YB-1 orchestrates onset and resolution of renal inflammation via IL10 gene regulation

Jialin Wang a, Sonja Djudjaj b, Lydia Gibbert a, Vera Lennartz a, Daniel M. Breitkopf a, Thomas Rauen a, Daniela Hermert a, Ina V. Martin a, Peter Boor b, Gerald S. Braun a, Jürgen Floege a, Tammo Ostendorf a, #, Ute Raffetseder a, #, *

a Department of Nephrology and Clinical Immunology, University Hospital RWTH-Aachen, Aachen, Germany
b Institute of Pathology, University Hospital RWTH-Aachen, Aachen, Germany

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Abstract

The Y-box-binding protein (YB)-1 plays a non-redundant role in both systemic and local inflammatory response. We analysed YB-1-mediated expression of the immune regulatory cytokine IL-10 in both LPS and sterile inflammation induced by unilateral renal ischaemia–reperfusion (I/R) and found an important role of YB-1 not only in the onset but also in the resolution of inflammation in kidneys. Within a decisive cis-regulatory region of the IL10 gene locus, the fourth intron, we identified and characterized an operative YB-1 binding site via gel shift experiments and reporter assays in immune and different renal cells. In vivo, YB-1 phosphorylated at serine 102 localized to the fourth intron, which was paralleled by enhanced IL-10 mRNA expression in mice following LPS challenge and in I/R. Mice with half-maximal expression of YB-1 (Yb1+/C0) had diminished IL-10 expression upon LPS challenge. In I/R, Yb1+/2/C0 mice exhibited ameliorated kidney injury/inflammation in the early-phase (days 1 and 5), however showed aggravated long-term damage (day 21) with increased expression of IL-10 and other known mediators of renal injury and inflammation. In conclusion, these data support the notion that there are context-specific decisions concerning YB-1 function and that a fine-tuning of YB-1, for example, via a post-translational modification regulates its activity and/or localization that is crucial for systemic processes such as inflammation.

Keywords: renal inflammation ● renal fibrosis ● YB-1 ● IL-10 ● fourth intron ● ischaemia–reperfusion ● LPS

Introduction

The YB-1 is a transcriptional and translational factor, which regulates many cellular processes such as cell proliferation, DNA repair, cellular stress response, cell differentiation, embryonic development and inflammation [1–3]. In both, systemic and localized inflammatory responses to infectious stimuli [4] as well as during ‘sterile’ inflammation [3, 4], YB-1 regulates expression of pro-inflammatory mediators. In the onset of inflammation, YB-1 up-regulates the expression of pro-inflammatory factors such as interleukin (IL)-6 [5] and CCL5 [4, 6–8]. CCL5 is an important inducer of immune cell infiltration, which is transcriptionally and translationally [4] regulated by YB-1 in monocytes/macrophages [8] as well as in human arterial smooth muscle cells [7]. However, anti-inflammatory properties of YB-1 via a trans-repressive capacity on the Ccl5 promoter upon macrophage differentiation are also described [8]. Thus, YB-1 influences the early-phase of inflammation and also seems to contribute to its termination in the later phase. However, so far, it is unknown whether this dual role of YB-1 also encompasses the regulation of proteins with anti-inflammatory properties such as IL-10.

Experimental renal ischaemia–reperfusion (I/R) allows to analyse the onset of acute kidney injury (AKI) and late sequelae including renal regenerative processes [9, 10]. Following lack of blood supply with hypoxia (ischaemia) and restoration of the circulation (reperfusion), reversible renal inflammation occurs. Experimental I/R models one of the leading cause of AKI in both native and transplant kidneys [11, 12], which is still associated with high mortality and morbidity [13, 14]. In I/R-induced AKI, an immune response, is initiated following the ischaemic insult via the extravasation of immune cells through the disrupted endothelium. Early inflammatory events occur in the microvascular and tubulointerstitial compartment of the medulla with leukocytes and platelets blocking renal microcirculation. Subsequently, this strong inflammatory response further spreads to the...
renal cortex and chemokine expression is still high during the repair phase [10].

Both, the early- and the late-phase of kidney I/R are characterized by infiltration of immune cells including different subtypes of T lymphocytes [15], macrophages and dendritic cells [16] that can facilitate injury but also promote repair.

Immune regulatory mediators, such as IL-10, have been described as renoprotective, as they alleviate kidney damage in nephritis [17, 18]. IL-10 suppresses activation and accumulation of innate immune cells. Recently, regulatory T cell (Treg)-derived IL-10 has been described to exert an important protective role in murine models of I/R [17] and crescentic glomerulonephritis [18]. Beyond immune cells, resident renal cells, that is mesangial [19] and tubular [20] cells, are also able to produce IL-10.

In this study, we unravel molecular mechanisms underlying YB-1-induced IL-10 production during immune responses to microbial PAMPs as well as during sterile inflammation and clarify the general capacity of YB-1 to mediate pro- and anti-inflammatory processes in these models in wild-type (WT) and Yb1+/− mice. As it controls IL-10 expression, we demonstrate that YB-1 orchestrates both onset and resolution of the inflammatory responses.

