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*J Immunol* published online 19 July 2013
http://www.jimmunol.org/content/early/2013/07/18/jimmunol.1300416

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2013/07/22/jimmunol.1300416.DC1

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YB-1 Is an Early and Central Mediator of Bacterial and Sterile Inflammation In Vivo

Lydia Hanssen,*1 Christina Aeidousty,*1 Sonja Djujdaj,*1 Björn C. Frye,*1 Thomas Rauen,*1 Peter Boor,*2+,† Peter R. Mertens,§ Claudia R. van Roeyen,*1 Frank Tacke,*† Felix Heymann,*‡ Andre P. Tittel,‖ Alexander Koch,*§ Jürgen Floege,*† Tammo Ostendorf,*1+ and Ute Raffetseder*1

In vitro studies identified Y-box–binding protein (YB)-1 as a key regulator of inflammatory mediators. In this study, we observed increased levels of secreted YB-1 in sera from sepsis patients. This led us to investigate the in vivo role of YB-1 in murine models of acute peritonitis following LPS injection, in sterile renal inflammation following unilateral ureteral obstruction, and in experimental pyelonephritis. LPS injection enhanced de novo secretion of YB-1 into the urine and the peritoneal fluid of LPS-treated mice. Furthermore, we could demonstrate a significant, transient upregulation and posttranslational modification (phosphorylation at serine 102) of YB-1 in renal and inflammatory cells. Increased renal cytoplasmic YB-1 amounts conferred enhanced expression of proinflammatory chemokines CCL2 and CCL5. Along these lines, heterozygous YB-1 knockout mice (YB-1+/d) that display 50% reduced YB-1 levels developed significantly lower responses to both LPS and sterile inflammation induced by unilateral ureteral obstruction. This included diminished immune cell numbers due to impaired migration propensities and reduced chemokine expression. YB-1+/d mice were protected from LPS-associated mortality (20% mortality on day 3 versus 80% in wild-type controls); however, immunosuppression in YB-1+/d animals resulted in 50% mortality. In conclusion, our findings identify YB-1 as a major, nonredundant mediator in both systemic and local inflammatory responses. The Journal of Immunology, 2013, 191: 000–000.

Mediators involved in the immune defense against pathogens include specialized cells and the innate immune system as well as chemokines, cytokines, and their cognate receptors (1). Most acute inflammatory processes, for example, infections, are cleared by these processes, and tissue architecture is restored. However, when the initial stimulus sustains, a transition of chronic inflammation into fibrosis may prevail. Similar to normal wound healing, organ fibrosis develops in response to an initial insult with the intent to restore tissue architecture and to regain organ function (2). For example, bacterial infections of the urinary tract bare the risk of permanent renal damage and development of fibrosis (3). Conversely, renal damage by obstructive nephropathy is characterized by an inflammatory state within the kidney that is not initiated by microbial pathogens, but through cytokines and growth factors produced by damaged tubular cells, infiltrating macrophages, and activated myofibroblasts. The identification of mediators that determine the course of both acute and chronic inflammatory processes can potentially lead to new therapeutic approaches.

The highly conserved cold-shock protein Y-box–binding protein (YB)-1 plays an important role in inflammatory processes (4). YB-1 accomplishes pleiotropic functions in the cell by virtue of its binding capacities to nucleic acids as well as direct protein–protein interactions. YB-1 is composed of an alanine/proline-rich N terminus, the central cold-shock domain, which mediates DNA and RNA binding and a C-terminal charged zipper that defines alternating positively and negatively charged amino acids. At the transcriptional level, YB-1 regulates the expression of numerous cytokine and chemokines (4–6) and their receptors (7, 8). Because YB-1 is a major component of messenger ribonucleoprotein particles in mammalian cells, it also regulates the stabilization of mRNA and translation of mediators such as TGF-β and CCL2/MCP-1 (9, 10). Furthermore, YB-1 is involved in mRNA processing (11).

YB-1 also bears chemotactic properties following active nonconventional secretion by monocyteic immune and mesangial cells (12). We previously proved that extracellular YB-1 binds to the receptor Notch-3, which is temporally upregulated upon mesangial cell activation in conjunction with YB-1 and Ets-1 in a rat model resembling mesangioproliferative glomerular diseases (anti-Thy1.1 nephritis) (13–15).
Homozygous YB-1<sup>−/−</sup> mice reveal embryonic/perinatal lethality underscoring the crucial role of YB-1 in essential cellular functions during development (16). In contrast, heterozygous mice (YB-1<sup>+</sup>/<sup>−</sup>) do not display a spontaneous phenotype. These mice exhibit a 50% reduction in cellular YB-1 content (13, 16), and gene dosage effects in comparison with wild-type (WT) littermates have not been investigated to date. To address the role of YB-1 in the inflammatory response in vivo, we induced two renal inflammatory models in WT and YB-1<sup>−/−</sup> mice, as follows: 1) acute peritonitis following LPS injection (17, 18), and 2) unilateral ureteral obstruction (UUO), in which sterile inflammation occurs secondary to mechanical renal damage and enables the study of long-term inflammatory effects.

Materials and Methods

Human serum samples

The study protocol was approved by the local ethics committee (ethics committee of University Hospital Aachen, RWTH Aachen), and written informed consent was obtained from each patient. The study was conducted according to the principles expressed in the Declaration of Helsinki. Patients who met the criteria proposed by the American College of Chest Physicians and the Society of Critical Care Medicine Consensus Conference Committee for severe sepsis and septic shock were categorized as sepsis patients, and the others as nonsepsis patients (19). Demographic and clinical information on patients’ characteristics is given in Table 1.

