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Pneumococcal wall teichoic acid is required for the pathogenesis of *Streptococcus pneumoniae* in murine models

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Pneumococcal asymptomatic colonization of the respiratory tracts is a major risk for invasive pneumococcal disease. We have previously shown that pneumococcal wall teichoic acid (WTA) was involved in pneumococcal infection of sepsis and adherence to epithelial and endothelial cells. In this study, we investigated the contribution of pneumococcal WTA to bacterial colonization and dissemination in murine models. The result showed that nasopharynx colonizing D39 bacterial cells have a distinct phenotype showing an increased exposure of teichoic acids relative to medium-grown bacteria. The WTA-deficient mutants were impaired in their colonization to the nasopharynx and lungs, and led to a mild inflammation in the lungs at 36 h post-inoculation. Pretreatment of the murine nares with WTA reduced the ability of wild type D39 bacteria to colonize the nasopharynx. In addition, the WTA-deficient strain was impaired in its ability to invade the blood and brain following intranasal administration. WTA-deficient D39 strain was reduced in C3 deposition but was more susceptible to the killing by the neutrophils as compared with its parent strain. Our results also demonstrated that the WTA enhanced pneumococcal colonization and dissemination independently of the host strains. These results indicate that WTA plays an important role in pneumococcal pathogenesis, both in colonization and dissemination processes.

Keywords: *Streptococcus pneumoniae*, wall teichoic acid, colonization, pathogenesis

Introduction

Streptococcus pneumoniae remains one of the leading pathogens causing pneumonia, sepsis and meningitis, especially in children and the elderly (Liu *et al.*, 2008; Montagnani *et al.*, 2008). Asymptomatic colonization of the upper respiratory tract with pneumococci is a key step in disease development and horizontal transmission within populations (Bogaert *et al.*, 2004). Despite the implementation of the immunization program worldwide, invasive pneumococcal disease has been reported most frequently in children younger than 5 years (Hsu *et al.*, 2009).

Teichoic acid (TA) is an essential component of the cell wall (Weidenmaier and Peschel, 2008). In Gram-positive bacteria, wall teichoic acid (WTA) is covalently attached to the peptidoglycan; whereas lipoteichoic acid (LTA) is linked to the glycolipid anchor in the membrane. Of note, the chemical composition of TA displays species-specific variation, and it is uncommon for WTA and LTA to have the same chemical composition (Weidenmaier and Peschel, 2008). In *Staphylococcus aureus*, LTA is a glycerol phosphate polymer (Grundling and Schneewind, 2007), while WTA is composed of ribitol phosphate repeating units (Weidenmaier *et al.*, 2004). Compared to the TAs of other Gram-positive bacteria, pneumococcal TA has a more complex chemical composition. In addition, pneumococcal LTA and WTA share the same sugar backbone consisting of several different sugar residues and phosphorylcholine (*P*-Cho) modification (Draing *et al.*, 2006; Bui *et al.*, 2012; Gisch *et al.*, 2013).

TA plays an important role in physiological processes including bacterial growth, autolysis and binding of divalent cations (Weidenmaier and Peschel, 2008). In *Bacillus subtilis*, WTA is implicated in bacterial growth, cell division and morphogenesis (Grundling and Schneewind, 2007; Weidenmaier and Peschel, 2008). Although pneumococcal WTA is structurally different from that of the *B. subtilis* WTA, we showed that it was also important for bacterial division and morphogenesis (Wu *et al.*, 2014). In addition, it was recognized that, as an alternative non-protein adhesin, WTA was involved in nasal colonization in *S. aureus* (Weidenmaier *et al.*, 2004; Kawai *et al.*, 2011). In pneumococci, however, due to the lack of a TA mutant, evidence for the role of pneumococcal TA in virulence has been limited to studies with phase variants or on the *P*-Cho moiety of TAs (Tomasz, 1968; Weiser *et al.*, 1994; Cundell *et al.*, 1995; Gehre *et al.*, 2009). A previous study demonstrated that nasopharynx colonizing bacteria commonly have a higher amount of TAs than bacteria grown in the blood (Kim and Weiser, 1997), suggesting the possible involvement of TAs in colonization. Interaction between *P*-Cho and the platelet activated factor receptor indicated the role of *P*-Cho in pneumococcal adherence to the cytokine-activated cells (Cundell *et al.*, 1995). Nevertheless, the role of pneumococcal TAs in disease pathogenesis has not been systemically investigated.

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RafX (SPD_1672), a hypothetical protein, is composed of 397 amino acids and has been reported to be involved in the biosynthesis of WTA (Wu *et al.*, 2014). We have also shown that pneumococcal WTA contributes to infection of sepsis and is important for pneumococcal adherence to epithelial and endothelial cells (Wu *et al.*, 2014). Here, we focus on more the *in vivo phenotypes of the ΔrafX* mutant. The most striking changes in phenotype, including colonization and dissemination, C3 deposition, susceptibility to neutrophils and virulence were found for Δ*rafX* mutant.

