Original Article

Host Langerin (CD207) is a receptor for *Yersinia pestis* phagocytosis and promotes dissemination

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*Yersinia pestis* is a Gram-negative bacterium that causes plague. After *Y. pestis* overcomes the skin barrier, it encounters antigen-presenting cells (APCs), such as Langerhans and dendritic cells. They transport the bacteria from the skin to the lymph nodes. However, the molecular mechanisms involved in bacterial transmission are unclear. Langerhans cells (LCs) express Langerin (CD207), a calcium-dependent (C-type) lectin. Furthermore, *Y. pestis* possesses exposed core oligosaccharides. In this study, we show that *Y. pestis* invades LCs and Langerin-expressing transfectants. However, when the bacterial core oligosaccharides are shielded or truncated, *Y. pestis* propensity to invade Langerhans and Langerin-expressing cells decreases. Moreover, the interaction of *Y. pestis* with Langerin-expressing transfectants is inhibited by purified Langerin, a DC-SIGN (DC-specific intercellular adhesion molecule 3 grabbing nonintegrin)-like molecule, an anti-CD207 antibody, purified core oligosaccharides and several oligosaccharides. Furthermore, covering core oligosaccharides reduces the mortality associated with murine infection by adversely affecting the transmission of *Y. pestis* to lymph nodes. These results demonstrate that direct interaction of core oligosaccharides with Langerin facilitates the invasion of LCs by *Y. pestis*. Therefore, Langerin-mediated binding of *Y. pestis* to APCs may promote its dissemination and infection.

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*Yersinia pestis*, a Gram-negative bacterium, is the causative agent of bubonic plague, passed into the annals of history as Black Death epidemics.12 A hallmark of plague is the rapid dissemination of *Y. pestis*, which leads to systemic infection in susceptible individuals.3,4

Many Gram-negative bacterial pathogens contain lipopolysaccharides, which consist of three structural regions: (i) the lipid A backbone, (ii) core oligosaccharides, and (iii) the O-antigen (Figure 1). Gram-negative bacteria are classified as smooth or rough based on the presence or absence of the O-antigen, respectively. Rough Gram-negative bacteria bear a shortened lipopolysaccharides—referred to as lipooligosaccharides (LOS)—of which the oligosaccharide core is exposed to the extracellular environment.

Innate immune system functions are initiated in the skin by antigen-presenting cells (APCs) such as dendritic cells (DCs), or a subset of immature APCs,5,6 such as Langerhans cells (LCs). APCs either phagocytose and kill invading pathogens or deliver the pathogens to other types of host immune cells for further elimination. APCs in the skin express at least three immunoreceptors that belong to the calcium-dependent (C-type) lectin family: DC-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN, CD209), DEC-205 (CD205), and Langerin (CD207). Human LCs (hLCs) mostly express Langerin but do not express DC-SIGN.7 Pathogens such as HIV exploit DC-SIGN-mediated uptake by APCs to efficiently disseminate throughout a host, travelling to the host’s lymph nodes.6,9 On the other hand, human Langerin (hLangerin; CD207), an innate immune receptor for HIV-1 on LCs, may function as a natural barrier to the transmission of HIV-1 and certain viruses.10-12

In this study, we investigated the interaction between Langerin and *Y. pestis* core oligosaccharides. Our results suggest that after overcoming the first line of host defence (the skin) via a fleabite, *Y. pestis*
Y. pestis invades LCs

One of the mechanisms by which Y. pestis invades human DCs involves its naturally exposed core oligosaccharides and plasminogen activator. The expression of O-antigen shields bacterial core oligosaccharides and blocks the interaction of Y. pestis, as well as several other Gram-negative bacteria, with DCs. In light of these findings, we assessed whether Y. pestis strains KIM10 (rough strain, exposed core oligosaccharides), KIM10- core (smooth strain, core oligosaccharides covered with an O-polysaccharide) and KIM10-core (deep rough strain, expressing a truncated LOS outer core) could invade hcbLCs (human cord blood LCs) and hLCs (primary LCs) in a manner similar to E. coli. We used three corresponding Escherichia coli K-12 strains, CS180 (rough strain, exposed core oligosaccharides), CS1861 (smooth strain, CS180 expressing an O-antigen) and CS2429 (deep rough strain, outer core oligosaccharides deleted) as controls. These E. coli strains have been used previously to demonstrate that the exposure of the E. coli core oligosaccharides is essential for bacterial interaction with DC-SIGN (Table 1). Both the Y. pestis and E. coli rough strains (KIM10 and CS180), but not the corresponding smooth strains (KIM10-core and CS1861) or the corresponding deep rough strains (KIM10-core and CS2429) effectively invaded hcbLCs and hLCs, suggesting that bacterial uptake is mediated by their core oligosaccharides (Figure 2). Y. pestis invades Chinese hamster ovary (CHO) cells that express Langerin

Whereas LCs do not express DC-SIGN, the C-type lectin Langerin is one of their major receptors. Therefore, we tested whether the ability of Y. pestis to invade hcbLCs and hLCs depended on the bacterium’s interaction with Langerin. The same Y. pestis (KIM10, KIM10-core and KIM10-core) and E. coli strains (CS180, CS1861 and CS2429) were tested for their ability to invade CHO transfectants stably expressing two C-type lectin receptors, CHO-hLangerin and CHO-hDEC-205 (human CD205) (Figure 3a). We selected hDEC-205 because recent data have shown that murine DEC-205 serves as the receptor for plasminogen activator of Y. pestis. We used CHO-NEO as a control-transfected cell line, as it expresses only the neomycin gene. The results show that the rough Y. pestis strain KIM10 invades CHO-hLangerin cells, but not CHO-NEO and CHO-hDEC-205 cells, indicating that hLangerin is a receptor for Y. pestis (Figure 3b). In addition, the rough strains KIM10- and CS180 but neither the smooth strains (KIM10-core or CS1861) nor the deep rough strains (KIM10-core or CS2429) promoted the invasion of CHO-hLangerin cells, indicating that core oligosaccharides of both Y. pestis and E. coli K-12 are ligands for hLangerin. Of note, hDC-SIGN displayed a similar ability to bind the bacterial core oligosaccharides.

