

Differential innate immune responses of a living skin equivalent model colonized by *Staphylococcus epidermidis* or *Staphylococcus aureus*

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Abstract

Staphylococcus epidermidis is a commensal on skin, whereas *Staphylococcus aureus* is a transient pathogen. The aim was to determine whether the skin's innate defence systems responded differently to these microorganisms. Differential gene expression of a human skin equivalent (SE) model was assessed by microarray technology, in response to colonization by *S. epidermidis* or *S. aureus*. Only a small number of transcripts were significantly ($P < 0.0001$) increased (12) or decreased (35) with gene expression changes of > 2 -fold on SEs colonized with *S. epidermidis* compared with controls (no colonization). Expression of one innate defence gene, pentraxin 3 (PTX3), was upregulated, while psoriasin, S100A12, S100A15, β defensin 4, β defensin 3, lipocalin 2 and peptidoglycan recognition protein 2 were downregulated. In contrast, large numbers of transcripts were significantly increased (480) or decreased (397) with gene expression changes of > 2 -fold on SEs colonized with *S. aureus* compared with controls. There was upregulation in gene expression of many skin defence factors including Toll-like receptor 2, β defensin 4, properdin, PTX3, proinflammatory cytokines tumour necrosis factor- α , IL-1 α , IL-1 β , IL-17C, IL-20, IL-23A and chemokines IL-8, CCL4, CCL5, CCL20 and CCL27. These differences may partly explain why *S. epidermidis* is a normal skin resident and *S. aureus* is not.

Introduction

The resident commensal microorganisms found on human skin are predominantly Gram-positive species. The commensal *Staphylococcus epidermidis* is the most prevalent staphylococcal species, whereas *Staphylococcus aureus* is a transient, opportunistic pathogen causing superficial as well as serious invasive infections. Skin is the first line of defence against pathogens and understanding the interactions between the microbial communities and epidermis is essential.

The surface of human skin is continually exposed to a variety of microorganisms/pathogens, but infections are not common in healthy individuals, suggesting the presence of a robust and effective cutaneous defence system. Originally, microbial colonization was considered to be controlled by (1) the adverse physical and chemical properties of skin, including a relatively dry surface, acidic pH, fluctuations in temperature, salt/ion concentration, nutrient availability

and inhibitory lipid moieties (Bojar & Holland, 2002); (2) the continual loss of microorganisms attached to desquamating corneocytes; and (3) the presence of a physical/chemical permeability barrier composed of an intact stratum corneum with interlocking corneocytes and a hydrophobic extracellular lipid-rich matrix organized in lamellar bilayers (Elias, 2005). However, it is now recognized that epidermal keratinocytes also play an active role in the host's innate defences by the constitutive expression and induction of pattern recognition receptors (PRRs), antimicrobial peptides/proteins (AMPs), chemokines and proinflammatory cytokines. These mechanisms of defence provide rapid recognition of and protection against microbial assault mediated via the sensing abilities of the PRRs and the killing properties of AMPs.

PRRs have a broad specificity and recognize conserved molecular patterns, known as pathogen-associated molecular patterns, which are shared by large groups of

microorganisms (Janeway, 1989). These ligands are frequently bacterial structural components such as lipoproteins/lipopeptides, lipoteichoic acid, peptidoglycans or viral RNA. After ligand binding, PRRs initiate signalling pathways involved in the generation of AMPs, proinflammatory cytokines and chemokines, which mediate the recruitment of leucocytes and stimulation of the adaptive immune response.

As effectors of innate immunity, AMPs are able to directly kill or inactivate a wide spectrum of microorganisms through membrane disruption, essential trace element sequestration or indirectly through the activation of dermal endothelium and chemotaxis of leucocytes such as phagocytes, dendritic cells and memory T cells. Disruption of the skin's permeability barrier and the presence of proinflammatory cytokines also stimulate epidermal keratinocytes to induce the expression of AMPs. However, many AMPs, the most abundant being lysozyme, psoriasin, RNase 7 and human β defensins 1, 2 and 3 are expressed constitutively in healthy skin in the upper keratinocyte layers of the stratum granulosum and stratum corneum forming a protective 'chemical barrier', which inhibits microbial growth and invasion at the skin surface (Schroder & Harder, 2006).

