

Virulence factors of *Yersinia pestis* are overcome by a strong lipopolysaccharide response

Sara W Montminy¹, Naseema Khan¹, Sara McGrath², Mitchell J Walkowicz³, Fiona Sharp¹, Joseph E Conlon¹, Koichi Fukase⁴, Shoichi Kusumoto⁴, Charles Sweet¹, Kensuke Miyake⁵, Shizuo Akira⁶, Robert J Cotter², Jon D Goguen³ & Egil Lien¹

At mammalian body temperature, the plague bacillus *Yersinia pestis* synthesizes lipopolysaccharide (LPS)–lipid A with poor Toll-like receptor 4 (TLR4)–stimulating activity. To address the effect of weak TLR4 stimulation on virulence, we modified *Y. pestis* to produce a potent TLR4-stimulating LPS. Modified *Y. pestis* was completely avirulent after subcutaneous infection even at high challenge doses. Resistance to disease required TLR4, the adaptor protein MyD88 and coreceptor MD-2 and was considerably enhanced by CD14 and the adaptor Mal. Both innate and adaptive responses were required for sterilizing immunity against the modified strain, and convalescent mice were protected from both subcutaneous and respiratory challenge with wild-type *Y. pestis*. Despite the presence of other established immune evasion mechanisms, the modified *Y. pestis* was unable to cause systemic disease, demonstrating that the ability to evade the LPS-induced inflammatory response is critical for *Y. pestis* virulence. Evading TLR4 activation by lipid A alteration may contribute to the virulence of various Gram-negative bacteria.

Early recognition of invading bacteria by the innate immune system has a crucial function in antibacterial defense by triggering inflammatory responses that prevent the spread of infection and suppress bacterial growth. For many pathogens, early innate responses slow the progress of infection, which allows for adaptive immune responses to develop. Pathogens with exceptional ability to evade or suppress innate responses may cause the death of the host before adaptive responses become effective. This is demonstrated by the activity of the Gram-negative pathogen *Yersinia pestis*, the causative agent of plague. In nature, *Y. pestis* depends mainly on the bite of infected fleas for transmission between mammalian hosts and to cause bubonic plague¹. The development of bubonic plague in a suitable host requires that *Y. pestis* establish a systemic infection distinguished by very high bacteremia, exceeding 1×10^8 bacteria per ml of blood in some human plague patients, after inoculation of often only a few bacteria in the skin delivered by a flea bite. Early foci of infection contain large numbers of bacteria and are often entirely devoid of inflammatory cells^{2,3} despite the presence of multiple and redundant mechanisms that normally trigger innate immunity. To suppress local inflammation, *Y. pestis* uses several parallel mechanisms that individually are necessary for virulence, including a complex type III secretion system (T3SS), which delivers multiple effector proteins to the cytoplasm of host cells in contact with the bacteria, and the

expression of Pla, an outer membrane protease with plasminogen activator activity^{1,2,4}. Extracellular release of the T3SS-associated protein LcrV has also been suggested to contribute to immune suppression mediated by the pathogenic yersiniae³.

Toll-like receptors (TLRs) are central to the host innate immune response against invading microbes^{5,6}. To achieve the profound suppression of inflammation found *in vivo*, *Y. pestis* either must avoid inducing proinflammatory responses via the TLRs or must actively suppress the effects of such responses. A combination of those two approaches probably has the greatest potential to be effective. Several of the T3SS effectors have activities that could serve to blunt the effects of TLR stimulation, including derangement of intracellular signaling, induction of apoptosis and interference with phagocytosis^{1,2,4}. A key pathway for the induction of inflammation in response to Gram-negative bacteria is the activation of TLR4 signaling by lipopolysaccharide (LPS), a chief component of the Gram-negative outer membranes^{7–10}. Many types of LPS are extraordinarily potent stimulators of TLR4, activating immune cells at concentrations of picograms per milliliter. The immune-activating moiety of LPS is lipid A, a diglucosamine unit with covalently attached acyl chains that interacts with the host LPS receptor complex consisting of TLR4 and its coreceptor MD-2 to induce cellular responses^{7–12}. The glycosylphosphatidylinositol-anchored molecule CD14 binds LPS to the cell

¹Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA.

²Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA. ³Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655, USA. ⁴Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka 560-0043, Japan. ⁵Division of Infectious Genetics, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan. ⁶Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan. Correspondence should be addressed to E.L. (egil.lien@umassmed.edu).

Received 20 June; accepted 1 August; published online 17 September 2006; doi:10.1038/ni1386

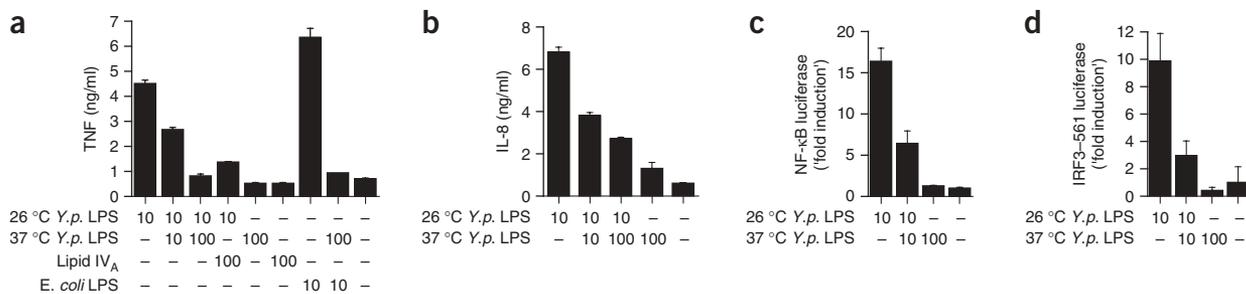


Figure 1 LPS from *Y. pestis* grown at 37 °C has inhibitory activity. **(a)** ELISA of TNF in supernatants from human PBMCs treated with LPS from *Y. pestis* grown at 37 °C (37 °C *Y.p.* LPS) or synthetic tetra-acyl lipid IV_A, followed immediately by incubation for 16 h with LPS from *Y. pestis* grown at 26 °C (26 °C *Y.p.* LPS) or LPS from *E. coli*. **(b)** ELISA of IL-8 in supernatants from HEK293 cells expressing human TLR4–MD-2 exposed to LPS from *Y. pestis* grown at 37 °C followed by incubation for 16 h with LPS from *Y. pestis* grown at 26 °C. **(c,d)** Luciferase activity of cells transfected with NF-κB–luciferase **(c)** or IRF3-dependent 561–luciferase **(d)** reporters treated with LPS from *Y. pestis* grown at 37 °C and/or 26 °C and then incubated for 18 h. Lipid concentrations (below graphs), ng/ml. Data are from one representative experiment of three (error bars, s.d.) and represent the mean of duplicates **(a)** or triplicates **(b–d)**.

surface, transfers LPS to TLR4–MD-2 and enhances cellular activation^{12,13}, whereas the TLR adaptors MyD88, Mal (also called Tirap), TRIF and TRAM initiate the intracellular signaling cascade after interaction with the Toll–interleukin 1 (IL-1) resistance domain of TLR4 (ref. 5).

