Methicillin-Resistant *Staphylococcus aureus* Adaptation to Human Keratinocytes

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**IMPORTANCE**

Human skin is a major site of *Staphylococcus aureus* infection, and keratinocytes actively participate in eradication of current infections. Keratinocytes and recruited immune cells participate in skin defense against infection. We postulated that *S. aureus* is able to adapt to the milieu within human keratinocytes to avoid keratinocyte-mediated clearance. From a collection of *S. aureus* isolates from chronically infected patients with atopic dermatitis, we noted 22% had an *agr* mutant-like phenotype. Using several models of human skin infection, we demonstrate that toxin-deficient, *agr* mutants of methicillin-resistant *S. aureus* (MRSA) USA300 are able to persist within keratinocytes by stimulating autophagy and evading caspase-1 and inflammasome activation. MRSA infection induced keratinocyte autophagy, as evidenced by galectin-8 and LC3 accumulation. Autophagy promoted the degradation of inflammasome components and facilitated staphylococcal survival. The recovery of more than 58% *agr* or RNAIII mutants (*P < 0.0001*) of an inoculum of wild-type (WT) MRSA from within wortmannin-treated keratinocytes compared to control keratinocytes reflected the survival advantage for mutants no longer expressing *agr*-dependent toxins. Our results illustrate the dynamic interplay between *S. aureus* and keratinocytes that can result in the selection of mutants that have adapted specifically to evade keratinocyte-mediated clearance mechanisms.

**ABSTRACT**

Skin is the commonest site of *Staphylococcus aureus* infection, which is a major cause of skin and soft tissue infections in the United States (1, 2), usually infecting patients with no underlying immune defects (3). While these skin infections are typically local, they provide a source of organisms for recurrent/persistent colonization and a reservoir for systemic dissemination. The host response to skin infection is shared by local keratinocytes and immune cells that are recruited to the disruption in the epithelial barrier (4, 5). Despite the recruitment of neutrophils once infection is established at a cutaneous site, it can be difficult to clear and may require surgical drainage, even if appropriate antibiotics are employed (6).

Human skin is a complex immune and physical barrier (4) composed of multiple layers of proliferating and differentiating keratinocytes linked by tight junctions (7). Keratinocyte production of antimicrobial peptides to kill bacteria is well established, whereas exactly how keratinocytes kill ingested bacteria, including staphylococci, is not fully understood. *S. aureus*-induced keratinocyte death may result in death of the bacteria or release of organisms to be cleared by recruited phagocytes. Compensation for keratinocyte loss induced by infection is ongoing, as human keratinocytes are continually in the process of proliferation, maturation, and cell death (7). Keratinocytes undergo pyroptosis, a caspase-1-dependent activation of the NLRP3 inflammasome resulting in cell death (8–10). This is triggered by α-hemolysin (Hla), additional staphylococcal toxins, including the Panton-Valentine leukocidin (PVL) (11) and other two-component toxins (12) which are under control of the *agr* locus (13). Caspase-1-dependent pyroptosis results in the production of interleukin 1β (IL-1β) to recruit neutrophils, a process facilitated by the constitutive expression of pro-IL-1β in keratinocytes (8). Activation of the inflammasome functions to eradicate infecting organisms and to recruit neutrophils to eliminate extracellular bacteria (14, 15).

*S. aureus* has evolved multiple mechanisms to promote survival within the context of human skin (16, 17). Differentiating keratinocytes are actively undergoing autophagy (18), a process that is often important in the clearance of intracellular pathogens and provides a source of nutrients through catabolism (19, 20). Autophagy can also serve to limit the availability of inflammasome components and decrease proinflammatory signaling.
If keratinocytes contribute significantly to S. aureus defense, it is likely that these organisms have acquired mechanisms to evade keratinocyte-mediated clearance (16). We postulated that MRSA USA300 evades keratinocyte-mediated clearance through the selection of toxin-deficient mutants that can persist intracellularly. Using both laboratory-derived mutants of the epidemic MRSA USA300 strain LAC and S. aureus isolated from atopic dermatitis (AD) patients, we demonstrate selection of toxin-deficient agr mutants within autophagic keratinocytes that have increased ability to persist within human skin.

RESULTS

Characterization of S. aureus from atopic dermatitis patients.

