Endotoxic shock in AUF1 knockout mice mediated by failure to degrade proinflammatory cytokine mRNAs

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Excessive production of proinflammatory cytokines, particularly tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β), plays a critical role in septic shock induced by bacterial endotoxin (endotoxemia). Precise control of cytokine expression depends on rapid degradation of cytokine mRNAs, mediated by an AU-rich element (ARE) in the 3′ noncoding region and by interacting ARE-binding proteins, which control the systemic inflammatory response. To understand the function of the ARE-binding protein AUF1, we developed an AUF1 knockout mouse. We show that AUF1 normally functions to protect against the lethal progression of endotoxemia. Upon endotoxin challenge, AUF1 knockout mice display symptoms of severe endotoxic shock, including vascular hemorrhage, intravascular coagulation, and high mortality, resulting from overproduction of TNFα and IL-1β. Overexpression of these two cytokines is specific, and shown to result from an inability to rapidly degrade these mRNAs in macrophages following induction. Neutralizing antibodies to TNFα and IL-1β protect AUF1 knockout mice against lethal endotoxic shock. These and other data describe a novel post-transcriptional mechanism whereby AUF1 acts as a crucial attenuator of the inflammatory response, promoting the rapid decay of selective proinflammatory cytokine mRNAs following endotoxin activation. Defects in the AUF1 post-transcriptionally controlled pathway may be involved in human inflammatory disease.

[Keywords: AU-rich element; ARE; AUF1; mRNA decay; endotoxic shock; cytokines]

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Septic shock accounts for 9% of U.S. annual deaths and is a leading cause of hospital-related mortality (Tracey et al. 1987; Dinarello 1994; Hotchkiss and Karl 2003; Martin et al. 2003). Typically initiated by a bacteria infection, septic shock results from the uncontrolled expression of proinflammatory cytokines, primarily those produced by macrophages (Tracey et al. 1987; Dinarello 1994; Hotchkiss and Karl 2003; Martin et al. 2003), which leads to an overwhelming systemic inflammatory response culminating in multiple organ failure. Lipopolysaccharide (LPS) endotoxin, a component of the bacterial cell wall, stimulates macrophages to produce proinflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β), which are critical mediators of septic shock (Glauser et al. 1991). The excessive production of these proinflammatory cytokines leads to systemic capillary leakage and vascular hemorrhage, tissue destruction, and ultimately lethal organ failure and death (Tracey et al. 1987; Glauser et al. 1991; Dinarello 1994; Hotchkiss and Karl 2003; Martin et al. 2003). Thus, the expression of TNFα and IL-1β cytokines needs to be tightly regulated during an inflammatory response.

Regulation of cytokines occurs at multiple levels, including the stability of their encoding mRNAs (Chen and Shyu 1995; Guhaniyogi and Brewer 2001; Wilusz et al. 2001). The mRNAs encoding most inflammatory cytokines are short-lived, with instability conferred by an AU-rich element [ARE] in the 3′ noncoding region (Chen and Shyu 1995; Guhaniyogi and Brewer 2001). The ARE consists of multiple copies of an AUUAU pentamer, either adjacent or interrupted by other sequences, or even noncanonical AU-rich motifs (for review, see Chen and Shyu 1995; Guhaniyogi and Brewer 2001). The ARE promotes rapid cytoplasmic degradation of mRNAs (Guhaniyogi and Brewer 2001) and in some cases translation arrest (Grosset et al. 2004). It is thought that the different arrangements and types of ARE sequences may confer these different activities and may be responsible for different types of control of ARE-mRNA decay (Guhaniyogi and Brewer 2001). ARE-mRNAs can also be rapidly sta-
Endotoxic shock in AUF1 knockout mice

