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Relative contribution of Panton-Valentine leukocidin to PMN plasma membrane permeability and lysis caused by USA300 and USA400 culture supernatants

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Abstract

Panton-Valentine leukocidin (PVL) is a cytolytic toxin associated with severe community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections. However, the relative contribution of PVL to host cell lysis during CA-MRSA infection remains unknown. Here we investigated the relative contribution of PVL to human polymorphonuclear leukocyte (PMN) plasma membrane permeability and lysis in vitro by using culture supernatants from wild-type and isogenic *lukS/F-PV-negative* (Δpvl) USA300 and USA400 strains. Using *S. aureus* culture conditions that favor selective high production of PVL (CCY media), there was on average more PMN plasma membrane permeability and cell lysis caused by supernatants derived from wild-type strains compared with those from Δpvl strains. Unexpectedly, plasma membrane permeability did not necessarily correlate with ultimate cell lysis. Moreover, the level of pore formation caused by culture supernatants varied dramatically (e.g., range was 0.32–99.09% for wild-type USA300 supernatants at 30 min) and was not attributable to differences in PMN susceptibility to PVL among human blood donors. We conclude that PMN pore formation assays utilizing *S. aureus* culture supernatants have limited ability to estimate the relative contribution of PVL to pathogenesis (or cytolysis in vitro or in vivo), especially when assayed using culture media that promotes selective high production of PVL.

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Keywords

STAPHYLOCOCCUS AUREUS; VIRULENCE; LEUKOCIDINS

1. Introduction

Staphylococcus aureus is a significant cause of human infections worldwide. The organism also acquires antibiotic resistance readily and methicillin resistant *S. aureus* (MRSA) are endemic in healthcare settings in many countries [1]. Prior to the early 1990s, MRSA infections were almost exclusively associated with healthcare settings and disease occurred in individuals with known risk factors for infection. Although healthcare-associated MRSA (HA-MRSA) remain a major problem, MRSA are a leading cause of community-associated bacterial infections in some industrialized countries, such the United States and Canada [2]. These so-called community-associated MRSA (CA-MRSA) infections occur in seemingly healthy individuals with no predisposing risk factors for infection, suggesting that they have enhanced virulence by comparison. Experimental data with animal infection models using CA-MRSA strains provides strong support to this notion [3,4]. The molecular basis for the enhanced virulence phenotype of CA-MRSA strains, especially USA300 and USA400, which predominate in North America, is incompletely defined.

A methicillin-resistance element known as staphylococcal cassette chromosome (SCC) *mec* type VI (SCC*mec*IV) and genes encoding Panton-Valentine leukocidin (PVL) (*lukS-PV* and *lukF-PV*) are elements common among many CA-MRSA strains worldwide [2]. PVL is a cytolytic toxin comprised of LukF-PV and LukS-PV subunits that assemble into an octameric pore on the surface of myeloid cells, including polymorphonuclear neutrophils (PMNs). Although sublytic concentrations of PVL cause PMN apoptosis [5], sufficient pore formation causes a change in the cellular levels of normally impermeable solutes, such as K⁺, which can lead to osmotic cell lysis (necrosis) [6,7]. The leukocidin is linked by epidemiology to specific types of severe skin infection and severe necrotizing pneumonia [8,9]. Inasmuch as PMNs are the primary cellular defense against *S. aureus* infections, molecules such as PVL that have potential to eliminate neutrophils and/or alter neutrophil function might therefore contribute to pathogenesis. USA300 and USA400 strains contain genes encoding multiple pore-forming toxins with high homology or identity to *lukS-PV* and *lukF-PV*. The relative contribution of these molecules to CA-MRSA virulence has not been determined, although this matter has been the subject of intense research over the past several years [10–15]. In addition, high expression of PVL in vitro appears optimal only during growth in specific *S. aureus* culture media, which may limit the utility of such assays in predicting activity in vivo.

As a step toward understanding the relative contribution of PVL to lysis of PMNs caused by USA300 and USA400 strains, we evaluated human PMN plasma membrane permeability and lysis using culture supernatants from multiple *S. aureus* growth conditions in vitro.

