



# Three-dimensional human skin models to understand *Staphylococcus aureus* skin colonization and infection

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*Staphylococcus aureus* is both a major bacterial pathogen as well as a common member of the human skin microbiota. Due to its widespread prevalence as an asymptomatic skin colonizer and its importance as a source of skin and soft tissue infections, an improved understanding of how *S. aureus* attaches to, grows within, and breaches the stratified layers of the epidermis is of critical importance. Three-dimensional organotypic human skin culture models are informative and tractable experimental systems for future investigations of the interactions between *S. aureus* and the multi-faceted skin tissue. We propose that *S. aureus* virulence factors, primarily appreciated for their role in pathogenesis of invasive infections, play alternative roles in promoting asymptomatic bacterial growth within the skin. Experimental manipulations of these cultures will provide insight into the many poorly understood molecular interactions occurring at the interface between *S. aureus* and stratified human skin tissue.

**Keywords:** *Staphylococcus aureus*, MRSA, skin, colonization, organ culture

*Staphylococcus aureus* is simultaneously a pathogen of significant morbidity and mortality and a common member of the skin microbiota. As a major human bacterial pathogen, *S. aureus* infections cause tens of thousands of deaths and millions of outpatient and emergency room visits in the United States annually (1). Although the disease caused by *S. aureus* infections manifests in a wide range of clinical presentations, the vast majority are skin and soft tissue infections (2, 3).

However, in spite of the pathogenesis associated with *S. aureus*, it is also acknowledged to be a typical member of the complex community of microbes living on and within our skin (4, 5). An estimated 20% of American adults are persistently colonized in the anterior nares with *S. aureus*, and an additional 30% are intermittently carrying *S. aureus* in the nares (6, 7). For neonates and children, the prevalence of *S. aureus* positive skin cultures is thought to be even higher (8, 9). Furthermore, evidence from recent prevalence studies in which non-nasal skin sites are surveyed suggests that traditional screening approaches may be underestimating a larger skin colonization burden of *S. aureus* by focusing on one niche, the anterior nares (10–12). Little is known about the transition from the asymptomatic colonization state to an invasive infection, despite the fact that persistently colonized individuals have nearly triple the risk of developing *S. aureus* bacteremia and are most often infected by their own colonizing strains (13–16).

Whether one considers *S. aureus* as an invasive pathogen or from the perspective of its role as a common colonizer, an appreciation of how *S. aureus* interacts with the skin environment is central to its biology. Much remains unknown about how *S. aureus* persists in the skin over time and what bacterial factors it may use to

actively modify the host skin environment to persist in its replicative niche. We propose that three-dimensional (3D) human skin culture models are an informative and tractable experimental system for future investigations of the interactions between *S. aureus* and the multi-faceted skin tissue. In this perspective, we consider the current experimental model systems for studying the *S. aureus* skin interface, and describe advantages of utilizing organotypic 3D human skin models for future investigations into *S. aureus* biology.

Historically, many of the molecular biology studies into *S. aureus* skin interactions have been done using *in vitro* infections of keratinocytes cultured as a two-dimensional (2D) monolayer of cells. Such studies have provided insight into many aspects of bacterial attachment and the innate immune response mounted by keratinocytes upon encountering bacteria (17, 18). However, the bi-dimensional nature of these assays completely eludes the complex stratification and terminal differentiation process central to how keratinocytes form a multilayered epidermal tissue with barrier functionality. To study determinants of *S. aureus* interaction with a stratified skin tissue (as opposed to isolated keratinocytes), investigators have relied on a variety of animal models. Epicutaneous or superficial epidermal inoculation of *S. aureus* in rodent skin models have proven ineffective for reproducible observations of *S. aureus* growth on skin over extended periods of time (19–22). Further, there are considerable differences between human skin and rodent skin in both histology and immunology, which complicate interpretation of data with respect to bacterial localization and replication in the skin. A recently developed murine model of *S. aureus* skin and soft tissue infections uses small allergy test needles coated in bacteria to introduce the inoculum precisely

and superficially into the outer ear pinna (23). This model has been used to examine the immune response to a superficial *S. aureus* skin infection (23). With this notable exception, nearly all of the existing *S. aureus* animal skin models require severe mechanical disruption of the skin to facilitate bacterial growth, such as the subcutaneous foot pad model, the scalpel wound model, or the subcutaneous skin abscess model (24–27). While these models are extremely useful for studying pathogenesis, they neither facilitate observation of the replicative niche of *S. aureus* within an intact tissue, nor address how the transition of *S. aureus* from an asymptomatic colonization state to a more invasive soft tissue or systemic infection might occur.