Lipid A structure is conserved among Gram-negative bacteria, making it an archetypical example of a pathogen-associated molecular pattern; however, it is not invariant. For example, the number, location and composition of acyl chains varies among species, is influenced by the environment and is often heterogeneous even in a single species^{10,14,15}. Based on *in vitro* observations, it has been hypothesized that the production of a weakly stimulatory LPS could be involved in the virulence of several Gram-negative pathogens¹⁵. Hexa-acylated lipid A structures are found in *Y. pestis* grown at 21 °C to 27 °C (flea temperatures), but these are mainly tetra-acylated at 37 °C (host temperature)^{16–18}. Although hexa-acylated LPS–lipid A is normally a strong activator of human cells, tetra-acylated lipids have lower stimulatory activity^{8,16,17,19,20}. It has been suggested that the temperature-dependent remodeling of lipid A structure could be necessary for *Y. pestis* to achieve the high bacterial load in mammalian blood required for efficient flea infection before the induction of lethal shock. It is also possible that *Y. pestis* may need to minimize TLR4 stimulation early in infection to prevent containment by local inflammation.

Y. pestis provides an excellent model for testing the idea that LPS modification contributes to Gram-negative virulence through evasion of TLR4 signaling. Here we have studied a genetically modified strain of *Y. pestis* expressing a potent TLR4-activating hexa-acylated LPS at 37 °C. This strain was avirulent in wild-type mice, despite the presence of other well established virulence determinants. Hence, both active immune suppression and stealth are critical for evasion of antibacterial host responses during plague. Our results emphasize the essential function of TLR4-mediated innate immunity in protection against life-threatening infections and have broad implications for understanding bacterial virulence in *Y. pestis* and other organisms.

RESULTS

LPS from *Y. pestis* grown at 37 °C inhibits TLR4 activation

In contrast to bacteria grown at 26 °C, *Y. pestis* grown at 37 °C poorly activated TLR4 signaling (**Supplementary Methods and Supplementary Fig. 1** online), supporting the idea that LPS from *Y. pestis* grown at 37 °C has low stimulatory potential^{16,17}. Because tetra-acylated LPS–lipid A may antagonize activity induced by potent endotoxin^{8,19,20}, we also analyzed the anti-inflammatory function of LPS

from *Y. pestis* grown at 37 °C. LPS purified from the *Y. pestis* KIM5 strain grown at 26 °C but not that grown at 37 °C activated human peripheral blood mononuclear cells (PBMCs; **Fig. 1a**) and HEK293 cells expressing human TLR4 and MD-2 (**Fig. 1b**). Furthermore, activation induced by LPS from *Y. pestis* grown at 26 °C and LPS from *Escherichia coli* was inhibited by LPS from *Y. pestis* grown at 37 °C and by the synthetic tetra-acylated lipid IV_A in human PBMCs (**Fig. 1a**). Stimulation of HEK293 cells expressing human TLR4 and MD-2, as assessed by release of IL-8 (**Fig. 1b**) and activation of reporters dependent on transcription factors NF-κB and IRF3 (**Fig. 1c,d**), by LPS from *Y. pestis* grown at 26 °C was also inhibited by LPS from *Y. pestis* grown at 37 °C. Two central branches of TLR4 signaling result in activation of NF-κB and IRF-3, transcription factors necessary for the induction of proinflammatory cytokines and type I interferons, respectively^{5,6}. Those observations suggested that LPS from *Y. pestis* grown at 37 °C has a general defect in activation of TLR4 signaling, possibly reflecting a failure to form oligomers with the receptor complex¹². We also noted repression of cytokine release in PBMCs from cynomolgus macaques (*Macaca fascicularis*; **Supplementary Fig. 2** online), a model organism for studies of plague in primates. Bacteria may well contain a mixture of stimulatory and nonstimulatory LPS species, especially during transition between flea and host temperatures, with the antagonistic activity of tetra-acylated LPS ‘blunting’ the induction of primate immune signaling by the active LPS species.

Y. pestis expressing acyltransferase LpxL makes potent LPS

Mass spectroscopy^{16–18} (**Supplementary Fig. 3** online) demonstrated mainly tetra-acylated lipid A in *Y. pestis* grown at 37 °C, with hexa-acylated structures present only at lower temperatures (**Fig. 2a,b**). LpxL (also called HtrB) and LpxM (also called MsbB) are ‘late’ acyltransferases in *E. coli* and several other Gram-negative bacteria and are required for addition of the secondary acyl chains to the tetra-acylated precursor lipid IV_A (ref. 10). LpxP, a third late *E. coli* acyltransferase, is active at low temperatures^{10,21,22}. Inspection of published sequences for the *Y. pestis* genome^{23–25} has identified only genes encoding two late acyltransferases, LpxP and LpxM, suggesting that the lack of LpxL in combination with the temperature sensitivity of LpxP is responsible for the absence of hexa-acylated lipid A at 37 °C.

We cloned *lpxL* from the K12 strain of *E. coli* and expressed the gene in *Y. pestis* KIM5 under control of its own promoter on the vector pBR322 (‘pLpxL’), generating the strain ‘*Y. pestis* KIM5-pLpxL’. Mass spectroscopy (**Supplementary Fig. 3**) showed that this strain