RESULTS

Y. pestis invades LCs

One of the mechanisms by which Y. pestis invades human DCs involves its naturally exposed core oligosaccharides and plasminogen activator. The expression of O-antigen shields bacterial core oligosaccharides and blocks the interaction of Y. pestis, as well as several other Gram-negative bacteria, with DCs. In light of these findings, we assessed whether Y. pestis strains KIM10 (rough strain, exposed core oligosaccharides), KIM10-core (smooth strain, core oligosaccharides covered with an O-polysaccharide) and KIM10-core (deep rough strain, expressing a truncated LOS outer core) could invade hcbLCs (human cord blood LCs) and hLCs (primary LCs) in a manner similar to E. coli. We used three corresponding Escherichia coli K-12 strains, CS180 (rough strain, exposed core oligosaccharides), CS1861 (smooth strain, CS180 expressing an O-antigen) and CS2429 (deep rough strain, outer core oligosaccharides deleted) as controls. These E. coli strains have been used previously to demonstrate that the exposure of the E. coli core oligosaccharides is essential for bacterial interaction with DC-SIGN (Table 1). Both the Y. pestis and E. coli rough strains (KIM10 and CS180), but not the corresponding smooth strains (KIM10-core and CS1861) or the corresponding deep rough strains (KIM10-core and CS2429) effectively invaded hcbLCs and hLCs, suggesting that bacterial uptake is mediated by their core oligosaccharides (Figure 2).

The core oligosaccharides of E. coli mediate the interaction with hLangerin

Although the data shown in Figures 2 and 3 suggest that Y. pestis utilises its core oligosaccharides as a ligand to interact with hcbLCs, hLCs and hLangerin-expressing cells, we cannot exclude the possibility that another outer membrane component may function as an additional ligand. The O-antigen may indeed prevent access to many of the surface structures that are exposed in the rough E. coli and Y. pestis mutants. For example, recent data from our lab have demonstrated that the expression of O-antigen blocks the interaction of murine DEC-205 (mCD205, another receptor for Y. pestis) with plasminogen activator. Although core oligosaccharide mutants of Y. pestis are not presently available, well-characterised core oligosaccharide mutants of E. coli, Salmonella and Neisseria gonorrhoeae have been used to demonstrate that Gram-negative bacteria use core oligosaccharide components to interact with hDC-SIGN. Therefore, we examined the abilities of several E. coli core oligosaccharide mutants (waaR, waaO, waaG and waaC) (Figure 1) to interact with CHO-hLangerin cells (Table 1). Figure 4 shows that the waaR, waaO, waaG and waaC mutants, which express core oligosaccharides of decreasing lengths, lose the ability to promote phagocytosis by CHO-hLangerin cells, suggesting that the epitope recognised by hLangerin resides in the main chain of the outer LOS core. Although the waaR, waaO and waaG mutants show increased resistance to phagocytosis by CHO-hLangerin cells, all three mutants are less resistant to phagocytosis compared with the waaC mutant, suggesting that some sugars in the core between the first heptose and the terminus of the LOS core participate in the LOS–Langerin interaction.

The data from the E. coli lipopolysaccharide mutants isolate show that an outer-core saccharide has an important role in the hLangerin interaction. This mirrors the data between core oligosaccharides and hLangerin (Figures 2, 3 and 4) and between core oligosaccharides and hDC-SIGN.

Inhibition of Langerin-mediated phagocytosis of Y. pestis by purified FLAG-Langerin, an anti-Langerin antibody, mannann, oligosaccharides and a DC-SIGN-like molecule

hcbLCs express both hLangerin and hDC-SIGN (Figure 5a). We examined whether host–pathogen interactions could be inhibited by anti-hLangerin and anti-hDC-SIGN antibodies in these cells (Table 1). As shown in Figure 5b, when used individually, neither anti-hLangerin nor anti-hDC-SIGN antibodies affected the phagocytosis of Y. pestis KIM10 by hcbLCs. However, when these two antibodies were combined, the phagocytosis of KIM10 by hcbLCs was significantly reduced. Nevertheless, this reduction was not complete, suggesting that another outer membrane component may function as an additional ligand.
that additional receptors for \textit{Y. pestis} are present on the hcbLCs (Figure 5b).

Primary hLCs predominantly express hLangerin (Figure 5c) and an anti-Langerin antibody was indeed sufficient to inhibit \textit{Y. pestis} phagocytosis by hLCs (Figure 5d). This suggests that the hLangerin receptor has a major role in the interaction between LCs and \textit{Y. pestis}.

