Sublytic concentrations of Staphylococcus aureus Panton-Valentine leukocidin alter human PMN gene expression and enhance bactericidal capacity

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ABSTRACT
CA-MRSA infections are often caused by strains encoding PVL, which can cause lysis of PMNs and other myeloid cells in vitro, a function considered widely as the primary means by which PVL might contribute to disease. However, at sublytic concentrations, PVL can function as a PMN agonist. To better understand this phenomenon, we investigated the ability of PVL to alter human PMN function. PMNs exposed to PVL had enhanced capacity to produce O2− in response to fMLF, but unlike priming by LPS, this response did not require TLR signal transduction. On the other hand, there was subcellular redistribution of NADPH oxidase components in PMNs following exposure of these cells to PVL—a finding consistent with priming. Importantly, PMNs primed with PVL had an enhanced ability to bind/ingest and kill Staphylococcus aureus. Priming of PMNs with other agonists, such as IL-8 or GM-CSF, altered the ability of PVL to cause formation of pores in the plasma membranes of these cells. Microarray analysis revealed significant changes in the human PMN transcriptome following exposure to PVL, including up-regulation of molecules that regulate the inflammatory response. Consistent with the microarray data, mediators of the inflammatory response were released from PMNs after stimulation with PVL. We conclude that exposure of human PMNs to sublytic concentrations of PVL elicits a proinflammatory response that is regulated in part at the level of gene expression. We propose that PVL-mediated priming of PMNs enhances the host innate immune response. J. Leukoc. Biol. 92: 361–374; 2012.

Introduction
S. aureus is a Gram-positive bacterium that causes a significant number of infections worldwide [1]. Indeed, MRSA is currently a leading contributor to HA infections [2, 3]. MRSA infections that occur outside of hospital settings, known as CA-MRSA infections, were reported in the early 1990s and have become widespread in the United States and Canada [4, 5]. In contrast to HA-MRSA, CA-MRSA causes infections in individuals with no known risk factors for infection. The ability of these strains to cause disease in otherwise healthy individuals suggests that they have enhanced virulence compared with traditional HA-MRSA strains. In vitro and in vivo work supports this hypothesis [6, 7]. Although progress has been made, the molecular basis of the enhanced virulence phenotype of CA-MRSA remains incompletely determined.

Genes encoding PVL are present in the genome of many CA-MRSA strains, including the epidemic USA300 strain [8, 9]. PVL consists of two subunits, LukS-PV and LukF-PV, whose
genes are transcribed as an operon; it is a cytolytic toxin specific for myeloid cells, including PMNs [10]. The presence of both subunits is required for formation of pores within the PMN plasma membrane. Inasmuch as PMNs are the most prominent cellular component of the innate immune system and thus, the primary defense against *S. aureus* infections, it has been proposed that PVL contributes to virulence by causing lysis of PMNs and other myeloid cells. However, previous studies have shown that cytolysis in vitro requires a concentration of PVL that may not be achieved in vivo [11, 12].

Sublytic concentrations of PVL elicit numerous cellular responses, including release of MPO and chemotactic molecules, such as IL-8 and LTB4 [13–16]. PMNs exposed to PVL undergo granule exocytosis and produce ROS following stimulation with fMLF [17]. These observations suggest that sublytic levels of PVL prime PMNs for enhanced activation by a secondary stimulus, although the molecular basis for PVL-mediated PMN priming remains unknown.

To gain a better understanding of the molecular basis of PVL-mediated PMN priming, we investigated mobilization of the NADPH oxidase components to the plasma membrane and measured PMN gene expression following exposure of these cells to sublytic concentrations of PVL. In addition, we identified proinflammatory molecules secreted by PMNs following exposure to the leukotoxin. Our results provide new insight into a possible role played by PVL during human infection.

**MATERIALS AND METHODS**

**Human PMN isolation**

PMNs were isolated from venous whole blood of healthy individuals, as described previously [18]. Purity of PMNs was 99.7 ± 0.2%, and viability was 99.2 ± 1.1%, as determined from a sampling of >40 PMN preparations during the course of the studies. PMN preparations typically contain 95–98% neutrophils, and virtually all of the remaining cells are eosinophils. Each subject gave informed consent prior to participation in the study, and all work was approved by the Institutional Review Board for Human Subjects, NIAD, NIH (Hamilton, MT, USA), or by the Institutional Review Board for Human Subjects, Montana State University (Bozeman, MT, USA).

