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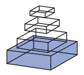

Intracellular *Staphylococcus aureus*: live-in and let die

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Staphylococcus aureus uses a plethora of virulence factors to accommodate a diversity of niches in its human host. Aside from the classical manifestations of *S. aureus*-induced diseases, the pathogen also invades and survives within mammalian host cells. The survival strategies of the pathogen are as diverse as strains or host cell types used. *S. aureus* is able to replicate in the phagosome or freely in the cytoplasm of its host cells. It escapes the phagosome of professional and non-professional phagocytes, subverts autophagy, induces cell death mechanisms such as apoptosis and pyronecrosis, and even can induce anti-apoptotic programs in phagocytes. The focus of this review is to present a guide to recent research outlining the variety of intracellular fates of *S. aureus.*

Keywords: *Staphylococcus aureus*, phagocytosis, phagosomal escape, autophagy, host cell death, bacterial persistence

INTRODUCTION

Most manifestations of *Staphylococcus aureus* disease involve extracellular bacteria (furuncles, carbuncles, impetigo, abscesses, septicemia, necrotizing pneumonia) or biofilm formation (catheter-induced infective endocarditis, atherosclerosis). Aside from this *S. aureus* infections have a second face: there is accu- [illegible] strategy thereby escaping detection by professional phagocytes.

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INTERNALIZATION OF *S. aureus* BY HOST CELLS

Invasion of non-professional phagocytes by *S. aureus* is mediated by a zipper-type mechanism. To date many bacterial adhesins have been identified with Fibronectin (Fn)-binding proteins A and B (FnBPA, FnBPB) constituting the major staphylococcal adhesins for non-professional phagocytes such as epithelial, endothelial cells, fibroblasts, osteoblasts, and keratinocytes (Dziewanowska et al., 1999; Jevon et al., 1999; Lammers et al., 1999; Peacock et al., 1999; Sinha et al., 1999; Fowler et al., 2000; Ahmed et al., 2001; Kintarak et al., 2004; Sinha and Fraunholz, 2010; Edwards et al., 2011; **Figure 1**, Map Item 1). Fibronectin-bridging between FnBPs and $\alpha_5\beta_1$ integrins on the host cell surface is sufficient to induce zipper-type uptake of staphylococci (Sinha et al., 2000). However, FnBPs also have been shown to directly bind to human heat shock protein 60 (Hsp60) exposed on the cellular surface (Dziewanowska et al., 2000). FnBP-independent invasion was observed in *S. aureus* Newman, which produces C-terminally truncated FnBPs that are not covalently anchored to the cell wall of *S. aureus.* It has been shown that strain Newman gets internalized by epithelial cells and fibroblasts mediated by extracellular adherence protein (Eap; Harraghy et al., 2003) with its cellular receptor still not identified to date. Recently, the staphylococcal autolysin (Atl) was identified to function as adhesin/invasin with heat shock cognate protein Hsc70 being the direct cellular receptor (Hirschhausen et al., 2010). Further, wall teichoic acids (WTA) seem to be important for establishment of nasal colonization and there is evidence that a scavenger receptor is involved in WTA binding (Weidenmaier et al., 2004, 2005, 2008). Clumping factor B (ClfB) has been shown to bind to cytokeratins in the extracellular matrix (ECM) of host cells (O'Brien et al., 2002; Wertheim et al., 2008; Haim et al., 2010), and staphylococcal protein A can directly interact with tumor necrosis [illegible] cells is not known thus far.

Since FnBPs contribute to the adherence of *S. aureus* to intact endothelium *in vivo* (Laschke et al., 2005; Kerdudou et al., 2006; Edwards et al., 2010), we can assume that staphylococcal invasion of epithelia or the endothelium is relevant in natural infections. The interaction of FnBP with ECM Fn is mediated by tandem β zipper structures via the binding of multiple fibronectin molecules by the repetitively arranged modules within a single FnBP (Schwarz-Linek et al., 2003; Rudino-Pinera et al., 2004; Bingham et al., 2008). As a result FnBP/Fn sequester $\alpha_5\beta_1$ integrins on the host cell surface. The resulting receptor clustering relays signals that result in cytoskeletal rearrangements (Agerer et al., 2005; Schröder et al., 2006b). The rearrangements initiated at focal adhesions, which are remodeled to fibrillar adhesions by loss of focal adhesion kinase (FAK), paxillin, and vinculin. The rearrangement is accompanied by a centripetal movement of *S. aureus* on the host cell surface that were observed by videomicroscopy (Schröder et al., 2006a). The repeated generation of actin comet tails beneath adherent staphylococci or FnbA-coated beads and the formation of actin cups without internalization of staphylococci is interpreted by the authors as a delay of phagocytosis (Schröder et al., 2006a; **Figure 1**, Map Item 2). Invasion signaling further involves src kinase (Agerer et al., 2003). Extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinase (JNK) but not mitogen-activated protein kinase (MAPK) p38 are required in osteoblasts (Ellington et al., 2001), whereas in HEp-2 cells p38 MAPK was found to be upregulated

