

Supplementary Methods

Methods

1. Research Ethics Approval

All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Henan University and were approved by the Henan University Animal Ethics Committee.

2. Animals

Six-week-old female C57BL/6J mice were purchased from SPF (Beijing) Biotechnology Co. Mice were maintained on a standard light: dark cycle (12hr light, 8:00 a.m- 8:00 p.m, 12hr dark 8:00 pm-8:00 am) , with food and water provided ad libitum. After acclimatising for one week, behavioural experiments were conducted.

3. Model establishment

Intradermal Injection of AREG Protein into the Mouse Cheek: Sixteen mice were randomly assigned into either the vehicle control and AREG treatment groups in equal numbers. To facilitate intradermal (i.d) injection of AREG, the mice's cheeks were shaved one day prior. The treatment group received an i.d injection of AREG protein (989-AR, R&D Systems; diluted in PBS) at doses of 2.5, 5, or 10 µg/10 µL, while the control group received 10 µL of PBS. Injections were performed at an angle of approximately 15 ° relative to the cheek skin. Following i.d injection, scratching behaviour was recorded for 1 hour. Mice were euthanised 4 hours post-injection, and the skin at the injection site was collected and divided into two portions: one stored at -80 °C for transcriptomic analysis and the other fixed in 4% paraformaldehyde for immunohistochemical staining.

Construction of a Calcipotriol (MC903)-Induced AD-like Mouse Model and Injection of AREG Neutralizing Antibody (anti-AREG): Ten mice were randomly assigned to two groups: MC903 + AREG neutralising antibody or MC903 + vehicle control. The left ear of mice were treated topically with 20 µl of a 2 nmol MC903 solution (C4369, Sigma-Aldrich) for 13 days to induce an AD-like lesion, while the right ear received a vehicle control treatment (20 µL anhydrous ethanol). On day 10, the experimental group received an intraperitoneal (i.p.) injection of anti-AREG (25 µg, reconstituted in 70 µL PBS) (AF989, R&D Systems), while the vehicle control group received 70 µL PBS. One hour after injection, 2 nmol/20 µL MC903 was applied to the left ear, and 20 µL anhydrous ethanol was applied to the right ear. We recorded behaviour for one hour, and repeated the procedure for 4 days.

Construction of a 2,4-Dinitrofluorobenzene (DNFB)-Induced ACD Mouse Model and Injection of AREG Neutralising Antibody: Ten mice were randomly assigned to two groups: DNFB + AREG neutralising antibody or DNFB + vehicle control. To facilitate DNFB treatment, abdominal hair was shaved ($\approx 2 \times 2$ cm) one day before model induction to expose the skin. On Day 1, 25 µL of 0.5% DNFB (dissolved in 4:1 acetone:olive oil) was applied to the abdomen, and body weight was recorded (**Figure 2g**). Starting from day 2, the AREG-neutralising antibody group received an (i.p) of an anti-AREG antibody (5 µg/70 µL), while the vehicle control group received an i.p injection of 70 µL of PBS. The antibody injection was conducted once daily till day 9. Vehicle control injection was performed similarly. On Day 7,

one hour after the antibody or vehicle control injection, 10 μ L of 0.3% DNFB was applied to the right ear, with vehicle applied to the left ear, and scratching behaviour was recorded. On Day 9, four hours after AREG antibody injection, mice were euthanised, and ear tissues were collected for further analysis.

4. RNA-seq

RNA sequencing was performed by Beijing Genomics Institution (Shenzhen, China). Total RNA was extracted using Trizol (Invitrogen) and mRNA purified with oligo(dT)-magnetic beads. Purified mRNA was fragmented, reverse-transcribed into cDNA, and second-strand cDNA was synthesized. cDNA fragments underwent end repair, A-tailing, adapter ligation, and PCR amplification, followed by purification with Ampure XP beads. Quality control was performed using an Agilent 2100 bioanalyzer. Double-stranded PCR products were circularised to generate the final library, amplified into DNA nanoballs, and sequenced on a DNBseq platform to produce 50-base single-end reads. Raw reads were filtered with SOAPnuke (-l 15 -q 0.2 -n 0.05) and aligned to the reference genome using HISAT2. Mapping statistics were assessed, and gene expression analyses, including PCA, correlation, differential expression, heatmaps, and pathway analysis (Ingenuity Pathway), were performed using BGI's Dr Tom platform. Differentially expressed genes were defined as $\log_2$ fold change ≥ 1.0 and FDR < 0.05 , with FDR significance indicated as * (0.01–0.05), ** (0.001–0.01), *** (0.0001–0.001), and **** (< 0.0001).

