

## 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.<sup>1,2</sup> A hallmark of plague is the rapid dissemination of *Y. pestis*, which leads to systemic infection in susceptible individuals.<sup>3,4</sup>

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,<sup>5,6</sup> 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.<sup>7</sup>

Pathogens such as HIV exploit DC-SIGN-mediated uptake by APCs to efficiently disseminate throughout a host, travelling to the host's lymph nodes.<sup>8,9</sup> 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.<sup>10–12</sup>

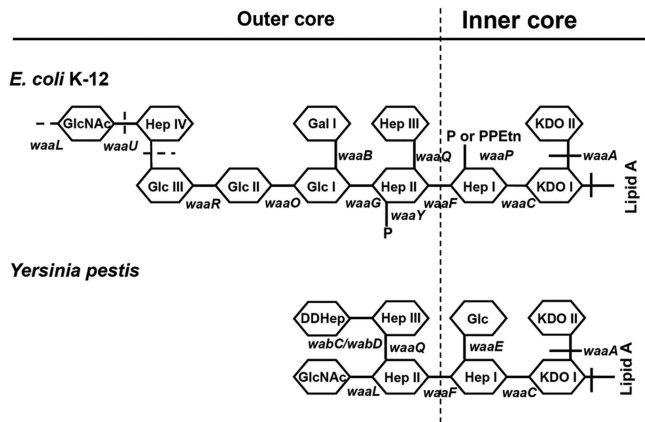
In this study, we investigated the interaction between Langerin and *Y. pestis* core oligosaccharides. Our results suggest that after overcoming the first line of host defence (the skin) via a fleabite, *Y. pestis*

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**Figure 1** Structures of the inner-core and outer-core regions of the core oligosaccharides of *E. coli* K-12 and *Y. pestis* and the genes involved in their synthesis. Genes encoding enzymes that are involved in the biosynthesis of core oligosaccharides are shown along with lines that indicate the approximate sites at which the enzymes exert their activity (solid line). Sites that are variably substituted or still under investigation are indicated by dashed lines. GlcNAc, *N*-acetyl-glucosamine; Glc, glucose; Hep, heptose; Gal, galactose; P, phosphate; PPEtn, phosphoethanolamine; KDO, 2-keto-3-deoxyoctonate. *Y. pestis* and *E. coli* K-12 naturally do not possess O-antigens.

may exploit LCs to spread throughout the host and consequently may trigger bacterial dissemination and infection.

## RESULTS

### *Y. pestis* invades LCs

One of the mechanisms by which *Y. pestis* invades human DCs involves its naturally exposed core oligosaccharides<sup>13</sup> and plasminogen activator.<sup>9</sup> 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.<sup>13–16</sup> In light of these findings, we assessed whether *Y. pestis* strains KIM10<sup>-</sup> (rough strain, exposed core oligosaccharides), KIM10<sup>-</sup>O<sup>+</sup> (smooth strain, core oligosaccharides covered with an O-polysaccharide) and KIM10<sup>-</sup>-core<sup>-</sup> (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 hDCs.<sup>13</sup> 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).<sup>13–16</sup> Both the *Y. pestis* and *E. coli* rough strains (KIM10<sup>-</sup> and CS180), but not the corresponding smooth strains (KIM10<sup>-</sup>O<sup>+</sup> and CS1861) or the corresponding deep rough strains (KIM10<sup>-</sup>-core<sup>-</sup> 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,<sup>7</sup> 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<sup>-</sup>, KIM10<sup>-</sup>O<sup>+</sup> and KIM10<sup>-</sup>-core<sup>-</sup>) 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*.<sup>9</sup> 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<sup>-</sup> 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<sup>-</sup> and CS180 but neither the smooth strains (KIM10<sup>-</sup>O<sup>+</sup> or CS1861) nor the deep rough strains (KIM10<sup>-</sup>-core<sup>-</sup> 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.<sup>13–16</sup>

### 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.<sup>9,17</sup> 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.<sup>14</sup> 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* lipopolysaccharides mutant isolates 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.<sup>13,14,16</sup>

### Inhibition of Langerin-mediated phagocytosis of *Y. pestis* by purified FLAG-Langerin, an anti-Langerin antibody, mannan, 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<sup>-</sup> by hcbLCs. However, when these two antibodies were combined, the phagocytosis of KIM10<sup>-</sup> by hcbLCs was significantly reduced. Nevertheless, this reduction was not complete, suggesting