2. Materials and Methods

2.1. Bacterial strains and culture

USA300 (LAC and SF8300) and USA400 (MW2) wild-type and isogenic *lukS-PV* and *lukF-PV* deletion strains (LAC Δ *pvl*, SF8300 Δ *pvl*, and MW2 Δ *pvl*) were described previously [11, 12]. Bacteria from frozen, low passage stocks were cultured overnight in trypticase soy broth (TSB, Difco, Detroit Michigan), CCY medium (3% wt/vol yeast extract, 2% Bacto-Casamino acids, 2.3% sodium pyruvate, 0.63% Na₂HPO₄, and 0.041% KH₂PO₄, pH 6.7), or 100% pooled human serum. Overnight cultures were either used to generate supernatants directly or diluted 1:200 into fresh culture media and incubated for 8 h (to early stationary phase of growth,

OD₆₀₀ = 0.75) with shaking at 225 rpm at 37°C. Bacteria were removed from the culture media by centrifugation (2061 g for 10 min at 4°C). Culture supernatants were sterilized by filtration and stored in aliquots at -80°C for future use.

2.2. Purification of PVL subunits from USA300 culture medium

LukS-PV and LukF-PV subunits were purified from culture supernatants of USA300 strain LAC containing deletion of *hlgA*, *hlgB*, and *hlgC* (LAC Δ *hlgABC*) as described previously [16,17], but with a few modifications. Briefly, LAC Δ *hlgABC* was cultured to early stationary phase of growth in CCY medium and cultures were centrifuged to remove bacteria. Following sterile filtration, supernatant proteins were precipitated with ammonium sulfate (80% saturation) at 4°C for 16 h. Precipitates were centrifuged at 15000 g for 20 min at 4°C and resuspended in Buffer 1 (30 mM sodium phosphate buffer, pH 6.5). Proteins were dialyzed against Buffer 1 for 5 h, subjected to ion-exchange chromatography using a HiPrep 16/10 CM FF sepharose column (GE Healthcare Life Sciences, Piscataway, New Jersey), and eluted with a linear gradient of 0 to 0.5 M NaCl in Buffer 1. Fractions containing LukS-PV were subjected to a second round of ion-exchange chromatography using a Mono S 5/50 GL column (GE Healthcare Life Sciences) and LukS-PV was eluted with a linear gradient of 0 to 0.25 M NaCl in Buffer 1. Ammonium sulfate was added to LukF-PV and LukS-PV fractions to 1.5 M and these samples were subjected to hydrophobic interaction chromatography using a HiTrap Butyl HP column (GE Healthcare Life Sciences). PVL subunits were eluted with a linear gradient of 1.5 to 0 M ammonium sulfate and aliquots of each subunit were stored at -80°C in 0.2 M NaCl-Buffer 1. Identity and purity of LukS-PV and LukF-PV were evaluated initially by SDS-PAGE and immunoblot analysis, and then by liquid chromatography tandem mass spectrometry (LC-MS/MS) at the NIAID Mass Spectrometry Unit, Bethesda, Maryland.

2.3. Human PMN assays

PMNs were isolated from venous whole blood of healthy individuals using a published method [18] in accordance with a protocol approved by the NIAID Institutional Review Board for Human Subjects. Each human subject included in the study gave informed consent. Lysis of PMNs was assessed by the release of lactate dehydrogenase (LDH) using a Cytotoxicity Detection Kit (Roche Applied Sciences, Pleasanton, California) as described previously [3, 12]. Culture supernatants were thawed on ice and diluted in RPMI 1640 medium (Invitrogen) buffered with 10 mmol/l HEPES (RPMI/H, pH 7.2). PMNs (1×10^6) in 100 μ l RPMI/H were combined with 100 μ l of diluted supernatants in 96-well round-bottom plates. Cells were incubated for the indicated times (3–18 h) at 37°C with 5% CO₂. At designated time points, plates were centrifuged at 587 g for 7 min at 4°C. Aliquots (100 μ l) from each well were transferred to a 96-well flat-bottom plate and percent LDH release was determined according to the manufacturer's instructions.

PMN plasma membrane permeability (formation of plasma membrane pores) was measured by ethidium bromide (EtBr) uptake as described essentially by Gauduchon et al. [19]. Culture supernatants were diluted in RPMI/H as described above and mixed 1:1 with human PMNs (1×10^6) and 4 μ mol of EtBr as described [19]. At designated time points, PMNs were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, California).

