

Role of α_2 -macroglobulin in fever and cytokine responses induced by lipopolysaccharide in mice

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Gourine, Alexander V., Valery N. Gourine, Yohannes Tesfaigzi, Nathalie Caluwaerts, Fred Van Leuven, and Matthew J. Kluger. Role of α_2 -macroglobulin in fever and cytokine responses induced by lipopolysaccharide in mice. *Am J Physiol Regulatory Integrative Comp Physiol* 283: R218–R226, 2002. First published March 7, 2002; 10.1152/ajpregu.00746.2001.— α_2 -Macroglobulin (α_2 M) is not only a proteinase inhibitor in mammals, but it is also a specific cytokine carrier that binds pro- and anti-inflammatory cytokines implicated in fever, including interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α (TNF- α). To define the role of α_2 M in regulation of febrile and cytokine responses, wild-type mice and mice deficient in α_2 M (α_2 M^{-/-}) were injected with lipopolysaccharide (LPS). Changes in body temperature as well as plasma levels of IL-1 β , IL-6, and TNF- α and hepatic TNF- α mRNA level during fever in α_2 M^{-/-} mice were compared with those in wild-type control mice. The α_2 M^{-/-} mice developed a short-term markedly attenuated (ANOVA, $P < 0.05$) fever in response to LPS (2.5 mg/kg ip) compared with the wild-type mice. At 1.5 h after injection of LPS, the plasma concentration of TNF- α , but not IL-1 β or IL-6, was significantly lower (by 58%) in the α_2 M^{-/-} mice compared with their wild-type controls (ANOVA, $P < 0.05$). There was no difference in hepatic TNF- α mRNA levels between α_2 M^{-/-} and wild-type mice 1.5 h after injection of LPS. These data support the hypotheses that 1) α_2 M is important for the normal development of LPS-induced fever and 2) a putative mechanism of α_2 M involvement in fever is through the inhibition of TNF- α clearance. These findings indicate a novel physiological role for α_2 M.

thermoregulation; proteinase inhibitor; interleukin; tumor necrosis factor; endotoxin

A REGULATED RISE in body temperature (T_b) or fever is an adaptive response of the organism to infection, injury, or trauma aimed at facilitating host resistance and slowing the growth of the pathogen (22). Considerable evidence indicates that many circulating cytokines, such as interleukin (IL)-1 β , IL-6, tumor necrosis factor- α (TNF- α), and others, act as endogenous pyrogens and are responsible for the induction and maintenance

of fever by raising the “set point” for T_b regulation (22, 23). On the basis of the extensive evidence indicating crucial roles for IL-1 β , IL-6, TNF- α , and other cytokines in the development of the febrile response, we hypothesized that any endogenous factor involved in the mechanisms of cytokine production or clearance may also play an important role in fever. In the present study, we investigated the role in fever of α_2 -macroglobulin (α_2 M), one of the major plasma proteinase inhibitors in mammals and a specific cytokine carrier that binds and possibly regulates metabolism of cytokines implicated in fever, including IL-1 β , IL-6, and TNF- α .

α_2 M is a tetrameric glycoprotein (Mr ~750 kDa) present in human plasma at high concentrations (~2–4 mg/ml) and a proteinase inhibitor that binds proteinases from all major classes (5, 13, 34, 40). The major source of plasma α_2 M is the hepatocyte (5, 34, 40); however, other cells including monocytes and macrophages synthesize and secrete α_2 M (3, 11, 21). Proteinase cleavage of the sensitive peptide bonds in the α_2 M “bait region” induces a conformational change in the α_2 M molecule, which irreversibly traps the reacting proteinase (12, 34, 40). Conformational change of the α_2 M also exposes binding sites for the α_2 M receptor/low-density lipoprotein receptor-related protein (LRP) (2, 36), which is present on the surfaces of many different cell types, including hepatocytes and macrophages (30). After binding to LRP, α_2 M-proteinase complexes rapidly undergo endocytosis, indicating that LRP is responsible for plasma clearance of conformationally modified α_2 M (2, 13, 30, 36). α_2 M in its native form does not bind LRP and has a prolonged half-life in circulation (34).

In humans, α_2 M is constantly present in plasma, and the changes in plasma concentrations are moderate and rarely diagnostic for any disease [for discussion, see Umans et al. (37)]. There is evidence, however, that lipopolysaccharide (LPS) may increase as well as suppress (perhaps depending on the experimental condi-

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tions) production of α_2 M by human monocytes and macrophages in vitro (3, 11, 21). These data indicate that during inflammation in humans, concentration of α_2 M may change significantly on the tissue or cellular level, although alterations in plasma concentrations are negligible.

The ability to bind practically all cytokines, hormones, and growth factors is an intriguing feature of α_2 M, and it indicates the potential role for this plasma protein in fever. Several studies demonstrated that α_2 M is a specific cytokine carrier, which binds major pro- and anti-inflammatory cytokines, such as IL-1 β (6, 7), IL-6 (29), TNF- α (9, 20, 42, 44), and others. Many cytokines can bind both native and proteinase-activated forms of α_2 M (9). Importantly, binding of cytokines to the protease-activated α_2 M does not affect the interaction of protease- α_2 M-cytokine complex with LRP (25). However, the functional role of α_2 M in regulation of cytokine metabolism in vivo is far from clear. As it was discussed by LaMarre et al. (25) and Crookston et al. (9), the function of α_2 M as a cytokine-binding molecule is complicated due to the different conformational states of α_2 M. By functioning as a cytokine carrier, α_2 M in its native form may protect bound cytokines from proteolytic degradation and, therefore, lengthen the plasma half-life. Most of the cytokines bound to the native α_2 M retain their biological activity [for review, see LaMarre et al. (25)]. On the other hand, only proteinase-activated α_2 M is recognized by LRP, and cytokines bound to the proteinase-activated α_2 M may be targeted to the cells expressing LRP for endocytosis and rapid clearance [for discussion, see LaMarre et al. (25)].

