Mannose-binding lectin enhances Toll-like receptors 2 and 6 signaling from the phagosome

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Innate immunity is the first-line defense against pathogens and relies on phagocytes, soluble components, and cell-surface and cytosolic pattern recognition receptors. Despite using hard-wired receptors and signaling pathways, the innate immune response demonstrates surprising specificity to different pathogens. We determined how combinatorial use of innate immune defense mechanisms defines the response. We describe a novel cooperation between a soluble component of the innate immune system, the mannose-binding lectin, and Toll-like receptor 2 that both specifies and amplifies the host response to *Staphylococcus aureus*. Furthermore, we demonstrate that this cooperation occurs within the phagosome, emphasizing the importance of engulfment in providing the appropriate cellular environment to facilitate the synergy between these defense pathways.

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Abbreviations used: LBP, LPS-binding protein; LTA, lipoteichoic acid; MBL, mannose-binding lectin; MFI, mean fluorescence intensity; MOI, multiplicity of infection; PGN, peptidoglycan; SP, surfactant protein; TAMRA, tetramethyl-6-carboxyrhodamine; TLR, Toll-like receptor.

The innate immune system represents an evolutionarily conserved defense against infectious agents that senses pathogenic microorganisms through "pattern-recognition receptors," molecules that recognize the conserved molecular patterns on the surface of microbes (1–3). Over the past decade, much of the focus of innate immune research has been to understand the role of the archetypal pattern-recognition molecules, the Toll-like receptors (TLRs). The TLRs are key sensors of pathogens that trigger signal transduction events that converge to activate mitogen-activated protein (MAP) kinases and transcription factors such as NF-κB and interferon regulatory factors, leading to proinflammatory responses (4). However, although TLRs are canonical, germline-encoded receptors that signal via essentially hard-wired pathways, it is evident that the final response induced by a pathogen displays surprising specificity. This specificity is in part caused by the complexity of pathogens, which carry a variety of TLR ligands that defines their distinct "molecular signature" and results in combinatorial ligation of TLR heterodimers and contributes to specify pathogen-appropriate responses (4, 5). More recently, work has begun to explore the importance of signaling from non-TLR receptors in contributing to defining pathogen-specific responses (6, 7).

Emerging data indicate that coordinated integration of signals from alternative defense pathways such as the Nod-like receptors, scavenger receptors, and C-type lectins contributes to innate immune discrimination. The best defined example is the recognition of endotoxin that occurs via a cooperation of molecules, including LPS-binding protein (LBP) (8), CD14 (9), and MD2 (10), that enhance TLR4 signaling in response to LPS (11). Recent work has demonstrated that other TLRs can be similarly modified: CD36 recognizes Gram-positive bacterial cell wall products and modulates TLR2/6 signaling, and the C-type lectin, Dectin-1, can either signal alone to induce IL-10 or in cooperation with TLR2 to induce IL-12 in response to yeast (12–15). These examples demonstrate how combinatorial recognition by multiple receptors and integration of signaling establishes an innate immune network that provides robust but pathogen-specific responses.

The evolutionarily ancient defense pathways mediated by soluble molecules such as

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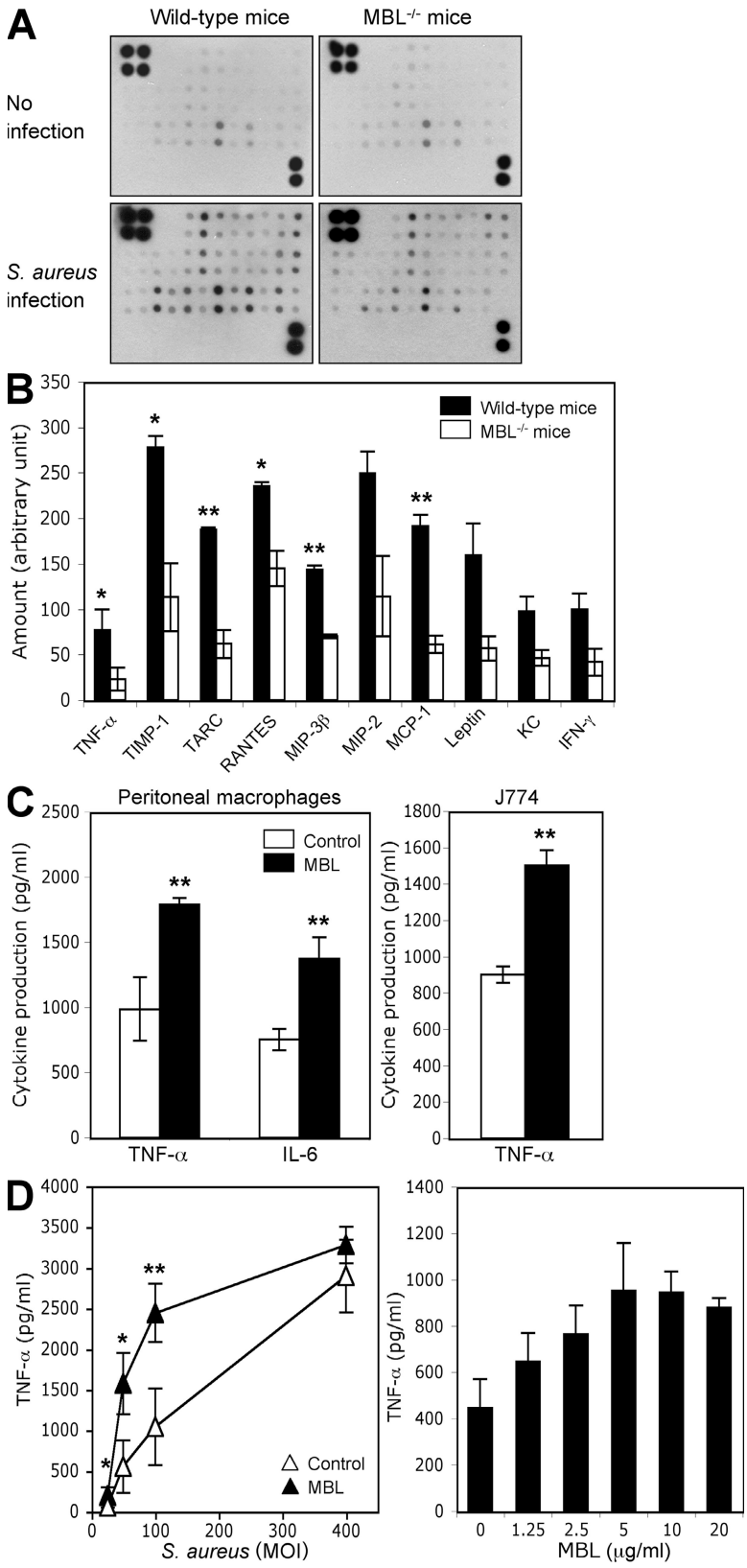

Figure 1. MBL regulates the cytokine responses upon *S. aureus* infection. (A) Protein array blots showing the relative amounts of specific cytokines present in the pooled serum samples obtained from wild-type ($n = 3$) and MBL$^{-/-}$ ($n = 3$) mice on a C57BL/6 background at 2 h after i.v. inoculation in the tail vein with 2×10^7 *S. aureus* or saline (no infection; Fig. S1 A shows the map of the protein array). (B) Expression levels of cytokines in serum

complement and collectins are also integral components of innate immune defense. A key example is the mannose-binding lectin (MBL), a circulating protein that recognizes a variety of infectious agents and, hence, functions as a prototypic pattern-recognition molecule (1, 16). MBL belongs to the collectin family and functions as an opsonin (17, 18) that is also able to activate the complement cascade via the lectin pathway (19–21). Association studies have linked low serum levels of MBL or *mbl2* genotypes with particular diseases (22), including an increased susceptibility to certain infections (18, 23–25). In addition, accumulating in vitro data show that MBL is able to modulate inflammatory responses (26–28). These observations suggest that MBL may act as an important natural modifier of human defense pathways. Supporting this hypothesis, our recent in vivo studies have demonstrated that mice lacking MBL are highly susceptible to pathogens such as *Staphylococcus aureus* (29, 30). However, the molecular mechanism by which MBL modulates inflammatory responses remains undefined.

