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Tape-stripping alters the microbe-host correlations in mouse skin

To the Editor,

Allergic diseases usually begin early in life with atopic dermatitis (AD), progressing to food allergies, asthma, and/or rhinitis later in life. AD is characterized by itching and scratching, dermal infiltration with T helper 2 (Th2) cells, and elevated serum IgE and eosinophilia.¹ Continuous scratching causes skin injury and induction of wound repair mechanisms from inflammatory reactions (0-48 hours) and tissue proliferation (2-14 days) to remodeling (<1 year).² Abnormal host-microbe interactions have been shown to associate with cutaneous disorders like AD, acne, and psoriasis, but the exact mechanisms underlying the microbial contributions to disease development and progression are currently unknown.³ Our aim was to study how skin injury induced by tape-stripping changes mouse skin microbiota, genomewide gene expression, and how these two coexisting systems interact during epithelial recovery phase up to 29 days.

To study microbiota-host interactions, standardized skin injury was induced to BALB/c mouse skin by eight consecutive tape-stripping (Figure S1). After 1, 5, 8, 11, and 29 days, skin swabs and biopsies were collected for 16S rRNA sequencing and genome-wide arrays, respectively. For detailed methodological information, please see online repository. Histologically, both epidermis and dermis showed enhanced thickening, peaking at 8 days, and reaching the original thickness in 29 days (Figures 1A, S2). Neutrophils were recruited to the site of tape-stripping at the early time points (1 day), and small number of eosinophils was present in the skin at later time points (5-11 days) (Figure 1A).

As an injury disturbs the cutaneous homeostasis, we investigated dynamics of the skin microbiota during microbial recolonization by 16S amplicon sequencing. Microbial alpha-diversity increased until 11 days and then started to decrease but did not reach the original diversity seen in the untreated controls (Figure 1B). The operational taxonomical units (OTUs) that changed significantly in relative abundances formed four main clusters in the heatmap containing samples from time points 5d, 11d, ctrl-1d-8d, and ctrl-29d (Figure 1C). The intact skin of the control mice had high proportional

abundance of Burkholderiales/Proteobacteria (Figure S3), which are shown to play an important role in skin homeostasis.⁴ Alekseyenko et al⁵ divided Proteobacteria-associated cutaneotype into healthy individuals and Actinobacteria/Firmicutes-associated cutaneotypes into psoriatic and lesional skin specimens. Along with this, Firmicutes was the major phylum represented on mouse skin at 5 days in our studies, represented primarily by the order Gemellales, which is also categorized into the AD-prone signature microbes in humans.⁶ The distribution of bacterial OTUs and their relative abundances did not fully recover to the original level at our last time point (29 days), suggesting that skin irritation by intensive scratching and following barrier disturbance cause long-lasting effects on microbiota composition on the involved epidermal area.

In reverse to microbial changes, a month was enough for the skin transcriptome to recover. The differentially expressed genes (DEGs, Figure S4) formed two separate clusters at 1 day and at 5 days, and samples at time points 8 and 11 days shared the third cluster, whereas gene expressions of the untreated controls and samples at d29 together formed the last cluster (Figure 1D). Gene functions related to inflammation (IL-6/8/17, AHR), antigen presentation, chemotaxis, and acute-phase responses were highly activated, while helper T-cell pathways (Th1/Th2) and co-stimulation signaling (ICOS, PPAR, and LXR/RR) were decreased in canonical pathway analysis (IPA) especially at the 1 day time point (Figures 1E, S5). Inflammation and proliferation phases were recognized in our transcriptomics analysis, and similar pathways have been reported to be activated also in previous studies.⁷ Almost all stages of wound healing are affected by various matrix metalloproteinases (MMP), and also in our data, MMPs were highly upregulated especially at 1 day and in lesser amount at 5 days (Figure S6). Tightly regulated expression of MMPs is crucial for proper re-epithelialization and regeneration, and their dysregulation leads to prolonged inflammation and delayed wound healing.⁸ The microarray results were validated by RT-PCR (Figure S7).

To study the interactions between microbiota and host gene expression, we performed regularized canonical correlation analysis,