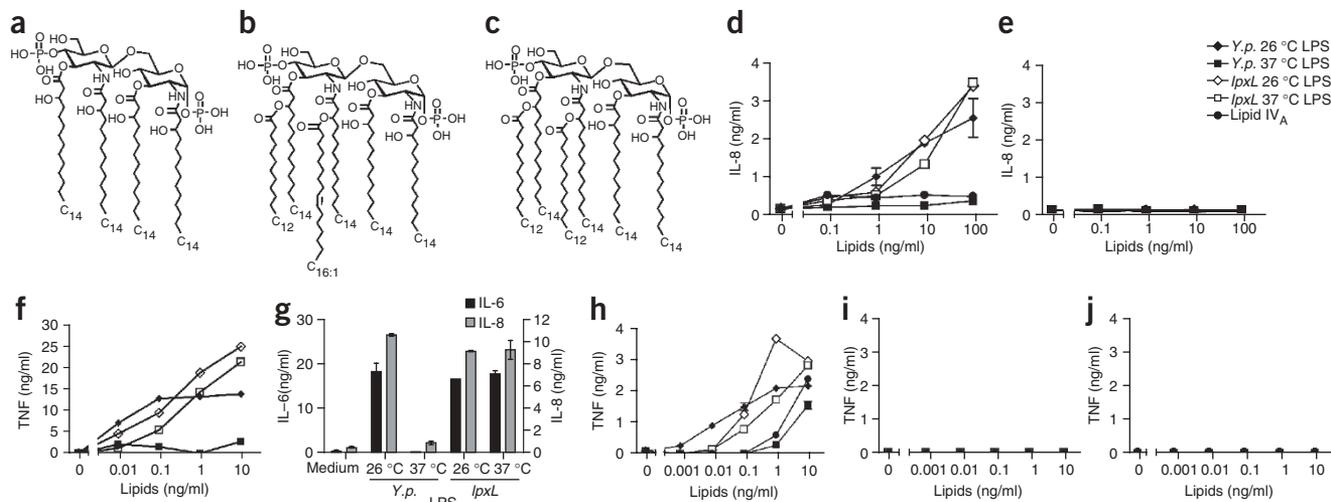


Figure 2 *Y. pestis* pLpxL synthesizes a potent LPS. (a–c) Main lipid A structures found after isolation of lipid A from *Y. pestis* KIM5 grown in liquid culture at 37 °C (a) or 26 °C (b) or in *Y. pestis* KIM5-pLpxL grown at each temperature (c). These were determined by MALDI-TOF mass spectrometry; a mixture of different compounds may be present at a given temperature (Supplementary Fig. 3). (d,e) ELISA of IL-8 in supernatants of HEK293 cells expressing human TLR4–MD-2 (d) or human TLR2 (e) stimulated for 18 h with LPS from *Y. pestis* KIM5 grown at 26 °C (*Y.p.* 26 °C LPS) or 37 °C (*Y.p.* 37 °C LPS); *Y. pestis* KIM5-pLpxL grown at 26 °C (*lpxL* 26 °C LPS) or 37 °C (*lpxL* 37 °C LPS); or synthetic lipid IV_A (Lipid IV_A). (f–g) ELISA of TNF (f) or IL-6 and IL-8 (g) in supernatants of human PBMCs isolated from a healthy donor and exposed to LPS for 16 h as described in d,e. Similar results were obtained with cells from nine people. (h–j) ELISA of TNF in supernatants of peritoneal macrophages isolated from wild-type mice (h), TLR4-knockout mice (i) and MD-2-knockout mice (j) and then stimulated for 16 h with various forms of *Y. pestis* LPS and lipid IV_A as described in d,e. Data are from one representative experiment of three to nine (error bars, s.d.) and represent the mean of triplicates (d,e) or duplicates (f–j).

contained previously unknown hexa-acylated structures at both 37 °C and 26 °C (Fig. 2c), indicating that, as in *E. coli*, LpxL mediates the addition of one 2' secondary C_{12:0} acyl chain to lipid IV_A. Presumably, a 3' secondary C_{12:0} acyl moiety is added by the endogenous LpxM at each temperature, suggesting that the function of the LpxM enzyme is temperature independent in *Y. pestis*. In contrast to the results obtained with KIM5 (Figs. 1 and 2d), LPS from *Y. pestis* KIM5-pLpxL grown at either 37 °C or 26 °C strongly activated HEK293 cells expressing human TLR4 and MD-2 (Fig. 2d) but not those expressing TLR2 (Fig. 2e). We obtained similar results with human PBMCs: only the LPS from *Y. pestis* KIM5 grown at 37 °C showed profoundly reduced induction of release of tumor necrosis factor (TNF), IL-6 and IL-8 (Fig. 2f,g). The responses of human PBMCs from several healthy volunteers ($n = 9$) followed a similar pattern (data not shown), as did PBMCs from cynomolgus macaques (Supplementary Fig. 4 online), indicating that primates have very limited ability to respond to native LPS from *Y. pestis* grown at 37 °C. Rodent cells have a higher capacity to respond to tetra-acylated lipid A than do human cells, a phenomenon attributed to species differences in TLR4–MD-2 (refs. 8,26–28). Indeed, mouse macrophages released small amounts of TNF in a TLR4- and MD-2-dependent way when exposed to the LPS from *Y. pestis* KIM5 grown at 37 °C, but this response was much weaker than that obtained with LPS from *Y. pestis* KIM5-pLpxL grown at 37 °C from or with LPS from either strain grown at 26 °C (Fig. 2h–j). In summary, a considerable deficit in response to LPS from *Y. pestis* grown at 37 °C was common to cells from all species tested, and the expression of *E. coli* LpxL in *Y. pestis* resulted in the production of a hexa-acylated LPS at 37 °C, with considerable ability to activate TLR4 signaling.

Y. pestis with potent LPS cannot produce bubonic plague

Mammals have multiple surveillance pathways that can trigger anti-bacterial responses. However, *Y. pestis* has also sophisticated active

mechanisms for suppressing these responses, including a T3SS that 'intoxicates' host cells in contact with the bacteria^{1,4}. To determine the importance of LPS stimulatory activity in this complex environment, we did infection experiments in mice. Many rodents are natural hosts for *Y. pestis*¹, and mice are a well established model for the study of plague. Fully virulent *Y. pestis* (the KIM1001 strain²) contains all known virulence factors; thus, we were able to evaluate the effect of potent LPS in the context of all those other activities. *Y. pestis* KIM1001 produced 100% mortality in mice after subcutaneous injection (a model in which the resulting disease is similar to bubonic plague) of 1×10^3 colony-forming units (CFU; Fig. 3a), whereas infection with 1×10^2 CFU resulted in 80% mortality. In contrast, the same doses of *Y. pestis* KIM1001 expressing pLpxL ('*Y. pestis* KIM1001-pLpxL') did not cause disease (Fig. 3a). The effect of LpxL was profound: at doses of up to 1×10^7 CFU, which is approximately 1×10^6 mean lethal doses for the KIM1001 parent strain, we detected no mortality or apparent signs of illness (Table 1). There was limited swelling at the infection site, particularly at the higher dose of *Y. pestis* KIM1001-pLpxL. The phenotype of *Y. pestis* KIM1001-pLpxL was not confined to the C57BL/6 mouse strain, as four of four 129Sv mice died when infected with 1×10^3 CFU *Y. pestis* KIM1001, whereas four of four 129Sv mice survived infection with *Y. pestis* KIM1001-pLpxL (data not shown). Control experiments indicated that other *Y. pestis* virulence determinants were not affected by pLpxL (Supplementary Note online). The presence of pLpxL had a minimal effect on bacterial growth rate (Supplementary Fig. 5 online), and the T3SS remained fully functional, as exemplified by retention of calcium-dependent growth at 37 °C (data not shown), retention of T3SS-dependent cytotoxicity toward HeLa cells (data not shown) and normal secretion of the central T3SS virulence factor YopM (Supplementary Fig. 5). Furthermore, resistance to serum-dependent bacterial killing was preserved (data not shown), the activity of the Pla protease remained normal (Supplementary Fig. 5) and the presence of the