Materials and methods

Cell culture

Rat mesangial cells (rMCs) and human T lymphocytes (HUT78T) were cultured in RPMI 1640 medium, and human tubular epithelial cells (HK-2) were incubated in low-glucose DMEM medium with 1% MEM-NEAA and 10 μg/ml insulin. All media were supplemented with 10% FCS, 100 units/ml penicillin and 100 units/ml streptomycin. All cell lines were maintained at 37°C in humidified air with 5% CO₂. Some cells were incubated with ochratoxin A (OTA, Santa Cruz Biotechnology, Heidelberg, Germany, 20 μM) for the indicated times or with 10 ng/ml LPS (Sigma-Aldrich, Steinheim, Germany) or with PBS alone for 6 hrs. To prevent phosphorylation of YB-1, cells were pre-incubated with 10 μM Ly294002 (Calbiochem, Darmstadt, Germany) for 2 hrs. Results were confirmed in at least three independent experiments.

Plasmids

Plasmids encoding the luciferase gene solely or the luciferase gene fused with the IL10 fourth intron (630 bp) were described before [21]. A full-length YB-1 expression plasmid (YB-1-pSG5) was kindly donated by J.Ting (Lineberger Comprehensive Center, University of North Carolina).

Transient transfection

Transient transfections in HK-2 cells were performed using calcium phosphate precipitates as described previously [22]. rMCs (2 × 10⁵ cells/well) were transiently transfected with the lipid-based transfection reagent FuGENE 6 HD (Promega, Madison, WI, USA) according to the manufacturer’s instruction. Human HUT78T cells were transfected by means of electroporation; 2.5 × 10⁶ cells resuspended in 1 ml of RPMI 1640 medium supplemented with 20% FCS were added to electroporation cuvettes (0.4 cm gap, Bio-Rad, Hercules, CA, USA) together with a total amount of 20 μg of plasmid DNA, respectively. The mixture was incubated for 5 min. on ice, and cells were electroporated at 250 V/1100 μF in a Gene Pulser II electroporation system (Bio-Rad). Cells were incubated another 5 min. on ice prior to resuspension in 2 ml of RPMI 1640 medium supplemented with 20% FCS, transferred to six-well tissue culture plates and incubated at 37°C and 5% CO₂.

Luciferase reporter assay

Luciferase reporter constructs were introduced into HUT78T, HK-2 cells and rMCs by the indicated transfection protocols mentioned above. Luciferase assay using the Dual-Luciferase® Assay System (Promega) was performed according to the manufacturer’s instruction and as described before [6]. Results were confirmed in at least three independent experiments.

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 Yb1 locus (Yb1−/+), 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). Homozygous YB-1-deficient mice are not viable and die before birth due to neuronal disorders, which precludes their use [23].

Ischaemia/Reperfusion (I/R)-induced inflammation model

For the unilateral renal I/R-induced inflammation model, 13- to 16-week-old male Yb1+/− mice and their age-matched WT littermates (Yb1−/+; WT) were anaesthetized with i.p. injection of 0.01% xylazine hydrochloride and 1% ketamine hydrochloride mixture diluted in 0.9% NaCl acclimatized on a warmed electrical plate for 15 min. (constant plate temperature of 37°C). The abdominal cavity was exposed through left lateral incision, and the left renal pedicle was carefully isolated. The pedicle occlusion was performed using non-traumatic vascular clamps for 30 min. or 25 min., and the efficacy of occlusion was confirmed by colour changing in the entire kidney. Sham-operated mice underwent the same procedure without clamping the vessels. During the whole procedure, mice were maintained at 37°C using a warmed electrical plate. Mice were killed by cervical dislocation on days 1, 5 and 21 after renal reperfusion. The left kidney tissues were taken for immunohistochemical analyses, protein and mRNA extraction.

LPS (Lipopolysaccharide)-induced inflammation model

For the LPS-induced inflammation model, 18- to 22-week-old Yb1+/− mice and their age-matched WT littermates received a single dose of
for each reaction in a total of 25
Core kit with SYBR Green I; Eurogentec, Seraing, Belgium) were used.
Gapdh was used as an internal standard. The primer sequences for
rRNA (Hs99999901_s1) as an internal control from Applied Biosys-
tems.

For Ngal detection, 0.75 µl cDNA and 12.5 µl PCR Master Mix (qPCR
Core kit with SYBR Green I; Eurogentec). The conditions for all Taqman
PCRs were 50°C for 2 min., followed by 40 cycles of 95°C for 15 sec.
and 60°C for 1 min.

Quantitative real-time RT-PCR
For quantitative real-time RT-PCR, total RNA was purified from corti-
cal kidney tissue by TRizol reagent (Life Technologies, Darmstadt,
Germany), according to the manufacturer’s protocol. First-strand
cDNA was synthesized with Moloney murine leukemia virus reverse
transcriptase (Invitrogen, Carlsbad, CA). qRT-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 mouse il10 (Mm00439614_m1), mouse chemokine Ccl5
(Mm01302428_m1), rat il10 (Rn00563409_m1), human il10
(Hs00961622_m1), human YB1 (Hs02742754_g1) and eukaryotic 18S
rRNA (Hs99999901_s1) as an internal control from Applied Biosys-
tems.

For Ngal detection, 0.75 µl cDNA and 12.5 µl PCR Master Mix (qPCR
Core kit with SYBR Green I; Eurogentec) was used as an internal standard. The primer sequences for Ngal and
Gapdh were as follows: Gapdh, 5'-GATGGTGATGGGCTTCCC-3'; and 5'-TCACCACCCATTCAGTTGTCA-3'.
The amount of gene expression was determined by the comparative deltaCT methodology.