In this paper, we confirm that MBL plays an essential role in *S. aureus* infection, making a contribution comparable to that of TLR2. MBL modifies cytokine responses, not through its capacity to act as an opsonin or to activate complement, but via a novel cooperation with TLR2/6. We demonstrate that MBL binds to lipoteichoic acid (LTA) and complexes with TLR2 to increase ligand delivery, resulting in up-regulation of TLR2 responses. Furthermore, we demonstrate the essential contribution of the phagosome in coordinating these responses. These data indicate a novel mechanism by which soluble innate immune molecules such as MBL integrate with TLRs in the innate immune network to specify and amplify host defense responses.

RESULTS

MBL modifies the cytokine responses after *S. aureus* infection

We have previously observed that mice lacking MBL have reduced TNF-α and IL-6 responses during infection to *S. aureus,* suggesting a role for MBL in regulating cytokine release in vivo (30). To determine whether MBL enhanced response or defined a unique cytokine signature after bacterial infection, wild-type and MBL-null mice were infected with *S. aureus* i.v., and serum cytokines were determined using protein microarrays. These arrays demonstrated significant differences between wild-type and MBL-null mice 2 h after inoculation (Fig. 1, A and B); specifically, MBL augmented production of a subset of proinflammatory cytokines and chemokines such as TNF-α, tissue inhibitor of metalloproteinase 1, and monocyte chemotactic protein 1 (Fig. 1 B) but did not modify production of others (GCSF, IL-2, or IL-12p70; Fig. S1 B, available at http://www.jem.org/cgi/content/full/jem.20071164/DC1). MBL also down-regulated production of the immunoregulatory cytokine IL-10 (Fig. S1 B). Collectively, these data demonstrate that MBL contributes to modify the host response to *S. aureus* in vivo and specifies a unique inflammatory cytokine signature. Similar results were observed in thioglycollate-elicited peritoneal macrophages and J774 macrophages stimulated in vitro with heat-inactivated bacteria opsonized with or without MBL (Fig. 1, C and D). Consistent with our in vivo data, MBL significantly enhanced TNF-α and IL-6 production by macrophages in response to *S. aureus* in a dose-dependent manner (Fig. 1, C and D).

Altered response to *S. aureus* in the presence of MBL is not caused by increased bacterial uptake

MBL functions as an opsonin and, consistent with previous reports, was able to accelerate phagocytic uptake of different particles, including pathogens (e.g., *S. aureus*; Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20071164/DC1) and apoptotic cells (not depicted). To determine if the altered response to *S. aureus* in the presence of MBL was caused by opsonization and increased uptake, we assessed macrophage responses to a variety of particles opsonized by MBL. MBL opsonization of neutral particles such as mannose-coated beads and apoptotic cells failed to induce cytokine production, demonstrating that MBL opsonization alone was insufficient to induce or alter proinflammatory cytokine production (Fig. S3). In addition, despite increasing uptake of heat-inactivated group B *Streptococcus*, *Escherichia coli*, or *Salmonella montevideo* (unpublished data), we observed no effect of MBL on TNF-α production in response to these proinflammatory particles (Fig. S4). Thus, increased bacterial uptake by MBL opsonization did not correlate with increased cytokine production. We next determined if the ability of MBL to regulate cytokine response to *S. aureus* was unique to MBL by examining the effect of opsonization with two related collectins, surfactant protein A (SP-A) and SP-D. SP-A increased uptake but not TNF-α response (Fig. S5), again demonstrating that increased phagocytosis is insufficient to enhance the proinflammatory response to *S. aureus*. Interestingly, SP-D shared with MBL the ability to increase TNF-α production (Fig. S5 B). Collectively, these data provide evidence that MBL plays a specific role in regulating response to *S. aureus* beyond that of simple opsonization or increased bacterial uptake.

after *S. aureus* infection. Data are mean intensity ± SD of duplicate signals obtained from the protein array blots shown in A. (C and D) Cytokine production by peritoneal macrophages (from wild-type C57BL/6J mice) and J774 macrophages after in vitro stimulation with *S. aureus*. Cells were incubated with heat-inactivated *S. aureus* (MOI = 50 or as indicated), which were opsonized without (control) or with MBL at 10 μg/ml or the indicated concentrations. Induction of cytokine responses at 2 (TNF-α) or 4 (IL-6) h was measured by ELISA in culture supernatants. Data are representative of four independent experiments. Data indicate mean ± SD of triplicates. *, $P \leq 0.05$; **, $P < 0.01$.

To definitively test if the altered response to *S. aureus* in the presence of MBL could be explained by increased bacterial uptake, flow cytometry and intracellular cytokine staining were used to simultaneously measure bacterial engulfment and cytokine production at a single-cell level. These assays allowed cytokine production to be normalized for the number of bacteria internalized. At 3 h, equivalent numbers of control and MBL-opsonized bacteria were internalized by macrophages (Fig. 2 A). However, when macrophages carrying equivalent bacterial-derived fluorescence (and hence, bacterial loads) were examined, MBL opsonization was associated with a 1.5–2-fold increase in TNF-α, as determined by the mean fluorescence intensity (MFI) of intracellular cytokine staining (Fig. 2 B). Such increases were observed as early as 90 min after internalization of the bacteria (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20071164/DC1). These data unequivocally indicate that increased bacterial uptake per se cannot explain the increased cytokine production associated with MBL opsonization and suggest that MBL plays a specific role in regulating response to *S. aureus*.

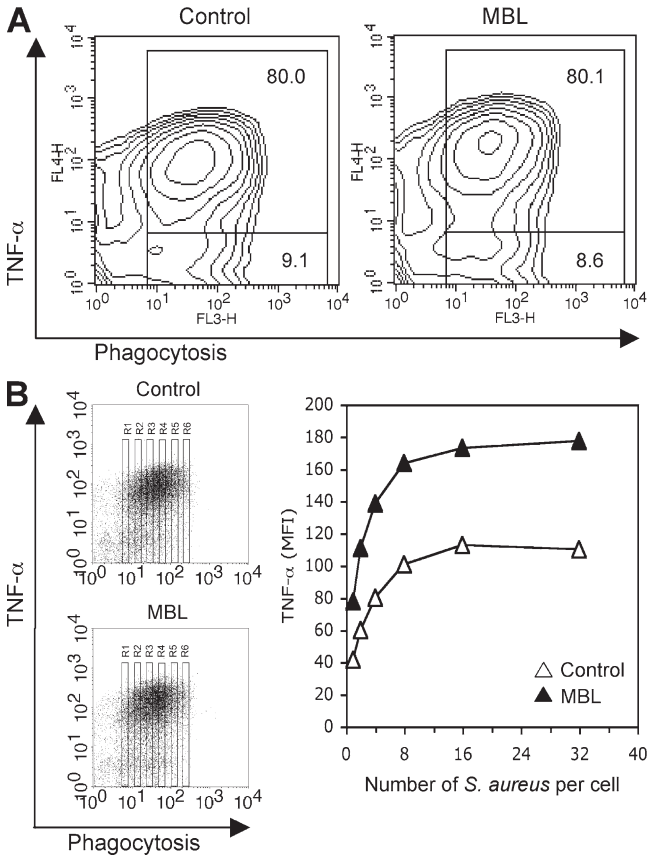

Figure 2. MBL enhances TNF-α response in macrophages with equivalent bacterial loads. (A) Single-cell analysis by flow cytometry determining bacterial engulfment and intracellular TNF-α production. TAMRA-labeled *S. aureus*, unopsonized (control) or opsonized with MBL at 10 μg/ml, were incubated with adherent C57BL/6J peritoneal macrophages at an MOI of 25 for 1–3 h. Phagocytosis and intracellular TNF-α production were measured simultaneously by flow cytometry. Contour plots show the percentages of TNF-α–producing (top) or –nonproducing (bottom) cells at 3 h after internalization of the bacteria. (B) TNF-α production in macrophages with defined bacterial loads. The number of bacteria engulfed by macrophages was estimated with the MFI obtained for a single bacterial particle. Cells that contain one- or twofold increasing numbers of bacteria were identified in regions R1–6 (density plots; left), thus allowing normalization of cytokine production for defined bacterial loads (right). Data are the MFI of intracellular TNF-α production from corresponding regions and representative of three independent experiments.