The RNA-sequencing results from the ear skin samples used in our previous study were reanalysed and presented in this study. In brief, the samples were from the following models.

For the RNAseq models establishment: MC903-induced ear model (GSE230812): The right cheek skin of the mice in the experimental group was smeared with 20 μ L of 4 nmol MC903, and the right cheek of the mice in the control group was smeared with 20 μ L of solvent (absolute ethanol). After 7 days, after 4 hours of treatment, the cheek were collected for RNA-Seq. N=5 mice/group. DNFB-induced ear model (GSE237038): Mice were applied 25 μ L 0.5% DNFB (D1529, Sigma-Aldrich, dissolved in 4:1 acetone: olive oil) on the hairless part of the mouse abdomen (Ogawa et al, 2020). From day 6 to day 10, 10 μ L of 0.3% DNFB was applied to the left ear of the mouse, and an equal dose of acetone/olive oil (4:1) mixture was applied to the right ear. On day 11, the mouse ear skin tissues were harvested for RNA sequencing. AEW-induced dry skin cheek model (GSE237113): The right cheek hair was shaved until the skin was completely exposed. Cotton pads were soaked in an acetone:ethanol mixture and applied to the shaved area every day at 9 am and 5 pm for 30 seconds, then a cotton pad soaked in distilled water was applied to the same area for 30 seconds for 11 consecutive days. On day 11, after 4 hours of treatment, we harvested the mice's ear skin for RNA sequencing.

5. Single Cell Transcriptomics

A publicly available single-cell RNA transcriptomic data (GSE153760) was downloaded from the NIH database. This dataset consisted of blister material from AD lesional skin (n=4) and healthy control skin (n=5) resected from the antecubital fossa. The data were processed using the Seurat package (v5.3.1) in RStudio. For preprocessing and quality control, we initially

excluded doublets using Tukey's fences for outlier detection. We then processed the data with DoubletFinder to retain only singlets.

We first log-normalised the datasets using the NormalizeData function, then identified 3,000 highly variable genes in 15 human patient samples using the Seurat FindVariableFeatures function. After running DoubletFinder, we merged the data by disease state and performed batch correction with Harmony. We then assigned cells to clusters based on gene-expression similarity using the Leiden method and projected them using Uniform Manifold Approximation and Projection (UMAP). Differential gene expression between clusters was calculated by likelihood-ratio test as well as Wilcoxon rank-sum test with Bonferroni correction implemented in FindMarkers function. We annotated each cluster based on canonical marker genes and DEG analysis.

6. Behaviour Scoring

Scratch bouts and behaviours were assessed by observers blinded to the experimental groups. For itch evaluation, mice were acclimated for at least 1 hour in transparent, multi-compartment enclosures (10 cm × 10 cm; Ugo Basile). Scratching behaviour was recorded using EthoVision XT (Noldus) without human presence. A scratching event was defined as a hind limb movement directed toward the target site, counted when the limb returned to the ground or mouth. Continuous scratching within 1 second was considered a single bout. Assessments were conducted shortly after administration of pruritic compounds or allergen/irritant challenges, and mice were euthanised at the end of the study. All animals were monitored daily using a welfare scoring sheet. A score of 3 in either behavioural or respiratory categories indicated the need for humane euthanasia. Mice exhibiting severe pain, itch, or distress not intrinsic to the model, as determined in consultation with the veterinary team, were withdrawn from the study and humanely euthanised.

7. Culture of Primary Human Keratinocytes (NHEKs) and murine primary keratinocytes (NMEKs)

NHEKs (00192627B, Lonza) were cultured in KBM (keratinocyte basal medium)-Gold medium supplemented with KBM-Gold SingleQuot KC (Lonza) and maintained for 3 days before use (Meng et al., 2018).

Mouse skin was washed in 2% antibiotic PBS, and the epidermis was separated from the dermis by scalpels. The basal side of the epidermis was digested in 0.05% trypsin-EDTA (in PBS) at 37 °C for 8 minutes, and digestion was stopped with DMEM containing 10% serum. Keratinocytes were released by rubbing the epidermis with scalpels, filtered through a 100 µm strainer, centrifuged at 700 rpm for 5 minutes, and resuspended in complete medium before plating. Cells were washed 2–3 times with PBS, cultured in complete medium, and the medium was changed every other day according to cell density and condition.