Animal experiments

The local review board approved all animal studies according to prevailing guidelines for scientific animal experimentation. Animals were held in cages with constant temperature and humidity with drinking water and food ad libitum. Mice heterozygously targeted for a disruption of the YB-1 locus (YB-1<sup>+/−</sup>, C57BL/6 background) were kindly donated by Timothy J. Ley (Section of Stem Cell Biology, Division of Oncology, Washington University Medical School, St. Louis, MO) (16).

LPS model. For the LPS-induced inflammation model, 18- to 22-wk-old WT mice and their age-matched WT littermates (YB-1<sup>+/−</sup>, WT) received a single dose of LPS, dissolved in PBS, by i.p. injection at a concentration of 1.5 mg/kg body weight (BW). Control mice were injected with an equivalent volume of PBS alone. All animals appeared healthy during the experiment (17) and were sacrificed at 18 h after injection (n = 5–6 for each time point and genotype), blood samples were removed and immediately frozen. For renal protein extraction, cortical tissue was homogenized in Triton X-100 lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM ethyleneglycoltetraacetic acid, 10% glycerol, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF chloride, 0.1 mM sodium orthovanadate) at 4˚C for 30 min, followed by repetitive sonification treatment. For RNA preparation, the tissue was incubated in RNeater (Life Technologies) and RNA was isolated via the TRIzol method. For immunohistochemical analyses, renal tissue was fixed in methyl Carnoy’s solution at 4˚C for 24 h.

LPS mortality. To determine the mortality rate under LPS treatment, 9-wk-old male and female YB-1<sup>−/−</sup> mice (n = 5) and WT littermates (n = 5) were injected i.p. with a single dose of 20 mg/kg LPS dissolved in PBS. The mice were monitored daily. Ten days after injection, the experiment was terminated.

Immunosuppression model. The 15-wk-old YB-1<sup>−/−</sup> (n = 17) and WT (n = 5) mice received 30 mg/kg BW cyclosporine A s.c. (0.9%/sodium chloride solution-diluted Sandimmun concentrate [Novartis, Basel, Switzerland]) for 2 wk daily. As controls, YB-1<sup>−/−</sup> and WT mice were injected solely with a control solution (33% Cremophor EL [Sigma-Aldrich Chemie, Steinheim, Germany], 33% ethanol, and 40% NaCl solution [0.9%]) (n = 5 each). At sacrifice, blood samples were taken and used for microbiological analyses and an automated hemogram.

Urinary tract infection model. Pyelonephritis was induced, as previously described (20). Briefly, uropathogenic Escherichia coli bacteria (UPEC strain 536) were grown in Luria–Bertani medium, collected by centrifugation (1200 × g, 10 min), and resuspended in 2 ml Luria–Bertani medium to a final concentration of 1 × 10<sup>9</sup> CFU/ml. Mice (C57BL/6, n = 5) 10 wk of age were anesthetized and injected intraperitoneally with 1 × 10<sup>9</sup> UPEC using a flexible polyethylene catheter (outer diameter 0.6 mm) coated with Instillagel (Farco Pharma, Cologne, Germany). Three hours later, the procedure was repeated to induce pyelonephritis. Before analysis, mice were perfused with sterile PBS. The number of ascended bacteria was quantified by scoring CFU after overnight culture of kidney collagenase digest or of homogenates prepared in PBS with an Ultra Turrax at 37˚C on chrome-plating system identification plates (Biomerieux, Nürtlingen, Germany). Kidneys were removed and shocked frozen, and protein extracts were prepared, as described before (21).

UUO model. A model of UUO was performed with 20-wk-old female littermates (22). The left ureter of the animals was ligated for 5 d. Both kidneys were collected for preparation of cortical RNA, protein, and immunohistochemical analyses. None of the animals died before sacrifice at day 5.

Isolation of primary cells, cell culture, and stimulation

For granulocyte isolation, 2.5 ml dextran solution (5%) was added to 10 ml PBS. Cells were sedimented by centrifugation (1200 × g, 10 min, 4˚C), and resuspended in 2 ml RPMI 1640 medium (Biochrom, Berlin, Germany). Cells were counted.

Bone marrow (BM) cells were harvested by flushing the bones with MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany), and a single-cell suspension was prepared by passing it through a 21-gauge needle and subsequently through a 40 μm nylon mesh. Cells from four animals were combined, centrifuged at 1200 rpm for 5 min (room temperature), resuspended in 10 ml MACS buffer, and counted.

Lymphocytes were isolated from four mice per group were isolated from the hind limbs of 26-wk-old YB-1<sup>−/−</sup> and WT mice, respectively. BM was harvested by flushing the bones with MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany), and a single-cell suspension was prepared by passing it through a 21-gauge needle and subsequently through a 40 μm nylon mesh. Cells from four animals were combined, centrifuged at 1200 rpm for 5 min (room temperature), resuspended in 10 ml MACS buffer, and counted.

Mice were injected i.p. with a single dose of 20 mg/kg LPS dissolved in PBS. The mice were monitored daily. Ten days after injection, the experiment was terminated.

Isolation of primary mesangial cells, kidneys of 8-wk-old female C57BL/6 mice were perfused with magnetic Dynabeads M-450 (Life Technologies), digested with a 1 mg/ml collagenase type I (Life Technologies) for 30 min at 37˚C, and passed through a 100 μm cell strainer. Glomeruli were isolated out of the flow-through by a high-power magnet and cultured in modified RPMI 1640 medium (Biochrom, Berlin, Germany) containing no L-valine but 90 mg/l D-valine, supplemented with 20% FCS, 1% penicillin/streptomycin, and 0.5% fungizone. All cell culture supplements were obtained from Life Technologies. Mesangial cells grew out of the glomeruli within 1 wk.

