Molecular mechanism of leukocidin GH–integrin CD11b/CD18 recognition and species specificity

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Edited by Victor J. Torres, New York University Langone Medical Center, New York, NY, and accepted by Editorial Board Member John Collier November 15, 2019 (received for review August 20, 2019)

Host–pathogen interactions are central to understanding microbial pathogenesis. The staphylococcal pore-forming cytotoxins hijack important immune molecules but little is known about the underlying molecular mechanisms of cytotoxin–receptor interaction and host specificity. Here we report the structures of a staphylococcal pore-forming cytotoxin, leukocidin GH (LukGH), in complex with its receptor (the α-I domain of complement receptor 3, CD11b-I), both for the human and murine homologs. We observe 2 binding interfaces, on the LukG and the LukH protomers, and show that human CD11b-I induces LukGH oligomerization in solution. LukGH binds murine CD11b-I weakly and is inactive toward murine neutrophils. Using a LukGH variant engineered to bind mouse CD11b-I, we demonstrate that cytolytic activity does not only require binding but also receptor-dependent oligomerization. Our studies provide an unprecedented insight into bicomponent leukocidin–host receptor interaction, enabling the development of antitoxin approaches and improved animal models to explore these approaches.

host–pathogen interaction | pore forming toxins | receptor recognition | leukocidin | integrin

The pathogen Staphylococcus aureus is a versatile human pathogen with the unique ability to cause a wide range of diseases, such as skin and soft tissue infections, sepsis, or pneumonia, attributed to its immense diversity of host-targeting virulence factors (1). The secreted leukocidins, a family of bicomponent pore-forming toxins, are believed to be at the core of S. aureus immune evasion by lysing phagocytic cells, mainly neutrophil granulocytes but also monocytes and macrophages (2, 3). S. aureus produces up to 5 different leukocidins—γ-hemolysins HlgAB and HlgCB, LukSF-PV (PVL), LukED, and LukGH (also called LukAB) (2)—with their cell type and species specificity driven by binding to different proteinaceous receptors on the surface of the immune cells (2, 3). Following receptor binding, the toxins oligomerize to form a lytic, octameric, β-barrel pore on the cell membrane. Although the steps involved in the leukocidin structural changes occurring during the pore formation are at least partly understood, less is known about the role of the receptors in this process (4, 5).

The cellular receptors of all bicomponent toxins, except LukGH, are transmembrane-spanning G protein-coupled receptors (2, 3, 6). LukGH, however, binds to the extracellular α-I domain of the α5β2 integrin (CD11b/CD18, macrophage-1 antigen, or complement receptor 3) (7). CD11b/CD18 is a member of the CD18 integrin family and is expressed on professional phagocytic cells (8) with a central role in the immune system, binding more than 40 protein ligands, including human fibrinogen and the complement fragment iC3b (9–11). Both the α and β-subunits contain large ectodomains, one transmembrane domain each, and short cytoplasmic domains, which enable communication with the extracellular environment. The 2 ectodomains, supported by their upper and lower legs, come together to form the integrin head, which comprises the α-I domain, the canonical ligand binding site in the integrins. Integrin activation, the so-called “inside-out signaling,” results in an allosteric switch in the CD11b/CD18 ectodomain from a resting, bent state to the extended form, with the corresponding activation of the α-I domain (conversion to open form, see below) and ligand recruitment (12).

The human α-I domain (CD11b-I) was expressed recombinantly, independently of the other integrin subunits (13), and to date 13 crystal structures of CD11b-I in complex with natural ligands, antagonists, antibodies, or alone, have been solved (13–20). However, despite the critical role of CD11b-I in the immune system of different mammals (21), all available crystal structures were obtained with the human CD11b-I (huCD11b-I). Two different conformations have been observed: The so-called inactive (closed or low affinity) and active (open or high affinity) forms of CD11b-I. The latter involves the rearrangement of the metal coordinating residues at the metal ion-dependent adhesion site of the corresponding activation of the α-I domain (conversion to open form, see below) and ligand recruitment (12).

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Staphylococcus aureus is one of the most virulent bacterial pathogens and, in particular, has the richest repertoire of cytotoxins: A single bacterium can secrete 6 different β-barrel pore-forming toxins, with different cell type and species specificities. Each toxin engages specific receptors on target cells, but the role the receptor plays in the pore-formation process is poorly understood. Here, we determine the crystal structures of a very potent S. aureus leukocidin (LukGH) in complex with its receptor (CD11b-I) from a sensitive (human) and an insensitive (murine) host, and track the receptor involvement in different steps on the pore-formation pathway. These results advance the knowledge of receptor-mediated leukocidin pore formation and open ways for anti-leukocidin and anti-S. aureus approaches.

Significance

Staphylococcus aureus is one of the most virulent bacterial pathogens and, in particular, has the richest repertoire of cytotoxins: A single bacterium can secrete 6 different β-barrel pore-forming toxins, with different cell type and species specificities. Each toxin engages specific receptors on target cells, but the role the receptor plays in the pore-formation process is poorly understood. Here, we determine the crystal structures of a very potent S. aureus leukocidin (LukGH) in complex with its receptor (CD11b-I) from a sensitive (human) and an insensitive (murine) host, and track the receptor involvement in different steps on the pore-formation pathway. These results advance the knowledge of receptor-mediated leukocidin pore formation and open ways for anti-leukocidin and anti-S. aureus approaches.