Results

Generation of AUF1-null mutant mice

To assess the function of AUF1 in vivo, we generated AUF1 knockout mice through homologous recombination in mouse embryonic stem cells. The construct targeted the third exon of AUF1, which contains two RNA-binding motifs, and disrupted the remainder of the reading frame by homologous recombination [Fig. 1A]. The wild-type and targeted AUF1 alleles were identified by Southern blot DNA hybridization analysis using probes 5′ and 3′ to the homology arm employed in the targeting construct (Fig. 1B), and by PCR amplification of genomic DNA (Fig. 1C). Furthermore, a Southern blot analysis using a probe specific for the neomycin resistance (neo+) cassette confirmed the presence of only one copy of the neo+ cassette in the genome after homologous recombi-
nation, ruling out the possibility of a random integration (data not shown). Immunoblot analysis of protein extracts of mouse organs with high levels of AUF1 [Lu and Schneider 2004] demonstrated abrogation of AUF1 expression, while the level of another RNA-binding protein [KSRP] remained unaltered [Fig. 1D], indicating the successful disruption of the AUF1 locus. Matings of AUF1−/− mice produced homozygous AUF1 mutant animals in a Mendelian ratio (25% of progeny) [Supplementary Fig. S1a] with no embryonic lethality detected. AUF1−/− newborns were indistinguishable from wild-type littermates except they were smaller and had reduced body weight [Supplementary Fig. S1b]. The growth retardation phenotype was equally distributed in both sexes. AUF1−/− mice survived to adulthood and were fertile. Other than smaller size and reduced body weight, macroscopic and histological examination did not reveal any morphological abnormalities in major organs, of young adult mice. Further analysis of AUF1−/− embryos found no evidence for major developmental defects [Supplementary Fig. S2] or significant alteration in fetal hematopoietic organs including thymus, spleen, and liver (data not shown). Therefore, disruption of AUF1 did not cause any severe defect in mouse development.

Disruption of AUF1 causes a fivefold increase in mortality during LPS-induced endotoxemia

To determine whether AUF1 regulates the inflammatory response, we examined the effect of AUF1 deficiency on the survival of mice subjected to endotoxin (LPS)-induced endotoxemia. AUF1 knockout mice were injected intraperitoneally with a sublethal dose [20 mg/kg] of bacterial LPS endotoxin. At this dose, LPS potently stimulates proinflammatory cytokine expression and induces a systemic inflammatory response, but typically without provoking significant mortality. While the majority of wild-type mice (90%) survived the endotoxin challenge and showed only a slight reduction in activity, AUF1 knockout mice displayed severe manifestations of endotoxemia, including diarrhea, tachypnea, lethargy, and piloerection [Fig. 2B]. By 72 h post-LPS challenge, AUF1−/− mice displayed about a fivefold increase in mortality compared with wild-type mice (47.4% vs. 10%) [Fig. 2A]. Survival analysis showed that the survival rate of AUF1 knockout mice is statistically significantly lower than that of the wild-type mice after LPS challenge [Fig. 2A, p < 0.01]. The surviving AUF1−/− mice required more than twice as long to recover compared with wild-type animals [3–4 d vs. 1–1.5 d, respectively] (data not shown). Thus, AUF1 knockout mice are much more susceptible to the endotoxin challenge than wild-type mice.

AUF1−/− mice undergo more severe endotoxic shock

One of the essential features of endotoxic shock is disseminated intravascular coagulation, characterized by widespread blood coagulation and vessel hemorrhage, particularly in the kidney and lung [Parrillo 1993]. Disseminated intravascular coagulation is tightly associated with pathological activation of proinflammatory cytokines TNFα and IL-1β [Dinarello 1997]. Histological examination found widespread hemorrhage in the kidneys of AUF1−/− mice following LPS challenge [Fig. 2C], indicating a massive capillary leakage. In contrast, there was only mild hemorrhage in the kidney of wild-type control mice. To assess the intensity of hemorrhage, the mean number of hemorrhage lesions per field at 10× magnification was determined. Compared with wild-type mice, AUF1−/− mice had a significantly greater number of hemorrhagic lesions in the kidney (mean lesion number/field ± SD: 9.8 ± 2.9 for AUF1−/− and 4.1 ± 1.6 for wild-type mice, p < 0.001), which were more extensive in size. Moreover, sampling of peripheral blood of AUF1−/− mice demonstrated a fivefold increase in blood urea nitrogen (BUN) levels and a 2.5-fold increase in the serum levels of released liver enzyme aspartate aminotransferase (AST) [Fig. 2D], markers of tissue destruction that correlate with a severe loss of renal and liver func-
tion, respectively. Further, higher resolution showed that intravascular coagulation was common in kidney, lung, and liver of AUF1−/− mice, but not in wild-type mice following endotoxin challenge, with an average of four coagulation lesions per 10× magnification field in AUF1−/− mice and less than one lesion per field in control animals [Fig. 2E]. Loss of AUF1 expression therefore leads to widespread intravascular coagulation associated with organ failure, a major cause of fatal endotoxic shock after endotoxin challenge.