**S. aureus culture conditions**

USA300 strain LAC was cultured overnight in TSB (Difco, Detroit, MI, USA) from frozen bacterial stocks. Overnight cultures were diluted 1:200 in fresh TSB media and cultured to a midlogarithmic growth phase (OD600=0.75), as described [11]. Bacteria (10^6 CFUs) were centrifuged at 8000 rcf for 2 min, washed once with PBS, and centrifuged again to pellet bacteria. For assays that measured opsonophagocytic killing by PMNs, bacteria were resuspended in PBS and then opsonized with human serum as described by Gauduchon et al. [19], but with modifications [11]. Purified PVL subunits (LukF-PV and LukS-PV) were diluted at the desired concentrations in RPMI/H. PVL-mediated pore formation was evaluated by incubating human PMNs (1×10^6) with 4 μM EtBr and 1 nM, 2 nM, or 5 nM active PVL for 30 min. LukF-PV and LukS-PV were boiled at 95°C for 10 min to produce iPVL, which was used as a negative control for PMN assays where indicated. Alternatively, PMNs in RPMI/H + 4 μM EtBr were electroporermobilized with a Gene Pulser II (Bio-Rad, Hercules, CA, USA) using a single pulse at 2 kV with the capacitor set at 25 μF (2 kV pulse in a 0.4-cm cuvette). EtBr uptake was analyzed by flow cytometry (FACS-Calibur, BD Biosciences, San Jose, CA, USA).

Human IL-8 and GM-CSF were purchased from eBioscience (San Diego, CA, USA). fMLF, PMA, and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). PMN agonists, 20 μM IL-8, 100 ng/ml GM-CSF, 1 μM fMLF, 1 μg/ml PMA, 100 ng/ml LPS, or hLAC (2.5×10^9 cfu), were diluted in RPMI/H and added to human PMNs (5×10^5), suspended in RPMI/H, containing 4 μM EtBr. Samples were incubated for 30 min at 37°C. LPS was sonicated at a frequency of 40 kHz in ice water for 15 min before use (Branson 2200, Branson Ultrasonics, Danbury, CT, USA). PMNs were then incubated with 1 nM PVL (LukF-PV and LukS-PV) for the indicated times, and EtBr uptake was assessed by flow cytometry.

PMN lysis was determined by LDH release using the cytotoxicity detection kit (Roche Applied Sciences, Pleasanton, CA, USA), as described previously [6, 20]. Human PMNs (100 μl, 1×10^6) were combined with 1 nM, 2 nM, or 5 nM active PVL or iPVL in a 96-well plate (Costar, Cornung, NY, USA) and incubated at 37°C for 5 h (total 200 μl/well). Plates were centrifuged at 1600 rpm for 7 min at 4°C, and 100 μl aliquots of each well were transferred to a new 96-well plate.

**PMN assays for priming, activation, and bactericidal activity**

Release of O_2^- was measured as described previously by DeLeo et al. [21] with modifications. PMNs (1×10^6/ml) were incubated at 37°C for 30 min with fMLF, LPS, IL-8, GM-CSF, active PVL (LukF-PV and LukS-PV), iPVL, or PVL subunits separately at concentrations described above. Electroporermobilized PMNs were used in some assays (see Fig. 3). Each agonist (1 μM fMLF, 20 μM IL-8, 100 ng/ml GM-CSF, or 1 nM PVL final concentration) was aliquoted into wells of a 96-well microtiter plate before the addition of primed PMNs (1×10^6). LPS is a known PMN-priming agent and was used as a positive control for this purpose. PepTidoglycan (InvivoGen, San Diego, CA, USA) is a known TLR2 agonist and was used as a positive control (at 10 μg/ml) for TLR2-priming assays that contained anti-TLR2-blocking antibody. Quiescent PMNs were activated with 1 μg/ml PMA as a positive control for production of O_2^- . All wells contained ferricytochrome c (Sigma-Aldrich) at a final concentration of 100 μM, and assays were performed in triplicate (± 40 μg/ml SOD (Sigma-Aldrich)). O_2^- production was determined by measuring the SOD-inhibitable reduction of ferricytochrome c at 550 nm for 20 min using a microplate spectrophotometer (Synergy MX, BioTek, Winooski, VT, USA).

Neutralizing IgA2 mAb, specific for human TLR2 (clone B4H2), TLR4 (clone W7C11), and CD14 (clone D5B8), and an isotype control IgA2 mAb were obtained from InvivoGen. Rabbit polyclonal antibody specific for Luk-PV was generated by immunization of rabbits with purified, native Luk-PV using standard methods. Anti-LukS-PV IgG was purified using two HiTrap Protein A affinity columns (GE Healthcare, Piscataway, NJ, USA), connected in series. In brief, columns were equilibrated with 10 column vol of a 20 mM sodium phosphate-binding buffer (buffer 1, pH 8.0) at 4°C. Anti-PVL serum was diluted with Buffer 1 (v/v) and subsequently passed through the columns for 3 h using a flow rate of 1.0 ml/min. Columns
were washed with Buffer 1 until the OD (OD₇₀₀ nm) of the flowthrough reached background levels. An additional wash with 10 mM sodium phosphate buffer (pH 8.0) was performed for 10 min at 1.0 mL/min. Bound polyclonal antibody was eluted with 0.1 M glycine buffer (pH 2.7) and collected in tubes containing 0.1 mL 1 M Tris-HCl. Total protein concentration was quantified with a protein assay from Bio-Rad. PMNs (10⁶ cells/mL) were incubated with each mAb (5–10 μg/mL final concentration) at room temperature for 30 min with gentle agitation, prior to priming with LPS or PVL. O₂⁻ production was determined as described above.

