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Yersinia pestis-Host Immune Cells Interactions at Early Events During Bubonic Plague Infection

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Abstract Plague is a fatal disease caused by *Yersinia pestis* and is initiated with the introduction of the bacteria into the skin by the bite of an infected flea. In the dermis, *Y. pestis* can evade the innate immune response of the host and then disseminate to the draining lymph node, where it replicates leading to formation of the pathognomonic bubo of bubonic plague. However, the early events that occur immediately after *Y. pestis* entrance until it infects deeper tissues remain poorly understood. Recently, advanced microscopy techniques have been useful to follow bacterial dissemination during plague infections and to characterize *Yersinia*-host interactions. This review focuses on the major events that occur early after different routes of *Y. pestis* infection, as well as the role of the host's innate immune cells in *Y. pestis* dissemination.

Keywords *Yersinia* · Dermis · Neutrophils · Macrophages · Lymph node · Dissemination

Introduction

Plague Is a Historic Disease that is Re-emerging

Plague, caused by the bacterium *Yersinia pestis* (*Y. pestis*), is a severe zoonotic disease with a remarkable place in history as it is infamous for having caused 100 to 200 million of deaths in the course of three pandemics during the last 15 centuries, making it one of the leading infectious disease killers of humans [1]. Although plague is often classified as an eradicated disease which was only present during ancient ages, the truth is that it is still present in endemic regions of the modern world. Plague is characterized by occasional outbreaks or epizootics following periods of low prevalence, sometimes spanning ~30–50 years. Around 1000 to 2000 cases of plague are reported each year, though the true number is likely much higher. Plague epidemics have been reported in the USA in the 1920s, India during the 1950s and 1960s, and in Vietnam during the Vietnam War of the 1960s and 1970s. Since the 1990s, more than 40,000 cases have been recorded; most human cases in 2000s have occurred in Africa, where plague has been reported in 6 countries (Congo, Madagascar, Malawi, Mozambique, Uganda and Tanzania). Plague cases in these areas now account for over 95 % of world reported cases [2–8]. A steady incidence of plague is maintained in Asia, where the disease is present in China, India, Kazakhstan, Mongolia and Vietnam, representing 1 to 5 % of total world cases. In the Americas, Peru and the USA are the countries that have reported plague cases. It is for these reasons that plague has been classified by the Centers for Disease Control (CDC) as a re-emerging disease and is still a matter of study. In fact, between 1900 and 2012, 1006 confirmed or probable human plague cases have been reported by CDC to have occurred in the US states of New Mexico, Arizona, Colorado, California, Georgia, Oregon, and Nevada.

Plague: the Disease

Among the 17 species included in the genus *Yersinia*, three are important pathogens of humans and other mammals: *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, and *Y. pestis*.

This article is part of the Topical Collection on *Rickettsia*

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Interestingly, the former two are enteric pathogens that are transmitted by a fecal-oral route and cause an intestinal infection, called yersiniosis, whereas *Y. pestis* is a vector-borne pathogen that produces the fatal disease plague. The clinical presentation of plague in its hosts differs considerably from yersiniosis, although *Y. pestis* diverged from *Y. pseudotuberculosis* and maintains a great genetic relatedness to its clonal ancestor [9].

Plague is mainly transmitted by fleas but also by consumption or handling of infectious host tissues or through inhalation of respiratory droplets or aerosols. Man is an accidental host for *Y. pestis*, and although domestic and urban rats were the initial animal reservoirs, nowadays wild animal reservoirs such as prairie dogs, squirrels, marmots, and infected larger mammals like cats and coyotes are the cause of cross-infections responsible of the majority of human plague cases [1, 10]. Plague can present in three forms: bubonic, septicemic, and pneumonic. Bubonic plague is the predominant form (90 % of suspected cases), with mortality around 15 or 50 % if untreated [3, 10]. This form arises following transmission by flea bite. Fleas acquire the bacteria by feeding on a highly bacteremic host. Approximately 80 species of fleas have been found to be infected with *Y. pestis* in nature or to be susceptible to experimental infection, although they vary in their ability to transmit the bacteria. The rat flea *Xenopsylla cheopis* is identified as the most efficient vector [11]. Inside the flea, the bacteria first have to adapt to the temperature downshift from 37 °C (in the mammal host) to 26 °C, as well as to the many natural physicochemical properties of the flea gut environment. *Y. pestis* colonizes the flea gut and replicates within the flea midgut and proventriculus (a valve connecting the flea esophagus and the midgut), forming a thick coherent biofilm that may eventually occlude (block) the foregut proventriculus and esophagus [12]. Although blockage formation is typically observed at 2–3 weeks post-infection, it can occur as early as 5 days post-infection [13, 14]. This occlusion impedes the fresh blood meal ingestion, and the so-called blocked fleas become starved. Desperate starving fleas increase their attempts to acquire a blood meal by biting new hosts. These fleas are however unable to satisfy its hunger because the fresh blood cannot pass to the midgut due to the blockage. Instead, this results in reflux of the blood meal together with dislodged bacteria from the blocked foregut into the bite site of a naïve host [12, 14–16]. This is the biological mechanism of transmission [15]. An alternate mechanism of transmission from fleas occurs when the flea feeds on a new host shortly after taking a blood meal from a highly septicemic host. This is predicted to be a mechanical transmission occurring through inoculation of residual bacteria from the flea mouth parts that remains shortly after infectious blood feeding [16, 17].

