Enhancement of bacterial virulence by antibody neutralization of immune-activating toxins

Pauline Yoong
Channing Laboratory; Department of Medicine; Brigham and Women’s Hospital; Harvard Medical School; Boston, MA USA

Bacterial pathogens release a number of toxins that are able to form pores in target host cells, which can result in their destruction. Due to this property of this subgroup of toxins, they are considered virulence factors. A lesser known ability of these toxins when present at lower concentrations that are insufficient for host cell lysis to occur, is their activation of host immune cells. The Panton-Valentine Leukocidin (PVL) secreted by an alarming percentage of Staphylococcus aureus causing community-acquired infections, is one such toxin. Due to the low inoculum of S. aureus we used to establish skin abscesses in a murine model of infection, lower amounts of PVL are likely to be present early in infection, hence, the proinflammatory properties of PVL may be more evident. Our data not only suggested that this was indeed the case, but we also showed that antibodies directed to PVL neutralized immune activation by this toxin resulting in a less robust host innate immune response. Thus, given the high levels of antibodies to PVL present in healthy individuals, these antibodies may directly enhance the virulence of PVL-producing S. aureus by dampening the innate immune response to infection. Since many pore-forming toxins share this dual property of concentration-dependent host cell lysis and immune activation, it is interesting to speculate that antibodies raised to some bacterial toxins may have the opposite intended outcome of directly enhancing bacterial virulence instead of controlling infection.

Bacterial pathogens enumerate a slew of toxins that can disable the host, hence allowing the bacteria to gain a foothold to initiate infection, proliferate and cause disease. On the surface, most bacterial toxins have clear-cut, defined functions indicating the mechanism wherein they disable host defenses and damage tissues. A prime example would be pore forming toxins that punch holes in target host cells, resulting in the eventual lysis of those cells. The Panton-Valentine leukocidin (PVL) is one such pore forming toxin produced by an inordinately high percentage of methicillin-resistant Staphylococcus aureus (MRSA) strains that cause community-acquired (CA-MRSA) infections. PVL is made up of multimers of two protein subunits, LukS and LukF, which assemble into a pore-like complex that lyses polymorphonuclear (PMN) and monocytic cell lineages of certain mammals, including those of humans. Based on this known function of PVL, it seems simple to assume that it would be a virulence factor for S. aureus by disabling a key host factor needed to control infection. However, studies in the past several years have yielded contradictory findings as to whether PVL is a virulence factor for CA-MRSA, putting into question the role that PVL plays in staphylococcal infections despite, or in addition to, its pore forming capabilities. A threshold needs to be reached for PVL to cause lysis, as sufficient numbers of LukS and LukF subunits are required for pore formation to occur. Interestingly however, studies using PMNs incubated with PVL amounts that are below the threshold needed for cell lysis (sub-lytic concentration) have...
demonstrated that pro-inflammatory cytokine release is activated in cells,\textsuperscript{10-12} suggesting that in early stages of infection when PVL levels are low, the primary response to this toxin is activation of innate immunity and increased resistance to infection.

Several research groups have established animal models for infection by PVL-producing S. aureus but generally must use a relatively high inocula on the order of $10^7$–$10^{10}$ CFU.\textsuperscript{6} In some of those studies, PVL behaved as a virulence factor, as it enhanced the pathogenicity of S. aureus.\textsuperscript{7,14,15} This result was not surprising given the high inocula of PVL-producing S. aureus used in those infections, where PVL levels likely rapidly rose to those needed to exceed the lytic threshold, and the pore-forming, cytotoxic functions of PVL were likely dominant. We established a skin abscess infection model in mice that required significantly lower inocula of S. aureus (on the order of $10^4$–$10^5$ CFU per animal). When an infection was established with these lower inocula of MRSA, we found PVL-producing S. aureus to be less virulent than the isogenic $\Delta pvl$ strains, as reflected in the comparatively poorer replication of PVL-producing strains within the abscesses compared with $\Delta pvl$ strains (Fig. 1). At this lower inoculum, it is possible that PVL was at a sub-lytic concentration where its pro-inflammatory, immune-enhancing properties predominated.

