Specific targeting and killing of Gram-negative pathogens with an engineered phage lytic enzyme

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Phage lytic enzymes have potential as new inroads toward novel antibiotics. Until now this approach has only been promising for Gram-positive bacteria because in Gram-negatives the target of lytic action is protected by an outer envelope. Information gleaned from the structural studies of two plague proteins—pesticin and FyuA—allowed us to engineer a “hybrid” protein to address this problem. This hybrid consisted of T4 phage lysozyme linked to a FyuA targeting domain and was capable of killing Gram-negative cells. This work therefore presents a proof of principle that phage lytic enzymes can be engineered to cross the outer envelope. Furthermore, hybrid engineering handed us a tool for the mechanistic investigation of TonB mediated membrane transport. This commentary describes our recent efforts to test the efficacy of the hybrid in a mouse infection model and the directions this work might take in the future.

In an age of rising antibiotic resistance humanity desperately needs to investigate new paths to antimicrobial chemotherapy. There are now several reports that show that lytic enzymes isolated from phages may work well as therapeutic agents against diseases caused by Gram-positive bacteria (Fischetti, Trends Microbiol 2005). Yet there is a problem in applying the same strategy to Gram-negative pathogens. The target of the toxic action of the lytic enzymes is a structural layer known as peptidoglycan which in Gram-positive bacteria is exposed but in Gram-negative organisms is sequestered beneath a protective outer membrane where the lytic enzyme cannot reach it.

Unexpectedly we found a way of addressing this problem by using X-ray crystallography to study a system of two interacting proteins from the plague bacterium (Lukacik et al., Proc Natl Acad Sci U S A 2012). One of these proteins was a bacterial toxin called pesticin, whose toxic efficacy depends on the presence of a second protein FyuA in the outer membrane (Fig. 1). The crystal structures revealed FyuA to be a classical outer membrane TonB-dependent iron transporter consisting of a β barrel whose pore was blocked by a plug domain (Noinaj et al., Annu Rev Microbiol 2010). Pesticin on the other hand was comprised of two distinct domains and we noticed that the structure of the C-terminal domain resembled the well-studied lysozyme from T4 phage. Guided by the structure we switched the pesticin lysozyme-like domain for T4 lysozyme to construct a “hybrid” protein. The hybrid protein now consisted of a lytic domain linked to an N-terminal pesticin domain that we determined to be responsible for FyuA binding. The addition of this hybrid to the archetypal Gram-negative organism—E. coli (modified to express fyuA in the outer membrane)—resulted in direct killing without the need for physical disruption of the outer membrane using chelators, pressure, temperature changes or eukaryotic antimicrobial peptides. Like with pesticin this killing was fyuA-dependent. Overall this experiment provides a proof of principle that phage lytic enzymes such as T4 lysozyme can be engineered to target Gram-negative bacteria.

It would be misleading to interpret this experiment as a simple replacement of a homologous domain. In fact the functional and structural differences between the two are large enough to broaden the native antimicrobial spectrum of pesticin to include other bacteria, namely ones that are normally immune to pesticin by virtue of a pesticin immunity protein—Pim. We have demonstrated this both in an E. coli model where the fyuA and pim genes were artificially expressed and in pathogenic Yersinia pestis strains.

The construction of the hybrid protein also provided us with a first tool to chip away at the long elusive mechanism of TonB-mediated transport. An important finding from this study was that the presence of engineered disulfides (Matsumura and Matthews, Science 1989) within the T4 lysozyme domain of the hybrid that join residues far apart in the sequence did not completely eliminate the import. This finding was quite surprising since the FyuA barrel pore is too narrow to accommodate a fully folded lytic domain even if the pore-blocking plug domain is displaced. Within the field, toxin unfolding is generally accepted to be a pre-requisite of TonB facilitated import (Cascales et al., Microbiol Mol Biol Rev 2007). We expect that this potentially controversial finding will raise a response in the literature and indeed Patzer et al. (J Biol Chem 2012) have recently shown that similar introduction of disulfide bonds has led to a complete loss of hybrid activity. The conflicting results could simply be due to differences in the constructs and experimental setup. For example we tested a hybrid mutant that contained two disulfide bonds while the closest match that Patzer et al. tested contained one of the equivalent disulfide bonds but lacked the other. There are other differences between our constructs such as the presence and position of the affinity tag, the junction between the N-terminal pesticin and T4 lysozyme domains and a point mutation in the T4 lysozyme domain. For the experimental setups, we performed our killing assay in broth and then plated the survivors for counting while Patzer et al. performed a plate assay and observed zones of lysis. If Patzer et al. are correct, the most plausible explanation is that a small population of our mutant hybrid was reduced and therefore still active. However we found no evidence of such a population either by Coomassie gel staining or by mass spectrometry.