Author contributions: N.T. and A.B. designed research; N.T., D.M., and M.A.G. performed research; N.T., D.M., and M.A.G. contributed new reagents/analytic tools; N.T., D.M., M.A.G., and A.B. analyzed data; H.R., D.S., K.D.-C., E.N., and A.B. provided supervision; all authors contributed to interpreting the data and reviewed the manuscript; and N.T. and A.B. wrote the paper with input from D.M. and M.A.G.

Competing interest statement: A.B. and H.R. are employees of X4 Pharmaceuticals GmbH, the legal successor of Arsanis Biosciences GmbH, which has developed an antileukocidin antibody.

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 6RHV and 6RHW) and the Small Angle Scattering Biological Data Bank, https://www.sasbdb.org/ (accession nos. SASDF55 and SASDF46).

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1913690116/-/DCSupplemental.

First published December 18, 2019.

www.pnas.org/cgi/doi/10.1073/pnas.1913690116
Crystallize moCD11b-I in complex with the full-length LukGHK319A.

Mouse PMNs are resistant to LukGH at concentrations up to 30 μM, by 10- to 15-fold increased cytotoxicity toward rabbit PMNs (27), a variant with increased affinity toward rbCD11b-I paralleled activity toward mouse PMNs, at concentrations up to 20 μM (without rbCD11b-I) (Fig. 1A). However, these mutants display no activity toward rbCD11b-I (Fig. 1C). The core of the interface is well conserved between the mouse and human receptors (Fig. 2A, Left and SI Appendix, Table S2). Central to these interactions is the MIDAS site, where the LukH residue E323 completes the octahedral coordination sphere around the metal ion, together with the conserved CD11b-I residues (S144, S142, and T209) and 2 water molecules, as seen for the active conformation of huCD11b-I (Figs. 1F and 2A, Left, and SI Appendix, Fig. S1) (13, 14). The importance of this interaction is supported by the lack of receptor binding and cytolytic activity of the LukGHK322A variant (27, 30) and by the fact that Mg2+ substitution at the MIDAS site by Ca2+ impairs LukGH binding (SI Appendix, Table S3). Additional interactions involve the salt bridges between the side chains of E244 (CD11b-I) and R294 (LukH) and the side chain of R208 (CD11b-I) and the C-terminal carboxyl group of G324 (LukH). Polar contacts between R208 (CD11b-I) and H188 and Y321 (LukH), van der Waals contacts between F246 (CD11b-I) and D114, H188, and Y321 (LukH), and a hydrophobic interaction between P249 (CD11b-I) and W187 (LukH) are also observed (Fig. 2A, Left). Mutagenesis studies at these positions in LukH confirm their involvement in
Fig. 1. Binding and activity of LukGH wild-type and mutants to CD11b-I and crystal structure of LukGH-CD11b-I. (A) Steady-state analysis of LukGH wild-type binding to moCD11b-I. The steady state $K_d$ is shown in the Inset. (B) Binding of LukGH to hu- or moCD11b-I expressed as response units (mean of 2 to 10 independent experiments ± SEM) and $K_d$ (mean of 2 to 10 independent experiments ± SD). EC_{50} values of LukGH mutants toward differentiated HL-60 cells or mouse PMNs assessed in a luminescent cell viability assay measuring cellular ATP content (mean of 2 to 8 independent experiments ± SEM). For variants that had limited or no cytotoxicity (could not kill >75% of cells at the highest toxin concentration used), EC_{50} is not shown. (C) Cytotoxicity of LukGH, LukGH^K319A, and LukED toward mouse PMNs assessed in a luminescent cell viability assay measuring cellular ATP content at cytotoxin concentrations of 30 μM, 20 μM, and 100 nM, respectively (mean of 3 independent experiments ± SEM). (D) Front and top view of LukGH^K319A-moCD11b-I crystal structure. Dark blue and light green cartoons represent LukH and LukG from dimer 1 and dark green and light blue cartoon represent LukG and LukH from dimer 2, respectively. moCD11b-I is shown as an orange cartoon. Other dimers forming the octamer pore and bound CD11b-I molecules, are shown as a gray cartoon. Red spheres represent bound DMSO molecules from one asymmetric unit (dark red sphere represents DMSO 2). Comparison of moCD11b-I secondary structure (E) and MIDAS residues (F) from LukGH^K319A-moCD11b-I structure (orange ribbon) with the active (1IDO, light pink ribbon) and inactive (1JLM, light gray ribbon) form of huCD11b-I. Structures are aligned on moCD11b-I and MIDAS residues in E and F, respectively. The metal ions from the moCD11b-I structure and the inactive form of CD11b-I (1JLM) are shown as orange and gray spheres, respectively.
binding and activity (27). While residues E244, R208 and F246 are conserved between human, rhesus macaque, pig, rabbit, and mouse CD11b-I variants, residues R208 and F246 are replaced by Q and Y, respectively, in guinea pig (SI Appendix, Fig. S2B). The LukH–CD11b-I complex is stabilized by several salt bridges and polar interactions (SI Appendix, Table S2), explaining why the LukGH affinity for CD11b-I decreases with increasing the ionic strength, even though protein stability is not affected (SI Appendix, Fig. S2A and Table S4). At the extremities of the interface (Fig. 2A, Right), the LukH–CD11b-I interactions vary in the 2 species. The main driver is the S277 huCD11b-I residue, which is K in the mouse variant. K277 forms a salt bridge with D316 (LukH) in the LukGHK319A–moCD11b-I complex (not present for the human complex) (Fig. 2A, Right Lower). It appears that reduction of the size and removal of the positive charge (K319A) is needed to prevent steric clashes and electrostatic repulsion between K277 and K319, explaining the increased binding of the LukGHK319A variant to moCD11b-I. Instead, S277 from huCD11b-I forms hydrogen bonds with the side chains of LukH residues Y314 and...
D316, which brings the main chain of huCD11b-I closer to LukH (Fig. 2A, Right Lower).