MBL enhances the response to *S. aureus* independently of complement activation via C3

One mechanism by which MBL could increase cytokine response is through activation of complement via the lectin pathway. To address this possibility, we used serum derived from MBL-null or MBL × C3–null mice. Consistent with our in vivo data, less TNF-α was produced when *S. aureus* were opsonized with serum from MBL-null mice than with wild-type serum (Fig. 3 A), and this defect could be rescued by the addition of exogenous MBL (Fig. 3 A). Importantly, exogenous MBL also restored TNF-α production when serum from MBL × C3–null mice was used (Fig. 3 B), indicating that the ability of MBL to increase TNF-α occurred independently of C3 activation. Furthermore, MBL augmented TNF-α response to *S. aureus* in macrophages obtained from C3-deficient mice (Fig. 3 C) and complement receptor 3 (Mac-1)–deficient mice (Fig. 3 D). Collectively, these data support our previous in vivo observations (30, 31) and demonstrate conclusively that MBL is sufficient to enhance the proinflammatory cytokine responses to *S. aureus* independently of complement activation.

MyD88- and TLR2-dependent responses to *S. aureus* are up-regulated by MBL

TLR2 is known to play a key role in response to *S. aureus* (14, 32). Based on the observations that MBL functions to regulate cytokine responses induced by *S. aureus* independently of opsonization or complement, we hypothesized that MBL might function to directly modify this host defense pathway. To address this possibility, we examined the in vivo response of MBL and TLR2-null animals after infection with *S. aureus*. Consistent with our published work (30), MBL-null mice showed a profound defect in their ability to fight *S. aureus* infection (Fig. 4 A). Moreover, this defect was comparable to that of the TLR2-null mice (Fig. 4 A). These data indicate that MBL and TLR2 make comparable contributions to host defense to *S. aureus* in vivo.

To establish if MBL and TLR2 function in the same pathway of host defense, we examined the effect of MBL opsonization in macrophages from MyD88-, TLR2-, and TLR4-deficient mice. Macrophages isolated from wild-type or $TLR4^{-/-}$ mice responded normally to *S. aureus*, and as expected, MBL opsonization enhanced TNF-α production (Fig. 4 B). In contrast, $MyD88^{-/-}$ macrophages failed to respond to heat-inactivated *S. aureus*, and TNF-α was not produced even when the bacteria were opsonized with MBL

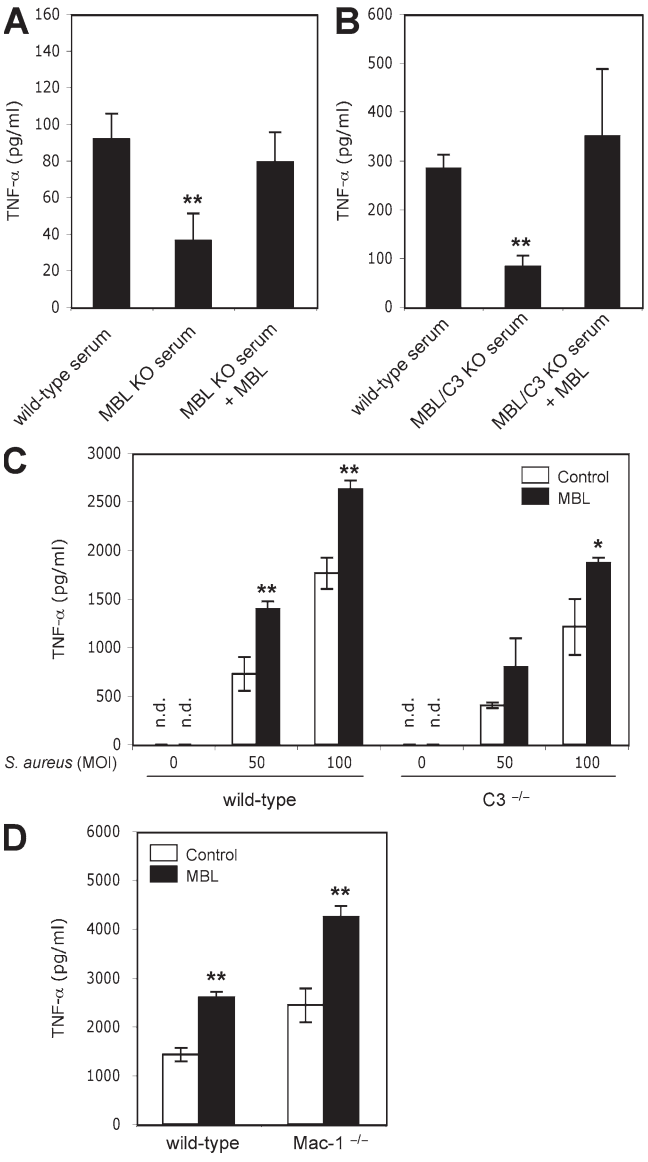

Figure 3. MBL enhances proinflammatory response to *S. aureus* independently of complement activation. (A and B) TNF-α response to *S. aureus* opsonized with mouse serums. C57BL/6J peritoneal macrophages were cultured in the presence of heat-inactivated *S. aureus* (MOI = 50), which were opsonized with wild-type serum (from wild-type mice), MBL KO serum (from MBL knockout mice; A), or MBL/C3 KO serum (from MBL × C3 knockout mice; B), or the KO serums supplemented with exogenous MBL at 20 µg/ml. TNF-α response at 2 h was measured by ELISA in culture supernatants. (C and D) TNF-α production by control (wild-type), $C3^{-/-}$, or complement receptor $3^{-/-}$ macrophages in response to *S. aureus* opsonized with or without MBL. Peritoneal macrophages from control C57BL/6J mice, $C3^{-/-}$ mice (C), or complement receptor $3^{-/-}$ ($Mac\text{-}1^{-/-}$) mice (D) were cultured in the presence of heat-inactivated *S. aureus* (at the indicated MOIs [C] or MOI = 50 [D]), which were opsonized without (control) or with 10 µg/ml of MBL. TNF-α response at 2 h was measured. Data are representative of two independent experiments and indicate the mean ± SD of triplicates. *, $P \leq 0.05$; **, $P < 0.01$. n.d., no detectable cytokine.

(Fig. 4 C). Similarly, $TLR2^{-/-}$ macrophages showed a significant, but not total, impairment in TNF-α production in response to *S. aureus* (Fig. 4 B). Importantly, the residual TNF-α produced in the absence of TLR2 was not enhanced by MBL (Fig. 4 B). These results demonstrate that MBL enhances TNF-α released in response to activation of the TLR2–MyD88-dependent pathway. Furthermore, as MBL is epistatic to TLR2–MyD88 activation, these data place MBL and TLR2 in the same pathway of host defense.

MBL binds TLR2/6 ligands and modulates signaling from this heterodimer

To determine whether MBL-regulated signaling induced by TLR2 via binding to TLR ligands, we characterized the ligand that MBL bound on the surface of *S. aureus*. We first tested the ability of MBL to bind to *S. aureus*–derived ligands in solid phase by ELISA. MBL was able to bind to both LTA and peptidoglycan (PGN) in solid phase (Fig. 5 A). However, it is important to note that the binding to LTA is Ca^{2+} dependent (Fig. 5 A), indicating that MBL interacts with LTA using its carbohydrate recognition domain (Ca^{2+} dependent). In contrast, binding to PGN is partially Ca^{2+} dependent (Fig. 5 A), suggesting that MBL may bind PGN via other domains such as the collagenous region or tail (Ca^{2+} independent). To further determine the ligand that MBL bound on the surface of *S. aureus*, flow cytometry was used to measure the binding of MBL to the bacteria. Cyanine 3–labeled MBL (Cy3-MBL) bound strongly to heat-inactivated *S. aureus*, and the binding was highly Ca^{2+} dependent and, hence, completely blocked by EDTA (Fig. 5 B). However, despite binding of MBL to both LTA and PGN in solid phase, only soluble LTA, and not PGN, was able to compete and inhibit Cy3-MBL binding to heat-inactivated *S. aureus* (Fig. 5 B). This inhibition was comparable to the effects of mannan. Importantly, even when used at high concentrations, PGN did not block Cy3-MBL binding to *S. aureus*. The inhibition by LTA was also confirmed with fluorescence microscopy (Fig. 5 C). Collectively, these data suggest that MBL binds via its carbohydrate recognition domain to LTA exposed on the surface of intact bacteria.

