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A Quantitative Trait Loci Analysis to Map Genes Involved in Lipopolysaccharide-Induced Inflammatory Response: Identification of Macrophage Scavenger Receptor 1 as a Candidate Gene

William B. Fulton,* Roger H. Reeves,† Motohiro Takeya,‡ and Antonio De Maio2*†

Septic shock, which is a major complication observed after trauma and other human diseases, is likely the product of a prolonged and poorly controlled systemic inflammatory response. Symptoms of sepsis can be partially reproduced by injection of bacterial LPS in mice. Differences in mortality between C57BL/6J and A/J mice after LPS injection have been previously observed and correlated with differences in the inflammatory response between these two inbred strains. In the present study, we have mapped four loci responsible for differences in levels of LPS-induced IL-10, named modifier of IL-10, between the two strains. A locus within mouse chromosome 8 was confirmed using chromosome 8 consomic mice. This locus was further reduced in size by haplotype analysis and evaluated by the presence of potential candidate genes. The macrophage scavenger receptor 1 (Msr1) within this locus emerged as a candidate gene based on differences at the expression and structural levels between C57BL/6J and A/J mice. In comparison with wild-type (C57BL/6J) mice, Msr1 knockout mice displayed reduced levels of LPS-induced IL-10, but not of TNF-α or IL-6, confirming a specific role for this gene in the regulation of IL-10. These results suggest that Msr1 is involved in the regulation of the anti-inflammatory process, thus offering a new perspective on the molecular mechanisms involved in endotoxemia and sepsis. The Journal of Immunology, 2006, 176: 3767–3773.
Using an intercross strategy, we previously identified loci on mouse chromosomes (Chr) 13 and 5 showing an epistatic relationship for the infiltration of polymorphonuclear leukocytes in the liver after LPS challenge (7). In the present study, we mapped several loci for plasma levels of IL-10 as part of the inflammatory response induced by LPS. A candidate gene associated with LPS-induced IL-10 plasma levels was identified using a combination of genetic mapping and informatics.

Materials and Methods

Experimental animal model of endotoxemia

Male mice (A/J, AKR/J, BALB/cJ, B6, DBA/2J, B6AF1/J (BXA), AXB/BXA recombinant inbred, B6-Chr 8°/NaJ, and B6-Chr 9°/NaJ) were obtained from The Jackson Laboratory. AB6F1/J (AXB) mice were bred in our animal facility, and macrophage scavenger receptor (Msr1) knockout mice were donated by Dr. M. Freeman (Massachusetts General Hospital, Boston, MA). Mice were maintained under identical environmental conditions in a pathogen-free animal facility. All mice used in this study were *Helicobacter* negative. All procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University School of Medicine. At 8 wk of age, animals were fasted for 16 h before injection of *E. coli* LPS serotype 026:B6 (15 mg/kg; Difco Laboratories) under aseptic conditions. After injection, animals were given food and water ad libitum. At 1.5 h postinjection, mice were sacrificed under isoflurane anesthesia; blood was drawn by cardiac puncture using a 22-gauge needle, placed into K2EDTA, and centrifuged for 1 h. Nonadherent cells were washed off, and PM were isolated as described above, washed twice with PBS, and centrifuged for 5 min. Plasma (supernatant) was removed, aliquoted, and frozen at −80°C until use. Plasma cytokine levels were measured by ELISA (BioSource International).

**Linkage analysis**

Linkage analysis was performed using MapManager QTbX20 (10). IL-10 plasma levels were averaged from five animals, log-transformed, and entered as QTL. Mean values of IL-10 were weighted by SE (log-transformed) and compared against the AXB/BXA set for QTL mapping (11) that was corrected based on updated genomic mapping. Suggestive, significant, and highly significant threshold levels were determined by the permutation test function of MapManager, which is based on the statistical methods developed by Churchill and Doerge (12). A likelihood ratio statistic (LRS) value of 4.6 is equivalent to one logit of the odds (LOD). A two-LOD support interval was defined for loci as the region at which the LRS value was within 9.2 U (2 LOD U) of the peak value.

