Deadly plague versus mild-mannered TLR4

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Yersinia pestis **evades lipopolysaccharide (LPS)-induced inflammation and establishes deadly infection mediated by myriad virulence factors. Virulence can be completely neutralized, however, if *Y. pestis* expresses a Toll-like receptor 4–stimulating LPS.**

Plague is one of the deadliest diseases in human history. It decimated the population of Europe and North Africa, killing over 200 million people in two pandemics (Justinian's plague of 541–767 and Europe's Black Death of 1347–1350). The plague bacillus *Yersinia pestis*, transmitted to humans by fleas, has several potent virulence factors and often kills a human host before a protective immune response can be mounted. In this issue of *Nature Immunology*, Lien and colleagues describe a mechanism by which *Y. pestis* evades innate immunity during early stages of infection, which allows the bacillus to mount its deadly attack[1]. In a mammalian host, *Y. pestis* produces tetra-acylated lipopolysaccharide (LPS), which is antagonistic to Toll-like receptor 4 (TLR4), the main innate pattern recognition receptor that detects Gram-negative bacteria. Nonstimulatory tetra-acylated LPS prevents induction of the initial TLR4-mediated inflammatory response. When Lien and colleagues modified *Y. pestis* to express stimulatory hexa-acylated LPS, not only were the bacteria completely avirulent but they also were able to induce protective immunity against fully virulent, wild-type *Y. pestis*.

Plague is predominantly a zoonosis. In the wild, the reservoir of *Y. pestis* is maintained in rodents, such as ground squirrels and prairie dogs, and is transmitted by fleas (sylvatic plague) (**Fig. 1**). Fleas can also transmit *Y. pestis* to urban rodents, such as rats (urban plague), and to humans. Transmission to humans by fleabites results in bubonic plague, named after the early manifestation of painful swollen lymph nodes, called 'buboes'. The disease then progresses to a systemic infection and often death. If *Y. pestis* reaches the lungs, purulent pneumonia (pneumonic plague) develops, which is rapidly fatal if untreated, and which can be easily transmitted to other people, who develop pneumonic plague without the initial bubonic plague. Although now rare (about 1,000 cases per year world-wide), plague can potentially be spread rapidly in the form of pneumonic plague, which has made it one of the most feared agents of biowarfare and bioterrorism.

What makes the plague bacillus so deadly? The strategy of *Y. pestis* is to evade innate immunity and paralyze immune cells. Its type III secretion system (which is a syringe-and-needle–like structure mounted in the bacterial cell wall) injects toxic effector proteins, called Yops (for *Yersinia* outer proteins), into innate immune cells, mainly macrophages, dendritic cells and granulocytes[2,3]. YopE, YopT and YopO prevent phagocytosis by targeting Rho GTPases and destroying the cytoskeleton[2,4]. YopE also inhibits caspase-1–mediated maturation and release of interleukin (IL)-1β. YopH prevents phagocytosis and release of a chemokine, MCP-1, through its protein tyrosine phosphatase activity, which disrupts several signaling pathways[4]. YopJ shuts down MKK6 and NF-κB signaling and cytokine production, and induces apoptosis of macrophages through the caspase cascade[2,4,5]. YopM interacts with protein kinase C–like 2 and ribosomal protein S6 kinase, which are also involved in proinflammatory signaling[2,4]. These assaults on the immune cells effectively block phagocytosis and induction of proinflammatory cytokines. Another Yop, LcrV, which is the anchoring unit on the tip of the type III secretion needle[6], is also secreted and induces anti-inflammatory IL-10 through TLR2 (refs. 2,4). And there are other virulence factors, such as plasminogen activator, murine toxin, and capsular and fimbrial proteins.

If that was not enough, *Y. pestis* has one more trick up its sleeve: Lien and colleagues find that *Y. pestis* evades detection by TLR4. TLR4, a prototypical pattern-recognition receptor for LPS from the outer membrane of Gram-negative bacteria[7], triggers induction of proinflammatory cytokines such as tumor necrosis factor, IL-1, IL-6 and IL-8 (ref. 8). TLR4-deficient mice do not respond to LPS and are resistant to endotoxin shock[8]. But how important is TLR4 (and the nine other human TLRs) in resistance to infections? For one thing, high conservation of TLRs in vertebrates suggests that they are important. For another, mutations in TLR4 and the elements of its signal-transduction pathways are associated with increased sensitivity to infections[9]. Also, TLR4-deficient mice are more susceptible to infections with several Gram-negative bacteria[8]. However, some studies have found no association of TLR4 polymorphism with sensitivity to infections in humans, and other studies failed to show higher sensitivity of TLR4-deficient mice to Gram-negative bacteria. Thus, despite extensive work on TLR4-induced signal transduction, its importance in resistance to infections is less certain.

