Brain angiogenesis inhibitor 1 (BAI1) is a pattern recognition receptor that mediates macrophage binding and engulfment of Gram-negative bacteria

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Edited* by Roy Curtiss, Arizona State University, Tempe, AZ, and approved December 27, 2010 (received for review October 1, 2010)

Bacterial recognition by host cells is essential for initiation of infection and the host response. Bacteria interact with host cells via multiple pattern recognition receptors that recognize microbial products or pathogen-associated molecular patterns. In response to this interaction, host cell signaling cascades are activated that lead to inflammatory responses and/or phagocytic clearance of attached bacteria. Brain angiogenesis inhibitor 1 (BAI1) is a receptor that recognizes apoptotic cells through its conserved type I thrombospondin repeats and triggers their engulfment through an ELMO1/Dock/Rac1 signaling module. Because thrombospondin repeats in other proteins have been shown to bind bacterial surface components, we hypothesized that BAI1 may also mediate the recognition and clearance of pathogenic bacteria. We found that preincubation of bacteria with recombinant soluble BAI1 ectodomain or knockdown of endogenous BAI1 in primary macrophages significantly reduced binding and internalization of the Gram-negative pathogen *Salmonella typhimurium*. Conversely, overexpression of BAI1 enhanced attachment and engulfment of *Salmonella* in macrophages and in heterologous nonphagocytic cells. Bacterial uptake is triggered by the BAI1-mediated activation of Rac through an ELMO/Dock-dependent mechanism, and inhibition of the BAI1/ELMO1 interaction prevents both Rac activation and bacterial uptake. Moreover, inhibition of ELMO1 or Rac function significantly impairs the proinflammatory response to infection. Finally, we show that BAI1 interacts with a variety of Gram-negative, but not Gram-positive, bacteria through recognition of their surface lipopolysaccharide. Together these findings identify BAI1 as a pattern recognition receptor that mediates nonopsonic phagocytosis of Gram-negative bacteria by macrophages and directly affects the host response to infection.

innate immunity | lipopolysaccharide-binding | TNF-α

Recognition of bacteria by host cells is essential for initiation of the innate immune response, in which pattern recognition receptors (PRRs) play an important role (1). PRRs represent a family of molecules that include Toll-like receptors (TLRs), scavenger receptors, C-type lectin receptors, and cytosolic sensors such as NOD1 and NOD2 that allow cells to recognize and clear unwanted particles or foreign molecules (2). For example, TLR2 recognizes the surface peptidoglycan (PG) of Gram-positive bacteria, TLR4 recognizes the surface lipopolysaccharide (LPS) of Gram-negative bacteria, while TLR5 recognizes conserved determinants in bacterial flagellin (1). A second class of PRR includes the scavenger receptor CD36, which recognizes a wide range of proteoglycan and lipid-containing pathogen associated molecular patterns (PAMPs) and also helps in the clearance of apoptotic cells (3, 4). Although surface PRRs are important in stimulating the host response to infection, the internalization of bacteria may modify the host response further as the bacterial PAMPs are displayed to intracellular sensing mechanisms.

BAI1 (brain-specific angiogenesis inhibitor 1) is a member of the so-called adhesion-type family of 7-transmembrane receptors (3). Its name derives from an initial observation that an extracellular fragment of the receptor inhibited neovascularization in an experimental brain tumor model (5). More recently it was discovered that BAI1 is expressed on macrophages, where it acts as a receptor for the clearance of apoptotic cells (6). A key feature of BAI1 is the presence of five extracellular type 1 thrombospondin repeats (TSRs) that bind to surface-exposed phosphatidylserine on apoptotic cells (6).

TSRs in other proteins have been shown to bind a variety of bacterial products including LPS from Gram-negative bacteria and PG from Gram-positive bacteria (7, 8). Importantly, the ligand binding specificities of different TSRs appear to vary; whereas thrombospondin-1 (TSP1) interacts only with PG (8), the single TSR in mindin/spondin-2 can also bind LPS and lipoteichoic acid (LTA) (7). Although BAI1 contains five TSR motifs, it is not known whether BAI1 recognizes bacterial products and, if so, what responses are triggered by this interaction.

We report here that BAI1 recognizes a range of Gram-negative, but not Gram-positive, bacterial pathogens through an interaction between the BAI1 TSRs and bacterial surface LPS. Binding of bacteria to BAI1 triggers activation of the Rho-family GTPase Rac1 through an ELMO1/Dock/Rac signaling module, which binds directly to the cytoplasmic domain of BAI1. Interestingly, BAI1-mediated Rac activation is necessary not only for engulfment of bound bacteria, but also for an efficient downstream proinflammatory response. Together these observations suggest that BAI1 functions not only as a receptor for apoptotic cells, but also as a unique PRR for Gram-negative bacterial pathogens that contributes directly to their internalization and the immunopathogenesis of infection.

