Heat Shock Transcription Factor 1 Inhibits Expression of IL-6 through Activating Transcription Factor 3

Ryosuke Takii, Sachiyi Inouye, Mitsuaki Fujimoto, Tamami Nakamura, Toyohide Shinkawa, Ramachandran Prakasam, Ke Tan, Naoki Hayashida, Hitoshi Ichikawa, Tsonwin Hai and Akira Nakai

J Immunol published online 16 December 2009
http://www.jimmunol.org/content/early/2009/12/16/jimmunol.0902579

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/12/15/jimmunol.0902579.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Heat Shock Transcription Factor 1 Inhibits Expression of IL-6 through Activating Transcription Factor 3

Ryosuke Takii,* Sachiyu Inouye,*1 Mitsuaki Fujimoto,* Tamami Nakamura,* Toyohide Shinkawa,* Ramachandran Prakasam,* Ke Tan,* Naoki Hayashida,* Hitoshi Ichikawa,† Tsonwin Hai,‡ and Akira Nakai*

The febrile response is a complex physiological reaction to disease, including a cytokine-mediated increase in body temperature and the activation of inflammatory systems. Fever has beneficial roles in terms of disease prognosis, partly by suppressing the expression of inflammatory cytokines. However, the molecular mechanisms underlining the fever-mediated suppression of inflammatory gene expression have not been clarified. In this study, we showed that heat shock suppresses LPS-induced expression of IL-6, a major pyrogenic cytokine, in mouse embryonic fibroblasts and macrophages. Heat shock transcription factor 1 (HSF1) activated by heat shock induced the expression of activating transcription factor (ATF) 3, a negative regulator of IL-6, and ATF3 was necessary for heat-mediated suppression of IL-6, indicating a fever-mediated feedback loop consisting of HSF1 and ATF3. A comprehensive analysis of inflammatory gene expression revealed that heat pretreatment suppresses LPS-induced expression of most genes (86%), in part (67%) via ATF3. When HSF1-null and ATF3-null mice were injected with LPS, they expressed much higher levels of IL-6 than wild-type mice, resulting in an exaggerated febrile response. These results demonstrate a novel inhibitory pathway for inflammatory cytokines. The Journal of Immunology, 2010, 184: 000–000.

Inflammatory cytokines, such as IL-1 and -6 and TNF-α, are produced in response to bacterial infections and disease and elicit the febrile response, a complex physiological reaction to disease, including a cytokine-mediated increase in body temperature and the activation of inflammatory systems (1, 2). Fever plays beneficial roles in the clinical prognosis of disease (3, 4). Experimentally, the pretreatment of animals with heat shock also increased survival in a LPS-injected endotoxic model (5, 6). These beneficial roles of fever are mediated partly by a strengthening of immune surveillance through lymphocyte trafficking (7), as well as by the suppression of pyrogenic and inflammatory cytokines, such as IL-1 and TNF-α, in the cell (8–10) and body (6, 11). However, it is unclear whether hyperthermia inhibits the expression of IL-6 as it affects IL-6 and TNF-α expression differently in macrophages (10).

One major adaptive response to high-stress temperature in all living organisms is the production of heat shock proteins (HSPs) that assist protein folding and inhibit protein denaturation (12). This response is regulated mainly at the level of transcription by heat shock transcription factor 1 (HSF1) (13–15). HSF1 protects cells from exposure to extreme temperatures, by inducing the expression of HSP (16–19), and from various pathophysiological conditions, such as age-related neurodegenerative diseases (20–23). Because HSF1 cannot sense even a mild increase in temperature, it has been suggested to play roles in the regulation of genes for inflammatory cytokines (24). In fact, analysis in vivo using an LPS-injected endotoxic model showed that mortality is high in mice lacking HSF1, which is associated with an increase in the serum TNF-α level (25). Furthermore, HSF1 inhibits the expression of cytokines by binding directly to the TNF-α promoter (26) or by physically interacting with NF-κB, an activator for IL-1β (27). However, we do not know the involvement of heat shock or HSF1 in the expression of hundreds of inflammatory genes, including IL-6, or the mechanisms by which HSF1 suppresses the expression of these genes.

We previously found that HSF1 directly binds to the IL-6 promoter to open the chromatin structure in the absence of heat shock (28, 29). Thus, HSF1 is required for the LPS-mediated maximal expression of IL-6 in macrophages and spleen cells. In this study, we extended our previous findings and examined the molecular links between heat shock and inflammatory responses. Unexpectedly, we found that activation of HSF1 inhibited LPS-induced IL-6 expression in macrophages and mouse embryonic fibroblast (MEF) cells. The activated HSF1 induced the expression of activating transcription factor (ATF) 3, a negative regulator of the IL-6 gene (30). Furthermore, the HSF1-ATF3 pathway was required for heat-mediated suppression of inflammatory genes, including IL-6, in cultured cells and in mice.

Materials and Methods

Cell cultures and treatments

Primary cultures of wild-type, HSF1−/− (19) and ATF3−/− (31) MEFs were prepared and maintained at 37°C in 5% CO2 in DMEM containing 10% FBS. Macrophages were collected as adherent peritoneal cells (28).
Cells were treated with heat shock at 42˚C for 1 h and then with LPS Escherichia coli 0127:B8 (Sigma-Aldrich, St. Louis, MO) (1 µg/ml) for specific periods. IL-6 levels in culture media were determined using an ELISA kit (Invitrogen, Carlsbad, CA).

