

Single-cell transcriptome profile of mouse skin undergoing antigen-driven allergic inflammation recapitulates findings in atopic dermatitis skin lesions



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Background: Allergic skin inflammation elicited in mice by epicutaneous (EC) sensitization with antigen shares characteristics with human atopic dermatitis (AD). **Objective:** We characterized gene expression by single cells in mouse skin undergoing antigen-driven allergic inflammation and compared the results with findings in AD skin lesions. **Methods:** Mice were EC sensitized by application of ovalbumin (OVA) or saline to tape-stripped skin. Single-cell RNA sequencing was performed on skin cells 12 days later. Flow cytometry analysis was performed to validate results. **Results:** Sequencing identified 7 nonhematopoietic and 6 hematopoietic cell subsets in EC-sensitized mouse skin. OVA sensitization resulted in the expansion in the skin of T cells, dendritic cells, macrophages, mast cells/basophils, fibroblasts, and myocytes cell clusters, and in upregulation of T_H2 cytokine gene expression in CD4⁺ T cells and mast cells/basophils. Genes differentially expressed in OVA-sensitized skin included genes important for inflammation in dendritic cells and macrophages, collagen deposition, and leukocyte migration in fibroblasts, chemotaxis in endothelial cells and skin barrier integrity, and differentiation in KCs—findings that recapitulate those in AD skin lesions. Unexpectedly, mast cells/basophils, rather than T cells, were the major source of *IL4* and *IL13* in OVA-sensitized mouse skin. In addition, our results suggest novel pathways in fibroblast and endothelial cells that may contribute to allergic skin inflammation.

Conclusion: The gene expression profile of single cells in mouse skin undergoing antigen-driven shares many features with that in AD skin lesions and unveils novel pathways that may be involved in allergic skin inflammation. (*J Allergy Clin Immunol* 2022;150:373-84.)

Key words: Atopic dermatitis, single-cell RNA-Seq, allergic skin inflammation

Atopic dermatitis (AD) is the most common skin inflammatory disease in infants and young children. It is characterized by a defective skin barrier, epidermal hyperplasia, dermal infiltration by T cells and eosinophils, and type 2–dominated local and systemic immune responses evidenced by increased cutaneous expression of type 2 cytokines, eosinophilia, elevated levels of serum IgE, and the presence of IgE antibodies to environmental and food allergens.^{1,2}

The type 2 cytokines IL-4 and IL-13 are responsible for many of the pathologic features of AD.¹⁻³ IL-4 and IL-13 drive the expression of chemoattractants for eosinophils, basophils, and T_H2 cells, inhibit keratinocyte (KC) differentiation and production of antimicrobial peptides, impair skin barrier function, promote lipid abnormalities in the epidermis, increase endogenous protease activity, and drive skin remodeling.^{2,4-7} A key role for IL-4 and IL-13 in allergic skin inflammation has been documented in several mouse models⁸⁻¹⁰ and has been demonstrated in patients with AD by the beneficial effect of monoclonal antibody against the IL-4Rα chain shared by the IL-4 and IL-13 receptors.¹¹⁻¹³

Our understanding of the pathophysiology of AD has benefited enormously from the use of animal models.⁸⁻¹⁰ Our laboratory has developed a mouse model of allergic skin inflammation induced by repeated epicutaneous (EC) sensitization of tape-stripped skin with OVA or peanut antigens.^{8,9,14-16} This model shares several characteristics with human AD. These include skin barrier disruption, scratching behavior, local and systemic type 2 immune response, epidermal thickening, and dermal infiltration by CD4⁺ T cells and eosinophils.^{8,9,15} In addition, mice EC sensitized with OVA develop food allergy after oral antigen challenge and airway inflammation after intranasal antigen challenge,^{14,16-18} thus mimicking the atopic march. These shared features have made our mouse model widely used in investigating allergic skin inflammation in AD.^{8,9,19-23}

Single-cell RNA sequencing (scRNA-Seq) allows measuring gene expression profiles at the single-cell level in tissues by combining barcoding technology, sequencing, and computational analysis.²⁴⁻²⁶ As a result of the complex cellular composition of the skin and technically challenging

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Abbreviations used

AD:	Atopic dermatitis
DC:	Dendritic cell
EC:	Epicutaneous
KC:	Keratinocyte
MC:	Mast cell
OVA:	Ovalbumin
scRNA-Seq:	Single-cell RNA sequencing

disruption of this tissue into single-cell suspension, most scRNA-Seq studies performed on skin to date have focused on limited cell types,²⁷⁻³⁴ with few studies evaluating global cell populations in skin.³⁵⁻³⁷ These studies have been instrumental in determining the diversity of different skin cell types, such as epidermal KCs, fibroblasts, endothelial cells, and immune cells. Two recent studies have reported scRNA-Seq of lesional and nonlesional skin from AD patients, showing that T-cell subsets in AD lesional skin upregulate markers for T_H2/T_H22 cells, such as *Il13* and *Il22*, and demonstrated increased infiltration of myeloid cells with inflammatory phenotypes, including dendritic cells (DCs), macrophages, and Langerhans cells.^{36,37} One of the studies further identified a population of $COL6A5^+COL18A1^+$ fibroblasts in lesional AD skin that express chemokines, cytokines, and extracellular matrix products induced in type 2 inflammatory conditions.³⁶

We performed scRNA-Seq analysis of full-thickness mouse skin subjected to EC sensitization with OVA or saline as control. The results show striking similarities with the results of scRNA-Seq studies and other studies of human AD lesions, providing further support for the utility of this mouse model in studying disease mechanisms in AD. Unexpectedly, mast cells (MCs)/basophils, rather than T cells, were the major source of *Il4* and *Il13* in OVA-sensitized skin. In addition, fibroblasts in OVA-sensitized mouse skin expressed genes involved in the mesenchymal-epidermal transition and regulation of NF- κ B activation, and endothelial cells expressed genes that stimulate angiogenesis, suggesting novel pathways that may contribute to allergic skin inflammation.

METHODS**EC sensitization**

Female BALB/c mice were purchased from Charles River Laboratories (Wilmington, Mass). *Il13^{eGFP/+}* mice in BALB/c background were a gift from Andrew N. J. McKenzie (Medical Research Council, Laboratory of Molecular Biology, Cambridge, United Kingdom). *Foxp3^{eyfp-cre}* (C57BL/6) mice were purchased from The Jackson Laboratory (Bar Harbor, Me). Six- to 8-week-old mice were epicutaneously sensitized for 10 days as described previously.¹⁵ Briefly, mice were anesthetized, and their back skin was shaved and tape-stripped with a film dressing (Tegaderm, 3M, Maplewood, Minn), followed by the application of 200 μ g OVA (Sigma-Aldrich, St Louis, Mo) or saline every other day. Analyses were performed at day 12. All mice were kept in a pathogen-free environment and fed an OVA-free diet. All procedures were performed in accordance with the animal care and use committee of the Children's Hospital Boston.