After the flea bite, *Y. pestis* colonizes the host intradermally and then migrates to the regional lymph node (LN), causing the swelling of LN in the victim, typically in the axillae or groin with the resulting inflammation and formation of the classic lesions known as "buboes" [18]. Diarrhea, nausea, and vomiting are frequent manifestations at this stage. Bacteria can spread systemically through the blood [18], liver, spleen, and other organs then causing the septicemic plague, which has 30 to 50 % mortality rate, even with antibiotic administration [19]. Disseminated intravascular coagulopathy, meningitis, and multiorgan failure are common signs in this plague form. Occasionally, fleas can also deposit bacteria directly into the bloodstream of a mammalian host resulting in primary septicemic plague infection which occurs without the appearance of buboes and is observed in a small proportion of patients (15 %) [20, 21, 22••]. The fact that bacteria can be detected sometimes in spleens 1 h after the flea feeding supports the idea that they can be introduced directly into the bloodstream during flea feeding [22••]. Bacteria can then also reach the lungs, causing secondary pneumonic plague, which can be transmitted directly from person to person, through infectious aerosols. Pneumonic plague can then be developed by people as primary infection by direct inhalation of infectious droplets or aerosols from pneumonic plague sufferers that have developed the disease from either primary or secondary infections [23]. Primary pneumonic plague is the rarest form of the disease but has the highest mortality rate, which can reach 100 % if untreated or >50 % even with antimicrobial treatment [3, 20, 24]. The initial symptoms are flu-like, which rapidly progress to pneumonia with bloody and watery sputum production.

Successful colonization of the host depends on the expression of bacterial virulence factors such as a type III secretion system (T3SS), pH 6 antigen, F1 antigen, and *pgm* locus. These are upregulated at 37 °C and prevent phagocytosis [3, 25] and are minimally expressed in the flea midgut [26, 27]. *Y. pestis* maintains a virulence plasmid termed pCD1 (or pYV in enteropathogenic *Yersinia*) that encodes a T3SS and effector proteins called Yops (for *Yersinia* outer proteins), which are injected into host innate immune cells via the T3SS [28] and confer resistance to phagocytosis by neutrophils and macrophages, reduction in proinflammatory cytokine production, and triggering apoptosis in host cells [22••, 29, 30].

Despite the extensive study of the pathogenesis of plague, very little is still known about the major events occurring early during infection, this is, immediately after inoculation of *Y. pestis* into the skin. How *Y. pestis* moves from the initial site of contact in the dermis into deeper tissues and how these events affect bacterial dissemination are largely speculative. Being the host and pathogen interactions crucial events during dissemination, early events are key to understand the disease and to design strategies to control plague, especially in those areas where the disease is endemic. This review focuses on recent findings about the bacterium-host interactions during early dissemination from the inoculation site to the LN, with special emphasis on the intradermal models of infection, which have been greatly improved thanks to advances in in

vivo microscopy techniques. Important events cited throughout the text are summarized in Fig. 1.

Bubonic Plague Progression: What We Have Learnt About Infection Routes Using Recent In Vivo Imaging Techniques

Until recent years, the conventional method to follow plague disease progression in animal models was to experimentally infect mice with *Y. pestis* WT or mutant strains, sacrifice groups of animals at various time intervals, and determine bacterial counts in their tissues. Although this method has provided very useful information about the pathogenesis in *Y. pestis*, it remains laborious and time consuming. In the past 5 years, new imaging techniques using fluorescence or luminescence have been explored and used for the deeper understanding of *Y. pestis*-host interactions and bacterial dissemination during progression of bubonic plague.

Bioluminescence imaging (BLI) allows for the in vivo visualization of a pathogen in a host through the course of infection. This technique uses *Y. pestis* strains transformed with a plasmid containing the *luxCDABE* genes, which encode a bacterial luciferase and other enzymes that are necessary to generate the substrate for luciferase [31, 32]. In the presence of its substrate, luciferase catalyzes a reaction generating bioluminescent light. The light emitted by the recombinant microorganism is measured and imaged using an in vivo BLI system that uses a high-sensitivity camera that detects very small amounts of light. This system has been successfully used to follow *Yersinia* dissemination over time after mice are infected subcutaneously (SC), intradermally (ID), and intranasally (IN) [33•, 34•, 35]. It was also demonstrated to be useful at detecting mutants with defects in colonization or dissemination during infection.

Intravital microscopy (IVM) enables the study of cellular and molecular events in living organisms. This technique uses genetically encoded fluorescent protein (FP) tags, live cell dyes, and other methods to fluorescently label proteins that are imaged using confocal microscopy [36]. In recent years, intravital confocal microscopy has been used to study the bacterium-immune cells dynamic interactions in the dermis. For this, green fluorescent protein (gfp-) or red fluorescent protein (rfp-) expressing *Y. pestis* have been used to inoculate mice and immune cells have been stained by using fluorescently labeled antibodies [37••, 38••]. In addition, transgenic mice expressing different fluorescent proteins in each immune cells like macrophages, neutrophils, and dendritic cells have been used to track those cells recruited to the dermis in response to bacteria inoculation [22••, 39••]. These methods have allowed the revelation of which factors play a major role in the early events that defines a *Y. pestis* infection.

SC model of infection has been most commonly used to study plague in vivo. Mice models of *Y. pestis* SC experimental infection using BLI have shown that *Y. pestis* can remained confined at the site of injection during the course of infection, up to 6 days [33•, 34•], as reported also for ear intradermal infection [40]. It has suggested that the main targets of *Y. pestis* multiplication are the lymph nodes, spleen, and liver. LN are the first organs to be colonized [33•, 34•], in agreement with the notion that during bubonic plague *Y. pestis* travels from the inoculation site (IS) to the proximal LN prior to dissemination [18]. The bacteria reach the proximal and distal LN from the inoculation site within the first 3 days, apparently via the lymphatic stream. Spleen and liver are colonized almost simultaneously at day 4, after a slight and transient bacteremia (Fig. 1). Colonization of other organs in live animals is not significant until the terminal stage of the infectious process, after septicemic phase. But once the bacteria reaches the LN, the disease progresses rapidly leading to the invasion of the entire body (septicemia) within 2 days (at day 5) and the death at day 6 [33•] (Fig. 1).

Although SC model has certainly shed light around the host-pathogen interaction, in recent years, the ID route has been gaining impact as a model of infection, which is understandable as this is the biologically relevant route for the entry of *Yersinia* into the host. Being a flea-borne pathogen, *Y. pestis* has to surpass the skin epithelium as the first barrier to penetrate into deeper tissues and then disseminate systemically. In the skin, the dermis layer is the first tissue where initial interactions between host and *Yersinia* occur, because the first layer, the epidermis, is crossed through the mechanical action of the fleas' mouth parts and it has been shown that fleas do not deliver *Y. pestis* into the subcutaneous space either [11, 21, 41].