Northern blot analysis and RT-PCR

Total RNA was isolated from MEF cells or tissues using TRizol (Invitrogen); a Northern blot analysis was performed, as described previously, using [32P]-labeled cDNA probes for mouse IL-6, HSP70-1, and β-actin (28) and a [32P]-labeled BamHI/EcoRI fragment of pcDNA3.1-mATF3 (29). An autoradiographic image on Fuji medical X-ray film (Super HR-HA, Fujiﬁlm, Tokyo, Japan) was scanned, and levels of mRNA were estimated using the NIH Image program. RT-PCR analysis of mRNA levels was performed essentially as described previously (28), using specific primers for inflammatory genes (Supplementary Table I). The amplified DNA was stained with ethidium bromide and photographed using an Epi-Light UV FA1100 system (Aisin Cosmos R&D, Kariya, Japan).

Western blot analysis

Cell extracts were prepared from MEF cells and peritoneal macrophages in NP-40 lysis buffer (29) and subjected to Western blotting using a rabbit antiserum for hHSF1 (α-mHSF1) (32), a rabbit polyclonal IgG for ATF3 (C-19; Santa Cruz Biotechnology, Santa Cruz, CA), a mouse monoclonal IgG for HSP70 (W27; Santa Cruz), GFP (Nacalai Tesque, Kyoto, Japan), or β-actin (AC-15; Sigma-Aldrich). Signals were detected using ECL Western blotting detection reagents (General Electric, Fairfield, CT).

Adenoviral infection. Primary cultures of MEF cells were plated on 60-mm dishes and incubated in 2 ml serum-free DMEM containing adenosine (2 × 10−5 PFU/ml) for GFP, human HSF1 (hHSF1), an active hHSF1 (hHSF1ΔRDRT), or an inactive hHSF1 (hHSF1/R17G or hHSF1ΔAB) (29) for 2 h. After adding 2 ml DMEM containing 20% FBS, cells were maintained for 48 h and then treated with LPS for 2 or 4 h. Cell extracts were prepared in NP-40 lysis buffer for Western blot analysis, or total RNA was isolated from the cells for Northern blot analysis. To restore ATF3 expression, ATF3-null MEF cells were infected with an adenosine expressing ATF3 (29), as described above, and treated with heat shock at 42˚C and/or LPS (1 µg/ml).

Reporter analysis

A DNA fragment of region 1 (~418 to +69) relative to the transcription start site in the ATF3 promoter was isolated by PCR using genomic DNA of mouse T24 ES cells as a template. Primers used were ATF3 pro5-2: 5′-CTA AGC TTT TAG AGA AAG GTC GTG CCC GC -3′ and ATF3 pro3: 5′-GTG TCG ACA GCG CCG GTG TGC ACC CCT TT-3′ (boldface nucleotides are HindIII and SalI recognition sites, respectively). The fragments were inserted upstream of the HSV-thymidine kinase promoter of ptk-galp3-luc at the HindIII/SalI site to generate a pATF3-luc reporter plasmid. We deleted a region containing putative heat shock elements (HSEs) HSE2 to HSE4 (~390 to ~356) (pATF3-luc/ml) or a region containing putative HSE5 to HSE9 (~305 to ~249) (pATF3-luc/ml). A DNA fragment of the HSP70 promoter (~517 to +21) was amplified by PCR and inserted into ptk-galp3-luc at the SalI/BamHI site to generate the hHSF70-luc reporter plasmid. HEK293 cells were transfected with 2 µg per plate of the reporter plasmid and 2 µg pGut-Z as an internal control by the calcium phosphate method. At 4 h after the transfection, cells were washed with PBS and incubated for an additional 48 h in normal medium. After heat shock at 42˚C for 1 h and recovery at 37˚C for 8 h, cell extracts were prepared, and luciferase and β-galactosidase activities were examined. To examine the combined effects of LPS treatment and physiological heat shock, HEK 293 cells stably expressing TLR 4 (a gift from Dr. K. Miyake, the Institute of Medical Science, the University of Tokyo) were transfected with plasmids as described above, heat-shocked at 40˚C for 1 h, and allowed to recover for 4 h in the absence or presence of LPS.

Microarray analysis

Total RNA was prepared from two MEF cell cultures derived from two independent embryos at embryonic day 15.5 (ICR mice). Cells were maintained at 37˚C (control), treated with LPS for 4 h, or treated with LPS for 4 h after pretreatment with heat shock at 42˚C for 1 h. Gene expression was analyzed using a GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) containing ~45,000 probe sets. Target cRNA was prepared from 1 µg total RNA with a One-Cycle cDNA Synthesis Kit and 3′-Amplification Reagents for IVT Labeling (Affymetrix). Hybridization to the microarrays, washing and staining, and scanning were performed according to the manufacturer’s instructions. The scanned image data were processed using the GeneChip Operating Software version 1.4 (Affymetrix), and the change (increase or decrease) for each probe set was evaluated by a Comparison Analysis with the software.