For ChIP analyses, real-time qPCR was performed using a qPCR
Core Kit for SYBR Green I (Eurogentec). The conditions for all Taqman
PCRs were 50°C for 2 min., followed by 40 cycles of 95°C for 15 sec.
and 60°C for 1 min.

Chromatin immunoprecipitations (ChiPs)
ChiP assay was performed using SimpleChip Plus Enzymatic Chromatin
IP Kit (Cell Signaling, Danvers, MA, USA) according to the manufac-
turer’s protocol and as described before [24]. Antibodies specific to his-
tone H3 (Cell Signaling) as a positive control, YB-1 (Eurogentec) and
p-YB-1S102 (Cell Signaling), were added for immunoprecipitation and
compared to control non-specific IgG (Cell Signaling). A proportion
(20%) of the samples was kept as ‘input’ to represent the PCR amplifi-
cation of the total sample.

The real-time qPCR primer sequences used to amplify the regions
within the murine il10 fourth intron were as follows: 5'-TGTGGGAACC-
CAGCAAATG-3'; 5'-TGTAACTGAGGTGGTGGCTTTA-3'. The amount of
immunoprecipitated DNA was subtracted from the amplified DNA that
was bound by the non-specific IgG control and calculated relative to
the respective input DNA. Further, real-time qPCR products were
separated on a 3% agarose gel containing 0.1% GelRed (Biotium,
Hayward, CA).

Electrophoretic mobility shift analyses
The biotinylation of synthetic DNA probes corresponding to the anti-
sense strands of the human IL10 fourth intron sequences was per-
formed with the Biotin 3’ End DNA Labeling Kit (Thermo Fisher
Scientific, Rockford, Illinois, USA) according to the manufacturer’s
instructions. Nuclear cell extract preparation and EMSA with Light-Shift

Cortical kidney lysates and Western blot analysis
Cortical kidney lysates were prepared as described before [4]. Protein
concentrations were determined using BC Assay Protein Quanti-
tation Kit (Uptima Interchim, Montluçon, France) according to the manufac-
turer’s protocol; 10 to 30 µg protein of kidney extracts was subjected to
SDS-PAGE. Proteins were subsequently transferred to nitrocellu-
lose membranes and visualized by Lumi-Light and Lumi-LightPLUS Western
blotting substrate (Roche Diagnostics GmbH, Mannheim, Germany),
respectively. The primary antibodies anti-YB-1 against the protein
C-terminus (Sigma-Aldrich) and anti-p-YB-1 against phosphorylated
Ser-102 (Cell Signaling) and antibody against NGAL (R&D systems, USA)
were used. To ensure equal protein loading, the blots were additionally
incubated with a monoclonal GAPDH-specific antibody (Novus Biologi-
cals, Littleton, CO).

Immunohistochemistry/immunofluorescence
Immunohistochemistry was performed in 1-µm-thick paraffin sections of
methyl Carnoy’s-fixed specimens using the Vectastain Avidin/Biotin Sys-
tem (Vector Laboratories, Burlingame, CA), as described before [25].
Renal tissues were stained using the following primary antibodies: anti-
human Col1A1 (Southern Biotech, Birmingham, AL), anti-Kim1 (R&D
systems, USA), anti-mouse F4/80 (Serotec, Düsseldorf, Germany), anti-
proliferating cell nuclear antigen (PCNA) (Leinco Technologies, St.
Louis, MO) and anti-Ly6G (BD Biosciences, San Jose, CA). Negative controls
were stained for the immunohistochemical procedures consisted of substi-
tution of the primary antibody with non-immune IgG. Quantification of
Ly6G was described before [4]. Total number of PCNA-stained nuclei in renal tubuli
was counted in 25 randomly selected fields at ×200 magnification. For
staining quantification of collagen and F4/80, 20 consecutive images of
tubular cortex were taken per section. The percentage of the positively
stained area was extracted for intensity using ImageJ software (Wayne
Rasband, NIH) and a mean area was calculated. Periodic acid–Schiff
(PAS) staining was performed as described before [25], and the tubular
injury was scored on a scale of 0–4: 0 = none; 1 = 0–25%; 2 = 25–
50%; 3 = 50–75%; 4 = more than 75%. The total score is the calcu-
lated average of all tubular scores.

Statistical analysis
Values are expressed as means ± S.D. Statistical significance was eval-
uated using the Student’s t-test or ANOVA and Bonferroni post hoc
test, whenever more than two groups were compared, with significance
accepted when P value was less than 0.05. All in vitro experiments were
performed at least in triplicate.

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Results

**YB-1 regulates IL10 expression via a regulatory element within the IL10 gene locus**

To examine the impact of YB-1 on IL10 expression, we inspected the fourth intron (4th intron), which constitutes an important intronic enhancer element within the human IL10 gene locus [21], for potential YB-1 binding sites. We detected a putative Y-box with an adjacent inverted repeat motif (indicated by asterisks in Fig. 1A) starting at 4346 bp within the IL10 gene. This element displayed strong homologies with known YB-1-binding motifs within the matrix metalloproteinase2 (Mmp2) [26] and DNA polymerase-α (DPA) [27] gene loci (Fig. 1B).

