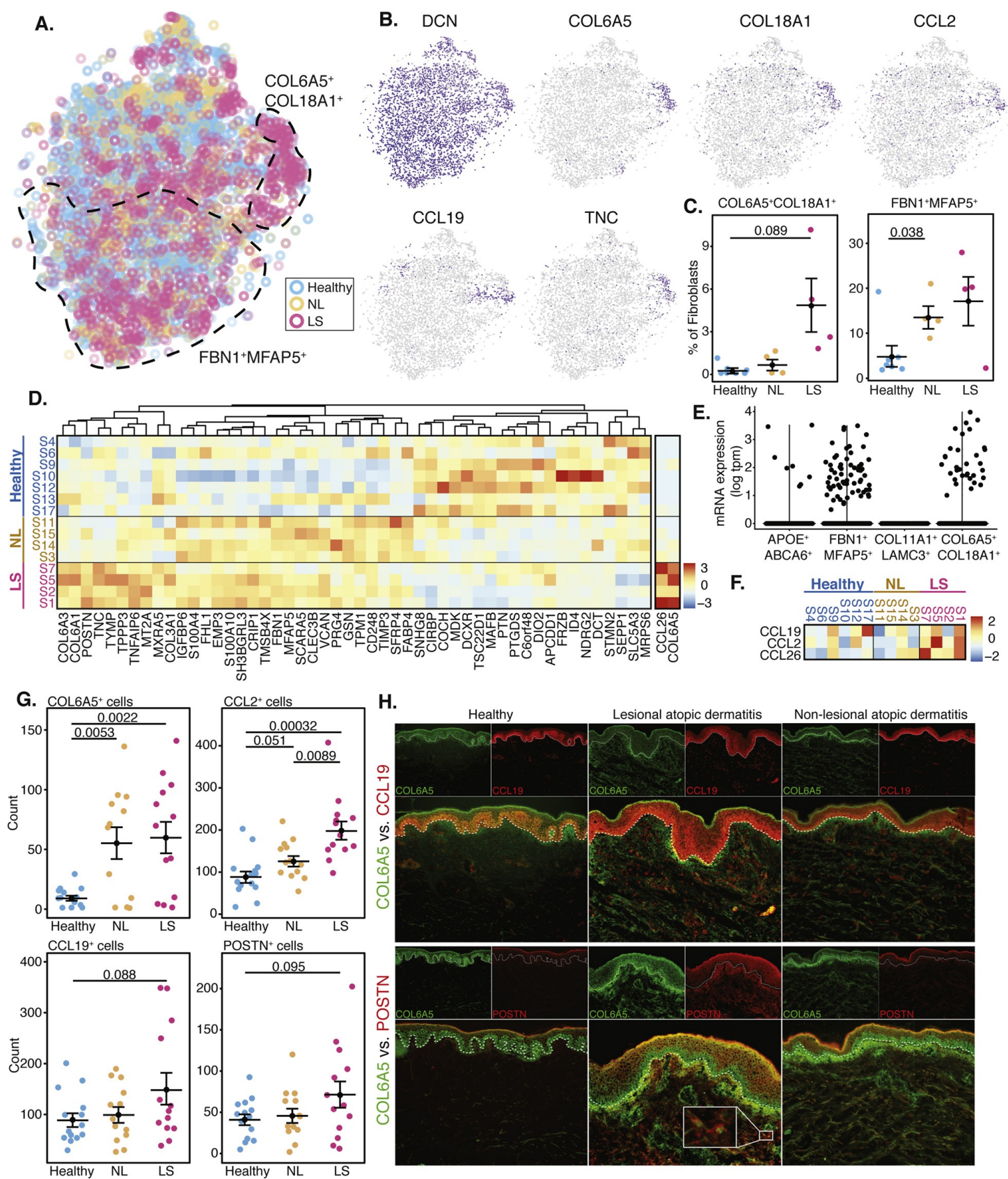


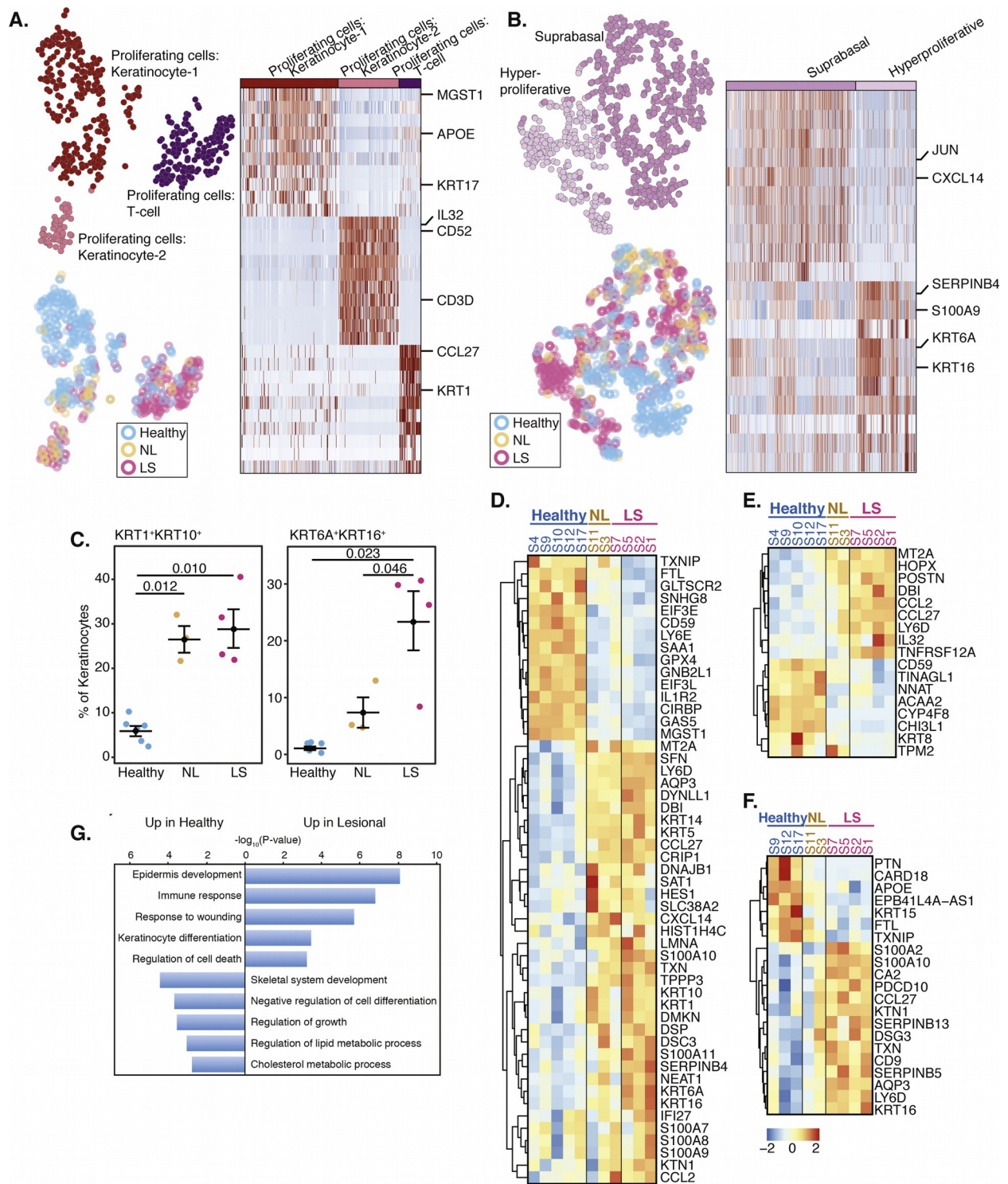
G. Macrophages, Dendritic cells

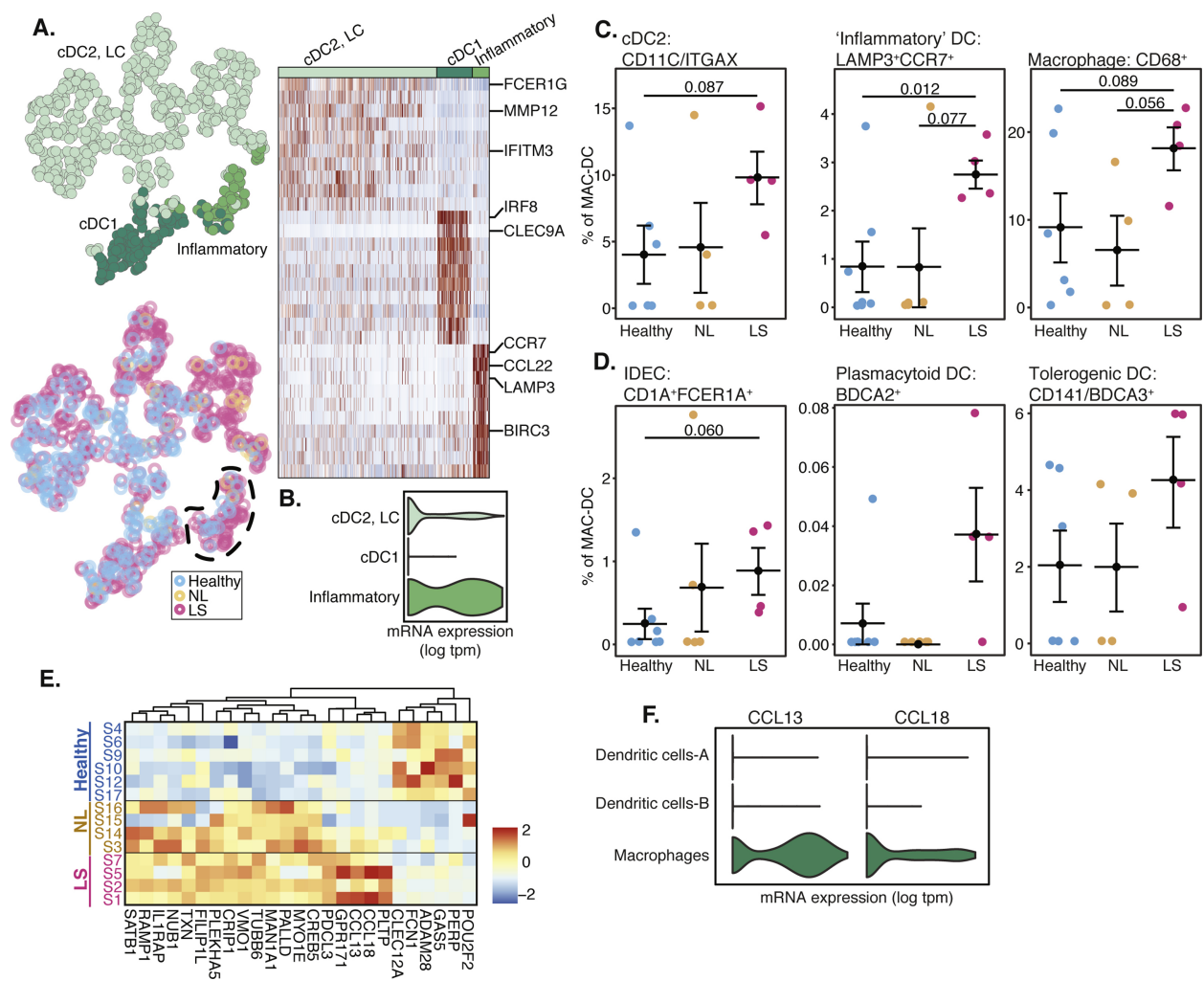


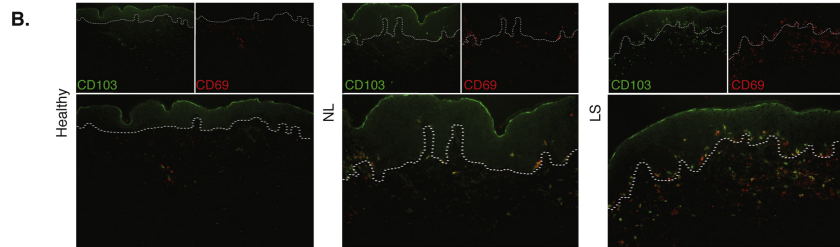
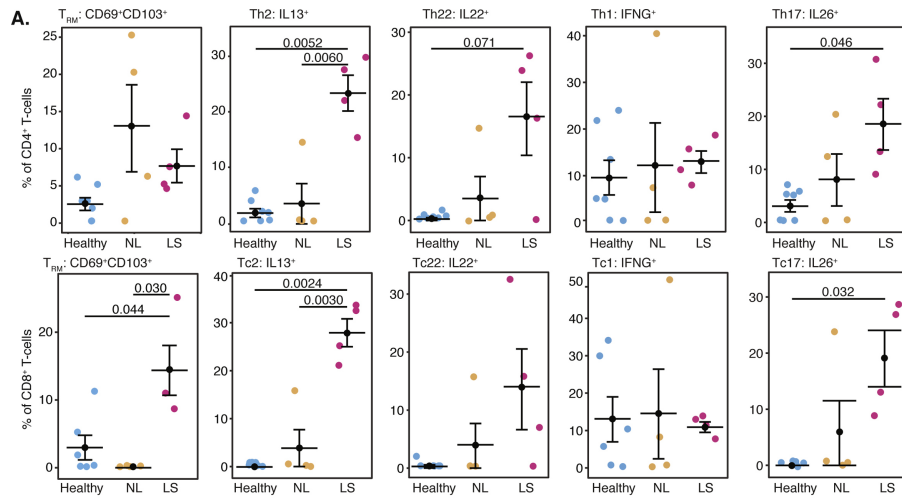
H. T-cells











C.

	All	Healthy	NL	LS
IL13 ⁺	2.66	11.94	22.91	
IL22 ⁺	0.95	12.69	20.08	
IL26 ⁺	4.38	11.94	17.63	
IFNG ⁺	5.07	5.97	5.92	
IL13 ⁺ /IFNG ⁺	0.53	2.00	3.87	
IL22 ⁺ /IFNG ⁺	0.19	2.13	3.39	
IL26 ⁺ /IFNG ⁺	0.86	2.00	2.98	

	CD4 ⁺	Healthy	NL	LS
Th2	3.42	12.73	19.10	
Th22	0.99	12.73	16.58	
Th17	4.38	11.82	16.40	
Th1	2.76	2.73	3.42	
Th2/Th1	1.24	4.67	5.58	
Th22/Th1	0.36	4.67	4.84	
Th17/Th1	1.88	4.33	4.79	

	CD8 ⁺	Healthy	NL	LS
Tc2	0.00	11.76	25.41	
Tc22	1.18	11.76	17.21	
Tc17	0.00	17.65	15.57	
Tc1	9.41	11.76	9.02	
Tc2/Tc1	0.00	1.00	2.82	
Tc22/Tc1	0.13	1.00	1.91	
Tc17/Tc1	0.00	1.50	1.73	

