Pilus Adhesin RrgA Interacts with Complement Receptor 3, Thereby Affecting Macrophage Function and Systemic Pneumococcal Disease

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ABSTRACT

Pneumococcal pili have been shown to influence pneumococcal colonization, disease development, and the inflammatory response in mice. The role of the pilus-associated RrgA adhesin in pneumococcal interactions with murine and human macrophages was investigated. Expression of pili with RrgA enhanced the uptake of pneumococci by murine and human macrophages that was abolished by antibodies to complement receptor 3 (CR3) and not seen in CR3-deficient macrophages. Recombinant RrgA, but not pilus subunit RrgC, promoted CR3-mediated phagocytosis of coated beads by murine and human macrophages. Flow cytometry showed that purified CR3 binds pneumococcal cells expressing RrgA, and purified RrgA was shown to interact with CR3 and its I domain. In vivo, RrgA facilitated spread of pneumococci from the upper airways and peritoneal cavity to the bloodstream. Earlier onset of septicemia and more rapidly progressing disease was observed in wild-type mice compared to CR3-deficient mice challenged intranasally or intraperitoneally with pneumococci. Motility assays and time-lapse video microscopy showed that pneumococcal stimulation of macrophage motility required RrgA and CR3. These findings, together with the observed RrgA-dependent increase of intracellular survivors up to 10 h following macrophage infection, suggest that RrgA-CR3-mediated phagocytosis promotes systemic pneumococcal spread from local sites.

IMPORTANCE Streptococcus pneumoniae is a major contributor to morbidity and mortality in infectious diseases globally. Symptomatology is mainly due to pneumococcal interactions with host cells leading to an inflammatory response. However, we still need more knowledge on how pneumococci talk to immune cells and the importance of this interaction. Recently, a novel structure was identified on the pneumococcal surface, an adhesive pilus found in about 30% of clinical pneumococcal isolates. This pilus has been suggested to be important for successful spread of antibiotic-resistant pneumococcal clones globally. Here we sought to identify mechanisms for how the pneumococcal pilin subunit RrgA contributes to disease development by interacting with host immune cells. Our data suggest a new way for how pneumococci may cross talk with phagocytic cells and affect disease progression. An increased understanding of these processes may lead to better strategies for how to treat these common infections.

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cells in vitro and virulence in vivo in murine models (1, 4). The crystal structure of RrgA was recently solved (5). It was demonstrated that the 893-residue-long adhesin formed an elongated structure composed of four domains of which the major domain, the D3 domain, adopts an integrin I collagen recognition domain suggested to interact with extracellular matrix (ECM) proteins. Indeed, purified RrgA has been shown to bind fibronectin, laminin, and collagen I, but not to vitronectin (6).

The innate immune system involves effectors and immune cells and constitutes the first line of defense against invading pathogens. In the lungs, phagocytosis mediated by resident macrophages plays a central role in clearance of pneumococci early in infection, and bacterium-induced Toll-like receptor 9 (TLR9)-NF-κB signaling has been suggested to enhance the phagocytic capacity of alveolar macrophages (AMs) (7, 8). It has also been reported that influenza virus sensitization to pneumococcal infection might operate via an interferon-induced inhibition of bacterial clearance, mediated by AMs in the lungs (9).

Numerous surface receptors and associated signal transduction pathways are involved in the phagocytic machinery, leading to bacterial killing and later to the induction of an adaptive immune response. The complement system acts as a part of the innate immune response by opsonizing microbes in a specific manner. Complement receptors (CRs) on the surfaces of phagocytes recognize and internalize the opsonized pathogens. Opsonization of bacteria by immunoglobulins leads to similar enhanced uptake of bacterial clearance, mediated by AMs in the lungs (9).

To identify a putative receptor on BMDMs, we considered CR3, also named integrin CD11b/CD18 or Mac-1. Pretreatment of BMDMs with anti-CD11b antibodies decreased the uptake of pneumococcal strain T4 and the T4ΔrgaV(lacE::rrgA) strain to the level of uptake of the T4ΔrgaA mutant (P < 0.001) (Fig. 1A). Antibody treatment had no effect on the uptake of the T4Δrga mutant. We also treated murine BMDMs with anti-CD11c antibodies and found that they did not influence uptake of T4, thereby confirming the antibody specificity in inhibition (Fig. 1A).

