**Skin programming of inflammatory responses to *Staphylococcus aureus* is compartmentalised within epidermal keratinocytes differentiation status.**

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1. **What’s already known about this topic?**

The microbiome exists as a dynamic feature alongside host epidermal responses aimed at maintenance of commensal species and clearance of pathogenic species. During acute cutaneous inflammation in diseases such as atopic eczema there are alterations in the microbiome as well as histological and ultrastructural changes to the stratified epidermis that disturb host-microbe interactions.

1. **What does this study add?**

Using patient-derived *ex vivo* biopsies alongside parallel bulk and single-cell transcriptomic and secreted proteomic profiling of epidermal models, this investigation suggests that pathogen-associated remodelling of the epidermis is compartmentalised to different keratinocyte populations. Single-cell transcriptomic analysis distinguished cells from basal, spinous, and granular layers which could further be distinguished in relation to model exposure, with important implications for long-term epidermal health and microbiome handling.

1. **What’s the translational message?**

Features of diseases such as atopic eczema are acute inflammation, microbiome alterations and epidermal ultrastructural changes. Heterogeneous perturbation of undifferentiated keratinocytes to dysbiosis indicates non-transient responses that may persist after pathogen clearance. However, unaffected basal keratinocytes remain, providing a target for epidermal homeostasis. Elucidating the molecular mechanisms regulating bacterial sensing-triggered inflammatory responses within tissues will enable increased understanding of the links between microbiome dysbiosis and inflammatory skin diseases.

**Summary**

**Background**Acute cutaneous inflammation causes microbiome alterations as well as ultrastructural changes in epidermis stratification. However, the interactions between keratinocyte proliferation and differentiation status and the skin microbiome has not been fully explored.

**Objectives**Hypothesising that the skin microbiome contributes to regulation of keratinocyte differentiation and can modify antimicrobial responses, we examined the effect of exposure to commensal (*S. epidermidis*, SE) or pathogenic (*S.* aureus, SA) challenge on epidermal models.

**Methods**

Explant biopsies aimed to demonstrate species-specific anti-microbial effect of host factors; further investigated in reconstituted epidermal models by bulk transcriptomic analysis alongside secreted protein profiling. Single-cell RNA-seq analysis was performed to explore the keratinocyte populations responsible for SA inflammation. A dataset of 6391 keratinocytes from control (2044 cells), SE challenged (2028 cells) and SA challenged (2319 cells) was generated from reconstituted epidermal models.

**Results**

Bacterial lawns of SA, not SE, were inhibited by human skin explant samples and microarray analysis of 3D-epidermis models showed that host AMP expression was induced by SE but not SA. Protein analysis of bacterial co-cultured models showed that SA exposure induced inflammatory mediator expression indicating keratinocyte activation of other epidermal immune populations.

Single-cell DropSeq analysis of unchallenged naïve, SE challenged, and SA challenged epidermis models was undertaken to distinguish cells from basal, spinous, and granular layers, and be interrogated in relation to model exposure. In contrast to SE, SA specifically induced a sub-population of spinous cells which highly expressed transcripts related to epidermal inflammation and antimicrobial response. Furthermore, SA, but not SE, specifically induced a basal population which highly expressed IL-1 alarmins.

**Conclusions**

These findings suggest that pathogen-associated remodelling of the epidermis is compartmentalised to different keratinocyte populations. Elucidating the mechanisms regulating bacterial sensing-triggered inflammatory responses within tissues will enable further understanding of microbiome dysbiosis and inflammatory skin diseases, such as atopic eczema.

**Introduction**

The stratified epidermis maintains cutaneous homeostasis by mediating keratinocyte turnover, stratum corneum formation, microbiome handling and anti-microbial defence. Keratinocytes progress through the epidermis forming distinct basal, spinous, granular and cornified strata. Terminal differentiation of outer layer keratinocytes forms the epidermal cornified barrier, eventually shed by desquamation, and constant tissue renewal by proliferation and differentiation is critical for skin function1. However, precisely if or how epidermal differentiation is modified by skin microbiome changes is not yet fully characterised.

