Imaging Early Pathogenesis of Bubonic Plague: Are Neutrophils Commandeered for Lymphatic Transport of Bacteria?

David M. Bland, Deborah M. Anderson

Department of Veterinary Pathobiology, University of Missouri, Columbia, Missouri, USA

ABSTRACT Vector-borne infections begin in the dermis when a pathogen is introduced by an arthropod during a blood meal. Several barriers separate an invading pathogen from its replicative niche, including phagocytic cells in the dermis that activate immunity by engulfing would-be pathogens and migrating to the lymph node. In addition, neutrophils circulating in the blood are rapidly recruited when the dermal barriers are penetrated. For flea-borne disease, no insect-encoded immune-suppressive molecules have yet been described that might influence the establishment of infection, leaving the bacteria on their own to defend against the mammalian immune system. Shortly after a flea transmits *Yersinia pestis* to a mammalian host, the bacteria are transported to the lymph node, where they grow logarithmically and later spread systemically. Even a single cell of *Y. pestis* can initiate a lethal case of plague. In their article, J. G. Shannon et al. [mBio 4(5):e00170-13, 2013, doi:10.1128/mBio.00170-13] used intravital microscopy to visualize trafficking of *Y. pestis* in transgenic mice *in vivo*, which allowed them to examine interactions between bacteria and specific immune cells. Bacteria appeared to preferentially interact with neutrophils but had no detectable interactions with dendritic cells. These findings suggest that *Y. pestis* infection of neutrophils not only prevents their activation but may even result in their return to circulation and migration to distal sites.

Yersinia pestis is the causative agent of plague, a rapidly progressing and frequently lethal flea-borne disease. The bacteria colonize the flea midgut and are transmitted to mammals after forming an infectious biofilm that lodges in the proventriculus of the flea (1). Once deposited in the dermal layer of the mammalian host, the bacteria will migrate to the draining lymph node, establish a replicative niche, and eventually spread systemically, causing multiorgan failure and death of the host. *Y. pestis* can invade epithelial cells and survive and replicate in macrophages, but tissue damage and disease are primarily caused by its rapid extracellular growth and toxicity to host cells (2, 3).

The infection is believed to progress as an initial anti-inflammatory response mediated at least in part by the type III secretion system (T3SS) (4). When grown at low temperature and in the flea, the T3SS is poorly expressed, requiring 37°C for maximal transcriptional induction. The molecular mechanism and timing of how this transition occurs *in vivo* have not been well characterized, and it appears likely the initial interactions with host cells would proceed with bacteria vulnerable to immune activation. Furthermore, *Y. pestis* lipopolysaccharide (LPS) is immunostimulatory at low temperature and must undergo a biosynthetic change at 37°C that provides stealth and attenuation of Toll-like receptor signaling such that inflammation can be controlled. The question is, since these virulence factors are thermally regulated, what happens in the early stage during the transition from stimulatory to less stimulatory life cycles?

Following adherence of bacteria to host immune cells, the type III secretion system delivers effector proteins, collectively known as Yops, to the host cell cytosol. *In vivo*, *Y. pestis* preferentially targets phagocytic cells for injection of Yops, thus preventing their activation (5, 6). *Y. pestis* is nonmotile but invasive due to the activity of an extracellular broad-spectrum protease (plasminogen activator [Pla]) whose cleavage of fibrin and plasminogen enhances adhesion and promotes growth in tissues (7). There is little information available on the mechanism or kinetics of bacterial dissemination from the skin to the lymph node during the early stage of infection, and no surface proteins have yet been described that function as homing receptors which *Yersiniae* could use to traffic to the lymph node. Prevailing models for bacterial trafficking to the primary lymph node involve intracellular transport via the lymphatic system or extracellular vascular dissemination.

In their article, Shannon et al. (8) visualize host-pathogen interactions shortly after infection to identify possible host cell vehicles that might transport *Y. pestis* to the lymph node in a murine intradermal model of bubonic plague. Infection of transgenic mice expressing fluorescently labeled neutrophils (LysM-GFP, where GFP is green fluorescent protein) or dendritic cells (CD11c-YFP, where YFP is yellow fluorescent protein), with *Y. pestis* strains constitutively expressing dsRed, allowed the investigators to visualize the early stage of infection by intravital microscopy. Their provocative findings suggest that interactions between bacteria and neutrophils dominate the early stage and may contribute to systemic circulation of the infection.

During an inflammatory response initiated by tissue injury as well as recognition of *Y. pestis* pathogen-associated molecular patterns (PAMPs), neutrophils and monocytes are recruited from the peripheral blood (9). Neutrophils are by far the largest cell population and quickly migrate to the infection, where they are believed to mediate bacterial clearance. Inflammatory monocytes also enter infected tissue from the blood, where they mature to carry out macrophage or dendritic cell functions in host defense, including bacteriocidal activity, tissue repair, and antigen presentation (10). In addition, dendritic cells routinely traffic to the lymph node to display antigen to B and T cells and have previously been shown to

Published 5 November 2013

Citation Bland DM, Anderson DM. 2013. Imaging early pathogenesis of bubonic plague: are neutrophils commandeered for lymphatic transport of bacteria? mBio 4(6):e00837-13. doi:10.1128/mBio.00837-13.