Changes in intracellular Ca²⁺ were measured with a FlexStation II scanning fluorometer using fluorescent dye Fluo-4 AM (Invitrogen, Life Technologies, Grand Island, NY, USA), as described previously [22]. PMNs, suspended in HBSS without Ca²⁺ and Mg²⁺, were loaded with Fluo-4 AM dye (1.25 μg/mL final concentration) and incubated for 30 min in the dark at 37°C. After dye loading, the cells were washed with HBSS, resuspended in HBSS²⁻, and aliquoted into the wells of flat-bottom, half-area-well microtiter plates (2 x 10⁵ cells/well). The source plate contained MLF or dilutions of PVL (LukFP⁺+LukSPV) in HBSS²⁻. Changes in fluorescence were monitored (excitation wavelength = 485 nm; emission wavelength = 538 nm) every 15 s for 15 min at room temperature after automated addition of control buffer, 100 nM MIF (positive control), or PVL at the specified concentration. Response curves are shown as relative fluorescence units measured over the 15-min monitoring period.

Surface expression of CD11b was determined after PMNs (1 x 10⁶) were exposed to 1 nM PVL for 0, 15, and 30 min at 37°C. In some assays, PMNs were exposed to iPVL for 30 min or pretreated for 30 min at 37°C with 10 μM SB203580, a p38/reactivating kinase MAPK inhibitor (InvivoGen), and then incubated with PVL for an additional 30 min. After exposure to PVL or iPVL, cells were washed twice with stain buffer (BD Biosciences) and incubated on ice for 60 min with PE-conjugated anti-human CD11b primary antibody or PE-labeled mouse IgG1 isotype control (BD Biosciences). PMNs were washed three times with stain buffer and analyzed by flow cytometry.

Redistribution of gp91phox and p47phox from PMN granules and cytosol to the plasma membrane was determined as described [23]. In brief, proteins in human PMN plasma membrane-enriched fractions were resolved by 12.5% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked overnight, and gp91phox and p47phox were detected using mAb specific for gp91phox (clone 54.1) and p47phox (clone 43.27) and peroxidase-conjugated anti-rabbit donkey IgG secondary antibody and ECL (SuperSignal West Pico, ThermoFisher Scientific, Pittsburgh, PA, USA).

Phagoscytosis and killing of S. aureus by human PMNs were determined using a method described previously but with modifications [24]. Bacteria in PBS (10⁶ CFUs) were diluted 1:1 in 100% serum (50% serum final concentration) and then opsonized for 30 min at 37°C. Opsonized S. aureus were centrifuged for 2 min at 8000 rpm, supernatant was aspirated, and bacteria were resuspended in RPMI/H at 10⁵ CFUs/mL. S. aureus suspension (100 μl; 10⁵ CFUs) was combined with 10³ PMNs in a final volume of 200 μl, and the assay was rotated gently for 2 h at 37°C. Aliquots of the assays were evaluated by microscopy (cytospin followed by Wright-Giemsa stain) or mixed with saponin on ice for 15 min and subsequently plated on trypti-

case soy agar. CFUs were enumerated the following day and were used to determine percent S. aureus survival relative to a time-matched control assay that did not contain PMNs. To quantitate the percent PMNs with bound ingested S. aureus, 250 PMNs from at least five fields of view were scored for associated bacteria (includes bound and ingested).

Identification of molecules released from human PMNs following incubation with PVL was performed by RBM (Austin, TX, USA). In brief, human PMNs from four different blood donors (1 x 10⁶) were incubated ≥ 1 nM PVL at 37°C for 4 h. Cell suspensions were centrifuged at 1800 rpm for 10 min at 4°C. Supernatants were analyzed by RBM (HumanMAP v. 1.6), as described by the vendor http://www.myriadrbm.com/products-services/humanmap-services/humanmap/? (http://www.rulesbasedmedicine.com/products-services/human-maps.aspx). Data were analyzed using a paired t test, and a zero was used for samples that were not measurable on the standard curve. A complete set of RBM data is provided as supporting information on the website (Supplemental Table 1).

Alternatively, the concentration of MPO and VEGF in PMN culture supernatants after a 4-h exposure to PVL or iPVL was determined using an EnzChek MPO activity assay kit and VEGF ELISA kit, according to the manufacturer’s instructions (Invitrogen, Life Technologies).

PMN apoptosis assays

PMNs were exposed to 1 nM PVL or individual PVL subunits (LukF-PV or LukSPV) as described above, and apoptosis was determined using published methods [18, 25]. PMNs (1 x 10⁶) were analyzed using a Cytospin 4 (Thermo Shandon, Waltham, MA, USA), as described by the manufacturer. PMNs were stained with Wright-Giemsa (Sigma-Aldrich), and condensed nuclei were visualized by light microscopy (Axioskop 2 Plus, Carl Zeiss, Thornwood, NY, USA) at ×100 magnification. A total of 250 cells was scored from five fields of view for each sample. Images were acquired with an AxioCam digital camera (Carl Zeiss). Alternatively, PMN apoptosis was assessed using a modified TUNEL assay (Apo-BrdU apoptosis detection kit, BD Biosciences), as described by Kohyashii et al. [18].