Keratinocytes interact with the innate and adaptive immune system2-4. Their primary role favours tolerance, dampening inflammation in the context of a healthy skin microbiome5. *S. epidermidis* (SE)is a ubiquitous cutaneous coloniser and is almost always non-pathogenic. SE-induced immune training and dampening arises, in part, via host TLR3 sensing of bacterial products such as lipoteichoic acid and LP786, 7. *S. aureus* (SA), however, can be highly pathogenic but mostly transiently colonises human skin, being found on approximately one-third of the population8. Keratinocyte sensing of SA by pattern recognition receptors such as TLR2 and -9, and NOD1 and -2, leads to inflammatory responses, notably dysregulated in atopic skin9. These are, partly, a host defence mechanism. TLR2-mediated activation of NFκB transcription pathways induces expression of hBD3 and RNase710. SA dominates the microbiota of lesional skin of atopic eczema, and significantly alters immune signalling, leading to more severe disease. Indeed, SA density correlates with disease severity, and lesion resolution associates with microbial clearance11.

Understanding the programming of the regulatory-versus-immunostimulatory axis in the skin is of great interest. We hypothesised that functional differences in keratinocyte differentiation arise from challenge with commensal or pathogenic bacteria. Addressing this, we utilised a 3D epidermis culture, allowing investigations of the effects of bacterial colonisation on keratinocytes at different states. Alterations of transcriptional programmes were captured by measuring gene expression by microarray and single-cell DropSeq RNA-sequencing12.

**Materials and Methods**

**Patient samples**

Explant skin tissue was donated from discard surgery and consented under ethical approval (LREC Number: 07/Q1704/46). The explant skin tissue was placed epidermis down on agar plates spread with 106 CFU bacteria and cultured overnight. Inhibition zones were calculated using ImageJ.

**Reconstituted human epidermis models**Primary keratinocytes were seeded in culture inserts (Millicell, 0.4μm pore size, 12mm diameter) (Merck Millipore) to confluence for 48-72 hours. For differentiation, culture media was replaced with KGM2 with 2% FBS and 1.8mM CaCl2 (Sigma). Air-liquid interface (ALI) cultures were generated over 14-15 days.

**Bacterial Challenge**

*Staphylococcal* strains used were *S. aureus* NCTC 8325-4 and *S. epidermidis* ATCC 1222813, 14. Bacterial challenge was performed by adding 100ul of either PBS, *S. aureus* or *S. epidermidis* (1x107 CFU/ml in PBS) to the model apical aspect. Models were incubated for three hours, followed by PBS wash and returned for a further 21 hours.

**Undernatant proteome profiling**The Proteome ProfilerTM Human XL Cytokine Array Kit (R&D systems) was used to semi-quantitatively assay undernatant media. ImageJ was used to measure the relative spot intensity (RSI), and duplicates were averaged for each analyte. Results of <10% the maximum intensity were considered background signal.

**Microarray analysis of epidermal models**Model RNA was obtained by addition of RLT lysis buffer (QIAGEN) and purified using the RNeasy plus mini kit (QIAGEN) using manufacturer’s instructions. The purified RNA quality was assessed by Agilent 2100 Bioanalyser using the RNA 6000 Nano kit (Agilent).

The SurePrint G3 Human Gene Expression v3 8x60K Microarray Kit (Agilent) was used for bulk transcriptomic analysis following all manufacturer’s instructions. The numerical expression data of each array was acquired by the feature extraction software and further analysis was performed in GeneSpring v14.9 (Agilent) and Ingenuity Pathway Analysis software (QIAGEN).

**CIBERSORT analysis of model composition:** Models were assessed by *in silico* quantification for consistent epidermal stratification from bulk microarray data using CIBERSORT15 (performed online at https://cibersort.stanford.edu/). Re-optimisation for CIBERSORT deconvolution of skin/epidermis was previously done by us16. The LM22 reference panel was replaced by data from Cheng *et al.* (2018). Single-cell basal, mitotic, and spinous keratinocyte data from healthy trunk skin were collapsed to pseudobulk profiles by gene-wise summation to provide reference signatures. All run settings were kept at default.