**Isolation of mouse peritoneal macrophages (PMΦ)**

Mice were euthanized by cervical dislocation under isoflurane anesthesia, injected with 5 ml of cold RPMI 1640 medium supplemented with 1 U/ml heparin. The medium was gently flushed five times within the peritoneum and withdrawn. Cells were centrifuged and resuspended at a concentration of 1 × 10^6 cells/ml in RPMI 1640 medium (Mediatech Cellgro), supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 50 μM penicillin, 50 μg/ml streptomycin (Invitrogen Life Technologies), plated in 10-cm² tissue culture dishes, and maintained at 37°C in 5% CO₂, 95% air for 1 h. Nonadherent cells were washed off, and PMΦ were collected and resuspended with cold RIPA buffer (20 mM HEPES (pH 7.4), 1% Nonidet P-40, 50 mM NaCl, 1 mM EGTA, 5 mM β-glycerophosphate, 30 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 0.1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A). The lysate was centrifuged at 2000 × g for 2 min and the supernatant was used for SDS-PAGE and Western blotting. Proteins immobilized on a polyvinylidene difluoride (PVDF) membrane were quenched for 1 h with TBS-TBST (20 mM Tris, 500 mM NaCl, 0.1% Tween 20) containing 5% powdered milk, and incubated with anti-mouse Msr1 (1/5000) for 16 h at 4°C. This Ab was raised in rabbit against a synthetic peptide (KEEEQAHVEEQVKQEQVR) corresponding to the α helical coiled-coiled domain of mouse Msr1 common to various mouse strains (13). Membranes were washed three times with TBST and incubated with HRP-conjugated anti-rabbit-IgG (1/2500; Amersham Pharmacia Biotech) in blocking buffer for 1 h. Membranes were washed again with TBST and the signal was visualized using Super Signal West Dura (Pierce Biotechnology).

**Immunostaining**

PMΦ were fixed with 4% paraformaldehyde for 10 min, then washed twice with PBS. Non-specific binding was blocked by incubation with 10% FCS in PBS for 30 min at 25°C. Fixed cells were incubated with anti-mouse Msr1 (1/200) in PBS-5% FCS for 1 h at 25°C. Cells were washed three times with PBS and incubated with anti-rabbit IgG Cy3 Conjugate (Fab′, 1/1000; Sigma-Aldrich) in PBS-5% FCS for 40 min at 25°C. Cells were washed three times with PBS, then mounted and visualized with a fluorescent microscope.

**FACS analysis**

PMΦ were isolated as described above, washed twice with PBS, and resuspended in PBS containing 5% FCS and 1 mg NaN₃. Cells were incubated at 25°C with anti-mouse Msr1 (1 μg/ml × 10⁶ cells) for 1 h and washed twice with PBS. Cells were resuspended in PBS containing 5% FCS and 1 mm NaN₃ and incubated with anti-rabbit IgG FITC Conjugate (Fab′, 1/1000; Sigma-Aldrich) for 40 min at 25°C. Cells were then washed twice with PBS, resuspended in PBS-EDTA, and analyzed by flow cytometry.

**Data analysis**

Statistical significance was determined by one-way ANOVA with Dunn’s correction or ANOVA on Ranks with Dunn’s correction or Student-Newman-Keuls method and Mann-Whitney *U* rank sum test or the Student *t* test; *p* < 0.05 was considered significant. Data are expressed as mean ± SEM.

**Results**

Several QTLs for LPS-induced IL-10 plasma levels were identified using recombinant inbred mouse strains

We previously showed that the frequency of mortality of C57BL/6j (B6) mice was higher than that of A/J mice after injection with LPS. This difference in mortality was correlated with differences in the inflammatory response between the two strains (6). Because cytokines are excellent markers of the inflammatory process, we evaluated the plasma levels of IL-10 in A/J and B6 mice 1.5 h after LPS injection. This time point corresponds to the maximal detectable levels of this cytokine after administration of LPS. Significantly higher levels of LPS-induced IL-10 plasma levels were observed in B6 in comparison with A/J mice (Fig. 1). This analysis was performed several times over a period of a year to ensure statistical significance.

**FIGURE 1.** IL-10 plasma levels following LPS injection in A/J, B6, and AxB generation. Male A/J (n = 15), B6 (n = 17), AxB (n = 15), and BxA (n = 15) mice were fasted for 16 h and injected i.p. with LPS (15 mg/kg). Blood was drawn via cardiac puncture 1.5 h after the injection, and IL-10 levels were measured in plasma by ELISA. IL-10 plasma levels in B6, AxB, and BxA mice were significantly higher than in A/J mice (*p* < 0.05 by ANOVA on Ranks, Dunn’s correction). Each group represents three or more experiments to account for seasonal variation.
overcome any potential seasonal variability in LPS-induced cytokine levels. The difference in response between B6 and A/J mice was observed regardless of any seasonal variability. Analysis of the first generation (F1) between B6 and A/J mice (AXB/BXA) showed that LPS-induced IL-10 levels were similar to those of B6 mice, suggesting that this trait is neither sex-linked nor imprinted and is likely B6 dominant. Recombinant inbred (RI) mouse strains were used to map the loci contributing to these phenotypes. Male mice from the AXB/BXA set (26 RI strains) were injected with LPS and levels of IL-10 were measured (Fig. 2). The log-transformed data, weighted for variance, was analyzed by MapManager QTXb20. Several contributing loci, named modifier of IL-10 (Milt), were obtained for the IL-10 plasma level phenotype. Two loci, one on Chr 8 (Milt3) and the other on Chr 13 (Milt4), displayed significant linkages, whereas loci on Chr 2 (Milt1) and 4 (Milt2) exhibited suggestive linkages (Table I).