It is important not to neglect FyuA in the discussion of this work. Presence of the fyuA
gene is a recurring theme in the literature investigating heightened virulence of human and animal pathogenic E. coli strains. Recent reports also tell that fyuA appears to be associated with relapse and persistence of urinary tract infections (Ejrnaes et al., Virulence 2011) and multidrug resistance in animal and human infections (de Verdier et al., Acta Vet Scand 2012; Platell et al., Antimicrob Agents Chemother 2012). Targeting fyuA for antimicrobial therapy now offers some very tantalizing prospects. Since bacteria producing FyuA are not part of a healthy bacterial flora we can in theory selectively kill only the virulent organisms and leave the rest unharmed. The broad killing by commonly used antibiotics probably promotes the spread of resistance genes among the human microbiota. More than this, targeting FyuA now has the potential to eliminate the infections that are most persistent and difficult to treat, such as ones caused by drug resistant strains. When considering limitations, it is clear that this approach will not be applicable to all Gram-negative pathogens since not all express FyuA. This is not a great concern because the power of this approach is its selectivity rather than indiscriminate killing. However a possible problem is loss of FyuA due to selective pressure.

This may be especially pertinent since Gram-negative pathogens possess a number of alternative iron acquisition mechanisms of which FyuA is only one. On the other hand, FyuA is required for virulence in the early stages of bubonic plague so loss of this iron transporter would result in decreased infectivity/toxicity of the strain.

An obvious and challenging future path of investigation would be to demonstrate that the antimicrobial approach presented by the hybrid protein has medical relevance by first showing that it is effective in a mouse infection model. We have already attempted to carry out initial animal studies where mice were infected intranasally with 1,000 cfu Y. pestis C092. Following infection, high concentrations of the hybrid were administered also intranasally. A number of hybrid instillation intervals were tested in order to identify an optimum protocol—co-infusion, a single dose after 1.5 h or two doses after 2 and 24 h. Since no such optima were identified these experiments were grouped into a single hybrid treated cohort and the survival results are presented in Table 1. Because the sample sizes were small, the Fisher Exact test was used to compare the two groups. The p value is 0.13, above the cut-off of 0.05, so the difference in the survival rates is not statistically significant. Table 1 shows the exact 95% confidence intervals (CI) for the two percentages. These CI overlap, which is consistent with the p value. Nevertheless our data hint that increasing the number of animals in this experiment might have given a small but significant difference in favor of a protective effect conferred by the hybrid. It is difficult to speculate on the reasons for this low efficacy. Perhaps the TonB-dependent transport of a large hybrid molecule is very slow compared with the speed of bacterial adhesion and subsequent infection. Additionally, in carrying out these experiments we were faced with a number of severe technical challenges in particular with the production of large amounts of hybrid protein and intranasal administration of large hybrid protein droplets which were concentrated to their protein solubility limit. Maybe these experiments could be revisited once the bactericidal activity of the hybrid toxin has been improved as is suggested in the original manuscript.

Instead of improving hybrid activity one might take a different approach altogether. A different toxic domain unrelated to T4 lysozyme could be attached while retaining the FyuA targeting capacity of the N-terminal pesticin domain. Only further research will tell which of these approaches, if any, will be successful in the future. In the meantime the pressing urgency for antibiotic discovery remains.

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Table 1. Survival of mice following infection with Y. pestis C092

| N mice | Survivors | % Surv | Exact 95% CI |
|--------|-----------|--------|--------------|
| Control | 28 | 14 | 50.0% | 31–69% |
| Hybrid treated | 37 | 26 | 70.3% | 53–84% |
Immunomodulation by the Panton-Valentine leukocidin can benefit the host during *Staphylococcus aureus* infections

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*Staphylococcus aureus* infections have recently spread beyond healthcare settings into the general population. A majority of community-acquired strains are multidrug-resistant (CA-MRSA) and elaborate Panton-Valentine leukocidin (PVL). Despite the potent lytic activity of PVL on immune cells, its role in pathogenesis remains inconclusive. This is likely attributed to the ability of lower, sublytic levels of PVL to activate protective, proinflammatory properties of a wide range of host cells beyond those it targets for lysis. This was highlighted in an animal pneumonia model comparing isogenic PVL+ and Δpvl CA-MRSA strains, where animals infected with PVL-producing strains exhibited better survival. In addition to triggering cell activation for increased production of proinflammatory cytokines and antibacterial factors, there were also indications that PVL modulated the immune response by downregulating TNFα and inducing apoptosis, potentially contributing to improved outcomes of infection for the host. The continuous shift in balance between *S. aureus* and the host underscores the challenges in treating and predicting outcomes of infection.

