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The *Staphylococcus aureus* polysaccharide capsule and Efb-dependent fibrinogen shield act in concert to protect against phagocytosis

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Staphylococcus aureus has developed many mechanisms to escape from human immune responses. To resist phagocytic clearance, *S. aureus* expresses a polysaccharide capsule, which effectively masks the bacterial surface and surface-associated proteins, such as opsonins, from recognition by phagocytic cells. Additionally, secretion of the extracellular fibrinogen binding protein (Efb) potently blocks phagocytic uptake of the pathogen. Efb creates a fibrinogen shield surrounding the bacteria by simultaneously binding complement C3b and fibrinogen at the bacterial surface. By means of neutrophil phagocytosis assays with fluorescently labelled encapsulated serotype 5 (CP5) and serotype 8 (CP8) strains we compare the immune-modulating function of these shielding mechanisms. The data indicate that, in highly encapsulated *S. aureus* strains, the polysaccharide capsule is able to prevent phagocytic uptake at plasma concentrations <10 %, but loses its protective ability at higher concentrations of plasma. Interestingly, Efb shows a strong inhibitory effect on both capsule-negative and encapsulated strains at all tested plasma concentrations. Furthermore, the results suggest that both shielding mechanisms can exist simultaneously and collaborate to provide optimal protection against phagocytosis at a broad range of plasma concentrations. As opsonizing antibodies will be shielded from recognition by either mechanism, incorporating both capsular polysaccharides and Efb in future vaccines could be of great importance.

INTRODUCTION

Staphylococcus aureus is a major human pathogen responsible for many community- and hospital-acquired infections. Disease conditions may range from mild wound infections to more severe invasive illnesses such as endocarditis and bacteraemia (Lowy, 1998; Tong *et al.*, 2015).

The innate immune system is of high significance for the clearance of invading pathogens such as *S. aureus* (van Kessel *et al.*, 2014). Neutrophils, the predominant phagocytic cells of the innate immune system, rapidly engulf bacteria via phagocytosis and kill them intracellularly. Neutrophils recognize bacteria via specific receptors that are directed against bacterium-bound opsonins such as antibodies and complement components. The complement system is a complex proteolytic cascade of human plasma proteins that recognize surface-associated antibodies and specific bacterial surface structures (Gros *et al.*, 2008; Ricklin *et al.*, 2010). Activation of the cascade will result in deposition of several complement proteins at the bacterial surface. Complement component C3b is the major opsonin responsible for phagocytosis of bacteria by neutrophils and other phagocytic cells. Additionally, interaction of the Fc domain of bacterium-bound antibodies (IgG) with Fc receptors on the neutrophil contributes to effective phagocytosis.

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Abbreviations: CP5, capsular polysaccharide 5; CP8, capsular polysaccharide 8; Efb, extracellular fibrinogen binding protein.

One supplementary figure is available with the online Supplementary Material.

To resist phagocytic clearance, *S. aureus* has evolved various immuno-modulatory mechanisms that frustrate the process of phagocytosis (Foster, 2005; Itoh *et al.*, 2010; Foster *et al.*, 2013; Stemerding *et al.*, 2013; Kang *et al.*, 2013). For instance, *S. aureus* produces several proteins that modulate binding of IgG to the bacterial surface (protein A and Sbi) or inhibit recognition of surface-bound IgG by Fc receptors (FLIPr). Also, *S. aureus* secretes multiple proteins that block activation of complement (e.g. SCIN, Ecb, Efb, Cna, SSL10). Furthermore, *S. aureus* has developed several ways to shield its surface from recognition by the host immune system. The first shielding mechanism is represented by the formation of a capsule, a polysaccharide structure surrounding the bacterial cell wall (O'Riordan & Lee, 2004). The two main serotypes produced by clinical *S. aureus* strains are the serotype consisting of capsular polysaccharide 5 (CP5) and capsular polysaccharide 8 (CP8), accounting for ~75 % of all clinical isolates, of which CP8 strains are the most prevalent (Sompolinsky *et al.*, 1985; Hochkeppel *et al.*, 1987; Albus *et al.*, 1988; Lee *et al.*, 1990). These capsules comprise trisaccharide repeating units of N-acetyl mannosaminuronic acid, *N*-acetyl L-fucosamine and *N*-acetyl D-fucosamine and are identical except for the glycosidic linkages between the sugars and the sites of *O*-acetylation (Jones, 2005). The CP5 and CP8 strains form non-mucoid colonies that are indistinguishable from colonies formed by unencapsulated strains. CP5 and CP8 are not only found among clinical isolates but are also expressed by commensal strains (Sompolinsky *et al.*, 1985; Albus *et al.*, 1988). The expression of CP5 or CP8 has been shown to enhance virulence and survival of *S. aureus in vivo* (Thakker *et al.*, 1998; Nilsson *et al.*, 1997; Watts *et al.*, 2005). Next to inhibition of phagocytic uptake, CP5 expression has been described to provide protection against intracellular killing of the bacterium (Nilsson *et al.*, 1997). However, *S. aureus* capsule expression (and therefore capsule size) is highly variable and depends on the presence or absence of certain environmental factors, such as CO_2 (Herbert *et al.*, 2001). Therefore, capsule density and thus inhibition of phagocytosis are subject to the location of the bacterium in the body.

As a second shielding mechanism against phagocytosis, *S. aureus* secretes a protein that links specific plasma proteins to its surface. This extracellular fibrinogen binding protein (Efb) is a 16 kDa protein that binds to complement C3b on bacteria and simultaneously attracts fibrinogen to the surface. In doing so, Efb covers bacteria with a thick layer of fibrinogen that potently prevents recognition of surface-associated antibodies and C3b by phagocytic cells (Ko *et al.*, 2013).

Currently, it is not well understood why *S. aureus* evolved two separate mechanisms for shielding its surface from phagocytosis. In this study we further analyse the anti-phagocytic properties of both the capsule and Efb. Our findings indicate that these two shielding mechanisms can work in concert to enhance the resistance of *S. aureus* against phagocytosis.