Y. pestis is a typical Gram-negative bacterium, with an outer membrane LPS that, at first blush, would seem likely to activate TLR4, which is normally stimulated by hexa-acylated LPS found in *Escherichia coli*, *Salmonellae* and other *Enterobacteriaceae*.

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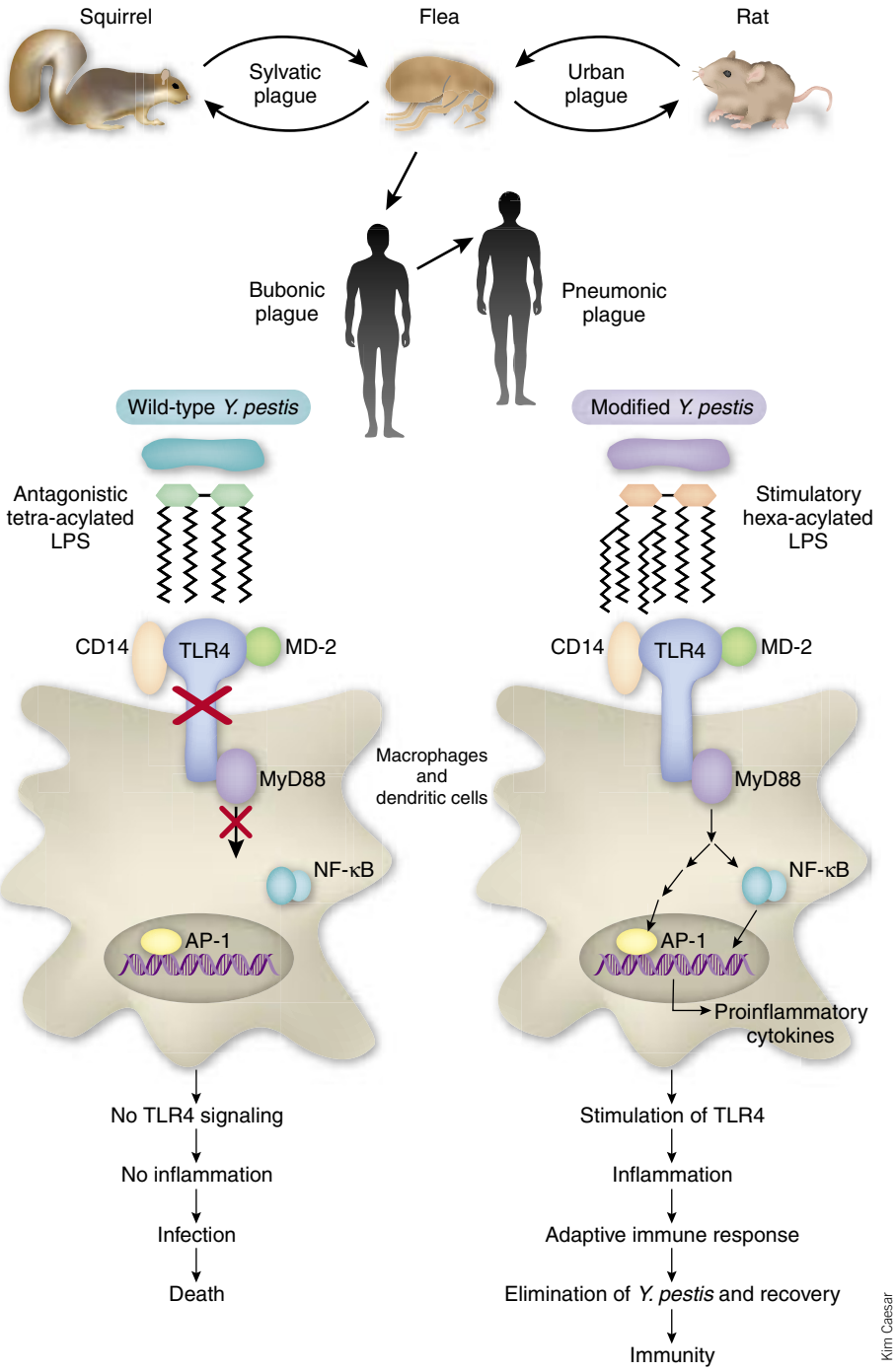

