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Plasminogen Activator Pla of *Yersinia pestis* Utilizes Murine DEC-205 (CD205) as a Receptor to Promote Dissemination*

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***Yersinia pestis*, a Gram-negative bacterium that causes bubonic and pneumonic plague, is able to rapidly disseminate to other parts of its mammalian hosts. *Y. pestis* expresses plasminogen activator (PLA) on its surface, which has been suggested to play a role in bacterial dissemination. It has been speculated that *Y. pestis* hijacks antigen-presenting cells, such as macrophages (MΦs) and dendritic cells, to be delivered to lymph nodes to initiate dissemination and infection. Both alveolar MΦs and pulmonary dendritic cells express a C-type lectin receptor, DEC-205 (CD205), which mediates antigen uptake and presentation. However, no ligand has been identified for DEC-205. In this study, we show that the invasion of alveolar MΦs by *Y. pestis* depends both *in vitro* and *in vivo* on the expression of PLA. DEC-205-expressing MΦs and transfectants, but not their negative counterparts, phagocytosed PLA-expressing *Y. pestis* and *Escherichia coli* K12 more efficiently than PLA-negative controls. The interactions between PLA-expressing bacteria and DEC-205-expressing transfectants or alveolar MΦs could be inhibited by an anti-DEC-205 antibody. Importantly, the blockage of the PLA-DEC-205 interaction reduced the dissemination of *Y. pestis* in mice. In conclusion, murine DEC-205 is a receptor for PLA of *Y. pestis*, and this host-pathogen interaction appears to play a key role in promoting bacterial dissemination.**

Yersinia pestis is the Gram-negative bacterium that causes bubonic and pneumonic plague. *Y. pestis* has evolved directly from *Yersinia pseudotuberculosis* within the last 10,000–20,000 years (1, 2). The *Yersinia* genus is composed of 12 species, and 3 species of *Yersinia* are pathogenic to humans (3, 4). *Y. pseudotuberculosis* and *Yersinia enterocolitica* cause mild enteric diseases, whereas the hallmark of *Y. pestis* is its rapid dissemination in the mammalian host resulting in bubonic and pneumonic plague and high mortality.

All three pathogenic *Yersinia* share a virulence plasmid, pCD1 (pYV), which is essential for pathogenesis (5, 6). This plasmid encodes a type III secretion system (7), YadA {an adherence and virulence surface molecule, although *yadA* is a pseudogene in *Y. pestis* (8, 9)}, and a number of secreted effector proteins that inhibit bacterial phagocytosis and specific innate immune responses (10).

Y. pestis harbors two additional plasmids, pPCP1 (9.6 kb) and pMT1 (pFra) (102 kb), which encode the plasminogen activator (PLA)[3] and the F1 protein, respectively. The products of these genes are necessary for tissue invasion (11), capsule formation (12), and infection of the plague flea vector (13, 14). Capsule formation by *Y. pestis* has been proposed to confer anti-phagocytic capabilities (15, 16). PLA is an outer membrane protease that also plays a role in bacterial attachment and invasion of one eukaryotic epithelial cell line, but the invasive function does not involve its proteolytic activity (17). Recent studies have further confirmed the important roles of PLA in the progression of bubonic and pneumonic plague, in which PLA contributes to dissemination of *Y. pestis*, especially to bubonic plague (14, 18).

Lipopolysaccharide (LPS) of many Gram-negative bacterial pathogens mediates toxicity and resistance to serum killing and phagocytosis and generally consists of three structural regions as follows: (i) the lipid A backbone, (ii) an oligosaccharide core (core LPS), and (iii) O-antigen (19, 20). However, *Y. pestis* does not produce an O-antigen (21, 22). The shortened LPS is also referred to as lipo-oligosaccharide and is presumably exposed to the external environment. In general, Gram-negative bacteria are classified as smooth or rough based on the presence or lack of the O-antigen, respectively. An interesting observation

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[3] The abbreviations used are: PLA, plasminogen activator; MΦ, macrophage; APC, antigen-presenting cell; DC, dendritic cell; LPS, lipopolysaccharide; CFU, colony-forming unit; FCS, fetal calf serum; CHO, Chinese hamster ovary; HIV-1, human immunodeficiency virus, type 1.

supports the idea that *Yersinia* spp. expressing O-antigen blocks enzymatic activity of PLA (23). Therefore, it is also possible that expression of O-antigen in *Y. pestis* could physically shield interactions between PLA and potential host receptors.

Antigen-presenting cells (APCs) such as macrophages (MΦs) play an essential role in the host defense against invading pathogens and in the control and maintenance of innate and adaptive immunity (24, 25). Innate immune functions are initially carried out by the ability of APCs to phagocytose and kill invading pathogens or to deliver them to other types of host immune cells for further elimination of the pathogens. APCs express members of the C-type lectin family. For example, alveolar MΦs express a C-type lectin receptor, DEC-205 (CD205). Although DEC-205 participates in an important role in the antigen-presenting process (26, 27), no ligand for this receptor has yet been identified.

Some pathogens may pirate innate immune receptors for their dissemination. It has been speculated that after entering the host via a bite from an infected flea (10), *Y. pestis* may use innate immune receptors present on APCs to traffic to lymph nodes where they encounter host lymphocytes (10). This hypothesis is reminiscent of the mechanism HIV-1 uses to target APCs. HIV-1 may hijack another C-type lectin receptor, DC-specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN, CD209), so as to be captured and trafficked to target cells such as CD4 lymphocytes in lymph nodes (28–30). Human DC-SIGN is also expressed by a certain class of MΦs (31), including human alveolar MΦs.

Recently, we demonstrated that human DC-SIGN and mouse SIGN-R1 can be used as receptors for the core LPS of *Y. pestis*, promoting bacterial adherence and phagocytosis to human DCs and murine MΦs (20).[4] Based on these studies, we have hypothesized that after *Y. pestis* enters the lungs through aspiration, it invades alveolar MΦs via core LPS-DC-SIGN-SIGN-R1 interaction (20).[4] In this study, we explored whether *Y. pestis* can exploit this interaction to be disseminated to spleens in a mouse model. Unexpectedly, we found that in fact PLA can also mediate the binding of *Y. pestis* with host DEC-205, and the interaction may play a role in bacterial dissemination.

EXPERIMENTAL PROCEDURES

Bacterial Strains—Escherichia coli K12 strain CS180 contains core LPS but lacks O-antigen (33). CS1861 is an isogenic strain of CS180 harboring pSS37, a plasmid containing all the genes necessary for the expression of the *Shigella dysenteriae* 1 O-antigen (33–35) (Table 1). *E. coli* strains were cultured on Luria-Bertani medium (LB) supplemented with 1.5% agar at 37 °C overnight.

Y. pseudotuberculosis (Y1) is a serotype O:1a strain and lacks the virulence plasmid (pYV) and does not express the Ail protein.[5] The strain was obtained from the CDC and used as a control strain for invasion (20, 36), because this bacterium invades almost all epithelial cell lines via an invasin-integrin interaction (37).

All strains of *Y. pestis* used in this study are derived from the KIM strain (38). There are nine derivatives of *Y. pestis* used in this study as follows. 1) KIM6$^+$ was derived from wild-type KIM by curing the virulence plasmid (pCD1 or pYV). 2) KIM10$^+$ was derived from KIM6$^+$ by curing the pPCP plasmid. 3) KIM6$^-$ is a derivative of KIM6$^+$ obtained after spontaneous deletion of the *pgm* (pigmentation) locus (38). 4) KIM10$^-$ is a derivative of KIM6$^+$ obtained after curing the pPCP plasmid and deletion of the *pgm* locus. 5) KIM6$^+$-O$^+$ is KIM6$^+$/pAY100.1 that expresses the O-antigen of *Y. enterocolitica* serotype O:3 from the O-antigen gene cluster cloned in plasmid pAY100.1 (39). 6) KIM10$^+$-PLA is the KIM10$^+$ containing a plasmid that expresses the PLA. 7) KIM10$^-$-Δail is the KIM10$^-$, whose *ail* gene is deleted (40). 8) KIM6$^+$-pBR322 is KIM6$^+$ that carries the pBR322 plasmid. 9) KIM10$^+$-pBR322 is KIM10$^+$ that carries pBR322 (Table 1). Strains were cultured at 26 °C on GC-based plates (Difco) supplemented with 1% hemoglobin (U. S. Biochemical Corp.) (20).