**Background**

*Staphylococcus aureus* is a bacterium that causes widespread hospital- and community-acquired infections. Its success as a pathogen is attributed in part to its acquisition of resistance to optimally effective antibiotics and to an arsenal of virulence factors designed to attack or evade every level of host immune defenses. *S. aureus* secretes a number of cytotoxins capable of targeted killing of select host cells. With its capacity for host cell and tissue destruction, these toxins seem to be ideal weapons for establishing and maintaining staphylococcal infections. Among the array of *S. aureus* toxins is a bi-component, pore-forming toxin known as the Panton-Valentine leukocidin (PVL). PVL consists of two different protein subunits, which multimerize into a multivalent pore structure.

**Figure 1.** Schematic of PVL pore formation, and the different cellular pathways it uses to activate host cells. Steps involved in immune activation are outlined in solid boxes, while steps in pore formation are outlined in gray dashed boxes. Sublytic levels of PVL activate human neutrophils by stimulating calcium ion channels, followed by an influx of calcium into the cell. This occurs prior to PVL pore formation. PVL has also been shown to activate murine macrophages via TLR2. While sublytic PVL do not require TLR to prime human neutrophils, it has been suggested that PVL lysis of host cells releases host damage-associated molecular patterns (DAMPs) that in turn is recognized by TLR to activate human neutrophils and macrophages/microbes.
A β-barrel structure that inserts into target host cells, effectively creating channels in the cell membranes (see Fig. 1 for a schematic of PVL pore formation, steps outlined in gray dashed boxes). The resulting osmotic dysregulation eventually leads to cell lysis. The cytolytic activity of PVL seems to be confined to a sub-set of primary human immune cells, including neutrophils, monocytes and mast cells. In ex vivo experiments using purified PVL, cytotoxic activity was demonstrated down to the nM range on target cells, suggesting that PVL is a highly potent toxin. Given that PVL elicits contradictory effects on immune cells, depending on its concentration, any potential effects it may have on staphylococcal pathogenesis could be occluded. Indeed, scientific and clinical reports on the role of PVL in infection are contradictory and highly controversial.

Role of PVL in a Model of MRSA Pneumonia

We sought to delineate the role of PVL in pneumonia by comparing isogenic wild-type (WT) and Δpvl methicillin-resistant S. aureus (MRSA) strains in a mouse pulmonary infection model. Unexpectedly, the outcome was contrary to conventional reasoning, wherein a cytolytic toxin would be expected to enhance bacterial virulence. We found the Δpvl strains were significantly more virulent than the corresponding PVL-producing WT parental strains, with higher mortality among the mice infected with the Δpvl MRSA (Fig. 2A). Consistent with those observations, increasing levels of PVL expression from the same three strains of MRSA from pvl ( lukSF-PV ) genes on a high copy number plasmid decreased the mortality rates and/or prolonged the time to death (Fig. 2B). Using ELISA to measure the amounts of PVL within the lungs of infected mice, we observed a loose correlation whereby the higher the concentration of PVL detected within the lung, the lower the rate of mortality (Fig. 2C).

Outcomes from our pneumonia infection models indicate that PVL may have a protective role within the host, which is consistent with the ability for PVL to activate immune cells. Mouse models of infections are ideally suited

Figure 2. Comparison of the virulence of PVL-producing MRSA strains with their respective isogenic Δpvl mutants in a mouse pneumonia model. (A) Comparison of the percent mortality at 48 h in mice infected with three different MRSA strains, their Δpvl isogenic strains and, in the case of strain MW2, the PVL-complemented strain (pvl comp). p values were determined by Chi-square analysis. Differences with strain NRS193 were not significant. (B) Survival curves comparing outcomes in mice infected with WT PVL+ MRSA or isogenic strains expressing higher levels of PVL from pOS1-pvl. p values by log-rank test. (C) Trend correlating levels of PVL production and survival in a mouse model of S. aureus pneumonia. (D) Pathology of selected murine lung sections 8 h post infection with MRSA strain MW2, and its isogenic Δpvl mutant.
to study the proinflammatory aspect of PVL, as mouse cells are relatively resistant to PVL’s lytic activity. Indeed, lung pathology from infected mice 8 h after infection showed the PVL-producing WT MW2 strain induced extensive inflammation, while inflammation was notably absent from samples infected with MW2 Δpvl (Fig. 2D), suggesting that PVL elicits proinflammatory reactions from mouse cells as well. The fact that WT MW2 infected mice fared considerably better than mice infected with MW2 Δpvl would indicate that the inflammation at an early time point soon after infection was beneficial, and favored a positive outcome of infection for the host.

