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Erythropoietin attenuates acute kidney dysfunction in murine experimental sepsis via activation of the β-common receptor

Sina M. Coldewey[1, 2]*,Areeg I. Khan[1]*, Amar Kapoor[1], Massimo Collino[3], Mara Rogazzo[3], Michael Brines[4], Anthony Cerami[4], Anthony P. Hall[5], Michael Sheaff[7] Julius E. Kieswich[1], Muhammed M. Yaqoob[1, 6], Nimesh S.A. Patel[1], Christoph Thiemermann[1]

Author affiliations

[1]Queen Mary University of London, Barts and The London School of Medicine & Dentistry, The William Harvey Research Institute, London, United Kingdom; [2]Hannover Medical School, Department of Anaesthesia and Intensive Care Medicine, Hannover, Germany; [3]University of Turin, Department of Drug Science and Technology, Turin, Italy; [4]Araim Pharmaceuticals, Ossining, NY 10562, USA; [5]Innovative Medicines Global Safety Assessment, AstraZeneca, Cheshire, United Kingdom; [6]Department of Nephrology, Barts Health NHS Trust, The Royal London Hospital, London, United Kingdom, [7] xxx

* SMC and AIK contributed equally to this study

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Addresses for correspondence:

Professor C Thiemermann, MD (Dr. med.) PhD (LON) FPharmacolS FRCP FMedSci Queen Mary University of London Barts and The London School of Medicine & Dentistry William Harvey Research Institute Centre for Translational Medicine and Therapeutics Charterhouse Square, London, EC1M 6BQ, UK Phone: + 44 (0) 20 7882 2121; Fax: +44 (0) 20 700 3870; E-mail: c.thiemermann@qmul.ac.uk	Dr. Sina Maren Coldewey Queen Mary University of London Barts and The London School of Medicine & Dentistry William Harvey Research Institute Centre for Translational Medicine and Therapeutics Charterhouse Square, London, EC1M 6BQ, UK Phone: + 44 7412594597; E-mail: s.m.coldewey@qmul.ac.uk

Abstract There is evidence that the β-common receptor (βcR) plays a pivotal role in the tissue-protective (non-hematopoietic) effects of erythropoietin (EPO). This study was designed to investigate whether EPO reduces the acute kidney injury (AKI) caused by sepsis in the mouse and whether this effect is mediated by the βcR. In two months old C57BL/6 wild type and βcR knockout mice, lipopolysaccharide (9 mg/kg for 18 h) caused a significant increase in urea and creatinine and, hence, AKI. This AKI was not associated with any overt morphological alterations in the kidney. The AKI was attenuated by EPO (1000 IU/kg, 1 h after lipopolysaccharide) in wild type mice, but not in βcR knockout mice. In kidneys of endotoxemic wild type mice, EPO enhanced the phosphorylation of Akt, glycogen synthase kinase-3β and endothelial nitric oxide synthase and inhibited the activation of nuclear factor-κB. All of these effects of EPO were lost in βcR knockout mice. Sepsis is more severe in older animals or patients. Eight months old wild type and βcR knockout mice that underwent cecal ligation and puncture developed AKI at 24 h, which was attenuated by EPO (1000 IU/kg, 1 h post cecal ligation and puncture) in wild type mice, but not in βcR knockout mice. In conclusion, we report, for the first time, that the activation of the βcR by EPO is essential for the observed reduction in AKI in either endotoxemia (young mice) or polymicrobial sepsis (old mice), and for the activation of well-known signaling pathways by EPO.

Introduction

Sepsis is a complex clinical entity caused by an individual's systemic response to an infection with a wide range of clinical symptoms often leading to multiple organ dysfunction syndrome (MODS) and ultimately multiple organ failure (MOF). Despite a substantially improved knowledge of the pathophysiology of sepsis, the treatment of this condition is still a clinical challenge. To date, therapies have been mostly supportive and all specific therapeutic approaches, except early administration of antibiotics and "early goal directed therapy" [1, 2], have failed to be translated successfully into the clinical setting. Hence, new pharmacological strategies are urgently needed to improve this condition. In sepsis some organs can escape relatively unscathed, whereas others become affected both early and severely [3]. The kidney is one such organ that can be affected early with the development of acute kidney injury (AKI). This dysfunction is one of the most frequent and serious complications of sepsis and septic shock [4]. According to the Acute Kidney Injury Network consensus conference in 2007, AKI is diagnosed as an abrupt (within 48 hours) reduction in kidney function defined as an absolute increase in serum creatinine (≥ 26.4 µmol/l), a percentage increase in serum creatinine (≥ 1.5-fold from baseline), or a reduction in urine output (≤ 0.5 ml/kg per h for more than 6 h) [5]. It is interesting to note that this definition does not take into consideration histological evidence of tissue injury and is solely based on rise in creatinine or fall in urinary output - even though the word injury may imply the existence of morphological evidence of injury [6].

Interestingly, the incidence of AKI increases with the severity of sepsis, occurring in 19 % of patients with sepsis, 23 % patients with severe sepsis and 51 % patients with septic shock [7]. Sepsis occurs more frequently and severely in older patients [8] and there is some evidence that the elderly septic patient is more susceptible for the development of AKI [9]. Similarly, in animal models of experimental sepsis systemic inflammation and the release of pro-inflammatory cytokines are greater in older animals [10-12] and the severity of septic AKI was recently reported to be age-dependent in female mice [13]. It has been proposed that the

increase in the severity of sepsis in aging animals is secondary to changes in gut flora (Hyde et al., 1990).

Emerging evidence suggests that the pathogenesis of septic renal dysfunction involves distinct mechanisms when compared to a non-septic etiology [14]. There is evidence in man, that the AKI caused by sepsis is not associated (or at least solely explained) by significant morphological changes such as extensive tubular necrosis [6, 15]. The proposed pathophysiological mechanisms of sepsis-associated AKI include systemic vasodilatation, intra-renal vasoconstriction, inflammation and bioenergetics failure [14, 16 6, 7, 17].