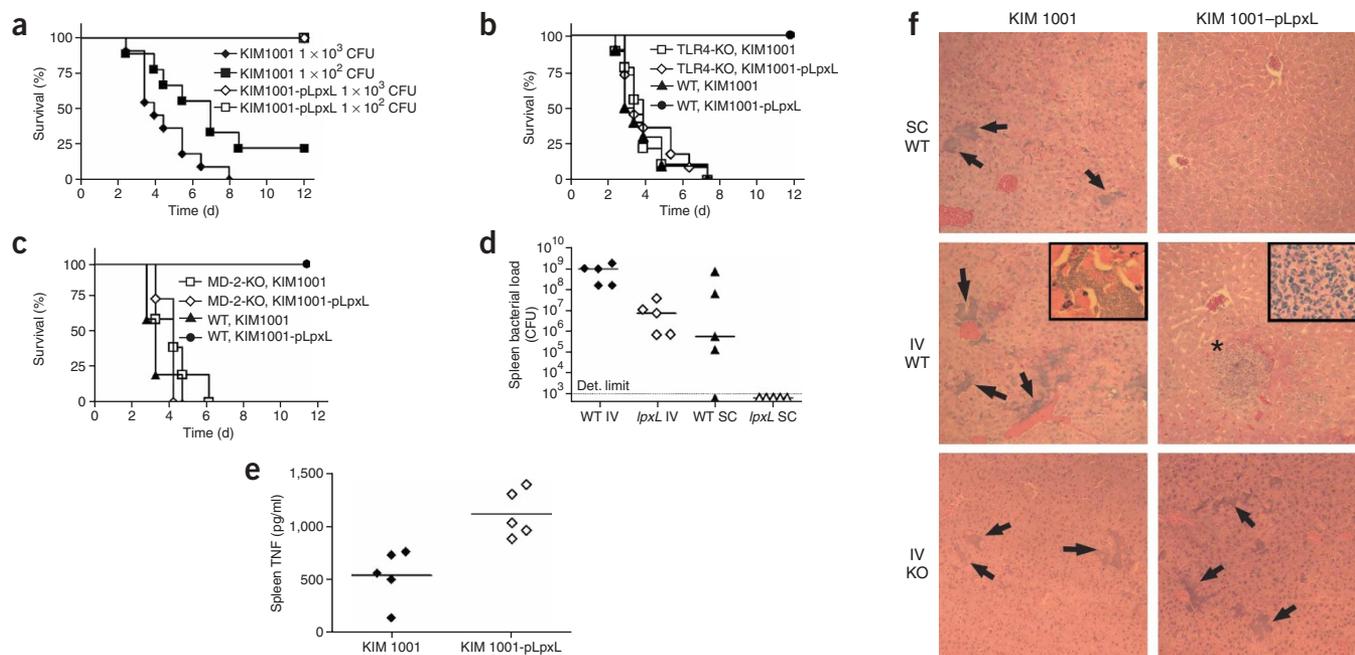


Figure 3 *Y. pestis* pLpxL is avirulent in wild-type mice. (a–c) Lethality of infection with *Y. pestis* strains. (a) Survival of wild-type mice ($n = 10$ mice per infection group) infected subcutaneously with *Y. pestis* KIM1001 or KIM1001-pLpxL at doses of 1×10^3 CFU or 1×10^2 CFU. $P < 0.0001$, KIM1001 versus KIM1001-pLpxL at 1×10^3 CFU. (b) Survival of wild-type mice (WT) and TLR4-knockout mice (TLR4-KO; $n = 10$ mice per infection group) infected subcutaneously with *Y. pestis* KIM1001 or KIM1001-pLpxL at a dose of 1×10^3 CFU. $P < 0.0001$, wild-type versus TLR4-knockout, for KIM1001-pLpxL. (c) Survival of wild-type mice (WT) and MD-2-knockout mice (MD-2-KO; $n = 8$ mice per infection group) infected subcutaneously with *Y. pestis* KIM1001 or KIM1001-pLpxL at a dose of 1×10^3 CFU. $P < 0.0001$, MD-2-knockout versus wild-type, for KIM1001-pLpxL. (d) CFU in spleen homogenates from wild-type mice infected intravenously (IV) or subcutaneously (SC) with wild-type *Y. pestis* KIM1001 (WT) or KIM1001-pLpxL (*lpxL*). Det. limit, detection limit. (e) ELISA of TNF in spleen homogenates from intravenously infected mice from d. $P = 0.008$. Small horizontal lines indicate median (d) or mean (e). (f) Immunohistology of liver sections from wild-type mice (WT) or TLR4-knockout mice (KO) infected subcutaneously or intravenously with 1×10^3 CFU of *Y. pestis* KIM1001 or KIM1001-pLpxL, stained with hematoxylin and eosin. Arrows indicate bacteria-containing lesions; *, microabscess containing infiltrating inflammatory cells. Insets, magnification of bacterial mass (KIM1001) or microabscess (KIM1001-pLpxL). Original magnification, $\times 100$ (main images) or $\times 1,000$ (insets). Data are representative of six (a,b) or three (c–f) experiments.

plasmid vector by itself had no effect on virulence (Supplementary Fig. 6 online). Although the rodent TLR4–MD-2 system has a limited but notable response to tetra-acylated LPS–lipid A^{8,26–28} (Fig. 2h), the resulting innate immune signaling was apparently insufficient to protect infected mice. From these data, we hypothesized that the development of systemic disease after infection at peripheral sites, as occurs in bubonic plague, is dependent on the production of LPS with reduced TLR4–MD-2 stimulating activity.

