Humanisation of the zebrafish C5a receptor confers targeting by human-specific staphylococcal virulence factors.

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Summary

Staphylococcus aureus (*S. aureus*) possesses high host-specificity, including many human-specific virulence factors. Current efforts towards vaccine development have largely failed, citing inappropriate infection models and insufficient understanding of staphylococcal virulence. We sought to create a humanised zebrafish infection model susceptible to human-specific virulence factors, focusing on the human C5a receptor (C5AR1) which is targeted by three human-specific staphylococcal virulence factors, CHIPS, PVL, and HlgCB. We demonstrated that the zebrafish C5a receptor (C5aR) responds to serum-derived zebrafish C5a, mediates phagocyte recruitment, and is not targeted by any adapted staphylococcal virulence factors. *In vivo* expression of C5AR1 in zebrafish neutrophils conferred susceptibility to PVL and HlgCB and enhanced *S. aureus* infection. Lastly, we designed a humanised zebrafish C5aR with only three amino acid changes that maintains endogenous signalling capability yet gained sensitivity to CHIPS-mediated inhibition. We show that a partially humanised zebrafish is a valuable model for investigating host-specific virulence factors.

Introduction

Host-specialisation is a major trait observed in many bacterial pathogens and represents a major obstacle towards understanding infection *in vivo* (Bäumler and Fang, 2013). Among these host-specific pathogens is the gram-positive opportunistic pathogen *Staphylococcus aureus*, which 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 (Hersh et al., 2008; Graves et al., 2010; Kallen, 2010; Federspiel et al., 2012). 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 single infectious agent (Klevens et al., 2007), emphasising a need for alternative therapies. Despite promising results from *in vivo* studies targeting bacterial surface components and toxins (Shinefield et al., 2002; Weems et al., 2006; Moustafa et al., 2012; Fowler et al., 2013), these targets have largely failed to translate from traditional infection models into humans. This is attributed to the variety of virulence factors *S. aureus* uses to evade the immune system, as well as shortcomings that exist in current infection models (Mestas and Hughes, 2004; Salgado-Pabón and Schlievert, 2014a).

Many of these virulence factors exhibit a high degree of human specificity, notably, a single amino acid change in the secreted chemotaxis inhibitor CHIPS causes as much as a 100-fold loss in binding affinity to its target receptor *in vitro* (Haas et al., 2004a). These virulence factors perform many different functions that enhance bacterial virulence and many 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 (de Haas et al., 2004a; Rooijakkers et al., 2005; Spaan et al., 2013a, 2014; de Jong et al., 2017).

Three *S. aureus* virulence factors interact with the human C5a receptor (C5AR1, CD88), a seven-transmembrane loop G-protein coupled receptor (GPCR) that is highly expressed on the surface of neutrophils (Laumonnier et al., 2017) 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 would appear to be specifically focused against neutrophils, which form an essential line of defence against staphylococcal infection (Spaan et al., 2013b). 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., 2004b). In addition, 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 surface (Spaan et al., 2013a, 2014). These toxins are secreted in 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 chemotactic signalling by competitively inhibiting receptor signalling in a manner resembling CHIPS (Spaan et al., 2013a, 2014). The high-affinity protein-protein interactions between virulence factors and C5AR1 at the amino-acid level produce a highly human-specific interaction and, consequently, understanding of the roles that they play during infection is limited due to a lack of suitable humanised infection models (Proctor, 2012; Fowler and Proctor, 2014; Salgado-Pabón and Schlievert, 2014b). To fully understand the relative contributions of these and other virulence factors during natural infection, a new humanised infection model is urgently required.

The zebrafish (*Danio rerio*) is a useful model organism for investigating bacterial infections (Elks et al., 2013; Harvie et al., 2013; Torraca et al., 2017) and the innate immune system (Renshaw and Loynes, 2006; Hall et al., 2007; Henry et al., 2013). Due to its optical transparency, ability to produce high numbers of offspring, and genetic tractability, it offers many unique approaches over existing infection models. Zebrafish have a fully-developed innate immune system by 2 days post-fertilisation with mature phagocytic cells (Bennett et al., 2001; Lieschke et al., 2001; Ellett et al., 2011) and a complement system that is highly homologous to humans (Zhang and Cui, 2014). While components from all arms of complement (classical, alternative, lectin) are present in larval zebrafish (Wang et al., 2009;

Yang et al., 2014; Earley et al., 2018) and confer early humoral immunity to the embryo via maternal transfer (Wang et al., 2009; Ricklin et al., 2010), the zebrafish complement system is not well characterised, particularly in terms of function (Zhang and Cui, 2014).

In this study, we demonstrate that the zebrafish C5a receptor (C5aR) is a functional receptor that responds to recombinant and serum-derived zebrafish C5a *in vitro*, suggesting a role in zebrafish immunity resembling the human receptor. Next, by generating a human C5AR1 knock-in zebrafish model we were able to confer sensitivity of zebrafish neutrophils to the bicomponent toxins PVL and HlgCB *in vivo*, resulting in enhanced sensitivity of these zebrafish to *S. aureus* infection. Finally, we generated a minimally humanised C5aR variant that maintained functional C5a responses yet was robustly inhibited by CHIPS. Together, our studies show that partially humanised zebrafish represent a valuable platform to investigate the activities of human-specific virulence factors *in vivo* and emphasises the central role of pore forming toxins during infection *in vivo.*

Methods

Cells, Lentiviral transductions

Human monocytic U937 cells and HEK293T cells were obtained from ATCC (American Type Culture Collection) and grown in RPMI or DMEM medium, respectively, supplemented with glutamine, penicillin/streptomycin and 10% 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 (van de Weijer et al., 2014; 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 two days post transduction, and transgene expression was confirmed by flow cytometry using a mouse anti-FLAG M2 antibody (F1804, Sigma-Aldrich) together with an APC or PE-conjugated secondary anti-mouse antibody (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).

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, then washed and suspended to 3 million cells per mL in RPMI/0.05% HSA followed by data acquisition on a FACSVERSE (BD Biosciences). Basal fluorescence level for each sample was determined during the first ten seconds, followed by addition of the stimulus while continuing the

acquisition to measure signalling-induced cytosolic Ca^{2+} release by increased Fluo-3AM fluorescence.

Cell permeability assays

Cells were resuspended in 100 µL RPMI/0.05% HSA and incubated with for 30 min at 37 °C with 10 µg/mL PVL or HlgCB (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 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes/Thermo Fisher) and analysed on a FACSVERSE (BD Biosciences). Pore formation was defined as the percentage of cells positive to 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., 2013a, 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 glycines (6xHis-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 6xHis-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., 2004b; Haas et al., 2005).

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 minutes on ice. After washing the samples were analysed on a FACSVERSE (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 HEPES buffer was incubated with zymosan for 30 minutes at 37 °C to activate the alternative complement pathway that results in C5a generation. The activated serum was centrifuged at 10,000 rpm and the supernatant containing the anaphylatoxins was stored at -80°C.

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 14/10 hour light/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. 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 10ng/µl of Tol2 transposase mRNA, according to published protocols (Nüsslein-Volhard and Dahm, 2002). At three days post-fertilisation, positive transgenic larvae were selected and raised to maturity, then screened for successful germline integration of the construct.

Injection of the zebrafish otic vesicle

1% agarose dishes supplemented with E3 were cast using the triangular moulds described in (Bernut et al., 2015). 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 media was then removed with a pipette to minimise movement during injection. Larvae were then injected from the dorsal side into the otic vesicle. Four hours after injection, larvae were fixed for 1 hour in 4% paraformaldehyde, and later stained with Sudan Black B to indicate neutrophils. Proteins were injected at the highest available concentration, which were 10µM and 90µM for hsaC5a (human) and dreC5a (zebrafish) respectively, and 30.3µM and 16.7µM for PVL and HlgCB respectively.

Systemic infection of zebrafish embryos and *irf8* knockdown

Zebrafish larvae at 30 or 54 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 using microcapillary pipettes filled with the bacterial suspension of known concentration. 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).

Sudan Black B Staining

Sudan Black B staining was performed according to previously published methods (Buchan et al., 2019a).

Bacterial Culture Preparation

To prepare a liquid overnight culture of *S. aureus*, 5ml of BHI broth medium (Oxoid) was inoculated with a colony of *S. aureus* strain USA300 (Fey et al., 2013) and incubated at 37°C overnight with shaking. To prepare *S. aureus* for injection, 50ml of BHI media was inoculated with 500µl of overnight culture and incubated for roughly 2 hours at 37°C with shaking. The OD_{600} of each culture was measured and 40ml of the remaining culture harvested by centrifugation at 4500g for 15 minutes 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.

Statistics

All data were analysed in Prism 7.0 (GraphPad Software, San Diego, CA, USA).