8. Intracellular Ca²⁺ Measurement

For intracellular Ca²⁺ measurements, NHEKs (Lonza) were loaded with 3 µM Fluo-4 AM (F14201, Thermo) for 20–30 min. Images were acquired every 2 s using MateXpress 6 software on an ImageXpress Micro 4 system (Molecular Devices) after applying recombinant

human AREG (300 ng/ml, 262-AR, R&D Systems). Intracellular calcium changes were quantified as F/F_0 , where F represents fluorescence intensity and F_0 the baseline fluorescence, and plotted over time. In some analyses, changes were expressed as F/F_0 , where F denotes the fluorescence relative to baseline, and graphed over time. Cells exhibiting $\geq 20\%$ increase in fluorescence ($F\Delta/F_0 \geq 0.2$) were classified as responders (Larkin et al., 2021).

9. Cytokine Release Array

Murine primary keratinocytes were cultured in hydrocortisone-free medium (Lonza) for at least 24 h, then treated with murine AREG (300 ng/mL, 989-AR, R&D Systems) or vehicle for 24 h. Supernatants were collected and analysed using the Proteome Profiler Mouse XL Cytokine Array (R&D Systems) according to the manufacturer's instructions. Cytokine signals were quantified with ImageJ, and fold changes were calculated relative to controls.

10. Quantitative Real-time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from murine cells using TRIzol, and quantified using a NanoDrop 2000 (Thermo Fisher). cDNA was synthesised from 1 μ g RNA using the High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo). RT-qPCR was performed with SYBR Green Master Mix (309155, Thermo) and gene-specific primers. Expression levels were normalised to GAPDH. Primer sequences are listed below:

Targets	Primers or oligos (5'→3')
<i>mAreg</i>	F: GCAGATACATCGAGAACCTGGAG; R: CCTTGTCATCCTCGCTGTGAGT
<i>mTrpv3</i>	F: ATCCTGCTGAGGAGTGGCAACT; R: TTGATCTCGCGGCTGAGGATGT
<i>mGapdh</i>	F: CATCACTGCCACCCAGAAGACTG; R: ATGCCAGTGAGCTTCCCGTTCAG
<i>mTslp</i>	F: GCAAATCGAGGACTGTGAGAGC; R: TGAGGGCTTCTCTTGTCTCCG
<i>ml13</i>	F: AACGGCAGCATGGTATGGAGTG; R: CTGTCCTCAGACCGATGTTCTC
<i>hTRPV3</i>	F: ATCCTACTGCGGAGTGGCAACT; R: CGCTTCTCCTTGATCTCACGAC

11. Immunofluorescence Staining

Paraffin-embedded mouse skin sections were deparaffinized, rehydrated, permeabilised with 0.2% Triton X-100 in PBS (PBS-T), and blocked with 5% normal donkey serum in PBS for 1 h at room temperature. Sections were incubated overnight at 4 °C with rabbit anti-AREG antibody (1:200, Abs124825, Absin), followed by Alexa Fluor-conjugated secondary antibodies (1:500). After washing, sections were mounted with ProLong Antifade Reagent with DAPI (Thermo Fisher Scientific). Images were captured using an Olympus IX73 inverted microscope with CellSens software.

12. Effect of Gefitinib on AREG-induced TRPV3 Transcription in HaCaT

HaCaT cells (BioCat GmbH) were serum-starved for 3 hours, followed by incubation in serum-free medium containing Gefitinib (2 μ M; SML1657, Merck) for 2 hours. The medium was then replaced with serum-free medium containing Gefitinib (2 μ M) and/or AREG (300 ng/mL; 100-55B, Thermo Fisher Scientific) or their corresponding vehicle controls. Specifically, cells were treated with Gefitinib plus AREG vehicle (HBSS containing 0.1% BSA), Gefitinib plus AREG, or Gefitinib vehicle (DMSO) plus AREG. The cells were then incubated for an additional 24 hours. Cells were then harvested and processed for RT-qPCR for TRPV3 transcription.