Chromatin immunoprecipitation

Primary MEF cells were exposed to heat stress at 42˚C for 1 h, and a chromatin immunoprecipitation (ChIP) assay was performed using a kit (Upstate Biotechnology, Lake Placid, NY), according to the manufacturer’s instructions (31). An antiserum for HSF1 (α-HSF1c) was used to precipitate complexes of HSF1 and DNA fragments (29). ChIP-enriched DNA was amplified using primers corresponding to region 1 in the ATF3 promoter (Supplemental Fig. 2) or primers corresponding to the IL-6 promoter (~565 to ~827) (29). A rabbit polyclonal IgG for ATF3 (C-19)
was used to detect the binding of ATF3 to the IL-6 promoter (−230 to +31) in vivo (29). The amplified DNA was stained with ethidium bromide, photographed, scanned, and quantified by NIH Image.

**LPS treatment in mice**

HSF1+/− (19), ATF3+/− (32), and IL-6−/− mice (The Jackson Laboratory, Bar Harbor, ME) mice were maintained through crosses with ICR mice. The animals were kept at 24°C with lights on from 08:00 to 20:00. To analyze responses to LPS treatment, 12-wk-old mice were injected i.p. with PBS as a control or with 40 µg/kg LPS and 400 mg/kg D-(-)-galactosamine hydrochloride (GalN) (Sigma-Aldrich) (33) at zero time (10:00AM). Mice were killed by cervical translocation, and tissues were removed for Northern blot analysis. Serum IL-6 levels were determined using an ELISA kit, as described previously (28). All experimental protocols were reviewed by the Committee for Ethics on Animal Experiments of Yamaguchi University Graduate School of Medicine.

**Measurement of body temperature**

Core body temperature was monitored using telemetry. We used a commercially available data-acquisition system composed of an implantable telemetry transmitter (PhysioTel TA10ETA-F20, Data Science International, St. Paul, MN) and a receiver (PhysioTel Receiver RPC-1) placed under the cage of each animal and connected to a data-acquisition matrix. Ten days before the experiments, 6-wk-old male mice were anesthetized with an i.p. administration of Nembutal (500 mg/kg) (Dainippon Pharmaceutical, Osaka, Japan) and implanted with a transmitter. Mice were injected i.p. with LPS at 50 or 150 µg/kg (34) at 10:00 AM. Body temperature was recorded at 10-min intervals, beginning ≥24 h before, and continuing for ≥24 h after the injection of LPS.

**Statistical analysis**

Values were tested for significance with the Student t test or two-way ANOVA. p < 0.05 was considered significant.

**Results**

**LPS-induced IL-6 expression is suppressed through HSF1 activation**

We found that the LPS-induced expression of IL-6 was suppressed by pretreatment with heat shock at 42°C for 1 h like that of TNF-α and IL-1β in macrophages (Fig. 1A). LPS-induced IL-6 mRNA expression and protein production were also inhibited by the same heat pretreatment in MEFs for 12 and 24 h, respectively (Fig. 1B, 1C). We next examined the involvement of HSF1 in the heat-mediated suppression of LPS-induced IL-6 expression. Overexpression of the wild-type or actively mutated HSF1ARDT suppressed the LPS-induced expression of IL-6 mRNA, similar to the pretreatment with heat shock, whereas overexpression of two nonfunctional mutants, HSF1R71G and HSF1ΔAB, had no effect (Fig. 1D). These results demonstrate that LPS-induced IL-6 expression is suppressed through the activation of HSF1.

**FIGURE 2.** Synergistic effect on ATF3 expression by heat shock and LPS. A, Expression of ATF3 after heat shock. Wild-type and HSF1-null primary MEF cells were heat-shocked at 42°C for the indicated periods, and a Northern blot analysis was performed (upper panel). Cells were heat-shocked at 42°C for 1 h and then kept at 37°C for 0, 1, or 2 h to recover. Cell extracts were prepared in NP-40 lysis buffer and subjected to a Western blot analysis (lower panel). B, Total RNA was prepared from MEF cells heat-shocked at 40°C (HS), treated with LPS at 37°C for 2 h (LPS), or treated with LPS at 40°C (HS+LPS) for 2 h. A Northern blot analysis was performed (n = 3). sp < 0.05 versus control (C); Student t test. C, Cell extracts were prepared in NP-40 lysis buffer from MEF cells heat-shocked at 40°C (HS), treated with LPS at 37°C (LPS), or treated with LPS at 40°C (HS+LPS) for the indicated periods. Levels of ATF3 protein were estimated by Western blot analysis; mean ± SD compared with levels in untreated cells are shown (n = 3). sp < 0.05 versus LPS; ANOVA. D, MEF cells were infected with an adenovirus expressing GFP, HSF1, or an hHSF1 mutant (ARDT, R71G, or ΔAB) for 48 h and then treated with LPS for 2 h. A Northern blot analysis was performed; ATF3 mRNA levels compared with those in untreated GFP-expressing cells are shown (n = 3). sp < 0.05 versus GFP with LPS for 2 h; Student t test. E, ChIP-enriched DNA was prepared using preimmune serum (p.i.) or anti-HSF1 Ab (1c) from MEF cells not treated (Cont.) or treated with heat shock at 42°C for 1 h (HS). DNA fragments of the R1 region in the ATF3 promoter (a, −425 to −276; b, −321 to −159; c, −210 to +80; d, −396 to −299) were amplified by PCR. F, Reporter analysis of the ATF3 promoter. HEK293 cells transfected with reporter plasmids were not treated (Cont.) or were incubated at 42°C for 1 h and then allowed to recover for 4 h (HS). Luciferase (LUC) activity relative to that in cells transfected with ptk-luc is shown (n = 3). sp < 0.05; Student t test. G, HEK293 cells expressing TLR4 were transfected with reporter plasmids for 48 h and were left untreated (Cont.), incubated at 40°C for 1 h and allowed to recover for 4 h (HS), incubated with LPS for 4 h (LPS), or incubated with LPS at 40°C for 1 h and left for 4 h to recover (HS+LPS). Luciferase (LUC) activity relative to that in untreated cells is shown (n = 3). sp < 0.05; Student t test.
Treatment of cells with LPS and heat shock synergistically induces ATF3 expression