Purified FLAG-Langerin, mannan (an antagonist of mannose receptors), several oligosaccharides, a DC-SIGN-like molecule (Mermaid) and the anti-hLangerin antibody were tested to determine whether any of these molecules could block the interaction between \textit{Y. pestis} and CHO-hLangerin cells. A specific set of oligosaccharides (found in the core oligosaccharides) and a recombinant form of the C-type lectin Mermaid (His-Mermaid) were selected based on their ability to inhibit the core oligosaccharide–hDC-SIGN interaction.13,14,16

\textit{Yersinia pseudotuberculosis} serotype O:1a (Y1), which displays a Langerin-independent interaction with epithelial cells, served as a control strain for the invasion assay. Because of its strong invasive ability, only one-third of the bacterial suspension of Y1 was used for infection relative to the rest of strains (Table 1). In addition, the anti-hLangerin antibody appeared to inhibit the interaction between KIM10+ and CHO-hLangerin cells compared with the ability of the anti-Langerin antibody to inhibit the \textit{Y. pestis}–hLC interaction. The recovery rates of Y1 and KIM10+ bacteria were reduced in the presence of lactoferrin, indicating that some bacteria are susceptible to the lactoferrin peptide, which is well known for its antibacterial properties.18

Purified FLAG-Langerin and DC-SIGN-like molecules bind to rough but not smooth \textit{Y. pestis}

Purified FLAG-Langerin and His-Mermaid have previously been shown to inhibit the interaction between \textit{Y. pestis} core oligosaccharides and hLangerin. To determine whether this inhibition was due to competition between these two lectins and hLangerin for binding to core oligosaccharides, the abilities of purified FLAG-Langerin and His-Mermaid to bind to KIM10− and KIM10−O+ were tested. \textit{E. coli} strains CS180 and CS1861 were included as positive and negative controls, respectively, for the His-Mermaid-binding experiments. Figure 6 shows that purified fluorescein isothiocyanate (FITC)-Langerin and FITC-His-Mermaid bind more strongly to KIM10− and CS180 than to KIM10−O+ and CS1861, indicating that these two molecules directly interact with the core oligosaccharides of \textit{Y. pestis}.

\begin{table}[h]
\centering
\caption{Bacterial strains and cell lines used in this study}
\begin{tabular}{lll}
\hline
\textbf{Strains} & \textbf{Genotypes (phenotypes)} & \textbf{References} \\
\hline
\textit{Y. pestis} & & \\
KIM6+ & Wild type (rough) & 37 \\
KIM6−O+ & KIM6+ expressing O-antigen (smooth) & 19 \\
KIM10− (KIM10−\textit{Δail}) & Derivative of KIM5 in which the \textit{ail} gene has been deleted and both plasmid pCD1 and pPCP1 have been cured & 13,38 \\
KIM10−O & KIM10−O-antigen & 19 \\
KIM10−core (\textit{gmhA}-deleted KIM10−) & Deep rough mutant derivative of KIM10−, the \textit{gmhA} allelic exchange plasmid pCBD41 was mobilised from \textit{E. coli} SM10\textit{pir}/pCBD41 into KIM10− & This study \\
\textit{Y. pestis} 1418 & KIM D27 (Lcr−, \textit{pgm}−, \textit{pst}+) & 46,47 \\
\textit{Y. pestis} 1418−O+ & \textit{Y. pestis} 1418 expressing O-antigen (smooth) & This study \\
\textit{Y. pseudotuberculosis} & & \\
Y1 & O:1a, wild-type expressing invasin but with pYV plasmid naturally cured (smooth) & 17,48 \\
\hline
\textit{E. coli} K-12 & & \\
CS180 & Wild-type (rough) & 35,36,49 \\
CS1861 & CS180-O-antigen & 35,36,49 \\
CS2429 & waaC & 35,36,49 \\
CS2198 & waaR & 35,36,49 \\
CS2488 & waaO & 35,36,49 \\
CS1943 & waaG & 35,36,49 \\
\hline
\textbf{Cell lines} & \textbf{Characteristics} & \\
\hline
hcbLCs & Langerhans cells derived from human cord blood cells were purchased from MatTek Corporation (Ashland, MA, USA) & \\
hLCs & Isolated from skin biopsy samples obtained from healthy patients undergoing plastic surgery & \\
CHO-NEO cells & Control cell line, which expresses the neomycin resistance gene only & \\
CHO-hLangerin cells & Generated by transfecting CHO cells with human Langerin cDNAs for stable surface expression & \\
CHO-hDEC-205 cells & Generated by transfecting CHO cells with human DEC-205 cDNAs for stable surface expression & \\
\hline
\end{tabular}
\end{table}
Expression of O-antigen reduces the ability of *Y. pestis* to disseminate to lymph nodes and delays death in a murine model

We speculated that the interaction of *Y. pestis* with C-type lectin receptors promotes bacterial dissemination.9,13 Given that the interaction appears to be mediated by core oligosaccharides and hLangerin, we hypothesised that shielding the exposed core oligosaccharides of *Y. pestis* would reduce its dissemination and consequently delay death in a murine model. To test this hypothesis, the *Y. pestis* strain KIM6, as well as the strain KIM D27 with and without O-antigen expression, were injected into mouse metacarpal paw pads. The KIM6 and KIM D27 strains were used in this experiment rather than KIM10 because KIM10 does not survive in mice.9,13 For the dissemination assay, subiliac lymph nodes were collected, and the bacterial counts were determined, which allowed for the calculation of *Y. pestis* dissemination rates into the lymph nodes. In mice challenged with the strain KIM D27, the amount of time that the animals took to succumb to infection was recorded following inoculation. As indicated in Figure 7, the dissemination (Figure 7a) and death (Figure 7b) of O-antigen-expressing *Y. pestis* were significantly reduced. Notably, the expression of O-antigen did not affect *Y. pestis* infectivity when delivered
intravenously, indicating that *Y. pestis* uptake by skin APCs mediate *Y. pestis* dissemination. In addition, the growth rate of *Y. pestis* was not dependent on O-antigen expression.