Little is known about aspects of the natural infection that affect disease progression. These imaging methods have clearly shown differences between in vivo and in vitro approaches to study host-pathogen interactions. Even more, they allowed comparisons between different routes of inoculation and highlight the importance of using a biologically relevant model when studying host-pathogen interactions in vivo. Comparisons of ID and SC models of infection have shown there are differences in the progression of the disease depending on the route used to inject *Y. pestis*. When inoculated intradermally, bacteria colonize the host faster than via the SC route of infection, as they are detected as early as 6 hpi in the draining LN, despite most mice did not have detectable lymph node colonization only by 12 hpi [38••, 42•]. Bacteria were first detected in the spleen at 24 hpi, and in comparison with the SC route, much higher numbers of bacteria are found in LN and spleen 48 hpi [38••, 42•] (Fig. 1); however, the SC route produces higher mortality regardless of the inoculated dose [38••].

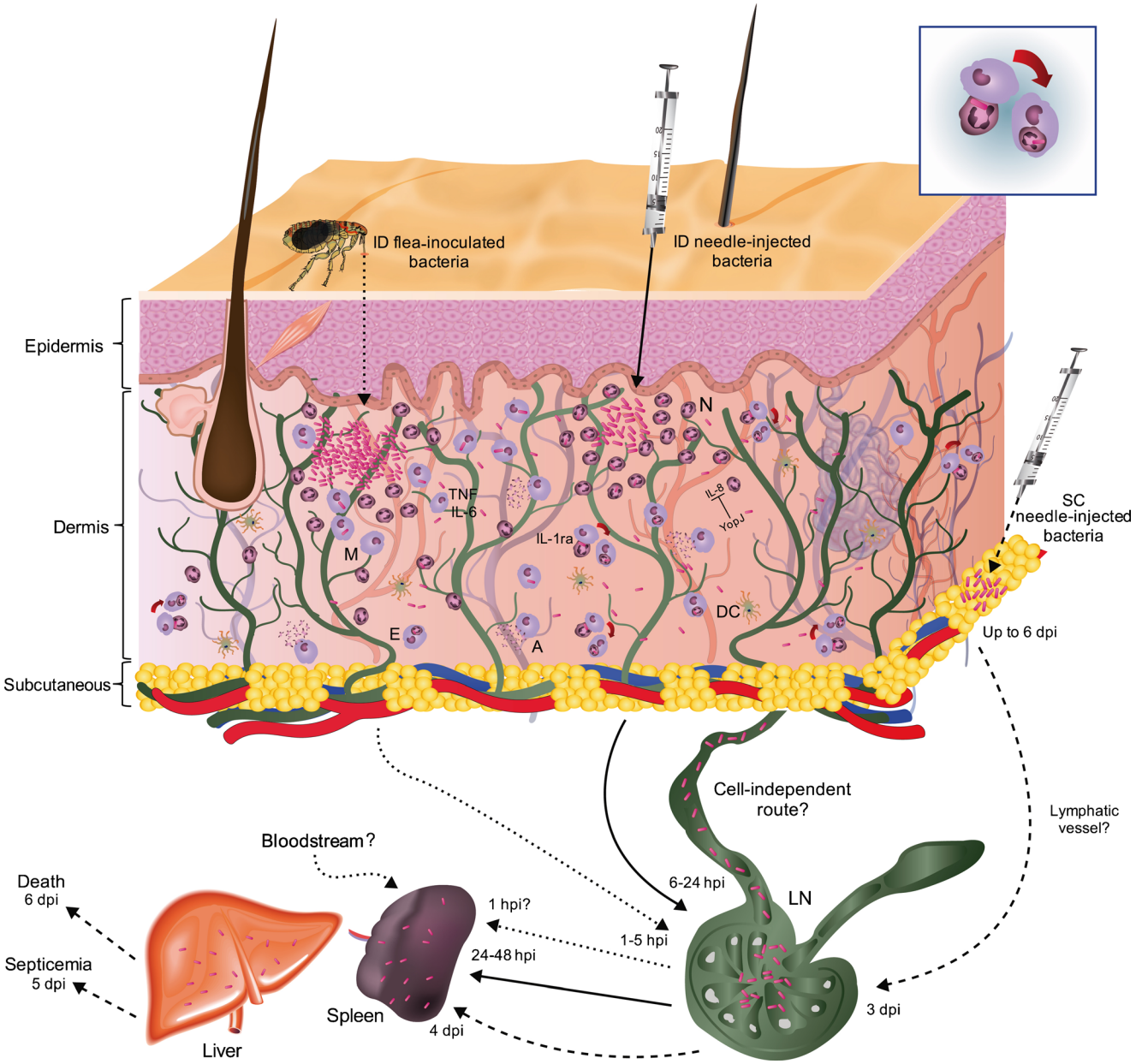

Fig. 1 Schematic representation of the *Y. pestis*-host interactions at early events during infection by different routes. There are differences in the progression of bubonic plague depending on the route of infection. Subcutaneously (*SC*) inoculated bacteria can remain up to 6 days in the inoculation site and reach lymph node (*LN*) (apparently via lymphatic stream) and systemic organs days after infection (*dpi*), while the ID-inoculated bacteria reach LN and systemic organs some hours after infection (*hpi*). There are differences even between the two intradermal (*ID*) routes of infection, as the injected bacteria disseminate slower than those that are inoculated by the natural vector, the flea. In the ID flea bite inoculation route, bacteria can be seen in the spleen 1 h after flea bite, suggesting bacteria can be introduced directly into the bloodstream. Flea-transmitted *Y. pestis* is more likely associated with macrophages (*M*), whereas those needle-inoculated recruit more neutrophils (*N*) and are more likely associated with these cells. Bacteria can be phagocytosed and destroyed by neutrophils, which suffer apoptosis (*A*) and the rests are then cleared by macrophages. Bacteria can also survive inside neutrophils which are recognized by macrophages, then internalized and partially degraded. This process is called efferocytosis (*E*) (indicated with the *red arrows* and magnified in the *right upper box*). Cytokine response is importantly influenced during *Y. pestis* infection. Direct contact of bacteria with macrophages induces secretion of TNFα and IL-6, but when macrophages are in contact with neutrophil-associated bacteria, there is an increase in secretion of IL-1ra instead. The *Y. pestis* T3SS-encoded effector YopJ suppresses IL-8 secretion by neutrophils. There is minimal recruitment of dendritic cells (*DC*), and they show minimal interaction with bacteria. Some not cell-associated bacteria can enter lymphatic vessels, suggesting a phagocyte-independent route of dissemination. Two bottlenecks seem to be defining the bacterial population that reaches the LN and deeper organs, as the number of bacteria that initially infect decreases throughout the dissemination. See text for details