In some experiments, PMNs were incubated with 1 nM purified PVL (LukS-PV + LukF-PV) or individual PVL subunits and EtBr and LDH release were determined as described above.

2.4. SDS-PAGE and LukS/F-PV Western Blots

Proteins present in CCY and TSB culture supernatants were resolved by 12.5% SDS-PAGE and transferred to nitrocellulose membranes using an iBlot Dry Blotting System (Invitrogen, Carlsbad, California). Nitrocellulose membranes were blocked in Tris-buffered saline

containing 10% goat serum and 1% Tween 20 overnight. Immunoblots were rotated for 1 h at ambient temperature in diluted blocking buffer containing affinity-purified rabbit IgG specific for a peptide region of LukF-PV or LukS-PV (GenScript USA Inc., Piscataway, New Jersey) or rabbit IgG specific for Hla (Sigma-Aldrich, St. Louis, Missouri). Membranes were washed three times for 10 min each in wash buffer (250 mM NaCl, 10 mM HEPES, 0.2% Tween-20, pH 7.4) and then incubated with peroxidase-conjugated anti-rabbit donkey IgG secondary antibody for 1 h. Membranes were washed twice in wash buffer and once in Tris-buffered saline and LukF-PV and LukS-PV were visualized using enhanced chemiluminescence (SuperSignal West Pico, Fisher Scientific, Pittsburgh, Pennsylvania). LukS-PV was quantified by densitometry with a standard curve of purified LukS-PV using Quantity One Software (Bio-Rad Laboratories, Hercules, California). There was 0.8 µg/ml, 3.8 µg/ml, and 3.6 µg/ml LukS-PV produced by MW2, LAC, and SF8300 in TSB at early stationary phase of growth, and 0.9 µg/ml, 2.0 µg/ml, and 1.2 µg/ml for these strains after overnight growth in the same media. By comparison, there was 30.6 µg/ml, 100.4 µg/ml, and 101.4 µg/ml LukS-PV produced by MW2, LAC, and SF8300 in CCY at early stationary phase of growth, and 31.8 µg/ml, and 32.8 µg/ml for LAC and SF8300 after overnight growth in CCY. PVL subunits were frequently not detectable in MW2 culture supernatants after overnight growth in CCY.

2.5. *S. aureus* genomic DNA extraction

USA300 strain LAC was cultured in TSB to early stationary phase of growth and then bacteria were pelleted by centrifugation as described above. Bacteria were resuspended in 450 µl of P1 buffer (Plasmid Prep Kit, Qiagen, Inc., Valencia, California), to which 50 µl of 1 mg/ml lysostaphin was added. Samples were incubated for 3 h at 37°C to complete lysis. After lysis of bacteria, DNA was isolated using the DNeasy Tissue Kit (Qiagen) and as recommended by the manufacturer.

2.6. RNA extraction and TaqMan Real-Time RT-PCR analysis

S. aureus strains were cultured as indicated and lysed using FastPrep (FP 120, MP Biomedicals, Solon, Ohio). RNA isolation was completed using the RNeasy Mini Prep Kit (Qiagen) as described previously [20]. Each strain and growth condition was assayed in triplicate by TaqMan real-time RT-PCR analysis using an ABI 7500 thermocycler (Applied Biosystems Inc., Foster city, California). Change in expression of target genes was determined by comparison to known quantities of *S. aureus* genomic DNA and relative expression of the housekeeping gene *gyrB*. The primer-probe sequences are as follows: *gyrB* forward primer 5'-CAAATGATCACAGCATTTGGTACAC-3', *gyrB* probe 5'-AA TCGGTGGCGACTTTGATCTAGCGAAAG-3', *gyrB* reverse primer 5'-CGGCATCAG TCATAATGACGAT-3', *LukF-PV* forward primer 5'-TTGCTTTTGCTATCC AATACA GTTG-3', *LukF-PV* probe 5'-TGCAGCTCAACATATCACACCTGTAAGT-3', *LukF-PV* reverse primer 5'-TCGGAATCTGATGTTGCAGTTG-3', *LukS-PV* forward primer 5'-AATAACGTATGGCAGAAATATGGATGT-3', *LukS-PV* probe 5'-ACTCATGCTACTAGAAGAACAACACTATGG-3', *LukS-PV* reverse primer 5'-CAAATGCGTTGTGTATTCTAG ATCCT-3'.