We previously identified genes whose expression increased after heat shock in wild-type and HSF1-null cells (28). Among them, ATF3 had much higher mRNA and protein levels after heat shock at 42°C in wild-type cells than in HSF1-null cells (Fig. 2A, Supplemental Fig. 1). Maximal ATF3 expression was induced in cells treated with severe heat shock at 42°C, irrespective of LPS treatment (data not shown). However, mRNA and protein levels were significantly elevated in cells treated with LPS and heat shock at 40°C compared with levels in cells treated with LPS or heat shock alone (Fig. 2B, 2C). Furthermore, overexpression of wild-type or actively mutated HSF1 promoted LPS-induced expression of ATF3 mRNA similar to the pretreatment with heat shock, whereas the overexpression of non-functional HSF1 mutants had no effect (Fig. 2D), indicating a synergistic induction of ATF3 expression by treatment with LPS and heat shock.

There are ≥17 conserved HSE-like sequences in the mouse and human ATF3 promoter (up to −500 bp) (Supplemental Fig. 2). To identify the HSF1-binding region, we performed a ChIP assay and found that HSF1 binds to at least region d (−396 to −299) of the ATF3 promoter (Fig. 2E), which corresponds to three overlapping HSEs (HSE2–4). Reporter activity was induced after heat shock at 42°C for 1 h in the presence of HSE2–4, but not when HSE2–4 was deleted (Fig. 2F). Furthermore, HSE2–4 is necessary for the synergistic effect on reporter activity of LPS treatment and heat shock at 40°C (Fig. 2G).

ATF3 is required for suppression of LPS-induced IL-6 expression by heat shock

We next examined the expression of IL-6 in ATF3-null MEF cells (32). Levels of IL-6 mRNA were slightly higher up to 6 h after LPS treatment in ATF3-null cells than in wild-type cells (30), and pretreatment at 42°C for 1 h resulted in a marked difference in the levels of IL-6 mRNA between wild-type and ATF3-null cells (Fig. 3A). Surprisingly, pretreatment of ATF3-null cells with heat shock suppressed neither LPS-induced IL-6 mRNA expression nor LPS-induced IL-6 production in the medium (Fig. 3B, 3C). Re-expression of ATF3 in ATF3-null cells partially suppressed the LPS-induced IL-6 mRNA expression and fully reversed the suppressive effects of heat pretreatment on the expression of IL-6 mRNA and protein. Endogenous ATF3 induced to express by heat shock or heat shock plus LPS treatment bound to the IL-6 promoter.

**FIGURE 3.** ATF3 is required for heat-mediated suppression of IL-6 expression. A, Expression of IL-6 in wild-type and ATF3-null MEF cells treated with LPS. Total RNA was isolated from wild-type (ATF3+/+) and ATF3-null (ATF3−/−) cells treated with LPS for the indicated periods (left panel) or with LPS after the pretreatment at 42°C for 1 h (right panel). Northern blot analysis was performed: IL-6 mRNA levels relative to the IL-6 mRNA level in untreated wild-type cells are shown (n = 3). *p < 0.05; ANOVA. B, Wild-type and ATF3-null MEF cells infected with an adenovirus expressing GFP (+Ad-GFP) or ATF3 (+Ad-ATF3) were left untreated (Control), incubated at 42°C for 1 h and allowed to recover for 4 h (HS), incubated with LPS for 4 h (LPS), or incubated with LPS at 42°C for 1 h and left for 4 h (HS+LPS). A Northern blot analysis was performed; relative IL-6 mRNA levels are shown (n = 3) (left panel). IL-6 production in each medium was evaluated by ELISA (n = 3) (right panel). *p < 0.05 versus ATF3−/− cells with LPS; **p < 0.05 versus ATF3−/− cells with HS+LPS; ANOVA. C, Cell extracts were prepared from cells in A and subjected to a Western blot analysis. D, MEF cells were treated as described in B, and a ChIP assay was performed using anti-ATF3 Ab. A DNA fragment of IL-6 (−230 to +31) was amplified by PCR (lower panel) and quantified. Mean ± SD are shown (n = 3) (upper panel). *p < 0.05; Student t test. E, Peritoneal macrophages were incubated with LPS at 37°C for the indicated period with or without heat shock pretreatment at 42°C for 1 h. Cell extracts were subjected to a Western blot analysis. Levels of ATF3 were compared with those in MEF cells infected with Ad-ATF3 (Ad-ATF3) or in cells incubated at 42°C for 1 h and allowed to recover for 4 h. F, Wild-type and ATF3-null peritoneal macrophages infected with an adenovirus expressing GFP (+Ad-GFP) or ATF3 (+Ad-ATF3) were treated as described in B. IL-6 production in each medium was evaluated by ELISA (n = 3) (left panel). *p < 0.05 versus ATF3−/− cells with HS+LPS; ANOVA. Levels of ATF3 protein in wild-type cells treated with LPS after heat shock and in ATF3-null cells infected with Ad-ATF3 were examined by Western blotting (right panel).
in vivo at 4 h after the heat treatment (Fig. 3D). However, the overexpression of ATF3 was not sufficient for the binding, and heat shock promoted the binding to the IL-6 promoter, probably because the nuclear translocation of ATF3 is accelerated by heat shock (Supplemental Fig. 3). We next examined the expression of ATF3 and IL-6 in peritoneal macrophages and found that pretreatment at 42˚C for 1 h markedly promoted LPS-induced ATF3 expression (Fig. 3E) and that pretreatment with heat shock barely suppressed LPS-induced IL-6 production in macrophages lacking ATF3 (Fig. 3F). These results clearly indicate that ATF3 is required for the suppression of LPS-induced IL-6 expression by heat shock and exclude the possibility that the heat pretreatment impaired gene expression or signal transduction in a nonspecific manner.