**Table 1** Bacterial strains and cell lines used in this study

Strains	Genotypes (phenotypes)	References
<i>Y. pestis</i>		
KIM6 <sup>+</sup>	Wild type (rough)	37
KIM6 <sup>+</sup> -O <sup>+</sup>	KIM6 <sup>+</sup> expressing O-antigen (smooth)	19
KIM10 <sup>-</sup> (KIM10-Δ <i>ail</i> )	Derivative of KIM5 in which the <i>ail</i> gene has been deleted and both plasmid pCD1 and pPCP1 have been cured	13,38
KIM10 <sup>-</sup> -O	KIM10 <sup>-</sup> -O-antigen	19
KIM10 <sup>-</sup> -core <sup>-</sup> ( <i>gmhA</i> -deleted KIM10 <sup>-</sup> )	Deep rough mutant derivative of KIM10 <sup>-</sup> , the <i>gmhA</i> allelic exchange plasmid pCBD41 was mobilised from <i>E. coli</i> SM10λpir/pCBD41 into KIM10 <sup>-</sup>	This study
<i>Y. pestis</i> 1418	KIM D27 (Lcr <sup>+</sup> , <i>pgm</i> <sup>-</sup> , <i>pst</i> <sup>+</sup> )	46,47
<i>Y. pestis</i> 1418-O <sup>+</sup>	<i>Y. pestis</i> 1418 expressing O-antigen (smooth)	This study
<i>Y. pseudotuberculosis</i>		
Y1	O:1a, wild-type expressing invasin but with pYV plasmid naturally cured (smooth)	17,48
<i>E. coli</i> K-12		
CS180	Wild-type (rough)	35,36,49
CS1861	CS180-O-antigen	35,36,49
CS2429	<i>waaC</i>	35,36,49
CS2198	<i>waaR</i>	35,36,49
CS2488	<i>waaO</i>	35,36,49
CS1943	<i>waaG</i>	35,36,49
<i>Cell lines</i>		
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	

that additional receptors for *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 *Y. pestis* phagocytosis by hLCs (Figure 5d). This suggests that the hLangerin receptor has a major role in the interaction between LCs and *Y. pestis*.

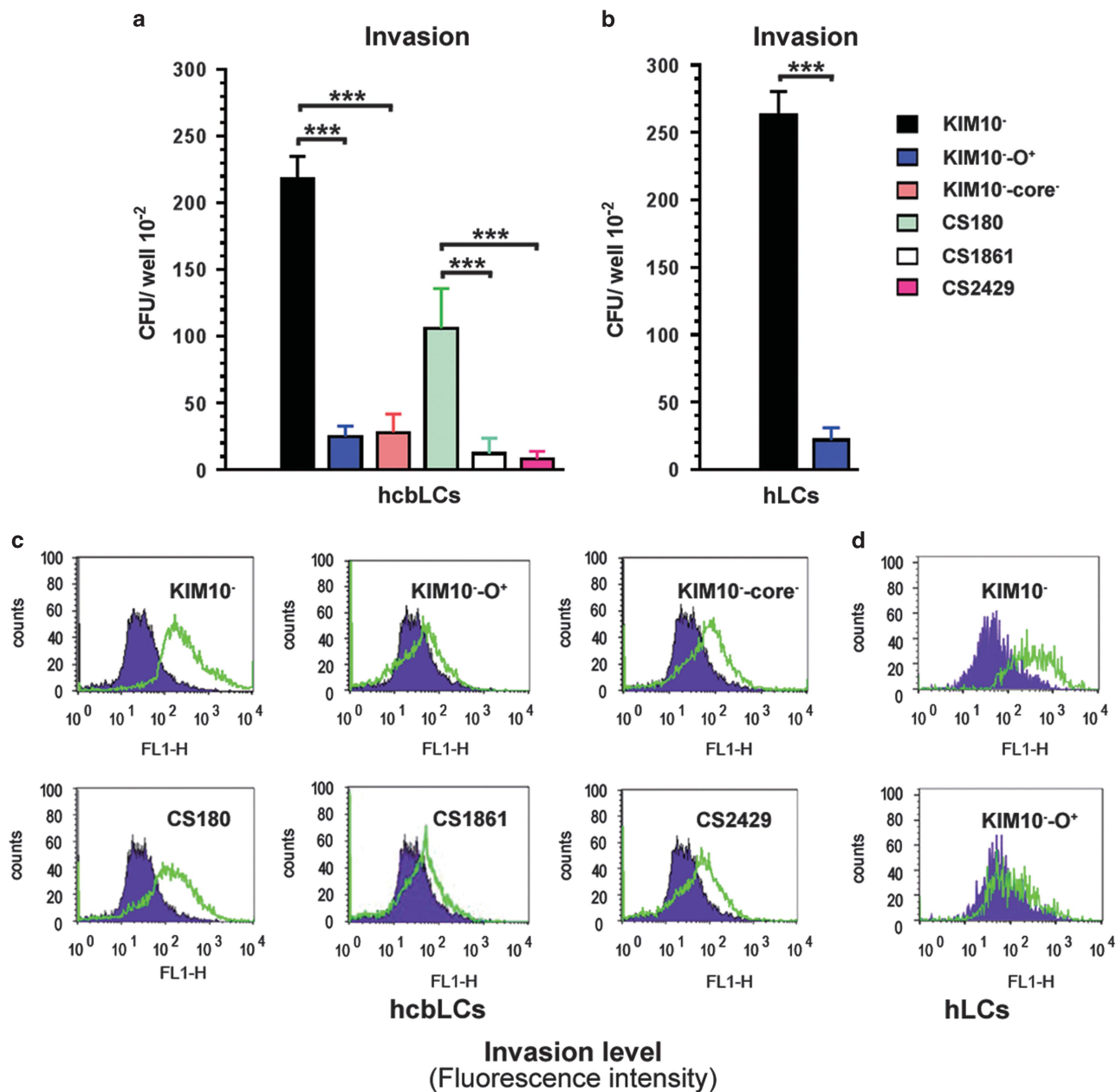
Purified FLAG-Langerin, mannan (an antagonist of mannose receptors), several oligosaccharides, a DC-SIGN-like molecule (Mermaid) and the anti-hLangerin antibody were tested to determine whether any of these molecules could block the interaction between *Y. pestis* and CHO-hLangerin cells. A specific 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.<sup>13,14,16</sup> *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).

