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The Staphylococcal Toxin Panton-Valentine Leukocidin Targets Human C5a Receptors

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SUMMARY

Panton-Valentine Leukocidin (PVL) is a staphylococcal bicomponent pore-forming toxin linked to severe invasive infections. Target-cell and species specificity of PVL are poorly understood, and the mechanism of action of this toxin in *Staphylococcus aureus* virulence is controversial. Here, we identify the human complement receptors C5aR and C5L2 as host targets of PVL, mediating both toxin binding and cytotoxicity. Expression and interspecies variations of the C5aR determine cell and species specificity of PVL. The C5aR binding PVL component, LukS-PV, is a potent inhibitor of C5a-induced immune cell activation. These findings provide insight into leukocidin function and staphylococcal virulence and offer directions for future investigations into individual susceptibility to severe staphylococcal disease.

INTRODUCTION

In the early 1930s, Panton and Valentine described a powerful leukocidal toxin produced by multiple *Staphylococcus aureus* isolates. This leukocidin, now known as Panton-Valentine leukocidin (PVL), is cytotoxic to neutrophils and, to a lesser extent, to monocytes and macrophages, but not to lymphocytes (Gauduchon et al., 2001; Meyer et al., 2009; Panton and Valentine, 1932). PVL is a prophage-encoded, bicomponent, pore-forming exotoxin comprising the protein subunits LukS-PV and LukF-PV (Diep et al., 2006; Panton and Valentine, 1932). Initial binding of LukS-PV to the surface of target cells, which is an essential step, triggers secondary binding of LukF-PV (Colin et al., 1994). This subsequently induces the assembly of lytic pore-forming hetero-octamers (Jayasinghe and Bayley, 2005). At sublytic concentrations, PVL enhances host responses by priming of susceptible cells (Graves et al., 2012; Yoong and Pier, 2012). The in vitro susceptibility of neutrophils to PVL differs considerably among mammalian species (Hongo et al., 2009; Löffler et al., 2010; Olsen et al., 2010). While human and rabbit neutrophils are highly susceptible, murine and macaque neutrophils are resistant to PVL. The molecular basis for the selectivity of PVL for certain cell types and species is not understood, although the involvement of a myeloid-specific receptor has been suggested (Gauduchon et al., 2001).

S. aureus causes various diseases ranging from superficial skin and soft tissue infections (SSTI) to severe invasive disease (DeLeo et al., 2010). In the 1990s, methicillin-resistant *S. aureus* (MRSA) was found to infect previously healthy individuals. Since then, community-associated (CA) MRSA strains have rapidly emerged worldwide (Vandenesch et al., 2003). The majority of CA-MRSA isolates carry prophages with the genes encoding PVL, partially due to the successful spread of the PVL-carrying clone USA300 in the United States (DeLeo et al., 2010; Vandenesch et al., 2003). Based on epidemiological studies, noticeably those conducted in countries with a low prevalence of CA-MRSA, PVL is associated with skin and soft tissue infections (Shallcross et al., 2013) and severe necrotizing pneumonia in otherwise healthy individuals (Gillet et al., 2002; Hidron et al., 2009; Lina et al., 1999). However, the role of PVL is still under debate, partly due to the difficulty of addressing this issue in experimental mice models of disease (Bubeck Wardenburg et al., 2007; Labandeira-Rey et al., 2007; Voyich et al., 2006). In rabbit models of necrotizing pneumonia, PVL was recently confirmed to contribute to the severity of disease (Diep et al., 2010).

Species specificity is a common characteristic of other immune modulators secreted by staphylococci. This selectivity can often be reduced to high-affinity protein-protein interactions, depending on unique amino acid sequences of host targets (Rooijakkers et al., 2005). The chemotaxis inhibitory protein of *S. aureus* (CHIPS) targets the extracellular N terminus of the human C5a receptor (C5aR, CD88), a seven-transmembrane G protein-coupled receptor (GPCR) (Postma et al., 2005). C5a, a powerful anaphylatoxin, is released during complement activation. Detection of invading bacteria by phagocytes via the C5aR is considered one of the earliest innate recognition events (Woodruff et al., 2011). By inhibiting the human C5aR, CHIPS efficiently blocks neutrophil activation and recruitment in vitro (de Haas et al., 2004). C5a is a ligand for another seven-transmembrane receptor known as C5L2 (GPR77).

Much of the function of the latter receptor remains elusive. Although closely related to the C5aR, C5L2 is not coupled to a G protein and therefore has no direct intracellular signaling activity (Monk et al., 2007).

Here, we report the identification of the human C5a receptors, C5aR and C5L2, as receptors for LukS-PV and mediators of PVL-induced cytotoxicity. We illustrate that expression and interspecies variations of the C5a receptors drive cell and species specificity of PVL toxicity.

RESULTS

LukS-PV Binds the Human C5aR and C5L2

Figure 1A confirms that LukS-PV binds to human neutrophils and monocytes but not to lymphocytes (Colin et al., 1994; Gauduchon et al., 2001; Jayasinghe and Bayley, 2005), suggesting the involvement of a specific receptor. LukF-PV alone does not bind to any of the cell populations (Colin et al., 1994). Both components of PVL were tested for interference with the binding of monoclonal antibodies directed toward 56 different receptors on leukocytes as described earlier (Bardoel et al., 2012). Incubation of neutrophils and monocytes with LukS-PV specifically decreased the binding of three antibodies recognizing different epitopes of the human C5aR (Figure 1B), suggesting an interaction between LukS-PV and this receptor. As expected, LukF-PV alone did not inhibit the binding of any of the monoclonal antibodies. No interference was observed for binding of antibodies directed toward other receptors, as illustrated by the antibody for an unrelated GPCR, CXCR2 (Figure 1B). Binding of LukS-PV to neutrophils was inhibited by the ligand of the C5aR, C5a, and by the staphylococcal C5aR antagonist CHIPS (de Haas et al., 2004) (Figure 1C), further indicating an interaction between LukS-PV and the C5a receptor.

To confirm the interaction between LukS-PV and the C5aR, we tested LukS-PV binding to U937 promyelocytic cells stably transfected with the human C5aR. LukS-PV specifically bound to cells transfected with the human C5aR, but not to nontransfected cells or cells stably transfected with CXCR2 (Figure 1D). Since C5a is the ligand for the C5aR and the closely related receptor C5L2, LukS-PV binding to human embryonic kidney (HEK) cells stably transfected with the C5L2 was investigated. Indeed, LukS-PV bound to C5L2 transfected cells (Figure 1D). The half-maximal effective binding concentration of LukS-PV was calculated to be 6.2 nM (SD ±5.1) for the C5aR-transfected cells, 15.2 nM (SD ±6.2) for the C5L2-transfected cells, and 2.8 nM (SD ±1.2) for human neutrophils, without statistically significant differences (Figure 1E).

As LukS-PV binding is mediated by both C5a receptors, we evaluated relative receptor expression levels of the C5aR and C5L2 on leukocyte populations freshly isolated from different donors. Both receptors were present on neutrophils and monocytes but not detectable on lymphocytes (Figure 1F). In agreement with previous studies (Gao et al., 2005; Woodruff et al., 2011), we found that the ratio of C5aR to C5L2 was 68 on neutrophils (SD ±18.1, $p < 0.001$) and 33 on monocytes (SD ±13.1, $p < 0.01$). Thus, of both receptors, the C5aR will be most readily available for LukS-PV. Relative receptor expression levels of the C5aR on transfected cells used in this study were comparable to neutrophils. The C5L2 on the transfected cells was expressed in relatively high amounts compared to neutrophils (Figure S1A available online). The low level of C5L2 expression on neutrophils indicates that binding of LukS-PV to neutrophils is mainly driven by the C5aR.