The pleiotropic hormone erythropoietin (EPO) is known to possess organ protective properties, which are independent from its well-established hematopoietic effects [18]. Endogenous EPO is primarily produced by renal cortical fibroblasts, and the fact, that erythropoietin receptors (EPO-R) are expressed in glomerular, endothelial and tubular epithelial cells [19, 20], suggests endogenous EPO may function in a paracrine manner to limit the extent of injury after a noxious stimulus [19]. Furthermore, studies have proposed that the tissue protective effects of EPO are mediated through a 'tissue-protective' receptor, which is pharmacologically distinct from the classical hematopoietic EPO-R [21]. This tissue protective receptor is a heterocomplex composed of EPO-R and the β-common receptor (βcR), which exhibits a lower affinity for EPO [22]. Over the years, EPO has been shown to be protective in various animal models of injury including cerebral ischemia [23, 24], myocardial ischemia [25-27], and renal injury [28-30]. Indeed, in a model of bilateral renal artery occlusion in the rat, EPO inhibits apoptotic cell death, promotes renal functional recovery, and enhances tubular epithelial regeneration [19].

It should be noted that the effects of EPO in sepsis-induced AKI are controversial [31-36]. Some studies report no beneficial effect of EPO in models of endotoxemia in the rat or pig [31]; [32]. In contrast, other studies have shown that EPO exerts beneficial effects in experimental sepsis. In 2007, Mitra et al. reported for the first time that pretreatment with EPO reduces the renal dysfunction in the mouse [34]. Moreover, administration of EPO given 1 h after endotoxin or CLP

reduces renal dysfunction and mortality in mice [35]. Pessoa de Souza et al. recently reported their extensive investigation that pre- or post-treatment with high-dose EPO (4000 IU/kg) exhibited anti-inflammatory effects, improved survival as well as renal function in a rat model of sepsis. However, none of the above (positive) studies investigated the role of the βcR in either the observed effects or the signaling events initiated by EPO.

This study investigates the effects of EPO and specifically, the role of the βcR in the renal dysfunction caused by endotoxemia [lipopolysaccharide (LPS)] or polymicrobial sepsis [cecal ligation and puncture (CLP)].

Results

Effect of EPO on renal dysfunction in endotoxemic WT and βcR KO mice

When compared to sham mice treated with saline, WT mice subjected to 18 h of endotoxemia demonstrated a significant increase in both serum urea (7.3 ±. 0.27 vs. 33.1 ±.1.75 mmol/l, $P<0.05$, Figure 1A) and serum creatinine (25.9 ± 0.92 vs. 57.1 ± 4.72 µmol/l, $P<0.05$, Figure 1C), indicating the development of AKI. Administration of EPO significantly attenuated the rise in serum urea (33.1 ± 1.75 vs. 25.9 ± 3.2 mmol/l, $P<0.05$, Figure 1A) and creatinine (57.1 ± 4.72 vs. 38.2 ± 2.47 µmol/l, $P<0.05$, Figure 1C). To investigate the role of the proposed tissue protective receptor in LPS-induced AKI for the beneficial properties of EPO reported above, we have evaluated the effect of EPO on serum urea and creatinine in βcR KO mice. When compared to sham βcR KO mice treated with saline, βcR KO mice subjected to 18 h of endotoxemia demonstrated a significant increase in serum urea (8.3 ± 0.55 vs. 33.0 ± 1.94 mmol/l, $P<0.05$, Figure 1B) and creatinine (27.8 ± 0.81 vs. 59.4 ± 5.06 µmol/l, $P<0.05$; Figure 1D). Most notably, the renal dysfunction caused by endotoxemia in βcR KO mice was not attenuated by EPO (urea 33.0 ± 1.94 vs. 34 ± 1.27 mmol/l, $P>0.05$, Figure 1B; creatinine 59.4 ± 5.06 vs. 54.9 ± 3.06 µmol/l, $P>0.05$, Figure 1D).

Effects of endotoxemia and/or EPO on renal morphology (light microscopy) and immunohistochemical staining for apoptosis [cleaved caspase-3 (CCR3)] kidney injury molecule-1 (KIM-1)

To gain a better understanding of the mechanisms underlying the observed renal dysfunction in endotoxemia, we carried out an extensive histological analysis in all experimental groups. Evaluation by light microscopy (HE-staining) revealed that there was no overt morphological evidence of proximal tubular epithelial cell injury, interstitial edema, interstitial inflammation, vasculopathy or glomerular abnormality in any of the study groups (Figure 2A). Therefore the results of the HE pathology evaluation produced no significant treatment-related changes.

To further investigate the potential role of apoptosis in the observed renal dysfunction, we analyzed all sections for the active fragment of caspase-3 (CCR3), which plays a key role in apoptosis. Immunohistochemical analysis for CCR3 revealed that CCR3 staining was characterized by generally minimal or very minimal staining of individual or small clumps of cells predominantly within the medulla but scattered positively stained cells were also visible throughout the kidney within glomeruli/tubulointerstitial spaces and blood vessels. Overall, we found no staining in sham-operated animals and a small increase in staining in all animals subjected to endotoxemia with no significant treatment-related changes. (Figure 2B, $P>0.05$).

To further investigate the potential role of proximal tubular injury in the observed renal dysfunction, we analyzed all sections for KIM-1, an early biomarker of proximal tubular injury. KIM-1 staining was characterized by generally diffuse staining of the whole kidney with positive staining presenting as areas of multi-focal to diffuse increased staining intensity within the pars recta region of the kidney. Compared with sham operated WT animals. The results of the Kim-1 IHC pathology evaluation showed slightly increased staining in the endotoxemic WT mice, which appeared reduced after treatment with EPO. However, the magnitude of the changes was small. This evaluation was, therefore, repeated by blind re-reading of groups by the original study pathologist and by a second pathologist (data not shown) and confirmed that there was indeed a small difference. There was no treatment-related change in the βcR KO

mice. Overall, none of the observed changes in KIM-1 immunohistochemistry were statistically significant (P>0.05, Figure 2 C).

Effect of EPO on the phosphorylation of Akt, glycogen synthase kinase-3β (GSK)-3β and endothelial nitric oxide synthase (eNOS) and on the nuclear translocation of the p65 nuclear factor (NF)-κB subunit in the kidneys of endotoxemic WT and βcR KO mice

To gain a better insight into the potential mechanism(s) underlying the observed beneficial effects of EPO, we investigated the effects of this hormone on cell signaling pathways in the kidneys of WT and βcR KO mice, known to confer tissue protection or to inhibit inflammation. When compared to kidneys of sham mice treated with saline, kidneys of WT and βcR KO mice subjected to LPS demonstrated no change in the total Akt content and the phosphorylation of Akt on Ser^{473} (Figure 3A), total GSK-3β content and the phosphorylation of GSK-3β on Ser^{9} (Figure 3B) or total eNOS content and phosphorylation of eNOS on Ser^{1177} (Figure 3C). Treatment of endotoxemic WT mice with EPO, however, resulted in a substantial increase in the phosphorylation of serine residues on Akt (Figure 3A), GSK-3β (Figure 3B), and eNOS (Figure 3C). In contrast, administration of EPO to endotoxemic βcR KO mice caused no change in the phosphorylation of Akt (Figure 3A), GSK-3β (Figure 3B), and eNOS (Figure 3C). When compared to kidneys from sham mice treated with saline, the kidneys of endotoxemic WT and βcR KO mice exhibited significant increases in the nuclear translocation of the p65 NF-κB subunit (Figure 3D), indicating the activation of NF-κB. Treatment of endotoxemic WT mice with EPO resulted in a significant reduction in nuclear translocation of p65 and, hence, inhibition of activation of NF-κB in the kidney (Figure 3D). In contrast, administration of EPO to endotoxemic βcR KO mice did not attenuate the nuclear translocation of p65 caused by LPS (Figure 3D).