LTA is a known TLR2/6 ligand and, thus, led us to hypothesize that MBL regulates heterodimer TLR2/6 signaling in response to *S. aureus*. To test if MBL modifies TLR2/6 signaling, HEK293 cells stably expressing GFP-TLR2 (TLR2-HEK293) and transfected with an NF-κB–luciferase reporter system were used. Soluble MBL showed no effect on NF-κB activation in this system (Fig. 5 D), supporting our observations that MBL alone is insufficient to induce proinflammatory response (Fig. S3). In addition, cells expressing TLR2 alone failed to respond to heat-inactivated *S. aureus* with or without MBL opsonization. However, when TLR2-HEK293 cells were cotransfected with TLR6, MBL caused a significant increase in *S. aureus*–induced activation of NF-κB (Fig. 5 D), indicating that MBL can enhance signaling initiated via the TLR2/6 heterodimer. To further determine if the enhanced signaling by MBL was via the interaction with LTA on the bacterial surface, we examined the effect of

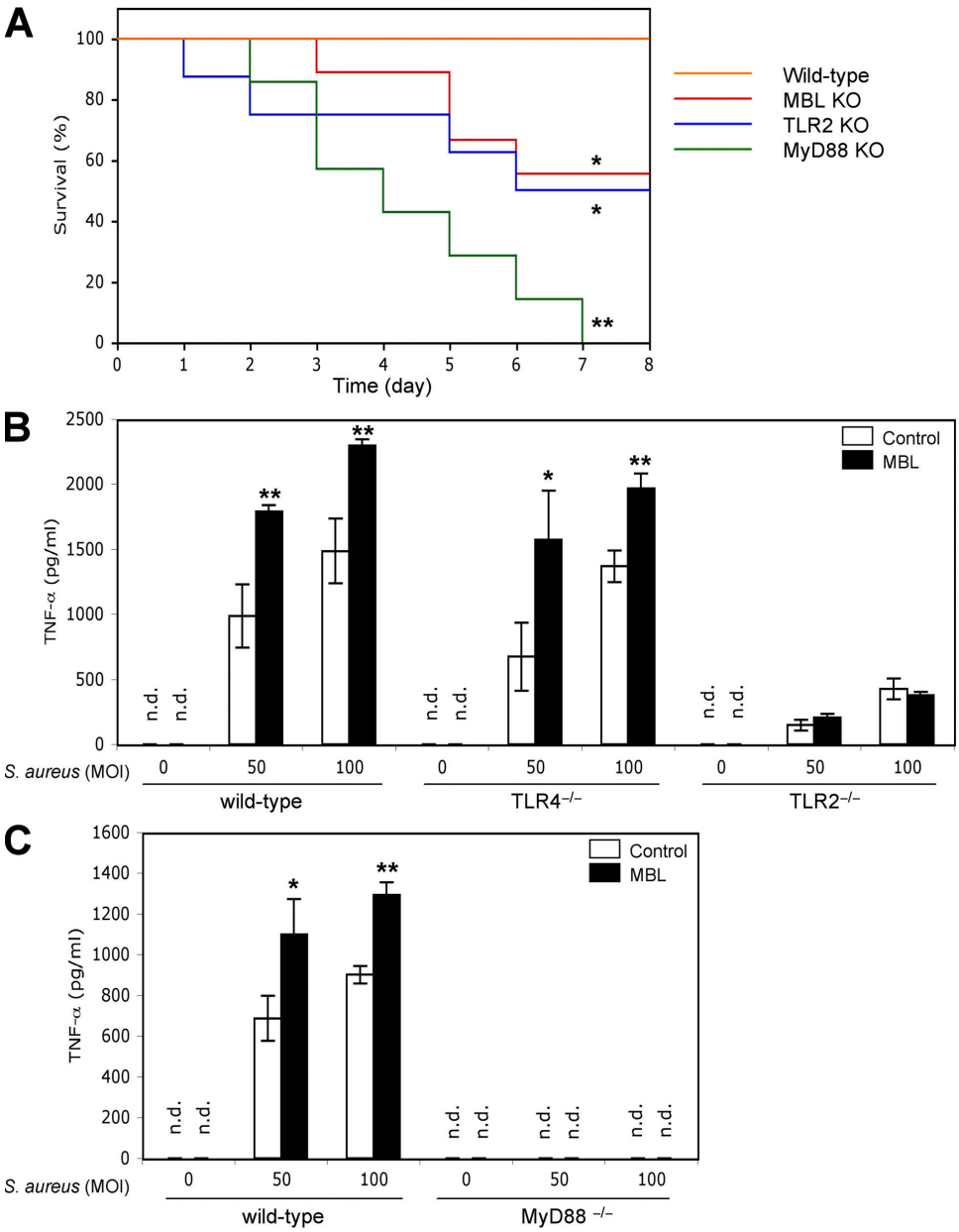

Figure 4. MBL up-regulates TLR2–MyD88–dependent response to *S. aureus*. (A) Survival of wild-type ($n = 8$), MBL$^{-/-}$ ($n = 9$), TLR2$^{-/-}$ ($n = 8$), and MyD88$^{-/-}$ ($n = 7$) mice on a C57BL/6 background after i.v. inoculation in the tail vein with 1.5×10^7 *S. aureus*. (B and C) In vitro TNF-α response to *S. aureus* opsonized with or without MBL in control (wild-type), TLR2$^{-/-}$, TLR4$^{-/-}$, or MyD88$^{-/-}$ macrophages. Peritoneal macrophages from TLR2$^{-/-}$, TLR4$^{-/-}$, or control B6129PF2/J mice (B), or MyD88$^{-/-}$ or control C57BL/6J mice (C), were cultured in the presence of heat-inactivated *S. aureus* (at the indicated MOIs), which were opsonized without (control) or with 10 μg/ml of MBL. Induction of TNF-α response at 2 h was measured by ELISA in culture supernatants. Data are representative of three independent experiments and indicate the mean ± SD of triplicates. *, $P \leq 0.05$; **, $P < 0.01$. n.d., no detectable cytokine.

MBL on the response to LTA. MBL was able to enhance the response to LTA only when immobilized on the surface of a particle (LTA-coated beads) but had no effect on soluble LTA (Fig. S7, available at http://www.jem.org/cgi/content/full/jem.20071164/DC1). Thus, MBL only augments signals to particulate TLR2/6 ligands that are delivered to phagosomes.

MBL complexes with TLR2 and modulates the signaling within phagosomes

The observed cooperation between MBL and TLR2/6 suggested a possible physical association between these molecules. To investigate this possibility, we first examined the localization of MBL and TLR2 during phagocytosis of heat-inactivated *S. aureus* using immunofluorescence microscopy.