Bone marrow (BM)–derived cells from four mice per group were isolated from the hind limbs of 26-wk-old YB-1<sup>−/−</sup> and WT mice, respectively. BM was harvested by flushing the bones with MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany), and a single-cell suspension was prepared by passing it through a 21-gauge needle and subsequently through a 40 μm nylon mesh. Cells from four animals were combined, centrifuged at 1200 rpm for 5 min (room temperature), resuspended in 10 ml MACS buffer, and counted.

Table 1. Clinical data of patients with sepsis

<table>
<thead>
<tr>
<th></th>
<th>Sepsis</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n)</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Age [years] (range)</td>
<td>55 (20–79)</td>
<td>46 (18–58)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>5/4</td>
<td>6/2</td>
</tr>
<tr>
<td>Height [cm] (range)</td>
<td>172 (156–188)</td>
<td>177.5 (159–183)</td>
</tr>
<tr>
<td>Weight [kg] (range)</td>
<td>90.4 (42.0–109.6)</td>
<td>76.5 (67.0–103.0)</td>
</tr>
<tr>
<td>BMI [kg/m²] (range)</td>
<td>30.6 (15.3–44.4)</td>
<td>26.3 (21.2–29.7)</td>
</tr>
<tr>
<td>ICU days</td>
<td>10 (3–56)</td>
<td>APACHE II 12.5 (7–26)</td>
</tr>
<tr>
<td>SAPS II</td>
<td>40 (20–54)</td>
<td>Death rate 33%</td>
</tr>
</tbody>
</table>

APACHE, Acute Physiology and Chronic Health Evaluation; BMI, body mass index; ICU, intensive care unit; SAPS, Simplified Acute Physiology Score.

YB-1 IS A MEDIATOR OF INFLAMMATION IN VIVO
streptomycin. The HUT78 T cell line was grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. The human liver hepatocellular carcinoma cell line (Hepg2) was grown in DMEM-nut mix F12 supplemented with 10% FCS, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were incubated for different times with 10 ng/ml LPS or with solvent PBS alone as a control.

Proteins in the cell supernatant were precipitated by addition of ice-cold TCA (20% v/v; Sigma-Aldrich). Precipitated proteins were pelleted by centrifugation at 20,000 × g for 45 min at 4°C, washed twice with ice-cold 70% ethanol, air dried, and resuspended in 30 μl distilled water. All cell culture media and supplements were purchased from Life Technologies, unless otherwise stated.

**Transwell migration assays**

Granulocyte migration assays were performed using FluoroBlok cell culture inserts (pore size 3.0 μm) in 24-well plates (Falcon; BD Biosciences, San Jose, CA). After staining with calcein-AM (Calbiochem, Darmstadt, Germany), Ly6G-positive neutrophils were washed twice and resuspended in RPMI 1640 medium (Life Technologies) containing 0.1% BSA (Sigma-Aldrich). For the migration assay, 7 × 10^5 granulocytes were given in the upper well in a volume of 250 μl. The lower well contained 500 μl RPMI 1640 (Life Technologies) supplemented with the chemokinome CCL5 (200 ng; ImmunoTools, Friesoythe, Germany). After incubation of the plates at 37°C for 1 h, cell culture inserts were removed and the fluorescence was measured at 535 nm with Wallac Victor 1420 multilabel counter (PerkinElmer, Waltham, MA).

**Immunohistochemistry**

Immunohistochemistry was performed in 1 μm paraffin sections of methyl Carnoy’s-fixed specimens using the Vectastain Avidin/Biotin System (Vector Laboratories, Burlingame, CA), as described before (14). Renal tissues were stained using the following primary Abs: rat monoclonal Ly6G IgG (BD Biosciences, San Jose, CA), rat monoclonal anti-mouse F4/80 IgG (Serotec, Düsseldorf, Germany), and rabbit polyclonal anti-human YB-1 C-terminal IgG (Sigma-Aldrich Chemie). Negative controls for the immunohistochimical procedures consisted of substitution of the primary Ab with nonimmune IgG. Quantification of F4/80 staining was performed by counting the positively stained cells in 3 randomly selected fields at original magnification ×100 in the renal cortex. Quantification of Ly6G immunostaining was performed by counting positively stained cells in 20 randomly selected fields at original magnification ×100 on Olympus BX41 at room temperature with aperture Achromat 0.9 (Olympus, Hamburg, Germany). Camera Color View II (Soft Imaging Solution; Olympus), and Software Analysis Pro (Olympus).

**Cortical kidney lysates and Western blot analysis**

Cortical kidney lysates of LPS- and PBS-treated YB-1+/d and WT mice were prepared, as described before (21). Unless stated otherwise, 10 μg protein of cortical kidney extracts and 2 μg/μl TCA-precipitated urine proteins were subjected to SDS-PAGE. Western blotting was performed, as described earlier (11). The following Abs were used: rabbit polyclonal anti-YB-1 C-terminal IgG (Sigma-Aldrich Chemie), rabbit polyclonal anti-phosphorylated YB-1 Ser413 IgG (Cell Signaling, Beverly, MA), and rabbit polyclonal anti-kidney injury molecule (KIM)-1 (Acris, San Diego, CA). Blots were reprobed with a mouse anti-GAPDH–specific mAb (Novus Biologicals, Littleton, CO), whereas bands of nuclear extracts were incubated with a rabbit anti-histone H3–specific Ab (Cell Signaling, Beverly, MA) to ensure equal protein loading.

