Infusion of IL-10–expressing cells protects against renal ischemia through induction of lipocalin-2

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Ischemia/reperfusion injury is a leading cause of acute renal failure triggering an inflammatory response associated with infiltrating macrophages, which determine disease outcome. To repair the inflammation we designed a procedure whereby macrophages that overexpress the anti-inflammatory agent interleukin (IL)-10 were adoptively transferred. These bone marrow–derived macrophages were able to increase their intracellular iron pool that, in turn, augmented the expression of lipocalin-2 and its receptors. Infusion of these macrophages into rats after 1 h of reperfusion resulted in localization of the cells to injured kidney tissue, caused increases in regenerative markers, and a notable reduction in both blood urea nitrogen and creatinine. Furthermore, IL-10 therapy decreased the local inflammatory profile and upregulated the expression of pro-regenerative lipocalin-2 and its receptors. IL-10–mediated protection and subsequent renal repair were dependent on the presence of iron and lipocalin-2, since the administration of a neutralizing antibody for lipocalin-2 or administration of IL-10 macrophages pretreated with the iron chelating agent deferoxamine abrogated IL-10–mediated protective effects. Thus, adoptive transfer of IL-10 macrophages to ischemic kidneys blunts acute kidney injury. These effects are mediated through the action of intracellular iron to induce lipocalin-2.

Several researchers have studied the effects of delivering genetically modified macrophages to injured tissue, focusing primarily on the use of anti-inflammatory cytokines for therapeutic purposes.[1–3] Wilson *et al.*[2] developed a system of locally introduced bone marrow–derived macrophages (BMDM) into inflamed glomeruli during nephrotoxic nephritis.[2] In this system, they were able to demonstrate that the injection of interleukin (IL)-10–overexpressing macrophages reduced glomerular inflammation. Despite the confirmed therapeutic role of IL-10 therapy in these renal diseases, it remains unclear whether this therapy may also prove to be effective in renal ischemia/reperfusion injury (IRI). We previously highlighted that macrophages have a critical role in IRI,[4] indicating that renal repair and inflammation are closely interlinked. We demonstrated that macrophages fostered regeneration under anti-inflammatory conditions, but were unable to exert this pro-reparative effect in an inflammatory environment. In view of these findings, we overexpressed IL-10 in BMDMs and adoptively transferred them to ischemic kidneys during the inflammatory phase with the aim of testing whether these predominantly anti-inflammatory macrophages were able to modulate inflammation and injury.

Lipocalin-2 (Lcn-2) is a 25-kDa protein substantially upregulated following renal tubular injury and may have a role in limiting kidney damage.[5,6] Recent evidence suggests that Lcn-2 functions as a growth and differentiation factor in multiple[7] cells. Application of exogenous Lcn-2 to epithelial cells induced proliferation and expression of genetic markers of early epithelial progenitors. Some of the biological effects of Lcn-2 may depend markedly on its association with the iron complex. It has also been shown that Lcn-2 may induce apoptosis as a result of depletion of intracellular iron pools. This effect, however, is abolished if iron-loaded Lcn-2 is used.[8] What is of great significance is that it appears to be the presence of iron that accounts for the distinct biological effects of Lcn-2. Furthermore, Lcn-2 was recently described as being regulated by iron.[9]

In vivo evidence has outlined the role of IL-10 in enhancing intracellular iron[10] and also inducing the

expression of heme oxygenase (HO),[11] leading to excessive heme degradation and the resultant increase in iron. In addition, previous studies revealed profound effects of anti-inflammatory cytokines on iron homeostasis.[12]

Considering that intracellular iron is a biological response of IL-10 and an inducer of the synthesis of the potentially pro-reparative Lcn-2, we hypothesized that macrophages overexpressing IL-10 exert their reparative function by means of an increase in intracellular iron and its action upon Lcn-2.

RESULTS

Maturity and specificity of BMDMs

We determined the mature macrophage marker CD11b on the surface of BMDMs by flow cytometry. Results showed a differentiation rate of about 80% (CD11b-positive cells in %: isotype 1.32 ± 0.42; CD11b 82.44 ± 5.41). In addition, BMDMs were stained for the mature macrophage marker ED1. Immunofluorescence showed a high expression profile of nearly 100% of total BMDMs (Figure 1).

IL-10 macrophages protect against IRI

Blood urea nitrogen (BUN; Figure 2a) and creatinine (Figure 2b) showed an increase after IRI. Administration of control macrophages was unable to prevent injury, whereas IL-10 macrophages significantly reduced creatinine levels up to control levels. In addition, BUN levels were substantially diminished, but still remained higher than the control group. Histological analysis (Figure 2c and d) indicated that renal injury could be observed at 24 h of reperfusion. Treatment with IL-10 macrophages showed significantly higher tissue integrity, whereas transfer of control macrophages did not improve injury. Tissue injury was significantly decreased with respect to the I/R_β-gal group, but was still significantly higher than sham animals. Thus, IL-10–overexpressing BMDMs were highly protective against injury, but still higher cell doses might be needed to achieve the complete reversion to control levels.

Transduction efficiency was evaluated via adenovirus GFP, with adenovirus IL-10 macrophages showing dose- and time-dependent increases in IL-10 expression. We also measured mannose receptor, IL-1β, and nitric oxide production. Results show an upregulation of anti-inflammatory mediators in IL-10 macrophages, even when stimulated with lipopolysaccharides (Supplementary Figure S1 online). This may also occur because of autocrine effects of IL-10 itself, as IL-10 is known to be able to polarize macrophages toward an anti-inflammatory M2 phenotype.

TNF-α and IL-1β expression was reduced upon administration of IL-10 macrophages (Figure 3a), whereas the anti-inflammatory mediator profile was increased in the ischemic kidney (Figure 3b).

Ki-67 and proliferation cellular nuclear antigen (PCNA) (Figure 3c) showed a clear increase in the IL-10 macrophage-treated groups. We performed staining for PCNA and stathmin[13–15] as known regenerative markers. Few cells were found to express stathmin and PCNA under sham conditions
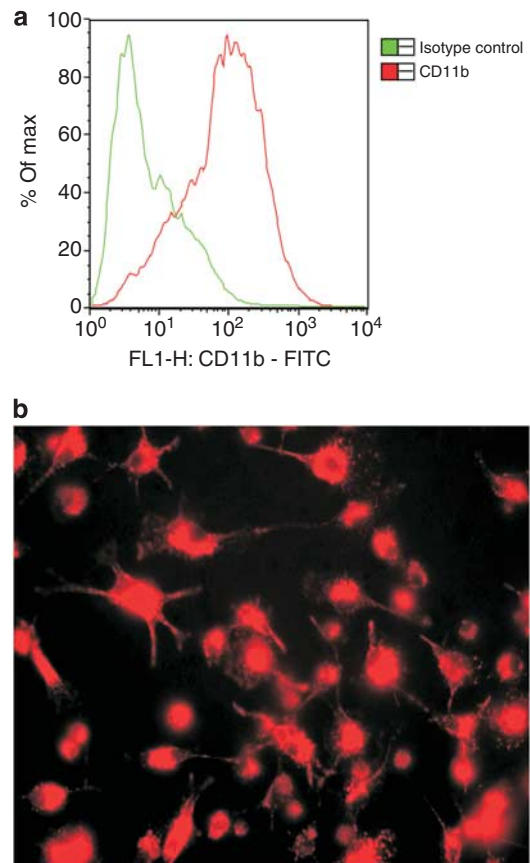
**Figure 1 | Maturity of bone marrow–derived macrophages.**
(**a**) The assessment of CD11b levels by flow cytometry demonstrates clear maturity and differentiation of precursor cells from the bone marrow toward macrophages after incubation with granulocyte-macrophage colony-stimulating factor (10 ng/ml) for 7 days (CD11b-positive cells in %: isotype 1.32 ± 0.42; CD11b 82.44 ± 5.41). (**b**) Immunofluorescent staining of ED1 (CD 68) of bone marrow–derived macrophages showed high numbers of ED1-positive cells out of the total number of cells differentiated from whole bone marrow preparations, thus confirming the maturity status of differentiated macrophages. A representative cross-section of five independent experiments is illustrated. FITC, fluorescein isothiocyanate.

or in ischemia/reperfusion (I/R) group (Figure 3d). Treatment with control macrophages showed similar results, whereas IL-10 macrophages increased their expression.

Importantly, BMDMs localized efficiently to injured renal tissue, indicated by PKH-26GL staining of infused macrophages (Supplementary Figure S2 online).