In the present study, the role of α_2 M in fever and cytokine responses induced by LPS was investigated using α_2 M gene knockout (α_2 M $-/-$) mice developed by Umans et al. (37). Murine α_2 M is a close homolog of human α_2 M. Similar to human α_2 M, murine α_2 M is a tetrameric glycoprotein with the molecular mass of \sim 720 kDa constantly present in plasma in a concentration of \sim 2 mg/ml. Murine α_2 M also inhibits proteases from all known classes and undergoes identical human α_2 M conformational changes upon reaction with protease. Although during experimental inflammation in mice moderate changes in plasma α_2 M can be observed (1, 19), similar to human α_2 M, murine α_2 M is not an acute phase protein. Similarities between human and murine α_2 M suggest that α_2 M $-/-$ mice represent an adequate animal model for determining the role of this plasma protease inhibitor in fever and cytokine responses during experimental inflammation.

We hypothesized that the role of α_2 M in fever depends on whether this protease inhibitor facilitates or inhibits clearance of the "major" endogenous pyrogens, e.g., IL-1 β , IL-6, or TNF- α . In this study, LPS-induced fevers in α_2 M $-/-$ mice were compared with those in wild-type (WT) control mice. To investigate possible involvement of cytokines, changes in plasma levels of IL-1 β , IL-6, and TNF- α were determined during fever in α_2 M $-/-$ and α_2 M WT mice. Because initial experiments showed that during fever plasma concentration

of TNF- α , but not of IL-1 β or IL-6, was significantly lower in the α_2 M $-/-$ mice compared with their WT controls, LPS-induced changes in hepatic TNF- α mRNA levels in knockout and WT mice were studied as well.

MATERIALS AND METHODS

Animals

All studies on conscious mice were conducted in facilities of the Lovelace Respiratory Research Institute, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International, and were approved by the Institutional Animal Care and Use Committee. Mice homozygous for a null mutation in the α_2 M gene were created by homologous recombination in embryonic stem cells. The generation and characterization of this strain have been described in detail (37, 38). The original α_2 M $-/-$ mice [C57BL \times 129J random hybrids (37)] were back-crossed into the C57BL/6J mouse strain for at least seven generations. Mice with a C57BL background of at least 98.5% were obtained, which alleviated problems with differences in genetic background between experimental and control (C57BL/6J) mice. Age-, weight-, and sex-matched specific pathogen-free male C57BL/6J mice (\sim 3–4 mo of age, weighing 25–35 g) were purchased from Jackson Laboratories (Bar Harbor, ME) to serve as WT controls for all experiments. On arrival, mice were housed one per cage in specific pathogen-free animal quarters, in a room maintained at a constant temperature of $30 \pm 1^\circ\text{C}$, a temperature within the thermoneutral zone of mice, and in a 12:12-h light-dark cycle with lights on at 0600. Drinking water and laboratory rodent chow were provided ad libitum. At the end of the experiments, the animals were humanely killed by an overdose of anesthetic (10% halothane in an air mixture).

Surgery

Mice were anesthetized with 4% halothane in an air mixture. An incision was made in the abdomen, and a miniature battery-operated, temperature-sensitive telemetry transmitter (model VMFH, Minimitter, Sunriver, OR) was placed into the abdominal cavity for continuous monitoring of T_b and motor activity. The muscle and skin levels of the abdomen were separately sutured. The wound area was swabbed with Furacin, and the animals were returned to their cages where they were allowed to recover for at least 7 days. Experiments were begun once each animal demonstrated a normal circadian variation in T_b .

T_b and Motor Activity Measurements

Core T_b ($\pm 0.1^\circ\text{C}$) and motor activity were monitored with implanted telemetry units (Minimitter). The animal's T_b was proportional to the signal frequency emitted by the implanted transmitter. Any change in the position of the implanted transmitter relative to the antenna under the cage was recorded as a pulse of activity. Recordings were made at 5-min intervals by use of a peripheral processor (Datacol III System, Minimitter) connected to an IBM personal computer.

Body Weight and Food Intake Measurements

Body weight and food intake were measured at 0900 each day on a top-loading balance with accuracy to ± 0.1 g. Changes in body weight and food consumption were calculated by subtracting the values obtained for each successive 24-h time point after injection from the value obtained im-

mediately before injection. Thus changes in body weight and food intake were relative to preinjection values.

Induction of Fever

Purified LPS (*Escherichia coli* endotoxin 0111:B4, Sigma Chemical, St. Louis, MO) was dissolved in pyrogen-free saline and injected intraperitoneally at a dose of 2.5 mg/kg. Control mice received an equivalent volume of sterile, pyrogen-free saline. Commercial-grade, steam-distilled turpentine (Sunnyside, Wheeling, IL) was injected intramuscularly into the left hindlimb at a volume of 20 μ l/mouse. Control animals were injected with 20 μ l of sterile saline intramuscularly into the same injection site. All mice were anesthetized with halothane during the injection procedure.

ELISA

Blood for cytokine analysis was collected from anesthetized (4% halothane in air mixture) mice by cardiac puncture. Blood was drawn into heparinized syringes, and plasma was separated by centrifugation (12,000 rpm, 5 min, 20°C) of the freshly drawn blood and stored at -20°C until assayed. Cytokines were not separated from the plasma carrier proteins before assays. IL-1 β , IL-6, and TNF- α concentrations in plasma were measured using mouse IL-1 β (R&D Systems, Minneapolis, MN), IL-6 (Endogen, Woburn, MA), and TNF- α (Endogen) immunoassay kits according to manufacturer's instructions. These assays detect IL-1 β , IL-6, and TNF- α at concentrations as low as 3.0, 7.0, and 10.0 pg/ml, respectively.