Since the TLR2/CD14 receptor complex was recently reported to be involved in PVL-induced lung inflammation in mice (Zivkovic et al., 2011), we tested binding of LukS-PV to HEK cells cotransfected with the human TLR2 and CD14. However, no binding was observed (Figure S1B).

These data collectively demonstrate that LukS-PV binds both the human C5a receptors C5aR and C5L2. Binding of LukS-PV to neutrophils is mainly driven by the C5aR.

PVL-Induced Pore Formation Is Mediated by the Human C5aR and C5L2

To evaluate whether binding of LukS-PV to the C5aR and C5L2 mediates cytotoxicity of target cells, we tested induction of cell permeability using a membrane-impermeant fluorescent dye. Only cells transfected with the C5aR or C5L2 were susceptible to PVL-induced pore formation, while nontransfected cells or cells transfected with the CXCR2 were fully resistant (Figures 2A and 2B). We confirmed C5a-receptor-mediated cytotoxicity of PVL using crude bacterial supernatants of wild-type *S. aureus* USA300-LAC, a common CA-MRSA isolate in the United States, and its isogenic PVL mutant (*luks/f-pv* knockout) strain (Voyich et al., 2006). Pore formation was observed when supernatant of the wild-type bacterium was applied to C5aR- and C5L2-transfected cells. No lysis was induced by culture supernatant of the PVL-negative mutant strain (Figure 2C). Since PMA-differentiated THP-1 macrophages are sensitive to PVL-induced pore formation, we used small hairpin RNA (shRNA)-mediated silencing of C5aR expression to study the effect on PVL cytotoxicity. Silencing C5aR expression led to a strong decrease in LukS-PV binding and a concomitant reduction of PVL-induced pore formation in THP-1 macrophages (Figure 2D). In line with the negative binding experiments of TLR2/CD14-cotransfected cells, no pore formation was detected when PVL was applied to these cells (Figure S1C).

If the C5aR is the major PVL receptor, C5aR competition should interfere with toxicity. Indeed, pretreatment of human neutrophils with C5a or CHIPS shifted the half-maximal effective lytic concentration of PVL from 0.9 nM (SD ±0.2) for untreated cells to 3.1 nM (SD ±0.6) for C5a-treated cells ($p < 0.001$) and 3.5 nM (SD ±1.0) for CHIPS-treated cells ($p < 0.001$). The staphylococcal protein FLIPr-like, binding two unrelated GPCRs expressed on neutrophils (Prat et al., 2009), did not influence susceptibility of the cells (Figure 2E). However, because high concentrations of CHIPS were needed to antagonize PVL-induced pore formation (Figure S2A), we studied the expression of both CHIPS and PVL in the culture supernatant of *S. aureus* USA300-LAC (Figures S2B and S2C). In different broths, CHIPS was produced in insufficient amounts to compete with PVL for interaction with the C5aR (Figures S2C and S2D). The induction of pore formation by various PVL-carrying clinical *S. aureus* isolates was similar, irrespective of the presence of the gene encoding CHIPS (Figure S2E). Antagonism of PVL by CHIPS under physiological circumstances therefore seems unlikely.

Taken together, these data demonstrate that PVL uses both the C5aR and C5L2 to induce pore formation. Since

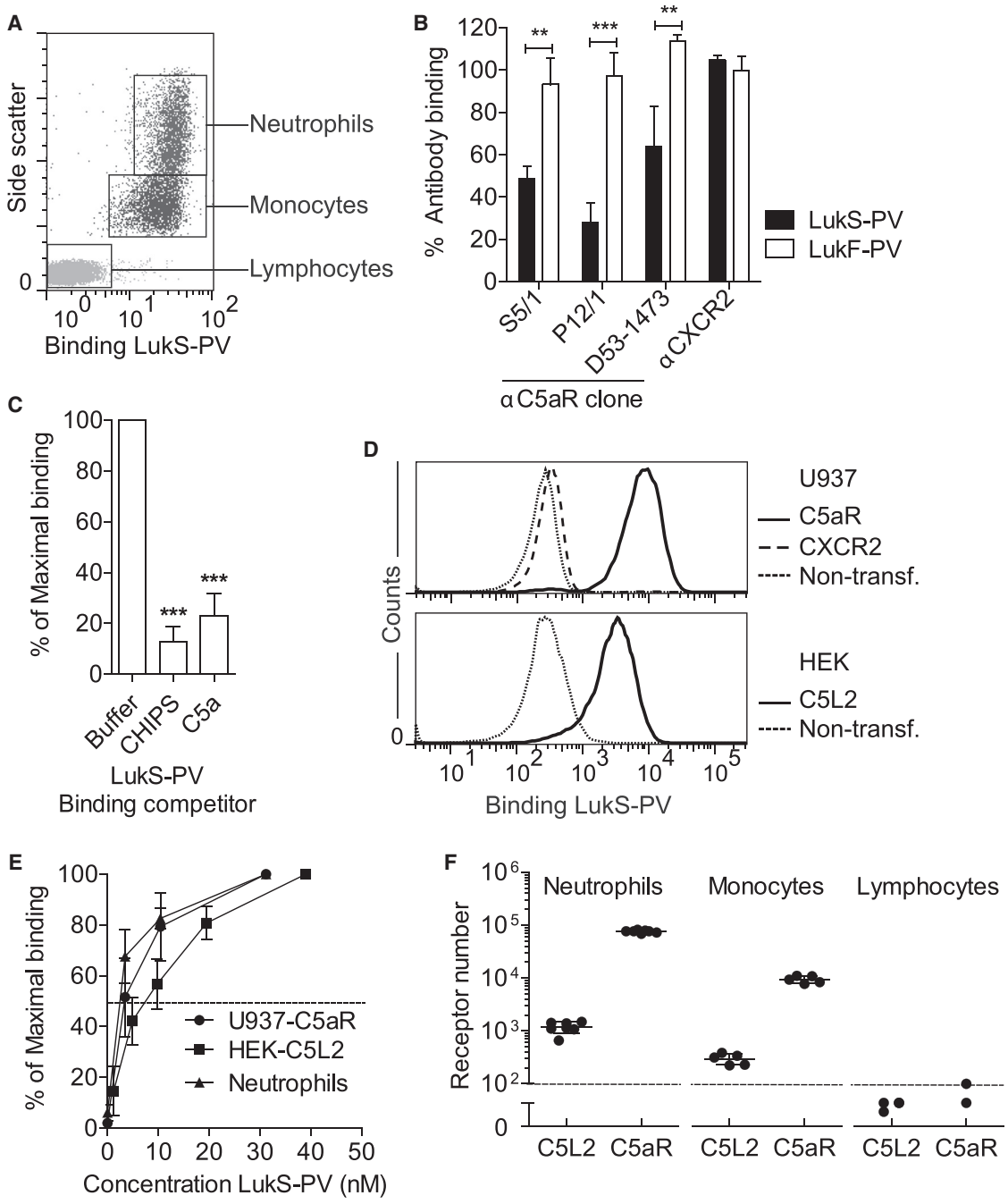

Figure 1. LukS-PV Binds the Human C5aR and C5L2

(A) LukS-PV (12 nM) binds human neutrophils and monocytes but not lymphocytes.