The development of renal dysfunction in aging mice following cecal ligation and puncture (CLP).

The development of renal dysfunction in our short and severe model of polymicrobial sepsis via CLP is strongly age-dependent. Compared to sham-operated animals (26.3 ± 0.72 μmol/l),

we could not determine a significant renal dysfunction (determined by changes in serum creatinine) 24 h after CLP in two (25.7 ± 1.13 µmol/l) and five months (38.1 ± 6.2 µmol/l) old WT mice, which underwent CLP surgery. However, when compared to sham-operated mice, eight months old WT mice, that underwent CLP surgery, developed a significant and reliable increase in serum creatinine (26.3 ± 0.72 vs. 84.6 ± 7.34 µmol/l, $P<0.05$, Figure 4).

Effect of EPO on renal dysfunction in WT and βcR KO mice that underwent CLP

When compared to sham-operated WT mice, eight months old WT and βcR KO mice that underwent CLP surgery demonstrated a significant increase in serum creatinine (WT mice 26.3 ± 0.72 vs. 76.3 ± 8.01 µmol/l, $P<0.05$, Figure 5A; KO mice 23.2 ± 6.44 vs. 82.1 ± 11.77 µmol/l, $P<0.05$, Figure 5B). Administration of EPO to septic WT mice significantly attenuated the rise in serum creatinine (76.3 ± 8.01 vs. 49.9 vs. 6.54 µmol/l, $P<0.05$, Figure 5A). Most notably, the renal dysfunction caused by CLP in βcR KO mice was not attenuated by EPO (82.1 ± 11.77 µmol/l, 71.4 ± 10.81, $P>0.05$, Figure 5B).

Expression of *CSf2rb* (encoding the βcR) mRNA in medulla and cortex of the kidneys of WT sham mice.

To gain a better insight into where the βcR was expressed in the mouse kidneys, we attempted to detect the *CSf2rb* gene (which encodes the βcR, which was modified in the βcR knockout mice used in this study). We, therefore, isolated mRNA from medulla and cortex of the kidneys of WT sham mice and subjected the cDNA obtained after reverse transcription of mRNA to a PCR protocol with appropriate primers (see Tab. S1 online supplementary). We could clearly detect presence of βcR mRNA in both cortex and medulla (Figure 6).

Discussion

Here we demonstrate that the administration of a large dose (1000 IU/kg s.c.) of EPO 1 h after LPS administration or CLP attenuates AKI. Most notably, we describe for the first time that the observed beneficial effects of EPO in WT mice subjected to either endotoxemia or sepsis were not observed in mice, which lacked the βcR. In 2004, Brines and colleagues proposed that the tissue protective effects of EPO and its derivatives might be mediated (at least in part), by the βcR [22]. The βcR is a signal transduction subunit, which is shared by the α-chain subunits of the IL-5, IL-3 and GM-CSF receptors [37]. The α-chains are able to bind their ligand with low affinity (1-100nM), but the hetero-receptors are not able to signal in the absence of the βcR. On the other hand, the βcR does not measurably bind a ligand by itself, but it amplifies a signal once a ligand has bound (in the picomolar range) [38, 39]. The βcR is not required for erythropoiesis, as βcR KO mice have normal erythrocyte maturation [22]. The EPOR functionally associates with the βcR [40, 41] to generate an EPOR-βcR complex, the latter of which has been proposed to mediate the tissue-protective effects of EPO. In mice, the βcR was expressed (mRNA) in both cortex and medulla of the kidney. Our finding that the renoprotective effects of EPO in endotoxemia/sepsis are lost in βcR KO mice supports our hypothesis that the βcR is essential for the 'tissue-protective' effects of EPO in sepsis.

There is now increasing evidence that the renal dysfunction (AKI) associated with sepsis in patients is secondary to functional rather than structural changes. Although some morphological abnormalities have been reported in kidneys from patients, which died from sepsis, these changes were relatively small and are unlikely to account for the profound renal dysfunction observed in these patients. For instance, despite the high prevalence of clinical renal dysfunction (65%) in 20 patients, which died from sepsis and multiple organ dysfunction, only one septic patient had evidence of kidney necrosis. No renal tubular or glomerular cell apoptosis was seen in any of these septic patients. Hence, in patients without preexisting renal disease, renal histology did not reflect the severity of renal injury indicated by the decrease in kidney function (Hotchkiss CCM, 1999). We show here that mice subjected to endotoxemia

for 18 h have a profound renal dysfunction in the absence of overt morphological alterations or even increases in biomarkers of apoptosis (cleaved caspase-3) or proximal tubular injury (or KIM-1) in the kidney. As EPO did not affect any of the above (minimal) signs of injury, it is unlikely that the reduction in renal dysfunction afforded by EPO in sepsis is secondary to prevention of renal injury.

What, then, are the signaling events that are activated by EPO via the βcR? There is evidence that the beneficial effects of EPO are secondary to the activation of the survival kinase Akt [42-45]. Akt is a member of the phosphoinositide 3-kinase signal transduction enzyme family, which regulates cellular activation, inflammatory responses, chemotaxis, and apoptosis [46]. When phosphorylated by its upstream regulator, phosphoinositide-dependent kinase, Akt modulates cell survival and growth [46]. We document here that EPO causes the activation of Akt in WT mice subjected to endotoxemia, while the activation of this pathway by EPO was lost in βcR KO mice. The above findings support the view that both, the βcR and the activation of Akt, are essential for the reported beneficial effects of EPO.