**Quantitative real-time PCR and PCR**

For quantitative real-time PCR, total RNA was purified from sorted cells, liver, and cortical kidney tissue via TRIzol reagent (Life Technologies), according to the manufacturer’s protocol. First-strand cDNA was synthesized with Moloney monkey leukemia virus reverse transcriptase (Life Technologies). Quantitative real-time PCR was carried out with the 7300 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). TaqMan master mix and TaqMan primer sets were obtained for chemokine ligand 1 (Mm04270470_m1), chemokine ligand 2 (Mm00999056_m1), chemokine ligand 5 (Mm01302428_m1), CCR-motivic chemokine receptor 5 (Mm01216171_m1), TLR4 (Mm0045273_m1), and human YB-1 (Hs07247254_g1) from Applied Biosystems. Eukaryotic 18S rRNA (Hs99999901_s1) served as an internal control. For KIM-1 detection, 0.75 μl cDNA and 12.5 μl PCR Master Mix (qPCR Core kit with Sybr Green I; Eurogentec, Seraing, Belgium) were used for each reaction in a total of 25 μl volume. GAPDH cDNA amplification was used as an internal standard. The conditions for all TaqMan PCRs were 50°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All quantitative RT-PCR (qRT-PCR) data were calculated using the ΔΔ cycle threshold method. The primer sequences for KIM-1 and GAPDH are as follows: GAPDH, 5'-GGC AAA TAC ACC ACA GCA ACA GT-3' and 5'-AGATGGA TGA TGG GCT TCC C-3'; KIM-1, 5'-TGG TTG CCT TTC CGT GTC TCT-3' and 5'-TCT TCA GCT CCG GAA TGC A-3'.

**Coimmunoprecipitation of mRNA associated with YB-1**

Prior to immunoprecipitation, polyclonal anti-YB-1 Ab and irrelevant anti-rabbit IgG (Cell Signaling) as negative control were covalently linked to protein A-Sepharose beads (50% suspension; Invitrogen), as described before (23). A total of 20 μg protein A-Sepharose coupled either with polyclonal anti-YB-1 Ab or nonspecific IgG incubated with 200 μg kidney cell extract from LPS-stimulated or control-untreated recombinant mesangial cells for 1.5 h at 4°C in immunoprecipitation buffer (20 mM HEPES [pH 7.4], 100 mM potassium chloride, 5 mM magnesium acetate, 1 mM DTT; 0.025% Triton X-100, and protease inhibitors). Sepharose beads were washed three times in immunoprecipitation buffer, and precipitated material was resuspended in TRIZol reagent (Invitrogen). Associated RNA was isolated and reverse transcribed, and PCR analysis was performed, as described above.

**ELISA**

To quantify the CCL5 protein content of blood plasma and cortical kidney lysates, quantitative sandwich enzyme immunoassay technique with reagents from R&D Systems (Minneapolis, MN; DuoSet ELISA development kit DY278) was performed according to the manufacturer’s instructions. The OD was measured using a microplate reader set to 450 nm. All standards (dilution 1:2 and 1:10) and samples (blood plasma dilution, 1:10; kidney lysates dilution, 1:20) were assayed as duplicates.

**Sorting of leukocytes from peritoneal lavage**

Peritoneal cells of two mice per group (PBS/LPS) were pooled and stained directly using mAbs for CD45, Ly6G, CD115, CD11b, CD4, CD8, and NK1.1 (eBioscience, San Diego, CA), and dead cells were excluded using
Hoechst33258 (Merck). Sorting was performed using a FACS Aria-II SORP (BD Biosciences) using a 70 μm nozzle. Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Chemokine array
To measure the amount of different chemokines in the lavage of LPS-treated YB-1−/− and WT mice, Bio-Plex Pro assays were performed, according to manufacturer’s protocol (instruction manual for cytokines, chemokines, and growth factors). The lavage samples were diluted 1:4 in Bio-Plex standard diluent; measurement was performed using a Luminex 100 system (Qiagen).

Statistical analysis
Data were analyzed using the software GraphPad Prism 5 (San Diego, CA). Results are presented as means ± SD. We used t test to compare two experimental groups; whenever more than two groups were compared, ANOVA and Bonferroni post hoc test were applied to determine significance levels. Log-rank test was used for survival curves. A p value <0.05 was considered statistically significant.

Results
YB-1 is detected in serum samples of sepsis patients, and LPS challenge induces YB-1 expression and activation during the inflammatory response in mice

First, we investigated whether extracellular YB-1 is present in systemic bacteremia. Serum samples from nine patients with severe sepsis and eight hospitalized nonsepsis patients were analyzed for the presence of extracellular YB-1 by Western blot analysis (Fig. 1A). Patients’ clinical characteristics are outlined in Table I (median values with ranges in brackets). Sepsis patients displayed medium-range Acute Physiology and Chronic Health Evaluation (APACHE) II (median 12.5; range 7–26) and Simplified Acute Physiology Score II (median 40; range 20–54) and had been treated in the intensive care unit for a median duration of 10 d (range 3–56). Notably, extracellular YB-1 was only detectable in significant amounts in the sera of sepsis patients and was absent in sera obtained from nonsepsis patients. Equal protein loading within the groups was ascertained by Coomassie stainings (data not shown).