Atopic dermatitis (AD) is a common inflammatory skin condition affecting 20% of the population. AD patients typically have chronic or recurrent S. aureus infection (22); thus, they are a likely source for S. aureus strains that have adapted to human keratinocytes. As part of an ongoing study to correlate S. aureus genotypes, phenotypes, and clinical outcomes in AD patients, 85 S. aureus isolates from superficial skin cultures of 133 patients were characterized, and 22% were noted to be toxin deficient, as defined by lack of hemolysis on sheep blood agar plates, likely indicating mutation in the agr or hla locus (23). We postulated that strains lacking toxin production would be less likely to activate keratinocyte clearance mechanisms and might have increased intracellular persistence. The first 10 of these isolates for which whole-genome sequences were available were screened for survival within keratinocytes using a gentamicin protection assay and for induction of IL-1β/H9252 as a marker of inflammasome activation compared with wild-type (WT) MRSA USA300 LAC and an agr null mutant control (Fig. 1A). The results of four typical AD strains are shown; both the agr control strain and strain AD4 had significantly increased intracellular persistence at 24 h compared to the WT USA300. Strain AD7 had an intermediate phenotype, whereas...
strains AD5 and AD10 were more similar to the USA300 control and did not accumulate to any appreciable extent within the keratinocytes. The strains with increased intracellular persistence (AD4 and AD7), as well as the agr control, induced less IL-1β, a marker of inflammasome activation (Fig. 1B). This correlated with lack of detectable Hla (α-hemolysin) (Fig. 1C) and significantly decreased expression of RNAIII by AD4 (Fig. 1D). AD7 was found to have a stop codon mutation in the hla locus consistent with its lack of Hla production (Fig. 1E). Comparison of whole-genome sequences of these strains to the appropriate agr reference strains revealed no additional mutations in the agr loci. While AD5 behaved much like the prototypic USA300 reference strain, AD10, which was efficiently cleared from the keratinocytes, expressed relatively less Hla and induced modest amounts of IL-1β, suggesting additional mechanisms of clearance from within the keratinocyte.

To ascertain whether additional hemolysins contribute to clearance from keratinocytes, we screened for expression of delta-hemolysin (Hld) (phenol-soluble modulin gamma [Psm-γ]). The *S. aureus* delta-hemolysin has been suggested to be important in the pathogenesis of AD through its targeting of mast cells (24), and Hld mutants have been linked to chronic bone infection (25). Accordingly, the AD isolates were all screened for the presence and expression of the *hld* locus. The clinical isolates, including AD10, contained the *hld* locus (Fig. 1F). Expression of Hld in the total collection of AD isolates was assessed by enhancement of hemolysis in the presence of the β-hemolysin (Hlb) (strain RN4220) determined by cross-streaking (with the caveat that this is not completely specific for Hld) (26). Hld production in the characterized AD strains did correlate with intracellular persistence. To better characterize how *S. aureus* induced keratinocyte-mediated clearance, we more critically analyzed the intracellular uptake and persistence of the common WT USA300 LAC and defined agr and toxin-deficient mutants.

**Increased intracellular persistence of toxin-deficient mutants.** The relative ability of the WT and USA300 mutants to persist intracellularly was quantified using a gentamicin protection assay in the HaCaT keratinocyte cell line and in primary keratinocytes (HEKn) (Fig. 2). The *agr* locus regulates expression of several toxins, including Hla and Psm, and affects PVL production (*lukS* and *lukF*) as well (27, 28). Uptake of WT and agr null mutant strains into the HaCaT keratinocytes was equivalent and mediated by the fibronectin binding proteins (29), primarily FnBPA (fibronectin binding protein A) (Fig. 2A and B). USA300 mutants lacking *hla* or *agr* accumulated to a significantly greater extent within keratinocytes than WT organisms did, and a small but significant fraction persisted intracellularly for more than 48 h (Fig. 2C); lack of PVL expression did not enhance intracellular persistence under these conditions. Psmα mutants, but not Psmβ or Psmδ mutants, had increased intracellular persistence at 24 h (Fig. 2C). *agr* mutants with other genetic backgrounds had significantly increased intracellular survival followed for up to 96 h (Fig. 2D). Controls for keratinocyte viability using trypan blue exclusion (Fig. 2E) or lactate dehydrogenase (LDH) release (Fig. 2F) after a 24-h incubation with the various *S. aureus* strains were not significantly different, suggesting that only a small fraction of the cells in culture are infected and killed by staphylococci.

