INFECTION AND IMMUNITY, Feb. 2009, p. 615–621 Vol. 77, No. 2
0019-9567/09/$08.00+0 doi:10.1128/IAI.00931-08
Copyright © 2009, American Society for Microbiology. All Rights Reserved.

Humoral Immunity against Capsule Polysaccharide Protects the Host from *magA*$^{+}$ *Klebsiella pneumoniae*-Induced Lethal Disease by Evading Toll-Like Receptor 4 Signaling[▽]

Ming-Fang Wu,[1] Chih-Ya Yang,[1] Tzu-Lung Lin,[2] Jin-Town Wang,[2,3] Feng-Ling Yang,[4] Shih-Hsiung Wu,[4] Bor-Shen Hu,[5] Teh-Ying Chou,[6] Ming-Daw Tsai,[4,7] Chi-Hung Lin,[1] and Shie-Liang Hsieh[1,7,8]*

Department and Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan[1]; Department of Microbiology[2] and Department of Internal Medicine,[3] National Taiwan University Hospital, Taipei, Taiwan; Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan[4]; Section of Infectious Diseases, Department of Internal Medicine, Taipei City Hospital Heping Branch, Taipei, Taiwan[5]; Section of Surgical Pathology, Department of Pathology and Laboratory Medicine, Taipei Veterans General Hospital, Taipei, Taiwan[6]; Genomics Research Center, Academia Sinica, Taipei, Taiwan[7]; and Immunology Research Center, National Yang-Ming University and Taipei Veterans General Hospital, Taipei, Taiwan[8]

Received 26 July 2008/Returned for modification 27 August 2008/Accepted 3 November 2008

Klebsiella pneumoniae magA **(for mucoviscosity-associated gene A) is linked to the pathogenesis of primary pyogenic liver abscess, but the underlying mechanism by which *magA* increases pathogenicity is not well elucidated. In this study, we investigated the role of the capsular polysaccharides (CPS) in the pathogenesis of *magA*$^{+}$ *K. pneumoniae* by comparing host immunity to *magA*$^{+}$ *K. pneumoniae* and a Δ*magA* mutant. We found that Toll-like receptor 4 recognition by *magA*$^{+}$ *K. pneumoniae* was hampered by the mucoviscosity of the *magA*$^{+}$ *K. pneumoniae* CPS. Interestingly, monoclonal antibodies (MAbs) against *magA*$^{+}$ *K. pneumoniae* CPS recognized all of the K1 strains tested but not the Δ*magA* and non-K1 strains. Moreover, the anti-CPS MAbs protected mice from *magA*$^{+}$ *K. pneumoniae*-induced liver abscess formation and lethality. This indicates that the K1 epitope is a promising target for vaccine development, and anti-CPS MAbs has great potential to protect host from K1 strain-induced mortality and morbidity in diabetic and other immunocompromised patients in the future.**

Klebsiella pneumoniae is an enteric gram-negative bacillus that causes human nosocomial infections in immunocompromised patients and accounts for a significant proportion of hospital-acquired infections in neonatal wards. *K. pneumoniae* is well known as a major cause of nosocomial bacterial pneumonia and urinary tract infection (22, 26).

The majority of clinical isolates of *K. pneumoniae* express a pronounced capsular polysaccharide that is essential to the virulence of *Klebsiella* (22). It has been demonstrated that strains with capsule (such as serotype K1 and K2) were virulent in animal model, whereas serotypes without capsule are less virulent or without virulence (21, 22). R. W. Tsay et al. (33) found that capsular serotype K1 was the most common serotype (23.4% versus 14%) found in the community-acquired and nosocomial *K. pneumoniae* infections.

Recently, a new type of invasive *K. pneumoniae* (K1 strain) has become the main agent causing primary liver abscesses in community-acquired infections (16, 35), and 10 to 12% of these cases were complicated by either metastatic meningitis (5) or endophthalmitis (6, 13, 19). Such infections occur not only in Taiwan but also in Western countries (2, 3, 18, 25). Even though the *K. pneumoniae* strains responsible for these infections are sensitive to aminoglycosides and cephalosporins, the mortality rates of primary liver abscess and metastatic meningitis are 10% (35) and 30 to 40% (11, 32), respectively. This reflects the ineffectiveness of the current antibiotic therapy alone for this infection-related organ failure.

Among the 77 serotypes of *Klebsiella*, strains expressing the K1 capsular antigen account for the majority (63.4%) of liver abscess isolates of *K. pneumoniae* (13). This suggests that K1 capsular antigens confer survival advantage to bacteria and is an important indicator for the occurrence of liver abscess and endophthalmitis in *K. pneumoniae* infection. However, the molecular mechanism rendering the K1 strain more invasive has not been well elucidated.

Recently, a novel gene *magA* (named for mucoviscosity-associated gene A) located in the K1 capsular gene cluster was identified from a Taiwan *K. pneumoniae* strain (NTUH-K2044), and the presence of *magA* correlated with the K1 serotype of *K. pneumoniae* (7, 12). Moreover, MagA is essential for the synthesis of NTUH-K2044 capsular polysaccharide (CPS), which is associated with high mucoviscosity and interferes with complement deposition. This feature makes *magA*$^{+}$ *K. pneumoniae* resistant to complement-mediated lysis when incubated with nonimmune human serum (NHS) (12). It is interesting that a *magA*-deficient mutant of NTUH-K2044 (Δ*magA* mutant) completely loses mucoviscosity and becomes susceptible to complement deposition and phagocytosis (12).

* Corresponding author. Mailing address: Department of Microbiology and Immunology, National Yang-Ming University, Taipei 11221, Taiwan. Phone: 886-2-28267161. Fax: 886-2-28277933. E-mail: slhsieh@ym.edu.tw.
▽ Published ahead of print on 17 November 2008.

Downloaded from iai.asm.org at National Taiwan Univ. on June 11, 2009

This indicates that *magA*$^+$ *K. pneumoniae* CPS plays an essential role in pathogen resistance to host immunity. Therefore, we sought to determine whether the *magA*$^+$ *K. pneumoniae* CPS can mask underlying lipopolysaccharide (LPS) and interferes with host recognition by Toll-like receptor (TLR) (34).