Due to the need for a physiologically relevant *in vitro* model system to dissect the interactions of *S. aureus* with intact human skin, we have modified an existing 3D organotypic human skin tissue model to examine the processes of staphylococcal skin colonization and infection (28, 29). An appreciation of the inherent limitations of 2D cultures for understanding skin biology has motivated the development of many tools to study stratified human skin tissue over the past several decades (30–32). Only recently, microbiologists have begun to capitalize on these advancements and utilize 3D organotypic human skin models to examine the specific interactions between the human skin and clinically relevant viral, bacterial, and fungal species (31, 33–36).

Established models for studying stratified human skin fall into two main categories, namely *ex vivo* human skin explant cultures and regenerated 3D organotypic models derived from primary cells and/or human cell lines. All 3D skin models are relatively labor and time intensive when compared to traditional 2D skin models using keratinocyte-derived cell lines. *Ex vivo* human skin explants, typically acquired from neonatal foreskin, surgical, or cadaveric tissues, can be maintained in cell culture media directly or on supports in an air–liquid interface and remain viable in culture for up to 2 weeks. Skin explants have the advantage of containing all resident cell types of the epidermis and dermis as well as skin appendages; however, there are limited options for experimental manipulation of host genetics as well as restricted availability of such tissue samples. Organotypic 3D skin models (sometimes referred to as reconstructed skin models) are generally comprised of primary or immortalized human keratinocytes, grown at an air–liquid interface on an extracellular support matrix, which can be seeded with fibroblasts (32, 37, 38). Other relevant cell types have been incorporated into organotypic skin models including melanocytes, Langerhans cells, as well as endothelial and nervous cells [reviewed in Ref. (30)]. Organotypic 3D stratified human skin cultures comprised of immortalized human cell lines such as the widely studied HaCaT cell line do not reflect the intrinsic genetic variability of cultures cultivated using primary keratinocytes, leading some to argue that models built using the former cells are more reproducible (32). On the other hand, HaCaT 3D organotypic cultures exhibit differentiation and stratification deficiencies when compared to primary keratinocyte 3D organotypic skin cultures (39–41).

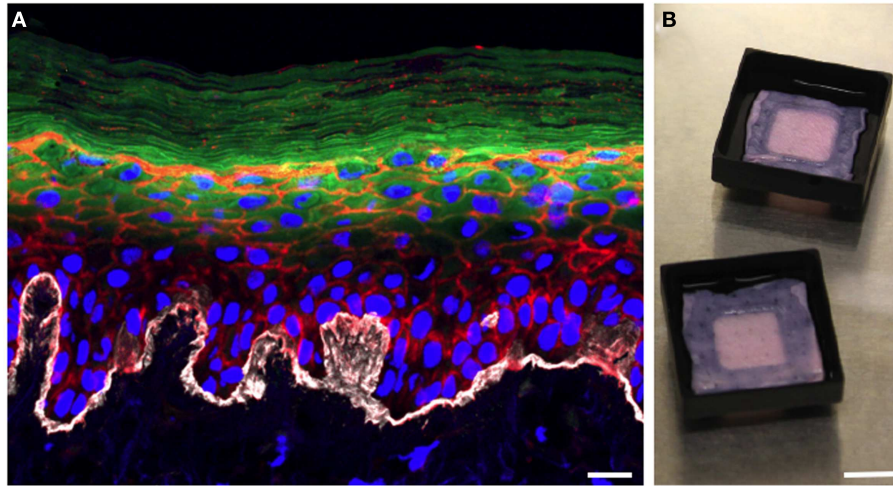
In the 3D organotypic human epidermal tissue model we have modified to study *S. aureus* colonization and infection, primary human keratinocytes and fibroblasts are isolated from fresh discarded neonatal foreskin specimens (28, 29). Fibroblasts are