The use of animal models for investigations of host–microbial interactions on human skin is of limited use due to the intrinsic differences between human and animal skin, but it is also undesirable in terms of ethical considerations. In this study, a human living skin equivalent (SE) model was used to determine by gene array technology whether the skin responds differently when exposed to a resident commensal (*S. epidermidis*) or a transient opportunistic pathogen (*S. aureus*). The SE model was developed *in house* and had a dermal matrix of fibrin and viable fibroblasts, a basement membrane and a stratified epidermis with a stratum corneum, closely reflecting the environment found in *in vivo* skin. It supported the colonization of *S. epidermidis* or *S. aureus* for up to 72 h of incubation with consistent recovery densities from replicate SEs and with an intact and undamaged surface (Holland *et al.*, 2008), and therefore the model was considered to have the potential to predict the defence mechanisms involved in microbial interactions in human skin.

Materials and methods

SEs

Adult human dermal fibroblasts (Cascade Biologics) and neonatal foreskin primary human keratinocytes (Invitrogen) used in the construction of SEs were pooled from several donors to provide greater gene diversity. Cells were cultured and dermal equivalents (DEs) and SEs were prepared according to Holland *et al.* (2008).

Bacterial strains

The staphylococcal strains were *S. epidermidis* (strain S9) isolated from the volar forearm (Farrell *et al.*, 1993) and *S. aureus* (strain SH 1000) donated by Prof. Simon Foster, Sheffield University, UK.

Colonization of SEs with *S. epidermidis* or *S. aureus*

Staphylococcus epidermidis and *S. aureus* were grown overnight in 2% (w/v) tryptone at 37 °C up to the late exponential phase of growth. The inoculum density was obtained by dilution of the culture in sterile distilled water immediately before inoculation. Replicate SEs (4 ×) were inoculated with 100 μ L of 10⁴ CFU per SE *S. epidermidis* (coded SEP 1-4); SEs (4 ×) were inoculated with 10⁴ CFU per SE *S. aureus* (coded SAU 1-4) or control SEs (4 ×) were inoculated with 100 μ L sterile distilled water (coded C1-4). After 2 h incubation, the surface of each SE was blotted dry to remove surplus fluid and nonadherent bacteria from the inoculum. After a total of 24-h incubation, three replicate SEs from each group were individually soaked in Prep Protect (Miltenyi Biotec), an RNA stabilizer, at 4 °C for 24 h before sending to Miltenyi Biotec for microarray analysis. Microorganisms were recovered from the remaining SEs as described by Holland *et al.* (2008).

Incubation conditions

All cells, DEs, SEs, control SEs and microbial colonizations were incubated at 37 °C in 5% (v/v) CO₂ and air, unless otherwise stated.

Microarray

Miltenyi Biotec carried out the microarray work using dye swap replicates and Agilent's comparative genomic hybridization technology. Differential gene expression was determined by comparative analyses of (1) SEs colonized with *S. epidermidis* vs. controls; (2) SEs colonized by *S. aureus* vs. controls; and (3) SEs colonized with *S. aureus* vs. SEs colonized with *S. epidermidis*.

Total RNA was extracted from each SE sample using an RNeasy Kit (Machery and Nagel). RNA purity was determined using the Agilent 2100 BioAnalyser system. All samples had ratios of rRNA genes (28S/18S) > 1.5, confirming the integrity of the RNA. Equal amounts of RNA from SEP 1, 2 and 3 were pooled to give sample SEP, from SAU 1, 2 and 3 to give sample SAU and from controls 1, 2, and 3 to give sample C. Pooling the RNA provided each sample with the greatest transcript diversity. RNA samples were linearly amplified using the Low RNA Input Linear Amp Kit (Agilent). Half of each reaction mixture was used for cRNA synthesis and fluorescent labelling using either

cyanine 3-CTP or cyanine 5-CTP. Appropriate volumes of Cy3- and Cy5-labelled cRNAs were pooled, and after fragmentation the mixtures were hybridized overnight on whole human genome 60-mer oligo microarrays according to Agilent's processing protocol. The hybridization schedules included dye swap replicates giving six microarrays: (1) SEP-Cy5 and C-Cy3; (2) SAU-Cy5 and C-Cy3; (3) SAU-Cy5 and SEP-Cy3; (4) C-Cy5 and SEP-Cy3; (5) C-Cy5 and SAU-Cy3; and (6) SEP-Cy5 and SAU-Cy3. The microarrays were scanned using Agilent scanner technology and analysed using LUMINATOR software.