To further understand the role of CPS in the pathogenesis of *K. pneumoniae*, we compared the differential immune response to *magA*$^+$ and Δ*magA K. pneumoniae* strains and generate anti-*magA*$^+$ *K. pneumoniae* CPS monoclonal antibodies (MAbs) to test their effect to protect mice for *magA*$^+$ *K. pneumoniae*-induced lethality. We report here that *magA*$^+$ *K. pneumoniae* CPS is sensitive to heat treatment, and disruption of CPS structure increases the host response to *magA*$^+$ *K. pneumoniae* to a level similar to that of the Δ*magA* strain. Moreover, anti-CPS MAbs could agglutinate all of the K1 strains tested, enhance phagocytosis, and protect mice from *magA*$^+$ *K. pneumoniae*-induced lethality. This suggests that CPS is a promising target for vaccine development, and anti-CPS MAbs are the potential therapeutic agents to protect host against the complications induced by *K. pneumoniae* of K1 serotype.

MATERIALS AND METHODS

Reagents. Human macrophage–colony-stimulating factor was purchased from R&D Systems. The other chemicals were purchased from Sigma Chemical, including LPS (isolated from *Escherichia coli* serotype O111:B4), 5(6)-carboxytetramethyl-rhodamine *N*-hydroxy-succinimide ester [5(6)-TAMRA], and other chemicals.

Cell cultures. Human monocyte-derived macrophages (hMDMs) were cultured as previous described (4). Briefly, human peripheral blood samples were isolated from the blood of normal individuals by standard density gradient centrifugation with Ficoll-Paque (Amersham Biosciences, Piscataway, NJ). CD14$^+$ cells were subsequently purified from human peripheral blood cells by high-gradient magnetic sorting using the VARIOMACS technique with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14$^+$ monocytes were cultured in complete RPMI 1640 medium (JRH) supplemented with 10 ng of human macrophage–colony-stimulating factor/ml at 37°C in 5% CO_2.

HeNC2 (with functional TLR4) and GG2EE cells (lacking functional TLR4) are bone marrow-derived J2 virus-transformed macrophage cell lines as described previously (1), were propagated in RPMI 1640 medium (JRH) supplemented with 10% heat-inactivated fetal calf serum, and cultured in a 37°C, 5% CO_2 incubator.

Bacterial strains and growth conditions. The parental strain of the Δ*magA* strain, NTUH-K2044 (*magA*$^+$ *K. pneumoniae*), was a clinical isolate; the construction of Δ*magA* strain was described in a previous study (12). Bacteria strains were grown in Luria-Bertani (LB) medium at 37°C. Selected antibiotics were added for the culture of the Δ*magA* strain (kanamycin, 50 μg/ml) (12) and *K. pneumoniae* strains carrying plasmid GFPuv gene (chloramphenicol, 100 μg/ml), respectively (12). Other clinical bacterial strains were obtained from the Department of Internal Medicine Taipei City Hospital (Heping Branch, Taipei, Taiwan).

String test for hypermucoviscosity. The string test was performed as described previously (12). Hypermucoviscosity was defined by the formation of viscous strings >5 mm in length when a loop was used to stretch the colony on an agar plate (positive string test).

Negative staining for bacterial capsule. The capsule of *K. pneumoniae* is detected by negative staining. Briefly, the bacterial suspension is mixed with an equal volume of nigrosin (10%), spread onto a glass slide, and heat fixed for several seconds. The slide was further incubated with 1% crystal violet for 2 min. The expression of capsule was observed as the exclusion of nigrosin and crystal violet around the bacteria.

Stimulation of hMDMs. The hMDMs were seeded in 24-well plates at a density of 6×10^5/ml. The bacteria were either inactivated by exposure to UV light at 20 J/cm^2 or to heat at 95°C for 30 min. After three washes with phosphate-buffered saline (PBS), the bacteria were resuspended in PBS and added to hMDMs at a multiplicity of infection (MOI) of 5 and incubated for 24 h. LPS (1 μg/ml) was the positive control. The macrophage cell lines HeNC2 and GG2EE (6×10^5/ml) were incubated with bacteria at an MOI of 0.5 in 24-well plates, respectively. LPS (1 ng/ml) was used as a positive control. All experiments were performed at least in triplicate.

Preparation of MAbs. BALB/c (8-week-old) mice were immunized subcutaneously with *K. pneumoniae* (2×10^3 CFU per mice, the 50% lethal dose was 5×10^2 CFU in our study) three times, with 2 weeks between each injection before sacrifice. The spleen cells from survival mice were fused with murine myeloma cells (NS-1) using polyethylene glycol. After incubation in hypoxanthine-aminopterin-thymidine for 2 weeks, hybridoma secreting antibodies against immobilized CPS (100 ng of CPS from *magA*$^+$ strain per well) were selected by enzyme-linked immunosorbent assay (ELISA). The isolation of bacterial CPS was as described in a previous study (37), and the LPS level in the CPS samples (<1 EU/ml in the CPS) is determined by the *Limulus* amebocyte lysate assay.

ELISA. To determine the tumor necrosis factor alpha (TNF-α) levels in the culture supernatants of macrophages (hMDMs or mouse macrophage) incubated with UV or heat-inactivated *magA*$^+$ or Δ*magA K. pneumoniae* strains, 200 μl of supernatant was harvested at 24 h after incubation, and the concentrations of TNF-α were determined by using a ELISA kit (R&D Systems).

Rapid agglutination assay. *K. pneumoniae* was diluted to an optical density at 600 nm of 0.1 with PBS. An aliquot (25 μl, 3×10^6 to 6×10^6 CFU) was incubated with anti-CPS MAb (7.5 μg in 25 μl) and shaken on a rotary platform (130 rpm) for 1 h at room temperature. Agglutination was observed and photographed. The original magnification under the microscope was ×200.

Double immunodiffusion. A double immunodiffusion test was performed as described previously (7). Briefly, the anti-CPS ascites (10F8G4 clone) (1 μl) was loaded into the central well, while the CPS extract (20 μg) was loaded into peripheral wells. After an overnight incubation at 37 °C, the gels were incubated with 1% azocarmine (dissolved in 2% glacial acid; Chroma) for 2 h, followed by incubation with 2% glacial acid for destaining.