2.7. Statistical analyses

Data were evaluated using a paired Student's t-test (GraphPad Prism 5, GraphPad Software, Inc., San Diego, California). PMN lysis data in Fig. 4 from wild-type CCY culture supernatants were also compared using a one-way analysis of variance (ANOVA) and Tukey's posttest.

3. Results

3.1. Membrane pore formation caused by *S. aureus* culture supernatants is highly variable

To estimate the relative contribution of PVL to formation of membrane pores in leukocytes, we evaluated the ability of culture supernatants from USA300 and USA400 wild-type and Δpvl strains to promote uptake of ethidium bromide (EtBr) by human PMNs (Fig. 1 and Fig. 2). EtBr uptake has been used widely to estimate PVL-mediated membrane pore formation with human neutrophils, as the diameter of the pores (2–2.4 nm or 20–24 angstroms) allow free diffusion of EtBr (0.8 nm or 8 angstroms) into cells [21–26]. We first tested pore-forming capacity of CCY culture supernatants, since *S. aureus* can produce up to 100 milligrams of PVL per liter of CCY media [16,27,28]. There was time-dependent formation of PMN membrane pores with all growth conditions and supernatant concentrations (dilutions) tested (Fig. 1 and Fig. 2). On average, there was significantly more uptake of EtBr by PMNs exposed to wild-type culture supernatants compared with those from Δpvl strains (Fig. 1 and 2). However, in many of the individual assays, especially those in which PMNs were exposed to culture supernatants for 30 min, the level of pore formation was comparable between wild-type and Δpvl strains (Fig. 1B and C, and Fig. 2C). We found that culture supernatants from bacteria grown in trypticase soy broth (TSB), a standard culture medium for *S. aureus*, had little or no PMN pore-forming capacity at the highest concentration used for CCY (1:500 dilution) (data not shown). Membrane pore formation was highly varied using CCY culture supernatants (e.g., the range of pore formation was 0.6–99.9% for the MW2 wild-type strain at 30 min using a 1:500 dilution) (Fig. 1C). Although there was variation in PMN pore formation among individuals using the same batch of culture supernatant, some of the observed variation overall was due to differences among separate batches CCY media (Fig. 1 and Fig. 2, symbol colors and fills).

3.2. Correlation of membrane pore formation and PMN lysis

Formation of plasma membrane pores by PVL and other two-component toxins of *S. aureus* is generally considered to result in host cell lysis. To test this notion, we evaluated the ability of culture supernatants from USA300 and USA400 wild-type and Δpvl strains to cause release of lactate dehydrogenase (cell lysis) from human PMNs over time (Fig. 3 and 4). PMN lysis caused by exposure to CCY or TSB culture supernatants from USA300 and USA400 strains was time and concentration dependent (Fig. 3 and 4). In addition, there was significantly more lysis of PMNs exposed to wild-type CCY culture supernatants compared with those from Δpvl strains at the highest concentrations tested (1:250 and 1:500 dilutions) (Fig. 3A and B). In contrast, there was no difference in PMN lysis between TSB culture supernatants from wild-type and Δpvl strains (e.g., PMN lysis was 87.1 ± 14.5 and $87.5 \pm 9.1\%$ after a 3-h exposure to LAC wild-type and Δpvl TSB supernatants from overnight culture (1:10 dilution) (Fig. 4).

Although there was some concordance between pore formation and cell lysis assays, especially with CCY culture supernatants from wild-type strains, there were noted differences. First, at the lowest concentration of CCY culture supernatant used (1:2000 dilution), there was little or no correlation between pore formation and PMN lysis (compare Fig. 2C and 3C). Further, PMN pore formation was at or near 100% after 30 min of exposure to the highest concentration of CCY culture supernatants from Δpvl strains (Fig. 1C), whereas the corresponding cell lysis was < 10% at all time points tested (up to 18 h) (Fig. 3B). We also note that culture supernatants from the MW2 wild-type strain often caused significantly less lysis than those from USA300 wild-type strains (Fig. 3). Collectively, the data indicate that pore formation caused by *S. aureus* culture supernatants does not necessarily correlate with (or result in) cell lysis.