Materials and Methods

Ethic statement

All animals were bred in the specific pathogen free condition. All experimental protocols were approved by the Ethics Committee of Chongqing Medical University (Reference number: 2011-032). All animals were purchased from the Laboratory Animal Center of Chongqing Medical University (certificate number: SYXK(yu)2007-0001). The experiments were performed in accordance with the Declaration of Helsinki and with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Bacterial growth conditions

S. pneumoniae D39 strain (NCTC 7466, serotype 2) and R6 were purchased from the National Collection of Type Cultures (London, UK). Pneumococcal strain CMCC(B)31203 (serotype 3) was purchased from the China Medical Culture Collection (CMCC, China). Δ*rafX* mutants were constructed as described in our previous study (Wu *et al.*, 2014). *S. pneumoniae* strains were grown in semi-synthetic casein hydrolysate medium supplemented with 5% yeast extract (C+Y) medium or blood agar plates at 37°C in an atmosphere with 5% CO_2.

Flow cytometric analysis

For TA assays, D39 bacteria were administrated intranasally to the nasal cavity of Balb/c mice and bacteria in nasal washes were collected for flow cytometric analysis at 72 h post-inoculation. Nasal wash samples were collected from the mice after sacrifice. The trachea was exposed and a 23-gauge blunt needle was placed into the distal trachea. Nasal washes were collected from the tip of the nose by flushing with 2 ml PBS through the trachea. Bacteria were pelleted and a total of 5×10^6 bacterial cells in phosphate-buffered saline (PBS) supplemented with 5% fetal calf serum (Hyclone) were incubated with anti-cell wall associated polysaccharide (CWPS) antibodies (1:200; Statens Serum Institut) in a 200 µl mixture for 60 min at 37°C. The bacterial pellets were washed twice with PBS, resuspended in 1:200 dilution of secondary antibodies [phycoerythrin (PE)-labeled goat anti-rabbit IgG, BD Biosciences] and incubated for 60 min at 4°C. C3 deposition assays were performed according to a published procedure (Shainheit *et al.*, 2014). After additional thrice washes, bacteria were re-suspended in PBS containing 1% bovine serum albumin and 1% paraformaldehyde before detection by a flow cytomerty (FACSVantage; Becton Dickinson Biosciences).

Growth of WTA-deficient mutant in the blood

Pneumococcal survival in mouse whole blood was performed by inoculating 2×10^2 CFU of bacteria (in 10 µl) into 90 µl of fresh heparinized mouse blood and incubated without rotation at 37°C. After 2 h, reactions were plated onto the blood agar plates and bacterial numbers were determined after overnight culture at 37°C. The proliferation index was determined by dividing bacterial counts at 2 h post-incubation by those at time of inoculation (0 h). Three independent experiments were performed.

Opsonophagocytic killing assays

Neutrophils were isolated according to previously established protocol (Standish and Weiser, 2009). In brief, phagocytes were collected by lavage of the peritoneal cavity (8-10 ml/animal with PBS containing 20 mM EDTA) of mice treated 24 h and again 3 h before cell harvest by intraperitoneal injection of 1 ml 10% sterile casein in PBS. Cells collected from the peritoneal cavity were separated by a 5 min centrifugation at 275 × *g* with 42% and 51% Percoll. Neutrophils were collected and washed with 5 ml of Hank's buffer without Ca^{2+} or Mg^{2+} (Invitrogen) with 0.1% gelatin. The cell purity and number of viable cells were determined by Wright's stain and trypan blue dye, respectively. Cells were adjusted to a density of 10^7 cells/ml in Hank's buffer with Ca^{2+} and Mg^{2+} (Gibco), and 0.1% gelatin.

The opsonophagocytic killing assay using neutrophils was conducted as previously described (Standish and Weiser, 2009). Briefly, bacteria cultured to mid-log phase were collected, PBS-washed, and resuspended in Hank's buffer with Ca^{2+} and Mg^{2+} (Gibco), and 0.1% gelatin. Ten thousand CFU *S. pneumoniae* was pre-opsonized with 10 µl of guinea pig complement in a total volume of 100 µl for 60 min at 4°C, and the purified neutrophils (10^6 cells in 100 µl) were added and incubated for another 90 min at 37°C with rotation. Killing was stopped by spinning the cells onto ice after addition of 1 ml distilled water containing 0.01% BSA. The number of recovered bacteria was determined by plating serial dilutions on blood agar plates.