**PVL and Immune Activation**

Low, sublytic concentrations of PVL can activate primary human immune cells to secrete an array of proinflammatory cytokines, including interleukin (IL)-8 and leukotriene B4. These cytokines act as chemoattractants, further recruiting more activated neutrophils to the site of infection. Additionally, activated primary immune cells turn on an array of pathways designed to contain microbial infections, including the secretion of antimicrobial factors, and enhanced phagocytosis.

Our next aim was to dissect the basis, on a molecular level, for the differences in the survival outcomes in the pneumonia infections. To do so, we used a mouse lung epithelial cell line (MLE 12) originating from the same strain of mice used in the infection models (FVB), as well as mouse neutrophils isolated from the bone marrow of FVB mice. Cell activation was measured using three benchmarks, the secretion of the proinflammatory cytokine KC (mouse homolog of human IL-8), phosphorylation of the p38 mitogen-activated protein kinase (phospho-p38 MAPK, a signaling molecule in the phospho relay pathway leading to increased transcription of proinflammatory genes), and the release of antimicrobial factors. Both mouse neutrophils and lung epithelial cells responded by all measures to stimulation by PVL (Fig. 3A–C). Despite the resistance of mouse cells to PVL cytotoxicity, these cells responded to the proinflammatory inductive activities of PVL. This is consistent with the outcome of the mouse pneumonia infections, based on the assumption that the activation of immune defenses by PVL protected mice from MRSA infections.

**PVL Mediated Resolution of Inflammation**

To ascertain further differences in host immune responses elicited by the WT and Δpvl MRSA strains, pulmonary homogenates were analyzed for differential cytokine production at several time points post infection. Of the panel of cytokines tested, a meaningful difference was observed in the levels of TNFα. WT MW2 infected mice seemed to maintain a steady level of TNFα, whereas TNFα levels were significantly higher in mice infected with the Δpvl counterpart (Fig. 3D). It has been well documented that uncontrolled increases in TNFα can result in death arising from symptoms akin to those associated with septic shock, one of which being neutropenia, which is consistent with the absence of PMNs or inflammation in the lungs of mice infected with the Δpvl strain. The elevated levels of TNFα could explain the increased mortality of mice infected with the Δpvl strain. These findings would suggest that PVL could possibly be regulating the levels of TNFα at the site of infection. Consistent with that, downregulation of TNF transcripts in human PMNs exposed to sublytic concentrations of PVL has been reported.

Apoptosis is a form of programmed cell death crucial to resolving inflammation. The apoptotic marker Caspase 3 was used as an indicator of cells undergoing apoptosis.
could still be activated by PVL, we wondered if mouse cells not normally susceptible to PVL could respond in a similar manner. To test this, a human alveolar basal epithelial cell line (A549), which is resistant to lysis by PVL, was used. The human cells did indeed respond similarly, with PVL stimulating the secretion of IL-8, phosphorylation of p38 MAPK, as well as release of antimicrobial factors (Fig. 4A–C). These results suggest that host cell activation by PVL could potentially be much more far-reaching than its lytic activity, which is limited to a subset of immune cells. This may explain why MRSA infections with PVL-producing strains are often associated with better outcomes, should its beneficial proinflammatory properties outweigh its lytic effect.

Like the mouse cells, increased levels of Caspase 3 in human neutrophils incubated with sublytic levels of PVL would suggest that these cells were undergoing apoptosis and in turn downregulating the immune response in response to PVL (Fig. 4D). However, the human alveolar cells did not induce Caspase 3 at any amounts of PVL tested (up to 13 nM, not shown). The levels of IL-8 released by A549 cells by PVL were many orders of magnitude lower than that from human neutrophils, suggesting the possibility that low levels of proinflammatory cytokines may activate basal levels of inflammation that does not necessitate downregulating.