Results

Zebrafish and human C5a-C5aR signalling is species specific

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 (Figure 1A), and measured the receptor's ability to bind and respond to recombinant zebrafish and human C5a (dreC5a/hsaC5a) using previously established methods (Spaan et al., 2013a). For clarity, human C5a will be referred to as 'hsaC5a', and zebrafish C5a as 'dreC5a'. As controls, we generated cells stably expressing the human C5a receptor (U937-hsaC5aR), or an empty vector control (U937-EV). Firstly, flow-cytometric analysis of recombinant, FITC-labelled C5a-binding capacities showed that U937-dreC5aR cells specifically bound dreC5a, but not hsaC5a (Figure 1B). Conversely, U937-hsaC5aR cells strongly bound hsaC5a and only marginally interacted with dreC5a, suggesting that both receptors retain the ability to bind C5a in a species-specific manner. Activation of GPCRs, including the C5a receptor, results in the activation of intracellular signalling cascades culminating in the cytosolic release of intracellular calcium 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 resulted in robust calcium release, indicative of successful receptor ligation and activation, whereas U937-hsaC5aR expressing cells responded minimally to dreC5a (Figure 1C). 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. Conversely, U937-hsaC5aR cells responded robustly to hsaC5a or human activated serum treatment, but not dreC5a treatment. Taken together, these data show 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 not targeted by 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 C5aR's expressed by several other species (Spaan et al., 2013a, 2014). However, their ability to target zebrafish complement components is unknown. Therefore, 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 signalling ability in the presence of CHIPS at concentrations that completely inhibited U937-hsaC5aR cells, indicating that CHIPS is ineffective at targeting the zebrafish receptor (Figure 2A). Furthermore, addition of purified, recombinant leukocidin components to U937-dreC5aR cells showed that these cells were insensitive to concentrations of PVL and HlgCB that readily permeabilised U937-hsaC5aR cells (Figure 2B,C). These data indicate that the zebrafish C5aR is insensitive to the inhibitory and/or lytic activity of *S. aureus* virulence factors CHIPS, PVL, and HlgCB at the concentrations tested.

Establishing a transgenic line expressing C5AR1-Clover in zebrafish neutrophils

As is the case for other animal models (de Haas et al., 2004b; Spaan et al., 2013a, 2014), zebrafish express a functional C5a-C5aR signalling axis that is insensitive to human-specific virulence factors, preventing the study of the role of these virulence factors in a non-humanised model. We therefore asked whether humanisation of zebrafish C5aR can confer sensitivity to C5a-targeting evasion molecules, thereby creating an *in vivo* model to study the role of these bacterial virulence factors during natural *S. aureus* infection. To this end, a transgenic construct directing expression of a fluorescent, Clover-tagged C5AR1 driven by the zebrafish neutrophil-specific *lyz* promoter (Hall et al., 2007) was introduced into the zebrafish

genome by Tol2 transgenesis (Kwan et al., 2007), producing the transgenic line *Tg(lyz:hsaC5AR1-Clover)sh505*. Expression of C5AR1-Clover in zebrafish neutrophils was confirmed by crossing this line to the established transgenic zebrafish line *Tg(lyz:nfsB-mCherry)sh260* that displays neutrophil-specific mCherry expression (Buchan et al., 2019a). Double-transgenic larvae have neutrophils positive for both mCherry and Clover, confirming that the C5AR1-Clover protein is expressed specifically in zebrafish neutrophils (Figure 3A,B). While 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 (Figure 3C,D). Notably, the total number of neutrophils in these fish was unaffected by transgene expression (Figure S2).

Zebrafish neutrophils expressing C5aR1 migrate to human C5a

Next, we investigated functional activity of human C5AR1 in the transgenic zebrafish line by injecting recombinant dreC5a and hsaC5a into the otic vesicles, two sac-like invaginations in the head of the fish that form part of the neural endoderm that later becomes the zebrafish inner ear (Alsina and Whitfield, 2017) and is a preferred site for assessing phagocyte migration (Benard et al., 2012; Deng et al., 2013). As expected, injection of dreC5a into non-humanised *lyz:*nfsB-mCherry fish resulted in migration of neutrophils to the injection site, while injection of hsaC5a did not affect neutrophil migration (Fig. 4A, B). In contrast, expression of the human *C5AR1* transgene in zebrafish neutrophils conferred the ability to migrate towards hsaC5a, showing that C5AR1 acts as a functional C5a receptor in zebrafish neutrophils that can direct neutrophil migration.

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

As human C5AR1 acted as a functional receptor in our zebrafish model and was targeted by human-specific virulence factors *in vitro*, we next asked whether neutrophil-specific expression of hsaC5aR increases the susceptibility of humanised fish to staphylococcal infection. To investigate this, we first separated 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 30hpf according to previously published methods (Prajsnar et al., 2008). Initially, we observed no difference in mortality between C5AR1-positive and -negative fish (Figure 5A). However, in previous work, it was described that bacteria present in the zebrafish circulation are predominantly phagocytosed and cleared by macrophages, while neutrophils phagocytose surface-associated bacteria at other sites (Colucci-Guyon et al., 2011). To specifically study the effect of humanised neutrophils in clearing *S. aureus* from the fish circulation, we silenced *irf8* expression using an *irf8* morpholino to alter zebrafish haematopoiesis and induce the differentiation of macrophages into neutrophils (Li et al., 2011). This results in zebrafish with a neutrophil-replete phenotype and minimal macrophage presence. By performing the same experiment in these neutrophil-replete zebrafish, we found that C5AR1-positive zebrafish were significantly more susceptible to staphylococcal infection than C5AR1-negative fish, both when infected at 30hpf or 50hpf (Figure 5B,C). Taken together, this suggests that expression of human C5AR1 in zebrafish neutrophils enhances susceptibility to staphylococcal infection.

Humanised zebrafish neutrophils are targeted by PVL and HlgCB

Having established that human C5AR1 expression in zebrafish neutrophils confers sensitivity to *S. aureus* infection, we next investigated whether C5AR1-expressing neutrophils are targeted by the human-specific *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 hours 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 C5AR1-negative fish (Figure 6A,B). This showed that C5AR1 expression sensitises zebrafish neutrophils to PVL *in vivo*. Similarly, we observed a reduced number of neutrophils at the injection site in humanised larvae injected with USA300+HlgCB (Figure 6C,D). Since the S-subunit of HlgCB (as well as PVL) can competitively inhibit C5AR1 signalling at low concentrations (Spaan et al., 2013a, 2014), we also evaluated the effect of injecting only HlgC on neutrophil numbers (Figure 6C,D). The absence of a significant difference in neutrophil numbers in the presence of HlgC only suggests that the cause of the reduced neutrophil number at the injection site is due to pore-formation and cell lysis rather than inhibition of C5aR signalling (Figure 6C,D). Together, these data indicate that the *Tg(lyz:hsaC5AR1-Clover)sh505* line exhibits susceptibility to human-specific pore-forming leukocidins PVL and HlgCB, and can be used to study the roles of these toxins *in vivo*.

A modified zebrafish c5ar1 with minimal residue changes becomes targeted by the human-specific factor CHIPS

While the gain-of-function approach of introducing human C5AR1 into zebrafish neutrophils conferred sensitivity to PVL/HlgCB, this strategy cannot be used to confer sensitivity to the third C5AR1-targeting *S. aureus* virulence factor CHIPS, as inhibition of the transgenic human C5aR by CHIPS will not prevent activation of the endogenous zebrafish C5aR. The CHIPS binding site in hsaC5aR was mapped to a stretch of 21 amino acids at the N-terminus ($C5AR1_{7\text{-}28}$) (Postma et al., 2005), and within this site the inhibitory activity of CHIPS is reliant on the presence of two O-sulfated tyrosine residues (Y_{11}, Y_{14}), four aspartic acid residues (D_{10}, D_{15}, D_{18}, D_{21}) which maintain a turn motif with CHIPS and stabilise hydrogen bonding, and two

leucine residues (L_{20}, L_{22}) which support hydrogen bonding and possess side chains that are in direct contact with the β-sheet surface of CHIPS (Chen et al., 1998; Ippel et al., 2009). We next aimed to harness this detailed knowledge of the CHIPS targeting site on C5AR1 to introduce minimal changes in dreC5aR to confer targeting by CHIPS while retaining normal receptor physiology. Notably, this may be complicated by the fact that hsaC5a also interacts with the receptor N-terminus (as well as the core region) via its C-terminal domain (Gerard and Gerard, 1994), suggesting that N-terminal changes in the CHIPS binding site may affect the ability of the receptor to respond to its dreC5a ligand. We therefore generated 10 variant *dreC5aR* receptors with 'human' residues in key regions of the CHIPS interaction site (Figures 7A). All variants maintained surface expression upon introduction into U937 cells (Figure S1) and all, with the exception of the fully humanised control, retained responsiveness to dreC5a similar to the wild type dreC5aR (Figure 7B). Importantly, two receptor variants (H and I, in addition to the fully humanised receptor), gained susceptibility to CHIPS-mediated inhibition of signalling upon recombinant dreC5a treatment (Fig. 7C). The presence of Y_{11}, D_{21} and L_{22} was enough to confer targeting (Figure 7C, variant H) and further deletion of N_{18} enhanced this effect (Figure 7C, variant I). Interestingly, deletion of N_{18} results in an aspartic acid (D) at position 18, replicating the importance of the D_{18} residue observed in other studies (Postma et al., 2005). Thus, we generated a minimally adapted humanised dreC5aR variant that retains dreC5a responsiveness yet is potently inhibited by CHIPS. Introduction of these three amino acid changes into the endogenous zebrafish *c5ar1* gene will be an important next step to study the contribution of CHIPS to *S. aureus* infection *in vivo*.