The LUMINATOR software provided gene lists with the complete raw data, normalized Cy5/Cy3 \log_{10} ratios, Cy5/Cy3 fold changes, sequence descriptions and *P*-values. The software combined data sets derived from two corresponding dye swap replicates using a proprietary 'combine algorithm'. The polarity of the second dye swap experiment was changed to synchronize the upregulation or downregulation of the genes. The combined results included 1+4, 2+5 and 3+6 to give merged dye swap data for SEP-Cy5 and C-Cy3; SAU-Cy5; and C-Cy3; SAU-Cy 5 and SEP-Cy3, respectively. Differentially expressed transcripts were selected using conservative filters for a *P*-value < 0.0001 and a > 2-fold change.

Results

Comparative microarray analysis

Colonization of SEs

Microarray analyses were performed on SEs after either no colonization or colonization by *S. epidermidis* and *S. aureus* for 24 h at 37 °C, allowing adequate time for interactions and feedback mechanisms to occur between the bacteria and stratum corneum/keratinocytes. There was no colonization on control SEs, while colonization densities were 8.21 \log_{10} CFU per SE and 7.37 \log_{10} CFU per SE for *S. epidermidis* and *S. aureus*, respectively, showing a 10-fold lower colonization density for *S. aureus*. The surface of each SE was examined for damage macroscopically and microscopically and found to be intact.

SEP-Cy5 and C-Cy3

Human commensals are maintained on the skin under normal homeostatic conditions, but whether or which factors of the innate defence system exert control over their colonization is not understood. When SEs were colonized with the commensal *S. epidermidis* for 24 h and compared with noncolonized control SEs, it was found that the expression of many genes remained unchanged (33 164). Differential expression of only a small number of genes were

significantly ($P < 0.0001$) increased (466) or decreased (341), and of these very few were significantly increased (12) or decreased (35) with expression changes of > 2-fold (Fig. 1a). Those relating to innate defence of skin were identified from the preselected genes ($P < 0.0001$ and > 2-fold change). The only transcript to be upregulated ($P < 0.0001$ and > 2-fold change) was pentraxin 3 (PTX3). PTX3 is a soluble PRR. It binds to selected Gram-positive and Gram-negative bacteria, facilitates bacterial recognition by phagocytes and binds the complement factor C1q, activating the classical complement pathway and mediating bacteria lysis and cell death (Mantovani *et al.*, 2008). Several transcripts were downregulated, however, including psoriasin (S100A7), calgranulin C (S100A12), S100A15 protein, β defensin 4 (DEFB4), β defensin 3 (DEFB103A), lipocalin 2 (LCN2) and peptidoglycan recognition protein 2 (PGLYRP2) (Fig. 1b). All have a broad spectrum of antibacterial activity against both Gram-positive and Gram-negative microorganisms, although there are differences in their specificity. S100 proteins and LCN2 have greater activity against Gram-negative organisms such as *Escherichia coli* and are considered to be bactericidal by essential trace element zinc deprivation and by sequestration of the iron-laden siderophore enterochelin, respectively (Flo *et al.*, 2004; Buchau *et al.*, 2007), while PGLYRP2 has greater bactericidal activity against staphylococci by hydrolysis of cell wall peptidoglycan. Thus, the effect of *S. epidermidis* on SEs is the upregulation of a single defence molecule and decreased antimicrobial responses.

SAU-Cy5 and C-Cy3

It is important to understand which factors of the innate defence system are involved in protecting human skin from this opportunistic pathogen. When SEs colonized with *S. aureus* for 24 h were compared with noncolonized control SEs, many genes remained unchanged (28 939), but large numbers of transcripts were significantly ($P < 0.0001$) increased (2569) or decreased (2459) with many of these genes having increased (480) or decreased (397) expression changes > 2-fold (Fig. 2a). A large number of differentially expressed genes associated with innate defence and inflammatory responses were significantly ($P < 0.0001$) increased > 2-fold. There was upregulation in gene expression of the skin defence factors Toll-like receptor 2 (TLR2), β defensin 4 (DEFB4), properdin (BF), pentraxin 3 (PTX3), peptidoglycan recognition proteins (PGLYRP2 & 4), calgranulin C (S100A12) and S100A15; proinflammatory cytokines including interleukins IL-1 β , IL-1 α , IL-17C, IL-20, IL-23A, tumour necrosis factor (TNF) and lymphotoxin β (LTB); growth factors including granulocyte colony-stimulating factors (CSF2 & 3); chemokines including IL-8, CCL 3, 4, 5, 19, 20 and 27 and CXCL 1, 2, 3, 10 and 14, some of which