RT-PCR

RNA was isolated from the livers of α_2 M -/- and WT mice treated with either LPS or saline using phenol-free total RNA isolation kits (Ambion, Austin, TX) according to the manufacturer's manual. First-strand cDNA was synthesized from 3 μ g of total RNA primed with poly dT₂₁ by using the Superscript Preamplifications System (Life Technologies, Gaithersburg, MD). To eliminate the possibility of false positives by residual genomic DNA, samples were treated with DNase (Roche Biochemicals, Indianapolis, IN). Primers for murine TNF- α and β -actin were purchased from Stratagene (La Jolla, CA) and used at a concentration of 1 μ M. After cDNA synthesis, PCR reactions were performed parallel in 50- μ l reaction volumes. PCR amplification reaction included a 5-min denaturation at 94°C and a 5-min annealing at 60°C, followed by 35 cycles of 1.5 min at 72°C, 45 s at 94°C, and 45 s at 60°C, with a final extension of 10 min at 72°C. Primer pair for TNF- α was 5'-ATGAGCACAGAAAGCATGATC-3' (sense) and 5'-TACAGGCTTGCTCACTCGAATT-3' (antisense). Twenty microliters of amplified products were analyzed by electrophoresis in a 2% agarose gel. Each RT-PCR assay was repeated at least once for confirmation. The bands of the PCR products on the agarose gel were quantified via densitometry using a Fluor-S MAX Imager and the Quantity One software (BioRad, Hercules, CA). The band intensities of TNF- α were normalized with the corresponding band intensities for β -actin.

Experimental Design

Experiment 1. LPS-induced fever in α_2 M gene knockout mice. Mice were assigned to one of four groups: α_2 M -/- mice treated with either LPS ($n = 16$) or pyrogen-free saline ($n = 11$) and α_2 M WT mice injected with either LPS ($n = 20$) or pyrogen-free saline ($n = 14$). All injections were performed at 0900. T_b and motor activity were monitored for 3 h before and 48 h after LPS or saline injections. Body weight and food

intake were measured up to 4 days postinjection in six α_2 M -/- mice and eight WT mice treated with LPS and in six α_2 M -/- mice and eight WT mice injected with saline.

Experiment 2. Turpentine-induced fever in α_2 M gene knockout mice. To test the ability of α_2 M -/- mice to mount a normal thermogenic response, we compared fevers in α_2 M -/- and WT mice developed during localized inflammation (induced by turpentine injection). Mice were assigned to one of four groups: α_2 M -/- mice treated with either turpentine ($n = 4$) or pyrogen-free saline ($n = 3$) and α_2 M WT mice injected with either turpentine ($n = 5$) or pyrogen-free saline ($n = 4$). Because of the limited availability of α_2 M -/- mice for our studies, all α_2 M -/- and WT mice were those previously used in *experiment 1*. To circumvent any effect of previous injections, only those mice previously injected with sterile saline were used. All injections were performed between 0900 and 1000. T_b was monitored for 48 h after turpentine or saline injections.

Experiment 3. LPS-induced changes in plasma levels of IL-1 β , IL-6, and TNF- α in α_2 M gene knockout mice. Plasma concentrations of cytokines were measured at 1.5, 4, and 27 h after injection of LPS because of the following reasons. In mice and rats, at 1.5 h after the LPS challenge, high plasma concentrations of both IL-6 and TNF- α are observed, whereas TNF- α level is at or near its peak (8, 41). In addition, results of *experiment 1* showed that, at 1.5 h after LPS injections, T_b is significantly lower in the α_2 M -/- mice compared with their WT controls. At 4 h after LPS treatment, both moderate increase in plasma concentration of IL-1 β and still high plasma level of IL-6 are observed, whereas plasma TNF- α concentration decreases to control levels (8). Because in some studies in mice fever was observed to last up to 30 h after LPS injections (26, 27), we also measured plasma levels of cytokines at 27 h after LPS challenge (at the middle of the next day after LPS injections). The design for *experiment 1* was used. Mice were placed into one of four groups: LPS injected α_2 M -/- ($n = 5$), saline injected α_2 M -/- ($n = 3$), LPS injected WT ($n = 6$), or saline injected WT ($n = 3$). Mice were injected with either LPS (2.5 mg/kg) or saline between 0900 and 1000. Blood for cytokine analysis was collected from anesthetized (4% halothane in air mixture) α_2 M and WT mice by cardiac puncture at 1.5, 4, and 27 h after the injections. Immediately after the collection, plasma was separated by centrifugation and stored at -20°C until assayed.

Experiment 4. LPS-induced changes in hepatic TNF- α mRNA levels in α_2 M gene knockout mice. There is evidence that, in response to LPS challenge, different hepatic cells are capable of TNF- α expression, whereas Kupffer cells are the predominant source of circulating TNF- α (8, 18, 28). Therefore, to compare LPS-induced production of TNF- α in α_2 M -/- and WT mice, hepatic TNF- α mRNA levels were measured. Taking into account that the decay in hepatic TNF- α mRNA level is slow [hepatic TNF- α mRNA level only slightly decreases between 1 and 3 h after LPS challenge (33)], whereas half-life of TNF- α protein in circulation is very short (4, 10), and because of the limited availability of the α_2 M -/- mice for our studies, hepatic TNF- α mRNA levels were measured at the same time as the plasma TNF- α concentration, i.e., 1.5 h after injections of LPS or saline. The design for *experiment 1* was used. Animals used in *experiment 3* for blood collection were also used to harvest liver tissue. Immediately after blood collection, a portion of the liver was removed, immediately frozen in liquid nitrogen, and stored at -80°C until total RNA extraction and assay for TNF- α mRNA levels.