Skin cell preparation

Skin pieces (1 cm²) from EC sensitized mice were obtained. Skin pieces were finely chopped using scissors after fat removal and digested for 90

minutes in the media containing Liberase (0.2 mg/mL, Roche, Basel, Switzerland) and DNase II (Sigma-Aldrich), with continuous shaking at 37°C. Digested skin homogenates were passed through a 100 μ m cell strainer, washed, and resuspended in 0.05% fetal calf serum in phosphate-buffered saline.

Droplet-based scRNA-Seq

Experiments were performed on the 10x Genomics Chromium platform, with the Chromium Single Cell 3' Library and Gel Bead Kit v2 and Chromium Single Cell 3' Chip kit v2 according to the manufacturer's instructions in the Chromium Single Cell 3' Reagents Kits v2 user's guide (10x Genomics, Pleasanton, Calif). Briefly, ~6000 cells resuspended in phosphate-buffered saline supplemented with 0.05% fetal calf serum were loaded in each channel. Cells were then partitioned into gel beads in emulsion in the Chromium controller, where cell lysis and barcoded reverse transcription of RNA occurred, followed by amplification, shearing, and 5' adaptor and sample index attachment. Barcoded single-cell libraries were sequenced with 38 bp paired-end reads on an Illumina NextSeq 500 instrument (Illumina, San Diego, Calif).

Single-cell transcriptome analysis

The analysis was performed with the computational resources provided by Research Computing Group at Boston Children's Hospital and Harvard Medical School (Boston, Mass), including High-Performance Computing Cluster Enkefalon 2 (E2) and BioGrids scientific software made available for data analysis. The raw sequencing data were preprocessed to generate the read count per gene per cell matrices using Cell Ranger v3.1.0 (10x Genomics), including BCL-to-FASTQ file conversion, data multiplexing according to cell barcodes, genome alignment to mouse genome mm10 with STAR, and read counting per gene per cell into matrices. A pipeline for the single-cell profiling was built based on the Seurat R toolkit (R v3.6.2, Seurat v3.1.2),^{38,39} including cell filtering, clustering, annotation, differential gene expression, and visualization. Cells expressing 200 to 2500 genes that were detected in at least 3 cells and a percentage of mitochondrial genes below 5% in droplets were considered viable and nonmultiplets, and were included for analysis. After performing this quality control step to extract cells of good quality, the 3 replicates per group were merged, and the 2 experimental groups were integrated for analysis by identifying common sources of variation using canonical correlation analysis.^{38,39} Cell clustering was performed with a shared nearest-neighbor graph-based method, followed by the original Louvain algorithm for modularity optimization (resolution = 2.5) after data dimension reduction using principal component analysis (number of dimensions = 35). After the cell clusters were determined, their marker genes were identified with the FindMarkers function. For cluster annotation, the top marker genes based on adjusted *P* values were manually curated to match canonical cell types and their marker genes based on a literature research and the resources in ImmGen (Immunological Genome Project, www.immgen.org). Finally, the differential gene expression between groups was calculated by likelihood-ratio test⁴⁰ as well as Wilcoxon rank-sum test with Bonferroni correction implemented in FindMarkers function in Seurat for further study. Raw and processed scRNA-Seq data for all samples have been deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession code GSE194254.

Flow cytometry

Skin cells were preincubated with Fc γ R-specific blocking mAb (2.4G2) and washed before staining with the following mAbs: CD3 (17A2), CD4 (GK1.5), CD45 (30F11), and TCR δ (ebioGL3) from eBioscience (San Diego, Calif); CD11b (M1/70) and CD117 (2B8) from BioLegend (San Diego, Calif), and anti-IgE (R35-72) from BD Biosciences (San Jose, Calif). For cytokine staining, cells were stimulated with ionomycin (0.5 μ g/mL, Sigma-Aldrich), phorbol 12,13-dibutyrate (1 μ g/mL, Sigma-Aldrich), brefeldin A (eBioscience), and monensin (eBioscience) in complete RPMI

1640 for 3 hours before surface staining. Then cells were fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences) and stained in permeabilization solution with IL-22 (1H8PWSR) from eBioscience. Cells were analyzed by flow cytometry using an LSRFortessa machine (BD Biosciences). The data were analyzed by FlowJo software version 10 (Treestar, Ashland, Ore).

Statistical analysis

The differential gene expression between groups was calculated by likelihood-ratio test⁴⁰ as well as the Wilcoxon rank-sum test with Bonferroni correction. Statistical significance in the proportion of cell types was determined by the 2-tailed Student *t* test. *P* < .05 was considered statistically significant.

RESULTS

Cell composition of EC-sensitized mouse skin by scRNA-Seq

We previously showed that EC sensitization of mouse skin with OVA for 12 days results in allergic skin inflammation that recapitulates many pathologic features of AD lesional skin.^{15,22} As previously reported, mouse skin EC sensitized with OVA showed increased transepidermal water loss, increased epidermal thickness, and increased infiltration with mononuclear cells compared to mouse skin EC sensitized with saline (see Fig E1, B and C, in this article's Online Repository at www.jacionline.org). In addition, OVA-sensitized skin exhibited increased infiltration of CD45⁺ T cells, including CD4⁺ T cells and eosinophils, and upregulation of *Il4* and *Il13* messenger RNA levels compared to saline-sensitized skin (Fig E1, D-G). To perform scRNA-Seq, the skin of BALB/c mice EC sensitized with OVA or saline (*n* = 3 per group, for a total of 6 samples) was dissociated into a single-cell suspension by enzymatic digestion, followed immediately by barcoding the cells and scRNA-Seq. We profiled 6,200 ± 1,010 cells per skin sample with ~18,000 ± 2,940 reads per cell and 1,250 ± 52 unique genes detected per cell. After performing quality control to remove cells with low-quality profiles and suspected doublets, 14,862 high-quality single-cell profiles from the 6 samples were retained for subsequent analysis.