IL-10 macrophages induce Lcn-2

Levels of Lcn-2 mRNA (Figure 4a), protein (Figure 4b), receptor Lcn-2 mRNA (Figure 4c), and megalin mRNA expression (Figure 4d) were measured and showed significant increase in all ischemia/reperfusion groups, although these levels were further increased following treatment with IL-10–overexpressing macrophages but not with untransduced macrophages (e.g., up to threefold for Lcn-2 protein
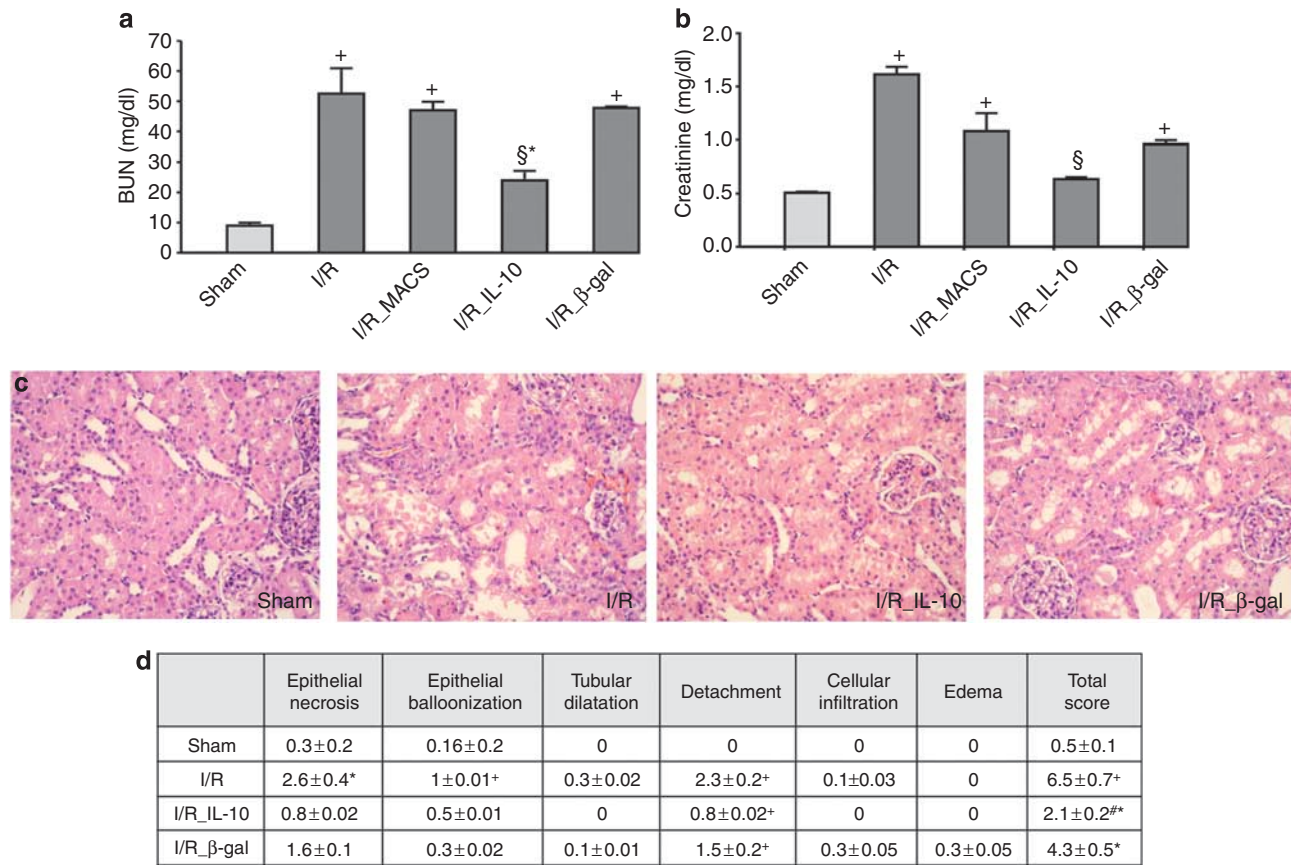
Figure 2 | Interleukin (IL)-10-transduced macrophages protect against ischemia/reperfusion injury. Animals were subjected to 45 min of bilateral ischemia or were sham-operated and killed at 24 h of reperfusion. The effect on (**a**) blood urea nitrogen (BUN) and (**b**) creatinine in plasma of adoptive transfer of either β-gal- or IL-10–treated macrophages was assessed. Macrophage infusion was performed for each condition at 1 h of reperfusion. The following groups were analyzed: Sham: control group without infusion of macrophages; ischemia/ reperfusion (I/R): 45 min of bilateral ischemia followed by 24 h of reperfusion; I/R_MACS: ischemia/reperfusion group with injection of untreated bone marrow–derived macrophages; I/R_IL-10: ischemia/reperfusion group with adoptive transfer of *ex-vivo*–modified macrophages overexpressing IL-10; I/R_β-gal: ischemia/reperfusion group with administered control β-gal-expressing macrophages. Data are represented as means ± s.e.m.; $n = 5$; *$P < 0.05$ vs. Sham, $^{+}P < 0.01$ vs. Sham, $^{§}P < 0.01$ vs. IR_β-gal. (**c**) Conventional histological analysis of hematoxylin and eosin-stained kidney sections (original magnification × 400) of the corticomedullary region confirmed protective effects of IL-10 administration compared with Adβ-gal-BMDM infusion. Representative images of sham rats are shown and compared with ischemia/reperfusion groups. (**d**) Histological damage was quantified in a blinded manner by a skilled expert in the field. *$P < 0.05$ vs. Sham, $^{+}P < 0.01$ vs. Sham, $^{#}P < 0.05$ vs. IR_β-gal.

compared with a twofold increase in the I/R group alone or in the presence of untransduced macrophages). Despite these results, it is difficult to draw concrete interferences concerning the origin of Lcn-2 at this point as especially the mRNA data point toward the involvement of other cells in the production of total Lcn-2 in the kidney.

Table 1 shows the highest range of macrophage density (ED1-positive cells) in the corticomedullary area of renal tissue, although significance was not achieved.

We stained for Lcn-2 and macrophages (ED1; Figure 5). In the I/R group, macrophages infiltrated the kidney, but did not show co-localization with Lcn-2. When IL-10 macrophages were infused, a great number of BMDMs could be observed co-localizing with Lcn-2, suggesting that Lcn-2 was produced by the administered BMDMs. This might hold true because of the fact that IL-10–overexpressing macrophages were transduced with an adenoviral vector, so that a

continuous production of Lcn-2 was found and detected in these macrophages. Tubular cells produce Lcn-2, but as it is a secreted protein released immediately into urine, immunological detection of Lcn-2 coming from tubular cells is difficult to achieve by immunohistochemistry. In this sense, it can only be stated that IL-10 macrophages contribute to the overall Lcn-2 protein content in the kidney.