To further confirm a role for CR3 in pneumococcal phagocytosis, we performed phagocytosis assays with BMDMs from CD11b−/− mice, which lack functional CR3. These macrophages showed a 40% reduction in the uptake of strain T4 expressing pili with RrgA (Fig. 1B) (P < 0.0012), which is similar to the level of uptake of the RrgA-deficient bacteria by wild-type macrophages. When comparing uptake of the different pneumococcal strains in CD11b−/− macrophages, no statistically significant differences were observed (Fig. 1B). Targeting CD11b by antibodies or its complete absence only partially inhibited BMDM phagocytosis, testifying to the role of other phagocytic receptors in pneumococcal uptake by macrophages.

Next we used human macrophages (THP-1 cells) in phagocytosis assays of the T4, T4Δrga, and T4ΔrgaV(lacE::rrgA) strains (Fig. 1C). A moderate but significant decrease of T4ΔrgaA phagocytosis compared to the wild type and the RrgA-complemented mutant was found. A monoclonal antibody specific for human CD11b decreased THP-1 phagocytosis only for strain T4 expressing wild-type pili but had no effect on the uptake of the T4ΔrgaA mutant (Fig. 1C).

Recombinant RrgA, but not RrgC, promotes CR3-mediated phagocytosis of coated beads by murine and human macrophages. To directly demonstrate a role for the RrgA adhesin in phagocytosis, we conjugated full-length recombinant RrgA (rRrgA) to fluorescent microspheres and monitored uptake by murine BMDMs and by human THP-1 cells (Fig. 2A). For a relevant control, we made use of rRrgC, an ancillary pilus 1 component believed to anchor the completed pilus to the peptidoglycan cell wall. In both BMDMs and THP-1 cells, phagocytosis was three and four times higher for beads coated with rRrgA than for beads coated with rRrgC and bovine serum albumin (BSA), respectively. Antibodies specific to murine and human CD11b completely inhibited phagocytosis of rRrgA-coated beads by murine BMDMs and human THP-1 cells, respectively (Fig. 2B).

Purified CR3 binds directly to pneumococcal cells expressing pilus-associated RrgA and to recombinant RrgA. The RrgA adhesin has been demonstrated to bind to a number of extracellular
matrix proteins, including collagen I, fibronectin, and laminin in contrast to the other two pilus 1 constituents, RrgB and RrgC (6). To find evidence for a direct interaction between RrgA-expressing pneumococci and CR3, we performed flow cytometry analyses of purified human CR3 (CD11b/CD18) binding to *S. pneumoniae* T4, T4ΔrgA, and T4ΔrgA(lacE::rgA) strains, making use of

**FIG 1** The pneumococcal pilus subunit RrgA enhances uptake by murine and human macrophages through interaction with CD11b (CR3). (A and B) The bacteria were labeled with FITC and incubated with monolayers of bone marrow-derived macrophages (BMDMs) for 1 h at an MOI of ca. 60 bacteria per cell, and the fluorescence intensity was measured by FACS. The phagocytic index for each sample was calculated by multiplying the percentage of internalized cells by the mean fluorescence intensity and dividing this by the mean fluorescence intensity of the wild-type (T4) sample multiplied with the percentage of FITC-positive cells. (A) Phagocytosis assays were performed with murine BMDMs. The mutant lacking RrgA (T4ΔrgA) was taken up to a significantly lower extent than the bacteria expressing RrgA (T4 and T4ΔrgA(lacE::rgA) strains). BMDMs were pretreated with antibodies (Ab) against mouse CD11b. This inhibited the uptake of the T4 and T4ΔrgA(lacE::rgA) strains, which under these conditions reached levels similar to those of the uptake of the T4ΔrgA mutant. The antibody treatment did not, however, affect the uptake of the T4ΔrgA mutant. Antibodies directed against mouse CD11c did not influence the uptake of T4. Each condition was done in triplicate and was repeated at least three times. Values that were significantly different (*P* < 0.001) are indicated by a bar and three asterisks. Values that were not significantly different are indicated by a bar and n.s. (for not significant). (B) CR3-deficient (CD11b/CD18−−) BMDMs exhibited lower levels of uptake of T4 than macrophages from wild-type mice (Wt). The decrease was comparable to the levels of uptake of the T4ΔrgA mutant by wild-type macrophages. There were no statistically significant differences in the levels of uptake of the different pneumococcal strains by the CR3-deficient macrophages. Each experiment was done in triplicate and was repeated at least three times. Values that were significantly different (*P* < 0.01) are indicated by a bar and two asterisks. Values that were not significantly different are indicated by a bar and n.s. (for not significant). (C) In concordance with the findings in murine macrophages, a mutant lacking RrgA (T4ΔrgA) was taken up by human phorbol myristate acetate (PMA)-differentiated THP-1 cells to a significantly lower extent than bacteria expressing RrgA. Furthermore, a specific antibody against human CD11b inhibited the uptake of the T4 strain but did not significantly affect the uptake of the T4ΔrgA mutant. Values are means plus standard errors of the means (SEM) (error bars). Values that were significantly different (*P* < 0.05) are indicated by a bar and one asterisk. Values that were not significantly different are indicated by a bar and n.s. (for not significant).
Absence of RrgA and/or murine expression of CR3 decreased the number of bacteria in the bloodstream early after intranasal and intraperitoneal challenge. We next investigated whether pneumococcal expression of RrgA and host expression of CR3 affects virulence and dissemination of pneumococci from a local site to the bloodstream. As intranasal (i.n.) inoculation of pneumococci in mice mimics the natural route of infection in humans, we first investigated whether RrgA and CR3 influence disease kinetics in an infection model of pneumococcal pneumonia. We inoculated 5 × 10^6 CFU i.n. into C57BL/6 mice and monitored the animals up to 72 h postinfection (Fig. 4A to C). Wild-type mice infected with the T4 or T4ΔrrgA (lacE::rrgA) strain showed increased mortality compared to wild-type mice infected with the T4ΔrrgA strain (Fig. 4A) (P = 0.0187 in the survival analysis with the Kaplan-Meier log rank test). Furthermore, the two strains expressing RrgA could be detected in blood samples from wild-type mice as early as 12 h postinfection, whereas no T4ΔrrgA bacteria were recovered from blood samples from any mice at this early time point (Fig. 4B). CD11b^-/- mice inoculated i.n. with the T4 strain succumbed later to infection than wild-type mice challenged with the same strain (Fig. 4A), and CR3-deficient mice also exhibited a delay in the onset of bacteremia (Fig. 4B and C).