PMN microarray analysis

For the gene expression assays, purity of the PMNs (from three PMN donors) was 99.8 ± 0.2%, and viability was 99.6 ± 0.1%, as assessed by flow cytometry. PMNs (1 x 10⁶) in RPMI/H were cultured with 1 nM PVL or iPVL at 37°C for 30, 60, or 180 min. At each indicated time-point, PMNs were lysed with RLT buffer, and RNA was purified and used to generate ≥12 μg biotin-labeled cRNA target, as described previously [18]. Samples from three different PMN donors were hybridized on HU135 + two GeneChips (Affymetrix, Santa Clara, CA, USA). cRNA labeling, GeneChip hybridization, and scanning were completed according to the manufacturer’s protocols (http://media.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf). GeneChip hybridization and subsequent scanning were performed by the Genomics Unit, Rocky Mountain Laboratories, NIAID, NIH. Vehicle controls were analyzed to determine levels of background signal for each donor at every time-point.

Microarray data were normalized using GeneChip Operating Software (v1.4). At each time-point, data from PMNs, cultured with active PVL, were compared directly with those treated with iPVL using Partek Genomics Suite (Partek, St. Louis, MO, USA). Genes were defined as differentially expressed if they were significantly different from the iPVL control (P ≤ 0.01, two-way ANOVA), changed twofold in expression, and had signal levels above background. The Venn diagram was generated by the Genomics Unit, Rocky Mountain Laboratories, NIAID, NIH. Analysis of signal transduction pathways was performed with Ingenuity Pathway Analysis (Redwood City, CA, USA). Microarray data have been posted online at http://www.ncbi.nlm.nih.gov/projects/geo/ under series number GSE3939 and are Minimum Information About a Microarray Experiment-compliant.

Statistical analyses

Data (see Figs. 1–6 and Supplemental Fig. 1) were compared using a one-way ANOVA and Dunnett’s or Tukey’s post-test to correct for multiple comparisons or a paired t test (GraphPad Prism 5, GraphPad Software, San Diego, CA, USA), as indicated in the legend. Data in Supplemental Table 1 were analyzed using a paired t test (GraphPad Prism 5).

RESULTS

PVL-mediated pore formation and cytolysis

We first determined the concentration at which purified PVL from S. aureus culture supernatant (USA300 strain LAC) caused formation of PMN plasma membrane pores but limited cytolysis (Fig. 1A–C). By 30 min, >90% of PMNs were EtBr-
positive at each of the PVL concentrations tested (pore formation was 95%, 98%, and 99% for 1 nM, 2 nM, and 5 nM PVL, respectively; Fig. 1A). PMN lysis was measured by release of LDH using conditions identical to the pore-formation assays, but incubation time was extended to 3 h (Fig. 1B). Despite high levels of pore formation with 1 nM and 2 nM PVL, subsequent cell lysis was limited (9±2% and 15±6%, respectively). We note that cell lysis increased significantly between 2 nM and 5 nM (e.g., lysis was 52±3% using 5 nM PVL). iPVL caused no pore formation and had no cytolytic capacity (Fig. 1A and B).

PVL primes human PMNs for enhanced production of O$_2^-$

In accordance with previous studies [13, 17, 26], 1 nM PVL caused an increase in free intracellular Ca$^{2+}$ concentration and enhanced release of O$_2^-$ after subsequent stimulation with iMLF (Supplemental Fig. 1A and Fig. 2A). The ability of PVL to prime for iMLF-mediated release of O$_2^-$ was optimal at 1 nM PVL (Fig. 2B) and required both PVL subunits in their native form, as neither individual PVL subunit nor iPVL primed PMNs for iMLF-mediated O$_2^-$ production (Fig. 2A). The ability of PVL to prime PMNs for an enhanced response to iMLF was not a result of an increase in surface-expressed FPR1, as surface expression of FPR1 was similar in the presence or absence of PVL priming (mean FL1 by flow cytometry was 24.2±7.7 and 25.9±7.0 for control and PVL-primed PMNs, respectively; n = 4). As with other priming agents, such as LPS [27], PVL failed to elicit O$_2^-$ production when added subsequently to iMLF (Fig. 2A). We note also that a polyclonal antibody specific for LukS-PV blocked PVL-mediated priming of PMNs in these assays—findings consistent with studies by Yoong and Pier [28] (Fig. 2C).