(B) Binding inhibition of different antibodies directed toward the human C5aR and CXCR2 by LukS-PV or LukF-PV (protein subunit concentrations, 313 nM).

(C) Binding competition of LukS-PV (60 nM) by C5a or CHIPS (600 nM) on neutrophils.

(D) LukS-PV (31 nM) binding to U937 and HEK cells.

(E) Concentration-dependent binding of LukS-PV to neutrophils and transfected cells, expressed in relation to maximal binding at 31 nM. The dashed line indicates 50% of maximal fluorescence.

(F) Receptor expression levels on neutrophils as compared to isotype control, quantified by calibration to defined antibody binding capacity units. The dashed line indicates the detection threshold. Bars represent SD, with n = 3. Statistical significance is displayed as $^*p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ using two-way ANOVA with Bonferroni posttest correction for multiple comparison. Histograms depict a representative example. See also Figure S1.

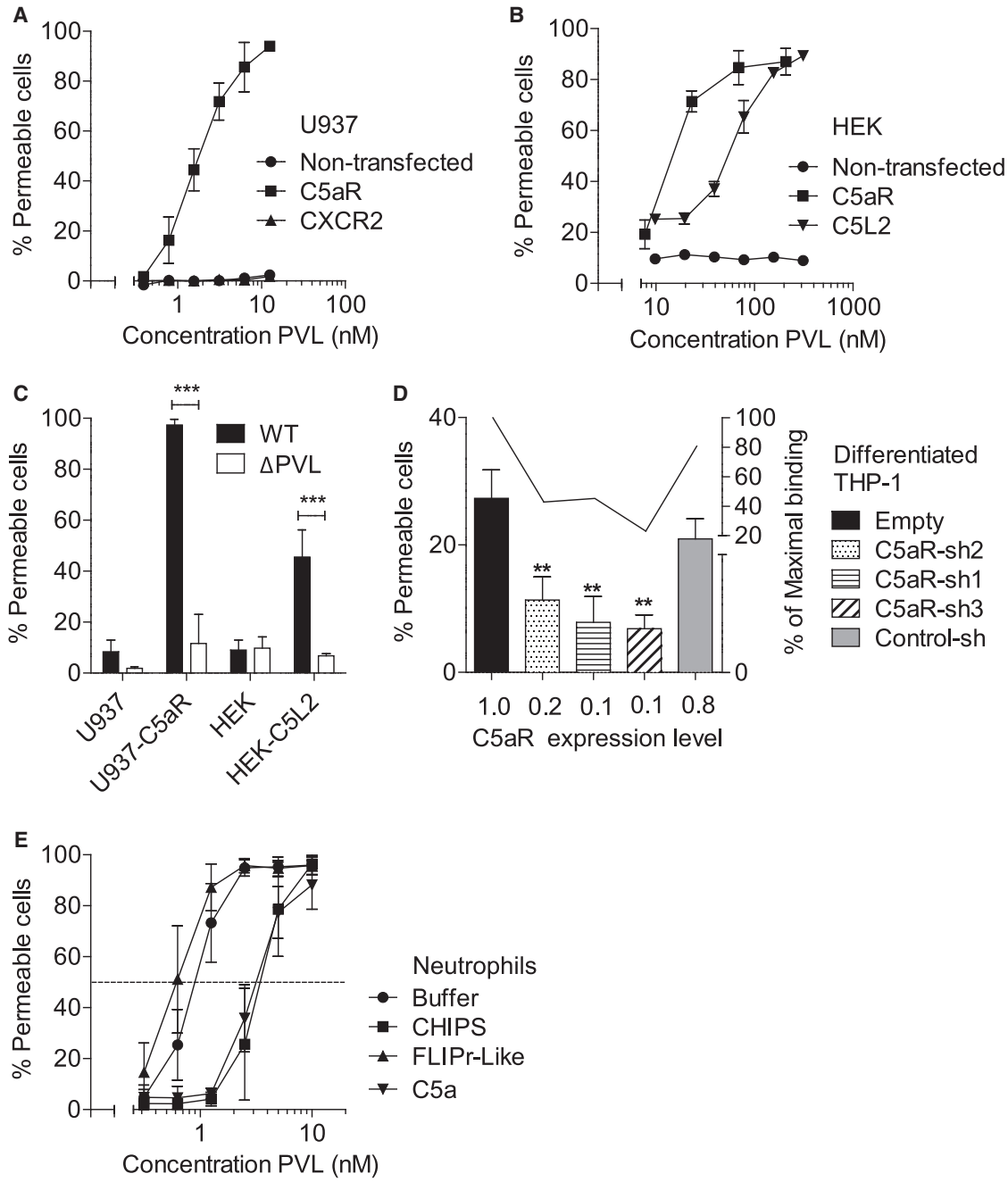

Figure 2. PVL-Induced Pore Formation Is Mediated by the Human C5aR and C5L2

(A and B) Pore formation in transfected U937 (A) and HEK (B) cells using different concentrations of PVL.

(C) PVL in bacterial supernatant induces C5aR- and C5L2-mediated lysis. Pore formation induced by 5% overnight CCY-based bacterial supernatant applied to transfected U937 and HEK cells.

(D) Binding of LukS-PV (LukS-PV concentration 31 nM, line) to and pore formation (PVL concentration 2.8 nM, columns) in PMA-differentiated THP-1 cells expressing different *c5ar1*-targeting shRNA, with relative C5aR mRNA expression levels as determined by quantitative RT-PCR. Binding of LukS-PV expressed as maximal binding to cells transduced with an empty construct.

(E) Pore formation inhibition in neutrophils by C5a (100 nM), CHIPS (710 nM), or FLIPr-like (833 nM). The dashed line indicates 50% permeable cells. Permeable cells are PI or DAPI positive. Bars express SD, with n = 3. Statistical significance is displayed as $^{**}p < 0.01$ using Student's t test. See also Figure S2.

the C5aR is most abundantly present, antagonizing the C5aR can protect human neutrophils against PVL-induced pore formation.