Loss of AUF1 promotes excessive production of TNFα and IL-1β

We determined whether the increased susceptibility to endotoxemia of AUF1−/− mice is due to deregulation of proinflammatory cytokine expression, particularly TNFα and IL-1β, two critical mediators of endotoxic shock. For this purpose, we measured the serum TNFα levels in wild-type and AUF1−/− mice after LPS challenge. The level of TNFα was elevated by 30% in peripheral blood of AUF1−/− mice after endotoxin injection [Fig. 3A]. The serum TNFα level is likely to be an underestimate of the level of TNFα produced and locally accumulated because of rapid retention in tissues, catabolism, and incomplete release into circulating blood [Finkelman et al. 1993]. In addition, the high level of blood clotting in endotoxin-challenged AUF1−/− mice makes
Compared with wild-type cells, TNFα cytokine levels at time points following LPS challenge was also examined to monitor the dynamic change of endotoxin challenge.

AUF1 LPS-stimulated of AUF1 increased TNFα and IL-1β levels in and IL-1β after LPS stimulation showed that production of TNFα was almost threefold higher in AUF1 knockout mice (Fig. 5E, below). Primary macrophages derived from wild-type and AUF1 knockout mice. No significant differences were found, apart from the absence of AUF1 in knockout mice (Fig. 5E, below). Primary macrophages were stimulated with LPS and the levels of cytokines secreted into medium were determined by ELISA. Comparison of the accumulated levels of cytokine secretion after LPS stimulation showed that production of TNFα and IL-1β were increased by 40% and 70%, respectively, in AUF1-deficient macrophages (Fig. 3B). Therefore, loss of AUF1 increased TNFα and IL-1β levels in response to endotoxin challenge.

The kinetics of TNFα and IL-1β cytokine expression was also examined to monitor the dynamic change of cytokine levels at time points following LPS challenge. Compared with wild-type cells, TNFα production in LPS-stimulated AUF1−/− macrophages was increased by 2.5-fold, and IL-1β by twofold overall (Fig. 3C). Importantly, TNFα and IL-1β expression in AUF1−/− macrophages remained elevated at 24 h, therefore for a longer duration of time (Fig. 3C) compared with the wild-type macrophage controls. While the expression level of TNFα declined by 24 h in wild-type macrophages, it was still almost threefold higher in AUF1−/− macrophages. IL-1β expression continued to increase over 24 h, to a level twofold greater in AUF1−/− macrophages than in wild-type cells. Expression of IL-1β reached a plateau by 30 h in wild-type macrophages but continued to increase slowly in AUF1 knockout mice (data not shown). Thus, there is an increased and more sustained expression of TNFα and IL-1β in AUF1−/− macrophages compared with wild-type macrophages, which is associated with the severe pathophysiological reaction of AUF1−/− animals to endotoxin. These data suggest that AUF1 functions to attenuate the proinflammatory cytokine response after endotoxin challenge.

Abnormal stabilization of TNFα and IL-1β mRNAs results from disruption of AUF1

Given the reported function of AUF1 in promoting rapid degradation of ARE-mRNAs (Guhaniyogi and Brewer 2001), we asked whether increased TNFα and IL-1β expression is due to an alteration in mRNA stabilities. To monitor the decay of ARE-mRNAs, actinomycin D was applied to block transcription after primary macrophages were stimulated with LPS. The level of remaining mRNA transcripts was determined by real-time quantitative RT–PCR at times post-inhibition of transcription. We found that TNFα and IL-1β mRNAs in wild-type macrophages was increased by 40% and 70%, respectively, in AUF1-deficient macrophages (Fig. 3B). Therefore, loss of AUF1 increased TNFα and IL-1β levels in response to endotoxin challenge.