LPS-triggered secretion of YB-1 has been shown already for different cell types such as mesangial cells and monocytes (12). In this study, we investigated other cell types for their abilities to secrete YB-1 following endotoxin challenge. In supernatants obtained from cultivated T lymphocytes, kidney, and hepatic cells as well as from primary human granulocytes, YB-1 was detected either after 2 or 4 h of LPS treatment (Fig. 1B).

Next, we investigated YB-1 expression and secretion in a LPS model in vivo. Six hours following i.p. LPS injection in mice, YB-1 mRNA expression in cells obtained from the peritoneal cavity...
increased >4-fold, and remained >2-fold increased after 48 h, as compared with peritoneal cavity cells from PBS-instilled control animals (Fig. 1C). Furthermore, we detected de novo secretion of full-length YB-1 protein into the peritoneal lavage fluid 6 h after LPS induction (Fig. 1D, upper right panel). Enhanced YB-1 expression could be assigned to neutrophils, because only FACS-sorted neutrophils exhibited >25-fold upregulation of YB-1 mRNA amount compared with PBS control (Supplemental Figs. A/B, Supplemental Table I). In contrast, YB-1 amounts in other cells such as CD115high macrophages, CD115low macrophages and pooled lymphocytes, including CD4 and CD8 T cells as well as NK cells, were not elevated. Secretion of YB-1 protein did not persist at 48 h after LPS application, and no YB-1 was detectable in PBS control mice (Fig. 1D, lower panel).

Next, we aimed to analyze whether the peritoneal LPS injection affected the inflammatory response in the kidneys. Forty-eight hours after LPS challenge, we observed enhanced YB-1 expression with >3-fold induction of YB-1 transcript numbers (Fig. 2A) and protein amount (Fig. 2B, lower panel) in the renal cortex. At 6 h after LPS induction, YB-1 was not induced at the mRNA, but only at the protein level (Fig. 2A, 2B, upper panel), suggesting posttranscriptional mechanisms. To investigate whether LPS-triggered inflammation influences YB-1 activation by posttranslational modification in vivo, we used a polyclonal anti–YB-1 Ab that specifically detects YB-1 at its phosphorylated serine residue 102, which is located within the highly conserved cold-shock domain. YB-1 phosphorylation occurred already in the early phase of inflammation (Fig. 2B, 6 h, middle panel), but was no longer detectable 48 h after LPS treatment (Fig. 2B, 48 h, middle panel). More specifically, we proved that, following LPS exposition, YB-1 was also upregulated and transiently phosphorylated in primary mesangial cells isolated from C57BL/6 mice (Fig. 2C). Our finding of an increased renal YB-1 expression after LPS was confirmed by immunohistochemistry, showing that YB-1 was focally overexpressed in the renal cortex, with a strong nuclear localization in tubular, tubulointerstitial, and glomerular cells. Cytoplasmic expression of YB-1 was also focally enhanced (Fig. 2D).

To investigate functional consequences of the elevated YB-1 expression following LPS application, we analyzed the physical interaction of YB-1 with mRNA of chemokines CCL2 and CCL5 in the kidneys. To this end, YB-1 was immunoprecipitated from kidney protein extracts of mice with and without prior LPS challenge. Coimmunoprecipitated mRNA was isolated and detected by subsequent RT-PCR (Fig. 2E, upper panels). In comparison with a nonspecific IgG control, >6-fold increases of YB-1–bound CCL2 and CCL5 transcript numbers, respectively, were detected in mice stimulated with LPS (Fig. 2E, upper panels, lanes 4). We did not observe YB-1 binding to GAPDH mRNA (Fig. 2E, lower panel).

To confirm upregulation of YB-1 during localized bacterial tissue infection, we established an additional murine model of pyelonephritis by instilling uropathogenic E. coli into the bladder (20). A marked upregulation and phosphorylation of renal YB-1 occurred 20 h following instillation of bacteria (Fig. 2F).

**FIGURE 3.** Cyto- and chemokines are expressed differently in YB-1+/+ mice, and their LPS-triggered secretion is altered compared with WT littermates, resulting in an impaired chemotaxis. (A) Transcript numbers of chemokines CCL5, CCL2, and CXCL1 and chemokine receptor CCR5 were determined by qRT-PCR in kidneys of YB-1+/− in comparison with WT animals (n = 6–10). (B) Serum concentrations of CCL5 protein levels measured by ELISA were diminished in kidneys (left panel) and serum (right panel) of YB-1+/− mice (n = 5). (C) Secretion of immunomodulatory factors following LPS challenge (6 h) is changed in YB-1+/− compared with WT mice. (D) The number of infiltrating immune cells in the peritoneal cavity was reduced in the lavage of YB-1+/− mice following LPS injection (n = 12). (E) F4/80-positive cells in lavage were strongly elevated 48 h following LPS treatment in YB-1+/+, but not in YB-1+/− mice. Shown are cells per visual field (mean of 10 fields, n = 4). Data represent means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. +/+, WT; +/-, YB-1 heterozygous mice; P, PBS, L, LPS.
We conclude that YB-1 is upregulated and phosphorylated at its serine residue 102 in response to endotoxin and that it contributes to enhanced chemokine expression in these instances.

**Half-maximal YB-1 expression results in altered expression of chemokines and impaired chemotaxis**

Next, we analyzed the role of YB-1 during the innate immune response using heterozygous YB-1–deficient mice (YB-1+/d). To investigate gene dosage effects in nonmanipulated mice, we first compared the known YB-1 target gene CCL5/RANTES and two additional chemokines with relevance for acute kidney injury (AKI), namely CCL2/MCP-1 (24) and KC/CXCL1 (25), in YB-1+/d and WT littermates. Compared with WT animals, YB-1+/d mice exhibited a constitutive reduction in renal CCL5 and CCL2 gene expression (Fig. 3A, left and middle panels), whereas CXCL1 transcript numbers were markedly increased (Fig. 3A, right panel). CCL5 receptor (CCR5) mRNA was also reduced in kidneys of YB-1+/d mice (Fig. 3A). Low CCL5 protein levels were confirmed in YB-1+/d mice in kidney and serum samples (Fig. 3B).