PVL-Induced Priming Is Mediated by the Human C5aR

Since sublytic concentrations of PVL were recently reported to prime neutrophil responses (Graves et al., 2012; Yoong and

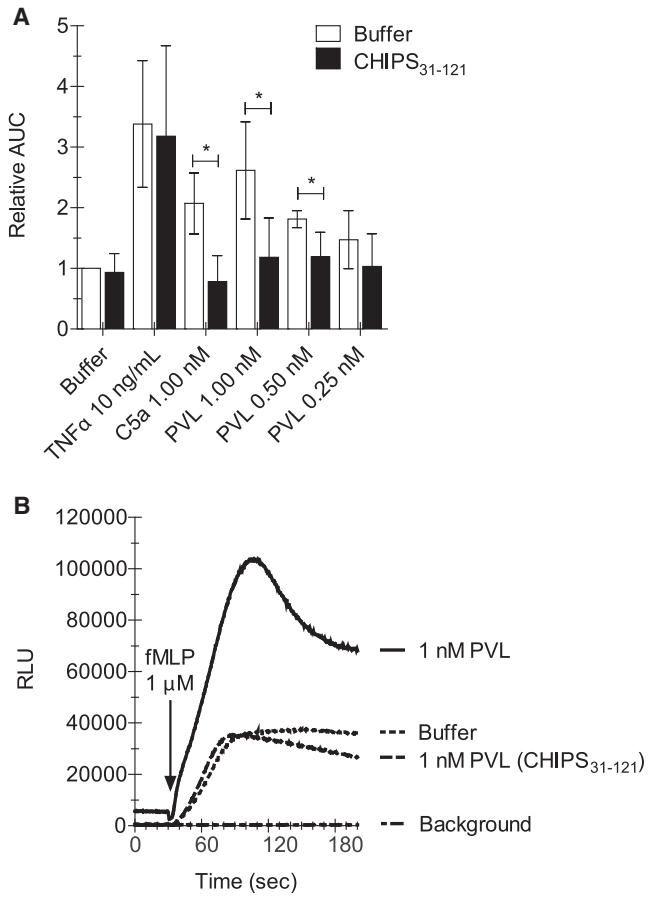

Figure 3. PVL-Induced Priming of Neutrophils Is Mediated by the Human C5aR

(A and B) Priming was antagonized by pretreatment of cells with 700 nM of the specific C5aR antagonist $CHIPS_{31–121}$. Shown in (A): oxidative burst of neutrophils induced by 1 μM fMLP after priming with 10 ng/ml TNF-α, 1 nM C5a, and 0.25 to 1 nM PVL. Oxidative burst, detected by luminescence, is expressed as relative area under the curve (AUC). Shown in (B) is a representative example of C5aR-dependent priming by PVL. The oxidative burst is expressed as relative light units (RLU) induced by an injection of 1 uM fMLP (arrow) after priming cells with 1 nM PVL. Bars express SD, with n = 2–6. Statistical significance is displayed as *p < 0.05 using Student's t test.

Pier, 2012), we tested if the immune-activating properties of PVL are mediated by the C5aR as well. Indeed, C5a, tumor necrosis factor α (TNF-α), and low concentrations of PVL increased the N-formyl-methionine-leucine-phenylalanine (fMLP)-induced oxidative burst by neutrophils. Priming by C5a and PVL, but not by TNF-α, was fully antagonized after preincubation of cells with the specific C5aR antagonist $CHIPS_{31–121}$ (Haas et al., 2005) (Figures 3A and 3B). This shows that priming induced by sublytic concentrations of PVL is mediated by the C5aR.

The C5aR Determines Species Specificity of PVL

Murine and macaque neutrophils do not bind LukS-PV and are resistant to PVL-induced pore formation, while human and rabbit neutrophils bind LukS-PV and are fully susceptible to PVL cytotoxicity (Figure 4) (Hongo et al., 2009; Löffler et al., 2010; Olsen et al., 2010). All species tested showed expression of the C5aR

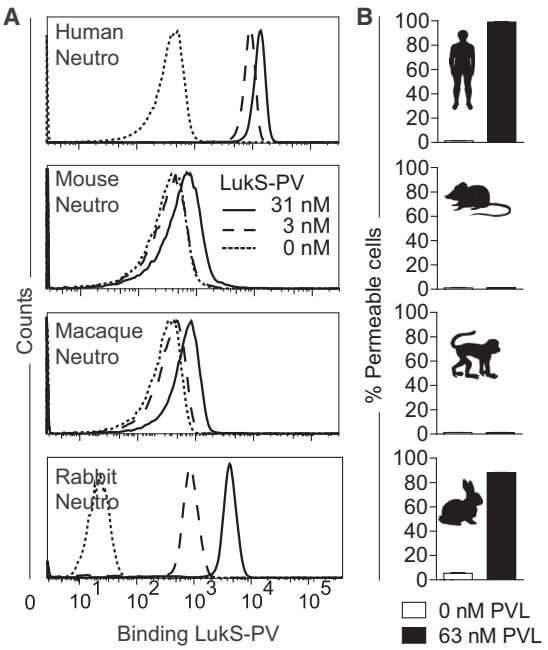

Figure 4. PVL Shows Species Specificity for Both Binding to and Pore Formation In Neutrophils

(A and B) LukS-PV binding to (A) and PVL-induced pore formation in (B) freshly isolated human, mouse, macaque, and rabbit neutrophils. Permeable cells are PI or DAPI positive. Bars express SD, with n = 2. Histograms depict a representative example. See also Figure S3.

on the surface of neutrophils (Figure S3). Therefore, species specificity of PVL could not be explained simply by the presence or absence of the C5aR. Because of significant interspecies variation within the amino acid sequences of the C5aR, we tested whether binding of LukS-PV to the C5aR could explain the previously observed species specificity of PVL (Hongo et al., 2009; Löffler et al., 2010; Olsen et al., 2010). Cells transfected with the murine or macaque C5aR showed no binding of LukS-PV (Figure 5A) and were fully resistant to PVL-induced pore formation (Figure 5B). However, cells expressing the rabbit C5aR bound LukS-PV comparable to the human C5aR and were highly susceptible to PVL-mediated pore formation. As murine and macaque neutrophils are intrinsically resistant to PVL (Löffler et al., 2010), the interaction of PVL with the C5L2 of both mammals was not further studied. In summary, these experiments show that interspecies variations of the C5aR determine species specificity of PVL.

The C5aR Core Region Is Critical for PVL-Induced Pore Formation

To evaluate which regions of the C5a receptors are targeted by PVL, we tested different receptor constructs in HEK cells for their ability to mediate LukS-PV binding and PVL-induced pore formation (Figures 6A and 6B). Cells transfected with the extracellular N termini of C5aR or C5L2 showed only weak binding of LukS-PV at high concentration. Moreover, binding to these N termini was insufficient to cause pore formation. This observation was confirmed by experiments with cells expressing a chimeric receptor composed of the C3a receptor (C3aR) core

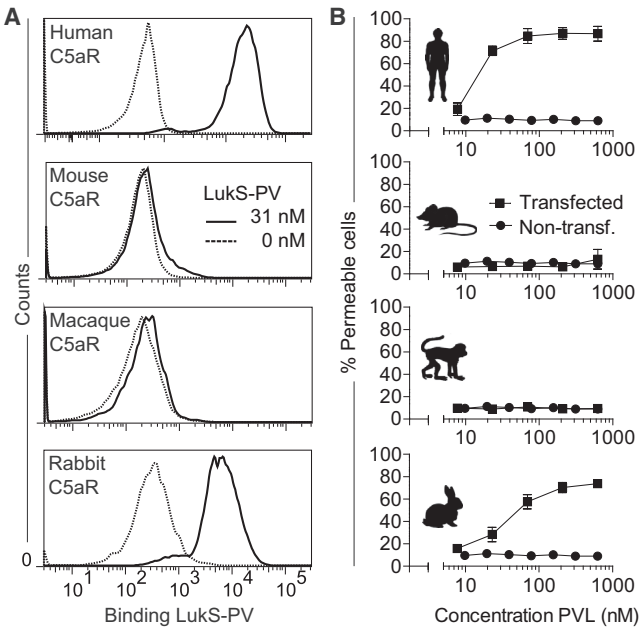

Figure 5. The C5aR Determines Species Specificity of PVL

(A) LukS-PV binding to HEK cells transfected with the human, mouse, macaque, and rabbit C5aR.

