Deficiency of Mannose-Binding Lectin Greatly Increases Susceptibility to Postburn Infection with *Pseudomonas aeruginosa*


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Burn injury disrupts the mechanical and biological barrier that the skin presents against infection by symbionts like the *Pseudomonas aeruginosa*, a Gram-negative bacteria. A combination of local factors, antimicrobial peptides, and resident effector cells form the initial response to mechanical injury of the skin. This activity is followed by an inflammatory response that includes influx of phagocytes and serum factors, such as complement and mannose-binding lectin (MBL), which is a broad-spectrum pattern recognition molecule that plays a key role in innate immunity. A growing consensus from studies in humans and mice suggests that lack of MBL together with other comorbid factors predisposes the host to infection. In this study we examined whether MBL deficiency increases the risk of *P. aeruginosa* infection in a burned host. We found that both wild-type and MBL null mice were resistant to a 5% total body surface area burn alone or s.c. infection with *P. aeruginosa* alone. However, when mice were burned then inoculated s.c. with *P. aeruginosa* at the burn site, all MBL null mice died by 42 h from septicemia, whereas only one-third of wild-type mice succumbed ($p = 0.0005$). This result indicates that MBL plays a key role in containing and preventing a systemic spread of *P. aeruginosa* infection following burn injury and suggests that MBL deficiency in humans maybe a premorbid variable in the predisposition to infection in burn victims. *The Journal of Immunology*, 2006, 176: 1769–1775.

The successful restriction of early infection is achieved by a well-orchestrated cooperative action of a network that intertwines overlapping and circulating molecules and cell-associated receptors. These include TLRs, lectin-like receptors, serum antimicrobial molecules, natural Abs, complement proteins, and collectins that include mannose-binding lectin (MBL), also known as mannose-binding protein or mannan-binding lectin (1–3). MBL is a pattern recognition molecule that recognizes certain carbohydrate moieties common to certain Gram-positive and Gram-negative bacteria, fungi, viruses, protozoa, and apoptotic cells (4–9). MBL, upon recognition of infectious agents, activates complement via the Ab independent lectin pathway where the MBL-associated serine protease-2 cleaves C4 and C2 (10, 11). This action generates the C3 convertase, C4bC2a, which is a critical step in the production of the downstream complement effector molecules. The skin is the largest and most important organ of the innate immune system and provides a physical barrier consisting of epidermis and dermis. The former is packed with keratinocytes that make up a mechanical barrier whereas the latter contains capillaries and a variety of immune cells including Langerhans cells, dendritic cells, and macrophages. When skin is damaged by burn injuries the mechanical barrier is ruptured resulting in a complex interplay between pro- and anti-inflammatory responses that create an ideal milieu for bacterial invasion (12–17). The local inflammatory responses include a dynamic response by epithelial cells and keratinocytes, which modulates antimicrobial peptide gene expression (18) and orchestrate the diapedesis of neutrophils. These reactions appear to play a critical role in the local production of a variety of biologically active molecules, including proteases and metalloproteases that each play important roles in wound healing (19, 20). Other local inflammatory responses also increase endothelial cell permeability and leakage of plasma into tissue to form edema and blisters (21, 22). The edema provides a nutrient-rich environment for local bacterial growth and increases risk of bacterial escape into the blood stream leading to systemic infection (12, 14–17). We postulated that MBL, a serum protein, might play a key role as an innate immune molecule able to limit the systemic spread of locally infecting pathogens in burned tissue. This idea was informed by the knowledge that MBL is deployed quickly to sites of infection as has been shown in the lung (23) in addition to our previous work demonstrating that MBL null mice are highly susceptible to infection with *Staphylococcus aureus* (24) and HSV-2 (25).

In this study we investigated the roles of MBL against bacterial infection following a dry burn involving 5% of the total body surface area (TBSA). We chose *P. aeruginosa* because infection with this bacterium is a major cause of life-threatening complications in burn patients (16, 17, 26, 27). This opportunistic pathogen rarely infects immunocompetent hosts but exploits a weakened
immune system caused by burns, cancer, AIDS, or other immunocompromising conditions (28, 29). We demonstrate that mice lacking both MBL-A and MBL-C (MBL null mice) are highly susceptible to postburn infection with *P. aeruginosa*. MBL has not previously been studied in association with thermal injuries, and our data raise the possibility that MBL deficiency may be a risk factor in burn patients.