Lcn-2 expression was induced in a dose-dependent manner *in vitro*. Lcn-2 protein (Figure 6a) from IL-10 macrophages and both Lcn-2 receptors (Figure 6b and c) showed an increase in line with the transduction of IL-10 to rat BMDMs. External stimulation with Lcn-2 (80 ng/ml; Figure 6d) showed a significant upregulation of both Lcn-2 and its receptors. Incubation of BMDMs with green fluorescent protein (GFP)-tagged Lcn-2 revealed direct protein–receptor interactions given that the fluorescent signal was determined on the surface of the cells (Figure 6eB).
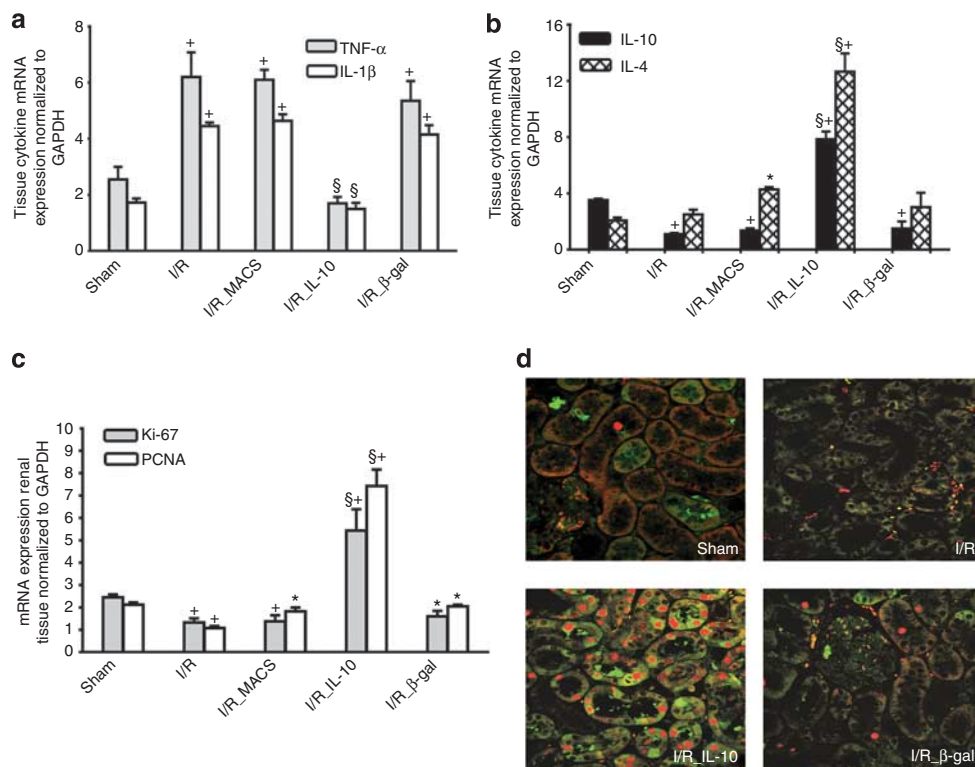
Figure 3 | Interleukin (IL)-10-transduced macrophages modify the inflammatory milieu and induce tubular cell proliferation after ischemic injury *in vivo*. Animals were subjected to 45 min of bilateral ischemia or sham-operated and killed at 24 h of reperfusion, with macrophage infusion at 1 h of total reperfusion time where indicated. The following groups were analyzed: Sham: control group without infusion of macrophages; ischemia/reperfusion (I/R): bilateral ischemia of 45 min with subsequent reperfusion of 24 h; I/R_MACS: ischemia/reperfusion group with injection of untreated bone marrow–derived macrophages; I/R_IL-10: ischemia/reperfusion group with adoptive transfer of *ex-vivo*–modified macrophages overexpressing IL-10; I/R_β-gal: ischemia/reperfusion group administered control β-gal-expressing macrophages. (**a**) Tumor necrosis factor (TNF)-α and IL-1β kidney expression profiles as representative inflammatory milieu and (**b**) IL-10 and IL-4 expression profiles as representative anti-inflammatory milieu of the different study groups was measured by mRNA expression via reverse transcription (RT)-PCR. Results show the downregulation of pro-inflammatory milieu via the adoptive transfer of IL-10–overexpressing macrophages, indicating a crucial role for IL-10 in macrophage phenotype determination and inflammation outcome. (**c**) The effect of Ki-67 and proliferation cellular nuclear antigen (PCNA) mRNA expression (tested via quantitative RT-PCR in total renal tissue homogenates) and (**d**) immunostaining of stathmin (green) and PCNA (red) expression (original magnification × 400), in renal tissue of adoptive transfer of either β-gal- or IL-10–treated macrophages, was assessed. Results show a clear increase of regenerative/proliferative parameters in the IL-10 macrophage-treated groups, thereby indicating the pro-proliferative effect of IL-10 macrophages in kidney repair. Real-time PCR data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as arbitrary units of relative expression. Data are represented as means ± s.e.m; $n = 5$; $*P < 0.05$ vs. Sham, $^+P < 0.01$ vs. Sham, $^§P < 0.01$ vs. IR_β-gal.

Addition of Lcn-2 without GFP-tag reduced the staining (Figure 6eC), indicating the ability of Lcn-2 to compete for its receptor.

Lcn-2 expression from IL-10 BMDMs depends on iron

Cellular iron increased following AdIL-10 transduction, revealing the iron-retaining role of IL-10. The iron chelator, desferroxamine (DFO), reduced cellular iron in BMDMs (Figure 7a). Lcn-2 mRNA expression and Lcn-2 protein content (Figure 7b and c) showed a significant increase where cellular iron content was high, thus indicating the likely function of Lcn-2 as an iron target gene.

Inflammatory markers are modulated by way of the administration of iron and the iron chelator DFO. Results indicate an increased expression of anti-inflammatory markers in the groups with induced Lcn-2 (Supplementary Figure S3 online).

IL-10 rescues viability via an iron-dependent mechanism

NRK52e cells were subjected to anoxia, and viability was measured by fluorescence-activated cell sorting via propidium iodide (PI)/annexin V 16 h after treatment with conditioned media (CM) from differentially stimulated rat BMDMs. Figure 8a shows a protection of renal epithelial cells upon the administration of CM from high-iron-content cells such as IL-10–transduced or Fe-treated macrophages. A detailed analysis is represented in Figure 8b. Ki-67 and PCNA mRNA expression showed a pro-proliferative profile. This evidence was in line with previous results, in that high-iron-content cells, whether by way of overexpressing IL-10 or through direct treatment with Fe, released a soluble factor into the CM capable of rescuing renal epithelial cells from injury (Figure 8c). The application of CM to a different cell line revealed the same tendencies (Supplementary Figure S4 online). To underline the role of Lcn-2 in CM, cells were
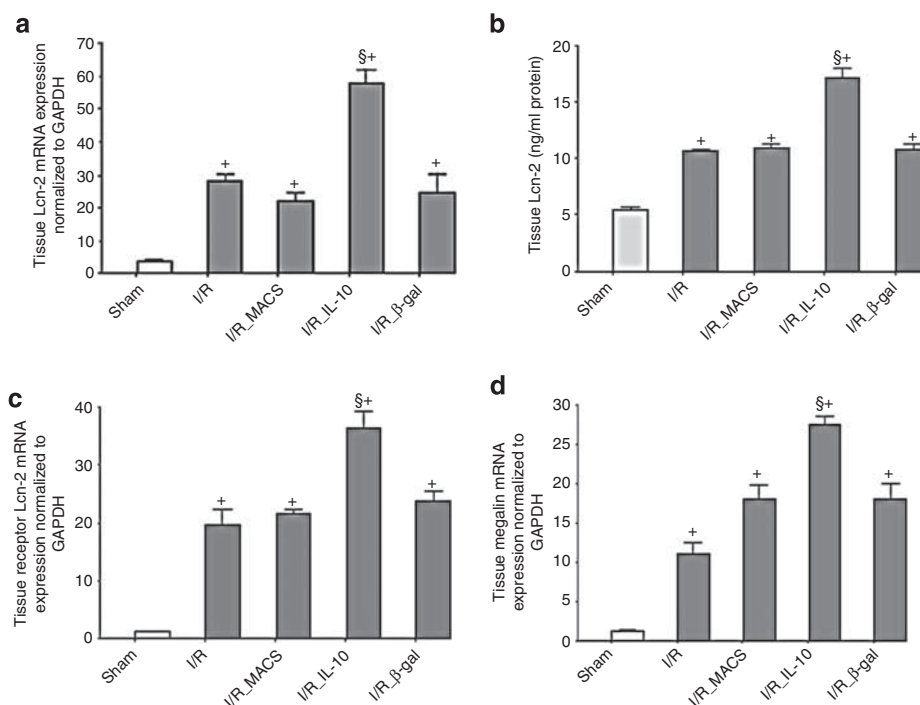
Figure 4 | Adoptive transfer of interleukin (IL)-10 macrophages modulates lipocalin-2 expression, the tissue receptor lipocalin-2, and megalin expression in ischemic kidneys *in vivo*. Macrophages were infused, where indicated, at 1 h of total reperfusion time (24 h) following 45 min of bilateral ischemia. The following experimental groups were included in the study: Sham: control group without infusion of macrophages; ischemia/reperfusion (I/R): bilateral ischemia for 45 min followed by 24 h of reperfusion; I/R_MACS: ischemia/reperfusion group with injection of untreated, bone marrow–derived macrophages; I/R_IL-10: ischemia/reperfusion group with adoptive transfer of *ex-vivo*–modified macrophages overexpressing IL-10; I/R_β-gal: ischemia/reperfusion group administered control β-gal-expressing macrophages. (**a**) Lipocalin-2 mRNA levels, (**b**) lipocalin-2 protein, and (**c**) receptor lipocalin-2 mRNA and (**d**) megalin mRNA expression were measured, and showed a significant increase in all ischemia/reperfusion groups, although fold induction after treatment with IL-10–overexpressing macrophages was even higher than that with untransduced macrophages. Real-time reverse transcription PCR data were normalized to relative expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and presented as arbitrary units of relative expression. ELISA data were expressed as nanogram per milligram of protein. Data are represented as means ± s.e.m.; $n = 5$, [+]$P < 0.01$ vs. Sham, [§]$P < 0.01$ vs. IR_β-gal.

Table 1 | Quantification of ED1-positive macrophages in renal kidney sections

	Sham	I/R	I/R_IL-10	I/R_β-gal
Macrophage density, counts/ × 400 field (ED1-positive cells)	2–8	50–120	80–150	70–130

Abbreviations: I/R, ischemia/reperfusion; IL, interleukin.
The effect of the different treatments with adoptive transfer of *ex-vivo*–modified macrophages on interstitial macrophage infiltration in renal ischemia reperfusion injury outcome was evaluated by quantification of ED1-positive macrophages in the corticomedullary area of renal tissue sections. Positive cells were counted from eight different fields per sample, averaged, and expressed as a range of macrophage density.

tested with CM in the presence of Lcn-2–neutralizing antibody or control IgG antibody (Table 2). Results showed a decrease in viability upon blockage of Lcn-2 in CM.

