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A Novel Role for Plasmin-Mediated Degradation of Opsonizing Antibody in the Evasion of Host Immunity by Virulent, but Not Attenuated, Francisella tularensis

Deborah D. Crane, Shayna L. Warner, and Catharine M. Bosio

Opsonization by Abs represents a critical component of the host immune response against many pathogens. The mechanisms by which virulent microbes evade this protective response are not completely understood. In disease mediated by Francisella tularensis, Ab can effectively protect against infections with attenuated strains, for example, LVS, but not virulent strains such as SchuS4. Thus, it is likely that SchuS4 has mechanisms, which are not present in LVS, that allow evasion of opsonization by Ab, dampening the protective effects of these host molecules. Here we demonstrate that evasion of Ab-mediated opsonization and phagocytosis by the highly virulent SchuS4 is associated with its ability to bind the host serine protease plasmin. SchuS4, but not the closely related LVS, bound active plasmin. Plasmin bound SchuS4 degraded exogenous and opsonizing Abs, whereas LVS failed to do so. Furthermore, plasmin-mediated inhibition of Ab opsonization by SchuS4 also inhibited Ab-mediated uptake of this bacterium by macrophages. Ab-mediated uptake of uncoated and opsonized SchuS4 elicited a strong proinflammatory response in infected macrophages. However, plasmin-coated, opsonized SchuS4 poorly elicited production of these protective proinflammatory cytokines. This unique host-pathogen interplay is a novel immune evasion strategy utilized by virulent F. tularensis, and it provides one explanation for the ability of Ab to protect against attenuated, but not virulent, strains of F. tularensis. This mechanism may also represent a more common hereto unrecognized strategy by which virulent bacteria evade detection and clearance by Ig. The Journal of Immunology, 2009, 183: 4593–4600.

Antibody is recognized as an essential host factor for control of multiple microbial pathogens. Thus, it is not surprising that virulent microbes, unlike their more attenuated counterparts, have developed a number of strategies to evade the protective effects of Abs in the host. One example of this dichotomy in the protective capabilities of Ab among attenuated and virulent bacterial infections is found in infections mediated by Francisella tularensis. Recent studies have demonstrated that passive transfer of either immune serum or mAbs can protect against a lethal challenge with the attenuated F. tularensis strain LVS (1, 2). However, experiments testing protective efficacy of immune serum and mAbs against challenge with virulent F. tularensis strain SchuS4 failed to reciprocate this protection and resulted in only a modest extension of the mean time to death among treated animals (1, 2).

There are a number of explanations for the difference in Ab-mediated protection between attenuated and virulent bacteria, including variation in surface Ags and affinity or avidity of the Abs to the targeted bacterial Ag. However, an additional explanation is direct interference with the opsonic activity of Abs by the bacteria themselves. This interference could be mediated by proteases generated directly by the bacteria or by the microbe co-opting host proteases, such as the serine protease plasmin.

Plasmin is normally generated in the host following conversion of the precursor plasminogen (Plg) to plasmin via the enzyme urokinase plasminogen activator (uPA) (as reviewed in Ref. 3). Generation of plasmin is a tightly regulated process and has been primarily associated with tissue remodeling in the host. However, plasmin has also been demonstrated to have proteolytic activity on host Ig (4). Several species of bacteria have been shown to either bind or participate in the generation of plasmin (5). However, the contribution of plasmin and its effect on Ab-mediated protection in bacterial pathogenesis has remained largely unexplored.

In this report, we provide evidence that, in contrast to attenuated LVS, virulent SchuS4 directly interferes with the activity of opsonizing Abs in a process requiring plasmin. Taken together, our data describe one mechanism by which virulent bacteria evade Ab-mediated protection and also serve as evidence for a novel strategy by which plasmin may be used as a virulence factor for other bacterial pathogens.

Materials and Methods

Bacteria

F. tularensis SchuS4 was provided by Dr. J. Peterson and Dr. M. Schriefer (Centers for Disease Control, Fort Collins, CO). F. tularensis strain LVS was provided by Dr. J. Celli (Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT). LVS and SchuS4 were cultured as previously described (6, 7). Briefly, LVS and SchuS4 were cultured in modified Mueller-Hinton broth (Mueller-Hinton broth supplemented with

4 Abbreviations used in this paper: Plg, plasminogen; anti-FT, anti-Francisella LPS Ab; LW, last wash; Pla, plasminogen activator protein; uPA, urokinase plasminogen activator.
CalC1a, MgCl2, 0.1% glucose, 0.025% ferric pyrophosphate, and 2% Iso-Vitalex; BD Biosciences) at 37°C with constant shaking overnight, aliquoted into 1 ml samples, frozen at −80°C, and thawed just before use. Frozen stocks were titered by enumerating viable bacteria from serial dilutions plated on modified Mueller-Hinton agar as previously described. The number of viable bacteria in frozen stock vials varied <5% over a 10-mo period.