Unsupervised clustering and *post hoc* annotation integrating saline- and OVA-sensitized skin identified 47 clusters spanning 13 distinct cell types (Fig 1, A; and see Fig E2 and Table E1 in the Online Repository at www.jacionline.org). Fibroblasts, identified by the expression of *Lum*, *Dcn*, and *Col1a2*, were the most frequent cell type (~46%), followed by KCs (~26%), identified by the expression of *Krt1*, *Krt5*, *Krt14*, and *Krt15*. Endothelial cells (~10%) were identified by the expression of *Cldn5* and *Vwf*, macrophages (~4%) by the expression of *Lyz2* and *Cd14*, pericytes (~3.7%) by the expression of *Rgs4*, *Rgs5*, and *Kcnj8*, DCs (~3.0%) by the expression of *Cd74* and *H2-Eb1*, Schwann cells (~2.3%) by the expression of *Mbp* and *Kcna1*, T cells (~2.3%) by the expression of *Trbc1*, *Trbc2*, and *Cxcr6*, neutrophils (~0.6%) by the expression of *S100a9* and *S100a8*, Langerhans cells (~0.4%) by the expression of *Cd207* and *Ltc4s*, melanocytes by the expression of *Oca2*, *Sox10*, and *Pax3*, myocytes (~0.2%) by the expression of *Csrp3*, *Apobec2*, and *Ckmt2*, and MCs/basophils (~0.1%) by the expression of *Fcer1a*, *Cpa3*, and *Mcpt8* (Fig 1, B and C, and Table E1).

Consistent with the development of allergic skin inflammation, OVA-sensitized skin demonstrated an increase in the total number

of cells recovered compared to saline-sensitized skin. This was caused by expansion of both immune cells, including T cells, macrophages, and DCs, and nonhematopoietic cells, including fibroblasts and pericytes, as well as by the appearance of neutrophils and MC/basophil populations (Fig 1, D, Table 1, and Fig E2).

Characterization of fibroblast populations in mouse EC-sensitized skin

Fibroblast spanned 16 clusters (Fig 2, A, and see Table E2 in the Online Repository at www.jacionline.org). Cells in clusters 1 to 6 were collagen I-producing fibroblasts characterized by the expression of *Col1a1* and *Col1a2* and comprised ~44% of the fibroblast population. Clusters 7 to 10 were collagen VIII/IX-producing fibroblasts characterized by the expression of *Col8a1*, *Col8a2*, *Col9a1*, *Col9a2*, and *Col9a3* and comprised ~37% of the fibroblast population. Cells in clusters 11 and 12 expressed *Sst*, *Hhip*, and *Sox2* characteristic of dermal papilla fibroblasts and comprised ~7% of the fibroblast population. Cells in cluster 13 expressing *Myod1* and *Lypd2* characteristic of myofibroblasts comprised ~1% of the fibroblast population. Cells in clusters 14 to 16 expressing *Mfap5*, *Cygb*, and *Apod* characteristic of lipofibroblasts comprised ~11% of the fibroblast population (Fig 2, B and C).

There were important differences between OVA-sensitized and saline-sensitized skin in the expression of key genes in fibroblast subsets. Collagen I-producing fibroblasts from OVA-sensitized skin showed increased *Ccl8*, *Col5a2*, *Cxcl5*, *Il33*, and *Postn* expression and diminished *Sfn* and *Il1r2* expression compared to those in saline-sensitized skin (Fig 2, D, and see Table E3 in the Online Repository at www.jacionline.org). In addition, collagen VIII/IX-producing fibroblasts from OVA-sensitized skin showed increased *Ccl2*, *Ccl7*, and *Fnl* expression (Fig 2, E, and Table E3). After OVA sensitization, dermal papilla fibroblasts showed increased *Lgr4* expression and diminished *Hes1* and *Mmp3* expression (Fig 2, F, and Table E3). Lipofibroblasts from OVA-sensitized skin showed increased *Ccl8*, *Col5a2*, and *Cxcl5* expression (Fig 2, G, and Table E3). These results suggest that fibroblasts play an important role in skin remodeling and the attraction of immune cell into allergic inflamed skin.

Characterization of the KC population in mouse EC-sensitized skin

The KC population spanned 13 clusters, tracking with the different layers of the epidermis, the various parts of the hair follicle, and the sebaceous gland (Fig 3, A, and Table E2). Specifically, cells in clusters 1, 2, and 3 comprised ~26% of the KC population and showed high expression of *Krt14*, *Krt5*, and *Krt15*, characteristic of the basal layer of the epidermis. Cells in cluster 4 comprised ~13% of KCs and expressed *Krt1* and *Krt10*, characteristic of the spinous layer. Cells in cluster 5 comprised ~9% of KCs and expressed *Flg* and *Lor*, characteristic of the granular layer (Fig 3, B and C). Cells in cluster 6 and 7 comprised ~14% of the population and expressed *Krt17* and *Lrg5*, characteristic of the hair follicle outer bulge. Cells in cluster 8 expressed the hair follicle inner bulge gene group 1 (*Krt25*, *Krt27*, and *Krt28*), and clusters 9 to 11, expressing characteristics of hair follicle inner bulge gene group 2 (Fig 3, B and C),

