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RESEARCH ARTICLE

SPECIAL ISSUE: CELL BIOLOGY OF HOST–PATHOGEN INTERACTIONS

Human-specific staphylococcal virulence factors enhance pathogenicity in a humanised zebrafish C5a receptor model

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ABSTRACT

Staphylococcus aureus infects ∼30% of the human population and causes a spectrum of pathologies ranging from mild skin infections to life-threatening invasive diseases. The strict host specificity of its virulence factors has severely limited the accuracy of *in vivo* models for the development of vaccines and therapeutics. To resolve this, we generated a humanised zebrafish model and determined that neutrophil-specific expression of the human C5a receptor conferred susceptibility to the *S. aureus* toxins PVL and HlgCB, leading to reduced neutrophil numbers at the site of infection and increased infection-associated mortality. These results show that humanised zebrafish provide a valuable platform to study the contribution of human-specific *S. aureus* virulence factors to infection *in vivo* that could facilitate the development of novel therapeutic approaches and essential vaccines.

KEY WORDS: Host-pathogen interactions, Immunology, Microbiology, Staphylococcus aureus, Zebrafish, *In vivo* models

INTRODUCTION

Staphylococcus aureus is a highly specialised pathogen that colonises ∼30% of the human population and causes a variety of mild to severe illnesses ranging from skin and soft-tissue infections to necrotising pneumonia, endocarditis and septicaemia (Tong et al., 2015). In the USA, as many as 50% of *S. aureus* infections are caused by antibiotic-resistant strains (Moran et al., 2006), with methicillin-resistant *S. aureus* (MRSA) among the leading causes of death by a bacterial agent (Klevens et al., 2007), emphasising the need for development of alternative therapies or vaccines. Despite promising results from vaccine studies utilising bacterial surface components and toxins as antigens, these candidates have largely failed to translate from traditional infection models to humans (Salgado-Pabón and Schlievert, 2014). A likely reason for this is the inability of current *in vivo* models to accurately recapitulate human infections, as *S. aureus* expresses a variety of strictly human-specific virulence factors that are ineffective in these models (Salgado-Pabón and Schlievert, 2014). Although their contribution to natural infection remains poorly understood, these virulence factors broadly target the host innate immune system to impair the complement system, oxidative enzymes, chemotactic proteins and phagocytic cells in order to evade recognition and destruction (Buchan et al., 2019a). To properly recapitulate human infections and to study the contributions of *S. aureus* virulence factors to infectivity and pathogenesis *in vivo*, a new humanised infection model is required.

Three *S. aureus* virulence factors interact with the human C5a receptor (C5AR1, also known as CD88), a seven-transmembrane loop G-protein-coupled receptor (GPCR) that is highly expressed on the surface of neutrophils (Spaan et al., 2013b) and recognises host anaphylatoxin C5a released during complement activation to control phagocyte activation and chemotaxis. The targeting of C5AR1 by multiple *S. aureus* virulence factors appears to be specifically focused against neutrophils, which form an essential line of defence against staphylococcal infection (Spaan et al., 2013a). The two bicomponent pore-forming toxins Panton–Valentine leukocidin (PVL) and γ-haemolysin CB (HlgCB) target C5AR1 to recognise and lyse phagocytic cells by forming β-barrel pores in the cell membrane (Spaan et al., 2013b, 2014). The toxins are secreted as two subunits, known as the S- (slow, LukS-PV/HlgC) and F- (fast, LukF-PV/HlgB) subunits due to their chromatography elution profiles. Besides inducing cell lysis, S-subunits also disrupt chemotaxis by competitively inhibiting receptor signalling (Spaan et al., 2013b, 2014). In addition, the chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a 14 kDa protein that prevents C5a-mediated chemotaxis by binding directly to the N-terminus of C5AR1 (Haas et al., 2004). The high-affinity protein–protein interactions between virulence factors and C5AR1, which have been characterised at the amino acid level, are highly human specific and consequently our insight into the roles they play during infection is limited due to a lack of suitable humanised infection models (Fowler and Proctor, 2014; Salgado-Pabón and Schlievert, 2014).

The zebrafish *Danio rerio* is a widely used model organism for investigating bacterial infections and the innate immune system (Henry et al., 2013; Torraca and Mostowy, 2018). Owing to their optical transparency, ability to produce high numbers of offspring and genetic tractability, zebrafish offer many unique approaches over existing infection models. Zebrafish have a fully developed innate immune system by 2 days post fertilisation (dpf), characterised by the presence of mature phagocytic cells (Lieschke et al., 2001) and a complement system that is highly homologous to that in humans (Zhang and Cui, 2014).

In this study, we developed a humanised C5AR1 knock-in zebrafish infection model and determined the contribution of the *S. aureus* toxins PVL and HlgCB to infection *in vivo*. Whereas

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wild-type zebrafish neutrophils were resistant to toxin-mediated lysis, we show that neutrophil-specific C5AR1 expression confers sensitivity of zebrafish neutrophils to PVL and HlgCB-mediated lysis *in vivo*. Humanised zebrafish displayed reduced neutrophil abundance at the sites of infection and increased *S. aureus*-associated mortality as a result of the direct interaction between *S. aureus* and the human C5a receptor when expressed by zebrafish neutrophils. In conclusion, our studies not only illustrate the critical contribution of PVL and HlgCB to *in vivo* infection and pathogenesis, but also show the significance of humanised zebrafish as a novel platform to investigate the activities of human-specific virulence factors *in vivo* and to accurately recapitulate natural human infection in a model organism.

RESULTS

Zebrafish possess a functional C5aR that is responsive to serum-derived C5a

To study the C5a–C5aR signalling axis in zebrafish, we first expressed the zebrafish C5a receptor (*c5ar1*) in the human monocyte-like cell line U937 (U937-dreC5aR) by lentiviral transduction and measured the ability of the receptor to bind and respond to recombinant zebrafish and human C5a (denoted dreC5a and hsaC5a, respectively) using previously established methods (Spaan et al., 2013b). As controls, we generated cells stably expressing the human C5a receptor (U937-hsaC5aR) or an empty vector control (U937-EV). First, flow cytometric analysis of recombinant, FITC-labelled C5a-binding capacities showed that U937-dreC5aR cells specifically bound dreC5a, but not hsaC5a (Fig. 1A). Conversely, U937-hsaC5aR cells strongly bound hsaC5a and only minimally interacted with dreC5a, suggesting that both receptors bind C5a in a species-specific manner. Activation of GPCRs, including the C5a receptor, results in the induction of intracellular signalling cascades culminating in the cytosolic release of intracellular Ca^{2+} stores (Tawk et al., 2015). Accordingly, we evaluated the signalling ability of the receptors by measuring intracellular Ca^{2+} release following receptor stimulation using the Fluo-3AM probe. Treatment of U937-dreC5aR cells with recombinant dreC5a provoked robust Ca^{2+} release, indicating successful receptor ligation and activation, whereas U937-hsaC5aR-expressing cells responded minimally to dreC5a (Fig. 1B). Importantly, zymosan-activated zebrafish serum induced a response similar to recombinant C5a, indicating that the zebrafish C5aR responds to physiological concentrations of zebrafish C5a in activated fish serum under these conditions (Fig. 1B). Conversely, U937-hsaC5aR cells responded robustly to hsaC5a or human activated serum treatment, but not dreC5a treatment. Taken together, these data show that the zebrafish *c5ar1* gene encodes a functional surface receptor that interacts with, and is activated by, physiological concentrations of C5a. Furthermore, the human and zebrafish C5a receptor and ligand pairs are species specific and are not interchangeable.