Even more interesting, we found that antibodies directed to PVL were able to enhance, not diminish, the virulence of PVL$^+$ S. aureus strains in our skin abscess infection model (Fig. 2A). This appeared to occur by neutralization of the pro-inflammatory effects of PVL. Our results went beyond questioning the value of anti-staphylococcal antibodies in prevention of infections by S. aureus (as some studies have found that high titers of antibodies to S. aureus are not effective in preventing re-infection\textsuperscript{16-20}), but supported the hypothesis that the manner by which PVL plays a role in CA-MRSA pathogenesis is by antibody-mediated neutralization of the host’s innate response activated by sub-lytic amounts of PVL produced early in the course of infection. Mechanistically, we confirmed that sub-lytic concentrations of PVL induced the opening of calcium ion channels in PMNs,\textsuperscript{21,22} a hallmark of PMN-mediated activation,\textsuperscript{23} and showed that antibodies directed to PVL inhibited this activation (Fig. 2B). As activated PMNs secrete a number of antimicrobial factors,\textsuperscript{24,25} we compared the anti-bacterial activity of PMNs exposed to sublytic PVL released into cell culture supernatants in the presence of antibodies to PVL (Fig. 2C). We found that in the presence of antibody to PVL, as compared to non-immune serum, PMN supernatants had lower anti-bacterial activity, which is consistent with PVL antibodies preventing sublytic PVL-mediated activation of PMNs.

Naturally occurring antibodies to PVL are high in humans, even without preceding, clinically apparent S. aureus infection.\textsuperscript{26,27} These antibodies are likely raised to commensal S. aureus strains expressing PVL and/or related, cross-reactive leukocidins.\textsuperscript{5} The ubiquitous nature of antibodies to PVL in human sera may in part explain why antibodies against S. aureus appear not to be useful in preventing recurring infections. Results from our animal models raise the question as to whether naturally occurring PVL antibodies may even be promoting infections with MRSA, possibly explaining the prevalence of PVL-production by CA-MRSA strains.\textsuperscript{3} In particular, our findings raise concerns in the proposed use of the LukS subunit of PVL as a vaccine component, which is currently in phase 1 clinical trial (http://clinicaltrials.gov/ct2/show/NCT01011335?term=NABI,rank = 5). If our findings in the animal model extrapolate to humans, then antibodies raised to LukS may have the opposite effect to that intended by vaccination by enhancing the pathogenicity of PVL-producing S. aureus, as opposed to preventing staphylococcal infections. It is provocative to consider that the contribution of PVL to S. aureus virulence is dependent on host antibodies directed to PVL, but our data suggest this possibility. This would also indicate a previously unobserved level of sophistication in the proposed use of the LukS subunit of PVL as a vaccine component.