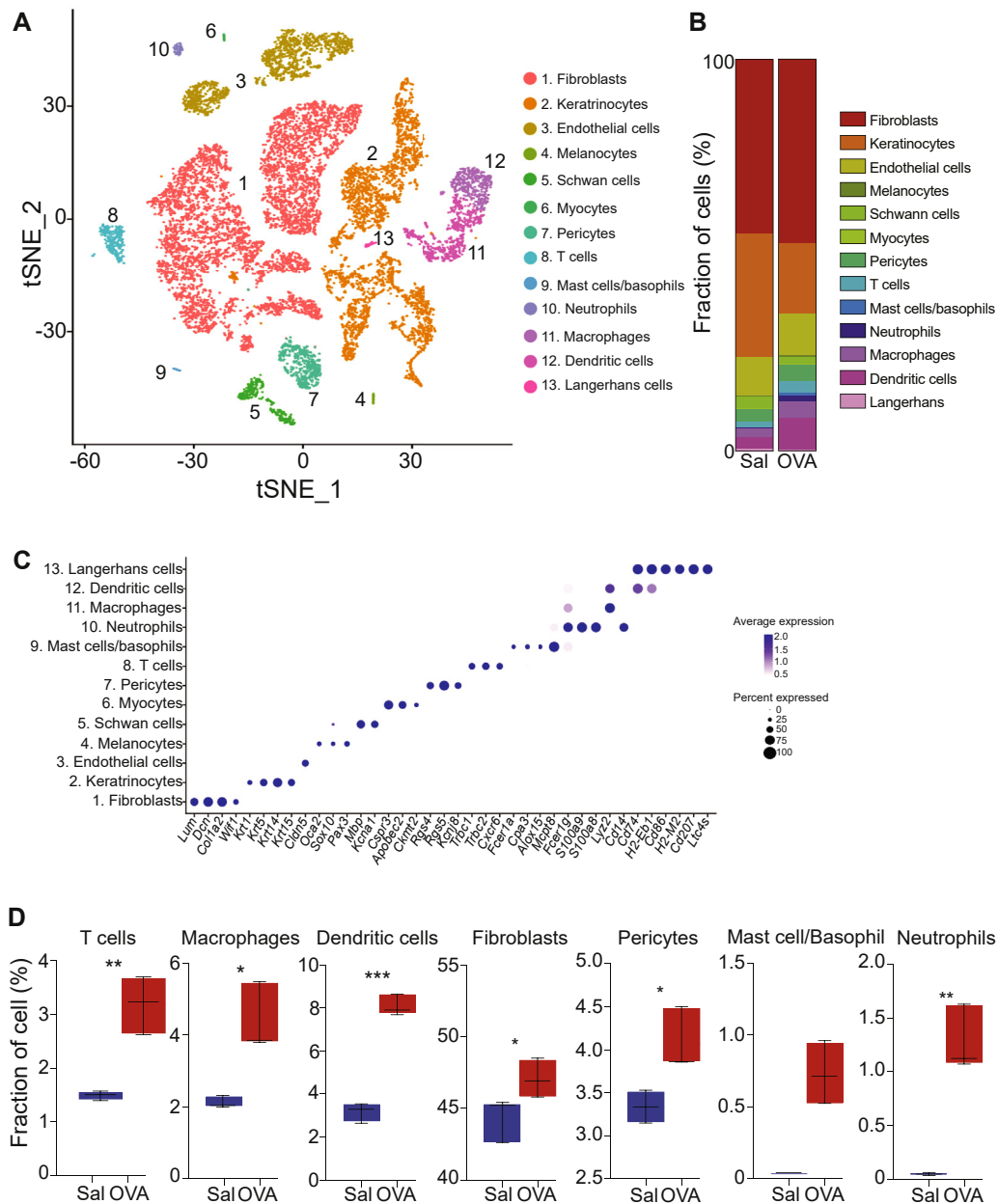


FIG 1. Cell composition of EC-sensitized mouse skin by scRNA-Seq. **A**, *t*-Distributed stochastic neighbor embedding (tSNE) plot for 14,211 high-quality single-cell transcriptomes from mouse skin EC sensitized with saline ($n = 3$) and OVA ($n = 3$), revealing 13 different cell populations. **B**, Fractions of cell populations identified in EC-sensitized skin with saline (Sal) and OVA. **C**, Dot plot displaying top expressed genes in each cell population compared to the other cell populations identified in EC-sensitized skin. **D**, Box plot of the distribution of 7 selected cell populations in mouse skin EC sensitized with OVA or saline. * $P < .05$, ** $P < .005$, and *** $P < .001$.

comprised ~26% of the population. Cells in cluster 12 comprised ~4% of KCs and expressed *Awat2*, *Far2*, *Acot5*, and *Pnpla5*, characteristic of the sebaceous gland (Fig 3, B and C). Cells in cluster 13 comprised ~8% of KCs and expressed *Defb6* and *Cst6*, characteristic of the upper hair follicle (Fig 3, B and C).

As with fibroblasts, KCs showed cell-intrinsic differences between OVA and saline sensitization. KCs from OVA-sensitized skin showed diminished *Krt10* and *Krt2* and increased

Ccl27a and *Atg5* expression in the basal layer, increased *S100a11* and *Krt14* expression in the spinous layer, and decreased expression of *Flg*, *Lor*, and *Tgm3* in the granular layer compared to those in saline-sensitized skin (Fig 3, D-F, and Table E3). Furthermore, hair follicle inner and outer bulge as well as upper hair follicle KCs from OVA-sensitized skin showed diminished *Krt1*, *Krt10*, and *Krt2* expression (Fig 3, G-I, and Table E3). Together, these results suggest that allergic skin inflammation impairs KC differentiation.

TABLE I. Cell numbers and percentages of the different cell populations identified in our analysis

No.	Cluster	No. of cells		% of total	
		EC saline	EC OVA	EC saline	EC OVA
1	Fibroblasts	2885	3895	44.3	46.25
2	KCs	2053	1739	31.62	20.78
3	Endothelial cells	656	864	10.10	10.32
4	Melanocytes	22	32	0.34	0.38
5	Schwann cells	188	173	2.90	2.07
6	Myocytes	11	31	0.17	0.37
7	Pericytes	216	328	3.33	3.92
8	T cells	95	233	1.46	2.78
9	MCs/basophils	1	48	0.02	0.57
10	Neutrophils	3	81	0.05	0.97
11	Macrophages	133	322	2.05	3.85
12	DCs	197	595	3.03	7.11
13	Langerhans cells	33	28	0.51	0.33
	Total	6493	8369	100	100

Characterization of endothelial cell populations in mouse EC-sensitized skin

The endothelial cell population spanned 4 clusters (Fig 4, A, and Table E2). Cells in cluster 1 comprised ~32% of endothelial cells and were characterized by the expression of the lymphatic endothelium genes *Mmrn1*, *Flt4*, and *Lyve1*. Cells in clusters 2 to 4 comprised ~68% of endothelial cells and were characterized by the expression of the vascular endothelium genes *Vwf*, *Esam*, and *Ly6c1* (Fig 4, B and C). Vascular endothelial cells from OVA-sensitized skin showed increased *Selp* and *Cxcl2* expression (Fig 4, D, and Table E3) compared to those from saline-sensitized skin, whereas lymphatic endothelial cells from OVA-sensitized skin showed increased expression of *Selp* and *S100a6* (Fig 4, E, and Table E3), which encode for a protein essential for cell-cycle progression in endothelial cells. These results suggest that endothelial cells play an important role in the attraction of immune cells into allergic inflamed skin.

Characterization of immune cell populations in mouse EC-sensitized skin

The T-cell cluster in OVA-sensitized skin demonstrated increased expression of *Cd4* and decreased expression of *Tcrd* compared to saline-sensitized skin (Fig 5, A, and Table E3). Flow cytometry analysis of CD3⁺ cells revealed an increased percentage of CD4⁺ T cells at the expense of the percentage of TCR $\gamma\delta$ ⁺ T cells in OVA-sensitized skin compared to saline sensitized control (Fig 5, B). These results indicate a shift in CD4⁺/TCR $\gamma\delta$ ⁺ T-cell ratio at sites of allergic skin inflammation.

T cells from OVA-sensitized skin further showed increased expression of genes associated with T_H22 (*Ahr*), T_H2 (*Il13* and *Tnfrs4*), and regulatory T cells (*Ctla4* and *Tnfrs25*) compared to saline-sensitized skin (Fig 5, A, and Table E3), suggesting accumulation of these cell populations in skin undergoing allergic inflammation. Consistent with these results, intracellular staining showed an increase in the percentage of IL-22-producing CD4⁺ T cells in OVA-sensitized skin compared to saline-sensitized skin (Fig 5, C). Furthermore, analysis of EC-sensitized skin from

Il13^{eGFP/+} and Foxp3^{Cre-YFP} showed respectively increased percentages of CD4⁺ T cells expressing IL-13 and FOXP3 in OVA-sensitized skin compared to saline-sensitized skin (Fig 5, D and E).