As a first step toward understanding the molecular basis of the PMN response to PVL, we tested whether formation of plasma membrane pores per se primes PMNs for iMLF-mediated release of O$_2^-$ (Fig. 3A–C). With the use of electroporation conditions that cause EtBr uptake (pore formation) in 91.1 ± 2.3% of the cells (comparable with pore formation caused by 1 nM PVL; see Fig. 1A), iMLF-mediated O$_2^-$ production was similar to that in PMNs primed with PVL [e.g., O$_2^-$ production at 20 min was 6.4±0.1 nmoles for electroporamebulated PMNs vs. 6.5±2.1 nmoles for PMNs primed with PVL (Fig. 3A–C)]. These findings indicate that formation of pores in the PMN plasma membrane can prime human PMNs for enhanced fMLF-mediated O$_2^-$ production. Although these findings are consistent with the idea that formation of membrane pores by PVL contributes to PVL-mediated priming of PMNs (rather than—or in addition to—signal transduction through a surface receptor), there must still exist a link between formation of membrane pores and biochemical events that ultimately lead to the production of O$_2^-$ after secondary stimulation with fMLF.

As much as LPS or PVL similarly primed human PMNs for iMLF-mediated release of O$_2^-$, it is possible that there is an overlap in signal transduction pathways following priming with these agents. In accordance with this notion, Zivkovic et al. [29] used a mouse alveolar macrophage cell line (MH-S) and transfected human embryonic kidney 293 cells to show that PVL binds to TLR2 and affects proinflammatory signal transduction through TLR2, CD14, and MyD88. This group also reported that LukS-PV alone was sufficient to elicit proinflammatory responses in vitro or in mice in vivo, which is at variance with our finding that both PVL subunits are required to prime human PMNs for iMLF-mediated O$_2^-$ release. To determine whether PVL priming of human PMNs is mediated by a similar signal transduction process, we primed PMNs with LPS or PVL for 30 min and then measured iMLF-stimulated O$_2^-$ release in the presence or absence of antibodies known to block binding of ligands to TLR2, TLR4, or CD14 (Fig. 4A–C). Anti-TLR2, anti-TLR4, and anti-CD14 antibodies inhibited the ability of LPS to prime human PMNs for enhanced iMLF-stimulated O$_2^-$ release (e.g., at 20 min, iMLF-stimulated O$_2^-$ generation was 8.2 nmoles/10$^6$ cells for LPS-primed PMNs in the presence an isotype control antibody vs. 1.6 nmoles/10$^6$ cells for those in the presence of anti-CD14 antibody; Fig. 4A and B). The inhibition of LPS priming by anti-TLR2 antibody is likely explained by the known contamination of commercial LPS preparations with lipopeptide, which signals through TLR2 [30, 31]. The anti-TLR2 antibody also inhibited PMN
priming by a *S. aureus* peptidoglycan, a known TLR2 agonist (Supplemental Fig. 1B). By comparison, these antibodies failed to inhibit PVL-mediated priming of PMNs for production of O$_2^-$ in response to fMLF (Fig. 4C). Taken together, these data suggest that PVL priming of human PMNs involves a process other than signal transduction through TLR2, TLR4, or CD14.

**PVL causes granule exocytosis and primes human PMNs for enhanced microbicidal capacity**

Inasmuch as priming of PMNs by LPS causes granule exocytosis and enrichment of NADPH oxidase components at the plasma membrane [23], which explains, in part, the ability of LPS to prime PMNs for enhanced production of O$_2^-$ following stimulation with fMLF, we tested the ability of PVL to induce up-regulation of CD11b at the cell surface and subcellular redistribution of NADPH oxidase components (Fig. 5A–C). PMNs exposed to PVL had a significant increase in the surface expression of CD11b, a finding most compatible with the fusion of secretory vesicles and/or specific granules with the plasma membrane (Fig. 5A and B). These results are consistent with the known ability of priming agents, such as LPS, to up-regulate PMN surface expression of CD11b [32, 33]. SB203580, a widely used inhibitor of p38 MAPK that has been shown to block priming of PMNs by LPS [34], partially inhibited PVL-mediated up-regulation of CD11b surface expression (Fig. 5C).

There was time-dependent association of gp91phox and p47phox with the plasma membrane of human PMNs following exposure of these cells to 1 nM PVL (Fig. 5D). The ability of PVL to increase surface expression of CD11b (and other receptors present in specific granules) and cause partial assembly of the NADPH oxidase suggests that exposure to the leukocidin can prime for enhanced PMN phagocytosis and killing of *S. aureus*. To test this notion, we measured binding/uptake and killing of *S. aureus* (the USA300 epidemic clone) by PMNs (Fig. 5E and F). Priming of PMNs by PVL significantly increased binding/uptake and killing of *S. aureus* by PMNs (e.g., survival of USA300 was 41.8 ± 3.5% and 9.3 ± 3.1% in the presence and absence of PVL priming;
Collectively, these results provide strong support for the idea that PVL can function as a PMN-priming agent.