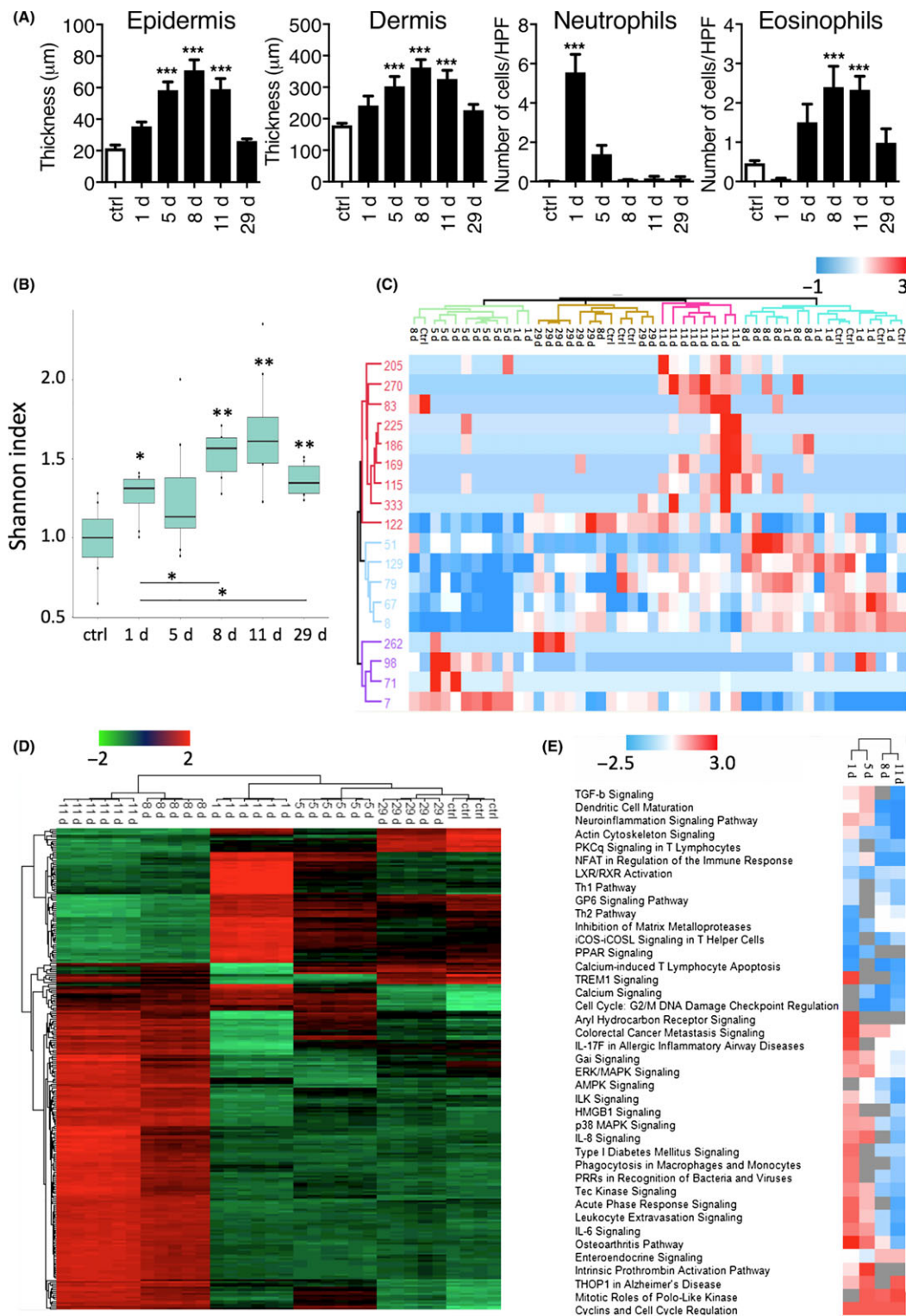


FIGURE 1 Tape-stripping thickens skin layers, changes microbiome, and modifies gene expressions. A, Skin thicknesses were measured, and number of infiltrating cells was counted from the H&E-stained sections. B, Shannon diversity index. A-B, For comparisons, nonparametric Mann-Whitney *U* test was used, and results are expressed as mean $\pm$ SEM. **P* = 0.05; ***P* = 0.01; ****P* = 0.001. C, The most significantly changed 18 microbial operational taxonomic units (OTUs) were clustered based on their normalized (Z-scored) abundances according to time with Pearson distance method. D, Differentially expressed genes (DEGs) were hierarchically clustered by similarity based on Euclidean correlation. E, Ingenuity pathway analysis (IPA) of DEGs on Canonical pathways analysis