Animal experiments

To investigate the role of WTA in nasopharyngeal colonization, six weeks old Balb/c female mice were randomly divided into designed groups. Mice were anesthetized with pentobarbital sodium (1.5%) and then intranasally inoculated with 6×10^6 CFU in 30 µl PBS of wild type D39 strain (serotype 2), D39Δ*rafX* mutant, or complemented strain (WL; D39Δ*rafX* mutant complemented with the full length of *rafX* using pEVP3 plasmid). Six mice from each group were killed after 12 h, 24 h, 72 h, and the numbers of pneumococci in the nasal wash, lungs, blood and brain were determined. For histological examination, lungs of infected mice were isolated at 36 h postinoculation and fixed in 4% paraformaldehyde. Fixed tissues were embedded in paraffin, sliced, and stained with hematoxylin and eosin.

To examine whether the role of WTA on nasopharyngeal colonization and dissemination affected by the host strain, groups of mice were administrated intranasally with wild type CMCC(B)31203 strain (serotype 3) or CMCC(B)31203Δ*rafX*

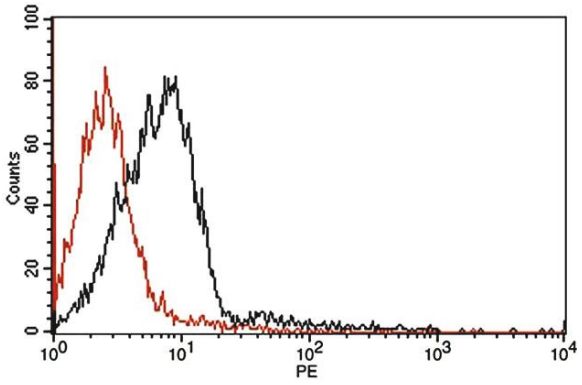

Fig. 1. Teichoic acid was surface-exposed during nasopharyngeal colonization. A representative flow cytometric result demonstrating binding of CWPS antiserum to medium-grown D39 strain (Red) or pneumococci collected from nasal washes (Black).

mutant at a dose of 1×10^8 CFU. Six mice from each group were killed after 12 h, 24 h, 72 h, and the numbers of pneumococci in the nasal wash, lungs, blood, and brain were counted. For intraperitoneal infection models, six-week-old female Balb/c mice were challenged with CMCC(B)31203 strain or CMCC(B)31203Δ*rafX* mutant at a dose of 1×10^8 CFU. Survival was monitored every four hours in the first three days, and then daily to the observation end.

For inhibition assays, Balb/c mice were pretreated with 300 μg of monoclonal mouse IgA antibody specific for *P*-Cho (Clone ID TEPC-15, Cat# M1421; Sigma-Aldrich) or CWPS (Statens Serum Institut) at the indicated amounts five min before inoculation of bacteria of 10^5 CFU. Isotype IgA (Abcam) or PBS treated mice were used as the controls. Cell wall-associated polysaccharide (CWPS) is structurally a peptidoglycan-attached teichoic acid complex of pneumococci (Skovsted