Phagocytosis assay. To observe the ability of hMDMs to uptake anti-CPS MAb-coated bacteria, cells (2×10^5) were labeled with TAMRA [5(6)-carboxytetramethyl-rhodamine *N*-hydroxy-succinimide ester] and incubated with *K. pneumoniae* strains (*magA*$^+$ and Δ*magA*) stably expressing green fluorescent protein at 37°C for 1 h (MOI = 5) in glass-bottom culture plates. The cells were washed with PBS and then fixed in 1% paraformaldehyde at room temperature for 1 h before observation under a confocal microscope (Leica TCS-SP5).

Serum resistance assay. A serum resistance assay was performed as described previously (12). The bacteria (3×10^6 to 6×10^6 CFU) were incubated with anti-CPS MAb (clone 10F8G4, 7.5 μg) at room temperature for 1 h, followed by addition of NHS or heat-inactivated serum (HIS; 56°C for 30 min) to a final concentration of 25%, and were incubated at 37°C for another 1 h. Samples were diluted serially and plated on LB plates to determine the colony numbers after incubation at 37°C overnight.

Protection assays. For the protection assay, groups of 8-week-old mice (12 mice for each group) were injected intraperitoneally with 100 μg of purified MAbs (9E9F11 and 10F8G4) or isotype control antibody (mouse immunoglobulin M [IgM]) or PBS. After 24 h, mice were inoculated with *magA*$^+$ *K. pneumoniae* strain (2×10^3 CFU) intraperitoneally and were observed for 1 month to determine the mortality rate. The surviving mice were sacrificed at the end of the fourth week. For histochemical staining, livers were removed from infected mice at day 12 postinfection. The weights of the spleens were also measured at day 12 after infection.

Statistical analysis. A Student *t* test was used to analyze the statistical significance of differences using the Prism software package (GraphPad), and a *P* value of <0.05 was considered significant. The survival rate was determined by Kaplan-Meier analysis with a log-rank test, and statistical significance was accepted at a *P* value of <0.05.

RESULTS

Differential effects of heat and UV on the CPS of *magA*$^+$ *K. pneumoniae*. Before incubation of hMDMs with *magA*$^+$ and Δ*magA K. pneumoniae*, bacteria were inactivated by heat (95°C for 30 min) or UV (20 J/cm^2). The capsule of the Δ*magA* mutant was thin without obvious mucoviscosity and was easily pelleted by centrifugation. In contrast, the capsule of *magA*$^+$ *K. pneumoniae* was thick with high mucoviscosity and could not be pelleted by centrifugation. Heat treatment reduced the mucoviscosity as determined by the string test (Fig. 1A), while UV

Downloaded from iai.asm.org at National Taiwan Univ. on June 11, 2009

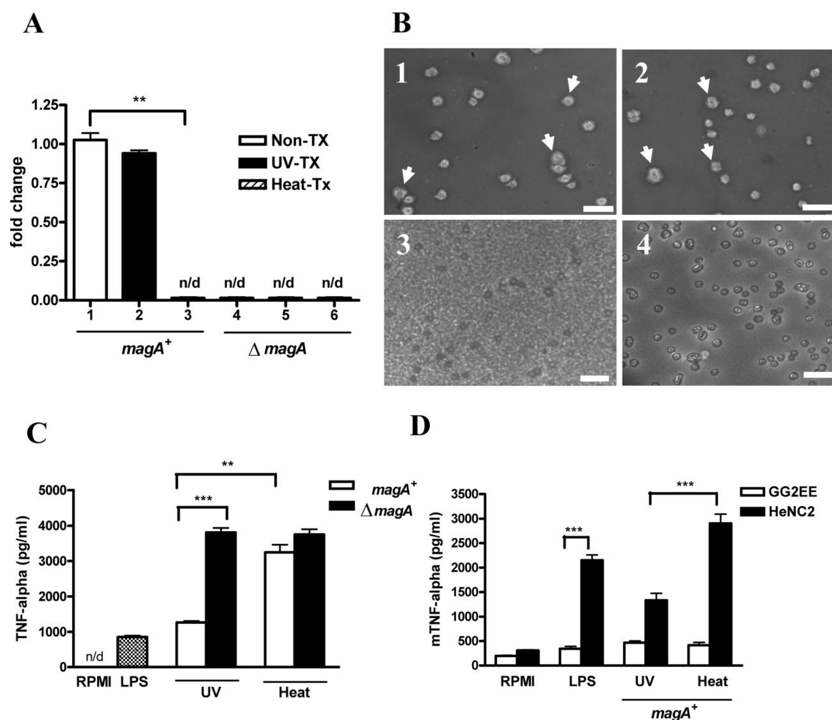

FIG. 1. Differential effect of heat and UV on the CPS of *magA*$^+$ and Δ*magA K. pneumoniae* strains. (A) After heat (95°C for 30 min) and UV irradiation (20 J/cm^2) treatment (TX), bacterial colonies were subjected to the string test determine the viscosity. The string length of *magA*$^+$ *K. pneumoniae* is about 50 mm, and the *y* axis denotes the fold change after either heat or UV treatment compared to untreated *magA*$^+$ *K. pneumoniae*. Samples 1 to 3, *magA*$^+$ *K. pneumoniae*; samples 4 to 6, Δ*magA K. pneumoniae*. n/d, Not detectable. The significance of the coupled difference was determined by using the Student *t* test. **, $P < 0.01$. (B) The *magA*$^+$ *K. pneumoniae* (5×10^7 to 1×10^8 CFU in 10 μl) was either untreated or inactivated by UV or heat inactivated. The capsule structure was visualized by negative staining. Panels: 1, untreated; 2, UV inactivated; 3, heat inactivated; 4, Δ*magA* strain. Scale bar, 1 μm. (C) hMDMs (6×10^5/ml) were incubated with UV-irradiated or heat-treated *magA*$^+$ and Δ*magA* strains (MOI = 5) for 24 h. Culture supernatants were harvested and subjected to ELISA to measure the secretion of TNF-α. LPS (1 μg/ml) was used as a positive control. (D) HeNC2 and its TLR4-defective mutant GG2EE were incubated with UV-inactivated or heat-inactivated *magA*$^+$ bacteria (MOI = 0.5) for 24 h to measure TNF-α secretion. LPS (1 ng/ml) is used as a positive control. The data are expressed as the means ± the standard deviations from three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (Student *t* test).

treatment still maintained the mucoviscosity of *magA*$^+$ *K. pneumoniae*, suggesting that UV treatment did not disrupt *magA*$^+$ *K. pneumoniae* CPS structure. This argument is supported by the observation that the capsule of *magA*$^+$ *K. pneumoniae* is maintained after UV treatment (Fig. 1B2), whereas heat inactivation disrupted the capsule (Fig. 1B3). We therefore decided to use UV for bacteria inactivation in the subsequent experiments.