Since S277 is conserved between different species, except for mouse, we performed “humanizing mutations” (i.e., we introduced a K277S P278E double mutation in moCD11b-I) to confirm the above hypothesis. The LukGH variants with mutations in the region involved in the interaction with K277 (LukGHK264A, LukGHK265A) showed a similar binding pattern for moCD11b-I K277S P278E and huCD11b-I, with decreased binding affinity for LukGHK294A and LukGHK310A, while those with mutations remote from this interaction site (LukGR119A, LukGR121A, LukGD312A) were not distinguished by the CD11b-I variant (Fig. 2B). The decreased binding affinity of LukGHK310A for huCD11b-I is probably due to loss of a salt bridge between K319 and E244 (CD11b-I). Additionally, we “humanized” the neighboring Q279 in moCD11b-I (moCD11b-I Q279K), which makes a N-H···π interaction with the aromatic side chain of Luk H Y314 in the mouse but not in the human complex (the corresponding K279 residue is oriented away from the interface). The moCD11b-I Q279K variant had significantly increased affinity toward LukGHK310A compared to moCD11b-I K277S P278E (Fig. 2B and SI Appendix, Fig. S2C). Additional interactions are present in the moCD11b-I complex only, including the salt bridge at the top of the interface (D251 [moCD11b-I]-R119 [LukH]) (Fig. 2A, Right Upper) and a hydrogen bond at the bottom (N146 [moCD11b-I]-K322 [LukH]) (Fig. 2A, Right Lower).

While the LukH residues forming the salt bridges in human and mouse complexes are mostly conserved, except for LukH R119 and K319, only 2 positions from CD11b-I involved in salt bridge formation are conserved between human, rabbit, mouse, pig, rhesus macaque, and guinea pig (SI Appendix, Fig. S2A and Table S2). The conservation of the CD11b-I residues involved in the binding epitope in the mouse and human CD11b-I complex structure between different species reveals the highest similarity between human and rhesus macaque (89% identity), which correlates with activity toward macaque PMNs (26%) and the highest divergence between human and guinea pig (63% identity).

**Second Binding Interface between LukG and CD11b-I.** Unexpectedly, besides the LukH–CD11b-I epitope, we observed a second binding region between CD11b-I and LukG from an adjacent dimer of the LukGH octamer (Figs. 1D and 2C and SI Appendix, Supplementary Table S5). This interface is partly conserved between the mouse and human complexes: For example, the hydrogen bond between R66 (LukG) guanidinium group and L205 (CD11b-I) main-chain carbonyl group. However, most of the residues contacting the 2 loops in LukG in this interface differ between the 2 species: For example, N33 (LukG) side chain makes a hydrogen bond with the carbonyl group of D178 in moCD11b-I and E178 in huCD11b-I. Particularly interesting is the interaction of moCD11b-I with the loop 68-72 in LukG: Due to steric hindrance by K203 in moCD11b-I, the loop is flipped up to ~180° compared to the uncomplexed structures (PDB ID codes 5G31 [huCD11b-I] and 5G30 to the complex with the human receptor (SI Appendix, Fig. S3), which in turn flips the side chain of LukG D69, allowing formation of a salt bridge with R181 in moCD11b-I (Fig. 2C, Right). The flip is presumably kinetically unfavorable, as D69 loses hydrogen bonds with 3 residues from the adjacent β-sheet. The difference electron density map suggests flexibility of this loop and the presence of some other minor alternate conformations, which we have not been able to model satisfactorily (SI Appendix, Fig. S3). This disorder is not observed in the complex with huCD11b-I, which has T at position 203 and does not appear to interact with the LukG 68-72 loop.

All LukG residues involved in the second binding interface are variable in the currently available LukG sequences (75 to 80% conservation level), in contrast to the main interface, where more than half of the residues are fully conserved (the remaining show 76 to 99% conservation) (SI Appendix, Tables S2 and S5).