**MRSA USA300 bacteria escape from the endosome to cause keratinocyte pyroptosis.** For *S. aureus* to gain access to the keratinocyte cytosol, the staphylococci must first escape from the endosome, a process associated with toxin and Psm expression (30). *S. aureus* can be cleared by the phagolysosome (31) or escape to the cytosol where organisms expressing toxin could activate caspase-1-mediated pyroptosis through the NLRP3 inflammasome (9, 10), resulting in keratinocyte death (32). Alternative mechanisms of cell death induced by staphylococci include apoptosis, a caspase-3-dependent process, and necroptosis, a RIP1-RIP3-MLKL (RIP1 stands for receptor-interacting protein 1 and MLKL stands for mixed-lineage kinase domain-like) mode of cell death (33). Using keratinocytes in primary culture as well as the HaCaT cell line, we found that caspase-1 inhibition, but not caspase-3 inhibition, resulted in significantly increased intracellular survival of *S. aureus* USA300 at 24 h (Fig. 3A). Caspase-1 inhibition also increased the intracellular persistence of the *agr* mutant (Fig. 3B), suggesting that additional non- *agr*-dependent gene products may contribute to inflammasome activation and that *agr* mutants can escape from the keratinocyte endosome. The effect of caspase-1 inhibition on staphylococcal persistence suggested that many of the endocyted staphylococci readily gain access to the cytosol. Treating HaCaT cells with chloroquine or bafilomycin to limit endosomal acidification had no significant effect upon the numbers of staphylococci retained (Fig. 3C). Imaging studies were performed using organotypic cultures; human skin grafts were grown on a feeder layer of fibroblasts to determine the location of *S. aureus* USA300 within the multiple strata of normal human skin as well their distribution within individual skin cells (Fig. 3D). *S. aureus* was observed within the stratum granulosum. In electron micrographs, both WT and agr mutant staphylococci appeared to be proliferating within the keratinocytes, not necessarily within membrane-bound compartments. Confocal imaging using either EEA-1, a fluorescent marker for the early endosome, or monodansyl cadaverine (MDC), a marker for the late autophagosome (34), demonstrated colocalization of WT and agr mutants within autophagosomal compartments; however, some organisms were apparently free in the cytosol, indicating that the *agr* null mutants are not limited to the endosome, and the WT USA300 bacteria are not exclusively cytosolic in human keratinocytes (Fig. 3F).

**WT MRSA USA300 and agr null mutant induce autophagy in human skin grafts.** The ingestion of staphylococci by keratinocytes is a likely stimulus for autophagy, a process which generates energy in response to metabolic or other types of stress by inducing degradation of cellular organelles, including those containing pathogens (35, 36). To characterize the interplay between *S. aureus* and autophagy in human keratinocytes, we used human skin grafts maintained on SCID mice to follow the consequences of *S. aureus* infection (see Fig. S1 in the supplemental material). These grafts reflect the expected architecture of healthy skin with a basal proliferative layer, the differentiating keratinocytes of the stratum spinosum and stratum granulosum, and the terminally differentiated stratum corneum at the surface (37) (Fig. 4A). Staphylococci were visualized intercalating in between adjacent corneocytes, indicating that they could infect the epidermis without major disruption of the epithelial barrier (Fig. 4B). Pathology was apparent 72 h following the application of 5 × 10⁶ CFU of strain USA300 in 10 μl of phosphate-buffered saline (PBS) onto the surface of the intact graft (Fig. 4C). Infection with the WT organisms resulted in hyperkeratosis of the stratum corneum with small mounds of serum and neutrophilic crust, with very few bacterial colonies apparent. Many more clusters of bacteria are seen in
the **agr** mutant-infected graft (Fig. 4D). Neutrophil infiltration is apparent in WT MRSA-infected grafts (Fig. 4E); hyperkeratosis is observed, but bacteria are not visualized. The stratum corneum of the **agr** mutant-infected grafts also shows hyperkeratosis and parakeratosis (Fig. 4F). Significantly, there is broad erosion of the epidermis, covered by fibrin and numerous neutrophils seen at higher magnification, and bacteria are observed within vacuoles (Fig. 4G). Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining revealed the fragmented DNA of dead cells in the infected grafts, but not in PBS.
controls, consistent with keratinocyte death as a mechanism of staphylococcal clearance (Fig. 4H).

**S. aureus** infection induces autophagy in human skin. Confocal imaging of the infected grafts was done to assess accumulation of the markers of autophagy. Galectin-8 which is recruited to damaged endosomes, especially those damaged by pathogens and targets them for autophagosomal clearance (38), colocalized with the organisms in the infected skin grafts (Fig. 5A) and was more abundant in the *agr* null mutant infections. LC3, a marker for autophagosome formation (34), was detected in grafts infected with WT *S. aureus* and more prominently with *agr* null mutant *S. aureus*, but it did not colocalize with the organisms at this time point, 3 days postinoculation. Images of *spa* null mutant, *agr* mutant, and WT staphylococci are shown (see Fig. S2 in the supplemental material) to demonstrate that protein A-associated binding of the antistaphylococcus antibody is not responsible for apparent differences in the numbers of fluorescence-labeled WT and *agr* mutant staphylococci. Control images using the secondary antibody alone were entirely negative.

Electron micrographs of infected human keratinocytes in or-
ganotypic cultures revealed *S. aureus* USA300 *agr* mutant within a characteristic double-membrane-bound compartment, seen in apposition to mitochondria, typical of the autophagosome (20) (Fig. 5B). To determine how induction of autophagy affects the intracellular persistence of *S. aureus*, we treated keratinocytes with the autophagy inhibitor wortmannin or 3-methyladenine (3-MA), which when used for a limited time, has a similar effect (39), and quantified USA300 staphylococci by gentamicin protection assay (Fig. 5C). For both the WT and *agr* mutant, there was significantly decreased intracellular survival in the HaCaT and primary keratinocytes treated with inhibitors of autophagy, suggesting that these organisms may be exploiting autophagy to persist within the keratinocytes. Immunoblots performed with HaCaT cells at early time points after *S. aureus* infection indicated induc-
tion of galectin-8 by 2 h postinfection, but no consistent changes in LC3II (Fig. 5D).