Zebrafish C5aR is resistant to human-specific virulence factors

The three *S. aureus* C5AR1-targeting virulence factors, CHIPS, PVL and HlgCB, are known to display strict human specificity and are unable to interact with C5aRs expressed by several other species (Spaan et al., 2013b, 2014). However, their ability to target zebrafish complement components is unknown. To test this, we first assessed the functionality of the zebrafish C5a receptor in the absence and presence of the inhibitory protein CHIPS. We observed that U937-dreC5aR cells retained complete responsiveness to dreC5a in the presence of CHIPS at concentrations that completely inhibited U937-hsaC5aR cells, indicating that CHIPS is ineffective at targeting the zebrafish receptor (Fig. 1C). Based on our detailed molecular understanding of the interactions between CHIPS and the 21-amino-acid-binding site in the N-terminus of the

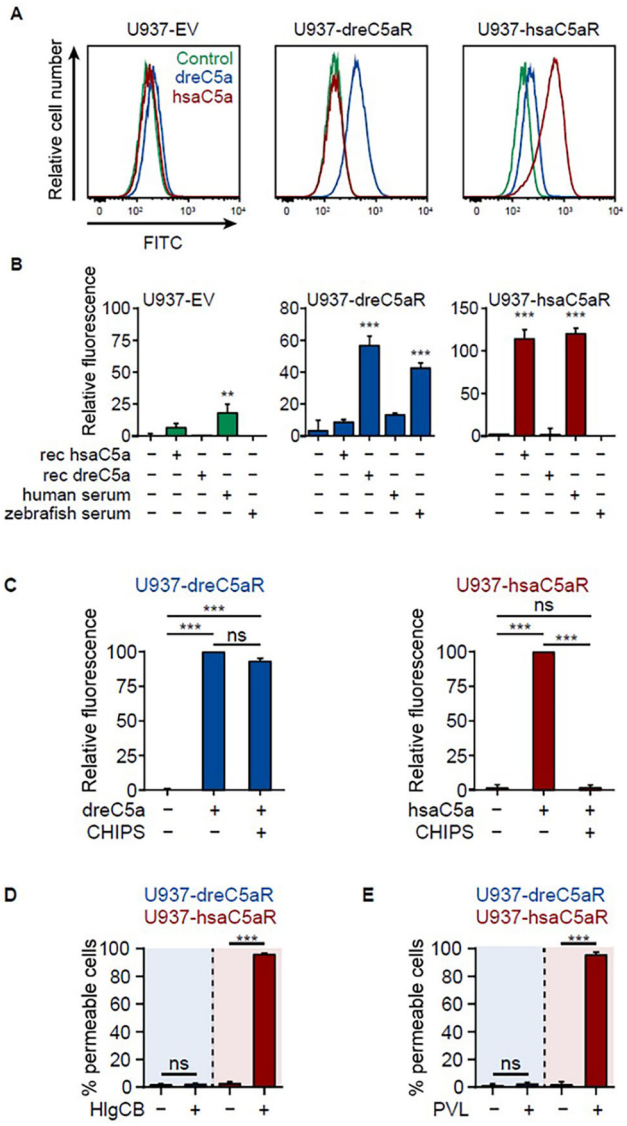

Fig. 1. Zebrafish possess a functional C5aR that is insensitive to targeting by *S. aureus* virulence factors CHIPS, PVL and HlgCB. (A) Binding of FITC-labelled zebrafish C5a (dreC5a) or human C5a (hsaC5a) to U937 cells stably expressing the zebrafish C5a receptor (dreC5aR), the human C5a receptor (hsaC5aR) or an empty vector control (EV) was determined by flow cytometry. (B) Relative C5aR activity following treatment with recombinant human or zebrafish C5a or zymosan-activated serum was determined by measuring cytosolic Ca^{2+} release using a Ca^{2+}-sensitive fluorescent probe (Fluo-3AM) and displayed as a percentage relative to the positive control (10 µM ionomycin treatment, set at 100%). (C) C5aR activity following treatment with recombinant human or zebrafish C5a with or without 10 µg/ml recombinant CHIPS was determined by measuring cytosolic Ca^{2+} release using a Ca^{2+}-sensitive fluorescent probe (Fluo-3AM) by flow cytometry and displayed as percentage relative to the C5a-treated sample without CHIPS (middle bar, set at 100%). (D,E) Pore formation in U937-dreC5aR (blue) or U937-hsaC5aR (red) cells following 30 min incubation with 10 µg/ml HlgCB (D) or 10 µg/ml PVL (E), as measured by percentage DAPI-positive cells by flow cytometry. Data are presented as means±s.d. [n=2 (for B–D) or n=3 (for E)]. ***P<0.001; ns, not significant [two-way ANOVA with Dunnett's multiple comparisons correction of each sample versus the untreated control (B,C) or unpaired two-tailed t-test (D,E)].

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human C5aR, we predicted that a few amino acid changes may be sufficient to render the zebrafish C5aR sensitive to the inhibitory actions of CHIPS. To test this, we generated ten rationally designed variants of the zebrafish receptor, substituting human residues at key points within the CHIPS-binding site (Postma et al., 2005). All ten variants showed normal surface expression and endogenous signalling activity in response to recombinant dreC5a, similar to the wild-type zebrafish receptor (Fig. S1A). Notably, activation of three partly humanised variants (Fig. S1B–D, variants A, H and I) by dreC5a was effectively inhibited in the presence of CHIPS, the most conservative change requiring only three amino acid substitutions (Fig. S1B–D, variant H). These data indicate that sensitisation of dreC5aR to CHIPS can be achieved with only three amino acid changes in the endogenous receptor.