**CD11b-I Promotes LukGH Oligomerization in the Absence of a Cell Surface.** The ability of CD11b-I to bind to the oligomerization interface indicates that the receptor alone (in absence of a cell surface) may promote oligomerization. To further investigate this, we developed a non invasive oligomerization assay using dynamic light scattering (DLS), by mixing LukGH with CD11b-I in a 1:1 molar ratio and monitoring the molecular size of the mixture, expressed as radius, over time. The hydrodynamic radius of LukGH and CD11b-I alone are ~5 nm and ~2 nm, and do not change for up to 36 to 48 h (SI Appendix, Fig. S4A). When the 2 components were mixed, we observed a time-dependent increase in radius from ~5.5 nm to ~11 to 12 nm over several hours, after which a plateau was reached (Fig. 3A). We assign the lower radius (~5.5 nm) to the LukGH–CD11b-I complex, based on data with an oligomerization-deficient variant, the LukGH dimer, which binds huCD11b-I, but is lacking cytolytic activity (28), and shows no change in size when mixed with CD11b-I (Fig. 3A). The higher (~11 nm) radius corresponds to the final oligomerization product, a relatively stable structure that does not aggregate in the time frame of the experiment (up to 96 h), which is, most probably, an assembly similar to the octameric pore found in the crystal. We observed oligomerization of LukGH in the presence of human and rabbit CD11b-I, but not mouse CD11b-I, which parallels the activity data (Fig. 3A). Moreover, the LukGHK310A variant, which shows strong binding to moCD11b-I is still unable to oligomerize in the presence of moCD11b-I, explaining its lack of cytolytic activity.

When the oligomerization rate was approximated to a first-order rate constant, we observe that huCD11b-I–induced oligomerization of LukGH is ~3x faster than that induced by rbCD11b-I at physiological NaCl concentrations (150 mM) (Fig. 3B and SI Appendix, Fig. S4B). There is, however, a marked dependence of oligomerization rate on NaCl concentration (i.e., it increases with increasing NaCl concentration from 0 to 150 mM), with some variations at higher salt concentrations for different receptors (Fig. 3B and SI Appendix, Fig. S4B).

In order to investigate the stoichiometry requirements for CD11b-I–mediated oligomerization of LukGH, we measured the oligomerization efficiency and rate at different CD11b-I to LukGH ratios. The oligomerization appears complete at ratios as low as 1:1 (one CD11b-I molecule per LukGH octamer), with the oligomerization rate increasing almost linearly with increasing the ratio to 1:1 (4 CD11b-I molecules per LukGH octamer), indicative of a catalytic role of CD11b-I in this process (Fig. 3C).

Using site-directed mutagenesis, as described in the SI Appendix, Supplementary Results and Discussion, we could clearly confirm the involvement of LukG residues N33, R66, D69, P70, and N71 in both oligomerization and activity with the human system (Fig. 3 D and E), and for R66 and D69 also with rabbit cells (SI Appendix, Fig. S5A), in agreement with structural data. The most striking loss of activity was seen when LukG N33 was mutated to the negatively charged E, presumably due to repulsion at the second interface (LukG N33 interacts with E178 in huCD11b-I) (Figs. 2C, Right and 3D). Importantly, all of the tested variants showed no change in binding to huCD11b-I, confirming that loss of activity was not due to decreased binding affinity (SI Appendix, Fig. S5B).

None of the oligomerization site mutants, coexpressed with LukHK310A showed any activity toward mouse PMNs up to cytotoxin concentrations of 800 to 1,000 nM, no improved affinity toward moCD11b-I, and no increase in radius in presence of moCD11b-I, when tested by DLS (Fig. 3F and SI Appendix, Fig. S5 C and D).

**Fab Binding to the LukG Subunit of LukGH–huCD11b-I Prevents Its Cell Membrane Independent Oligomerization.** In order to gain insight into the structural organization of the LukGH–receptor complex...
Fig. 3. Oligomerization of LukGH in solution, binding and activity of LukGH oligomerization variants. (A) Change of LukGH, LukG1H, and LukGHK319A (at 5 mg/mL) plus hu-, rb-, or moCD11b-I (at 2.5 mg/mL) cumulant radius, over time, measured in 25 mM Hepes, pH 7.5, 1 mM MgCl₂, 150 mM NaCl (mean of 1 to 2 replicates ±SEM). The dotted lines represent fitting of the data to a one-phase association model with fixed $y_0 = 5$ at $x_0 = 0$ h (GraphPad Prism). (B) Oligomerization rate constant ($k$) and plateau for LukGH, LukGHK319A, and LukG1H (at 5 mg/mL) plus huCD11b-I (2.5 mg/mL) in 25 mM Hepes, pH 7.5, 1 mM MgCl₂, 0 to 300 mM NaCl (mean of 1 to 2 replicates ±SEM). Data were fitted as in A giving $R^2 > 0.93$. (C) Oligomerization rate constant ($k$) of LukGH (4.5 mg/mL) plus increasing amounts of huCD11b-I (2.3 mg/mL) in 25 mM Hepes, pH 7.5, 1 mM MgCl₂, 150 mM NaCl (mean of 2 replicates ±SEM). Linear regression fit (GraphPad Prism) is shown in red with equation in Inset. (D) Activity of LukGH mutants toward differentiated HL-60 cells expressed as EC₅₀ and percent cell viability at maximal toxin concentration (100 nM) (mean of 2 independent experiments ±SEM). Red and black line represent EC₅₀ value and percent cell viability of LukGHK319A mutant, respectively. Variants that had limited or no cytotoxicity (could not kill >75% of cells at the highest toxin concentration used) are marked with "#." (E) Oligomerization rate constant ($k$) of LukG oligomerization mutants coexpressed with LukHK319A (at 4.5 mg/mL) plus huCD11b-I (2.3 mg/mL) (mean of 2 replicates ±SEM). Data were fitted as in A, in all cases, except for LukGQ31A LukHK319A (#, ambiguous fit), yielding $R^2 > 0.94$. (F) Cumulant radius of LukGH variant-moCD11b-I complexes (at 4.5 mg/mL for LukGH and 2.3 mg/mL for CD11b-I) and individual LukGH variants at 36 h of incubation in 25 mM Hepes, pH 7.5, 1 mM MgCl₂, 150 mM NaCl (mean of 1 [circled] or 2 replicates ±SEM). In case the sample shows increased radius at time 36 h, earlier time points are shown (24 and 12 h). Dotted lines represent ±10% change from a 5.5-nm radius. Samples with sum of squares error >10 are marked with "#."
in solution, we used solution small-angle X-ray scattering (SAXS) for LukGH and the LukGH–huCD11b-I complex in the presence of the Fab fragment of a LukGH neutralizing antibody (Fig. 4 and SI Appendix, Table S6). The Fab was used to stabilize the dimer and to allow the elution of the complex from the size-exclusion chromatography column to ensure the sample’s monodispersity. First, the complex of the Fab fragment with LukGH was analyzed in the absence of the receptor, and compared to the crystal structure of the complex we have previously determined (29). The computed distance distribution \( p(r) \) (Fig. 4B) indicates that the molecule is a multidomain (distinctive bumps) and an elongated particle [skewed \( p(r) \) shifted to shorter distances]. Furthermore, the overall structural parameters derived from SAXS (molecular mass, radius of gyration \( R_g \)), and maximum dimension \( D_{\text{max}} \) (SI Appendix, Table S6) are fully compatible with a monomeric construct and strongly support that the binding of the Fab fragment prevents the oligomerization of LukGH. Moreover, the experimental data are in good agreement with the theoretical curve calculated from a structural model derived from the available crystal structure (PDB ID code 5K59) with a discrepancy \( \chi^2 = 1.8 \) (Fig. 4A).