Next, we studied the LPS-triggered acute peritonitis model in YB-1+/d versus WT mice. Six hours after LPS injection, cyto- and chemokine levels were analyzed in the peritoneal lavage. Upregulation of IL-1β, IL-6, IL-10, IL-17, G-CSF, as well as CXCL1, CCL2, and CCL5 protein levels was observed in WT and YB-1+/d mice upon LPS challenge (Fig. 3C). However, the increase of IL-6, IL-17, CCL2, and CCL5 expression was significantly dampened, and CXCL1 levels were enhanced in the peritoneal lavage fluid in YB-1+/d mice as compared with WT mice (Fig. 3C, gray columns).

Altered concentrations of cyto- and chemokines in YB-1+/d mice were associated with altered chemotactic properties. Forty-eight hours after LPS challenge, we detected elevated numbers of infiltrating cells in the peritoneal cavity of WT, but not YB-1+/d mice (Fig. 3D). The same trend was observed when infiltrating F4/80-positive cells in the peritoneal fluid were counted, confirming reduced cell numbers in YB-1+/d mice (Fig. 3E). Lavage cells in WT mice additionally comprised B and T lymphocytes (Supplemental Fig. 1C, 1D).

Of note, in contrast to WT mice (Figs. 1B, 2A), YB-1+/d animals failed to increase their YB-1 transcripts in peritoneal cavity cells and in the kidneys at 6 and 48 h after LPS application (data not shown).
Amelioration of AKI in LPS-induced systemic inflammatory response in YB-1+/d mice

Because i.p. LPS injection results in AKI (see below), we compared renal chemokine expression in the LPS model. Of note, YB-1 transcript numbers and protein were markedly lower in kidneys obtained from YB-1+/d mice as compared with kidneys from WT littermates (13). Transcript numbers of CCL5, CCL2, and CXCL1 were upregulated following LPS treatment in kidneys of both WT and YB-1+/d mice, but CCL5 and CXCL1 gene expression was significantly dampened in YB-1+/d mice (Fig. 4A). Neutrophil cell numbers at 6 h following LPS injection increased ~2-fold in WT mice, but not in YB-1+/d mice (Fig. 4B). Monocyte/Macrophage numbers in the kidneys 48 h following LPS treatment also tended to be lower in YB-1+/d mice as compared with WT animals (Fig. 4C, 4D), whereas neutrophils were not detectable any longer at this time point (Supplemental Fig. 1E, 1F).

Next, we assessed the renal damage in YB-1+/d and WT mice following LPS challenge. LPS-treated mice displayed only minor tubular vacuolization and marginal loss of tubular brush borders in both groups. We therefore assessed the expression of KIM-1, an early and sensitive marker of tubular cell damage. In WT mice, renal KIM-1 mRNA increased 6 h after LPS treatment (Fig. 4E), as did KIM-1 protein secretion into the urine (Fig. 4F). YB-1+/d mice revealed only a moderate upregulation of renal KIM-1 mRNA (Fig. 4E) upon LPS application, and no KIM-1 protein was detected in urine (Fig. 4F). In line with this, no secreted YB-1 was detected in the urine of LPS-challenged YB-1+/d mice in contrast to WT mice (Fig. 4G).

Impaired chemokine/chemokine receptor expression and diminished granulocyte infiltration into the liver in YB-1+/d mice

To clarify the role of YB-1 as a mediator of multiorgan failure in systemic endotoxemia, we extended our studies to the liver. As demonstrated in the kidneys (13), heterozygous YB-1 knockdown mice revealed markedly lower YB-1 transcript numbers and protein content in the liver (Fig. 5A). Similar to the kidney (Fig. 4A), hepatic upregulation of CCL5, CCL2, and CXCL1 mRNA was attenuated in YB-1+/d animals following i.p. LPS application in comparison with WT littermates (Fig. 5B). In line with this, infiltration of neutrophils into the liver was significantly reduced in liver tissues from LPS-challenged YB-1+/d mice (Fig. 5C, 5D). Of note, hepatic CXCL1 transcript expression in unchallenged YB-1+/d mice was significantly elevated, again similar to our observations in the kidneys (data not shown).
CCL5 expression and granulocyte migration propensities are diminished in YB-1+/d mice

To further analyze the mechanism(s) responsible for the reduced migration capacities of immune cells in YB-1+/d animals, BM cells were isolated, and transcript numbers of YB-1, CCL5, CCL2, CXCL1, CCR5, and TLR4 were quantified. Expression of LPS receptor TLR4 was markedly decreased in BM cells from YB-1+/d animals as compared with WT mice (Fig. 5E). Similar to the results obtained in the kidneys, CCL5 expression was reduced by ~50% in YB-1+/d BM cells, whereas expression of CCR5 was not impaired (Fig. 5E). CCL2 and CXCL1 mRNA levels were below the detection limit.

Next, we analyzed the capability of BM-derived granulocytes from YB-1+/d mice to migrate toward a chemoattractant gradient evoked by CCL5. Granulocytes from YB-1+/d mice exhibited impaired migration capacities along the CCL5 gradient as compared with WT littermates (Fig. 5F).