(B) PVL-induced pore formation in transfected HEK cells. Permeable cells are DAPI positive. Bars express SD, with n = 2–3. Histograms depict a representative example. See also Figure S4.

domain and the C5aR extracellular N terminus. Binding of LukS-PV to this chimera was exclusively observed at high concentration, and cells were resistant to PVL-induced pore formation. Transfected cells expressing the opposite chimera of the C5aR core domain and the C3aR extracellular N terminus showed similar binding but were partially susceptible to pore formation. These data show that LukS-PV binds both N terminus and core regions of the C5aR, while the latter of these two is critical for PVL to induce pore formation.

Since LukS-PV binds the N terminus of the C5aR, interaction of LukS-PV with the N terminus was further investigated in solid phase. Binding of LukS-PV to the N terminus was weaker as compared to CHIPS (Figure 6C) and dependent on sulphation of the receptor on residues 11 and 14 (Figure 6D). Sulphation of these residues is crucial for CHIPS to bind as well (de Haas et al., 2004). Using isothermal titration calorimetry, a K_D of 127 nM (SD ±17 nM) was calculated for LukS-PV to bind the C5aR N terminus (Figure 6E). This K_D is approximately 10-fold higher than that of CHIPS (de Haas et al., 2004).

Altogether, these findings indicate a complex interaction between LukS-PV and the C5aR as a whole but demonstrate that cytotoxic activity of PVL is highly dependent on the core regions of the C5aR.

LukS-PV Is a Potent Inhibitor of the Human C5aR

Activation of the C5aR by C5a results in a transient rise of intracellular calcium concentrations. When applied alone, LukS-PV and LukF-PV are not cytotoxic (Colin et al., 1994). As LukS-PV targets the C5aR, we tested if LukS-PV acts as a functional inhibitor of cellular activation by detection of intracellular calcium mobilization. LukS-PV, but not LukF-PV, strongly antagonized activation induced by C5a on both human neutrophils (Figure 7A) and U937 cells transfected with the C5aR (Figure 7B). Inhibition was independent of the N terminus of the C5aR, since LukS-PV also antagonized activation induced by the C-terminal C5a peptide Tyr65, Phe67 65–74 (Figure 7C). This peptide only interacts with the core region of the C5aR (Chen et al., 1998). In contrast, CHIPS binds the extracellular N terminus of the C5aR and therefore can only inhibit full-length C5a (de Haas et al., 2004). Collectively, these data identify LukS-PV as an inhibitor of the human C5aR, which is at least as potent as CHIPS. These experiments further reveal a complex interplay between the protein and the receptor, confirming an interaction of LukS-PV with the core region of the C5aR.

DISCUSSION

We show that LukS-PV binds the human C5a receptors C5aR and C5L2. As a functional bicomponent toxin, PVL uses the C5a receptors to induce pore formation in the target cell membrane. Receptor expression profiles of the C5a receptors on different cell populations explain 80-year-old observations in which PVL was reported to target neutrophils, monocytes, and macrophages, but not lymphocytes, since the latter are negative for expression of both C5aR and C5L2 (Gao et al., 2005; Gauduchon et al., 2001; Gerard and Gerard, 1991; Meyer et al., 2009; Monk et al., 2007; Panton and Valentine, 1932; Woodruff et al., 2011). The number of LukS-PV receptors estimated in a more recent study matches the combined expression level of both C5a receptors on neutrophils (Gauduchon et al., 2001). Although some nonmyeloid cells express both C5a receptors, expression levels are low compared to myeloid cells (Haviland et al., 1995). The abundant expression, notably on neutrophils, makes the C5aR a readily available target for the toxin and allows the toxin to distinguish phagocytic cells from other cells. In our experimental setup, the protection of human neutrophils against PVL-induced pore formation by the specific C5aR inhibitor CHIPS indicates that the C5aR is the major receptor for PVL on neutrophils, and C5L2 the minor receptor. Decreased susceptibility of C5aR-silenced THP-1 macrophages for PVL cytotoxicity supports the notion that the C5aR is the major PVL receptor and further indicates that the existence of another major PVL receptor is highly unlikely.

In a recent report, TLR2 and CD14 were presented as an essential receptor complex for PVL-induced lung inflammation in mice (Zivkovic et al., 2011). However, in another publication, antibodies specific for TLR2, TLR4, and CD14 failed to inhibit PVL-mediated priming of human neutrophils for enhanced function (Graves et al., 2012). We have shown that HEK cells stably transfected with the human TLR2/CD14 do not bind LukS-PV and are resistant to PVL-induced pore formation. Although we cannot exclude secondary effects mediated by the TLR2/CD14 receptor complex, it is clearly not involved in direct cytotoxicity of PVL since mice as well as mouse neutrophils are intrinsically resistant (Hongo et al., 2009; Löffler et al., 2010).

Our findings show that the immune-activating properties of PVL at sublytic concentrations, as observed by others (Graves et al., 2012; Yoong and Pier, 2012), are mediated by the human C5aR as well. Using in vitro infection experiments,