Mice—BALB/CJ mice were bred at the University of Illinois at Chicago-Rockford animal facilities and used in this study. BALB/CJ mice were selected, because of the expression of SIGN-R1 and DEC-205 in peritoneal MΦs and alveolar MΦs, respectively, in these mice (41–43).

Biological Reagents—Anti-mouse DEC-205, anti-mouse SIGN-R1, and anti-human DC-SIGN antibodies were purchased from SeroTec (Raleigh, NC). YTH71.3, a rat antibody that recognizes CEACAM1 (CD66a), CEACAM6 (CD66c), and CEACAM3 (CD66d), was purchased from Roche Applied Science.

Isolation of Mouse Peritoneal and Alveolar Macrophages—After euthanizing a mouse, its intact abdomen was exposed, cleaned with 70% ethanol, and opened. 5 ml of RPMI medium was injected into the intraperitoneal cavity. The mouse abdomen was gently massaged for 3 min, and then the lavage fluid was collected. The suspension containing the peritoneal MΦs was seeded in flasks and placed in a CO_2 incubator for 2 h. The cell layers were washed three times to remove nonadherent cells. MΦs were removed from the plastic surface by incubating with citrate saline and re-seeded for interaction assays or stained with antibodies to check the expression level of receptors.

Alveolar MΦs were obtained using the following procedures. After euthanizing a mouse, its bronchial tract was opened, and 1 ml of RPMI medium was injected into the lungs via a tiny tube. The mouse chest was gently massaged for 3 min, and then the lavage fluid was collected. The purification processes followed the same process as the peritoneal MΦs.

Host Cell Lines—Mouse C-type lectin transfectants, CHO-mDC-SIGN, CHO-mSIGN-R1, CHO-mSIGN-R3, and CHO-mDEC-205 (CD205), were generated by transfecting CHO cells with mouse corresponding C-type lectin cDNAs, followed by selection for stable surface expression as originally described (41). CHO-NEO was used as a control cell line, which expresses the neomycin resistance gene only.

Adherence and Phagocytosis Assays—The assays for adherence and phagocytosis have been described previously (44, 45).

[4] S. Zhang, C. G. Park, S. S. Bartra, A. Shetty, P. Zhang, G. Zheng, S. Bulgheresi, Z. Yu, G. V. Plano, J. Klena, M. Skunilk, J. Hinnebusch, and T. Chen, submitted for publication.

[5] T. Chen, unpublished data.

Briefly, host cells (CHO, HeLa, dendritic cells, or macrophages) were plated in 24- or 96-well plates. Cells were suspended in RPMI medium with 2% FCS at a concentration of 4×10^5/ml. One-half ml of each of these cell suspensions were added to 24-well plates, and after addition of 50 μl of bacterial suspensions at a concentration of 1×10^7 colony-forming units (CFU)/ml, the cells were allowed to incubate for 2.5 h (2 h for dendritic cells and alveolar MΦs) at 37 °C in the presence of 5% CO_2. The cell monolayers were then washed three times with phosphate-buffered saline. The number of associated bacteria (adherent and internalized) per cell was quantified by washing the cells three times with RPMI medium containing 2% FCS and plating the culture after the cells were lysed by the addition of 0.5% saponin (Calbiochem).

To determine the internalization of bacteria, gentamicin, which kills extracellular bacteria but cannot penetrate into host cells, was added into each well to a final concentration of 100 μg/ml, and the cultures were incubated for 60 min. Cells were washed twice to remove the antibiotic. Then the cells were suspended in phosphate-buffered saline containing 0.5% saponin, diluted, and plated on LB as well as the *Y. pestis* plates. The level of internalization of bacteria in these host cells was calculated by determining the CFU recovered from lysed cells. All experiments were performed in triplicate, and each experiment was repeated three times. The data were expressed as mean ± S.E. Statistical significance was calculated using the Student's *t* test.

For the inhibition assay, anti-DEC-205 antibody was added 20 min prior to the addition of bacteria at a concentration of 5 μg/ml. The concentration used was based on our preliminary data and was selected based on the fact that at these concentrations there was no influence on the survival of bacteria and host cells, as previously and recently shown (19, 20, 46).

Determination of Phagocytosis by Flow Cytometry—The following method was used to supplement the survival-based phagocytosis assay described above because APCs are known to kill some phagocytosed bacteria (20, 47). Briefly, bacteria were suspended in RPMI medium containing 5- and 6-carboxyfluorescein diacetate, succinimidyl ester (CFDA-S.E.; Molecular Probes, Eugene, OR), for 40 min and washed twice with RPMI medium to remove the excess dye. Labeled bacteria were added to MΦ cultures for 2 h. Cell cultures were washed twice to remove unbound bacteria. MΦs plus 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 (47) to quench the fluorescence from extracellular labeled bacteria. Trypan blue blocks fluorescence but cannot penetrate host cells; therefore, fluorescence from internalized bacteria was not influenced by addition of trypan blue. The rate of bacterial internalization was determined by comparing the intensity of fluorescence-positive MΦs with various bacteria. The greater the fluorescence intensity, the more bacteria are phagocytosed by MΦs. All of the results were evaluated in triplicate.

In Vivo Phagocytosis Assays—After anesthetizing a mouse, its bronchial tract was opened, and about 20 μl of bacterial suspension ($A_{600} = 0.1$) was injected deep into the lungs using a tiny tube. After 2 h, mice were euthanized and alveolar MΦs were collected as described in the procedure for alveolar MΦs above. The numbers of cells in lavage fluid were counted for each collection. 1×10^5 mouse cells were seeded into each well of 96-well plates, containing RPMI medium with 2% FCS and gentamicin at a concentration of 100 μg/ml and were then incubated for 1.5 h to allow the MΦs to adhere to plates and kill the extracellular bacteria. Each well was washed three times with RPMI medium containing 2% FCS to remove nonadherent cells and lysed with saponin, following the same procedures as used in the *in vitro* phagocytosis assays.

Dissemination Assay—In this study, the dissemination was defined such that *Y. pestis* was transported from lungs to spleens. In detail, mice were inoculated with *Y. pestis* KIM6$^+$-pBR322, KIM6$^+$-O$^+$, KIM10$^+$-pBR322 and KIM10$^+$-PLA, at a concentration of $A_{600} = 1.5$ in phosphate-buffered saline (0.2 ml) via the intranasal route with a small tube after anesthesia (18, 48, 49). Mice were injected intravenously with ampicillin at the same concentration in each of following days. After the 1st day, mice were euthanized, and both the lungs and spleens were removed daily. Collected lungs and spleens were homogenized. The homogenized tissues were then lysed with 1% Triton X-100 to release the bacteria 10 min before they were plated onto agar plates containing ampicillin. The total isolated CFU per spleen was defined as the dissemination rate.

Three points should be noted. 1) 30 min before inoculation, mice were injected with ampicillin at a final concentration of 50 μg/g of mouse body weight to maintain the plasmid-based expression of O-antigen in KIM6$^+$-O$^+$ and PLA in KIM10$^+$-PLA. The rationale for the high dose of bacteria ($\sim 10^9$/ml) in the inoculum is discussed in depth under "Discussion." 2) To meet biosafety and regulatory requirements, the tetracycline marker on the original pBR322 has been deleted. 3) The *Y. pestis* strains used in this study were cultured at 26 °C; at this temperature, *Y. pestis* does not produce the F1 capsule, which is capable of blocking interactions with host cells (15, 16, 20).