**DISCUSSION**

LCs belong to a subset of immature DCs localised in the skin and express the major C-type lectin receptor Langerin instead of DC-SIGN. In this study, using a well-defined set of core oligosaccharides mutants, we demonstrated that, similar to hDC-SIGN, hLangerin binds the core oligosaccharides of *Y. pestis*. This interaction is likely important for the initiation of *Y. pestis* pathogenesis in a manner analogous to HIV-1 infection mechanisms. HIV-1 has been reported to hijack DC-SIGN to ensure its capture and transmission to CD4+ lymphocytes target cells located in the lymph nodes.

The interaction of *Y. pestis* with hDC-SIGN from monocyte-derived dendritic cells (MDDCs) supports this idea. The interaction of *Y. pestis* with MDDCs can be reduced by combined treatment with anti-hDC-SIGN and anti-hLangerin antibodies, suggesting that hLangerin could act as a redundant receptor for *Y. pestis* in MDDCs. The commercially available LCs employed in this study were generated from human cord blood cells and express both hLangerin and, to a lesser extent, DC-SIGN (Figure 5a). These LCs behaved similarly to MDDCs as a combination of anti-hDC-SIGN and anti-hLangerin antibodies significantly decreased their ability to bind *Y. pestis*. Several Gram-negative bacteria, including *Y. pestis*, utilise their exposed core oligosaccharides to interact with hDC-SIGN. The functional similarity between hLangerin and hDC-SIGN is consistent with a recent study from Chatwell *et al.*, which showed that the carbohydrate recognition domain of hLangerin is structurally very similar to that of hDC-SIGN.

Oligosaccharides were selected based on their predicted ability to inhibit *Y. pestis*-C-type lectin interactions. Several Gram-negative bacteria, including *Y. pestis*, might utilise their core oligosaccharides to interact with hDC-SIGN. In addition, HIV uses the gp120–DC-SIGN interaction to initiate capture by DCs and transmission to CD4+ T cells. Therefore, blockage of DC-SIGN-mediated transmission of HIV is recognised as a valid therapeutic strategy to fight HIV infection. For example, Lewis X oligosaccharides have been shown to prevent DC-mediated HIV-1 transmission by blocking the DC-SIGN–gp120 interaction. Our studies confirm that some oligosaccharides inhibit the interactions between *Y. pestis* and transfectants expressing hDC-SIGN or hLangerin (Figure 5). Although Lewis X components do not interact with hLangerin well, they also inhibit the hLangerin–*Y. pestis* interaction (Figure 5e). This may be due to the fact that Lewis X components may bind both the ligand, the core lipopolysaccharides on *Y. pestis* and Langerin-expressing host cells.

HIV surface proteins, such as GP120, are highly glycosylated and bind to DC-SIGN and Langerin, likely via their carbohydrate moieties. Although extensive biochemical, structural and functional studies have been performed to understand the interactions between GP120 and C-type lectin receptors, it remains unclear whether DC-SIGN and Langerin bind the same sugar residues on GP120. Given that hDC-SIGN and hLangerin bind the same bacterial carbohydrate structures of core oligosaccharides from several Gram-negative bacteria, we speculate that hDC-SIGN and hLangerin might also bind the same sugar residues on HIV-1 GP120. In contrast to GP120, the core oligosaccharides are amenable to manipulation, and thus their analysis may shed light on HIV–host interactions.
Most cells that express C-type lectins function in innate immunity. Langerin is an innate immune receptor for HIV-1 that is present on LCs and may function as a natural barrier to the transmission of HIV-1 and other viruses. However, the LD_{50} of certain strains of Y. pestis, such as CO92, has been reported to be as low as one colony-forming unit (CFU) in a murine model. Based on these observations, it is not surprising that Y. pestis causes the death of over one-third of the European population during the Black Death epidemic. Therefore, we speculate that the natural barrier function provided by Langerin against certain viruses does not apply to Y. pestis.

Taken together, this study demonstrates that Langerin is a cellular receptor for Y. pestis core oligosaccharides and suggests that Langerin-mediated uptake by APCs may promote bacterial dissemination. We speculate that Y. pestis may hijack APCs to promote bacterial transportation to the lymph nodes, employing a mechanism similar to that of HIV, which has been shown to interact with DC-SIGN. This acquired knowledge should spur novel strategies to combat Y. pestis infection by blocking its interaction with host cell receptors.

METHODS

Declaration of ethical approval

All experiments were approved by the Medical Ethics Committee of Tongji Hospital and were conducted in accordance with the institutional guidelines.

Bacterial strains

E. coli K-12 strain CS180 synthesizes core oligosaccharides but lacks an O-antigen. CS1861 is a derivative of CS180 that harbours pSS37, a plasmid containing all of the genes necessary for the expression of the Shigella dysenteriae type 1 O-antigen. CS2429 (waaC) is a deep rough isogenic mutant of CS180 that lacks both the O-antigen and most of the core. Additional isogenic mutants of CS180 used in this study include: CS1918 (waaR), CS2488 (waaO), CS1943 (waaG) and CS2429 (waaC) (Figure 1) by CHO and CHO-hLangerin cells was determined using the same procedures described in Figure 2. The data presented were pooled from three independent experiments. The data represent the means ± s.e.m. N = 9. Statistical analysis was performed using one-way analysis of variance and the Newman–Keuls test. ***P < 0.001.