**Differential roles of HSF1 in inflammatory gene expression**

HSF1 directly or indirectly inhibits the expression of TNF-α and IL-1β (26, 27). Therefore, we next examined whether the HSF1-ATF3 pathway is involved in heat-mediated suppression of inflammatory gene expression. We performed a comprehensive analysis of gene expression using a DNA microarray to identify inflammatory genes whose expression is suppressed by heat shock. We found 100 genes that showed a >3-fold increase in expression on LPS treatment (Fig. 4A). Remarkably, 86 genes (86%) exhibited a <2-fold increase after combined treatments with heat shock and LPS in MEF1 and MEF2 cells. Although IL-6 was not one of these 86 genes, its expression increased 6.8-fold in MEF1 cells treated with LPS but only 2.6-fold in the cells exposed to heat shock and LPS (GEO record GSE16266). Among 40 genes whose expression increased >8-fold in the cell culture treated with LPS but <2-fold in response to heat shock and LPS, 24 genes were confirmed by RT-PCR to be markedly suppressed in their LPS-induced expression on pretreatment with heat shock (Fig. 4B). The LPS-induced mRNA expression of 16 (67%) of these genes was not suppressed in ATF3-null cells, even in the presence of heat pretreatment like that of IL-6, and re-expression of ATF3 in ATF3-null cells partially suppressed the induction (Fig. 3B).

**FIGURE 4.** Heat shock suppresses LPS-induced expression of inflammatory genes. A, Identification of inflammatory genes suppressed by heat shock. Total RNA was isolated from two independent MEF cells maintained at 37˚C (Control), treated with LPS for 4 h, or treated with LPS for 4 h after heat shock at 42˚C for 1 h (HS+LPS). One hundred genes that exhibited a >3-fold increase (LPS/control) (upper panel) and 116 genes that showed a >3-fold increase (HS+LPS/control) (lower panel) in expression were analyzed using a microarray. B, Identification of inflammatory genes whose expression was suppressed by ATF3. Wild-type (ATF3+/+) and ATF3-null (ATF3−/−) MEF cells were left untreated (C), incubated with LPS for 4 h (L), or incubated with LPS at 42˚C for 1 h and allowed to recover for 4 h (H+L) (left panel). ATF3-null cells were infected with an adenovirus expressing GFP or ATF3 and then left untreated (C) or treated with LPS for 4 h (L) (right panel). RT-PCR was performed, and representative data are shown. Seventeen ATF3-dependent genes (green and red) and eight ATF3-independent genes (blue) are indicated. The level of β-actin mRNA is shown as an internal control. C, HSF1 is required for maximal expression of a set of inflammatory genes. Wild-type (HSF1+/+) and HSF1-null (HSF1−/−) MEF cells were left untreated (C) or treated with LPS for 4 h (L). Results of RT-PCR are shown. Maximal expression of NOS2, ICAM1, and IL-6 genes required HSF1 (red), whereas that of PTGES did not (green). D, Wild-type and HSF1-null MEF cells were left untreated (control) or treated with tri-chostatin A (TSA) for 48 h and then incubated with LPS for 6 h. An RT-PCR assay of IL-6, NOS2, and ICAM1 mRNAs was performed, and levels of mRNA relative to the level in untreated cells are shown (n = 3). *p < 0.05 versus HSF1−/− cells with LPS; ANOVA. E, A ChIP assay was performed using anti-HSF1 Ab. DNA fragments of the IL-6, NOS2, and ICAM1 promoters were amplified by PCR. F, A ChIP assay was performed using anti-H3K9Ac Ab or H3K9Me2 Ab. DNA fragments of IL-6, NOS2, and ICAM1 promoters were amplified by PCR and quantified (n = 3). *p < 0.05; Student t test.
These results demonstrate that the expression of the 16 genes is ATF3 dependent. In contrast, a deficiency of ATF3 only slightly affected the LPS-induced expression of the other eight genes (33%), including TNFα and IL-1β, and re-expression of ATF3 in ATF3-null cells had no effect on the LPS-induced expression of these genes (Fig. 3B).

Furthermore, we found that HSF1 is required for the maximal mRNA expression of 8 ATF3-dependent genes (33%) (Fig. 4B, 4C). Pretreatment of HSF1-null MEF cells with trichostatin A, a histone deacetylase inhibitor, restored the LPS-induced expressions of NOS2, ICAM1, and IL-6 mRNAs (Fig. 4D). Because the promoters of NOS2 and ICAM1 have been characterized (35, 36), we analyzed them and found that HSF1 bound directly to them (Fig. 4E, Supplemental Fig. 4), promoting the acetylation and inhibiting the methylation of histone H3K9 (Fig. 4F). These results indicate that HSF1 regulates the expression of a set of inflammatory genes indirectly, by activating ATF3, and directly, by binding to the promoters, which facilitates the binding of ATF3 to the promoters through chromatin opening.