Discussion

Complete understanding of staphylococcal infection depends on the thorough characterisation of its virulence factors and an appreciation of their physiological relevance and synergistic role during the onset of infection. Due to a lack of appropriate animal models, human-specific

virulence factors have been difficult to adequately study *in vivo*, creating a gap in our understanding that hampers the experimental validation of effective *S. aureus* vaccine candidates. In this study, we showcase the versatility of the zebrafish as a model for investigating the roles of host-specific virulence factors during *S. aureus* infection.

The zebrafish complement system is only partly explored, although many genes orthologous to human complement genes have been identified genetically (Zhang and Cui, 2014). In addition, central components of all three complement pathways are found in zebrafish and related species (Wang et al., 2009; Hu et al., 2010; Yang et al., 2014; Earley et al., 2018) and have been shown to provide bacteriolytic defences in the developing embryo (Ricklin et al., 2010). The anaphylatoxins C3a and C5a and their cognate receptors C3aR and C5aR mediate phagocyte activation, chemotaxis to sites of infection and microbial clearance in neutrophils and macrophages (Ricklin et al., 2010). We demonstrate for the first time that the zebrafish C5a receptor (dreC5aR) acts as a functional G-protein coupled receptor in zebrafish and binds C5a in a host-specific manner. We determined that recombinant C5a and zymosan-activated zebrafish serum induces a comparable level of receptor signalling in *c5ar1*-expressing cells, indicating that receptor activation occurs at physiological levels of C5a. Additionally, we observed that zebrafish neutrophils are recruited to zebrafish C5a, suggesting that the dreC5a-dreC5aR signalling axis mediates neutrophil chemotaxis in zebrafish.

As a gain-of-function approach, we then expressed the human C5a receptor (C5AR1) in zebrafish neutrophils, establishing the transgenic line *Tg(lyz:hsaC5AR1-Clover)sh505*. C5AR1 was functionally expressed on the surface of humanised zebrafish neutrophils, and conferred responsiveness to human C5a, indicating that it acts as a functioning receptor in the zebrafish. Interestingly, double transgenic neutrophils were also observed migrating to zebrafish C5a, most likely due to signalling by the endogenous dreC5aR that remains intact in these fish. In addition to recruitment to hsaC5a, C5AR1 expressing fish also became more susceptible to staphylococcal infection when studied in a neutrophil-replete zebrafish. This

suggests that expression of C5AR1 significantly modifies the immune environment of the fish, as a result of targeting by human-specific virulence factors.

We also observed a C5AR1-dependent reduction in the number of neutrophils at the injection site, suggesting that *Tg(lyz:hsaC5AR1-Clover)sh505* neutrophils are effectively targeted by these toxins. We opted to inject purified proteins into the fish as it is unclear whether PVL or HlgCB would be produced at sufficient levels to induce pore-formation and cell lysis *in vivo*, however PVL is known to be produced in high quantities in abscesses (Badiou et al., 2008). A recent study showed that only the S-subunit of PVL targets C5AR1 while the F-subunit targets the protein tyrosine phosphatase CD45, an enzyme present on all nucleated immune cells (Tromp et al., 2018). Neutrophil reductions were only observed in the presence of both toxin components, suggesting that it is the result of cell lysis rather than inhibition of chemotaxis by the toxin's S-subunit alone. The fact that we still observe targeting in a non-human model possessing only C5AR1 suggests that zebrafish CD45 retains sufficient similarity to the human protein to confer receptor targeting by PVL.

To verify the host-specificity of C5AR1-targeting *S. aureus* virulence factors, we purified CHIPS, PVL and HlgCB and co-incubated them with U937-dreC5aR cells. We observed that compared with the human receptor, dreC5aR is insensitive to these virulence factors at concentrations that effectively inhibited C5AR1, consistent with previous studies that demonstrate their striking human specificity (Haas et al., 2004b; Spaan et al., 2013a, 2014). We then aimed to identify the minimal number of amino acid changes to the dreC5aR required to confer targeting by CHIPS by making single amino acid changes to the N-terminus, informed by our prior knowledge of CHIPS binding (Postma et al., 2005; Ippel et al., 2009). The CHIPS binding site in C5AR1 consists of a 21-residue region in the N-terminus (Postma et al., 2005) that includes two O-sulfated tyrosine residues (Y_{11}, Y_{14}), four aspartic acid residues (D_{10}, D_{15}, D_{18}, D_{21}) and two leucine residues (L_{20}, L_{22}). While nearly all variants we tested retained endogenous signalling in response to recombinant dreC5a, most of these variants remained insensitive to inhibition by CHIPS. Importantly, however, dreC5a-mediated

activation of two partly humanised variants was effectively inhibited by CHIPS. $Y_{11}DL_{21-22}$ (Figure 7, variant H) showed an ~80% reduction in signalling, and Y_{11}-$N_{17}DL_{21-22}$ (Figure 7, variant I) was inhibited by ~95%, similar to the hsaC5aR. This recapitulates the importance of the O-sulfated tyrosines in CHIPS binding and suggests that D_{21} and L_{22} are important in CHIPS binding in this context. Notably, $Y_{11}DL_{21-22}$ also retained endogenous signalling matching the wild type, producing a zebrafish receptor with only three amino acid changes that retains signalling while conferring almost complete inhibition of receptor signalling by CHIPS.

Our work shows that selective humanisation of the zebrafish can be a powerful approach towards studying host-restricted virulence of bacterial pathogens. There are several other GPCR's targeted by *S. aureus* in this manner. For example CXCR2 is targeted by three virulence factors, SSL5, Staphopain A and Leukotoxin ED, of which Staphopain A exhibits human-specific activity (Bestebroer et al., 2009; Laarman et al., 2012; Reyes-Robles et al., 2013; Buchan et al., 2019b). Additionally, the formyl peptide receptors (FPR1, FPR2) are targeted by CHIPS, FLIPr and SSL13 (de Haas et al., 2004a; Prat et al., 2006; Zhao et al., 2018).

The zebrafish shows much potential as a tractable model for studying species-specific pathogenesis. We show here, for the first time, that zebrafish have a functional C5a-C5aR signalling axis that mediates neutrophil recruitment. After humanising zebrafish neutrophils through transgenic expression of the human C5a receptor (C5AR1), we observed recruitment to human C5a, targeting by human-specific virulence factors PVL and HlgCB, and reduced survival during staphylococcal infection. Finally, we identified a variant of the zebrafish C5a receptor (dreC5aR) that was susceptible to targeting by a third human-specific virulence factor, CHIPS, after changing only three amino acids. These results show that highly specific host-pathogen interactions that are well understood *in vitro* are successfully recapitulated *in vivo* using this model and should lead to broader use of the zebrafish to study species-specific host-pathogen interactions.

Conclusion

In this study, we have successfully characterised the zebrafish C5a receptor, showing that it signals in a species-specific manner in response to physiological concentrations of zebrafish C5a. We also show that the receptor functions in a similar fashion to the human receptor on the surface of zebrafish neutrophils, that it can induce neutrophil recruitment and that it is insensitive to targeting by human-specific virulence factors. Conversely, we found that zebrafish neutrophils expressing the human receptor are targeted by human-specific virulence factors by generating a transgenic zebrafish line expressing C5AR1 on the neutrophil surface. These fish become significantly more susceptible to staphylococcal infection due to targeting by pore-forming leukocidins PVL and HlgCB, suggesting that humanisation of the zebrafish model to confer sensitivity to human-specific virulence factors is a viable approach for studying pathogenicity. Finally, we produced a signalling dreC5aR variant with only three amino acid substitutions that is targeted by the human-specific virulence factor CHIPS, suggesting that a partially humanised infection model is also a viable approach to studying pathogenesis in this model.

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Figure Legends

Figure 1. Zebrafish and human C5a-C5aR signalling is species specific.