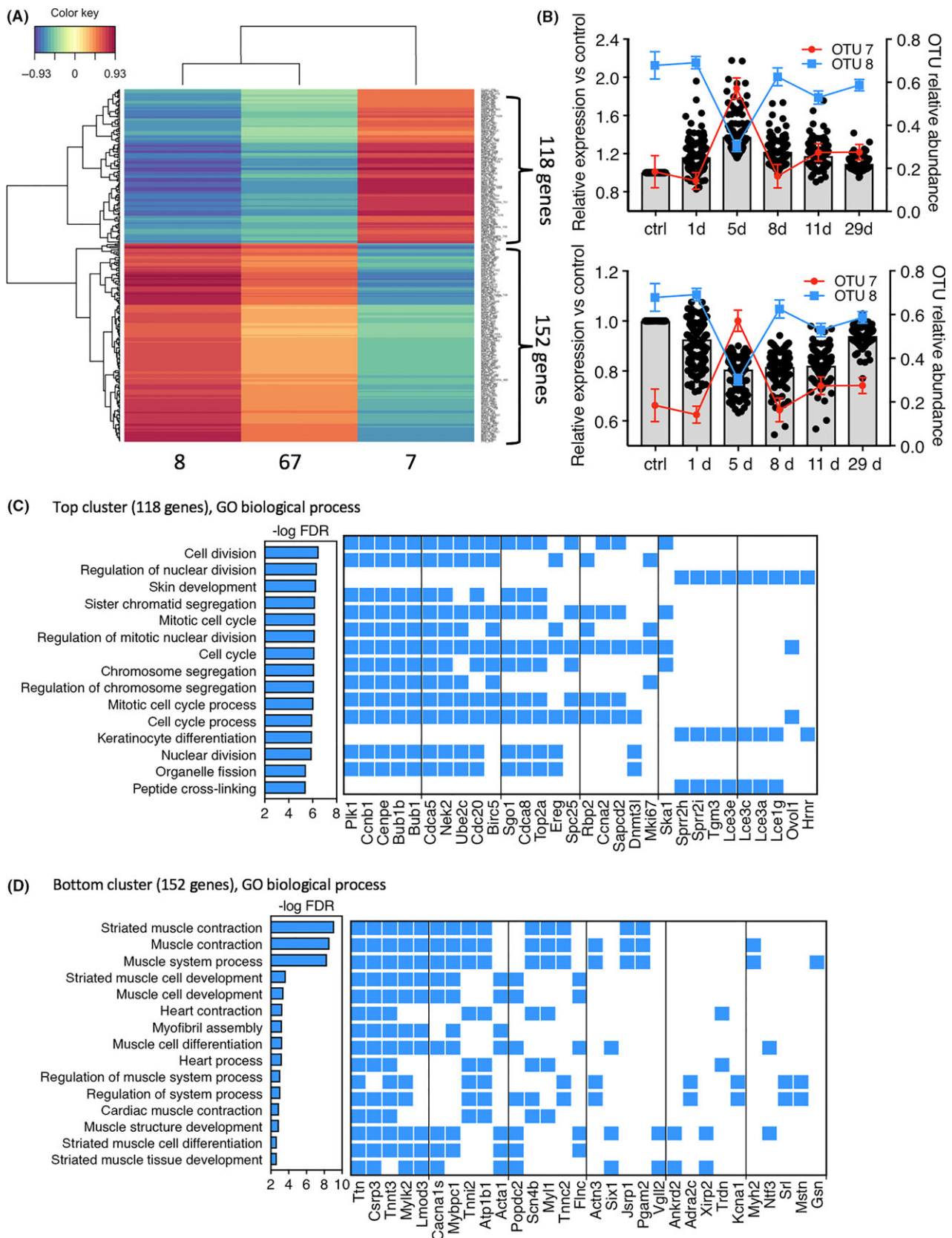


FIGURE 2 Data integration shows correlation between microbiota and skin transcriptome. A, Heatmap and hierarchical clustering of interacting 270 differentially expressed genes (DEGs) correlating with three microbial operational taxonomic units, OTUs 7, 8, and 67. B, Relative expression of top and bottom clusters of the DEGs in (A) with relative abundance of OTUs 7 and 8. Fifteen of the most significant GO biological processes and their interrelating genes in the top (C) and bottom (D) clusters according to the PANTHER Classification System

which showed a significant correlation between 3 OTUs and 270 DEGs throughout the time points (Figure 2A). OTUs 7 (*Gemella*/Firmicutes) and 8 (*Ralstonia*/Proteobacteria) made up 78.9%–87.4% of the skin microbiota composition at all time points. Enhanced abundance of the pathobiont *Gemella* was previously associated with AD,⁶ whereas abundance of *Ralstonia* was shown to be decreased in the skin of allergic dogs.⁹ Especially at 5 days, *Gemella* positively correlated with the upper cluster of 118 genes, and anticorrelated with the bottom cluster of 152 genes, whereas *Ralstonia* showed correlations in the opposite manner (Figure 2B). According to the PANTHER Classification System, the upper cluster genes play roles either in cell division and chromosome organization (Bub1) or in formation of cornified envelope (Lce genes) during keratinocyte differentiation (Figure 2C). In reverse, the lower cluster of genes was involved in muscle cell development and muscle construction, and many of these genes were related to titin (Ttn), troponin (Tnnt3, Tnni2), or actin/myosin (Acta1, Mylk2, Mybpc1, and Myl1) filaments (Figure 2D). These genes act mainly during the proliferation phase, when activated fibroblasts and keratinocytes divide and migrate into the injury site to establish a viable barrier, and lesioned tissue area is diminished by wound contraction. In addition to the impact on wound healing and allergic sensitization, better understanding of these interactions might offer instruments for plastic surgery and subcutaneous drug delivery systems.