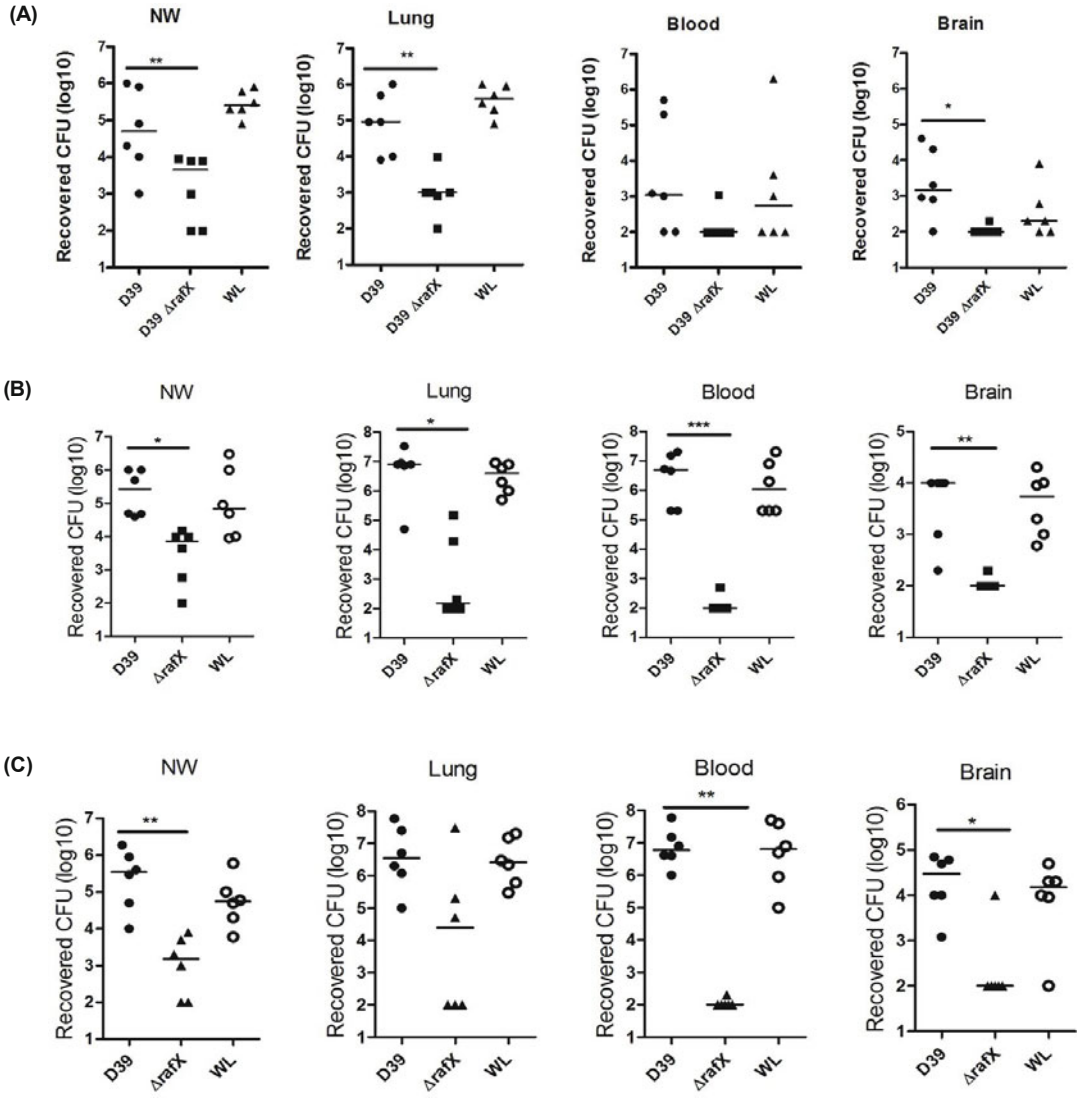

Fig. 2. Bacterial numbers in the nasal washes (NW), lungs, blood, and brain following challenge with pneumococcal strains. Mice were infected intranasally with D39, D39Δ*rafX*, or complemented strain (WL) at a dose of 6×10^6 CFU. Six mice were killed after 12 h (A), 24 h (B) or 72 h (C), and bacterial numbers in the nasal washes, lungs, brain and blood were determined. The limit of detection was determined to be 100 CFU. Statistical significance was examined with Student's *t* test, and *P* values were recorded. * $P < 0.05$; ** $P < 0.01$. The bars indicate the median bacterial numbers.

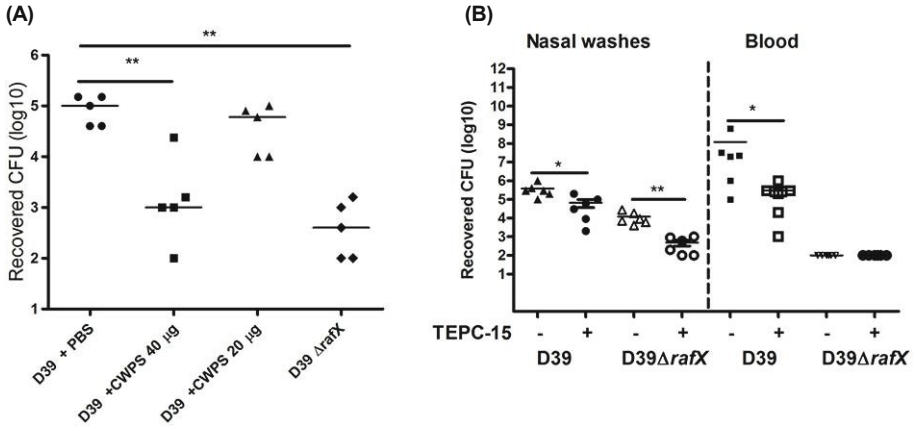

Fig. 3. Specific inhibition of TAs protects mice from colonization and dissemination to the blood. (A) Inhibition with CWPS protected mice against pneumococcal nasopharyngeal colonization. Balb/c mice were inoculated with CWPS at the indicated doses or PBS and then challenged with 10^5 CFU of D39 or D39Δ*rafX* mutant. Pneumococci were recovered three days after challenge. (B) Mice were pretreated with TEPC-15 (monoclonal mouse IgA antibody) five minutes before intranasal administration with 10^5 CFU of wild type D39 strain and D39Δ*rafX*. Bacteria were recovered three days after infection by nasal washing, and heart puncture in a separate experiment. Statistical significance was examined with Student's *t*-test, and *P* values were recorded. * $P < 0.05$; ** $P < 0.01$. The bars indicate the median bacterial numbers.

et al., 2007). Three days after inoculation, bacteria in the nasal wash or blood were collected, and dilutions were plated on blood plates to determine viable colonies.

Statistical analysis

Statistical difference between groups were compared with Student's *t* test, the non-parametric Mann-Whitney or Wilcoxon test using GraphPad Prism 5 (GraphPad Software). Statistical significance was defined as $P < 0.05$.