When we challenged C57BL/6 mice intraperitoneally (i.p.) with the T4 strain and the different isogenic mutants, we observed that mice infected with the T4ΔrrgA mutant lacking RrgA survived significantly longer than mice infected with either the T4 or T4ΔrrgA (lacE::rrgA) strain both expressing RrgA (Fig. 5A) (P = 0.0025 in the survival analysis with the Kaplan-Meier log rank test) (4). The prolonged survival of mice infected i.p. with the T4ΔrrgA mutant was accompanied by lower levels of bacteria in the bloodstream (Fig. 5B).

To rule out the possibility that the prolonged survival of mice infected with the T4ΔrrgA mutant either i.n. or i.p. was due to slower growth of the bacteria in blood, we infected mice intravenously (i.v.) with the T4 strain and the T4ΔrrgA mutant. Using the i.v. infection route, there were no differences in the survival rate or bacterial growth in blood samples from mice infected with the two strains (Fig. 5C and D).

Finally, we investigated whether a lack of CR3 expression affected the survival of mice after i.p. inoculation with the T4 or T4ΔrrgA strain. When we performed i.p. experiments, we observed that there was a delay in the onset of symptoms and a significantly longer overall survival time of CR3-deficient mice inoculated i.p. with the T4 or T4ΔrrgA strain.
(CD11b−/−) mice compared to wild-type mice (P = 0.0051 in the survival analysis with the Kaplan-Meier log rank test) (Fig. 5E). CD11b−/− mice had lower numbers of CFUs in blood when infected with both T4 and T4ΔrrgA strains compared to wild-type mice infected with T4, arguing that the dissemination of RrgA-expressing pneumococci is impaired in CD11b−/− mice (Fig. 5F).

Bacterial expression of the pilus-associated RrgA adhesin results in higher numbers of intracellular survivors at late time points after phagocytosis. The results presented so far suggest that the RrgA adhesin, an ancillary protein of the pneumococcal 1 pilus, enhances phagocytosis through a direct interaction with the CR3 receptor expressed on murine and human macrophages. We expected that enhanced phagocytosis in vitro would translate into decreased virulence in vivo. In contrast, expression of RrgA in the bacteria together with CR3 in the host promoted bacterial virulence in mice and an early appearance of bacteria in the bloodstream after local infection.