What, then, are the downstream targets of Akt? There is good evidence that activation of Akt results in Ser^9 phosphorylation of GSK-3β [47-49]. GSK-3β is a serine-threonine kinase that was originally recognized as a kinase that phosphorylates glycogen synthase. In contrast to most other kinases, GSK-3β is active in a resting cell state and is regulated by multiple signaling pathways including the Akt pathway, which inhibits this kinase by causing Ser^9 phosphorylation [47, 48]. Specific inhibitors of the activation GSK-3β attenuate the AKI caused by co-administration of LPS and peptidoglycan [50] and improve survival in endotoxemia [51]. Agents that activate the PI3K/Akt pathway [47] also inhibit GSK-3β. We have previously reported that insulin activates Akt, inhibits GSK-3β and reduces AKI caused by LPS and peptidoglycan [52]. We report here that EPO enhances the phosphorylation of the Ser^9 residue of GSK-3β resulting in the inhibition of this kinase, which attenuates the renal dysfunction caused by LPS and peptidoglycan [53-55]. This effect of EPO was lost in βcR KO mice.

Downstream of GSK-3β, several studies have now reported an association between GSK-3β

and NF-κB activity *in vitro* [56, 57] and *in vivo* [50, 52]. Treatment of TNF-α stimulated hepatocytes with a specific GSK-3β inhibitor resulted in a decrease of the NF-κB–dependent gene transcription [58]. NF-κB is a transcriptional factor that plays an important role in regulating the transcription of a number of genes (e.g. iNOS, cyclooxygenase-2, IL-1, TNF, IL-6), especially those involved in producing mediators involved in local and systemic inflammation, such as cytokines, chemokines, cell adhesion molecules, apoptotic factors, and other mediators [59]. The protective effects of inhibitors of GSK-3β in endotoxemia/sepsis have been attributed to inhibition of NF-κB secondary to either phosphorylation of Ser^{536} on the p65 subunit of NF-κB [50] or prevention of the association of cAMP response element-binding (CREB) with p65 [51]. In addition, GSK-3β may also inhibit the activation of NF-κB by phosphorylating and degrading IκBα, which is required to prevent NF-κB translocation [57]. All of these effects may contribute to the reported beneficial effects of inhibitors of GSK-3β in sepsis. We report here that EPO prevents the nuclear translocation of p65 and, hence, the activation of NF-κB. This effect of EPO was lost in βcR KO mice. All of the above findings support the view that EPO enhances the activation of Akt resulting in inhibition of GSK-3β and inhibition of the activation of NF-κB in a βcR-dependent fashion

In addition to inhibiting the activation of GSK-3β, activation of Akt results in the phosphorylation of eNOS on Ser^{1177}, which, in turn, causes activation of eNOS resulting in an enhanced formation of NO in the microcirculation. There is evidence that EPO enhances the phosphorylation of Ser residues on eNOS resulting in its activation [36, 60, 61]. In endothelial cells, EPO causes eNOS activation, while inhibition of EPOR or βcR by neutralizing antibodies or small interfering RNA abolished the EPO-induced NO formation [61]. Most notably, inhibition of the βcR abolished the EPO-induced increase and the phosphorylation of eNOS, Akt, Src or Janus kinase 2 [61]. The findings support the view that βcR plays a key role in the activation of eNOS by EPO in endothelial cells. We report here that the increase in Ser^{1177} phosphorylation of eNOS by EPO in the kidney is lost in βcR KO mice. While our study was under way, de Souza and colleagues also showed that the attenuation of sepsis-induced AKI by EPO in the

rat is associated with an increased expression of eNOS and inhibition of the activation of NF-κB, which the authors attributed to an anti-inflammatory and endothelial protective effect. [36]. In line with this study Rodrigues et al. reported recently in the same animal model that 24 h pre-treatment with continuous erythropoietin receptor activator (CERA) protected against sepsis induced AKI at 24 h, which was in part, attributable to a suppression of the inflammatory response. Interestingly, their results revealed that renal EpoR expression, which was down regulated in their model of sepsis, was preserved by pretreatment with CERA [16].

In conditions associated with sepsis, activation of eNOS is beneficial as the enhanced formation of NO causes local vasodilation, inhibits adhesion of platelets and neutrophils, and regulates angiogenesis [62, 63]. Agents that release NO or enhance the formation of endogenous NO may attenuate excessive intrarenal vasoconstriction and reduce renal dysfunction [64, 65]. Thus, activation of eNOS (possibly secondary to activation of Akt) may contribute to the beneficial effects of EPO reported here.

All of the above findings support the view that the βcR is essential for the following effects of EPO in mice with endotoxemia: 1) reduction of AKI; 2) activation of Akt; 3) activation of eNOS; 4) inhibition of GSK-3β; and 5) inhibition of the activation of NF-κB.

In order to investigate whether the βcR is also essential for the effects of EPO in polymicrobial sepsis, we developed a model of CLP induced AKI. We found that the degree of renal dysfunction increased with the age of the animals. We were unable to document a significant degree of AKI at 24 h after CLP in young (two and five months old) mice, while eight months old mice exhibited a very large increase in creatinine within 24 h after CLP. Most notably, administration of EPO (at 1 h after CLP) attenuated the AKI caused by polymicrobial sepsis in aging mice. In order to investigate the role of the βcR in this effect of EPO, we bred aged-matched βcR KO mice. Using these animals, we demonstrate that the beneficial effects of EPO were lost in eight months old βcR KO mice.

In conclusion, our findings show convincingly for the first time that the βcR is essential in the reduction of the acute renal dysfunction caused by EPO in sepsis as well as all of the signaling events traditionally associated with the well-documented beneficial effects of EPO.

Methods

Animals

The animal protocols followed in this study were approved by the local *Animal Use and Care Committee* in accordance with the derivatives of both the *Home Office guidance on the Operation of Animals (Scientific Procedures Act 1986)* published by Her Majesty's Stationary Office and the *Guide for the Care and Use of Laboratory Animals* of the National Research Council. This study was carried out on 73 male WT C57BL/6 mice (Harlan Laboratories, Wyton, UK) and 73 male βcR KO mice on a C57/BL-6J genetic background (bred and maintained at Queen Mary University of London Biological Services Unit) receiving a standard diet and water *ad libitum*. We employed young (2 months) mice for the model of endotoxemia and old (8 months) mice for the model of polymicrobial sepsis.