In the case of the murine host, whose cells are resistant to lysis by PVL, it nonetheless appeared to harness the beneficial properties of PVL during MRSA infections, as reflected by lower mortality rates of mice infected with PVL-producing strains. However, it is more challenging to envision the role PVL plays in the human host, since PVL can be detrimental or beneficial to the host, depending on the concentrations of PVL. Low, sublytic levels of PVL may serve to amplify host immunity during the initial stages of infection, where bacterial numbers and toxins elaborated by them are still low. Once an infection is established, there is conceivably a gradient of PVL, with the highest concentration near the site of infection, and a decrease in PVL as distance from the infection increases, although it is uncertain if the concentration of PVL achieved in vivo during active infection would even be sufficient to cause cell lysis (Fig. 2C).

**Implications of PVL on S. aureus Infections**

Given that PVL has such potent lytic activity on primary human immune cells, which are essential in controlling infections, it is unexpected that a PVL-dependent virulence phenotype is not readily observed. The study of PVL virulence in mouse models of S. aureus infections could pose some problems because of the relative resistance of mouse cells to PVL-mediated lysis. However, mouse models aside,

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**Figure 4.** Immunomodulatory effects of PVL on human cells. (A and B) Detection of phospho-p38 and human IL-8 produced by purified PMNs (A) and cultured A549 human alveolar basal epithelial cell line (B) exposed to indicated concentrations of purified PVL. (C) Percentages of viable MRSA strain MW2 after addition of supernatants from the indicated cells that were first incubated with purified PVL, compared with bacterial count in cells lacking exposure to PVL. Bacterial counts from cell supernatant killing assays are averaged from a minimum of 3 independent experiments. Error bars denote SEM. Statistical analyses were performed by the t-test (**p < 0.01; *p < 0.05; *p < 0.01). (D) Production of Caspase 3, as determined by immunoblot, from indicated cells 6 h after exposure to indicated concentration of PVL. ◆ denotes PVL concentrations that stimulate release of antibacterial factors by cells.
there is example after example in the clinical setting whereby PVL makes no contribution to pathogenesis, and even in some cases, infections with PVL- *S. aureus* are associated with better infection outcomes.

The findings reported here reaffirm that PVL has strong proinflammatory capabilities that could well influence the outcome of infection in favor of the host. PVL has been shown to activate mammalian cells by at least two mechanisms: by inducing calcium influx into host cells, and via host recognition of bacterial molecular patterns by mammalian toll-like receptors (TLR) (Fig. 1, steps involved in immune activation are outlined with solid boxes). Both pathways trigger signal cascades that switch on transcription of proinflammatory cytokine genes and activate antibacterial mechanisms. TLRs are expressed widely on many cells, suggesting a potential means by which PVL can activate cells that are resistant to its lytic activity. Inflammatory cytokines elicited by PVL could in turn activate other cells to secrete additional cytokines, potentially augmenting inflammation exponentially. Additionally, PVL can also synergize with other *S. aureus* factors to amplify the host inflammatory response. Could these proinflammatory, immune-activating properties of PVL be alleviating *S. aureus* infection?

As is always the case, there are important caveats to the activation of inflammation. Overactive or even uncontrolled immune activation has detrimental effects on the host due to the release of toxic substances such as low pH vesicles, TNFα and reactive oxygen species. Thus, to minimize damage to the host resulting from immune activation, inflammation needs to be controlled and downregulated once the initial threat has been neutralized. Intriguingly, data garnered from our mouse infections would suggest that PVL can also downregulate inflammation, consistent with reports that human PMNs downregulated genes involved in the inflammatory response upon prolonged incubation with sublytic PVL.

In the same manner by which bacteria are constantly changing to enhance their colonization and infectivity potentials, the host may have developed the means to battle these organisms by targeting one of its most potent virulence factors. In treatment of any patient with a *S. aureus* infection, both the factors elaborated by the pathogen, as well as the host response to it, must be considered. Activation of innate immunity is not limited to PVL, but has also been shown to occur with α-toxin, γ-hemolysin, LukAB/GH and LukED, other toxins elaborated by *S. aureus*. Additionally, certain combinations of these toxins can amplify the inflammatory response even further. In light of multiple *S. aureus* toxins having properties potentially beneficial to the host, the use of therapeutic antibodies should be cautioned. The neutralization of *S. aureus* toxins, and its proinflammatory effects, could inadvertently have the opposite outcome, as evidenced previously by our group in a skin abscess infection model. Clearly, more study is needed for a better understanding of the delicate host-pathogen interaction to more effectively control the increasing cases of devastating staphylococcal infections.