Next, we determined the sensitivity of zebrafish C5aR-expressing cells to pore-formation and lysis by the *S. aureus* toxins PVL and HlgCB. Whereas U937-hsaC5aR cells were efficiently permeabilised in the presence of the recombinant leukocidin components of PVL or HlgCB, U937-dreC5aR cells were resistant to these same concentrations of PVL and HlgCB (Fig. 1D,E). We then aimed to identify the minimal amino acid changes in the zebrafish C5aR that are sufficient to gain sensitivity to PVL and/or HlgCB using the same strategy as we used to gain CHIPS sensitivity. We tested a set of 15 dreC5aR variants with many combinations of (partly) humanised intracellular and extracellular loops and individual amino acids that are known to be involved in the PVL or HlgCB interaction with the hsaC5aR (Fig. S2). Unfortunately, we failed to achieve toxin sensitivity while simultaneously maintaining surface expression and signalling capability for any of these humanised dreC5aR variants. In conclusion, we found that the wild-type zebrafish C5aR is insensitive to the lytic activity of *S. aureus* virulence factors CHIPS, PVL, and HlgCB at the concentrations tested. While CHIPS sensitivity can be achieved with only three amino acid changes in dreC5aR, we were unable to identify dreC5aR variants that gained PVL and/or HlgCB sensitivity.

Generation of transgenic zebrafish with neutrophil-specific human C5AR1 expression

Next, we sought to develop an *in vivo* infection model to study the role of *S. aureus* pore-forming toxins during natural infection. Because we were unable to generate a humanised dreC5aR sensitive to PVL and/or HlgCB *in vitro*, we instead introduced the complete human C5a receptor into zebrafish neutrophils. To this end, a transgenic construct directing expression of a fluorescent Clover-tagged C5AR1 driven by the zebrafish neutrophil-specific *lyz* promoter (Buchan et al., 2019b) was introduced into the zebrafish genome by Tol2 transgenesis (Kwan et al., 2007), producing the transgenic line *Tg(lyz:hsaC5AR1-Clover)sh505*. To verify whether C5AR1–Clover expression was restricted to zebrafish neutrophils, we crossed this line to the established transgenic zebrafish line *Tg(lyz:nfsB-mCherry)sh260*, which displays neutrophil-specific mCherry expression (Buchan et al., 2019b). In the double-transgenic larvae, we observed Clover expression exclusively in the mCherry-positive cells, confirming that the C5AR1 protein is expressed specifically in zebrafish neutrophils (Fig. 2A,B). Furthermore, whereas mCherry showed general cytoplasmic localisation, the C5AR1-associated Clover signal was enriched at the cell membrane of neutrophils from *Tg(lyz:hsaC5aR1-Clover)sh505* zebrafish, suggesting that the cell-surface expression of C5AR1 observed in human neutrophils is correctly recapitulated in the humanised zebrafish system (Fig. 2C,D; Movie 1). Notably, the total number of neutrophils in these fish was unaffected by transgene expression (Fig. S3).

Human C5AR1 is functional in humanised zebrafish

Next, we investigated functional activity of human C5AR1 in the transgenic zebrafish line by assessing neutrophil migration to recombinant dreC5a and hsaC5a injected into the otic vesicles, two sac-like invaginations in the head of the fish that are a preferred site for assessing phagocyte migration (Benard et al., 2012). In the non-humanised *lyz:*nfsB-mCherry fish, dreC5a injection resulted in migration of neutrophils to the injection site, as expected due to endogenous receptor function, while hsaC5a injection did not induce neutrophil migration (Fig. 3A,B). In contrast, neutrophils expressing the human *C5AR1* transgene displayed robust migration towards the site of hsaC5a injection, showing that C5AR1 acts as a functional C5a receptor in zebrafish neutrophils that is able to direct neutrophil migration.

Humanised zebrafish neutrophils are targeted by PVL and HlgCB *in vivo*

Having established that human C5AR1 is expressed as a functional receptor on the surface of zebrafish neutrophils, we next investigated whether C5AR1-expressing neutrophils are targeted by the C5AR1-targeting *S. aureus* toxins PVL and HlgCB *in vivo*. To this end, the community-acquired MRSA strain USA300 was injected with or without recombinant PVL into the otic vesicle of wild-type or C5AR1-transgenic larvae, and the number of neutrophils present at the injection site was determined 4 h later. Whereas injection of USA300 alone resulted in similar numbers of neutrophils at the injection site in both C5AR1-negative and -positive larvae, the addition of PVL significantly reduced neutrophil numbers specifically in C5AR1-expressing larvae, while not affecting neutrophil migration in C5AR1-negative fish (Fig. 4A,B). Similarly, we observed a reduced number of neutrophils at the injection site in humanised larvae injected with USA300 together with HlgCB (Fig. 4C,D). These results showed that C5AR1 expression sensitises zebrafish neutrophils to PVL and HlgCB *in vivo*.

The S-subunit of HlgCB (HlgC) has been reported to competitively inhibit C5AR1 signalling at low concentrations (Spaan et al., 2013b, 2014). However, a HlgC-only injection did not significantly affect neutrophil abundance at the injection site, suggesting that the reduced neutrophil counts observed in the presence of HlgCB injection are due to the pore-forming activity of HlgCB and cell lysis rather than inhibition of C5aR signalling by HlgC alone (Fig. 4C,D). In conclusion, our data indicate that human C5AR1 expression in zebrafish neutrophils conferred sensitivity to the *S. aureus* toxins PVL and HlgCB, and showed that the presence of these toxins reduces neutrophil numbers at the sites of infection *in vivo*.

Zebrafish expressing human C5AR1 are more susceptible to *S. aureus* infection

Given that human C5AR1 acted as a functional receptor that was targeted by the *S. aureus* toxins PVL and HlgCB in our zebrafish model, we next sought to determine whether neutrophil-specific expression of C5AR1 increases the susceptibility of humanised fish to staphylococcal infection. To investigate this, we first separated the fish into non-humanised (*lyz:*nfsB-mCherry only) and humanised (*lyz:*hsaC5AR1-Clover; *lyz:*nfsB-mCherry) groups and injected the community-acquired MRSA strain USA300 into the circulation valley of the fish at 30 hours post fertilization (hpf) according to previously published methods (Prajsnar et al., 2008). In this infection model, macrophages are able to clear *S. aureus* from the fish circulation, so to specifically study the effect of neutrophils, we silenced *irf8* expression using an *irf8* morpholino to