A) Schematic of the stable introduction of C5aR-encoding plasmids into U937 cells by lentiviral transduction. Lentiviral particles used to transduce U937 cells were produced in HEK293T cells. Two days after transferring the virus-containing supernatant to U937 cells, successfully transduced cells were selected to >95% purity by treatment with 400 µg/ml zeocin. **B)** 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. **C)** Relative C5aR activity following treatment with recombinant human or zebrafish C5a or zymosan-activated serum was determined by measuring cytosolic calcium release using a Ca^{2+}-sensitive fluorescent probe (Fluo-3AM) and displayed as percentage relative to the positive control (10 µM

ionomycin treatment, set at 100%). Data are presented as means ± SD; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ANOVA with Dunnett's multiple comparisons correction of each sample versus the untreated control).

Figure 2. Zebrafish C5aR is insensitive to targeting by *S. aureus* virulence factors CHIPS, PVL, and HlgCB.

A) 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 mean percentage ± SD relative to the C5a-treated sample without CHIPS (middle bar, set at 100%). **B,C)** Pore formation in U937-dreC5aR (blue) or U937-hsaC5aR (red) cells following 30 min incubation with 10 µg/ml HlgCB (B) or 10 µg/ml PVL (C), as measured by percentage DAPI-positive cells by flow cytometry and expressed as means ± SD; ns, not significant; *, $p<0.05$; ***, $p<0.001$ (ANOVA with Dunnett's multiple comparisons correction (A) or *t*-test (B, C)).

Figure 3. Creation of a transgenic zebrafish expressing C5AR1-Clover.

A) A double-transgenic *Tg(lyz:hsaC5AR1-Clover)sh505; Tg(lyz:nfsB-mCherry)sh260* larva at 3dpf. Dashed white box indicates the enlarged region shown in B), scale bar = 250µm. **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 bar = 100µm. **C)** Close-up of Clover (green) and mCherry (purple) expression in a double-transgenic neutrophil in the caudal haematopoietic tissue of a 3dpf larva, scale bar = 10µm. **D)** Line intensity profile of the fluorescent signal of Clover (green) and mCherry (purple) across the yellow arrow shown in C).

Figure 4. Human C5AR1 is functionally expressed in transgenic zebrafish and confers sensitivity to hsaC5a.

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

Figure 5. Humanised C5AR1-positive zebrafish are more susceptible to staphylococcal infection.

A) Zebrafish larvae were separated into non-humanised (*lyz:*nfsB-mCherry only) and humanised (*lyz:*hsaC5AR1-Clover; *lyz:*nfsB-mCherry) groups and infected with ~1,800cfu of USA300 intravenously via the circulation valley. Survival was monitored up to 96 hrs post infection and data was analysed using a Log-rank Mantel-Cox test, (n=79-81 over three independent experiments) ns, not significant. **B, C)** Neutrophil-replete zebrafish were generated by silencing *irf8* expression through injecting an *irf8* morpholino at the single-cell stage. Following intravenous infection with USA300 at (b) 30hpf (~700cfu), or (c) 50hpf (~2,000cfu), zebrafish survival was monitored for 96 hrs. (b) n= 80-81 over three independent experiments; ***, $p<0.001$**.** (c) n=86-103 over three independent experiments; ****, $p<0.0001$.

Figure 6. Neutrophils of C5AR1-expressing zebrafish are susceptible to pore-formation by *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 ~3,500cfu of *S. aureus* USA300 with or without 30.3 µg/ml PVL. **B)** Number of neutrophils present at the otic vesicle at 4hpi, blue points denote the representative images in (a). The white outline indicates the otic vesicle. **C)** Representative images of neutrophil abundance in zebrafish larvae injected into the otic vesicle as in (a) with a vehicle control, or ~3,500 *S. aureus* USA300 with or without 16.7 µg/ml HlgCB or HlgC, as indicated. **D)** Number of neutrophils present at the otic vesicle at 4hpi, blue points denote the representative images in (c). Error bars shown are mean ± SD, groups were analysed using a two-way ANOVA and adjusted using Bonferroni's multiple comparisons test. (b) n=22-26 over two independent experiments and (d) n=32-41 over three independent experiments. ns, not significant; *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$. Scale bars = 200µm.

Figure 7. Three amino-acid changes in the CHIPS binding site of dreC5aR confer susceptibility to inhibition by CHIPS.

A) Overview of the ten dreC5aR (A-I and fully humanised) variants with (partially) humanised N-termini tested, with the altered amino acids indicated in red; numbers refer to amino acid positions in dreC5aR1. **B)** Relative C5aR activity in U937 cells stably expressing an empty vector control (Con), the wild type dreC5aR (WT), or the dreC5aR variants described in (A), assessed by measuring calcium mobilisation by flow cytometry using the fluorescent probe Fluo-3AM following incubation with 3.3 µM recombinant His-dreC5a. Data is presented as relative fluorescent signal compared to the wild type U937-dreC5aR (WT, set at 100%). **C)** C5aR activity determined as in (b) following incubation with 3.3 µM recombinant His-dreC5a in the absence or presence of 10 µg/ml recombinant CHIPS presented as percentage

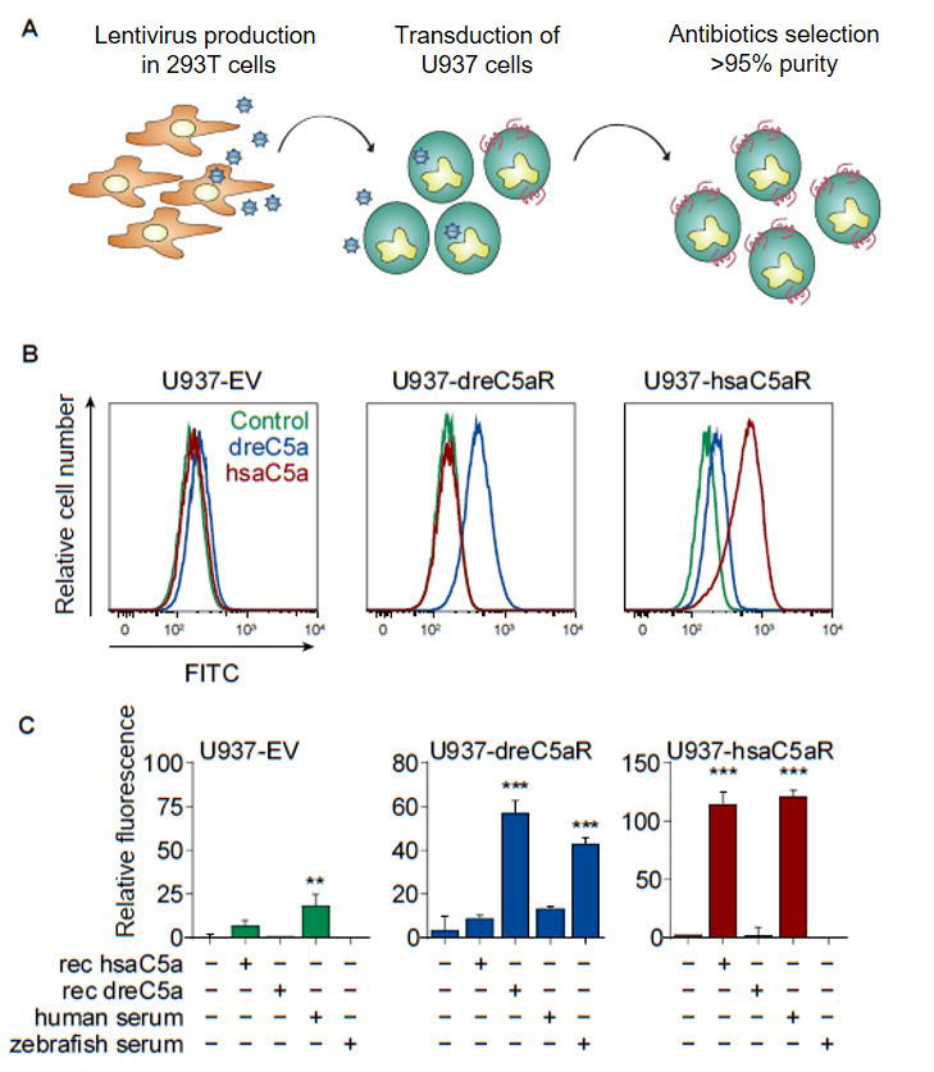
fluorescent signal of the CHIPS-treated sample relative to the CHIPS-negative control. All data are presented as means ± SD and were analysed using a two-way ANOVA with Dunnett's multiple comparisons correction; *, $p<0.05$; **, $p<0.01$; ns, not significant.