IL-10 rescues from IRI via iron and Lcn-2
BUN (Figure 9a) and creatinine (Figure 9b) indicated an essential role for iron and Lcn-2 in IL-10 protection. Lcn-2 blockade or DFO treatment in IL-10 macrophages caused an increase in both markers. Hematoxylin and eosin staining (Figure 9c) and histological analysis (Table 3) further corroborated these results. Pro- (Figure 9d) and anti-inflammatory (Figure 9e) cytokine profiles showed an upregulation of inflammation in IL-10–treated animals when

Lcn-2 was either blocked or was in the group in which DFO-treated, IL-10–transduced macrophages were infused. Such observations highlighted the crucial roles for Lcn-2 and iron on IL-10 therapy (Figure 9f). Ki-67 and PCNA indicate that Lcn-2 blockade abolished IL-10–dependent protective effects. DFO data support previous evidence. Further immunostaining for PCNA and stathmin corroborated the expression data (Figure 9g).

To study the actions specific to anti-Lcn-2, incubation with an isotype-matched IgG antibody was performed. Results revealed the role of anti-Lcn-2 in reducing both Lcn-2 and its receptors *in vitro*. Incubation with anti-IgG had no effect on the expression of Lcn-2 and its receptors, thus
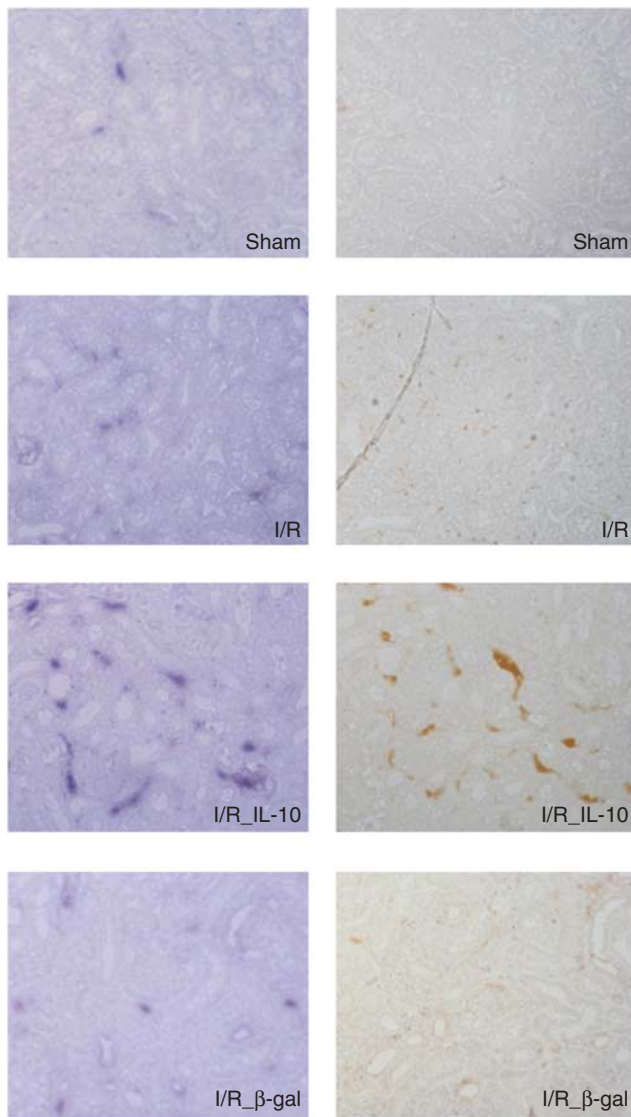
Figure 5 | Immunohistochemical staining for lipocalin-2 (right panel) and macrophages (ED1; left panel) in renal tissue sections. Immunohistochemical staining for lipocalin-2 (right panel) and macrophages (ED1; left panel) in renal tissue sections of the different study groups revealed the source of lipocalin-2 *in vivo*. In the ischemia/reperfusion (I/R) group, a great number of macrophages infiltrated the inflamed/injured kidney, but did not co-localize with lipocalin-2 expression. On the contrary, when interleukin (IL)-10-transduced macrophages were infused at 1 h of total reperfusion time, there was a significant number of co-localized macrophages, plus increased lipocalin-2 immunoreactivity, indicating lipocalin-2 production by the macrophages administered. Evaluation was performed with light microscopy (original magnification × 40). Microphotographs show representative images of five independent experiments for each condition.

indicating the specific activity of the neutralizing antibody (Supplementary Figure S5 online). In addition, in order to show that DFO treatment does not affect the viability of the macrophages, control macrophages were treated with DFO with and without iron. As shown in Supplementary Figure S6, cell viability measured by Hoechst/PI remained

unaffected by any of the treatments. However, when iron was added after treatment with the chelator, both Lcn-2 and iron levels were recovered (Supplementary Figure S6 online).

DISCUSSION

Previous work of our group indicated that macrophages were able to promote repair under anti-inflammatory conditions.[4] This previous study revealed that the administration of resting macrophages was unable to induce regeneration at the time points in which tissue was inflamed, and were only able to promote regeneration when macrophages were reinjected during the period of no inflammation (at 72 h of reperfusion). We concluded that macrophages induce renal regeneration after ischemia/reperfusion, but that this function depends on the inflammatory milieu. Recently, the biphasic effect of macrophages in the injured kidney has been observed and attributed to a macrophage switch toward an alternatively activated macrophage phenotype, leading to the suppression of the inflammatory response and promotion of a proliferative repair phase.[16] Furthermore, it has been suggested that the targeting of macrophage activation and phenotype is leading to new therapies in the treatment of many acute and chronic kidney diseases.[17]

Results of the present study reinforce these findings, as administration of untreated macrophages during the inflammatory phase did not influence repair. We report here that IL-10–overexpressing macrophages are effective during the inflammatory phase, but it should still be noted that probably higher cell doses would be needed to achieve full protection. Although it may be evident that these macrophages give rise to anti-inflammation, they also foster the expression of pro-regenerative factors such as Lcn-2. Administration of IL-10 macrophages augments repair owing to the fact that (1) they are primed toward M2 and (2) are able to reprogram the inflammatory environment. Alternatively, they might be able to reprogram resident macrophages toward M2, but this has not been demonstrated in the present study. The clinical use of IL-10 has been studied in a great variety of inflammatory and infectious disorders,[18] and alterations in the production of IL-10 have been linked to numerous disease states.[19–22] With regard to the kidney, Huang *et al.*[23] reported that IL-10 inhibits macrophage-mediated glomerular injury, including the reduction of proteinuria, histological severity, and the production of pro-inflammatory cytokines. IL-10 has also been reported to reduce inflammation and mesangial cell proliferation in acute glomerulonephritis induced by anti-Thy-1 antibody.[24] In addition, it has been observed to suppress glomerulosclerosis formation in FGS/Kist, a rat model of spontaneous focal segmental glomerulosclerosis.[25] IL-10 administration in animal models for nephrotoxic nephritis underlined the protective effects of IL-10. Interestingly, gene knockout models for IL-10 in nephritic mice confirmed an increased glomerular injury profile compared with wild-type mice.[26] In addition, IL-10 administration significantly reduced ischemia-associated renal injury and dysfunction, indicated by renal function markers and
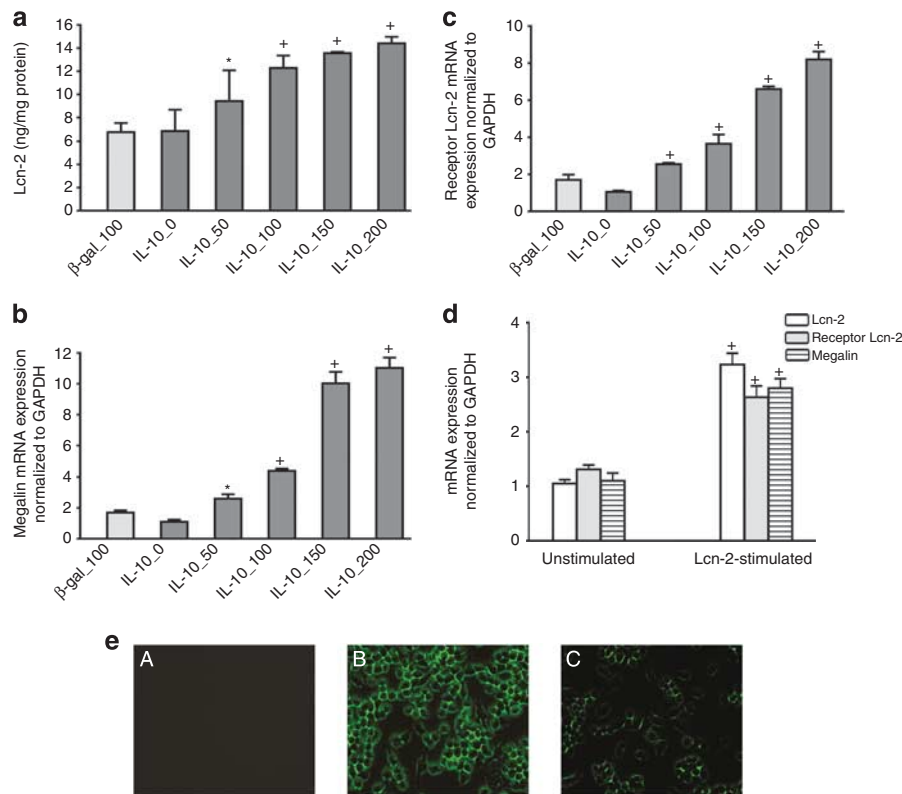
Figure 6 | Lipocalin-2 (Lcn-2) and its receptors are synthesized from adenovirus IL-10 (AdIL-10)–transduced, rat bone marrow–derived macrophages in a dose-dependent manner *in vitro*. (**a**) Lcn-2 protein content was measured by ELISA from cellular supernatant of AdIL-10–transduced primary macrophages. mRNA levels of both Lcn-2 receptors, megalin (**b**), and receptor Lcn-2 (**c**) were measured by real-time reverse transcription (RT) PCR, and showed a significant increase in a dose-dependent manner, which was in line with the transduction of IL-10 to rat bone marrow–derived macrophages. (**d**) External stimulation of rat bone marrow–derived macrophages with recombinant Lcn-2 protein (80 ng/ml) for a 24-h period showed a significant upregulation of both Lcn-2 and its receptors, measured by real-time RT-PCR. Such is indicative of a positive feedback mechanism of Lcn-2 in and of itself along with its receptors. (**e**) Bone marrow–derived macrophages (A) were incubated with 5 μg of green fluorescent protein (GFP)-tagged recombinant Lcn-2 protein for 24 h (B) or with recombinant Lcn-2 protein without GFP-tag (50 μg) for competitive analysis (C). GFP staining can be observed surrounding the cells, resulting in a marked increase in staining after incubation with the GFP-tagged recombinant Lcn-2 protein alone, unlike the case of the competitive test (C). Cells were viewed on a Leica TCS NT laser microscope. Magnification ×40; $n = 5$. Real-time RT-PCR data were normalized to relative expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were presented as arbitrary units of relative expression. ELISA data were expressed as nanogram per milligram protein. Data are represented as means ± s.e.m. from macrophages isolated from four different rats. Each femur was used separately and evaluated at least in triplicate for each group; $*P < 0.05$ vs. IL-10_0/unstimulated; $^{+}P < 0.01$ vs. IL-10_0/unstimulated.