Results

BAI1 Recognizes Bacteria Through Its TSRs. BAI1 has been shown to recognize apoptotic cells by binding to exposed phosphatidylserine (6). To determine if BAI1 can also bind bacteria, we examined its ability to recognize the Gram-negative enteric pathogen *Salmonella enterica* serovar Typhimurium. For most of our studies, we used a genetically engineered *S.* Typhimurium strain (Δ*invG*) that cannot actively invade host cells. Bacterial attachment in the absence of internalization was measured in murine J774 macrophages overexpressing BAI1, in which cells were pretreated with cytochalasin D to block phagocytosis. As

Author contributions: S.D., K.A.O., K.T.L., P.B.E., and J.E.C. designed research; S.D., K.A.O., and K.T.L. performed research; D.P., S.G.B., J.M.W., C.D.S., and K.S.R. contributed new reagents/analytic tools; S.D., K.A.O., K.S.R., P.B.E., and J.E.C. analyzed data; and S.D., K.S.R., P.B.E., and J.E.C. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1014775108/-/DCSupplemental.

shown in Fig. 1*A*, cells overexpressing BAI1 bound 63% more bacteria than controls (4.05×10^5 cfu vs. 2.49×10^5; Fig. S1).

Because macrophages express other surface receptors that may bind Gram-negative bacteria (e.g., scavenger receptor or TLRs) (9), we assayed *Salmonella* binding to nonphagocytic fibroblastic (i.e., CHO) cells (which do not express endogenous BAI1) in the presence and absence of exogenous BAI1. As shown in Fig. 1*B*, expression of BAI1 enhanced binding of *S.* Typhimurium by 2.3-fold relative to mock-transfected cells. Taken together, these data indicate that BAI1 can recognize determinants on the surface of *S.* Typhimurium and mediate its binding to macrophages and heterologous cells.

TSRs in other proteins have been shown to bind a variety of bacterial products including LPS from Gram-negative bacteria and PG and LTA from Gram-positive bacteria (7, 8). Importantly, the ligand binding specificities of different TSRs appear to vary; whereas TSP1 interacts only with PG (8), the single TSR in mindin/spondin-2 can bind PG, LTA, and LPS (7). As BAI1 contains five TSR motifs, we tested whether BAI1 recognizes bacterial products through interactions with its TSRs. Previous work has shown that binding of apoptotic cells to cultured macrophages can be inhibited by preincubation with a soluble BAI1 ectodomain fragment, which blocks binding to endogenous receptors (6); this fragment contains the N-terminal arginyl-glycyl-aspartic acid (RGD) motif and the five TSRs (RGD-TSR; Fig. S2). To determine if this holds true for bacteria, *S.* Typhimurium (ΔinvG) was preincubated with GST alone, GST-RGD-TSR, or a GST fusion containing the RGD motif but lacking the five TSRs (RGD-ΔTSR). Bacterial binding to macrophages was then measured as described earlier in the presence of cytochalasin D. As shown in Fig. 1 *C* and *D*, preincubation with GST-RGD-TSR inhibited *Salmonella* binding to J774 macrophages (50.5 ± 16%) and primary bone marrow-derived macrophages (BMDMs; 67 ± 5%) relative to GST alone. Importantly, GST-RGD-ΔTSR had no inhibitory effect, indicating that inhibition required the presence of the TSRs. A comparable level of inhibition was observed in macrophages freshly isolated from murine small intestine (10), demonstrating the physiological relevance of this interaction (Fig. 1*E*).

To confirm that the observed inhibition was caused by competition for binding to endogenous BAI1, BMDMs were depleted of BAI1 by using siRNA. As shown in Fig. 1*G*, BAI1 knockdown reduced surface binding of *S.* Typhimurium by 45 ± 5.8%. In contrast, knockdown of ELMO1, which is necessary for BAI1-mediated engulfment (6), had no effect on surface binding.

Binding of Bacteria to BAI1 Triggers Engulfment. To assay bacterial internalization, we used a standard gentamicin protection assay. Briefly, cells were exposed to bacteria for 1 h at 37 °C, washed, and incubated for an additional 90 min in the presence of the membrane-impermeable antibiotic gentamicin. This treatment kills extracellular bacteria, but intracellular bacteria remain viable and are quantified as described earlier by measuring colony-forming units in cell lysates. As shown in Fig. 2*A*, overexpression of BAI1 in J774 cells increased internalization of the noninvasive *Salmonella invG* mutant strain. Similarly, expression of BAI1 in CHO cells resulted in a more than fourfold increase in bacterial internalization, relative to vector controls (Fig. 2*B*). Together these findings indicate that BAI1 binds *S.* Typhimurium at the cell surface and mediates internalization of the bound bacteria, even in heterologous, nonphagocytic cells.

Preincubation of noninvasive *S.* Typhimurium with GST-RGD-TSR reduced internalization by 61 ± 16% in J774 macrophages, whereas incubation with GST alone or GST-RGD-ΔTSR had no effect (Fig. 2*C*). A corresponding inhibition was observed in BMDMs depleted of endogenous BAI1 by siRNA (Fig. 2*D*). Importantly, knockdown of ELMO1, which did not inhibit bacterial binding to the cell surface (Fig. 1*G*), did inhibit bacterial uptake, consistent with its known role in coupling BAI1 to the phagocytic machinery (6). Surprisingly, although an isogenic, invasive *Salmonella* strain (SL1344) bound BMDMs more efficiently than the noninvasive strain, knockdown of BAI1 or