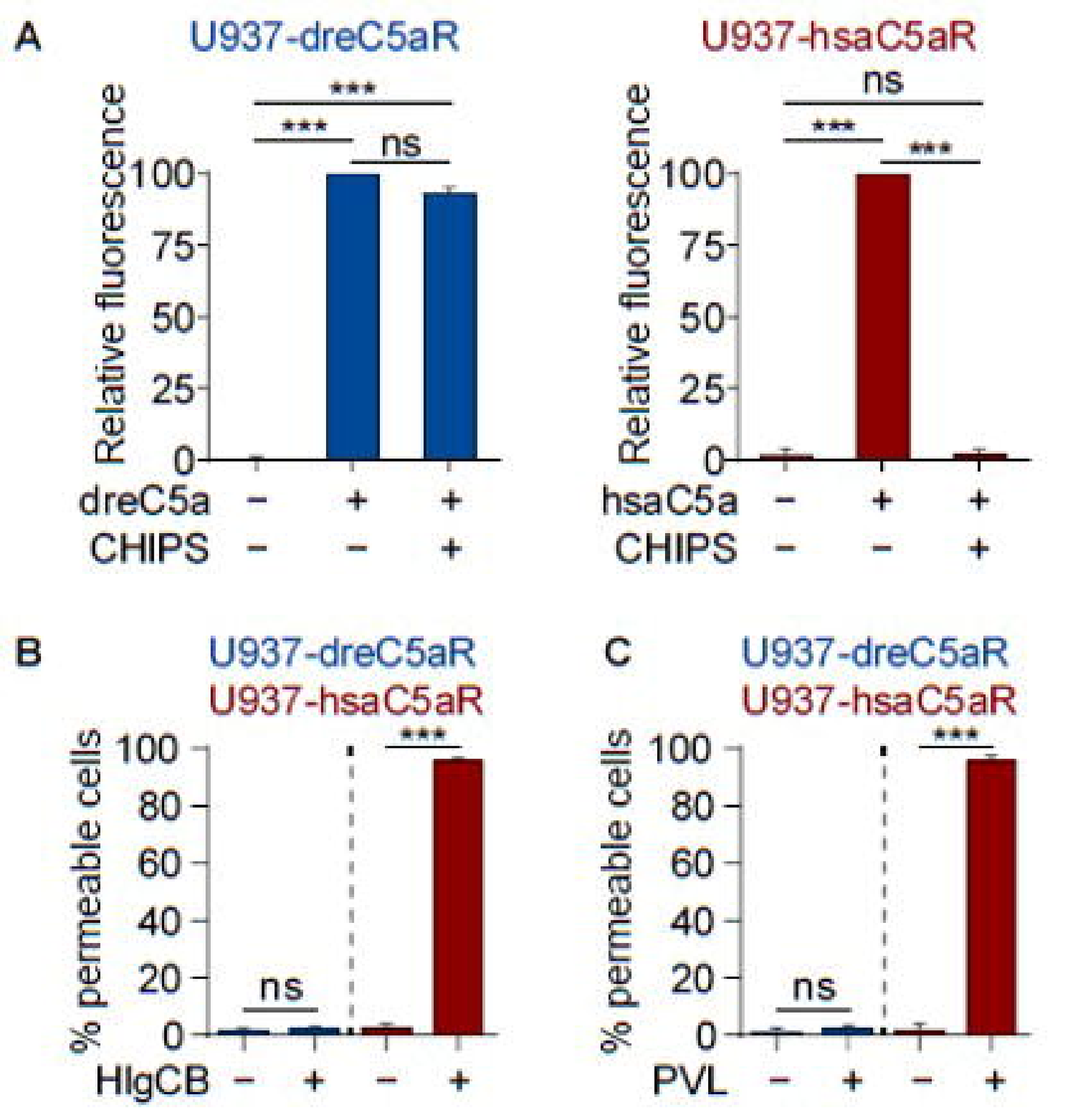
Supplemental Figure 1. Expression of receptor variants in U937 cells

Expression of FLAG-tagged wild type, partially humanised dreC5aR receptor variants A-I, and dreC5aR with a fully humanised CHIPS binding site ('humanised') on transduced U937 cells was evaluated by flow cytometry following staining with an anti-FLAG antibody and an APC-conjugated secondary antibody (blue). Control, empty-vector transduced U937 cells. Staining without the primary antibody (red) was included as negative control.

Supplemental Figure 2. Expression of the human C5a receptor does not interfere with zebrafish haematopoiesis

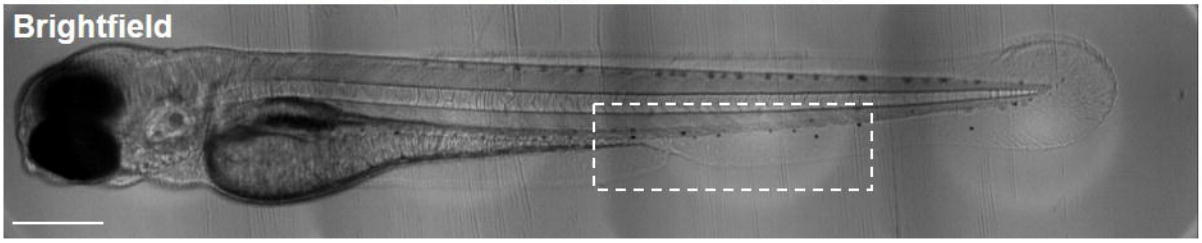
A) 4dpf larvae from a *Tg(lyz:hsaC5AR1-Clover)sh505 x Tg(lyz:nfsB-mCherry)sh260* cross, separated into non-humanised (*lyz:*nfsB-mCherry only) and humanised (*lyz:*hsaC5AR1-Clover; *lyz:*nfsB-mCherry) groups and stained with Sudan Black B to detect neutrophils. The white dashed box indicates the enlarged view alongside. **B)** Total body neutrophil counts from both groups. Values shown are mean ± SEM (n=50 over two experiments); groups were analysed using an unpaired *t*-test (two-tailed). ns, not significant.


A
Lentivirus production in 293T cells
Transduction of U937 cells
Antibiotics selection >95% purity
B
U937-EV
U937-dreC5aR
U937-hsaC5aR
Control
dreC5a
hsaC5a
Relative cell number
FITC
C
U937-EV
U937-dreC5aR
U937-hsaC5aR
Relative fluorescence
rec hsaC5a
rec dreC5a
human serum
zebrafish serum

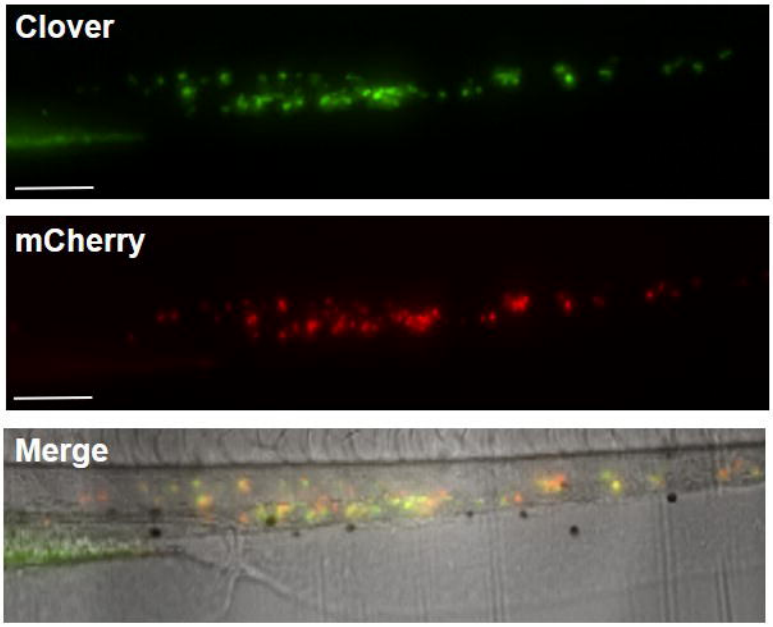

A
U937-dreC5aR
U937-hsaC5aR
Relative fluorescence
100
75
50
25
0
***
***
ns
ns
***
***
dreC5a − + +
CHIPS − − +
hsaC5a − + +
CHIPS − − +
B
U937-dreC5aR
U937-hsaC5aR
% permeable cells
100
80
60
40
20
0
ns
***
HlgCB − + − +
C
U937-dreC5aR
U937-hsaC5aR
% permeable cells
100
80
60
40
20
0
ns
***
PVL − + − +