histological analysis.[27] The fact that IL-10 may inhibit ischemia-associated renal injury is of relevant clinical importance as ischemia is one of the major causes of acute renal failure following renal transplantation. Nevertheless, data from unbiased searches for urinary molecules in humans revealed that the presence of IL-10 is not a relevant factor in kidney injury in humans, owing to the fact that IL-10 was not upregulated in patients with acute postoperative kidney injury[28] compared with those without. In addition, BMDMs genetically modified to produce IL-10 reduced injury in experimental glomerulonephritis.[2]

However, the therapeutic role of IL-10 macrophages in IRI had not been addressed until now. Consequently, there are mechanisms and mediators that are yet to be elucidated.

One possible regenerative mediator is Lcn-2, which is produced at sites of injury and may act by modulating repair.[29,30] We were able to verify that the administration of

IL-10 macrophages induces Lcn-2 and its receptors in the kidney. In addition, we found that increases in renal Lcn-2, produced as a consequence of IL-10 therapy, were mainly due to the infiltration of macrophages, as observed by co-localization. Sheng *et al.* demonstrated that the regulation of Lcn-2 and its receptor is mediated through distinct transcription and signaling pathways, which may act simultaneously.[31] The beneficial effects of IL-10 therapy could be attributed to increases in Lcn-2 release and function, which could be inferred by the action of Lcn-2 on its receptors. Lcn-2 activation of its receptors could be able to increase Lcn-2 uptake, thereby favoring its reparative functions in renal epithelial cells. It could be suggested that Lcn-2 activities are similar to those of growth factors[7] capable of modulating various cellular responses, including apoptosis, proliferation, and differentiation. The administration of Lcn-2–neutralizing antibodies reversed IL-10–mediated
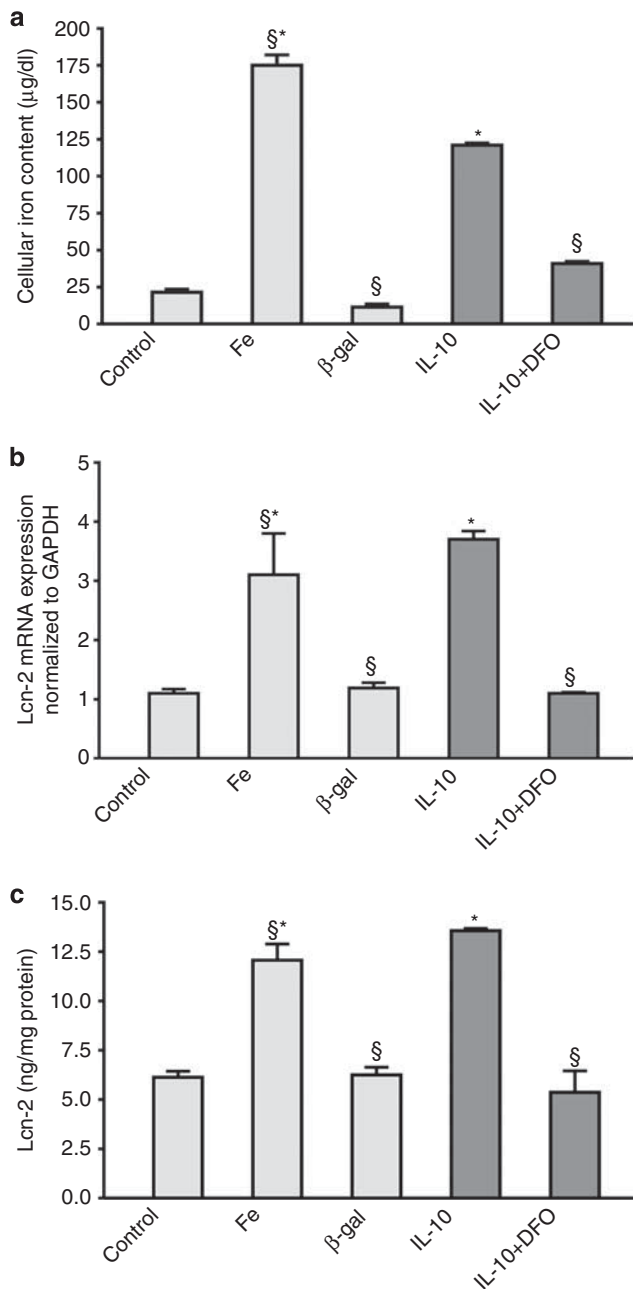
protection against IRI, indicating that the protective effects may depend to some extent on the production of Lcn-2.

Tilg *et al.*[10] provided evidence for the involvement of IL-10 in anemia during inflammatory diseases. A direct effect of IL-10 on ferritin translation and the storage of iron within macrophages was described.

Iron accumulation could be attributed to the induction of HO, described in previous studies,[11] which indicated that IL-10 induces expression of HO-1, a stress-inducible protein with potential anti-inflammatory effect, via a p38 mitogen-activated protein in murine macrophages. HO-1 induction via IL-10 could lead to excessive heme degradation and, consequently, increases in iron. In addition, the administration of genetically modified macrophages overexpressing HO-1 has been proven effective in improving the outcomes in kidney ischemia/reperfusion.[32]

Our results are consistent with these observations, given the fact that the increase in cellular iron after AdIL-10 transduction elucidates the role of IL-10 to accumulate iron. The highly specific iron chelator DFO[33] provokes a decrease in intracellular iron when administered to IL-10 macrophages

Figure 7 | Lipocalin-2 (Lcn-2) expression from interleukin (IL)-10-transduced, rat bone marrow–derived macrophages is dependent on cellular iron content. (**a**) Transduced rat bone marrow–derived macrophages were incubated with desferroxamine (DFO; 100 μmol/l) for 24 h. Macrophage control groups included treatment with iron (20 mg/ml), untreated macrophages, and β-gal-transduced macrophages. Cellular iron content shows a significant increase after treatment with iron and after adenovirus IL-10 (AdIL-10) transduction, indicating the iron-retaining role of IL-10. The iron chelator DFO significantly reduced cellular iron content. (**b**) Lcn-2 mRNA expression and (**c**) Lcn-2 protein content was measured in these groups and showed a significant increase where cellular iron content was high, thus indicating the likely function of Lcn-2 as an iron target gene. Real-time reverse transcription PCR data were normalized to relative expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were presented as arbitrary units of relative expression. ELISA data were expressed as nanogram per milligram protein. Data are represented as means ± s.e.m. from macrophages isolated from four different rats; each femur was used separately and evaluated at least in triplicate for each group, *$P < 0.01$ vs. Control, §$P < 0.01$ vs. IL10.