METHODS

Bacterial strains and fluorescence labelling. In this study we used various wild-type *S. aureus* strains expressing different capsular polysaccharides: wild-type CP5-expressing strains include strain Reynolds (Jean Lee, Boston, MA, USA), COL (Andreas Peschel, Tübingen, Germany), USA100 (Jean Lee) and Newman (Jean Lee); wild-type CP8-expressing strains include Sanger 252 (Tim Foster, Dublin, Ireland), Becker and MN8 (Jean Lee). Capsule-negative strains included are USA300 (Frank Deleo, NIAID, Hamilton, MT, USA), 8325–4 (Tim Foster) and Wood 46 (ATCC-10832). 'Isogenic capsule-negative mutants of strains Reynolds, Newman and MN 8 were created by deletion of the *cap5* or *cap8* as described (Watts *et al.*, 2005; Pohlmann-Dietze *et al.*, 2000). The CP8-negative mutant of strain Becker was created via transposon mutagenesis using Tn551.' CP8-expressing strain Reynolds was generated by substitution of the *cap5* region with the *cap8* region (Watts *et al.*, 2005). Capsular serotypes were verified by flow cytometry analyses using specific CP5 and CP8 antisera (see below). Strains were fluorescently labelled by transformation with the pCM29 plasmid, constitutively expressing either GFP or mCherry under regulation of the sarA promoter as previously described (Pang *et al.*, 2010; Schenk & Laddaga, 1992). Alternatively, strains were fluorescently labelled with FITC (Sigma). To this end, bacteria were grown on Columbia agar (Oxoid) supplemented with 2 % (w/v) NaCl (CSA) for 24 h at 37 °C, suspended, washed and resuspended in PBS. FITC (0.5 mg ml^{-1} in DMSO) was added and incubated for 30 min on ice. Bacteria were washed twice and resuspended in RPMI containing 0.05 % human serum albumin (RPMI-HSA). All strains were grown on CSA for 24 h at 37 °C to guarantee optimal capsule expression (Thakker *et al.*, 1998; Pohlmann-Dietze *et al.*, 2000) and stored at −20 °C in RPMI-HSA before use.

Protein purification. Recombinant Efb proteins were generated in *E. coli* as described previously (Ko *et al.*, 2011). Briefly, the *efb* gene from *S. aureus* strain Newman (Mal Horsburgh, Liverpool, UK) (without the signal peptide) was amplified by PCR and ligated into the pGEX-5x-1 vector (GE Healthcare) for N-terminal fusion with glutathione *S*-transferase (GST). Mutations of the fibrinogen and C3 binding domains were introduced in pGEX plasmids containing full-length GST-Efb as described previously (Ko *et al.*, 2011, 2013). The mutant EfbΔFg lacks both fibrinogen binding domains and was previously described as EfbΔFg1+2 (Ko *et al.*, 2013). EfbΔC3 has been altered in the C3d binding site and therefore lacks C3-binding ability. Recombinant proteins were expressed and purified according to the manufacturer's manual (GE Healthcare).

Purification of human plasma and neutrophils. To prepare plasma, blood was collected in 3 ml blood tubes (Roche) containing recombinant hirudin (15 μg ml^{-1}) from four healthy volunteers. After centrifugation for 10 min at 2080 ***g*** plasma was collected, pooled and stored at −80 °C. For isolation of human neutrophils, blood from a healthy donor was collected in heparin vacutainers (BD) and cells were isolated using the Ficoll-Histopaque gradient method (Bestebroer *et al.*, 2007).

Capsule visualization with transmission electron microscopy. GFP-labelled *S. aureus* strains Reynolds (CP5) and its isogenic CP-negative mutant (5×10^7 ml^{-1}) were incubated with rabbit CP5 antiserum (Watts *et al.*, 2005, 1 : 100) in PBS-0.5 % BSA for 45 min at 4 °C and washed twice with PBS-0.5 % BSA. Subsequently, bacteria were adsorbed to 100 mesh hexagonal Formvar-carbon-coated copper grids (Stork-Veco). Samples were contrasted with 0.4 % uranyl acetate (pH 4.0) and 1.8 % methylcellulose (Slot & Geuze, 2007) and analysed in a Tecnai 12 transmission electron microscope (FEI) at 80 kV.

Capsule quantification by flow cytometry. GFP- or FITC-labelled *S. aureus* strains (5×10^7 ml^{-1}) were incubated with rabbit CP5 and CP8 antiserum (Watts *et al.*, 2005, 1 : 100) in PBS-0.5 % BSA for 45 min at 4 °C and washed twice with PBS-0.5 % BSA. Bacteria were incubated

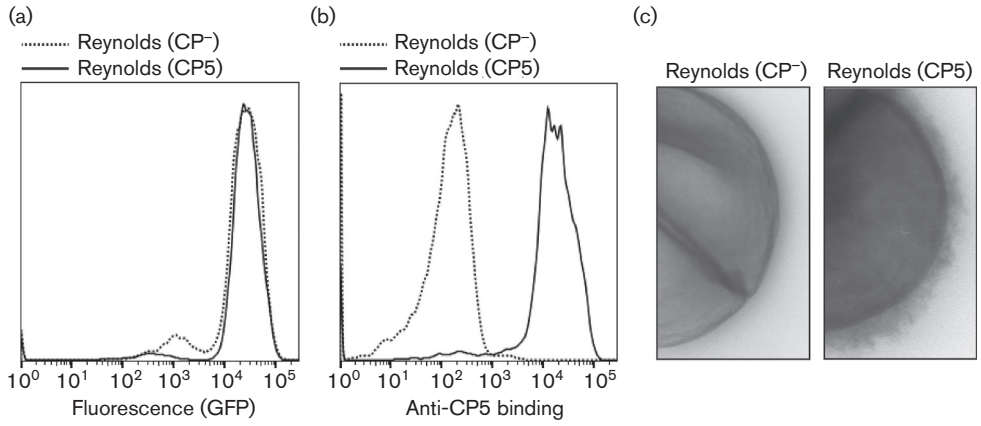

Fig. 1. (a) Flow cytometry histogram showing the fluorescence of *S. aureus* strain Reynolds (CP5) and its isogenic capsule-negative mutant (CP⁻) after transformation with pCM29-GFP plasmid. (b) Flow cytometry histogram showing binding of rabbit anti-CP5 antibodies to GFP-labelled Reynolds (CP5) and isogenic mutant (CP⁻), detected with Alexa647-conjugated protein A. (c) Transmission electron micrographs showing the GFP-labelled Reynolds (CP5) and mutant (CP⁻) strain. Strains were pretreated with anti-CP5 antibodies to enhance stability and electron density of the capsule. Representative images are shown.