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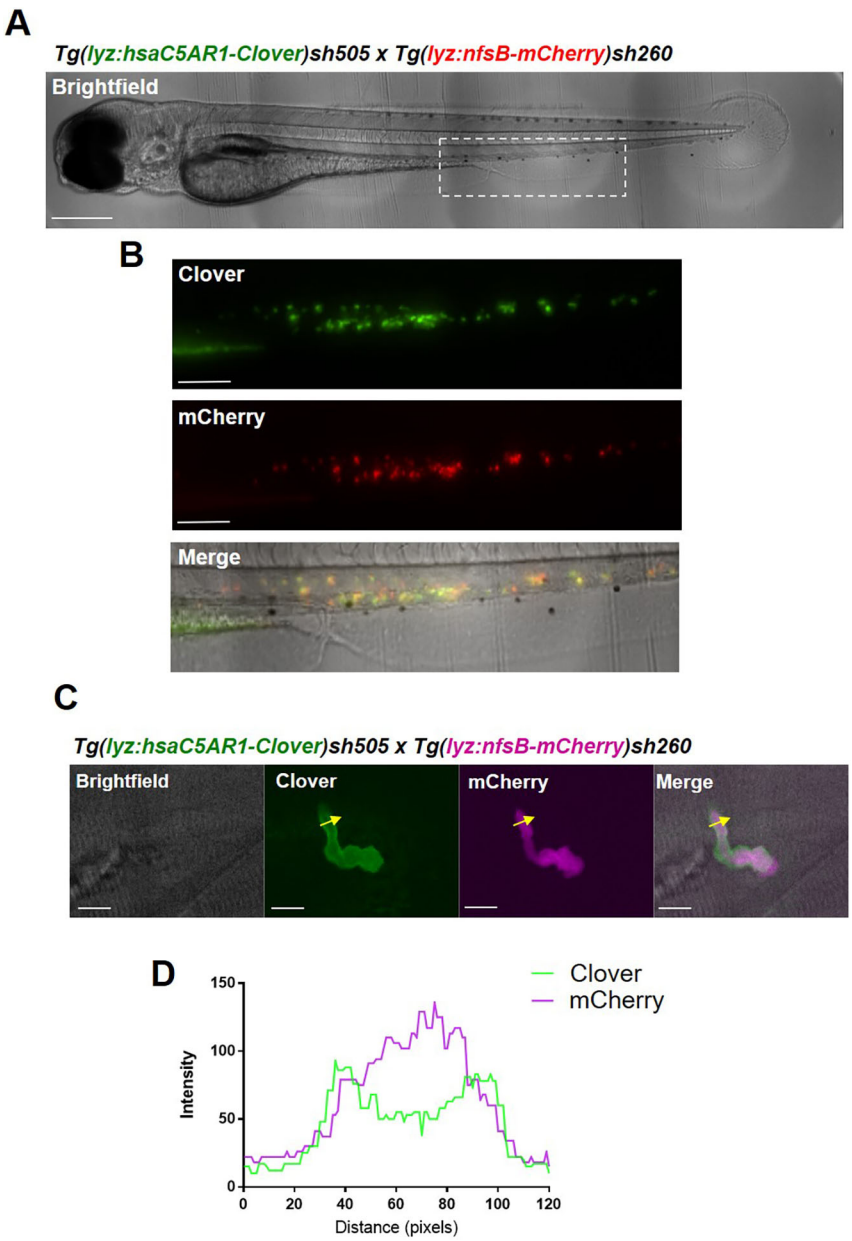

Fig. 2. Generation of a transgenic zebrafish expressing human C5AR1-Clover. (A) A double-transgenic *Tg(lyz:hsaC5AR1-Clover)sh505; Tg(lyz:nfsB-mCherry)sh260* larva at 3 dpf. Dashed white box indicates the enlarged region shown in B. Scale bar: 250 µm. (B) Microscopy analysis of neutrophil-specific mCherry (red) and Clover (green) transgene expression in the enlarged view of the caudal haematopoietic tissue shown in A. Scale bars: 100 µm. (C) Close-up of Clover (green) and mCherry (purple) expression in a double-transgenic neutrophil in the caudal haematopoietic tissue of a 3 dpf larva. Scale bars: 10 µm. (D) Line intensity profile of the fluorescent signal of Clover (green) and mCherry (purple) across the yellow arrow shown in C. Results shown are representative of five neutrophils from three individual larvae.

alter zebrafish haematopoiesis and favour the differentiation of neutrophils over macrophages (Li et al., 2011). In this way, we observed significantly higher mortality following staphylococcal infection for the C5AR1-positive zebrafish compared to the C5AR1-negative fish, when infected at 30 hpf or 50 hpf (Fig. 5A, B). This demonstrates that expression of human *C5AR1* in zebrafish neutrophils enhances susceptibility to staphylococcal infection and suggests that the C5aR-targeting toxins PVL and HlgCB play crucial roles in determining the severity of *S. aureus* infection *in vivo*.

DISCUSSION

Complete understanding of staphylococcal infection requires a thorough characterisation of staphylococcal virulence factors and an appreciation of their physiological relevance and synergistic role during natural infection. Owing to the host-specificity of the virulence factors, human-specific virulence factors have been difficult to adequately study in animal models, creating a gap in our understanding that has disrupted the experimental validation of effective *S. aureus* vaccine candidates and novel therapeutic approaches. In this study, we addressed this problem by generating a humanised zebrafish model that allowed us to study the important contributions of human-specific *S. aureus* toxins PVL and HlgCB to infection-related mortality in a relevant *in vivo* system.

S. aureus expresses three virulence factors that target the human complement receptor C5aR. Although components from all arms of the complement system (classical, alternative and lectin) have been found in larval zebrafish and are known to confer early humoral immunity to the embryo via maternal transfer (Earley et al., 2018; Wang et al., 2009; Yang et al., 2014), the zebrafish complement system including C5aR has barely been characterised at the functional level. To facilitate our studies of the C5aR-targeting virulence factors, we first established functionality of the C5a–C5aR axis in zebrafish. By expressing the zebrafish C5aR on U937 cells, we showed that dreC5aR ligation by recombinant zebrafish C5a or zymosan-activated zebrafish serum resulted in similar Ca^{2+}

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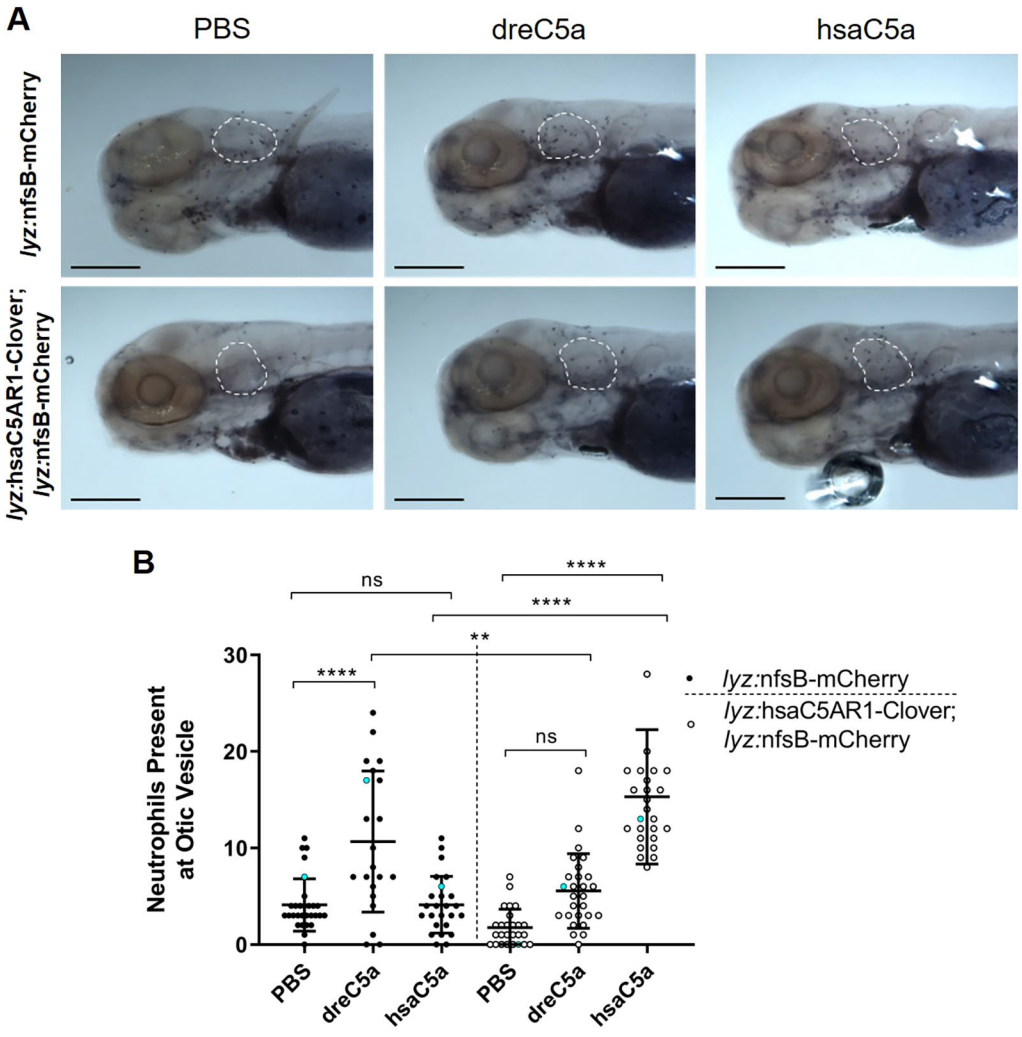