We focused on the two loci that displayed significant QTL (Milt3 and Milt4). Consomic mice were used to confirm these loci for Chr 8 (B6-Chr8A/NaJ) and Chr 13 (B6-Chr13A/NaJ), respectively. These consomic mouse strains contained the chromosome of interest from A/J and all remaining chromosomes from B6 mice (14). Observed LPS-induced IL-10 plasma levels in B6-Chr8A/NaJ mice were very similar to those of A/J mice (Fig. 3), whereas B6-Chr13A/NaJ behaved as B6 mice (data not shown). An unrelated consomic mouse strain, B6-Chr9A/NaJ, was also shown to display a response similar to B6 mice. These observations confirm the influence of a gene within Milt3 on levels of LPS-induced IL-10. In contrast, Milt4 may have an epistatic relationship with other genes.

Refinement of Milt3 position by haplotype/single nucleotide polymorphism analysis

Based on the consomic mouse results, Milt3 was further refined because this locus can be analyzed independently of other alleles.

The number of plausible candidate genes contained within the 95% confidence interval around this locus (40 cM) is very large, minimizing the chance for successful systematic analysis. Consequently, we attempted to reduce the number of candidate genes by using a haplotype comparison among inbred mouse strains (http://snp.gnf.org) (15). We analyzed the IL-10 response in inbred strains (AKR/J, BALBc/J, DBA/2J, 129/SvImJ(129/SJ)) that shared haplotype information on Chr 8 with A/J but differed from B6 mice (15). LPS-induced IL-10 plasma levels were similar among AKR/J, BALBc/J, DBA/2J, 129/SJ with respect to A/J mice and significantly lower than B6 mice (Fig. 4A). Based on the haplotype comparison among "A/J-like" strains, we were able to reduce the size of Milt3 from 40 cM to ~24 cM, decreasing the number of potential candidate genes from ~160 to 68 (Fig. 4B).

Msr1 as a candidate gene

Evaluation of possible candidate genes within the reduced 24 cM region on Milt3 indicated only five genes with known polymorphisms between A/J and B6: Msr1, Rasl5–2, Nat1, Lpl, and Ucp1. Msr1 (SR-A) was chosen as the primary candidate gene for several reasons. Msr1 encodes for a cell surface glycoprotein that is specifically expressed in macrophages (16). These cells are central to the activation and regulation of the inflammatory response. Msr1 is apparently involved in the clearance of numerous substances, including LPS, pathogens, and apoptotic cells (17). Polymorphisms within the coding region of Msr1 have already been identified between B6 and A/J mice, although no functional differences were previously attributed to these allelic variants (18). To investigate the possible role of Msr1 in the differential response to LPS, Msr1−/− mice bred onto a B6 background for at least six generations (provided by Dr. M. Freeman; Ref. 19) were injected with LPS, and IL-10 plasma levels were measured 1.5 h post-injection. LPS-induced IL-10 plasma levels were reduced by ~50%

### Table I. QTLs that modify the LPS-induced IL-10 response

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chr</th>
<th>Support Interval (2-LOD)*</th>
<th>Relative Size of Support Interval (cM)</th>
<th>Significance/LRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milt1</td>
<td>2</td>
<td>D2mit31-Iaps2–3</td>
<td>3</td>
<td>Suggestive 16.6</td>
</tr>
<tr>
<td>Milt2</td>
<td>4</td>
<td>D4mit31-D4mit251</td>
<td>14.7</td>
<td>Suggestive 14.9</td>
</tr>
<tr>
<td>Milt3</td>
<td>8</td>
<td>Telomere-D8mit45</td>
<td>37</td>
<td>Significant 20.8</td>
</tr>
<tr>
<td>Milt4</td>
<td>13</td>
<td>D13mit122-D13mit11</td>
<td>4</td>
<td>Significant 25.7</td>
</tr>
</tbody>
</table>