Electrophoretic mobility supershift assays (EMSA) were performed with oligonucleotides that encompass the Y-box within the fourth intron of the human IL10 gene. Indeed, we observed protein–DNA complexes (indicated by arrowheads in Fig. 1C) when nuclear extracts of renal cells, namely rat mesangial cells (rMCs, left) and human tubular cells (HK-2, right) that are known to produce IL-10 [19, 20], were included. Specifically, addition of an antibody against the C-terminus of YB-1 protein confirmed its binding to this site. Consequently, YB-1 overexpression enhanced the IL10 fourth intron activity in rMCs (Fig. 1D) and HK-2 cells (Fig. 1E).

For some genes, Akt-mediated YB-1 phosphorylation at serine residue 102 (p-YB-1S102) [28] is a prerequisite for its trans-activating capacities [6, 29] and previous studies have revealed YB-1 phosphorylation in LPS-challenged primary MCs and in a model of acute LPS-triggered nephritis in vivo [4]. Thus, we next asked whether targeting Akt/PKB kinase activity also affects IL10 expression upon exposition to microbial PAMPs. Pre-incubation with kinase inhibitor Ly294002 resulted in a complete blockade of LPS-triggered elevated expression of IL-10 (Fig. 2A) and activation of the fourth intron measured by a reporter assay in rMCs (Fig. 2B). Comparable results were obtained in T lymphocytes (HUT78T) (Fig. 2C).

Next, we addressed the impact of (phospho)-YB-1 on renal Il10 expression in mice. We compared binding of YB-1 (Fig. 2D and E) and p-YB-1S102 (Fig. 2D and F) to the Il10 fourth intron in kidneys of three mice challenged with LPS or with PBS as control. Particularly, binding of p-YB-1S102 to the fourth intron was enhanced 12 hrs following LPS treatment (Fig. 2F) along with elevated renal IL-10 mRNA expression (Fig. 2G). The importance of YB-1 for Il10 gene expression during LPS-triggered inflammation

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Fig. 1 (A) Inspection of fourth intron within the human IL10 gene locus revealed a putative YB-1-binding site starting at 4346 bp. (B) Comparison with known YB-1-binding motifs of the matrix metalloproteinase2 (Mmp2) and DNA polymerase-α (DPA) genes revealed strong homologies and an inverted repeat motif (indicated by stars and arrows). (C) Nuclear protein extracts from rMCs and HK-2 cells were prepared, and complex formation with the oligonucleotide that encompasses the Ybox within the IL10 fourth intron was assessed. Two strong high-mobility nucleoprotein complexes appeared (<) which were strongly weakened especially in the presence of C-terminal YB-1-specific polyclonal antibody but not with non-specific IgG antibody as control. (D/E) Relative IL10 fourth intron luciferase activity was enhanced after YB-1 overexpression in rMCs (D) and in HK-2 cells (E). Experiments were performed in at least three independent experiments, each performed in triplicate. Data are expressed as mean values ± S.D. *P < 0.05. NE, nuclear extract.

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was further underlined by a reduced mRNA expression (Fig. 2H) in kidneys of Yb1+/- mice that display half-maximal expression of YB-1 at both the transcript and protein levels [4, 23, 30] compared to their wild-type (WT) littermates. Vice versa, an increased YB-1 content enhanced IL10 fourth intron activity in renal mesangial cells (Fig. 2I) and in T lymphocytes (Fig. 2J) following LPS activation.

Thus, (phospho)-YB-1 binds to IL10 fourth intron and hereby regulates early adjustment of inflammation via expression of immune regulatory mediator IL-10 in renal and immune cells.
Half-maximal YB-1 expression results in decreased tubular damage in the early-phase of I/R but hinders renal regeneration at later stages

To further investigate the regulatory role of YB-1 not only in driving acute inflammation but also to monitor progression and resolution of renal inflammation/regeneration, we used a second model of kidney damage associated with sterile inflammation, the unilateral renal ischaemia–reperfusion injury model (I/R) in the early (day 1 after I/R), established (day 5) and advanced disease stage (day 21). In this model, treatment with IL-10 has been previously shown to ameliorate both renal and systemic inflammation [31]. During the time course of I/R, YB-1 mRNA was significantly up-regulated in kidney cortex on day 1 (Fig. 3A), returned to normal levels on day 5 and was significantly reduced on day 21. Western blot analyses confirmed transient upregulation of renal YB-1 (Fig. 3B/C).

In order to elucidate YB-1’s function in this model, we used Yb1−/− mice and compared these to WT littermates. Histological examination revealed tubular damage in all I/R groups, indicated by loss of the brush border, cast formation (arrow), acute tubular dilatation (d) and necrosis (arrowhead) (Fig. 3D). Compared to WT animals, Yb1−/− mice exhibited less pronounced damage on days 1 and 5. However, 21 days after I/R, mice with half-maximal YB-1 expression exhibited significantly stronger damage compared to WT mice, demonstrated by the tubular damage score (Fig. 3D/E) and by areas of exceeding inflammation/fibrosis especially in Yb1−/− mice (Fig. 3D, boxed region with a higher power mage; marked by asterisk).