**Febrile response is augmented in HSF1-null and ATF3-null mice**

We next examined the impact of HSF1 on IL-6 expression in mice injected i.p. with LPS. The IL-6 mRNA level in each tissue reached a peak at 2 h after the injection in wild-type mice and then declined quickly (Supplemental Fig. 5). Remarkably, these levels were much higher in the heart, liver, and kidney of HSF1-null mice than in the same tissues of wild-type mice, whereas they were only slightly higher in the spleen, probably as a result of the effect of HSF1 on chromatin (28, 29). As a result of elevated levels of IL-6 mRNA in most tissues of HSF1-null mice, serum IL-6 concentrations were extremely high in HSF1-null mice, like in ATF3-null mice (30), compared with those in wild-type mice (Fig. 5A).

The major systemic effects of an elevated serum IL-6 level are an acute-phase response and the febrile response (1, 37), both of which are impaired in IL-6–null mice (38, 39). We found the mRNAs of acute-phase proteins, α1-macroglobulin and serum amyloid A1 and A2, to be expressed more in the liver of HSF1-null mice (Supplemental Fig. 6). Furthermore, body temperatures of HSF1-null and ATF3-null mice were significantly higher after the injection of a low dose of LPS (50 μg/kg) compared with wild-type mice, whereas body temperatures of IL-6–null mice did not increase after the injection of a low dose of LPS (150 μg/kg) (Fig. 5B). These results demonstrate that HSF1 and ATF3 suppress febrile responses in mice injected with LPS, at least by inhibiting the expression of IL-6.

Consistent with the finding that the body temperature of IL-6-null mice did not increase, levels of HSF1 bound to the ATF3 promoter were much lower at 2 and 4 h after the LPS injection in IL-6-null mice than in wild-type mice (Fig. 6A). Furthermore, levels of ATF3 translocated into the nucleus in the liver were reduced (Fig. 6B), and levels of ATF3 bound to the IL-6 promoter were also much lower (Fig. 6C). These results indicate that an IL-6–mediated increase in body temperature promotes the activation of HSF1 and ATF3.

**Discussion**

We identified the ATF3 gene as a major target of HSF1. ATF3 is a member of the ATF/CREB family of transcription factors and was induced in response to various types of stress, including serum stimulation, inhibition of protein synthesis, ionizing radiation, and UV radiation (40), in part through the ERK, JNK, and p38 pathways (41, 42), and was involved in cell growth and survival (31, 43). However, ATF3 played no role in the survival of MEF cells at high temperature or in cell growth after a single exposure to high temperature, whereas HSF1 did (Supplemental Fig. 7). More recently, it was shown that ATF3 inhibits inflammation in various tissues (30, 44–46). Surprisingly, in this study, we demonstrated that ATF3 is required for heat-mediated suppression of the LPS-induced expression of IL-6 and many other inflammatory genes.

This finding led us to conclude that heat shock inhibits LPS-induced IL-6 expression in mouse cells. Previous reports showed that IL-6 expression was not inhibited by hyperthermia in human macrophages treated with LPS (10) and that hyperthermia promoted IL-6 production in human enterocytes (47). Furthermore, treatment with hyperthermia in mice resulted in an increase in serum IL-6 levels (48). However, we clearly demonstrated the suppressive effect of hyperthermia on IL-6 expression in mouse macrophages and MEF cells (Fig. 1). This result was further confirmed by the finding that LPS-induced IL-6 expression in ATF3-null MEF cells was not affected by hyperthermia (Fig. 3).

Because HSF1 is required for maximal expression of IL-6, partially opening the chromatin structure of the IL-6 promoter in spleen cells and MEF cells (29), we wondered whether the serum level of IL-6 increased or decreased in mice deficient in HSF1. We found that serum IL-6 levels and IL-6 mRNA levels in many tissues were extremely high in HSF1-null mice compared with wild-type mice (Fig. 5, Supplemental Fig. 5). These findings indicate for the first time that HSF1 plays a major role in the negative regulation of IL-6 expression in mice.

It is clear that heat pretreatment inhibited the LPS-induced expression of ~86 genes (86%) (Fig. 4). These genes were divided into an ATF3-independent group containing eight genes, including TNFα and IL-1β, whose promoters may be directly inhibited by HSF1 (26, 27), and an ATF3-dependent group containing 16 genes in addition to IL-6, the expression of half of which is not maximally induced by LPS treatment in the absence of HSF1. The NOS2, ICAM1, and IL-6 promoters are bound by HSF1, and their chromatin structure is more open in the presence of HSF1. Thus,
HSF1 affects chromatin status directly and also inhibits the expression of inflammatory genes directly by activating ATF3 (Supplemental Fig. 8).

HSF1 was originally identified as a transcriptional activator of heat shock proteins, and a lot of attention has focused on its role in protecting cells from lethal thermal stress (16–19, 49, 50). However, most cells in the body do not encounter such extreme temperatures (i.e., >42°C). Rather, cells are repeatedly exposed to febrile-range temperatures, which are usually elicited by exercise, feeding, infection, and disease (1, 2). In this study, we showed for the first time that HSF1 induces the expression of another transcription factor, ATF3, to regulate a unique set of genes, such as inflammatory genes. Inflammatory cytokines support the invasion of inflammatory cells, and promote tissue repair; however, they are detrimental at excessively high levels. This novel negative regulatory loop may inhibit an excessive febrile response and injuries to tissues.