Purified FLAG-Langerin, mannan, several oligosaccharides (Gal-GalNAc, GlcNAc-Gal-Glc (A8297), Gal-GlcNAc (A7916) and especially the 3'-Sialyl-Lewis-X tetrasaccharide, (S1782)), His-Mermaid and one single-sugar residue (GlcNAc) inhibited the interaction between KIM10<sup>-</sup> and CHO-hLangerin cells (Figure 5e;

Table 1). In addition, the anti-hLangerin antibody appeared to inhibit the interaction between KIM10<sup>-</sup> and CHO-hLangerin cells compared with the ability of the anti-Langerin antibody to inhibit the *Y. pestis*-hLC interaction. The recovery rates of Y1 and KIM10<sup>-</sup> 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.<sup>18</sup>

#### Purified FLAG-Langerin and DC-SIGN-like molecules bind to rough but not smooth *Y. pestis*

Purified FLAG-Langerin and His-Mermaid have previously been shown to inhibit the interaction between *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<sup>-</sup> and KIM10<sup>-</sup>-O<sup>+</sup> were tested. *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<sup>-</sup> and CS180 than to KIM10<sup>-</sup>-O<sup>+</sup> and CS1861, indicating that these two molecules directly interact with the core oligosaccharides of *Y. pestis*.