Our findings were further demonstrated by expression of the tubular damage markers neutrophil gelatinase-associated lipocalin (NGAL) (Fig. 3F/G). In WT mice, a transiently elevated expression of NGAL protein (Fig. 3F) and NGAL mRNA (Fig. 3G) was observed that peaked on day 1 and continuously decreased in the subsequent observation period until day 21. In contrast, in Yb1−/− animals, NGAL mRNA expression significantly decreased on day 5, but significantly increased again on day 21 compared to WT mice (Fig. 3G, white bars). Elevated NGAL protein was detectable on day 21 only in Yb1−/− mice (Fig. 3F, far right). Augmented tubular damage on day 21 in Yb1−/− mice was additionally confirmed by immunofluorescence of kidney injury molecule (KIM)-1 (Fig. 4A).

Temporal mRNA expression of CCL5, a key chemokine in tissue inflammation, was lower in Yb1−/− compared to WT animals at earlier time-points in I/R, and however was significantly elevated on day 21 (Fig. 4B). This shifted immune response was reinforced by the analysis of infiltration of Ly6G+ and F4/80+ immune cells. In I/R, early inflammatory events start from the medulla and further spreads to the renal cortex and this is triggered among others by neutrophils. In the medulla of Yb1−/− mice, markedly fewer Ly6G+ cells were detected on day 1; however at later time-points, increased numbers of medullary Ly6G+ cells were detected in none of the two genotypes (data not shown). However, significant more renal cortical Ly6G+ cells were detected on day 21 in Yb1−/− compared to WT mice (Fig. 4C). On day 5, the number of cortical F4/80+ monocytes/macrophages was significantly higher in cortices of WT compared to Yb1−/− mice whereas on day 21 no statistical significant difference was observed between the two genotypes (Fig. 4D).

Following I/R, renal cortical deposition of collagen type 1A (Col1A), a major extracellular matrix (ECM) component in renal fibrotic tissue, was significantly higher in WT compared to Yb1−/− animals on day 5, however significantly lower on day 21 (Fig. 4E/F). In line with this, expression of PCNA in tubules as an index of tubular proliferation but also of tubular repair and renal regeneration was significantly higher in Yb1−/− than in WT animals on day 1 but lower on day 21 (Fig. 4G/H).

To further explore differences during the regeneration processes in both genotypes, ischaemia time was shortened from 30 min. to 25 min. and expected kidneys analysed on day 21 exhibited considerably less tubular damage (Fig. 4I compared to Fig 3E). Advanced regeneration was also reflected in PCNA+ tubular nuclei (Fig. 4J) and of F4/80+ cells (Fig. 4K) that was already lowered again in WT, however still significantly elevated in Yb1−/− mice. Furthermore, expression of NGAL tended to be higher in Yb1−/− mice but did not reach significance (Fig. 4L).

Taken together, Yb1−/− mice exhibit an ameliorated inflammatory response and kidney damage at the earlier stages following I/R but exhibit aggravated kidney injury, inflammation, fibrosis and less regeneration on day 21.

YB-1 modulates renal IL-10 expression following I/R

To directly address anti-inflammatory properties of YB-1 in the different phases after I/R, we monitored presence of p-YB-1S102 and its binding to the Il10 fourth intron region on days 1, 5 and 21 by ex vivo ChIP analyses of murine kidneys. Analyses of kidney extracts from WT animals exhibited phosphorylation of YB-1 especially at the two early time-points following I/R (Fig. 5A/B). On day 1, enhanced binding of p-YB-1S102 (about fourfold) to the fourth intron of Il10 was observed that decreased only slightly on day 5 but clearly dropped until day 21 (Fig. 5C, broken line). In contrast to p-YB-1S102, binding of total YB-1 to the fourth intron was already strongly diminished on day 5 (Fig. 5C, solid line). During the time course of I/R, a transiently elevated renal expression of IL-10 mRNA was observed, peaking on day 5 (Fig. 5D, black bars). Thus, at the time of high Il10 gene activation, the ratio between p-YB-1S102 and total YB-1 shifted towards p-YB-1S102. In Yb1−/− mice compared to WT mice, a strongly and significantly reduced expression of renal IL-10 mRNA was observed in the acute phase of I/R, especially on day 5 (Fig. 5D, white versus black bars). However, on day 21, Yb1−/− mice exhibited significantly enhanced IL-10 expression compared to their WT littermates that was paralleled by highest renal p-YB-1S102 content on day 21 (Fig. 5E/F) and binding of YB-1/p-YB-1S102 to the Il10 fourth intron (Fig. 5G).