Taken together, YB-1+/d mice exhibit a reduced basal expression of proinflammatory chemokines, including CCL5 and CCL2, in multiple organs and fail to upregulate these in response to LPS challenge. Additionally, granulocytes from these mice display aberrant capabilities to migrate along a CCL5 gradient.

Genetic ablation of YB-1 protects from UUO-dependent inflammation

To investigate whether YB-1 also contributes to noninfection-mediated renal damage, we extended our studies to the UUO model that is accompanied by a sterile inflammatory response. Following UUO, kidneys of WT mice exhibited an increased influx of F4/80-positive monocytes/macrophages (Fig. 6C) that was significantly lower in YB-1+/d animals (Fig. 6D, 6E). Of note, neutrophils were not detectable in kidneys 5 d after UUO (data not shown).

Chemokines propagating monocyte influx, that is, CCL5 and CCL2, were elevated up to 50-fold (CCL5) and 25-fold (CCL2) in obstructed WT kidneys as compared with nonobstructed kidneys (Fig. 6F, 6G). Comparable to our results within the LPS model, we observed significantly less upregulation of chemokines CCL2 and CCL5 in YB-1+/d animals. In contrast, CXCL1 expression was significantly elevated in YB-1+/d mice compared with their WT littermates.

Lower YB-1 expression in YB-1+/d mice results in better survival following LPS-induced mortality, but immunosuppression enhances mortality rates

Despite reduced expression of proinflammatory chemokines, YB-1+/d mice exhibit a normal lifespan as compared with their WT littermates (L. Hanssen and U. Raffetseder, unpublished observations). Following LPS challenge, mice deficient for YB-1 exhibited a markedly better survival as compared with WT littermates (Fig. 7A). However, when mice were treated with the immunosuppressant cyclosporine A (CsA) (30 mg/kg BW), mortality rates increased exclusively in YB-1+/d mice within the first 10 d of treatment (Fig. 7B).

Collectively, these results point to a crucial role for YB-1 in the control of the inflammatory response.

FIGURE 6. Genetic YB-1 ablation protects animals from UUO-depnedent immune response. (A–E) Immunostaining for infiltrating monocytes/macrophages in WT (A and C) and YB-1+/d mice (B and D) was performed by means of the F4/80 Ab. (E) Quantification was performed by assessing the positively stained cortical area (%). Diagrams on the right show data obtained by computer-based morphometric analyses on day 5 of UUO (C and D). “Healthy” contralateral kidneys are shown in (A) and (B). Values indicate the relative area (in %) of tissue that immunostained positively. Scale bars, 50 μm. Relative transcript numbers for CCL5 (F), CCL2 (G), and CXCL1 (H) were quantified by qRT-PCR in obstructed and contralateral kidneys of WT and YB-1+/d mice 5 d after UUO. Transcript numbers were normalized against 18S rRNA content. Significance was calculated by comparing WT with YB-1+/d animals. Data represent means ± SD (n = 5 for each group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, +/-, WT; +/-d, YB-1 heterozygous mice.
FIGURE 7. YB-1+/d mice are protected from LPS-induced mortality, whereas immunosuppression results in higher mortality rates. (A) Increased survival of YB-1+/d compared with WT mice after endotoxic shock induced by injection of 20 mg LPS/kg BW (n = 5). (B) Daily application of CsA (s.c. 30 mg/kg BW) or solvent as control to WT and YB-1+/d mice for 2 wk led to a marked increase in mortality rates of YB-1+/d animals (+/+ control, n = 5) (+/d, CsA [n = 17]). *p < 0.05, **p < 0.01. +/+ WT; +/d, YB-1 heterozygous mice.

Discussion

The first major finding of the current study was that YB-1 was upregulated in acute inflammatory processes in vivo, as demonstrated in the LPS-triggered model that has been widely used to characterize reversible renal inflammation processes in rodents (17, 18) and additionally in a pyelonephritis model. We and others have previously demonstrated that YB-1 is involved in the onset of inflammation (4, 26–28). A transient upregulation of YB-1 protein occurs during experimental mesangioproliferative nephritis (13), especially in the acute proliferative phase, and YB-1 predominantly localizes to the mesangial compartment. In other studies, LPS exposure induced YB-1 expression in the fetal brain (26) and liver (27). Furthermore, in hearts recovering from ischemia/reperfusion injury, YB-1 upregulation occurred transiently in a situation of sterile inflammation (4). We now demonstrate that this upregulation is accompanied by enhanced activation of YB-1 through a posttranslational phosphorylation step at serine residue 102 during the early phase of the inflammatory process. It has been shown that Akt-mediated phosphorylation of YB-1 at serine residue 102 is required for the nuclear translocation of YB-1 (7) and that this constitutes a prerequisite for downstream signaling events, such as the expression of chemokines (e.g., CCL5) by subsequent binding of activated YB-1 to its specific gene promoters (21). In accordance, loss-of-function mutations at YB-1 serine residue 102 prevent growth induction and nuclear trafficking (29) as well as proper promoter binding (30).