**Excessive production of proinflammatory cytokines is not due to an increase in the monocyte/macrophage population in AUF1−/− mice**

Hematopoietic cells, especially monocytes and macrophages, are major sources of proinflammatory cytokines TNFα and IL-1β. To determine whether lack of AUF1 affected the development and circulating levels of hematopoietic cells, we performed a complete blood count and a differential count of blood cells for wild-type and AUF1−/− mice, and analyzed their macrophage population in the peritoneum and spleen by flow cytometric analysis. Red blood cell parameters, leukocyte, and platelet counts in peripheral blood were comparable and within normal ranges in AUF1−/− and wild-type littermates. Our analysis also confirmed the presence of all major hematopoietic lineages in AUF1−/− mice without significant alteration in macrophage numbers (Fig. 4A). Flow cytometry analysis of the macrophage population (F4/80 staining for murine macrophages) in peritoneum and spleen did not reveal significant difference between AUF1−/− and wild-type mice (Fig. 4B). Thus, the excessive production of proinflammatory cytokines in LPS challenged AUF1−/− mice is not due to an increase in the number of source cells.

**Figure 4.** Analysis of peripheral blood and macrophage population in AUF1−/− mice. (A) Red blood cell parameters and numbers of leukocyte subpopulations were obtained by complete blood cell count and differential cell count of peripheral blood. No significant difference was detected between AUF1 knockout and wild-type mice. (B) Numbers of macrophages in peritoneum and spleen were determined by flow cytometry analysis of F4/80 surface expression (a cell surface marker specific for murine macrophages). Representative histograms are shown [n = 5 in each group]. No significant difference was observed.
macrophages were unstable and rapidly degraded with half-lives of 31 and 26 min, respectively (Fig. 5A–B). However, in AUF1−/− macrophages, the half-lives of TNFα and IL-1β mRNAs were 2.5-fold [60 min for IL-1β mRNA] and twofold greater [63 min for TNFα mRNA] compared with wild-type macrophages. The levels of TNFα and IL-1β mRNAs in unstimulated wild-type and AUF1 knockout cells were barely detectable [data not shown]. There was no change in the stability of ARE-containing IL-6 mRNA in AUF1−/− macrophages [Fig. 5C], or in the stability of cyclophilin A [Cyp A] mRNAs [Fig. 5C, inset; data not shown], which was used as a control for normalization. These results indicate that AUF1 specifically promotes decay of select mRNAs including TNFα and IL-1β. To confirm the binding of AUF1 to TNFα and IL-1β mRNAs, immunoprecipitation-quantitative RT–PCR [IP qRT–PCR] was performed to determine the intracellular AUF1 protein–RNA interaction. AUF1 proteins were immunoprecipitated from cytoplasmic lysates of macrophages, from which mRNAs were isolated. The amount of each target mRNA bound by AUF1 was determined by real-time qRT–PCR using specific primers. TNFα, IL-1β, and Cyclooxygenase 2 (Cox2) mRNAs were detected in both AUF1 and elf4G immunoprecipitate (Fig. 5D), the latter of which was consistent with the interaction of elf4G with actively translated mRNAs. In contrast, IL-6 mRNA and granulocyte-colony stimulating factor [G-CSF] mRNA, a non-ARE mRNA, were found associated only with elf4G, but not with AUF1 (Fig. 5D). These results indicate that AUF1 proteins selectively bind to TNFα, IL-1β, and Cox2 mRNAs, all of which contain overlapping AUUUA pentamers. The 3’ untranslated regions of TNFα and IL-1β mRNAs differ from that of IL-6 in containing multiple consecutive copies of the AUUUA pentamer, whereas IL-6 contains only two pentamers, and they are interrupted by intervening sequences. It is likely that in a natural physiological setting in the absence of AUF1 ectopic overexpression, AUF1 binding requires the consecutive placement of multiple AUUUA sequences in vivo.