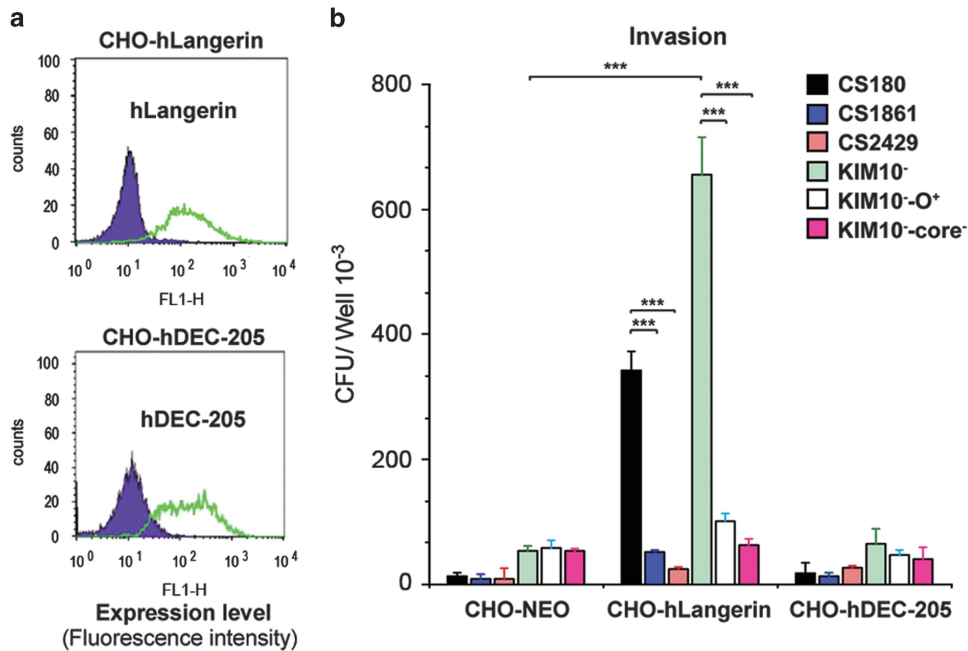


**Figure 2** *Y. pestis* core oligosaccharides interact with hcbLCs or hLCs. (a and b) Gentamicin protection and (c and d) flow cytometry assays were used to determine the invasion rates of two sets of the Gram-negative bacteria *Y. pestis* (KIM10<sup>-</sup>, KIM10<sup>-</sup>O<sup>+</sup> and KIM10<sup>-</sup>-core<sup>-</sup>) and *E. coli* K-12 (CS180, CS1861 and CS2429) into hcbLCs or hLCs. These *Y. pestis* and *E. coli* K-12 strains are also described in Zhang *et al.*<sup>13</sup> (a and b) The data presented were pooled from three independent experiments. The data represent the means  $\pm$  s.e.m.  $N=9$ . Statistical analysis was performed using one-way analysis of variance and the Newman-Keuls test. \*\*\* $P<0.001$ . (c and d) Labelled and unlabelled bacteria are indicated by open and filled symbols, respectively.

**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.<sup>9,13</sup> 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.<sup>9,13</sup> 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





**Figure 3** *Y. pestis* invades CHO-hLangerin cells but not other transfectants. The expression levels of each of the CHO transfectants are shown in panel (a), with the transfectants and CHO-NEO cells represented by open and filled curves, respectively. The phagocytosis (b) of two sets of bacteria *E. coli* K-12 (CS180, CS1861 and CS2429) and *Y. pestis* (KIM10<sup>-</sup>, KIM10<sup>-O+</sup> and KIM10<sup>-core-</sup>) by CHO-hLangerin and CHO-hDEC-205 cells were analysed. Bacteria and CHO transfectants were incubated together for 2 h, and extracellular bacteria were killed with 100 µg ml<sup>-1</sup> (final concentration) gentamicin. CHO-NEO cells were used as the negative control cell line. The number of phagocytosed bacteria was determined by counting CFU recovered following gentamicin treatment. 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.

intravenously, indicating that *Y. pestis* uptake by skin APCs mediate *Y. pestis* dissemination.<sup>19</sup> 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<sup>20</sup> and express the major C-type lectin receptor Langerin instead of DC-SIGN.<sup>7</sup> 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<sup>+</sup> lymphocytes target cells located in the lymph nodes.<sup>21–23</sup>

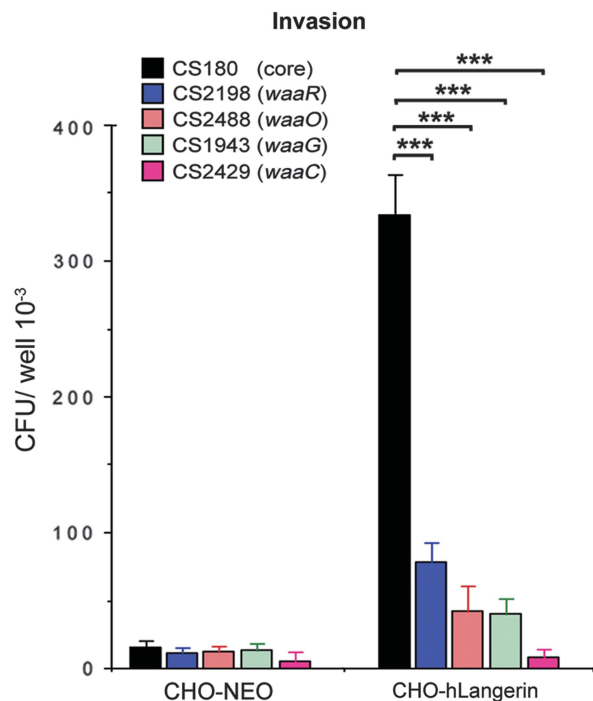
The interaction of *Y. pestis* with hDC-SIGN from monocyte-derived dendritic cells (MDDCs) supports this idea.<sup>13</sup> The interaction of *Y. pestis* with MDDCs can be reduced by combined treatment with anti-hDC-SIGN and anti-hLangerin antibodies, suggesting that hLangerin could act as a redundant receptor for *Y. pestis* in MDDCs.<sup>13</sup> The commercially available LCs employed in this study were generated from human cord blood cells and express both Langerin 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*.<sup>13,14</sup>