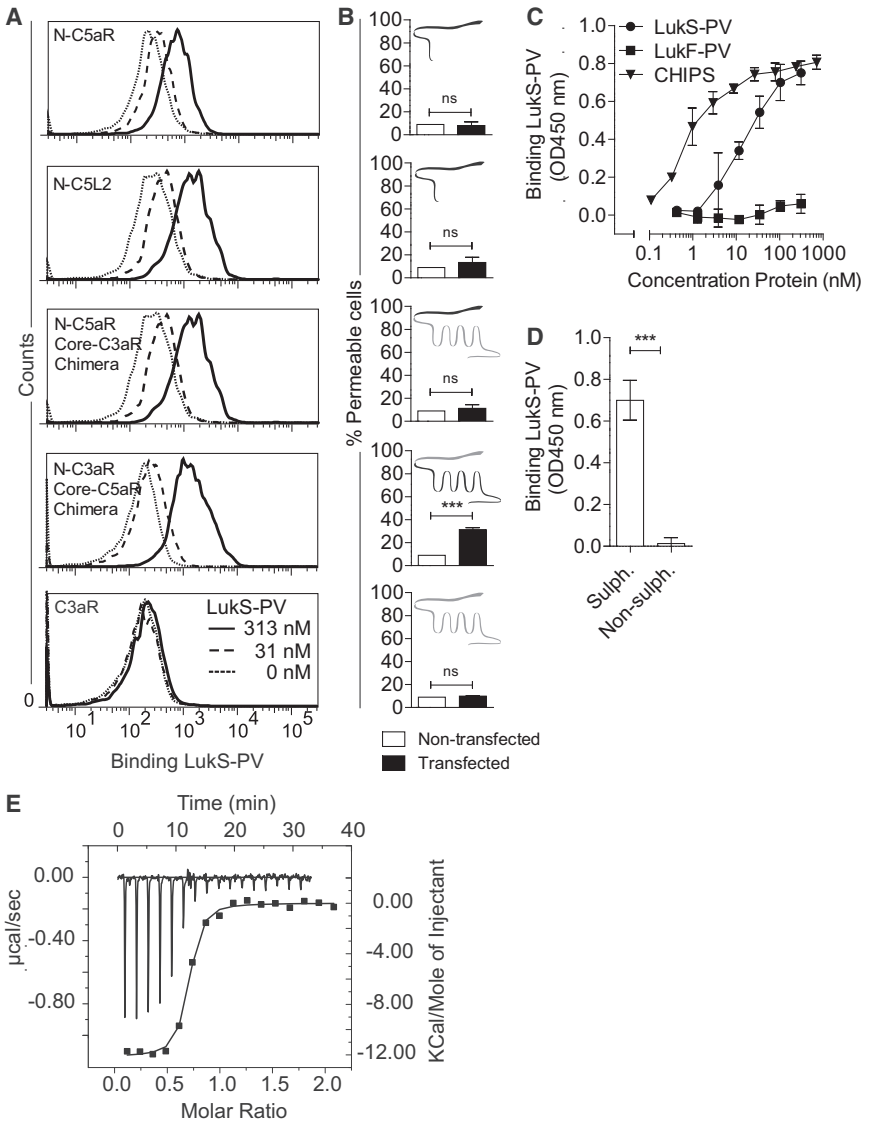

Figure 6. The C5aR Core Region Is Critical for PVL-Mediated Lysis

(A) LukS-PV binding to HEK cells transfected with the N termini of the C5aR or C5L2 or chimera receptors composed of the C5aR and C3aR.

(B) PVL-induced pore formation (PVL concentration, 625 nM) in transfected HEK cells.

(C and D) Binding of LukS-PV to the solid-phase C5aR N terminus on an ELISA plate (C) is dependent on sulphation of residues Y11 and Y14 (100 nM LukS-PV) (D).

(E) Isothermal titration calorimetry plot showing the association of the C5aR N terminus with LukS-PV. Permeable cells are DAPI positive. Bars express SD, with n = 3–4. Statistical significance is displayed as $^{**}p < 0.01$ or $^{***}p < 0.001$ using Student's t test. Histograms depict a representative example. See also Figure S5.

a PVL-dependent or C5aR-mediated phenotype of direct killing by *S. aureus* could not be obtained (data not shown). As shown by others, direct killing of neutrophils by *S. aureus* is independent of PVL (Ventura et al., 2010).

LukS-PV binds to both the N-terminal and core domain of the human C5aR. However, binding of LukS-PV to the host cell in itself is not sufficient to trigger pore formation. A specific interaction with the core domain, likely resulting from conformational changes, is required for cytotoxic activity. Because of the limited sequence homology of only 35% between the C5aR and C5L2, prediction of relevant amino acids is difficult (Monk et al., 2007). Most probably, major determinants will be present in the three extracellular domains. This hypothesis is supported by our observations that LukS-PV antagonizes stimulation with a C-terminal C5a peptide (Tyr65, Phe67 65–74), which only interacts with the core region of the C5aR (Chen et al., 1998). The affinity of LukS-PV for the C5aR as a whole, therefore, is predicted to be considerably higher than the one we calculated for the N-terminal C5aR alone. Next to being part of a two-component pore-forming toxin, we identified LukS-PV as a potent inhibitor of the human C5aR by antagonizing C5a-induced activation of neutrophils. This indicates that, next to CHIPS, *S. aureus* produces another molecule to interact with the C5aR (de Haas et al., 2004). When applied alone, LukS-PV and LukF-PV are not cytotoxic (Colin et al., 1994). Due to its genetic arrangement in an operon structure, secretion of LukS-PV by the pathogen is accompanied by the other protein subunit, LukF-PV (Colin et al., 1994; Diep et al., 2006), allowing formation of the cytotoxin on the target cell. Although LukS-PV antagonizes C5a-induced activation of neutrophils in vitro, it remains unclear if functional inhibition of the C5aR by LukS-PV alone contributes to pathogenesis of *S. aureus*. Since CHIPS is expressed at insufficient amounts by CHIPS-PVL coproducing *S. aureus* strains, antagonism of PVL-induced pore formation by CHIPS seems unlikely under physiological circumstances. Insight into the interaction of LukS-PV with the C5a receptors will be of value for the development of anti-inflammatory drugs in C5a-mediated diseases (Woodruff et al., 2011).

Recently, the staphylococcal leukocidin combination LukED was reported to target the human immunodeficiency virus coreceptor CCR5, which like the C5aR is a member of the GPCR family (Alonzo et al., 2013). Although not fully explaining the cell selectivity of LukED, the interaction of LukED with CCR5 highlights the relevance of CCR5-positive leukocytes in the pathogenesis of *S. aureus*. Together with the identification of the C5aR and C5L2 as the PVL receptors presented here, the report on LukED and CCR5 suggests an apparent common interaction of staphylococcal bicomponent pore-forming toxins with GPCRs.

PVL is an example of bacterial toxins and modulators, especially of staphylococcal origin, exhibiting pronounced species

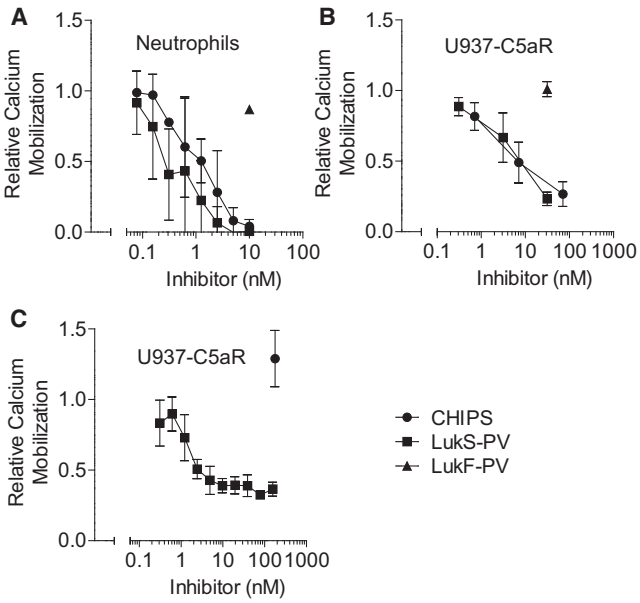

Figure 7. LukS-PV Inhibits the C5a-Induced Cell Activation

(A–C) Calcium mobilization with 1 nM C5a (A and B) in human neutrophils (A) and U937-C5aR cells (B) or 2.5 μM C-terminal C5a (C) in U937-C5aR cells inhibited by increasing concentrations of LukS-PV and CHIPS. Bars express SD, with n = 3.