To determine whether disruption of AUF1 affects the expression of other ARE-binding proteins that have been implicated in regulating ARE-mRNA stability, we examined their expression levels in isolated peritoneal macrophages by the immunoblot analysis, as described above. The expression levels of major ARE-binding proteins [TTP, HuR, KSRP, TIA-1] and control protein [β-tubulin] remained unaltered in AUF1−/− macrophages [Fig. 5E]. Thus, the increased stability of TNFα and IL-1β mRNAs appears to be a direct consequence of loss of AUF1. Collectively, these data demonstrate that AUF1 governs the inflammatory response and prevents the excessive production of proinflammatory cytokines TNFα and IL-1β by promoting decay of their mRNAs.

Blocking TNFα and IL-1β activity protects AUF1 knockout mice from severe endotoxic shock

To confirm that the LPS-induced endotoxic shock phenotype in AUF1−/− mice is due to overproduction of TNFα and IL-1β, animals were injected with neutralizing antibodies against TNFα and IL-1β prior to a sublethal LPS challenge. The general condition of each mouse was monitored, and a condition score was derived based on numeric manifestations of endotoxemia, including lethargy, diarrhea, tachypnea, piloerection, and death, as described in the Materials and Methods section. Whereas IgG-injected AUF1−/− mice showed typical signs of endotoxic shock observed earlier, animals treated with an-
IgG- or antibody-injected AUF1 shock by TNF. Blood clot formation. Note the dramatically reduced blood clot
sections of organs 24 h following LPS challenge to visualize in IgG-injected score is plotted. Asterisks indicate death of two out of four mice
48-, and 76-h time points (p significantly lower than those of the other three groups at the 42-, 3180 GENES & DEVELOPMENT

Figure 6. Protection of AUF1+ mice from severe endotoxic shock by TNFα and IL-1β antibodies. (A) The condition score of
IgG- or antibody-injected AUF1 knockout (KO-IgG; KO-Ab) and wild-type (WT-IgG; WT-Ab) mice after LPS challenge. Mice were given either IgG or antibody against TNFα and IL-1β prior to LPS challenge as described in Materials and Methods [n = 4 in each group]. The general condition of each mouse was monitored and scored based on the manifestations of endotoxemia including lethargy, diarrhea, tachypnea, and piloerection [score scale 0–10: 10 for normal activity of wild-type mice and 0 for death]. The condition score of IgG-treated AUF1+ group is significantly lower than those of the other three groups at the 42-, 48-, and 76-h time points [p < 0.01]. Mean value of condition score is plotted. Asterisks indicate death of two out of four mice in IgG-injected AUF1+ group. (B) Representative H&E-stained sections of organs 24 h following LPS challenge to visualize intravascular coagulation in mice injected with antibodies to TNFα, IL-1β, or IgG [n = 4 in each group]. Arrowheads indicate blood clot formation. Note the dramatically reduced blood clot formation in AUF1+ mice treated with TNFα and IL-1β antibody compared with those treated with IgG. (C) BUN and AST levels in mice 24 h following challenge with LPS [n = 3, p < 0.08 for AST levels in AUF1 knockout mice, p < 0.06 for BUN levels in AUF1 knockout mice].

Discussion

In this study we report that AUF1 protects animals from fatal progression of endotoxemia to endotoxic shock by attenuating the expression of proinflammatory cytokines TNFα and IL-1β through facilitated degradation of their mRNAs. This was shown by the abnormal stabilization of TNFα and IL-1β mRNAs in AUF1−/− macrophages after LPS stimulation compared with wild-type macrophages. These data indicate that AUF1 specifically promotes TNFα and IL-1β mRNA decay. We further confirmed that excessive proinflammatory cytokine production is due to loss of AUF1, and is responsible for development of endotoxic shock in AUF1−/− mice. The role of AUF1 in regulating inflammatory cytokine expression in vivo was shown by targeted disruption of the mouse AUF1 gene, which resulted in increased mortality during experimentally induced endotoxemia. The increased lethality of AUF1 knockout animals was shown to result from excessive production of TNFα and IL-1β, two critical mediators of endotoxic shock. Overexpression of these two proinflammatory cytokines following LPS challenge was found to be due to the inability to rapidly degrade TNFα and IL-1β mRNAs following their induction. Thus, we have identified AUF1 as a critical attenuator of proinflammatory cytokine expression.