Macrophages in OVA-sensitized skin demonstrated increased expression of antigen presentation genes (*H2-Ab1*, *H2-Aa*, *H2-Eb1*, *H2-DMb1*, *H2-DMa*, and *Cd74*), the chemokines *Ccl8* and *Ccl6*, and the fatty acid transporter *Fabp5*, but decreased expression of inflammatory macrophage markers such as the chemokines *Ccl2*, *Ccl3*, *Cxcl1*, and *Cxcl2* and the inflammatory cytokine *Tnfa* compared to saline-sensitized skin (Fig 5, F, and Table E3). These results suggest that allergic skin inflammation promotes macrophages with an alternative activation phenotype.

DCs in OVA-sensitized skin demonstrated increased expression of *Irf8*, *Il12b*, *Fscn1*, *Cts2*, and *Cd24a*, but decreased expression of *Areg*, *Cd14*, *Ctss*, *Fcer1g*, and *Fcgr3* compared to saline-sensitized skin (Fig 5, G, and Table E3). These data suggest that allergic skin inflammation promotes an inflammatory phenotype in skin DCs.

Cells in the MC/basophil cluster in OVA-sensitized skin exhibited expression of *Il4*, *Il13*, *Mcp8*, and *Ccr12* (Fig 5, H, and Table E3), suggesting increased infiltration of MCs and basophils in allergic inflamed skin. Flow cytometry analysis of skin cells confirmed the increase in basophils (identified as CD3⁺IgE⁺CD117⁺ cells) and MCs (identified as CD3⁺IgE⁺CD117⁺ cells) in OVA-sensitized skin compared to saline-sensitized skin (Fig 5, I).

Finally, the T_H2 cytokine *Il4* was detected only in OVA-sensitized skin with MCs/basophils being the only contributors. More *Il13* was detected in OVA-sensitized than in saline-sensitized skin. In both samples, MCs/basophils, rather than T cells, were the major *Il13* contributors (Fig 5, J).

DISCUSSION

We describe the scRNA-Seq profile of mouse skin undergoing acute allergic inflammation elicited by EC sensitization with antigen.

Our unsupervised clustering analysis revealed 7 nonhematopoietic and 6 hematopoietic cell types in mouse skin. The identification in our study of these 13 different cell types in mouse skin is consistent with the recently published scRNA-Seq analysis of full-thickness unmanipulated mouse skin.³⁵ In addition, our analysis identifies MC/basophil and neutrophil populations in OVA-sensitized skin that were not detected in saline-sensitized skin and that were not previously reported in unmanipulated mouse skin.³⁵ This likely reflects the cutaneous recruitment and/or expansion of MC/basophil and neutrophil populations in allergic skin inflammation.

We detected expansion of immune cells, fibroblasts, and pericytes in mouse skin undergoing antigen-driven acute allergic inflammation. We identified 16 different clusters of fibroblasts in mouse skin, consistent with the heterogeneity of dermal fibroblasts,^{31,35,41} as well as 4 subsets of endothelial cells. Analysis of differentially expressed genes in dermal fibroblasts from OVA-sensitized skin reveals increased expression of genes coding for chemokines (*Ccl8*, *Ccl2*, and *Cxcl5*) and molecules involved in tissue remodeling (*Tnc*, *Postn*, *Col5a2*, and *Col6a3*). In addition, fibroblasts from OVA-sensitized skin expressed higher levels of *Il33* and *Prgh4*—molecules implicated in the suppression of