Next, we analyzed the LukGH–Fab complex bound to huCD11b-I. Noticeable increases observed for the overall parameters \( (R_g, \text{from about 4.8 to about 5.1 nm, } D_{\text{max}} \text{ from 16 to 18 nm}) \) and an increase by about 20 kDa in the molecular mass are in line with the stable 1:1 complex formation (Fig. 4 and SI Appendix, Table S6) corresponding to LukGH–Fab complex bound to one huCD11b-I. No concentration-dependent alterations in the SAXS data are observed, indicating that the receptor is tightly bound to LukGH also in the presence of the Fab fragment. Moreover, the experimental data are in very good agreement \( (\chi^2 = 1.4) \) (Fig. 4A) with the scattering curve computed from a model combining the LukG:Fab interface (PDB ID code 5K59) and the LukH:huCD11b-I interface (crystal structure described here) (Fig. 4B). To further improve the fit, the program CORAL was used. Here, the missing amino acids (44 N-terminal residues of LukH and 22 C-terminal residues of CD11b-I) were modeled as dummy residues. With this approach, a \( \chi^2 \) value of 1.0 was achieved (SI Appendix, Fig. S6). Comparison of 20 individual runs suggests that the N-terminal of LukH is rigid and elongated.

Binding of the \( \alpha \)LukGH-mAb\#5.H1H2 Fab to the rim region of the LukG protomer (Fig. 5A) (29) in the LukGH dimer did not prevent binding of CD11b-I to LukGH (via the LukH protomer) in solution, but prevented oligomerization, as predicted from the crystal structure (\( \alpha \)LukGH-mAb\#5.H1H2 binds to the oligomerization interface) (29) and confirmed by DLS measurements in the presence of the Fab (Fig. 5B). However, on the cell surface, when LukGH is bound to the receptor, the \( \alpha \)LukGH-mAb\#5.H1H2 epitope is no longer accessible (Fig. 5A) and no \( \alpha \)LukGH-mAb\#5.H1H2 binding to cell-bound LukGH was detected (29).

We have also determined the effect of anti-CD11b-I antibodies with known epitopes (SI Appendix, Fig. S2A) on the activity of LukGH on lipopolysaccharide (LPS)-activated human PMNs. In the presence of the LM2/1 antibody, whose epitope is in the proximity of the LukGH binding epitope (SI Appendix, Fig. S2A), we observed an inhibition of LukGH activity (Fig. 5C), in agreement with a previous report (7). In contrast, the CBRM1/5 antibody, which recognizes a conformational epitope present only on the active form (31), enhances LukGH activity (Fig. 5D), presumably due to an allosteric activation. This is particularly interesting since an opposite effect (i.e., inhibition of binding) was observed with other CD11b-I ligands, ICAM-1 and fibrinogen, in the presence of CBRM1/5 (31).

**Discussion**

LukGH is a unique member of the bicomponent cytotoxin family, as it dimerizes in solution before receptor and target cell binding (28, 30). This feature has been proposed to be responsible for the very high cytotoxic activity of LukGH, which also correlates well with receptor up-regulation and activation on target cells (25, 28). At “high” receptor densities, on activated PMNs, the activity of LukGH is up to 3 orders of magnitude higher than on resting PMNs (25). Here, we provide the molecular basis for this correlation. A single receptor molecule is able to bind 2 adjacent dimers in the octamer, and implicitly a single LukGH dimer can bind 2 receptor molecules, via separate LukH and LukG interfaces. In addition, LukGH binds to the active form of the I-domain of CD11b, as all of the other bona fide CD11b ligands.