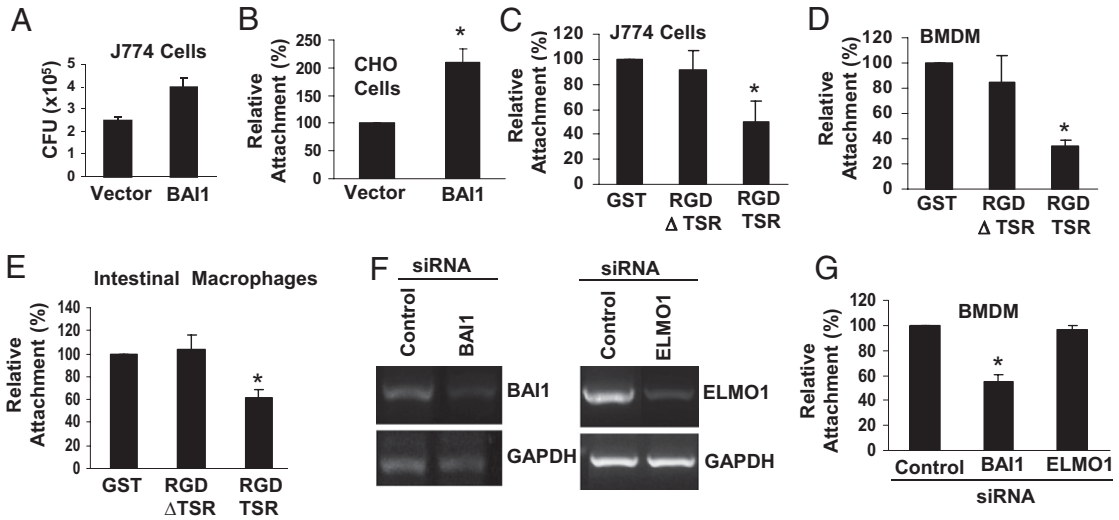

Fig. 1. Bacterial recognition mediated by the TSR domains of BAI1. (*A*) J774 macrophages expressing exogenous BA1 or transfected with empty vector were incubated with a noninvasive *Salmonella* Typhimurium strain (Δ*invG*) in the presence of cytochalasin D to measure adhesion in the absence of internalization. Numbers of bound bacteria (in cfu) were determined as described in *Materials and Methods*. (*B*) CHO cells were transfected with empty vector or a plasmid expressing untagged full-length BAI1. Bacterial attachment was assayed as in *A*. (*C–E*) Inhibition of *Salmonella* attachment by the recombinant BAI1 ectodomain. Bacteria were preincubated for 15 min with 10 ng/μL GST alone, GST fused to an N-terminal BAI1 fragment containing the RGD motif and all five TSRs (GST-RGD-TSR), or a similar fusion lacking the TSRs (GST-RGD-ΔTSR). Attachment to J774 cells (*C*), BMDMs (*D*), and intestinal macrophages (*E*) was assayed in the presence of cytochalasin D as described in *Materials and Methods*. (*F* and *G*) Down-regulation of BAI1 inhibits bacterial attachment. BMDMs were depleted of endogenous BAI1 or ELMO1 by using siRNA, and knockdown efficiency was determined by RT-PCR (*F*). Attachment of *S.* Typhimurium was assayed as described earlier (*G*). In *B–G*, data represent the means (±SD) of three independent experiments. In *A*, cfu values (mean ± SD) indicated are from a representative experiment with triplicate wells (*$P \leq 0.05$).

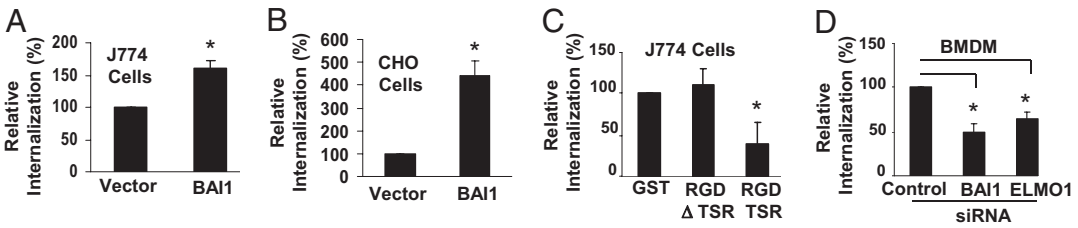

Fig. 2. BAI1 promotes bacterial internalization. (*A* and *B*) Bacterial internalization was measured by using the gentamicin protection assay in J774 cells (*A*) or in CHO cells (*B*) expressing exogenous BAI1. Cells were incubated with *S.* Typhimurium (Δ*invG*) for 1 h at 37 °C in the absence of cytochalasin D and internalization measured as described in *Materials and Methods*. (*C*) Competitive inhibition of bacterial internalization by the BAI1 ectodomain. *S.* Typhimurium (Δ*invG*) was preincubated with GST alone, GST-RGD-ΔTSR, or GST-RGD-TSR, then incubated with J774 cells for 1 h at 37 °C. (*D*) Knockdown of BAI1 or ELMO1 inhibits bacterial internalization. BMDMs were depleted of endogenous BAI1 or ELMO1 as described in Fig. 1. Internalization of *S.* Typhimurium (Δ*invG*) was assayed as described earlier. In *A*–*D*, data represent the mean ± SD of three separate experiments (*$P \leq 0.05$).