Figure 4 Interaction of core oligosaccharide E. coli mutants with CHO-hLangerin cells. Internalisation of E. coli K-12 strains CS180, CS2198 (waaR), CS2488 (waaO), CS1943 (waaG) and CS2429 (waaC) (Figure 1) by CHO and CHO-hLangerin cells was determined using the same procedures described in Figure 2. The data presented were pooled from three independent experiments. The data represent the means ± s.e.m. N = 9. Statistical analysis was performed using one-way analysis of variance and the Newman–Keuls test. ***P < 0.001.

Reagents

(1) Anti-hLangerin and anti-DC-SIGN antibodies were purchased from Abcam (Cambridge, UK). (2) The following oligosaccharides and mannan were purchased from Sigma (St Louis, MO, USA). (3) The monosaccharides, GlcNAc and GalNAc, were purchased from Sigma (St Louis, MO, USA). (4) Mermaid is a DC-SIGN-like molecule expressed by the mouse nematode Caenorhabditis elegans. The carbohydrate recognition domain of Mermaid shares both structural and functional similarity with that of DC-SIGN. A recombinant form of Mermaid (His-Mermaid) was expressed and purified as described previously. (5) The extracellular domain of hLangerin tagged with the FLAG epitope used in this study was generated and purified according to a previously described protocol for mouse Langerin. (6) Purified core oligosaccharides from an N. gonorrhoeae lgtB variant were used in this study because our previous data showed that the core oligosaccharides from this bacterium, unlike E. coli K-12 core oligosaccharides, inhibited the core oligosaccharide–hDC-SIGN interaction. In addition, our unpublished data have shown that the purified core oligosaccharides from KIM10 do not inhibit hLangerin– and hDC-SIGN–Y. pestis interactions. The loss of the ability to inhibit these interactions is likely due to changes in conformation generated during the purification process. The purification procedures of core oligosaccharides have been described previously.

Human cord blood LCs

hCBLCs derived from human cord blood cells were purchased from MatTek Corporation (Ashland, MA, USA). However, the methods used to generate these LCs are proprietary and are not available from the manufacturer. Based on the information provided by the manufacturer, this LC line expresses Langerin, which was confirmed in Figure 5a. Although previous studies have suggested that LCs usually do not express DC-SIGN, our data show that the hCBLCs purchased from this company express a low level of DC-SIGN (Figure 5a).

Isolation and purification of hLCs

hLCs were obtained from healthy patients who were undergoing plastic surgery and provided informed consent. Briefly, skin biopsies were freed from fatty tissues and split-cut with a keratome set. Skin slices were then incubated with 0.05% trypsin containing EDTA in Hanks buffer without Ca^{2+} and Mg^{2+} for 1 h at 37°C. Then the epidermis was detached from the dermis using fine...
Figure 5 Inhibition of hLangerin-mediated phagocytosis of *Y. pestis* by anti-hLangerin antibody, mannan and oligosaccharides. The expression levels of hcbLCs and hLCs are shown in panels (a) and (c), respectively. *Y. pestis* KIM10<sup>−</sup> cultured at 26 °C was incubated with hcbLCs (b) and hLCs (d) for 1.5 h and CHO-hLangerin cells for 2 h (e) in the presence or absence of purified FLAG-hLangerin, anti-CD66/-hLangerin/-hDC-SIGN antibodies, mannan, various oligosaccharides and a DC-SIGN-like protein (His-Mermaid). The experiments with hcbLCs and hLCs were performed using antibody treatment only. All reagents were added to the media 20 min before the addition of bacteria. The concentration of each reagent used in this experiment was based on previously published data.<sup>14–16</sup> The phagocytosis rate of *Y. pestis* was determined by the recovery of bacteria following gentamicin treatment. *Y. pseudotuberculosis* serotype O:1a was used as a control strain that shows core-independent invasion of CHO cells. The data presented were pooled from three independent experiments. The data represent the means ± s.e.m. N=9. Statistical analysis was performed using one-way analysis of variance and the Newman–Keuls test. ***P<0.001.
forceps, after which it was placed in Hank’s balanced salt solution supplemented with 10% heat-inactivated foetal calf serum. Repeat pipetting was used to disrupt the epidermal sheets, and single-cell suspensions were achieved with repeat pipetting and forceful passage through sterile gauze. Consecutive density gradient centrifugation with lymphocyte separation was used to enrich the LCs fractions, as previously described. CD1a and CD207 monoclonal antibodies were used to determine the purity or the expression levels of freshly isolated hLCs.

Human C-type lectin transfectants
CHO-hLangerin and CHO-hDEC-205 cell lines were generated by transfecting CHO cells with corresponding human C-type lectin cDNAs. Transfection was followed by G418 (1.5 mg ml\(^{-1}\)) selection and screening for stable surface expression, as illustrated in Figure 3a. CHO-NEO cells were used as a control cell line that expresses the neomycin resistance gene only.

Adherence and phagocytosis assays
The assays for adherence and phagocytosis have been described previously. Briefly, host cells (hcbLCs, hLCs and CHO) were plated in 24- or 96-well plates. The cells were suspended in RPMI medium supplemented with 2% foetal calf serum at a concentration of 4 × 10^5 ml\(^{-1}\) (hLCs at a concentration of 8 × 10^4 ml\(^{-1}\)). In all, 500 μl of each of these cell suspensions was added to 24-well plates, and after the addition of 50 μl of bacterial suspensions at a concentration of 5 × 10^6 CFU ml\(^{-1}\), the cells were allowed to incubate for 2 h at 37 °C in the presence of 5% CO\(_2\).
To determine the internalisation of bacteria, gentamicin, which kills extracellular bacteria but cannot penetrate into host cells, was added to each well at a final concentration of 100 μg ml\(^{-1}\), and the cultures were incubated for 60 min. The cells were washed twice to remove the gentamicin. The cells were suspended in phosphate-buffered saline containing 0.5% saponin, after which the cells were diluted and plated on Luria–Bertani or GC-based media plates. The level of internalisation of bacteria in the host cells was calculated by