Several Gram-negative bacteria,<sup>14–16</sup> including *Y. pestis*,<sup>13</sup> 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.*,<sup>24</sup> 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.<sup>25</sup> Several Gram-negative bacteria, including *Y. pestis*, might utilise their core oligosaccharides to interact with hDC-SIGN.<sup>13,14</sup> <sup>26</sup> In addition, HIV uses the gp120–DC-SIGN interaction to initiate capture by DCs and transmission to CD4<sup>+</sup> T cells.<sup>21</sup> 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.<sup>27</sup> Our studies confirm that some oligosaccharides inhibit the interactions between *Y. pestis* and transfectants expressing hDC-SIGN<sup>13</sup> 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,<sup>28</sup> 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,<sup>13–16</sup> and thus their analysis may shed light on HIV–host interactions.



**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  $\pm$  s.e.m.  $N=9$ . Statistical analysis was performed using one-way analysis of variance and the Newman-Keuls test. \*\*\* $P<0.001$ .

Most cells that express C-type lectins function in innate immunity.<sup>29,30</sup> 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.<sup>10–12</sup> However, the LD<sub>50</sub> 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.<sup>31,32</sup> Based on these observations, it is not surprising that *Y. pestis* caused the death of over one-third of the European population during the Black Death epidemic.<sup>33</sup> Therefore, we speculate that the natural barrier function provided by Langerin against certain viruses does not apply to dissemination and infection by *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 synthesises 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* serotype 1 O-antigen.<sup>34–36</sup> 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: CS2198 (*waaR*), CS2488 (*waaO*), and CS1943 (*waaG*) (Figure 1)<sup>35,36</sup> (Table 1).<sup>22,24</sup> The *E. coli* strains were cultured on Luria-Bertani agar. *Y. pseudotuberculosis* (Y1) is a serotype O:1a strain that lacks the virulence plasmid (pYV). The *Y. pestis* strains used in this study originated from KIM5 (KIM D27), a strain in which the *pgm* (pigmentation) gene has been deleted.<sup>37</sup> These strains are avirulent when delivered by peripheral routes of infection. KIM10-*Δail* is a KIM5 derivative in which the *ail* gene has been deleted<sup>38</sup> and the pCD1 and pPCP1 plasmids have been cured.<sup>13,38</sup> To simplify the nomenclature, KIM10-*Δail* has been abbreviated as KIM10<sup>-</sup> throughout this manuscript. KIM10<sup>-</sup>O<sup>+</sup> expresses an O-antigen from *Y. enterocolitica* serotype O:3<sup>19</sup> and is an isogenic derivative of KIM10<sup>-</sup>. KIM10<sup>-</sup>core<sup>-</sup> is also an isogenic derivative of KIM10<sup>-</sup>, in which the outer core oligosaccharides have been deleted as described below (Table 1). The *Yersinia* strains were cultured on GC-based plates (Difco, Sparks, MD, USA) supplemented with 1% haemoglobin (USB Co., Cleveland, OH, USA). All strains of *Yersinia* spp. used in this study were cultured at 26 °C.