**Single-cell RNA-seq analysis**

Single-cell RNA-analysis was carried out as per DropSeq. Models were dissociated to suspension using TrypLE Express (Gibco). Briefly, 1ml pre-cooled TrypLE Express, and 500μl pre-warmed TrypLE Express were added into the inserts for 15 minutes, then 1ml cold PBS, 10% FBS was added. Cells were collected after 70μm filtration, centrifugation and resuspension in fresh KGM2.

Models from the same challenge condition were pooled to reduce inter-model variation. Multiplexing across challenge conditions was achieved by tagging cells with barcoded antibodies following the Cell Hashing protocol17. Control samples were tagged with TotalSeq-A0251 (GTCAACTCTTTAGCG); SA samples, TotalSeq-A0252 (TGATGGCCTATTGGG); and SEsamples, TotalSeq-A0253 (TTCCGCCTCTCTTTG). The DropSeq co-encapsulation with primer-coated micro-bead procedure was followed. After droplet breakage and microbead purification, cDNA libraries were generated as detailed in18, 19. Sequencing was performed on an Illumina NextSeq using a paired-end run of 3.6-5.0x104 reads/cell, at the Wessex Investigational Sciences Hub laboratory, University of Southampton, UK.

**Data pre-processing and bioinformatic analysis**Illumina-generated BCL (base call files) were converted to fastq using the bcl2fastq tool. The resulting read files were aligned using Kallisto (v.0.46.1) and Bustools (v.0.39.3).

Transcriptomic data from scRNA-seq was analysed, unless otherwise stated, using the python-based Scanpy framework20 (v1.4.6). Quality control covariates were established such as removal of empty barcodes (EmptyDrops21), counts per cell, genes per cell and per centage of mitochondrial genes. Expression data were normalised by SCRAN22.

Sequencing batches were integrated using the batch-balanced k-nearest-neighbour method (BBKNN)23. The data were visualised by scatter plot in the reduced dimensional space from Uniform Manifold Approximation and Projection (UMAP). Calculation of embedded density to aided selection of clustering resolution for optimal granularity explaining cell biology. Leiden clustering was performed using a resolution of 0.5 for the whole dataset and a resolution of 0.2 on a restricted subset to properly reflect difference observed from bacterial challenge24.

Layer-defining epidermal makers were used for annotation of basal (*KRT5*, *KRT14*, *COL17A1)*, spinous (*KRT1*, *KRT10*) and granular layers (*KRT6A*, *KRT6B*, *KRT16*, *S100A7*, *S100A8*, and *S100A9*). Differential gene expression analysis was performed using MAST (BH p-value <0.01, |log FC|>1)25. Gene ontology analysis was undertaken using the tool ToppFun within the ToppGene suite.

**Data availability**

Sequencing data for the microarray and scRNA-seq is stored in the Gene Expression Omnibus database, submission numbers GSE192454 and GSE192641, respectively.

**Results**

Epidermal models [**Figure 1A**] were processed for bulk RNA sequencing. Computational deconvolution by CIBERSORT revealed the models to be highly stratified and consistent [**Figure 1B**]. Modelled keratinocyte populations recapitulated of approximately 90% cells of human epidermis, excluding the non-keratinocyte populations [**Figure 1C**].

**Epidermal inhibition of bacterial growth is species specific**

To examine skin anti-microbial action, we cultured human skin explant models on agar plates with bacterial lawns. Growth inhibition rings for SA significantly larger than SE, indicating species-specific regulation by secreted factors [**Figure 2A**] (Supplementary figure 1).

We next investigated the effect of pathogenic or commensal *Staphylococcus* infection of established epidermal models after 24 hours. Models were challenged with either SA or SE at 102 or 106 CFU each and bacterial growth measured at three hours and again at 24 hours. Both bacterial species indicated similar bacterial CFU after 24 hours post-challenge regardless of initial inoculation, however SE experienced greater proliferation **[Figure 2B]**. The time course confirmed active inhibition of SA from either inoculum to similarly low levels (p>0.9999) [**Figure 2C**, red]. In contrast, both SE challenge doses demonstrated proliferation to similar levels at 24 hours (p=0.9322) [**Figure 2C**, blue] indicating that epidermal colonisation is tolerated at around 108 CFU per model.