Results

TAs was surface accessible during nasopharynx colonization in pneumococcal D39 strain

We first aimed at validating whether TAs are surface accessible in *S. pneumoniae* D39 strain (a virulent strain with thick capsule). To examine the exposure of TAs during colonization, encapsulated D39 bacteria were administrated intranasally to the nasal cavity and bacteria in nasal washes were collected at 72 h post-inoculation. Expression of TAs on the surface of pneumococci was examined by flow cytometric analysis using rabbit anti-CWPS sera. Preimmune rabbit sera showed no reactivity with the bacteria (data not shown). As shown in Fig. 1, TA was only partially exposed at the pneumococcal surface when the encapsulated D39 strain was grown in C+Y medium. In contrast, nasopharynx colonizing D39 bacteria showed a significant increase in PE-recorded events with a right shift relative to medium-grown cells (Fig. 1), indicating a distinct phenotype for nasopharynx colonizing pneumococci that shows substantially increased amounts of surface-exposed TAs compared to those grown in C+Y medium.

Pneumococcal WTA was necessary for successful colonization and dissemination

Groups of Balb/c mice were inoculated intranasally with equal numbers of D39, D39Δ*rafX* or complemented strain at a dose of 6×10^6 CFU. Bacterial numbers in the nasal washes (NW), lungs, brain and blood were determined at 12 h (Fig. 2A), 24 h (Fig. 2B) or 72 h (Fig. 2C) after inoculation, respectively. Compared with the wild type strain, the D39Δ*rafX* showed a statistical decrease in numbers of pneumococci colonizing the nasopharynx at each time point ($P < 0.05$; Fig. 2), respectively. In addition, D39Δ*rafX* mutant was reduced in its capacity to invade the brain and blood at 24 h and 72 h respectively (Fig. 2). D39Δ*rafX* mutant was reduced in its ability to invade the blood at 12 h and to colonize the lungs at 72 h postinoculation relative to the wild-type strain, despite showing any significant difference. These results suggest that pneumococcal WTA is involved in bacterial virulence, both in colonization and invasion.

Pretreatment of mouse nares with WTA and TEPC-15 inhibited nasal colonization of pneumococci

To confirm the role of WTA in pneumococcal pathogenesis, mice were inoculated with CWPS five minutes before intranasal administration with 10^5 CFU of D39 or D39Δ*rafX*. Bacteria were recovered 72 h after infection by nasal washing and heart puncture. Pretreatment with 40 µg CWPS led to a 100-fold reduction in numbers of nasopharyngeal colonization pneumococci in murine model ($P < 0.05$; Fig. 3A). Pretreatment with TEPC-15 also significantly inhibited pneumococcal nasopharyngeal colonization and translocation to the blood ($P < 0.05$, Student's *t*-test). There were 3×10^5 CFU bacteria (median number) recovered from nasal washes when mice were pre-inoculated with isotype IgA; whereas 4.5×10^4 CFU (median number) was recovered from mice

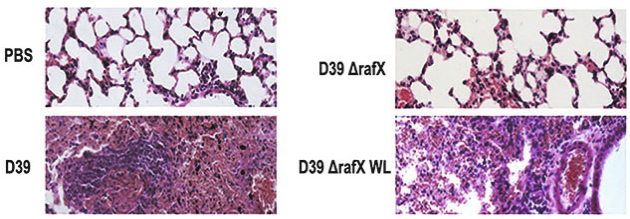

Fig. 4. Histopathological analysis of lungs from mice intranasally challenged with wild-type D39 strain, D39Δ*rafX* mutant or complemented strain (D39 Δ*rafX* WL). Mice were infected intranasally with 6×10^6 CFU of D39, D39Δ*rafX* mutant or the D39 Δ*rafX* WL strain. Mice inoculated with PBS were served as negative control. Lung samples were prepared and stained with hematoxylin and eosin, viewed at × 200.

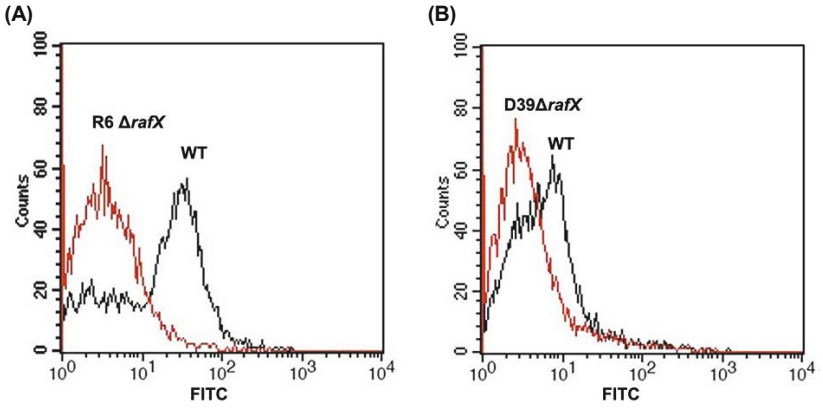

Fig. 5. Representative histogram of C3 deposition to wild type D39 and R6 and their respective mutants. The representative histogram demonstrating the binding of C3 to R6 (left) and D39 (Right) and their mutants.