To begin explaining these results, we first monitored the fate of RrgA-expressing and non-RrgA-expressing pneumococci after phagocytosis. The uptake of bacteria by macrophages leads to formation of phagosomes, where bacteria confront intracellular killing mechanisms. We monitored intracellular survival by performing gentamicin protection assays where T4, T4ΔrrgA, and T4ΔrrgA(lacE::rrgA) strains were incubated with macrophages (BMDMs). After antibiotic killing of extracellular bacteria, the number of internalized viable pneumococci was determined by quantitative plating at various time points. There were significantly fewer CFUs of the T4ΔrrgA mutant inside the macrophages at the beginning of the experiment (Fig. 6) (P = 0.0011), which further supported the differences in uptake observed in the fluorescence-activated cell sorting (FACS)-based phagocytosis assay (Fig. 1). The rate of bacterial killing was similar for all pneumococcal strains (Fig. 6). Interestingly, the increased uptake of RrgA-expressing bacteria and the unaffected intracellular killing rate resulted in significantly higher numbers of survivors for the T4 and T4ΔrrgA(lacE::rrgA) strains compared to the T4ΔrrgA strain even 10 h after infection (P < 0.05).

The interaction between RrgA and CR3 leads to increased motility and migratory behavior of murine BMDMs and human THP-1 cells. CR3 is a highly versatile pattern recognition receptor that when ligated activates leukocytes via signaling complexes and actin re-
This activation not only mediates phagocytosis but also promotes leukocyte transmigration. The motility/migration of immune cells affects infection, inflammation, and wound healing, and pathogens may utilize this cellular function in order to disseminate within the host (21–24). Therefore, we investigated whether the identified interaction between RrgA and CR3 affected the motility/migration of murine BMDMs (Fig. 7A) and human THP-1 cells (Fig. 7B). Motility/migration assays were performed using a Transwell system, in which we determined that strain T4 expressing RrgA increased the motility of BMDMs and THP-1 cells approximately twofold compared to untreated control cells (Fig. 7A, \( P < 0.01 \), and Fig. 7B, \( P < 0.05 \)). Interestingly, the T4\( \Delta rrgA \) strain, lacking RrgA, did not induce the motility/migration of macrophages, while induction of motility/migration was restored in the complemented strain, T4\( \Delta rrgA \)\( \Delta (lacE::rrgA) \). Both CR3-expressing and CR3-deficient (CD11b\(^{-/-}\)) macrophages migrated across the Transwell chamber when macrophage chemoattractant 1 (MCP-1) was present in the bottom chamber. However, the two RrgA-expressing strains failed to induce motility/migration in CR3-deficient (CD11b\(^{-/-}\)) BMDMs (Fig. 7A), supporting the role for this receptor in macrophage motility/migration upon interaction with pneumococcal RrgA. Finally, when BMDMs were preincubated with antibodies against CD11b, there were no differences in motility/migration between untreated control cells and cells challenged with RrgA\(^+\) or RrgA\(^-\) bacteria (data not shown).

To further confirm that the motile/migratory behavior of BMDMs is induced by RrgA and CR3, we performed additional motility assays using time-lapse video microscopy. Single-cell tracking revealed that the motility/migration of BMDMs was induced.
by pneumococci expressing RrgA [T4 and T4ΔrggA(lacE::rrgA) strains], but not by the T4ΔrggA strain (P < 0.001 [Fig. 7C and D]). CR3-deficient BMDMs were not affected by the addition of any of the pneumococcal strains; their motility/migration remained at control levels (Fig. 7C and D). Also, the velocity of the cells was measured using this method, which revealed that cells infected with T4 and T4ΔrggA(lacE::rrgA) strains moved faster than uninfected control cells (Fig. 7D) (P = 0.00015). In contrast, BMDMs infected with the T4ΔrggA mutant did not move faster than the control cells. The velocity of the CR3-deficient BMDMs did not increase after bacterial challenge and remained at control levels throughout the experiment.

**DISCUSSION**

We show for the first time that expression of pili on the pneumococcal surface leads to enhanced phagocytosis by murine BMDMs and human macrophages (THP-1 cells). Furthermore, we provide evidence that the pilus-associated adhesin RrgA, rather than the pilus-forming RrgB or pilus-associated RrgC subunits, promotes phagocytosis (25). Moreover, we show that a specific antibody...
H2O2, and therefore, prolonged intracellular survival time might last longer time period (at least 10 h). Pneumococci produce several extracellular matrix proteins, including CR3 binding to RrgA-expressing pneumococci was not shown to interact with a number of extracellular matrix proteins, such as PspC, which interact with CR3. This process is likely to enhance bacterial clearance. However, the simultaneous activation of motility and migratory behavior of macrophages may tilt the balance in favor of the ingested microbe. The bistability of pilus expression in a pneumococcal population (29, 30) and the fact that only a subset of pneumococcal strains harbor the pilus (27). Also, the migration of activated macrophages from the peritoneal cavity to the lymph nodes and blood has been shown to depend on CR3. (28). Thus, activated macrophages carrying pneumococci might migrate in a CR3-dependent manner to regional lymph nodes, and from there, infected phagocytic cells or liberated live bacteria are thought to reach the bloodstream, thereby contributing to systemic spread from local sites. However, further in vivo studies are needed to demonstrate that pneumococci can be translocated into the bloodstream via infected immune cells. Also, it is important to stress that other pneumococcal virulence properties, such as PspC, might mediate a direct translocation of bacteria across biological barriers.