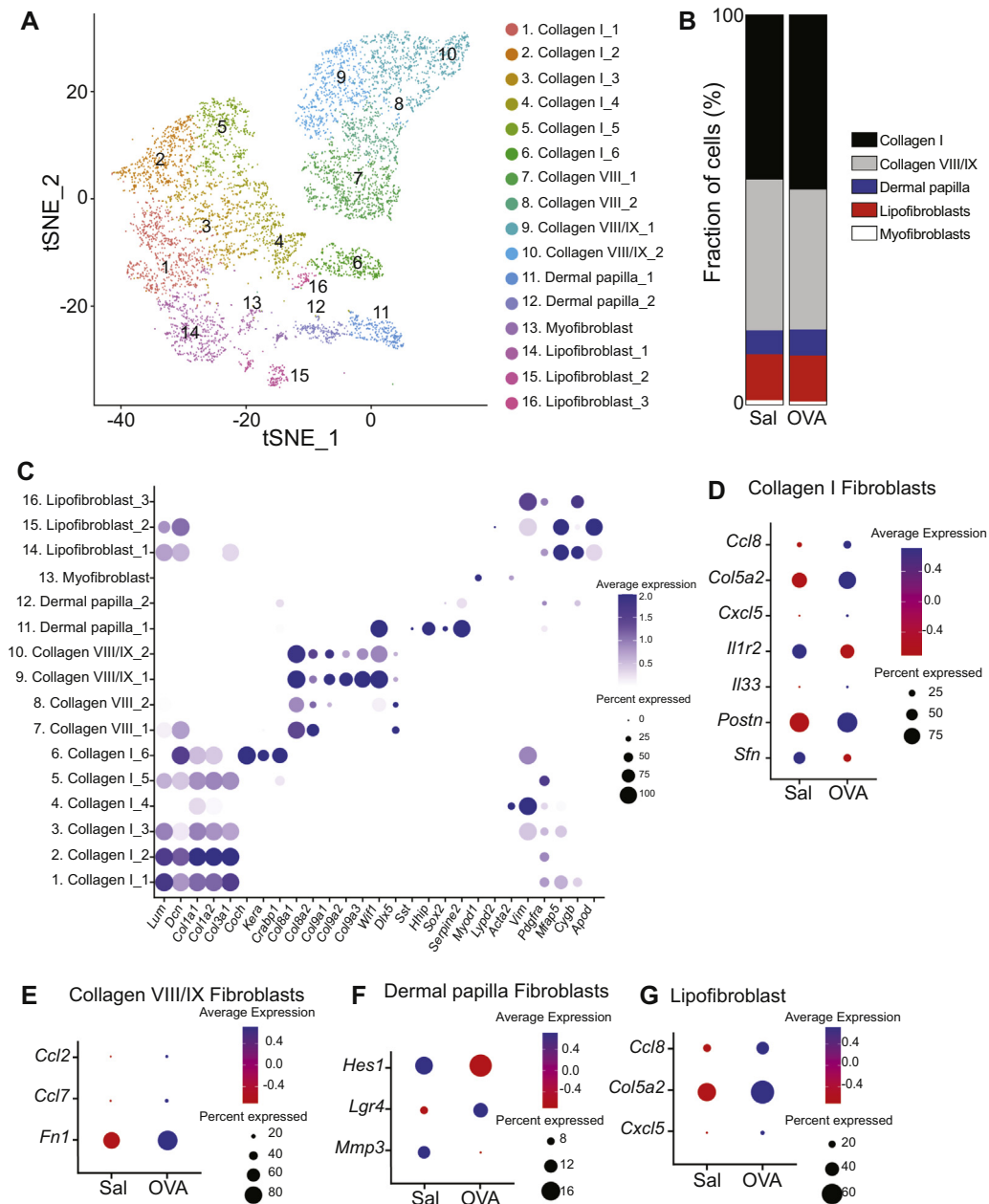


FIG 2. Characterization of the fibroblast population in mouse EC-sensitized skin by scRNA-Seq. **A**, t-Distributed stochastic neighbor embedding (tSNE) plot for fibroblast cluster from EC-sensitized mouse skin, revealing 16 clusters. **B**, Distribution of fibroblast populations identified in mouse skin EC sensitized with OVA or saline. **C**, Dot plot displaying top expressed genes in each fibroblast cluster. **D-G**, Dot plot of differentially expressed genes in the collagen I-producing fibroblasts (**D**), collagen VIII/IX-producing fibroblasts (**E**), dermal papilla fibroblast (**F**), and lipofibroblast (**G**) clusters from skin EC sensitized with saline or OVA.

NF- κ B activation induced by IL-1 β and Toll-like receptor activation.^{42,43} Our results suggest that in addition to their known role in attracting immune cells and skin remodeling,⁴⁴ fibroblasts may play a role in the susceptibility of cutaneous infection observed in AD patients.

Endothelial cells from OVA-sensitized skin showed increased expression of *Cxcl2*, *Sele*, and *Selp*, consistent with the role of endothelial cells in the cutaneous attraction of immune cells.⁴⁵

In addition, they exhibited differential expression of genes regulating endothelial cell growth and sprouting, suggesting that allergic skin inflammation activates an autocrine loop that stimulates angiogenesis in endothelial cells.

AD is characterized by a defective skin barrier due to impaired KC differentiation, lipid abnormalities, and increased protease activity in the epidermis.⁴⁶⁻⁴⁹ Our analysis reveals that KCs from OVA-sensitized skin exhibit dysregulated expression of keratins

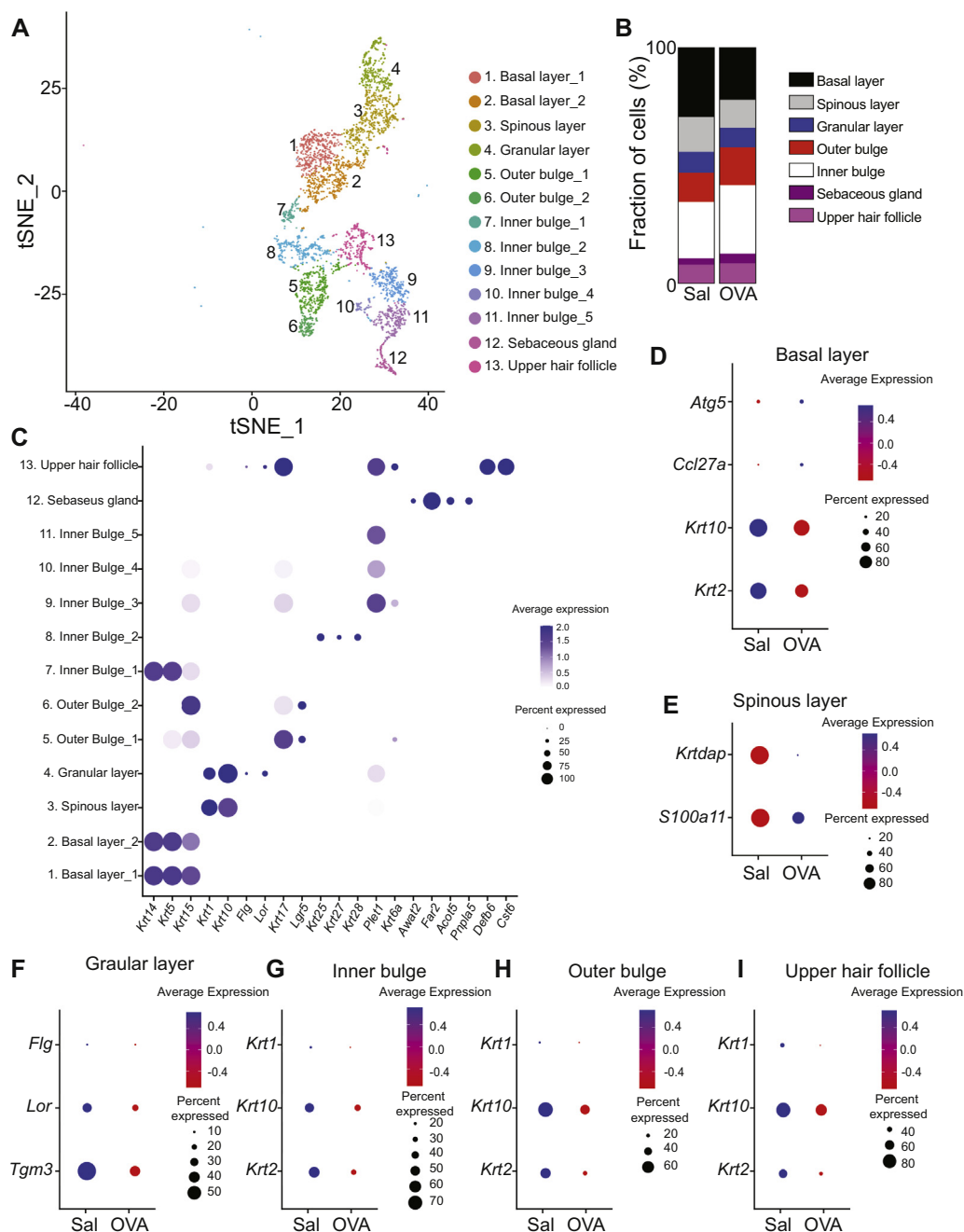


FIG 3. Characterization of the KC population in mouse EC-sensitized skin by scRNA-Seq. **A**, *t*-Distributed stochastic neighbor embedding (tSNE) plot for KC cluster from EC-sensitized mouse skin, revealing 13 clusters. **B**, Distribution of KC populations identified in mouse skin EC sensitized with OVA or saline. **C**, Dot plot displaying top expressed genes in each KC cluster. **D–I**, Dot plot of differentially expressed genes in the KC basal layer (**D**), spinous layer (**E**), granular layer (**F**), inner bulge (**G**), outer bulge (**H**), and upper hair follicle (**I**) clusters from skin EC sensitized with saline or OVA.