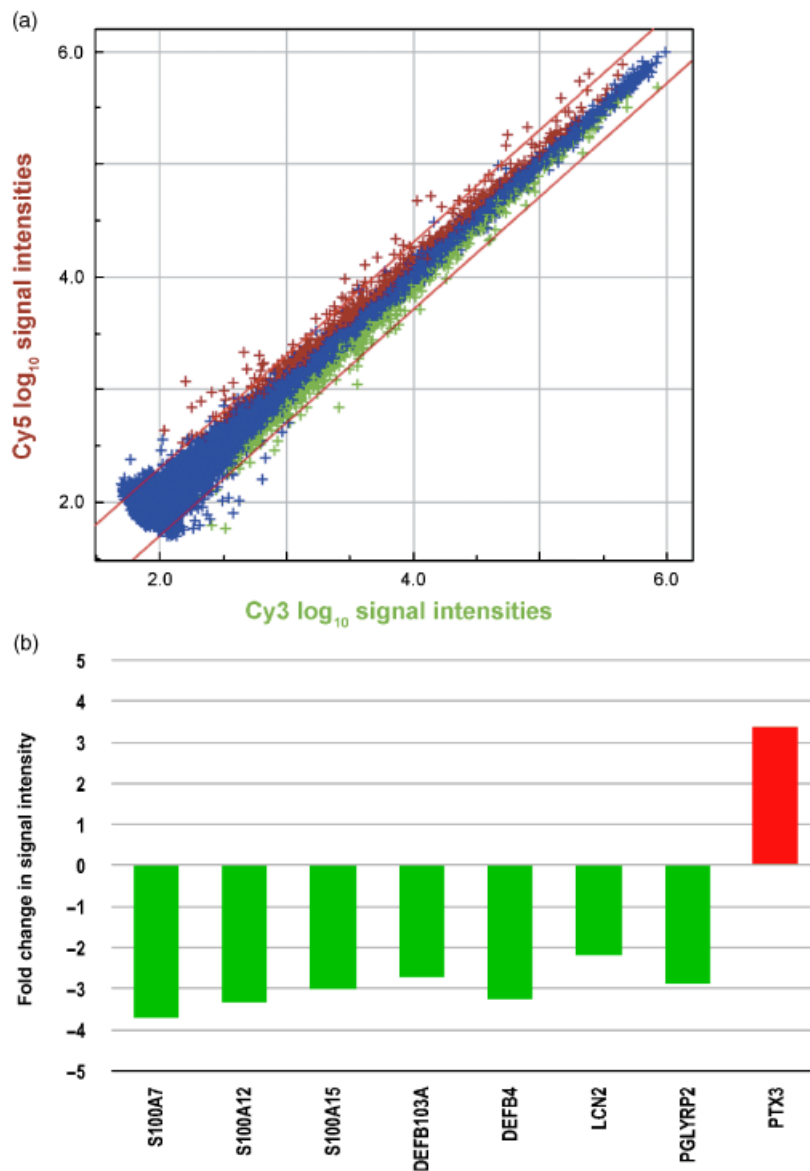


Fig. 1. Gene expression of SEs colonized with *Staphylococcus epidermidis* (SEP) compared with control SEs not colonized with bacteria (C). (a) Scatterplot of signal intensities of differential gene expression from SEP-Cy5 and C-Cy3. (b) Differential expression of innate defence genes from SEP-Cy5 and C-Cy3, which were either downregulated, i.e. psoriasin (S100A7), S100 protein 12 (S100A12) and S100 protein 15 (S100A15), β defensin 4 (DEFB4), β defensin 3 (DEF103A), lipocalin 2 (LCN2), peptidoglycan recognition protein 2 (PGLYRP2) or upregulated, i.e. pentraxin 3 (PTX3).

have antimicrobial properties as well as mediate the migration of leucocytes to sites of inflammation; and adhesion factors including ICAM-1, which again are important for recruitment of inflammatory cells to infected sites. The protease inhibitor transcripts elafin (PI3) and serine proteinases (SERPINA 3 and B1) were upregulated. They have antimicrobial activity against Gram-positive and Gram-negative bacteria, inhibit target enzymes such as neutrophil elastase and prevent microbial invasion of skin. Also many signal transduction and transcription factors including MAPKs, SMAD1&3, TRAF1&4, NF κ B, IRF-1 and SOCS-1 transcripts were upregulated, providing evidence that a diverse selection of innate immune responses had been activated.