Subsequent studies in damaged proximal tubular HK-2 cells showed that a higher YB-1 expression enhanced Il10 fourth intron activity (Fig. 5H) with a profound increase in IL-10 mRNA expression...
Progression of chronic renal inflammation/kidney diseases critically depends on the decision between resolution and persistence of inflammation. Recently, we demonstrated that Yb1+/− mice are protected in a renal inflammation/fibrosis model with persistent a pathological condition, namely unilateral ureteral obstruction (UUO) [24]. On the contrary, in I/R, the insult is initial and trans- sient, which allows to analyse not only the onset and the progression of inflammation but also repair mechanisms that are more efficient at shorter ischemia times. Countervailing effects of YB-1 were observed during the course of this model and the perception that YB-1 is involved not only in the onset but also in the resolution of inflammation is supported by the different outcome in WT and Yb1+/− mice following I/R. While the expression of markers of inflammation (CCL5) and of fibrosis (Col1A1) was alleviated in the early-phase of inflammation (days 1 and 5) in Yb1+/− mice compared to their WT littermates, they were increased in the later phase (day 21). Thus, factors induced at early time-points might be responsible for the limited regeneration in the subsequent course of the disease. This applies, for NGAL, a well-established marker of both renal injury and cell regeneration, which was expressed to a lesser extent at day 5 in the established inflammatory phase after I/R in Yb1+/− mice compared to their WT littermates, they were increased in the later phase (day 21). Hence, factors induced at early time-points might be responsible for the limited regeneration in the subsequent course of the disease. This applies, for NGAL, a well-established marker of both renal injury and cell regeneration, which was expressed to a lesser extent at day 5 in the established inflammatory phase after I/R in Yb1+/− mice compared to their WT control mice. Furthermore, a key factor for a rapid and effective response to hypoxia includes HIF-1α that is regulated by YB-1 at the gene and/or mRNA level [38, 39]. HIF-1α protein expression is enhanced by direct YB-1 binding to and subsequent activation of the translation of HIF-1α mRNA [38].

Another important feature to suppress the immune response is the generation of IL-10. Expression of IL-10 is achieved among others by regulatory T cells (Tregs). An adoptive transfer of these cells in renal I/R exerts protective effects that were lost when IL-10-deficient Tregs were transferred [17]. However, Tregs are not the only cell population that produces IL-10 in kidneys. Thus, in Treg-depleted mice, renal IL-10 expression was still markedly increased in the late renal recovery phase after reperfusion. This is in line with our observation in the hearts recovering from I/R injury [3], where the expression of YB-1 in the myocardium was markedly increased in the early-phase after reperfusion. This is in line with our observation in the renal I/R model, where YB-1 mRNA and protein expression increased during the onset of inflammation, albeit to a lower extent as compared to other models. Recently, a decrease of YB-1 protein content 24 hrs after I/R in kidneys was described [35]. Conceivably, these opposing observations relate to differences in the experimental setting, for example bilateral [35] versus unilateral clamping that can cause different systemic effects with impact on kidney function.

Opposite effects of YB-1 during inflammatory processes have been described for CCL5 gene regulation in monocytes and in different macrophages in the course of inflammatory responses [6-8] and also for IL-6 generation following LPS challenge. In the latter, YB-1 acts by differential control of IL-6 mRNA levels as an inducer in dendritic cells and as a repressor in infiltrating macrophages [5]. Obviously, the phosphorylation status of YB-1 decides on its compartment-specific functions, as this influences its subcellular location [6, 28]. Akt/PKB-mediated phosphorylation at serine 102 (pYB-1s102) [28] promotes nuclear YB-1 translocation [6, 36, 37] and affects YB-1-dependent gene activity, as demonstrated for the promoter of epidermal growth factor receptor [29] as well as for CCL5 [6] and—as we demonstrate now—for the fourth intron region within the IL10 gene, an important anti-inflammatory mediator.

Discussion

In this study, we identified YB-1 as an important regulator of the immune regulatory cytokine IL-10 via its fourth intron region in endo- taxemia and in I/R-induced sterile inflammation and demonstrated that YB-1 exerts both pro- and anti-inflammatory properties in the course of inflammation.

Over the last years, YB-1 turned out to be an important player in modulating inflammatory events [3, 5–8, 22, 24, 33, 34]. Transient upregulation of YB-1 occurs during the acute phase of inflammation in experimental inflammation models in kidneys [4] and in hearts recovering from I/R injury [3], where the expression of YB-1 in the myocardium was markedly increased in the early-phase after reperfusion. This is in line with our observation in the renal I/R model, where YB-1 mRNA and protein expression increased during the onset of inflammation, albeit to a lower extent as compared to other models. Recently, a decrease of YB-1 protein content 24 hrs after I/R in kidneys was described [35]. Conceivably, these opposing observations relate to differences in the experimental setting, for example bilateral [35] versus unilateral clamping that can cause different systemic effects with impact on kidney function.

Opposite effects of YB-1 during inflammatory processes have been described for CCL5 gene regulation in monocytes and in differentiated macrophages in the course of inflammatory responses [6-8] and also for IL-6 generation following LPS challenge. In the latter, YB-1 acts by differential control of IL-6 mRNA levels as an inducer in dendritic cells and as a repressor in infiltrating macrophages [5]. Obviously, the phosphorylation status of YB-1 decides on its compartment-specific functions, as this influences its subcellular location [6, 28]. Akt/PKB-mediated phosphorylation at serine 102 (pYB-1s102) [28] promotes nuclear YB-1 translocation [6, 36, 37] and affects YB-1-dependent gene activity, as demonstrated for the promoter of epidermal growth factor receptor [29] as well as for CCL5 [6] and—as we demonstrate now—for the fourth intron region within the IL10 gene, an important anti-inflammatory mediator.