(*Krt1*, *Krt2*, *Krt10*, and *Krt14*) in multiple layers, increased expression of *Atg5* in the basal layer, and decreased expression of genes important for barrier integrity (*Flg*, *Lor*, and *Tgm3*). These changes have been reported in skin from AD patients and in our mouse model of antigen-driven allergic skin inflammation.^{5,47,50–56}

OVA-sensitized mouse skin exhibited increased numbers of T cells that expressed *Cd4* as well as genes associated with T_H2

cells, including *Il13* and *Tnfrsf4*, and demonstrated increased expression of genes associated with Treg cells, including *Tnfrsf25* and *Ctla4*. These findings are in line with the previously reported increase in $CD4^+$ T cells and in T_H2 cytokine expression in OVA-sensitized skin in our model and in AD skin lesions,^{4,14,15,57,58} as well as with results of scRNA-Seq analysis of AD skin lesions.^{36,37} IL-4 and IL-13 play a major role in AD pathophysiology. Importantly, our analysis identifies the MC/basophil

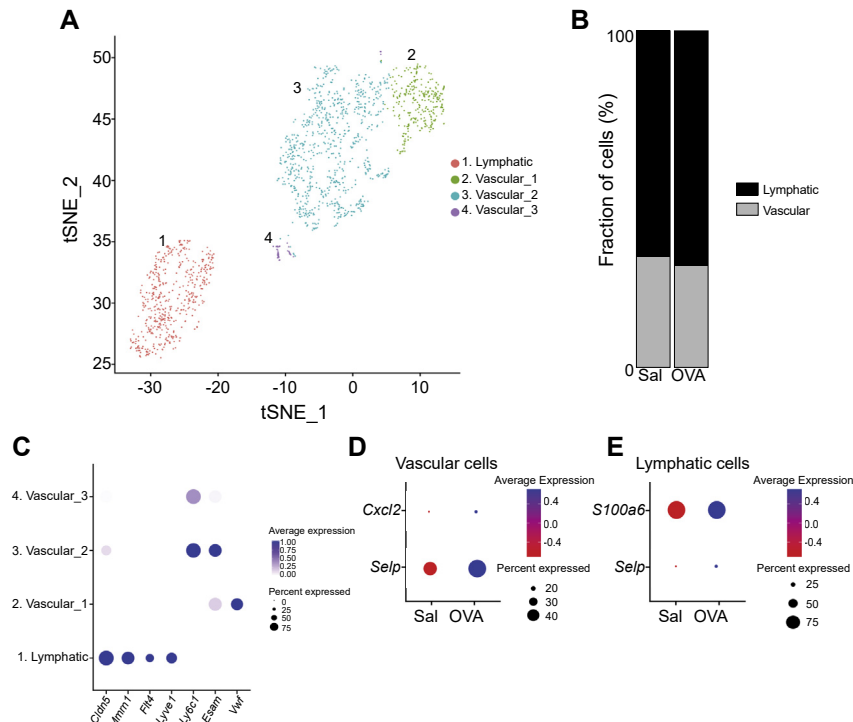


FIG 4. Characterization of the endothelial cell population in mouse EC-sensitized skin by scRNA-Seq. **A**, *t*-Distributed stochastic neighbor embedding (tSNE) plot for endothelial cell cluster from EC-sensitized mouse skin, revealing 4 clusters. **B**, Distribution of endothelial cell populations identified in mouse skin EC sensitized with OVA or saline. **C**, Dot plot displaying top expressed genes in each endothelial cell cluster. **D** and **E**, Dot plot of differentially expressed genes in the endothelial cell vascular (**D**) and lymphatic (**E**) clusters from skin EC sensitized with saline or OVA.

population, rather than T cells, as the major source of *Il4* and *Il13* in acute allergic skin inflammation.⁵⁹ This is in line with our recent findings using flow cytometry analysis of cells from OVA-sensitized mouse skin.¹⁵

DCs and macrophages play an important role in AD.^{60,61} Our results show that skin acutely sensitized with OVA exhibits an increased number of macrophages and DCs, but not Langerhans cells. This is consistent with the increase in inflammatory DCs and macrophages in AD skin lesions previously demonstrated by immunohistochemistry and digital imaging⁶² and by scRNA-Seq.^{36,37} The macrophage cluster from OVA-sensitized skin shows an increased expression of genes involved in antigen presentation and alternative macrophage activation. This is consistent with the increased number of CD163⁺ cells, a marker for alternatively activated macrophages, and the presence of cells expressing markers for both macrophages and DCs in AD lesional skin.^{59,62} In addition, DCs from OVA-sensitized skin downregulated genes coding for proteins highly expressed in monocytes (*Cd14*, *Fcrlg*, and *Fcgr3*). Taken together, these results suggest that the type 2 cytokine environment promotes *in situ* differentiation of monocytes to DCs at sites of allergic skin inflammation.

Our study has several limitations. First, we were unable to identify cell populations, such as eosinophils and type 2 innate lymphoid cells, or detect cytokines, such as *Il17a* and *Il22*, previously described in OVA-sensitized mouse skin and in AD skin lesions,^{63–66} possibly because of the small number of hematopoietic cells recovered and some limitations of droplet-based scRNA-Seq at capturing eosinophils and neutrophils. Second, the small

number of hematopoietic cells analyzed did not allow us to perform a more granular analysis of their different states. Third, we were unable to distinguish between T-cell subsets with clustering, such that both Treg cells and T_H2 are members of a single T-cell cluster or split MC and basophil populations. Fourth, our analysis is limited to a single point in time, providing only a snapshot of the disease and thus does not provide information about the dynamics of the cutaneous response during the course of allergic skin inflammation. Future studies are needed to overcome these limitations.

Importantly, our study demonstrates that our mouse model of antigen-driven allergic skin inflammation elicited by EC sensitization shares at the level of gene expression by single cells many features found in AD lesional skin by scRNA-Seq and demonstrates general, although not total, agreement with the results of published scRNA-Seq and global gene expression studies of AD lesional skin (Table II; and see Fig E3, Fig E4, and Table E4 in the Online Repository at www.jacionline.org).^{67–81} Our comparative analysis of our data and data on gene expression in AD lesional skin is based on the publicly available data sets in human AD, with the limitations that skin samples were collected and processed under different conditions in different laboratories, and analyzed using different bioinformatic pipelines.

In summary, the application of scRNA-Seq analysis to our mouse model of antigen-driven allergic skin inflammation further enhances the utility of this model in investigating potential disease mechanisms, and it identifies potentially novel therapeutic targets in AD.