The most important or most highly upregulated transcripts of innate defence are shown in Fig. 2b and it is clear that the antimicrobial responses upregulated by *S. aureus* were dominated by IL-23. Purified peptidoglycan from *S. aureus* is known to induce the production of IL-23, which in turn induces the production of IL-17. IL-17 was also upregulated and induces the production of proinflammatory cytokines and the recruitment of neutrophils to the site of infection resulting in bacterial clearance (McKenzie *et al.*, 2006). IL-23 also acts on memory T cells to induce IFN γ expression and thus these results indicate that *in vivo* IL-23 would be important in providing a rapid innate response, and the development of the slower adaptive immune response to *S. aureus*. Other upregulated transcripts

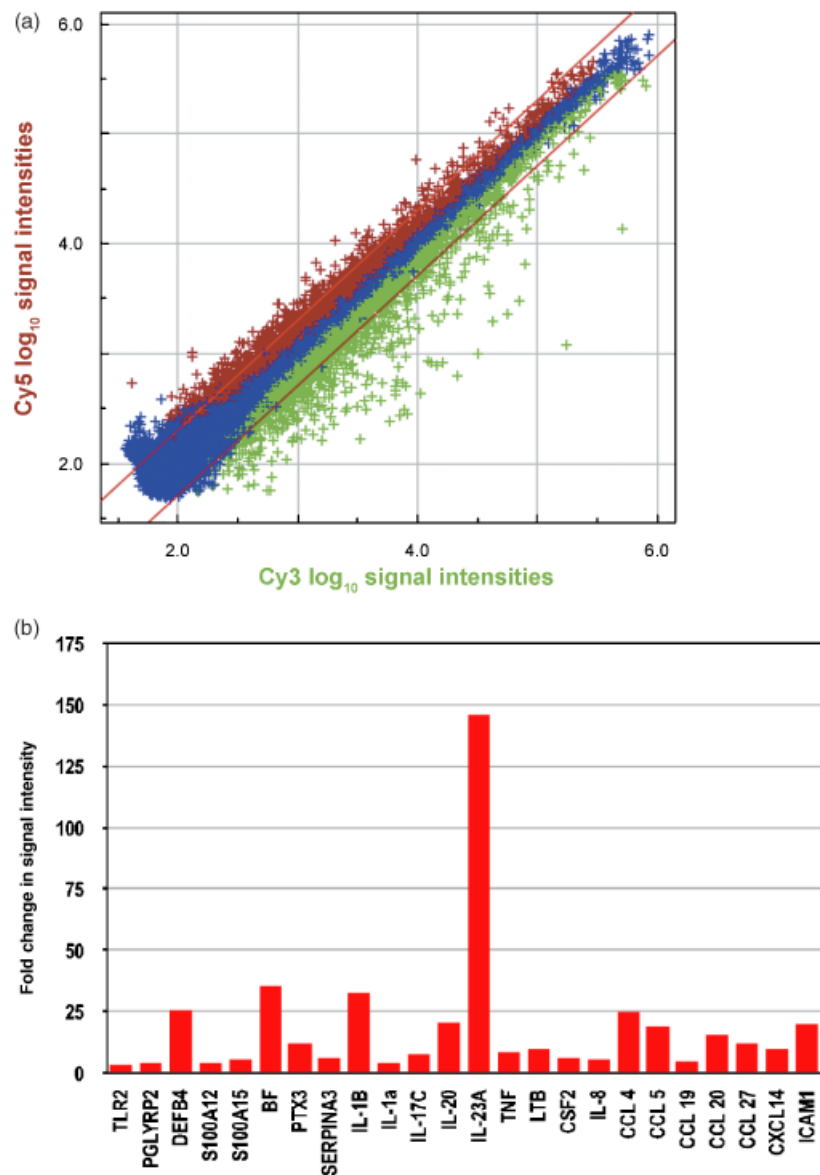


Fig. 2. Gene expression of SEs colonized with *Staphylococcus aureus* (SAU) compared with control SEs not colonized with bacteria (C). (a) Scatterplot of signal intensities of differential gene expression from SAU-Cy5 and C-Cy3. (b) Differential gene expression from SAU-Cy5 and C-Cy3 showing the most important or highly upregulated innate defence transcripts including TLR2, peptidoglycan recognition protein 2 (PGLYRP2), β defensin 4 (DEFB4), properdin (BF), pentraxin 3 (PTX3), S100 proteins (S100A12 and S100A15), proinflammatory cytokines and chemokines.

responsible for rapid humoral responses and bacterial clearance *in vivo* are PTX3 and BF. PTX3 binds to its bacterial target and C1q, and activates the classical complement pathway, while BF acts as a recognition factor, directly binding to microbial target, forming a surface for the assembly of C3 convertases and playing a major role in the activation of the alternative complement cascade (Spitzer *et al.*, 2007). Schroder & Harder (2006) were unable to detect DEFB4 in human skin, but its upregulated gene expression in SEs suggests that it would have strong antimicrobial activity *in vivo* against *S. aureus*. Another important innate defence factor to be upregulated was TLR2, although not surprising, as the lipoproteins/lipopeptides in

S. aureus cell wall are known ligands for this receptor (Stoll *et al.*, 2005). After ligand binding, TLR2 signalling pathways induce the production of proinflammatory cytokines and chemokines, and *in vivo* these factors would recruit phagocytes, dendritic cells and T cells to the site of *S. aureus* infection. The upregulation of the IL-20 transcript was also important as it is responsible for the induction of a number of defence responses including chemokines, S100 family proteins and β -defensins in keratinocytes (Sa *et al.*, 2007). Thus, multiple and diverse innate defence mechanisms have been induced by the colonization of *S. aureus* on SEs, the significance of which may be to provide a 'fail-safety' mechanism to counter evasion of any individual responses by this pathogen.