**Differential epidermal response to *Staphylococcal* colonisation is caused by altered expression of keratinocyte-derived factors**

We hypothesised that epidermal inhibition of SA may derive from anti-microbial peptides (AMPs). Therefore, the key cutaneous AMPs were analysed from bulk transcriptomic analysis of skin models. Surprisingly, SA colonisation did not significantly alter model expression of key AMPs at 24 hours compared to naïve controls, except for *S100A15* [**Figure 3A**]. In contrast, SE induced the expression of LL-37, RNase 5, S100A8, S100A15, hBD2, hBD3, and hBD4 genes relative to control (log2 fold-change >1) at 24 hours [**Figure 3A**].

We reasoned that SA inhibition may derive from immune cell recruitment and activation via keratinocyte-derived cytokines, chemokines, and growth factors. To explore immune-related soluble factors synthesised by keratinocytes in response to bacterial exposure, culture undernatants from bacteria challenged models were harvested for proteomic analysis of 101 analytes (Supplementary figure 2). SA challenged models showed dominantly pro-inflammatory mediators compared to naïve controls, including CD14, CD30, GROα, IL-16 and VEGF [**Figure 3B**].

**Single-cell sequencing of epidermal models revealed stratified responses to *Staphylococcal* challenge**

To address our primary objective to characterise bacterial responses within epidermal strata, we generated a single-cell RNA-sequencing dataset from naïve control, SE challenged, and SA challenged models. Genes expressed characteristically in basal (40% cells), spinous (9.7%) and granular (50.2%) keratinocytes were used as markers to identify stratification [**Figure 4A**].

Density clustering by bacterial challenge demonstrated clear pathogen-specific differences in keratinocyte transcriptomes [**Figure 4B**]. Considering individual epidermal strata, within these transcriptomic sub-clustering was highly correlated with challenge conditions for both basal and spinous populations, but not granular keratinocytes [**Figure 4C**]. These challenge-related strata sub-populations were further confirmed by unbiased clustering using the Leiden algorithm 24, indicating the existence of transcriptome-based sub-stratification within each layer independent of the proliferation-differentiation axis [**Figure 4D**]. This suggests that the pathogen induced sub-clustering within epidermal strata were not a result of keratinocyte differentiation and may be reversible.

***S. aureus* challenge of epidermal models induces a specific spinous keratinocyte sub-population**

Based on *KRT1*/*10* expression and challenge-annotated density plotting of UMAP projections, we considered Spinous-1 (S1) to represent a challenge-naïve cluster, Spinous-2 (S2) a commensal-related cluster, and Spinous-3 (S3) to be pathogen-associated [**Figure 4D**]. Furthermore, visualisation of an epidermal differentiation score across the whole dataset indicated S3 highly expressed differentiation markers [**Figure *5*A**].

We performed differential gene expression analysis comparing these three clusters (Supplemtary figure 3). A total of eight genes distinguished naïve and commensal spinous clusters, including canonical spinous markers KRT1 and KRT10, found downregulated in S2 keratinocytes. [**Figure *5*B, C**].

However, SA exposed keratinocytes from the S3 cluster, compared to the naïve and commensal spinous clusters, upregulated 27 [**Figure *5*E**] and 26 [**Figure *5*G**] genes, respectively (Supplementary figure 3). Of these, 19 were common between comparisons, and suggested activation of antimicrobial humoral responses (GO: 0019730, BH-adjusted p-value = 8.711E-7) and epidermis development (GO: 0008544, BH-adjusted p-value = 2.539E-6). SA as compared to naïve, also induced striking markers of inflammation with upregulation of: *CD24*, *IL1RN*, *SERPINB1* and *NEAT1*26-30.This indicated that the genes upregulated by SA challenge are unique to pathogenic challenged rather than general microbial colonisation. Comparing between SA and SE exposed keratinocytes, revealed the role of SA in upregulation of the epidermal differentiation complex (EDC) genes such as *IVL* and *SPRR1B*; associated barrier genes: *DMKN*, *KRT6B*, and *SBSN*, and the anti-microbial serpin, *SERPINB3* [**Figure *5*G**].

**Bacterial challenge induces two distinct basal keratinocyte populations**

Basal keratinocytes from each challenge could be assigned to two of four clusters. Basal-1 and -2 (B1 and B2) were evident in unchallenged naïve conditions. SE challenged models induced Basal-2 and -3 (B2 and B3) whereas SA also contributed to B3, and additionally Basal-4 (B4) [**Figure *4*C, D**].