A

Tg(lyz:hsaC5AR1-Clover)sh505 x Tg(lyz:nfsB-mCherry)sh260



B



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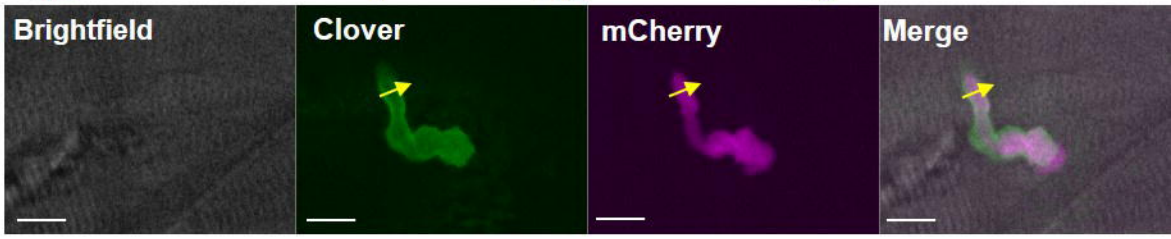
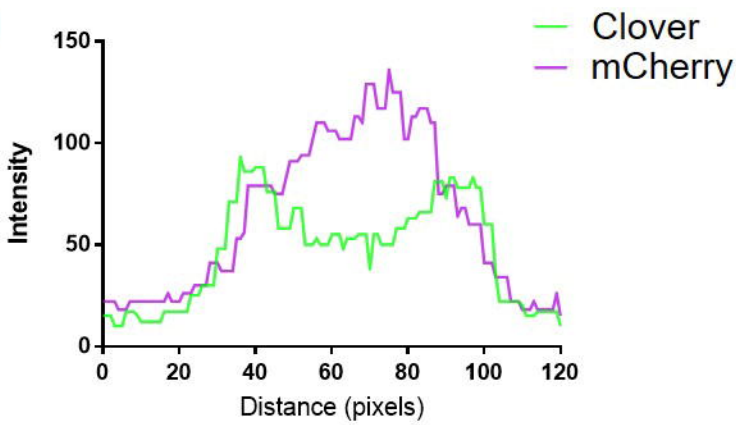
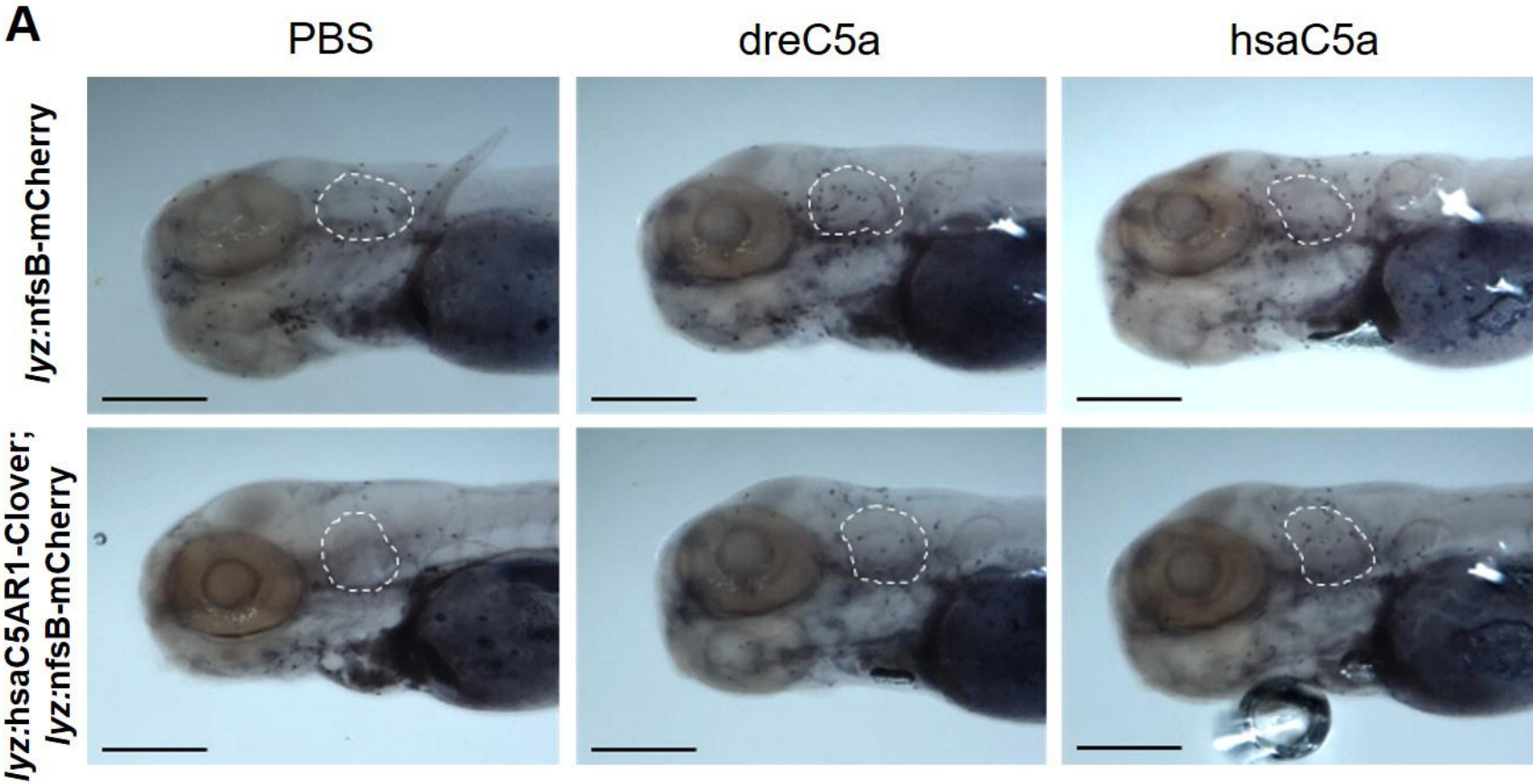
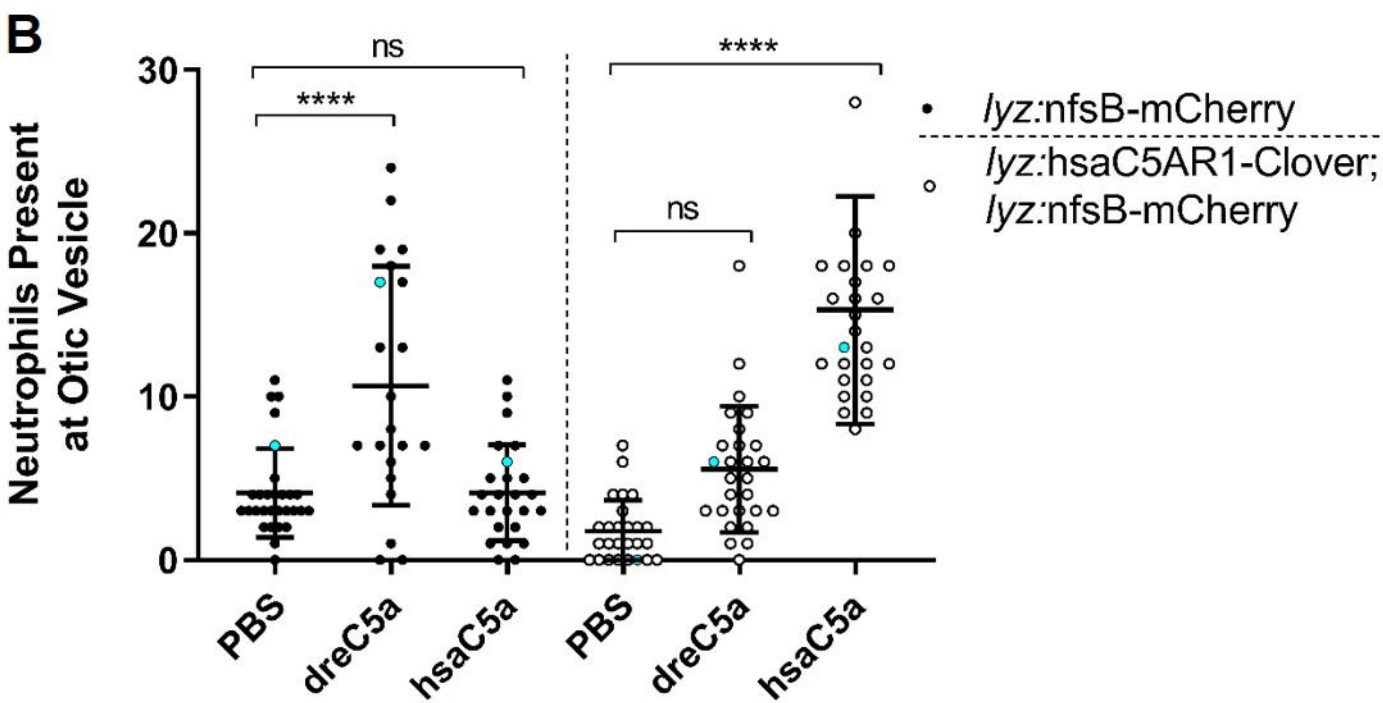
Tg(lyz:hsaC5AR1-Clover)sh505 x Tg(lyz:nfsB-mCherry)sh260