Fig. 3 (A) Transient upregulation of YB-1 mRNA expression in the time course of renal I/R model (n = 5–10). (B/C) Western blot analyses (B) of protein lysates for YB-1 obtained from control (ctrl) and ischaemic kidneys at days 1, 5 and 21 and quantification thereof (C). (D/E) Tubular damage in PAS-stained cortical tissue in I/R groups. Representative images (D) and quantification (E) of renal damage indicated by acute tubular dilatation (d), necrosis (arrowhead) and loss of proximal tubule brush borders revealed less pronounced damage on day 5 in Yb1+/− compared to WT I/R mice but more on day 21 (n = 5–10). (F) Western blot analyses of renal protein extracts of sham, I/R WT and Yb1+/− mice with anti-NGAL Ab and quantification thereof (lower panel). (G) Transcript level of NGAL following I/R in WT and Yb1+/− mice. Band intensities were quantified by densitometry, and values were normalized against GAPDH. Relative band intensities are depicted in histograms (n = 5–8). Scale bars, 50 µm. Data are expressed as mean values ± S.D. *P < 0.05; ****P < 0.0001.
phase following I/R [15]. Along these lines, observations from others [19, 20] and our group proved that mesangial and tubular cells also account for IL-10 production upon an (inflammatory) insult. The regulation of IL-10 expression through YB-1 in both resident renal and immune cells via the fourth intron was demonstrated in the present study via cell culture assays and two in vivo models of renal inflammation, namely in LPS-triggered and sterile inflammation following renal I/R.

In summary, our study provides further evidence for the indispensability of YB-1 as an adaptor for the immune response and its decisive role for IL-10 expression. Thus, fine-tuning of the cellular YB-1 content and its subcellular localization is crucial in systemic inflammatory processes.

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Conflict of interests

The authors confirm that there are no conflict of interests.