Figure 1 *Y. pestis* life cycle, inhibition of TLR4 activation by wild-type *Y. pestis*, and activation of TLR4 by genetically modified *Y. pestis*. Wild or urban rodents maintain a reservoir of *Y. pestis* that is transmitted by fleas (sylvatic plague and urban plague). Transmission by fleabites to humans results in bubonic plague, whereas person-to-person transmission by aerosol causes pneumonic plague (top). Wild-type *Y. pestis* at 37 °C produces tetra-acylated LPS, which inhibits TLR4 activation and allows *Y. pestis* to evade protective inflammatory response and establish often fatal infection (lower left). Genetically modified *Y. pestis* expressing stimulatory hexa-acylated LPS activates TLR4 and induces inflammation and adaptive immune response, which eliminate *Y. pestis* and result in recovery and immunity to reinfection with wild-type *Y. pestis* (lower right).

When grown at 21–26 °C (in the flea, for example), *Y. pestis* produces a typical hexa-acylated LPS of this sort; however, at 37 °C (in mammalian hosts), *Y. pestis* produces tetra-acylated LPS that does not stimulate TLR4 (ref. 10). Lien and colleagues show that not only is tetra-acylated LPS nonstimulatory for TLR4, but it also acts as an antagonist for the stimulatory hexa-acylated form of LPS. Thus, immediately after transmission from the flea into the mammalian host, *Y. pestis* begins to produce the antagonistic tetra-acylated LPS, which inhibits TLR4-mediated activation of the host cells by any hexa-acylated LPS still present in the transmitted bacteria. This strategy prevents activation of macrophages and secretion of proinflammatory cytokines, and also prevents activation and maturation of dendritic cells, which are needed for induction of adaptive immunity (**Fig. 1**).

To test the *in vivo* role of this switch from stimulatory hexa-acylated LPS to inhibitory tetra-acylated LPS in *Y. pestis* infection, Lien and colleagues cloned the *E. coli* gene responsible for the synthesis of the stimulatory hexa-acylated LPS into *Y. pestis*. They found that the chimeric *Y. pestis* expressed stimulatory hexa-acylated LPS at 37 °C, instead of the inhibitory tetra-acylated LPS (**Fig. 1**). That single change in *Y. pestis* was sufficient to cause complete loss of virulence in subcutaneously infected mice, which are a model of bubonic plague. The surprising finding is that virulence was lost despite the presence of all other *Y. pestis* virulence factors: although 1,000 wild-type *Y. pestis* bacteria produced 100% mortality in wild-type mice, challenges with as many as 10^7 of the chimeric *Y. pestis* bacteria with the stimulatory hexa-acylated LPS produced no mortality. Notably, the difference in virulence between the two *Y. pestis* strains was not seen in TLR4-deficient mice, which were equally sensitive to *Y. pestis* expressing tetra-acylated or hexa-acylated LPS. These results demonstrate that the protection induced by hexa-acylated LPS is mediated by TLR4. Moreover, the sites of infection with the chimeric *Y. pestis* strain showed infiltrates of inflammatory cells, confirming the proinflammatory capacity of hexa-acylated LPS-expressing bacteria *in vivo*. The requirement for TLR4 signaling was further confirmed by results showing a requirement for MD-2, a necessary component of the TLR4 receptor complex, and for MyD88, the main adaptor that transduces the TLR4 signal.

These results unequivocally show that tetra-acylated LPS is a potent virulence factor of *Y. pestis* that facilitates the development of infection through evasion of LPS detection by TLR4, thus preventing the induction of an early proinflammatory response (**Fig. 1**). The results also clearly demonstrate the pivotal role of TLR4 in *in vivo* protection against infection. Together with other virulence factors, expression of nonstimulatory

LPS allows *Y. pestis* to reach high numbers in the initial stages of infection. This then allows the organism not only to spread and produce its devastating disease, but also to complete its life cycle, as large numbers of bacteria are needed for successful transmission from a mammalian host to fleas and then to the next host. Although humans are totally unresponsive to tetra-acylated (non-stimulatory) LPS, mice do show a modest response that is not sufficient for protection against infection. That difference may make mice more resistant to plague than are humans, thus allowing a reservoir of *Y. pestis* to be maintained in wild rodents.