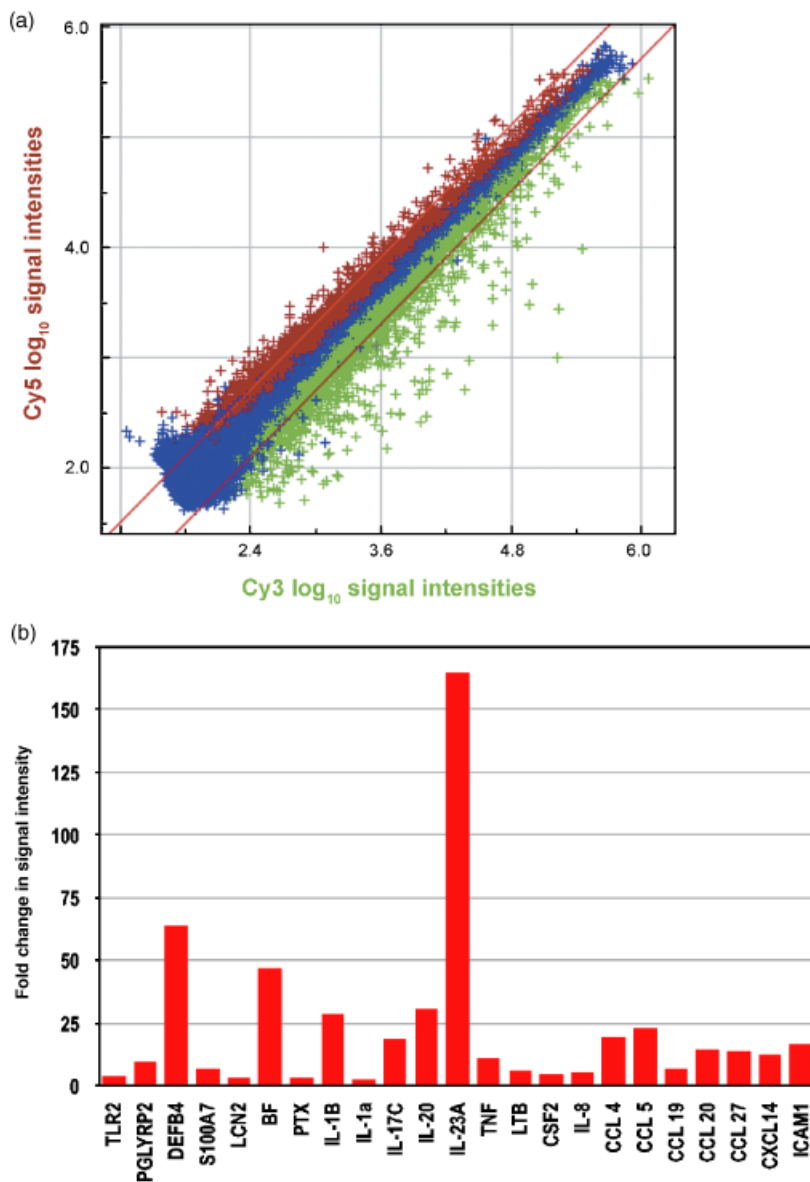


Fig. 3. Gene expression of SEs colonized with *Staphylococcus aureus* (SAU) compared with SEs colonized with *Staphylococcus epidermidis* (SEP). (a) Scatterplot of signal intensities of differential gene expression from SAU-Cy5 and SEP-Cy3. (b) Differential gene expression from SAU-Cy5 and SEP-Cy3 showing the most important or highly upregulated innate defence transcripts including TLR2, peptidoglycan recognition protein 2 (PGLYRP2), β defensin 4 (DEFB4), psoriasin (S100A7), lipocalin 2 (LCN2), properdin (BF), pentraxin 3 (PTX3), proinflammatory cytokines and chemokines.

SAU-Cy5 and SEP-Cy3

To identify the differences between colonization of SEs with a commensal or pathogen, a direct comparison of the differential gene expression was made. Large numbers of transcripts were significantly increased (567) or decreased (474) with gene expression changes >2 -fold on SEs colonized with *S. aureus* compared with SEs colonized with *S. epidermidis* (Fig. 3a). The main differences when comparing SAU-Cy5 vs. SEP-Cy3 in contrast to SAU-Cy5 vs. C-Cy3 were the increased expression of AMPs including DEFB4, BF, PGLYRP2, S100A7 and LCN2 and proinflammatory cytokines TNE, IL-17C, IL-20 and IL-23A (Fig. 3b). Thus, the results of this comparison were similar to those when

S. aureus colonization of SEs was compared with controls and confirm the data that *S. epidermidis* had little effect on SE gene expression, while *S. aureus* had a large effect.

Discussion

In this study, gene array technology was used to examine the diversity of human genes, regulated in epidermal keratinocytes to predict the behaviour of resident commensals (*S. epidermidis*) or transient opportunistic pathogens (*S. aureus*). An SE model system was used and it had the advantage that the expression of innate defence factors of a single microbial colonization could be identified, whereas *in vivo* skin testing

always exists against a resident microbial community background, making specificity of responses hard to detect.