MAST DEG analysis showed that SE did not induce any significant alteration in transcriptome as compared to the naïve control (Basal-2 compared B1). Whereas, comparing Basal-3 (shared SE, SA) to B1, induced a basal keratinocyte wounding response (GO: 009611) (BH-adjusted p-value 2.626E-2) [**Figure 6A**] (Supplementary figure 3)*.*

B4 was highly associated with *S. aureus* challenge. The basal biology of these B4 keratinocytes was disturbed, as evident by reduced mRNA expression of basal keratins 5 and 14, alongside reduced *CXCL14* compared to naïve B1 cells [**Figure 6B**]. Transcriptomic changes compared to naïve basal keratinocytes (B1) showed upregulation of intermediate filament formation of the basal cytoskeleton (*KRT5, KRT14, KRT6A, KRT17, CXCL14* and *DST*), matrix metalloproteinase genes (*MMP1*, *-9* and *-10*) and laminin genes (*LAMA3,* -*B3* and -*C2*) in B4 keratinocytes [**Figure 6C**]. These transcripts were not upregulated in the B3 populations indicating their involvement in epidermal perturbation specific to SA. In addition, B4 cluster keratinocytes upregulated both immediate and delayed release alarmins, IL-1α and IL-1β, indicative of attempted pro-inflammatory activation of other potential cutaneous populations of the epidermis.

**Discussion**

Staphylococci microbiota help govern cutaneous microbiome composition and are key species colonising human skin31, 32. Single-cell analysis of our models revealed stratified responses to microbial challenge. The stratified morphology describes the classical epidermal layers but also compartmentalises proliferation/differentiation functions, tissue homeostasis and inflammation. Although this anatomy is well described, its contribution to epidermal homoeostasis and function is not yet fully understood.

Recent studies have used single cell analysis to investigate this question, however, these are almost exclusively focussed on the role of non-keratinocyte inflammatory and immune populations underscoring epidermal heterogeneity33, 34. The compartmentalisation of processes involved in transcriptional programming of intercellular communication, inflammatory regulation and, particularly, follicular WNT signalling across healthy and inflamed epidermis from various anatomical sites has also been recently described35. Here the authors showed that almost one-eighth of the epidermal transcriptome aligns with classical differentiation patterns, yet their cutaneous function and biological mechanisms are unknown but confirmed the utility of a single cell approach to classify keratinocyte biology. However, it did not explore the effect of biologically relevant variables on keratinocyte differentiation, as we report.

We investigated the response of 3D-model keratinocytes to topical commensal or pathogen challenge by bulk and single-cell transcriptomic analysis and secreted protein factors. This identified an involucrin-expressing spinous populations that was almost unique to SAchallenged epidermal models. Previously, it was demonstrated that differentiated involucrin-positive keratinocytes are critical to epidermal microbial regulation36. We demonstrate that SA growth is strongly inhibited but SA challenge did not upregulate hBD2 expression, reported as a key protective factor released by keratinocytes against SA proteases37. We postulate that through co-evolutionary mechanisms, host surveillance against SA is highly sensitive and activated by low bacterial load. The reason for this is likely to facilitate bacterial clearance before adverse infection occurs. Early SA clearance is necessary to prevent reaching a tipping point that leads to microbiome disruption, preferential overgrowth of SA and loss of cutaneous microbial diversity. Indeed, SA-induced dysbiosis is a key driver of cutaneous inflammation in atopic eczema38. Our keratinocyte-only model demonstrates without the presence of the classical immune system, keratinocytes can inhibit SA growth, proving advantageous in preventing established colonisation before other immune populations are recruited. It is plausible that this SA clearance begins soon after colonisation and expression of effector keratinocyte-derived AMPs

is not captured at the timepoint we investigated. In contrast, commensal colonisation with SE primes the epidermis to expression AMPs, in what can be considered a symbiotic relationship between host and bacteria to prevent colonisation of pathogens.