## Reagents

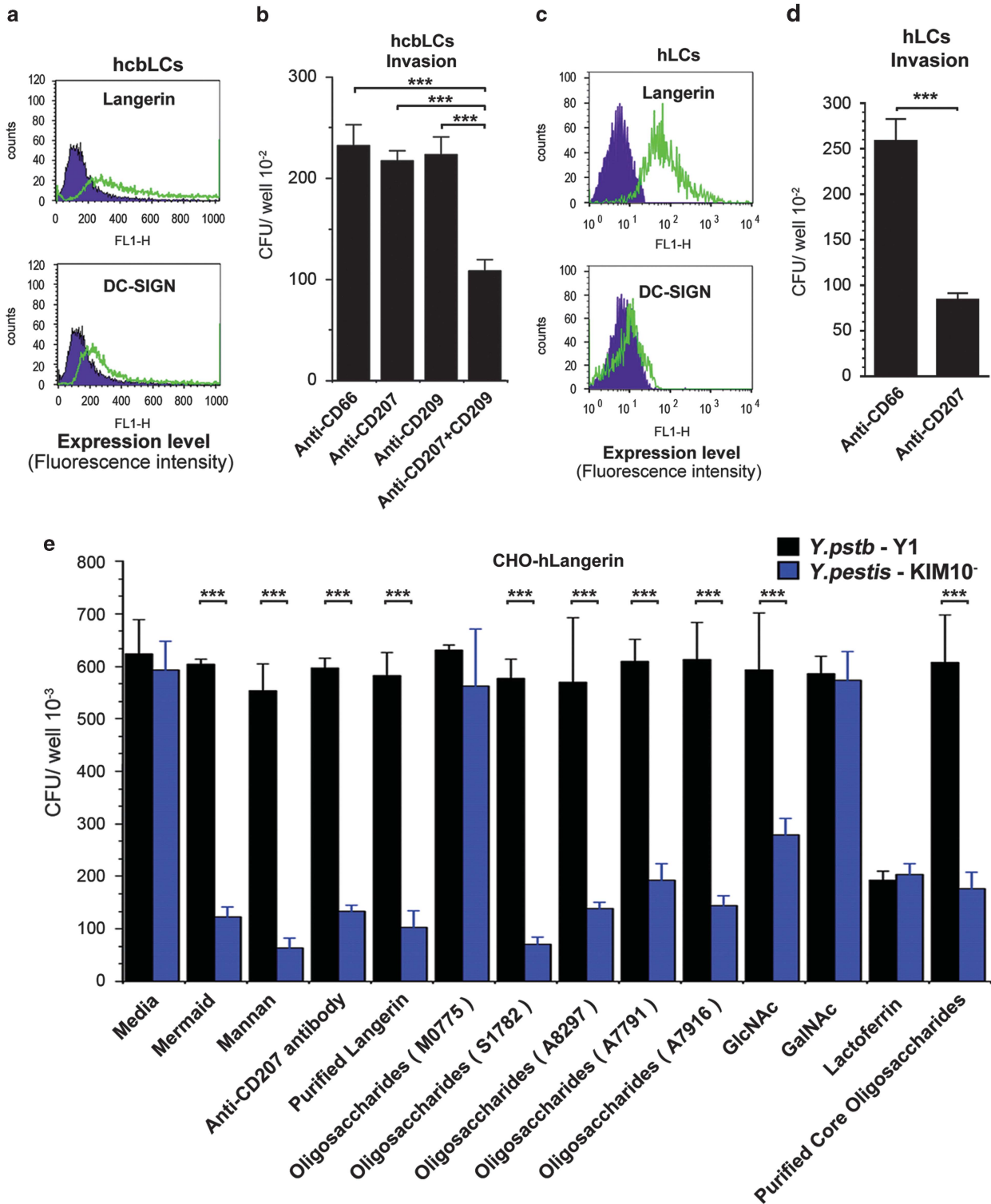
(1) Anti-hLangerin and anti-DC-SIGN antibodies were purchased from Pharmingen (San Diego, CA, USA). YTH71.3, a rat antibody that recognises CEACAM1 (CD66a), CEACAM6 (CD66c) and CEACAM3 (CD66d) was purchased from Roche (Indianapolis, IN, USA). The anti-FLAG antibody conjugated to FITC was purchased from Sigma (St Louis, MO, USA). (2) The following oligosaccharides and mannan were purchased from Sigma:  $\beta$ -D-GlcNAc-(1  $\rightarrow$  3)- $\beta$ -D-Gal-1  $\rightarrow$  OMe {Methyl 3-O-(N-acetyl- $\beta$ -D-glucosaminyl)- $\beta$ -D-galactopyranoside, M0775},  $\alpha$ -NeuNAc-(2  $\rightarrow$  3)- $\beta$ -D-Gal-(1  $\rightarrow$  4) ( $\alpha$ -L-Fuc-[1  $\rightarrow$  3])-D-GlcNAc {3'-Sialyl-Lewis-X tetrasaccharide, S1782},  $\beta$ -D-GlcNAc-(1  $\rightarrow$  6)- $\beta$ -D-Gal-(1  $\rightarrow$  2)-D-Glc { $\beta$  6'-GlcNAc-lactose, A8297},  $\beta$ -D-Gal-(1  $\rightarrow$  4)-D-GlcNAc {N-Acetyl-D-lactosamine, A7791} and  $\beta$ -D-Gal-(1  $\rightarrow$  6)-D-GlcNAc {2-Acetamido-2-deoxy-6-O-( $\beta$ -D-galactopyranosyl)-D-glucopyranose, A7916}. (3) The monosaccharides, GlcNAc and GalNAc, were purchased from Sigma. (4) Mermaid is a DC-SIGN-like molecule expressed by the marine nematode *Laxus oneistus*. The carbohydrate recognition domain of Mermaid shares both structural and functional similarity with that of DC-SIGN.<sup>39</sup> A recombinant form of Mermaid (His-Mermaid) was expressed and purified as described previously.<sup>39</sup> (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.<sup>40,41</sup> (6) Purified core oligosaccharides from an *N. gonorrhoeae* *igtB* 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.<sup>14</sup> In addition, our unpublished data have shown that the purified core oligosaccharides from KIM10<sup>-</sup> 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.<sup>14</sup>