seeded into pieces of devitalized human dermal tissue derived from cadaveric donors to provide the underlying support matrix. Keratinocytes are then seeded on top of the fibroblast-populated dermis and grow at the air–liquid interface (Figure 1B). After several days of growth, the keratinocytes fully differentiate, generating a basement membrane and all of the stratified epidermal layers, including the outermost squames of the stratum corneum (Figure 1A). The resulting 3D human organotypic tissues are composed entirely of human protein and cells, and unlike murine skin these human organotypic tissues recapitulate the thickness and most of the cellular architecture of the human epidermis and underlying dermis. A limitation of this model is the high genetic variability of skin cultures due to the use of primary cells from heterogeneous donors. Another feature of the model that needs to be improved in the future is the absence of cell types other than keratinocytes and fibroblasts, and skin appendages such as hair follicles and apocrine and eccrine sweat glands.

Epicutaneous infections of these 3D human skin cultures with *S. aureus* USA300 constitutively expressing GFP allow us to follow the bacteria during the colonization process over time. Overnight colonies of bacterial cultures grown on agar plates are re-suspended in Hanks buffer, and the inoculum is applied to the air interface of the skin culture using a pipette tip. At various times after infection, the skin cultures are harvested into paraformaldehyde fixative and further processed for cryosectioning. Using confocal microscopy to generate 3D images of infected skin tissue, we can examine the bacterial skin interface both from a “top–down” view (Figures 2B,D) as well as by looking at cross-sectional slices (Figure 2C). We have tested whether the bacteria are capable of growing on the regenerated human epidermis without exogenous addition of nutrients or media. By starting with a very small inoculum so that mainly single bacteria adhere to the corneocyte surface, we can follow the fate of the microbes over time and determine whether they grow and their preferential replication niche. By 2 days post-infection, the bacteria have expanded to form microcolonies localized at different levels within the stratum corneum of the epidermis (Figure 2, compare Figures 2A,B to later stages of growth in Figures 2C,D). Experiments done with antibiotics added to the basal media reveal this bacterial growth depends on direct interactions with the keratinocytes and not from contact with the cell culture media in the basolateral compartment of the skin cultures (data not shown).

This model system will allow us to query multiple poorly understood aspects of the interactions between *S. aureus* and skin tissue. For example, we can more precisely identify the replicative niche of *S. aureus* within the skin. Does a deeper, quiescent reservoir population of bacteria seed a more rapidly expanding population of surface associated bacteria in the stratum corneum? In addition to examining the location of bacterial attachment and growth during colonization, we can use this experimental system to observe a more pathogenic bacterial invasion of the epidermis. By modulating the maturity of the skin cultures at the time of infection, we can create conditions where bacteria are interacting with nucleated keratinocytes localized to more basal layers of the epidermis.

Unlike human or porcine skin explant models, a key advantage of an *in vitro* 3D epidermal organotypic system is that it allows the study of skin colonization and infection processes by



**FIGURE 1 | Three-dimensional organotypic human epidermal tissues recapitulate the stratified structure of the epidermis. (A)** Cross-sectional view of a fully differentiated 3D human organotypic epidermal tissue. Collagen VII (white) comprising the basement membrane is visible at the interface between the dermis and epidermis. Nuclei of keratinocytes and fibroblasts are blue, filamentous actin is red, and loricrin (green) localizes to the granular