RESULTS

Y. pestis Invades Mouse Alveolar Macrophages—We recently showed that human DC-SIGN and mouse SIGN-R1 can be used as receptors for the core LPS of *Y. pestis*, promoting bacterial adherence and phagocytosis to human DCs and MΦs, including human alveolar MΦs and murine peritoneal MΦs (20).[4] Therefore, we have speculated that *Y. pestis* might utilize the interaction of core-LPS and SIGN-R1 to invade murine alveolar MΦs in the case of the mouse model. To investigate this hypothesis, *Y. pestis* KIM6$^+$ (a natural rough strain with the core LPS presumably exposed) and its isogenic derivative KIM6$^+$-O$^+$ (a smooth strain in which the outer core LPS is shielded by O-antigen) were examined for their ability to invade alveolar MΦs. Two corresponding *E. coli* K12 strains CS180 (rough) and CS1861 (CS180 expressing an O-antigen, smooth) were used as controls. We have used these sets of strains to demonstrate that exposure of the core-LPS of *E. coli* and *Y. pestis* is essential to initiate the interaction of core-LPS and DC-SIGN/SIGN-R1 (19, 20, 47, 50)[4] (Table 1). In addition, mouse peritoneal MΦs were used as control host cells that can bind *E. coli* and *Y. pestis* through the interaction of core LPS and SIGN-R1.[4] We reasoned that, like murine peritoneal MΦs,

TABLE 1
Bacterial strains used in this study

Strains	Genotypes + phenotypes	Refs.
***E. coli* K-12**		
CS180	Original type (rough)	33–35
CS1861	CS180 expressing O-antigen (smooth)	33–35
DH5α	Original type (rough), similar to CS180	14
DH5α-PLA	DH5α expressing the PLA	14
Y. pestis		
$KIM6^+$	The natural type (rough)	38
$KIM10^+$	$KIM6^+$ with pPCP1 plasmid cured	38
$KIM6^-$	$KIM6^+$ with deletion of *pgm* locus	38
$KIM10^-$	$KIM6^+$ with pPCP1 plasmid cured and deletion of *pgm* locus	38
$KIM6^+$-O^+	$KIM6^+$ expressing the O-antigen (smooth)	39
$KIM10^+$-PLA	$KIM10^+$ expressing the PLA	14, 38
$KIM10^-$-Δail	$KIM10^-$ with deletion of *ailC*	40
$KIM10^-$-Δail-PLA	$KIM10^-$-Δail expressing the PLA	14, 38, 40
$KIM6^+$-pBR322	$KIM6^+$ carrying pBR322 plasmid	This study
$KIM10^+$-pBR322	$KIM10^+$ carrying pBR322 plasmid	This study
Y. pseudotuberculosis		
Y1, O:1Aa	Wild-type expressing the invasin, but with naturally pYV plasmid cured and deletion of *ail* (smooth)	37

mouse alveolar MΦs might use the same mechanism to phagocytose *Y. pestis*. The results presented in Fig. 1 show three phenomena. 1) *Y. pestis* $KIM6^+$ invades alveolar and peritoneal MΦs equally well (Fig. 1*A*). 2) Phagocytosis of *Y. pestis* strain $KIM6^+$-O^+ by peritoneal and alveolar MΦs was reduced compared with *Y. pestis* strain $KIM6^+$ (Fig. 1, *A* and *B*), presumably as a result of expression of O-antigen. 3) The most interesting result was observed with our control infection using the *E. coli* strains. Peritoneal MΦs generated an effective phagocytic response to *E. coli* K12 CS180 (rough), but not to CS1861 (with expression of O-antigen), which was not the case when using alveolar MΦs. Both CS180 and CS1861 can only promote a limited phagocytic response in alveolar MΦs, indicating a clear difference in the interaction of rough Gram-negative bacteria with alveolar MΦs and peritoneal MΦs.

We have recently developed an *in vivo* phagocytosis assay using peritoneal MΦs,[4] which lead us to examine the *in vivo* phagocytosis of *Y. pestis* by alveolar MΦs. The *in vivo* phagocytosis of $KIM6^+$ and $KIM6^+$-O^+ (Fig. 1*C*) shows a similar pattern to the *in vitro* phagocytosis (Fig. 1*A*), although the numbers of recovered bacteria were much lower *in vivo*. We believe that the low recovery of bacteria *in vivo* is because of the technical difficulty in achieving high concentrations of bacteria in the lungs of live mice. It should be noted that the concentration of inoculating bacteria was 100 times higher, in comparison with *in vitro* invasion assay. For this experiment, we did not use CS180/CS1861, because *E. coli* K12 are very sensitive to serum killing. In short, the results indicate that the phagocytosis of *Y. pestis* by alveolar MΦs is involved with the molecule(s) other than core LPS on *Y. pestis*.

DEC-205, Expressed on Mouse Alveolar Macrophages, Is a Receptor for Y. pestis—The data presented in Fig. 1 indicate that the invasion of alveolar MΦs by *Y. pestis* might not be mediated by exposure of core LPS. This was initially surprising, as core LPS is a ligand for the SIGN-R1 receptor, expressed on peritoneal MΦs.[4] However, studies from several other investigators have suggested that mouse alveolar MΦs in fact do not express SIGN-R1 (43) but may express another receptor, DEC-205 (51). To explain these contrasting experiments, we examined the expression of DEC-205 and SIGN-R1 on alveolar and peritoneal MΦs. Our results confirm that mouse alveolar MΦs and peritoneal MΦs predominantly express DEC-205 and SIGN-R1, respectively (Fig. 2). However, in comparison with its CHO transfectants (Fig. 3*B*), the expression of DEC-205 in nonactivated alveolar MΦs is moderate. Our data indicated that LPS-activated alveolar MΦs did not enhance the expression of DEC-205 and their ability to phagocytose the *Y. pestis* (data not shown).

Because alveolar MΦs do not express SIGN-R1, we examined whether the invasion of *Y. pestis* into alveolar MΦs results from the interaction between *Y. pestis* and DEC-205 (Fig. 2). Four CHO transfectants stably expressing the mouse C-type lectin receptors mDC-SIGN, mSIGN-R1, mSIGN-R3, and mDEC-205 (CD205) (41, 42) were infected with *Y. pestis* $KIM6^+$ and $KIM6^+$-O^+. CHO-NEO was used as a negative control cell line. CS180 and *Y. pseudotuberculosis* (Y1) were used as the positive control strains, because *Y. pseudotuberculosis* (Y1) grown at 26 °C invades most epithelial cell lines, including CHO (36) via the invasin protein (52), and CS180 was shown to interact with mCHO-SIGN-R1 via its core LPS.[4] In addition, in comparison with other strains, only half the numbers of *Y. pseudotuberculosis* were loaded, because of its ability to promote an aggressive invasion into epithelial cells. The expression of each lectin on CHO cells is shown in Fig. 3*B*.[4] Several phenomena can be observed from these results (Fig. 3*A*). *Y. pestis* $KIM6^+$ interacts with CHO-mDEC-205 but not the CHO-NEO, showing that DEC-205 is a receptor for *Y. pestis*. The interactions of CHO-mSIGN-R1 with *Y. pestis* $KIM6^+$ and CS180 are most likely because of the exposure of core LPS on the bacteria as the isogenic derivative of both $KIM6^+$ and CS180 expressing an O-antigen no longer effectively interact with this transfectant, as also shown recently.[4] We observed that the expression of O-antigen on $KIM6^+$ was still able to block the interaction of this *Y. pestis* with CHO-mDEC-205, indicating that other potential ligands may also be shielded by expression of O-antigen.

It should be noted that our preliminary data showed that there is also a SIGN-R1- or DEC-205-independent interaction, which can be inhibited by addition of heparin, a synthetic form of heparan sulfate. We present this information, because heparin was included in the assays measuring bacteria-CHO-transfectant interaction to reduce some non-DEC-205 or SIGN-R1-specific interaction between *Y. pestis* and epithelial cells.[4] The rationale for the addition of heparin is addressed in detail under "Discussion."