CPS of *magA*$^+$ *K. pneumoniae* attenuates macrophage responses by hindering recognition of LPS by TLR4. After incubation with hMDMs, UV-inactivated Δ*magA K. pneumoniae* was more potent than the UV-inactivated *magA*$^+$ strain to stimulate TNF-α production (Fig. 1C). In contrast, the ability of heat-inactivated *magA*$^+$ *K. pneumoniae* was similar to that of the heat-inactivated Δ*magA* strain to stimulate TNF-α production from hMDMs (Fig. 1C). That heat treatment disrupts the capsule integrity of the *magA*$^+$ strain (Fig. 1B3) and restores its ability to stimulate TNF-α production (Fig. 1C) suggests that the integrity of CPS decides the stimulatory effect of *magA*$^+$ *K. pneumoniae*, and CPS may function as a barrier to hinder hMDM recognition to other underlying bacterial components, such as LPS. A critical role of TLR4 in the recognition of the microbial component LPS was initially characterized by the evidence that C3H/HeJ mice with mutation in the *tlr-4* gene impede LPS signal transduction, and the mice become resistant to LPS and yet are highly susceptible to gram-negative bacterial infection (23). Two mouse macrophage cell lines (HeNC2 and GG2EE) containing the functional TLR4 and nonfunctional TLR4, respectively, were used to investigate whether TLR4 is involved in host recognition to *K. pneumoniae*. As expected, *magA*$^+$ *K. pneumoniae* only has a weak stimulatory effect on TNF-α production from GG2EE. Compared to UV-treated *magA*$^+$ *K. pneumoniae*, heat-treated *magA*$^+$ *K. pneumoniae* more strongly stimulates TNF-α production from HeNC2 cells (Fig. 1D). This suggests that TNF-α secretion is via interaction of TLR4 and underlying LPS, and disruption of CPS by heat exposes the underlying LPS to stimulate TNF-α secretion via interaction with TLR4 on HeNC2 cells. It has been shown that OmpA can bind and activate macrophages and synergize with LPS, leading to maximal TNF-α production from macrophages (30). Therefore, we cannot rule out the possibility that CPS also reduces TNF-α secretion by interfering with the interaction of OmpA with a not-yet-defined pattern recognition receptor on host cells.

Agglutination of K1 strains by anti-*magA*$^+$ *K. pneumoniae* CPS MAbs. We further sought to determine whether CPS is the target for the development of vaccine against the invasive *magA*$^+$ *K. pneumoniae* strains. To address this question, hy-