ELMO1 reduced internalization efficiency to a similar extent (Fig. S3). Together, these findings demonstrate that BAI1 not only recognizes bacteria at the cell surface, but that engagement of BAI1 triggers bacterial internalization.

BAI1-Mediated Bacterial Internalization Requires Rac1 Activation Through an ELMO1-Dependent Mechanism. The ELMO1/Dock180 complex acts as a bipartite guanine nucleotide exchange factor for the Rho-family GTPase Rac1, which coordinates the formation of membranous pseudopods that drive particle internalization during phagocytosis (11). Previous work showed that ELMO1 binds to a conserved helical region in the cytoplasmic domain of BAI1, and that ligation of BAI1 by apoptotic cells triggers the activation of Rac1 in an ELMO1- and Dock180-dependent manner (6). Mutation of three charged residues within this α-helix (RKR-AAA) significantly reduces the binding of ELMO1 to BAI1 (6). As shown in Fig. 2*D*, knockdown of ELMO1 attenuated bacterial uptake by macrophages to the same extent as BAI1 knockdown, suggesting that BAI1 and ELMO1 are also functionally linked during bacterial internalization. To test this hypothesis, we expressed WT BAI1 and mutant BAI1 (RKR-AAA) to comparable levels (Fig. S4) in CHO cells and measured Rac1 activity by using a pull-down assay, following addition of noninvasive *S.* Typhimurium to the culture. As shown in Fig. 3*A*, addition of bacteria to control CHO cells transfected with empty vector induced a small but detectable activation of Rac1 during a 30-min incubation. In cells expressing WT BAI1, the basal level of Rac1 activation was higher, but was dramatically increased upon addition of bacteria. In contrast, no activation of Rac1 was detected in cells expressing mutant BAI1 (RKR-AAA) that cannot couple to ELMO1. Rather, the mutant appeared to act as a dominant negative, preventing even the low level of activation observed in control cells. Importantly, the failure of this mutant to activate Rac1 correlated with impaired internalization of bacteria (Fig. 3*B*). These data support the notion that the binding of bacteria to BAI1 triggers their internalization through a mechanism involving the activation of Rac1 by the ELMO/Dock180 complex.

Bacterial Interaction with BAI1 Triggers Proinflammatory Responses. The clearance of apoptotic cells is typically noninflammatory, and in fact often results in the production of anti-inflammatory molecules such as TGF-β1 (12, 13) or IL-10 (14). In contrast, recognition of bacterial PAMPs by TLRs or other PRRs typically results in proinflammatory signaling, which is an important factor in bacterial clearance from infected tissue. To determine if bacterial recognition by BAI1 induces a proinflammatory response, we measured the production of a major proinflammatory cytokine, TNF-α, in BMDMs. As expected, incubation of noninvasive *Salmonella* (ΔinvG) with control BMDMs led to a robust induction of TNF-α (Fig. 3*C*). In contrast, BAI1-mediated internalization of the bacteria induced only a small increase in the release of IL-10 (Fig. S5). The mean value for IL-10 induced by infection was 132 pg/mL, compared with more than 3,000 pg/mL reported in studies following the engulfment of apoptotic cells (14). Remarkably, knockdown of BAI1 in BMDM cells reduced the level of TNF-α release nearly 50% and the knockdown of ELMO1 expression decreased TNF-α release even further. A similar reduction of TNF-α was observed when BMDMs depleted of BAI1 or ELMO1 were infected with invasive *Salmonella* (SL1344; Fig. S6).

BAI1 Preferentially Recognizes Gram-Negative Bacteria. As described earlier, the TSRs in other proteins exhibit distinct ligand specificities: whereas TSP1 interacts only with the surface PG of Gram-positive bacteria (8), the single TSR in mindin/spondin-2 can bind PG and LTA of Gram-positive bacteria and the LPS of Gram-negative species (7). To assess the binding preferences for BAI1, CHO fibroblasts were engineered to stably express an epitope-tagged form of the receptor. These cells were then incubated with a number of different Gram-negative [*S.* Typhimurium, *Escherichia coli* (DH5α), and *Campylobacter jejuni*] and Gram-positive (*Staphylococcus aureus, Streptococcus pneumoniae*, and group A *Streptococcus*) bacteria. For this assay, FITC-labeled bacteria were incubated with parental CHO cells or their BAI1-expressing derivatives, and bacterial binding was assessed by flow cytometry. As shown in Fig. 4*A*, binding of all three Gram-negative species was significantly enhanced in the presence of BAI1. In contrast, none of the Gram-positive species exhibited binding greater than background, suggesting that BAI1 preferentially recognizes Gram-negative bacteria.