Plasmid pPCP1 Is Involved in the Interaction of Y. pestis with DEC-205 Receptor—As suggested above, core LPS, a ligand for mouse SIGN-R1,[4] may not participate in the interaction of $KIM6^+$ *Y. pestis* with alveolar MΦs, which express the DEC-205. It should be recognized that *Y. pestis* $KIM6^+$ also contain the pPCP1 plasmid as well as the *pgm* locus. This led us to speculate that the ligands for the DEC-205 receptor might be encoded either on the pPCP1 plasmid, which encodes nine expressible genes, or the *pgm* locus containing many important genes that are related to pathogenesis of *Y. pestis* (53–55). Four strains, $KIM6^+$, $KIM6^-$ (lacking the *pgm* locus), $KIM10^+$ (lack-

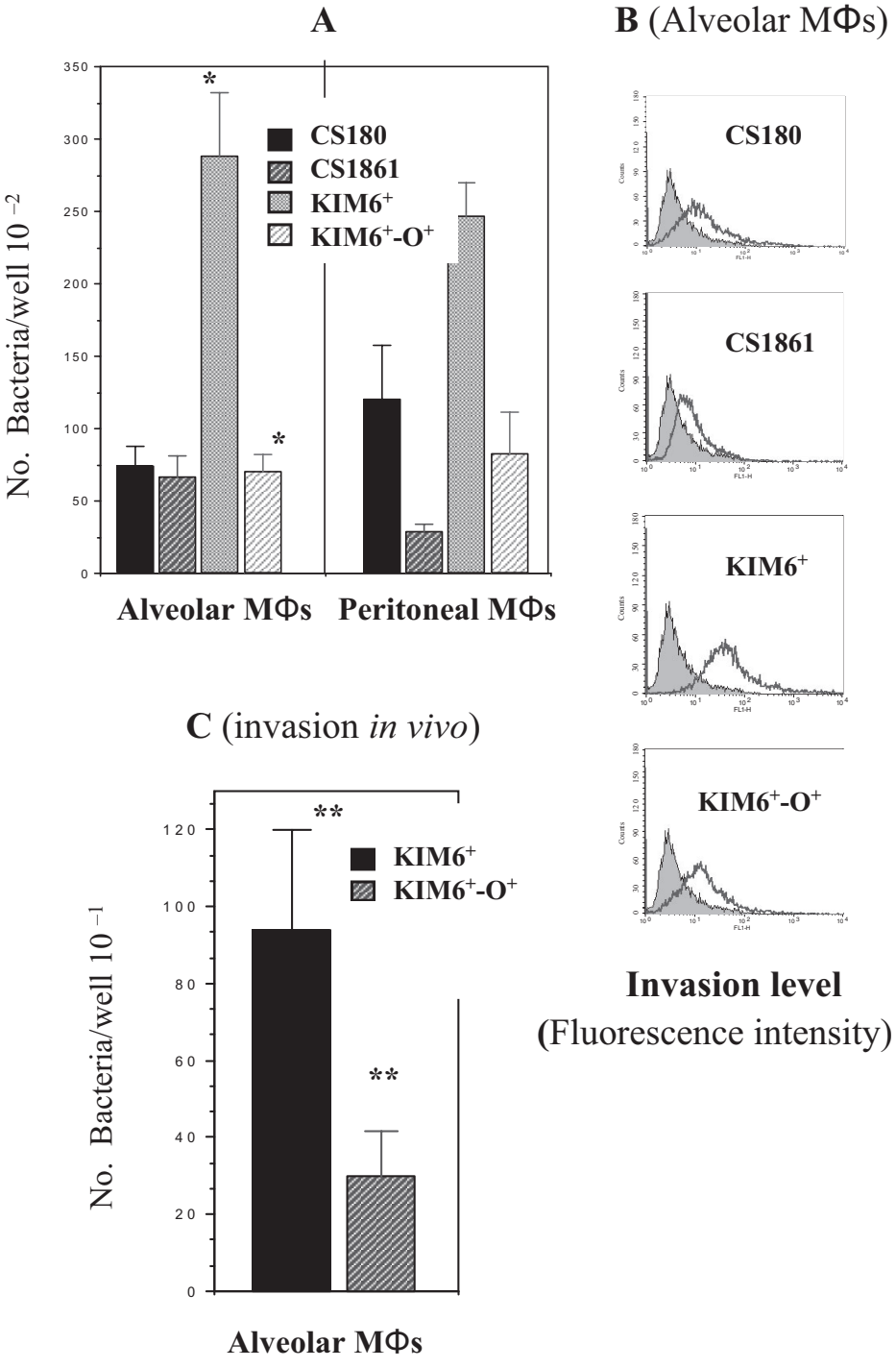

FIGURE 1. **Phagocytosis of *Y. pestis* by mouse alveolar macrophages occurs *in vitro* and *in vivo*.** Gentamicin protection (*A*) and flow cytometry-based assays (*B*) were used to determine the invasion ability of *Y. pestis* KIM6$^+$, KIM6$^+$-O$^+$, *E. coli* K12 CS180, and CS1681 (expression of O-antigen) into purified mouse alveolar MΦs and peritoneal MΦs, and the latter is shown Footnote 5. The invasive nature of the *E. coli* strains with human APCs has been described recently (19, 20, 47, 50). The *in vivo* phagocytosis of *Y. pestis* KIM6$^+$ and KIM6$^+$-O$^+$ is shown in *C*. *, $p < 0.001$, calculated by Student's *t* test in the comparison of the invasion of alveolar MΦs by *Y. pestis* KIM6$^+$ to KIM6$^+$-O$^+$. **, $p < 0.005$ in the *in vivo* comparison of invasion between these two strains. Labeled and unlabeled bacteria are indicated by *open* and *filled symbols*, respectively (*B*). The greater the fluorescence intensity, the more bacteria are phagocytosed by MΦs.

ing pPCP plasmid), and KIM10$^-$ (lacking both *pgm* locus and pPCP plasmid), were examined for their ability to interact with CHO-mDEC-205. *Y. pseudotuberculosis* (Y1) was used as a positive control. As shown in Fig. 4, *Y. pestis* KIM6$^+$ and KIM6$^-$ but not the KIM10$^+$ and KIM10$^-$ interact with CHO-DEC-205, indicating that pPCP1 rather than the *pgm* locus is necessary for the interaction with DEC-205. Again, heparin was present in this assay. Our unpublished data[5] further show that KIM10$^+$ binds to CHO cells better than KIM6$^+$ in the absence of heparin, indicating that the presence of the pPCP1 plasmid may inhibit other adherence factors.

Plasminogen Activator of Y. pestis Is a Ligand for DEC-205 and Plays a Role in Interaction with Alveolar Macrophages—Although the pPCP1 plasmid contains nine potential expressible genes, PLA is the most studied and notable component. In addition, work from other investigators indicates that expression of O-antigen in *Yersinia* spp. blocks the activity of PLA encoded by the pPCP1 plasmid (23). The data described in Fig. 3*A* also show that expression of O-antigen inhibits the interaction of *Y. pestis* KIM6$^+$ containing pPCP1 with CHO-mDEC-205. Therefore, KIM10$^-$-Δail, KIM10$^-$-Δail-PLA (40), *E. coli* K12 DH5α, and *E. coli* K12 DH5α-PLA were tested for their interactions with CHO-mDEC-205 and alveolar MΦs. CHO-NEO was used as the negative control. We find that removal of the *ail* gene reduces the non-C-type lectin-mediated interaction[5] as described and discussed under "Experimental Procedures" and "Discussion." Fig. 5 shows that KIM10$^-$-Δail-PLA and *E. coli* K12 DH5α-PLA, but not the KIM10$^-$-Δail and *E. coli* K12 DH5α, interact with CHO-DEC-205, demonstrating that PLA of *Y. pestis* is a ligand for DEC-205. In addition, expression of PLA in KIM10$^-$-Δail and DH5α enhances the ability to interact with alveolar MΦs, indicating that PLA participates in the interaction of *Y. pestis* with alveolar MΦs.