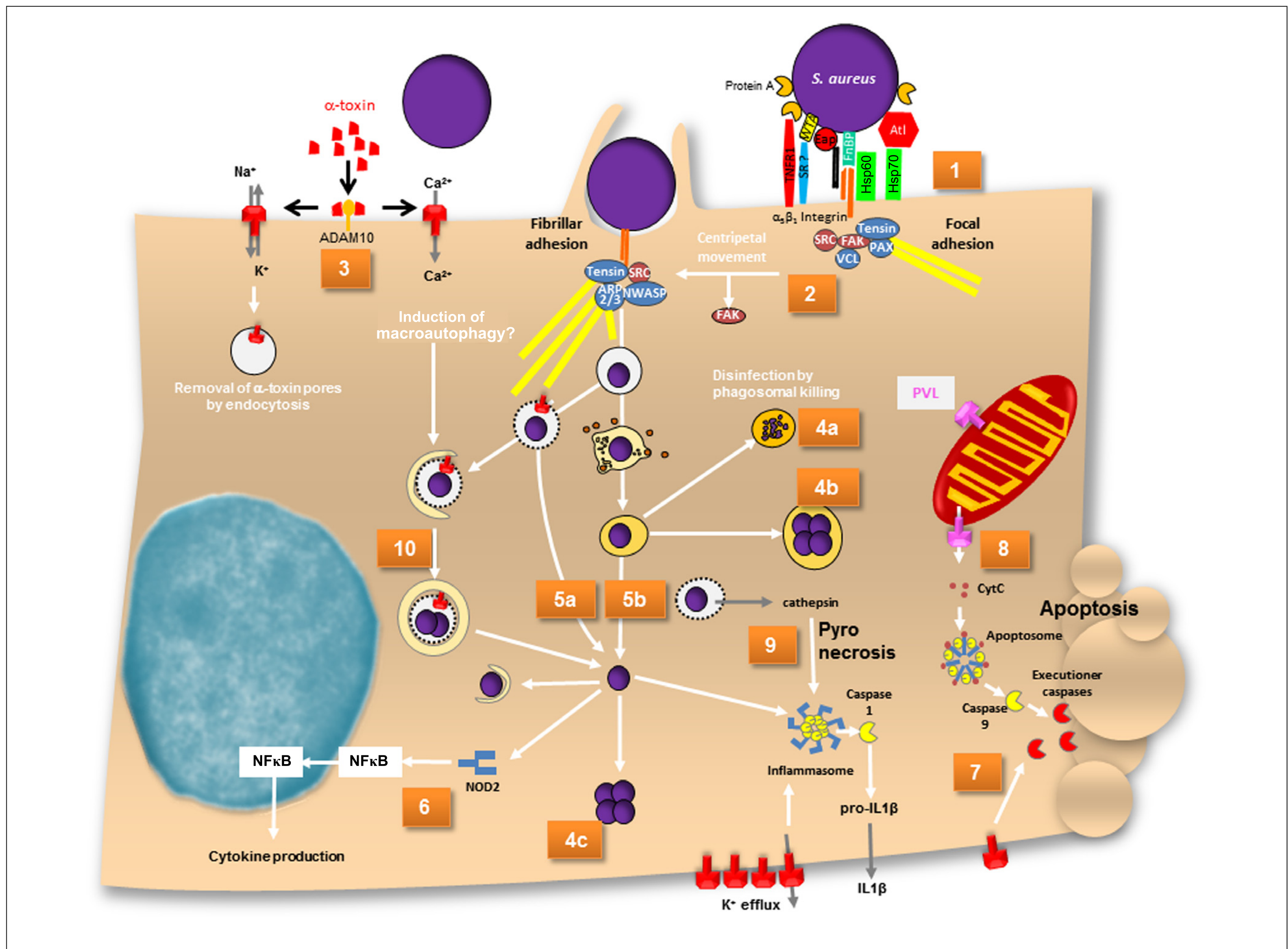

FIGURE 1 | A map of intracellular fates of *S. aureus*. (1) $\alpha_5\beta_1$ integrins are sequestered by FnBP-dependent fibronectin cross-linking at focal adhesions. (2) Centripetal movement and loss of FAK lead to development of fibrillar adhesions, at which phagocytic cups are formed and bacteria are eventually endocytosed. (3) Assembly of α-toxin pores on the plasma membrane of host cells leads is dependent on ADAM10. α-Toxin pores are permeable for cations. Ca^{2+} has been reported to induce macroautophagy. (4a) Bacteria are disinfected by phagolysosomes or (4b) survive and grow within endosomes or (4c) in the cytoplasm after phagosomal escape. (5a) Phagosomal escape can be mediated by α-toxin in cystic fibrosis cells and (5b) also by a combination of phenol-soluble modulins and phospholipases. (6) Cytoplasmic *S. aureus* peptidoglycan is recognized by NOD2, which activates NFκB and results in cytokine production. (7) The mode of cell death induced by *S. aureus* is not completely understood. While caspase-independent cell death exists, α-toxin is capable of inducing extrinsic apoptosis. Upon alpha-toxin induced potassium efflux caspase 2 has recently been shown to lead to mitochondrial outer membrane permeabilization. (8) PVL has been reported to permeabilize mitochondrial outer membrane thereby releasing cytochrome *c* and thus inducing the apoptosome in a Bax-independent pathway of intrinsic apoptosis. Caspase 9 subsequently activates executioner caspases. (9) Cathepsin release from permeabilized phagosomes activates the inflammasome. Activated caspase 1 leads to IL1β maturation and inflammatory pyronecrotic cell death. (10) Toxin-permeabilized endocytic vesicles are targeted by autophagy. During autophagy an isolation membrane engulfs leaky endosomes or cytoplasmically located bacteria. Within these autophagosomes bacterial replicate and eventually escape the organelle ultimately leading to host cell death. ADAM, a metalloprotease and disintegrin; ARP2/3, actin-related protein 2 and 3; Atl, autolysin; CytC, cytochrome *c*; Eap, extracellular adherence protein; FAK, focal adhesion kinase; FnBP, fibronectin-binding protein; HSP, heat shock protein; IL, interleukin; NFκB, nuclear factor κB; NWASP, neural Wiskott–Aldrich syndrome protein; PAX, paxillin; SR, scavenger receptor; VCL, vinculin; WTA, wall teichoic acid.