Statistical Analysis

Data are reported as means \pm SE. Experimental groups were compared using two-way ANOVA followed by the post hoc Fisher's test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Experiment 1. LPS-induced fever in α_2M gene knockout mice. The febrile response of $\alpha_2M^{-/-}$ and WT mice is shown in Fig. 1. The injection procedure caused profound short-term, stress-induced rises in T_b . Interestingly, the magnitude of this initial stress-induced rise in T_b was significantly higher in WT mice compared with $\alpha_2M^{-/-}$ mice, regardless of whether they were injected with LPS or saline (Fig. 1). Both groups of mice injected with saline returned to the normal level of T_b within 90 min with no difference between $\alpha_2M^{-/-}$ (Fig. 1, \blacktriangle) and WT (Fig. 1, \triangle) animals. As shown in Fig. 1, $\alpha_2M^{-/-}$ mice developed short-lived and markedly attenuated fever in response to LPS compared with their WT counterparts. α_2M WT mice responded to LPS with an $\sim 0.9^\circ\text{C}$ fever that began 90 min postinjection and lasted up to 7 h (Fig. 1, \circ). In $\alpha_2M^{-/-}$ mice, LPS induced a short-term $\sim 0.6^\circ\text{C}$ rise in T_b , which returned to the values observed in $\alpha_2M^{-/-}$ and WT mice treated with saline 4 h after the injection (Fig. 1, \bullet). Thus febrile response to LPS in $\alpha_2M^{-/-}$ mice was shorter by 3 h compared with fever in α_2M WT mice. There was no difference in T_b between $\alpha_2M^{-/-}$ and WT mice treated with either LPS or saline 9–48 h after the injections (data not shown).

During the 24-h period after injection, LPS induced a significant reduction in body weight (Fig. 2A) and food intake (Fig. 2B) in $\alpha_2M^{-/-}$ and WT mice relative to saline-injected controls. There was no significant difference in body weight and food intake between α_2M

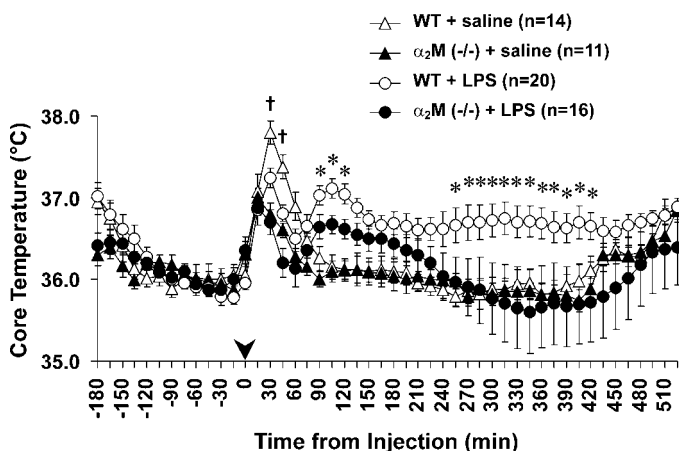


Fig. 1. Effect of lipopolysaccharide (LPS; 2.5 mg/kg) or pyrogen-free saline (equivalent volume) injected intraperitoneally on body temperature in α_2 -macroglobulin gene knockout ($\alpha_2M^{-/-}$) and wild-type (WT) mice. Data are presented as means \pm SE. Numbers in parentheses indicate sample sizes. Arrowhead indicates time of LPS and saline injections. \dagger Significant difference between $\alpha_2M^{-/-}$ and WT mice injected with LPS; between $\alpha_2M^{-/-}$ and WT mice injected with saline, $P < 0.05$. * Significant difference between LPS-injected $\alpha_2M^{-/-}$ and WT mice, $P < 0.05$.

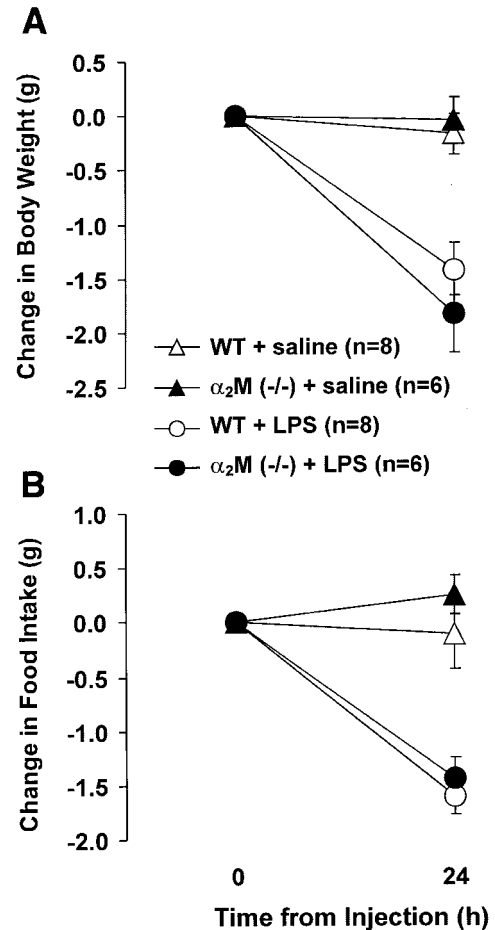


Fig. 2. Effect of LPS (2.5 mg/kg) or pyrogen-free saline (equivalent volume) injected intraperitoneally on change in body weight (A) and food intake (B) in $\alpha_2M^{-/-}$ and WT mice. Data are presented as means \pm SE. Numbers in parentheses indicate sample sizes.

$-/-$ and WT mice injected with LPS (Fig. 2). Injection of LPS also resulted in a complete suppression of locomotor activity in both $\alpha_2M^{-/-}$ and WT mice (data not shown). However, changes in activity in response to LPS did not differ between $-/-$ and WT mice.

Experiment 2. Turpentine-induced fever in α_2M gene knockout mice. Local tissue injury following turpentine administration was accompanied by significant fever on the next day. There was no difference in turpentine-induced changes in T_b between $\alpha_2M^{-/-}$ and WT mice (Fig. 3).