Interactions of CHO-DEC-205 and Alveolar Macrophages with PLA-expressing Y. pestis and E. coli Is Inhibited by DEC-205 Antibody—To verify the specificity of the Y. pestis-alveolar MΦ interaction, we examined whether the interaction of PLA-expressing *Y. pestis* KIM10$^-$-Δail and *E. coli* DH5α with CHO-DEC-205 and alveolar MΦs could be inhibited by anti-DEC-205 antibody. Anti-CD66 antibody was

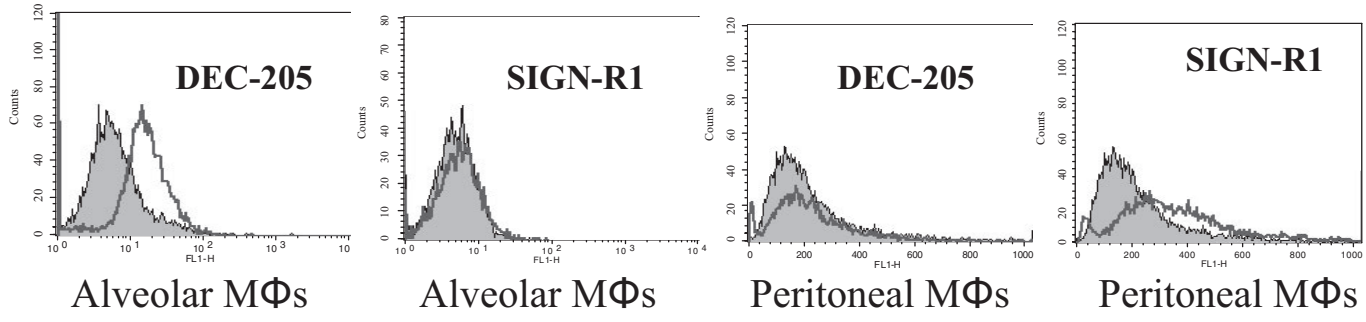

FIGURE 2. **The expression of DEC-205 and SIGN-R1 in alveolar and peritoneal macrophages.** Both alveolar and peritoneal MΦs were examined for their expression of DEC-205 and SIGN-R1 with flow cytometry. The level of expression for each receptor is shown with fluorescence intensity. The anti-human DC-SIGN antibody was used as a negative control, which is shown as *filled symbols*.

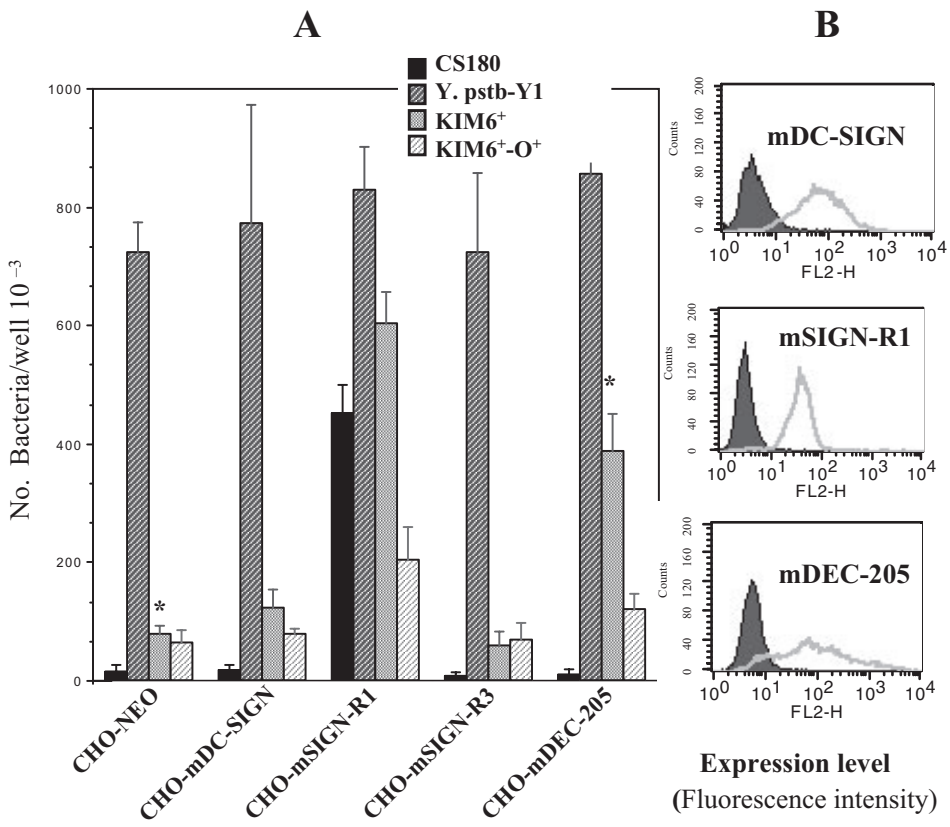

FIGURE 3. ***Y. pestis* but not *E. coli* K12 invades CHO-mDEC-205.** *A*, invasion of *E. coli* K12 CS180, *Y. pseudotuberculosis* (Y1), and *Y. pestis* KIM6$^+$ and KIM6$^+$-O$^+$ into CHO cell lines transfected by the mouse C-type lectins; CHO-mDC-SIGN, CHO-mSIGN-R1, CHO-mSIGN-R3 and CHO-mDEC-205 (CD205) were quantified by incubating the cell lines for 2.5 h with the bacterial strains and by killing the extracellular bacteria with 100 μg/ml of gentamicin. The number of phagocytosed bacteria was determined by counting CFU recovered following gentamicin treatment. The expression levels of each transfectant are shown in *B*. C-type lectin and CHO-NEO transfectants are shown in the *open* and *filled curves*, respectively. The level of expression for each receptor is shown with fluorescence intensity. Based on the Student's *t* test, *, $p < 0.001$, comparing the interaction of CHO-mDEC-205 with KIM6$^+$ to the interaction of CHO-NEO with the same bacterium.

employed as a control antibody. Again, we used *Y. pseudotuberculosis* serotype O:1a grown at 26 °C, which mediates a DEC-205-independent interaction, as a control strain. Fig. 6 shows that anti-DEC-205 antibody blocks the invasion of the PLA-KIM10$^-$-Δail and -DH5α to CHO-DEC-205. Although this antibody reduced the phagocytosis of PLA-KIM10$^-$-Δail and DH5α by alveolar MΦs (Fig. 6*A*), the blockage was not complete, indicating that other receptors on alveolar MΦs might be involved in this interaction. Also, the dynamic nature of APCs, which presumably phagocytose most antigens, could lead to specific or nonspecific phagocytosis. It should be noted that the concentrations of antibody used in this study did not influence the viability of either bacteria or host cells (19) as also found with our control strain *Y. pseudotuberculosis* (20). Together, these data indicate that there is a specific interaction between DEC-205 of alveolar MΦs and PLA of *Y. pestis.*

Expression of PLA and O-antigen Promotes and Inhibits Early Dissemination of Y. pestis, Respectively—PLA plays an important role in promoting dissemination of Y. pestis (14, 18). We have presented data showing that DEC-205 is a receptor for the PLA ligand. Expression of O-antigen by *Y. pestis* inhibits with the PLA-DEC-205 interaction. The obvious question is whether the PLA-DEC-205 interaction enhances the dissemination of *Y. pestis* and whether blockage of this interaction would reduce the dissemination. Therefore, *Y. pestis* KIM6$^+$, KIM10$^+$, and KIM10$^+$-PLA (PLA-expressing *Y. pestis* KIM10$^+$) were examined for their ability to translocate from lungs to spleens. Briefly, mice were infected with these strains via nasal inoculation to mimic pneumonic plague (18, 48, 49). Then mice lungs and spleens were isolated, homogenized, and spread onto *Y. pestis* plates. Our results (Fig. 7, *A* and *B*) show that both KIM6$^+$ and KIM10$^+$ could be transmitted to spleens, but the KIM6$^+$ is more effective, as KIM10$^+$ was retained in the lung more. The most interesting result is that the single gene-expressing KIM10$^+$-PLA partially regained