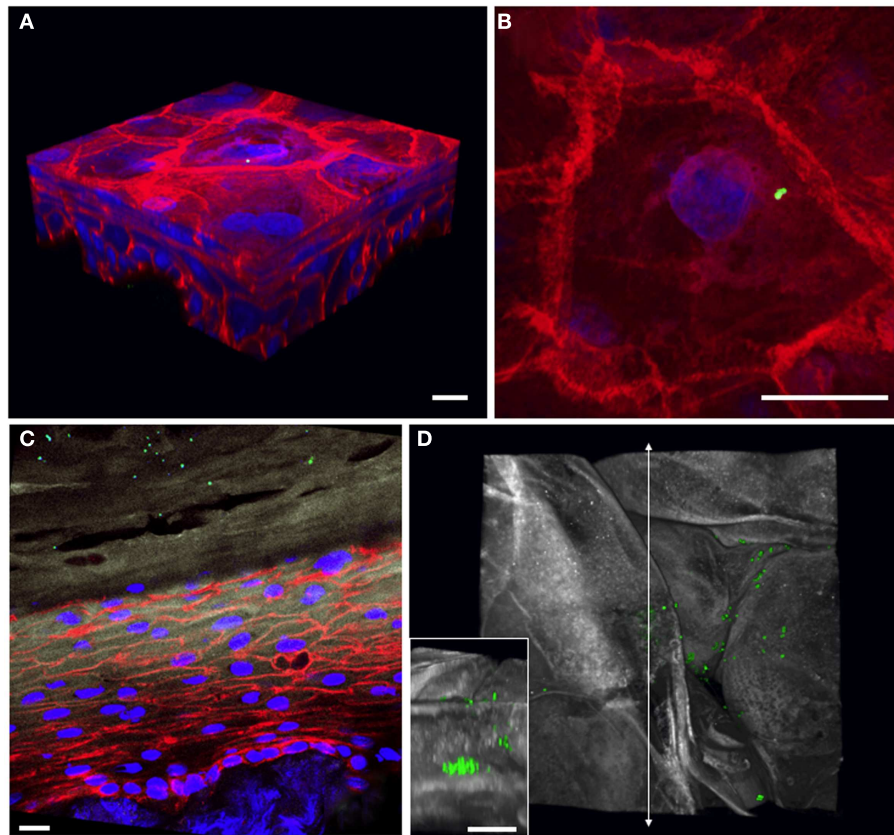
layer and the squames of the stratum corneum. Scale bar is 10  $\mu\text{m}$ . **(B)** Top-down macroscopic view of the 3D organotypic human epidermal tissue system. Pieces of devitalized human dermis are seeded with primary human keratinocytes and fibroblasts. Tissue culture support trays raise the organotypic human epidermal tissue and promote keratinocyte differentiation by facilitating growth at the air–liquid interface. Scale bar is 1 cm.

experimentally controlling both the skin tissue as well as the bacteria (42–44). In this model, the contribution of specific host keratinocyte factors to generate an effective epidermal barrier or to provide critical innate immune responses can be examined. Human keratinocytes treated with RNAi against a gene of interest prior to seeding the dermis generate skin tissue, which is stably knocked-down for a particular gene of interest (28, 29). By then infecting these “custom knocked-down” skin tissues, in the future we can examine how tissues deficient in a particular structural or innate immune product may alter the replicative niche of *S. aureus*. Specifically, this approach could prove useful to assess the role of the host protein filaggrin in staphylococcal infections of skin cultures (45). A better understanding of staphylococcal interactions with filaggrin-depleted skin is relevant due to the contribution of filaggrin to the pathology of atopic dermatitis, a dermatological condition, which is known to enhance *S. aureus* skin colonization as well as pre-dispose patients to staphylococcal skin infections (46–48). Indeed, 3D organotypic human skin cultures depleted for filaggrin using siRNA technology have been reported in the dermatological literature (49).

In addition to experimentally manipulating the host side of the system, we can query the impact of specific bacterial virulence factors in promoting growth and invasion processes. The role played by *S. aureus* virulence determinants in skin persistence is an area ripe for inquiry. A handful of staphylococcal virulence factors have known host targets within the skin, most notably the exfoliative toxins, which cleave epidermal desmogleins, leading to staphylococcal scalded skin syndrome (50, 51). However, the vast majority of *S. aureus* virulence factors have been investigated solely for their role in contributing to invasive disease and remain largely ignored from the perspective of asymptomatic colonization. Considering the evolutionary pressure on *S. aureus* for