The ID needle inoculation model has been widely used previously to mimic the natural route of transmission, that of the bite of an infected flea. However, flea saliva also contains molecules that could be influencing the host innate immune

response [43]. Forthwith, it was hypothesized then that the course of the plague disease transmitted by the natural plague vector might differ from the needle inoculation model. To evaluate this, Shannon et al. developed tools for IVM to image *Y. pestis* deposited in the dermis of an infected mouse by flea bite. They first identified the exact areas at which fleas have fed on the mouse dermis using a fluorescent DNA marker that stains damaged cells at the flea bite site caused by fleas inserting their mouth parts into the skin. A strain of transgenic mouse, the LysM-eGFP mice that expresses eGFP in neutrophils, was used for these experiments. This allowed characterization of the neutrophil migration to these sites [22••]. In this model, dissemination of bacteria from the dermis to the LN can occur within the first 5 h after flea feeding, although small numbers of bacteria were also found in the LN approximately 1 h post-feeding (Fig. 1). This suggests very rapid *Y. pestis* dissemination to the spleen and LN and indicates that migration from inoculation site to the LN is more rapid than previously thought [22••]. Sometimes bacteria could be detected in the spleen 1 h after flea bite, suggesting that bacteria can be introduced directly into the bloodstream during flea feeding and that very rapid dissemination to the spleen and LN is common after flea transmission of *Y. pestis*.

How Does *Y. pestis* Traffic to Reach the Lymph Node?

Very little is known about the events that follow inoculation and how pathogens move from the initial site of contact into deeper tissues. Since some patients develop systemic plague without prior history of buboes, it has been proposed that *Y. pestis* is able to use both lymphatic drainage and the bloodstream to establish itself in the host [44]. Although initial immune events in the dermis have not been well elucidated, so far, it is well accepted that after the entry into the host, *Y. pestis* first disseminates from the inoculation site in the skin into the draining LN [18, 34•] and then moves to the bloodstream causing the septicemic process [45]. In vivo image analyses have supported this notion as it has been shown that at 6–12 hpi bacteria began to disseminate from the dermis to LN [39••], and within about 12 to 24 h, *Y. pestis* has colonized the draining LN. From there, the pathogen disseminates to other target organs in the host, such as the spleen and liver [42•] (Fig. 1). Henceforth, efforts have been directed at elucidating how *Y. pestis* disseminates from the skin to the LN.

In a natural plague infection, the flea deposits *Y. pestis* into the dermis layer. In contrast to the epidermis and the subcutaneous space, the dermis is rich in terminal lymphatic vessels [46], which are more permeable and subjected to high pressure, anatomical characteristics that enable it to readily take up an antigen. So, *Y. pestis* inoculation into the dermis could contribute to its efficient movement to the LN through these vessels, which would be in agreement with the suggestion that *Y. pestis* could travel freely in the lymph fluid [37••]. Gonzalez et al. used an intradermal model of infection inoculating a small dose and volume of *Y. pestis* (200 CFU in 2 μl) that closely mimics that deposited during natural flea bite transmission [38••]. They found that *Y. pestis* does not replicate in the skin, but larger numbers of bacteria in the skin are detected at later stages of infection and are likely derived from systemically circulating *Y. pestis* [38••]. Bacteria were seen within lymphatic vessels attached to the LN after 24 hpi [37••]. Interestingly, within the first 12 hpi appears to be a bottleneck that defines the population that will reach the LN and then disseminate systemically. This was concluded from a dissemination assay that used bacteria that were chromosomally bar-tagged with different oligonucleotide sequences as the ID inoculums. At different time points post-inoculation, the bacterial population was recovered and sequenced to identify which strains disseminated from the IS [37••]. After 12 hpi, they recovered from LN only a small fraction of the population they inoculated intradermally. Furthermore, they recovered from the spleens, only those bacteria that were also recovered from LN, implying that after the ID inoculation *Y. pestis* has to pass through the LN before disseminating throughout body, and that this LN bacterial population is defined by the initial bottleneck. This bottleneck effect was only notable for the ID inoculation route, impressing that inherent skin factors delimit bacterial numbers that reach the LN and then spread systemically [37••]. Using the same dissemination assay and tagged strains, in a SC infection model, a second bottleneck affecting the bacterial dissemination from LN to deeper organs was suggested, as many tagged strains present in the LN were later absent in the spleen [37••].

There is also the perception that *Y. pestis* traffics from the skin to the LN within phagocytic cells produced as part of the early host innate immune response. Some studies have elucidated this phenomenon using flow cytometry and fluorescent microscopy. These studies have analyzed which host cell types are recruited to the dermis early after ID infection of *Y. pestis*. This has shed light on the response of the host innate immune cells to bacteria early after ID infection of *Y. pestis* and their role in bacterial dissemination to the LN.

The Role of Neutrophils and Macrophages in *Y. pestis* Dissemination

Ever since Janssen and Surgalla first reported that *Y. pestis* could be found inside both macrophages and neutrophils after IP infection [47], there has been interest in intracellular pathogenesis of *Y. pestis*. Neutrophil recruitment following ID inoculation along with induced expression of *Y. pestis* genes essential for combating neutrophil-derived reactive nitrogen species has been shown in rats [18, 21]. It is thought that after SC infection, *Y. pestis* are phagocytosed and quickly killed by

neutrophils, whereas those taken up by macrophages are able to survive, replicate, and disseminate [48]. Despite these observations, the role of these cells during the initial phases of bubonic plague pathogenesis and their requirement for *Y. pestis* dissemination from the skin into the LN remains vague.

