Intracellular escape strategies of *Staphylococcus aureus* in persistent cutaneous infections

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Abstract

Pathogenic invasion of *Staphylococcus aureus* is a major concern in patients with chronic skin diseases like atopic dermatitis (AD), epidermolysis bullosa (EB), or chronic diabetic foot and venous leg ulcers, and can result in persistent and life-threatening chronic non-healing wounds. *Staphylococcus aureus* is generally recognized as extracellular pathogens. However, *S. aureus* can also invade, hide and persist in skin cells to contribute to wound chronicity. The intracellular life cycle of *S. aureus* is currently incompletely understood, although published studies indicate that its intracellular escape strategies play an important role in persistent cutaneous infections. This review provides current scientific knowledge about the intracellular life cycle of *S. aureus* in skin cells, which can be classified into professional and non-professional antigen-presenting cells, and its strategies to escape adaptive defense mechanisms. First, we discuss phenotypic switch of *S. aureus*, which affects intracellular routing and degradation. This review also evaluates potential intracellular escape mechanism of *S. aureus* to avoid intracellular degradation and antigen presentation, preventing an immune response. Furthermore, we discuss potential drug targets that can interfere with the intracellular life cycle of *S. aureus*. Taken together, this review aimed to increase scientific understanding about the intracellular life cycle of *S. aureus* into skin cells and its strategies to evade the host immune response, information that is crucial to reduce pathogenic invasion and life-threatening persistence of *S. aureus* in chronic cutaneous infections.

Keywords

antigen-presenting cells; intracellular bacteria; skin; *Staphylococcus aureus* ; wounds

1 | INTRODUCTION

Staphylococcus aureus is a facultative anaerobic Gram-positive coccus. About 30% of the human population carries this commensal microorganism on their skin without any clinical manifestations.[1] Cutaneous microbiome studies have also shown that *S. aureus* colonizes regions of the skin with higher humidity and temperature, such as the groyne, axillary vault and toe web.[2] However, when the skin barrier is compromised, *S. aureus* can colonize the wound regardless of location and become pathogenic. *Staphylococcus aureus* infections can cause a range of illnesses from minor skin infections, such as pimples, impetigo and abscesses, to persistent chronic wounds to life-threatening diseases, such as pneumonia, endocarditis and sepsis.[1,3,4] Infection often challenges the host's immune system, and if the immune system fails to fight bacterial invasion, *S. aureus* can spread from the wound area to other tissues and ultimately lead to sepsis and death. Pathogenic invasion of *S. aureus* is, therefore, particularly a major concern in immune-compromised patients, such as elderly, diabetic and paediatric population, but also in patients with chronic skin diseases like atopic dermatitis (AD) and epidermolysis bullosa (EB).[4]

There is a great deal of genetic variation within the *S. aureus* species due to its fast-co-evolutionary adaptation skills.[5] With molecular typing, at least 17 800 different strains of *S. aureus* have been identified of around the world, and a distribution map was generated of the most prevalent *S. aureus* protein A (*spa*, an immunoglobulin-binding protein) types in different continents.[6] Furthermore, wounds, from EB patients can be colonized with multiple (ranging from 2–6) types of *S. aureus*, and the type of *S. aureus* mirrors the geographic location of the patient and can vary over time.[1,7,8] Specific *S. aureus* strains have also found to be associated with non-healing diabetic foot ulcers.[9] Nowadays, *S. aureus*–related infections are treated by a limited panel of antibiotics, but life-threatening resistance is rising, particularly towards pathogenic methicillin-resistant strains (MRSA).[5] Therefore, there is an urgent need to develop alternative treatment options.

Generally, *S. aureus* is recognized as an extracellular pathogen, but increased evidence is rising that *S. aureus* can also invade, hide and persist into various types of cells, which can be classified into professional- and non-professional antigen-presenting host cells.[10–12] The intracellular niche increases survival chances of *S. aureus*. However, host cells may also have the ability to kill intracellular bacteria and present antigens on the cell surface for the induction of an adaptive immune response.[13] Currently, the intracellular life cycle of *S. aureus* is incompletely understood. The aim of this review was to discuss scientific knowledge on the intracellular life cycle of *S. aureus* in skin host cells and its strategies to evade the host immune system. This knowledge may be useful to help reduce *S. aureus* infections, reoccurrence and resistance to antibiotic treatment.

2 | INTRACELLULAR LIFE CYCLE OF *Staphylococcus aureus*

2.1 | Phenotypic switching of *Staphylococcus aureus*

Tight bacterial adherence to host structures is a crucial step in infection development. *Staphylococcus aureus*–derived fibronectin-binding proteins (FnBps) bind to host cell–derived integrin $\alpha5\beta1$, inducing changes in the cytoskeleton that lead to the uptake

of bacteria into phagosomes.[10,14] Following invasion, *S. aureus* can induce a strong inflammatory and cytotoxic response or remain silent and hide intracellularly. The response mainly depends on the expression of the bacterial accessory gene regulator (*agr*) system. The *agr* system in *S. aureus* controls expression of many virulence factors, exoenzymes and other cytotoxic factors that kill the host cell.[10,15] However, phenotypic switching within the *agr* system occurs in about 10% of the colonizing strains. These *agr*-deficient colonies, frequently associated with hospitalizations and persistent infections, exhibit a high rate of cellular internalization where they induce less toxicity within cells.[10,12,16] *S. aureus agr*-deficient colonies are recognized as "small colony variants" (SCVs), which are characterized by small colony size, slow growth, reduced metabolism, downregulated virulence genes and low cytotoxicity (Figure 1), allowing *S. aureus* to escape the host's immune system and reside in certain cell types. In line with this, studies have shown that SCVs can persist into host cells for several weeks.[17] Furthermore, clinical cases have shown that *S. aureus* infections can remain dormant with relapses occurring months or years later after apparent successful antimicrobial treatment.[18–20] Environmental factors, such as an increase in pH or exposure to nutrient-rich medium, can restore phenotype of SCVs back into a virulent and fast proliferating,[10,17] suggesting that these variants may serve as a hidden source for persistent/reoccurring chronic infections. Interestingly, when host cells are infected with aggressive wild-type *S. aureus*, these bacteria induce inflammation and cytotoxicity in the intracellular location, which correlates with high *agr* expression (Figure 1A).[10] Over the course of a couple of days, host cells activate certain degradation systems, which allow them to combat bacterial infection. Several weeks later, a small amount of *S. aureus* seems to be able to resist cellular degradation and persist inside host cells for long periods of time. A scheme of the difference between wild-type cytotoxic (*agr*-positive) and SCV-like dormant (*agr*-deficient) *S. aureus* strains is depicted in Figure 1A.