alongside ERK (Li et al., 2009). Further, phosphorylation of transcription factor c-Jun, but not of Elk-1 or ATF-2 has been demonstrated during invasion of osteoblasts (Ellington et al., 2001). The phosphoinositide-3-kinase (PI3K)-Akt pathway is active during *S. aureus* internalization by bovine endothelial cells (Oviedo-Boyso et al., 2011).

Eventually, *S. aureus* gets endocytosed by professional as well as non-professional cells where the pathogen faces a variety of intracellular fates.

Staphylococcus aureus INTRACELLULAR PERSISTENCE AND GROWTH

The fates of the pathogen and the infected host cell depend on staphylococcal isolate and genotype (Krut et al., 2003) as well as differential susceptibility of host cells to virulence factors, host cell gene expression, etc. For example, *S. aureus* produces different hemolysins. The majority of bovine mastitis strains were phenotypically positive for the sphingomyelinase β-toxin, whereas only a minority of human strains isolated from cases of septicemia

or nasal carriage was positive for β-toxin (Aarestrup et al., 1999). There seems to exist a selective pressure for *S. aureus* strains colonizing humans to acquire β-toxin converting phage (Goerke et al., 2009). This is most likely due to staphylococcal complement inhibitor SCIn and the chemotaxis inhibitor protein CHIPS (and additional factors) that are usually found to be encoded by the respective phage genomes. Purified β-toxin, selectively kills monocytes (Bhakdi et al., 1996) and destroys platelets, but barely affects other cell types (Wadstrom and Mollby, 1972). Similarly, most human cell types are fairly insensitive to the pore-former α-toxin, whereas human leukocytes and cells from other mammalian species are highly susceptible (Bhakdi and Tranum-Jensen, 1991). The metalloprotease ADAM10 has been recently identified as receptor of α-toxin monomers (Wilke and Bubeck Wardenburg, 2010; Inoshima et al., 2011; **Figure 1**, Map Item 3). The differential specificities of α-toxin possibly reflect ADAM10 expression differences in the respective cells or might result from differential capability of host cells to remove α-toxin pores by endocytosis (Husmann et al., 2009).

Staphylococcus aureus survival within host cells was highly dependent on multiplicity of infection (MOI; e.g., Mohammed et al., 2007; Schwartz et al., 2009; Pang et al., 2010) and also the growth phase of the bacteria used for infection (Schwartz et al., 2009). Green-fluorescent protein (GFP)-expressing *S. aureus* displayed bleaching of the fluorescent protein, which indicated degradation of the bacteria in polymorphonuclear neutrophils (PMN; **Figure 1**, Map Item 4a). The loss of fluorescence was not strain-specific and was seen in each of several different strains of *S. aureus*, including nosocomial and community-associated methicillin-resistant strains. When rapidly growing *S. aureus* was used for infection, the bacteria were found to be more susceptible to GFP bleaching (Schwartz et al., 2009). This indicated that these bacteria were cleared more efficiently. Bacterial disinfection was mainly dependent on hypochlorous acid (HOCl; Schwartz et al., 2009). Phagosomal acidification and digestion of *S. aureus* within professional phagocytes is required for MyD88-dependent toll-like receptor (TLR) responses to infection (Abdelzaher et al., 2010).