For a commensal pathogen like Streptococcus pneumoniae, where healthy carriage vastly dominates over disease, each interaction between microbe and host, may have dual effects, sometimes favoring the host and other times the microbe. Here we demonstrate that the pilus-associated adhesin RrgA promotes phagocytosis by BMDMs in a process requiring CR3. This process is likely to enhance bacterial clearance. However, the simultaneous activation of motility and migratory behavior of macrophages may tilt the balance in favor of the ingested microbe. The bistability of pilus expression in a pneumococcal population (29, 30) and the fact that only a subset of pneumococcal strains harbor the pilus (27). Also, the migration of activated macrophages from the peritoneal cavity to the lymph nodes and blood has been shown to depend on CR3. (28). Thus, activated macrophages carrying pneumococci might migrate in a CR3-dependent manner to regional lymph nodes, and from there, infected phagocytic cells or liberated live bacteria are thought to reach the bloodstream, thereby contributing to systemic spread from local sites. However, further in vivo studies are needed to demonstrate that pneumococci can be translocated into the bloodstream via infected immune cells. Also, it is important to stress that other pneumococcal virulence properties, such as PspC, might mediate a direct translocation of bacteria across biological barriers.

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**FIG 6** Killing by macrophages is not affected by the presence of RrgA, but intracellular bacteria are found up to 10 h postchallenge. Bacteria were incubated onto monolayers of BMDMs for 1 h. Extracellular bacteria were killed by the addition of gentamicin to the assay medium. At various time points, cells were lysed with 2% saponin, and viable bacteria were determined by plating serial dilutions. The rate of bacterial death was similar for all strains, but bacteria expressing RrgA, i.e., T4 and T4ΔrrgA(lacE::rrgA) strains, were taken up to a greater extent, which led to higher numbers of intracellular bacteria after 10 h. Each treatment was done in triplicate and was repeated at least three times. Values that are significantly different are indicated by asterisks as follows: *P < 0.05; **P < 0.01.

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Against murine CD11b decreased phagocytosis by BMDMs and THP-1 cells of pneumococci expressing RrgA to a similar level of phagocytosis as that of RrgA− bacteria, suggesting that RrgA promotes phagocytosis via CR3. Finally, interaction between RrgA and CR3 appears to be direct and mediated by the CD11b integrin domain of CR3, thereby representing the first identified cellular receptor for a Gram-positive pilus. Even though RrgA has been shown to interact with a number of extracellular matrix proteins, purified CR3 binding to RrgA-expressing pneumococci was not affected by the addition of extracellular matrix proteins, like collagen 1, known to interact with either of these two proteins (6).

Our data demonstrate that internalization of RrgA+ and RrgA− bacteria follows a similar route, leading to phagosome formation and intracellular killing. However, pneumococci expressing RrgA were phagocytosed to a greater extent and BMDMs harbored higher numbers of viable RrgA+ than RrgA− bacteria over a longer time period (at least 10 h). Pneumococci produce several components toxic to macrophages, such as pneumolysin and H2O2, and therefore, prolonged intracellular survival time might lead to death of infected macrophages prior to the complete eradication of ingested bacteria.

When pneumococci expressing RrgA were injected intranasally and intraperitoneally into wild-type mice, they reached the lungs after infection with S. pneumoniae (26, 27). AMs are central to the normal surveillance of pneumococci. They may, however, have a dual function in host defense, as it was recently demonstrated that AMs migrate from the lungs to the lung-draining lymph nodes early after infection with an S. pneumoniae 6B isolate (27). Also, the migration of activated macrophages from the peritoneal cavity to the lymph nodes and blood has been shown to depend on CR3 (28). Thus, activated macrophages carrying pneumococci might migrate in a CR3-dependent manner to regional lymph nodes, and from there, infected phagocytic cells or liberated live bacteria are thought to reach the bloodstream, thereby contributing to systemic spread from local sites. However, further in vivo studies are needed to demonstrate that pneumococci can be translocated into the bloodstream via infected immune cells. Also, it is important to stress that other pneumococcal virulence properties, such as PspC, might mediate a direct translocation of bacteria across biological barriers.