13. Murine Peritoneal Mast Cell Culture and Degranulation (Beta-Hex) Assay

Primary mast cells were isolated from 8–10-week-old C57BL/6 mice. Briefly, the peritoneal cavity was lavaged with ice-cold sterile PBS containing 1% BSA and 0.1% EDTA. The recovered lavage fluid was centrifuged at $300 \times g$ for 5 min at 4 °C, and the cell pellet was resuspended in PBS. Mast cells were enriched by density-gradient centrifugation using BeyoColl™ separation medium (C546, Beyotime). Briefly, a discontinuous gradient consisting of 3 mL each of 100%, 40% and 30% separation medium was prepared, and 1 mL of the cell suspension was carefully layered onto the 30% fraction. Samples were centrifuged at $800 \times g$ for 20 min at 4 °C with the centrifuge brake disabled. Cells recovered from the 30%/40% and 40%/100% interfaces were collected, washed twice with ice-cold PBS ($300 \times g$, 5 min, 4 °C), and resuspended in complete medium consisting of high-glucose DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, 20 ng/mL stem cell factor (SCF; TMPJ-00166, Taoshushu) and 20 ng/mL interleukin-3 (IL-3; TMPK-00049, Taoshushu). Cells were seeded in 10-cm culture dishes and incubated at 37 °C for 2 h. Non-adherent cells were subsequently collected, gently resuspended and seeded into 48-well plates. Cells were stimulated with AREG (300 ng/mL; URPA006Mu01, Wuhan Yunkelong Biotechnology) or the corresponding vehicle for 15 min. Mast cell degranulation was assessed by measuring β -hexosaminidase (β -Hex) activity using a degranulation assay ELISA kit (CS-1295E, Beyotime). Degranulation percentage is calculated by removing background activity by subtracting the blank control from both the experimental sample and the Triton X-100 total-release control. The resulting blank-corrected experimental value is then expressed as a proportion of the blank-corrected Triton X-100 value to represent the fraction of total β -hexosaminidase released.

14. Data Analysis

Data are presented as mean $\pm$ SEM. Statistical significance was assessed using unpaired Student t-test, with $P < 0.05$ considered significant. Analyses were performed using Prism (GraphPad Software, La Jolla, CA). For RNA-seq, differentially expressed genes (DEGs) were defined as those with $|\log_2 \text{fold change}| \geq 1.0$ and a false discovery rate (FDR, Q-value) < 0.05 ; genes not meeting the fold-change threshold were not considered DEGs, even if $\text{FDR} < 0.05$. FDR values were further categorized as < 0.001 , $0.001 - < 0.01$, $0.01 - < 0.05$, or ≥ 0.05 .

For scRNAseq analysis of GSE153760, patients suffered from AD since childhood and did not receive topical (2 weeks) or systemic therapy (4 weeks) prior to skin sampling. Lesions were free of clinical signs of superinfection (including strong oozing or crusting). Nine AD patients (mean age 29.4, range 19–49; mean EASI 26.2, range 5.0–44.7) and eight HC individuals (mean age 39.1 years, range 28–49) were subjected to suction blistering. Four additional AD patients (mean age 29.8 years, range 22–42; mean EASI 35.4, range 24.1–46.5) and two HC individuals (27 and 42 years) donated skin biopsies.