**Figure 8 | Interleukin (IL)-10 rescues renal epithelial cell viability from anoxic injury *in vitro* via an iron-dependent mechanism.** (**a**) Fluorescence-activated cell sorting analysis of propidium iodide (PI) binding and annexin V binding of renal epithelial cells 16 h after treatment with conditioned media from differentially stimulated rat bone marrow–derived macrophages under anoxic conditions. The plots indicate the proportion of live, viable cells (annexin V-negative, PI-negative), early apoptotic cells (annexin V-positive, PI-negative), necrotic cells (annexin V-negative, PI-positive), and dying cells (annexin V-positive, PI-positive). Untreated control cells were treated with equal volume of diluent (binding buffer) without staining. Experiments were conducted in triplicate in five independent experimental settings. Results show a clear protection of renal epithelial cells upon the administration of conditioned media from high-iron-content cells such as IL-10–transduced or Fe-treated macrophages. (**b**) The proportion of viable cells, early apoptotic cells, necrotic cells, and dying cells is represented in the table as percentages of the total cell number. Untreated control cells were treated with an equal volume of diluent without any staining. Experiments were conducted in triplicate in five independent experimental settings and represented as means ± s.e.m. #$P < 0.05$ vs. CM normoxia, *$P < 0.05$ vs. CM anoxia, +$P < 0.01$ vs. CM anoxia. The effect of conditioned media upon (**c**) Ki-67 and proliferation cellular nuclear antigen (PCNA) mRNA expression, assessed through quantitative reverse transcription (RT) PCR in total renal cell homogenates, showed a pro-proliferative profile. This evidence was in line with previous results, in that high-iron-content cells, be it by way of overexpressing IL-10 or through direct treatment with Fe, release a soluble factor into the conditioned media capable of rescuing renal epithelial cells from injury. Real-time RT-PCR data were normalized to relative expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were presented as arbitrary units of relative expression. Data are represented as means ± s.e.m.; $n = 8$, *$P < 0.05$ vs. CM IL-10, #$P < 0.01$ vs. CM IL-10; §$P < 0.01$ vs. Control anoxia.

in line with a decrease in Lcn-2 expression, indicating a direct link between IL-10, iron accumulation, and Lcn-2 induction. Our results corroborate previous reports that Lcn-2 is regulated by iron. It could be concluded that the biological effects of IL-10 are dependent on iron, and thus the expression of Lcn-2. The role of iron as an inducer of Lcn-2 is consistent with previous studies that have indicated that the presence of iron appears to account for the distinct

biological effects of Lcn-2.[8] Furthermore, a secondary effect of Lcn-2 on iron accumulation cannot be discarded, as it is known that Lcn-2 could bind to iron and deliver it to the cell through a process requiring endocytosis by a pathway differing from that used by transferring.[34]

In conclusion, the adoptive transfer of IL-10 macrophages to ischemic kidneys protects against acute kidney injury. The protection is mediated through the action of IL-10 on
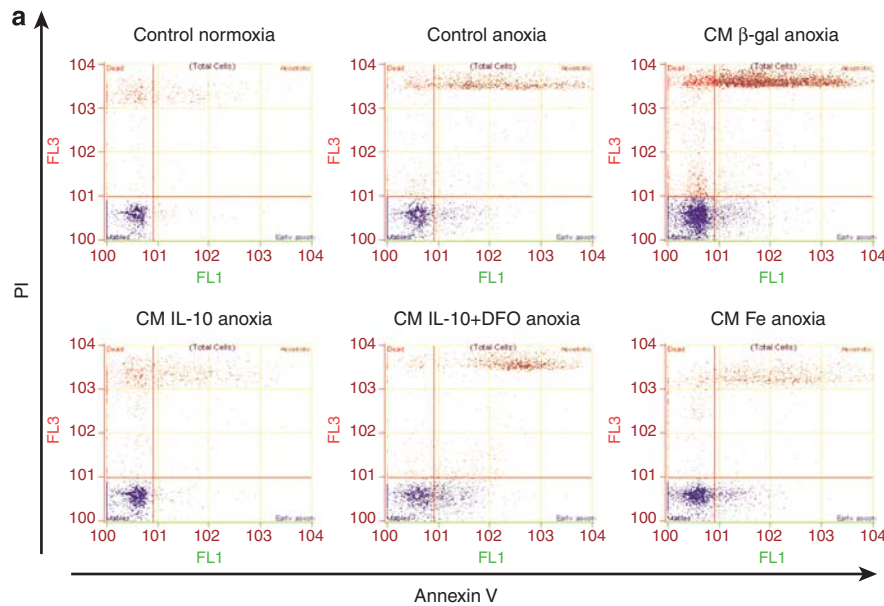
	CM normoxia	CM anoxia	CM β-gal anoxia	CM IL-10 anoxia	CM IL-10+DFO anoxia	CM Fe anoxia
Dead cells (%)	15.7 ± 1.3	11.5 ± 0.8[#]	14.2 ± 0.8[+]	15.0 ± 2.4[+]	6.5 ± 0.9[§,#]	8.0 ± 0.1[§]
Apoptotic cells (%)	7.7 ± 1.2	41.7 ± 3.7[#]	34.4 ± 2.9[#]	12.1 ± 1.0[§]	40.5 ± 1.9[#]	22.4 ± 0.9[#,+]
Viable cells (%)	74.7 ± 2.9	36.3 ± 6.0[#]	45.2 ± 1.8[+,§]	70.9 ± 3.7[§]	32.4 ± 3.8[#]	59.8 ± 1.9[§,*]
Early apoptotic cells (%)	1.7 ± 0.5	10.4 ± 1.5[#]	6.1 ± 0.6[+,#]	1.8 ± 0.6[§]	20.4 ± 1.0[§,#]	9.6 ± 1.0[#]
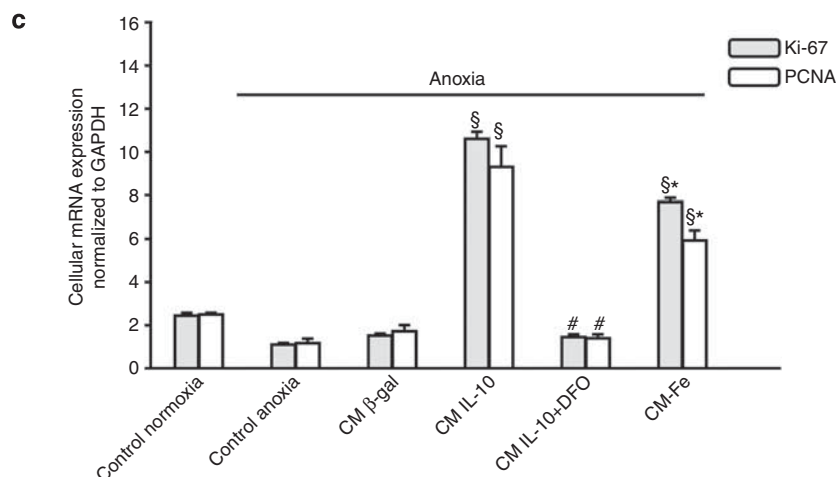
intracellular iron, which is able to induce Lcn-2, and in turn act as a mediator of the beneficial effects of IL-10 therapy.

MATERIALS AND METHODS
Animal model
The study was conducted using male Sprague–Dawley rats (Charles River, Wilmington, MA). Experiments were conducted with the approval of our institution's Research Commission, and by following European Union guidelines for handling and care of laboratory animals. Animals were anesthetized with isolfurane and placed in a supine position with body temperature maintained at 37 °C. The abdominal area was covered to minimize dehydration.

After laparotomy and dissection of renal pedicles, bilateral ischemia was induced by occluding the renal pedicles with an atraumatic microvascular clamp for 45 min. During reperfusion, the clamps were removed.

Experimental groups (*n* = 5 per group)
Sham: control without infusion of macrophages; I/R: 45 min of bilateral ischemia followed by 24 h of reperfusion; I/R _MACS: I/R group with injection of untreated BMDMs; I/R_β-gal: I/R group injecting β-gal macrophages; IL-10: I/R group with adoptive transfer of BMDMs overexpressing IL-10; IL-10 + AbLcn-2: after 45 min ischemia, following 50 min of reperfusion, a neutralizing anti-Lcn-2 (75 µg per animal) antibody was administered intravenously. At 1 h of reperfusion, IL-10 macrophages were transferred; IL-10 + AbIgG: as in the previous group, but using anti-IgG (75 µg per animal) isotype control antibody; IL-10 + DFO: IL-10 macrophages were treated *ex vivo* with DFO (Sigma, Madrid, Spain; 100 µmol/l) for 24 h; DFO-treated IL-10 macrophages were then injected intravenously at 1 h of reperfusion; AbLcn-2: bilateral ischemia was induced for 45 min. At 1 h of reperfusion, a neutralizing anti-Lcn-2 antibody (75 µg per animal) was injected intravenously without the additional transfer of macrophages; total reperfusion time was 24 h.