Immune cell kinetic studies using flow cytometry, immunohistochemistry, and IVM have shown that neutrophils are the predominant cell type recruited to the injection site (Fig. 1). These cells arrive within minutes even in response to a trauma or break in the skin [39••]. Irrespective of strain virulence, large numbers of neutrophils are seen 4 h after ID needle inoculation of *Y. pestis* followed by slower accumulation of macrophages that peaks 6 hpi [39••, 42•]. Around 80 % of bacteria were associated with neutrophils and many trafficked *Y. pestis* away from the injection site, but a large proportion of the remaining bacteria were associated with macrophages. Decreased activation of neutrophils and macrophages was notable after infection with strains harboring the pCD1 plasmid, indicating that pCD1 is important for the evasion of neutrophil and macrophage response [39••, 42•]. However, notable differences in the inhibition of the neutrophil activation are still observed among attenuated strains harboring the pCD1 plasmid [39••], indicating that *Y. pestis* may possess pCD1-independent mechanisms that can account for the reduction in neutrophil activation in vivo. More than 95 % of the neutrophil-associated bacteria were intracellular, pointing to neutrophil interactions with *Y. pestis* being important during early establishment of bubonic plague. However, dissemination of virulent strains in neutrophil-depleted mice was not affected, indicating that neutrophils are not essential for dissemination from the dermis to the LN [39••].

After the first bottleneck that defines the population that will reach the LN, bacteria that did not pass to the LN remain in the skin throughout the infection. Somehow these bacteria activate the skin to be responsive to future inoculations resulting in detrimental effects to bacteria. Neutrophil recruitment is not responsible for activation either as although these cells form clusters in the skin and are concentrated at the injection site, when depletion of neutrophils was induced with a specific depletion antibody, there was no impact on bacterial trafficking to LN. However, although neutrophils are not able to clear infection, they appear important for restricting bacterial proliferation in the skin [37••].

One important role of the neutrophils is to act as intermediate hosts for subsequent non-inflammatory infection of macrophages. In the ID injection model of infection, a portion of *Y. pestis* may be unable to survive phagocytosis by neutrophils, but it has been also reported that up to 10 % of *Y. pestis* may survive after phagocytosis by neutrophils [30, 49] and they can replicate within neutrophils even after 10 h of incubation. Previous studies demonstrated that neutrophils that contain *Y. pestis* [50, 51] and *Y. pseudotuberculosis* [52] initiate apoptosis and present phosphatidylserine (PS), a marker of an early apoptosis, on their surfaces [53–55]. PS is then recognized by macrophages, which results in the uptake and clearance of the apoptotic infected neutrophils by macrophages within 1–2 h, a process called efferocytosis [56–58] (Fig. 1). The neutrophils so internalized are partially degraded by the macrophage. Some *Y. pestis* are also killed, whereas some are able to survive and replicate within macrophages that become infected by efferocytosis [59]. Normally, the contact of macrophages with *Y. pestis* induces secretion of the inflammatory cytokines TNF-α and IL-6 (Fig. 1), but addition of macrophages to neutrophils containing *Y. pestis* resulted in decreased levels of both cytokines [59]. Recently, the contribution of the pCD1 plasmid-encoded effector Yops, in alteration of cytokine production by human neutrophils, has been evaluated and it has been reported that YopJ is the major effector protein responsible for suppressing IL-8 production by neutrophils, although other effector proteins could be potentiating YopJ-mediated inhibition to aid disruption of PMN signaling pathways leading to IL-8 production [60••] (Fig. 1). This suggests that *Y. pestis* is able to inhibit secretion of anti-inflammatory cytokines, not only by active pCD1-dependent inhibition but also by infecting macrophages through non-inflammatory efferocytosis. On the other hand, there is secretion of the anti-inflammatory cytokine IL-1ra when macrophages take up *Y. pestis* containing neutrophils (Fig. 1), which could mostly occur from macrophages, although both the neutrophils and macrophages are able to secrete this cytokine [61–63]. It seems therefore that phagocytosis by neutrophils prior to uptake by macrophages may be an evasive strategy for *Y. pestis* to infect macrophages, a strategy that subsequently prevents immune response signaling from macrophages, thereby facilitating continued bacterial survival and dissemination. The efferocytosis also prevents the release of the neutrophil's microbicidal agents into the host tissues.

When using the flea bite inoculation, important differences in the numbers or composition of innate immune cells recruited to the inoculation site are seen in comparison with the other needle inoculation routes. Despite blocked fleas making repeated unsuccessful attempts to feed which results in more damaged skin, neutrophil response was highly variable and did not correspond with the amount of skin damage. Fleas transmitted a highly variable number of bacteria into the skin, and number of neutrophils recruited to bite sites was high and correlated with bacteria numbers at the bite site. This shows that any suppressive effect that flea saliva may have on neutrophil recruitment could not override the response to bacteria in the dermis. In this model, macrophages also migrated toward flea bite sites and interacted with small numbers of flea-transmitted bacteria. In contrast to observations from using the ID injection, very little translocation of bacteria was observed inside the neutrophils, and instead the few observed to be moving were frequently associated with macrophages [22••] (Fig. 1). This suggests that flea-transmitted *Y. pestis* may more

likely associated with macrophages, whereas those needle inoculated are more likely associated with neutrophils. This would have implications for *Y. pestis* pathogenesis as it has been shown that macrophages are much more permissive for *Y. pestis* survival and growth than neutrophils are [48]. Flea saliva contains molecules homologous or analogous to salivary proteins in other blood-feeding arthropods that are known to be anti-inflammatory [43], which would explain that the needle inoculated bacteria recruit more neutrophils due to lack of accompanying anti-inflammatory effects.