Not all bacterial cells are disinfected by the phagolysosomes. *S. aureus* has been reported to persist inside phagocytes or endothelial cells for prolonged periods (Hamill et al., 1986; Lowy et al., 1988; Vann and Proctor, 1988; Buisman et al., 1991; Hiemstra et al., 1992; Schröder et al., 2006a; Garzoni et al., 2007; Kubica et al., 2008; Tuchscherr et al., 2011; reviewed in Sendi and Proctor, 2009). Persistence is most often attributed to small colony variants (SCVs) of *S. aureus*. SCVs often present a metabolically quiescent, non-hemolytic, non-pigmented phenotype characterized by reversible auxotrophies in heme biosynthetic pathways or in oxidative phosphorylation (Proctor et al., 1994) as well as a defined transcriptome (Garzoni et al., 2007) and proteome (Kriegeskorte et al., 2011). Also, SCVs are generally found to be mutants in the accessory gene regulator locus (*agr*), thus failing to produce a variety of quorum sensing-controlled virulence factors. SCVs grow slowly and hence are more resistant to a variety of antibiotics (reviewed in Sendi and Proctor, 2009). Further, SCVs display a thick cell wall (Bulger and Bulger, 1967) and an up-regulation of alternative sigma-factor σB (Moisan et al., 2006), which enables *S. aureus* to cope with a variety of environmental stressors (Horsburgh et al., 2002). There is increasing evidence that *S. aureus* can persist *in vivo* in human infections presumably owing to its extreme durability and resistance against a variety of environmental conditions and thus can serve as a potential source for recurrent infection (Proctor et al., 1995; von Eiff et al., 2001; Kipp et al., 2003; Schröder et al., 2006a; reviewed in Garzoni and Kelley, 2009; Sendi and Proctor, 2009). SCVs of *S. aureus* even have been shown to survive and grow within host cell phagosomes (Schröder et al., 2006a; **Figure 1**, Map Item 4b). Also, the complementation of *rsb*U in laboratory strains restored activity of the alternative sigma-factor σB and led to intracellular growth of *S. aureus* within phagolysosomes of THP-1 phagocytes (Olivier et al., 2009). Contrasting these reports, staphylococcal growth has been described after pathogen translocation to the host cell cytoplasm (**Figure 1**, Map Item 4c): *S. aureus* strain Newman is able to escape the phagosome and persists within human monocyte-derived macrophages (hMDM) which resulted in host cell lysis on day 5 after infection (Kubica et al., 2008). The authors postulate that this survival within phagocytes might constitute a route for dissemination of staphylococcal infection. This is further corroborated by the identification of cytoprotective effects on macrophages after phagocytosis of *S. aureus*. Thus, the up-regulation of anti-apoptotic factors upon staphylococcal infection is responsible for extended phagocyte lifetime (Koziel et al., 2009). Both studies suggest that *S. aureus* might penetrate deeper into the tissue and even disseminate to different sites within "Trojan horse" phagocytes (Koziel et al., 2009). Survival within PMN is reported to depend on the accessory regulator Sar1, which was crucial to *S. aureus* survival inside spacious vacuoles, whereas *sar*$^-$ strains were localizing to so-called "tight vacuoles" (Gresham et al., 2000). Such different vacuoles can also be observed in non-professional phagocytes (Sinha and Fraunholz, 2010). The large vacuoles also are reminiscent of spacious *Listeria*-containing phagosomes, which were found to be non-acidified and non-degradative niches in macrophages (Birmingham et al., 2008). However, a more thorough characterization of the different vacuolar locations in intracellular *S. aureus* infections is lacking.

Staphylococcus aureus PHAGOSOMAL ESCAPE

Phagocytosed bacterial pathogens evade lysosomal killing, e.g., by disintegration of the organelle membrane in order to translocate into the host cell cytoplasm. *Listeria monocytogenes* co-opts the pore-forming toxin (PFT) listeriolysin O (LLO) and phospholipases (reviewed in Dramsi and Cossart, 2002; Schnupf and Portnoy, 2007), and Group A streptococci use the PFT streptolysin O (Hakansson et al., 2005). Phagosomal escape of *S. aureus* initially has been described by Bayles et al. (1998) and the *agr*-dependency of this process has been demonstrated (Qazi et al., 2001; Shompole et al., 2003; Jarry and Cheung, 2006; Kubica et al., 2008). The membrane destructive function of staphylococcal α-toxin thus suggested an involvement of the pore-former in phagosomal membrane disruption. The requirement for phagosomal escape and intracellular bacterial survival has been reported in CFT-1, a cystic fibrosis (CF) lung cell line (Jarry and Cheung, 2006) as well as in macrophages (Kubica et al., 2008; **Figure 1**, Map Item 5a). In CFT-1, *S. aureus* translocates into the cytoplasm in an α-toxin-dependent manner and the bacteria replicate within the