Address correspondence to Deborah M. Anderson, andersondeb@missouri.edu.

shuttle pathogens from the skin to the lymph node through the lymphatic system or the vasculature. During *Y. pestis* infection, Shannon et al. (8) observed robust and active recruitment of LysM-expressing cells to the infected area, indicating neutrophils and/or inflammatory macrophages are recruited shortly after the initiation of the infection. Furthermore, bacteria appeared to colocalize with these cells and subsequently mobilized and migrated away from the plane of vision. Strikingly, neutrophil recruitment and apparent phagocytosis of bacteria occurred even when the T3SS was present.

In agreement with previous observations, Shannon et al. (8) reported that phagocytosis of T3SS$^+$ *Y. pestis* does not activate neutrophils, with no upregulation of the activation marker CD11b (11). The T3SS is believed to be largely inactive when bacteria are in the phagolysosome, and whether this is due to an environment that downregulates expression of the T3SS or because the translocation pore cannot assemble across the phagosomal membrane is not known (12). It is therefore likely that these neutrophils were not activated because of a soluble anti-inflammatory signal induced by the activity of the T3SS on other cells rather than a direct effect of Yop injection by intracellular bacteria. This interpretation is consistent with the lung model, where T3SS$^+$ *Y. pestis* establishes an anti-inflammatory state that is permissive for growth of avirulent T3SS$^-$ bacteria (4).

Y. pestis infects multiple tissues, with continuous bacterial growth at the inoculation site, as well as seeding of primary and secondary immune tissues followed by rapid bacterial growth in these sites. As bacteria continue to grow in the inoculation site and lymph node and as both sites become diseased, neutrophils are recruited from the circulation. Although the data are consistent with a model whereby neutrophils with lowered activation and altered programming might carry live bacteria to the lymph node, the mechanism whereby this would occur is not clear. Neutrophils are not known to express lymph node-homing receptors, and their reentry into the vasculature following phagocytosis may occur only under special circumstances (9). Nevertheless, live bacterial transport from subcutaneous tissue to the lymph node via neutrophils has been observed previously during vaccination of animals with *M. tuberculosis* BCG, and additional investigation into the mechanism underlying these observations is warranted (13).

Activated neutrophils undergo apoptosis after ingesting their prey, and apoptotic neutrophils are cleared by macrophages in a process known as efferocytosis (14). Efferocytosis triggers an altered programming of macrophages that results, in part, in increased migration of macrophages carrying their cargo to the lymph node and spleen (15, 16). Could macrophages transport *Y. pestis* bacteria that had been engulfed by efferocytosis of infected neutrophils? In the Shannon et al. study (8), bacteria continue to fluoresce after the cell dies, making it difficult to distinguish the fate of the intracellular bacteria shown in the intravital images. Furthermore, inflammatory macrophages that are recruited from the blood also express the LysM promoter and therefore would be likely to be labeled with GFP in the transgenic mouse used in these studies (17). Both macrophages (F480$^+$) and neutrophils (Gr-1$^+$) harbored intracellular bacteria in the skin, and, overall, some open questions remain about the specific mechanism whereby the bacteria are initially brought to the primary lymph node.

Dendritic cells are present in the dermal layer of the skin, placing them in close proximity with bacteria shortly after infection. While an intracellular life cycle within macrophages is well documented, there is little information available on intracellular bacteria in dendritic cells. There are, however, several lines of evidence suggesting that dendritic cells are inactivated by *Y. pestis* virulence factors, and, overall, the *in vitro* data suggest dendritic cell migration mediated by its homing receptor CCR7 is prevented (18, 19). Shannon et al. (8) provide *in vivo* evidence supporting this model and found no interaction between dendritic cells and bacteria in the dermis by intravital microscopy or flow cytometry. Furthermore, they show that CCR7 deletion has no effect on bacterial colonization of the lymph node, regardless of the presence of the T3SS or other *Yersinia*-specific virulence factors. This suggests that dendritic cells may play little to no role in transport of Gram-negative bacteria, not just *Y. pestis*, to the lymph node.

The use of intravital microscopy by Shannon et al. (8) provides exciting insights into host pathogen interactions that occur *in vivo*. Technology limitations required the use of high challenge doses to complete this study, and the presence of large numbers of bacteria likely facilitated their systemic spread through multiple mechanisms. A flea bite that transmits even one bacterium to the human host can subsequently lead to lethal bubonic plague. It is clear that the inflammatory response to a single cell would be vastly different than it is when 10^5 bacteria are injected, and access to the primary lymph node may be more limited at a low challenge dose. Although questions remain about the mechanism(s) whereby bacteria transit to the lymph node, it is clear that LysM-expressing cells mobilize following phagocytosis of Gram-negative bacteria. Do intracellular *Y. pestis* bacteria survive in neutrophils and later escape to further the infection? Will these neutrophils reenter circulation and migrate to the primary lymph node? And how does flea saliva influence the response in the dermis? With the further development of the sensitivity of intravital microscopy and the availability of genetically engineered mice expressing cell-specific markers, these questions will soon be answered. This exciting technology has the ability to address fundamental questions of trafficking where tissue architecture influences subsequent host-pathogen interactions that may be critical to the development of disease or immunity and have been missed by standard methods of analyzing homogenized tissues.