## Human cord blood LCs

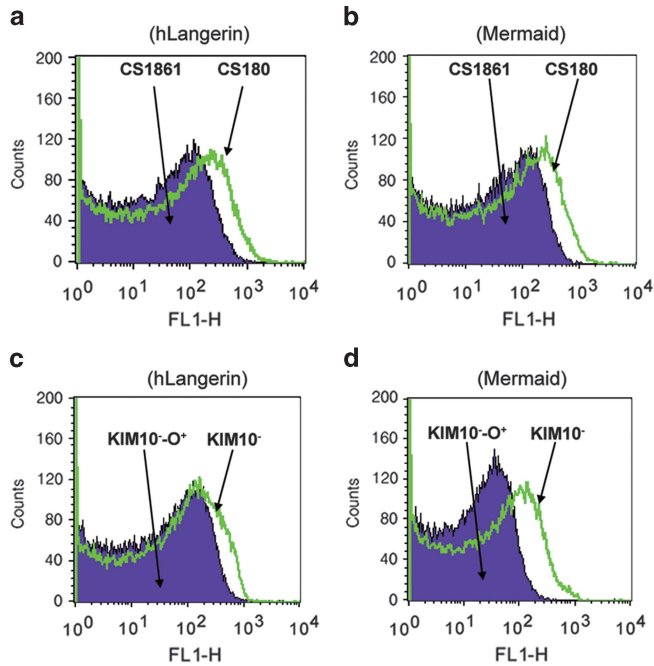
hcbLCs derived from human cord blood cells were purchased from MatTek Corporation (Ashland, MA, USA). However, the methods used to generate these LCs are proprietary and are not available from the manufacturer. Based on the information provided by the manufacturer, this LC line expresses Langerin, which was confirmed in Figure 5a. Although previous studies have suggested that LCs usually do not express DC-SIGN,<sup>7</sup> 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.<sup>42</sup> Skin slices were then incubated with 0.05% trypsin containing EDTA in Hanks buffer without Ca<sup>2+</sup> and Mg<sup>2+</sup> 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.



**Figure 6** Purified FLAG-hLangerin and DC-SIGN-like molecules bind to *Y. pestis* core oligosaccharides. *Y. pestis* KIM10<sup>-</sup>, KIM10<sup>-</sup>O<sup>+</sup>, CS180 and CS1861 were incubated with 10  $\mu$ g 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<sup>-</sup> or CS180 and KIM10<sup>-</sup>O<sup>+</sup> 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.<sup>14,45</sup>

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.<sup>42</sup> CD1a and CD207 monoclonal antibodies were used to determine the purity or the expression levels of freshly isolated hLCs.