cytoplasm (Kahl et al., 2000; Jarry et al., 2008). However, in the LCSFN cell line complemented with the wild-type CF transmembrane conductance regulator, CFTR, α-toxin has no effect (Jarry and Cheung, 2006). Further, it has been demonstrated that neither α-toxin overexpressing strains (Lám et al., 2010) nor laboratory strains inducibly expressing α-toxin (Giese et al., 2009, 2011) are capable of releasing staphylococci into the host cell cytoplasm. By expression of the amphiphilic 26 amino-acid (AA) peptide δ-toxin in the non-cytotoxic laboratory strain *S. aureus* RN4220 *S. aureus* was capable of escape in rates similar to that of heterologously expressed LLO (Giese et al., 2011). δ-Toxin is encoded by the agr-effector RNAIII and is translated about 1 h after transcription of RNAIII (Balaban and Novick, 1995). It is capable of lysing bacterial protoplasts, lysosomes, lipid spherules, mitochondria, and erythrocytes *in vitro* in a temperature-independent manner. Its activity and mode of action is comparable to that of non-ionic detergents (Kreger and Bernheimer, 1971; Kreger et al., 1971; Rahal Jr., 1972; Kapral, 1976; reviewed in Verdon et al., 2009). δ-Toxin is encoded by RNAIII, the *agr* effector, and thus might constitute an immediate response to space limitation by phagosomal engulfment (**Figure 1**, Map Item 5b). However, membrane disruption by δ-toxin depended on the presence of the staphylococcal sphingomyelinase, β-toxin (Giese et al., 2011), which cleaves sphingomyelins (SM) to phosphorylcholine and ceramide moieties. δ-Toxin hardly binds to negatively charged phospholipids, binds strongly to liquid-disordered domains and poorly to cholesterol and sphingomyelin liquid-ordered raft domains (Pokorny et al., 2006). In one model, β-toxin thus may cleave SM to ceramides, which tend to accumulate in membrane microdomains. The hydrophobic nature of ceramide-rich domains thus might constitute regions of δ-toxin assembly, which eventually lead to target membrane permeabilization (**Figure 2**). *S. aureus* strain USA300 LAC, however, is escape proficient yet does not encode a functional β-toxin due to lysogeny of a β-converting phage (Diep et al., 2006). We thus have to hypothesize alternative factors that can act in phagosomal escape, such as a variety of lipases encoded by the staphylococcal genome or phenol-soluble modulins (PSMs; see below). Alternatively, prophages might be lost, e.g., during exposure to phagosomal reactive oxygen species, and then might contribute to phagosomal escape. A similar activation mechanism is used by *Streptococcus pneumoniae*, which produces hydrogen peroxide and thereby lyses *S. aureus* by a "remote control" prophage activation (Selva et al., 2009).

For complete hemolysis of erythrocytes in sheep blood agar plates either a shift to 4°C (hot–cold hemolysis), osmotic stress, or synergistic toxins are necessary. δ-Toxin belong to the class of PSMs, which initially had been shown to be present within a hot-phenol extraction of *S. epidermidis* extracts (Otto et al., 2004) and only later had been identified in *S. aureus* by sequence homology (Wang et al., 2007). The major groups of PSMs are divided into two operons referred to as PSMα and PSMβ. The PSMα operon is comprised of four open reading frames (ORFs) with approximately 20 AA. PSMβ encodes two ORFs, which are about 40 AA in length (Wang et al., 2007). The expression of PSMβ has