![Figure 6](image)

**Figure 6** Purified FLAG-hLangerin and DC-SIGN-like molecules bind to *Y. pestis* core oligosaccharides. *Y. pestis* KIM10\(^{-}\), KIM10\(^{-}\)-O\(^{+}\), CS180 and CS1861 were incubated with 10 μg of FITC-His-Mermaid and purified FLAG-Langerin for 30 min. The ability of FITC-His-Mermaid and Langerin to bind the bacteria was measured using flow cytometry. The fluorescence intensities of KIM10\(^{-}\) or CS180 and KIM10\(^{-}\)-O\(^{+}\) or CS1861 are represented by non-filled and filled curves, respectively. *E. coli* strains CS180 and CS1861 should be regarded as positive and negative controls, respectively, as shown previously.

![Figure 7](image)

**Figure 7** Expression of *Y. pestis* O-antigen reduces pathogen dissemination and host death. (a) KIM6\(^{+}\) and KIM6\(^{-}\)-O\(^{+}\) (O-antigen expressing) were inoculated in mice following the procedures described in the Methods section. After 24 h, the mice were killed, and the subiliac lymph nodes were separated, homogenised and spread on GC-base plates. The dissemination rate represents the CFU recovered from whole subiliac lymph nodes. The data presented were pooled from three independent experiments. The data represent the means ± S.E.M. N = 9. Statistical analysis was performed using Student’s t-test. ***\(P<0.001\). (b) Mouse survival in a dissemination model challenged with *Y. pestis* 1418 (KIM D27) or *Y. pestis* 1418-O\(^{+}\). The survival curve shows the animal survival time from the inoculation. The data presented were pooled from three independent experiments. Statistical analysis was performed using the log-rank test. *\(P<0.05\).
determining the CFU recovered from lysed cells. All experiments were performed in triplicate, and the data are expressed as the means ± s.e.m.

For the inhibition assay, purified FLAG-Langerin (100 µg ml⁻¹), anti-human Langerin (5 µg ml⁻¹) antibody, anti-DC-SIGN (5 µg ml⁻¹) antibody and carbohydrates, including core oligosaccharides (500 µg ml⁻¹), oligosaccharides (500 µg ml⁻¹), a DC-SIGN-like protein (10 µg ml⁻¹) and mannan (500 µg ml⁻¹), were added 20 min prior to the addition of bacteria. The concentrations used were determined based on our preliminary data and were selected based on the fact that, at these concentrations, the compounds exerted no effects on the survival of bacteria or host cells, as previously shown.13,14

Determination of phagocytosis by flow cytometry

The following method was used to supplement the survival-based phagocytosis assay described above, as APCs are known to kill some phagocytosed bacteria.13,15 Briefly, the bacteria were suspended in RPMI medium containing 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes, Eugene, OR, USA) for 40 min and then washed twice with RPMI to remove the excess dye. The labelled bacteria were added to the cell cultures and allowed to interact for 2 h. Cell cultures were washed twice to remove the unbound bacteria. The LCs and associated bacteria were fixed with 2% paraformaldehyde. Before flow cytometry, a 1:10 dilution of trypan blue (0.4%, Sigma) was added to the fixed cell cultures, and the mixture was incubated at ambient temperature for 10 min15 to quench the fluorescence from the extracellular-labelled bacteria. Trypan blue blocks fluorescence but cannot penetrate host cells. Therefore, fluorescence from internalised bacteria is not influenced by the addition of trypan blue. The rate of bacterial internalisation was determined by comparing the intensity of fluorescent cells infected with the various bacteria. Greater the fluorescence intensity indicates that more bacteria were phagocytosed by the LCs.

Binding of FITC-conjugated His-Mermaid and purified FLAG-Langerin to bacteria

Mermaid is secreted by L. inermis onto the posterior, bacteria-associated region of this marine nematode. The interaction of Mermaid with bacteria is thought to induce symbiont aggregation.16 FITC-conjugated His-Mermaid (FITC-His-Mermaid) was generated using the FITC Labeling Kit (Calbiochem Corp., San Diego, CA, USA) according to the manufacturer’s instructions and as described previously.14 E. coli CS180/CS1861 and Y. pestis KIM10/KIM10-·O-, suspended in phosphate-buffered saline at OD₆₀₀ = 0.02, were incubated with FITC-His-Mermaid or purified FLAG-Langerin at a concentration of 20 µg ml⁻¹ for 30 min. For FITC-His-Mermaid binding, the bacteria were washed once with phosphate-buffered saline before flow cytometry analysis. For Langerin binding, FITC-conjugated anti-FLAG antibody was added to the bacterial suspensions after one wash. The binding of FITC-His-Mermaid and purified FLAG-Langerin to the bacteria were measured based on fluorescence intensity using flow cytometry.

In vivo dissemination assay

The in vivo dissemination assay is similar to an assay we previously developed.8 KIM6- and KIM6-·O- (expressing O-antigen) were suspended in phosphate-buffered saline at OD₆₀₀ = 1.5. In all, 50 µl of the Yersinia suspension was injected into both of the hind paws of mice. The mice were also injected intravenously with ampicillin at a final concentration of 50 µg g⁻¹ body weight to maintain the plasmid-based expression of O-antigen. The mice were killed, and the subiliac lymph nodes were isolated 24 h post-injection. The isolated buboes were then homogenised and lysed with 1% Triton X-100 to release the bacteria prior to plating onto agar plates containing ampicillin. The total isolated CFU of the subiliac lymph nodes per mouse was defined as the dissemination rate.