2.2 | Lysosomal pathway of *Staphylococcus aureus*

Skin cell types can be classified into professional and non-professional antigen-presenting cells (APCs). Professional APCs, such as macrophages, dendritic cells and B cells, are efficient in internalizing foreign antigen including *S. aureus*. After host cell internalization, bacteria generally route through the phagosomal degradation pathway that terminates into a highly degradative organelle with a low pH, the phagolysosome (Figure 1B). Via this vesicular pathway, many internalized microorganisms are efficiently eliminated by professional APC. Recently, research showed that the SCV-like (*agr*-deficient) *S. aureus* phenotype is induced by low pH. These low-pH–resistant bacteria are able to hijack the phagolysosome and remain dormant in these organelles. However, phenotypically switched SCV-like bacteria have the potential to revert back to fully aggressive wild-type phenotype by neutral pH.[21] Therefore, treatment with lysosomal alkalizing agents, such as chloroquine together with antibiotics, may be a novel effective strategy to fight persistent chronic *S. aureus* infections.

2.3 | Autophagy pathway of *Staphylococcus aureus*

Non-professional APCs, such as keratinocytes, endothelial cells and fibroblasts, also have the capacity to internalize bacteria.[11,12,22,23] However, these cells do not have an optimal

lysosomal capacity to degrade bacteria. Instead, non-professional antigen APCs combat intracellular pathogens by inducing autophagy.[12]

Autophagy is a conserved membrane trafficking pathway in eukaryotic cells that sequesters cytoplasmic contents by double-membrane autophagosomes and eventually delivers them to autophagolysosomes. Autophagy is regulated by the AKT pathway and can be pharmacologically induced by rapamycin, which is an inhibitor of the serine/threonine kinase Tor. In contrast, inhibition of type III phosphatidylinositol 3-kinase (PI3K) by the compound wortmannin inhibits autophagy.[24] Rapamycin increases co-localization of *agr*-positive *S. aureus* with the autophagy marker LC3, while wortmannin drastically impairs bacterial co-localization with LC3 in non-professional APCs.[25] Furthermore, rapamycin increases the intracellular growth of cytotoxic *agr*-positive *S. aureus*, ultimately leading to killing of host cells. This is in contrast to wortmannin, which drastically impairs the growth of intracellular *S. aureus* (Figure 1B). Interestingly, SCV-like/*agr*-deficient *S. aureus* did not localize in LC3-positive autophagosomes. However, when autophagy was induced by rapamycin, *agr*-deficient *S. aureus* were observed in autophagosomes enabling bacterial replication and killing of host cells.[25] More recently, studies have shown that intracellular *S. aureus* is targeted by selective autophagy (also called xenophagy) involving ubiquitination and autophagy receptors such as SQSTM1 in non-professional APCs.[26,27] Ubiquitination is also a protein modification used by host cells to tag proteins that are destined for proteasomal degradation (ubiquitin proteasome system [UPS]). Selective autophagy, however, is mediated by autophagy receptor molecules, like SQSTM1, that deliver ubiquitinated bacteria to autophagosomes.[28] Therefore, inhibition of selective autophagy receptors might prevent *S. aureus* from routing through the autophagy machinery, and/or recruitment of proteasomes to these ubiquitin-surrounded bacteria might encourage cytosolic degradation.

2.4 | Perforin-2 and *Staphylococcus aureus*

Perforin-2 is an innate antibacterial effector essential for elimination of intracellular bacteria in both professional and non-professional phagocytic skin cells (Figure 1B).[11,23] First described in macrophages in vertebrates, it was initially named macrophage-expressed protein 1 (Mpeg1) before being renamed Perforin-2 due to its membrane attack complex/perforin (MACPF) domain.[11,29] Perforin-2 is located within the membranes of endosomal vesicles which traffic to and fuse with the bacteria containing phagosome. Acidification triggers a conformational change in the MACPF and P-2 domains which allows membrane insertion and pore formation on bacterial surfaces, enabling reactive oxygen species, nitric oxide and lysozyme to enter and perform bacterial killing.[11,29–32] Perforin-2 was shown to be critical for the clearance of a variety of Gram-positive and Gram-negative pathogens. Perforin-2–deficient mice are unable to clear *S. aureus* upon epicutaneous infection, highlighting its indispensable role in the host's innate antimicrobial response.[31,33] Multiple obligatory intracellular pathogens have developed mechanism to counteract Perforin-2,[11,34] while recent data have shown *S. aureus* ability to suppress Perforin-2 in skin cells (Figure 1B),[23] suggesting a novel mechanism of invasion and its possible role in chronic wounds in both elderly and paediatric EB population.

3 | INDUCTION OF AN ADAPTIVE IMMUNE RESPONSE BY

Staphylococcus aureus

Pathogens invading the host are first controlled by a rapid and broad targeting of the innate immune response. After a couple of days of fighting, the innate immune response is followed by a second line of defense, "the adaptive immune response." During this response, APCs generate highly specific T and B cells that can recognize *S. aureus* peptides and kill pathogen-infected cells. After resolution of an immune response, antigen-specific memory T cells persist in many sites of the body, including the skin. Long-lived memory cells provide an effective quick first line of defense against a secondary infection of a particular *S. aureus* strain. Importantly, the majority of adults contain detectable levels of circulating antigen-specific memory T cells to *S. aureus*.[33] This indicates that most adults have previously been exposed to *S. aureus* through either their commensal colonization or subclinical infection. Furthermore, this implies that *S. aureus* invades APCs where bacteria are proteo-lytically cleaved in phagolysosomes and peptides are loaded and presented on major histocompatibility complex (MHC) molecules for T-cell priming.