Acknowledgments
We thank Dr. S. Calderwood, G. Santro, and S. Kitajima for helpful discussions and Dr. K. Miyake for reagents.

Disclosures
The authors have no financial conflicts of interest.

References


Fig. S1

Fold change (HS/C)

ATF3 Jun Fas Rrad Egr1 Edn1 Dusp1 Dusp2 Dusp8

HSF1+/+ HSF1−−
ATF3 promoter R1 regions

Fig. S2
Fig. S3
Fig. S5
Fig. S6
**Fig. S7**

(A) Relative cell number (%) over time at 42°C (h) for HSF1 WT and HSF1 KO. Asterisks indicate significance: *p < 0.05.

(B) Cell number (10⁵) over days after heat shock for HSF1 WT (0h), HSF1 KO (0h), HSF1 WT (1h), and HSF1 KO (1h).
Fig. S8

Activation of Inflammatory genes

Suppression of Inflammatory genes

PTGES
CXCL1
CD83
TLR2
SLCL15A
RIPK2
CD69
CXCL16

HSF1-dependent chromatin opening

NF-κB activation

IL-6

Inflammatory response

Fever ↑

Negative regulatory loop

Suppression of Inflammatory genes

TNF
IL1β

ATF3

HSF1

NOS2
ICAM1
IL-6
Supplementary Figure Legends

FIGURE S1. A DNA microarray analysis identified nine genes whose expression increased more than 3-fold in both wild-type and HSF1-null primary MEF cells treated with heat shock at 42°C for 1 h compared with that in untreated cells (Inouye et al. 2004). Fold changes in wild-type and HSF1-null MEF cells are shown.

FIGURE S2. Alignment of R1 nucleotide sequences in mouse and human ATF3 promoters. Asterisks show identical sequences and boxes indicate HSE-like sequences within the mouse (+90 to -457) and human (+1 to -546) sequences. The Number of HSE-like sequences is 24 in the mouse promoter and 26 in the human promoter. Seventeen regions (boxes) were conserved in both promoters. Boldfaced characters indicate conserved G nucleotides in the consensus sequence nGAAn. Two deletions (ΔHSE2-4 and ΔHSE5-9) used for a reporter analysis are shown in red and with arrows. A TATA box, cAMP-responsive element (CRE), and TPA-responsive element (TRE) are indicated.

FIGURE S3. Heat shock promotes nuclear translocation of ATF3. (A) ATF3-null MEF cells (ATF3-/-) were transfected with an adenovirus expressing ATF3, left untreated (C) or treated at 42°C for 1 h, and allowed to recover at 37°C for 0, 2, and 4 h. A ChIP assay was performed using anti-ATF3 antibody. DNA fragments of IL-6 (-230 to +31) were amplified by PCR. The same cell extracts were subjected to a Western blot analysis. (B) ATF3-null MEF cells transfected with an adenovirus expressing ATF3 were treated as described in A.
Whole cell extracts and nuclear and cytoplasmic extracts were prepared, and subjected to a Western blot analysis.

**FIGURE S4.** Schematic representation of the IL-6, NOS2, and ICAM1 promoters. Amplified fragments for the ChIP analysis are indicated by arrows.

**FIGURE S5.** HSF1 deficiency promotes IL-6 expression in mice. (A) Expression of IL-6 and Hsp70 mRNA in various tissues of mice injected with LPS. Northern blot analysis using total RNA isolated from mice injected with LPS plus GalN for 0, 2, 6 h. Br, brain; Lu, lung; Li, liver; Sp, spleen; Ki, kidney; In, intestine; He, heart; Mu, skeletal muscle. (B) Mice were treated as described in A. Total RNA was isolated from the liver, kidney, heart, and spleen at the time points indicated, and a Northern blot analysis was performed. Levels of IL-6 mRNA were quantified and means and standard deviations are shown (n=3). Stars indicate these are significant (p < 0.05).

**FIGURE S6.** Acute phase response in the liver of HSF1-null mice. Total RNA was isolated from the liver of wild-type and HSF1-null mice injected with LPS and GalN for the indicated periods. RT-PCR was performed and representative results are shown. mRNA expression relative to that in the control liver was quantified and means and standard deviations are shown (n=3). α2M, α2-macrogloblin; Fib, fibrinogen; SaA1, serum amyloid A1; SaA2, serum amyloid A2; Hp, haptoglobin; Act, β-actin.
FIGURE S7. ATF3 did not contribute to HSF1-mediated cell protection. (A) Survival of cells incubated at high temperature. Wild-type (HSF1 WT) and HSF1-null (HSF1 KO) primary MEF cells (left), or Wild-type (ATF3 WT) and ATF3-null (ATF3 KO1 and KO2 from different embryos) primary MEF cells (right) were incubated at 42°C for the indicated periods, and numbers of attached cells were counted. Means and standard deviations are shown (n=3). (B) Cell growth after a single exposure to high temperature. Cells were treated with heat shock at 42°C for 1 h, and then incubated at 37°C for 6 days. Numbers of cells were counted, and means and standard deviations from three independent experiments are shown.