Using a combination of X-ray crystallography and SAXS, we were able to capture 2 intermediates in the receptor-mediated LukGH pore-formation pathway. The LukGH dimer–CD11b-I complex, stabilized by an oligomerization inhibitory Fab fragment, was analyzed by SAXS. This ternary complex involves interactions between CD11b-I and the cap domain of the LukH subunit, close to the LukGH oligomerization site, as also indicated.

![Fig. 4. SAXS analysis of complex formation. (A) Scattering data as log(|S|) vs. s plot compared to the theoretical scattering of the respective models. These comprise the interfaces as retrieved from the crystal structures; \( \chi^2 \) values are indicated. Curves are shifted along the y axis for better visualization. (B) Distance distribution profile of LukGH-Fab (black) and LukGH-Fab–huCD11b-I (red). The Inset shows the expected complex formation as cartoon representation, with the LukG, LukH, huCD11b-I, and Fab subunits in green, blue, orange, and purple cartoons, respectively.](image-url)
by previous data generated with site-directed mutagenesis (27). Since there are no major structural changes in the LukGH dimer compared to the unligated form, this is presumably one of the first intermediates in the pathway. The second intermediate is the fully formed LukGH octamer complexed with 4 CD11b-I molecules, which in addition to the LukH interface (the binding interface), involves interactions with 2 LukG loops from a neighboring LukGH dimer (across the oligomerization interface). This is likely one of the final intermediates before insertion of the pore into the target cell membrane, although it is possible that not all 4 sites need to be occupied for pore formation to occur (see below).

Interestingly, while the LukGH dimer–CD11b-I binding interface has 6 salt bridges, none is present in the oligomerization interface, at least with the human receptor. Accordingly, the ionic strength requirements for the 2 processes also appear to follow different trends (i.e., increase in ionic strength favors oligomerization but impairs binding). This corroborates the electrostatic nature of the LukGH dimer–CD11b-I interaction, and hydrophobic nature of the oligomerization interface, and may indicate different preferences for diverse microenvironments.

Based on all of the structural, mutagenesis, antibody inhibition, and cytotoxicity enhancement data presented here, we propose a mechanism of pore formation by LukGH on activated PMNs (Fig. 6). LukGH binds to its integrin receptor, CD11b/CD18, in an extended conformation, induced as a result of inside–out signaling following activation (12). This agrees with the potentiation of LukGH activity on LPS-stimulated PMNs by CBRM1/5, an anti–CD11b-I antibody that targets an epitope shielded in the bent integrin (Fig. 5 A and D). According to this model, initially LukGH binds an active CD11b-I domain on the cell surface, via its LukH subunit, presumably with concomitant recruitment of an adjacent CD11b-I domain (which may already have an occupied LukH site) via the LukG subunit (Fig. 6). Homodimerization of integrin α-domains, triggered by interactions between the homologous transmembrane domains, has been reported for the activated form of integrin αIIbβ3 (32). Recruitment of 2 additional LukGH dimers to form the octamer may not necessarily involve other I-domains (Fig. 6), since octameric pore formation is thought to be a highly cooperative process (as shown for S. aureus γ-hemolysin) (33). Moreover, DLS oligomerization data in solution suggest that there is no effect of additional receptor domains on oligomerization efficacy and only a small increase in oligomerization rate from 2 to 4 CD11b-I equivalents per LukGH octamer is observed.

β-Barrel pore formation is a 2-step process, and the final step of insertion into the membrane is thought to occur after complete oligomerization of the cap domain (4). In the extended form of the integrin, the I-domain is ~20 nm from the cell surface, so the integrin would have to bend to allow for the insertion of the pore (Fig. 6). Alternatively, the receptor may dissociate before pore insertion, via an unidentified mechanism, similar to the proposed receptor dissociation after pore formation/oligomerization in the case of another bicomponent leucocidin, LukSF (5). Ligand
binding to the active I-domain of CD11b of the bent CD11bCD18 integrin is also not unprecedented: ICAM-1 binding was shown to have antiinflammatory effects (34). Another aspect is the orientation of the LukGH pore relative to the cell surface when LukGH binds the receptor, as the alignment of CD11b-I on the available ectodomain crystal structures (PDB ID codes 3K71, 5ES4, 4NEH, 3K6S) does not result in a LukGH pore oriented perpendicular to the membrane (SI Appendix, Fig. S7). In principle, the region linking the I-domain with the rest of the α-chain in integrins is flexible (35, 36) and may allow the rotation of the LukGH pore toward the membrane. An intriguing possibility is that LukGH pores are able to kill adjacent cells, or that such a mechanism is used for LukGH-dependent bacterial escape from intracellular compartments (37). Being able to specifically engage activated CD11b on the PMN surface is not the only advantage of the bivalent toxin–receptor interaction. CD11b/CD18 is known to bind a variety of endogenous ligands (SI Appendix, Table S7), with affinities in the high nanomolar range, some with epitopes overlapping with LukGH binding [e.g., C3d (19), iC3b (9), or human fibrinogen (10)] (SI Appendix, Fig. S24). The concentration of these ligands varies with tissue type, but is particularly high in the blood (e.g., 1.5 to 4.0 mg/mL for fibrinogen). It is not yet clear whether LukGH is active in S. aureus bacterial sepsis, but the avid binding of LukGH to the CD11b receptors certainly provides a competitive advantage over the monovalent endogenous ligands. Following the same principle, the anthrax toxin protective antigen (PA) binds to its von Willebrand type I domain receptor, to the MIDAS site, via the PA IV domain, but forms additional interactions using a neighboring domain (PA II), leading to an ~1,000-fold higher affinity compared to a typical integrin–ligand complex (38). Moreover, it was shown that the protonation of a histidine residue on the receptor, at the edge of this additional binding pocket, controls the pH-dependent dissociation from PA II and subsequent pore formation, reminiscent of the CD11b interaction with LukG, where reduced interactions appear to favor oligomerization (38).