Resistance to *Y. pestis* pLpxL depends on TLR4 signaling

A critical experiment to test the hypothesis mentioned above was verification that resistance to *Y. pestis* KIM1001-pLpxL depends on functional TLR4 signaling. Accordingly, we infected wild-type and TLR4-deficient mice subcutaneously with *Y. pestis* KIM1001 or KIM1001-pLpxL at a dose of 1×10^3 CFU. Both mouse strains succumbed to infection with *Y. pestis* KIM1001, whereas only TLR4-deficient mice died when infected with *Y. pestis* KIM1001-pLpxL (Fig. 3b). Progression of disease (median time to death; Fig. 3b), bacterial growth *in vivo* and/or systemic dissemination of disease, as reflected by bacterial titers in the spleen (Supplementary Fig. 5) and the development of symptoms (activity, posture and appearance of buboes), were similar in TLR4-deficient mice infected with either *Y. pestis* KIM1001 or KIM1001-pLpxL. These results demonstrated that in the absence of TLR4, *Y. pestis* KIM1001-pLpxL remains fully virulent, and were consistent with other evidence suggesting that the KIM1001-

pLpxL strain retains all other virulence factors found in *Y. pestis* KIM1001 except for the ability to produce mainly tetra-acylated LPS.

In contrast to those data, three of three TLR4-deficient mice survived subcutaneous infection with 1×10^5 CFU of a *Y. pestis* KIM1001-based strain defective for the surface protease known as Pla (data not shown), a strain that does not cause systemic infection by the subcutaneous route². Containment of the Pla-defective strain is also dependent on a substantial inflammatory reaction, although the mechanism by which Pla normally suppresses the inflammatory response is not known. Our results suggested that TLR4 deficiency does not grossly compromise innate responses to *Y. pestis* in general

Table 1 Survival after infection with KIM1001 or KIM1001-pLpxL

Inoculum	Survival	
	KIM1001	KIM1001-pLpxL
1×10^2 CFU	4 of 20	20 of 20
1×10^3 CFU	0 of 30	29 of 29
1×10^5 CFU	–	14 of 14
1×10^7 CFU	–	5 of 5

Survival of wild-type mice after subcutaneous infection with increasing doses of *Y. pestis* KIM1001 or KIM1001-pLpxL, presented as surviving mice of total mice. –, not done. Data are representative of two to six experiments.

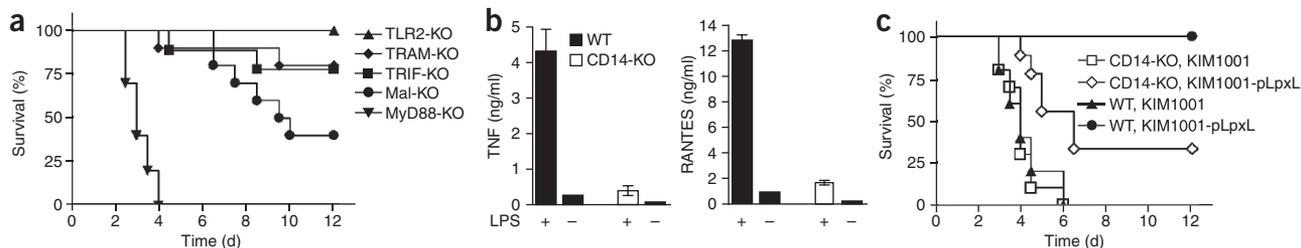


Figure 4 MyD88, Mal and CD14 contribute to the LPS-mediated survival of mice infected with KIM1001-pLpLx. (a) Survival of mice deficient (-KO) in MyD88, Mal, TRAM, TRIF or TLR2 ($n = 10$ mice per group), infected subcutaneously with 1×10^3 CFU of *Y. pestis* KIM1001-pLpLx. $P < 0.0001$, MyD88 versus Mal; $P = 0.003$, Mal versus TLR2. (b) ELISA of TNF and RANTES in supernatants from wild-type (WT) or CD14-knockout (CD14-KO) peritoneal macrophages left unstimulated (-) or stimulated for 18 h with 10 ng/ml of LPS from *Y. pestis* KIM5-pLpLx grown at 37 °C (+). (c) Survival of wild-type or CD14-knockout mice infected subcutaneously with *Y. pestis* KIM1001 ($n = 10$ wild-type; $n = 10$ CD14-knockout) or with *Y. pestis* KIM1001-pLpLx ($n = 10$ wild-type; $n = 9$ CD14-knockout), at a dose of 1×10^3 CFU. $P = 0.002$, CD14-knockout versus wild-type, for KIM1001-pLpLx. Data are representative of three experiments. Cytokine analysis (b) was done in triplicate (error bars, s.d.).

and that loss of Pla and the production of stimulatory LPS permit effective innate responses through distinct pathways.

MD-2, a small molecule attached noncovalently to the extracellular portion of TLR4, is a critical component of the LPS receptor complex^{11,12,29,30}. We therefore expected the phenotype of mice deficient in MD-2 and infected with *Y. pestis* KIM1001-pLpLx to resemble that of TLR4-deficient mice (Fig. 3b). When we infected mice deficient in MD-2 with 1×10^3 CFU of *Y. pestis* KIM1001 or KIM1001-pLpLx, all mice in both groups died (Fig. 3c). Hence, the protection from disease induced by KIM1001-pLpLx requires both TLR4 and MD-2.

Foci of infection containing virulent *Y. pestis* are often devoid of inflammatory cells, reflecting the ability of this pathogen to evade immune responses^{2,3}. Intravenous infection is a useful model for examining inflammation in tissues remote from injection site trauma. Mice infected intravenously with *Y. pestis* KIM1001-pLpLx had an enhanced antibacterial response, as demonstrated by their extended survival time (Supplementary Fig. 7 online) and significantly lower spleen bacterial titers ($P = 0.008$; Fig. 3d). Despite having a lower bacterial load, mice infected with *Y. pestis* KIM1001-pLpLx had twofold more TNF in spleen homogenates than did mice infected with *Y. pestis* KIM1001 (Fig. 3e). Livers of mice infected with wild-type *Y. pestis* were characterized by masses of free bacteria, without distinct indications of local inflammation (Fig. 3f). In contrast, *Y. pestis* KIM1001-pLpLx caused microabscess formation in livers of wild-type mice but not those of TLR4-deficient mice (Fig. 3f). Liver tissue was normal in mice receiving 1×10^3 CFU of *Y. pestis* KIM1001-pLpLx subcutaneously (Fig. 3f), and we detected no bacteria in spleens (Fig. 3d). These data indicated that subcutaneous administration of *Y. pestis* KIM1001-pLpLx fails to establish systemic infection, presumably because of containment by effective local immunity at the infection site.