with Alexa647-conjugated Protein A (1 : 1000, Molecular Probes) and, after another washing step, fixed with formaldehyde (1 %) before flow cytometry measurement with a FACS Verse device (BD).

Phagocytosis assays. All phagocytosis assays were performed in Falcon tubes (Corning). Freshly isolated human neutrophils (5×10^6 ml^{-1}) were stained with Vybrant DiD cell-labelling solution (1 : 1000, Molecular Probes), and washed three times with and resuspended in RPMI-HSA before use. GFP- or FITC-labelled *S. aureus* (5×10^7 ml^{-1}) were pre-incubated with human plasma in the presence or absence of Efb (0.5 μM) for 2 min at 37 °C. DiD-stained neutrophils (5×10^6 ml^{-1}) were added and phagocytosis was allowed for 15 min at 37 °C, with shaking (600 r.p.m.). Cold formaldehyde (1 %) in RPMI-HSA was added to stop the reaction and samples were analysed by flow cytometry measurement of the fluorescence of the neutrophils.

Confocal microscopy. *S. aureus* strains Reynolds (CP5 and CP⁻) (mCherry-labelled, 1×10^8 ml^{-1}) were pre-incubated with human plasma (3 %) for 30 min at 37 °C in Veronal buffer containing 5 mM $CaCl_2$ and 2.5 mM $MgCl_2$ (VBS^{++}) to deposit C3b on the bacterial surface. After a washing step with VBS^{++}–0.5 % BSA, bacteria were incubated with Efb or Efb mutants (0.5 μM) for 1 h at 37 °C, with shaking (600 r.p.m.). Following another washing step, a 1 h incubation with Alexa-488-conjugated fibrinogen (60 μg ml^{-1}, Invitrogen) at 37 °C shaking was performed, after which bacteria were fixed with formaldehyde (1 %). For visualization by confocal microscopy, samples were transferred onto poly-L-lysine-coated cover slips (0.45 μm; 12 mm diameter; Becton Dickinson) or, as a control, samples were analysed by flow cytometry. Confocal images were acquired using a Leica TCS SP5 inverted microscope equipped with HCX PL APO CS 63×/1.40–0.60 OIL objective (Leica Microsystems).

RESULTS

Fluorescence labelling and capsule expression of *S. aureus* strains

To compare the anti-phagocytic effects of the staphylococcal polysaccharide capsule with the Efb shield, we first performed *in vitro* neutrophil phagocytosis assays with different encapsulated *S. aureus* strains that show varying degrees of capsule expression (Fig. S1, available in the online Suplementary Material). Since the Reynolds strain expresses a thick CP5 capsule (Fig. S1; Thakker *et al.*, 1998; Watts *et al.*, 2005), we initially focused on this strain in our phagocytosis experiments and used its isogenic mutant (Reynolds CP⁻) as a capsule-negative strain. Both strains were fluorescently labelled by transformation with a pCM29-GFP plasmid that allows for intracellular production of GFP under a constitutive promoter (Pang *et al.*, 2010). Strains were grown on CSA for 24 h at 37 °C to guarantee that capsule expression was optimal (Thakker *et al.*, 1998). The fluorescence of the strains was measured by flow cytometry (Fig. 1a), which confirmed that GFP was properly expressed and that both strains were equally fluorescent. Furthermore, we confirmed expression of the polysaccharide capsule after fluorescence labelling by specific staining with a polyclonal antibody directed against CP5 (Fig. 1b). Finally, using transmission electron microscopy, we visualized the polysaccharide capsule of the GFP-labelled Reynolds (CP5) strain (Fig. 1c).

The polysaccharide capsule and Efb together protect against phagocytosis at a broad range of plasma concentrations

After confirming expression levels of both fluorescence and polysaccharide capsule, the strains were analysed in phagocytosis assays. We incubated the GFP-labelled *S. aureus* Reynolds (CP5) and mutant (CP⁻) strain with normal human plasma (as a source for antibodies, complement and fibrinogen) and freshly isolated human neutrophils. Phagocytosis of fluorescent bacteria by