Downloaded from iai.asm.org at National Taiwan Univ. on June 11, 2009

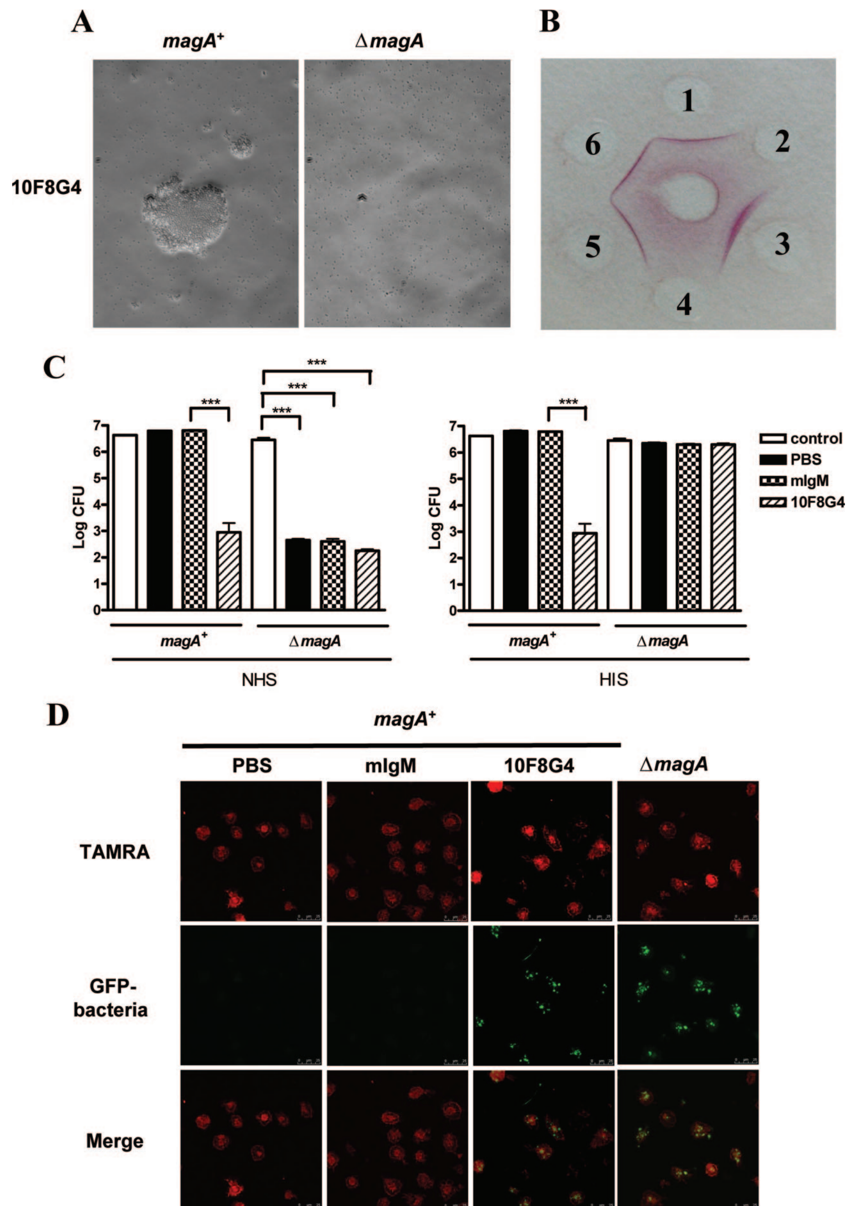

FIG. 2. Anti-CPS MAb inhibits the growth and enhances phagocytosis of *magA*$^+$ *K. pneumoniae* (A) Rapid agglutination assay. *magA*$^+$ and Δ*magA* strains were incubated with anti-CPS MAb (10F8G4) as described in Materials and Methods. Magnification, ×200. (B) Double immunodiffusion assay. An anti-CPS MAb (clone 10F8G4) was loaded into the central well, while CPS was loaded into peripheral wells of agar. The agar was incubated at 37°C overnight and dried in air before incubation with 1% azocarmine (dissolved in 2% glacial acid) for 2 h. The precipitation lines were visualized after incubation with 5% glacial acid for destaining. Spots: 1, *magA*$^+$ strain; 2, Δ*magA* strain; 3, 5, and 6, clinical K1 strains randomly selected from Table 2; 4, K62 from Table 3. (C) Bacteria (3×10^6 to 6×10^6 CFU, control) as described in panel A were incubated with anti-CPS MAb (7.5 μg/sample) for 1 h at room temperature, followed by the addition of NHS or HIS to a final concentration of 25%. The bacteria were then diluted (10-fold serial dilutions) and plated onto LB agar plates overnight to observe colony formation. The *y* axis of colony numbers was expressed as the log CFU. The data are expressed as the means ± the standard deviations from three independent experiments. ***, $P < 0.001$ (Student *t* test). (D) Phagocytosis was performed by incubating TAMRA-labeled hMDMs with bacteria stably expressing green fluorescent protein as described in Materials and Methods, followed by observation under a confocal microscope.

bridomas were generated by fusing myeloma cells with splenocytes isolated from mice immunized with *magA*$^+$ *K. pneumoniae*. All seven MAbs (IgM) recognized only the CPS of *magA*$^+$ *K. pneumoniae* but not that of the Δ*magA* mutant by agglutination assay (Fig. 2A). Moreover, all seven clones agglutinated *magA*$^+$ *K. pneumoniae* but not the Δ*magA* strain, as determined by both rapid agglutination assay (Table 1) and conventional double-immunodiffusion assay (Fig. 2B). Furthermore, all seven MAbs agglutinated all of the K1 strains of the clinical isolates (Table 2) and the reference strain but not the other serotypes (Table 3). This suggests that the anti-CPS MAbs recognize the K1 epitope(s) of all of the K1 strains tested, and the rapid serotyping without the need to extract CPS provided an alternative to the double-immunodiffusion assay.

Downloaded from iai.asm.org at National Taiwan Univ. on June 11, 2009

TABLE 1. MAbs to *magA*$^+$ strain

Clone	Isotype	Result[a] determined by:			
		ELISA		Agglutination	
		magA$^+$	Δ*magA*	*magA*$^+$	Δ*magA*
9E9C7	IgM	+	–	+	–
9E9D8	IgM	+	–	+	–
9E9F11	IgM	++	–	++	–
10F8C3	IgM	+	–	+	–
10F8E3	IgM	+	–	+	–
10F8F4	IgM	+	–	+	–
10F8G4	IgM	+++	–	+++	–

[a] "+" or "–" indicates that there was a positive or negative result, respectively, in the agglutination reactions and ELISA, "+++" indicates the strongest reaction, and "++" indicates an intermediate reaction.

Complement-independent killing effects of anti-CPS MAbs. It has been reported that *magA*$^+$ *K. pneumoniae* CPS interferes with complement deposition, and *magA*$^+$ *K. pneumoniae* is resistant to NHS-mediated lysis (12). Therefore, we sought to determine whether anti-CPS MAbs have a killing effect on *magA*$^+$ and Δ*magA K. pneumoniae* in the presence or absence of NHS or HIS. As previously observed, the Δ*magA* strain was sensitive, whereas the *magA*$^+$ *K. pneumoniae* strain was resistant, to NHS-mediated lysis (Fig. 2C, left panel). Interestingly, *magA*$^+$ *K. pneumoniae* was killed by anti-CPS MAb (clone 10F8G4), whether coincubated with NHS or HIS (Fig. 2C). This indicates that the anti-CPS MAb itself is enough to kill *magA*$^+$ *K. pneumoniae*. In addition, anti-CPS MAb also enhanced the uptake of *magA*$^+$ *K. pneumoniae* by MDMs (Fig. 2D). This suggests that anti-CPS MAbs may be useful in restricting bacterial survival and spread in vivo.

Anti-CPS MAbs protected mice from *magA*$^+$ *K. pneumoniae*-induced lethality. To investigate the potential therapeutic effects of anti-CPS MAbs in vivo, mice were injected with anti-CPS MAbs 24 h before intraperitoneal inoculation with *magA*$^+$ *K. pneumoniae* (2×10^3 CFU). At day 12 after infection, splenomegaly (Fig. 3A) and multiple liver abscesses with leukocyte infiltration (Fig. 3B) were observed in mice pretreated with PBS (Fig. 3A2) or isotype control antibodies (Fig. 3A3). In contrast, anti-CPS MAbs (clones 9E9F11 and 10F8G4) prevented splenomegaly (Fig. 3A4 and 5) and abscess formation (Fig. 3B4 and 5). The survival rates of mice treated with PBS or isotype control antibodies were 20 and 25%, respectively, while the survival rates in mice treated with anti-CPS MAbs (clones 10F8G4 and 9E9F11) were 100 and 95%, respectively (Fig. 3C). These data suggest that the K1 epitope of CPS is a promising target for vaccine development and that anti-CPS MAbs have great potential as therapeutic agents to protect against K1-induced lethality.