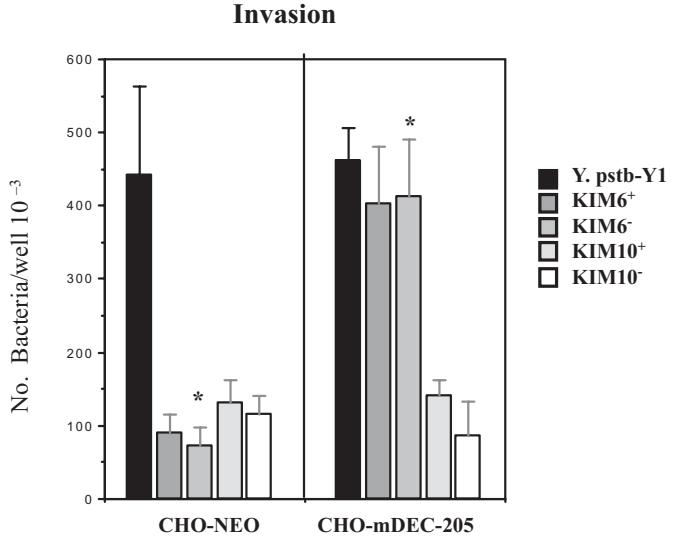

FIGURE 4. **Plasmid pPCP1 plays a role in mediating the interaction of *Y. pestis* with mDEC-205.** KIM6$^+$, KIM6$^-$ (lacking the *pgm* locus), KIM10$^+$ (lacking pPCP plasmid), and KIM10$^-$ (lacking both *pgm* locus and pPCP1 plasmid) were tested for their ability to invade alveolar MΦs (*left*) and CHO-mDEC-205 (*right*), to determine the involvement of plasmid pPCP1 or *pgm* locus. The invasion assay followed the same procedures as for the transfectants and MΦs in Figs. 1 and 3. *, both $p < 0.001$, calculated by Student's *t* test in the comparison of the invasion of MΦs (*left*) and CHO-mDEC-205 (*right*) by KIM6$^+$ to KIM10$^+$. It should be noted that the inoculum of *Y. pseudotuberculosis* was one-third that of the other strains.

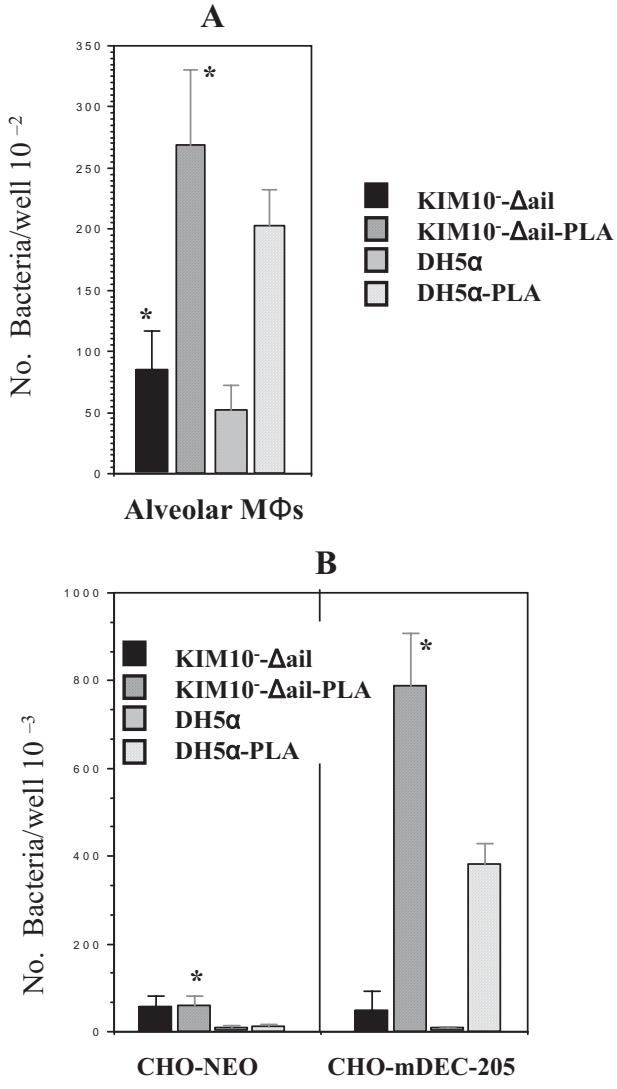

FIGURE 5. **PLA-expressing *Y. pestis* and *E. coli* invade CHO-mDEC-205 and alveolar macrophages.** KIM10$^-$-Δail, KIM10$^-$-Δail-PLA, *E. coli* K12 DH5α, and *E. coli* K12 DH5α-PLA were examined for their invasion into alveolar MΦs (*A*) and CHO-mDEC-205 (*B*). The invasion assay followed the same procedures as for transfectants and MΦs, described in Figs. 1 and 3. *, both are $p < 0.001$, calculated by Student's *t* test in the comparison of the invasion of MΦs (*A*) and CHO-mDEC-205 (*B*) by KIM10$^-$-Δail to KIM10$^-$-Δail-PLA.

its ability to be transmitted to spleens, but this only occurred on day 2. Furthermore, the retention rates in the lung for KIM10$^+$-PLA were dramatically reduced after day 1. Finally, our data also show that both KIM6$^+$ and KIM10$^+$ can persistently be disseminated to spleens for up to 9 days although the isolation rates are very low in the later stages (data not shown), indicating that the nonvirulent *Y. pestis* can stay in the lungs for a reasonably long period. However, if they are phagocytosed, these nonvirulent *Y. pestis* can only live about 2–3 days. In short, the results suggest that PLA plays a role in early *Y. pestis* dissemination during pneumonic plague.

On the other hand, we also speculated that the PLA-DEC-205 interactions, if blocked by expression of O-antigen, could reduce the dissemination. To investigate this hypothesis, *Y. pestis* KIM6$^+$ and its isogenic derivative KIM6$^+$-O$^+$ were examined for their ability to disseminate to mouse spleens. Four days after inoculation, the rate of dissemination was determined using the same procedures shown in Fig. 7, *A* and *B*. A high number of *Y. pestis* KIM6$^+$ (rough) but not KIM6$^+$-O$^+$ (smooth) were isolated from spleens (Fig. 7, *C* and *D*). In contrast, KIM6$^+$-O$^+$ bacteria were retained in the lungs in much higher numbers than KIM 6$^+$ bacteria. It is our presumption that the reduction in dissemination of *Y. pestis* KIM6$^+$-O$^+$ might be due to its inability to interact with DEC-205.

Because expression of O-antigen inhibits dissemination and the interaction between PLA and DEC-205, we suggest that the PLA-promoted dissemination is based on the ability of PLA to interact with DEC-205. Because KIM10$^+$ is still able to be disseminated, other factors must also have participated in the *Y. pestis* dissemination from lungs to spleens.