We also show that spinous keratinocytes are sensitive to SA challenge. At the gene expression level, this population was characterised by inflammatory signalling. Interestingly, we saw that SE challenge induced much higher expression of hBD2, yet our data and others’ microbiological studies37 confirmed that this did not inhibit SE growth. The SA-induced spinous population demonstrated greater expression of members of the IL-1 signalling pathway (*IL36G*, *IL1RN*, *S100)*. Wanke *et al.* (2011) reported that keratinocyte responses to SA infection via AMP release were dependent on IL-1 signalling. Our data suggests that this IL-1 signalling arises in a distinct basal proliferating population induced by SA also marked by overexpression of MMP and laminin genes. Alternatively, sample timing may reflect a time course of bacterial responses across the stratified model with those close to the apical surface demonstrating later AMP responses, and basal cells initiating earlier IL-1 responses to initiate AMP expression in this layer.

In the context of the whole epidermis, such IL-1 expressing basal cells will also provide danger signals to and prime other cutaneous immune populations, such as APCs. In the epidermis, IL-1α is constitutively expressed as a biologically inactive precursor for immediate release from keratinocytes upon receptor activation. Release of pro-IL-1α readily occurs during tissue trauma and infection to act as a damage-associated molecular pattern (DAMP) and is a potent trigger of IL-6 and TNFα expression39. In contrast, IL-1β is a later proinflammatory mediator in response to pathogenic stimulation40. Our results indicated that pathogenic infection of the epidermis induced basal keratinocyte IL-1α release, accompanied by IL-1β as early as 24 hours post-challenge. The shift in basal biology, therefore, is rapid but distinct from epidermal differentiation processes and potentially reversible. Reversal of such inflammatory alarmin responses, especially when arising inappropriately, might aid in restoring cutaneous homeostasis.

Basal keratinocyte responses to pathogen challenge were not homogenous. Approximately 60% of basal cells from SA challenged models expressed alarmin and tissue remodelling genes. The precise reason for sub-clusters within an epidermal compartment is not clear but may reflect yet unknown keratinocyte differentiation pathways or other regulatory factors. Spatial and temporal investigation of these sub-populations will help determine if the pro-inflammatory response represents a population constitutive to basal lineage (i.e., all basal keratinocytes can experience this phenotype) during pathogen challenge or is a distinct and transient subset arising in response to pathogen challenge.

One of the limitations of this work is the model system which relies on pure keratinocytes. However, we specifically chose this system so that we could characterise the keratinocyte compartment in detail in a human model. Addition of skin-resident T cells and dendritic cells, as well as extension of the model to contain full-thickness dermis, would be beneficial but such mixed cell skin models remain a challenge.

In summary, we have investigated keratinocyte responses in the context of commensal or pathogenic challenge of an organotypic epidermal model. These data from bulk transcriptomic investigation have shown that keratinocyte AMP expression challenged with commensal SE is increased without adverse effect on bacterial growth, unlike SA challenged models. Single cell investigation further established that pathogen challenged models express defensins from an inflammatory spinous population, whereas IL-1 alarmin signalling arose from basal cells. Therefore, we propose that epidermal microbial responses induce compartmentalised responses in the epidermal strata which in turn regulates the host inflammatory responses and modifies anti-microbial mediator expression. Further molecular and spatial investigation of these populations may help to elucidate their specific role in inflammatory cutaneous diseases associated with microbiome dysbiosis such as atopic eczema and could offer new therapeutic targets.

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**Figure Legends**

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| **Figure 1. Reconstituted Human Epidermis model culture. (A)** Representative H&E staining of an RHE model after 14 days of cultivation showing general morphology (top) and histology (bottom). **(B)** 21 models were analysed by bulk microarray analysis and subsequently resolved into relative basal (blue), mitotic (green), spinous (yellow) populations using machine-learning deconvolution **(C)** Basal, mitotic, and spinous populations for the deconvolution panel were derived from single-cell analysis of 25,000 keratinocytes from (n=3) healthy human epidermis. These three populations comprising 100% of the in vitro models composed 90% of the cells isolated and annotated from healthy human epidermis. |