CD11b/CD18 and the other β2 integrins (CD11a, CD11c, and CD11d) play important roles in immune defense mechanisms, at the same time regulating immune responses (39). Whereas reduction or lack of β2 integrins leads to higher susceptibility to infection and impaired inflammatory responses, increased expression or activation of integrins has been linked to autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, as well as inflammation-aggravated conditions, such as stroke (39). The extremely high specificity and avidity of LukGH toward activated CD11b, and the availability of structural information for the interaction, make LukGH a suitable candidate for engineering potential therapeutic candidates, with or without functional pores, targeting integrins in inflammatory diseases. The caveats of using a nonhuman therapeutic protein, particularly for chronic indications, typically arise from the short half-life and formation of antidrug antibodies. However, these could be potentially circumvented for LukGH by exploiting its own ability to blunt the adaptive immunity via dendritic cell targeting (40) and by making use of its numerous and diverse natural sequence variants (41).

Materials and Methods

Production of Recombinant LukGH Variants. LukGH variants were produced recombinantly in Escherichia coli, as described previously (27, 28), based on the wild-type sequence of the community-associated methicillin-resistant S. aureus (CA-MRSA)USA300 (ST8) TCH1516 strain. Protein concentration was calculated based on the UV absorbance at 280 nm using the extinction coefficient (ε280 = 112 000 M⁻¹ cm⁻¹) calculated with ProtParam tool (ExPaSy Server) (42) based on the LukGH protein sequence. Protein purity was determined by SDS-PAGE gels, stability by differential scanning fluorimetry, and the secondary structure by circular dichroism, as described in SI Appendix, Supplementary Material and Methods.

Production of moCD11b-I Variants and Expression and Purification of Recombinant huCD11b-I, rbCD11b-I, and moCD11b-I. The I-domains (amino acids 127 to 321) of huCD11b, rbCD11b, and moCD11b (huCD11b-I, rbCD11b-I, and moCD11b-I) were cloned into pET24a (Novagen) vector at Ndel/Xhol (Ndel/EbamHl for rbCD11b-I) sites and purified and biotinylated, as described in SI Appendix, Supplementary Material and Methods. Due to the lack of tryptophan in the amino acid sequence of hu- and mo-CD11b-I, protein concentration was determined based on the UV absorbance at 205 nm using the extinction coefficient [ε205(huCD11b-I) = 797 420 M⁻¹ cm⁻¹; ε205(rbCD11b-I) = 790 170 M⁻¹ cm⁻¹; ε205(moCD11b-I) = 794 570 M⁻¹ cm⁻¹] calculated with “A205 protein/peptide concentration webservice” (43). Protein purity and monomer content were assessed by nonreducing SDS-PAGE gel, stability by differential scanning fluorimetry and the secondary structure by circular dichroism, as described in SI Appendix, Supplementary Material and Methods.
Bio-Layer Interferometry. Binding of LukGH (wild-type and variants) to huCD11b-I, rbcD11b-I, or mCD11b-I (wild-type and mutants) was evaluated by Bio-Layer Interferometry (FortèBio Octet Red96 instrument, Pall Life Sciences) as described previously (27). In brief, biotinylated CD11b-I (2 to 4 µg/mL) was immobilized on streptavidin sensors (FortéBio, Pall Life Sciences). The association of LukGH (50 nM or 100 nM in assay buffer [PBS plus 1% BSA and 1 mM MgCl₂ or CaCl₂, or 25 mM Hepes, pH 7.5 plus 1% BSA plus 1 mM MgCl₂ and NaCl [150 to 1,000 mM]) to the immobilized receptor and dissociation in assay buffer were monitored for 5 min each. Response units (RU) and where possible (for monophasic binding curve) equilibrium dissociation constants (K_d) were determined using the Data Analysis 7 software (FortéBio, Pall Life Sciences) by simultaneously fitting the association and dissociation curves to a 1:1 binding model. The steady-state K_d values were determined for LukGH wild-type binding to mCD11b-I and rBCD11b-I by measuring binding at multiple LukGH concentrations (100 to 2,200 nM and 20 to 400 nM, respectively) and fitting the data to a steady-state equilibrium model (FortéBio Analysis Software, V7).