Detection of plasminogen bound to bacteria
The ability of LVS and SchuS4 to bind Plg was tested using a modified ELISA. SchuS4 and LVS were coated onto Immulon 2HB (Thermo Fisher Scientific) plates as previously described (8). Plates were then washed with TBS-T and blocked with 1% BSA (Pierce), and washed again (then, Plg (R&D Systems) or BSA (Sigma-Aldrich; negative control) was added at the indicated concentrations, in triplicate, and plates were incubated for 1 h at room temperature. Wells without bacteria served as negative controls to account for nonspecific binding of Plg and Abs. Bound Plg was detected using human-plasminogen Ab (clone 270409; R&D Systems) and HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Bound Abs were detected using One-Step TMB ELISA substrate (Pierce) and analysis at 450 nm using MRX Revelation and Revelation software (Dynex Technologies).

Detection of active plasmin bound to bacteria
Detection of active plasmin bound to the surface of LVS and SchuS4 was performed as previously described (9). Briefly, 4 × 10⁶ CFU SchuS4 were centrifuged and resuspended in 100 μl of PBS (Innolit) followed by addition of 40 μg of Plg and 30 IU of human uPA (American Diagnostica). Additional tubes of bacteria received either PBS (control), 40 μg of Plg or 30 IU of uPA. To account for any background activity of inactive plasmin adhering to the tube, a final tube that initially contained only uPA and Plg (no bacteria) was washed in an identical fashion to that done with Francisella-containing tubes. Each sample was incubated at 37°C/5% CO₂ for 90 min. Bacteria were then washed three times and each tube was resuspended in 400 μl of PBS. Three hundred microliters from each tube was divided into 100-μl aliquots into fresh microcentrifuge tubes, resulting in triplicate samples for each test condition. One hundred microliters (0.5 mg/ml) of the chromogenic substrate S-2251 (Chromogenix/DiaPharma) was added to each new tube and samples were incubated for 90 min at 37°C/5% CO₂. Samples were then centrifuged and the supernatants were transferred to an Immulon 2 HB plate for analysis at 405 nm using a MRX Revelation plate reader and Revelation software (Dynex Technologies).

Degradation of IgG
The ability of plasmin-bound LVS or SchuS4 to degrade IgG was assessed using a modified ELISA. Briefly, Immulon 2 HB plates were coated with purified human IgG (Sigma-Aldrich) in 0.1 M sodium bicarbonate. Plates were incubated at 4°C overnight, washed with PBS-0.05% Tween 20 and blocked with 1% BSA-PBS overnight at 4°C. Plasmin-coated bacteria and the indicated controls were prepared as described above. Bacteria were added to wells and incubated for 2 h at 37°C. The last wash (LW) from tubes containing bacteria served as negative controls. Supernatants from tubes that contained Plg and uPA alone (plasmin) served as positive controls. Degradation of IgG was detected as an inability of HRP rabbit anti-human Fc-specific Abs (Jackson ImmunoResearch Laboratories) to bind IgG in each well. Bound anti-IgG Abs were detected following addition of HRP goat anti-rabbit IgG (Sigma-Aldrich) and One-Step TMB ELISA substrate (Pierce) and analysis as described above. Total percentage degradation of IgG was calculated using the following formula: (ABS of wells containing only IgG − ABS of test well/ABS of wells containing only IgG) × 100 (where ABS is absorption).