The Role of Dendritic Cells in *Y. pestis* Dissemination

Dendritic cells (DC) are phagocytic antigen-presenting cells that reside in peripheral tissues such as the dermis when they are immature, but in response to antigens or tissue damage recognition, they undergo maturation, engulf antigens, and carry them to the lymphoid tissue to initiate an adaptive immune response [64]. Although ID DC numbers are very low compared with neutrophils, their migration has led to speculation that DC could play a role in the dissemination of *Y. pestis* from the dermis to the LN. However, studies demonstrate that irrespective of the virulence strain, DC are not recruited to the injection site and there is minimal interaction between *Y. pestis* and DC in the dermis in vivo (Fig. 1). Further, similarly to what is known for neutrophils, dissemination of *Y. pestis* from dermis to the LN is not dependent of dendritic cells [39••]. By flea bite, although DC appear to migrate towards flea bite sites that contained bacteria, they also showed minimal interaction with the flea-transmitted bacteria. However, as there is greater displacement of DC when flea bite-transmitted bacteria are present at the bite site but not to uninfected flea bites, it cannot be discarded that they can associate with bacteria later during the infection [22••].

Does *Y. pestis* Really Need an Intracellular Stage for Dissemination?

Y. pestis has some antiphagocytic factors such as T3SS, pH 6 antigen, and F1 antigen that are upregulated at 37 °C and are weakly expressed at 26 °C, then making it less susceptible to phagocytosis by macrophages inside the host. These antiphagocytic factors are predicted to be expressed at low levels during the first hours of infection. This gave rise to the hypothesis that an intracellular stage facilitates trafficking from skin to LN, and it was suggested that *Y. pestis* could traffic from the skin to LN inside macrophages and was supported by reports of intra-macrophage survival of *Y. pestis* [48]. However, bacteria grown at either 26 or 37 °C, i.e., not expressing or expressing antiphagocytic factors, respectively, are both efficient at reaching the LN [37••], implying that association with phagocytes is not necessary for dissemination. Moreover, those bacteria that are not cell-associated can enter lymphatic vessels like through a phagocyte-independent way, without the requirement for an intracellular stage (Fig. 1). In agreement with this, flea-transmitted bacteria have been found in the LN 1 h post-infection [22••] and needle injected *Y. pestis* could be found in the LN of some mice as early as 10 min post-infection [37••]. The implication is thus that bacteria may move so rapidly into the lymphatics that they bypass any significant interaction with phagocytes at the bite site.

Conclusions

There is little information available on the kinetics of bacterial dissemination from the inoculation site to the lymph node during the bubonic plague development. Here, we reviewed recent findings that shed light on the major events during the early stage of *Y. pestis* infections. The use of in vivo microscopy techniques allows following of the events that take place after the bacterial entrance into the body in order to accomplish dissemination. These strategies have demonstrated that there are important differences about the time it takes *Y. pestis* to reach the LN as well as the interactions with the host immune response cells, depending on which route of entry is evaluated (SC or ID) and even among different inoculation techniques using the same route of entry (needle-inoculated bacteria or flea-inoculated bacteria). The study of *Y. pestis*-host interactions at cellular level has led to determine that neutrophils and macrophages are the first line of innate immune host defense that *Y. pestis* contacts and evades in the skin in order to reach the draining LN. Current data suggest that phagocytosis by neutrophils can provide a host reservoir and a mechanism for non-inflammatory infection of macrophages via efferocytosis. Flea-transmitted *Y. pestis* is more likely associated with macrophages, whereas those needle-inoculated are more likely associated with neutrophils. Although both neutrophils and macrophages seem to be important to restrict bacterial proliferation in the skin, they do not seem to have impact on bacterial trafficking to LN. As bacteria have been found not cell-associated inside lymphatic vessels and inside systemic organs very shortly after inoculation, a possible phagocyte-independent way to reach the LN and subsequent dissemination have been suggested. The role of plasmid pCD1-encoded factors in altering anti-inflammatory cytokine production by phagocytes is important to suppress the activation of immune cells and therefore for *Y. pestis* virulence.

All these data contribute to a better understanding of the early events during *Y. pestis*-host cells interactions. They also emphasize the importance of using a biologically relevant route of infection that mimics better what occurs in a natural infection, when studying the pathogenesis of *Y. pestis*. Advances in this field could potentially result in strategies to control or prevent the plague bubonic plague.

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Compliance with Ethics Guidelines

Conflict of Interest Luary C. Martínez-Chavarría declares no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Papers of particular interest, published recently, have been highlighted as:
- • Of importance
- •• Of major importance