TABLE 2. Clinical *K. pneumoniae* strains[a]

Clinical diagnosis of patients	Total no. of isolates	K1 strains		Non-K1 isolates	
		No. of strains	Agglutination	No. of isolates	Agglutination
PLA	23	21	+	2	–
PLA plus fasciitis	6	5	+	1	–
Recurrent liver abscess	4	2	+	2	–
PLA plus endophthalmitis	7	6	+	1	–
Meningitis	4	3	+	1	–

[a] PLA, primary pyogenic liver abscess. "+" or "–" means that there was positive or negative result, respectively, in the agglutination reactions.

TABLE 3. Reference *K. pneumoniae* strains[a]

Bacterial strain	Serotype	Agglutination
ATCC 8045	K1	+
MGH78578	K52	–
K5	K5	–
K9	K9	–
K14	K14	–
K62	K62	–

[a] ATCC, American Type Culture Collection. K5, K9, K14, and K62 were isolated from patients with primary liver abscess; "+" means that there was a positive result in the agglutination reactions; "–" means that there was a negative result in the agglutination reactions.

DISCUSSION

We report here that the integrity of *magA*$^+$ *K. pneumoniae* CPS is not only essential to interfere with complement deposition (12) but also to determine the hMDM response to *magA*$^+$ *K. pneumoniae*. Furthermore, anti-*magA*$^+$ *K. pneumoniae* CPS MAbs could enhance the phagocytosis of *magA*$^+$ *K. pneumoniae*, prevent liver abscess formation, and protect mice from *magA*$^+$ *K. pneumoniae*-induced lethality. Since the anti-CPS MAbs can recognize all of the K1 strains tested, this suggests K1 epitope is a promising target for vaccine development against all of the K1 strains of *K. pneumoniae*.

Multiple *Klebsiella* components (e.g., fimbriae, siderophores, LPS, and capsules) have been considered to be potential virulence factors (22). Among these factors, LPS induces TNF-α (15). Although the CPS has been shown to play a major role in increasing pathogenicity, the mechanism(s) by which CPS increases the virulence of *K. pneumoniae*, in terms of its ability to induce liver abscess, is not well elucidated (9, 10).

Capsules can increase the virulence of *K. pneumoniae* by acting as a physical barrier between immunostimulatory bacterial products (such as fimbriae and LPS) and the host's immune system. The capsule, through a physical shielding mechanism, can inhibit fimbrial function (28) and impede the adhesion to invade epithelial cells by *K. pneumoniae* (27). This shielding of adhesion by the capsule has also been observed in other organisms, such as *E. coli* (29) and *Haemophilus influenzae* (31). The capsule can also mask the LPS molecules to prevent complement deposition (20). In the present study, we further demonstrated that, in addition to preventing complement-mediated lysis and uptake by macrophages, CPS plays an important role in attenuating hMDM responses via interference with the interaction of TLR4 with the underlying LPS, thus decreasing the induction of TNF-α, which is essential for the prevention of bacterial spreading and invasion (Fig. 1C). This elucidates a novel mechanism of CPS-mediated bacterial virulence.

In addition to the *magA*$^+$ *K. pneumoniae* CPS, the production of TNF-α was also suppressed by the *Salmonella enterica* serovar Typhi Vi capsular antigen (24, 36) and *Neisseria meningitidis* type C polysaccharide (17). The Vi capsule in the

Downloaded from iai.asm.org at National Taiwan Univ. on June 11, 2009

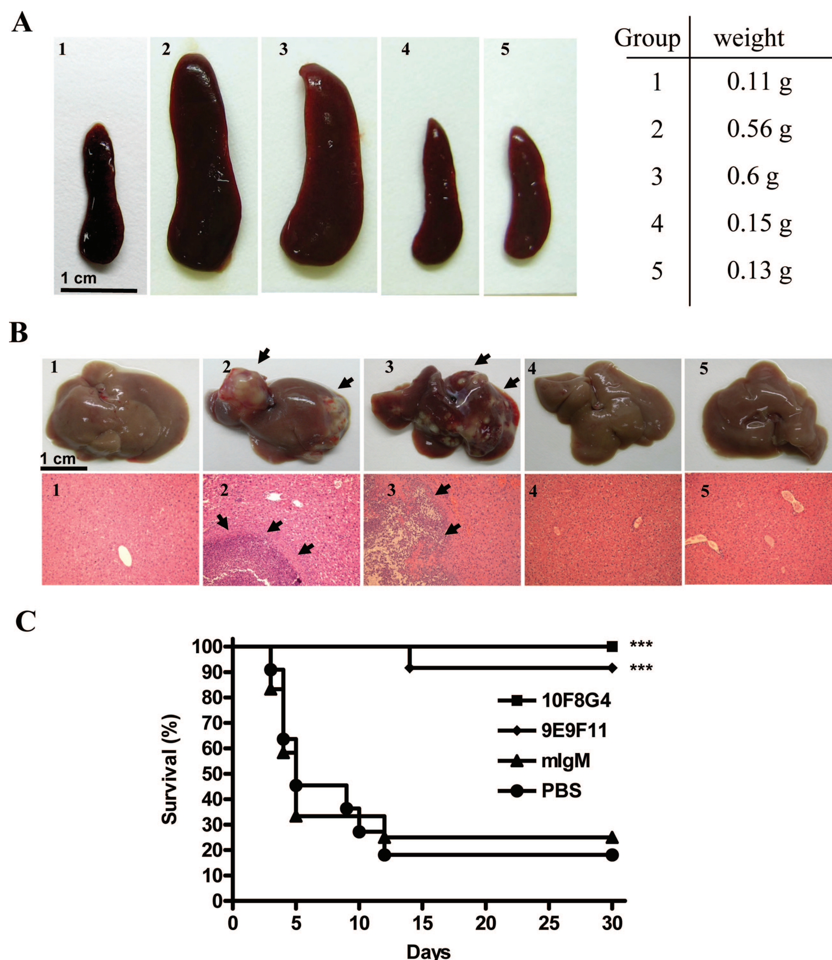

FIG. 3. Effects of anti-CPS MAbs on *magA*$^+$ strains in inducing pathological changes and lethality. At 24 h before inoculation with *magA*$^+$ strains (2×10^3 CFU), mice were injected with anti-CPS MAbs (100 μg/mouse), PBS, or isotype control (mIgM). At day 12 postinfection, mice were sacrificed, and the spleens (A) and livers (B) were removed and examined grossly, while liver abscesses were examined by hematoxylin and eosin staining and observed under a microscope (magnification, ×200). Panels: 1, no infection; 2, PBS; 3, mIgM; 4, anti-CPS MAb (clone 9E9F11); 5, anti-CPS MAb (clone 10F8G4). (C) The survival rate of each group was determined at 30 days after inoculation. ***, $P < 0.001$ (as determined by using Kaplan-Meier survival analysis and compared by log-rank test [$n = 12$ for each group]).