The relative induction of autophagy by WT and agr null mutant staphylococci was evaluated using a fluorescence microscopy and cytometric analysis of LC3 (Cyto-ID [Enzo Life Sciences]) (40) (Fig. 5E). Infection with WT USA300 induced Cyto-ID staining in 4.9% of the cells compared with 34% of the cells exposed to the agr null mutant. Treatment of the keratinocytes with wortmannin to inhibit autophagy decreased the numbers of either WT or agr null mutant staphylococci that were Cyto-ID positive as expected, and 3-MA essentially blocked autophagy altogether in this assay system. Chloroquine, by decreasing acidification of the endosome, resulted in increased Cyto-ID-positive cells, consistent with the interference of autophagosomal clearance (41). These data suggest that S. aureus that can persist within keratinocytes may exploit autophagy to evade clearance.

**Induction of autophagy enhances MRSA persistence through effects on the inflammasome.** In immune cells, induction of autophagy results in consumption of nonessential cellular components, including the inflammasome (42). Autophagic degradation of the NLRP3 component ASC (apoptosis-associated speck-like protein containing CARD [caspase activation and re-
cruitment domain], an adaptor protein that functions in inflammasome assembly, limits pyroptosis, IL-1β production (21), and inflammatory signaling (43) important in neutrophil recruitment (44) and critical for *S. aureus* clearance in vivo. *S. aureus*-infected HaCaT cells were pretreated with wortmannin to block autophagy, and relative amounts of ASC were assessed by immunoblotting. The wortmannin-treated keratinocytes were found to have increased amounts of ASC in *S. aureus*-infected cells compared to medium controls (Fig. 6A). To confirm that inhibition of autophagy increases inflammasome function, small interfering

FIG 6 Induction of autophagy decreases inflammasome activation. (A) Immunoblot of HaCaT cells detecting ASC in HaCaT cells in the presence (+) or absence (−) of wortmannin (Wm) after 2 h of infection with WT *S. aureus* USA300 or agr mutant and corresponding densitometry standardized to the GAPDH control. (B and C) Immunoblots of HaCaT cells detecting Nod2 (B) and ATG5 (C) knockdown by siRNA compared to nontargeting siRNA pool control in HaCaT cells exposed to media (med) or to WT *S. aureus* or agr mutant. (D and E) IL-1β production determined by ELISA in the corresponding Nod2 and ATG5 knockdowns and nontargeting siRNA-transfected controls all at 24 h postinfection. (F and G) Light microscopy of Gram-positive trichrome-stained (F) and confocal images (G) of sections obtained from *S. aureus* AD4- and AD10-infected human skin grafts on SCID mice after 72 h of infection using anti-*S. aureus* antibody (SA) (green), anti-galectin-8 (red), or anti-LC3 (red). Colocalization (yellow) of galectin-8 and the AD4 isolate is observed, but not with the AD10 strain. siRNA knockdowns were done twice, and the results of a representative experiment are shown.
also cleared from the human grafts at 72 h.

The AD10 strain that was efficiently cleared from HaCaT cells was marker galactose but not with LC3. This was in contrast to AD10; at 72 h postinoculation and was readily detected within the stratum granulosum. AD4, which had increased intracellular persistence in reus AD4 or AD10 infection (Fig. 6F and G). The Hla-deficient strain AD4, which had increased intracellular persistence in HaCaT cells (Fig. 1), was not effectively cleared from the skin graft at the 24-h time point.

RNA (siRNA) knockdown of two components of the autophagy pathway, Nod2, a cytosolic receptor for S. aureus cell wall components (45), and ATG5 (35) was performed, and the effects on IL-1β production were measured (Fig. 6B to E). Inhibition of autophagy resulted in increased induction of IL-1β, consistent with the proposed effect of autophagy on inflammasome-mediated signaling. To determine whether the clinical AD isolates of S. aureus similarly induce autophagy in human keratinocytes, we monitored infected SCID mouse-human (SCID-hu) grafts for autophagymediated signaling. To determine whether the clinical AD isolates of S. aureus similarly induce autophagy in human keratinocytes, we monitored infected SCID mouse-human (SCID-hu) grafts for the relative induction of galectin-8 and LC3 in response to S. aureus AD4 or AD10 infection (Fig. 6F and G). The Hla-deficient strain AD4, which had increased intracellular persistence in HaCaT cells (Fig. 1), was not effectively cleared from the skin graft at 72 h postinoculation and was readily detected within the stratum granulosum. AD4 colocalized with the autophagosomal marker marker granulin-8 but not with LC3. This was in contrast to AD10; the AD10 strain that was efficiently cleared from HaCaT cells was also cleared from the human grafts at 72 h.