3.1 | MHCI and MHCII vesicular transport

MHC class I molecules are expressed on both professional and non-professional APCs. For MHC class I peptide loading, intracellular cytosolic ubiquitinated non-self proteins, for example from viruses or mutated sequences, are destined for proteasomal degradation. Some of these trimmed peptides go into the endoplasmic reticulum via a peptide transporter called transporter associated with antigen processing (TAP). These peptides are loaded onto empty MHC class I molecules.[35] Loaded MHC class I:peptide complexes are presented on the cell surface where it can activate cytotoxic CD8 T cells. These T cells have the potential to directly kill infected host cells. Importantly, most of the steps of the MHC class I pathway are not essential for the viability of cells. In line with this, viruses (eg, cytomegalovirus) and maybe also *S. aureus* hiding in the intracellular can be expected to interfere with this pathway so that they can escape an immune response.[35] Therefore, *S. aureus* should be encouraged to leave vesicular compartments and go into the cytosol where it should be targeted to be ubiquitinated for proteasomal degradation and loaded onto MHC class I molecules for presentation (Figure 2).

In contrast to MHC class I molecules, MHC class II molecules are generally expressed on professional APCs, which can present lysosomal degraded peptides and activate CD4 T cells.[35] Binding of naïve CD4 T cells to loaded non-self peptide:MHC complexes results into rearrangement of T-cell receptors (TCR) and activation of CD4 T cells (Figure 2). Subsequently, primed CD4 T cells can activate B cells, allowing them to produce highly specific antimicrobial antibodies that specifically target pathogenic bacteria. Produced antibodies are helpful in the opsonization of bacteria and their engulfment by phagocytic cells, such as neutrophils and macrophages. CD4 T cells function mainly as helper cells and can be divided into Th1 and Th2 helper cells. Th1 cells produce IL-2 and interferon-gamma (IFN-y) and contribute to immune protection by helping macrophages, cytotoxic T cells, and B cells. Th2 cells, however, are characterized by IL-4 and IL-5 secretion and exacerbate disease by activating, mast cells, for example.[36] CD4 T cell can also differentiate into Th17

cells. These cells secrete IL-17, which is a pro-inflammatory substance and crucial for neutrophil recruitment. Importantly, IL-17A/F knockout mice develop spontaneous *S. aureus* skin abscesses and humans with IL-17 defects are prone to *S. aureus* skin infections.[37] In line with this, patients with a neutrophil disorder also suffer from an increased incidence of *S. aureus* infection.[37] This suggests that induction of an effective Th17 response plays an essential role in protection against *S. aureus* infection. However, we have found that neutrophils accumulate in chronic non-healing wounds of EB patients (unpublished data) which are very likely colonized with *S. aureus*.[8] Interestingly, IL-1β–/– mice, which have impaired neutrophil recruitment and host defense during an initial *S. aureus* infection, were found to be rescued during a secondary infection. Further mechanistic investigation showed that clonal expansion of TNF/IFN-γ–producing γσT cells induced by TLR2 signalling protected against additional *S. aureus* skin infections.[38] Furthermore, activation of γσT cells by commensal *Staphylococcus epidermidis* resulted in upregulation of Perforin-2 and enhanced ability of cells to eliminate intracellular *S. aureus*, suggesting the protective role of beneficial bacteria against persistent cutaneous infections.[39]

In another immune evasion strategy to avoid intracellular host cell clearance, *S. aureus* can manipulate the expression of co-stimulatory molecules on the cell surface. Appropriate co-stimulation of APC-derived CD80/86 with CD28 on T cells is essential to activate T cells. *Staphylococcus aureus*, however, can inhibit expression of activating co-stimulatory molecules infected cells affecting T-cell activation (Figure 2).[40] Additionally, *S. aureus* has been shown to induce programmed cell death 1 (PD-1) ligand expression in professional APCs.[40,41] PD-L1 binds to PD-1–expressing T cells induces T-cell exhaustion, which is characterized by loss of its effector functions. Therefore, blockade of PD-1/PD-L1 interactions might be an effective treatment option for intracellular *S. aureus*–infected cells. In fact, PD-1/PD-L1 blockade has been shown to protect against *S. aureus* infection after skin burn in mice.[41]

4 | INTRACELLULAR LIFE CYCLE IN PROFESSIONAL ANTIGEN-PRESENTING CELLS

4.1 | Macrophages

Macrophages are professional phagocytes that can ingest death cells, cellular debris, microorganisms and aged neutrophils. Skin-resident macrophages are the most frequent resident immune cell type in the dermis (Figure 3).[42] In response to local skin injury, resident macrophages and monocytes migrate to the affected site. Once arrived, monocytes differentiate into pro-inflammatory macrophages in response to local produced inflammatory cytokines. Macrophages play a key role in host defense by recognizing bacterial effectors and engulfing and phagocytosing them.[42] Therefore, macrophages play a critical role in innate immunity as they have the potential to directly kill ingested *S. aureus*. When macrophages ingest bacteria, the bacteria become trapped in the phagosome, which then fuses with the lysosome. Within this organelle, enzymes and toxic peroxides digest and clear the pathogen (Figure 1B).[43] However, macrophages have a limited capacity to degrade *S. aureus* and some bacteria can become resistant to this method of digestion and manage to prevent phagolysosomal maturation and acidification.[27,44] Eventually, infected macrophages

lyse and release viable bacteria, which can be phagocytosed by other macrophages. This continuous cycle of lysis and uptake maintains a viable pool of intracellular bacteria over time, leading to persistence of intracellular *S. aureus*.[27]

Macrophages express high basal levels of the antibacterial molecule Perforin-2.[11] Furthermore, it has been shown that Perforin-2–deficient macrophages infected with *Listeria monocytogenes* have increased acidification of vesicles resulting in bacterial replication and expression of virulence genes.[34] This suggests that Perforin-2 works upstream of the lysosomal degradation system. Therefore, investigation towards Perforin-2 expression in macrophages and their subtypes, from early phases of healing and chronic non-healing wounds will be informative. Furthermore, induction of Perforin-2 expression may be an effective therapeutic strategy to combat antibiotic-resistant *S. aureus* infections. Therefore, it is important to gain more knowledge into pathways that can induce expression of this protein, such as type I and type II interferon signalling pathways,[29] as well as Perforin-2 modulation by commensal microorganisms.[39]