specificity based on molecular compatibility with host targets (de Haas et al., 2004; Prat et al., 2009; Rooijakkers et al., 2005). Over the last few years, many studies have been published addressing the contribution of PVL to virulence of *S. aureus* in animal models of infection. Confusing reports on experiments in mice have provoked an intense international debate both for SSTI and necrotizing pneumonia (Bubeck Wardenburg et al., 2007; Labandeira-Rey et al., 2007; Voyich et al., 2006). With our proof of C5aR-mediated interspecies susceptibility for PVL-induced cytotoxicity, animal studies addressing the role of PVL in pathogenesis of *S. aureus* can now be better interpreted. We have demonstrated that cells transfected with the mouse and even macaque C5aR are fully resistant, while cells transfected with the rabbit C5aR are susceptible to PVL-induced pore formation. This is in line with previously described species-specific susceptibility of primary cells in vitro (Hongo et al., 2009; Löffler et al., 2010). Our data showing clear C5aR-mediated interspecies susceptibility for PVL-induced cytotoxicity offer a molecular explanation for the observed stronger relevance of rabbit versus mice models to study PVL-associated pathogenesis. Although the contribution of PVL to skin infections in rabbits is still under debate (Kobayashi et al., 2011; Lipinska et al., 2011), experiments by others in rabbits confirm PVL as a phagocyte-targeting virulence factor in *S. aureus* necrotizing pneumonia (Diep et al., 2010). Unfortunately, the staphylococcal C5aR antagonist CHIPS does not bind the rabbit C5aR (de Haas et al., 2004) and a monoclonal anti-human C5aR antibody does not protect against PVL-induced pore formation in rabbit neutrophils (data not shown), limiting us to investigate the contribution to staphylococcal pathophysiology of the interaction between PVL and the C5aR of a compatible species. It remains to be elucidated if lung epithelial cells are damaged indirectly by PVL-affected neutrophils and macrophages (Niemann et al., 2012; Perret et al., 2012) or directly via low expression of the C5aR (Haviland et al., 1995).

The underlying mechanisms for the predisposition of otherwise healthy human individuals to infrequent but highly lethal necrotizing pneumonia are poorly understood. Although often preceded by influenza-like symptoms contributing to susceptibility (Gillet et al., 2002; Hidron et al., 2009), human genetic factors might account for a poor outcome (Alcaïs et al., 2009). Variation in the expression of the C5a receptors is a likely candidate to explore for genetic determinism in PVL-associated disease.

EXPERIMENTAL PROCEDURES

Recombinant Protein Production and Purification

Polyhistidine-tagged LukS-PV and LukF-PV, CHIPS, $CHIPS_{31–121}$, and FLIPr-like were cloned and expressed as described elsewhere (de Haas et al., 2004; Haas et al., 2005; Perret et al., 2012; Prat et al., 2009). The *S. aureus* strains used as template are shown in Table S1.

Bacterial Strains and Culture

LAC is a USA300 pulsotype CA-MRSA isolate. LAC*Δpvl,* its isogenic PVL knockout strain, was donated by Frank R. DeLeo (Rocky Mountain Laboratories, Hamilton, MT, USA) (Voyich et al., 2006). All strains were cultured in casein hydrolysate and yeast extract (CCY) medium (optimal expression of leukocidins) (Lipinska et al., 2011; Woodin, 1959), tryptic soy broth, or Iscove's modified Dulbecco's medium (IMDM). Overnight supernatants were sterilized by a 0.2 μm filter and stored at −20°C. Experiments with clinical *S. aureus* isolates and quantification of CHIPS and PVL expression are described in Supplemental Experimental Procedures.

Cell Isolation, Cell Lines, and Transfections

Human leukocytes, obtained from healthy volunteers, and macaque (*Macaca fascicularis*) and rabbit (New Zealand white) leukocytes were isolated by Ficoll/Histopaque centrifugation (de Haas et al., 2004). Murine leukocytes (BALB/c) were derived from bone marrow. U937 cells (a human promyelocytic cell line) stably transfected with the C5aR, CXCR2, or an empty expression vector were obtained from Eric R. Prossnitz (University of New Mexico, Albuquerque, NM, USA) (Kew et al., 1997). HEK293T cells (a human embryonic kidney cell line) stably transfected with the C5L2 were donated by Peter N. Monk (Sheffield University Medical School, Sheffield, UK) (Kalant et al., 2003). HEK293T cells coexpressing TLR2 and CD14 were obtained from InvivoGen.

HEK293T cells were transfected according to the manufacturer's protocols with 4 μg DNA and 5 μl Lipofectamine 2000 (Invitrogen) for full-length receptors or with 2 μg DNA and 13 μl Polyethylenimine MAX (PolySciences) for N-terminal receptors. After 24–48 hr, transfected cells were harvested with 0.05% trypsin/0.53 mM EDTA. Cells transiently transfected with the full-length receptors were labeled with mouse anti-FLAG clone M2 (Sigma) followed by PE-labeled goat anti-mouse antibody (Dako). Cells transiently transfected with the N-terminal receptors were stained with mouse anti-hemagglutinin A clone 12CA5 (Abcam) followed by PE-labeled goat anti-mouse. Only receptor-expressing cells were included in analyses (Figures S5 and S6). Cells were cultured in Dulbecco's modified Eagle's medium (Lonza) supplied with 10% fetal calf serum (Invitrogen). For receptor expression determination on neutrophils of different species, see Supplemental Experimental Procedures. All in vitro experiments with cells were performed with RPMI (Invitrogen) supplemented with 0.05% human serum albumin (HSA; Sanquin), with cell concentrations adjusted to 5×10^6 cell/ml.

Design of Full-Length Receptor and N Terminus-Expressing Plasmids

Full-length human C5aR, C5L2, C3aR, mouse (C57BL/6) C5aR, macaque (*Macaca fascicularis*) C5aR, and rabbit (New Zealand white) C5aR were cloned into a pcDNA3.1 vector (Invitrogen) as described previously (Postma et al., 2005). Briefly, the amplification reactions were performed on QUICK-Clone

complementary DNA of human bone marrow or mouse liver (BD Biosciences Clontech) using PfuTurbo DNA polymerase (Stratagene). The C5aR of macaque and rabbit, which reside on a single exon, were amplified using chromosomal DNA isolated from blood. The following human C5aR/C3aR chimeras were constructed using overlap extension PCR: N-C3aR/TM-C5aR, in which amino acids 1–37 from the C5aR were substituted for amino acids 1–23 from the C3aR, and N-C5aR/TM-C3aR, in which amino acids 38–350 from the C5aR were substituted for amino acids 24–482 from the C3aR (Postma et al., 2005). To confirm surface expression of each described GPCR, an N-terminal FLAG tag (DYKDDDDK) was placed after the first methionine. An extra methionine was included directly after the FLAG tag to keep the N-terminal sequence intact. N termini of the human C5aR (amino acid 1–38) and C5L2 (amino acid 1–37), including a hemagglutinin A epitope tag, were cloned into a pDISPLAY plasmid (Invitrogen) (Postma et al., 2005). Primer designs, restriction sites, and accession numbers used in this study are displayed in Table S2.

THP-1 Differentiation and C5aR Downregulation

THP-1 monocytic cells were grown in RPMI 1640 (Life Technologies) supplemented with 10% fetal calf serum (Lonza), 2 mM glutamine, and 50 μM β-mercaptoethanol. THP-1 monocytes were differentiated into macrophages by the addition of 100 ng/ml phorbol myristate acetate (PMA; InvivoGen) for 48 hr. THP-1 monocytes were transduced with lentiviruses expressing human GIPZ shRNAmir targeting *c5aR1* (Thermo Fisher Scientific; shRNA1: clone ID V3LHS_635740; shRNA2: clone ID V3LHS_635738; shRNA3: clone IDV3LHS_635742) or targeting inflammasome adaptor *asc* (shRNA control) (Perret et al., 2012). Then 48 hr posttransduction, transduced cells were selected using puromycin (5 μg/ml) during a period of 4 days. Expression of shRNA in PMA-differentiated macrophages was checked by flow cytometry using GFP expression as a surrogate marker. To assess C5aR expression level in PMA-differentiated macrophages, C5aR mRNA levels were checked by quantitative RT-PCR with primers indicated in Table S2 using Improm (Promega) and LightCycler 480 SybrGreen I Master (Roche) kits on a LightCycler 480 II (Roche). C5aR expression level was normalized to GAPDH expression level.