Notably, loss of AUF1 did not cause a severe defect in mouse development. Targeted disruption of TTP, another ARE-binding protein that also regulates TNFα mRNA stability, causes a syndrome of cachexia, arthritis, and autoimmunity (Taylor et al. 1996). Although
AUFI and TTP are both ARE-binding proteins involved in regulating ARE-mRNA stability and both are found in immune cells (Carballo et al. 1998; Loflin et al. 1999; Sarkar et al. 2003; Lu and Schneider 2004), they demonstrate different phenotypes in knockout mice. TTP knockout mice develop several abnormalities in hematopoietic systems, particularly a smaller thymus without cortical/medullary organization (Carballo et al. 1998). In contrast, thymus development in AUFI-deficient mice is normal (data not shown). It has also been reported that in peripheral blood of TTP−/− mice, the white blood cell count is elevated by more than twofold over that of wild-type mice, and there is a marked increase in the number of circulating neutrophils and macrophages (Taylor et al. 1996). In contrast, in AUFI−/− mice, similar numbers of hematopoietic cells are found in peripheral blood compared with wild-type mice, and no change was observed in macrophages in peritoneum and spleen (Fig. 4). These observations suggest that AUFI may have different regulatory effects and different functions than TTP in the ARE-mRNA decay pathway. The phenotype of TTP−/− mice is likely the consequence of chronic excessive TNFα circulating in the blood (Taylor et al. 1996). Thus, TTP may constitutively target TNFα mRNA for degradation, and loss of TTP leads to a chronic and constant accumulation of TNFα mRNA and its overproduction. Our data from AUFI knockout mice suggests that AUFI may be a regulatory factor that normally does not execute its function until the activation of proinflammatory cytokine expression. In particular, in the absence of LPS-challenge, AUFI knockout mice did not display elevated levels of TNFα or IL-1β, and AUFI promoted the rapid decay of TNFα and IL-1β mRNAs only after their induction in response to endotoxin challenge. AUFI-directed mRNA degradation may therefore serve as a protective means to attenuate the inflammatory response post-stimulation, and to protect against deleterious effects of excessive inflammation.

The increased susceptibility of AUFI−/− mice to endotoxin challenge is reminiscent of the phenotype of mice lacking the TIA-1 gene (Piecyk et al. 2000), which encodes an ARE-binding protein that specifically binds the TNFα-ARE and represses TNFα translation. Increased production of TNFα in TIA-1−/− mice is mainly due to derepression of TNFα mRNA translation, as no alteration in TNFα mRNA stability is detected. In contrast, we found that abnormal stabilization of TNFα and IL-1β mRNAs contributes to their overexpression in AUFI−/− mice. The difference in the mechanisms by which AUFI and TIA-1 control TNFα production implies that a precise control of cytokine expression demands regulation at multiple levels.

At this time, we cannot formally rule out a hypothetical role for AUFI in regulating both the stability and translation of TNFα and IL-1β mRNAs. However, previous work on TTP and TIA-1 (Kontoyiannis et al. 1999, Piecyk et al. 2000) indicates that the translation repression and stability of ARE-mRNAs are independently controlled by different ARE-binding proteins. These findings suggest that AUFI may mainly control the stability of target mRNAs. Given the overlapping tissue distribution of decay-promoting AUFI and stability-promoting HuR (Lu and Schneider 2004), as well as the binding of AUFI and HuR to common target mRNAs (Lal et al. 2004), loss of AUFI might function in part by allowing HuR to play a more active role in stabilizing target mRNAs and, consequently, causing their overexpression.