In conclusion, our results show that tape-stripping disturbs the skin barrier, reversibly changes gene expression in the host, and has long-lasting effects on the skin microbiota. When the microbial OTUs and DEGs were integrated, we found a correlation of proliferation and muscle contraction gene functions with certain Firmicutes and Proteobacteria genera. Although deeper sequencing and identification of the interacting bacterial species is needed, we believe that *Gemella* and *Ralstonia* are examples of bacteria, which play a role in wound healing and (dys)regulation of skin homeostasis. Our results highlight the importance of skin homeostasis, and relevance to further investigate the interactions between microbiota and host gene expression as prolonged dysbiosis may lead to the establishment of skin diseases.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Genetic loci determining total immunoglobulin E levels from birth through adulthood

To the Editor,

Total immunoglobulin E (IgE) is an intermediate phenotype and a potential therapeutic target for allergic diseases. Recent genome-wide association studies (GWASs) have discovered single-nucleotide polymorphisms (SNPs) to be associated with IgE levels, including genes residing within the major histocompatibility complex (MHC) region across several populations.¹⁻⁵ However, limited susceptible genes from GWASs for IgE levels have been reported in Asian populations, while only Yatagai et al⁵ revealed SNPs within the human leukocyte antigen (*HLA*)-C region are associated with IgE levels in 1180 Japanese adults. Here, we aimed to investigate potential functional influence of susceptible genes associated with IgE levels by performing integrative analyses.

We conducted a GWAS of total IgE levels in a Taiwanese population (415 subjects recruited from the Prediction of Allergies in Taiwanese CHildren (PATCH) schoolchildren cohort⁶) and performed replication analyses in three independent cohorts: a Taiwanese birth cohort (431 newborns from the PATCH birth cohort,⁷ which is different to the children enrolled in the PATCH schoolchildren cohort), a Taiwanese schoolchildren cohort (another independent 838 PATCH schoolchildren), and a Caucasian adult cohort (1120 adults from the Chicago Food Allergy Study⁸). Characteristics of the discovery and replication samples are presented in Table S1. The study design and analysis pipeline are shown in Figure S1. Detailed methods are provided in the Supporting information.

Nine independent SNPs associated with IgE levels were identified at a suggestive significance level $P < 10^{-5}$ in the discovery sample (Table 1; Figure 1A) and only rs660895 was replicated in three independent samples ($P = 2.69 \times 10^{-3}$ for independent schoolchildren; $P = 1.90 \times 10^{-2}$ for birth cohort; $P = 3 \times 10^{-3}$ for adult cohort; $p_{\text{meta-analysis}} = 3.24 \times 10^{-10}$ for all study cohorts). The regional association plot for rs660895 in the *HLA-DRB1/DQA1/DQB1* gene region at 6p21.32 is presented in Figure 1B. We did not genotype those associated SNPs downstream *HLA-DQB1* for replication

because they are in strong linkage disequilibrium (LD) with SNP rs660895 ($r^2 > 0.8$). Lower IgE levels were found in subjects carrying one or two copies of the rs660895-G alleles in all study samples of schoolchildren, newborns, and adults (Figure 1C). No SNP at a genome-wide significance level was identified in the discovery cohort and only one of nine potentially associated SNPs was replicated in three independent cohorts, which might reflect the low power due to limited sample size.

We performed secondary analysis with IgE-related phenotypes, specifically, atopy, allergic rhinitis, asthma, eczema, and either one of allergic rhinitis, asthma, or eczema using 1235 PATCH schoolchildren. Rs660895 was associated with atopy ($P = 1.81 \times 10^{-3}$), allergic rhinitis ($P = 1.20 \times 10^{-4}$), and either one of allergic rhinitis, asthma or, eczema ($P = 2.31 \times 10^{-3}$) (Table 1). Seven of the 9 IgE-associated SNPs identified in the discovery analysis were associated with atopy, indicating their potential broader relevance to atopy (Table 1).

To evaluate potential functional influence, we mined two eQTL databases in four specific tissues (blood, skin, lung, and lymphoblastoid cells). The minor allele of rs660895-G was associated with lower expression of *HLA-DRB1*, *HLA-DQA1*, and *HLA-DQB1* genes in all four tissues, suggesting potential regulatory function of rs660895. We performed SNP-methylation associations using 34 945 CpG sites on chromosome 6 in 218 whole blood samples from the Chicago Food Allergy Study. We found that methylation levels at 27 of the 53 probes in the *HLA-DRB1/DQA1/DQB1* region were associated with genotypes at SNP rs660895 in the Chicago Food Allergy Study after correcting for multiple testing (Table S2). Three peanut-allergy-related differentially methylated probes (DMPs),⁸ cg15982117 ($p_{\text{FDR}} = 1.77 \times 10^{-2}$), cg17039645 ($p_{\text{FDR}} = 7.23 \times 10^{-4}$), and cg18111114 ($p_{\text{FDR}} = 1.20 \times 10^{-6}$) were associated with SNP rs660895, further supporting potential functional influence of SNP rs660895 not only on total IgE but also on allergic diseases (Table S2). Among the three peanut-allergy-related DMPs, cg15982117 and cg18111114 locate in the *HLA* gene region. In

Supporting document for:

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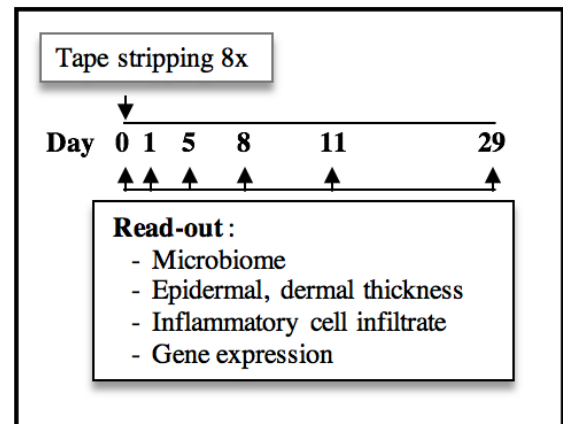
Supplementary Materials and methods

Animals and sensitization

Female Balb/c mice (6–8 weeks old) were purchased from Scanbur AB (Sollentuna, Sweden), and four mice were kept four per cage with food and drinking water ad libitum. All animal experiments were approved by the Social and Health Care Department of the State Provincial Office of Southern Finland (decision PH1238A).

Mice (balb/c) were anaesthetized with 4% Isofluran inhalation (Abbott Laboratories Ltd, Queenborough, UK), the skin on the back of the mice was shaved and then tape stripped eight times by a transparent adhesive tape (Tegaderm, 3M Health Care, St Paul, USA) to introduce a standardized skin injury. Two cages of mice (eight mice) were sacrificed at each timepoint. Samples from skin swabs (dipped in sterile 0.15M NaCl, 0.1% Tween) and biopsies from treated areas were obtained for RNA isolation, histology and immunohistochemical examination (Figure S1).

Figure S1. Study settings. The back of each mice was tape-stripped 8 times on day 0. Swabbing for microbiome studies and skin biopsies were taken 1d, 5d, 8d, 11d or 29d after the tape-strippings.



Histological analysis

Skin specimen were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4 µm sections were cut and stained with haematoxylin and eosin (H&E). Individual inflammatory cell types were counted in 5 high-power fields (HPF) from 3 different sections at x100 magnification with oil, and expressed as cells per HPF, with mean and standard error of the mean (SEM) calculated. The thickness of the epidermis and dermis was measured from 5-10 measurements/mouse using a 10x ocular lens on a light microscope. The measurements were adjusted for the magnification optics and expressed in micrometres with mean and SEM calculated.

Extraction of microbial DNA and sequencing

DNA was extracted from the mouse skin swabs using FastDNA Skin Kit for soil (MP Biochemicals) according to the instructions of the manufacturer. Each sample was homogenized twice with FastPrep Instrument (MP Biochemicals) at 6.0 m/s for 40 sec. Elution was done with 100 µl DES-H₂O. The V1–V3 regions of the 16S rRNA gene were amplified with modified universal bacterial primers with overhangs (F', ACACTCTTCCCTACACGACGCTCTTCCGATCTAGAGTTTGATCMTGGCTCAG and R', AGACGTGTGCTCTTCCGATCTGTATTACCGCGGCTGCTG). In PCR amplification, Phusion polymerase (Thermo Fisher Scientific/Finnzymes) with the HF buffer and 2.5% DMSO were used. Cycling conditions consisted of an initial denaturation at 98°C for 30 s, followed by 26-28 cycles of 98°C for 10 s, 65°C for 30 s, and 72°C for 10 s, and then a final extension at 72°C for 5 min. Between 10 and 15 ng of template was used for each reaction. PCR was done in triplicate and the products checked in agarose gel (approximately 550 nt in size) were mixed together. PCR products were purified with Ampure XP beads according to the instructions of the manufacturer. Purification was done in 1.5 ml tubes and magnetic rack. Extracted DNA was used for 16S gene sequencing on the Illumina MiSeq PE300 to obtain paired-end reads.

Analysis of the skin microbiota

Reads were preprocessed and aligned with USEARCH v.10.0¹ using parameters -fastq_maxdiffs 25, -fastq_pctid 25 ja -fastq_minovlen 5, and filtered with the criteria of maximum expected errors 2 (-fastq_maxee 2). The filtered sequences were dereplicated and all singleton sequences were discarded. To determine the taxonomy, OTUs were clustered (command -cluster_otus) and aligned with mothur v. 1.36.² using the SILVA rRNA gene database v. 123³. Data analysis was performed in R v. 3.3.2⁴ using the packages phyloseq⁵ and vegan⁶.

The microbial alpha diversity is shown by Shannon index. Differences between groups were analysed using the nonparametric Mann-Whitney U test. Pair-wise comparisons were corrected using the FDR-method ("false discovery rate") in p.adjust function in R.

Statistical analyses

Differences between groups in histological and rt-pcr analyses were performed with GraphPad Prism 7 Software (GraphPad Software Inc., San Diego, California). One-way ANOVA was followed by single group comparisons with nonparametric MannWhitney U-test. Results are expressed as mean $\pm$ SEM and p-values of <0.05 were considered to be statistically significant.