Inasmuch as in vitro culture media (CCY and TSB) are not likely representative of culture conditions during infection in humans, we tested the ability of normal human serum to promote

production and activity of *S. aureus* cytolytic toxins. In contrast to CCY and TSB, human serum used as culture media for USA300 and USA400 strains had zero capacity to cause PMN lysis at all concentrations tested (1:1–1:100 dilutions). This finding cannot be explained by the absence of bacterial growth in serum, since *S. aureus* grew reasonably well in this culture substrate (Fig. 6). Rather, the lack of PMN cytolysis in serum culture supernatants may be due to the ability of serum lipoproteins and apolipoprotein B to inhibit *agr* signaling [29] and thus, synthesis and secretion of PVL.

3.3. Cytolytic effects of purified PVL and human blood donor variability

We next used PVL subunits purified from USA300 to determine whether the variation in PMN pore formation noted in the assays with culture supernatant was due to individual susceptibility to PVL (Fig. 5). Neither subunit alone caused formation of membrane pores in human PMNs. By comparison, there was time-dependent uptake of EtBr using the combination of 1 nM LukF-PV+LukS-PV (Fig. 5). There was far less variation in the capacity of purified PVL to permeabilize human PMNs compared with culture supernatants (EtBr uptake was 76.1–96.1% at 30 min using purified PVL subunits) (Fig. 5C). Despite the high level of pore formation, there was essentially no corresponding PMN lysis at this time point (LDH release was $0.7 \pm 0.7\%$ at 30 min, $n = 4$ PMN donors) (Fig. 5D). These data indicate that the variation noted in assays with culture supernatants is largely independent of differences in PMN donor susceptibility to PVL.

3.4. Levels of *lukS-PV* and *lukF-PV* transcript and corresponding PVL protein subunits are highly varied depending on in vitro growth conditions

We next compared levels of *lukS-PV* and *lukF-PV* (*lukS/F-PV*) transcripts and corresponding protein subunits following culture of USA300 and USA400 strains in CCY, TSB, or human serum (Fig. 6A–H). Compared with strains cultured in TSB or human serum, there was more *lukS/F-PV* transcript made by LAC and MW2 following culture in CCY (Fig. 6A–C). In accordance with these findings, more LukS-PV and LukF-PV accumulated in CCY culture media compared with that in TSB (Fig. 6D–F). Most notably, there was selective high production of PVL in CCY media, since the level of alpha-hemolysin (Hla, another *agr*-regulated cytolytic toxin) present in CCY was not increased relative to that in TSB culture supernatants (Fig. 6F). Using a purified PVL standard, we estimate that USA300 strains accumulated ~3–4 µg/ml of LukS-PV in TSB and ~100 µg/ml in CCY at early stationary phase of growth (see Materials and Methods for details). The finding that there was little or no detectable *lukS/F-PV* transcript made by LAC cultured in human serum (Fig. 6C) is consistent with the observation that supernatants from neither USA300 nor USA400 strains cultured in human serum caused PMN lysis (data not shown) and that serum lipoproteins inhibit *agr* signal transduction [29].

There were also strain-dependent differences in *lukS/F-PV* transcript and PVL protein levels. For example, compared with LAC, there was less *lukS/F-PV* transcript made by MW2 in either CCY or TSB, and there was correspondingly less accumulated PVL protein in MW2 culture supernatants (e.g., there was ~42-fold more *lukF-PV* transcript made by LAC at early stationary phase of growth in CCY compared with MW2) (Fig. 6). These findings are compatible with differences noted between USA300 and USA400 strains in recent studies by Montgomery et al. [30]. Although differences in *lukS/F-PV* transcript and PVL protein levels may account in part for the differences in PMN lysis observed between MW2 and USA300 CCY culture supernatants (Fig. 3), there is limited correlation of transcript and protein levels with pore forming capacity of the strains, which was comparable (Fig. 1 and 2). Taken together, these data provide strong support to the notion that factors present in culture supernatants other than PVL are sufficient to cause formation of membrane pores in human PMNs.