**Materials and Methods**

**Mice**

MBL null mice were generated by crossing MBL-A null and MBL-C null mice as previously described (24). The MBL null and wild-type (WT) mice were maintained on a mixed background of 129Sv × C57BL/6d. Age- and gender-matched mice (8–10 wk of age) were used in each experiment. All animal experiments were performed under a protocol approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital (Boston, MA).

**Burn model**

One day before the burn, the mice were anesthetized by i.p. injection of avertin (30 mg/kg; Sigma-Aldrich) to remove fur from the dorsal side with a hair removal lotion (Marzena). The following day the mice were anesthetized as described, and the hairless skinfold of the dorsal side was elevated. Two brass bars (1 cm² in cross-section) were heated in boiling water and applied for 10 s, thus providing a dry, nonlethal full-thickness burn injury corresponding to ~5% of TBSA. Fluid replacement therapy was administered immediately after the burn by i.p. injection of 1 ml of saline. The mice were placed on a heating pad (38°C) and kept under observation during the recovery period from anesthesia.

**P. aeruginosa infection model**

Bioluminescent *P. aeruginosa*, a modified strain 180 (no. 19660; American Type Culture Collection) (30), were cultured at 37°C in brain heart infusion media (Microbiology System; BD Diagnostic Systems) overnight, inoculated into fresh brain heart infusion media the following morning, and cultured for 3 h at 37°C to mid-log phase conditions. The bacteria were washed in saline and resuspended in saline to 2 × 10⁶/ml (OD₆₀₀ = 0.6, which corresponds to 1 × 10⁶ CFU/ml in mid-log phase). The actual inoculation dose was determined by plating serial dilutions of the inoculum on tryptic soy agar plates (Sigma-Aldrich). Mice were anesthetized as described and inoculated s.c. with 2 × 10⁵ *P. aeruginosa* in 100 µl per mouse at the middle of the burned skin. Survival was recorded subsequently.

**Imaging**

To record progression of the burn wound, digital photographs were taken immediately after the burn injury and serially every day. *P. aeruginosa* infection was recorded by the low light imaging system (Hamamatsu Photonics) that has been described elsewhere in detail (30). Briefly, it consists in an intensified charge-coupled device camera mounted in a light-tight housing with a high-definition grayscale image of the entire mouse to be captured. In the photon-counting mode, an image of the emitted light was captured at a maximum of 1080 frames per second. Bound MBL was detected by centrifugation at 7000 × g for 1 h followed by centrifugation at 3700 × g for 10 min to collect the supernatant. MBL null and wild-type mice in the WT mouse serum and the supernatant were quantified to determine total MBL-A and MBL-C and unbound MBL-A and MBL-C using assays previously described (31). MBL in the supernatant was determined to quantify unbound rhMBL as previously described (32). Bacterial binding was expressed as the percentage of bound MBL. We examined the susceptibility of MBL null mice in an animal model of *P. aeruginosa* postburn infection. All WT and MBL null mice survived from burn injury alone (observed up to 3 wk) without showing any sign of illness, such as weight loss and ruffled fur. Likewise all unburned WT and MBL null mice survived from *P. aeruginosa* infection alone (s.c. inoculation of 2 × 10⁵ CFU per mouse) with no sign of illness. However, when the mice were inoculated s.c. with *P. aeruginosa* (2 × 10⁵ CFU per mouse) at the site of burn injury, all MBL null mice died within 42 h, compared with only 30% of the WT mice (*p = 0.0005*) (Fig. 1). Reconstitution of MBL null mice with rhMBL reversed survival to 50%,
which was significantly improved (p = 0.03) compared with that of MBL null mice and was not different from that of WT mice (p = 0.24) (Fig. 1). This result indicated that the high mortality in MBL null mice was indeed due to the lack of MBL.