The BAI1 TSRs Interact Directly with LPS. The most abundant component of the outer membranes of Gram-negative bacteria is LPS. To determine if the BAI1 TSRs can recognize LPS, we used a solid-phase assay in which GST, GST-RGD-TSR, or GST-RGD-ΔTSR were spotted onto nitrocellulose filters. These filters were then incubated with LPS from different sources, washed, and immunoblotted with antibody to LPS. As shown in Fig. 4*B*, the RGD-TSR construct can bind LPS from *S. enterica* serovar abortus equi and *E. coli* 055:B5 whereas the construct lacking the TSRs cannot. To confirm the ability of BAI1 to recognize LPS in intact cells, CHO cells were incubated with biotinylated ultrapure LPS (from *E. coli* 0111:B4) and surface binding was assayed by flow cytometry. Fig. 4*C* shows that cells expressing BAI1 bound significantly more LPS than nontransfected controls.

LPS consists of three parts: the membrane-anchored lipid A (which is recognized by TLR4), a positively charged "core" oligosaccharide region, and a highly variable terminal oligosaccharide chain referred to as O-antigen (Fig. S7). Our observation that *E. coli* DH5α (which lacks O-antigen) and *S. typhimurium SL1344* (which expresses O-antigen) showed comparable binding to BAI1 suggested that O-antigen is not necessary for this in-

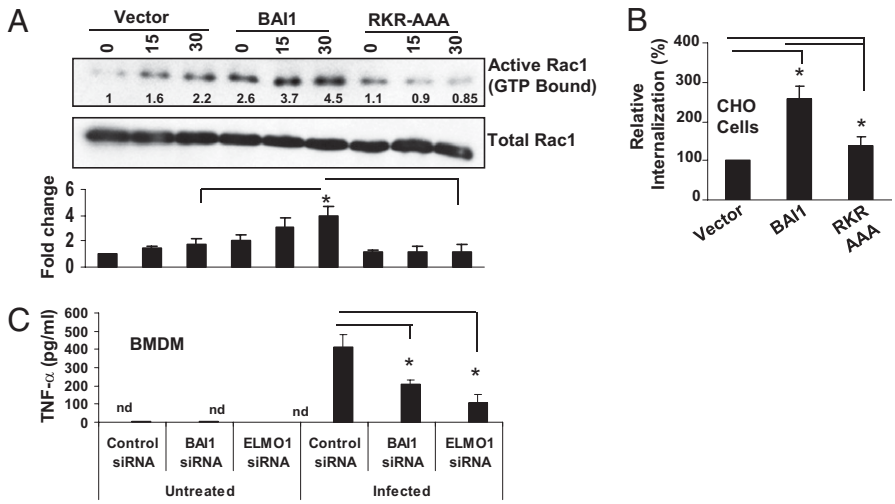

Fig. 3. Bacterial binding to BAI1 triggers Rac1 activation and bacterial internalization in an ELMO-dependent manner. (*A*) Rac1 activation was measured by pull-down assay after incubation with *S.* Typhimurium (Δ*invG*) for 0 min, 15 min, and 30 min. Pull-downs (*Upper*) and a fraction of the total lysates (*Lower*) were blotted for Rac1. The ratio of GTP-bound active Rac1 to total Rac1 is expressed relative to uninfected control cells (control value is set to 1). Data are representative of results from three experiments and the summary graph was plotted from the ratio of GTP-bound active Rac1 to total Rac1 of three independent experiments. (*B*) Cells expressing FLAG-BAI1 or FLAG-BAI1 (RKR-AAA) were incubated with *S.* Typhimurium (Δ*invG*) for 1 h at 37 °C and internalization was determined by using the gentamicin protection assay. (*C*) Knockdown of either BAI1 or ELMO1 impairs proinflammatory signaling in response to bacterial infection. TNF-α production was measured by ELISA in supernatants collected from control, BAI1-depleted, or ELMO1-depleted BMDMs after 6 h of incubation with *S.* Typhimurium (Δ*invG*). In *B* and *C*, data represent the mean ± SD of triplicate wells for each condition from three independent experiments (*$P \leq 0.05$).

teraction. To determine if BAI1 recognizes the charged core oligosaccharide, we tested the binding of recombinant BAI1 ectodomain to LPS variants (Fig. S7) containing the core [*E. coli* 055:B5 and *Salmonella* TV119 (Ra)] or lacking the core (*Salmonella* Minnesota Re595). For this assay, the LPS variants were spotted onto membranes and probed with recombinant BAI1 ectodomain. As shown in Fig. 4*D*, BAI1 bound efficiently to LPS species that contain the core oligosaccharide (Ra), but not to a form that lacks it (Re). Moreover, BAI1 failed to bind other bacterial products such as flagellin or the Gram-positive membrane component PG. In contrast, TSP1, which has three TSRs, bound PG but not LPS, as described previously by Rennemeier et al. (8). Together, these findings suggest that BAI1 mediates the binding of Gram-negative bacteria to macrophages through a direct interaction of the core oligosaccharides of LPS with the BAI1 TSRs.

Finally, LPS is well known to promote proinflammatory signaling through interactions with TLR4. To determine the extent to which BAI1 may contribute to the overall inflammatory response, we measured TNF-α release from control BMDMs or cells depleted of endogenous BAI1 by RNAi. Surprisingly, we found that down-regulation of BAI1 significantly attenuated TNF-α production in response to LPS treatment (Fig. 4*E*). These data suggest that BAI1 expression is required for efficient proinflammatory signaling, and that it may cooperate with TLR4 in the response to LPS.