Proinflammatory molecules alter PMN susceptibility to PVL

We next determined whether PVL primes PMNs for O$_2^-$ generation in response to IL-8 or GM-CSF and whether these proinflammatory agents prime PMNs for PVL-mediated O$_2^-$ production (Fig. 6A and B). IL-8, GM-CSF, or PVL alone had little or no capacity to elicit PMN O$_2^-$ production (Fig. 6A). However, IL-8 and GM-CSF primed PMNs for enhanced iMLF-mediated O$_2^-$ release, as described previously [35, 36] (Fig. 6B, solid triangles). By comparison, PVL elicited limited O$_2^-$ release (2.0–2.2 nmoles/10^6 cells) from IL-8- and GM-CSF-primed PMNs (Fig. 6B, open triangles).

Inasmuch as proinflammatory factors typically prime PMNs for enhanced function, which includes reorganization and increase of receptors and other molecules at the plasma membrane, proinflammatory stimuli could influence the ability of PVL to interact with neutrophils. To test this hypothesis, we stimulated human PMNs with multiple proinflammatory factors/activating agents and evaluated PVL-mediated pore formation (Fig. 6C and D). Stimulation of PMNs with iMLF or PMA caused a significant decrease in the ability of PVL to form pores in the PMN plasma membrane (e.g., 48±3% of the iMLF-stimulated cells were EtBr-positive following exposure to PVL vs. 81±3% EtBr-positive cells following exposure to PVL alone; Fig. 6C). By comparison, neither LPS nor hkUSA300 altered the ability of PVL to cause formation of membrane pores. Priming of PMNs with IL-8 or GM-CSF caused a transient but significant increase in PVL-mediated pore formation (e.g., by 15 min, 83±2% of the PMNs were EtBr-positive after pre-exposure to IL-8 compared with 65±3% of those incubated with PVL alone; P<0.01; Fig. 6D). These data indicate that remodeling of the PMN plasma membrane following exposure to specific proinflammatory agonists alters the ability of PVL to interact with PMNs. One possible explanation for these results is that PVL binds to a PMN receptor whose surface expression increases or decreases depending on the agonist, and this change in turn impacts the ability of PVL to form plasma membrane pores. Collectively, these data indicate that the ability of PVL to interact with human PMNs is influenced by the activation state of the cell.

PVL induces global changes in PMN gene expression

To gain further insight into the molecular basis of PVL-mediated PMN priming, we measured global changes in PMN gene expression following exposure to 1 nM PVL (Fig. 7). The number of differentially expressed genes increased in a time-dependent manner (i.e., there were 397 differentially expressed genes at 30 min, 1074 differentially expressed genes at 60 min, and 2850 differentially expressed genes at 180 min; Fig. 7A). Differentially expressed genes were categorized into biological pathways or grouped according to function (Fig. 7B). Only nine signal transduction pathways were significantly represented by differentially
expression of genes at 30 min (Fig. 7B). However, 30 and 114 signal transduction pathways were significantly represented by differentially expressed genes at 60 min and 180 min, respectively (the top 10 are shown). In general, proinflammatory pathways, such as those mediated by CD40 and EGF, were significantly represented early following the PVL-PMN interaction (30 min). By comparison, cell-fate pathways, such as death receptor signaling and Myc-mediated apoptosis signaling, were significantly represented by differentially expressed genes, 60 and 180 min after exposure of PMNs to PVL (Fig. 7B).

**Expression of PMN genes encoding major transcription regulators is increased following exposure to PVL**

Genes encoding proteins known to mediate the inflammatory response, such as STAT3, SOCS3, JUNB, FOS, FOSB, FOSL1, JUN, and TANK, were up-regulated within 120 min after PMN exposure to 1 nM PVL (Fig. 8). Expression of SOCS3 is regulated by the transcription factor STAT3, and therefore, up-regulation of SOCS3 is consistent with activation of STAT3. JUNB, FOS, JUN, FOSB, and FOSL1 encode proteins that dimerize and form the AP-1 transcription factor, which is known to associate with NF-κB as a result of signal transduction through proinflammatory pathways (reviewed in ref. [37]). In addition, genes involved in NF-κB signal transduction, such as NFKBID, MAP3K3, MALT1, BCL10, and BCL3, were differentially expressed, 180 min after exposure of PMNs to PVL (Fig. 8).

**Priming of PMNs with PVL alters expression of transcripts encoding surface receptors and proinflammatory molecules**

Genes encoding multiple PMN proinflammatory mediators, including CR1 (CD35), CXCL1 (GROα), CXCL2 (GROβ), FCAR (CD89) and OSM, were up-regulated 60 and/or 180 min after exposure to PVL (Fig. 8). FCAR encodes the IgA receptor CD89, CR1 encodes the serum CR CD35, and these molecules facilitate phagocytosis and PMN activation [40]. OSM, a member of the IL-6 family, is stored by mature PMNs as an active protein and released following priming or activation [41]. However, synthesis of OSM transcript by PMNs is known to occur under certain conditions, such as after culture with LPS and GM-CSF [41] or after phagocytosis [42].