DISCUSSION

Y. pestis, the causative agent of pneumonic plague, is able to rapidly disseminate from the lungs to other organs. Investigators have shown that PLA plays a crucial role in promoting *Y. pestis* dissemination. It is speculated that *Y. pestis* hijacks APCs to be delivered to lymph nodes, resulting in this dissemination. In this study, we found that the PLA of *Y. pestis* is a ligand for DEC-205. Our results also suggest that the PLA-DEC-205 interaction may mediate the dissemination of *Y. pestis.*

The DEC-205 receptor is expressed on many APCs in both humans and mice (56, 57). The main function of DEC-205 receptor is related to antigen presentation (26, 27, 58). Therefore, the DEC-205 molecule has been used to enhance host responses for vaccine development against the HIV GAG protein and V-antigen from *Y. pestis* (59–61). On the other hand, an important corollary to the ligand-receptor interaction is that

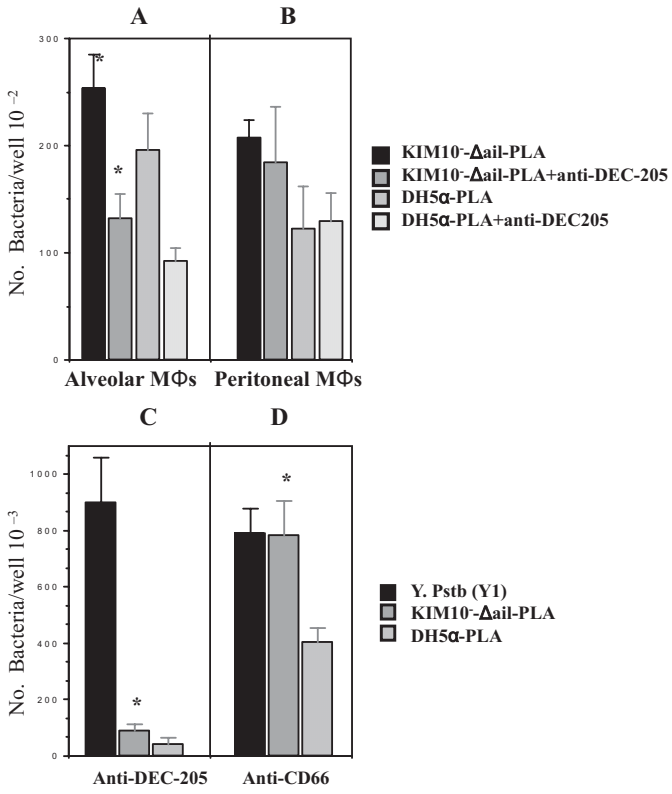

FIGURE 6. **Inhibition of mDEC-205-mediated phagocytosis of PLA-expressing *Y. pestis* and *E. coli* K12 by anti-mDEC-205.** The KIM10$^-$-Δail-PLA and *E. coli* K12 DH5α-PLA were incubated with alveolar MΦs (*A*) and peritoneal MΦs (*B*) for 1.5 h in the presence of anti-DEC-205 (5 μg/ml). CHO-mDEC-205 cells were also incubated with the KIM10$^-$-Δail-PLA, *E. coli* K12 DH5α-PLA, and *Y. pseudotuberculosis* for 2 h in presence or absence of anti-DEC-205 (5 μg/ml) (*C*) and anti-CD66 (5 μg/ml, the control antibody) (*D*). All reagents were added to media for 20 min before addition of bacteria. The concentration of each reagent used in this experiment was based on previously published data n (19, 20, 47, 50). The phagocytosis rate of *Y. pestis* was evaluated by the recovery of bacteria following gentamicin protection. *Y. pseudotuberculosis* serotype O:1a were used as control strains to show PLA-DEC-205-independent interaction with CHO. Based on the Student's *t* test, *, $p < 0.001$, compared the DEC-205-PLA interaction in the presence of anti-DEC-205 antibody to the interaction with the presence of anti-CD66 antibody.

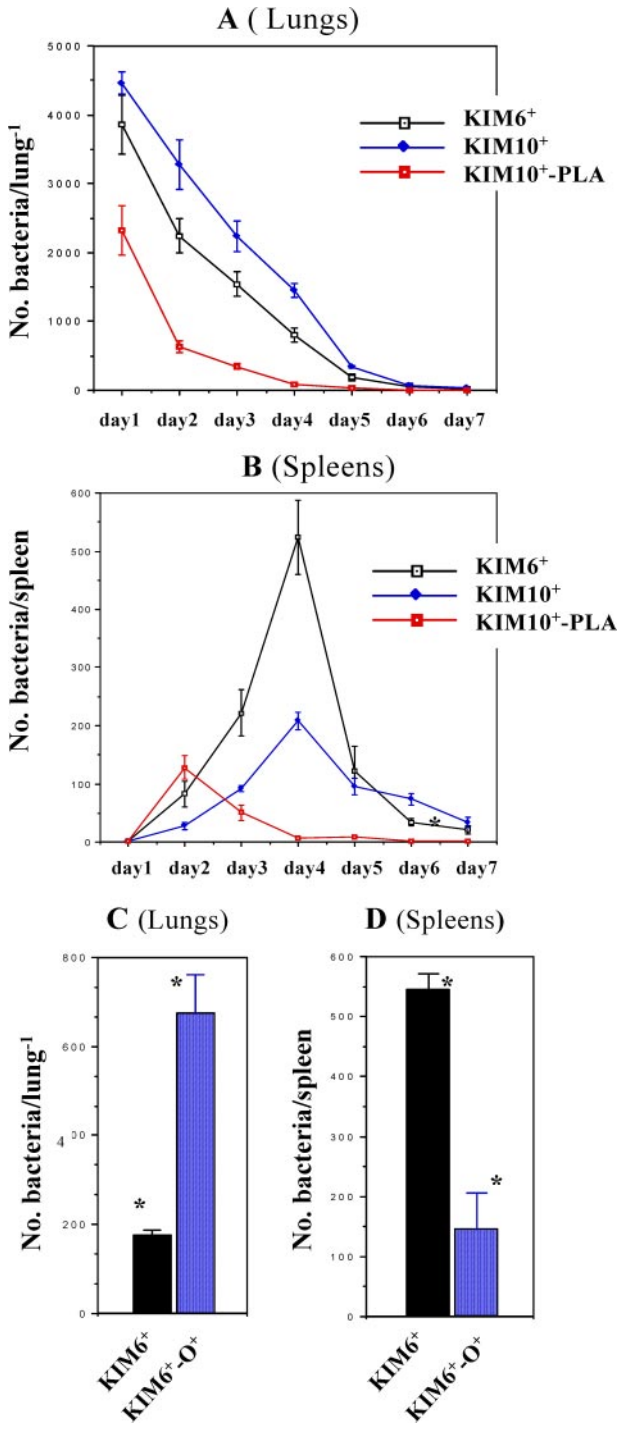

FIGURE 7. **Expression of PLA and O-antigen enhances and reduces the dissemination of *Y. pestis*, respectively.** After nasal inoculation of KIM6$^+$, KIM10$^+$ (lacking the pPCP plasmid), and KIM10$^+$-PLA, mice were euthanized. Both lungs (*A*) and spleens (*B*) were separated, homogenized, and spread on *Y. pestis* plates. The dissemination rate is the CFU recovered from whole spleens or lungs. *C* and *D*, KIM6$^+$ and KIM6$^+$-O$^+$ (expression of O-antigen) were inoculated to mice following the same procedures as in *A* and *B*. The data from day 4 were shown, and note the difference in scale between lungs and spleens.

some pathogens, including *Y. pestis*, have evolved mechanisms of exploiting those very host defense molecules designed to eliminate them, with the result of an expanded ability to disseminate in the host, as explored in this study. However, there are still several technical and scientific issues, which need to be addressed to validate our findings and conclusions.

In this study, nine *Y. pestis* KIM variants were chosen for investigating various aspects of the bacterial-host interaction, including dissemination; however, none of the selected strains were fully virulent (Table 1). This raises the issue of whether the conclusions we have reached are significant in understanding the pathogenic process of plague. Because of the virulent nature of *Y. pestis*, this pathogen is considered a "select agent," and its use in experiments are tightly restricted by IRBs and federal regulations; thus, it was not possible for us to use a virulent strain in our experiments. However, over the entire course of the dissemination experiments, the mouse fatality rate was zero. In fact, we did not even observe that there were any symptoms of discomfort, such as fever among the inoculated mice, suggesting that the innate immune system is very effective against these invading pathogens. These observations clearly confirm the reduced pathogenicity of these mutant strains.