serovar Typhi can lower the production of TNF-α and inhibit neutrophil infiltration by preventing the recognition of TLR4 to the serovar Typhi LPS (24, 36). Similarly, the capsule on *N. meningitidis* can inhibit TLR4 activation by interfering with the binding of the soluble components (CD14 and lipoprotein binding protein, the two members of the LOS receptor complex) to TLR4 binding (17). From these observations, evasion of innate immunity via inhibiting TLR4-mediated signaling may be a general feature of bacterial CPS.

Over the past two decades, CPS has been the obvious vaccine candidate for *Klebsiella*-induced pneumonia studies. Cryz et al. have demonstrated that active immunization with purified CPS protected rats against lethal *Klebsiella*-induced pneumonia (8). MAbs against K2 CPS reduced the inflammatory response in the lung and eliminated bacteria in a lung infection model (14). However, the ability of anti-CPS to prevent liver abscess formation has never been addressed. Our study demonstrates that anti-*magA*$^+$ *K. pneumoniae* CPS antibodies can agglutinate all of the K1 strains tested and prevent liver abscess formation induced by *magA*$^+$ *K. pneumoniae*. This indicates that the K1 epitope is a promising target for vaccine development and that anti-K1 MAbs have the potential to prevent liver abscess induced by all of the K1 strains.

ACKNOWLEDGMENTS

We thank BioLegend for antibody preparation and Hsien-Yeh Hsu for providing the HeNC2 and GG2EE cell lines. We are grateful to Chang-Phone Fung for providing the clinical strains of *K. pneumoniae* isolated from liver abscesses.

This study was supported mainly by the Department of Health, Executive Yuan, Republic of China (Taiwan) (grant DOH 94-TD-G-111-039), grant V97S5-005 from the Taipei Veterans General Hospital, and grant 94F008-5 from Academia Sinica.

REFERENCES

1. **Blasi, E., D. Radzioch, S. K. Durum, and L. Varesio.** 1987. A murine macrophage cell line, immortalized by v-raf and v-myc oncogenes, exhibits normal macrophage functions. Eur. J. Immunol. **17:**1491–1498.
2. **Cahill, M., B. Chang, and A. Murray.** 2000. Bilateral endogenous bacterial endophthalmitis associated with pyogenic hepatic abscess. Br. J. Ophthalmol. **84:**1436.
3. **Casanova, C., J. A. Lorente, F. Carrillo, E. Perez-Rodriguez, and N. Nunez.** 1989. *Klebsiella pneumoniae* liver abscess associated with septic endophthalmitis. Arch. Intern. Med. **149:**1467.