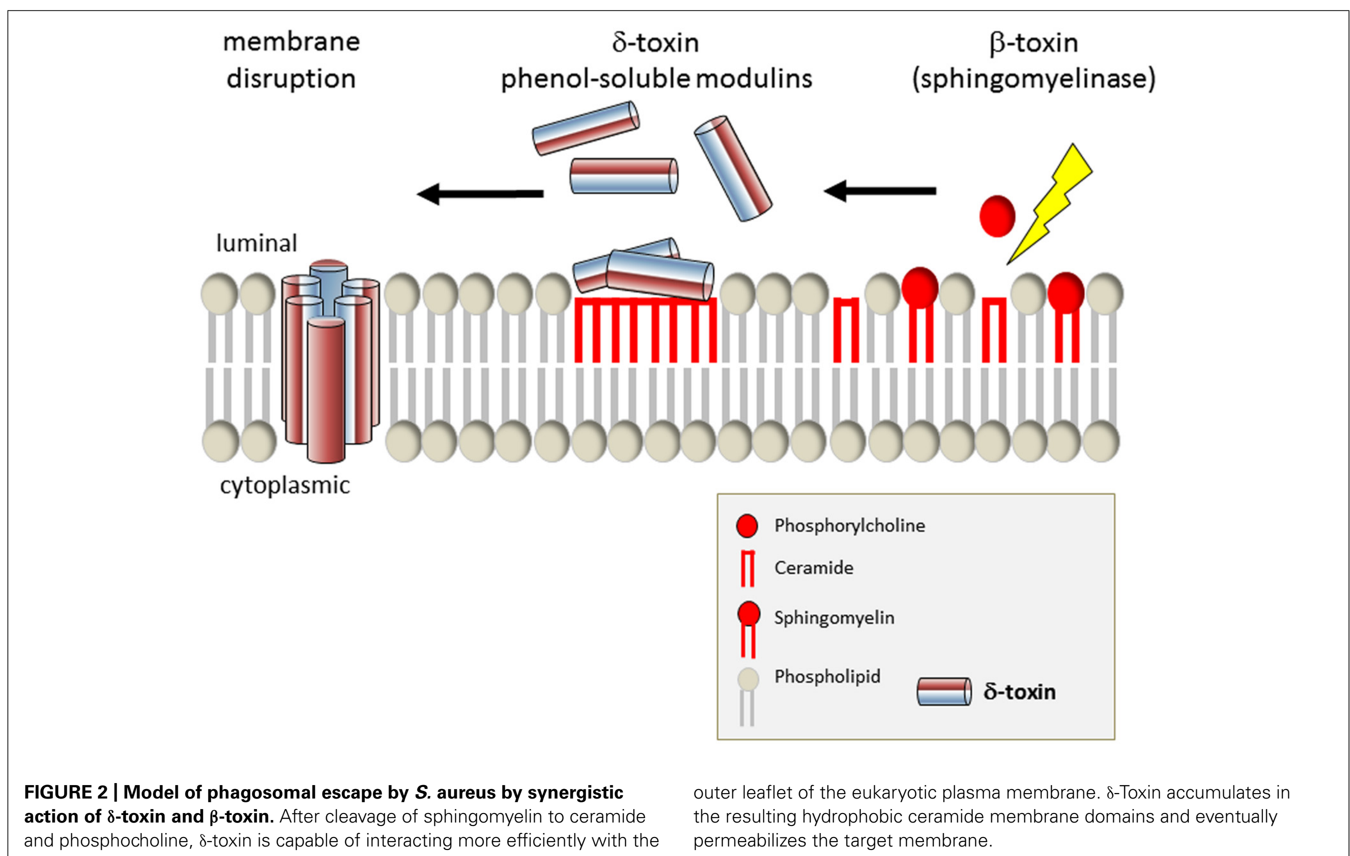

FIGURE 2 | Model of phagosomal escape by *S.* aureus by synergistic action of δ-toxin and β-toxin. After cleavage of sphingomyelin to ceramide and phosphocholine, δ-toxin is capable of interacting more efficiently with the outer leaflet of the eukaryotic plasma membrane. δ-Toxin accumulates in the resulting hydrophobic ceramide membrane domains and eventually permeabilizes the target membrane.

been demonstrated to result in phagosomal escape in a gain-of-function study (Giese et al., 2011). Just like δ-toxin, PSMα and PSMβ are *agr*-dependently expressed. It recently was shown that the staphylococcal *agr* system is confinement induced and thus comprises not only a quorum sensing system but also a diffusion sensing system active at the single cell level (Carnes et al., 2009). δ-Toxin and PSMs are hence produced upon phagosomal confinement and thus also low numbers of endocytosed staphylococci should be able to mount a toxin response in order to avoid lysosomal killing. Whereas gain of function assays demonstrated PSMβ activity in phagosomal escape (Giese et al., 2011), *in vivo* studies suggests a prominent role of PSMα, with the third ORF of the operon, PSMα3, being the most virulent principle (Wang et al., 2007). Despite these incongruities a common theme emerges: amphiphilic PSMs are involved in phagosomal escape. One possibility to explain the observed differences is the postulation of alternative pathways of phagosomal escape mechanisms for *S. aureus*.

Molecular patterns of pathogens that reside in the cytoplasm of their hosts are detected by nucleotide-binding and oligomerization domain proteins NOD1 and NOD2, which detect γ-D-glutamyl-diaminopimelic acid and muramyl dipeptide, peptidoglycan components of Gram-negative and Gram-positive bacteria. Peptidoglycan binding leads to a conformation change in NOD proteins, which initiates the recruitment of ubiquitin ligases and kinases and ultimately results in nuclear translocation of NFκB and activator protein 1 and expression of inflammatory genes (reviewed in Strober et al., 2006). NOD2 signaling upon *S. aureus* infection has been found to induce cytokine production (Kapetanovic et al., 2007) and thus might contribute to induction of inflammation, e.g., in the lung (Gomez and Prince, 2008; **Figure 1**; Map Item 6). NOD2-deficient mice exhibit a delayed inflammatory response and impaired bacterial clearance after infection with *S. aureus* (Hruz et al., 2009). α-Toxin facilitates NOD2-dependent recognition of *S. aureus* muramyl dipeptide (Hruz et al., 2009), possibly by interfering with phagosomal integrity.

The observation that *S. aureus* can translocate into the cytoplasm of host cells and grow without an immediately ensuing cell death (e.g., Kubica et al., 2008) illustrates that phagosomal escape is not identical with cytotoxicity. Thus, the link between phagosomal escape and host cell death still needs to be elucidated.