At 24 h of reperfusion, kidneys were harvested and snap-frozen at −80 °C, set in paraformaldehyde or embedded in an optical coherence tomograph and frozen in liquid nitrogen. Animals were killed and blood was collected.

Isolation, culture, and transfer of rat BMDMs
Rat BMDMs were isolated from the femur and matured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10 ng/ml granulocyte-macrophage colony-stimulating factor, 100 U/ml penicillin, and 100 µg/ml streptomycin for 7 days. For *in vivo* studies, BMDMs were transduced with adenoviral vectors to enable the expression of either IL-10 or β-gal. Where indicated, *ex-vivo*–stimulated BMDMs (1 × 10^6 cells per animal) were injected intravenously by puncture to the inferior cava at 1 h of reperfusion. BMDMs were tagged with the red fluorescent membrane label PKH-26GL (Sigma) in order to check their localization.[35]

Generation of CM from BMDMs
To obtain CM, macrophages were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. After transduction, BMDMs were starved for 24 h before the collection of CM. Supernatants from treated BMDMs were harvested via centrifugation and filtration through 0.2 µm pore filters.

Adenoviral vectors
Adenoviral vectors (AdIL-10, Adβ-gal, and adenovirus GFP) were kindly provided by Dr David Kluth and Dr Jeremy Hughes (MRC-CIR, Edinburgh, UK). For dose–response assays, 1 × 10^6 cells were transduced at different doses. An optimum multiplicity of infection of 50 was used for all experiments.

Caspase-3 activity assay
Caspase-3 activity was determined by measuring proteolytic cleavage of the substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Biomol, Lausen, Switzerland). A measure of 250 µg of protein and 12 mmol/l *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin was used to perform the assay, quantifying released amino-4-methylcoumarin by fluorospectrophotometry.

Griess assay
Nitrite accumulation was determined using the Griess reaction (Promega, Madison, WI) according to manufacturer's instructions.

Stimulation of BMDM
Rat BMDMs were incubated either with Lcn-2–neutralizing antibody (10 µg/ml; R&D, Minneapolis, MN) or with the isotype-matching IgG antibody (10 µg/ml; R&D) for 24 h. Transduced BMDMs were then incubated with lipopolysaccharides (15 ng/ml; Sigma) for 24 h. For Lcn-2-receptor studies, BMDMs were incubated with GFP-tagged Lcn-2 (5 µg/ml) for 24 h in the presence or absence of the competing Lcn-2 (50 µg/ml) without GFP-tag. Both recombinant proteins were supplied by PROTERA (Florence, Italy).

Iron determination
BMDMs were incubated with DFO (100 µmol/l) for 24 h. Control groups comprised treatment with iron (FERIV, G.E.S. GENERICOS ESPAÑOLES LABORATORIO, S.A., Madrid, Spain; 20 mg/ml), untreated cells, and β-gal-BMDM. Iron content was measured using

Table 2 | Renal epithelial cell viability after anoxic injury *in vitro* is dependent on lipocalin-2 in CM from IL-10 macrophages

	CM IL-10	CM IL-10+AbLcn-2	CM IL-10+AbIgG	CM β-gal	CM β-gal+AbLcn-2
% Viable cells	98.3 ± 2.28	49.04 ± 7.32*	95.13 ± 0.15	48.81 ± 2.76*	46.41 ± 2.78*

Abbreviations: CM, conditioned media; IL, interleukin; Lcn-2, lipocalin-2.
Trypan blue exclusion assay on NRK 52e cells incubated with conditioned media from adenovirus IL-10 (AdIL-10)–transduced macrophages under anoxic conditions. Cells were tested with conditioned media in the presence of either a lipocalin-2-neutralizing antibody or the isotype-matching control IgG antibody. Results confirm lipocalin-2–dependent rescue from anoxia-induced injury, and hence loss of viability, suggesting a key role of lipocalin-2 in protection. In contrast, treatment of renal epithelial cells with conditioned medium from Adβ-gal-transduced macrophages could not restore viability of renal epithelial cells under anoxic conditions. The presence or absence of a specific lipocalin-2-neutralizing antibody did not have any effect upon Adβ-gal-transduced macrophages, implying a link of IL-10 and lipocalin-2 in macrophages. Viability was measured in triplicate of five independent experiments. *P < 0.01 vs. CM IL-10.
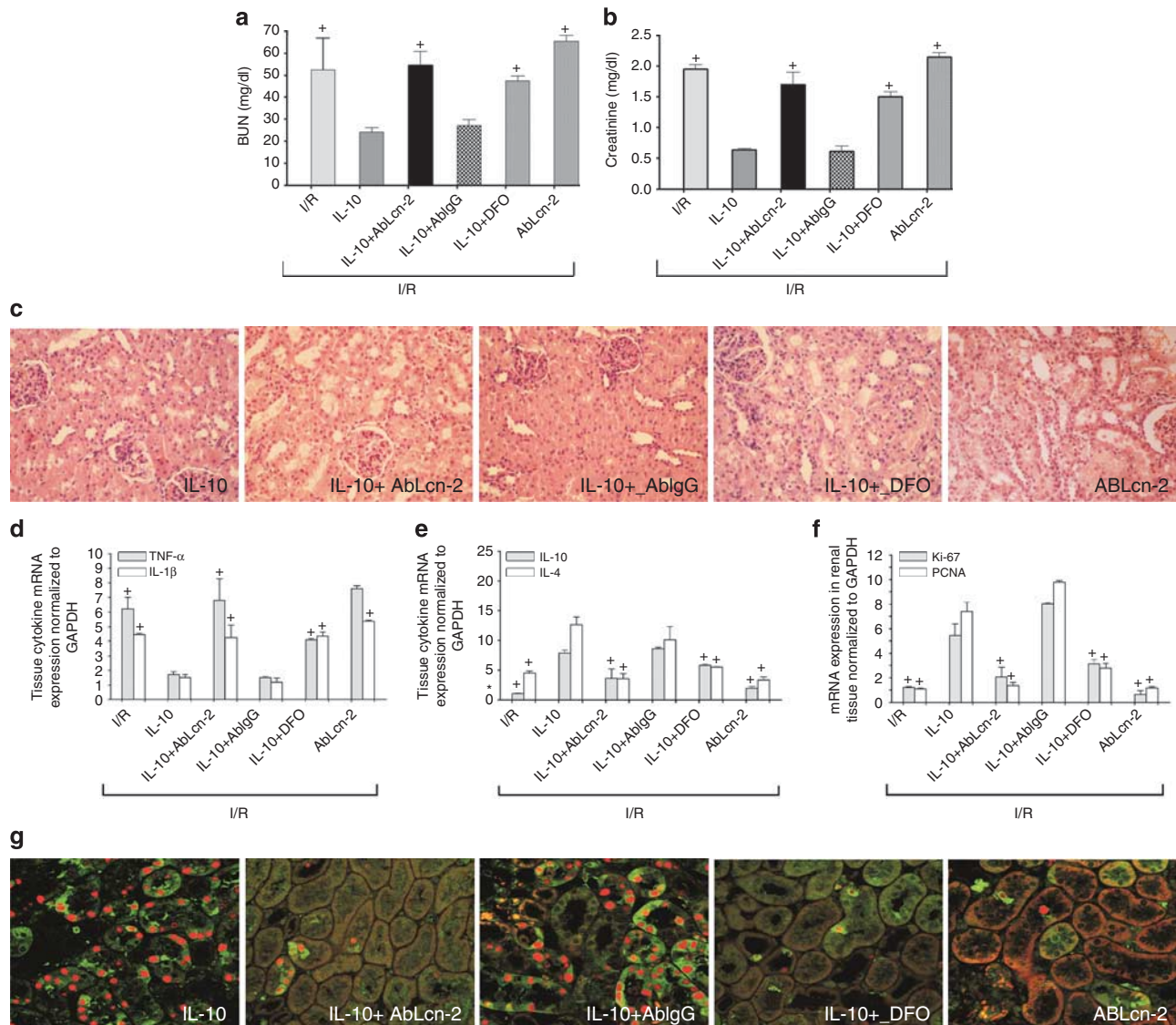
Figure 9 | Interleukin (IL)-10 rescues cell viability from ischemic injury *in vivo* via an iron- and lipocalin-2-dependent mechanism. Animals were subjected to 45 min of bilateral ischemia and were subsequently infused, where indicated, with previously stimulated bone marrow–derived macrophages at 1 h of total reperfusion time. Animals were killed at 24 h of reperfusion. The following study groups were analyzed: ischemia/reperfusion (I/R): bilateral ischemia of 45 min with subsequent reperfusion of 24 h; IL-10: ischemia/reperfusion group with adoptive transfer of *ex-vivo*–modified macrophages overexpressing IL-10; IL-10 + AbLcn-2: a neutralizing lipocalin-2 antibody (75 µg per animal) was administered intravenously to the ischemia/reperfusion group 10 min before IL-10–expressing macrophage transplantation; IL-10 + _AbIgG: a rat IgG antibody (75 µg per animal) was administered intravenously to the ischemia/reperfusion group 10 min before IL-10–expressing macrophage infusion; IL-10 + DFO: *ex-vivo*–modified macrophages overexpressing IL-10 were treated with the iron chelator deferoxamine (DFO; dose used: 100 µmol/l) before intravenous administration to the ischemia/reperfusion group; AbLcn-2: to evaluate the effect of the neutralizing lipocalin-2 antibody (75 µg per animal) on the injury outcome, 75 µg per animal was administered intravenously at 1 h of total reperfusion time (24 h) to the ischemia/reperfusion group. (**a**) Blood urea nitrogen and (**b**) creatinine in plasma clearly revealed a key role of lipocalin-2 expression in IL-10–mediated protection from injury. (**c**) Conventional histological analysis of hematoxylin and eosin-stained kidney sections (original magnification × 400) of the corticomedullary region corroborated the importance of iron-dependent lipocalin-2 expression in IL-10 therapy with regard to protection from ischemia/reperfusion injury. (**d**) Pro-inflammatory and (**e**) anti-inflammatory cytokine profiles of the different study groups highlighted the upregulation of pro-inflammatory milieu upon lipocalin-2 blockage with the neutralizing antibody, indicating a crucial role for lipocalin-2 in IL-10–mediated inflammation outcome. Data from the group in which DFO-treated, IL-10–transduced macrophages were infused confirm the *in vitro* data as pertaining to the function of IL-10–iron–lipocalin-2 in relation with protection against ischemia/reperfusion-associated kidney damage. (**f**) Pattern of normalized Ki-67 and proliferation cellular nuclear antigen (PCNA) mRNA expression evaluated via quantitative reverse transcription PCR indicates that lipocalin-2 has an important role in kidney repair following ischemia/reperfusion injury, given the fact that blockage of lipocalin-2 by its neutralizing antibody completely abolished IL-10–dependent protective and pro-proliferative effects on damaged renal tissue. DFO data supported previous data addressing the importance of iron in the expression of lipocalin-2 from IL-10–transduced macrophages and their associated protective effects on damaged kidney epithelia. PCR data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were represented as arbitrary units of relative expression. (**g**) Immunofluorescent staining of the regeneration markers PCNA (red) and stathmin (green) further confirmed PCR data and underlined the role of the IL-10/iron/lipocalin-2 axis in renal ischemia/reperfusion injury. Data are represented as means ± s.e.m.; *n* = 5; $^+P<0.01$ vs. I/R_IL-10.