**MBL null mice have higher bacterial loads in blood and organs**

To investigate whether the high mortality was caused by increased infection we examined the state of infection by determining bacterial loads both locally and systemically. Bioluminescent imaging system recorded local infection. Images of infected skin at 20 h after the postburn inoculation. Importantly, we found that bacterial titer in the blood of MBL null mice was significantly higher (p = 0.004) than that of WT mice (Fig. 2). Likewise, significantly elevated bacterial titers were also observed in kidney and liver (p = 0.045 and 0.033, respectively) of MBL null mice compared with that of WT mice (Fig. 2). The bacterial loads in lungs of MBL null mice were also elevated compared with that of WT mice, though this difference was not statistically significant. The elevated bacterial titers were significantly reduced in blood and kidneys (p = 0.03 for both) when MBL null mice were reconstituted with rhMBL and were not different in blood and kidneys (p = 0.33 and p = 0.1, respectively) from that of WT mice (Fig. 2). In contrast to the systemic infection, two independent methods, the observation by bioluminescent imaging of labeled bacteria (data not shown) and the colony counts (Fig. 2), indicated that there was no difference in local bacterial proliferation in the skin between MBL null and WT mice. Taken together these data suggested that MBL plays a role in inhibiting systemic spread of infection and subsequent seeding of distal organs rather than limiting the local infection at the site of inoculation.

**Whole blood of MBL null mice failed to restrict growth of P. aeruginosa in vitro**

*P. aeruginosa* was incubated with whole blood or plasma from WT, MBL null mice, and MBL null mice that were reconstituted with rhMBL. Growth of *P. aeruginosa* was 2-fold higher in whole blood of MBL null mice compared with the growth in blood from WT mice after 2 h incubation (p = 0.005) (Fig. 3a). The low growth inhibition in whole blood of MBL null mice was restored when the mice were injected with rhMBL before blood collection (p = 0.005 between MBL null and MBL null with rhMBL) (Fig. 3a). We also tested this phenotype in whole blood from an MBL-deficient human volunteer. The growth of *P. aeruginosa* in MBL-deficient blood was 3-fold reduced upon reconstitution with rhMBL (p = 0.006) (Fig. 3a). There was no difference in growth of *P. aeruginosa* when bacteria were incubated with plasma from WT, MBL null, or the reconstituted MBL null mice (Fig. 3a). Likewise there was no difference in the bacterial growth when human MBL-deficient plasma was incubated with or without rhMBL (data not shown). These results indicate that soluble factors alone were ineffective in curtailing the infection, and that both MBL and phagocytes are required for the killing of *P. aeruginosa*.

As there are conflicting reports whether MBL binds this bacterium (34, 35), we re-examined the binding of MBL to *P. aeruginosa* by quantitative methods. Under our assay conditions, ~50% of the MBL in WT mouse serum as well as rhMBL that was mixed into MBL null mouse serum bound to *P. aeruginosa* (Fig. 3b). This result corresponded to 4 ng of MBL-A, 33 ng of MBL-C, and 15 ng of rhMBL that bound to 1 × 10⁵ bacteria, which represent ~200 molecules of MBL bound per bacterium. A maximum of 2000 molecules of MBL could bind when an increased amount of MBL was used in the reaction mixture. The MBL was quantitatively recovered in the supernatant when mannose was added to the incubation with bacteria (Fig. 3b). Binding of MBL to *P. aeruginosa* could also be observed when the bacteria were coated on microtiter wells and the binding was inhibited by mannose or EDTA (OD₄₁₅ rhMBL = 0.596, with mannose = 0.082, and with EDTA = 0.131, which was background level). Thus we show that MBLS do bind to *P. aeruginosa* in a mannose-specific and calcium-dependent manner. When MBL binding to *P. aeruginosa* was analyzed by FACs, no binding was detected in agreement with a previously published report (34), whereas binding to *S. aureus* was obviously detected under the same condition (Fig. 3c). These results suggest that FACs analysis is not sensitive to detect MBL binding to *P. aeruginosa*.
IL-6 and TNF-α levels in blood and organs during postburn infection