Experiment 3. LPS-induced changes in plasma levels of IL-1 β , IL-6, and TNF- α in α_2M gene knockout mice. Plasma IL-1 β , IL-6, and TNF- α levels were measured in $\alpha_2M^{-/-}$ and WT mice at 1.5, 4, and 27 h following injection of LPS or saline (Fig. 4). At all time points tested following injection of saline, $\alpha_2M^{-/-}$ and WT mice showed low plasma IL-1 β , IL-6, and TNF- α concentrations that did not significantly differ between groups (Fig. 4). Although plasma levels of IL-1 β , IL-6, and TNF- α in $\alpha_2M^{-/-}$ and WT mice treated with saline were low, they were above the detection limits of the assays and were included in the statistical analysis. These baseline values of plasma IL-6 and TNF- α

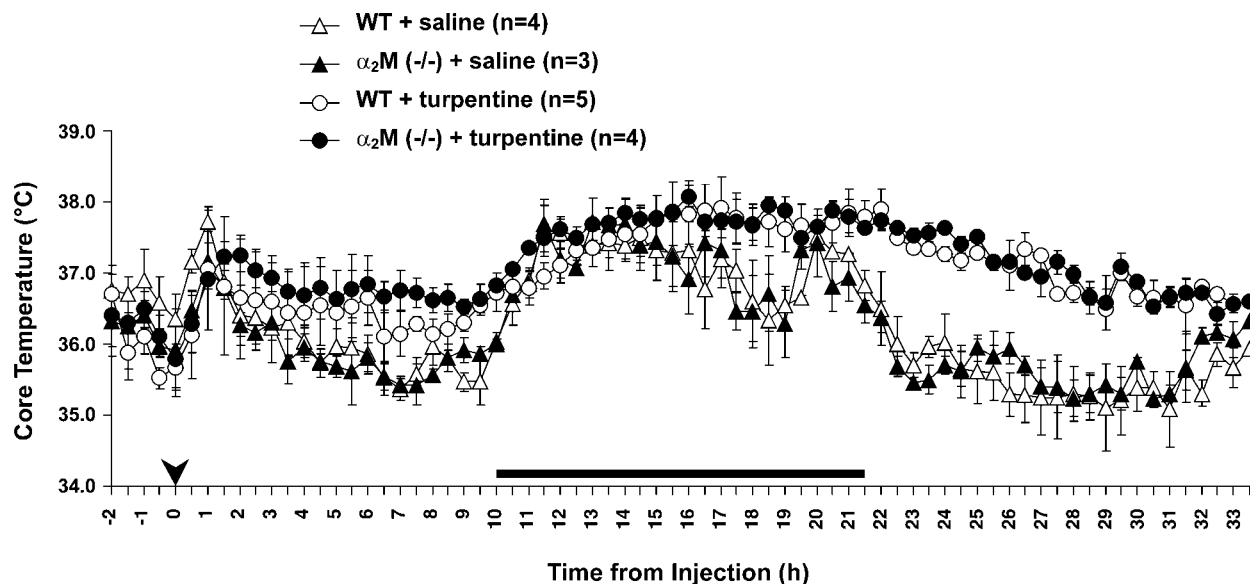


Fig. 3. Effect of turpentine (20 μ l) or pyrogen-free saline (20 μ l) injected intramuscularly into the left hindlimb on body temperature in α_2 M $-/-$ and WT mice. Data are presented as means \pm SE. Numbers in parentheses indicate sample sizes. Arrowhead indicates time of LPS and saline injections. Between 22 and 31 h after the injections, body temperature in α_2 M $-/-$ and WT mice treated with turpentine was significantly ($P < 0.05$) higher than in α_2 M $-/-$ and WT mice injected with saline.

concentrations are not adequately presented in Fig. 4, B and C, due to the large scale of the y-axis. The values of plasma TNF- α concentrations in α_2 M $-/-$ and WT mice 1.5 h after saline injection are given in the text below, as it is important for the comparison with the values of the TNF- α concentrations induced by LPS challenge.

Treatment with LPS resulted in a moderate but significant elevation in plasma concentration of IL-1 β in α_2 M $-/-$ and WT mice 4 h after the injection (Fig. 4A). There was no significant difference in plasma IL-1 β concentrations between $-/-$ and WT mice injected with LPS (Fig. 4A).

LPS injection resulted in a profound early elevation in plasma levels of IL-6 (Fig. 4B). A high concentration of IL-6 was observed in the plasma of α_2 M $-/-$ and WT mice 1.5 and 4 h after LPS injection (Fig. 4B). However, there was no significant difference in plasma IL-6 concentrations between α_2 M $-/-$ and WT mice injected with LPS at either time point tested (Fig. 4B).

Ninety minutes after saline injection, plasma concentrations of TNF- α in α_2 M $-/-$ and WT mice were 41 ± 28 and 55 ± 6 pg/ml, respectively ($P > 0.05$). LPS challenge resulted in a significant early elevation in the plasma level of TNF- α (Fig. 4C). Ninety minutes after LPS injection, plasma concentration of TNF- α was significantly lower in α_2 M $-/-$ mice compared with WT controls ($5,427 \pm 1,330$ vs. $12,817 \pm 1,477$ pg/ml, $P = 0.0037$; Fig. 4C). By 4 h after LPS injection, plasma TNF- α concentration decreased to control levels (within the range of 40–90 pg/ml), and there was no difference between α_2 M $-/-$ and WT mice (Fig. 4C).

Experiment 4. LPS-induced changes in hepatic TNF- α mRNA levels in α_2 M gene knockout mice. TNF- α mRNA levels in the liver were determined in α_2 M $-/-$

and WT mice at 1.5 h following injection of LPS or saline (when the difference in plasma concentration of TNF- α between α_2 M $-/-$ and WT mice was observed). α_2 M $-/-$ and WT mice injected with saline showed low TNF- α (Fig. 5) mRNA levels that did not significantly differ between groups. LPS led to a significant elevation in hepatic TNF- α (Fig. 5) mRNA levels at 1.5 h postinjection. There was no significant difference in hepatic TNF- α (Fig. 5) mRNA levels between α_2 M $-/-$ and WT mice 1.5 h after injection of LPS.