*Interval mapping for modifiers of IL-10 trait (Milt) QTL were obtained from Map Manager QTX analysis of the different RI strains. One LOD is equivalent to a 4.6 LRS, and 2-LOD support interval is within 9.2 U of the peak LRS value.
in the knockout mice compared with B6 mice (wild type). Moreover, LPS-induced IL-10 levels in the null mice were similar to those observed in A/J mice (Fig. 5A). In contrast, LPS-induced TNF-α and IL-6 plasma levels were similar between Msr1⁻/⁻ and B6 mice (Fig. 5, B and C). These results specifically segregate the effect of Msr1 on LPS-induced IL-10 from that on other cytokines.

The expression of Msr1 is different between B6 and A/J mice

We compared the presence of Msr1 in isolated naive PMΦ from A/J and B6 mice. Western blot analyses of protein lysates from PMΦ isolated from these mouse strains were performed using Abs specific for mouse Msr1 that did not differentiate between genetic variants (13). Higher levels of Msr1 were consistently observed in B6 with respect to A/J-isolated PMΦ (1.6-fold, *p < 0.005; Fig. 6A). This observation was confirmed to be involved in the regulation of LPS-induced IL-10 from a portion of the Msr1 region compared with B6 mice (wild type). More specifically, LPS-induced IL-10 plasma levels. IL-10 is an important cytokine in the inflammatory response (24–26). The expression of Msr1 in cells involved in the inflammatory process (i.e., macrophages). In addition, previous studies have suggested that Msr1 plays a role in the inflammatory response (24–26).

Discussion

We used QTL mapping in RI mouse strains as a direct approach to the identification of modifier genes of the inflammatory process, in particular, LPS-induced IL-10 plasma levels. IL-10 is an important component of the regulation of the inflammatory response. Four loci were identified by this approach, with only two, Milt3 (Chr 8) and Milt4 (Chr 13), displaying statistically significant levels. Milt3 was confirmed to be involved in the regulation of LPS-induced IL-10 levels by using recently developed consomic mice (14). In contrast, Milt4 could not be confirmed using the respective consomic mice, probably because it is involved in an epistatic relationship with other genes. Previous studies have mapped QTL to the same region on mouse Chr 8 for susceptibility to arthritis (20), colitis (21, 22), and experimental allergic encephalomyelitis (23). It is likely that these diseases share an inflammatory component with endotoxemia. The boundaries of Milt3 were delimited based on SNP haplotypes (http://snp.gnf.org); Ref. 15) and only five genes within this region were found to display polymorphisms (www.informatics.jax.org) between A/J and B6 mice. Of these, Msr1 was selected as a candidate gene because it is specifically expressed in cells involved in the inflammatory process (i.e., macrophages). In addition, previous studies have suggested that Msr1 plays a role in the inflammatory response (24–26).
The involvement of Msr1 in LPS-induced IL-10 plasma levels was sustained by using homozygote knockout mice, which display LPS-induced IL-10 levels similar to A/J and different from B6 (wild-type) mice. These knockout mice were generated using 129-derived ES cells (A3-1), and were backcrossed to B6 for at least six generations, providing a 99.2% B6 genomic background. Although we cannot disregard the possibility that other genes within the congenic area are involved in the regulation of LPS-induced IL-10 levels, the potential contribution of genes within this 129 congenic region is less likely because 129 mice showed a different LPS-induced cytokine profile (TNF-α and IL-6) than B6, A/J, and Msr1−/− mice.

MSR1 is an integral plasma membrane glycoprotein composed of three identical chains forming a collagenase-like structure. This glycoprotein is involved in the clearance of polyanionic macromolecules, including acetylated or oxidized low density lipoprotein, lipoteichoic acid, lipopolysaccharides, polynucleotides, and...
sulfated polysaccharides (17). In addition, MSR1 has been implicated in cell adhesion (27, 28) and the clearance of bacteria (24) and apoptotic cells (29). MSR1 is almost exclusively expressed on macrophages (16), which are the primary mediators of the inflammatory process. Polymorphisms within the Msr1 coding region have been reported between B6 and many other mouse strains, including A/J (18). We found that one of these polymorphisms resulted in a different number of N-glycosylation sites between the B6 and A/J variants, which may modify the function of this receptor. For example, an extra glycosylation site could affect the affinity of MSR1 for one or more of its several ligands or change the stability of the protein. The number of glycosylation sites observed in the B6 variant is conserved within other species, including humans (18). In addition, the content of MSR1 is higher in B6-derived PMΦ than in A/J cells. Thus, the IL-10 hyperresponse observed in B6 may be related to elevated abundance of MSR1. This explanation is consistent with decreased LPS-induced IL-10 levels in the knockout mice.