Purification of LukGH-huCD11b-I-Fab and LukGH-Fab Complexes and SAXS Analysis. LukGH and huCD11b-I purified as described above, and the Fab of αLukGH-mAb485.H122 (29) expressed in Chinese Hamster Ovary cells and purified by LC-αfinity chromatography (CaptureSelect, Thermo Scientific), were mixed in 1:1.15 molar ratio, respectively. For the LukGH-Fab complex, LukGH and Fab were added to Fab. Both complexes were concentrated by size-exclusion chromatography as described in SI Appendix, Supplementary Material and Methods. Synchrotron radiation X-ray scattering data were collected at the EMBL P12 beamline of the storage ring PETRA III (DESY, Hamburg, Germany) (44) for both complexes (LukGH-Fab and LukGH-Fab-huCD11b-I) from a dilution series to examine concentration-dependent alterations, as described in SI Appendix, Supplementary Material and Methods. The indirect inverse Fourier transform of the SAXS data and the corresponding probable real space-scattering pair distance distribution [P(r) versus r profile] were calculated using GNOM (45), from which the K_d and D_max were determined. The P(r) versus r profile was also used for volume and subsequent molecular weight estimates of the complexes, as described in Hajizadeh et al. (46). CRYSOL (47) was used to calculate the scattering profiles from the atomic coordinates of available crystal structures: The Fab fragment bound to LukGH, as deposited in PDB code 5K59, chains A and C (29) were used to create the Fab complex in 0.5-μL reservoir (25 to 30% [vol/vol] Jeffamine-600, 5 to 10% [vol/vol] DMSO) or 1-μL complex in 0.5-μL reservoir solution (30% [vol/vol] Jeffermine-600, 10% [vol/vol] DMSO), for LukGH-mAb510a-mCD11b-I and LukGH-huCD11b-I, respectively. The crystals were harvested from the crystallization drop using a nylon loop and frozen directly in liquid nitrogen without addition of a cryoprotectant.

Diffraction Data Collection, Structure Determination, Refinement, and Interpretation. Diffraction data were collected at 100 K at the European Synchrotron Radiation Facility at beamline ID30A-1 (MASSIF-1; wavelength 0.966 Å) for the LukGH-mAb510a-mCD11b-I complex and at beamline ID29 (wavelength 1.072 Å) for the LukGH-huCD11b-I complex. Both datasets were processed using the XDS program package (50). Due to significant anisotropic diffraction, the LukGH-huCD11b-I dataset was corrected and merged using the STARANISO Server (51) incorporating the programs MOSFLM (52), POINTLESS (53), and AIMLESS (54). The LukGH-mAb510a-mCD11b-I structure was solved by molecular replacement in Phaser (55) using LukG and LukH structures from their complex with a Fab fragment [PDB ID code 5K59, chains A and C (29)] as independent search models. After initial model building of LukGH in Coot (56) and 10 cycles of restrained refinement in REFMAC5 (57, 58), additional electron density corresponding to the mCD11b-I domain could be identified clearly and the missing component built in Coot and Buccaneer (59, 60). The structure of LukGH-huCD11b-I was solved by molecular replacement in Phaser (55) by searching sequentially with the LukGH dimer from the refined LukGH-mAb510a-mCD11b-I structure and then with the modified huCD11b-I domain (PDB ID code 1IDO) (13) lacking the C-terminal α7-helix (residues 303 to 315). Both structures of the complexes were finalized by model building and refinement in Coot and Phenix (61). Due to the anisotropic low-resolution diffraction data, the LukGH-huCD11b-I structure was refined by applying additional dihedral-angle restraints derived from the refined LukGH-mAb510a-mCD11b-I structure as a reference model. The data collection, refinement, and validation statistics are shown in SI Appendix, Table 51. The molecular interfaces and oligomeric states were analyzed in PISA (62) and the structures were superposed in the program LSOQB (63) as a part of the CCP4 program suite (64). The atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession codes 6HRV (LukGH-K319A-mCD11b-I (65) and 6HRW (LukGH-huCD11b-I (66).}

ACKNOWLEDGMENTS. We thank M. Bowler and D. de Sanctis at the European Synchrotron Radiation Facility, Grenoble, France for providing assistance; and D. Mantus and L. Stulik for critical reading of the manuscript. We thank M. Bowler and D. de Sanctis at the European Synchrotron Radiation Facility, Grenoble, France for providing assistance; and D. Mantus and L. Stulik for critical reading of the manuscript. We thank M. Bowler and D. de Sanctis at the European Synchrotron Radiation Facility, Grenoble, France for providing assistance; and D. Mantus and L. Stulik for critical reading of the manuscript.

The dynamic light scattering, circular dichroism, and differential scanning fluorimetry measurements were performed at the Vienna BioCenter Core Facilities Protein Technologies Facility (https://www.viennabiocenter.org/facilities/). K.D.-C.’s research was supported by the Christian Doppler Laboratory for Knowledge-based Structural Biology and Biotechnology, Federal Ministry of Economy, Family and Youth through the initiative “Laura Bassi Centres of Expertise,” funding the Centre of Optimized Structural Studies, 252275, COST action BM1405-Nonglobular proteins, and by the University of Vienna. M.A.G. received support from iNEXT, Grant 653706, funded by the Horizon 2020 programme of the European Commission. M.A.G. received support from iNEXT, Grant 653706, funded by the Horizon 2020 programme of the European Commission.
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