Fig. 3. Human C5AR1 confers sensitivity to hsaC5a in humanised zebrafish. (A) Neutrophil migration was assessed in non-humanised (*lyz:* nfsB-mCherry) and humanised (*lyz:* hsaC5AR1-Clover; *lyz*:nfsB-mCherry) zebrafish larvae at 3 dpf following injection with a PBS vehicle control, recombinant zebrafish C5a (dreC5a) or human C5a (hsaC5a) into the otic vesicle (area highlighted by dashed line). A 4 h post injection (hpi), larvae were fixed in 4% paraformaldehyde and stained with Sudan Black B to detect neutrophils. Scale bars: 200 µm. (B) Numbers of neutrophils present at the otic vesicle at 4 hpi in zebrafish treated as in A; blue points denote the representative images in A. Error bars shown are mean±s.d. (n=22–26 individual animals over two independent experiments). **P<0.01; ****P<0.0001; ns, not significant (two-way ANOVA and adjusted using Bonferroni's multiple comparisons test).

mobilisation to that observed upon activation of the human receptor. Furthermore, we observed neutrophil migration toward the sites of dreC5a injection *in vivo*. These results indicate that the C5a–C5aR axis is functional in zebrafish and, analogous to the human context, is involved in directing neutrophil migration towards invasive pathogens. Interestingly, both human and zebrafish C5a displayed strict species specificity and were not interchangeable with one another. We further found that, in agreement with studies in other species (Haas et al., 2004; Spaan et al., 2013b, 2014), *S. aureus* virulence factors CHIPS, PVL and HlgCB were ineffective against the zebrafish C5aR receptor, also corroborating their strict human specificity. Future studies should determine whether the other components and pathways of the human complement system are also functionally conserved in zebrafish.

Neutrophils are essential for protecting the body from acute bacterial infection, and play a prominent role in clearing *S. aureus* infections (Spaan et al., 2013a). In line with this, the redundant targeting of human neutrophils by multiple *S. aureus* virulence factors suggests that inhibition of neutrophil function is an important contributor to *S. aureus* infection success. Additionally, the C5aR is expressed at roughly tenfold higher levels on the surface of human neutrophils than on human monocytes, and is the primary target of Panton–Valentine leukocidin and γ-haemolysin CB (Spaan et al., 2013b, 2014), making neutrophils an ideal cell type for studying the importance of this receptor in controlling *S. aureus* infection.

Although the *S. aureus* pore-forming toxins are thought to exacerbate infection-associated morbidity and mortality (Naimi et al., 2003), their exact contribution to infection *in vivo* has remained elusive due to the well-characterised human specificity of the interaction between C5aR and these toxins (Buchan et al., 2019a). The introduction of the human C5aR into zebrafish neutrophils allowed us to study the contribution of PVL and HlgCB-mediated targeting of the C5aR on neutrophils during natural infection with high throughput. In this way, we found that the actions of both *S. aureus* pore-forming toxins resulted in reduced neutrophil presence at the site of infection and increased infection-associated mortality (Figs 4 and 5). The presence of HlgC alone, which was recently shown to inhibit neutrophil chemotaxis (Spaan et al., 2014), did not affect neutrophil numbers (Fig. 4D), suggesting that it is the cytotoxic activity of these toxins that leads to reduced neutrophil numbers in these fish, and not a blockade of neutrophil migration.

In our model, we observed equal neutrophil recruitment between the wild-type and humanised fish to sites of bacterial infection in the otic vesicle (Fig. 4B,D), indicating that overall dreC5aR and neutrophil functionality remained intact in the humanised fish. However, we also noticed a modest reduction in neutrophil recruitment to injected dreC5a in C5AR1-transgenic fish compared to wild type (Fig. 3) and to sites of inflammation (Fig. S4A,B), as well as slightly reduced neutrophil migration speed towards superficial