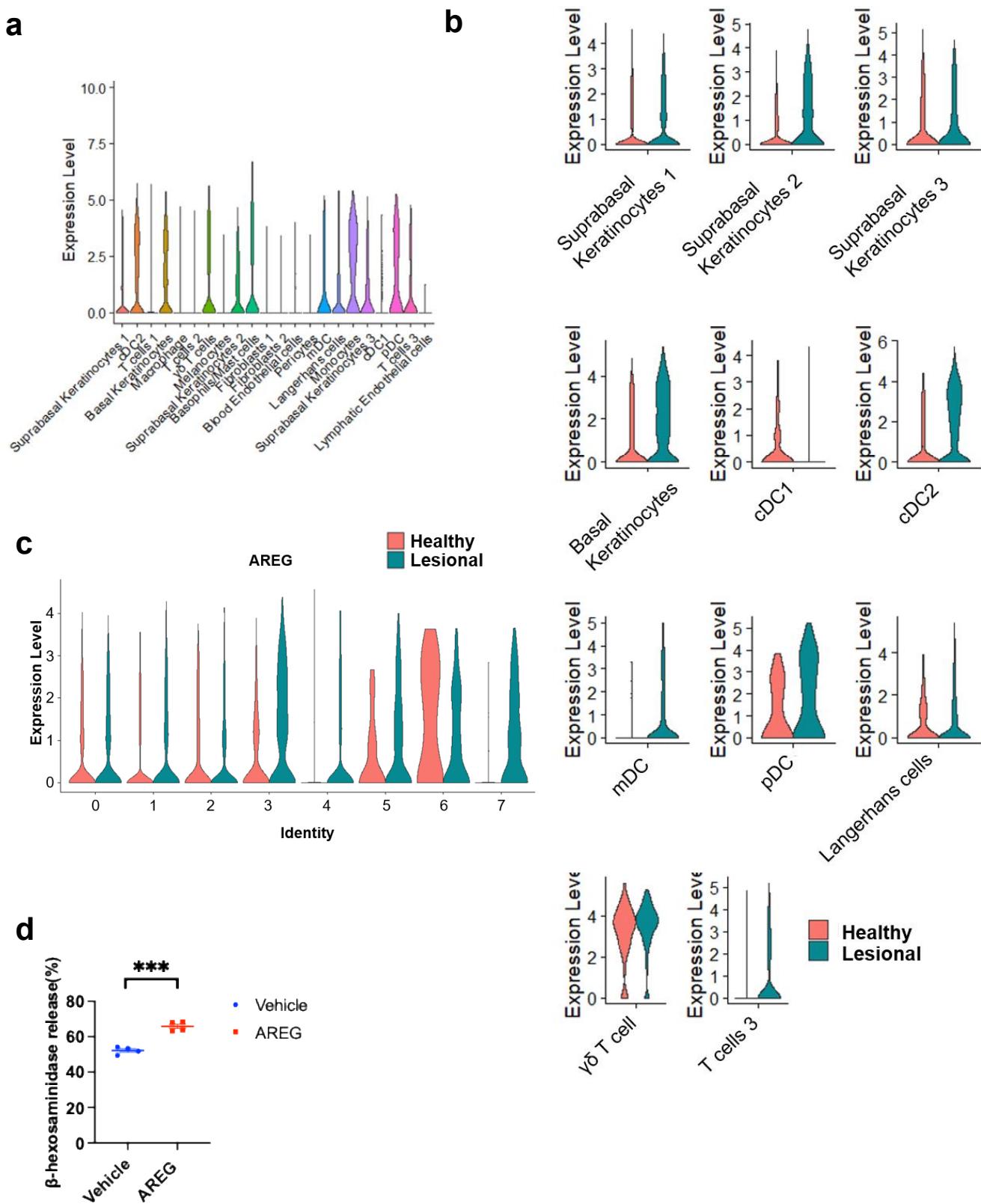
Reference

- Larkin C, Chen W, Szabo IL, Shan C, Dajnoki Z, Szegedi A, et al. Novel insights into the TRPV3-mediated itch in atopic dermatitis. *J Allergy Clin Immunol* 2021;147(3):1110–4 e5.
- Meng J, Moriyama M, Feld M, Buddenkotte J, Buhl T, Szollosi A, et al. New mechanism underlying IL-31-induced atopic dermatitis. *J Allergy Clin Immunol* 2018;141(5):1677–89 e8.

Supplemental Table S1

Data Source: GSE59275	
Samples	AREG-Counts Per Million:Cpm
Control keratinocytes Day 02 sample1	8.66
Control keratinocytes Day 02 sample2	7.54
Il-4-stimulated keratinocytes (3h) Day 02 sample1	2.74
Il-4-stimulated keratinocytes (3h) Day 02 sample2	5.29
Il-4-stimulated keratinocytes (24h) Day 02 sample1	1.63
Il-4-stimulated keratinocytes (24h) Day 02 sample2	8.12
Control keratinocytes (120h) Day 05 sample 1	36.72
Control keratinocytes (120h) Day 05 sample2	40.08
Il-4-stimulated keratinocytes (120h) Day 05 sample1	23.60
Il-4-stimulated keratinocytes (120h) Day 05 sample2	11.55

Supplemental Table S1. Reanalysed public dataset (Data Source:GSE59275) demonstrated that IL-4 treatment represses AREG expression in keratinocytes. Based on this finding, the induction of AREG transcription by IL-13 is relatively specific and is not a general consequence of type 2 cytokine stimulation. Thus, IL-13 and IL-4 may exert distinct effects on keratinocyte activation and AREG expression.



Supplemental Figure S1. Amphiregulin (*AREG*) expression in keratinocytes. Each cluster as illustrated by (a) violin plot and (b) feature plot for both health and AD skin. (c) Subclustering of suprabasal Keratinocyte 1 reveals 7 subclusters expressed AREG, with two showing increasing expression in lesional skin (Clusters 4 and 7). (d) AREG induces β -hexosaminidase release from murine peritoneal mast cells (n=4).