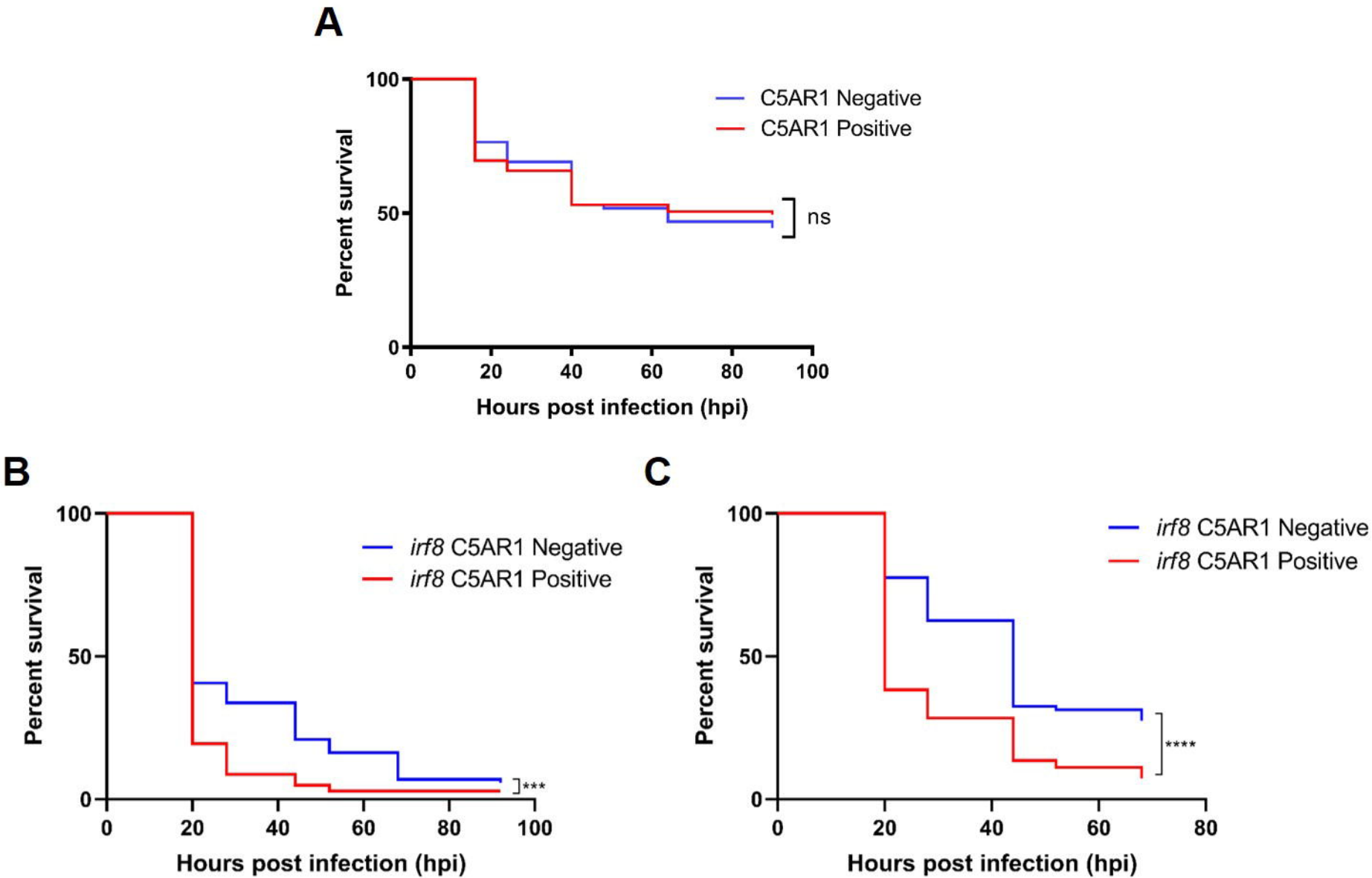


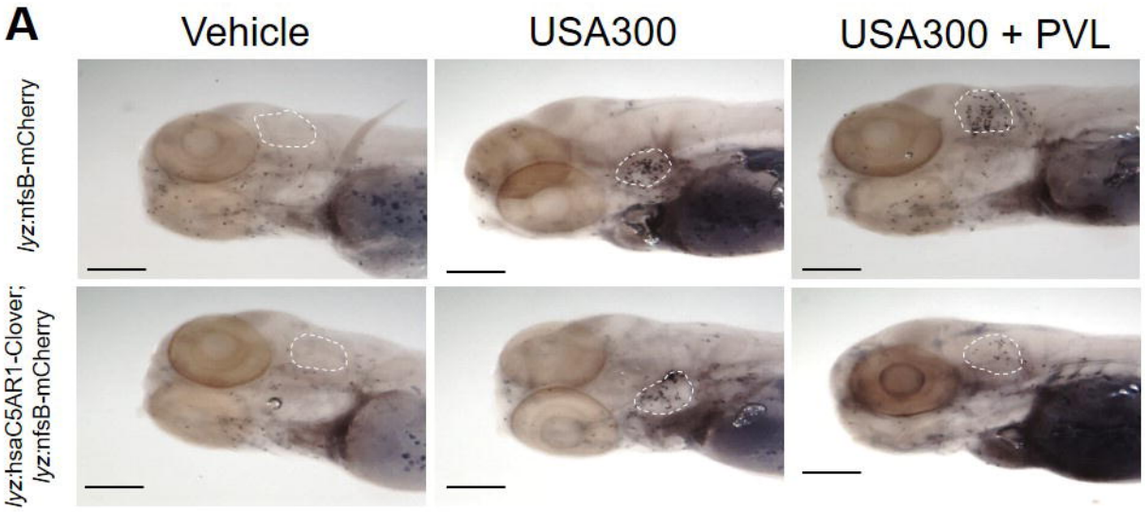
D

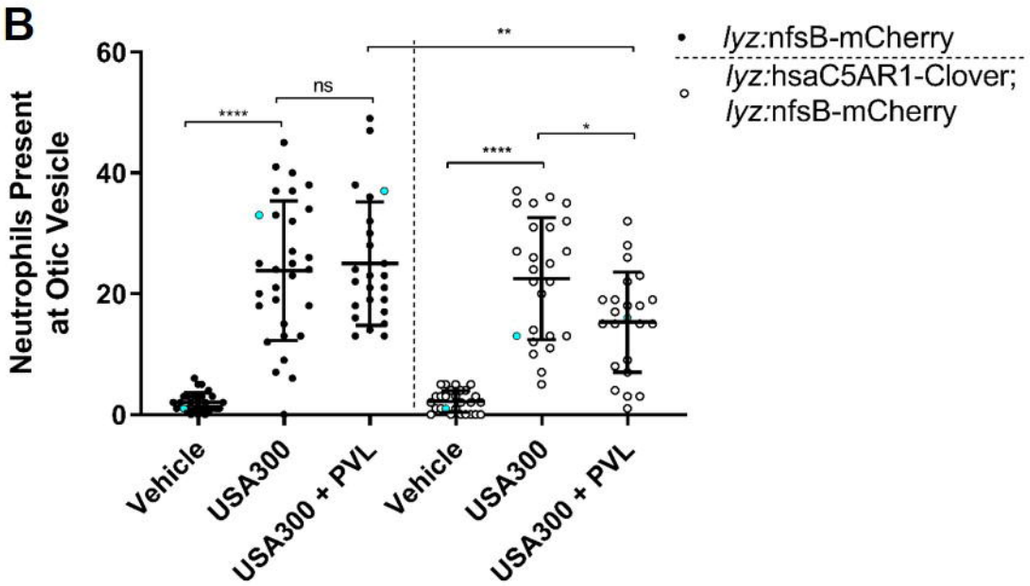


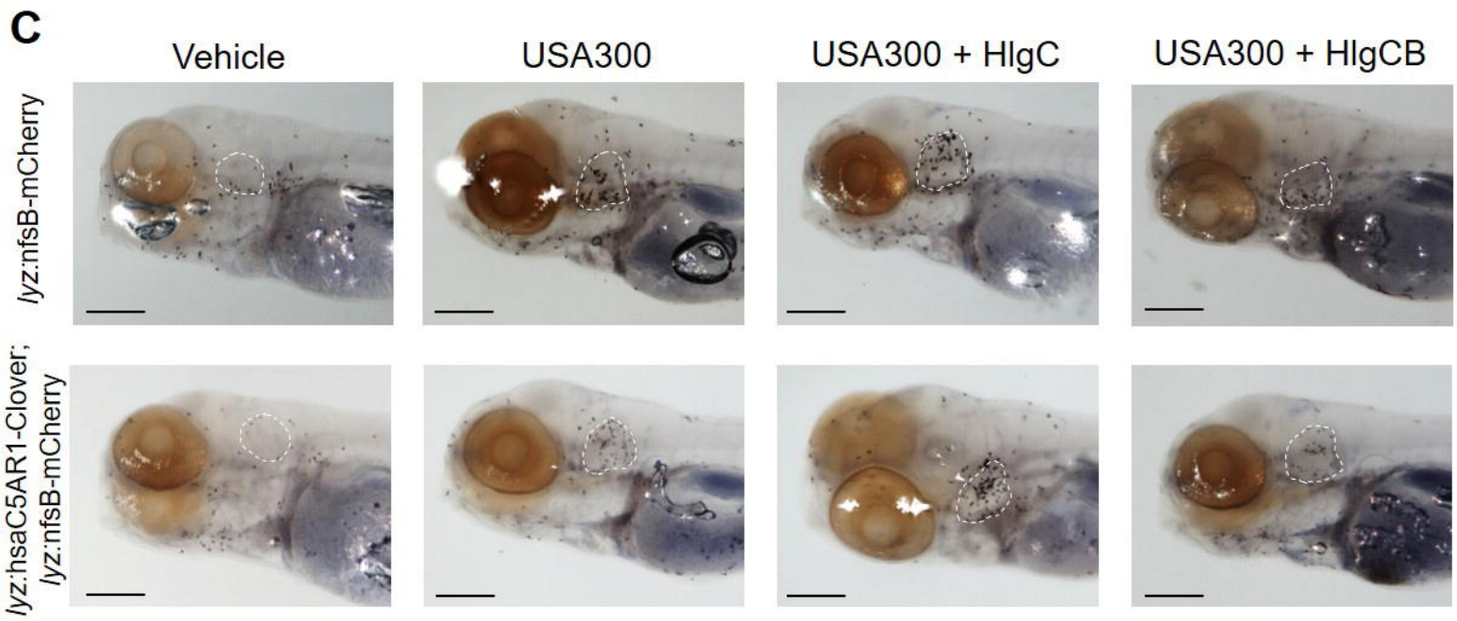

A
PBS
dreC5a
hsaC5a
lyz:nfsB-mCherry
lyz:hsaC5AR1-Clover;
lyz:nfsB-mCherry

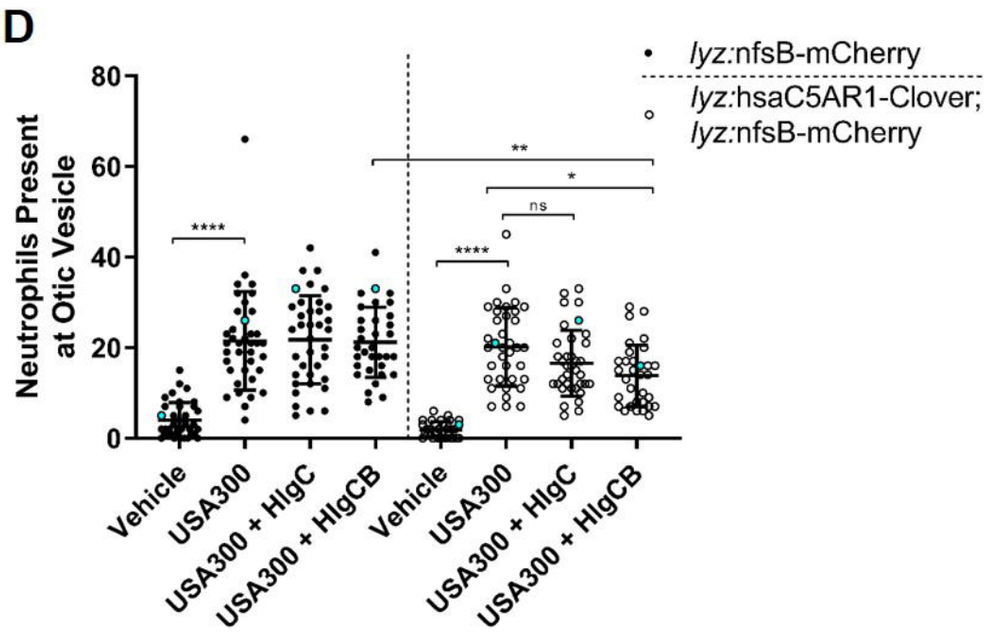

B
Neutrophils Present at Otic Vesicle
30
20
10
0
ns
****
****
ns
PBS
dreC5a
hsaC5a
PBS
dreC5a
hsaC5a
lyz:nfsB-mCherry
lyz:hsaC5AR1-Clover;
lyz:nfsB-mCherry


A
100
50
0
Percent survival
0
20
40
60
80
100
Hours post infection (hpi)
C5AR1 Negative
C5AR1 Positive
ns
B
100
50
0
Percent survival
0
20
40
60
80
100
Hours post infection (hpi)
irf8 C5AR1 Negative
irf8 C5AR1 Positive
***
C
100
50
0
Percent survival
0
20
40
60
80
Hours post infection (hpi)
irf8 C5AR1 Negative
irf8 C5AR1 Positive
****


A
Vehicle
USA300
USA300 + PVL
lyz:nfsB-mCherry
lyz:hsaC5AR1-Clover;
lyz:nfsB-mCherry


B
Neutrophils Present at Otic Vesicle
60
40
20
0
****
ns
**
****
*
Vehicle
USA300
USA300 + PVL