Assessment of Ab-mediated opsonization
Plasmin-coated F. tularensis was generated as described above. Bacteria incubated in the presence of PBS served as negative controls. Bacteria were diluted to 4 × 10⁷/100 μl and aliquoted into duplicate tubes. Alexa Fluor 488-conjugated anti-Francisella LPS Ab (anti-FT; US Biological) or FITC-conjugated mouse IgG2a (BD Biosciences) was added to the indicated tubes at 4.4 μg/ml. Bacteria were incubated for 2 h at 37°C/7% CO₂. Bacteria were centrifuged and the resulting pellets resuspended in 1 ml of 3% paraformaldehyde and incubated for 30 min at 37°C/7% CO₂. Then, bacteria were centrifuged again and pellets were resuspended in 100 μl of PBS before transfer to a 96-well plate (Corning). Fluorescence was assessed and reported as relative fluorescence units using a Tecan Safire2 microplate reader and XFFluor software (Tecan).

Culture and infection of bone marrow-derived macrophages
Bone marrow-derived macrophages were generated as previously described (10) with the exception that cells were differentiated into macrophages in the presence of 10 ng/ml recombinant murine M-CSF (Pepro-Tech). Macrophages were infected with a multiplicity of infection of 25 of F. tularensis as previously described (10), with the following modifications. F. tularensis was coincubated with macrophages at 37°C in 7% CO₂ for 1.5 h followed by treatment with 50 μg/ml gentamicin (Invitrogen) to eliminate extracellular bacteria. Cultures were incubated for an additional 45 min, washed with PBS, and fixed with 3% paraformaldehyde in PBS for 30 min at 37°C/7% CO₂ before analysis for bacteria as described below. The infection inoculum was confirmed by plating serial dilutions of stock F. tularensis on modified Mueller-Hinton agar plates immediately after addition to cell cultures.

Fluorescence microscopy
Macrophages were fixed and stained for F. tularensis and LAMP-1 as previously described (11). LAMP-1 and bacteria were visualized following incubation with rat anti-mouse LAMP-1 Abs followed by Alexa Fluor 568-conjugated anti-rat Abs (Invitrogen) and Alexa Fluor 488 mouse-conjugated anti-F. tularensis (US Biological), respectively. Samples were observed on a Zeiss Axio Imager M1 epifluorescence microscope for quantitative analysis. Approximately 75–100 cells per field and a minimum of three fields per coverslip were analyzed for presence of intracellular bacteria. The percentage of infected cells was calculated as follows: (no. infected cells/total no. of cells) × 100.

Detection of secreted cytokines
Culture supernatants were assayed for the presence of TNF-a and IL-6 by ELISA using commercially available kits according to the manufacturer’s instructions (R&D Systems).

Statistical analysis
Statistical differences between two groups were determined using an unpaired t test, with significance set at p < 0.05. For comparisons between three or more groups, analysis was done by one-way ANOVA, followed by Tukey’s multiple comparisons test, with significance determined for p < 0.05.

Results
F. tularensis binds plasminogen
Active plasmin is generated following its transformation from Plg by the enzyme uPA (as reviewed in Ref. 5). Therefore, the typical prerequisite for binding of plasmin to bacteria is the ability of the organism to bind Plg. Thus, we first determined if attenuated LVS and virulent SchuS4 could directly bind Plg. Both LVS and SchuS4 bound Plg in a dose-dependent manner (Fig. 1A). Samples that did not include bacteria (no LVS or no SchuS4, respectively) failed to bind anti-Plg Abs, indicating that Ab binding was specific for Plg bound to F. tularensis (Fig. 1A). Further evidence of a specific interaction of the bacteria with Plg was indicated by the failure of bacteria incubated with BSA to bind anti-Plg Abs. These controls confirmed that detection of Plg bound to LVS and SchuS4 was not a result of nonspecific interaction of the Abs used for detection with exogenous protein (Fig. 1A).