**MATERIALS AND METHODS**

**Bacterial strains and construction of mutants.** S. pneumoniae T4 (TIGR4) of serotype 4 belongs to a clone (ST205) with high invasive disease potential in Sweden (31–33). The insertion–deletion mutagenesis used for creating the rrgA mutant strain T4ΔrrgA, is described elsewhere (1, 34). Complementation in trans was achieved by inserting a second, intact copy of rrgA into the lacE lactose utilization operon, creating the T4ΔrrgA::lacE::rrgA (4, 35). Mutants were checked by PCR, sequencing, and immunogenicity using antibodies to pilin subunits. Murine BMDMs and human macrophage-like THP-1 cells. Murine BMDMs were extracted (7, 36), and cells were plated in 24-well plates (1 × 10⁶ cells per well) and incubated for 7 days at 37°C and 5% CO2. Human monocytic leukemia THP-1 cells (American Type Culture Collection [ATCC], Manassas, VA) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCSi), 2 mM l-glutamine, and 10 mM HEPES. To induce differentiation, THP-1 cells (5 × 10⁵ cells per well) were seeded onto a 24-well plate with 100 ng/ml of phorbol myristate acetate (PMA) (Sigma) for 30 h at 37°C and 5% CO2. Before use, cells were washed to remove nonadherent cells. Each condition was repeated at least three times. Values that are significantly different are indicated by asterisks as follows: *P < 0.05; **P < 0.01.
The interaction between RrgA and CR3 leads to enhanced motility of murine and human macrophages. Wild-type (Wt) and CR3-deficient (CD11b<sup>−/−</sup>) BMDMs were challenged with pneumococci. (A) Macrophage motility was measured using a Transwell system. The percentages of cells that migrated were calculated by dividing the output by the input. In wild-type BMDMs, the T4 strain increased the motility of macrophages approximately twofold compared to the untreated control (CTR), but the T4<sup>rrgA</sup> mutant failed to do so. The T4<sup>rrgA</sup> strain induced motility to levels similar to those of the T4 strain. No statistically significant difference was found between cells infected with T4. All strains failed to induce motility in CR3-deficient macrophages, but the addition
tion was performed in triplicate, and the experiments were repeated at least three times.

**Mouse challenge.** The mice were 6 to 10 weeks old and were matched by age and sex. The mice were inoculated intranasally, intraperitoneally, or intravenously with ca. 5 × 10⁶ CFU per mouse. Bacterial growth in blood was monitored by taking blood samples from the tail at various time points and plating the samples in serial dilutions on blood agar plates. The health status of the mice was carefully monitored, and clinical scores were given.

**In vitro phagocytosis assays.** Bacteria were resuspended in a solution containing 2.5 mg fluorescein isothiocyanate (FITC) (isomer I; Sigma), 1 ml dimethyl sulfoxide (DMSO) (Sigma), and 9 ml FITC buffer (0.05 M Na₂CO₃ [Merck, Darmstadt, Germany] and 0.1 M NaCl [Merck] in double-distilled water) and incubated on ice for 1 h. After the bacteria were washed 3 times, macrophages were challenged with FITC-labeled pneumococci at a multiplicity of infection (MOI) of about 60 bacteria per cell, spun down at 1,500 rpm for 5 min, and incubated for 60 min at 37°C and 5% CO₂. Serum-free RPMI 1640 (Gibco/Invitrogen) supplemented with 2 mM glutamine and 10 mM HEPES was used as assay medium. Following bacterial challenge, the cells were washed 3 times with cold phosphate-buffered saline (PBS). The cells were detached by 15- to 30-min incubation on ice with PBS supplemented with 5 mM EDTA at pH 8.0 and 4.0 mg ml⁻¹ ligandase (Sigma). The cells were transferred to 96-well V-bottom plates, washed in PBS, and resuspended in 2% paraformaldehyde (Sigma). Before data acquisition by FACS, extracellular fluorescence was quenched by adding trypan blue (Sigma) at pH 5.5. To inhibit phagocytosis, 10 µg ml⁻¹ of cytochalasin D (Sigma) was added 15 min prior to bacterial challenge. For antibody blocking experiments, the following affinity-purified antibodies were used: anti-mouse CD11c (N418), anti-mouse CD11b (M1/70) (eBioscience Inc., San Diego, CA) and anti-human CD11b (VIM12) (Invitrogen). BMDMs were pretreated with selected anti-mouse antibody and THP-1 cells were pretreated with selected anti-human antibody at a concentration of 20 µg ml⁻¹ for 30 min at 37°C and 5% CO₂ prior to bacterial challenge. During FACS acquisition, 1 × 10⁴ cells were counted in each sample. Each condition was added in triplicate, and the experiments were repeated at least three times. The phagocytic index for each sample was calculated by multiplying the percentage of FITC-positive cells with the mean fluorescence intensity (MFI), and dividing this by the mean MFI for the wild-type (T4) samples multiplied with the percentage of FITC-positive cells.