pretreated with TEPC-15. There was a 6.7-fold reduction in bacterial number following TEPC-15 treatment in mice challenged with wild type D39 strain. This inhibition was more impressive in mice infected with D39Δ*rafX* mutant, which resulted in a 20-fold reduction in bacterial number in nasopharynx following TEPC-15 treatment ($P < 0.01$; Fig. 3B). These findings confirm that WTA plays a role in colonization and invasion during pneumococcal infections.

WTA-deficient mutant induced a mild inflammatory response in lungs

Inspection of lungs from mice infected with wild-type D39 strain and complemented strain (D39Δ*rafX* WL) displayed much severer inflammation compared to the controls, characterized by neutrophils and monocytes infiltration and necrotic debris in one or more lobes of the lungs (Fig. 4). In contrast, D39Δ*rafX* infected mice only had a mild inflammation in lungs at 36 h post-inoculation (Fig. 4). PBS-treated mice were served as negative control.

C3 deposition was reduced in Δ*rafX* mutants

It has been reported that *S. pneumoniae* TA is an effective activator of the alternative pathway of the component (Hummell *et al.*, 1985). In addition, it is known that the CbpA (also known as PspC) is also necessary for the binding of complement protein C3 to pneumococcal surface (Smith and Hostetter, 2000). Our previous evidence showed that the CbpA protein is reduced on the bacterial surface of the R6Δ*rafX* mutant (Wu *et al.*, 2014). We therefore analyzed the C3 deposition on bacterial surface of the Δ*rafX* mutants. Mid-exponential bacteria were incubated with infant rabbit serum, probed with FITC-conjugated goat anti-rabbit C3 antibody, and followed by flow cytometry analysis. Compared to wild type strains, the Δ*rafX* mutants showed a significant decrease in FITC-recorded events (Fig. 5), indicating that Δ*rafX* mutants were reduced in C3 deposition on bacterial surface.

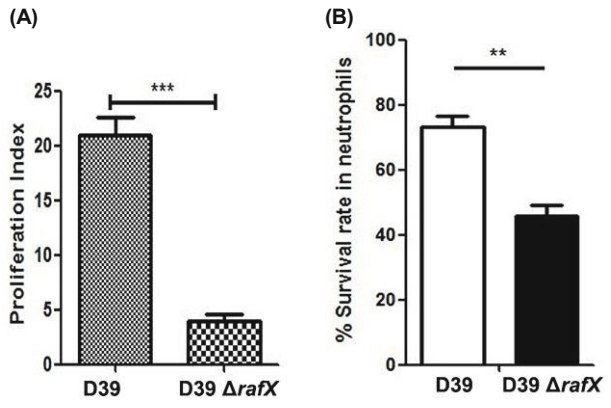

Fig. 6. Bacteria were grown in the mouse whole blood (A), or exposed to neutrophils (B). Bacteria were incubated with the mouse whole blood for 2 h at 37°C. The mixtures were plated onto the blood agar plates after 2 h incubation. Mean ± SD from three experiments. ** $P < 0.01$; *** $P < 0.001$.

The Δ*rafX* mutant proliferated much more slowly in blood than wild-type strain and has impaired resistance to phagocytic killing by neutrophils

We wondered whether the attenuated virulence of Δ*rafX* mutant could be the result of decreased proliferation in the blood. To address this question, wild-type D39 strain and the D39Δ*rafX* mutant were exposed to heparinized mouse blood for 2 h at 37°C. Wild-type D39 displayed a net growth of 20-fold compared to the inoculums in contrast to the D39Δ*rafX* mutant that showed a net growth of 3-fold compared to the inoculums after incubation ($P < 0.001$; Fig. 6A). These data suggest that the attenuated virulence observed in animals infected with the Δ*rafX* mutant is in part attributable to its reduced proliferation in the blood. Neutrophils are abundant in the blood and phagocytize pathogens in the early stage of infection. Therefore, viable wild-type D39 and Δ*rafX* mutant were incubated with neutrophils and survival rates were calculated following comparison to controls containing all reagents except the neutrophils. As shown in Fig. 6B, 73% wild-type D39 strain survived from the killing by neutrophils, whereas a markedly reduced number of the D39Δ*rafX* mutant (46%) survived from the killing ($P < 0.01$), suggesting an increased susceptibility of the WTA-deficient mutant to the bactericidal action of blood.