REFERENCES

1. **Hinnebusch BJ, Erickson DL.** 2008. *Yersinia pestis* biofilm in the flea vector and its role in the transmission of plague. Curr. Top. Microbiol. Immunol. **322:**229–248.
2. **Cowan C, Jones HA, Kaya YH, Perry RD, Straley SC.** 2000. Invasion of epithelial cells by *Yersinia pestis*: evidence for a *Y. pestis*-specific invasin. Infect. Immun. **68:**4523–4530.
3. **Straley SC, Harmon PA.** 1984. Growth in mouse peritoneal macrophages of *Yersinia pestis* lacking established virulence determinants. Infect. Immun. **45:**649–654.
4. **Price PA, Jin J, Goldman WE.** 2012. Pulmonary infection by *Yersinia pestis* rapidly establishes a permissive environment for microbial proliferation. Proc. Natl. Acad. Sci. U. S. A. **109:**3083–3088.
5. **Fields KA, Nilles ML, Cowan C, Straley SC.** 1999. Virulence role of V antigen of *Yersinia pestis* at the bacterial surface. Infect. Immun. **67:** 5395–5408.
6. **Marketon MM, DePaolo RW, DeBord KL, Jabri B, Schneewind O.** 2005. Plague bacteria target immune cells during infection. Science **309:** 1739–1741.
7. **Korhonen T, Haiko J, Laakkonen L, Jarvinen H, Westerlund-Wikstrom B.** 2013. Fibrinolytic and coagulative activities of *Yersinia pestis*. Front. Cell Infect. Microbiol. **3:**35.
8. **Shannon JG, Hasenkrug AM, Dorward DW, Nair V, Carmody AB,**

Hinnebusch BJ. 2013. *Yersinia pestis* subverts the dermal neutrophil response in a mouse model of bubonic plague. mBio **4**(**5**):e00170-13. doi: 10.1128/mBio.00170-13.
9. **Kolaczkowska E, Kubes P.** 2013. Neutrophil recruitment and function in health and inflammation. Nat. Rev. Immunol. **13:**159–175.
10. **Shi C, Pamer EG.** 2011. Monocyte recruitment during infection and inflammation. Nat. Rev. Immunol. **11:**762–774.
11. **Bosio CF, Jarrett CO, Gardner D, Hinnebusch BJ.** 2012. Kinetics of the innate immune response to *Yersinia pestis* after intradermal infection in a mouse model. Infect. Immun. **80:**4034–4045.
12. **Zhang Y, Murtha J, Roberts MA, Siegel RM, Bliska JB.** 2008. Type III secretion decreases bacterial and host survival following phagocytosis of *Yersinia pseudotuberculosis* by macrophages. Infect. Immun. **76:** 4299–4310.
13. **Abadie V, Badell E, Douillard P, Ensergueix D, Leenen PJ, Tanguy M, Fiette L, Saeland S, Gicquel B, Winter N.** 2005. Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. Blood **106:** 1843–1850.
14. **Korns D, Frasch S, Fernandez-Boyanapalli R, Henson P, Bratton D.** 2011. Modulation of macrophage efferocytosis in inflammation. Front. Immunol. **2:**57.
15. **Ariel A, Serhan C.** 2012. New lives given by cell death: macrophage differentiation following their encounter with apoptotic leukocytes during the resolution of inflammation. Front. Immunol. **3:**4.
16. **Schwab JM, Chiang N, Arita M, Serhan CN.** 2007. Resolvin E1 and protection D1 activate inflammation-resolution programmes. Nature **447:**869–874.
17. **Hume DA.** 2011. Applications of myeloid-specific promoters in transgenic mice support *in vivo* imaging and functional genomics but do not support the concept of distinct macrophage and dendritic cell lineages or roles in immunity. J. Leukoc. Biol. **89:**525–538.
18. **Robinson RT, Khader SA, Locksley RM, Lien E, Smiley ST, Cooper AM.** 2008. *Yersinia pestis* evades TLR4-dependent induction of IL-12$(p40)_2$ by dendritic cells and subsequent cell migration. J. Immunol. **181:** 5560–5567.
19. **Telepnev MV, Klimpel GR, Haithcoat J, Knirel YA, Anisimov AP, Motin VL.** 2009. Tetraacylated lipopolysaccharide of *Yersinia pestis* can inhibit multiple Toll-like receptor-mediated signaling pathways in human dendritic cells. J. Infect. Dis. **200:**1694–1702.

The views expressed in this Commentary do not necessarily reflect the views of the journal or of ASM.