Besides inducing an innate immune response, macrophages can also initiate an adaptive immune response and function as professional APCs. However, their antigen-presenting capacity is modest compared with dendritic cells. Furthermore, macrophages do not effectively migrate to skin draining lymph nodes.[42] After digesting *S. aureus* within phagolysosomes, macrophages can load bacterial antigen on MHC molecules and present them to local T cells.[45] Alternatively, macrophages may transfer antigen to dendritic cells, which can migrate and present antigen to highly organized lymphoid structures.[45]

When macrophages become dysregulated, wound healing complications and chronic infection may arise.[46–48] For example, the skin of AD patients presents a significant imbalance of the microbiome with high colonization of *S. aureus*.[14] Interestingly, macrophages from AD patients express significantly less Toll-like receptor 2 (TLR2) compared with healthy controls.[49] Since TLR2 plays an important role in pathogen recognition and activation of the innate immune system, reduced expression in macrophages may contribute to enhanced susceptibility to *S. aureus* skin infection. Patients with diabetes who have hyperglycaemia due to a lack of insulin are also more prone to *S. aureus* infections compared with healthy individuals. In line with this, diabetic patients have macrophages with reduced apoptotic clearance activity because of the effects of hyperglycaemia.[50] EB patients who develop mucocutaneous blisters after minimal trauma due to a genetic disorder are also prone to *S. aureus* infections.[51] Importantly, we have recently found a loss of macrophages in chronic non-healing wounds of EB patients (unpublished data).

4.2 | Dendritic cells and Langerhans cells

Dendritic cells differ from macrophages; in particular, because of their lower phagocytic capacity, they migrate more effectively to draining lymph nodes and have a better antigen-presenting capacity than macrophages.[42] Upon detection of microbes, dendritic cells upregulate MHC molecules, activate co-stimulatory molecules, produce inflammatory cytokines and migrate to draining lymph nodes to activate CD4 T cells. In human skin, dendritic cells are sub-classified into Langerhans cells, which reside exclusively in the epidermis, and interstitial dermal dendritic cells (DDCs), which reside in the adjacent dermis

(Figure 3). Importantly, compared with DDCs, Langerhans cells have a very low capacity to internalize and process bacteria like *S. aureus*. Furthermore, Langerhans cells have been shown to induce a very weak reactivation of memory CD4 T cells compared with DDCs.[52] A possible explanation for the prevention of an antibacterial response by Langerhans cells is that the epidermal immune tolerance provides symbiotic relationship with commensal bacteria. However, when bacteria penetrate from the epidermis into the dermal layer, DDCs will be activated resulting in an effective inflammatory immune response. Recent research showed that Langerhans cells can directly interact with *S. aureus* through the pattern recognition receptor langerin (CD207), and this interaction influenced Langerhans cell pro-inflammatory cytokine production.[53] In line with this, genome-wide association studies linked langerin to AD[54] making C-type lectin langerin-dependent signalling an interesting therapeutic target for *S. aureus*–associated skin infections. Furthermore, reduced number of Langerhans cell was associated with non-healing diabetic foot ulcers,[55] characterized with *S. aureus* colonization. Other studies followed engineered *S. aureus*–expressing *zsgreen* in DDCs that was colonized on a mouse skin. It was found that skin-derived migratory DC expressing high MHC molecules contained the majority of *S. aureus* antigen and that active CCR7-dependent transport of antigens to local lymph nodes is required for priming of bacteria-specific CD4+ T cells.[56] Based on the in vivo model used in this study, it would be of importance to quantitatively compare ability of different skin cells subtypes to up take *S. aureus*–expressing *zsgreen* or fluorescent protein during the course of a skin wound infection.[56–58]

Like macrophages,[49] AD skin–derived DDCs and Langerhans cells express less TLR2 compared with healthy skin.[59] Impaired TLR2-mediated signalling results in a less effective immune response, which may contribute to a less effective clearance of *S. aureus* invasion. In vitro, treatment of human monocytes with anti-TLR2–blocking antibodies and *S. aureus* has been shown to induce MHC class II expression,[39] demonstrating a link between TLR2 signalling and the presence of intracellular *S. aureus* during monocyte-derived dendritic cell maturation. Patients with diabetes have reduced DC maturation and migration to draining lymph nodes, likely due to hyperglycaemia[60] which can contribute to high prevalence of *S. aureus* infections and associated complications.[61,62]

4.3 | B cells

B-cell activation occurs in secondary lymphoid structures. Foreign proteins circulating through lymph fluid can activate B cells with help of T helper cells. Once a B cell receptor binds a foreign antigen, it is taken up by receptor-mediated endocytosis, degraded in lysosomes, and loaded onto MHC class II molecules for presentation to T cells (Figure 2). Subsequent T-cell receptor:MHC:peptide results in B-cell activation promoting proliferation and in antibody production inducing an humoral immune response.[4] Highly specific antibodies will bind the antigen present of the surface of *S. aureus*, which can subsequently be cleared by either the complement system or phagocytosing cells (eg, neutrophils, macrophages). Most adults carriers and non-carriers for *S. aureus* have a broad spectrum of *S. aureus*–specific serum immunoglobulins (IgG) antibodies.[37] Furthermore, the sera of EB patients with chronic non-healing wounds are colonized with higher levels of anti-*S. aureus* IgG than healthy controls. Additionally, EB patients who carry multiple *S.*

aureus types have even higher levels of anti-*S. aureus* antibodies than EB patients who carry only a single type of *S. aureus*.[63,64]

Unfortunately, recovery from a particular *S. aureus* infection does not appear to guarantee immunity against subsequent infections.[65] This may be explained by the highly polymorphic *spa* gene of *S. aureus*, of which currently more than 17 800 different types have been identified globally (SeqNet.org).[6] The primary binding site of *spa* is the Fc region of IgG produced by B cells interfering with effective opsonization and subsequent phagocytosis of *S. aureus*.[6] This increases the survival chance of *S. aureus* and circumvents B-cell memory. Furthermore, *S. aureus* produces various molecules with redundant functions, such that if one is eliminated (or targeted by an antibody), other *S. aureus* products may compensate for that loss of function.[37] Importantly, although healthy individuals have high titres to *S. aureus*, patients with defects in humoral immunity are not particularly prone to *S. aureus* infections.[37] Apparently, the lack of antibody against *S. aureus* is compensated by other adaptive immune mechanisms. This may explain why attempts to produce an effective *S. aureus* vaccine have failed.