Nevertheless, an advantage of using nonvirulent strains is that they allow us to apply genetically defined mutants to decode the complexity of host-pathogen interactions during *Y.*

pestis dissemination and infection. For example, we have used nonvirulent strains to examine the role of specific ligand-receptor pairs in the dissemination of *Y. pestis* to mouse spleens from the lungs in experiments mimicking pneumonic plague. If a virulent strain had been used, it would have been impossible to define the dissemination rate; these bacteria are fully capable of replicating in spleens. Also, we observed that the number of KIM10$^+$-PLA CFU retained in the lungs is dramatically reduced by day 2, with a corresponding rise in the isolation of KIM10$^+$-PLA from spleens. These results suggest that the high expression of PLA by *Y. pestis* induces the rapid phagocytosis by alveolar MΦs, resulting in bacterial clearance or dissemination and that the mutant strains can assist in modeling various stages of *Y. pestis* pathogenicity. As shown in Figs. 3 and 5, KIM10-PLA invades alveolar MΦs and CHO-DEC-205 more effectively than the KIM6$^+$, suggesting that the expression of PLA in KIM10-PLA might be higher than in KIM6$^+$.

For the same reason, we speculate that *Y. pestis* KIM6$^+$ bacteria can only survive for about 2–3 days after being phagocytosed or if they have initiated the dissemination process. Fig. 7 shows that after a short increase in dissemination on day 2, the recovery rates of KIM10$^+$-PLA from both lungs and spleens are dramatically reduced. The data thus suggest that the dissemination is initiated after bacteria are phagocytosed. We would also like to mention that we were unable to demonstrate spleen dissemination by KIM6$^-$ (data not shown) but do show for KIM6$^+$ in this study. We believe that the *pgm* locus may help *Y. pestis* to survive in the MΦs during infection, as shown in a recent study (55).

The overall dissemination rate is very low (Fig. 7). In whole spleens, an average of 500 KIM6$^+$ CFU was recovered at the peak of dissemination. Based on the study of Lathem *et al.* (18), the number of *Y. pestis* CO92 could reach 10^8–10^9 in spleens before these mice succumbed to the infection. It is possible that the nonvirulent strains are more susceptible to killing in the host, thus affecting overall dissemination numbers. Alternatively, it is possible that only a small fraction of the inoculum was able to reach the lungs. Two observations should be recognized here. First, the only difference between wild-type *Y. pestis* and KIM6$^+$ is the lack of the pYV plasmid in the latter, possibly affecting its ability to disseminate and/or survive. Second, under the experimental conditions described in this study, we were unable to isolate bacteria from blood samples of the infected mice (data not shown). We speculate that the bacteria transit in the bloodstream at concentrations too low or too rapidly for detection. It is also possible that the bacteria isolated from spleens have not entered there via the bloodstream but via an as yet unknown mechanism.

We also utilized the *Y. pestis* strain KIM10$^-$-Δail, which is highly attenuated. The deleted components of this strain include the *pgm* locus, pPCP1 plasmid, the pYV plasmid (encoding type III secretion system and other cytotoxic components), and the *ail* gene (40). The reason for selecting this strain was to reduce the interference of other known virulence components, which might mask aspects of the pathogen-host interaction under study. For example, although *Y. pestis* does not express invasin, it directly interacts with epithelial cells such as HeLa cells (52). We have observed that the interaction of *Y. pestis* with epithelial cells might involve the binding of the Ail protein with cell surface heparan sulfate proteoglycans.[5] As shown in Fig. 5, KIM10$^-$-Δail or KIM10$^-$-Δail-PLA has lost their ability to interact with epithelial cells in the absence of either SIGN-R1 or DEC-205.

Another issue of concern is why we used such a high concentration (OD = 1.5) of *Y. pestis* CFU as an inoculum. For the nonvirulent strains of *Y. pestis*, this large inoculum was required for us to recover any bacteria disseminating to the spleen. It is known that *Y. pestis* is an extremely virulent pathogen. Several experiments have indicated that LD_{50} of *Y. pestis* can be as low as 1 (62). However, to dissect the course of the bacterial infection, the use of attenuated *Y. pestis* mutants is more suitable.

In our recent studies (20),[4] we have utilized the anti-human DC-SIGN and anti-mouse SIGN-R1 antibodies to inhibit the interactions of *Y. pestis* with human monocyte-derived DCs and mouse MΦs, but neither of these antibodies was effective. In fact, we must combine both anti-human DC-SIGN and -human Langerin (CD207) (another host receptor for *Y. pestis*)[5] antibodies to show a moderate reduction in this interaction (20). As shown in Fig. 6*A* in this study, interaction of *Y. pestis* with alveolar MΦs was reduced by half following a single anti-DEC-205 antibody treatment, indicating that DEC-205 is a major receptor for *Y. pestis* and plays a specific role during the phagocytosis of *Y. pestis* by alveolar MΦs. However, this inhibition by anti-DEC-205 antibody was not complete, indicating that alveolar MΦs likely possess an additional receptor(s) for *Y. pestis.*

Both Welkos *et al.* (63) and Lathem *et al.* (18) indicated that PLA might not play a significant role in dissemination if the strain CO92 was infected via aerosol inhalation to non-human primates and intranasal route to mice, respectively. CO92, the most virulent strain, was used in their studies. Therefore, we suggest that binding of the bacteria to APCs, although actively blocking endocytosis via the type III secretion system, may facilitate the transport of viable bacteria to lymph nodes. As shown in Fig. 7, the KIM10$^+$, without expression of PLA, was still able to be disseminated, although not as efficiently at the KIM6$^+$.

What are the other possible mechanisms for promoting dissemination of *Y. pestis*? This study focused on the interactive roles between PLA of *Y. pestis* and DEC-205 of alveolar MΦs. However, it is likely that mouse pulmonary DCs, which express both DEC-205 and Langerin (CD207) rather than alveolar MΦs, are the cells responsible for migrating between tissues (64–66). Therefore, we would like to propose another model for pneumonic plague. After entering the lungs via aspiration, *Y. pestis* uses its Ail protein (40, 67) to bind to cell surface heparan sulfate proteoglycans and invade epithelial layers of bronchial tracts in the lungs. Then the *Y. pestis* encounters the pulmonary DCs via the SIGN-R1, DEC-205, or Langerin, which lead to the dissemination.

We have presented evidence *in vitro* and *in vivo* showing that *Y. pestis* may use the PLA-DEC-205 interaction to promote its invasion and possible dissemination. However, the fundamental question still remains as to whether the PLA-DEC-205 interaction plays a role in an *in vivo* infection.

The most straightforward approach would be to test whether a DEC-205 knock-out mouse model would be resistant to plague or reduce the dissemination of *Y. pestis*, an experiment that we are pursuing. The knock-out mouse model has been used successfully to identify viral receptors. For example, the CEACAM1 (CD66a) receptor knock-out mice have an increased resistance to mouse hepatitis viral infection (68), because mouse CEACAM1 is a receptor for mouse hepatitis virus (69). However, there are potential limitations of this approach for studying bacteria-host cell interactions. Strangely, there are no credible receptor knock-out models that are more resistant to bacterial infection. The reason might be simple; bacterial infections are more multifactorial than viral infections. Knock-out of one receptor might not be enough to determine the fate of a bacterial infection, a fact that might contribute to the failure of CCR5 knock-mice to resist *Y. pestis* infection (32, 70), even if CCR5 is a receptor for *Y. pestis.* Thus the mouse knock-out model may only provide unequivocal results if DEC-205 is the only receptor for *Y. pestis*. Unfortunately, many pathogens do not depend on only one receptor in their interactions with host cells, as we have indicated in our published or unpublished data[5] that *Y. pestis* interacts with human DC-SIGN, human Langerin, mouse SIGN-R1, mouse Langerin, cell surface heparan sulfate proteoglycan, and of course mouse DEC-205.

Taken together, this study has demonstrated that DEC-205 functions as a cellular receptor for *Y. pestis*, with PLA serving as a ligand. The data presented in this study indicate that interactions between PLA and DEC-205 may play a role in the dissemination of *Y. pestis*.

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