Staphylococcus aureus-INDUCED HOST CELL DEATH

The classical separation of host cell death into programed cell death (PCD; apoptosis) and accidental cell death or necrosis has long been superseded after the identifying a large variety of cell death mechanisms (for a reviews, see Taylor et al., 2008; Ting et al., 2008a; Bergsbaken et al., 2009). Many of which are defined by key factors that allow the assessment of death pathway activation following bacterial infection (e.g., reviewed in Rudel et al., 2010), although the synchronous activity of virulence factors from the staphylococcal arsenal renders unequivocal correlations between toxin and associated mode of cell death a daunting task. When virulent *S. aureus* strains are added to host cells in tissue culture, host cell death occurs via mechanisms that have been mainly identified as apoptotic (Bayles et al., 1998; Menzies and Kourteva, 1998, 2000; Wesson et al., 1998, 2000; Kahl et al., 2000; Nuzzo et al., 2000; Tucker et al., 2000; Haslinger et al., 2003; Genestier et al., 2005; **Figure 1**, Map Item 7). α-Toxin is both required and sufficient for induction of leukocyte cell death, either apoptotic or necrotic (Bantel et al., 2001; Essmann et al., 2003; Haslinger et al., 2003). Leukocytes are sensitive to staphylococcal α-toxin. Already low toxin doses induce apoptosis accompanied by a breakdown of the mitochondrial transmembrane potential (Bantel et al., 2001; Haslinger et al., 2003). A recent study suggests that caspase-2 acts as an initiator caspase during cell death of non-professional phagocytes. Caspase 2 was induced by potassium efflux due to pore-forming toxins such as staphylococcal alpha-toxin and aerolysin (Imre et al., 2012). By contrast, high doses induce necrotic cell death (Walev et al., 1993; Bantel et al., 2001; Essmann et al., 2003; Haslinger et al., 2003). Human endothelial cells are virtually insensitive to the action of *S. aureus* α-toxin, however, comparatively low numbers of *S. aureus* cells with a combined invasive and strongly hemolytic phenotype readily induce apoptotic cell death in HUVEC (Haslinger-Löffler et al., 2005). This suggests that cell death mechanisms are activated from within their intracellular location. The effect is highly specific, since fixed, non-hemolytic, rifampin-treated or weakly invasive staphylococci are not cytotoxic toward endothelial cells (Haslinger-Löffler et al., 2005). Multiple other studies indicate that *S. aureus* might kill its other types of host cells from within (Bayles et al., 1998; Menzies and Kourteva, 1998; Wesson et al., 1998; Nuzzo et al., 2000; Tucker et al., 2000; Krut et al., 2003; Haslinger-Löffler et al., 2005; Chatterjee et al., 2008; Jarry et al., 2008; Kubica et al., 2008; Lám et al., 2010). The virulence factors required for *S. aureus*-induced apoptosis in endothelial cells depend on *agr* and the alternative stress–response sigma-factor σB (Wesson et al., 1998; Qazi et al., 2001; Shompole et al., 2003; Jarry and Cheung, 2006; Kubica et al., 2008), but mainly seem to be independent of SarA (Haslinger-Löffler et al., 2005; Jarry et al., 2008) – although there are contradicting results on the involvement of SarA (Wesson et al., 1998).

Phage-encoded Panton-Valentine leukocidin (PVL) predominantly destroys leukocytes, although there also is some species specificity for human and rabbit PMN (Loffler et al., 2010). In PMN, PVL induced a rapid caspase-9/3-dependent cell death *in vitro* (Genestier et al., 2005). The authors further identified to a mitochondrial localization of the PVL toxin. Isolated mitochondria were permeabilized for pro-apoptotic factors such as cytochrome *c* (CytC) by PVL which suggested that PVL is able to create pores in the mitochondrial outer membrane and thus triggers a Bax-independent mitochondrial pathway of host cell apoptosis (Genestier et al., 2005; **Figure 1**, Map Item 8). During the intrinsic pathway of apoptosis release of CytC from injured mitochondria leads to activation of apoptosis-activating factor-1 (APAF-1). Oligomerizing CytC/APAF-1 recruits and subsequently activates pro-caspase 9. Caspase 9 then proteolytically activates effector caspases, which finally cleave their respective substrates resulting in membrane blebbing, and DNA fragmentation (reviewed in Rudel et al., 2010). Similarly, α-toxin has been described to activate caspases via the intrinsic death pathway (Bantel et al., 2001; Haslinger et al., 2003) independently of death receptor signaling (CD95/Fas/APO-1). Bcl-2 overexpressing Jurkat cells were protected from α-toxin mediated cell death (Bantel et al.,

2001; Essmann et al., 2003) although recent result suggest that the function of Bcl-2 in autophagy might be responsible for the observed phenomena (Schnaith et al., 2007; Mestre et al., 2010).

Interestingly, *S. aureus* also seems to be able to exert anti-apoptotic host cell responses. The pathogen was shown to suppress staurosporine-induced apoptosis in hMDM although early apoptotic features such as phosphatidylserine display at the outer plasma membrane leaflet, reduced mitochondrial membrane potential, CytC release, and caspase-3 activation are still observed (Koziel et al., 2009). *S. aureus* infection strongly upregulated the expression of mitochondrial membrane potential stabilizing Bcl-2 and Mcl-1 gene products. Since also heat-killed *S. aureus* was able to suppress apoptosis in the host cells staphylococcal products such as lipoteichoic acid or peptidoglycan might activate the macrophages through intracellular pattern recognition sensors such as nucleotide oligomerization domain receptors (NOD; Kapetanovic et al., 2007; Koziel et al., 2009).

Aside from apoptosis *S. aureus* is also able to induce pyronecrosis (**Figure 1**; Map Item 9). There, caspase-1 is activated as part of an inflammasome, which further consists of NOD-like receptor protein 3 (NLRP3) and the adaptor protein, apoptosis-associated speck-like protein containing a caspase-associated recruitment domain (ASC). *S. aureus* can function as a stimulus for NLRP3 (Munoz-Planillo et al., 2009), however, the molecular identity of the stimulating signal is not known thus far (Mariathasan et al., 2006; Ting et al., 2008b; Wright and Nair, 2010). Lysosomal permeabilization is one NLRP3-activating principle, wherein release of the lysosomal protease cathepsin B into the cytoplasm contributes to NLRP3 activation (reviewed in Willingham and Ting, 2008; Bergsbaken et al., 2009). Thus, it seems likely, that lysosomal rupture or permeabilization by *S. aureus* toxins releases cathepsin which then activates the inflammasome. Indeed staphylococcal α, β, and γ-hemolysins have been shown to be important activation of the NLRP3 inflammasome (Craven et al., 2009; Munoz-Planillo et al., 2009; Kebaier et al., 2012). The pore-forming α- and γ-toxins of *S. aureus* permeabilize membranes and thus might be involved in cathepsin release and subsequent inflammasome activation. α-Toxin further is known to permeabilize the plasma membrane for potassium ions. In turn, potassium efflux activates the inflammasome (Petrilli et al., 2007). β-Toxin has been shown to be involved in phagosomal escape (Giese et al., 2011) and thus also might act in cathepsin release.