Selection of agr mutants from within infected keratinocytes. S. aureus bacteria readily adapt to environmental immune pressure through the selection and proliferation of specific mutants (16). We postulated that WT organisms cleared by caspase-1-dependent pyroptosis in the cytosol would be at a selective disadvantage within keratinocytes compared to the agr mutant bacteria, which lacking toxin secretion, fail to trigger inflammasome-mediated clearance. Moreover, under autophagic conditions and diminished inflammasome activity, intracellular accumulation of sufficient numbers of WT organisms would enable selection of spontaneous agr-like mutants. To determine whether this selective process occurs in infected keratinocytes, we screened the phenotypes of S. aureus recovered from within keratinocytes infected with WT S. aureus alone or with inhibitors or with a mixed WT- agr mutant (1:1) infection using hemolysis on blood agar plates as a marker for WT (Hla toxin-producing) colonies (Fig. 7A). Following WT staphylococcal infection, 6.5% of the colonies recovered from within the keratinocytes at 24 h were nonhemolytic. In a mixed WT- agr mutant (1:1) infection, the staphylococci recovered were predominantly (83.3%) nonhemolytic (P < 0.0001 compared to the value for the WT). In wortmannin-treated cells, only 41.4% of the recovered staphylococci retained the WT phenotype, in contrast to 93.5% of the colonies infecting keratinocytes under control conditions. Control experiments were performed to verify that wortmannin or small amounts of gentamicin that might gain access to the cytosol of S. aureus (in the absence of the keratinocytes) did not induce the generation of agr mutants (see Fig. S3 in the supplemental material). The increased recovery of the toxin-deficient mutants from within keratinocytes is consistent with their ability to avoid inflammasome-mediated clearance, which would be enhanced in the absence of autophagy. Analysis of 10 nonhemolytic colonies isolated from keratinocytes originally infected with WT USA300 in wortmannin-treated cells revealed loss of Hla production, a major toxin associated with activation of the inflammasome (Fig. 7B) and/or lack of RNAIII, a mediator of agr signaling and Hla production (13) (Fig. 7C). Thus, there is active selection of toxin-deficient S. aureus within keratinocytes that is influenced by the effects of autophagy in enhancing intracellular survival.

**DISCUSSION**

Intact human skin is remarkably resistant to S. aureus infection, even by the highly virulent USA300 strains. Nonetheless, staphylococcal skin infection usually initiated through autoinoculation of inapparent breaks in the integrity of the epidermis or at sites of trauma, is a major clinical problem. Our data suggest that human keratinocytes participate in USA300 clearance through many of the same mechanisms as have been well described for immune cells. These mechanisms include uptake by α5β1 integrins, escape from the endosome that is not dependent upon expression of agr-associated toxins, and induction of pyroptosis, suggested by caspase-1-associated cytotoxicity and production of IL-1β. S. aureus infection stimulates keratinocyte autophagy, which does not appear to contribute to eradication, but instead facilitates intracellular persistence through suppression of inflammasome signaling, a mechanism of immune evasion shared by many human pathogens (46).

S. aureus clearance through endosomal acidification appears less important in keratinocytes than has been reported for immune cells and other types of epithelial and endothelial cells (30, 31, 47, 48). Endosomal escape and the ability to survive within the cytosol are likely dependent on both the strain and cell type. For
example, in studies using S. aureus RN4220 and HeLa cells, endosomal escape was mediated by the agr-dependent delta toxin synergizing with hla-toxin (30), whereas escape from the RHEK-1 keratinocyte cell line required PVL production in a community-acquired MRSA (CA-MRSA) strain from Taiwan (11). Studies with a number of more-toxigenic strains (MW2, LAC, and USA400) indicated that Psm expression is critical for endosomal escape and cytosolic replication in 293 (human embryonic kidney), THP-1, and endothelial cell lines (30). By using human keratinocyte lines, cells in primary culture, and human skin grafts in situ, we attempted to model more closely what occurs in differentiated human skin. Our data suggest that keratinocytes rapidly clear even a high inoculum of staphylococciiand that this occurs at least partially through staphylococcal escape from the endosome and activation of the inflammasome and pyroptosis. As human keratinocytes have constitutive expression of pro-IL-1β, they may be primed for inflammasome activation (8), and ongoing proliferation and differentiation compensate for loss of infected cells. TUNEL staining of the USA300-infected human grafts but not PBS controls delineated occasional TUNEL-positive dead keratinocytes, consistent with the induction of pyroptosis by the WT S. aureus infection, and consequent death of the host cell.