References

1. Lasham A, Print CG, Woolley AG, et al. YB-1: oncoprotein, prognostic marker and therapeutic target? Biochem J. 2013; 449: 11–23.
2. Kohno K, Izumi H, Uchiumi T, et al. The pleiotropic functions of the Y-box-binding protein, YB-1. BioEssays. 2003; 25: 691–8.
3. Raffetseder U, Liehn EA, Weber C, et al. Role of cold shock Y-box protein-1 in inflammation, atherosclerosis and organ transplant rejection. Eur J Cell Biol. 2012; 91: 567–75.
4. Hanssen L, Aldiouty C, Djudjaj S, et al. YB-1 is an early and central mediator of bacterial and sterile inflammation in vivo. J Immunol. 2013; 191: 2604–13.
5. Kang S, Lee TA, Ra EA, et al. Differential control of interleukin-6 mrna levels by cellular distribution of YB-1. PLoS ONE. 2014; 9: e112754.
6. Aldiouty C, Rauen T, Hanssen L, et al. Calcineurin-mediated YB-1 Dephosphorylation Regulates CCL5 Expression during Monocyte Differentiation. J Biol Chem. 2014; 289: 21401–12.
7. Krohn R, Raffetseder U, Bot I, et al. Y-box binding protein-1 controls CC chemokine ligand-5 (CCL5) expression in smooth muscle cells and contributes to neoointima formation in atherosclerosis-prone mice. Circulation. 2007; 116: 1812–20.
8. Raffetseder U, Rauen T, Djudjaj S, et al. Differential regulation of chemokine CCL5 expression in monocytes/macrophages and renal cells by Y-box protein-1. Kidney Int. 2009; 75: 185–96.
9. Hesketh EE, Czopek A, Clay M, et al. Renal ischaemia reperfusion injury: a mouse model of injury and regeneration. J Vis Exp. 2014; (88): 51816.
10. Stroo I, Stokman G, Teske GJ, et al. Chemokine expression in renal ischemia/reperfusion injury is most profound during the reparative phase. Int Immunol. 2010; 22: 433–42.
11. Tilney NL, Guttmann RD. Effects of initial ischemia/reperfusion injury on the transplanted kidney. Transplantation. 1997; 64: 945–7.
12. Devarajan P. Update on mechanisms of ischemic acute kidney injury. J Am Soc Nephrol. 2006; 17: 1503–20.
13. Star RA. Treatment of acute renal failure. Kidney Int. 1998; 54: 1817–31.
14. Mehta RL, Pascual MT, Soroko S, et al.; Program to Improve Care in Acute Renal D. Spectrum of acute renal failure in the intensive care unit: the PICARD experience. Kidney Int. 2004; 66: 1615–21.
15. Gandolfo MT, Jang HR, Bagnasco SM, et al. Foxp3 + regulatory T cells participate in repair of ischemic acute kidney injury. Kidney Int. 2009; 76: 717–29.
16. Li L, Huang L, Ye H, et al. Dendritic cells tolerateized with adenosine A(2)AR agonist attenuate acute kidney injury. J Clin Invest. 2012; 122: 3931–42.
17. Kinsey GR, Sharma R, Huang L, et al. Regulatory T cells suppress innate immunity in kidney ischemia-reperfusion injury. J Am Soc Nephrol. 2009; 20: 1744–53.
18. Ostmann A, Paust HJ, Panzer U, et al. Regulatory T cell-derived IL-10 ameliorates crescentic GN. J Am Soc Nephrol. 2013; 24: 930–42.
19. Sinuani I, Averbukh Z, Gileiman I, et al. Mesangial cells initiate compensatory renal tubular hypertrophy via IL-10-induced TGF-beta secretion: effect of the immunomodulator AS101 on this process. Am J Physiol Renal Physiol. 2006; 291: F384–94.
20. Dhande A, Ali Q, Hussain T. Proximal tubule angiotensin AT2 receptors mediate an anti-inflammatory response via interleukin-10: role in renoprotection in obese rats. Hypertension. 2013; 61: 1218–26.
21. Hedrich CM, Rauen T, Apostolidis SA, et al. Stat3 promotes IL-10 expression in lupus T cells through trans-activation and chromatin remodeling. Proc Natl Acad Sci U S A. 2014; 111: 13457–62.
22. Rauen T, Raffetseder U, Frye BC, et al. YB-1 acts as a ligand for Notch-3 receptors and modulates receptor activation. J Biol Chem. 2009; 284: 26928–40.
23. Lu ZH, Books JT, Ley TJ. YB-1 is important for late-stage embryonic development, optimal cellular stress responses, and the prevention of premature senescence. Mol Cell Biol. 2005; 25: 4625–37.
24. Wang J, Gibbert L, Djudjaj S, et al. Therapeutic nuclear shuffling of YB-1 reduces renal damage and fibrosis. Kidney Int. 2016; 90: 1226–37.
25. Djudjaj S, Chatziantoniou C, Raffetseder U, et al. Notch-3 receptor activation drives inflammation and fibrosis following tubulointerstitial kidney injury. J Pathol. 2012; 228: 286–99.
26. Mertens PR, Harendza S, Pollock AS, et al. Glomerular mesangial cell-specific transactivation of matrix metalloproteinase 2 transcription is mediated by YB-1. J Biol Chem. 1997; 272: 22905–12.
27. En-Nia A, Yilmaz E, Klinge U, et al. Transcription factor YB-1 mediates DNA polymerase alpha gene expression. J Biol Chem. 2005; 280: 7702–11.
28. Sutherland BW, Kucab J, Wu J, et al. Akt phosphorylates the Y-box binding protein 1 at Ser102 located in the cold shock domain and affects the anchorage-independent growth of breast cancer cells. Oncogene. 2005; 24: 4281–92.
29. Stratford AL, Habibi G, Astaneh A, et al. Epidermal growth factor receptor (EGFR) is transcriptionally induced by the Y-box binding protein-1 (YB-1) and can be inhibited with Iressa in basal-like breast cancer, providing a potential target for therapy. Breast Cancer Res. 2007; 9: R61.
30. Raffetseder U, Rauen T, Boor P, et al. Extracellular YB-1 blockade in experimental nephritis upregulates Notch-3 receptor expression and signaling. Nephron Exp Nephrol. 2011; 118: e100–8.
31. Soranno DE, Rodel CB, Altmann C, et al. Delivery of interleukin-10 via injectable hydrogels improves renal outcomes and reduces systemic inflammation following ischemic acute kidney injury in mice. Am J Physiol Renal Physiol. 2016; 311: 362–72.
32. Sorrenti V, Di Giacomo C, Acquaviva R, et al. Toxicity of ochratoxin a and its modulation by antioxidants: a review. Toxins. 2013; 5: 1742–66.
33. Hanssen L, Frye BC, Oostendorf T, et al. Y-box binding protein-1 mediates profibrotic effects of calcineurin inhibitors in the kidney. J Immunol. 2011; 187: 298–308.
34. van Roeyen CR, Eitner F, Martinkus S, et al. Y-box protein 1 mediates PDGF-B
effects in mesangioproliferative glomerular disease. J Am Soc Nephrol. 2005; 16: 2985–96.
35. Dong W, Wang H, Shahzad K, et al. Activated protein C ameliorates renal ischemia-reperfusion injury by restricting y-box binding protein-1 ubiquitination. J Am Soc Nephrol. 2015; 26: 2789–99.
36. Sinnberg T, Sauer B, Holm P, et al. MAPK and PI3K/AKT mediated YB-1 activation promotes melanoma cell proliferation which is counteracted by an autoregulatory loop. Exp Dermatol. 2012; 21: 265–70.
37. Wu J, Lee C, Yokom D, et al. Disruption of the Y-box binding protein-1 results in suppression of the epidermal growth factor receptor and HER-2. Cancer Res. 2006; 66: 4872–9.
38. El-Naggar AM, Veinotte CJ, Cheng H, et al. Translational Activation of HIF1alpha by YB-1 Promotes Sarcoma Metastasis. Cancer Cell. 2015; 27: 682–97.
39. Rauen T, Frye BC, Wang J, et al. Cold shock protein YB-1 is involved in hypoxia-dependent gene transcription. Biochem Biophys Res Commun. 2016; 478: 982–7.
40. Merline R, Moreth K, Beckmann J, et al. Signaling by the matrix proteoglycan decorin controls inflammation and cancer through PDCD4 and MicroRNA-21. Sci Signal. 2011; 4: ra75.
41. Tsuji-Takayama K, Suzuki M, Yamamoto M, et al. The production of IL-10 by human regulatory T cells is enhanced by IL-2 through a STAT5-responsive intronic enhancer in the IL-10 locus. J Immunol. 2008; 181: 3897–905.
42. Grant LR, Yao ZJ, Hedrich CM, et al. Stat4-dependent, T-bet-independent regulation of IL-10 in NK cells. Genes Immun. 2008; 9: 316–27.