Discussion

BAI1 was previously shown to function as a receptor for apoptotic cells, where it binds surface phosphatidylserine through its type I TSRs and triggers their engulfment via an ELMO1/Dock180/Rac1-mediated signaling pathway (6). Here we identify a unique function for BAI1 in the binding and internalization of Gram-negative bacteria through recognition of bacterial LPS by the BAI1 TSRs.

TSRs are conserved domains found in 41 human proteins, many of which are involved in cell adhesion, migration, communication, and tissue remodeling. The approximately 60 aa TSR domains are comprised of an elongated, three-stranded β-sheet (15). One face of the folded structure contains a helical groove that is rich in positively charged side chains, and is thought to represent the recognition face of the molecule. The density and distribution of these positive residues differs widely among TSRs from different proteins, and may have a significant impact on ligand specificity. As noted earlier, the single TSR in mindin/spondin-2 can bind a variety of ligands including LPS, LTA, and PG, whereas the TSRs of TSP1 appears to be selective for PG (7, 8) (Fig. 4*D*). The five BAI1 TSR domains vary significantly in surface charge distribution; the domain pI values vary from 5.2 to 8.7, suggesting that the different TSRs may recognize distinct ligands. Alternatively, two or more of the TSRs may act cooperatively to enhance binding affinity for multivalent ligands such as bacterial membranes. Our results indicate that the BAI1 TSRs bind Gram-negative, but not Gram-positive, organisms, and they are therefore unlikely to recognize LTA or PG, which are abundant in Gram-positive membranes. In fact, a direct assay for PG binding showed that TSP1 bound PG whereas BAI1 could not. Whether all five TSRs are important for BAI1 interaction with LPS, or any differential affinity for ligands exists among the TSRs, remain to be elucidated.

LPS is an important virulence factor for many Gram-negative pathogens (16). It comprises a lipid A region, which inserts into the membrane and contains five to seven acyl chains, depending on the organism. This is linked to an inner core complex of eight to 12 sugars, which is in turn linked to O-antigen, an oligosaccharide chain of variable length and composition. It is the composition of O-antigen that defines bacterial serotypes. TLR4 recognizes the lipid A moiety of LPS, in complex with the coreceptors CD14 and MD2 (17). In contrast, mindin/spondin-2 does not bind lipid A, but instead appears to recognize carbohydrate, as binding can be competed with simple sugars (e.g., mannose, glucose). We found that an *E. coli* strain (DH5α) lacking O-antigen in its LPS interacts with BAI1 in a cell-binding assay (Fig. 4*A*), suggesting that O-antigen is not a determinant of BAI1 binding. Moreover, we found that purified LPS containing the charged core oligosaccharide, but not O-antigen (Ra), bound BAI1