Keratinocyte ingestion of S. aureus, by either WT or agr mutants, stimulates autophagy. This was detected in the differentiated human skin grafts as well as in the keratinocyte lines. Keratinocytes are continually being replaced, thus there is ongoing autophagy as part of their self-replacement program (18). By immunoblotting and immunofluorescence imaging (Cyto-ID), we noted a background level of LC3-positive cells that was increased by WT and especially by agr mutant S. aureus infection. The agr mutant staphylococci that do not activate the inflammasome were occasionally observed within the classic double-membrane-bound compartment typical of the autophagosome. However, evidence of the classic autophagic pathway and staphylococcal removal through LC3-associated phagocytosis was not observed (35). A substantial number of ingested staphylococci colocalized with galetin-8, which labels the bacterially damaged endosome/autophagosome, as well as with MDC, a marker for the late autophagosome (34). As inhibitors of autophagy increased staphylococcal clearance from within the keratinocyte, it appears that this pathway is activated but ineffective in S. aureus eradication.

S. aureus adaptation to the intracellular milieu of human keratinocytes was enhanced by autophagy. Autophagic keratinocytes did not eradicate the infecting organisms; instead, they provided a substantial selective survival advantage that was not eradicated even after the agr-defective strains in patients with bloodstream infection as a skin pathogen. S. aureus survival within an intracellular niche has been well described at many sites, including neutrophils (52), osteoblasts, macrophages (53), sinus cells (54), mammary and pulmonary epithelial and endothelial cell lines (30, 48). Thus, finding a small population of S. aureus within human keratinocytes is not unexpected. The evolution of S. aureus during asymptomatic carriage documents ongoing adaptation of these pathogens to the host (55) and includes mutational variation in the agr loci (16). Clinical studies indicate that human nasal carriage of the same S. aureus strains can persist over weeks if not longer (56). The appearance of agr mutants, with predominantly single point mutations from clinical isolates has been interpreted to confer a short-term advantage for survival even if these mutations are only transient (57). The pathogenicity and clinical relevance of toxin-deficient strains have been recognized with the recovery of agr mutants from systemic human S. aureus infections (23). S. aureus agr locus is defective in patients with bloodstream infection was often nasal carriage (58), which is likely the source of autoinoculation for skin infection as well (59). While we found that only a small fraction of the infecting organisms appear to persist within keratinocytes, the increased recovery of the agr mutants from within skin cells, both from chronically infected patients and in a laboratory setting, suggests that these strains may be relevant to human skin colonization. Of note, decreased neutrophil-mediated killing of USA300 agr null mutant has been described, suggesting that these strains may be less likely to be eradicated in an in vitro setting (60).

Enhanced recovery of agr mutant staphylococci was also observed in infected human skin grafts. These grafts were resistant to superficial exposure to S. aureus USA300, and the vast majority of the inoculum was readily cleared. However, consistent with the data obtained using cell lines, there were substantially more agr null mutant organisms associated with the human skin grafts than the WT organisms, and activation of autophagy was clearly demonstrated by galetin-8 and LC3 immunofluorescence. The burden of infection served to stimulate autophagy that would enhance staphylococcal persistence by consuming ASC and diminishing pyroptosis. These skin graft models did not directly assess the role of recruited phagocytes in S. aureus clearance, an important factor in in vivo infection (14), but demonstrate the contribution of the secreted toxins and their effects in promoting staphylococcal clearance by keratinocytes (12, 49).

Multiple S. aureus USA300 toxins appear to contribute to keratinocyte-mediated clearance mechanisms, as would be expected due to the ability of the pore-forming toxins to mediate escape from the endosome and to activate inflammasome signaling (10, 30, 48). Keratinocyte uptake of the WT and various toxin-deficient strains was roughly equivalent, and increased intracellular survival was most consistently observed with the agr, hla, and psma null mutants. Although the PVL leukotoxin can activate inflammasome signaling, we did not observe intracellular persistence of the PVL-negative mutant, which still expresses other agr-dependent toxins. The agr locus, through sarA and RNAIII, regulates expression of hla and psma (13, 50). Hla-associated pathogenicity has been clearly documented in murine models of skin infection (51), and keratinocyte pyroptosis likely contributes to this process. Nonetheless, it is difficult to tease out the contributions of individual toxins, as there are multiple interactions between the different agr, psma, and hla loci (50), and the abundance of the various human toxin-specific receptors on keratinocytes has not been rigorously established.