**Figure 1.** Comparing bacterial counts from mouse abscesses induced with PVL$^+$ and isogenic $\Delta pvl$ strains. Comparison of the bacterial counts in 72 h old abscesses in the skin of mice induced by three wild-type MRSA strains (NRS193, NRS194 and LAC) with their respective isogenic $\Delta pvl$ counterparts. $p$ values are from unpaired t-tests. Bar represents means; error bars represent SEM.
Figure 2. Antibody mediated effects on action of PVL. (A) Bacterial counts from mice injected with non-immune rabbit sera (NIS) or PVL-immune rabbit antisera followed by induction of abscesses with different PVL+ S. aureus strains. (B) Influx of calcium into human PMNs exposed to sublytic amounts of purified PVL, in the presence of NIS or antibody to PVL, as measure of PMN activation. (C) Effect of antibody to PVL on production of an extracellular antibacterial factor by human PMNs exposed to PVL+ S. aureus. p values are from unpaired t-tests. Bar represents means; error bars represent SEM.
innate immunity are not limited to PVL. In fact, it appears to be a common theme that crosses bacterial species, as both Gram-positive and Gram-negative bacteria produce toxins that are also recognized by the host and activate innate immunity.28,29 Many of these toxins have clear roles in virulence; several examples include lysteriolysin O, which mediates the release of Listeria monocytogenes from the lysosome into the cytosol of an infected host cell.30,31 and alpha toxin of S. aureus,32 streptolysin O of Streptococcus pyogenes,33 alpha-hemolysin of Escherichia coli34 can all cause lysis of immune cells. However, many of these toxins can also modulate host immunity by inducing activation of immune cells, as evidenced by upregulation and subsequent release of cytokines, and increase in neutrophil chemotaxis as well as phagocytic ability.35-39 Overall, a delicate balance needs to be struck in order for the innate immune system to function optimally, allowing a bacterial infection to be cleared. Ideally the immune system needs to be activated to a level that allows efficient removal and/or killing of pathogens. However, an over-activated response can result in damage to the host by means of an overactive inflammatory response. It is not immediately clear if the pro-inflammatory effects of bacterial toxins favor one outcome over the other.

Pore-forming toxins can differ in their mechanisms of assembly and the nature of pores in which they form, examples of which are bi-component toxins such as PVL and thiol-activated cytolysins such as lysteriolysin O,31 which forms pores made up of multiple copies of a single chain polypeptide. Despite these differences, the pore-forming toxins still share pro-inflammatory properties at sub-lytic concentrations. The common ability for these toxins to lyse target host cells suggests that disturbing the integrity of the PMN membrane, even in the absence of pore formation and cell lysis, may often activate immunity and enhance host resistance to infections, particularly at the early stages of infection when toxin levels are likely to be lower. Other mechanisms have been described that work in a related fashion. For example, human complement proteins C5–C9 that form the membrane attack complex (MAC), which assembles into a pore in the membranes of infecting Gram-negative bacteria to mediate lysis, can do the same to neutrophils that have ingested bacteria.40 Subsequently, neutrophil lysis ensues which might be a means to control microbial infection by preventing intracellular survival of organisms that escape the PMN antibacterial process. But when the MAC complex is not inserted at a sufficient level to lyse the PMNs it can activate immunity as reflected in increased cytokine production and recruitment of additional neutrophils.41,42 Given the function and purpose of the MAC in immune eradication of pathogens, it would make perfect sense that this complex has dual functions on infected cells, sometimes mediating lysis at high concentrations to prevent an intracellular niche of surviving bacteria from forming, but also activating innate immunity at lower toxin levels. Less evident is the potential advantage these toxins may impart on bacteria by their activation of host immunity. Could it be a clever ploy devised by bacteria to lure target cells to the site of infection only to lyse them with a subsequent increase in toxin production? Crystal structures demonstrating striking similarities in certain domains of human MAC with domains of perforin-lysin and intermedilysin, both bacterial pore forming toxins,43 suggests the possibility that these pore forming molecules may have a common evolutionary origin. Overall, the activity of bacterial toxins on neutrophils may not just be one single predominant factor such as cell lysis but rather a more complex series of interactions encompassing initial events that activate host responses and later events with the ultimate goal of host cell destruction. Could host antibodies modify this, inadvertently contributing to a pathogen's overall means to disarm effective host immunity and increases its chances for survival during infection?

There are an endless number of parameters that can change the process and outcome of a bacterial infection. It seems that the levels of toxins present in an infected site can determine the fate of host cells susceptible to the effects of the toxin, indicating that the expression and rate of production of each toxin plays a role in infection outcomes. Furthermore, a lot remains to be learned about combinations of specific toxins and other virulence factors produced by bacteria that can modulate and alter the overall pathogenicity of the bacteria, and the effect of host immunity on the manifestations of disease. While the pro-inflammatory effects of toxins have been observed repeatedly under in vitro conditions, the potential benefits it may impart to the host have not been rigorously tested in animal models of infections. We appeared to have accomplished this in our skin abscess infections showing a lower virulence of PVL-expressing S. aureus as compared to isogenic PVL knockout strains, as well as an infection-enhancing effect of antibody to PVL. These observations may be applicable to other toxins and virulence scenarios, and open up new means to understand how bacteria are able to establish infections and persist to the point wherein meaningful manifestations of disease ensue.