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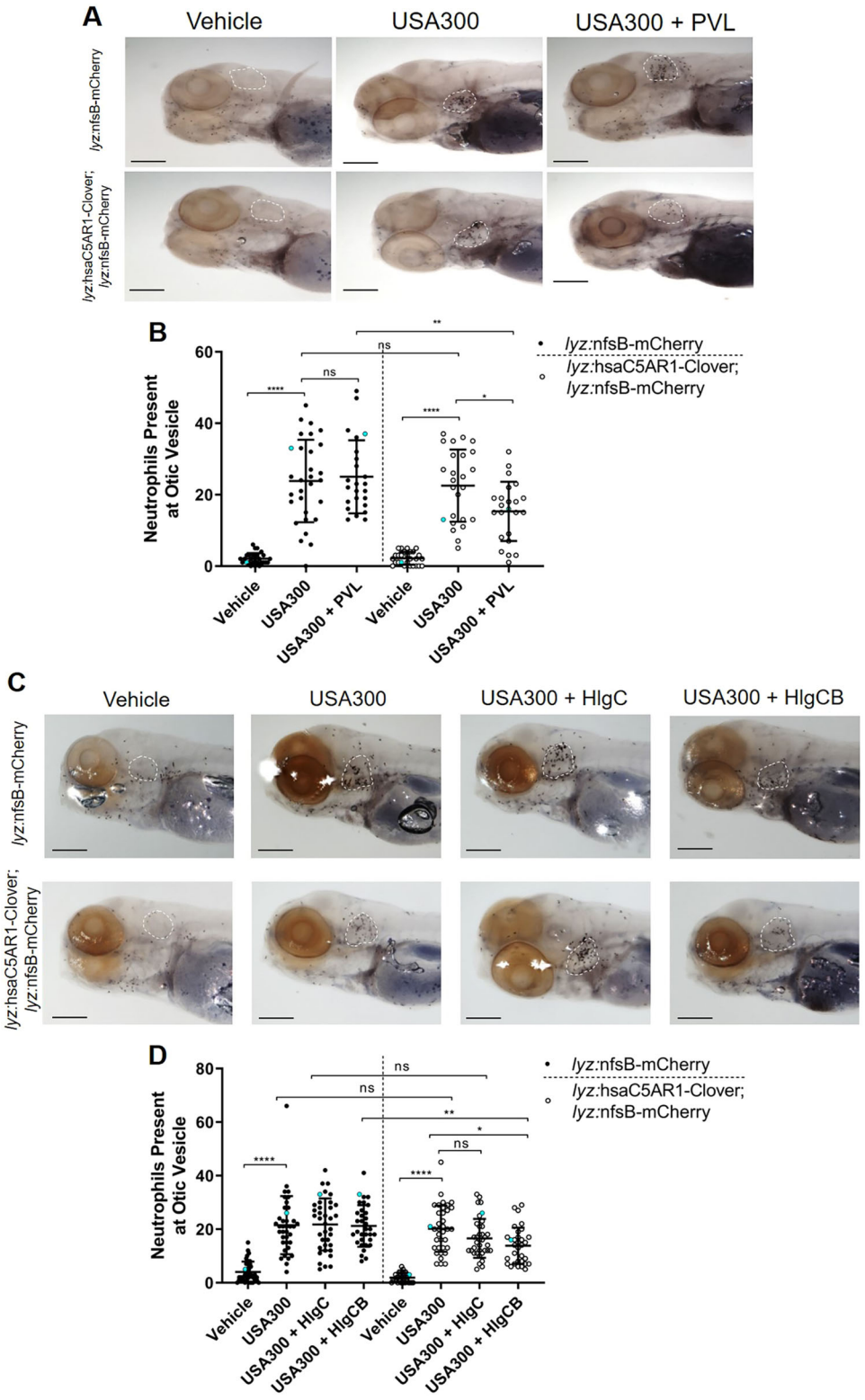

Fig. 4. Neutrophils of C5AR1-expressing zebrafish are susceptible to *S. aureus* toxins PVL and HlgCB. (A) Representative images of neutrophil abundance in zebrafish larvae at 3 dpf that were separated into non-humanised (*lyz*:nfsB-mCherry only) and humanised (*lyz*:hsaC5AR1-Clover; *lyz*:nfsB-mCherry) groups and injected into the otic vesicle with a vehicle control or ∼3500 colony-forming units (cfu) of *S. aureus* USA300 with or without 30.3 µg/ml PVL. The white outline indicates the otic vesicle. Scale bars: 200 µm. (B) Number of neutrophils present at the otic vesicle at 4 hpi; blue points denote the representative images in A. (C) Representative images of neutrophil abundance in zebrafish larvae injected into the otic vesicle as in A with a vehicle control, or ∼3500 *S. aureus* USA300 with or without 16.7 µg/ml HlgCB or HlgC, as indicated. Scale bars: 200 µm. (D) Number of neutrophils present at the otic vesicle at 4 hpi; blue points denote the representative images in C. Error bars shown are mean± s.d. For B, n=22–26 over two independent experiments and for D, n=32–41 over three independent experiments.; *P<0.05; **P<0.01; ****P<0.0001; ns, not significant (two-way ANOVA and adjusted using Bonferroni's multiple comparisons test).

injections (Fig. S4C–G). We suspect that this is due to competition between the C5AR1 and the endogenous receptor when expressed simultaneously at the cell surface. Despite these observations, the neutrophil response to infection was unaffected overall (Fig. 4B,D). Furthermore, we initially did not observe a difference in C5AR1-related infection-associated mortality in wild-type zebrafish (Fig. S5). However, in contrast to superficial infections where neutrophils play a critical role in containing and clearing infection, systemic infection in the zebrafish is primarily controlled by macrophages (Colucci-Guyon et al., 2011; Prajsnar et al., 2008). Therefore, to accurately study neutrophil targeting by these virulence factors, we required a neutrophil-dependent infection model.

To study the role of neutrophils specifically, we utilised *irf8* morpholinos to induce differentiation of zebrafish macrophages into

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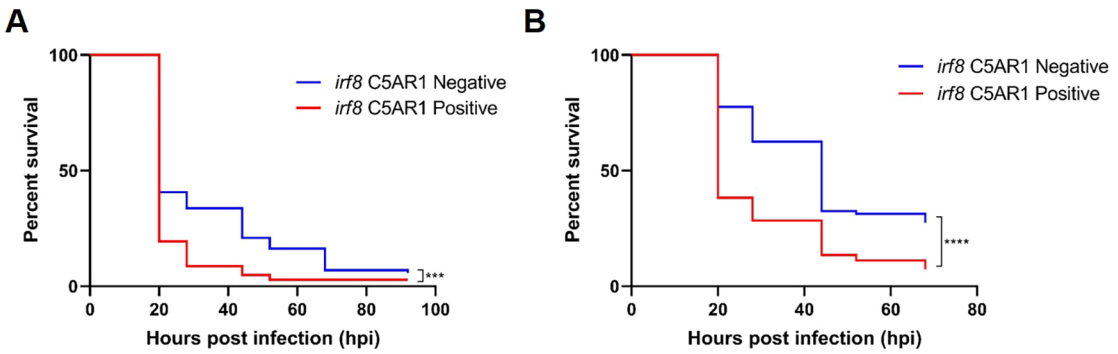

Fig. 5. Humanised neutrophil-replete zebrafish are more susceptible to staphylococcal infection. Neutrophil-replete zebrafish were generated by injecting an *irf8* morpholino at the single-cell stage, silencing *irf8* expression. Zebrafish larvae were then separated into non-humanised (*lyz*:nfsB-mCherry only) and humanised (*lyz*:hsaC5AR1-Clover; *lyz*:nfsB-mCherry) groups and infected with (A) 30 hpf [~700 colony-forming units (cfu)], or (B) 50 hpf (~2000 cfu). Survival was monitored up to 96 h post infection. For A, n=80–81 over three independent experiments; for B, n=86–103 over three independent experiments. ***P<0.001. ****P<0.0001. Data was analysed using a log-rank Mantel–Cox test.

neutrophils (Li et al., 2011). In this neutrophil-dependent model system, we clearly observed an increase in susceptibility of zebrafish expressing the human C5aR to *S. aureus* infection compared with C5AR1-negative siblings (Fig. 5). Our work thus shows that selective humanisation of the zebrafish is a powerful approach towards identifying the contribution of host-restricted virulence to bacterial infection and pathogenesis.