Reduced virulence and colonization and invasion of WTA-deficient CMCC(B) 31203 mutant

To examine whether the effects of WTA on pneumococcal virulence, colonization and invasion were influenced by host

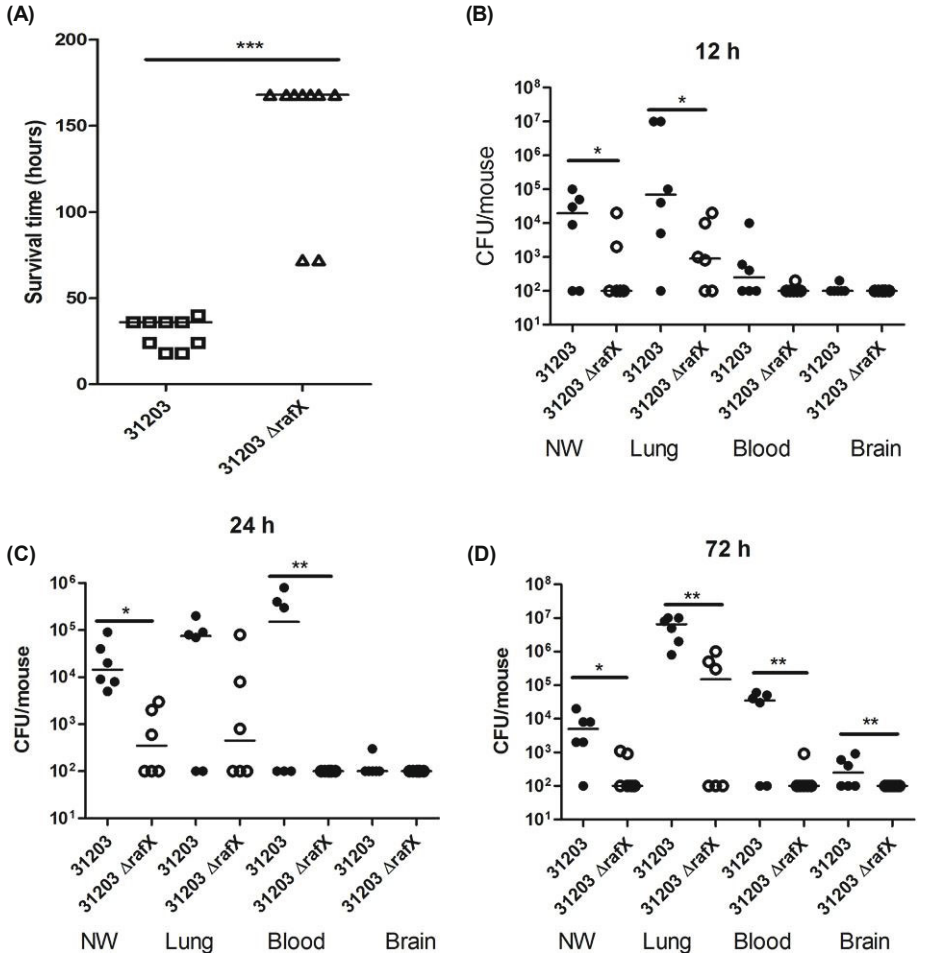

Fig. 7. Reduced virulence and reduced capacity to colonize the nasopharynx and lungs and invade the blood and brain of CMCC(B)31203Δ*rafX*. (A) The bars indicate the median survival time. Statistical significance was examined with Mann-Whitney U test, *** $P < 0.001$. Bacterial numbers in the nasal washes (NW), lungs, blood, and brain following bacterial inoculation were determined at 12 h (B), 24 h (C) or 72 h (D) post-infection. Statistical significance was examined with Student's *t* test, and *P* values were recorded. * $P < 0.05$; ** $P < 0.01$. The bars indicate the median bacterial numbers.

strain, *rafX* deletion mutation was constructed in the serotype 3 strain CMCC(B)31203. The reduced amount of WTA of the 31203Δ*rafX* mutant was confirmed before animal studies (data not shown). Wild-type strain or 31203Δ*rafX* mutant was administrated intraperitoneally into mice. Survival time was monitored over 7 days. As expected, the 31203Δ*rafX* mutant was significantly attenuated in bacterial virulence ($P < 0.001$; Fig. 7A). In addition, groups of mice were administered intranasally with either wild-type 31203 strain or 31203Δ*rafX* mutant at a dose of 1×10^8 CFU. Six mice from each group were killed after 12 h, 24 h, and 96 h, and the numbers of pneumococci in nasal washes and organs were quantified. Data show that this mutant was significantly reduced in colonization of the nasopharynx and lungs, and invasion to the blood and brain compared to the wild-type parent (Fig. 7B, C, and D). Besides, 31203Δ*rafX* mutant was also more susceptible to the killing by the neutrophils than the wild-type strain (data not shown).