Viralent, but not attenuated, F. tularensis binds active plasmin
Many pathogens have been noted to bind Plg (12–15); however, the ability of bacteria to interact with this protein may not correlate with their ability to bind the active protease plasmin. Thus, we next assessed the ability of Plg-coated LVS and SchuS4 to bind active plasmin. Because F. tularensis species do not encode any known Plg activator enzymes, we supplied exogenous, host-derived uPA as the catalytic enzyme. Supernatants from washed tubes in which Plg was incubated with uPA in the absence of bacteria (no bacteria; Plg plus uPA) accounted for any carryover of plasmin activity and served as a negative control. Additional controls included supernatants from the LW from tubes that contained bacteria, Plg, and uPA to account for residual plasmin that was not bound to the
bacteria. Despite the ability of LVS to bind Plg, in the presence of uPA, LVS failed to bind significant quantities of active plasmin. In contrast, Plg-coated SchuS4 readily bound active plasmin when uPA was present (Fig. 1B). Plasmin activity in samples containing LVS or SchuS4 incubated with either Plg or uPA alone was similar to that observed for the negative control, indicating that SchuS4 could not independently convert Plg to plasmin (Fig. 1B). SchuS4 and LVS incubated in the presence of PBS, but not Plg or uPA, also failed to have detectable levels of active plasmin (Fig. 1B). Furthermore, the plasmin activity observed in samples that contained all three components (SchuS4, Plg, and uPA) was significantly greater than that observed in supernatant from the LW of SchuS4 incubated in the presence of Plg and uPA (p < 0.001) (Fig. 1B). Therefore, the significant plasmin activity observed in SchuS4 samples incubated with Plg and uPA was not due to carryover of free plasmin in the supernatant. Given the proteolytic capabilities of plasmin, it was possible that this enzyme might negatively affect the viability of LVS and SchuS4. Thus, we compared viability of LVS and SchuS4 incubated with Plg and uPA to bacteria incubated in PBS. There were no differences in the number of viable bacteria present in PBS or uPA plus Plg samples, suggesting that the presence of plasmin did not affect viability of bacteria (data not shown). Taken together, these data demonstrate that, despite the apparent equivalent ability of attenuated LVS and virulent SchuS4 to bind Plg, only virulent SchuS4 was capable of binding significant concentrations of the active protease plasmin.

Plasmin-coated SchuS4 degrades Ig

Clearance of bacteria has been tightly associated with opsonization of the microbe with Ig. However, the strategies by which pathogens evade this opsonization have not been completely elucidated. Early studies examining the stability of Ig demonstrated that these molecules were susceptible to degradation by plasmin (4). Thus, plasmin associated with LVS or SchuS4 may endow the bacteria with the ability to directly degrade Ig and evade opsonization. To test this hypothesis, we next assessed the ability of plasmin-coated LVS and SchuS4 to degrade nonspecific IgG. Consistent with the poor association of plasmin with LVS, LVS incubated in the presence of uPA and Plg failed to degrade IgG. In contrast, plasmin-coated SchuS4 degraded significantly more IgG than did SchuS4 incubated in PBS, supernatant from the LW of bacteria incubated in the presence of Plg and uPA, and all LVS samples (p < 0.01) (Fig. 2). Although there was some carryover of IgG degradation in the supernatant from the LW of plasmin-coated bacteria, this was significantly less than the degradation observed with plasmin-coated bacteria themselves (Fig. 2). Thus, plasmin associated with SchuS4 resulted in degradation of host IgG, whereas attenuated LVS incubated under similar conditions was unable to mediate this proteolytic function.

Plasmin-coated SchuS4 interferes with opsonization and Ab-mediated phagocytosis

The primary function of host Ab directed against invading pathogens is opsonization. Binding of pathogen-specific Ab to microbes enables the host to more efficiently kill the pathogen via FcR-mediated uptake by phagocytic effector cells or formation of a complement-driven membrane attack complex on the surface of the bacterium. Since plasmin-coated SchuS4, but not LVS, readily degraded IgG (Fig. 2), we predicted that these bacteria would also be more resistant to opsonization by Francisella-specific Ab. To determine whether our hypothesis was correct, we compared the ability of SchuS4 and LVS incubated in PBS or Plg plus uPA with that of bacteria incubated in PBS or uPA alone.
Plasmin-coated bacteria degrade IgG. LVS and SchuS4 incubated with Plg and uPA to generate surface-bound plasmin were added to plates coated with human IgG. Wells incubated with PBS or SchuS4 alone served as negative controls, whereas supernatant from Plg treated with uPA served as positive controls. Plasmin-coated SchuS4 degraded significantly more human IgG compared with SchuS4 incubated in PBS, the LW from SchuS4 incubated with Plg and uPA, or LVS incubated under all conditions (*, p < 0.01). Error bars represent SEM. Data are representative of four experiments of similar design.