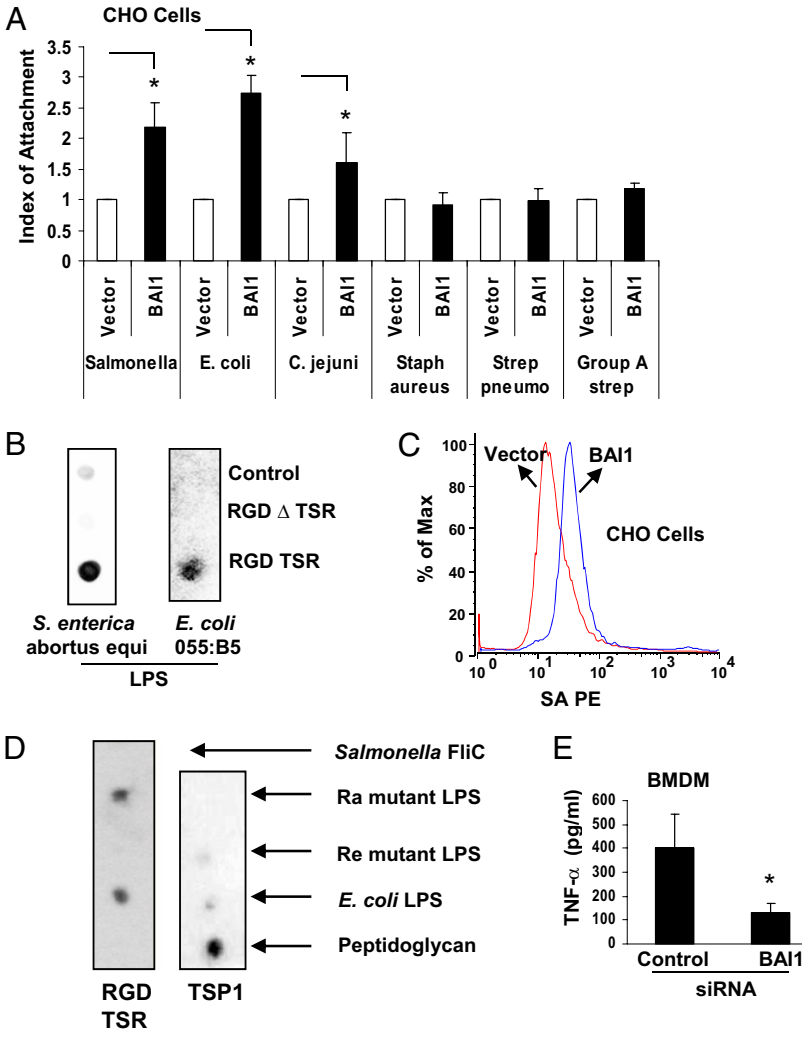

Fig. 4. BAI1 recognizes Gram-negative but not Gram-positive bacteria. (*A*) Equivalent numbers of *Salmonella* Typhimurium (*ΔinvG*), *E. coli* (DH5α), *C. jejuni*, *S. aureus*, *S. pneumoniae*, and group A *Streptococcus* were labeled with FITC and incubated at a multiplicity of infection (MOI) of 100 with vector control CHO cells (open bars) or CHO cells expressing FLAG-BAI1 (closed bars) for 1 h in the presence of cytochalasin D to prevent internalization. Binding of bacteria was analyzed by flow cytometry. For each bacterial species, an index of attachment was determined whereby background binding to non–BAI1-expressing CHO cells was arbitrarily set to a value of 1. Data represent the mean ± SD of three independent experiments. (*B–D*) The BAI1 TSRs bind directly to LPS. (*B*) Purified, recombinant GST (control), GST-RGD-ΔTSR, or GST-RGD-TSR (0.5 μg each) were spotted onto nitrocellulose filters. After blocking, filters were incubated with LPS from the indicated source and blotted with anti-LPS antibody. (*C*) CHO cells containing empty vector or expressing FLAG-BAI1 were incubated with biotin labeled LPS for 30 min at 37 °C. Cells were then washed, incubated with streptavidin–phycoerythrin, and analyzed by flow cytometry. (*D*) LPS from *E. coli* 055:B5, *Salmonella enterica* serotype Typhimurium TV119 (Ra mutant) and *Salmonella* enterica serotype Minnesota Re 595 (Re mutant), *S. aureus* PG, and *Salmonella* Typhimurium flagellin were spotted on nylon membranes, blocked, and incubated with GST-RGD-TSR (*Left*) or TSP1 (*Right*) followed by HRP-conjugated anti-GST or anti-TSP1 antibody. (*E*) Knockdown of BAI1 impairs proinflammatory signaling after LPS treatment. TNF-α production was measured by ELISA in supernatants collected from control or BAI1-depleted BMDMs after 6 h of *Salmonella* Typhimurium LPS (5 μg/mL) treatment. Data represent the mean ± SD of duplicate wells for each condition from three independent experiments (*$P \leq 0.05$).

efficiently whereas LPS lacking the core (Re) did not (Fig. 4*D*). This observation indicates that BAI1 interacts with a region of LPS distinct from the lipid A moiety that is recognized by TLR4. Whether BAI1 can recognize other carbohydrate-rich structures such as fungal glycans remains to be determined.

Professional phagocytes express an array of PRRs that recognize carbohydrate-based determinants, including multiple scavenger receptors (3) and the C-type lectin receptors (18). It is therefore not surprising that knockdown of BAI1 in macrophages did not completely abrogate bacterial uptake. However, our finding that *Salmonella* internalization was reduced by 50% in BAI1-depleted macrophages suggests that it is a quantitatively significant contributor to bacterial clearance.

The decrease in TNF-α production we observed in BAI1-depleted macrophages after bacterial infection or LPS treatment suggests that BAI1 also contributes significantly to proinflammatory signaling responses. TLR4 is also expressed in macrophages, and LPS signaling is known to require TLR4. However, it is also known that several phagocytic PRRs exhibit significant crosstalk with TLRs and can amplify or otherwise modify their signaling output (19, 20). It therefore seems likely that signals emanating from ligated BAI1 intersect with those derived from TLR4 and that BAI1 ligation positively regulates TLR4-mediated signaling. However, the mechanisms of this crosstalk remain to be defined.

In summary, we have identified a PRR, BAI1, that contributes to the uptake of Gram-negative bacterial pathogens by macrophages. Bacterial recognition occurs via a direct interaction between the BAI1 TSRs and the core oligosaccharide of bacterial LPS, and results in both bacterial engulfment and a proinflammatory signaling response. As LPS is an important virulence factor for Gram-negative pathogens, a more thorough knowledge of these aspects of microbial recognition may lead to new therapies that limit the survival of pathogenic bacteria and the tissue damage induced by local inflammatory responses.

Materials and Methods

Bacterial Strains, Cell Culture, Transfection, and Plasmids. *S. enterica* serovar Typhimurium (*ΔinvG*), a noninvasive isogenic mutant of WT strain SJW1103, was used to measure bacterial binding and internalization (21). In some experiments, the invasive *S. enterica* serovar Typhimurium strain SL1344 was also used. *SI Materials and Methods* includes further details of bacterial culture and cell maintenance.

Antibodies and Reagents. Antibodies used include the following: anti-LPS (Abcam), anti-Rac1 monoclonal antibody (Upstate Biotechnology), anti-FLAG (Sigma), anti-Tubulin (Abcam), HRP-conjugated anti-GST (GE Healthcare), antithrombospondin (Abcam), and streptavidin-HRP high sensitivity (Pierce). LPS from different bacterial species and *S. aureus* PG were purchased from Sigma-Aldrich and biotinylated ultrapure *E. coli* O111:B4 LPS and flagellin from *Salmonella* Typhimurium were obtained from InvivoGen. Recombinant TSP1 was purchased from Cell Sciences. Cytochalasin D (Sigma-Aldrich) was used at a concentration of 1 μg/mL to inhibit bacterial uptake by host cells.