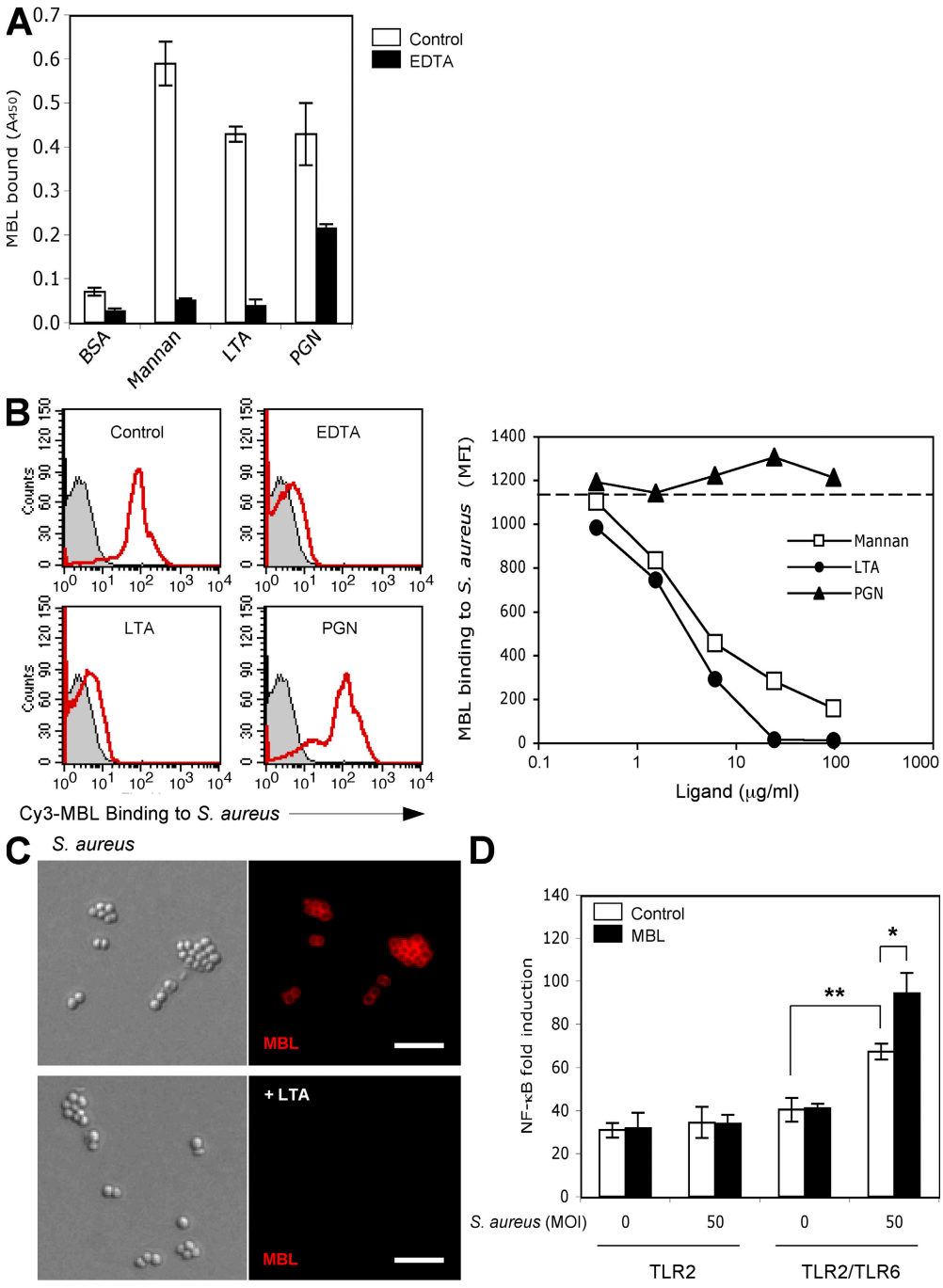

Figure 5. MBL recognizes *S. aureus* via binding to LTA and modules signaling from the TLR2/6 heterodimer. (A) MBL binding to LTA and PGN in solid phase. MBL was incubated at 10 μg/ml in microtiter plates coated with 100 μg/ml BSA, mannan, LTA, or PGN in the absence (control) or presence of EDTA for 2 h. MBL binding to the coated wells was determined by ELISA. Data are representative of at least three independent experiments and indicate the mean ± SD of triplicates. (B and C) Inhibition of MBL binding to *S. aureus* by LTA. Cy3-MBL at 10 μg/ml was pretreated without (control; B, left) or with 100 μg/ml LTA or PGN (B, left), or the indicated amount of mannan, LTA, or PGN (B, right), or 100 μg/ml LTA (C). Pretreated or nonpretreated Cy3-MBL at 10 μg/ml was incubated with heat-inactivated *S. aureus* in the presence or absence of EDTA for 30 min. Binding of Cy3-MBL (red) to the bacteria was analyzed by flow cytometry, where MBL binding was determined by MFI in the bacteria population (B), and fluorescence microscopy (C). The dashed line (B, right) indicates the binding to the bacteria by nonpretreated MBL. Bars, 5 μm. (D) NF-κB activation by *S. aureus* opsonized with or without MBL in HEK293 cells expressing TLR2 with or without TLR6. HEK293 cells stably expressing TLR2, cotransfected with the NF-κB reporter system, and with or without TLR6, were cultured in the presence of heat-inactivated *S. aureus* (MOI = 50), which were opsonized without (control) or with MBL at 10 μg/ml. Reporter gene activity at 4 h was measured by a luciferase assay system (see Materials and methods). Data are representative of two independent experiments and indicate the mean ± SD of triplicates. *, $P \leq 0.05$; **, $P < 0.01$.