DISCUSSION

In the present study, the role of α_2 M in LPS-induced fever and cytokine responses was studied. α_2 M $-/-$ mice developed a short-term markedly attenuated fever in response to LPS, suggesting that this plasma protease inhibitor is essential for normal development of LPS-induced fever in mice. There was no difference in plasma levels of IL-1 β and IL-6 between α_2 M $-/-$ and WT mice after injection of LPS. Plasma concentration of TNF- α shortly after LPS challenge was significantly lower in the α_2 M $-/-$ mice compared with their WT counterparts. No difference in the hepatic TNF- α mRNA levels between α_2 M $-/-$ and WT mice treated with LPS suggests augmented clearance of TNF- α in α_2 M $-/-$ mice. No difference in fever between α_2 M $-/-$ and WT mice following turpentine injection suggests that α_2 M $-/-$ mice can increase their T_b in response to other stimuli to the same extent as the control animals, indicating that α_2 M deficiency does not impair these animals' ability to mount an adequate thermogenic response. In view of the latter, an interesting observation is that the magnitude of the initial stress-induced rise in T_b (evoked by the injection procedure) was

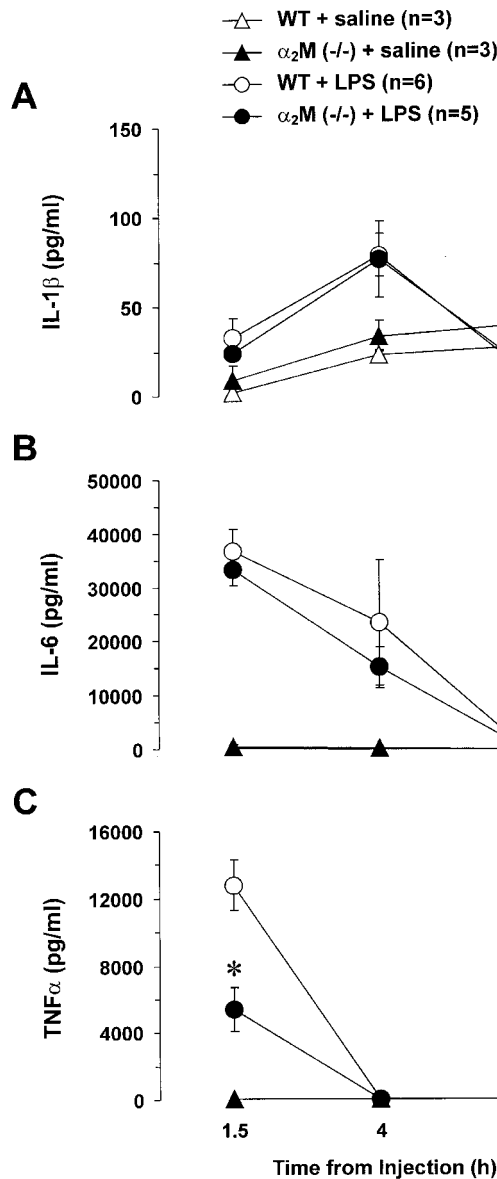


Fig. 4. Effect of LPS (2.5 mg/kg) or pyrogen-free saline (equivalent volume) injected intraperitoneally on plasma levels of interleukin (IL)-1 β (A), IL-6 (B), and tumor necrosis factor- α (TNF- α ; C) in α_2 M $^{-/-}$ and WT mice. Plasma IL-1 β , IL-6, and TNF- α concentrations were measured at 1.5, 4, and 27 h following injection of LPS or saline. Data are presented as means \pm SE. Numbers in parentheses indicate sample sizes. At 4 h after the injections, IL-1 β levels in plasma of α_2 M $^{-/-}$ and WT mice treated with LPS were significantly ($P < 0.05$) higher than in plasma of α_2 M $^{-/-}$ and WT mice injected with saline (A). At 1.5 and 4 h after the injections, IL-6 levels in plasma of α_2 M $^{-/-}$ and WT mice treated with LPS were significantly ($P < 0.05$) higher than in plasma of α_2 M $^{-/-}$ and WT mice injected with saline (B). *Significant difference in plasma TNF- α concentration between α_2 M $^{-/-}$ and WT mice injected with LPS, $P < 0.05$.

significantly lower in α_2 M $^{-/-}$ mice compared with WT mice, regardless of whether they were injected with LPS or saline (Fig. 1). This observation may suggest that α_2 M is also involved in the mechanisms of the development of stress-induced fever, and, to study the mechanisms of this involvement, the data have to be confirmed in "controlled" experiments, such as those

in which animals are exposed to an identical novel environment.

Successful targeting of the α_2 M gene and generation of the α_2 M $^{-/-}$ mice (37, 38) provided a valuable tool to dissect the role of this protease inhibitor in febrile and cytokine responses induced by LPS. Unfortunately, this transgenic model is not ideal, because mouse plasma, unlike plasma of humans and other mammals, contains two different types of α -macroglobulins: the tetrameric α_2 M and the monomeric or single-chain murinoglobulins, the in vivo function of which is still unclear [for detailed discussion, see Umans et al. (37)]. Although levels of murinoglobulins in the plasma of adult nonpregnant α_2 M $^{-/-}$ mice are unchanged compared with WT animals (37), we cannot completely exclude the possibility that, during development of the inflammatory response induced by either LPS or turpentine, murinoglobulins may, in part, functionally replace α_2 M in the α_2 M $^{-/-}$ mice. Therefore, as in any experiment involving gene knockout animals, caution should have been taken in the interpretation of the obtained data.