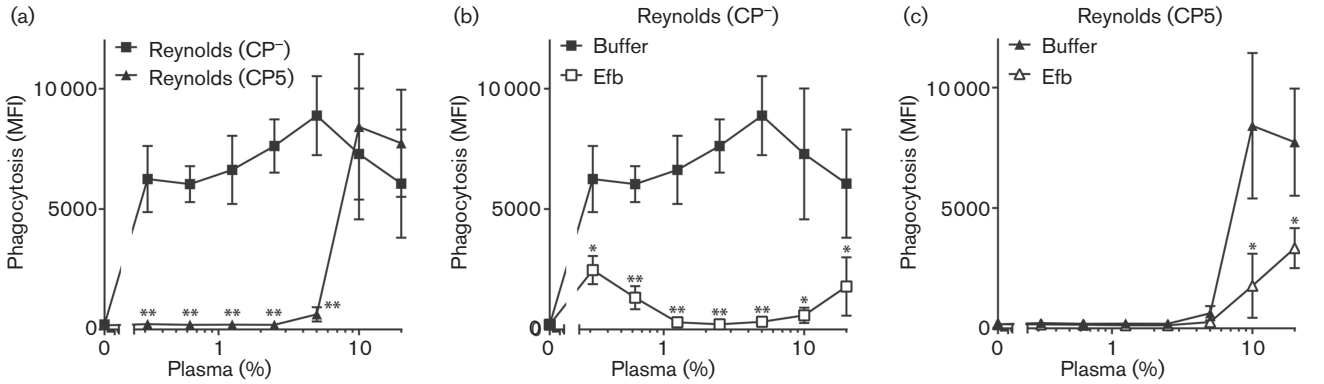

Fig. 2. Phagocytosis of GFP-labelled Reynolds (CP⁻ and CP5) by purified human neutrophils in the presence of human plasma alone (a) or plasma with 0.5 μM Efb (b, c), measured by fluorescence (geomean) of the neutrophils. (a) The polysaccharide capsule of *S. aureus* provides protection against phagocytosis at low plasma concentrations. (b) Addition of exogenous Efb inhibits phagocytic uptake of unencapsulated strain Reynolds (CP⁻) at all tested plasma concentrations. (c) Inhibition of phagocytosis of encapsulated strain Reynolds (CP5) is enhanced by addition of Efb. Graphs represent mean ± SD of three separate experiments. **P*<0.05, ***P*<0.005 for Reynolds (CP5) versus Reynolds (CP⁻) or Efb versus buffer (two-tailed Student's *t*-test). MFI, mean fluorescence intensity.

neutrophils was quantified using flow cytometry. As expected, we observed that the unencapsulated *S. aureus* strain was efficiently phagocytosed (Fig. 2a). As described previously (Cunnion *et al.*, 2003; Thakker *et al.*, 1998), the polysaccharide capsule of the Reynolds (CP5) strain potently blocked neutrophil phagocytosis at low plasma concentrations (Fig. 2a). However, at plasma concentrations ≥10 %, we observed little to no difference between the CP5-expressing Reynolds strain and its isogenic capsule-negative mutant. This suggests that CP5 does not protect against phagocytosis at higher plasma concentrations. As we previously observed that Efb prevents phagocytosis of *S. aureus* in plasma by shielding the bacterial surface with fibrinogen (Ko *et al.*, 2013), we wondered whether addition of Efb could also affect phagocytosis of encapsulated *S. aureus* strains. First, we found that purified GST-tagged Efb (0.5 μM) significantly blocked phagocytosis of the capsule-negative Reynolds (CP⁻) strain at all tested plasma concentrations (Fig. 2b). When the encapsulated strain Reynolds (CP5) was used, Efb also had an inhibitory effect on bacterial uptake at higher plasma concentrations, where the polysaccharide capsule itself is no longer protective (Fig. 2c). As a control, we showed that the GST-tag alone or GST-tagged Efb-N (the N-terminal domain of Efb) did not reduce phagocytic uptake (data not shown). These results suggest that the polysaccharide capsule and the Efb-dependent fibrinogen shield collaborate to fully protect *S. aureus* at an extensive range of plasma concentrations. Together, our findings indicate that these two anti-phagocytic mechanisms collaborate to fully protect *S. aureus* at an extensive range of plasma concentrations.

The Efb-dependent fibrinogen shield provides protection against phagocytosis on various encapsulated and capsule-negative *S. aureus* strains

Since we used the highly encapsulated Reynolds strain in these experiments, we wondered whether the presence of different plasma concentrations also influences phagocytosis inhibition by other capsule-expressing *S. aureus* strains. We therefore performed phagocytosis assays in 1, 3 and 10 % plasma using either unencapsulated *S. aureus* strains (USA300 and Wood 46), CP5-expressing strains (Col and USA100) and CP8-expressing strains (Sanger 252 and Reynolds CP8, the latter an isogenic mutant of strain Reynolds in which the *cap5* region was substituted with *cap8*). All strains were labelled with GFP and grown on CSA to ensure optimal capsule expression (Fig. S1). Since absolute fluorescence levels varied between strains, we expressed phagocytic uptake of each strain (Fig. 3a) as a relative value compared with the mean fluorescence intensity at 10 % plasma, at which phagocytosis had reached its maximum. Although this prohibits direct comparison between strains, this still allows us to analyse the effect of different plasma concentrations on phagocytosis efficiencies of each strain. Similar to the capsule-negative Reynolds strain, none of the other capsule-negative strains (USA300, 8325-4, Wood 46) showed a significant difference in phagocytic uptake at lower concentrations of plasma, compared with 10 % plasma (Fig. 3a). Notably, the other CP5- and CP8-expressing strains (COL, USA100, Sanger 252) did not show a substantial decrease in phagocytosis at the lower plasma concentrations. Only the isogenic Reynolds CP8 mutant showed a reduction in phagocytosis at 1 % plasma concentration. These experiments suggest that the anti-phagocytic effect of the *S. aureus*