Several other *S. aureus* virulence factors target GPCRs other than C5AR1. For example, CXCR2 is targeted by SSL5, staphopain A and leukotoxin ED (Bestebroer et al., 2009; Laarman et al., 2012; Reyes-Robles et al., 2013) and the formyl peptide receptors (FPR1 and FPR2) are targeted by CHIPS, FLIPr and SSL13 (de Haas et al., 2004; Prat et al., 2006; Zhao et al., 2018). We anticipate that more extensive humanisation of these and other receptors in zebrafish will lead to improved infection models that incorporate a multitude of human-specific virulence factors and even more closely resemble human infections, permitting detailed investigation of the interplay and relative importance of these virulence factors at different stages of infection. Our ultimate goal is to minimally interfere with zebrafish physiology by harnessing the detailed knowledge of the receptor interaction sites to design minimally humanised receptors that gain susceptibility to the *S. aureus* virulence factors while maintaining *in vivo* functionality. It is a promising start that only three amino acid changes were sufficient to confer CHIPS-mediated inhibition to an otherwise functional receptor *in vitro*. Unfortunately, we were so far unable to generate a partially humanised, functional zebrafish C5aR that displayed sensitivity to the pore-forming toxins. Even limited amino acid substitutions in the extracellular loops of the receptor abolished surface expression and thereby led to non-functional receptors, forcing us to introduce the entire human receptor into zebrafish neutrophils in this study.

It is worth noting however, that zebrafish are optimally suited to these studies while they remain optically accessible within the first few weeks of life. Within this time, they do not possess adaptive immunity (Herbomel et al., 1999), and so the zebrafish model is better suited to the study of innate immunity than adaptive.

In conclusion, we show that humanised zebrafish are a powerful tool to study the contribution of human-specific *S. aureus* virulence factors to infection outcome *in vivo*. Importantly, we believe that this model provides a starting point that can be further developed to incorporate additional human-specific pathogen–host interactions, functioning as an improved, expandable and translatable platform to accurately assess the efficacy of *S. aureus*-targeted therapeutic interventions.

MATERIALS AND METHODS

Cells and lentiviral transductions

Human monocytic U937 cells and HEK293T cells (purchased from and authenticated by ATCC) were grown in RPMI or DMEM (Lonza), respectively, supplemented with glutamine, penicillin/streptomycin and 10% fetal bovine serum (FBS). C5AR1 (CD88; NM_001736) and C5aR (XM_005159274) were cloned into a dual promoter lentiviral vector (BIC-PGK-Zeo-T2a-mAmetrine; RP172) described elsewhere (Tromp et al., 2018). This vector expresses the cloned transgene from an EF1A promoter as well as the fluorescent protein mAmetrine and the selection marker ZeoR from the PGK promoter. Third-generation lentiviral particles were produced in HEK293T cells following standard lentivirus production methods. Spin infection of U937 cells was performed by adding 100 µl virus supernatant with 8 µg/ml polybrene to 50,000 cells and spinning at 1000 ***g*** for 2 h at 33°C. Transduced cell lines were selected to high purity (>95%) by selection with 400 µg/ml Zeocin starting 2 dpf, and transgene expression was confirmed by flow cytometry using a mouse anti-FLAG M2 antibody (1:4000, F1804, Sigma-Aldrich) together with an APC or PE-conjugated secondary anti-mouse-IgG antibody (1:1000, Jackson) and acquisition on a FACSCantoII (BD Bioscience) cytometer. For expression of the zebrafish C5a receptor, the full-length mRNA coding sequence was used (XM_005159274.1). All cell lines used in this study have been regularly tested for potential mycoplasma contamination using the MycoAlert Kit (Lonza).

C5aR signalling assay

U937 cells were incubated with 2 mM Fluo-3AM (Thermo Fisher) in RPMI with 0.05% human serum albumin (HSA) at room temperature under constant agitation for 10 min following the manufacturer's instructions, then washed and suspended to 3×10^6 cells per ml in RPMI with 0.05% HSA followed by data acquisition on a FACSVERSE machine (BD Biosciences). Basal fluorescence level for each sample was determined during the first 10 s, followed by addition of the stimulus while continuing the acquisition to measure signalling-induced cytosolic Ca^{2+} release by increased Fluo-3AM fluorescence.

Flow cytometry analysis of C5a binding

Binding of FITC-labelled hsaC5a (human C5a) or dreC5a (zebrafish C5a) was determined by incubating U937 cells with 10 µg/ml FITC-C5a in RPMI supplemented with 0.05% HSA for 30 min on ice. After washing, the samples were analysed on a FACSVERSE machine (BD Biosciences).

Collection of zebrafish serum

Zebrafish blood was kindly supplied by Dr Astrid van der Sar (Amsterdam University Medical Centre) and serum collection was performed following a previously published protocol (Pedroso et al., 2012). Subsequently, 10% serum in 20 mM HEPES buffer containing 10 mM EGTA and 5 mM $MgCl_2$ was incubated with zymosan for 30 min at 37°C to activate the alternative complement pathway that results in C5a generation. The activated serum

was centrifuged at 10,000 ***g*** and the supernatant containing the anaphylatoxins was stored at −80°C.

Cell permeability assays

Cells were resuspended in 100 µl RPMI with 0.05% HSA and incubated for 30 min at 37°C with 10 µg/ml PVL or HlgCB (cloned and expressed as previously described in Spaan et al., 2013b, 2014; as PVL and HlgCB are two-component toxins, equimolar concentrations of polyhistidine-tagged LukS-PV, LukF-PV, HlgC and HlgB were used). Cells were then stained with 1 µg/ml DAPI (Molecular Probes/Thermo Fisher Scientific) and analysed on a FACSVERSE machine (BD Biosciences). Pore formation was defined as the percentage of cells positive for DAPI staining.

Recombinant protein production and FITC labelling

LukS-PV, LukF-PV, HlgC and HlgB were cloned and expressed as previously described (Perret et al., 2012; Spaan et al., 2013b, 2014). From the coding sequence of zebrafish C5 (XM_001919191.5) we identified a predicted C5a cleavage product (KFE DKA QKY GAF REY CLS GTR SSP TLE TCK DRA NRV TLP NKK TRR DYE KEK YCR LAF EQC CVF AKD LRK E) and included nine additional amino acids from an alignment with human C5 (NAE NII LSR). This sequence was codon-optimised for expression in *E. coli* K-12 and ordered as a gBlock (Integrated DNA Technologies). This was then ligated into the BamHI and NotI sites of the modified expression vector pRSETB (Invitrogen Life Technologies), containing a cleavable N-terminal poly-histidine tag and three glycine residues (6×His-TEV-GGG-dreC5a) or a non-cleavable N-terminal polyhistidine tag. Zebrafish C5a was expressed in *E. coli* strain Rosetta-gami(DE3)pLysS (Novagen; Merck Biosciences). Following cell lysis with 10 µg/ml lysozyme and three freeze–thaw sonication cycles in 20 mM sodium phosphate (pH 7.8), the His-tagged proteins were purified using nickel-affinity chromatography (HiTrap chelating, HP; GE Healthcare) with an imidazole gradient (10–250 mM; Sigma-Aldrich). Purified protein was stored in PBS at −20°C. Subsequently, the polyhistidine tag of 6×His-TEV-dreC5a was removed by incubation with TEV protease (Thermo Fisher Scientific) and the protein was FITC labelled at the N-terminus using the sortagging method (Popp et al., 2007). CHIPS protein was purified using previously published methods (de Haas et al., 2004; Haas et al., 2005).