5 | INTRACELLULAR LIFE CYCLE IN NON-PROFESSIONAL ANTIGEN-PRESENTING CELLS

5.1 | Keratinocytes

Healthy human skin acts as a physical barrier to prevent invasion of foreign pathogens while providing home to commensal microbiota. Structurally, the skin consists of several layers of keratinocytes, of which the outer horny layer (the epidermis) is constantly exposed to commensal bacteria, viruses and fungi (Figure 3). This top layer is composed of terminally differentiated, enucleated (squamous) keratinocytes that are chemically cross-linked to provide a barrier for the skin.[3,66] However, when the skin barrier is broken or when the balance between commensals and pathogens is disturbed, or in the case of skin disease or systemic disease, *S. aureus* can invade to the stratum granulosum, which contains less differentiated keratinocytes, or to the basal layer of keratinocytes (stratum spinosum), which contains undifferentiated keratinocytes (Figure 3).[3,66] Keratinocytes express several pattern recognition receptors (PRRs), which contribute to initial sensing of extracellular *S. aureus*.[3] Binding of *S. aureus* to PRRs results in NF-κB activation and induction of proinflammatory cytokine production, such as TNF-α.[67,68] Subsequently, an acute inflammatory response is initiated and inflammatory cells, such as professional APCs, are recruited.[69]

Keratinocytes are also able to phagocytose microorganisms, like *S. aureus*.[68] Invasion of keratinocytes by *S. aureus* requires high-affinity FnBPs, which interact with cell surface integrin α5β[70] and blockade of integrin α5β1; alternatively, disruption of FnBPs in *S. aureus* blocks internalization.[71] Furthermore, heat-killed *S. aureus* have been shown to be internalized into Rab5- and Rab7-positive phagosomes of mouse keratinocytes.[68] In HeLa cells, *S. aureus* has been shown to transit to LC3 positive autophagosomes where they are able replicate and inhibit fusion with lysosomes.[25] Other studies have shown that *S. aureus* rather becomes targeted by selective autophagy through ubiquitination.[26]

Several studies have demonstrated that keratinocytes can also express MHC class II molecules both under homeostasis and in the context of inflammation. However, induction of MHC class II, by, for example, IFN-y, on keratinocytes has been shown to control T-cell responses.[72,73] Moreover, it was recently shown that MHC class II–expressing keratinocytes control microbiota-induced Th1 cell responses.[74] This indicates that MHC class II–expressing keratinocytes avoid a local inflammatory reaction to commensal microorganisms. This effect might favour *S. aureus* infection and spreading. However, other studies have shown that keratinocytes can process extracellular antigen and induce MHC class II and ICAM expression, which can subsequently activate CD4 T cells. Pretreatment of keratinocytes with chloroquine inhibited this effect (Figure 1B), suggesting that the endosomal pathway for antigen processing is involved.[75] Whether intracellular *S. aureus* can induce or manipulate MHC class II expression and co-stimulatory molecules, such as CD80, CD86 or I-CAM, on keratinocytes is currently unknown.

It has been demonstrated that expression of antimicrobial Perforin-2 is induced during wound healing,[23] suggesting its role in protection against intracellular pathogens during barrier restoration. In vitro, Perforin-2 is constitutively expressed in keratinocytes.[11,29] However, Perforin-2 suppression was observed in *S. aureus*–infected wounds, while keratinocytes overexpressing Perforin-2 have demonstrated increased ability to eliminate intracellular *S. aureus*.[23] This suggests that *S. aureus* is able to manipulate Perforin-2 expression to reside inside the keratinocytes. Therefore, it is important to gain further scientific knowledge on pathways involved in Perforin-2 regulation. Ongoing studies will reveal the role of Perforin-2 in chronic wound infections of different aetiologies.

Staphylococcus aureus strains isolated from AD skin, but not control *S. aureus* strains, have been demonstrated to internalize into keratinocytes and accumulate into lysosomes.[76] This ability to internalize is very likely due to increased expression of FnBPs in AD-derived *S. aureus* strains as fibronectin antiserum partially inhibited uptake,[76] suggesting an SCV-like phenotype (Figure 1A). Moreover, Th2 cytokine expression was shown to be increased in lesional skin biopsies of patients with AD.[14] In line with this, dupilumab, which blocks Th2 signalling by blocking interleukin-4 treatment, has been shown to ameliorate dermatitis.[77] This indicates that evolved SCV-like *S. aureus* strains from AD skin induce an imbalanced Th1/Th2 adaptive immune response resulting in a Th2-shifted immune response. In theory, a Th2 adaptive immune response is induced by MHC class II molecules that present peptides derived from lysosomal degradation (Figure 2). It might therefore be possible that intracellular (SCV-like) *S. aureus* can induce MHC class II expression and peptide presentation in keratinocytes as well as co-stimulatory molecules in AD lesions, but this needs to be confirmed in an experimental setting. Keratinocytes from AD patients do not express significantly less TLR2 compared with healthy controls. However, keratinocytes from AD patients have been shown to respond less to TLR-2 stimulation compared with healthy control keratinocytes.[78]

5.2 | Endothelial cells

Endothelial cells are cells that line the interior surface of blood vessels (Figure 3) and lymphatic vessels, forming a passive barrier between blood or lymph in the lumen and

the vessel wall. Endothelial cells are not only a passive barrier, but they also play an important role in antimicrobial defense. For example, they attract and activate leucocytes to an inflammatory site, and help in firm adhesion of leucocytes to the endothelium, eventually resulting transendothelial migration towards the infected location. However, *S. aureus* can also invade endothelial cells and migrate to the vascular system from local infection sites. Entering the bloodstream can lead to infection of other tissues/organs, which is associated with poor prognosis.[79] Many *S. aureus* strains have been shown to be able to invade endothelial cells and, depending on virulence factors related to the *agr* system (Figure 1A,B), hide in endothelial cells in vitro.[80] Furthermore, SCVs of *S. aureus* within endothelial cells were demonstrated to withstand lysosomal degradation.[81] Additionally, Perforin-2 can be responsible for elimination of intracellular *S. aureus* from endothelial cells.[11,23,82]