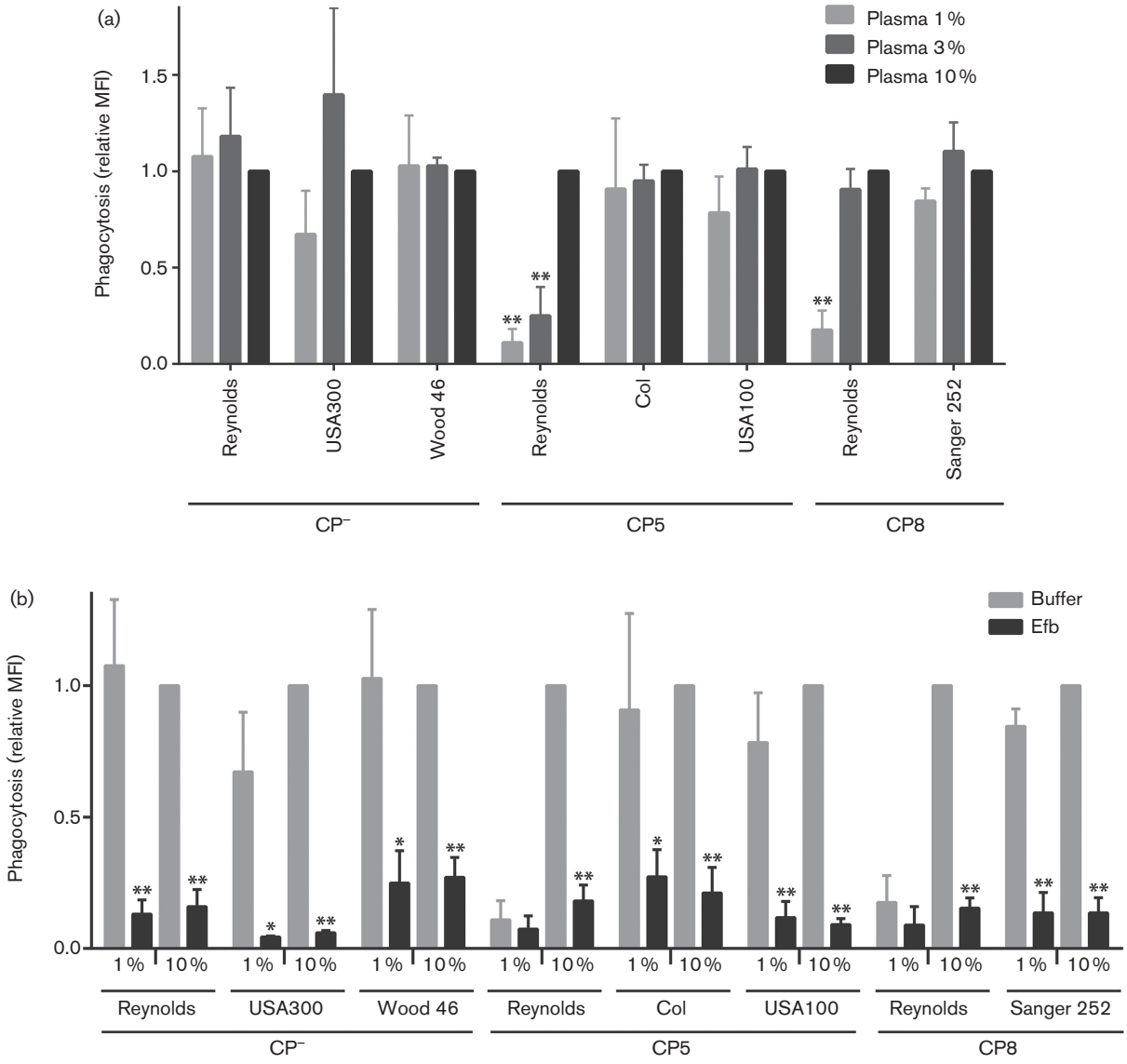

Fig. 3. (a) Phagocytosis of different GFP-labelled CP^{-}-, CP5- and CP8-expressing *S. aureus* strains in the presence of 1, 3 or 10 % human plasma. Phagocytosis is displayed as the relative fluorescence compared with the 10 % plasma condition of each strain. Graph represents mean ± SD of three separate experiments. *P<0.05, **P<0.005 for 1 or 3 % plasma versus 10 % plasma of the same strain (two-tailed Student's t-test). (b) Addition of Efb inhibits phagocytic uptake of different CP5 and CP8 encapsulated *S. aureus* strains at both 1 and 10 % human plasma. This was displayed by the relative fluorescence (geomean; compared with the buffer condition at 10 % plasma of the same strain) of the neutrophils. Graph represents mean ± SD of three separate experiments. *P<0.05, **P<0.005 for Efb versus buffer of the same strain (two-tailed Student's t-test). MFI, mean fluorescence intensity.

capsule depends both on the expression level of the capsule and on the plasma concentration. Next, we tested the anti-phagocytic effect of Efb on the other GFP-labelled CP5- and CP8-expressing strains. We observed that addition of Efb significantly reduced phagocytic uptake of all tested CP^{-}, CP5 and CP8 strains at both 1 and 10 % plasma (Fig. 3b). Since phagocytosis of strain Reynolds (CP5 and CP8) was already considerably reduced at 1 % plasma, an additional significant decrease in phagocytic uptake in the presence of Efb was not measured. This shows that the Efb-dependent fibrinogen shield can be created and function properly on capsule-negative as well as capsule-expressing *S. aureus* strains.

Interplay between Efb and capsule on other isogenic mutants of CP5 and CP8

The phagocytosis experiment in Fig. 3(b) suggests that capsule-mediated inhibition in our assay system is only

detectable for strain Reynolds, but not for other *S. aureus* strains. However, the exact contribution of the capsule in this experiment could not be studied due to the lack of isogenic capsule-negative mutants. Therefore, we decided to include three different capsule-expressing *S. aureus* strains [Newman (CP5), Becker (CP8) and MN8 (CP8)] in which the *cap5* and *cap8* loci are deleted (Watts *et al.*, 2005). These strains were fluorescently labelled and capsule expression was determined using specific CP5 and CP8 antibodies (Fig. 4a). When phagocytosis was analysed, results with FITC-labelled strain Reynolds (CP5 and CP$^-$) were comparable with previous assays; at 1 % plasma the encapsulated strain showed a decrease in phagocytic uptake compared with the capsule-negative strain but at 10 % plasma this inhibitory effect was not present (Fig. 4b). Strikingly, the polysaccharide capsule of strain Newman, showing 74 % CP5 expression compared with Reynolds, was not able to block phagocytosis at 1 and 10 % plasma (Fig. 4c). Also, the capsules of strain Becker and MN8 showed no significant inhibition of phagocytosis (Fig. 4d, e). As anticipated, addition of Efb blocked the phagocytic uptake of all tested strains, regardless of their capsule expression (Fig. 4b–e). Again, this shows that the Efb-dependent fibrinogen shield can be created and function properly on capsule-negative as well as capsule-expressing *S. aureus* strains.