Endotoxemia

In the model of endotoxemia-induced AKI, mice received either LPS (9 mg/kg in 5 ml/kg 0.9 % saline i.p.) or vehicle (5 ml/kg 0.9 % saline i.p.). One hour after induction of endotoxemia, mice were treated either with EPO (1000 IU recombinant human EPO in 10 ml/kg 0.9 % saline) or vehicle (10 ml/kg 0.9 % saline s.c.). Sham mice were not subjected to LPS-injection, but were otherwise treated in the same way. At 18 h the experiment was terminated and organ and blood samples were collected for quantification of organ injury. WT mice and βcR KO mice were randomly allocated into eight different groups. WT mice: (i) Sham + saline (n = 3); (ii) Sham + EPO (n = 3); (iii) LPS + saline (n = 9); (iv) LPS + EPO (n = 10); βcR KO mice: (v) Sham + saline (n = 3); (vi) Sham + EPO (n = 3); (vii) LPS + saline (n = 9); (viii) LPS + EPO (n = 20).

Polymicrobial sepsis

In this study we developed a 24 h model of septic AKI induced by CLP in aged mice. We followed the original CLP protocol introduced by Wichterman and co-workers [66] with slight modifications, such as the antibiotic therapy (Imipenem/Cilastin; 20 mg/kg) and analgesia (buprenorphine; 0.05 mg/kg), which were administered 6 h after surgery and every 12 h after that. The detailed surgical procedure is described in the online supplementary file. To evaluate the time course of the development of renal dysfunction in mice of different ages, serum creatinine was measured in two, five and eight months old WT mice 24 h after CLP surgery. Finally, we established a model of severe, polymicrobial sepsis in 8-months old male WT mice, which developed reliable renal dysfunction 24 h after CLP. This model was then used to verify the effects of EPO on renal function obtained in the model of endotoxemia. At the beginning of the experiment, mice underwent CLP surgery. Sham mice were not subjected to CLP, but were otherwise treated in the same way. One hour after CLP, mice were treated either with EPO (1000 IU recombinant human EPO in 10 ml/kg 0.9 % saline s.c.) or vehicle (10 ml/kg 0.9 % saline s.c.). WT mice and βcR KO mice were randomized into six different groups. WT mice: (i) Sham + saline (n = 3); (ii); CLP + saline (n = 10); (iii); CLP + EPO (n = 12). βcR KO mice: (iv) Sham + saline (n = 3); (v) CLP + saline (n = 6); (vi) CLP + EPO (n = 20).

Quantification of organ injury

All mice were anesthetized with 1.5 ml/kg i.p. of a ketamine (100 mg/ml)/xylazine (20 mg/ml) solution in a 2:1 ratio before being sacrificed. Approximately 1 ml of blood was collected by cardiac puncture into non-heparinized syringes and immediately decanted into serum gel S/1.3 tube (Sarstedt, Germany), after which the heart was removed to terminate the experiment. The samples were centrifuged at 9900 *g* for 5 min to separate serum, which was sent to an independent laboratory (IDEXX Laboratories, Buckinghamshire, UK) for analyses of serum creatinine and urea as markers of renal dysfunction as described in detail in the online supplement file. Additionally, kidney samples were taken, snap frozen and stored at -80 ^{o}C for western blot analysis or fixed in 10% neutral buffered formalin for histological

evaluation. The detailed description of the histological evaluation of kidney tissues are available in the online supplementary.

Western Blot Analysis

Quantitative Western blot analysis was carried out as described previously [67]. We assessed the degree of phosphorylation of Akt on Ser^{473}, GSK-3β on Ser^{9}, eNOS on Ser^{1177} and the nuclear translocation of the p65 subunit of NF-κB. The detailed method is described in the online supplementary file.

Detection *CSf2rb* (encoding the βcR) gene expression in medulla and cortex of the kidneys of WT sham mice.

Briefly, the medulla and the cortex were carefully dissected from the kidney and snap frozen. Samples were homogenized and RNA was extracted using RNeasy Mini Kit (Quiagen, Germany), according to manufacturers instructions. Reverse transcription polymerase chain reaction (RT PCR) was used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA. The detailed description of the detection *CSf2rb* (encoding the βcR) gene expression are available in the online supplementary.

Materials

Unless otherwise stated, all compounds in this study were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). Antibodies for western blot analysis were purchased from Santa Cruz Biotechnologies (Middlesex, UK). All solutions were prepared using non-pyrogenic saline (0.9 % [w/v] NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK). Recombinant human EPO (epoetin beta) was manufactured by Roche Diagnostics (Sussex, UK).

Statistical Analyses

All values described in the text and figures are presented as mean ± standard error of the mean (SEM) of *n* observations, where *n* represents the number of animals studied. Statistical analysis was performed using GraphPad Prism 5.0d (GraphPad Software, San Diego, California, USA). Data without repeated measurements were assessed by one-way ANOVA followed by Dunnett's *post hoc* test. A *P*-value of less than 0.05 was considered to be statistically significant.

Disclosure

MB and AC are officers of Araim Pharmaceuticals and currently hold stocks/shares in the company.

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Titles and legends

Figure 1. Effect of EPO on renal dysfunction in endotoxemic WT and βcR KO mice. Serum urea (**A**, **B**) and serum creatinine (**C**, **D**) were measured 18 h subsequent to sham-operation or LPS administration. Mice received either LPS (9 mg/kg in 5 ml/kg 0.9 % saline i.p.) or vehicle (5 ml/kg 0.9 % saline i.p.). One hour after induction of endotoxemia, mice were treated either with EPO (1000 IU EPO in 10 ml/kg 0.9 % saline s.c.) or vehicle (10 ml/kg 0.9 % saline s.c.). WT mice (**A**, **C**): Sham + saline (n = 3); Sham + EPO (n = 3); LPS + saline (n = 9); LPS + EPO (n = 10). βcR KO mice (**B**, **D**): Sham + saline (n = 3); Sham + EPO (n = 3); LPS + saline (n = 9); LPS + EPO (n = 20). Data are expressed as means ± S.E.M. for n number of observations. ★ $P < 0.05$ vs. LPS + saline of WT or KO animals respectively.