The ability of USA300 MRSA to actively adapt to conditions within human keratinocytes is likely an important factor in its success as a skin pathogen. S. aureus survival within an intracellular niche has been well described at many sites, including neutrophils (52), osteoblasts, macrophages (53), sinus cells (54), mammary and pulmonary epithelial and endothelial cell lines (30, 48). Thus, finding a small population of S. aureus within human keratinocytes is not unexpected. The evolution of S. aureus during asymptomatic carriage documents ongoing adaptation of these pathogens to the host (55) and includes mutation in the agr loci (16). Clinical studies indicate that human nasal carriage of the same S. aureus strains can persist over weeks if not longer (56). The appearance of agr mutants, with predominantly single point mutations from clinical infections has been interpreted to confer a short-term advantage for survival even if these mutations are only transient (57). The pathogenicity and clinical relevance of toxin-deficient strains have been recognized with the recovery of agr mutants from systemic human S. aureus infections (23). The source of agr defective strains in patients with bloodstream infection was often nasal carriage (58), which is likely the source of autoinoculation for skin infection as well (59). While we found that only a small fraction of the infecting organisms appear to persist within keratinocytes, the increased recovery of the agr mutants from within skin cells, both from chronically infected patients and in a laboratory setting, suggests that these strains may be relevant to human skin colonization. Of note, decreased neutrophil-mediated killing of USA300 agr null mutant has been described, suggesting that these strains may be less likely to be eradicated in an in vitro setting (60).
tion of agr defective *S. aureus* that persist intracellularly may be sufficient to maintain infection within human keratinocytes in areas such as the nares, as well as in diseased conditions such as atopic dermatitis. This small intracellular population may be especially refractory to eradication, as they are protected from many antibiotics, antibodies, and complement as well from the activity of the inflammasome and neutrophils. Therapeutic strategies to target the toxins associated with inflammasome activation and skin pathology (61) may also provide additional selective pressure for the intracellular persistence of these mutants.

**MATERIALS AND METHODS**

**Bacterial strains, human cells, and reagents.** MRSA USA300 FPR3757 (LAC) strains, specifically WT, spa null mutant (62), and mutant strains, were grown in LB broth overnight at 37°C, diluted 1:100, and grown to an optical density at 600 nm (OD_{600}) of 1.0 for keratinocyte infections. WT and alpha-hemolysin (Hla) mutant were provided by Juliane Bubeck-Wardenburg (University of Chicago) (63). WT and Panton-Valentine leucocidin (PVL) mutants were provided by Frank DeLeo (64) (National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, MT). Phenol-soluble modulin (Psm) (pmsA, pmsB, or hld) single and triple mutants (65) and agr mutants (13) were provided by Michael Otto (National Institute of Allergy and Infectious Diseases, MD). WT and agr-matched pairs in the RN and UAMS background were provided by Bo Shopsin (New York University Langone Medical Center, NY). The human keratinocyte HaCaT cell line was obtained from Angela Christiano (Columbia University, NY) and grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics.

**Human keratinocytes and organotypic cultures prior to MRSA infection.** Bacterial strains, human cells, and reagents. Cytotoxicity was determined using the LDH assay (Roche) using multiple frozen and thawed keratinocytes as a positive control. Alternatively, cell viability was determined using the Countess automated cell counter and trypan blue (Life Technologies).

**RNA analysis and siRNA.** RNA was isolated using the RNeasy total RNA kit (Omega Bio-Tek) followed by DNase treatment using a DNA-free DNA removal kit (Life Technologies). cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative reverse transcription-PCR (qRT-PCR) was performed using Power SYBR green PCR master mix (Applied Biosystems) in a StepOne Plus thermal cycler (Applied Biosystems). The primers for NALI were 5’ GGATGGCTTAAATACTCATAC 3’ and 5’ GGAAGGATGTATT CAAATG G 3’. ON-TARGETplus SMARTpool Human ATG5 siRNA, Nod2 and ON-TARGETplus nontargeting control pool siRNAs were purchased from Dharmacon and transfected into HaCaT cells using Lipofectamine RNAiMAX (Life Technologies) per the manufacturer’s instructions. After 72 h, the cells were stimulated with *S. aureus* USA300 (MOI of 10) in media containing fibronectin or PBS plus fibronectin for 2 h, treated with gentamicin as described above, and allowed to incubate for 24 h. Culture supernatants were collected and analyzed for IL-1beta and IL-18 by ELISA.

**Human keratinocytes and organotypic cultures after MRSA infection.** Bacillus anthracis was from the pathogen repository at the CDC. MRSA (WT or mutants) at a multiplicity of infection (MOI) of 10 and incubated at 37°C for 2 h. Extracellular bacteria were killed by the addition of gentamicin to a final concentration of 500 μg/ml. Intracellular infections were then allowed to continue in the presence of gentamicin for up to 96 h. To quantify intracellular bacteria, cells were disassociated with TrypLE express cell dissociation enzyme (Life Technologies), and dilutions were plated on LB agar plates for CFU enumeration. For assays with inhibitors (caspase-1, caspase-3, wortmannin, and 3-MA), controls containing the inhibitor in 5% DMSO in the absence of bacteria were included, along with controls containing just bacteria, to verify a lack of toxicity. Intracellular accumulation of staphylococci was quantified 24 h after the initial 2-h infection; additional time points are noted in the figures. Keratinocyte culture supernatants were collected for IL-1β enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Cytotoxicity was determined using the LDH assay (Roche) using multiple frozen and thawed keratinocytes as a positive control. Alternatively, cell viability was determined using the Countess automated cell counter and trypan blue (Life Technologies).