Despite previous reports that mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 signaling regulates IL-6 mRNA stability and its biosynthesis via the AU-rich 3′ noncoding region (Winzen et al. 1999; Neininger et al. 2002), we did not detect a significant alteration in IL-6 mRNA stability in AUFI-deficient cells (Fig. 5C). Our results suggest that AUFI more selectively regulates TNFα and IL-1β mRNA stability, and that IL-6 mRNA may not be controlled by AUFI under normal physiological conditions (without AUFI overexpression). mRNAs targeted for rapid decay by ARE elements, such as TNFα and IL-1β mRNAs, often contain three to five uninterrupted copies of an AUUUA pentamer in the 3′ noncoding region (Guhaniyogi and Brewer 2001). IL-6 mRNA, however, contains no consecutive AUUUA pentamers. Instead, its 3′ noncoding region contains two pentamers interspersed in an AU-rich region with varying U stretches of 2–5 nucleotides in length. We speculate that multiple consecutive AUUUA pentamers may be important for AUFI to interact with target ARE-mRNAs and regulate their stability. Consistent with this hypothesis, Cox2 mRNA, a class II ARE-mRNA harboring multiple overlapping AUUUA pentamers, was found to associate with AUFI in vivo (Fig. 5D) and its half-life was increased at least 50% in AUFI-deficient macrophages derived from AUFI knockout mice (N. Sadri and R. Schneider, unpubl.). It is also possible that IL-6 mRNA stability is regulated by different AUFI isoforms with opposing functions so that inactivation of all four AUFI isoforms may obscure the effect on the target mRNA. It has been suggested that the different AUFI isoforms possess different, or perhaps even opposing functions in regulating ARE-mRNA decay (Laroia et al. 1999; Loflin et al. 1999; Sarkar et al. 2003; Raineri et al. 2004). With the availability AUFI-deficient cells, these questions can now be experimentally addressed.

In summary, our results provide the first in vivo evidence for a novel post-transcriptional mechanism critical for controlling proinflammatory cytokine expression and protecting against fatal endotoxic shock after endotoxin exposure. Our findings indicate that after proinflammatory cytokine induction, AUFI promotes decay of TNFα, IL-1β, and possibly other inflammatory cytokine mRNAs as a means for controlling and tempering the inflammatory response. Defects in this post-transcriptional regulation may be involved in human inflammatory diseases.

Materials and methods

Generation of AUFI−/− mice

A 1.4-kb genomic fragment upstream of mouse AUFI exon 3, and a 7.9-kb genomic fragment downstream from AUFI exon 3,
were used as short and long recombination arms, respectively, in the construction of the targeting vector. A foxp2-flanked neo expression cassette was inserted between the two regions, resulting in a vector designed to delete the two RNA-binding motifs in exon 3. W4 embryonic stem (ES) cells were electroporated with the NotI-linearized targeting construct, selected in G418 (150 µg/mL) and gancyclovir (2 mM), and used for Southern blot DNA analysis. Five positive clones were identified using Southern blot and PCR analysis. Blastocyst injection was performed using two independent targeted ES cell clones. Germ-line transmission was obtained on further crossing of male chimeras with C57BL/6J females.

**Animal studies and histology**

Experiments were carried out in accordance with NIH guidelines for animal treatment, housing, and euthanasia. Mice were challenged by intraperitoneal LPS injection [low dose: 3 mg/kg weight, sublethal dose: 20 mg/kg] and monitored for general condition and survival. At indicated times, mice were euthanized and blood serum was collected. Blood BUN and AST levels were determined using Infinity BUN Reagent and AST Reagent (Sigma Diagnostics), respectively. For histological analysis, tissues were fixed in 10% buffered formaldehyde overnight at 4°C and paraffin-embedded. Sections (5 µm) were stained with hematoxylin and eosin (H&E). To determine the general condition of mice after LPS challenge, mice were monitored blindly (without knowing their genotypes) and individual scores were given based on the symptoms of endotoxemia [motor activity/coordination (0–2, normal being 2), strength (0–2, normal being 2), food intake (0–1, normal being 1), ocular exudate (0–2, no exudates denoting 2), diarrhea (0–1, no diarrhea being 1), piloerection/coat appearance (0–1, normal being 1), and breath rate (0–1, 0 denoting slowed rate)]. The condition score of a particular mouse is the summed score of individual symptom scores (10 being normal activity and 1 denoting close to death).