Animal challenging

Y. pestis 1418 (KIM D27) and its O-antigen-expressing derivative Y. pestis 1418-·O- were inoculated at OD₆₀₀ = 0.2 in a similar manner to that described for the in vivo dissemination assay. The death of the mice was recorded every 12 h up to 156 h post-infection.

Statistical analyses

All statistical analyses were conducted using the Prism software, version 6 (Graph Pad, San Diego, CA, USA). Statistical analyses for the in vitro studies were performed using one-way analysis of variance and Newman–Keuls test. Statistical analyses for the in vivo studies were performed using Student’s t-test. Survival group comparison was performed via log-rank test using Kaplan–Meier analysis. P<0.05 was considered as the threshold for statistically significant differences.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Prentice MB, Gilbert T, Cooper A. Was the Black Death caused by Yersinia pestis? Lancet Infect Dis 2004; 4: 72.
2 Box KL, Schuenemann VJ, Golding GB, Burbano HA, Waglechner N, Coombes BK et al. A draft genome of Yersinia pestis from victims of the Black Death. Nature 2011; 478: 506–510.
3 Latheem WW, Price PA, Miller VL, Goldman WE. A plasmogin-activating protease specifically controls the development of primary pneumonic plague. Science 2007; 315: 509–513.
4 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 2015; 11: e1004587.
5 Iodoya J, Cheong C, Suda K, Nada S, Kim YJ, Lee H et al. Cutting edge: langerin/CD207 receptor on dendritic cells mediates efficient antigen presentation on MHC I and II products in vivo. J Immunol 2008; 180: 3647–3650.
6 Iodoya J, Suda N, Suda K, Park CG, Steinman RM. Antibody to Langerin/CD207 controls the development of primary pneumonic plague. Science 2007; 315: 509–513.
7 Eberl S, Ehammer Z, Holzmann S, Schwinghamder F, Forstner M, Stoltzner P et al. Expression of C-type lectin receptors by subsets of dendritic cells in human skin. Int Immunol 2004; 16: 877–887.
8 Viboud G, Bliska JB. Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis. Annu Rev Microbiol 2005; 59: 69–89.
9 Zhang SS, Park CG, Zhang P, Bartra SS, Ploegh HL. Human Langerhans cells capture measles virus through Langerin and present viral antigens. Proc Natl Acad Sci USA 2009; 106: 1524–1529.
10 Ebner S, Ehammer Z, Holzmann S, Schwinghamder F, Forstner M, Stoltzner P et al. Expression of C-type lectin receptors by subsets of dendritic cells in human skin. Int Immunol 2004; 16: 877–887.
11 de Witte L, Nabatov A, Pion M, Fliotis D, de Jong MA, de Grujil T et al. Langerin is a natural barrier to HIV-1 transmission by Langerhans cells. Nat Med 2007; 13: 367–371.
12 de Witte L, de Witte L, de Vries RD, Lijters M, de Jong MA, Fliotis D et al. Human Langerhans cells capture measles virus through Langerin and present viral antigens to CD4(+) T cells but are incapable of cross-presentation. Eur J Immunol 2011; 41: 2619–2631.
13 van der Vlist M, de Witte M, Geijtenbeek TB. Langerin functions as an antiviral receptor on Langerhans cells. Immunoother 2010; 88: 410–415.
14 Zhang P, Skurnik M, Zhang SS, Schwartz O, Kalyanasundarm R, Bulgheresi S et al. Human dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (CD209) is a receptor for Yersinia pestis that promotes phagocytosis by dendritic cells. Infect Immun 2008; 76: 2070–2079.
15 Zhang P, Snyder S, Feng P, Azadi P, Zhang S, Bulgheresi S et al. Role of N-acetylglucosamine within core lipopolysaccharide of several species of gram-
negative bacteria in targeting the DC-SIGN (CD209). J Immunol 2006; 177: 4002–4011.

15 Zhang P, Schwartz O, Pantelic M, Li G, Knazze Q, Noble C et al. DC-SIGN (CD209) recognition of Neisseria gonorrhoeae is circumvented by lipooligosaccharide variation. J Leukoc Biol 2006; 79: 731–738.

16 Klena J, Zhang P, Schwartz O, Hull S, Chen T. The core lipopolysaccharide of Escherichia coli is a ligand for the dendritic-cell-specific intercellular adhesion molecule nonintegrin CD209 receptor. J Bacteriol 2005; 187: 1710–1715.

17 Chen T, Bellard RJ, Wilson J, Swanson J. Adherence of plus-Opag gonococci to epithelial cells in vitro involves heparan sulfate. J Exp Med 1995; 182: 511–517.

18 Valenti P, Antonini G. Lactoferrin: an important host defence against microbial and viral attack. Cell Mol Life Sci 2005; 62: 2576–2587.

19 Ostoen PF. Expression of heterologous O-antigen in Yersinia pestis KIM does not affect virulence by the intravenous route. J Med Microbiol 2003; 52: 289–294.

20 Marketon MM, DePaolo RW, DeBord KL, Jabri B, Schneewind O. Plaque bacteria target immune cells during infection. Science 2005; 309: 1739–1741.

21 Geijtenbeek TB, Kwon DS, Torselina R, van Wiet SJ, van Duijnhoven GC, Middel J et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell 2000; 100: 587–597.