Lien and colleagues further hypothesized that *Y. pestis* expressing hexa-acylated (stimulatory) LPS may serve as a vaccine against fully virulent bacteria. Mice that recovered from infection with the *Y. pestis* expressing hexa-acylated LPS were fully protected against subsequent subcutaneous (bubonic plague) or intranasal (pneumonic plague) challenge with fully virulent *Y. pestis*. The protection seen is presumably mediated by adaptive immunity, because T and B cell–deficient mice (*Rag1*$^{-/-}$) were protected only for a short time (9 d) against the challenge and eventually succumbed to infection. These results demonstrate the feasibility of the vaccine development approach by genetically engineering bacteria expressing TLR stimulants. Future research should focus on such vaccines not only against plague but also against other microorganisms, especially those that lack strong TLR stimulants.

Low-stimulatory LPS, although necessary, is obviously not sufficient for full virulence of *Y. pestis*, because other, less virulent *Yersinia* species (*Y. enterocolitica* and *Y. pseudotuberculosis*) also produce nonstimulatory LPS[10] as well as the type III secretory system with its many poisons[2,4]. Therefore, other virulence factors are also necessary for the deadly disease caused by *Y. pestis*[2,4]. The work of Lien and colleagues, however, brings us closer to a full understanding of the pathogenesis of plague. It shows that *Y. pestis* relies on evading innate immunity, in addition to paralyzing phagocytic and antigen-processing cells and inducing suppressive cytokines. Yet deadly plague can be prevented by a vaccine consisting of genetically engineered *Y. pestis* that produces a strong TLR4-dependent LPS response—a simple yet elegant defense against this scourge of humanity.

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Passera ou ne passera pas—accessibility is key

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Immunoglobulin and T cell receptor (TCR) germline transcription is associated with V(D)J recombination of these loci. New work formally demonstrates that transcriptional read-through of the TCR–J_α cluster indeed regulates TCR-J_α rearrangements.

The B and T lymphocytes, 'soldiers' of the immune system, exert their immune surveillance duty by means of the specific recognition of foreign pathogens through their highly polymorphic antigen receptors, the B and T cell receptors (BCR and TCR), respectively. The extensive heterogeneity of B and T cell repertoires is ensured by the somatic rearrangement of DNA encoding the various components—V (variable), D (diversity) and J (joining)—of immunoglobulins (Ig) and TCR variable domains by means of a specialized molecular mechanism: V(D)J recombination[1]. The recombination process is triggered in both B and T cells by unique enzymatic machinery that includes the recombinase-activating gene (RAG) factors 1 and 2. Complete Ig and TCR gene rearrangements are restricted to B and T cells, respectively, which argues for differential accessibility of the Ig and TCR chromosomal regions in B and T cells. This potential regulatory process represents the main component of the 'accessibility model' proposed by Yancopoulos and Alt in the mid-1980s[2]. For the last 20 years, we have lived with the formally unverified prediction that accessibility of Ig and TCR DNA loci could be a consequence of their germline transcription, a key feature of the accessibility model. In this issue of *Nature Immunology*, Abarrategui and Krangel demonstrate for the first time that transcriptional elongation over the TCR-J_α cluster constitutes a crucial prerequisite for TCR-J_α recombination[3]. This finding sheds new light on the mechanism of DNA accessibility; it also raises some new questions.

Ig and TCR molecules are encoded by seven different loci, whose structures are similar and have been conserved through evolution. Briefly, these loci are composed of multiple gene segments (V, D and J) scattered along the chromosomes (see **Fig. 1** for the structure of the TCRαδ loci) that are flanked by recombination-specific sequences (RSSs), which are the primary target of the site-specific V(D)J recombinase[1]. The lymphoid-restricted factors RAG1 and RAG2 bind to the RSSs and initiate the reaction, which consists of a DNA cut-and-paste mechanism that results in the assembly of VDJ functional exons. Although the various Ig and TCR genes are rearranged by the same RAG recombinase, the reaction is highly regulated spatially (B versus T cells), developmentally (TCRβ before TCRα, for example) and within individual cells, with only one allele being fully rearranged (a restriction known as 'allelic exclusion') in each cell except in the case of the TCRα

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