RNA isolation, cDNA synthesis and real-time quantitative PCR (RT-PCR)

The murine skin samples were homogenized in Trisure reagent using a FastPrep FP120 homogenizer (BIO 101; Thermo Savant, Waltham, Massachusetts) followed by phenol-chloroform protocol for RNA isolation. The quantity and purity of the total RNA was determined by NanoDrop spectrophotometer (ND-1000, Wilmington, Delaware) and Bioanalyzer 2100 instrument (Agilent Technologies, USA). Isolated total RNA was dissolved in nuclease free water and stored at -80°C .

cDNA was synthesized from 500 ng of total RNA in a 25 μl reaction using MultiScribe Reverse Transcriptase and random primers (The High-Capacity cDNA Archive Kit; Applied Biosystems, Foster City, California) according to the manufacturer's protocol. The synthesis was performed in a 2720 Thermal Cycler (Applied Biosystems, Carlsbad, California) starting with 25°C for 10 min and continuing with 37°C for 120 min.

Primers and probes (IL-1beta, IL-33 and S100A7a) for RT-PCR analysis were ordered as pre-developed assay reagents from Applied Biosystems. Endogenous 18S was used as housekeeping genes. Amplifications were performed in an 11 μl reaction volume with TaqMan universal PCR master mix (Applied Biosystems). The assays were performed in 96-well optical reaction plates with Relative Quantification 7500 Fast System (7500 Fast Real-Time PCR system, Applied Biosystems) according to the manufacturer's instructions.

Microarrays

DNA microarray analysis was performed using 4-6 samples from one group of mice. Average RIN value for RNA samples was 8.7 with lowest value of 7.3. Samples (200 ng total RNA) were labeled using a Two-Color Microarray-Based Gene Expression Analysis protocol (version 6.6). RNA primed with oligo-dT (T7) promoter primers was converted to cDNA with AffinityScript RNase Block (Low Input Quick Amp Labeling Kit, Two-color, Agilent Technologies), according to the instructions of the manufacturer. Primed cDNAs were labeled with Cy3 and Cy5 dyes (Agilent Technologies) and amplified using T7 RNA polymerase amplification method (Low Input Quick Amp Labeling Kit, Agilent Technologies), according to the instructions of the manufacturer. Amplified samples were cleaned-up with Qiagen's RNeasy mini kit (Qiagen, GmbH, Hilden, Germany). Primed cDNA was hybridized to the Agilent 2-color 60-mer oligo microarray slides (Agilent Technologies, SurePrint G3 Mouse GE 8x60K Kit, USA) according to the instructions of the manufacturer (Agilent Technologies). After washing and scanning the slides with Agilent Microarray Scanner G2505C (Agilent Technologies) raw intensity values were obtained with the Feature Extraction software (version 11.5.11, Agilent Technologies).

Analysis of microarray data

Data was imported to eUtopia (manuscript in preparation) software and the median intensities were log2 transformed and quantile normalized with Bioconductor package Limma⁷. SVA-package with Combat function was utilized for removal of batch effects, emerging from labelling and array variance⁸. Linear model was fitted to the batch-corrected data and pairwise comparisons were performed by empirical Bayes method. Genes with linear fold change ≥ 11.5 , and Benjamini-Hochberg adjusted P-value $\leq 10^{-5}$, were considered significantly differentially expressed when compared to samples of untreated naïve mice. Hierarchical clustering of differentially expressed genes was done within the Perseus data analysis platform⁹. Differentially expressed genes from different time points were independently Z-score normalized. The Euclidean method and the k-means algorithm were respectively used to determine the distance between two dataset points and to define the distance between two clusters. A hierarchical cluster of the top 250 differentially expressed genes (based on adj. p-values) are depicted in the form of heatmaps for each time point. Upregulated genes (red) are those genes that have a positive standard deviation from the genes mean expression across all exposures, and vice versa for downregulated genes (green). To evaluate the distribution of differentially expressed genes between specified contrast sets were created using Venny¹⁰.

Functional enrichment analyses were performed using web-based tools EnrichR (<http://amp.pharm.mssm.edu/Enrichr/>) and Panther from Gene Ontology Consortium (<http://www.geneontology.org>), and the QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, www.qiagen.com/ingenuity).

Integrative analysis of the microbiota and transcriptome

To identify associations between the microbiota (71 OTUs, >25 reads) and the transcriptome (1957 unique DEGs, $P \leq 10^{-5}$ and $FC \geq |1.5|$), we performed a Regularized Canonical Correlation Analysis utilizing the mixOmics package¹¹. The aim of the analysis was to apply a multivariate exploratory approach to highlight correlations between the two omics data sets acquired on the same experimental units/time points (1d, 5d, 8d, 11d and 30d). Regularization was applied since the number of variables in each set is greater than the number of experimental units. Before running rCCA, we tuned the regularization parameters λ_1 and λ_2 by using the leave-one-out cross validation procedure. A color-coded Clustered Image Maps ("heat maps") was generated to visualize all the associations with correlations greater than 0.75 (absolute value).