Figure 2. Effects of endotoxemia and/or EPO on renal morphology (light microscopy) and immunohistochemical staining for apoptosis [cleaved caspase-3 (CCR3)] kidney injury molecule-1 (KIM-1)

Morphological (HE staining, **A-F**) and immunohistochemical [CCR 3 (**G),** KIM-1 (**H**)] evaluations were performed in kidneys obtained from mice 18 h after sham-operation or LPS administration. Mice received either LPS (9 mg/kg in 5 ml/kg 0.9 % saline i.p.) or vehicle (5 ml/kg 0.9 % saline i.p.). One hour after induction of endotoxemia, mice were treated either with EPO (1000 IU EPO in 10 ml/kg 0.9 % saline s.c.) or vehicle (10 ml/kg 0.9 % saline s.c.). WT mice (**A**) Sham + saline (n = 4) (**B**) LPS + saline (n = 3); (**C**) LPS + EPO (n = 3). βcR KO mice (**D**): Sham + saline (n = 4); (**E**) LPS + saline (n = 4); (**F**) LPS + EPO (n =4). Evaluation by light microscopy (HE-staining) revealed that there was no overt morphological evidence of proximal tubular epithelial cell injury, interstitial edema, interstitial inflammation, vasculopathy or glomerular abnormality in any of the study groups and no treatment-related changes. (**A-F**). Analysis of CCR3 (**G**) sections (apoptosis) revealed for no staining in sham-operated animals and a small increase in staining in all animals subjected to endotoxemia with no significant treatment-related changes. ($P>0.05$). The results of the Kim-1 IHC pathology

evaluation (**H**) showed slightly increased staining in the endotoxemic WT mice, which appeared reduced after treatment with EPO. There was no treatment-related change in the βcR KO mice. Overall, none of the observed changes in KIM-1 immunohistochemistry were statistically significant (P>0.05).

Figure 3. Effect of EPO on the phosphorylation of Akt, GSK-3β and eNOS and on the nuclear translocation of the p65 NF-κB subunit in kidneys of WT and βcR KO mice with endotoxemia. Mice received either LPS (9 mg/kg in 5 ml/kg 0.9 % saline i.p.) or vehicle (5 ml/kg 0.9 % saline i.p.). One hour after induction of endotoxemia, mice were treated with either EPO (1000 IU EPO in 10 ml/kg 0.9 % saline s.c.) or vehicle (10 ml/kg 0.9 % saline s.c.). Each band is from a single western blot experiment that is representative of three separate experiments. Data are expressed as means ± S.E.M. for n number of observations. ★ $P < 0.05$ vs. LPS + saline of WT or KO animals respectively (white bars). • $P < 0.05$ vs. LPS + saline of WT or KO animals respectively (black bars). **Panel A–C:** Densitometric analysis of the bands (semi-quantitative Western blot analysis) is expressed for all groups studied as relative optical density (OD) of (**A**) phosphorylated Akt ($pSer^{473}$) and the corresponding total Akt content (Σ Akt); (**B**) phosphorylated GSK-3β (pSer9) and the corresponding total GSK-3β content (Σ GSK-3β); (**C**) phosphorylated eNOS ($pSer^{1117}$) and the corresponding total eNOS content (Σ eNOS). All values were corrected for the corresponding β-actin and normalized using the related sham-operated band. **Panel D:** Densitometric analysis of the bands is expressed for all groups studied as relative optical density (OD) for NF-κB p65 subunit levels in both, cytosolic and nuclear fractions normalized using the related sham operated band.

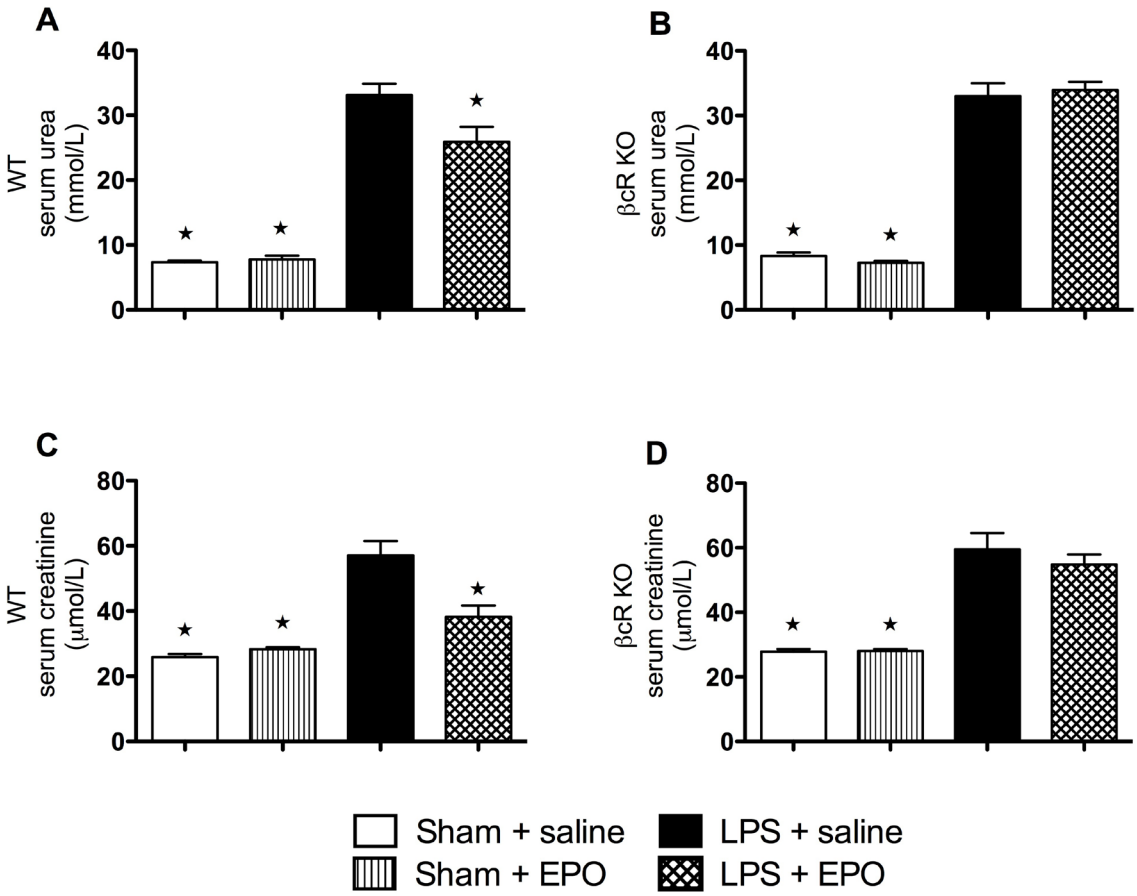
Figure 4. **The development of renal dysfunction in aging mice following CLP.** Serum creatinine levels were measured 24 h subsequent to sham-operation or CLP-surgery in WT mice at different ages (two, five and eight months). Data are expressed as means ± S.E.M. for n number of observations. ★ $P < 0.05$ vs. sham. Sham (2 months, n = 3); CLP (2 months, n = 3); CLP (5 months, n = 5); CLP (8 months, n = 10).