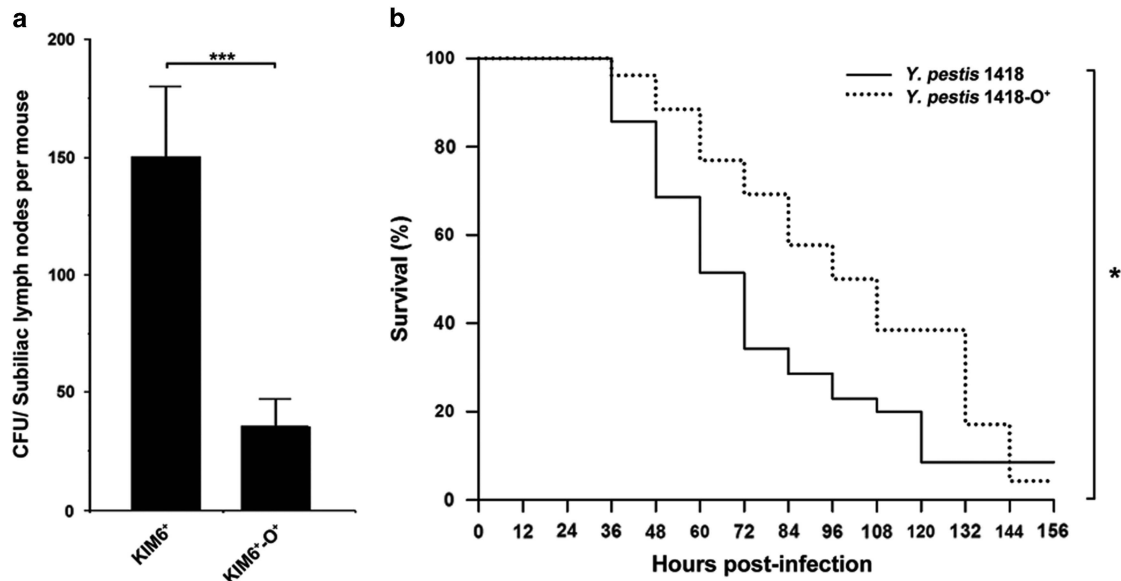
#### Human C-type lectin transfectants

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<sup>-1</sup>) selection and screening for stable surface expression, as illustrated in Figure 3a.<sup>43</sup> 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.<sup>44</sup> Briefly, host cells (hbcLCs, 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  $4 \times 10^5$  ml<sup>-1</sup> (hLCs at a concentration of  $8 \times 10^4$  ml<sup>-1</sup>). In all, 500  $\mu$ l of each of these cell suspensions was added to 24-well plates, and after the addition of 50  $\mu$ l of bacterial suspensions at a concentration of  $5 \times 10^6$  CFU ml<sup>-1</sup>, the cells were allowed to incubate for 2 h at 37°C in the presence of 5% CO<sub>2</sub>.

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  $\mu$ g ml<sup>-1</sup>, 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 7** Expression of *Y. pestis* O-antigen reduces pathogen dissemination and host death. (a) KIM6<sup>+</sup> and KIM6<sup>+</sup>-O<sup>+</sup> (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  $\pm$  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<sup>+</sup>. 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  $\pm$  s.e.m.

For the inhibition assay, purified FLAG-Langerin ( $100 \mu\text{g ml}^{-1}$ ), anti-hLangerin ( $5 \mu\text{g ml}^{-1}$ ) antibody, anti-DC-SIGN ( $5 \mu\text{g ml}^{-1}$ ) antibody and carbohydrates, including core oligosaccharides ( $500 \mu\text{g ml}^{-1}$ ), oligosaccharides ( $500 \mu\text{g ml}^{-1}$ ), a DC-SIGN-like protein ( $10 \mu\text{g ml}^{-1}$ ) or mannan ( $500 \mu\text{g ml}^{-1}$ ), 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.<sup>13,14</sup>

#### 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.<sup>13,15</sup> 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 min<sup>15</sup> 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. oneistus* onto the posterior, bacteria-associated region of this marine nematode. The interaction of Mermaid with bacteria is thought to induce symbiont aggregation.<sup>39</sup> 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.<sup>14</sup>

*E. coli* CS180/CS1861 and *Y. pestis* KIM10<sup>-</sup>/KIM10<sup>-</sup>-O<sup>+</sup>, suspended in phosphate-buffered saline at OD<sub>600</sub>=0.02, were incubated with FITC-His-Mermaid or purified FLAG-Langerin at a concentration of  $20 \mu\text{g ml}^{-1}$  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.<sup>9</sup> KIM6<sup>+</sup> and KIM6<sup>+</sup>-O<sup>+</sup> (expressing O-antigen) were suspended in phosphate-buffered saline at OD<sub>600</sub>=1.5. In all, 50  $\mu\text{l}$  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  $50 \mu\text{g g}^{-1}$  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<sup>+</sup> were inoculated at OD<sub>600</sub>=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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*Author contributions:* KY, CGP, GVP, MS, JDK and TC conceived and designed the experiments. KY, CC, SB, SZ, PZ, YXH, LYJ, HPH, HHD, LXX and SSB performed the experiments. KY, CGP, CC, SB, GVP, JDK and TC analysed the data. CGP, CC, SB, YPW, SGW, LZ, AYL, SSB, GVP and JDK contributed reagents/materials/analysis tools. KY, CGP, CC, SB, SSB, PZ, GVP, JDK and TC wrote the manuscript.

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