FIGURE S8. The feedback loop of IL-6 expression that negatively regulates the expression of inflammatory genes. Exogenous pyrogens such as LPS bind to Toll-like receptors (TLR), which induces activation of NF-κB through several steps (a dotted arrow). NF-κB induces the expression of many inflammatory genes including IL-6, leading to a febrile response. As a result, HSF1 is activated and directly suppresses the expression of some inflammatory genes. Simultaneously, it induces the expression of ATF3 which inhibits the expression of many inflammatory genes, including the IL-6.
Table S1 Primers for inflammatory genes

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX3CL1</td>
<td>5'-GTGCGGTTGCCCTCAGGCAGCCAGG-3'</td>
<td>5'-GGCCACCAGGACGTACCTTACTGCCC-3'</td>
</tr>
<tr>
<td>CD83</td>
<td>5'-CAGGGTGGCCTGAGGCTGCCCCG-3'</td>
<td>5'-CTCATACCGTTTCTGCTTATAGGGCCAGG-3'</td>
</tr>
<tr>
<td>CCL5</td>
<td>5'-CATGAGAAGTCTGACGCAGCTGCCC-3'</td>
<td>5'-CCCTCTATCCTAGCTCATTCTCC-3'</td>
</tr>
<tr>
<td>GBP2</td>
<td>5'-CGTACGGAAGGCTGAGGCTGCCCAGG-3'</td>
<td>5'-CCCTCAGAGATGATCAGGGATTTC-3'</td>
</tr>
<tr>
<td>GBP3</td>
<td>5'-CCGAGAAACATCTGCTAAAATCCAGG-3'</td>
<td>5'-GACTAAGCTTACTAGGGCCAGGCAGG-3'</td>
</tr>
<tr>
<td>TLR2</td>
<td>5'-CAATGATGCTATGCAACACTACCCG-3'</td>
<td>5'-GGATATTTGCAGTTTCAGTTTACC-3'</td>
</tr>
<tr>
<td>NOS2</td>
<td>5'-GCAGAACACCAAGGGTTGCTGCCCAGG-3'</td>
<td>5'-GTCAGAGCTTACGTTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>SLCL15A</td>
<td>5'-CGCTTACTCAGAAGCCACGGCTCC-3'</td>
<td>5'-CCATGACCTTACGCTGAGCCAGG-3'</td>
</tr>
<tr>
<td>RSAD2</td>
<td>5'-CTCCAGGAGAAATGGCCTGAGGCTGCCCAGG-3'</td>
<td>5'-CCTGGATACCTGGTCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>RIPK2</td>
<td>5'-CAGTGGATCCAGAAGTGGGATG-3'</td>
<td>5'-GGTGGTGACCTGGTCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>ICAM1</td>
<td>5'-GCAGATGGTGCCCTGCTGCCCAGGCTGCCCAGG-3'</td>
<td>5'-GTCTCATCCAGGCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>CD69</td>
<td>5'-GCGTACGAGGAAATGGCCTGAGGCTGCCCAGG-3'</td>
<td>5'-CCTGGATACCTGGTCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>PTGES</td>
<td>5'-GGCTAGCCGAGATGGCCTTCCCAGG-3'</td>
<td>5'-GCTGCTGCTGCTGCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>MPA21</td>
<td>5'-GAGGGACAGGCTGAGGCTGCCCAGG-3'</td>
<td>5'-GACTCAATCATCTACGCTTCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>CXCL16</td>
<td>5'-CGTACGAGGAAATGGCCTGAGGCTGCCCAGG-3'</td>
<td>5'-CCTGGATACCTGGTCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>TSLP</td>
<td>5'-CGACAGCATGGTGCTTCTGAGGCTGCCCAGG-3'</td>
<td>5'-GTGATTGGTTGTAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-CTGTTGACAAACCCAGGCTGCCCAGG-3'</td>
<td>5'-ATGCTTGGTCATACGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>CCL9</td>
<td>5'-GATGAAAGCCTTTCATCATCTGCCCAGG-3'</td>
<td>5'-GTTATTGGTCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>ICOSL</td>
<td>5'-GATGAAAGCCTTTCATCATCTGCCCAGG-3'</td>
<td>5'-CAGGGAAATGGCCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>CSF3</td>
<td>5'-CAGACACAGTGCTAAGGCCAGCAGGCTGCCCAGG-3'</td>
<td>5'-GTTATTGGTCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>CCL3</td>
<td>5'-CAGTATGGTCTTCCCACACTGCCCAGG-3'</td>
<td>5'-CAGTATGGTCTTCCCACACTGCCCAGG-3'</td>
</tr>
<tr>
<td>TNF</td>
<td>5'-GATGAGAAATGGCCTGAGGCTGCCCAGG-3'</td>
<td>5'-GTTATTGGTCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>IRG1</td>
<td>5'-GATGAAATAGCCATGACTCTAAAGG-3'</td>
<td>5'-GTTATTGGTCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>NFkBIE</td>
<td>5'-GGAGGAGGGCTGCTGAGGCTGCCCAGG-3'</td>
<td>5'-GTTATTGGTCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-GTGTGATGTTCCATTAGACAGC-3'</td>
<td>5'-TGAGAGGGCTGCTGAGGCTGCCCAGG-3'</td>
</tr>
<tr>
<td>Actin</td>
<td>5'-GACAGGATGCAAGGAGG-3'</td>
<td>5'-TTGCTGATCCACATCTGCTG-3'</td>
</tr>
</tbody>
</table>