Figure 5. Effect of EPO on renal dysfunction in septic WT and βcR KO mice. Serum creatinine levels were measured 24 h subsequent to sham-operation (no CLP) or CLP-surgery in WT (A) and βcR KO mice (B). One hour after CLP surgery mice were treated s.c. either with EPO (1000 IU recombinant human EPO in 10 ml/kg 0.9 % saline s.c.) or vehicle (10 ml/kg 0.9 % saline s.c.). WT mice: Sham + saline (n = 3); Sham + EPO (n = 3); CLP + saline (n = 10); CLP + EPO (n = 12). βcR KO mice: Sham + saline (n = 3); CLP + saline (n = 6); CLP + EPO (n = 20). Data are expressed as means ± S.E.M. for n number of observations. ★ $P < 0.05$ vs. CLP + saline of WT or βcR KO animals respectively.

Figure 6. Amplification of a CSf2rb gene fragment using cDNA from medulla or cortex of the kidneys of WT sham mice as template.

Messenger RNA was extracted from medulla or cortex of WT sham mice and subjected to reverse transcription. PCR performed with cDNA yielded amplicons with a calculated molecular size of 326 bp, when separated in a 1 % agarose gel. GAPDH served as extraction control. Furthermore, the intensity of the 175 bp GAPDH band confirmed comparative loading of the gel lanes.

FIGURE 1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C
WT
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(μmol/L)
80
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D
βcR KO
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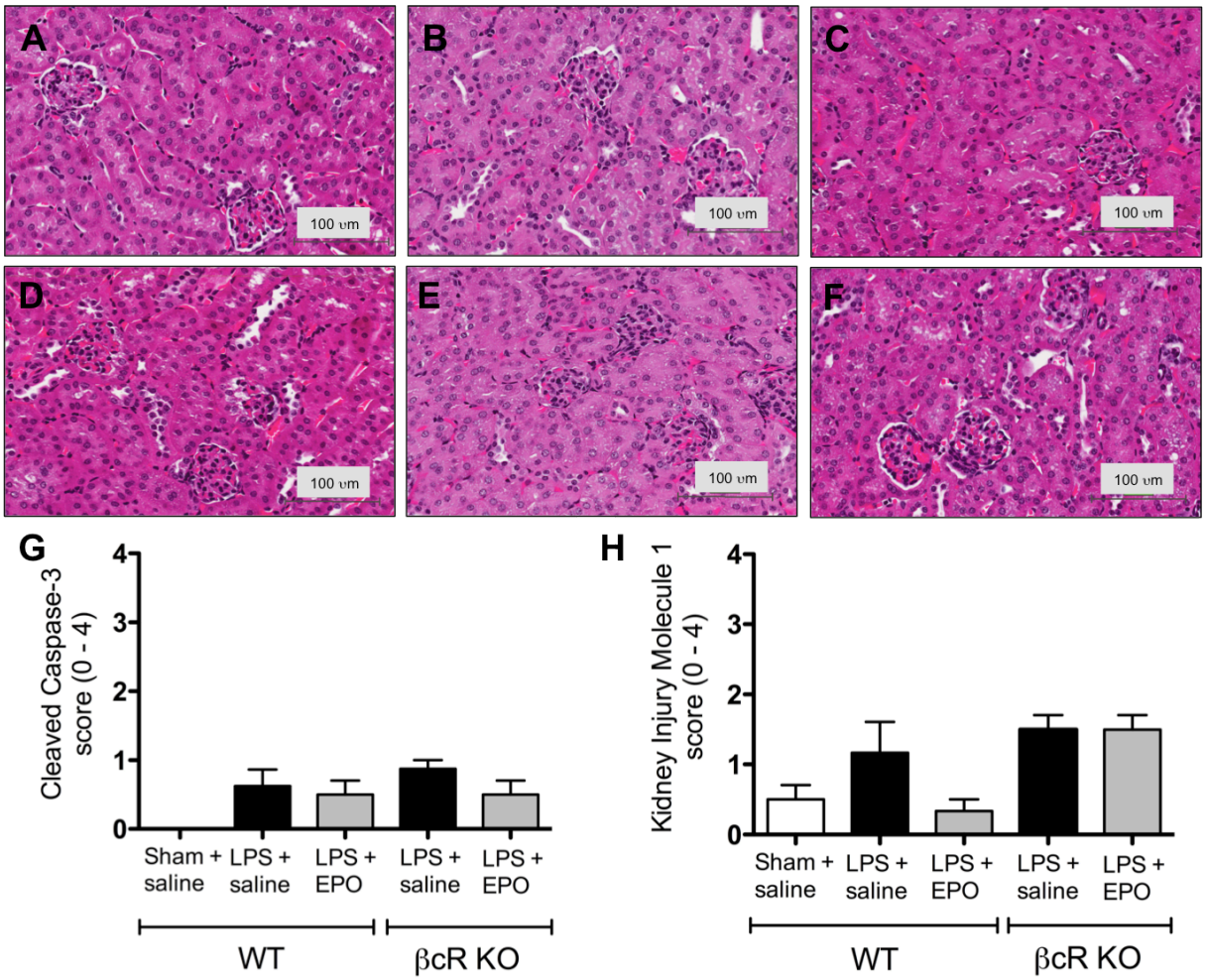
Sham + EPO
LPS + saline
LPS + EPO