We have correlated a hyperinflammatory response, in particular IL-10 levels, with an increase in mortality of B6 mice after endotoxic (6) and septic shock (8, 9), respectively. Other investigations have shown a correlation between overexpression of IL-10 and a detrimental outcome after infection (30). However, exogenous administration of IL-10 has been shown to be protective against LPS by down-regulating proinflammatory cytokines (3, 4). Kobayashi et al. (25) showed that the deletion of Msr1 resulted in protection from endotoxic shock, which was correlated with direct binding to LPS. This observation is consistent with the decrease in IL-10 levels observed in Msr1-deficient mice with respect to B6 wild-type mice. In contrast, Haworth et al. (31) showed that Msr1 knockout mice were more resistant to LPS after they were primed with bacillus Calmette-Guérin. The discrepancy between the studies could stem from the fact that the insults used were not identical, and that the genetic backgrounds of the null mice used in the two studies differed. In addition, these knockout mice have mixed genetic backgrounds of 129 and ICR due to a limited backcrossing strategy. In contrast, we used knockout mice that had been back-crossed to B6 mice for six generations, resulting in a more homogeneous genetic background (19). Moreover, we did not find a correlation between TNF-α and IL-10 and Msr1, as proposed by others (31, 32). We are, however, in agreement with the results of studies that used the same knockout mice (33), indicating that MSR1 does not play a direct role in the TNF-α-signaling pathway.

Internalization of Brucella spp. was reported to be mediated by MSR1 and blocked by the addition of LPS from the same species or from Salmonella, but not from E. coli LPS (24). Msr1 knockout mice displayed decreased protection from infection with Listeria monocytogenes and herpes virus (26), which correlates with A/J (susceptible) and B6 (resistant) phenotypes for these two infectious agents (34). These findings, and our own observations, strongly support a role for MSR1 in host defense and innate immunity. MSR1 has also been implicated in the pathological deposition of cholesterol during atherogenesis (35). Consistent with these results, A/J mice have been found to be more resistant than B6 mice in experimental models of atherosclerosis, diabetes, and obesity (34). These observations also suggest a possible link between MSR1 and IL-10 in many diseases besides sepsis.

MSR1 has also been implicated in the clearance of apoptotic cells (29), the appearance of which in immune organs (i.e., spleen and thymus), intestinal epithelial, and liver is a hallmark of sepsis (36). Therefore, it could be speculated that cells expressing different Msr1 genetic variants could clear apoptotic cells with different efficiencies. The prolonged presence of apoptotic cells may result in postapoptotic necrosis that induces a secondary inflammatory response, which may lead to the hyperinflammatory stage characteristic of septic shock. Recently, polymorphisms have been detected within the human MSR1 gene and correlated with the incidence of prostate cancer. The role of MSR1 in cancer may be related to observations indicating the infiltration of macrophages within the tumor (37). Thus, MSR1 appears to be involved in the inflammatory response associated with cancer. The involvement of MSR1 in sepsis introduces a possible new pathway in the septic process and contributes to a better understanding of the molecular mechanisms involved in this condition. Future research into the pathway involving MSR1 may reveal innovative therapeutic approaches to ameliorate the detrimental consequences of sepsis. Moreover, Msr1 may emerge as a genetic marker for the incidence of endotoxemia, sepsis, and related conditions. Finally, our study demonstrates the feasibility of using QTL to identify new components (genes) that are involved in the inflammatory process, which may be used as genetic markers to recognize a human population at risk for developing sepsis after trauma and infection.

**Acknowledgments**

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**Disclosures**

The authors have no financial conflict of interest.

FIGURE 7. Comparison of MSR1 surface expression on PMΦ isolated from A/J and B6 mice. A, PMΦ isolated from A/J and B6 mice were harvested by peritoneal lavage and allowed to attach to glass coverslips. Cells were immunostained with an anti-mouse MSR1 Ab and visualized by Cy3 IgG F(ab′)2. Figure depicts a representative field under ×100 objective. B, PMΦ isolated from A/J and B6 mice were harvested by peritoneal lavage and analyzed by FACS. Top row, Cells stained with anti-mouse MSR1 and as a secondary anti-rabbit IgG FITC conjugate F(ab′)2; bottom row, with secondary alone. Numbers in the top right quadrant represent number of cells gated that were positive for MSR1.
References