Exposure of PMNs to PVL also caused down-regulation of a subset of genes encoding surface receptors and cytokines involved in the inflammatory response (Fig. 8). For example, IL5RA, IL1R1, IL1A, CCL23, TNFRSF12A, TNFRSF10A, and TNF

![Figure 4. PVL-mediated priming of human PMNs does not involve TLR2 or TLR4. (A–C) Inhibition of LPS or PVL-mediated priming by antibodies specific for TLR2, TLR4, or CD14. PMNs were incubated with LPS (A and B) or PVL (C) as described above but after pretreatment with 5 μg/ml of the indicated antibodies. In some assays, antibodies specific for CD14 were used at 10 μg/ml (10), as indicated. Results are the mean ± se of three to 10 separate experiments or PMN donors. *p < 0.05 for the indicated comparisons using a one-way ANOVA and Tukey’s post-test.](image-url)
transcripts were decreased, 60 min after exposure of human PMNs to PVL. Down-regulation of transcripts encoding TNFRs and TNF-α at later time-points (60 and 180 min) provides support to the idea that there is eventual moderation of the pro-inflammatory response induced by PVL, a phenomenon consistent with induction of PMN apoptosis by PVL.

Mediators of the inflammatory response are released from PMNs following PVL priming

Inasmuch as PVL primed PMNs for enhanced function and caused up-regulation of transcripts involved in the inflammatory response, we next measured release of molecules involved in the inflammatory response following exposure of PMNs to 1 nM PVL. Immunoblots containing proteins from the plasma membrane-enriched fractions of PMNs, stimulated ± 1 nM PVL, were probed with antibodies specific for gp91phox (a gp91phox) or p47phox (p47phox). Results shown are representative of three separate experiments. Cyt., Cytosol-enriched fraction. (E) Binding/ingestion of USA300 by human PMNs following priming by 1 nM PVL or 100 ng/mL LPS for 30 min. Following priming, bacteria were combined with PMNs at a 1:1 ratio and rotated gently for 2 h, at which time, an aliquot of the assay was used to determine the percent PMNs with bound/ingested USA300. (F) Bactericidal activity of PMNs toward USA300 following priming by 1 nM PVL or 100 ng/mL LPS, as described for E. Results for E and F are the mean ± se of four separate experiments as indicated. *P < 0.05 for the indicated comparisons using a one-way ANOVA and Tukey’s post-test. S.a., S. aureus.

DISCUSSION

Recent multinational phase III clinical trials indicate that the presence of genes encoding PVL is not the primary determi-
nant of outcome in patients with MRSA skin and soft-tissue infections [44, 45]. Rather, individuals with PVL-positive infections were more likely to be cured [44, 45]. It is also noteworthy that the concentration of PVL achieved during S. aureus infection in vivo may be insufficient to cause PMN lysis [12, 46]—findings that bring into question the role of PVL as a cytolytic toxin. Antibodies against PVL are present in individuals who have had previous S. aureus infections, caused by PVL-positive or -negative strains [47, 48]. These observations suggest that some of the antibodies were originally elicited by two-component toxins other than PVL, but the antibodies cross-react with PVL. Recurrent infections with PVL-positive S. aureus strains occur in patients that have anti-PVL antibodies, suggesting that PVL has little or no role in establishment of infection [49]. Furthermore, a previous study demonstrated that administration of anti-PVL antibodies prior to USA300 or USA400 infection (PVL-positive) hindered clearance of infection [28]. This observation seems at variance with the presumed role of PVL during infection (i.e., cytolytic toxin that enhances virulence) but is consistent with the ability of the molecule to function as a PMN-priming agent.

The cytolytic properties of PVL are well-known from extensive work in vitro. However, there is a paucity of evidence to indicate that the primary function of PVL in vivo is cytolysis of host leukocytes. For example, Diep et al. [50] used a rabbit model to show that PMNs and release of protein tyrosine kinases partially inhibits PVL-mediated release of IL-8 from PMNs. Consistent with these previous studies, we found that PMNs incubated with 1 nM PVL re-
Figure 7. PVL causes global changes in PMN gene expression. PMNs (1×10⁷) were cultured with 1 nM native PVL or iPVL, and changes in transcript levels between the two conditions were measured using Affymetrix Hu133 + two GeneChips, as described in Materials and Methods. (A) Venn diagram depicting the total number of differentially expressed genes (i.e., as a result of exposure to PVL) at each time-point. (B) PMN signal transduction pathways represented by differentially expressed genes after cells were exposed to PVL. Pathways or processes were identified using Ingenuity Pathway Analysis, as described in Materials and Methods. The P value indicates the likelihood that genes are associated with a given pathway or process because of random chance. ATM, ataxia telangiectasia mutated; NRF2, NF-E2-related factor 2; FLT3, fms-like tyrosine-kinase 3; Hemato. Progen., hematopoietic progenitors; DHA, docosahexaenoic acid; EIF2, eukaryotic translation initiation factor 2.
mained largely intact for at least 3 h, as there was little or no release of LDH using these assay conditions (Fig. 1B). Taken together, the data provide strong support to the idea that PVL-mediated release of PMN proinflammatory molecules is caused by activation of signal transduction pathways rather than cytolysis.