Supplementary Results

Histology

Tape-stripping thickened the skin and enhances the recruitment of granulocytes (Figure S2).

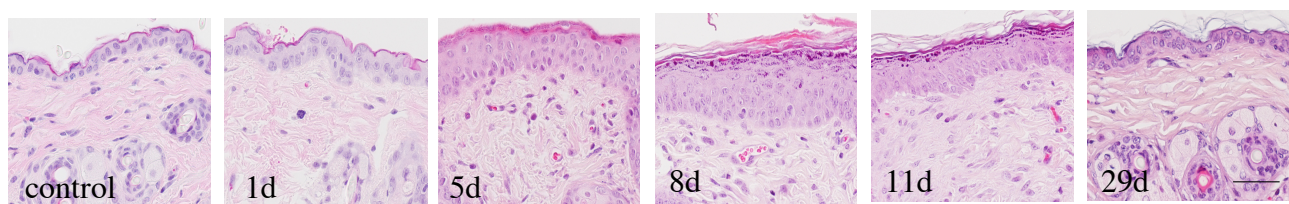


Figure S2. H&E stained skin sections at different time points, light microscope 40x magnification.

Tape-stripping modulates the microbiota on the skin

The shifts in the alpha diversity (Shannon index) were reflected also to the proportional abundances of bacterial composition, and Burkholderiales (Proteobacteria) was the predominant order in 83% of the timepoints (5/6). Gemellales (Firmicutes) became a highly predominant order at 5d, and sequences from Lactobacillales (Firmicutes) were present especially at 8d. Still, at the last time point (29d), the relative abundance of Gemellales (Firmicutes) was higher, and Burkholderiales (Proteobacteria) lower, than in the untreated control mice, indicating that changes of the microbiota are not fully restored in the followed 29 days. (Figure S3)

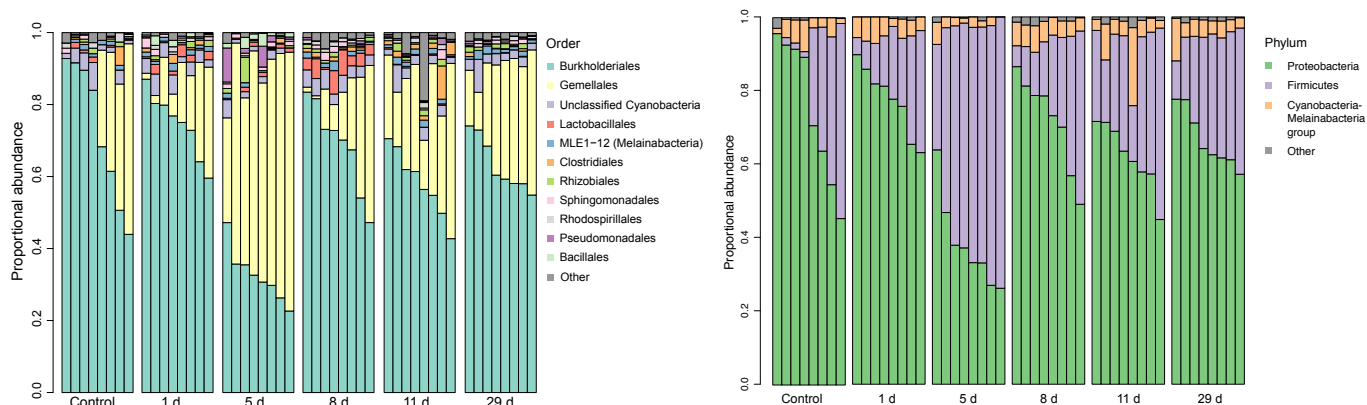


Figure S3: Microbiome characterization, proportional abundances of the 11 most abundant bacterial orders and 3 phyla at different time points.

Differential gene expression analysis, and pathway analyses

We found 702 DEGs at 1d, 662 DEGs at 5d, 815 DEGs at 8d and 1150 DEGs at 11d, when compared to control expressions in naïve mouse skin. There were no specific DEGs at the last time point (29d) (Figure S4). VENN diagram was used to form a general view of the distribution of common and specific DEGs (Figure S4), whose biological functions were roughly estimated at different time points by the EnrichR pathway analysis tool. The 384 genes specific for 1d played a role mostly in inflammatory responses, the 192 genes specific for 5d were involved in skin development and peptide cross-linking, whereas the shared 152 genes participated in muscle contraction and sliding during 120-11d (data not shown).