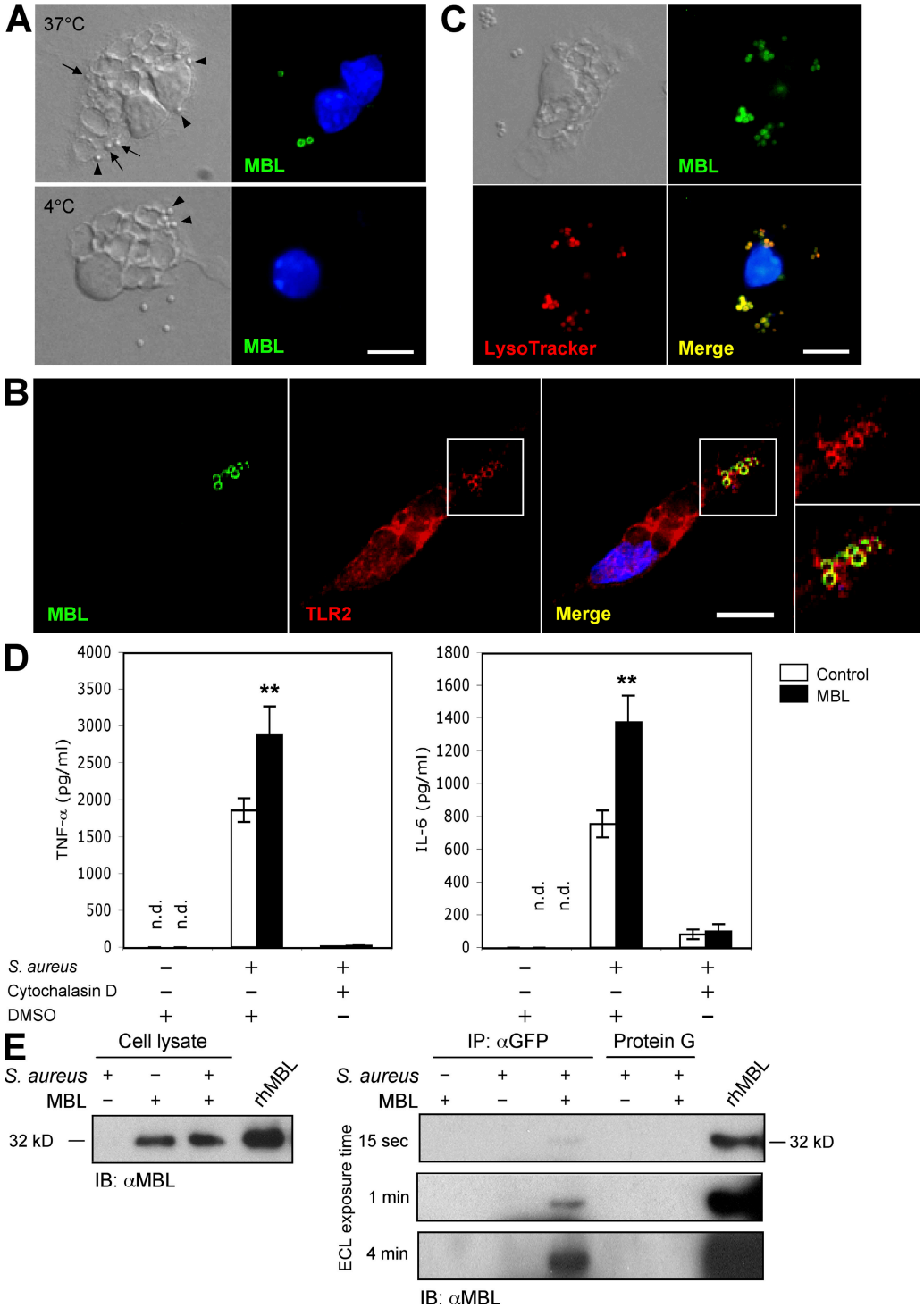

Figure 6. MBL traffics onto phagosomes and complexes with TLR2. (A–C) Localization of MBL on macrophage phagosomes. C57BL/6J peritoneal macrophages were incubated with Alexa Fluor 488–MBL–opsonized *S. aureus* for 10 min at 37 or 4°C (on ice; A), for 10 min at 37°C (B), or for 45 min at 37°C (C). Before intracellular staining for TLR2 or nuclei, cells were washed with EDTA to remove MBL from extracellular bacteria. Localization of MBL on phagosomes was shown by Alexa Fluor 488–MBL (green) on internalized bacteria (arrows), but not on noninternalized or cell surface–bound bacteria (arrowheads; A). Nuclei were stained with HOECHST (blue). Enrichment of TLR2 on phagosomes was shown by staining with anti-TLR2 (red), where TLR2s were colocalized with MBL in the merged image (B). (B, right) Images are magnifications of the insets. Localization of MBL on phagolysosome was shown in macrophages preloaded with LysoTracker (red; C). Data are representative of four independent experiments. Bars, 10 μm. (D) Requirement of phagocytosis for MBL-enhanced responses to *S. aureus*. C57BL/6J peritoneal macrophages were pretreated with 6 μM cytochalasin D or an equal volume of DMSO and incubated with heat-inactivated *S. aureus* (MOI = 100) opsonized without (control) or with 10 μg/ml MBL. Induction of cytokine responses at

TLR2- and Alexa Fluor 488–labeled MBL opsonized bacteria were both localized on the cell surface of macrophages during early phagocytosis (unpublished data). Previous work has demonstrated that TLR2 is recruited to macrophage phagosomes containing either zymosan or IgG-opsonized sheep red blood cells (33, 34), and similar to these observations, we also observed TLR2 enriched in *S. aureus* phagosomes. In addition, MBL trafficked into the early phagosomes (Fig. 6 A), where it colocalized with TLR2 (Fig. 6 B). MBL persisted within phagosomes for up to 45 min, at which time MBL could be colocalized with LysoTracker (an acidophilic dye that labels lysosomes), suggesting that MBL trafficked into mature phagolysosomes (Fig. 6 C). To further test if the colocalization between MBL and TLR2 in phagosomes correlates with their functional cooperation, we blocked bacterial engulfment in macrophages using cytochalasin D (Fig. S8 A, available at http://www.jem.org/cgi/content/full/jem.20071164/DC1) and measured cytokine responses (Fig. 6 D). Cytochalasin D inhibited *S. aureus*–induced TNF-α and IL-6 production and abrogated the ability of MBL to enhance cytokine release (Fig. 6 D). Importantly, these effects were not caused by adverse effects on cell viability, as production of TNF-α by macrophages in responses to LPS and zymosan was unaffected (Fig. S8 B). These data indicate that activation by *S. aureus* and, hence, the cooperation between MBL and TLR2 cannot occur on the cell surface but requires internalization.

To examine if the observed colocalization of MBL and TLR2 in the phagosome detected by immunofluorescence was caused by a physical association, we took advantage of the TLR2-HEK293T cells. HEK293T cells internalize *S. aureus* at low levels. TLR2-HEK293T cells were challenged with MBL-opsonized *S. aureus*, and using an anti-GFP antibody, TLR2 was immunoprecipitated and probed for association with MBL. MBL failed to bind to TLR2 when added alone, but when used to opsonize *S. aureus*, MBL could be detected as a coimmunoprecipitate with TLR2 (Fig. 6 E). These data support our microscopy data and suggest that *S. aureus* induces the association between MBL and TLR2 to form a complex within the phagosome.

Collectively, these data demonstrate an essential role of the phagosome in generating the signals required for induction of proinflammatory cytokine responses to *S. aureus*. These findings support a model in which *S. aureus* must be delivered into phagosomes to induce TLR2-mediated responses (Fig. S9, available at http://www.jem.org/cgi/content/full/jem.20071164/DC1). Furthermore, they suggest that modifiers of the TLR2 pathway such as MBL must also colocalize within this compartment to regulate the responses to bacterial-derived ligands (Fig. S9). Collectively, these data suggest that an important function of the phagosome is to provide an appropriate cellular compartment to concentrate ligands released from engulfed bacteria and coordinate signaling from different defense pathways.

DISCUSSION

The sensing of infectious agents is an essential component of first-line host defense (3). In this study, we confirm that MBL makes an important contribution to defining the host response after infection with *S. aureus* and demonstrate that this contribution occurs independently of its ability to increase uptake of *S. aureus* or activate complement. The model we suggest for the role of MBL in the host defense to *S. aureus* is based on the following observations. First, MBL and TLR2 make comparable contributions to host defense to *S. aureus* in vivo, and MBL is epistatic to TLR2–Myd88 signaling in vitro, suggesting that MBL-mediated microbial recognition and TLR2 sensing are functionally linked. Second, MBL binds to *S. aureus* via the known TLR2 ligand, LTA, and colocalizes and complexes with TLR2, potentially increasing ligand delivery and activation of the TLR2/6 signaling pathway. Finally, during phagocytosis of *S. aureus*, MBL traffics into early phagosomes, where it colocalizes with recruited TLR2. Intriguingly, when bacterial internalization is blocked, cytokine responses to *S. aureus* are abrogated, indicating the essential contribution of the phagosome to the activation of TLR2/6–MyD88–dependent signaling in response to *S. aureus*. These findings support a model in which MBL is delivered along with *S. aureus* into the phagosome, which provides the unique cellular environment required both for TLR2 signaling and for the augmentation by MBL. Collectively, these data describe a novel collaboration between MBL and TLR2/6 initiated upon engulfment of *S. aureus* (Fig. S9).

Although known as an opsonin, our data demonstrate conclusively that the ability of MBL to enhance the inflammatory response is not caused by increased phagocytosis. It is possible that complement activation, leading to the generation of C3 cleavage fragments or the formation of membrane attack complex, can facilitate enhanced signaling via complement receptors or presentation of bacterial ligands to TLRs. However, although an important function of MBL is to initiate

2 (TNF-α) or 4 (IL-6) h was measured by ELISA in culture supernatants. Data are representative of three independent experiments and indicate the mean ± SD of triplicates. *, $P \leq 0.05$; **, $P < 0.01$. n.d., no detectable cytokine. (E) Immunoprecipitation (IP) and immunoblot (IB) of lysates of HEK293T cells expressing TLR2, detecting association between MBL and TLR2. HEK293T cells stably expressing GFP-tagged TLR2 were transfected with TLR6 and were stimulated for 15 min with or without *S. aureus* (MOI = 50), which were opsonized with or without 10 μg/ml MBL. MBL was detected by the IB using anti-MBL antibody (αMBL) in the cell lysates (left) and in the immunoprecipitates of GFP-TLR2 with anti-GFP antibody (αGFP; right). 0.625 μg rhMBL was used as a control in the IB to indicate the size of a single subunit of the molecule. Proteins pulled down by protein G alone without αGFP were used as a control for the IP (see Materials and methods). The exposure time of enhanced chemiluminescence to develop the signals is indicated for the IB of the immunoprecipitates.

C3-dependent complement activation via the lectin pathway, C3 was not necessary to modify the inflammatory responses to *S. aureus*. These observations are consistent with our previous in vivo work using mice deficient in C3, MBL, and MBL/C3 that indicated that MBL has important effects in regulating responses to *S. aureus* independently of C3 (31). It remains to be addressed if MBL is involved in a C3-independent complement pathway such as that described recently (35). Furthermore, MBL modulation of cytokine response does appear to be relatively specific for *S. aureus*. We propose that this specificity is determined, in part, by the ability of MBL to bind LTA, a TLR ligand found within the cell wall of this Gram-positive organism, and facilitate its delivery to TLR2. Intriguingly, although two other collectins, SP-A and SP-D, also opsonize *S. aureus* (36–38), only SP-D modulated the response. MBL and SP-D, but not SP-A, share the ability to bind to LTA (39), and it is tempting to suggest that it is the ligand specificity of MBL and SP-D, and not their opsonic role, that determines the ability of these collectins to augment TLR signaling.