4. Discussion

It has long been known that PVL is cytolytic for myeloid cells and therefore, a putative virulence molecule of *S. aureus* [31–34]. Finck-Barbançon et al. provided direct evidence that PVL is a pore-forming toxin and reported that membrane pore size is dictated by ionic conditions of the extracellular environment [21]. Notably, these studies set a precedent for using an ethidium bromide uptake assay to evaluate membrane permeability following exposure to PVL [21]. Plasma membrane pores that allow ethidium bromide uptake by PMNs are in general considered to result ultimately in cell lysis through osmotic imbalance [6,7,21, 25]. The assay or modifications thereof have since been used to evaluate precise kinetics of pore formation caused by purified or recombinant PVL [19,23], pore-forming capacity of heterologous combinations of LukS and LukF proteins (PVL, gamma-hemolysin, and LukD and LukE subunits) [24], the ability of intravenous immunoglobulin or specific anti-PVL antibody to block pore formation [19], and to estimate the relative contribution of PVL to cytolytic capacity of *S. aureus* culture supernatants [12,19,35].

We reported previously that PMNs exposed to supernatants from Δpvl strains cultured overnight in YCP media, a culture media similar in composition to CCY, promoted uptake of EtBr at levels comparable to the wild-type strains [12]. Although these findings were unexpected, especially given the high level of PVL produced in either YCP or CCY media (up to 20% of the protein in culture filtrates [27]), they are perhaps explained by the high variation reported here as intrinsic to the pore formation assay. Some of the variation is due to differential levels of *lukS/F-PV* transcript and PVL protein levels produced in separate batches of the same media (Fig. 6A and G). Most importantly, there is not a direct correlation between EtBr uptake and LDH release by human PMNs, which is a widely accepted determination of cell lysis.

There is clearly more PMN lysis caused by CCY supernatants from wild-type USA300 strains compared to that from Δpvl mutant strains (not observed with MW2 wild-type and Δpvl strains), but this difference is presumably due to the very high concentration of PVL in CCY culture supernatants. Therefore, diluting CCY culture media to obtain PVL-specific cytolysis is in essence similar to using purified PVL, since such an approach decreases the level of other *S. aureus* molecules that would have otherwise contributed to pore formation and/or lysis. This notion is consistent with the observation that there are limited differences in PMN pore formation between wild-type and Δpvl strains using lower dilutions of CCY culture supernatant (Fig. 1C), and the finding that PVL is selectively overproduced in CCY media relative to Hla (Fig. 6F). Furthermore, the concentration of PVL produced in TSB (3–4 $\mu\text{g/ml}$), which is more representative of that found in human abscesses (0.3–1.8 $\mu\text{g/ml}$) [36], is perhaps a better gauge of the relative contribution of PVL to PMN lysis caused by *S. aureus*. In any case, factors present in TSB culture supernatants other than PVL, such as alpha-type phenol-soluble modulins [37] and/or gamma-hemolysin [17], were sufficient to cause lysis of human PMNs (Fig. 4).

We conclude that the PMN pore formation assays described herein, although appropriate to evaluate effects of purified PVL or estimate whether cytolytic capacity exists in culture supernatant, have limited ability to estimate the relative contribution of PVL to membrane pore formation or PMN lysis in vivo.

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Fig. 1.

Permeability of PMNs (plasma membrane pore formation) exposed to USA300 and USA400 CCY culture supernatants. PMNs (1×10^6) were incubated with a 1:500 dilution of CCY media alone (CCY) or CCY culture supernatants obtained from growth of *S. aureus* strains as indicated. Each symbol indicates a separate experiment and/or human PMN donor. Different batches of CCY media are indicated by symbol color or fill. Black bars indicate the mean percent ethidium bromide (EtBr) uptake by PMNs at each time point, (A) 5 min. (B) 15 min. (C) 30 min. *: $p \leq 0.05$ vs. wild-type (WT; i.e., MW2, LAC, or SF8300); **: $p \leq 0.01$ vs. WT; ***: $p \leq 0.001$ vs. WT; ns, not significant.

Fig. 2.