We then investigated inflammatory responses, as an overwhelming production of TNF-α and IL-6 has been associated with high morality (36–38). We determined levels of TNF-α and IL-6 in plasma and in homogenates of skin, liver, kidney, and lung at 20 h after burn and bacterial inoculation. IL-6 levels in plasma were significantly lower in MBL null mice (\(p < 0.004\)) compared with that of WT mice (Fig. 4). Similarly TNF-α was lower in lung (\(p = 0.05\)) and in skin (\(p = 0.05\)) of MBL null mice compared with that of WT mice (Fig. 4). The low TNF-α in lung and IL-6 in plasma were significantly restored when the mice were reconstituted with rhMBL (\(p = 0.08\) and 0.02, respectively) (Fig. 4), indicating that the differences can be ascribed to the presence or absence of MBL. In the liver the response was opposite as both IL-6 and TNF-α levels increased (\(p = 0.002\) and \(p = 0.04\), respectively) in MBL null mice compared with that of WT mice and the response was not reversed by reconstitution of mice with rhMBL (Fig. 4).

Reduced inflammatory response in MBL null mice following burn alone

We also examined the effect of burn alone on inflammatory responses locally and systemically in MBL null and WT mice. TNF-α and IL-6 were measured in skin homogenate and plasma from MBL null and WT mice following burn injury. No IL-6 was detected in plasma from either MBL null or WT mice but TNF-α in plasma was significantly lower (\(p = 0.01\)) in MBL null mice than that in WT mice at 20 h (Fig. 5). IL-6 could be detected in skin and was lower in MBL null mice compared with that of WT mice (\(p = 0.007\)), whereas there was no difference in TNF-α levels in skin between WT and MBL null mice (Fig. 5).

FIGURE 3. MBL-deficient blood fails to restrict growth of \(P.\) aeruginosa. a, Growth of \(P.\) aeruginosa was analyzed in ex vivo killing assay. Hirudin-treated whole blood or plasma from mice or whole blood from human donors was incubated with \(P.\) aeruginosa, and CFU per milliliter was determined at 20 min after the incubation at 37°C. Bars indicate mean ± SD of six results (three experiments in duplicates). **, \(p < 0.01\). One representative result of two repeated experiments is shown. b, \(P.\) aeruginosa express ligands for MBL binding. Binding of \(P.\) aeruginosa to human MBL and mouse MBL-A and MBL-C was determined using suspension phase binding assay as described in Materials and Methods. Result is presented as a percentage of bound MBL. Bars indicate mean ± SD of four binding experiments. c, Binding of Cy3-MBL to \(P.\) aeruginosa was examined by FACS. Shaded area and solid line indicate bacteria alone and binding of Cy3-MBL to bacteria, respectively. \(S.\) aureus was used as a positive control.

FIGURE 4. IL-6 and TNF-α levels following infection after burn. Levels of IL-6 and TNF-α were determined in plasma and in homogenates of skin, lung, and liver as described in Materials and Methods. Symbols represent individual mice. Two experiments were combined, and mice used were WT (\(n = 8\)), MBL null (\(n = 9\)), and reconstituted MBL null (\(n = 9\)) mice. Bars indicate mean. *, \(p < 0.05\); **, \(p < 0.01\).

FIGURE 5. Decreased cytokine levels in MBL null mice following burn injury. Levels of TNF-α and IL-6 were determined in plasma and skin homogenates at 20 h after burn as described in Materials and Methods. Symbols represent individual mice. Two experiments were combined, and WT (\(n = 6\)) and MBL null (\(n = 5\)) mice were used. Bars indicate mean. *, \(p < 0.05\); **, \(p < 0.01\).
Discussion