References
1. Schmitt CK, Meynick KC, O’Brien AD. Bacterial toxins: friends or foes? Emerg Infect Dis 1999; 5:224-34.
2. Gonzalez MR, Bischoffberger M, Pernot L, van der Glog FGL, Freche B. Bacterial pore-forming toxins: the (whole) story? Cell Mol Life Sci 2008; 65:493-507.
3. Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, Boyrud DJ, Etienne J, et al. Comparison of community- and health care-associated methicillin-resistant Staphylococcus aureus infection. JAMA 2003; 290:2976-84.
4. Finck-Barbancon V, Duportail G, Meunier O, Colin DA. Pore formation by a two-component leukocidin from Staphylococcus aureus within the membrane of human polymorphonuclear leukocytes. Biochim Biophys Acta 1993; 1182:275-82.
5. Kanelo J, Karnio Y. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: Structures, pore-forming mechanism and organization of the genes. Biosci Biotechnol Biochem 2004; 68:981-1005.
6. Voyich JM, Otto M, Mathema B, Braughton KR, Whitney AR, Welty D, et al. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant Staphylococcus aureus disease? J Infect Dis 2006; 194:1761-70.
7. Labandeira-Rey M, Couzon F, Boisset S, Brown EL, Bes M, Benito Y, et al. Staphylococcus aureus Panton-Valentine leukocidin causes necrotizing pneumonia. Science 2007; 315:1130-3.
8. Bubeck Wardenburg J, Bae T, Otto M, DeLeo FR, Schneewind O. Poring over Pores: _α_ hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia. Nat Med 2007; 13:1405-6.
9. Bubeck Wardenburg J, Palazzolo-Ballance AM, Otto M, Schneewind O, DeLeo FR. Panton-Valentine leukocidin is not a virulence determinant in murine models of community-associated methicillin-resistant Staphylococcus aureus disease. J Infect Dis 2008; 198:1166-70.
10. Konig B, Prevost G, Piroment Y, Konig W. Effects of Staphylococcus aureus leukocidins on inflammatory mediator release from human granulocytes. J Infect Dis 1994; 171:607-13.

11. Konig B, Koller M, Prevost G, Piroment Y, Aloff JE, Schreiner A, et al. Activiation of human effector cells by different bacterial toxins (leukocidin, alveolysin and erythrogenic toxin a): Generation of interleukin-8. Infect Immun 1994; 62:4831-7.

12. Hender T, Konig B, Prevost G, Piroment Y, Koller M, Konig W. Leukotriene B4 generation and DNA fragmentation induced by leukocidin from Staphylococcus aureus: protective role of granulocyte-macrophage colony-stimulating factor (GM-CSF) and -CSF for human neutrophils. Infect Immun 1994; 62:2529-35.

13. Deip BA, Chan L, Tartein P, Kajikawa O, Martin TR, Basiuk L, et al. Polymorphonuclear leukocytes mediate Staphylococcus aureus Panton-Valentine leukocidin-induced lung inflammation and injury. PNAS 2010; 107:5587-92.

14. Tieng CW, Kyme P, Low J, Rocha MA, Alahbe R, Miller LG, et al. Staphylococcus aureus Panton-Valentine leukocidin contributes to inflammation and muscle tissue injury. PLoS ONE 2009; 4:6387.

15. Brown EL, Diamitsos C, Thomas D, Badiou C, Koors EM, Chaudhury P, et al. The Panton-Valentine leukocidin vaccine protects mice against lung and skin infections caused by Staphylococcus aureus USA 300. Clin Microbiol Infect 2009; 15:156-64.