Plasmin inhibits opsonization of SchuS4 by Francisella-specific IgG (anti-F). Bacteria exposed to isotype control Ab served as negative controls for non-specific binding. There were no differences between bacteria incubated with PBS or uPA plus Plg (plasmin) and their interaction with isotype control Ab (Fig. 3). Similarly, regardless of preincubation conditions (PBS or uPA plus Plg), LVS bound similar concentrations of anti-F Abs. Compared with PBS-treated SchuS4, LVS (regardless of treatment) bound slightly less, but not significantly different, amounts of anti-F Abs. This suggests that opsonization with anti-F Abs may be slightly more efficient for SchuS4 bacteria. In marked contrast to plasmin-treated LVS, plasmin-coated SchuS4 bound significantly less (p = 0.0047) anti-FT Ab compared with PBS-treated SchuS4 (Fig. 3). Furthermore, plasmin-coated SchuS4 bound less anti-FT Abs than did LVS preincubated with either PBS or uPA plus Plg. Taken together, these data suggest that surface-bound plasmin aids in the interference of opsonization of virulent, but not attenuated, F. tularensis by Francisella-specific Abs.

Opsonization of bacteria by Ab enables more efficient uptake by host effector cells. As described for other bacteria, Ab-mediated uptake of F. tularensis results in compromised replication of the bacteria within macrophages (Refs. 16–18 and J. Celli, unpublished observation). Given that plasmin-coated SchuS4 interfered with the binding of anti-FT Ab, we next examined the impact that plasmin present on the bacterial surface had on Ab-mediated uptake of LVS and SchuS4 by macrophages. Bacteria were coated with plasmin and exposed to anti-FT Abs or isotype controls, as described above. Murine bone marrow-derived macrophages were infected with the prepared SchuS4 or LVS and uptake of bacteria was monitored by microscopy. As expected, LVS and SchuS4 treated with PBS and opsonized with anti-FT Abs were phagocytosed with greater efficiency than were bacteria incubated with isotype control Abs. This enhanced uptake of opsonized LVS and SchuS4 was evident as an increase in both the number of infected macrophages and in the number of bacteria per cell (Fig. 4). Interestingly, PBS-treated, Ab-opsonized SchuS4 were phagocytosed by more macrophages than were PBS-treated, Ab-opsonized LVS. Incubation of LVS and SchuS4 in uPA plus Plg (to generate plasmin) had no effect on the uptake of either LVS or SchuS4 in the presence of isotype control Ab (Fig. 4), and thus presence of plasmin alone did not affect phagocytosis of bacteria by macrophages. Similarly, preincubation of LVS with uPA plus Plg had no significant effect on the number of macrophages infected with opsonized LVS, nor the number of bacteria per cell (Fig. 4). In contrast, and in correlation with ~50% inhibition of opsonization by anti-FT Abs by plasmin-coated SchuS4 (Fig. 3), significantly fewer macrophages (~50% fewer) were infected with plasmin-coated SchuS4 opsonized with anti-FT Abs compared with PBS treated controls (p = 0.0081) (Fig. 4A). Furthermore, significantly fewer macrophages had 1–2 and 3–10 plasmin-coated, anti-FT opsonized SchuS4 compared with PBS, anti-FT opsonized SchuS4 controls (p = 0.0210 and 0.0100, respectively) (Fig. 4B). Thus, unlike more attenuated strains of Francisella, plasmin associated with virulent SchuS4 inhibited Ab-mediated opsonization and, subsequently, Ab-mediated phagocytosis by macrophages.

Plasmin-coated SchuS4 inhibit induction of proinflammatory cytokines by opsonized bacteria

One mechanism by which Ab-mediated phagocytosis aids in clearance and killing of invading pathogens is by enhancing the production of inflammatory cytokines (e.g., TNF-α) that trigger killing mechanisms in effector cells (19–21). Thus, in addition to plasmin-coated SchuS4 interfering with Ab-mediated phagocytosis, these bacteria may also limit the production of cytokines that are produced following uptake of Ab-opsonized bacteria. To test this hypothesis, we measured cytokines secreted by macrophages exposed to opsonized, plasmin-coated, or PBS control-treated LVS and SchuS4. As expected, all LVS and SchuS4 incubated in the presence of isotype control Abs failed to stimulate secretion of TNF-α or IL-6 from macrophages in quantities that were significantly different from uninfected controls. Ab-opsonized LVS and SchuS4 readily induced significantly greater amounts of TNF-α and IL-6 from macrophages compared with all other treatment groups (p < 0.01) (Fig. 5). Surprisingly, despite lack of evidence suggesting active plasmin bound to LVS (Figs. 2–4), plasmin-treated, Ab-opsonized LVS elicited significantly less TNF-α and IL-6 secretion from macrophages compared with PBS-treated, anti-FT-opsonized LVS (Fig. 5). However, even in the presence of plasmin, opsonized LVS elicited significantly more TNF-α and
IL-6 compared with unopsonized LVS controls and all SchuS4-infected cultures \((p > 0.01)\) (Fig. 5). Thus, although presence of plasmin effected the production of cytokines following phagocytosis of SchuS4 and LVS by macrophages was monitored by microscopy and was assessed as the total number of cells infected \((A)\) and the number of bacteria per cell \((B)\). A. Significantly fewer macrophages were infected with plasmin-coated SchuS4 opsonized with anti-FT compared with PBS-incubated, anti-FT-opsonized controls \((*, p = 0.0081)\). Preincubation with uPA and Plg did not significantly affect the ability of macrophages to take up anti-FT-opsonized LVS. B. Significantly fewer macrophages had 1–2 and 3–10 plasmin-coated, anti-FT-opsonized SchuS4 compared with PBS-treated, anti-FT-opsonized controls \((*, p = 0.0210 \text{ and } 0.0100, \text{ respectively})\).