In accordance with previous work, PVL had proinflammatory effects on human PMNs, including priming for enhanced...
release of $O_2^-$ and increased surface expression of CD11b. In addition, we discovered that PVL caused subcellular redistribution of NADPH oxidase components and promoted secretion of multiple inflammatory mediators (Figs. 5D and 9 and Supplemental Table 1). LPS priming of PMNs for enhanced production of $O_2^-$ is known to involve redistribution of NADPH oxidase components [23]. Our finding that PVL caused a similar redistribution of NADPH oxidase components likely explains the enhanced release of $O_2^-$ in PVL-treated PMNs that were subsequently stimulated with fMLF. Thus, at sublytic concentrations, PVL functions as a PMN-priming agent.

Despite the functional similarities of PVL to other priming agents, such as LPS, there are clear differences. For example, exposure of PMNs to sublytic concentrations of PVL caused release of MPO, a protein sequestered in azurophilic granules (Fig. 9A), and such a process is characteristic of PMN activation rather than priming by agents, such as GM-CSF, IL-8, or LPS. PVL also accelerated PMN apoptosis at a concentration (1 nM) that elicits a proinflammatory response (Fig. 9C and D), which is in variance with the ability of other priming agents to extend PMN survival in vitro. Thus, more work is needed to better understand the molecular basis of these PVL-

Figure 9. PVL-mediated release of proinflammatory molecules by PMNs. (A) Accumulation of proinflammatory mediators. Human PMNs were cultured for 4 h in the ±1 nM PVL and proinflammatory molecules in RPMI/H culture medium were determined by RBM, as described in Materials and Methods. A complete set of results is provided in Supplemental Table 1. Results are the mean ± s.d of four human PMN donors. *$P < 0.05$ versus samples minus PVL using a paired $t$ test. (B) Verification of MPO and VEGF release using a MPO activity assay (left panel) and a VEGF ELISA (right panel). Results are the mean ± s.d of four human PMN donors. *$P < 0.05$ for the indicated comparisons using an ANOVA and Tukey’s post-test. (C and D) PMN apoptosis. PMNs were incubated with 1 nM PVL for 3 or 6 h, and TUNEL-positive PMNs were determined by flow cytometry (C) or by assessment of condensed nuclei (D). Control assays at 6 h contained either subunit alone (1 nM LukS-PV or LukF-PV). Results in C and D are the mean ± s.e of four to eight separate experiments. *$P < 0.05$ using a paired $t$ test for assays at 3 h or an ANOVA with Dunnett’s post-test at 6 h.
mediated phenomena, and the microarray data presented here serve as a possible springboard for such work.

PMNs are exposed to a multitude of proinflammatory molecules during infection in vivo, and different combinations of stimuli are known to elicit different PMN responses. Application of this idea to PVL-positive S. aureus infections is perhaps reflected by our finding that FMLF, GM-CSF, and IL-8 alter the ability of PVL to interact with PMNs (Fig. 6C and D). These findings should not be interpreted to indicate that PVL binds to one of the receptors for these proinflammatory agents, although that is a possibility. Rather, many surface molecules, including receptors, are increased or decreased following exposure to activating agents or proinflammatory molecules, and expression of a putative PVL receptor on the PMN surface could thus be readily increased or decreased by activation or priming. Consistent with this idea, previous studies by Hensler et al. [32] suggested that heterotrimeric and low molecular-weight G-proteins are involved in PMN signal transduction following exposure to PVL. In addition, Gauduchon et al. [19] reported that PKC regulates the availability of the receptor for LukS-PV—findings that are in accordance with our data.

Importantly, our results demonstrate that subtle concentrations of PVL prime PMNs for enhanced microbial capacity. This notion is also supported by Yoong and Pier [28], who reported anti-PVL rabbit sera inhibited PMN killing of several PVL-positive S. aureus strains in vitro. By comparison, killing of isogenic lukS/F-PV deletion mutants by PMNs was similar in the presence or absence of anti-PVL antibody [28]. Collectively, these observations suggest that PVL can enhance rather than hinder the host innate immune response to S. aureus infection. Nonetheless, the ability of PVL to enhance PMN bactericidal activity in vivo merits further investigation. Elucidation of signal transduction mechanisms following such stimulation with PVL may provide new insight into the ability of specific agonists to elicit differential responses from human PMNs.

AUTHORSHIP

F.R.D. and S.D.K. conceived of and designed the study. S.F.G., K.R.B., A.R.W., D.E.S., D.L.R., and L.N.K. performed experiments. M.T.Q. contributed critical reagents. S.F.G., S.D.K., D.E.S., M.T.Q., and F.R.D. analyzed data. S.F.G., S.D.K., M.T.Q., and F.R.D. wrote the manuscript.

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