**Infection of keratinocytes and gentamicin protection assay.** HaCaT or HEKn cells grown to confluence and without antibiotics for 18 h prior to infection were pretreated for 1 h with 10 μg/ml fibronectin. Bacterial cultures grown to log phase (to an OD_{600} of 1.0) were pelleted and resuspended in media without antibiotics. HaCaT cells were infected with MRSA (WT or mutants) at a multiplicity of infection (MOI) of 10 and incubated at 37°C for 2 h. Extracellular bacteria were killed by the addition of gentamicin to a final concentration of 500 μg/ml. Intracellular infections were then allowed to continue in the presence of gentamicin for up to 96 h. To quantify intracellular bacteria, cells were disassociated with TrypLE express cell dissociation enzyme (Life Technologies), and dilutions were plated on LB agar plates for CFU enumeration. For assays with inhibitors (caspase-1, caspase-3, wortmannin, and 3-MA), controls containing the inhibitor in 5% DMSO in the absence of bacteria were included, along with controls containing just bacteria, to verify a lack of toxicity. Intracellular accumulation of staphylococci was quantified 24 h after the initial 2-h infection; additional time points are noted in the figures. Keratinocyte culture supernatants were collected for IL-1β enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Cytotoxicity was determined using the LDH assay (Roche) using multiple frozen and thawed keratinocytes as a positive control. Alternatively, cell viability was determined using the Countess automated cell counter and trypan blue (Life Technologies).

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**Human keratinocytes and organotypic cultures after MRSA infection.** Organotypic culture models were grown as previously described (8). NOD-2 null (NSG) mice (Jackson Laboratories) (66) were grafted with human neonatal foreskin skin per Columbia University institutional review board (IRB) protocol AAAAN1816. Freshly obtained skin was placed in DMEM containing penicillin and streptomycin, trimmed of dermal and subdermal compartments. The human skin was then allowed to mature for 3 to 6 weeks. Mouse bandages were inspected every day for the first 2 days after surgery and then weekly for the following 3 to 6 weeks. For
infections, mice were anesthetized with ketamine and xylazine, and S. aureus was applied dropwise in a 10-μl aliquot of PBS containing 10^6 CFU of S. aureus. Skin grafts were excised after 72 h and fixed in 4% paraformaldehyde for 24 h for paraffin sectioning and staining.

Animal work in this study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, the Animal Welfare Act, and U.S. federal law. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Columbia University (protocol AAAG7408).

Hla and Hld expression in atopic dermatitis strains. S. aureus strains, including clinical isolates (previously obtained from superficial skin swabs of atopic dermatitis patients and stored at −80°C), were grown in LB to stationary phase, and culture supernatants were harvested for Western immunoblots. Primary anti-α-hemolysin (anti-Hla) rabbit antibody was a gift from Juliane Bubeck-Wardenburg, University of Chicago, and used at 1:10,000 dilution. The presence of Hld in the clinical isolates was assessed by sequencing. Hemolysis was detected by growth on sheep blood agar plates (Becton, Dickinson) (5% in TSB). Synergistic hemolysis between Hld and Hlb was evaluated using the cross-streak method with S. aureus RN4220 by the method of Cheung et al. (26).

RNAIII expression in atopic dermatitis strains. Overnight cultures of S. aureus laboratory strains and 10 clinical isolates from atopic dermatitis patients were diluted 1:100 in LB broth and grown at 37°C to an OD600 of 1.0, and bacteria were pelleted, resuspended, and incubated for 2 h at 37°C in cell wall lysis mixture (6 μg/ml lysostaphin, 2.7 μg/ml mutanolysin, and 16.7 μg/ml lysozyme in 50 μM Tris-HCl—10 μM EDTA [pH 8]). RNA was extracted from bacteria, cDNA was synthesized, and qPCR was performed as described above to assess for S. aureus RNAIII expression.

Whole-genome sequencing. Clinical isolates of S. aureus were collected as part of an ongoing clinical study (approved by IRB AAJS956) and subjected to whole-genome sequencing. Genomic DNA was extracted from overnight cultures using the DNeasy blood and tissue kit (Qiagen) and subjected to whole-genome sequencing. Genomic DNA was prepared and subjected to whole-genome sequencing. Genomic DNA was prepared and subjected to whole-genome sequencing. Genomic DNA was prepared and subjected to whole-genome sequencing.

REFERENCES
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