![Figure 4](image-url)

**FIGURE 4.** Plasmin inhibits Ab-mediated uptake of SchuS4. LVS and SchuS4 were coated with plasmin following incubation with uPA plus Plg as described above. Bacteria then were incubated with anti-FT or matched isotype control Abs before addition to macrophages. Phagocytosis of SchuS4 and LVS by macrophages was monitored by microscopy and was assessed as the total number of cells infected \((A)\) and the number of bacteria per cell \((B)\). A. Significantly fewer macrophages were infected with plasmin-coated SchuS4 opsonized with anti-FT compared with PBS-incubated, anti-FT-opsonized controls \((*, p = 0.0081)\). Preincubation with uPA and Plg did not significantly affect the ability of macrophages to take up anti-FT-opsonized LVS. B. Significantly fewer macrophages had 1–2 and 3–10 plasmin-coated, anti-FT-opsonized SchuS4 compared with PBS-treated, anti-FT-opsonized controls \((*, p = 0.0210 \text{ and } 0.0100, \text{ respectively})\).

![Figure 5](image-url)

**FIGURE 5.** Plasmin-coated SchuS4 interferes with Ab-mediated induction of proinflammatory cytokines. LVS and SchuS4 were prepared as described above. Bacteria incubated in PBS served as negative controls (PBS). LPS-treated cells served as positive controls. Bacteria were incubated with anti-FT or matched isotype control Abs before addition to macrophages. Eighteen hours after infection culture supernatants were assessed for cytokines by ELISA. Opsonized, uncoated LVS induced secretion of significantly higher concentrations of TNF-\(\alpha\) and IL-6 \((*, p < 0.01)\) by macrophages compared with all other samples. Plasmin-coated, Ab-opsonized LVS induced significantly less TNF-\(\alpha\) from macrophages compared with opsonized,
PBS-treated controls \((p < 0.05)\) (Fig. 5). Plasmin-treated, Ab-opsonized SchuS4 also induced less IL-6 compared with PBS-treated, Ab-opsonized SchuS4, but these values were not significantly different. Furthermore, in the presence of plasmin, Ab-opsonized SchuS4 failed to induce TNF-\(\alpha\) or IL-6 at concentrations significantly different from uninfected controls (Fig. 5). Thus, phagocytosis of Ab-opsonized LVS and SchuS4 induced cytokine production from macrophages. However, the impact of plasmin on the interference of cytokine production following uptake of opsonized SchuS4 was significantly greater than that observed in cultures containing opsonized LVS \((p < 0.05)\). Taken together, this suggests that plasmin bound to the surface of SchuS4 modulates both the effect of Ab-mediated phagocytosis and induction of two key proinflammatory cytokines from macrophages.

**Discussion**

Successful colonization and replication of pathogens in the host requires evasion and/or manipulation of the host immune response. The most infectious pathogens have developed a number of mechanisms to avoid detection or destruction by both innate and adaptive host effector mechanisms. One such effector mechanism is Ab-mediated clearance of pathogens. Evasion of Ab-mediated immunity is an important hurdle for both extra- and intracellular pathogens to overcome. For example, opsonization of mycobacteria by Ab enhances uptake of the bacterium by phagocytic cells, activates the cells, and ultimately results in destruction of the microbe (22).