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| **Figure 2. Colonisation of epidermal models by Staphylococcal species.** **(A)** Calculated area of inhibition from the explant inhibition model for five donors (n=3/6 per donor). 106 CFU of SA or SE suspended in 100μl PBS were spread onto TSA plates to form overnight bacterial lawns. Shown are means with individual datapoints for each biopsy replicate. Inhibition zone was calculated by subtracting biopsy size (8mm) from gross inhibited area. Mann-Whiney tests for significance shown. **(B)** Models in duplicate/triplicate were infected with either 102 or 106 CFU of S. aureus (SA) or S. epidermidis (SE) initially for 3 hours to establish adherent/intracellular infection and left in culture for a further 21 hours. Bacteria were quantified by enumeration after serial-dilution and plating on TSA plates for colony counting after over-night incubation. Data expressed as mean ±SD. **(C)** Time course ofcolonisation of models by SA (red) and SE (black) over 24 hours at low (102 CFU, circles) and high (106 CFU, squares) inoculation loads at indicated time points (n = 4). Statistical significance within species comparisons across challenge loads were performed by Kruskal-Wallis tests, and across species significance independent of challenge load was performed by Mann-Whiney tests. |

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| **Figure 3. Secreted protein and cellular gene expression of keratinocytes induced by SA or SE colonisation. (A)** Heatmap of AMP gene expression from models colonised with Staphylococcal species at 3- and 24 hours expressed as a relative log2 fold-change to control models. (n­ = 3/4). **(B)** Heatmap of analyte expression secreted in response to Staphylococcal colonisation of RHE models. The Proteome Profiler Human XL Cytokine Array kit was used to analyse expression of analytes in culture undernatants harvested from models infected with 106 CFU of SA or SE and PBS control after 24 hours. Colonisations were performed in triplicate with undernatant pooled for analyte analysis over two independent experiments (average of duplicate experiments shown). Expression measured using ImageJ to determine average relative spot intensity between two spots per analyte. Analyte expression is shown as log2 fold-change relative to control, showing only those analytes which were significant for SA and/or SE comparisons (full heatmap available as Supplementary Figure 2). |

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| **Figure 4. Clustering of single cell RNA-seq of keratinocytes from bacteria challenged epidermal models. (A)** UMAP projection of 6391 keratinocytes from epidermal models left as microbe naïve control or challenged with S. epidermidis or S. aureus. Differentiation layers were defined using unbiased Leiden clustering at a resolution of 0.2 which matched approximate expression of canonical layer-defining markers [methods]. **(B)** The UMAP projection coloured by embedded density according to challenge conditions: microbe naïve (left), SE (middle) and SA (right), showing keratinocytes clustering from the same challenge condition by regions of more intense colour. **(C)** Embedded density plotting on the UMAP projection for separate basal (top), spinous (middle) and granular (bottom) layers according to challenge conditions: microbe naïve (left), SE (middle) and SA (right). **(D)** Highly granular clustering to define keratinocyte stratification (Basal, B; Spinous, S; Granular, G) and challenge-related sub-populations (numbered) defined by unbiased Leiden clustering at a resolution broadly matching observation in (A) and (C). |

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| **Figure 5. Gene expression of spinous keratinocytes. (A)** Expression score for the panel of epidermal differentiation complex genes across the whole single cell dataset represented in the UMAP space. **(B)** DEGs upregulated in Spinous-1 cluster compared to Spinous-2. **(C)** DEGs upregulated in Spinous-2 cluster compared to Spinous-1. **(D)** DEGs upregulated in Spinous-1 cluster compared to Spinous-3. **(E)** DEGs upregulated in Spinous-3 cluster compared to Spinous-1. **(F)** DEGs upregulated in Spinous-2 cluster compared to Spinous-3. **(G)** DEGs upregulated in Spinous-3 cluster compared to Spinous-2. Statistical significance for differential expression within the MAST algorithm was Benjamini-Hochberg-adjusted p-value <0.01, |logFC| >1. |

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| **Figure 6. Gene expression of basal keratinocytes. (A)** DEGs upregulated in Basal-3 cluster compared to Basal-1. **(B)** DEGs upregulated in Basal-1 cluster compared to Basal-4. **(C)** DEGs upregulated in Basal-4 cluster compared to Basal-1. Statistical significance for differential expression within the MAST algorithm was Benjamini-Hochberg-adjusted p-value <0.01, |logFC| >1. |