Efb attracts fibrinogen to the surface of both CP$^-$ and CP5 strains

To confirm that Efb can indeed create a fibrinogen shield around the surface of encapsulated strains, we visualized this shield by confocal microscopy. We pre-opsonized mCherry-labelled Reynolds (CP$^-$ and CP5) with 3 % plasma and incubated them with Alexa488-labelled fibrinogen in the presence of Efb (0.5 μM). As a control, we included two Efb mutant proteins that cannot form this shield due to the lack of fibrinogen (EfbΔFg) or C3b (EfbΔC3) binding motifs. First, we observed that bacteria incubated without the addition of Efb did not show binding of fibrinogen to the bacterial surface, which was to be expected as washed bacteria were used and therefore no endogenously produced Efb was present (Fig. 5a). In the presence of Efb, both the CP$^-$ and the CP5 strain were completely surrounded by a layer of fluorescent fibrinogen. This fibrinogen layer was not present when bacteria were incubated with the Efb mutant proteins. These results were confirmed by flow cytometry analyses of the samples used for confocal microscopy, showing a considerable increase of fibrinogen binding in the presence of full-length Efb (Fig. 5b). Remarkably, no significant difference was observed between the CP$^-$ and CP5 strain incubated with full-length Efb with both confocal microscopy and flow cytometry. This suggests that formation of the Efb-dependent fibrinogen shield is equally efficient on encapsulated and capsule-negative strains.

DISCUSSION

S. aureus has evolved many ways to evade and manipulate immune responses in order to survive inside the human host (Foster, 2005). As phagocytic uptake of *S. aureus* by neutrophils is crucial for clearance of the pathogen, suppressing this process will be of great importance to its persistence in the body. The polysaccharide capsule expressed by *S. aureus* has been shown to potently block killing by human neutrophils by covering C3b attached to the bacterial surface (Thakker *et al.*, 1998; Watts *et al.*, 2005). In this study, we observe that the capsule of strain Reynolds (CP5 and CP8) can efficiently block phagocytosis at low concentrations of plasma but that it loses its protective capacity at higher plasma concentrations. Furthermore, we have previously shown that Efb forms a shield of fibrinogen and thereby protects bacteria from phagocytosis (Ko *et al.*, 2013). Now, we demonstrate that the Efb-dependent fibrinogen shield can also effectively be formed on several encapsulated strains at a broad range of plasma concentrations. This shows that these two mechanisms of shielding can collaborate to ensure optimal protection against phagocytosis.

The results presented here suggest that the polysaccharide capsule of strain Reynolds has strong anti-phagocytic properties at lower plasma concentrations. Whereas the polysaccharide capsule has previously been shown to prevent recognition of staphylococcal surface-associated proteins by neutrophil receptors, it does not completely block the binding of specific antibodies nor the deposition of complement components at the bacterial surface (Cunnion *et al.*, 2001; Wilkinson & Sisson, 1979; Watts *et al.*, 2005). Complement activation was triggered by encapsulated *S. aureus*, which resulted in rapid deposition of C3b. However, purified capsular polysaccharides are not immunogenic and did not trigger complement activation (Watts *et al.*, 2005; Nemeth & Lee, 1995). Hence, deposition of C3b on an encapsulated strains does occur but was described to be located beneath the polysaccharide capsule on the bacterial cell wall and is thereby shielded from its surroundings (Watts *et al.*, 2005). Because C3b is able to deposit on top of other C3b molecules (Kinoshita *et al.*, 1988), it is possible that in high concentrations of plasma, C3b molecules can accumulate and eventually be displayed above the capsule, no longer shielded from complement receptors. This may explain the lack of phagocytic resistance by the polysaccharide capsule at high plasma concentrations in this study. An alternative explanation would be that antibodies against the polysaccharide capsule are present in normal human plasma, although concentrations have been shown to be too low to mediate phagocytic uptake (Thakker *et al.*, 1998; Fattom *et al.*, 1993). However, we previously showed that specific capsular antibodies can potently neutralize the anti-phagocytic effect of the polysaccharide capsule, as these antibodies enhance phagocytosis of encapsulated strains at low plasma concentrations (Ko *et al.*, 2013). This could indicate that at high plasma concentration, levels of antibodies directed against capsular polysaccharides are sufficient to efficiently activate complement, and thus C3b deposition, on top of the capsule leading to phagocytic