Zebrafish husbandry

Zebrafish (*Danio rerio*) were raised and maintained according to standard protocols (Nüsslein-Volhard and Dahm, 2002) in UK Home Office-approved aquaria at the Bateson Centre, University of Sheffield, and kept under a 14 h light–10 h dark regime at 28°C.

Creation of *Tg(lyz:hsaC5AR1-Clover)sh505* zebrafish

The plasmid used for introducing the transgene into the zebrafish genome (pDestTol2CG2 *lyz:*hsaC5AR1-Clover *cmlc2*:eGFP) was created by Gateway cloning (Kwan et al., 2007). The *C5AR1* gene was PCR amplified from the pIRES-C5AR1 plasmid with a truncated stop codon to allow C′ terminal fusion of clover and ligated into the middle-entry clone vector pDONR221 (Kwan et al., 2007). The final construct was created by an LR reaction combining a 5′ vector containing the *lyz* promoter, the middle entry vector pDONR221 C5AR1-Clover, a 3′ vector containing the Clover fluorophore, and the destination vector pDestTol2CG2. To induce transgenesis, plasmid DNA of pDestTol2CG2 *lyz:*hsaC5AR1-Clover *cmlc2*:eGFP was injected into zebrafish embryos at the one-cell stage with 10 ng/µl of Tol2 transposase mRNA, according to published protocols (Nüsslein-Volhard and Dahm, 2002). At 3 dpf, positive transgenic larvae were selected and raised to maturity, then screened for successful germline integration of the construct.

Injection into the zebrafish otic vesicle

For mounting and injection, agarose plates were cast by dissolving 1% agarose in 1× E3 medium (made according to standard protocols; Nüsslein-Volhard and Dahm, 2002) and placing into triangular molds. Before injection, larvae were anaesthetised by immersion in 0.02% (w/v) tricaine prior to transfer to the mounting dish. Larvae were then arranged laterally in rows. Excess medium was then removed with a pipette to minimise movement during injection. Larvae were then injected from the dorsal side into the otic vesicle. At 4 h after injection, larvae were fixed for 1 h in 4% paraformaldehyde, and later stained with Sudan Black B to indicate neutrophils (performed according to published methods; Buchan et al., 2019b). Proteins were injected at the highest available concentration, which were 10 µM and 90 µM for hsaC5a (human) and dreC5a (zebrafish), respectively, and 30.3 µM and 16.7 µM for PVL and HlgCB, respectively.

Systemic infection of zebrafish embryos and *irf8* knockdown

Zebrafish larvae at 30 or 50 hpf were microinjected into the circulation with bacteria as previously described (Prajsnar et al., 2008). Briefly, anaesthetised larvae were embedded in 3% (w/v) methylcellulose and injected individually with 1 nl bacterial suspension of known concentration using microcapillary pipettes. Following infection, larvae were observed frequently up to 122 hpf and numbers of dead larvae recorded at each time point. Morpholino-modified antisense oligomer against and *irf8* (splice MO) (Li et al., 2011) was injected using a method described previously (Prajsnar et al., 2008).

Bacterial culture preparation

To prepare a liquid overnight culture of *S. aureus*, 5 ml of BHI broth medium (Oxoid) was inoculated with a colony of *S. aureus* strain USA300 and incubated at 37°C overnight with shaking. To prepare *S. aureus* for injection, 50 ml of BHI medium was inoculated with 500 µl of overnight culture and incubated for roughly 2 h at 37°C with shaking. The OD_{600} of each culture was measured, and 40 ml of the remaining culture harvested by centrifugation at 4500 ***g*** for 15 min at 4°C. The pellet was then resuspended in a volume of PBS appropriate to the bacterial dose required. Once the pellets were resuspended they were then kept on ice until required.

Zebrafish tailfin transection

Zebrafish at 3 dpf were anaesthetised by immersion in E3 medium supplemented with 4.2% tricaine and complete transection of the tail was performed with a sterile scalpel. For imaging of larvae, a Nikon custom-build wide-field microscope was used [NikonTi-E with a CFI Plan Apochromat λ 10X, N.A. 0.45 objective lens, a custom built 500 µm Piezo Z-stage (MadCityLabs, Madison, WI, USA) and using Intensilight fluorescent illumination with ET/sputtered series fluorescent filters 49002 and 49,008 (Chroma, Bellow Falls, VT, USA)]. Analysis was performed using Nikon's NIS Elements software package.

Injection of the somite tail muscle

Prior to injection, Petri dishes containing ∼25 ml of solidified 1% agarose supplemented with E3 medium were used to mount larvae. Using a P10 Gilson pipette tip, regular circular indentations were made in the surface, which are large enough for the larvae's head to fit, securing them for injection. Larvae were then anaesthetised by immersion in 0.06% (w/v) tricaine; which is three times the normal dose of tricaine – this is necessary to prevent movement during somite injection. Larvae were then transferred to the mounting dish, and arranged by placing the heads of the larvae in the indentations. Typically, larvae were oriented facing right, and imaged facing left to minimise distance between the site of injection and the objective during microscopy. Once mounted, the larvae were injected into the somite adjacent to the end of the yolk extension. This was facilitated by orienting the needle in line with the somite to maximise the area of injection. Once injected, larvae were washed off the plate with E3 medium and kept in a Petri dish containing 0.02% (w/v) tricaine prior to mounting. Larvae were then placed in 0.8% low gelling point agarose (Sigma) supplemented with 0.02% (w/v) tricaine. Larvae were then mounted onto a circular dish with a Menzel-Gläser #0 cover slip fastened to the bottom using vacuum grease, and oriented facing left. Analysis of neutrophil migration to the infection site was performed in Volocity®.

Statistics

All data were analysed in Prism 7.0 (GraphPad Software, San Diego, CA, USA). Comparisons between groups were performed using a two-way ANOVA with different multiple comparisons tests depending on whether the group was compared with a control (Dunnett's test) or not (Bonferroni's test). Significance was assumed at $P<0.05$.

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Competing interests
The authors declare no competing or financial interests.

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Data availability
The raw data for this article is available at Mendeley Data; doi:10.17632/x55r5h6j4g.2.

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