Staphylococcus aureus AND THE SUBVERSION OF AUTOPHAGY

Autophagy sequesters cytoplasmic contents via an isolation membrane. Engulfment of cargo by the so-called phagophore forms double membrane-bound autophagic vesicles that eventually fuse with lysosomes to yield autolysosomes. Autophagy serves for degradation of organelles or self-digestion during nutrient limiting conditions such as starvation and is generally thought to constitute a cellular survival mechanism. During bacterial infections autophagy disposes of leaky vesicles or intracellular bacteria, however, bacterial pathogens have found multiple ways to subvert autophagy (reviewed in Dorn et al., 2002; Kirkegaard et al., 2004; Levine, 2005; Campoy and Colombo, 2009; Orvedahl and Levine, 2009; Ogawa et al., 2011).

Staphylococcus aureus was shown to interact with autophagosomes in a rather unique way (Schnaith et al., 2007) when compared to autophagy-subvertive strategies of other bacterial pathogens (Campoy and Colombo, 2009; Ogawa et al., 2011). *S. aureus* inhibits fusion of phagosomes with lysosomes. It permeabilizes HeLa phagosomes by a mechanism dependent on *Staphylococcus*-secreted toxins. The leaky phagosomes are targeted by autophagy and within autophagosomes *S. aureus* replicates. Eventually the bacteria escape from their intracellular confinement into the host cell cytoplasm in an *agr*-dependent manner (Schnaith et al., 2007; **Figure 1**, Map Item 9). Finally, host cell death is induced, which is independent of a caspase activation cascade but was blocked by overexpression of anti-autophagic Bcl-2. Induction of autophagy by rapamycin resulted in an increased number of recovered colony-forming units, whereas inhibition with wortmann in reduced the colonies recovered from the intracellular environment. *S. aureus*-induced autophagy resulted in a vacuolization of the host cell cytoplasm ("Swiss cheese phenotype"). *agr*-deficient *S. aureus* fail to induce autophagy, which results in maturation of bacteria-containing phagosomes followed by lysosomal degradation of the pathogens. α-Toxin is able to permeabilize membranes for Ca^{2+}, an inducer of autophagy (Brady et al., 2007) and autophagy targets phagosomes perforated by α-toxin in Chinese hamster ovary cells (Mestre et al., 2010). Whereas latter observation is in line with the results obtained by Schnaith et al. (2007) it contrasts findings that α-toxin is not sufficient to permeabilize HeLa phagosomes (Jarry and Cheung, 2006; Giese et al., 2009; Lám et al., 2010).

CONCLUSION

With about 200–300 virulence factors, *S. aureus* is able to exert a multitude of effects upon its eukaryotic host cells. Although many details have emerged through ground-breaking and recent research, only a minority of pathogenicity factors of *S. aureus* has been functionally annotated to date. Particularly the assessment of intracellular staphylococcal virulence is often hampered by the difficulty to discriminate between toxin effects that result from extracellular or intracellular bacteria, although inducible toxin-expressing might provide valuable tools for molecular dissection of host–pathogen interactions.

With our advancing knowledge of cell death mechanisms ground-breaking experiments will have to be re-evaluated in order to understand the mechanisms of *S. aureus*-induced host cell killing. When comparing experimental research originating from different labs the influence of a body of factors should be taken into account that could lead to different infection outcomes:

Staphylococcus aureus strain used for infection, its growth phase at the time of infection (and hence the bacterial growth medium), as well as MOI are important, whereas on the host side the cell type and hence the protein profile will drastically influence infection outcome by altering host cell susceptibility to bacterial toxins, response to pathogen-associated molecular patterns, expression of receptors, caspases, and other host factors.

In order to identify activities of single toxins or virulence factors gain-of-function studies can be useful, e.g., using the a pathogenic *S. carnosus* as toxin delivery vehicle. However, such strategies

will not easily identify pathways during which an orchestrated interplay of multiple virulence factors is required. However, novel high-throughput sequencing technologies of transposon insertions (Gawronski et al., 2009; van Opijnen et al., 2009) open up new possibilities for analysis of genome-wide mutant libraries of clinically relevant strains. Using such novel tools we will be able to address a lot of open questions with regard to intracellular staphylococcal infections: do different adhesins result in employment of different uptake mechanisms into non-professional phagocytes as these would subsequently result in different infection outcomes? What is the nature of tight and spacious vacuoles (Gresham et al., 2000) that are occupied by, for example, *sarA*− and *sar*A+ *S. aureus*, respectively? Which alternative pathways for phagosomal escape do exist? Which modes of cell death are activated by a single strain in different host cell types or different strains in a single host cell line? There is still a lot to learn about the versatile facultative intracellular pathogen, *Staphylococcus aureus*.

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