Ab has also recently been shown to be an important component for optimal protective responses against *F. tularensis*. Transfer of immune serum or mAbs uniformly protects animals against challenge with the attenuated strains of *F. tularensis* such as LVS (1, 2). In contrast, although transfer of immune serum or purified Abs extends the mean time to death, these treatments fail to increase overall survival following challenge with virulent *F. tularensis* (1, 2). There are a number of explanations for the failure of Ab to protect against infections with virulent *F. tularensis*. These include poor affinity or avidity of Ab directed against the bacterium, low abundance of the Ag targeted by the Ab associated with virulent organisms, direct suppression of proinflammatory responses typically elicited following Ab-mediated uptake of bacteria by phagocytes, or direct interference with Ab opsonization.

Data presented herein suggest the inability of Ab to efficiently protect against virulent *F. tularensis* infection is not due to the failure of Ab to bind bacteria under normal tissue culture conditions (Fig. 3), nor is the ineffectiveness of Ab-mediated protection due to the lack of production of proinflammatory cytokines following uptake of opsonized *F. tularensis* (Fig. 4). Rather, data presented herein suggest that the inability of Ab to adequately protect against virulent *F. tularensis* infections is directly related to the ability of virulent, but not attenuated, *F. tularensis* to more efficiently harness the proteolytic capability of host plasmin for degradation of *Francisella*-specific Abs (Figs. 2–5).

The interaction of SchuS4 and LVS with plasmin and Ab revealed a previously unappreciated difference in the ability of these bacteria to modulate the host immune response. For example, Ab opsonization of SchuS4 resulted in both greater numbers of infected macrophages and greater numbers of cells infected with 3–10 and >10 bacteria per cell compared with LVS (Fig. 4). This increase in phagocytosis may be a result of SchuS4 binding the anti-FT Ab with greater affinity than LVS. Alternatively, SchuS4 may have slightly greater amounts of the Ag recognized by the Ab used in these studies and thus bound more Ab on their surfaces. Experiments depicted in Fig. 3 suggest that SchuS4 was capable of binding modestly larger amounts of anti-FT than LVS. Regardless of the reason for increased phagocytosis of Ab-opsonized SchuS4 vs LVS, these data inferred that Ab-mediated clearance would be more efficient for SchuS4 infections than for LVS infections. However, previous reports demonstrate that passive transfer of immune serum or mAbs fails to protect infection with SchuS4 (1, 2). Thus, it is likely that functional differences in Ab-mediated uptake (outside of phagocytosis) may contribute to control of LVS, but not SchuS4, infections.

In this report we demonstrate that one such functional difference is the induction of proinflammatory cytokines following uptake of Ab-opsonized *Francisella*. Although more macrophages were infected with Ab-opsonized SchuS4 than opsonized LVS, production of TFN-\(\alpha\) and IL-6 from cells infected with opsonized LVS was significantly greater than that observed in cultures infected with opsonized SchuS4 (Fig. 5). This disparity in cytokine production between infections with either opsonized SchuS4 and LVS was even more dramatic in cultures infected with plasmin-coated, Ab-opsonized bacteria. uPA plus Plg-treated, Ab-opsonized LVS induced less TNF-\(\alpha\) and IL-6 than did PBS-treated, Ab-opsonized LVS. However, the concentration of cytokines detected in plasmin-treated, Ab-opsonized LVS cultures was significantly higher than that detected in any SchuS4 culture. In contrast, the presence of plasmin on the surface of SchuS4 resulted in nearly complete reversal of the Ab-mediated cytokine production observed in PBS-treated, Ab-opsonized SchuS4 infections. Thus, although the number of opsonized SchuS4 engulfed by macrophages was either greater or equivalent to that observed with opsonized LVS, virulent SchuS4 was less efficient at inducing cytokine responses. Furthermore, the presence of plasmin had a more profound effect on the induction of cytokines by Ab-opsonized SchuS4 compared with opsonized LVS.

