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Selective sensitization of human neutrophils to LukGH mediated cytotoxicity by *Staphylococcus aureus* and IL-8

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Summary *Objectives:* *Staphylococcus aureus* produces up to five bi-component leukocidins — LukSF-PV, gamma-hemolysins AB and CB, LukGH (LukAB) and LukED — to evade innate immunity by lysing phagocytic cells. Species specificity of these leukocidins limits the relevance of animal models, therefore we assessed their individual contribution using human neutrophils.
Methods: Human polymorphonuclear leukocytes (PMNs) were activated with stimuli relevant during bacterial infections and sensitivity to recombinant leukocidins was measured in cell-viability assays. Leukocidin receptor expression was quantified by flow cytometry.
Results: We observed greatly variable sensitivities of different PMN preparations towards LukGH. Activation of PMNs by lipopolysaccharide (LPS) or *S. aureus* culture supernatant (CS) lacking all leukocidins resulted in higher surface expression of CD11b, the LukGH receptor, and greatly enhanced the sensitivity towards LukGH, eliminating the variability observed with unstimulated cells. In contrast, CS induced a decrease in sensitivity of PMNs to the other four leukocidins and reduced surface staining for their cognate receptors (CXCR1, CXCR2, C5aR, C5L2). Delta-toxin and peptidoglycan mimicked the effect of CS. Moreover, IL-8, an important cytokine in neutrophil activation, also selectively increased LukGH sensitivity. Deletion of *lukGH*, but not other leukocidin genes, prevented PMN killing upon infection with USA300 CA-MRSA.

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Conclusion: Inflammatory signals enhance the susceptibility of human PMNs to lysis by LukGH rendering this toxin dominant among the *S. aureus* leukocidins *in vitro*.
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Introduction

Phagocytic cells, especially neutrophils are the cornerstones of host defense against *Staphylococcus aureus*.[1,2] *S. aureus* has developed multiple mechanisms to avoid its elimination by phagocytic cells. The most direct one is killing of neutrophils, monocytes and macrophages by secreted leukocidins. These cytolytic toxins – the two gamma-hemolysins (HlgAB, HlgCB), the Panton-Valentine leukocidin (LukSF-PV), LukED and LukGH (LukAB) – belong to the bi-component beta-barrel pore forming toxin family.[3,4] It is rather enigmatic that *S. aureus* has evolved to employ five different leukocidins and little is known about their individual contribution to human *S. aureus* infections. The *lukSF-PV* gene is carried on prophages in <10% of *S. aureus* isolates, while *lukED* is found in ~70% and *hlgACB* and *lukGH* in almost all clinical isolates.[3,4] Consequently, correlating gene prevalence with clinical epidemiology has been informative only for LukSF-PV. Therefore, methodological approaches that directly measure cytotoxic potencies associated with the individual leukocidins are needed to assess their contribution to *S. aureus* diseases.

Recently, receptors have been determined for all leukocidins and identified as important immune molecules that play crucial roles in activation and migration of immune cells. LukSF-PV and HlgCB both employ the complement receptors C5aR and C5L2 for cell targeting.[5,6] LukED and HlgAB both bind to the IL-8 receptors CXCR1 and CXCR2 and additionally target CCR5 or CCR2, respectively.[6–8] Based on the high amino acid sequence identity among the leukocidins (up to 82%), it is not surprising that some share receptors on their target cells. LukGH is a unique member of the bi-component staphylococcal toxin family with <40% amino acid sequence identity to the other leukocidins. Moreover, unlike the other four leukocidins, it forms a dimer in solution before binding to its target cells.[9,10] The LukGH receptor was identified to be CD11b, the alpha-subunit of the integrin αM/β2, also known as CD11b/CD18 or complement receptor 3 (CR3).[11]

Although the identification of these leukocidin receptors led to a better understanding of their cellular tropism, species specificity hinders delineation of their roles in pathogenesis *in vivo*.[3,4,12] Moreover, *in vitro* studies to date have mostly assessed the cytolytic activity of leukocidins towards myeloid cell lines or isolated neutrophils without the context of the inflammatory milieu that is shaped by the interplay between *S. aureus* and the host.

Therefore we employed *in vitro* assays with primary human neutrophils exposed to physiological priming/activating stimuli to assess the effect on leukocidin sensitivity and the relative contribution of the five leukocidins.

Materials and methods

Bacterial strains and culture supernatants (CS)

The USA300 CA-MRSA strain TCH1516 was obtained from ATCC (ATCC® BAA-1717™). Isogenic mutants lacking the *hla*, *lukED*, *lukSF-PV*, *hlgACB*, and *lukGH* genes were generated in the TCH1516 background by homologous recombination as described previously.[13] Wild type and mutant strains grown in RPMI (Gibco, Thermo Fisher Scientific) supplemented with 1% casamino acids (Amresco) at 37 °C under constant agitation (200 rpm) showed identical growth characteristics based on OD_{600nm} measurements and CFU counting on sheep blood agar plates. CS from cultures grown in RPMI-CAS to stationary phase (OD_{600nm}: ~1.7) were harvested by centrifugation at 5000 × g at 4 °C, followed by filter sterilization of the supernatant using 0.1 μm pore size PVDF syringe filters (Millipore).

PMN isolation and stimulation

PMNs were isolated from heparinized human blood, obtained from healthy volunteers or the Austrian Red Cross. Cells were purified using Percoll Plus (GE Healthcare) gradient centrifugation, as described previously.[10] Purification methods resulted in PMN purity of >95% determined microscopically by Giemsa staining as well as flow cytometric analysis of forward-versus side-scatter properties. Cell viability was >98% based on Trypan blue (Thermo Fisher Scientific) exclusion. Cells at a concentration of 1×10^6/mL were pre-incubated for 1 h at 37 °C, 5% CO_2 with LPS (purified from *Escherichia coli* O111, List Laboratories), *S. aureus* CS, *S. aureus* peptidoglycan (Sigma–Aldrich), *S. aureus* delta-toxin (synthetic peptide from AnaSpec) or human IL-8 (amino acids Ser28-Ser99, BioLegend) at concentrations indicated in the figures prior to use in cell-based toxicity assays or surface staining for leukocidin receptor expression by flow cytometry.

Recombinant leukocidins

Monomers of HlgAB, HlgCB, LukSF-PV and LukED, as well as the co-expressed LukGH dimer (all based on the TCH1516 genome sequences) were recombinantly expressed in *E. coli* as described previously.[10,13] Briefly, LukS-PV, LukF-PV, HlgA, HlgC and HlgB were expressed in soluble form with an N-terminal NusA/His6 tag, which was removed proteolytically after the first purification step. Purification typically involved three chromatographic steps: 1. IMAC (immobilized metal affinity column); 2. cation exchange or IMAC; and 3. size exclusion chromatography.[13] LukE and LukD were expressed without tag and purified by cation

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