5.3 | Fibroblasts

The main cells in the dermis of the skin are fibroblasts (Figure 3), which produce collagen and elastin proteins that provide structure and elasticity to the skin. Dermal fibroblasts also communicate with each other and other cell types playing an essential role in maintaining skin physiology.[42] Even though *S. aureus* has been demonstrated to invade fibroblasts in vitro,[10,26] invasion into dermal fibroblasts has not been studied. In mouse embryonic fibroblasts (MEFs) and 3T3 fibroblast–like cells, *S. aureus* has been shown effectively invade, multiply and induce apoptosis.[10] Specifically, in MEFs, *S. aureus* has been demonstrated to become targeted by selective autophagy through ubiquitination and the receptor proteins SQSTM1, OPTN and CALCOCO2 (Figure 2).[26] It is therefore worthwhile to study to what extend *S. aureus* invades and hides in dermal skin fibroblasts in human in vivo. This could be studied by, for example, examining immunostainings of skin biopsies derived from confirmed *S. aureus*–infected patients. However, keratinocytes and fibroblasts have been also shown to have intracellular defense mechanisms to eliminate *S. aureus* for which Perforin-2 expression is critical.[11,22] Like keratinocytes and endothelial cells, Perforin-2 expression in fibroblasts requires induction by interferons.[11] Therefore, it would be informative to study the expression levels of Perforin-2 in fibroblasts derived from early, established and chronic EB wounds. Alternatively, engineered wild-type/*agr*-positive or SCV-like/*agr*-deficient strains of *S. aureus*–expressing *zsgreen*[56] could be colonized on the back of a mouse skin and imaged over time.

5.4 | Effect of conventional

antibiotics and antimicrobial peptides on intracellular *Staphylococcus aureus*

—Treatment of intracellular *S. aureus* infections is a serious challenge, as conventional antibiotics tend to remain in the extracellular space. Most antibiotics, such as β-lactams and aminoglycosides, hardly penetrate into a eukaryotic cell.[83,84] Other types of antibiotics have an intracellular short time retention (macrolides), inadequate cellular distribution and/or low intracellular concentration (fluoroquinolones and vancomycin).[84] The use of carrier systems for delivery of antibiotics into the eukaryotic cells might be a way to kill intracellular *S. aureus* and studies towards this approach currently ongoing.[83,84]

Another serious treatment challenge is the phenotypic switching of *S. aureus* towards SCVs (Figure 1A,B). Recently, it has been shown that antibiotic pressure induces phenotypic switching of intracellular *S. aureus* towards non-growing persistent *S. aureus* in APC.[85] Intracellular phenotypic reversion towards the rapid growing wild-type phenotype was observed after removal of the antibiotic pressure.[85] This may explain the clinical observation of relapsing infections at the end of antibiotic therapy. It also indicates that persistence is highly reversible and it seems to correlate with the dose and duration of antibiotic exposure.[86,87] Therefore, additional studies towards the intracellular life cycle of *S. aureus* into skin cells are urgently needed.

Antibacterial peptide antibiotics are promising alternative drug candidates, because of their potential to cross the eukaryotic cell membrane and maintaining intracellular activity against *S. aureus*.[88] Several in vitro studies have shown good intracellular antibacterial activity against several *S. aureus* strains.[89–93] Importantly, two short antimicrobial synthetic peptides, WR12 and D-IK8, have been shown to kill non-growing persistent *S. aureus*.[90] Furthermore, topical application of these peptides on mice infected with MRSA was effective in reducing bacterial load.[90] Therefore, additional studies are needed to determine whether antimicrobial peptides are also effective to eliminate intracellular SCVs in vivo and whether there is a synergistic effect in combination with antibiotics and/or upregulation of Perforin-2.

6 | FUTURE PERSPECTIVES

Staphylococcus aureus skin infections remain a major challenge to the health of human societies around the world. Although most *S. aureus* strains are recognized as extracellular pathogens, *S. aureus* can also invade, hide and persist into various types of professional and non-professional APC hosts.[10] Consequently, the adaptive immune response towards *S. aureus* is affected in patients with chronic skin diseases, which may result in life threatening chronic non-healing wounds. Many in vitro studies have revealed intracellular immune escape routes taken by *S. aureus* (Figures 1, 2 and 3), but additional research is needed to confirm these findings in patients and in vivo animal models. Since *S. aureus* interferes with the AKT pathway for its own survival,[25,26] more clarification is needed about the role of this pathway and *S. aureus* persistence. To find novel manipulative *S. aureus* targets of the AKT pathway, a small interference RNA and a small molecule screen, as described for *Salmonella typhimurium*, might be utilized.[94] Another major open research question is whether ubiquitination of *S. aureus* can be manipulated so that it will be targeted for proteasomal degradation, which may result in MHC class I antigen presentation and immune recognition. Considering the intracellular life cycle of *S. aureus* in professional APCs, an open research question is how *S. aureus* affects TLR2 expression, which seems to result in enhanced susceptibility to *S. aureus* skin infection. Furthermore, since Perforin-2 is critical for intracellular killing of *S. aureus*, further investigation towards signalling pathways affecting the expression of this protein is necessary. Next to that, it would be important to gain more knowledge about the crosstalk between the interferon and the AKT pathway and *S. aureus* persistence. Gaining more knowledge into these pathways might result in the identification of molecules able to induce Perforin-2 expression in chronic non-healing wounds. These novel therapeutic approaches could be used in combination with

antibiotics to treat *S. aureus*–infected chronic non-healing wounds in both paediatric and adult population. Additionally, clarification is needed regarding which APC is used most by *S. aureus* to hide in vivo in different cutaneous pathologies utilizing in vivo preclinical models[56–58] and patient-derived biopsies. Furthermore, early detection of SCV-like strains from *S. aureus* skin wounds may be clinically important to predict which individuals are prone develop reoccurrence after antibiotic treatment. This could be done, for example, by analysing *agr*, *α-haemolysin* (*Hla*) or *FnBp* gene expression from human samples. Taken together, chronic wound infections are one of the most frequent complications of chronic dermatologic diseases and the prevalence of antibiotic and antimicrobial resistance is rising. Therefore, interfering with one or more of the intracellular escape strategies of *S. aureus* in persistent cutaneous infections will provide novel more efficient therapeutic approaches.