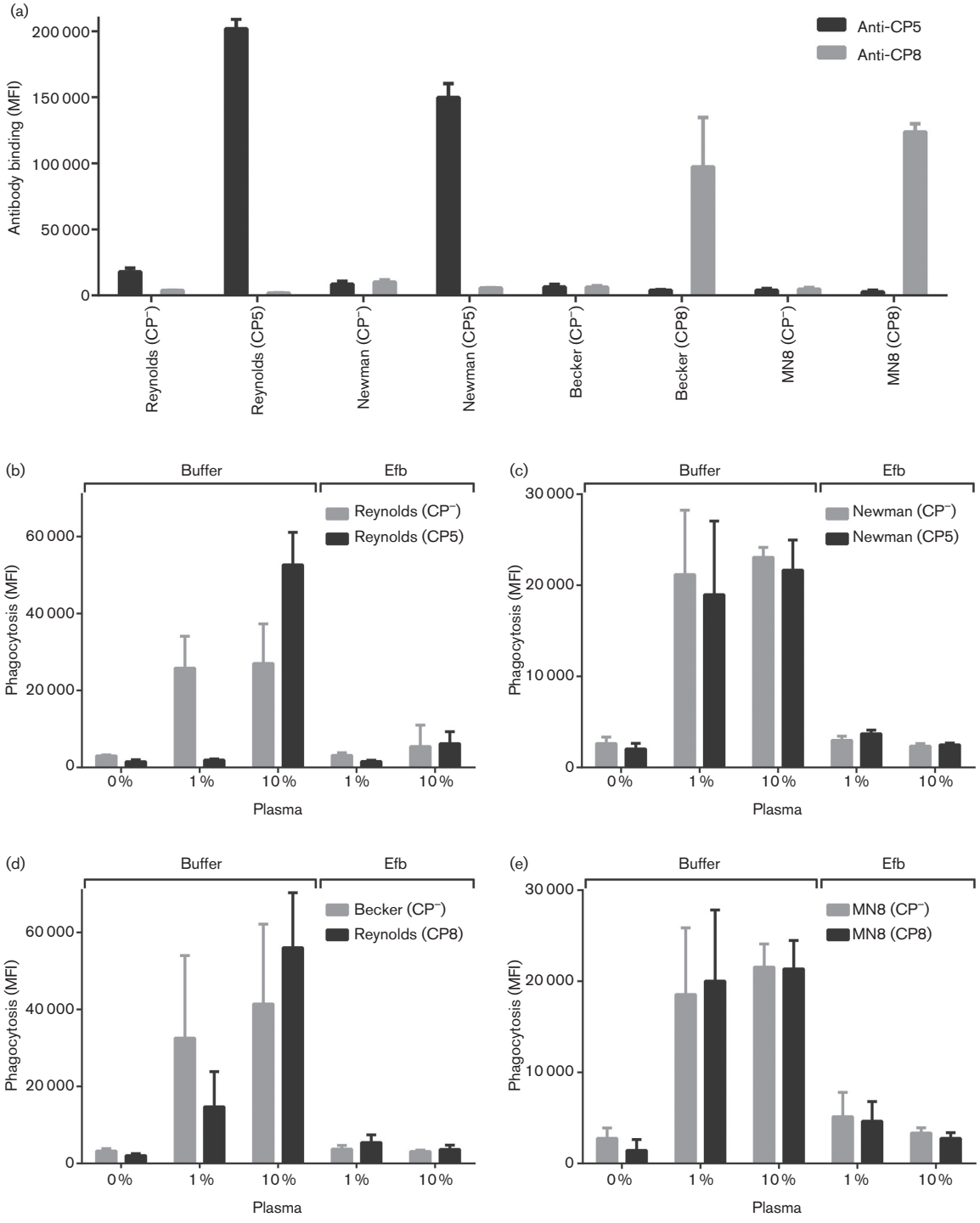

Fig. 4. Interplay between Efb and capsule on other isogenic mutants of CP5 and CP8. (a) Binding of rabbit CP5 and CP8 antibodies to different FITC-labelled CP5- and CP8-expressing *S. aureus* strains. (b–e) Phagocytosis of different FITC-labelled CP5- and CP8-expressing *S. aureus* strains by purified neutrophils in the presence of 1 or 10 % human plasma and 0.5 μM Efb. (b) Phagocytosis of strain Reynolds (CP5) and Reynolds (CP⁻). (c) Phagocytosis of CP5-expressing strain Newman and its isogenic mutant Newman (CP⁻) (buffer vs Efb: $P<0.05$ at 1 and 10 % plasma). (d) Phagocytosis of CP8-expressing strain Becker and its isogenic mutant Becker (CP⁻) (buffer vs Efb: n.s. at 1 % plasma, $P<0.05$ at 10 % plasma). (e) Phagocytosis of CP8-expressing strain MN8 and its isogenic mutant MN8 (CP⁻) (buffer vs Efb: $P<0.05$ at 1 and 10 % plasma). Graphs represent mean ± SD of three separate experiments. At 10 % plasma, the inhibitory effect of Efb was statiscally significant for all strains, but at 1 % plasma was significant only for strains Newman and MN8. MFI, mean fluorescence intensity.

uptake or to directly mediate phagocytosis through recognition by Fc receptors. Nevertheless, not all capsule-expressing strains tested in this study showed similar shielding capacities. Inhibition was most potent for the Reynolds (CP5) strain that is known for its thick capsule. CP8 and CP5 strains have been described to differ in their virulence, explained by the suggestion that CP5 strains commonly express more capsular polysaccharides than CP8 strains (Watts *et al.*, 2005). However, our *in vitro* data will not predict capsule expression inside the body and it is therefore also possible that these strains do produce a dense capsule *in vivo*, which is effective against phagocytic uptake. Interestingly, it was reported that the highly virulent USA300 isolates, prevalent in North America, lack the expression of capsular polysaccharide (Boyle-Vavra *et al.*, 2015). Also here, we observe that a USA300 isolate does not show impaired phagocytosis. Possibly, these strains use other mechanisms to circumvent phagocytic killing. For instance, expression of Efb and clumping factor A (ClfA) have been shown to be upregulated in USA300 strain LAC and therefore the fibrinogen binding capacity of this strain is high (Cheung *et al.*, 2011). Also, greater production of molecules that directly lyse neutrophils, such as Panton Valentine leukocidin (PVL) and phenol soluble modulin (PSM), could compensate for the lack of capsule expression by USA300 isolates (Otto, 2013; Cheung *et al.*, 2011).