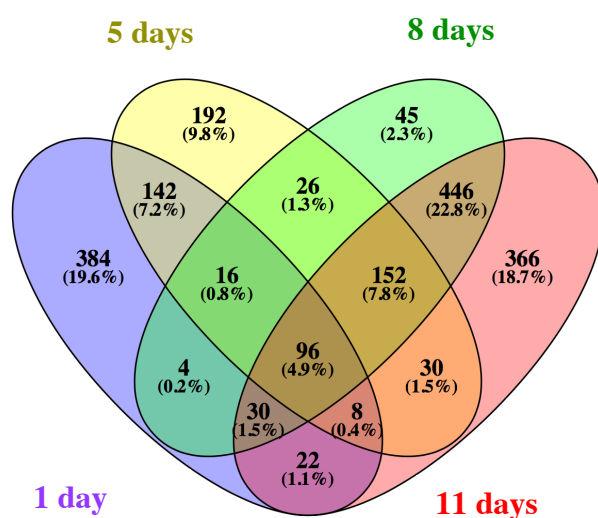


Figure S4: Specific and overlapping DEGs in VENN diagram at each time point.

Also, according to the Diseases and Biofunctions related pathways in IPA, chemotaxis, cell homing and cell activation as well as cytoskeleton organization were highly enhanced at 1d, while the apoptosis and cell necrosis functions were attenuated during time points 24-8d (Figure S5). At later time points (5d-11d), only functions related to cell survival or division and chromosomal alignment were activated (Figure S5).

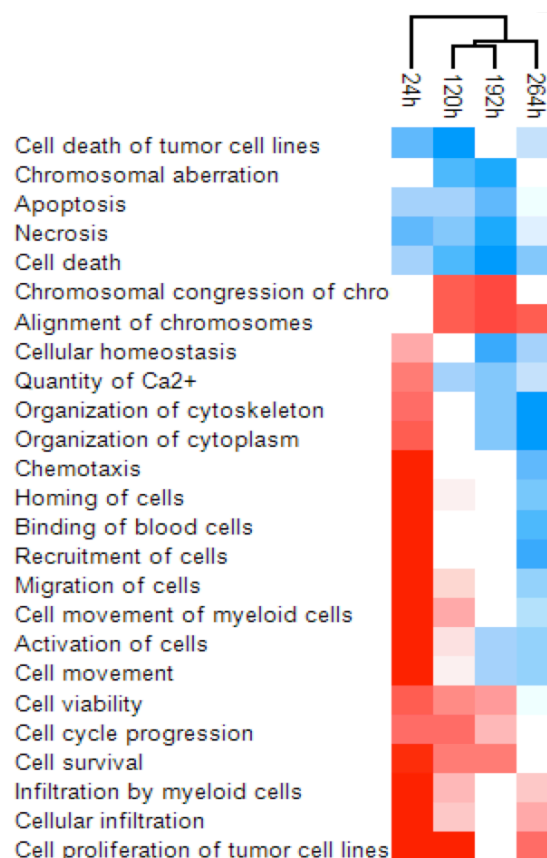


Figure S5: Ingenuity pathway analysis (IPA) of DEGs on Diseases and Biofunctions.

Also, expression of several matrix metalloproteinases including MMP3, MMP8-10, MMP13 and MMP19, were highly upregulated especially during 1d-5d (Figure S6). In order to validate the microarray results, we run RT-PCR for three target genes, IL-1beta, IL-33 and S100A7a, whose expressions were highly similar with obtained microarray results (Figure S7).

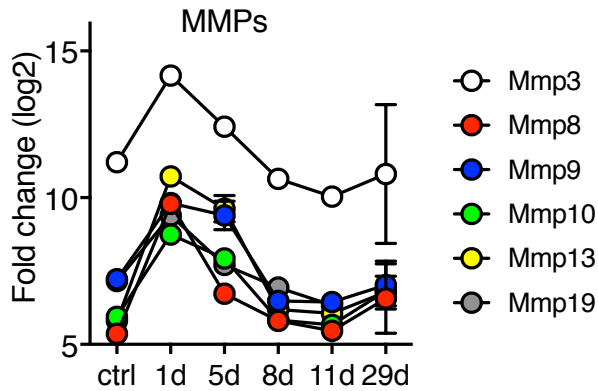


Figure S6. The expression of matrix metalloproteinases (MMP) at different time points in the array data.

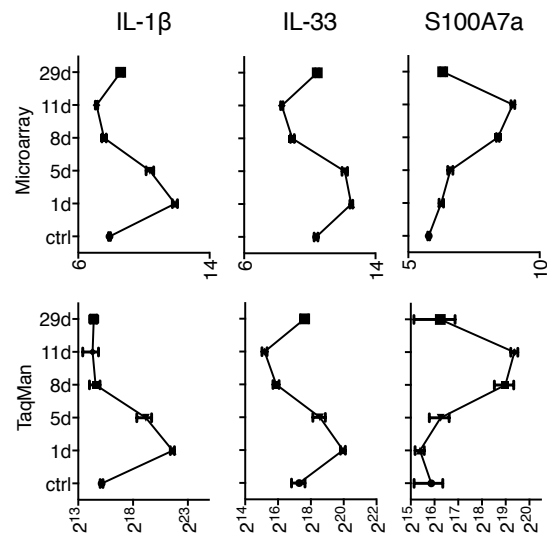


Figure S7. The microarray results were validated running RT-PCR for three target genes, IL-1beta, IL-33 and S100A7a by TaqMan. The expressions were highly similar.

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