Effect of TLR adapters and CD14

Four Toll-IL-1 receptor-containing adapter molecules, MyD88, Mal (also called TIRAP), TRIF and TRAM, participate in mediating cellular responses after exposure to LPS⁵. These adapters have different functions in inducing TLR4-dependent responses; the MyD88-Mal branch of TLR4 signaling leads to the early activation of NF- κ B and the release of several proinflammatory mediators, including TNF and IL-6 (ref. 5). In the MyD88-independent pathway, TRAM and TRIF mediate activation of IRF3 and the release of type I interferon and interferon-regulated genes such as *Ccl5* (which encodes the chemokine

RANTES), as well as late activation of NF- κ B⁵. Few studies have addressed the relative contribution of the four adapters in protection against Gram-negative pathogens. When we infected mice deficient in MyD88, Mal, TRIF or TRAM subcutaneously with 1×10^3 CFU of *Y. pestis* KIM1001-pLpLx, only mice deficient in MyD88 showed 100% mortality; 40% of mice deficient in Mal survived, whereas 80% of mice deficient in TRIF or TRAM survived (Fig. 4a). Mice deficient in TLR2 responded like wild-type mice after infection with *Y. pestis* KIM1001-pLpLx (Fig. 4a). These results suggested that MyD88-dependent antibacterial responses are essential for the TLR4-mediated protection against plague. Of the other Toll-IL-1 resistance-containing adapters, Mal was important, whereas TRAM- and TRIF-mediated signaling was less important.

Most members of the family enterobacteriaceae synthesize a 'smooth chemotype' of LPS that consists of lipid A, an oligosaccharide core and an O-specific polysaccharide containing 20–40 repeats of a defined unit¹⁰. In contrast, *Y. pestis* generates a 'rough' LPS³¹ with a core comprising five to eight saccharides lacking an O-specific polysaccharide^{18,32}. CD14, which is expressed as both a glycosylphosphatidylinositol-anchored protein and a soluble protein, enhances responses to LPS but is not absolutely necessary for LPS signaling via TLR4-MD-2. Studies have questioned the function of CD14 in responses leading to immune activation by 'rough chemotypes' of LPS^{33,34}. In particular, CD14 is suggested to be dispensable for TNF release and MyD88-dependent responses of 'rough' LPS³³. However, those conclusions have been based on experiments using a Re 'deep rough mutant' form of purified LPS containing only lipid A

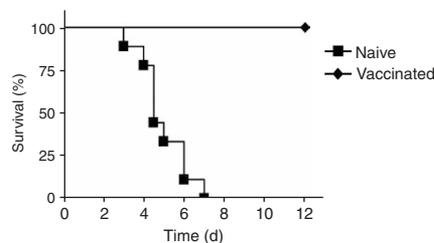


Figure 5 *Y. pestis* with potent TLR4-activating ability is an effective vaccine against plague. Survival of wild-type mice ($n = 10$ mice per group) challenged subcutaneously with 1×10^3 CFU of *Y. pestis* KIM1001-pLpLx (Vaccinated) or left untreated (Naive); 35 d later, mice were infected subcutaneously with 1×10^3 CFU of virulent *Y. pestis* KIM1001. Data are representative of five experiments.

Table 2 Survival of mice after subcutaneous challenge

Vaccination (KIM1001-pLpxL)	Challenge (KIM1001)	Survival
None	1×10^3 CFU	0 of 20
None	1×10^5 CFU	0 of 5
1×10^3 CFU	1×10^3 CFU	19 of 19
1×10^5 CFU	1×10^5 CFU	5 of 5
1×10^5 CFU	1×10^6 CFU	5 of 5

Survival of wild-type mice vaccinated subcutaneously with *Y. pestis* KIM1001-pLpxL and of naive mice (None) after subsequent subcutaneous challenge with increasing doses of *Y. pestis* KIM1001, presented as surviving mice of total mice. Data are representative of two to five experiments.

plus 3-deoxy-D-manno-oct-2-ulosonic acid saccharide moieties, which is not naturally found in pathogenic bacteria.

Similar to wild-type *Y. pestis*, the pLpxL-expressing *Y. pestis* strain produced 'rough' LPS (Supplementary Fig. 8 online). We were interested in determining if CD14 had any effect on the protection from lethal infection provided by the pLpxL strain. Macrophages from CD14-deficient mice were defective in their ability to respond to rough LPS from *Y. pestis* pLpxL grown at 37 °C, as assessed by the release of both TNF and RANTES (Fig. 4b). Subsequently, we inoculated CD14-deficient mice subcutaneously with 1×10^3 CFU of *Y. pestis* KIM1001-pLpxL to determine the function of CD14 in the protection against disease mediated by 'rough' LPS. Six of nine CD14-deficient mice infected with *Y. pestis* KIM1001-pLpxL died after infection, indicating a shift in the mean lethal dose of more than four orders of magnitude compared with that of wild-type mice (Fig. 4c). These results supported the hypothesis that CD14 is involved in transferring many types of 'rough' LPS to TLR4-MD-2 to amplify release of TNF and RANTES and various antibacterial responses.

Vaccination with *Y. pestis* expressing pLpxL

We hypothesized that the attenuated *Y. pestis* strain producing potent LPS might be effective as a vaccine against plague. To test that hypothesis, we vaccinated mice with a single subcutaneous dose (1×10^3 or 1×10^5 CFU) of *Y. pestis* KIM1001-pLpxL and then, 30–40 d later, challenged both vaccinated and naive mice subcutaneously with virulent *Y. pestis* KIM1001 at doses between 1×10^3 CFU and 1×10^6 CFU (the latter corresponding to approximately 1×10^5 mean lethal doses) or by intranasal administration, mimicking pneumonic disease, of 5×10^3 CFU or 5×10^4 CFU (the latter being approximately 150 mean lethal doses). All mice vaccinated with *Y. pestis* KIM1001-pLpxL survived, whereas all naive mice died (Fig. 5 and Tables 2 and 3), demonstrating that *Y. pestis* producing potent LPS is an effective vaccine against both bubonic and pneumonic plague.