Our studies provide evidence that a reduced YB-1 expression results in diminished immune responses in models of acute and chronic inflammation in vivo. Reduced YB-1 expression attenuated the response to bacterial endotoxin by modulating various players, for example, by reducing the expression of TLR4 and by dampening the upregulation of chemokines and their receptors. Other studies revealed that the blockade of one of these players results in reduced sepsis manifestations, as demonstrated for TLR4 (31), CCL5 (32), CXCL1, and CCL2 (33). The major involvement of TLR4 in immune cell recruitment and renal cell activation has been extensively described (31, 34) and is perpetuated by NF-κB chain enhancer of activated B cells (NFκB) (24). This activation results in the release of proinflammatory chemokines, such as CCL2, CCL5, and CXCL1, which mediate chemotaxis of immune cells during inflammatory processes (35). Reduced expression of TLR4 on immune cells in YB-1+/d mice may explain the reduced migration capacities of these cells. Gram-negative bacteria are recognized by TLR4 via the lipid A portion of LPS (36, 37), and deletion or blockade of TLR4 is associated with enhanced survival rates (31). In addition, renal expression of the receptor CCR5 is reduced in YB-1+/d mice, and may account for the reduced migration of inflammatory cells in this work. Consequently, at the site of LPS injection, as well as in the liver and the kidneys, YB-1 WT mice exhibited substantially more inflammatory cells than YB-1+/d mice. Furthermore, BM-derived neutrophils obtained from WT mice exhibited a stronger chemotactic potential evoked through CCL5 in comparison with neutrophils from YB-1+/d animals. Thus, impaired chemotaxis toward a CCL5 gradient in cells from YB-1+/d mice additionally contributes to a reduced inflammatory response to LPS in UUO. As a consequence, YB-1+/d mice exhibited fewer Ly-6G-positive neutrophils and monocytes/macrophages in the kidney and liver than WT animals following LPS challenge.

YB-1 is a cell type–specific regulator of protein expression. Depending on the cellular context, YB-1 may act as a transcriptional activator or repressor of the same gene (5, 21). Along these lines, YB-1+/d animals showed enhanced basal expression levels of CXCL1 in the kidneys and liver, whereas the inflammatory response to LPS included a decreased CXCL1 expression in the two organs compared with WT mice. However, the peritoneal lavage fluid following LPS injection (Fig. 3C) exhibited elevated CXCL1 levels as compared with WT littermates. Notably, enhanced presence of neutrophil chemoattractant CXCL1 in the sera from YB-1+/d mice did not result in stronger inflammatory response or chemotaxis in these mice.

Following LPS stimulation, genetic depletion of YB-1 also resulted in a diminished YB-1 secretion. Extracellular YB-1 had been previously shown to exhibit chemotactic and mitogenic activities when released from LPS-stimulated monocytes (12). In this study, we could extend the number of cell types that are capable of secreting YB-1 following LPS challenge, to granulocytes, T lymphocytes, and renal and hepatic cells. Thus, the potential of cells to secrete YB-1 seems to be a universal feature. Other inflammatory mediators, such as IL-1β, macrophage migration inhibitory factor, and high-mobility group box-1, follow similar secretion routes (38). High-mobility group box-1 has been described as an early mediator in sterile injury and a late mediator in infection (39). In serum samples of septic patients, extracellular YB-1 was detected (Fig. 1A). Thus, future studies will have to prove whether YB-1 is a suitable prognostic marker and/or a therapeutic target in sepsis patients. Because genetic depletion of YB-1 resulted in the reduced secretion and impaired function of extracellular YB-1 as well, the diminished immune cell transmigration seen in our study may be partially explained by the reduced amount of chemotactically active YB-1. Decreased YB-1 expression attenuated the inflammatory response. However, further suppression of the immune system by CsA finally resulted in a life-threatening situation in YB-1+/d mice. As a consequence thereof, half of the animals died within 10 d.

In conclusion, in this study we identified YB-1 as a crucial regulator of the innate immune response. Our data contribute to a better understanding of the onset and development of immune processes in vivo and may open new therapeutic perspectives.
Supplement Figure 1. (A and B) Flow cytometry sorting of peritoneal cells after intraperitoneal LPS challenge. Wild type mice were treated with LPS (1.5 mg/kg BW; i.p.) 6 hours before isolation by peritoneal lavage, lavaged cells of two mice per group were pooled. (A) Gates used for flow cytometry sorting of cells collected from peritoneal lavage. Cells were pregated for CD45 and dead cells were excluded using Hoechst33258. Four populations were used for sorting: Neutrophils, CD115$^{hi}$ macrophages, CD115$^{lo}$ macrophages and pooled lymphocytes including CD4 and CD8 T cells as well as NK cells. (B) Sorting purity control of sorted cell populations. NK cell population was determined from CD4-CD8-lymphocyte fraction. (C and D) Number of B- (C) and T-lymphocytes (D) is elevated only in lavage of LPS-triggered WT animals and not in YB-1$^{+/d}$ mice nor in PBS control. Shown are cytospins of lavage fluid 48 h following LPS/PBS treatment. (E and F) The number of neutrophils in kidney (E) and liver (F) is not elevated 48 h following LPS-treatment of YB-1$^{+/d}$ mice and WT littermates.

Table 1. Numbers of neutrophils/macrophages and upregulation of YB-1 mRNA expression following LPS challenge (6h). Lavage cells of two mice per group were pooled, FACS sorted and YB-1 mRNA content was determined by TaqMan analysis. For sorting, macrophages were divided in resident (CD115$^{lo}$) and inflammatory (CD115$^{hi}$) cells that were combined for TaqMan analysis. Antibody stained cells were counted by FACS sorter and on cytospins (10 visual fields per cytospin). Ly6G$^+$: neutrophils, F4/80$^+$: monocytes/macrophages.
Supplemental Table 1

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<th>Cell Sorting</th>
<th>YB-1 mRNA expression</th>
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<td></td>
<td><strong>cell number after sorting</strong></td>
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<td>Neutrophils</td>
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<tr>
<td>LPS, 6h</td>
<td>6.6x10&lt;sup&gt;5&lt;/sup&gt;</td>
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