This study provides for the first time in vivo evidence that MBL null mice are highly susceptible to *P. aeruginosa* infection following burn injury. The biological consequences of burn injuries vary as a result of combinations of heat source, length of input, and percentages of TBSA exposed. In this study we used a 5% TBSA, nonlethal dry burn. Burn alone had no obvious general health effect on either WT or MBL null mice. In striking contrast, when burn was followed by infection, all MBL null mice died within 42 h as a result of increased bacteremia and invasive infection in organs, which was demonstrated by significantly higher bacterial loads in MBL null mice compared with WT mice (Figs. 1 and 2). This bacterial spread from the skin triggered by burn injury is analogous to bacterial translocation from the gut following thermal injury (39, 40). The susceptibility phenotype was reversed by administration of rhMBL. In contrast there was no difference in bacterial proliferation at the local site between WT and MBL null mice. Furthermore, in the absence of concomitant burn injury MBL null mice contained *P. aeruginosa* and did not develop systemic infection. These results are consistent with the role of MBL as a circulating pattern recognition molecule that is rapidly deployed to local sites of infection with the precise purpose to limit and prevent systemic spread of the infection.

The skin is the largest innate immune organ as well as a physical barrier against noxious stimuli (41). Burn injury disrupts normal homeostasis and this results in predisposition to infection with a variety of potential pathogens, such as *S. aureus* and *P. aeruginosa*. In recognition of the high probability of local and systemic infection following burn injury, current standards of care involve the use of systemic and topical antimicrobial agents (42). There is an increasing awareness that the heterogeneous responses to infectious challenge may reflect genetic heterogeneity in the innate or the adaptive immune genotype of the individual. Although MBL has not been studied in relation to burn injuries or infections postburn, the results of our in vivo study indicate that MBL plays an important role in the first line host defense against *P. aeruginosa* infection, the most common pathogen affecting burn patients. McManus and colleagues (43) found that 10% of all burn patients admitted to burn units developed *P. aeruginosa* bacteremia and 80% of those died. The MBL gene is known to have polymorphisms in the promoter region and in exon 1 that determine serum MBL levels, which vary from a few nanograms per milliliter to several micrograms per milliliter (44, 45). Since the first clinical study that correlated an MBL-dependent opsonic defect in serum with a phenotype of recurrent infections (46), such patients were found to have one of three amino acid substitutions due to single nucleotide polymorphisms in exon 1 of the MBL gene that disrupt the collagen helix (44). More detailed analysis of the MBL gene revealed seven distinct MBL haplotypes in humans, four of which (LYPB, LYQC, HYPD, and LXPA) dictate low serum levels (45, 47). MBL deficiency, defined as <100 ng of MBL per milliliter in plasma, is very common in some ethnicities from 15% of the Caucasian population to as high as 30% in certain African populations (45, 47–50). Compelling clinical studies have suggested that MBL deficiency was associated with severe infections following chemotherapy, postoperative, and after stem cell transplantations (51–55). Other patient populations did not show such a relationship (56–58) and thus it appears that the effect of MBL deficiency is revealed only under certain clinical conditions. In our studies aimed at clarifying the role of MBL, we have found that MBL null mice are susceptible to infection with *S. aureus* (24) and HSV-2 (25). The present study adds *P. aeruginosa* to the list of pathogens that show increased infectivity in MBL-deficient animals.

MBL therefore appears to have a key role in the context of the acute inflammatory response, which includes the extravasation of serum and effector cells like neutrophils and monocytes. This idea is supported by our findings that MBL-dependent killing required serum factors as well as phagocytes. In this regard we did not observe a difference in the killing when bacteria were incubated in plasma alone from WT, MBL null mice, or MBL-deficient human plasma that was mixed with rhMBL (Fig. 3a), suggesting that MBL-mediated complement activation alone is not sufficient to restrict the growth of *P. aeruginosa*. By contrast, whole blood that contained effector cells of WT mice inhibited bacterial growth as compared with blood that contained MBL null mice effector cells (Fig. 3a). The inhibition of bacterial growth observed ex vivo in whole blood of MBL null mice was rescued by reconstitution with rhMBL (Fig. 3a). Likewise, MBL-deficient human blood efficiently inhibited bacterial growth when rhMBL was added (Fig. 3a). These results are consistent with previous work that demonstrated the importance of opsonophagocytosis in the effector arm of MBL against *S. aureus* (24, 59), and show that this mechanism is also operative against *P. aeruginosa*.