1. Perry RD, Fetherston JD. *Yersinia pestis*: etiologic agent of plague. Clin Microbiol Rev. 1997;10:35–66.
2. Bertherat E, Bekhoucha S, Chougrani S, Razik F, Duchemin JB, Houti L, et al. Plague reappearance in Algeria after 50 years, 2003. Emerg Infect Dis. 2007;13:1459–62.
3. Prentice MB, Rahalison L. Plague. Lancet. 2007;369:1196–207.
4. Andrianarisoa AC, Rakotoson J, Randretsa M, Rakotondravelo S, Rakotoarimanana RD, Rakotomizao J, et al. Madagascar: public health situation on the "Big Island" at the beginning of the 21st century. Med Trop. 2007;67:19–29.
5. Stenseth NC, Atshabar BB, Begon M, Belmain SR, Bertherat E, Carniel E, et al. Plague: past, present, and future. PLoS Med. 2008;5(1):e3.
6. Alvarez ML, Cardineau GA. Prevention of bubonic and pneumonic plague using plant-derived vaccines. Biotechnol Adv. 2010;28(1):184–96.
7. CDC Centers for Disease Control and Prevention. Final 1994 reports of notifiable diseases. Morbid Mortal Weekly Rep. 1995;44:537–43.
8. World Health Organization. Plague. Wkly Epidemiol Rec. 2005;80:138–40.
9. Achtman M, Zurth K, Morelli G, Torrea G, Guiyoule A, Carniel E. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. Proc Natl Acad Sci U S A. 1999;96(24):14043–8.
10. Rollins SE, Rollins SM, Ryan ET. *Yersinia pestis* and the plague. Am J Clin Pathol. 2003;119(Suppl):S78–85.
11. Hinnebusch BJ. The evolution of flea-borne transmission in *Yersinia pestis*. Curr Issues Mol Biol. 2005;7(2):197–212.
12. Hinnebusch B, Perry R, Schwan T. Role of the *Yersinia pestis* hemin storage (*hms*) locus in the transmission of plague by fleas. Science. 1996;273:367–70.
13. Engelthaler DM, Hinnebusch BJ, Rittner CM, Gage KL. Quantitative competitive PCR as a technique for exploring flea-*Yersinia pestis* dynamics. Am J Trop Med Hyg. 2000;62(5):552–60.
14. Perry RD. A plague of fleas-survival and transmission of *Yersinia pestis*. ASM News. 2003;69(7):336–40.
15. Hinnebusch BJ, Erickson DL. *Yersinia pestis* biofilm in the flea vector and its role in the transmission of plague. Curr Top Microbiol Immunol. 2008;322:229–48.
16. Eisen RJ, Gage KL. Adaptive strategies of *Yersinia pestis* to persist during inter-epizootic and epizootic periods. Vet Res. 2009;40:01.
17. Burroughs AL. Sylvatic plague studies. The vector efficiency of nine species of fleas compared with *Xenopsylla cheopis*. J Hygiene. 1947;45:371–96.
18. Sebbane F, Gardner D, Long D, Gowen BB, Hinnebusch BJ. Kinetics of disease progression and host response in a rat model of bubonic plague. Am J Pathol. 2005;166(5):1427–39.
19. Hull HF, Montes JM, Mann JM. Septicemic plague in New Mexico. J Infect Dis. 1987;155:113–8.
20. Inglesby TV, Dennis DT, Henderson DA, et al. Plague as a biological weapon: medical and public health management. Working group on civilian biodefense. JAMA. 2000;283:2281–90.
21. Sebbane F, Jarrett CO, Gardner D, Long D, Hinnebusch BJ. Role of the *Yersinia pestis* plasminogen activator in the incidence of distinct septicemic and bubonic forms of flea-borne plague. Proc Natl Acad Sci U S A. 2006;103:5526–30.
22.•• Shannon JG, Bosio CF, Hinnebusch BJ. Dermal neutrophil, macrophage and dendritic cell responses to *Yersinia pestis* transmitted by fleas. PLoS Pathog. 2015;11(3):e1004734. **This paper analyzes the host response to *Y. pestis* in the skin after transmission by its natural vector, the flea rat *X. cheopis*.**
23. Oyston PC, Williamson ED. Modern advances against plague. Adv Appl Microbiol. 2012;81:209–41.
24. Ratsitorahina M, Chanteau S, Rahalison L, Ratsifasoamanana L, Boisier P. Epidemiological and diagnostic aspects of the outbreak of pneumonic plague in Madagascar. Lancet. 2000;355:111–13.
25. Zhou D, Han Y, Yang R. Molecular and physiological insights into plague transmission, virulence and etiology. Microbes Infect. 2006;8:273–84.
26. Cornelis GR, Wolf-Watz H. The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. Mol Microbiol. 1997;23(5):861–7.
27. Vadyvaloo V, Jarrett C, Sturdevant DE, Sebbane F, Hinnebusch BJ. Transit through the flea vector induces a pretransmission innate immunity resistance phenotype in *Yersinia pestis*. PLoS Pathog. 2010;6(2):e10000783.
28. Marketon MM, DePaolo RW, DeBord KL, Jabri B, Schneewind O. Plague bacteria target immune cells during infection. Science. 2005;309:1739e41.
29. Cornelis GR. *Yersinia* type III secretion: send in the effectors. J Cell Biol. 2002;158:401–8.
30. Spinner JL, Cundiff JA, Kobayashi SD. *Yersinia pestis* type III secretion system-dependent inhibition of human polymorphonuclear leukocyte function. Infect Immun. 2008;76(8):3754–60.
31. Contag CH, Bachmann MH. Advances in in vivo bioluminescence imaging of gene expression. Annu Rev Biomed Eng. 2002;4:235–60.
32. Warawa JM, Lawrenz MB. Bioluminescent imaging of bacteria during mouse infection. Methods Mol Biol. 2014;1098:169–81.
33.• Nham T, Filali S, Danne C, Derbise A, Carniel E. Imaging of bubonic plague dynamics by in vivo tracking of bioluminescent *Yersinia pestis*. PLoS One. 2012;7(4):e34714. **This work used in vivo bioluminescence imaging to follow *Y. pestis* dissemination during bubonic plague.**
34.• Gonzalez RJ, Weening EH, Frothingham R, Sempowski GD, Miller VL. Bioluminescence imaging to track bacterial dissemination of *Yersinia pestis* using different routes of infection in mice. BMC Microbiol. 2012;12:147. **This work demonstrates the usefulness of BLI for monitoring dissemination of *Y. pestis* from multiple inoculation sites.**
35. Sha J, Rosenzweig JA, Kirtley ML, van Lier CJ, Fitts EC, Kozlova EV, et al. A non-invasive in vivo imaging system to study dissemination of bioluminescent *Yersinia pestis* CO92 in a mouse model of pneumonic plague. Microb Pathog. 2013;55:39–50.