Previous studies by us and others demonstrated that CD36 is a coreceptor for *S. aureus* and *S. aureus*–derived LTA and facilitates signaling via the TLR2/6 heterodimer (14, 15), suggesting that the function of CD36 is analogous to that of CD14 in TLR4 activation. However, our observations show that MBL can enhance *S. aureus*–induced TNF-α production in macrophages from CD36-null mice (unpublished data), indicating that MBL can act independently of CD36. It is possible that MBL may operate via MBL receptors such as calreticulin/CD91(40), CD93 (C1q receptor for phagocytosis) (41), and/or CD35 (complement receptor 1) (42). An alternative scenario is that MBL acts independently of any specific receptor, and these two possibilities remain to be explored.

A final but surprising observation is the requirement of engulfment for response to *S. aureus*. TLRs are not believed to function directly as phagocytic receptors but are recruited to phagosomes (33, 34), suggesting an important role of this organelle for initiating signaling by certain TLRs. In this regard, it will be of interest to determine if other known coreceptors of TLR2, such as CD36, are also colocalized with TLR2 in phagosomes. In conclusion, the results demonstrated in this study identify the mechanisms by which MBL collaborates with other host defense pathways and receptors to both specify and amplify the immune response to *S. aureus*. This novel collaboration between MBL and TLR2/6 makes a significant contribution to the host defense networks initiated in response to *S. aureus* infection in vivo and illustrates the essential role of accessory proteins in directing pathogen-specific responses. It is tempting to compare the role of MBL during phagosome degradation of *S. aureus* with that of LBP. However, unlike LBP, which recognizes LPS and enhances its sensing by TLR4 at the cell surface (11), MBL clusters LTA and presents it to TLR2/6 only within phagosomes. Importantly, this cooperation occurs within the phagosome of macrophages, emphasizing the role of engulfment in providing the appropriate cellular environment to facilitate synergy between defense pathways.

MATERIALS AND METHODS

Mice and cells. Wild-type mice, C57BL/6J and B6129PF2/J mice, and complement receptor 3 (Mac-1) knockout mice were purchased from the Jackson Laboratory. C3 knockout mice were provided by M. Carroll (CBR Institute for Biomedical Research, Boston, MA), MyD88 knockout mice were provided by M. Freeman (Massachusetts General Hospital and Harvard Medical School, Boston, MA), and TLR2 and TLR4 knockout mice were provided by R. Medzhitov (Yale University School of Medicine, New Haven, CT). MBL-null (MBL-A and MBL-C double knockout) and MBL × C3–null (MBL-A/-C/C3 triple knockout) mice were generated in our laboratory as previously described (30, 31). CD36 knockout mice were generated by K.J. Moore, as previously described (43). Animals were kept and handled under a protocol approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. Thioglycollate-elicited peritoneal macrophages were collected from mice by peritoneal lavage 3 d after intraperitoneal injection of 3% thioglycollate (Difco Laboratories), and maintained in DMEM (Invitrogen) containing 10% heat-inactivated FCS (Invitrogen) and penicillin-streptomycin (50 IU/ml and 50 μg/ml; Cellgro). Jurkat T cells were used as a source of apoptotic cells that were generated by the treatment of the cells with 500 ng/ml actinomycin D for 24 h in serum-free RPMI 1640 medium (Invitrogen) containing 0.4% BSA (Sigma-Aldrich).

Bacteria and reagents. The strain of *S. aureus* used was Reynolds capsular serotype 5 (provided by J.C. Lee, Brigham and Women's Hospital, Boston, MA). *S. aureus* were cultivated overnight at 37°C in 5 ml of Columbia media (Difco Laboratories) with 2% NaCl. The bacterial culture was diluted 1:40 in fresh medium and grown to the mid-exponential phase (OD_{600} = 0.6; 2 h). The strains of group B *Streptococcus*, *E. coli*, and *S. montevideo* used were a clinical isolate GBS type III CHO-1, K12 (American Type Culture Collection) and SH5770 (provided by H. Mäkelä, National Public Health Institute, Helsinki, Finland), respectively, and were grown as described previously (17, 44). The bacteria grown to the mid-exponential phase were heat inactivated at 65°C for 30 min, washed once (10,000 *g* for 10 min), and stored in aliquots at −20°C before use for cell stimulation in vitro. PGN and LTA, both derived from *S. aureus*, were purchased from InvivoGen and Sigma-Aldrich, respectively. Mannan-coated latex beads were prepared by passive adsorption of mannan on the bead surface. Approximately 2×10^9 polystyrene latex beads (Sigma-Aldrich), 1.1 μm in diameter, were washed twice (10,000 *g* for 10 min) in 0.1 M of carbonate-bicarbonate buffer, pH 9.6, and incubated with 500 μg of mannan from *Saccharomyces cerevisiae* (Sigma-Aldrich) in 500 ml of the carbonate-bicarbonate buffer for 1 h at 37°C. The beads were then washed twice with HBSS (Invitrogen), incubated in 5% BSA for 2 h at 37°C to block nonspecific binding sites, washed again, resuspended in HBSS, and stored at 4°C before use. LTA-coated latex beads were prepared by covalent coupling of Polybead Carboxylate Microsphates (Polyscience), 3 μm in diameter, with LTA (Sigma-Aldrich) using PolyLink EDAC (Polyscience), according to the manufacturer's instructions.

***S. aureus* infection in vivo.** All mice were between 6 and 12 wk old and on a C57BL/6 background. Mice were inoculated i.v. in the tail vein with 200 μl of a mid-exponential growth phase of *S. aureus* (1.5 or 2×10^7 CFU) and monitored for survival as described previously (30, 31). In some experiments, blood was collected from wild-type and MBL-null mice at 2 h after inoculation with *S. aureus* or control saline. Blood protein array was performed using the membranes included in a mouse protein cytokine array kit (Mouse Cytokine Antibody Array II; RayBiotech), in which 1 ml of five-fold-diluted serum samples were incubated with the membranes at 4°C overnight. The bound proteins were detected according to the manufacturer's instructions. The signal intensities of the proteins were determined using National Institutes of Health image analyzer software (available at http://rsb.info.nih.gov/nih-image/).

Bacterial stimulation in vitro. Macrophages were plated at 4×10^5 cells per well in 24-well tissue culture plates (Costar; Corning) in the DMEM the day before each experiment. Before the start of the assays, the cells were washed twice with PBS (Invitrogen) and cultured in fresh DMEM with 1% FCS. Stimulation of macrophages by heat-inactivated bacteria was performed at different multiplicities of infection (MOIs) ranging from 25 to 400. Heat-inactivated bacteria (e.g., for MOI = 100, 4×10^7 bacteria to each well of macrophages) were opsonized for 1 h at room temperature with or without recombinant human MBL (provided by NatImmune A/S, Copenhagen, Denmark), or SP-A or SP-D (provided by S. Gardai, National Jewish Medical and Research Center, Denver, CO) in some cases, at the indicated concentrations in 50 μl HBSS, or opsonized with 50% wild-type serum, MBL-deficient serum, or MBL and C3 double-deficient serum in 50 μl HBSS (serum were obtained and pooled from two to three wild-type mice, MBL-null mice, or MBL × C3–null mice). Opsonization with BSA was used as a control in some experiments. Opsonized microorganisms were added to the macrophages, and the cells were incubated at 37°C in 5% CO_2 for 2–4 h, after which culture supernatants were taken. The measurements of TNF-α and IL-6 levels in the supernatants were performed by ELISA (DuoSet ELISA Development System; R&D Systems) in accordance with the manufacturer's protocol. In some cases, mannan-coated latex beads ($\sim 4 \times 10^7$ particles to each well of macrophages) or 1.2×10^6 apoptotic cells opsonized with or without MBL were used to stimulate the macrophages in the presence or absence of LPS. All experiments were repeated in triplicate at least three times.