RNA Preparation and RT-PCR. Total RNA was extracted using the RNeasy kit (Qiagen) and reverse transcribed by using the SuperScript kit (Invitrogen), both according to the manufacturers' instructions. Primer sequences are included in *SI Materials and Methods*.

Preparation of Recombinant RGD-TSR. The RGD-TSR region of the BAI1 ectodomain (residues 202–585) was subcloned into the pGEX-4T2 vector and purified as described previously (6).

Peptide and Inhibitor Incubation. Where indicated, GST-RGD-TSR, GST-RGD-ΔTSR, or GST alone were preincubated with bacteria for 15 min at a concentration of 10 ng/μL before being added to cells in antibiotic-free DMEM in a 37 °C CO_2 incubator.

BMDM Preparation. Primary BMDMs were derived from the femurs and tibia of mice by using a modification of techniques described previously (10). Detailed methods are provided in *SI Materials and Methods*.

Preparation of Intestinal Macrophages. Gut antigen-presenting cells were isolated using techniques described previously (10, 22). Detailed methods are provided in *SI Materials and Methods*.

siRNA Transfections. Nucleofection was used to introduce BAI1 or ELMO1 siRNA (ON-Target Plus SMART pool; Dharmacon) into BMDM cells using the Amaxa mouse macrophage nucleofector kit (Lonza) using program Y-001. After 48 h, RNA was prepared to monitor the level of BAI1 and ELMO1 expression by RT-PCR.

Gentamicin Protection Assay. Quantification of intracellular bacteria was done by using the gentamicin protection assay as described previously (23). Detailed methods are described in *SI Materials and Methods*.

Bacterial Attachment Assays. Infection of bacteria was performed as described for the gentamicin protection assay but in the presence of 1 μg/mL cytochalasin D to block internalization. After incubation for 1 h, cells were washed to remove unbound bacteria and lysed in 1% Triton, and remaining surface-bound bacteria were quantified by plating of cell lysates on Luria–Bertani (LB) agar plates.

To measure bacterial attachment by flow cytometry, bacteria were labeled with FITC dye (Sigma-Aldrich) as previously described (24). Cells were incubated with labeled bacteria for 1 h at room temperature, washed, fixed with 1% paraformaldehyde, and analyzed by flow cytometry by using a Becton Dickinson FACSCalibur dual laser instrument as described (8).

Binding of LPS to BAI1. Biotinylated ultrapure *E. coli* O111:B4 LPS (5 μg; InvivoGen) was incubated with 10^6 cells in DMEM for 30 min at 37 °C. Cells were then washed and stained with streptavidin–phycoerythrin before flow cytometric analysis.

Dot Blot. Purified control GST, GST-RGD-ΔTSR, and GST-RGD-TSR (0.5 μg) were spotted onto nitrocellulose membrane and air-dried. Membranes were blocked with 3% BSA and incubated with LPS at 1 μg/mL overnight at 4 °C. Membranes were then probed with anti-LPS antibody in 3% BSA followed by HRP-conjugated secondary antibody, washed, and incubated in detection reagent.

In a similar assay, 0.5 μg of LPS from smooth (*E. coli*) and rough variants (Ra and Re mutants), PG (from *S. aureus*), or flagellin (from *S. typhimurium*) were spotted on nylon membrane, blocked, and incubated with 1 μg/mL of GST-RGD-TSR or recombinant TSP1 overnight at 4 °C. After washing, membranes were probed with HRP-conjugated anti-GST antibody (1:500) or anti-TSP1 antibody followed by HRP-conjugated anti-mouse IgG and developed by using Millipore detection reagent.

Western Blot. Cell lysates were separated by SDS/PAGE, transferred to a nitrocellulose membrane (Bio-Rad), and blocked in Tris-buffered saline solution with Tween (20 mM Tris-HCl/136 mM NaCl, pH 7.5, with 0.05% Tween 20) containing 5% nonfat dry milk. The binding of primary antibody was detected with HRP-conjugated secondary antibody (Amersham Biosciences).

Cytokine Measurement. Supernatants were collected from BMDMs infected with bacteria for the indicated times. Mouse TNF-α was measured by using an ELISA kit (BD Pharmingen) according to the manufacturer's instructions.

Assessment of Rac1 Activation. Rac1 activity was measured by a pull-down assay by using GST-PBD (p21-binding domain of Pak1) beads as described previously (25).

Statistical Analysis. Results are expressed as mean ± SD and were compared by using two-tailed Student *t* test; differences were considered significant if *P* values were lower than 0.05.

ACKNOWLEDGMENTS. We thank Joanne Lannigan and Michael Solga of the University of Virginia Flow Cytometry Core Facility for expert technical assistance in cell sorting and William Ross for flow cytometry. Research was supported by National Institutes of Health Grants DK058536 (to J.E.C.); AI08600, DK84063, and AI070491 (to P.E.); and GM64709 (to K.S.R.).

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