Downloaded from iai.asm.org at National Taiwan Univ. on June 11, 2009

4. **Chang, Y. C., T. L. Hsu, H. H. Lin, C. C. Chio, A. W. Chiu, N. J. Chen, C. H. Lin, and S. L. Hsieh.** 2004. Modulation of macrophage differentiation and activation by decoy receptor 3. J. Leukoc. Biol. **75:**486–494.
5. **Cheng, D. L., Y. C. Liu, M. Y. Yen, C. Y. Liu, and R. S. Wang.** 1991. Septic metastatic lesions of pyogenic liver abscess. Their association with *Klebsiella pneumoniae* bacteremia in diabetic patients. Arch. Intern. Med. **151:**1557–1559.
6. **Chiu, C. T., D. Y. Lin, and Y. F. Liaw.** 1988. Metastatic septic endophthalmitis in pyogenic liver abscess. J. Clin. Gastroenterol. **10:**524–527.
7. **Chuang, Y. P., C. T. Fang, S. Y. Lai, S. C. Chang, J. T. Wang, C. Struve, M. Bojer, E. M. Nielsen, D. S. Hansen, K. A. Krogfelt, R. Gierczynski, S. Kaluzewski, A. A. Zasada, W. Rastawicki, M. Jagielski, L. C. Ma, C. Z. Lee, C. T. Shun, F. C. Fang, N. Sandler, and S. J. Libby.** 2006. Genetic determinants of capsular serotype K1 of *Klebsiella pneumoniae* causing primary pyogenic liver abscess. J. Infect. Dis. **193:**645–654.
8. **Cryz, S. J., Jr., E. Furer, and R. Germanier.** 1986. Immunization against fatal experimental *Klebsiella pneumoniae* pneumonia. Infect. Immun. **54:**403–407.
9. **Cryz, S. J., Jr., F. Furer, and R. Germanier.** 1984. Experimental *Klebsiella pneumoniae* burn wound sepsis: role of capsular polysaccharide. Infect. Immun. **43:**440–441.
10. **Domenico, P., W. G. Johanson, Jr., and D. C. Straus.** 1982. Lobar pneumonia in rats produced by clinical isolates of *Klebsiella pneumoniae*. Infect. Immun. **37:**327–335.
11. **Fang, C. T., Y. C. Chen, S. C. Chang, W. Y. Sau, and K. T. Luh.** 2000. *Klebsiella pneumoniae* meningitis: timing of antimicrobial therapy and prognosis. QJM **93:**45–53.
12. **Fang, C. T., Y. P. Chuang, C. T. Shun, S. C. Chang, and J. T. Wang.** 2004. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. J. Exp. Med. **199:**697–705.
13. **Fung, C. P., F. Y. Chang, S. C. Lee, B. S. Hu, B. I. Kuo, C. Y. Liu, M. Ho, and L. K. Siu.** 2002. A global emerging disease of *Klebsiella pneumoniae* liver abscess: is serotype K1 an important factor for complicated endophthalmitis? Gut **50:**420–424.
14. **Held, T. K., M. Trautmann, M. E. Mielke, H. Neudeck, S. J. Cryz, Jr., and A. S. Cross.** 1992. Monoclonal antibody against *Klebsiella* capsular polysaccharide reduces severity and hematogenic spread of experimental *Klebsiella pneumoniae* pneumonia. Infect. Immun. **60:**1771–1778.
15. **Henderson, B., S. Poole, and M. Wilson.** 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60:**316–341.
16. **Ko, W. C., D. L. Paterson, A. J. Sagnimeni, D. S. Hansen, A. Von Gottberg, S. Mohapatra, J. M. Casellas, H. Goossens, L. Mulazimoglu, G. Trenholme, K. P. Klugman, J. G. McCormack, and V. L. Yu.** 2002. Community-acquired *Klebsiella pneumoniae* bacteremia: global differences in clinical patterns. Emerg. Infect. Dis. **8:**160–166.
17. **Kocabas, C., N. Katsenelson, S. Kanswal, M. N. Kennedy, X. Cui, M. S. Blake, D. M. Segal, and M. Akkoyunlu.** 2007. *Neisseria meningitidis* type C capsular polysaccharide inhibits lipooligosaccharide-induced cell activation by binding to CD14. Cell Microbiol. **9:**1297–1310.
18. **Lederman, E. R., and N. F. Crum.** 2005. Pyogenic liver abscess with a focus on *Klebsiella pneumoniae* as a primary pathogen: an emerging disease with unique clinical characteristics. Am. J. Gastroenterol. **100:**322–331.
19. **Liu, Y. C., D. L. Cheng, and C. L. Lin.** 1986. *Klebsiella pneumoniae* liver abscess associated with septic endophthalmitis. Arch. Intern. Med. **146:**1913–1916.
20. **Merino, S., S. Camprubi, S. Alberti, V. J. Benedi, and J. M. Tomas.** 1992. Mechanisms of *Klebsiella pneumoniae* resistance to complement-mediated killing. Infect. Immun. **60:**2529–2535.
21. **Mizuta, K., M. Ohta, M. Mori, T. Hasegawa, I. Nakashima, and N. Kato.** 1983. Virulence for mice of *Klebsiella* strains belonging to the O1 group: relationship to their capsular (K) types. Infect. Immun. **40:**56–61.
22. **Podschun, R., and U. Ullmann.** 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. **11:**589–603.
23. **Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler.** 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science **282:** 2085–2088.
24. **Raffatellu, M., D. Chessa, R. P. Wilson, R. Dusold, S. Rubino, and A. J. Baumler.** 2005. The Vi capsular antigen of Salmonella enterica serotype Typhi reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. Infect. Immun. **73:**3367–3374.
25. **Saccente, M.** 1999. *Klebsiella pneumoniae* liver abscess, endophthalmitis, and meningitis in a man with newly recognized diabetes mellitus. Clin. Infect. Dis. **29:**1570–1571.
26. **Sahly, H., and R. Podschun.** 1997. Clinical, bacteriological, and serological aspects of *Klebsiella* infections and their spondylarthropathic sequelae. Clin. Diagn. Lab. Immunol. **4:**393–399.
27. **Sahly, H., R. Podschun, T. A. Oelschlaeger, M. Greiwe, H. Parolis, D. Hasty, J. Kekow, U. Ullmann, I. Ofek, and S. Sela.** 2000. Capsule impedes adhesion to and invasion of epithelial cells by *Klebsiella pneumoniae*. Infect. Immun. **68:**6744–6749.
28. **Schembri, M. A., J. Blom, K. A. Krogfelt, and P. Klemm.** 2005. Capsule and fimbria interaction in *Klebsiella pneumoniae*. Infect. Immun. **73:**4626–4633.
29. **Schembri, M. A., D. Dalsgaard, and P. Klemm.** 2004. Capsule shields the function of short bacterial adhesins. J. Bacteriol. **186:**1249–1257.
30. **Soulas, C., T. Baussant, J. P. Aubry, Y. Delneste, N. Barillat, G. Caron, T. Renno, J. Y. Bonnefoy, and P. Jeannin.** 2000. Outer membrane protein A (OmpA) binds to and activates human macrophages. J. Immunol. **165:**2335–2340.
31. **St. Geme, J. W., III, and S. Falkow.** 1991. Loss of capsule expression by *Haemophilus influenzae* type b results in enhanced adherence to and invasion of human cells. Infect. Immun. **59:**1325–1333.
32. **Tang, L. M., and S. T. Chen.** 1994. *Klebsiella pneumoniae* meningitis: prognostic factors. Scand. J. Infect. Dis. **26:**95–102.
33. **Tsay, R. W., L. K. Siu, C. P. Fung, and F. Y. Chang.** 2002. Characteristics of bacteremia between community-acquired and nosocomial *Klebsiella pneumoniae* infection: risk factor for mortality and the impact of capsular serotypes as a herald for community-acquired infection. Arch. Intern. Med. **162:**1021–1027.
34. **Uematsu, S., and S. Akira.** 2006. Toll-like receptors and innate immunity. J. Mol. Med. **84:**712–725.
35. **Wang, J. H., Y. C. Liu, S. S. Lee, M. Y. Yen, Y. S. Chen, J. H. Wang, S. R. Wann, and H. H. Lin.** 1998. Primary liver abscess due to *Klebsiella pneumoniae* in Taiwan. Clin. Infect. Dis. **26:**1434–1438.
36. **Wilson, R. P., M. Raffatellu, D. Chessa, S. E. Winter, C. Tukel, and A. J. Baumler.** 2008. The Vi-capsule prevents Toll-like receptor 4 recognition of *Salmonella*. Cell Microbiol. **10:**876–890.
37. **Zamze, S., L. Martinez-Pomares, H. Jones, P. R. Taylor, R. J. Stillion, S. Gordon, and S. Y. Wong.** 2002. Recognition of bacterial capsular polysaccharides and lipopolysaccharides by the macrophage mannose receptor. J. Biol. Chem. **277:**41613–41623.

Editor: A. J. Bäumler

Downloaded from iai.asm.org at National Taiwan Univ. on June 11, 2009