Phagocytosis and detection of intracellular TNF-α. Macrophages plated at 10^6 cells per well in 12-well tissue culture plates in DMEM with 1% FCS were incubated with $\sim 2.5 \times 10^7$ heat-inactivated bacteria, labeled with tetramethyl-6-carboxyrhodamine (TAMRA; Invitrogen), and opsonized with or without 10 μl/ml of MBL, as described in the previous section, for 20 min on ice, allowing the synchronization of bacteria binding onto the cell. In all cases, before the opsonization and the incubation with macrophages, *S. aureus* clusters were disrupted by passing the bacteria through a 30-gauge needle. After 20 min on ice, the cells were further incubated for the times indicated in the figures at 37°C in the presence of GolgiStop (BD Biosciences) to accumulate intracellular TNF-α. The cells were washed twice with ice-cold PBS containing 5 mM EDTA (PBS/EDTA), detached with scrapers, and fixed in 3% paraformaldehyde. The cells were permeabilized and stained with allophycocyanin-conjugated anti–mouse TNF-α antibody (BD Biosciences) diluted in PBS with 0.2% saponin. After washing, the cells were analyzed by flow cytometry performed on FACSCalibur (Becton Dickinson); the analysis was performed with CellQuest Pro software (Becton Dickinson) to determine phagocytosis and intracellular TNF-α production at the single-cell level. GolgiStop alone did not stimulate TNF-α production (unpublished data). To estimate the number of bacteria engulfed by a single cell, TAMRA-labeled *S. aureus* used in the same experiment were also analyzed by flow cytometry to obtain the MFI of a single bacterial particle, which allowed to identify the cells that engulfed one- or twofold increasing numbers of bacteria and, therefore, to determine the means of intracellular TNF-α production in cells with the same bacterial loads.

MBL binding assays. MBL binding to bacterial cell wall components in solid phase was assessed by ELISA, as described previously (45). Flow cytometry was also used to measure MBL binding to the whole bacteria. In brief, Cy3-MBL (Cy 3 was obtained from GE Healthcare) at 10 μg/ml was preincubated with the indicated amounts of bacterial cell wall components (as shown in the figures) in liquid phase for 10 min at room temperature. Preincubated Cy3-MBL or Cy3-MBL without preincubation at 10 μg/ml was mixed with 2×10^7 heat-inactivated *S. aureus* in 100 μl of HBSS. The mixture was incubated for 30 min at room temperature. After washing once with HBSS (10,000 *g* for 2 min), the bacteria were analyzed for MBL binding by flow cytometry as described in the previous section. MBL binding was evaluated as MFI of the bacteria population. MBL binding to the bacteria was also analyzed by fluorescence microscopy, where the bacteria were cytospun onto microscope slides, and after mounting, images were captured as described in Immunofluorescence.

NF-κB–luciferase reporter assays. Dual luciferase reporter assays for NF-κB activation were performed in HEK293 cells that stably express TLR2 (provided by D. Golenbock, University of Massachusetts Medical School, Worcester, MA), as previously described (15). In brief, cells were transfected with NF-κB reporter construct and pcDNA3.1-TLR6 complementary DNA (cDNA) or mock pcDNA3.1 using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Before assays, the cells were washed with PBS and cultured in fresh DMEM with 1% FCS. Heat-inactivated *S. aureus* opsonized with or without MBL, as described in Bacterial stimulation in vitro, were added to the cells at an MOI of 50. Buffer alone with or without MBL was used as a control (MOI = 0). The cells were then incubated at 37°C in 5% CO_2 for 4 h and lysed, and reporter gene activity was measured using the Dual-Glo Luciferase Assay System (Promega) in accordance with the manufacturer's protocol. Data were normalized for transfection efficiency with the control reporter activity from the same sample and presented as the mean NF-κB fold induction of triplicate samples.

Immunofluorescence. Macrophages were plated at 8×10^5 cells per well on glass coverslips in sixwell tissue culture plates (Costar) and cultured overnight in the DMEM. Before incubation with heat-inactivated *S. aureus*, the cells were washed twice with PBS and cultured in fresh serum-free DMEM. In some experiments, 200 μM of LysoTracker Red DND-99 (Invitrogen), an acidophilic dye, was added to the cells for 30 min to label lysosomes. 8×10^6 heat-inactivated *S. aureus* were opsonized with 1 μg/ml of Alexa Fluor 488 (Invitrogen)–labeled recombinant human MBL (Alexa Fluor 488–rhMBL) in 50 μl HBSS for 30 min at room temperature. After incubation with opsonized bacteria for 10–45 min at 37°C or on ice, macrophages were washed twice with ice-cold PBS containing 5 mM EDTA (to remove Alexa Fluor 488–rhMBL from extracellular bacteria particles but not from those phagocytosed) and fixed in 3% paraformaldehyde. To detect TLR2, cells were permeabilized using 0.1% saporin in blocking buffer (HBSS containing 3% BSA, 0.2% gelatin, and 0.02% NaN_3) and stained with anti-TLR2 (clone H-175; Santa Cruz Biotechnology, Inc.) and Alexa Fluor 546–labeled goat anti–rabbit antibody (Invitrogen). HOECHST (Sigma-Aldrich) was used to stain nuclei. After mounting with Immu-Mount (Thermo Fisher Scientific), the cells were observed by fluorescent microscopy. Images were captured and analyzed using Openlab software (Improvision).

Immunoprecipitation and Western blotting. HEK293 cells that stably express GFP-tagged TLR2 were transfected with pcDNA3.1-TLR6 cDNA as described in NF-κB–luciferase reporter assays. Before stimulation, the cells were washed with PBS and cultured in fresh DMEM with 1% FCS. Heat-inactivated *S. aureus* opsonized with or without MBL in HBSS, as described in Bacterial stimulation in vitro, were added to the cells at an MOI of 50. HBSS alone with MBL was also used as a control. After incubation for 15 min at 37°C, cells were rinsed once in ice-cold PBS/EDTA and lysed with lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% NP-40, and 1× protease inhibitor cocktail [Complete Mini; Roche]). 500 μl of cell lysates at 2 mg/ml of protein concentration were precleared with 50 μl of 50% protein G–agarose–Sepharose beads (GE Healthcare) for 20 min at 4°C. The precleared lysates were then incubated without (control) or with 4 μg of mouse anti-GFP monoclonal antibody (clone 3E6; Invitrogen) for 2 h at 4°C, followed by incubation with 50 μl of the protein G beads overnight at 4°C. The beads were washed four times according to the manufacturer's protocol for using anti-GFP antibodies (Invitrogen) and then suspended in SDS sample buffer heated to 100°C for 3 min. The eluted GFP-TLR2 precipitates were applied to 12% SDS-PAGE and blotted to polyvinylidene fluoride membranes. MBLs were detected by immunoblotting using rabbit anti-MBL antibody (clone DB2) (46). Signals were developed using enhanced chemiluminescence (Millipore).

Inhibition assays. To assess the role of phagocytosis in the induction of TNF-α and IL-6 by heat-inactivated *S. aureus* in peritoneal macrophages, before the stimulation with heat-inactivated *S. aureus* (as described in Bacterial stimulation in vitro) cells were pretreated with 6 μM cytochalasin D

(Sigma-Aldrich) for 30 min to inhibit phagocytosis (47). Cells pretreated with the same volume of vehicle, DMSO, were used as a negative control for both inhibitors. The effect of these inhibitors in phagocytosis was also confirmed by the phagocytosis assays, as described in Phagocytosis and detection of intracellular TNF-α, in which cells were pretreated with the inhibitors before the incubation with the heat-inactivated bacteria.

Statistical analysis. A two-tailed and two-sample *t* test was performed with Excel (Microsoft) on triplicate ELISA measurements of cytokine levels to determine significance. Survival data were analyzed using log-rank and Wilcoxon tests using JMP software (version 5.0; SAS Institute Inc.).

Online supplemental material. Fig. S1 shows the protein array of blood cytokines in mice infected with *S. aureus*. Fig. S2 shows that MBL accelerates phagocytosis of *S. aureus*. Fig. S3 shows that MBL on apoptotic cells or latex beads is insufficient to induce TNF-α response. Fig. S4 shows that MBL has no effect on TNF-α response to group B *Streptococcus*, *E. coli*, or *S. montevideo*. Fig. S5 shows that SP-A enhances uptake but not TNF-α response to *S. aureus*. Fig. S6 shows that MBL enhances TNF-α response in macrophages with equivalent bacterial loads. Fig. S7 shows that MBL enhances the response to LTA only when immobilized on the surface of a particle (LTA-coated beads) but has no effect on soluble LTA. Fig. S8 shows that cytochalasin D blocks phagocytosis but has no adverse effects on production or secretion of TNF-α. Fig. S9 illustrates a model of MBL collaboration with TLR2/6 signaling. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20071164/DC1.

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