36. Ettinger A, Wittmann T. Fluorescence live cell imaging. Methods Cell Biol. 2014;123:77–94.
37.•• Gonzalez RJ, Lane MC, Wagner NJ, Weening EH, Miller VL. Dissemination of a highly virulent pathogen: tracking the early events that define infection. PLoS Pathog. 2015a;11(1):e1004587. **This work suggests that cells of the innate immune response are not required for *Y. pestis* to move into lymph nodes.**
38.•• Gonzalez RJ, Weening EH, Lane MC, Miller VL. Comparison of models for bubonic plague reveals unique pathogen adaptations to the dermis. Infect Immun. 2015b;83(7):2855–61. **This work reveals adaptations of *Y. pestis* to the dermis and emphasizes the importance of using ID route of infection when addressing *Y. pestis*-host interactions.**
39.•• Shannon JG, Hasenkrug AM, Dorward DW, Nair V, Carmody AB, Hinnebusch BJ. *Yersinia pestis* subverts the dermal neutrophil response in a mouse model of bubonic plague. mBio. 2013;48(5): e00170–13. **This work shows the role of neutrophils and pCD1 plasmid in the very early response to *Y. pestis*.**
40. Guinet F, AvÉ P, Jones L, Huerre M, Carniel E. Defective innate cell response and lymph node infiltration specify *Yersinia pestis* infection. PLoS One. 2008;3:e1688.
41. Chong SZ, Evrard M, Ng LG. Lights, camera, and action: vertebrate skin sets the stage for immune cell interaction with arthropod-vectored pathogens. Front Immunol. 2013;4:286.
42.• Bosio CF, Jarrett CO, Gardner D, Hinnebusch BJ. Kinetics of innate immune response to *Yersinia pestis* after intradermal infection in a mouse model. Infect Immun. 2012;80:4034e45. **This work describes the dynamics of the inflammatory response in the skin after *Y. pestis* infection.**
43. Andersen JF, Hinnebusch BJ, Lucas DA, Conrads TP, Veenstra TD, Pham VM, et al. An insight into the sialome of the oriental rat flea, *Xenopsylla cheopis* (Rots). BMC Genomics. 2007;8:102.
44. Frischknecht F. The skin as interface in the transmission of arthropod-borne pathogens. Cell Microbiol. 2007;9(7):1630–40.
45. Brubaker R. *Yersinia pestis* and bubonic plague. Prokaryotes. 2006;6:399–442.
46. O'Mahony S, Rose SL, Chilvers AJ, Ballinger JR, Solanki CK, Barber RW, et al. Finding an optimal method for imaging lymphatic vessels of the upper limb. Eur J Nucl Med Mol Imaging. 2004;31: 555–63.
47. Janssen WA, Surgalla MJ. Plague bacillus: survival within host phagocytes. Science. 1969;163:950–2.
48. Pujol C, Bliska JB. Turning *Yersinia* pathogenesis outside in: subversion of macrophage function by intracellular yersiniae. Clin Immunol. 2005;114(3):216–26.
49. Spinner JL, Carmody AB, Jarrett CO, Hinnebusch BJ. Role of *Yersinia pestis* toxin complex family proteins in resistance to phagocytosis by polymorphonuclear leukocytes. Infect Immun. 2013;81(11):4041–52.
50. Lukaszewski RA, Kenny DJ, Taylor R, Rees DG, Hartley MG, Oyston PC. Pathogenesis of *Yersinia pestis* infection in BALB/c mice: effects on host macrophages and neutrophils. Infect Immun. 2005;73:7142–50.
51. Spinner JL, Seo KS, O'Loughlin JL, Cundiff JA, Minnich SA, Bohach GA, et al. Neutrophils are resistant to *Yersinia* YopJ/P-induced apoptosis and are protected from ROS-mediated cell death by the type III secretion system. PLoS One. 2010;5: e9279.
52. Laws TR, Davey MS, Green C, Cooper IA, Titball RW, Lukaszewski RA. *Yersinia pseudotuberculosis* is resistant to killing by human neutrophils. Microbes Infect. 2011;13:607–11.
53. Kobayashi SD, Voyich JM, Buhl CL, Stahl RM, DeLeo FR. Global changes in gene expression by human polymorphonuclear leukocytes during receptor-mediated phagocytosis: cell fate is regulated at the level of gene expression. Proc Natl Acad Sci U S A. 2002;99: 6901–6.
54. Kobayashi SD, Braughton KR, Whitney AR, Voyich JM, Schwan TG, Musser JM, et al. Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. Proc Natl Acad Sci U S A. 2003;100:10948–53.
55. Bratton DL, Henson PM. Neutrophil clearance: when the party is over, clean-up begins. Trends Immunol. 2011;32:350–7.
56. Van Vre EA, Ait-Oufella H, Tedgui A, Mallat Z. Apoptotic cell death and efferocytosis in atherosclerosis. Arterioscler Thromb Vasc Biol. 2012;32:887–93.
57. Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. Nat Rev Immunol. 2007;7:964–74.
58. DeCathelineau AM, Henson PM. The final step in programmed cell death: phagocytes carry apoptotic cells to the grave. Essays Biochem. 2003;39:105–17.
59. Spinner JL, Winfree S, Starr T, Shannon JG, Nair V, Steele-Mortimer O, et al. *Yersinia pestis* survival and replication within human neutrophil phagosomes and uptake of infected neutrophils by macrophages. J Leukoc Biol. 2014;95(3):389–98.
60.•• Spinner JL, Hasenkrug AM, Shannon JG, Kobayashi SD, Hinnebusch BJ. Role of the *Yersinia* YopJ protein in suppressing interleukin-8 secretion by human polymorphonuclear leukocytes. Microbes Infect. 2015;pii: S1286-4579(15)00181-1. **This work describes the contribution of effector Yops in alteration of cytokine production by neutrophils.**
61. Jordan M, Otterness IG, Ng R, Gessner A, Rollinghoff M, Beuscher HU. Neutralization of endogenous IL-6 suppresses induction of IL-1 receptor antagonist. J Immunol. 1995;154:4081–90.
62. Schroder AK, von der Ohe M, Kolling U, Altstaedt J, Uciechowski P, Fleischer D, et al. Polymorphonuclear leucocytes selectively produce anti-inflammatory interleukin-1 receptor antagonist and chemokines, but fail to produce pro-inflammatory mediators. Immunology. 2006;119:317–27.
63. Rossol M, Heine H, Meusch U, Quandt D, Klein C, Sweet MJ, et al. LPS-induced cytokine production in human monocytes and macrophages. Crit Rev Immunol. 2011;31:379–446.
64. Platt AM, Randolph GJ. Dendritic cell migration through the lymphatic vasculature to lymph nodes. Adv Immunol. 2013;120:51–68.