Permeability of PMNs exposed to USA300 and USA400 CCY culture supernatants. PMNs (1×10^6) were incubated with a 1:2000 dilution of CCY media alone (CCY) or CCY culture supernatants obtained from growth of *S. aureus* strains as indicated. Each symbol indicates a separate experiment and/or human PMN donor. Different batches of CCY media are indicated by symbol color or fill. Black bars indicate the mean percent ethidium bromide (EtBr) uptake by PMNs at each time point, (A) 5 min. (B) 15 min. (C) 30 min. *: $p \leq 0.05$ vs. wild-type (WT; i.e., MW2, LAC, or SF8300); **: $p \leq 0.01$ vs. WT; ***: $p \leq 0.001$ vs. WT; ns, not significant.

Fig. 3.

PMN lysis after exposure to USA300 or USA400 CCY culture supernatants. PMNs (1×10^6) were incubated for 3, 6, 9, or 18 h with the indicated dilution of culture supernatant. Cell lysis was measured by LDH release as described in Materials and methods. Bars indicate mean $\pm$ SD of 8–11 PMN donors; *, $p \leq 0.05$ vs. WT; #, $p \leq 0.05$ using a one-way analysis of variance (ANOVA) with a Tukey's posttest. (A) 1:250 dilution. (B) 1:500 dilution. (C) 1:2000 dilution. PVL "+" indicates wild-type *lukS/F-PV*-positive strain; PVL "-" indicates isogenic *lukS/F-PV* negative strain. CCY alone caused no significant lysis of human PMNs over the 18 h culture period (range was 0–1.5% for 3–18 h, $n = 4$ PMN donors).

Fig. 4. PMN lysis after exposure to USA300 or USA400 TSB culture supernatants. PMNs (1×10^6) were incubated for 3, 6, 9, or 18 h with the indicated dilution of culture supernatant. Cell lysis was measured by LDH release as described in Materials and methods. Bars indicate mean $\pm$ SD of 6 PMN donors for panels A–C and 4–6 PMN donors for panel D; *: $p \leq 0.05$ vs. WT. (A) 1:10 dilution. (B) 1:50 dilution. (C) 1:100 dilution. (D) 1:500 dilution. PVL “+” indicates wild-type *lukS/F-PV*-positive strain; PVL “–” indicates isogenic *lukS/F-PV* negative strain. TSB alone caused no significant lysis of human PMNs over the 18 h culture period (range was 0–0% for 3–18 h, $n = 4$ PMN donors)

Fig. 5.

Purified PVL causes relatively consistent levels of plasma membrane pore formation in human PMNs. (A–C) PMNs (1×10^6) were incubated with 1 nM of purified PVL subunits (LukF-PV or LukS-PV) or the combination of both subunits for the indicated times. Each symbol color indicates a separate human PMN donor ($n = 9$). Black bars indicate the mean percent ethidium bromide (EtBr) uptake by PMNs at each time point. (D) PMNs (1×10^6) were incubated with 1 nM of purified PVL subunits (LukF-PV or LukS-PV) and PMN lysis was measured by release of LDH as described in Materials and methods. Red bar indicates mean $\pm$ SD of 4 PMN donors.

Fig. 6. Impact of culture media on *lukS-PV* and *lukF-PV* transcript levels and protein subunit secretion. (A–C) Relative expression of *lukS-PV* and *lukF-PV* transcripts during bacterial growth as indicated. Different colors or fill indicate separate batches of CCY media. Symbols are the mean of triplicate TaqMan samples. Black bars indicate mean of each strain group. (A) Growth in CCY. (B) Growth in TSB. (C) Growth in normal human serum. (D) PVL secretion during growth in CCY (upper panel) or TSB (lower panel). (E) Longer exposure of the immunoblot shown in the lower panel of (D). Results in panels D and E are representative of 3 experiments. (F) Immunoblot of PVL subunits (1.75 μ l of CCY or TSB culture supernatants) and Hla (35 μ l of CCY or TSB culture supernatants) produced in CCY or TSB culture media at early stationary phase of growth. Results are representative of 2 experiments. (G) Immunoblot of PVL subunits secreted during MW2 (USA400) and SF8300 (USA300) growth in different batches of CCY media. (H) Growth of LAC (USA300) in CCY, TSB, and normal human serum.