Discussion

S. pneumoniae is responsible for the majority of respiratory infectious diseases in the children younger than 5 years (O'Brien *et al.*, 2009). Our results demonstrate that the invading *S. pneumoniae* induced severe inflammation, as evidenced by recruitment and activation of neutrophils, and necrotic debris in lungs (Fig. 4). Moreover, septicemia occurred in all mice following intranasal challenge with the virulent *S. pneumoniae* (Figs. 2 and 7). During the course of an infection, pneumococci may fine-tune the expression of different virulence factors to adapt to different environments including nasopharyngeal mucosa, lung mucosa and blood (Kadioglu *et al.*, 2008). TA, an important component of the cell wall, is presented in all *S. pneumoniae* strains. There is evidence that the amount of TAs was significantly higher in the colonizing *S. pneumoniae* (transparent phenotype) than the opaque variant (Weiser *et al.*, 1994, 1996). In this study, by using flow cytometric analysis, we demonstrated that the increased surface-exposure of TAs of encapsulated D39 strain during nasopharyngeal colonization than medium-grown cells. This surface exposure supports the requirement of TAs during nasopharyngeal colonization. Of note, here we did not determine the amount of capsule expression, but according to the work by Hammerschmidt and coworkers showing capsule is down regulated during adherence (Hammerschmidt *et al.*, 2005), the increased exposure of TA may be partially due to the reduced expression of capsule.

Subsequently, the *in vivo* phenotype of the WTA-deficient mutant (Δ*rafX*) was evaluated. We show that the Δ*rafX* mutant has reduced ability to colonize the nasopharynx and lungs, and reduced ability to invade the blood and brain. Further-

more, there was reduced inflammation in the lungs of mice infected with the Δ*rafX* mutant. The low level of inflammation observed in Δ*rafX* mutant infected mice is likely due to the growth defect of the Δ*rafX* mutant or an impaired resistance of the Δ*rafX* mutant to the killing by the host. It is uncertain which TLR(s) is involved in this process, but cytokines such as IL-6, IFN-γ, and TNF-α in lung homogenates of mice infected with Δ*rafX* mutant were much lower at this time point than those in wild type strain infected mice (data not shown). A similar phenotype was seen with a second pneumococcal strain background of serotype 3 (Fig. 7). These results indicate that WTA is an important determinant for pneumococcal virulence, which is not influenced by pneumococcal host strain.

The invading *S. pneumoniae* is effective to colonize the nasopharynx and dissemination, whereas the Δ*rafX* mutants are impaired to colonize the nasopharynx and dissemination, suggesting that the presence of WTA is required to allow pneumococcal colonization and invasion in the host. The direct involvement of WTA in these processes was supported by the inhibition experiments with CWPS and TEPC-15 (Fig. 3). Of note, the reduced colonization and dissemination of Δ*rafX* mutants may in part result from the growth defect and reduced anchor of CbpA (Wu *et al*., 2014), an important adhesin of *S. pneumoniae*, on bacterial surface. Together, our study suggests that although the WTA-deficiency mutant has growth defect, WTA functions in cell-to-cell interaction which is important for colonization and hence dissemination.

In this work, we observed that the Δ*rafX* mutant could also be reduced in airway colonization in the presence of TEPC-15. This phenomenon occurred because the Δ*rafX* mutant still had a small amount of WTA as described in our previous study (Wu *et al*., 2014). It's precisely because Δ*rafX* mutant displays less WTA that a more pronounced inhibition could be observed than wild type strain in the presence of TEPC-15. This observation also helps to claim that pneumococcal colonization is related to the presence of the WTA.

A previous study based on phase variation evidence emphasized the importance of capsule during systemic infection (Kim and Weiser, 1997). In this work, our results show that the WTA-deficient mutant was less virulent than the wild-type strain during systemic infection, suggesting the essential role of TA in pneumococcal virulence when the bacteria are expressing equally large amounts of capsule in the bloodstream. It has been shown that resistance to complement deposition on pneumococcal surface has been shown to correlate with the enhanced virulence of bacteria (Sabharwal *et al*., 2009). In our study, we have demonstrated that although D39Δ*rafX* mutant was less susceptible to C3 deposition, it was strongly impaired in resistance to the killing by the neutrophils compared to wild-type D39 strain (Fig. 6A), suggesting the importance of WTA in defending host immune systems. This observation also suggests that, compared to C3 mediated effect, the integrity of cell wall plays a more fundamental role in resisting phagocytosis. Thus, the protective effects of WTA in systemic infection may result from its growth benefit and protection against phagocytosis.

In summary, we show that WTA-deficient D39 strain is less virulent than the wild-type strain and is impaired in nasal colonization, dissemination to and survival in the bloodstream in animal models. Furthermore, we demonstrate that the WTA-deficient mutant of pneumococcal serotype 3 has the same phenotype in virulence as WTA-deficient D39 mutant, thereby demonstrating that this is not a serotype specific effect and confirming that WTA is an essential factor for pneumococcal pathogenesis implicated in nasal colonization and invasive pneumococcal disease.

Acknowledgements

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