FIGURE 2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LPS + saline
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βcR KO
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Kidney Injury Molecule 1 score (0 - 4)
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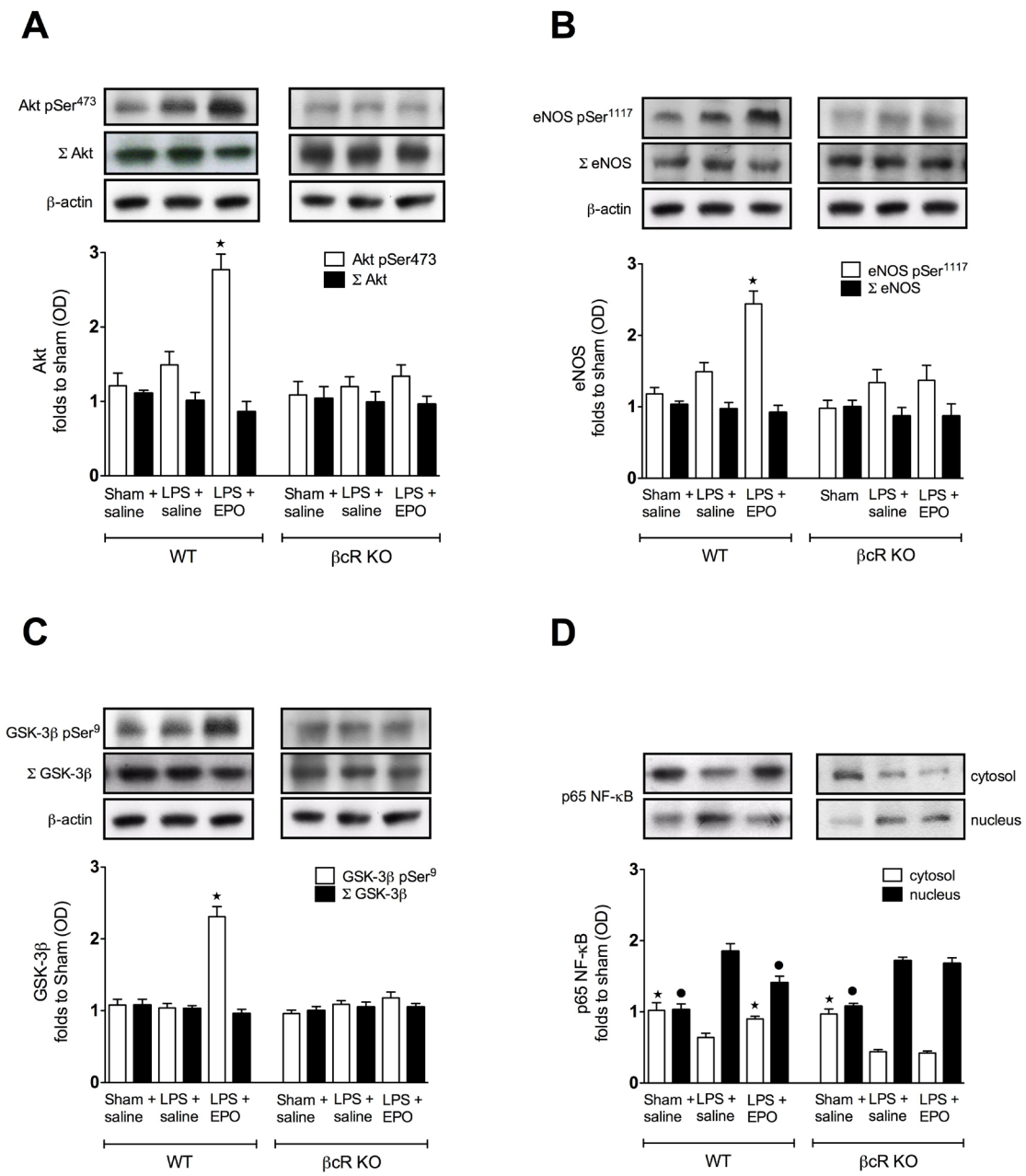
WT
βcR KO

FIGURE 3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Σ eNOS
β-actin
eNOS pSer1117
Σ eNOS
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C
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Σ GSK-3β
β-actin
GSK-3β pSer9
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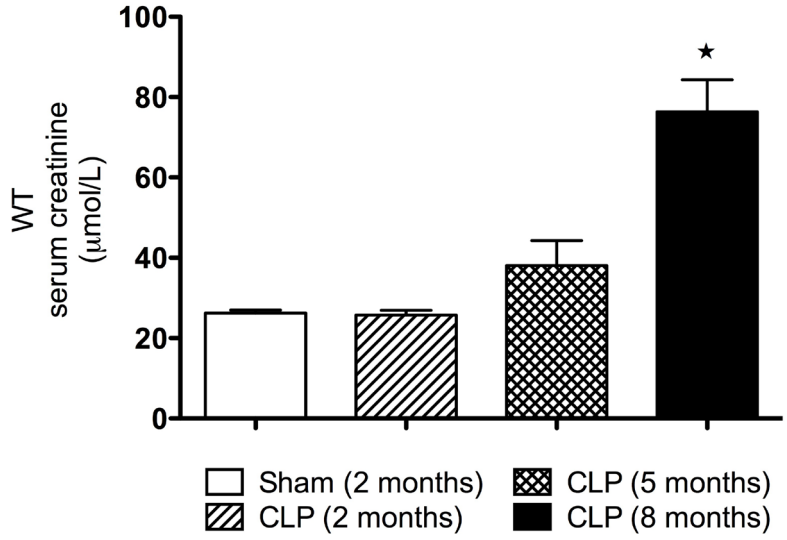
nucleus
p65 NF-κB folds to sham (OD)

FIGURE 4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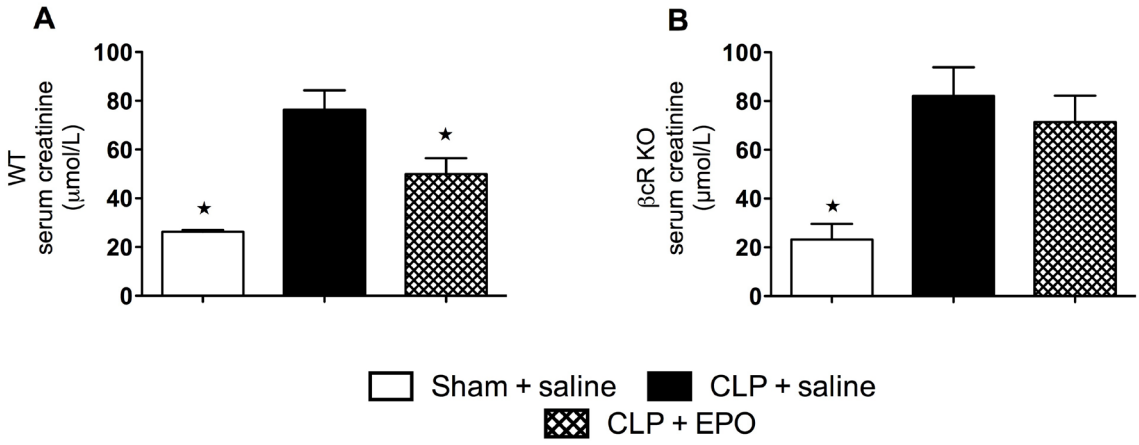
Sham (2 months)
CLP (2 months)
CLP (5 months)
CLP (8 months)

FIGURE 5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Sham + saline
CLP + saline
CLP + EPO

FIGURE 6