16. Huang SS, Dikrema DJ, Warren DK, Zuccotti G, Winokur PL, Tendolkar S, et al. Strain-relatedness of methicillin-resistant Staphylococcus aureus isolates recovered from patients with repeated infection. Clin Infect Dis 2008; 46:1241-7.

17. Miller LG, Quan C, Shyu A, Mousaie K, Bharadwa J, Tan N, et al. A prospective investigation of outcomes after hospital discharge for endemic, community-acquired methicillin-resistant -susceptible Staphylococcus aureus skin infection. Clin Infect Dis 2007; 44:483-92.

18. Nguyen DM, Mascara L, Branco E. Recurring methicillin-resistant Staphylococcus aureus infections in a football team. Emerg Infect Dis 2005; 11:526-32.

19. Sikitak D, Brown K, Hester J, Moore T, Czolby C, Musra HR, et al. Community-onset methicillin-resistant Staphylococcus aureus in an urban HIV clinic. HIV Med 2006; 7:361-8.

20. Huang SS, Plant R. Risk of methicillin-resistant Staphylococcus aureus infection after previous infection or colonization. Clin Infect Dis 2003; 36:281-5.

21. Baba Moussa L, Werner S, Colin DA, Mourey L, Pédeleaq JD, Samapa JP, et al. Discoupling the Ca2+-activation from the pore-forming function of the bi-component Panton-Valentine leukocidin in human PMNs. FEBS Lett 1999; 461:280-6.

22. Stadi L, Montiel H, Colin DA. The staphylococcal pore-forming leukotoxins open Ca2+ channels in the membrane of human polymorphonuclear neutrophils. J Membr Biol 1998; 162:209-16.

23. Schaff UY, Yamaohi I, Tse T, Griffin D, Kibarhi L, Simon SI. Calcium flux in neutrophils synchronizes beta2 integrin adhesive and signaling events that guide inflammatory recruitment. Ann Biomed Eng 2008; 36:632-46.

24. Koprinjak T, Weidenmaier C, Peschel A, Weiss JP. Wall teichoic acid deficiency in Staphylococcus aureus confers selective resistance to mammalian group IIa phospholipase A2 and human _-defensin 3. Infect Immun 2008; 76:2169-76.

25. Guan-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM. Antimicrobial peptides: general overview and clinical implications in human health and disease. Clin Immunol 2010; 135:1-11.

26. Brown EL, Bowden MG, Bryson RS, Hulten KG, Bord AS, Forbes A, et al. Pediatric antibody response to community-acquired Staphylococcus aureus infection is directed to Panton-Valentine leukocidin. Clin Vaccine Immunol 2009; 16:39-41.

27. Groze M, Daudwalder O, Diamitsos C, Badiou C, Gillet Y, Genestier AL, et al. Serum antibodies against Panton-Valentine leukocidin in a normal population and during Staphylococcus aureus infection. Clin Microbiol Infect 2009; 15:144-8.

28. Soderblom T, Oshamme C, Tottenpson E, Richter-Dahlfors A. Bacterial protein toxins and inflammation. Clin Microbiol Infect 2005; 35:628-31.

29. Backert S, König W. Interplay of bacterial toxins with host defense: molecular mechanisms of immunomodularity signalling. Int J Med Microbiol 2005; 295:419-30.

30. Role F, Zeller SA, Chakraborty T, Domann E, Machledt T, Kroekc M, et al. Human endothelial cell activation and mediator release in response to Listeria monocytogenes virulence factors. Infect Immun 2001; 69:897-905.

31. Billington SJ, Jost BH, Songer JG. Thiol-activated cytolysins: structure, function and role in pathogenesis. FEMS Microbiol Lett 2000; 18:217-205.

32. Schmeling DJ, Gemmell CG, Craddock PR, Quie PG, Peterson PK. Effect of staphylococcal alpha-toxin on neutrophil migration and adherence. Inflammation 1981; 5:313-22.