For the microbead phagocytosis assays, we used full-length wild-type rRrgA (amino acid [aa] 39 to aa 862) (1, 4, 37), full-length wild-type rRrgC (1, 4), and BSA conjugated to carboxylate-modified yellow-green fluorescent microsphere (fluoresphore carboxylate from Molecular Probes, Invitrogen) according to the manufacturer’s protocol. THP-1 and BMDM cells, differentiated onto chamber slides (Nunc, Rochester, NY), were treated with 10 µg ml⁻¹ of latex beads alone or beads covered with rRrgA, rRrgC, or BSA and incubated for 3 h at 37°C and 5% CO₂. The inhibition assay was performed as previously described using anti-mouse (M1/70) and anti-human (VIM12) anti-CD11b antibodies. Each treatment was repeated in duplicate. Each preparation was examined at ×100 magnification under oil immersion. At least 10 visual fields for each sample containing at least 10 or more cells were selected in a random fashion, and the number of internalized beads was counted manually, taking care to exclude beads that were only adherent to the cell surface. Experiments were repeated three times, and representative experiments are shown.

**ELISA.** To assess direct binding of CR3 (CD11b/CD18) and CD11b I domain-GST to the rRrgA protein, microtiter plates were coated overnight at 4°C with 10 µg/ml of purified CR3 (or CD11b I domain-GST). Subsequent steps were conducted at room temperature (RT) for 1 h each. Nonspecific binding sites were blocked with PBS-BSA (2%), followed by the addition of rRrgA (different concentrations from 0 to 200 ng per well) in PBS-Tween (0.01%) (PBST). Bound rRrgA was detected with a specific antibody against rRrgA (B -anti-rRrgA) and anti-rabbit serum conjugated to horseradish peroxidase (Sigma), both diluted 1:5,000 in PBST. Sigmafast-OPD (o-phenylenediamine dihydrochloride) (Sigma) was used for detection, and absorbance was measured at 492 nm. In inhibition assays, CR3 and CD11b I domain-GST were pretreated with 25 µg/ml of rRrgA.

**Purification of CR3 (CD11b/CD18).** Human CD11b/CD18 integrin was purified from human blood buffy coat cell lysates by affinity chromatography on MEM170 monoclonal antibody-Sepharose and eluted at pH 11.5 in the presence of 2 mM MgCl₂ and 1% octyl glucoside. Human blood buffy coats were from Finnish Red Cross Blood Transfusion Service, Helsinki, Finland. Integrin purity was checked by polyacrylamide gel electrophoresis in the presence of SDS (see Fig. S5 in the supplemental material).

**Flow cytometry analysis of CR3 binding to pneumococci.** FACS analysis was performed to quantify the binding of CR3 to RrgA-expressing pneumococci. Bacteria were grown overnight on solid medium and then harvested into PBS. Bacteria (10⁸ CFU) were incubated with 10 µg/ml of purified CR3 (CD11b/CD18) or with PBS as a negative control for 30 min at 37°C. After the bacteria were washed twice in PBS containing 0.1% BSA [PBS-BSA (0.1%)], binding was detected following incubation with an anti-CD11b MAb labeled with PerCP-Cy5.5 (BD BioScience) at a final concentration of 10 µg ml⁻¹ for 1 h on ice. The cells were washed twice in PBS-BSA (0.1%) and once in PBS and then fixed in 2% paraformaldehyde for 20 min at room temperature. Finally, the bacteria were washed, and the fluorescence intensity was analyzed by a flow cytometer (CyAn ADP; Beckman Coulter). Bacteria were detected using log forward and log side scatter dot plots, and a gating region was set to exclude debris and larger aggregates of bacteria. A total of 1 × 10⁶ bacteria (events) were analyzed for fluorescence using log scale amplifications. Comparison between the fluorescence intensity of the bacteria incubated with purified CR3 with that of bacteria incubated with PBS alone were illustrated by two-dimensional overlaid histograms representing fluorescence intensities calculated with voltage adjustment at FL4 linear channel. FACS results were also expressed as the change in the mean fluorescence intensity (ΔMFI), where ΔMFI was calculated by subtracting the mean fluorescence intensity of the bacteria incubated with purified CR3 from that of bacteria incubated with PBS alone.