Furthermore, our results indicate that Efb most potently prevents phagocytosis of the capsule-negative strain at plasma concentrations between 1 and 10 %. For Efb to completely cover *S. aureus* with a shield of fibrinogen and thus fully block phagocytosis, it not only requires simultaneous binding to both C3b and fibrinogen but also, very importantly, sufficient levels of these two plasma proteins. This explains the reduced efficiency of the Efb-dependent fibrinogen shield at very low plasma concentrations, as the layer generated at these levels of complement and fibrinogen will not be dense enough to completely mask the bacterial surface. We now show that Efb is also able to establish strong inhibition of phagocytosis on highly encapsulated strains. Therefore, binding of Efb seems not to be affected by the presence of capsular polysaccharides, even those of the highly encapsulated Reynolds strain (CP5). As the two shielding mechanisms provide protection at both low and high plasma concentrations, this could suggest that *S. aureus* has the ability to shield itself from phagocytic uptake at different locations inside the host, from tissue to bloodstream. Although we do not provide direct evidence that these shielding mechanisms occur during an infection *in vivo*, we believe that the concentrations of Efb used are relevant. Previously, we quantified the secretion of Efb in *S. aureus* (strain Newman) culture supernatants and found production levels of ~1 μM (Ko *et al.*, 2013). Although

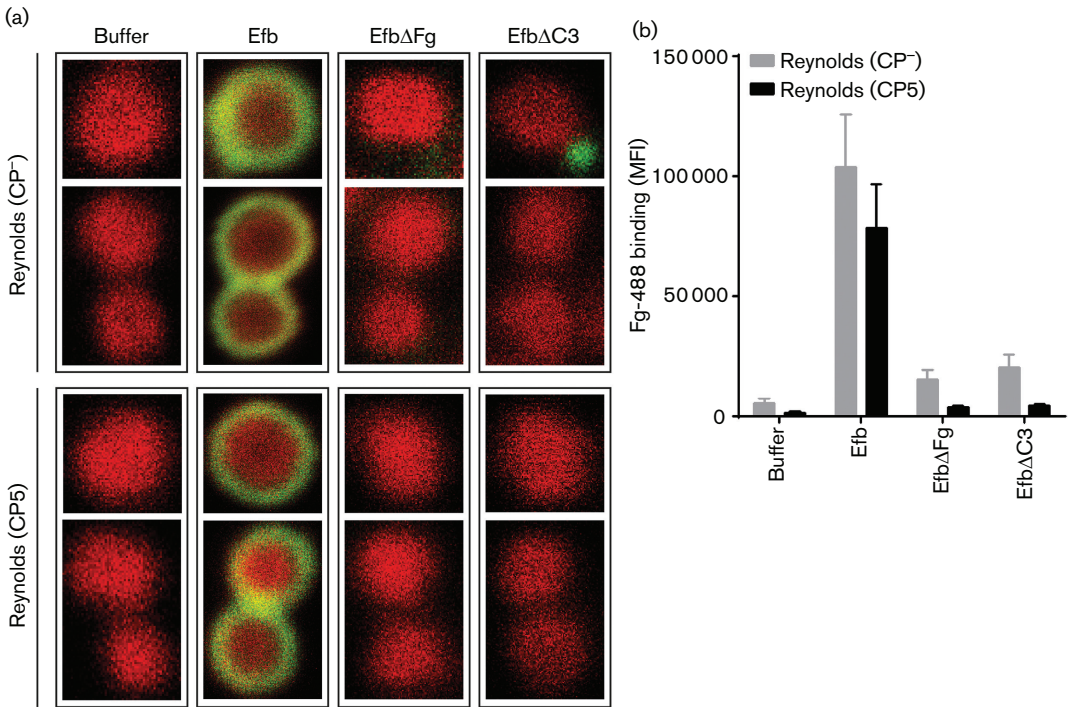

Fig. 5. (a) Confocal images of the binding of Alexa488-labelled fibrinogen to mCherry-labeled Reynolds (CP⁻ and CP5) strains, pre-opsonized with human serum (3 %), in the presence of Efb variants (0.5 μM). Representative images are shown. (b) Flow cytometry analyses of samples shown in (a). Graph represents mean ± SD of three separate experiments. $^{**}P<0.005$ for Efb versus buffer, EfbΔFg or EfbΔC3 (two-tailed Student's *t*-test). MFI, mean fluorescence intensity.

strain Newman has higher expression levels of Efb than most *S. aureus* strains due to a point mutation in the SaeR/S regulatory system (Voyich *et al.*, 2009), these levels are still more than 10 times higher than the calculated IC_{50} (0.08 μM) needed for inhibition of phagocytosis. Additionally, we showed that endogenously produced Efb mediates complex formation on the bacterial surface, as WT supernatants can attract fibrinogen to the bacterial surface whereas Efb-deficient supernatants do not introduce shield formation (Ko *et al.*, 2013). Furthermore, studies of the effect of Efb on the virulence of *S. aureus in vivo* show that the protein is expressed at levels high enough to be effective (Ko *et al.*, 2013; Palma *et al.*, 1996; Shannon *et al.*, 2005). Together, the data presented in this paper indicate that the balance between bacteria, plasma components and infiltrating immune cells can influence the anti-phagocytic properties of pathogenic *S. aureus*. Although it is generally believed that whole blood mimics a relevant physiological condition for *S. aureus* infections, we know that most *S. aureus* infections occur at localized sites of the body where bacteria encounter different concentrations of plasma and immune cells than in human whole blood. Furthermore, during an infection, the inflammatory response will alter the plasma-to-immune cell ratio because of rapid influx of immune cells. For this reason, the bacterium may have evolved additional mechanisms to subvert phagocytosis at different concentrations of plasma and neutrophils. This allows the bacterium to subvert immune clearance from different sites of the body and during different stages of an infection.

S. aureus is rapidly becoming more resistant to antibiotics (Rossolini *et al.*, 2014) and new therapeutic strategies are being explored (Vuong *et al.*, 2015). Despite interesting developments in preclinical studies (Lattar *et al.*, 2014; Wacker *et al.*, 2014; Park *et al.*, 2014), an effective vaccine against *S. aureus* is still not available. Although clinical studies in humans indicate that opsonic antibodies are successfully produced upon vaccination with different *S. aureus* antigens (including capsular polysaccharides) (Nissen *et al.*, 2015; Levy *et al.*, 2015), such antibodies fail to protect humans against *S. aureus* infections (Fattom *et al.*, 2015; Fowler *et al.*, 2013). Possibly, the shielding mechanisms described in this study complicate the effector mechanism of opsonic antibodies. Therefore, the inclusion of both capsular antigens and Efb could be important in the development of a protective *S. aureus* vaccine.

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