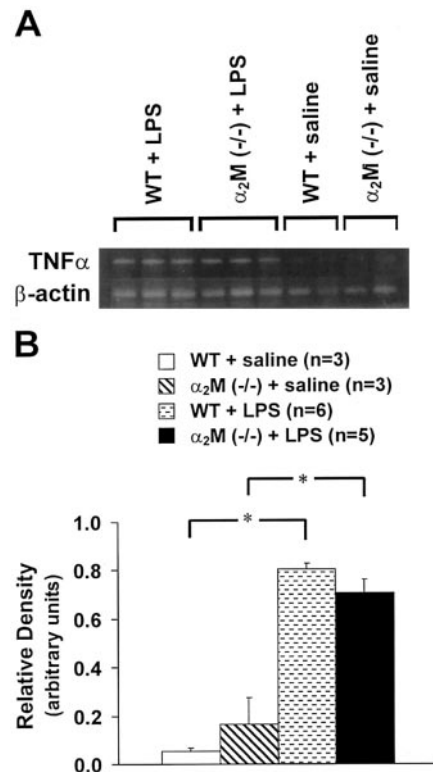


Fig. 5. A: detection of TNF- α and β -actin mRNAs by RT-PCR from RNA isolated from the liver of α_2 M $^{-/-}$ and WT mice 1.5 h after intraperitoneal injection of LPS (2.5 mg/kg) or pyrogen-free saline. B: quantification of the band intensities normalized for the housekeeping gene, β -actin, showed significant increases of hepatic TNF- α mRNA levels in α_2 M $^{-/-}$ and WT mice treated with LPS compared with their saline-injected counterparts. Samples of the hepatic RNA from 5 α_2 M $^{-/-}$ mice injected with LPS, 3 α_2 M $^{-/-}$ mice injected with saline, 6 WT mice injected with LPS, and 3 WT mice injected with saline were analyzed in 2 separate assays. Data are presented as means \pm SE. *Significant difference between α_2 M $^{-/-}$ mice injected with LPS and saline; between WT mice injected with LPS and saline, $P < 0.05$.

We reported recently that human α_2 M induces moderate fever ($\sim 0.5^\circ\text{C}$) in mice when injected intravenously in amounts similar to or even smaller than those observed during the development of the systemic inflammatory response (14). Furthermore, it was shown that 1 h after intravenous injection, human α_2 M induces moderate (smaller compared with LPS induced) but significant increase in plasma bioactivity of TNF- α (14), suggesting that TNF- α may mediate the pyrogenic effect of human α_2 M in mice. It is possible that exogenous α_2 M either inhibits rapid degradation of TNF- α , which is constitutively produced under basal "nonpathological" conditions (28, 33), or stimulates TNF- α synthesis, or both. Although the mechanisms of the increase in plasma TNF- α level induced by intravenous injection of exogenous α_2 M in mice have to be investigated, these findings coincide with the results of the present study, and, taken together, they suggest an important role for α_2 M in the mechanisms of fever development.

The present data also correlate well with the observations of Umans et al. (37), who reported that α_2 M $-/-$ mice are resistant to the lethal effects of LPS. However, although LPS-induced fever was lower in α_2 M $-/-$ mice, the other "signs" of sickness syndrome, i.e., body weight loss, decrease in food intake, and decrease in motor activity, were identical to those in control WT mice (Fig. 2). Thus, in terms of induction of anorexia and lethargy, α_2 M $-/-$ mice are equally sensitive to LPS. These unexpected observations suggest that although playing an important role in the development of fever, α_2 M probably is not involved in anorexia and lethargy induced by LPS in mice. In view of the results obtained in the present study, indicating that α_2 M is likely to be involved in LPS-induced fever through the inhibition of TNF- α clearance, these data are in agreement with the results obtained in the TNF double-receptor knockout mice, showing that TNF- α does not mediate LPS-induced anorexia and lethargy (27). However, one should apply some caution in interpretation of our results. It is possible, due to inherent redundancy in hormone/cytokine action, that in both cases knockout mice compensate for removal of one gene by increased action of another in the regulation of some aspects of the acute phase response to LPS.

Although α_2 M is not an acute phase protein in mice (unlike in rats and some other species), during experimental inflammation, moderate changes in plasma levels of murine α_2 M do occur (1, 19). LPS in a dose of 20 $\mu\text{g}/\text{mouse}$ (~ 1.0 mg/kg) induced a significant increase in plasma α_2 M concentration 24 and 48 h after intraperitoneal injection (19). It is impossible to compare these results with the data obtained in our study, because different strains of mice and doses of LPS were used. However, the study of Isaac et al. (19) is important to this discussion as it shows that LPS can induce α_2 M production in mice. Interesting data were obtained by LaMarre et al. (24), who showed that expression of LRP in murine macrophages can be markedly decreased by LPS. These data indicate that LPS is a natural regulator of the α_2 M/LRP system: it can in-

crease α_2 M production and, at the same time, suppress the expression of the α_2 M receptor.

We hypothesized initially that the effect of α_2 M on the febrile response depends on whether this protease inhibitor facilitates or inhibits clearance of the "major" endogenous pyrogens, e.g., IL-1 β , IL-6, or TNF- α . This hypothesis was supported by the vast amount of literature indicating that plasma α_2 M is a broad-spectrum protease inhibitor and a cytokine-binding protein and carrier at the same time (5–7, 9, 13, 20, 25, 29, 42, 44). Extrapolation from *in vitro* studies suggested that depending on the conformational state of the α_2 M molecule, its function in regulation of cytokine metabolism and, therefore, thermoregulatory febrile response could be different. As discussed by LaMarre et al. (25) and mentioned earlier in text, α_2 M in its native form may protect bound cytokine from proteolytic degradation by functioning as a cytokine carrier and, therefore, lengthen its plasma half-life. On the other hand, proteinase-activated α_2 M (that is recognized by LRP) may play an important role in the processes of cytokine clearance.