22 McDonald D, Wu L, Bahks SM, KawaiTamura VN, Unutma D, Hope TJ. Recruitment of HIV and its receptors to dendritic cell-T cell junctions. Science 2003; 300: 1295–1297.

23 Fahrbach KM, Barry SM, Ayehunie S, Lamore S, Klausner M, Hope TJ. Activated CD34− cells are a reservoir for HIV-1 in human bone marrow-derived Langerhans cells in vivo. Blood 2008; 112: 2256–2265.

24 Chatwell L, Holla A, Kaufer BB, Skerra A. The carbohydrate recognition domain of the proline-rich protein CRP-1 interacts with C1q. J Biol Chem 2007; 282: 13918–13925.

25 Thomas R, Brooks T. Attachment of Fcγ receptors to dendritic cells is mediated by the FcgammaRIIb receptor. FEBS Lett 2007; 581: 147–150.

26 Y. Yang, Y., M., Zhang Y, Higashide WM, McCormick BA, Chen J, Zhou D. The inactivation of mouse langerin/CD207. Mol Immunol 2006; 43: 369–379.

27 Gottfried RS. The Black Death: Natural and Human Disaster in Medieval Europe. Free Press: New York, USA, 1983.

28 de Witte L, Nabatov A, Geijtenbeek TB. Distinct roles for DC-SIGN+ dendritic cells and Langerhans cells in HIV-1 transmission. J Exp Med 2005; 203: 1741–17419.

29 Steinman RM. Dendritic cells in vivo: a key target for a new vaccine science. Immunol Today 2008; 29: 319–324.

30 Steinman RM. Decisions about dendritic cells: past, present, and future. Annu Rev Immunol 2012; 30: 1–22.

31 Welkos SL, Friedlander AM, Davis KJ. Studies on the role of plasminogen activator in systemic infection by virulent Yersinia pestis strain C922. Microb Pathog 1997; 23: 211–223.

32 Agar SL, Sha J, Foltz EM, Erova TE, Welter GB, Parham TE et al. Characterization of a mouse model of plague after aerosolization of Yersinia pestis C922. Microbiology 2008; 154: 1939–1948.

33 Gottfried RS. The Black Death: Natural and Human Disaster in Medieval Europe. Free Press: New York, USA, 1983.

34 Stumf J, Jann B, Jann K, Fortnagel P, Timmis KN. Genetic and biochemical analysis of Shigella dysenteriae 1 O antigen lipopolysaccharide biosynthesis in Escherichia coli K-12: structure and functions of the rfb gene cluster. Microb Pathog 1986; 1: 307–324.

35 Klena JD, Schnaitman CA. Function of the rfb gene cluster and the rfe gene in the synthesis of O antigen by Shigella dysenteriae 1. Mol Microbiol 1993; 9: 393–402.

36 Schnaitman CA, Klena JD. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol Rev 1993; 57: 655–682.

37 Fetherston JD, Schuetze P, Perry RD. Loss of the pigmentation phenotype in Yersinia pestis is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. Mol Microbiol 1992; 6: 2693–2704.

38 Barra SS, Sylar KL, D’O’Brien DM, Nilles ML, Hinnebusch BJ, Abalay A et al. Resistance of Yersinia pestis to complement-dependent killing is mediated by the All outer membrane protein. Infect Immun 2008; 76: 612–622.

39 Bulgheresi S, Schabosserova I, Chen T, Mullin NP, Maizels RM, Oll JA. A new C-type lectin similar to the human immunoreceptor DC-SIGN mediates siboution acquisition by a marine nematode. Appl Environ Microbiol 2006; 72: 2950–2956.

40 Park SH, Cheong C, Idoyaga J, Kim JY, Choi JH, Do Y et al. Generation and application of new rat monoclonal antibodies against synthetic FLAG and OLLAS tags for improved immunodetection. J Immunol Methods 2008; 331: 27–38.

41 Cheong C, Idoyaga J, Do Y, Park C, Park SH, Lee H et al. Production of monoclonal antibodies that recognize the extracellular domain of mouse langerin/CD207. J Immunol Methods 2007; 324: 48–62.

42 Tchou I, Sabido O, Lambert C, Miserly L, Garaud O, Genin C. Technique for obtaining highly enriched, quiescent immature Langerhans cells suitable for ex vivo assays. Immuno Lett 2003; 86: 7–14.

43 Y. Yang, Y., M., Zhang Y, Higashide WM, McCormick BA, Chen J, Zhou D. The inflammation-associated Salmonella SopA is a HECT-like E3 ubiquitin ligase. Mol Microbiol 2006; 62: 786–793.

44 Garcia E, Nedialkov YA, Elliot J, Motin VL, Brubaker RR. Molecular characterization of KatF (antigen 5), a thermoregulated chromosomally encoded catalase-peroxidase of Yersinia pestis. J Bacteriol 1999; 181: 3114–3122.

45 Skurnik M, Peippo A, Ervela E. Characterization of the O-antigen gene clusters of Yersinia pseudotuberculosis and the cryptic O-antigen gene cluster of Yersinia pestis shows that the plague bacillus is most closely related to and has evolved from Y. pseudotuberculosis serotype O:1b. Mol Microbiol 2000; 37: 316–330.

46 Isberg RR, Leong J. Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell 1990; 60: 861–871.

47 Klena JD, Ashford RS 2nd, Schnaitman CA. Role of Escherichia coli K-12 rfe genes and the rfp gene of Shigella dysenteriae 1 in generation of lipopolysaccharide core heterogeneity and attachment of O antigen. J Bacteriol 1992; 174: 7297–7307.