Monoclonal Antibody Binding Competition Assay

Neutrophils were incubated in a volume of 50 μl RPMI-HSA at 5 × 10^6 cell/ml with 313 nM protein for 30 min on ice. After washing, different antibodies were applied and incubated for 30 min on ice as described elsewhere (Bardoel et al., 2012). PE-conjugated mouse anti-human antibodies were used to detect the C5aR (clones: S5/1, BioLegend; P12/1, AbD Serotec; D53-1473, BD Biosciences) and CXCR2 (clone 48311.211, R&D Systems). Fluorescence was detected by flow cytometry and compared to cells incubated without protein.

Binding Assays

Binding of the polyhistidine-tagged proteins to cells was tested using detection of the polyhistidine tag as described previously (Prat et al., 2009). After incubation with the protein for 30 min in a total volume of 50 μl on ice, cells (5 × 10^6 cell/ml) were washed and resuspended with a fluorescein isothiocyanate (FITC)-conjugated mouse anti-his antibody (Life Span Biosciences). After 30 min incubation on ice, cells were washed and analyzed by flow cytometry. Analysis of binding to transiently transfected HEK cells was limited to receptor-positive cells. Half-maximal effective binding concentrations were calculated using nonlinear regression analyses.

Cell Permeability Assays

Cells were exposed to recombinant proteins or 5% crude bacterial supernatant in a volume of 50–100 μl RPMI-HSA at 5 × 10^6 cell/ml with 2.5 μg/ml propidium iodide (PI) or DAPI. Cells were incubated for 30 min at 37°C with 5% CO_2 and subsequently analyzed by flow cytometry. Pore formation was defined as intracellular staining by PI or DAPI. For competition experiments, cells were preincubated with C5a (Bachem) or CHIPS for 15 min at room temperature before challenge with PVL. As PVL is a two-component toxin, equimolar concentrations of polyhistidine-tagged LukS-PV and LukF-PV were used. Analysis of pore formation in transiently transfected HEK cells was limited to receptor-positive cells. Half-maximal effective lytic concentrations were calculated using nonlinear regression analyses. Detection of cell permeability induced by supernatants of clinical *S. aureus* isolates is described in Supplemental Experimental Procedures.

Quantification of Receptor Expression Levels

Freshly isolated human leukocytes or stably transfected cells were incubated in a total volume of 50 μl at 5 × 10^6 cell/ml with immunoglobulin G (IgG) 2a-κ mouse anti-C5aR antibody clone S5/1 (AbD Serotec) or IgG2a-κ mouse anti-C5L2 clone 1D9-M12 (BioLegend), followed by FITC-conjugated goat-anti-mouse antibody (Dako). Antibody binding was quantified by calibration to defined antibody binding capacity units using QIFIKIT (Dako).

Neutrophil Priming for the Oxidative Burst

Human neutrophils were resuspended in IMDM with 25 mM HEPES (without phenol red) supplemented with 0.1% HSA at a cell concentration of 1.25 × 10^6 cell/ml. Half of the cells were incubated for 5 min with 700 nM $CHIPS_{31–121}$ to block the C5aR. In a white 96-well microplate, 50 μl cells were primed for 30 min at 37°C with 10 ng/ml TNF-α (Sigma), 1 nM C5a (Bachem), and PVL (1, 0.5, or 0.25 nM) or with buffer as control. Subsequently, 100 μl HBSS-0.1% HSA containing 337 μM luminol was added and the chemiluminescence response recorded every 0.5 s for a total of 200 s. After 30 s of baseline recording, fMLP was injected (final concentration 1 μM) to stimulate the oxidative burst and recording continued as relative light units in a luminometer (LB960 Centro; Berthold). For each sample, the area under the curve (AUC) after addition of fMLP was calculated, setting recordings in the first 30 s as baseline. To compare various experiments, AUC values were expressed relative to buffer-treated cells stimulated with fMLP. Cells without fMLP stimulation gave a continuous baseline recording during the 200 s.

Peptide Synthesis, Isothermal Titration Calorimetry, and ELISA

To study the N-terminal part of the C5aR, peptides comprising of amino acids 7–28 (TTPDYGHYDDKDTLDLNTPVDK) were used, of which one was sulphated at both tyrosines at positions 11 and 14. Sulphated and nonsulphated C5aR peptides were synthesized on a 433A peptide synthesizer (Applied Biosystems) applying Fmoc/*t*Bu chemistry according to a method described elsewhere (Bunschoten et al., 2009). The final peptides were checked for purity (>98%) and composition by high-performance liquid chromatography and mass spectrometry. Peptide concentrations were determined by weight. Isothermal titration calorimetry with polyhistidine-tagged LukS-PV was performed on an ITC_{200} microcalorimeter (MicroCal) (Ippel et al., 2009). The data were analyzed using the MicroCal Origin software and fitted by nonlinear regression analysis. Three independent experiments were carried out. The experimental errors were estimated by Monte Carlo simulations. For ELISA, a 96-well plate (Nunc MaxiSorp) was coated with 3.6 μM sulphated or nonsulphated C5aR peptide. After blocking with 4% skimmed milk, polyhistidine-tagged proteins were applied. After washing, mouse monoclonal anti-his-tag antibody (Novagen) was added, followed by incubation with a horseradish-peroxidase-labeled goat anti-mouse IgG (1/10,000, SouthernBiotech) for 1 hr at 37°C. After washing, TMB was added as substrate, the reaction was stopped with H_2SO_4, and optical density at 450 nm was measured (Bardoel et al., 2012).

Calcium Mobilization Assays

Calcium mobilization in neutrophils and U937-C5aR cells was performed as previously reported (Postma et al., 2005). Briefly, cells were loaded with 2 μM Fluo-3AM in RPMI-HSA for 20 min at room temperature under constant agitation, washed with buffer, and suspended to 5 × 10^6 cell/ml in RPMI-HSA. Subsequently, the cells were preincubated with LukS-PV, LukF-PV, or CHIPS for 5 min at room temperature. Each sample of cells was first measured for approximately 10 s to determine the basal fluorescence level. Next, concentrated C5a (Sigma, final concentration 1 nM) or C5a C-terminal peptide (Bachem, Tyr65, Phe67 C5a 65–74: YSFKDMQLGR, final concentration 2.5 μM) was added and rapidly placed back in the sample holder to continue the measurement. Cells were analyzed using a flow cytometry gated on forward and side scatter to exclude dead cells and debris. The relative increase in fluorescence was expressed in comparison to noninhibited cells.

Ethics Statement

Human leukocytes were isolated after informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the UMC Utrecht, the Netherlands. Cells obtained from animals were acquired with permission of the animal ethics committees of the University of Lyon, France and the University Medical Center Utrecht, the Netherlands.

Graphical and Statistical Analyses

Flow cytometric analyses were performed with FlowJo (Tree Star Software). Statistical analyses were performed with Prism (GraphPad Software). Statistical significance was calculated using ANOVA and Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.04.006.

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