We have previously demonstrated that SchuS4, in the absence of Ab, is capable of actively suppressing cytokine responses in various host target cells and tissues (6, 23). It has also been reported that LVS interferes with cytokine production in host cells (24, 25). This suggested that SchuS4 and LVS are similar in their disruption of innate immune responses. However, data presented herein clearly demonstrate that (unlike LVS) in addition to disrupting innate immunity, SchuS4 is capable of profoundly modulating cytokine responses that are associated with adaptive immune responses as well, that is, production of cytokines following uptake of Ab-opsonized SchuS4. These data reveal an important advance in our understanding of the interplay between host immune responses and virulent *Francisella*. Furthermore, they describe an important, fundamental difference in LVS- and SchuS4-mediated infections of host cells. Taken together, these data confirm that Ab-mediated uptake is likely an important component of protective immunity against virulent *Francisella* infections, and that the bacteria has specific mechanisms in place to resist this host response. Moreover, our data also suggests that Ab-mediated protection against virulent *F. tularensis* may be improved following inhibition of specific components of the host plasminogen activating system.

Typically, bacteria interacting with the plasminogen activating system via binding of Plg also efficiently bind plasmin (as reviewed in Ref. 5). Thus, our observation that both LVS and SchuS4 bound Plg, but only SchuS4 bound active plasmin, was unexpected. To our knowledge, this is the first demonstration of a direct comparison of an attenuated and virulent strain of bacteria, with nearly equivalent ability to interact with Plg, to interact with plasmin in such a disparate way. The most likely explanation of the dissimilar nature in which LVS and SchuS4 interact with plasmin is that the molecule responsible for binding plasmin to the surface...
of the bacterium is either absent or present in low abundance on LVS compared with SchuS4. In a recent study, the only proteins present in outer membrane fractions of SchuS4 that were absent in LVS were homologs to the Yersinia autotransporter protein (Yap), designated as YapH-N and YapH-C (26, 27). In Yersinia, these proteins function as adhesins similarly to the plasminogen activator protein (Pla) that is also found on the surface of Yersinia (28–30). Pla is well known for its ability to interact with Plg and plasmin (31). However, the potential interaction of Yap with Plg and plasmin has not been examined. Thus, it is tempting to speculate that YapH-N and YapH-C proteins present on the surface of SchuS4 may play an important role in securing host plasmin to the bacterial surface.

In addition to differences in the species of outer membrane proteins present on LVS and SchuS4, it is also possible that small changes in the overall structure of LPS on these two related bacteria contributes to conversion of bound Plg to plasmin on SchuS4, but not on LVS. Previous studies conducted with Yersinia and Salmonella have shown that the structure of LPS associated with these organisms, specifically the ability of LPS to interact with arginine residues located in Yersinia and Salmonella proteins interacting with Plg (Pla and PgtE, respectively), was critical for the optimal conversion of Plg to plasmin on the surface of these bacteria (32). Although, LPS associated with LVS and SchuS4 are both poorly inflammatory, a direct comparison of their molecular structure and their ability to bind arginine residues located in outer membrane proteins has not been performed (6, 33). Therefore, in addition to specific differences in plasmin-binding proteins, small variations in the structure of SchuS4 LPS may contribute to the ability of outer membrane proteins present in this virulent bacterium to bind plasmin.

In conclusion, our data demonstrate a novel role for plasmin associated with virulent, but not attenuated, bacterial pathogens. With regard to Francisella-specific immunity, we demonstrate that Ab-mediated opsonization and phagocytosis of virulent F. tularensis results in the elicitation of proinflammatory cytokines by infected macrophages. This supports the hypothesis that Francisella-specific Ab plays an important role in protective immunity against this bacterium. Furthermore, our data provide a potential mechanism by which Ab may aid in the control of Francisella infections and how virulent strains utilize host systems to modulate this response. Our data also have important implications for many other bacterial diseases. Specifically, most studies examining the role of Plg and plasmin in bacterial infections have focused on the contribution these compounds make toward dissemination of the pathogens via degradation of extracellular matrix proteins. A direct demonstration of plasmin-mediated degradation of pathogen-specific Ab and the consequence of this activity for control of infection have not been addressed. Furthermore, a direct comparison of the ability of attenuated and virulent strains of a bacterial pathogen to harness the proteolytic potential of plasmin has not been reported. Considering the number of pathogens known to interact with plasminogen and plasmin (e.g., Neisseria, Haemophilus, and Borrelia), it is tempting to speculate that this strategy of plasmin-mediated degradation of Ig for interference of Ab-mediated opsonization and uptake by macrophages may occur for a number of pathogenic microbes (9, 34–36). Recognition and identification of the specific function of plasmin during bacterial infections will undoubtedly be a critical step in the development of effective therapeutics and vaccines directed against many infectious diseases, including those mediated by F. tularensis, where Ab is important.

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References