Contributions by adaptive immunity

The finding that a strong innate immune activation was essential for survival of acute infection with *Y. pestis* synthesizing a potent LPS (Figs. 3 and 4) did not indicate that adaptive immune responses are not also required for the production of sterilizing immunity. To determine the importance of adaptive responses in sterilizing immune protection, we infected mice deficient in the recombination-activating gene 1 product (RAG-1) with *Y. pestis* KIM1001-pLpxL. In contrast to infected TLR4-deficient mice, which died of acute disease within 6 d (Fig. 6), infected RAG-1-deficient mice remained healthy until day 9, when the first mouse seemed sick; subsequently, all mice died within the next few days (Fig. 6). These data supported the conclusion that

Table 3 Survival of mice after intranasal challenge

Vaccination (KIM1001-pLpxL)	Challenge (KIM1001)	Survival
None	5×10^3 CFU	0 of 20
1×10^3 CFU	5×10^3 CFU	19 of 19
1×10^5 CFU	5×10^3 CFU	5 of 5
1×10^5 CFU	5×10^4 CFU	5 of 5

Survival of wild-type mice vaccinated subcutaneously with KIM1001-pLpxL and of naive mice after subsequent intranasal challenge with increasing doses of KIM1001, presented as surviving mice of total mice. Data are representative of two to three experiments.

innate immunity contains the infection until adaptive responses sufficient for sterilization develop. Thus, even in the face of a potent TLR4-mediated innate immunity, adaptive responses are required for full clearance of infection by the *Y. pestis* pLpxL strain and are crucial for long-term survival of the host.

DISCUSSION

Our results have emphasized the 'double-edged sword' nature of the sensitive TLR4-dependent LPS-detection system. Injection of large amounts of purified LPS into humans and research animals induces the well established syndrome of endotoxic shock. As a result, potent endotoxin is often thought of as harmful during infection with Gram-negative bacteria. Our report has proposed that the expression of a potent LPS by some pathogens may in fact be beneficial for the host, providing early recognition of infection and effective onset of immune signaling. Subversion of TLR4-mediated immune responses by lipid A modification may be a central strategy for evasion of antibacterial mechanisms by various Gram-negative pathogens.

The general idea that emerges from the present understanding of *Y. pestis* virulence is that successful systemic infection depends on efficient and simultaneous suppression and/or evasion of multiple pathways ordinarily capable of eliciting protective local inflammatory responses. Our results have established that evading stimulation of TLR4 is an essential part of that strategy and have indicated that the increased capacity of *Y. pestis* pLpxL to induce TLR4 signaling is dominant over the antihost bacterial defenses provided by T3SS and other bacterial activities. Our findings have also emphasized the notable efficiency of TLR4-mediated responses in the control of infection by Gram-negative pathogens. Despite retention of the active means used by *Y. pestis* to suppress host responses, a strain producing hexa-acylated LPS and thus quickly detectable by means of TLR4-MD-2 failed to cause disease even when the subcutaneous inoculum

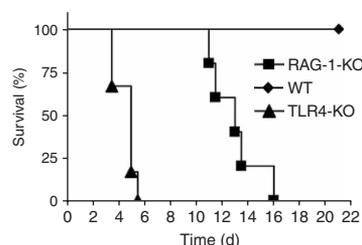


Figure 6 Adaptive immunity is critical for protection against KIM1001-pLpxL. Survival of RAG-1-knockout, TLR4-knockout or wild-type C57BL/6 mice ($n = 5$ mice per group) infected subcutaneously with 1×10^3 CFU of *Y. pestis* KIM1001-pLpxL. Data are representative of three experiments.

exceeded 1×10^6 mean lethal doses. This indicated that strong selection against TLR4 recognition probably exists and that adaptations to prevent LPS-TLR4 stimulation should be common among pathogens. Several other Gram-negative pathogens have been shown to synthesize lipid A with weak stimulatory activity, associated with differences in the number and composition of acyl chains^{14,15}. We have now established the importance of the synthesis of such modified lipid A forms for pathogenesis. It is also notable that *Y. pestis* LPS had reduced stimulatory activity in both murine and primate systems, reflecting the broad host range of this pathogen. Although we did not detect measurable stimulation in the primate system, mouse cells showed a limited but substantial response. However, those responses were not sufficient to protect mice during infection.

The production of a weakly stimulatory LPS is not the only mechanism needed by *Y. pestis* to escape destruction or containment by local inflammation. For example, key proteins injected into host cells by means of T3SS that block proinflammatory signaling and, in most *Y. pestis* strains, Pla protease activity are also required^{1,2,4}. Another potentially important activity is the antagonism of TLR4 stimulation by native *Y. pestis* LPS demonstrated above. Membranes may contain a mixture of stimulatory and nonstimulatory LPS species, especially immediately after the flea bite. In primates and possibly in other species, such antagonistic activity could ensure that membranes containing multiple LPS species become inert with respect to TLR4 stimulation as soon as the tetra-acylated LPS species achieves sufficient concentration in the host.

In addition to stimulating innate antibacterial defenses, activation of TLR signaling also leads to considerable induction of adaptive immune responses, which is mediated in part by substantial upregulation of costimulatory molecules on antigen-presenting cells⁵. In fact, many TLR ligands are promising constituents of vaccines, in which they serve as adjuvants³⁵. By enhancing activation of adaptive immunity, bacterial strains engineered to promote TLR stimulation may have useful properties as vaccine strains. Thus, incorporation of adjuvant activity into pathogens by LPS modification or other means may constitute a new general principle for vaccine development. As we have shown for *Y. pestis*, such modifications can substantially attenuate virulence. Along with the ability to stimulate immune responses, those are desirable characteristics for vaccine strains. Developing *Y. pestis* vaccines that provide good protection against both pneumonic and bubonic disease has been difficult. Effective humoral and cellular immunity are both thought to be necessary for optimal protection³⁶. Our results have provided a 'proof of principle' for the generation of attenuated strains of microorganisms with enhanced TLR-activating potential as a strategy for vaccine production.

Our findings also have implications for the development of therapeutics. Work to develop anti-infective agents based on the stimulation of innate immunity has shown promise^{37,38}. Our observations have provided support for that strategy by demonstrating that sufficient stimulation of innate responses can control infection by a highly virulent pathogen.

METHODS

Bacterial strains and growth conditions. *lpxL* of *E. coli* K12, including 435 base pairs upstream and 140 base pairs downstream of the coding region, was cloned using Pfu Ultra polymerase (Stratagene) and was ligated into the *Bam*HI and *Sal*I sites of pBR322, creating pMW::lpxL (or 'pLpxL'). The control plasmid pBR322Δtet was constructed by digestion of empty pBR322 with *Nae*I and *Eco*RV followed by ligation of the plasmid to remove the main part of the tetracycline-resistance gene. The resulting plasmids were electroporated into

Y. pestis KIM5 (ref. 39) or *Y. pestis* KIM1001 (ref. 2) and bacteria were selected by growth on tryptose–beef extract agar supplemented with 2.5 mM CaCl₂ and 0.6 mg/ml of glucose in the presence of 100 μg/ml of ampicillin. All strains containing either pBR322Δtet or pLpxL remained tetracycline sensitive. KIM1001 (pPCP1+, pCD1+, pMT1+) is highly virulent², whereas KIM5 bears the chromosomal deletion 'Δp_{gm}', which substantially attenuates virulence. KIM5 was used to limit risk of infection for *in vitro* studies for which a virulent strain was not required, and the *p_{gm}* locus contains no genes thought to affect LPS biosynthesis. For LPS preparations, KIM5 and KIM5-pLpxL were grown overnight in aerated tryptose–beef extract broth supplemented with CaCl₂ and glucose as described above.