**Far-Western blots.** Human CR3 (CD11b/CD18) or CD11b I domain-GST was blotted onto nitrocellulose membranes using the Manifold system in concentrations of 0.5, 1, 5, and 10 µg 5 µg of BSA and 5 µg of glutathione S-transferase (GST) were used as negative controls. The membranes were blocked with PBS with 0.01% Tween 20 [PBS-Tween (0.01%)] and 5% milk for 30 min at room temperature. The membranes were washed briefly and incubated with 2 g/ml of RrgA in the presence of PBS-Tween (0.01%) and 1% milk overnight at 4°C. The membranes were washed, primary antibody (Rb -anti-RrgA [1:1,000]) was added, and the membranes were incubated for 3 h at room temperature on a rocking platform. Finally, the membranes were incubated with secondary antibody (anti-Rb peroxidase [1:2,000]; Sigma) for 1 h. Experiments were repeated three times, and representative experiments are shown.

**Figure Legend Continued**

of macrophage chemoattractant protein 1 (MCP-1) in the bottom chamber led to increased migration of wild-type and CR3-deficient cells. (B) The presence of RrgA also enhanced the motility of human macrophages (THP-1 cells). (C to E) Motility was monitored using time-lapse video microscopy for up to 6 h. (C) Representative pictures illustrating all cell movements (red), FITC-labeled pneumococci (green), and BMDMs (gray). (D) Wild-type BMDMs infected with strains expressing RrgA were more motile than those infected with the T4ΔrrgA mutant. (E) T4 and T4ΔrrgA (lex:ExrrgA) strains, but not the T4ΔrrgA strain, moved faster than untreated controls. (D and E) CR3-deficient macrophages did not display either induction of motility or an increase in velocity when challenged with any of the pneumococcal strains. Values that are significantly different are indicated by bars and asterisks as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Values that were not significantly different are indicated by a bar and n.s. (for not significant).
anti-CD18 7E4 MAb and anti-CD11b MEM170 MAb, respectively, for 30 min at RT.

**In vitro gentamicin protection assay.** The gentamicin protection assay was performed as described elsewhere (7).

**In vitro motility assays.** Differentiated BMDM cells were seeded in serum-free medium (RPMI 1640 supplemented with 2 mM glutamine and 10 mM HEPES) in small petri dishes (35 by 10 mm) at a density of 1 × 10⁶ cells per dish. The cells were either left untreated (control) or challenged with pneumococci at an MOI of ca. 60 bacteria per cell, or recombinant mouse MCP-1 (10 ng ml⁻¹; Invitrogen) was added to the bottom chamber (38). After 1.5 h, gentamicin was added to the medium (400 μg ml⁻¹). After an additional 1.5 h, the cells were gently scraped off, counted (input), and transferred to Transwell inserts containing RPMI 1640, and supplemented with 2 mM glutamine, 10 mM HEPES, 100 μg ml⁻¹ gentamicin, and 10% FCS (time point zero). Additionally, samples were taken from the medium to ensure that the extracellular bacteria were killed. The cells were incubated for 15 h before the inserts were removed, and the cells in the lower wells were scraped off and counted (output). The percentage of migrated cells was calculated by dividing the output by the input. Each condition was added in triplicate, and the experiments were repeated at least three times.

**Time-lapse video microscopy.** Time-lapse video microscopy was performed by the method of Behnse et al. (39).

**Statistical analysis.** In vitro assays were analyzed using one-way analysis of variance (ANOVA) with Bonferroni’s posttest (mean fluorescence intensity, CFU numbers, and motile cells), unless otherwise stated. In vivo assays were analyzed using Kaplan–Meier log rank test (survival), Kruskal–Wallis test with Dunn’s posttest (CFU numbers), or chi-square test and Fisher’s exact test (proportion of bacteremic mice). Graph Pad Prism 4.0 software was used (Graph Pad Software, Inc.), and a P value of <0.05 was considered statistically significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00535-12/-/DCSupplemental.

**Figure S1, PDF file, 0.2 MB.**

**Figure S2, PDF file, 0.2 MB.**

**Figure S3, PDF file, 0.1 MB.**

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