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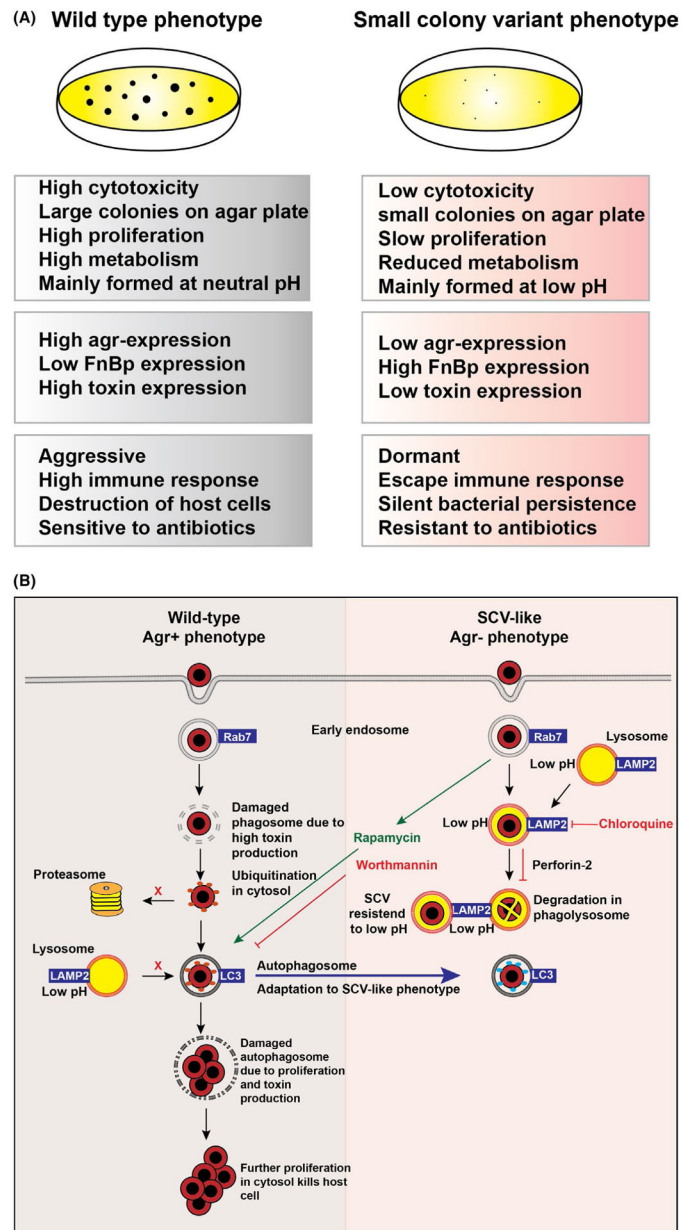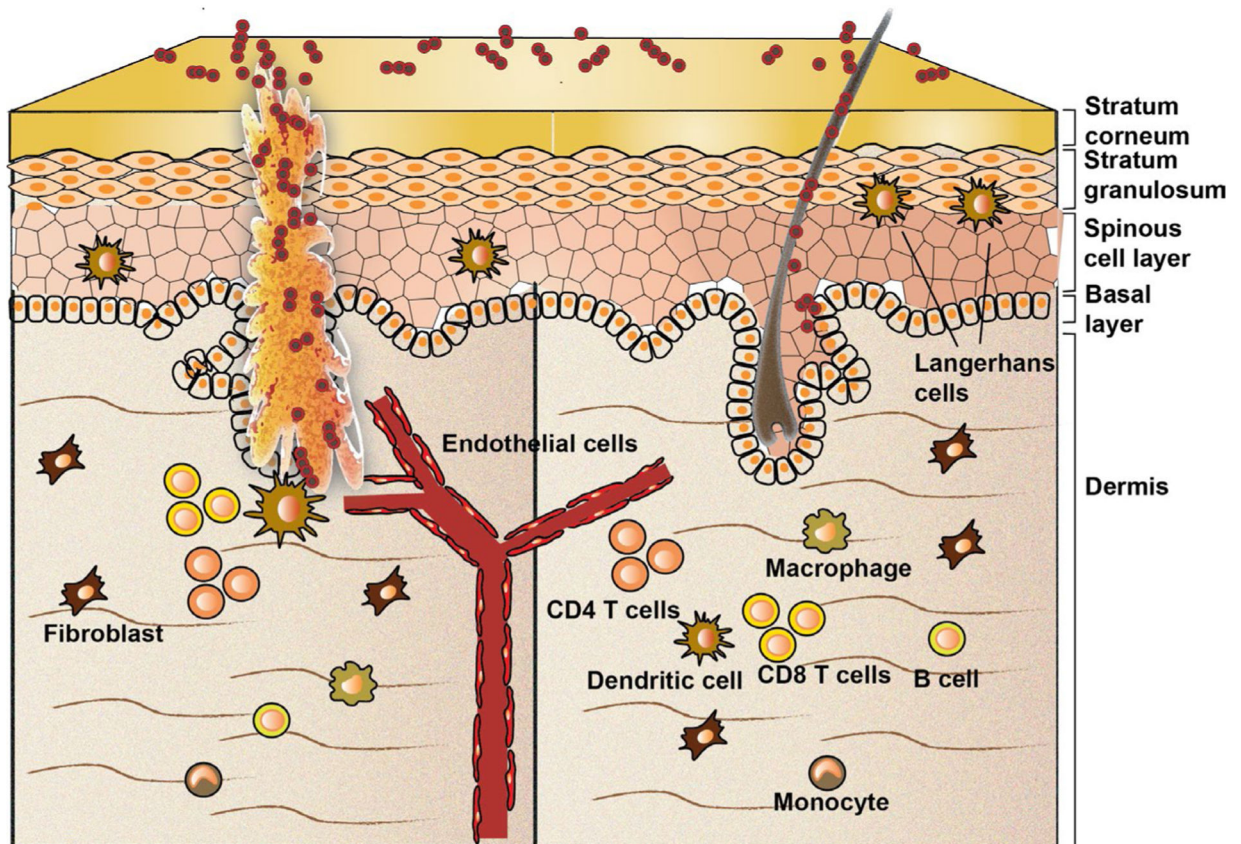
FIGURE 1.
(A) Difference between wild-type cytotoxic *agr*-positive and small colony variants (SCV)-like dormant *agr*-deficient *Staphylococcus aureus* colonies. (B) Intracellular routing of *S. aureus* wild-type cytotoxic *agr*-positive and SCV-like dormant *agr*-deficient *S. aureus* colonies
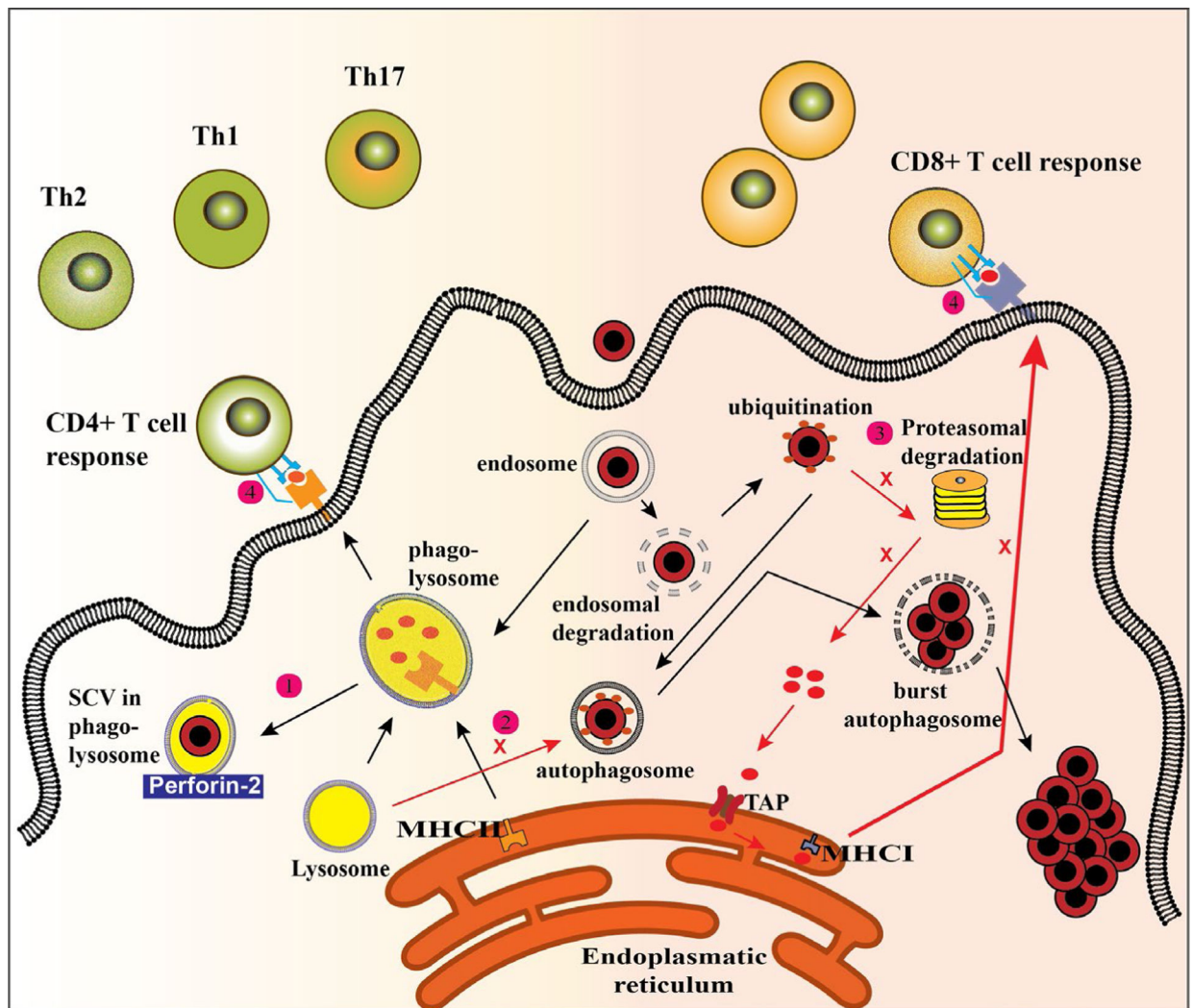
FIGURE 2.
Activation of different T-cell populations through different pathways of antigen presentation. After host cell internalization, *Staphylococcus aureus* generally route through the phagosomal degradation pathway that, under normal circumstances, terminates into a highly degradative organelle with a low pH, the phagolysosome. 1) To circumvent lysosomal degradation, *S. aureus* can adapt to a low pH tolerant small colony variants (SCV)-like phenotype. 2) *Staphylococcus aureus* can also hide in autophagosomes where it prevents fusion with lysosomes preventing degradation and presentation on major histocompatibility complex (MHC) class II molecules. 3) *Staphylococcus aureus* that end up in the cytosol are ubiquitinated but not targeted for proteasomal degradation and subsequent MHC class I presentation escaping a cytotoxic T-cell response. 4) *Staphylococcus aureus* can also manipulate the expression of co-stimulatory molecules preventing an effective T-cell response

FIGURE 3.

Potential antigen-presenting cells (APCs) that are present in the skin. When the skin barrier is disrupted, *Staphylococcus aureus* can colonize the open wound and become pathogenic. Professional APCs, such as macrophages, Langerhans cells, dendritic cells and B cells, can internalize and process *S. aureus* peptide fragments in lysosomes. Generated peptide fragments can be loaded onto major histocompatibility complex (MHC) class II molecules. Non-professional APCs, such as keratinocytes, fibroblasts and endothelial cells, also have the capacity to internalize and process peptide fragments of *S. aureus*. These cells activate their autophagy machinery as their lysosomal degradation capacity is limited. APC rely on Perforin-2–mediated kill of intracellular pathogens