The factors controlling the range of microorganisms and their densities on normal human skin is not fully defined, although physico-chemical variables and skin lipids have been implicated. An intriguing question is that why *S. epidermidis* is universally found on all skin sites and all people of all ages and the opportunistic pathogen is not? There cannot be a single mechanism that determines this observation, and the answer must be complex with the different resistance attributes of the two bacteria having some importance. Part of the complexity of the interactions is the different responses of normal skin to contact with the microorganism. These responses have evolved to allow the nonaggressive type to survive in the environment and the more aggressive type to evoke a protective response.

The work reported here strongly supports this argument. Unlike *S. epidermidis*, the colonization of *S. aureus* on intact SEs stimulated the increased expression of a diverse range of innate defence transcripts. These results indicate that the skin would protect itself by rapidly recognizing and responding to this pathogen, because *in vivo* these results would translate into a combination of rapid humoral responses with the production of the proinflammatory cytokines and chemokines, activation of the classical and alternative complement cascades, induction of AMPs and activation of PRRs, all of which would contribute to direct microbial killing, the influx of inflammatory cells, activation of phagocytosis and the mediation of the slower adaptive immune response.

Skin-microbial interactions are incompletely understood and these analyses have contributed to our knowledge of the host's responses to commensals and pathogens by showing that bacterial stimuli can induce different patterns of expression of antimicrobial effector molecules and that the different defensive repertoire may indicate in part why *S. epidermidis* is a commensal on skin and *S. aureus* is a transient pathogen.

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