Vehicle
USA300
USA300 + PVL
lyz:nfsB-mCherry
lyz:hsaC5AR1-Clover;
lyz:nfsB-mCherry

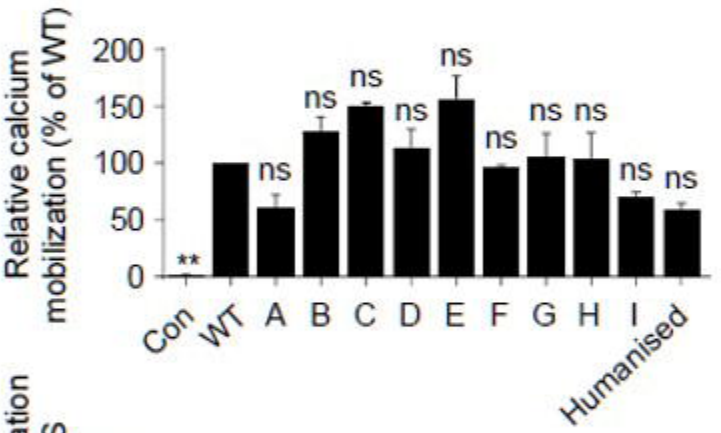
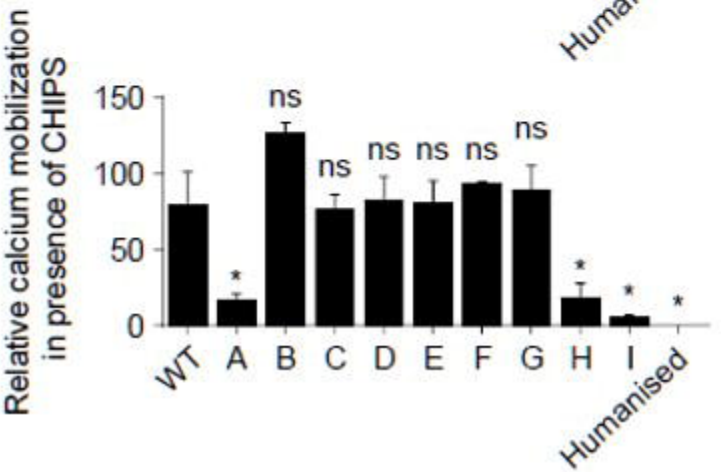

C
Vehicle
USA300
USA300 + HlgC
USA300 + HlgCB
lyz:nfsB-mCherry
lyz:hsaC5AR1-Clover;
lyz:nfsB-mCherry


D
Neutrophils Present at Otic Vesicle
80
60
40
20
0
****
**
*
ns
****
Vehicle
USA300
USA300 + HlgC
USA300 + HlgCB
Vehicle
USA300
USA300 + HlgC
USA300 + HlgCB
lyz:nfsB-mCherry
lyz:hsaC5AR1-Clover;
lyz:nfsB-mCherry

A

Position:	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Wildtype	D	W	T	S	Y	D	F	G	N	D	T	I	P	S	P
A	D	Y	G	H	Y	D	D	K	–	D	T	I	P	S	P
B	D	W	G	S	Y	D	F	G	N	D	T	I	P	S	P
C	D	W	T	S	Y	D	F	G	–	D	T	I	P	S	P
D	D	W	G	S	Y	D	F	G	–	D	T	I	P	S	P
E	D	Y	G	H	Y	D	F	G	N	D	T	I	P	S	P
F	D	W	T	S	Y	D	D	K	–	D	T	I	P	S	P
G	D	W	T	S	Y	D	F	G	N	D	T	I	D	L	P
H	D	Y	T	S	Y	D	F	G	N	D	T	I	D	L	P
I	D	Y	T	S	Y	D	F	G	–	D	T	I	D	L	P
Humanised	D	Y	G	H	Y	D	D	K	–	D	T	I	D	L	P

B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