Results of the present study suggest an augmented rate of TNF- α clearance in α_2 M $-/-$ mice. Several studies *in vitro* demonstrated binding of TNF- α to native and protease-activated α_2 M (9, 20, 42, 44). It has been shown that neither native nor protease-activated α_2 M reduces biological activity of TNF- α (44). Although TNF- α binds native α_2 M with lower affinity than protease-activated α_2 M, we hypothesize that under most conditions and particularly during LPS-induced fever, native α_2 M is more important in regulating TNF- α clearance than protease-modified α_2 M. First, our data suggest an augmented clearance of TNF- α in α_2 M $-/-$ mice. If protease-activated α_2 M played a significant role in LRP-mediated TNF- α clearance *in vivo*, we would expect to observe the opposite (i.e., higher plasma concentration of TNF- α in α_2 M $-/-$ mice compared with WT controls). Second, native α_2 M is the predominant form of α_2 M present in the plasma and in the extravascular microenvironments. In contrast, protease-activated α_2 M under most conditions is present only at trace levels. We hypothesize that native α_2 M, as a broad-spectrum protease inhibitor, protects bound TNF- α from proteolytic degradation and, therefore, lengthens its plasma half-life. This conclusion is supported by the evidence that TNF- α is efficiently destroyed by proteases released from activated polymorphonuclear neutrophils and that after proteolytic cleavage, TNF- α fragments lack any TNF- α -like cytotoxic activity (31, 39).

Our data do not support the observations of Hocheppied et al. (16), who showed an identical rate of clearance of injected TNF- α in α_2 M $-/-$ and WT mice. Both data are difficult to reconcile, especially because the same mice were used in both studies. Presumably, the clearance mechanisms of injected TNF- α are to some extent different from those of LPS-induced, endogenously produced TNF- α . The latter could be significantly affected by other responses (physiological and

humoral) induced by LPS and not mimicked by injection of TNF- α alone.

The lack of a difference in plasma levels of IL-1 β and IL-6 between α_2 M $-/-$ and WT mice after injection of LPS does not support the hypothesis that fever in α_2 M $-/-$ mice is attenuated due to decreased production or increased clearance of IL-1 β or IL-6. These data were somewhat unexpected in view of the evidence obtained in experiments in vitro suggesting that α_2 M is one of the major IL-1 β - and IL-6-binding plasma proteins (6, 7, 29). Data obtained in the present study reveal the limitations of a direct extrapolation from these in vitro studies and suggest that α_2 M may not play a significant role in the regulation of IL-1 β and IL-6 clearance during LPS-induced fever in mice.

Although the precise role of TNF- α in fever is still unresolved, we propose that the putative mechanism of α_2 M involvement in LPS-induced fever is through the inhibition of TNF- α clearance. Indeed, depending on the experimental conditions and animal species (and probably also strains) used, this cytokine can act as an endogenous pyrogen as well as an endogenous antipyretic. Injection or infusion of TNF- α appears to induce fever in several species (for review, see Refs. 22, 23, 35). When the actions of endogenous TNF- α were blocked experimentally, this cytokine showed antipyretic properties in rats and mice and pyrogenic activity in rats, rabbits, guinea pigs, and humans (for recent reviews, see Refs. 15, 32, 35). The following arguments supporting the above hypothesis should be mentioned: 1) TNF- α is a major proinflammatory cytokine, and its lower plasma levels indicate a smaller systemic inflammatory response. 2) We observed that intravenous injection of recombinant murine TNF- α (1 μ g) induces moderate (1°C) fever in C57BL/6J mice (A.V. Gourine and M.J. Kluger, unpublished observations) in mice of the same strain that was used in the present study. 3) It appears that, when TNF- α is not involved in fever, α_2 M is also without effect. Neither TNF- α (27) nor α_2 M is involved in fever induced by local inflammation (injection of turpentine). 4) Roth et al. (32) recently reported that in guinea pigs neutralization of TNF- α by treating the animals with its type 1 soluble receptor significantly attenuates the second phase of the LPS-induced febrile response without affecting its initial phase. Interestingly, neutralization of TNF- α by its type 1 soluble receptor affects the development of fever in guinea pigs in the same way as inactivation of the α_2 M gene affects fever in mice, i.e., resulting in a marked attenuation of the late phase of the febrile response without markedly affecting the initial phase.

In conclusion, results of the present study suggest that α_2 M is important for the normal development of LPS-induced fever in mice and that putative mechanism of α_2 M involvement in LPS-induced fever is through the inhibition of TNF- α clearance. We speculate that α_2 M serves as an inhibitor of proteinases (e.g., elastase) responsible for rapid degradation of TNF- α and, possibly, of other pyrogenic cytokines (but not IL-1 β or IL-6).

Perspectives

Protease inhibitors, α_2 M in particular, are often considered nonspecific defense molecules, with the function of protecting tissues from unwanted proteases released by pathogenic microorganisms or from the dying cells of the host. Data obtained in the present study indicate a novel physiological role for plasma α_2 M. We identified α_2 M as an endogenous factor involved in regulation of TNF- α clearance and essential for the normal development of fever in response to bacterial endotoxin. Because α_2 M has several diverse properties, it is possible that regulation of TNF- α clearance is not the only mechanism by which this protease inhibitor modulates the febrile response. For example, it has been shown that α_2 M is able to induce prostaglandin E₂ (17) and nitric oxide synthesis (43), suggesting that another putative mechanism of α_2 M involvement in fever could be via stimulation of prostaglandin E₂ and (or) nitric oxide production. Also, α_2 M is synthesized in the brain primarily by astrocytes, and expression of its receptor (LRP) has been identified in neurons and astrocytes in the central nervous system (30), suggesting that α_2 M can act directly in the brain to modulate fever induced by LPS. Further studies of the possible mechanisms of α_2 M involvement in fever may lead to the development of new approaches (based on modifying activity of this major protease inhibitor) of modulating febrile and inflammatory responses, which is particularly important in cases when overzealous fever or (and) excessive production of proinflammatory cytokines is harmful for the host.

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