**Quantification of cytokine expression**

Serum cytokine levels and cytokines secreted from primary macrophages were determined by ELISA. Equal numbers of peritoneal macrophages derived from three mice were pooled and seeded in three replicative wells (5 x 10^6 cells/well). Peritoneal cells were incubated at 37°C in an atmosphere of 5% CO₂ for 3 h to allow peritoneal macrophages to adhere. Nonadherent cells were removed by washing with PBS twice, and then macrophages were stimulated with 1 μg/mL LPS (Sigma) for the times indicated. Levels of TNFα and IL-1β were measured by ELISA (eBioscience) using an equal amount of culture supernatant at indicated time points (Fig. 3C), or at 8 h after LPS treatment for TNFα and 24 h after LPS treatment for IL-1β (Fig. 3B).

**Determination of mRNA half-life**

For decay studies, peritoneal macrophages were stimulated with LPS (1 μg/mL) for 4 h. After 4 h of LPS stimulation, actinomycin D (2 μg/mL) was added to block transcription, and total RNA was isolated after 0, 20, and 40 min post-actinomycin D treatment for TNF-α measurements, or after 0, 30, and 60 min post-actinomycin D treatment for IL-1β and IL-6 measurements. mRNA levels were determined by quantitative real-time RT–PCR (qRT–PCR, Roche Light Cycler), and were normalized to Cyc A mRNA as a control. PCR reactions were performed with gene-specific primers [primer sequences available upon request]. The plots (Fig. 5A–C) average three independent experiments for each mRNA to determine mRNA half-lives and standard deviation of results.

**TNF-α and IL-1β antibody administration studies**

AUFI–/– and AUFI+/+ male mice each received intraperitoneal injection of 200 μg of hamster monoclonal antibody to mouse TNFα [clone TN5-19.12, BD PharMingen] and 100 μg of hamster monoclonal antibody to mouse IL-1β [clone B122, BioLegend]. Mice of each genotype in the control group received 100 μg of hamster isotype IgG control [clone A19-3, BD PharMingen]. Six hours post-antibody injection, mice in both groups received an intraperitoneal injection of a sublethal dose of LPS (20 mg/kg). Mice were monitored for general condition and survival, as described in the above animal studies and histology section. Measurement of BUN and AST levels, and histological analyses were performed as described above.

**Immunoblot analysis and IP qRT–PCR**

Immunoblot analysis was performed following standard procedures. Polyclonal antibodies against AUFI, KSRP (from D. Black), and TIA-1 (from P. Anderson), and specific antibodies for HuR (Santa Cruz Biotechnology), TTP (Santa Cruz Biotechnology), and β-tubulin (Sigma) were used. ECL Western Blotting Detection Kit (Amersham Pharmacia) was used for detection. IP qRT–PCR was performed using anti-AUFI and anti-elf4G polyclonal antibody according to a protocol previously described (Sarkar et al. 2003).

**Analysis of immune cells**

Peripheral blood analysis was performed by Analytics, Inc. Spleen and peritoneal cells were made into single-cell suspensions in RPMI 1640 medium by passing through a 70-µm nylon cell strainer. The cell suspensions were depleted of erythrocytes by osmotic lysis, and cells were incubated on ice for 15 min with anti-CD16/CD32 [BD PharMingen] to block the Fc receptor. Subsequently, these cells were incubated with anti-F4/80-PE (clone A19-3, BD PharMingen) and 100 µg of hamster isotype IgG control [clone A19-3, BD PharMingen]. Six hours post-antibody injection, mice in both groups received an intraperitoneal injection of a sublethal dose of LPS (20 mg/kg). Mice were monitored for general condition and survival, as described in the above animal studies and histology section. Measurement of BUN and AST levels, and histological analyses were performed as described above.

**Statistical analysis**

Data are presented as mean ± SD for statistical comparison of two samples, the Student t-test was used for evaluation. Survival curves were generated using the Kaplan–Meier method, and significance was evaluated using the Log-rank test.

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Endotoxic shock in *AUF1* knockout mice mediated by failure to degrade proinflammatory cytokine mRNAs

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