These results led us to examine whether MBL indeed bound to these bacteria because there had been no consensus as to whether MBL binds to *P. aeruginosa*. Neth et al. (34) failed to detect MBL binding to *P. aeruginosa* by FACS analysis. In contrast, Kuipers et al. (35) demonstrated MBL-dependent complement activation by *P. aeruginosa* in their assay using innocent bystander lysis of chicken erythrocytes. In this study, we reconcile these differences by reporting results that MBL binding to *P. aeruginosa* was demonstrated via both the suspension phase binding assay and the solid phase ELISA type assay (Fig. 3b), although it was not the case by FACS analysis (Fig. 3c). Importantly, the binding was via the carbohydrate recognition domain of MBL as it was inhibitable by excess mannose and required calcium as was not observed in the presence of EDTA (60, 61).

Innate immune surveillance mechanisms in the skin involve keratinocytes and Langerhans cells in the epidermis as well as dermal fibroblasts, mast cells, dendritic cells, and macrophages that are repositories for a vast array of stored and inducible molecules that include antimicrobial peptides, chemokines, cytokines, and cytokine antagonists (62). Recognizing the complexity of the innate immune response to burn injury and subsequent infection, we decided to measure the local and systemic production of two key cytokines IL-6 and TNF-α as the levels of these two cytokines correlated with outcome in murine burn models and clinical studies (36–38). Indeed, both cytokines were detected in the skin following burn alone, although we did not identify the cellular source. IL-6 was significantly lower in MBL null mice compared with WT mice, whereas there was no difference in TNF-α (Fig. 5). In contrast, systemic TNF-α was significantly lower in MBL null mice whereas IL-6 could not be detected systemically in either WT or MBL null mice (Fig. 5). The failure of MBL null mice to produce systemic TNF-α by burn injury alone is of considerable interest as it suggests that MBL is a modulator of inflammation. In this regard it will be interesting to define whether MBL interacts with TLRs that play a key role in NF-κB-dependent secretion of TNF-α. It is clear from clinical studies with TNF-α antagonists and in TNF null mice that TNF-α plays a key role in host defense, thus the sluggish TNF-α response in MBL null mice is likely to contribute toward the susceptibility of these mice to infection (24, 63, 64).

Unlike burn injury alone the combination of burn injury and infection induced significantly higher levels of plasma IL-6 in WT mice compared with MBL null mice (Fig. 4). Similarly TNF-α was lower in the lung and the skin of MBL null mice following burn and infection (Fig. 4). In contrast, both IL-6 and TNF-α were
higher in the liver of MBL null mice than the liver in WT mice suggesting that cytokine responses differ depending on the organ. Of note, the reduced cytokine responses following the postburn infection could be reversed to WT levels by the reconstitution of MBL null mice with rhMBL, however, elevated cytokine responses in liver was not reversed (Fig. 4). Taken together, these results suggest that MBL modulates inflammatory responses not only to infectious challenge but also to sterile burn injury and that organ dependent mechanisms may be involved with the responses. MBL has a proinflammatory response when it discerns the fine molecular and macro molecular pattern of carbohydrates decorating the surface of pathogens (2). However MBL also recognizes altered-self in the context of necrotic and apoptotic cells (6–8). In this context MBL is reported to induce an anti-inflammatory response (6). MBL is a member of a group of molecules called collectins that include the lung collectins, surfactant protein-A and surfactant protein-D. Surfactant protein-A and -D have been described to have an anti-inflammatory role in the lung. However, under certain circumstances when the lung is infected, they may induce proinflammatory responses (65). The downstream signaling required to regulate these opposing responses is not well understood but is a focus of intense investigation.

In this study, we provide in vivo evidence that MBL is important in curtailing the systemic expansion of *P. aeruginosa* infection following burn injury. As the human MBL gene is polymorphic and certain haplotypes have been associated with increased risk to infection, we postulate that MBL deficiency may be an important risk factor among burn patients in susceptibility to systemic infection. Clinical studies in burn patients would be a natural extension of this work. Furthermore, the high incidence of carbapenem-resistant *P. aeruginosa* in burn patients (66) suggests that MBL therapy could be an important adjuvant in the antimicrobial armamentarium for this patient population.

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