effective transmission and the relatively infrequent occurrence of invasive infections relative to the high prevalence of colonization, it is likely that many staphylococcal virulence factors are involved in establishing and maintaining the colonization state. Clumping factor B is a clear example of a *S. aureus* virulence factor with dual roles in both the pathogenesis of a systemic infection and colonization. In bloodstream infections, clumping factor B binds to the  $\alpha$ -chain of fibrinogen, promoting platelet aggregation, and staphylococcal attachment to damaged tissue in endocarditis (52, 53). Nevertheless, it is one of the few known critical determinants of nasal colonization and has been shown to facilitate adhesion to the keratinized epithelium through its interaction with cytokeratin 10 expressed on squamous cells (18, 54). Similarly, the secreted toxin  $\alpha$ -hemolysin has been shown to promote the cleavage of E-cadherin in epithelia such as the lung and the skin in disease models (55). It seems very likely that the ability of *S. aureus* to modify host adherens junction biology may contribute to its ability to persist in the skin over time in a disease-free state. The 3D organotypic human epidermal tissue system will facilitate examination of the vast arsenal of *S. aureus* virulence factors with respect to the hypothesis that virulence factors may play unique roles in the biology of *S. aureus* colonization. One can assess the importance of a particular bacterial product through competition experiments where the same epidermal tissue is co-infected with wild type (WT) and isogenic mutant *S. aureus* strains. Each strain can be tracked via unique fluorophores, allowing observation of the fate of WT and mutant populations within the same stratified human tissue over time.

Examining the immune response to *S. aureus* cutaneous colonization and infections is an important potential application of a 3D human skin culture system. The bi-dimensional nature of traditional *S. aureus* infections of immune cell cultures *in vitro*



**FIGURE 2 | *Staphylococcus aureus* adherence, growth, and localization can be observed within 3D organotypic human epidermal tissues. (A)** 3D confocal microscopy reconstruction of an infected ( $T = 1$  h) 5-day-old 3D organotypic human epidermal tissue. *S. aureus* USA300 GFP-expressing bacteria (in green) are visible attached to the surface squames; filamentous actin is red, and nuclei are blue. Scale bar in all panels corresponds to 10  $\mu$ m. **(B)** Top-down view of bacterial inoculum in **(A)**, visualized immediately after infection. **(C)** Cross-sectional view of an 8-day-old 3D organotypic human

epidermal tissue, infected with *S. aureus* USA300 GFP for 2 days. Bacteria (in green) are visible throughout the squames in the stratum corneum (loricrin in white). Filamentous actin is red and nuclei are in blue. **(D)** After 2 days of growth on the organotypic epidermal tissue, *S. aureus* USA300 GFP has started to form visible microcolonies within the squames in the stratum corneum in this top-down view (loricrin in white). Inset depicts a cross-sectional view located along the vertical line with arrows revealing a large staphylococcal microcolony.

forces direct interactions between the bacteria and immune cells, which are not likely to represent the most physiologically relevant way in which an immune cell would encounter *S. aureus*. By incorporating relevant immune cell types such as Langerhans cells or neutrophils into the 3D skin culture system, future investigations could examine how *S. aureus* and immune cells interact with one another in stratified human epidermal tissue. Other investigators have successfully incorporated “Langerhans-like” immune cells into similar 3D dermatological skin cultures, although to our knowledge, these models have not yet been infected for use in microbiology studies (56–58). Additionally, treatment with antibodies directed against *S. aureus* or addition of recombinant cytokines in the basolateral media of the skin cultures could allow examining impact of immune response to *S. aureus* in the localized cutaneous setting. As more *S. aureus* vaccines move forward into clinical trials, it remains totally unknown how an efficacious antibody response to vaccination may impact *S. aureus* colonization burden (59). Studies using a 3D organotypic human epidermal tissue model will prove valuable in assessing how immunization

against *S. aureus* might alter bacterial population behavior on the epidermis or protect against invasive epidermal infections.

In order to better understand staphylococcal colonization and the transition to invasive infections, we must study *S. aureus* in its natural habitat: the human stratified squamous keratinized epithelium. Our group and others have begun to use organotypic 3D human skin equivalent cultures to experimentally probe *S. aureus* colonization and infections (36, 60). It is our hope that moving toward more physiologically relevant model systems, which capture the complex biology of the skin, will allow us to better understand the critical interface between our most important tissue barrier and *S. aureus*.

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