Lipid preparations. Pyrogen-free reagents and supplies were used as much as possible for lipid preparations. Bacteria were collected by centrifugation at 6,000g. LPS was purified from bacteria by hot water–phenol extraction⁴⁰, followed by two phenol re-extractions for removal of TLR2-activating contaminating lipoproteins⁴¹. The chemical synthesis of the tetra-acylated precursor lipid IVA (also called 406, LA-14-PP or precursor Ia) has been reported⁴². LPS from *E. coli* O111:B4 (Sigma) was re-extracted with phenol as described above. The absence of activation of TLR2-expressing cells (Fig. 2e) despite potent release of IL-8 by bacterial Pam3CysSK4 lipopeptide (data not shown) indicated the absence of contaminating lipoproteins in our highly purified LPS preparations.

Cell stimulation assays. Human PBMCs were obtained from healthy volunteers with informed consent and were isolated by centrifugation on Lymphoprep density media (Axis-Shield–Nycomed). The University of Massachusetts Medical School Institutional Review Board (Worcester, Massachusetts) approved the human subject protocol. HEK293 cells stably expressing human TLR4–yellow fluorescent protein and retroviral MD-2, or human TLR2–yellow fluorescent protein, or empty vector pCDNA3, were as published⁴³. Genejuice (Novagen) was used to transfect the NF-κB–luciferase (provided by K. Fitzgerald, University of Massachusetts Medical School, Worcester, Massachusetts) and IRF3-dependent 561–luciferase (a gift from G. Sen, Cleveland Clinic Foundation, Cleveland, Ohio⁴⁴) reporters into HEK293 cells expressing human TLR4 and MD-2. PBMCs were cultured in X-vivo 15 medium (Cambrex) containing ciprofloxacin and 1% FCS or 1% human serum, whereas HEK293 cells were stimulated in DMEM plus ciprofloxacin plus 10% FCS. Wild-type C57BL/6 or *Rag1*^{−/−} mice (backcrossed for ten generations onto the C57BL/6 strain) were from Jackson Laboratories. *Tlr4*^{−/−} mice (TLR4 knockout), *Myd88*^{−/−} mice (MyD88 knockout), *Tirap*^{−/−} mice (Mal knockout), *Ticam1*^{−/−} (TRIF knockout), *Ticam2*^{−/−} mice (TRAM knockout) and *Tlr2*^{−/−} mice (TLR2 knockout) were generated as described^{7,45–49} and were backcrossed for eleven (TLR4, TLR2 and MyD88), four (Mal) or five (TRIF and TRAM) generations onto the C57BL/6 strain. *Ly96*^{−/−} mice (MD-2 knockout)³⁰ (K.M. and Japan Science and Technology) and *Cd14*^{−/−} mice (CD14 knockout)⁵⁰, a gift from K. Moore and M. Freeman (Harvard Medical School, Boston, Massachusetts), were backcrossed for seven and ten generations, respectively, onto the C57BL/6 strain. Mouse peritoneal macrophages were collected from mice 3 d after injection of 2 ml thioglycollate (3%) and were cultured in RPMI 1640 medium containing 10% FCS. Cells were plated at a density of 2×10^4 cells per well (HEK293 cells), 5×10^4 cells per well (mouse macrophages) or 1×10^5 cells per well (PBMCs) in 96-well dishes and were stimulated for 16–18 h before collection of supernatants (for cytokine analysis) or cells for lysis (transfections and reporter assays; reagents from Promega). Cytokines were measured with ELISA kits from BD Pharmingen (mouse TNF and mouse RANTES) or R&D systems (human TNF, IL-6 and IL-8). Cell lysates from luciferase reporter assays were analyzed by the addition of luciferase substrate followed by luminometry.

In vivo infection. Mice were infected with *Y. pestis* KIM1001, KIM1001-pLpxL or KIM1001(pBR322Δtet) by subcutaneous injection of 50 μl into the nape of the neck, by intranasal infection with 50 μl dropped into the nostrils of mice anesthetized by ketamine-xylazine or by intravenous injection of 500 μl in the tail vein. Inocula contained various CFU suspended in PBS. Mice were killed by intraperitoneal pentobarbital overdose when moribund. Survival was monitored every 12 h during acute infection up to 21 or 28 d. *Y. pestis*

KIM1001-pLpxL strains generated by electroporation of pLpxL into KIM1001 in independent transformations had a similar phenotype during infection of mice. For collection of organs, mice were killed by pentobarbital overdose 48 h after intravenous infection or 72 h after subcutaneous infection and spleens were homogenized in PBS to obtain bacterial titers and for cytokine analysis. Separate infection experiments with detection limits of 1×10^3 or 1×10^1 CFU per spleen produced similar results, with an absence of detectable bacteria in spleens after subcutaneous infection with *Y. pestis* KIM1001-pLpxL. Livers were fixed in neutral buffered 4% formalin, were stained with hematoxylin and eosin and then were examined by microscopy. A Nikon Eclipse E400 instrument with a $10\times$ or $100\times$ oil objective was used. Images were captured with a Spot camera and corresponding software (Diagnostic instruments). All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and experiments followed its guidelines and regulations.

Statistical analysis. Differences in spleen CFU and TNF concentrations were analyzed by the Mann-Whitney U-test. Statistical differences in survival were analyzed by Kaplan-Meier survival analysis and the log-rank test.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank A. Yuan, N. Pan and K. Trimble for help with experiments; N. Deitemeyer and C. Lee for technical assistance; A. Cerny and J. Boulanger for animal husbandry; D.T. Golenbock for support; and R.R. Ingalls, N. Silverman and K. Fitzgerald for critically reading the manuscript. Supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (R01 AI057588 to E.L.) and the Diabetes Endocrinology Research Center (DK 32520).

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the Nature Immunology website for details).

Published online at <http://www.nature.com/natureimmunology/>

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