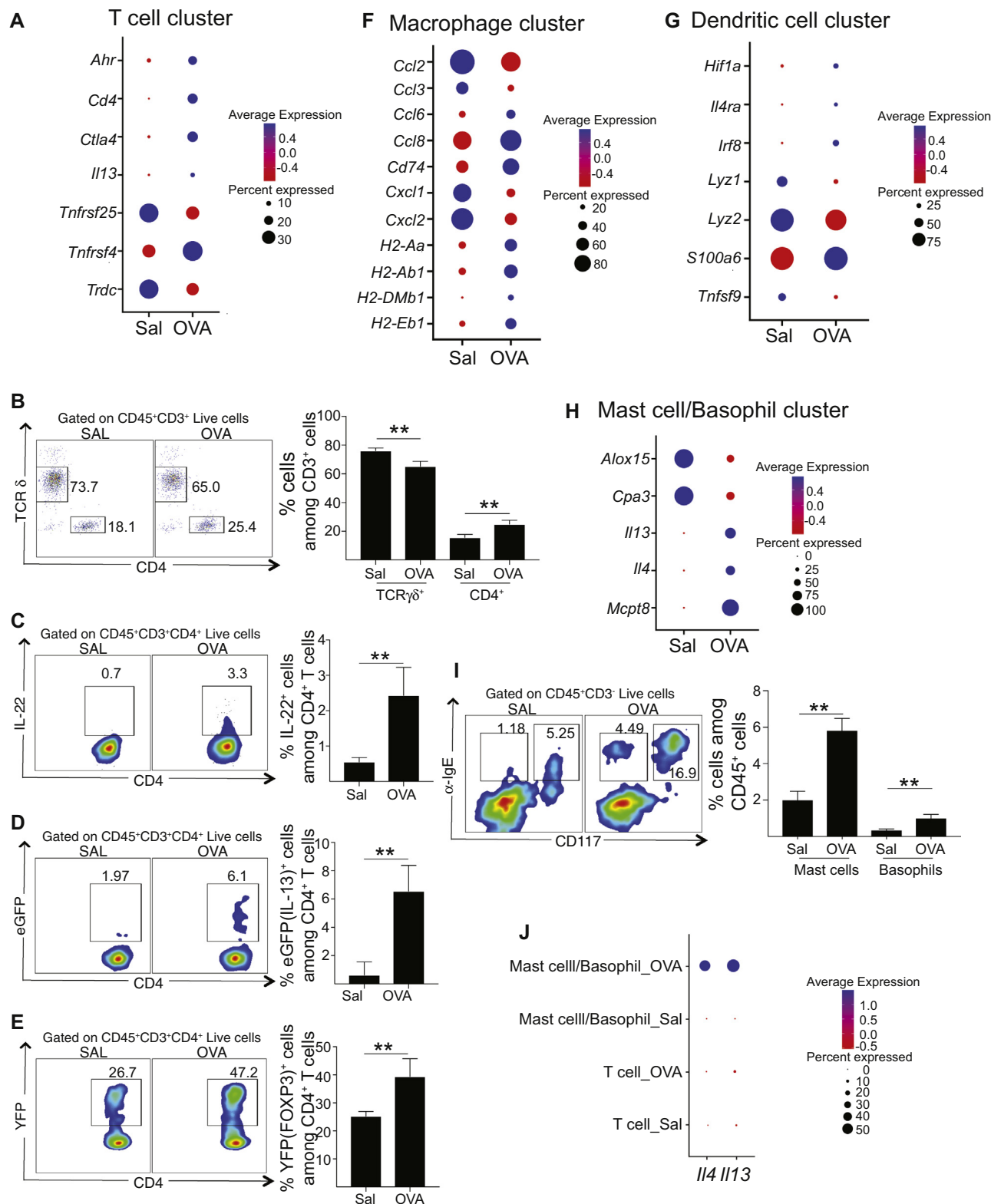


FIG 5. Characterization of immune cell populations in mouse EC-sensitized skin by scRNA-Seq. **A**, Dot plot of differentially expressed genes in T-cell cluster of skin EC sensitized with saline or OVA. **B–E**, Representative flow cytometry plots (left) and percentages of CD4⁺ T cells and TCR $\gamma\delta$ ⁺ T cells (**B**), CD4⁺IL-22⁺ T cells (**C**), CD4⁺eGFP(IL-13)⁺ T cells (**D**), and CD4⁺YFP(FOXP3)⁺ T cells (**E**) of skin EC sensitized with saline or OVA. **F–H**, Dot plot of differentially expressed genes in DC (**F**), macrophages (**G**), and MC/basophil (**H**) clusters of skin EC sensitized with saline or OVA. (**H**) Representative flow cytometry plots (left) and percentages of MCs and basophils of skin EC sensitized with saline or OVA. **I**, Dot plot displaying the relative *Il4* and *Il13* expression in the T-cell cluster and the MC/basophil cluster from skin EC sensitized with saline or OVA.

TABLE II. scRNA-Seq findings in mouse allergic skin inflammation shared with AD lesional skin

Characteristic	AD		Reference
	scRNA-Seq	Other	
Increased T-cell cluster		IHC	67
Increased DC cluster		IF	68
Increased macrophage cluster	X	IHC	36, 37, 62
Increased MC/basophil cluster		FACS, IF, histology	69, 70
Increased neutrophil cluster		IHC	71
Upregulation of <i>Il33</i> in collagen I-producing fibroblasts		IHC	72
Upregulation of <i>Ccl2</i> in fibroblasts	X		36
Upregulation of <i>Postn</i> in fibroblasts	X		36
Upregulation of <i>Ccl2</i> in fibroblasts	X		36
Upregulation of <i>Ccl27</i> in KC basal layer cluster	X		36
Downregulation of <i>Krt1</i> and <i>Krt10</i> in KC clusters		IF	73
Downregulation of <i>Flg</i> in KC basal layer cluster		PCR, IHC	74
Downregulation of <i>Lor</i> in KC basal layer cluster		PCR, IHC	50
Upregulation of <i>Selp</i> expression in endothelial cell clusters		IHC	75, 76
Upregulation of <i>Cd4</i> in T-cell cluster		IHC	67, 77
Upregulation of <i>Ctla4</i> and <i>Tnfrsf25</i> in T-cell cluster	X	IHC	36, 37, 78, 79
Upregulation of <i>Il13</i> in T-cell cluster	X	FACS	36, 37, 57
Upregulation of <i>Tnfrsf4</i> in T-cell cluster	X		36, 37
Upregulation of <i>Ahr</i> expression in T-cell cluster	X	FACS	36, 37, 66
Upregulation of <i>Fab5</i> , <i>Ccl8</i> and <i>ccl6</i> in macrophage cluster		IHC	59
Upregulation of antigen presentation genes in macrophage cluster		IHC	62

FACS, Fluorescence-activated cell sorting; IF, immunofluorescence; IHC, immunohistochemistry; X, scRNA-Seq data.

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Clinical implications: scRNA-Seq validates the use of the mouse model of antigen-driven allergic skin inflammation to investigate the pathogenesis of AD and unveils novel pathways in allergic skin inflammation.

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