Table 3 | Histological analysis of hematoxylin and eosin-stained kidney sections

	Epithelial necrosis	Epithelial balloonization	Tubular dilatation	Detachment	Edema	Total score
I/R-IL-10	0.6 ± 0.03	0.4 ± 0.03	0	0.5 ± 0.03	0	1.4 ± 0.02
I/R_IL-10_AbLcn-2	$2 \pm 0.1^+$	1.3 ± 0.04	0.8 ± 0.2	$2.9 \pm 0.4^+$	0.1 ± 0.03	$8.2 \pm 0.2^+$
I/R_IL-10_AbIgG	0.3 ± 0.05	0.2 ± 0.1	0	0.1 ± 0.01	0	0.8 ± 0.08
I/R_IL-10_DFO	$2.3 \pm 0.3^+$	0.6 ± 0.07	0.6 ± 0.2	$2.4 \pm 0.3^+$	0.4 ± 0.03	$7 \pm 0.2^+$
I/R _AbLcn-2	$1.8 \pm 0.6^+$	0.9 ± 0.08	1 ± 0.5	$2.3 \pm 0.06^+$	0.6 ± 0.06	$7.8 \pm 0.4^+$

Abbreviations: DFO, desferroxamine; I/R, ischemia/reperfusion; IL, interleukin.
Histological analysis indicated that animals treated with IL-10-overexpressing macrophages showed significantly higher tissue integrity, whereas adoptive transfer of IL-10–transduced macrophages in presence of the lipocalin-2-neutralizing antibody lost their protective effect upon ischemia-induced renal injury. Cellular infiltration, tubular cell balloonization, and severe necrosis could be observed. Furthermore, the transfer of IL-10–transduced macrophages previously treated with the iron chelator DFO did not improve injury outcome- thus suggesting a key role of iron-induced lipocalin-2 expression in IL-10–mediated protection in renal ischemia/reperfusion injury. $^+P < 0.01$ vs. I/R_IL-10.

Table 4 | Primer sequences

Gene (accession number)	Source	Primer sequence	Annealing Temperature (°C)	Amp length (bp)
Rn_Lcn-2 (NM_008491)	Invitrogen	Forward: 5′-CAAGTGGCCGACACTGACTA-3′ Reverse: 5′-GGTGGGAACAGAGAAAACGA-3′	48	192
Rn_GAPDH (NM_017008)	Invitrogen	Forward: 5′-CCGCCAATGTATCCG-TTG-TG- 3′ Reverse: 5′-TAGCCCAGGATGCCCTTTAGT-3′	48	207
Rn_Ki-67 (XM_00105622)	Invitrogen	Forward: 5′-AGACGTGACTGGTTCCCAAC-3′ Reverse: 5′-ACTGCTTCCCGAGAACTGAA-3′	48	197
Rn_PCNA (NM_022381)	Invitrogen	Forward: 5′-AGGACGGGGTGAAGTTTTCT-3′ Reverse: 5′-CAGTGGAGTGGCTTTTGTGA-3′	50	113
Rn_Mannose receptor (NM_001061231)	Invitrogen	Forward: 5′-CGAGGTGGTTTATGGGATGT-3′ Reverse: 5′-GGGTTCAGGAGTTGTTGTGG-3′	50	111
Rn_TNF-α (NM_012675)	Invitrogen	Forward: 5′-AACTCCGACAGCAAGCA-3′ Reverse: 5′-CGAGCAGGAATGAGAAGAGG-3′	55	211
Rn_IL-10 (NM_012854)	Qiagen	Quantitect primer assay	50	69
Rn_IL-1β (NM_031512)	Qiagen	Quantitect primer assay	48	91
Mm_IL-4 (NM_021283)	Qiagen	Quantitect primer assay	50	104
Mm_Megalin (001081088)	Qiagen	Quantitect primer assay	50	84
Rn_Lcn-2 receptor (NM_177421)	Qiagen	Quantitect primer assay	48	105

Sequences, annealing temperature, and expected fragment sizes of primers used in real-time quantitative reverse transcription PCR.

an ADVIA 2400 (Siemens Medical Diagnostics, Deerfield, IL) multichannel analyzer.

Anoxia experiments
Rat renal epithelial NRK52e cells and human embryonic kidney cells (HEK 293; ECACC, Salisbury, UK) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were subjected to anoxic (0.5% O_2) or normoxic conditions (20.7% O_2) for 16 h in the presence of either an Lcn-2–neutralizing antibody or an IgG isotype–matched antibody. Where indicated, cells were treated with CM from BMDMs.

Trypan blue assay
Cells were incubated with 0.2% Trypan blue (Sigma) in phosphate-buffered saline for 2 min at room temperature. After the reaction, Trypan blue solution was removed and both viable and dying cells were counted.

Annexin V/PI flow cytometry
Annexin V (Invitrogen, Grand Island, NY) staining was performed for 5 min. Cells were then centrifuged, resuspended, and incubated with PI for 5 min before taking measurement using a Becton Coulter flow cytometer (Becton Coulter, Fullerton, CA). Appropriate internal controls were performed for each experiment.

Hoechst/PI analysis
Cells were incubated with 0.1 μg/ml Hoechst 33342 (Invitrogen) and 1 μg/ml PI (Invitrogen) for 5 min at room temperature and analyzed using a fluorescence microscope.

Renal injury markers
BUN and creatinine were analyzed in plasma using an ADVIA 2400 (Siemens Medical Diagnostics) multichannel analyzer.

Histological analysis
Samples were embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin and eosin.

Immunofluorescent staining of kidney sections
Kidney sections were unmasked in sodium citrate buffer and blocked for 1 h. Staining of stathmin and PCNA was carried out as previously described.[15] Slides were incubated with anti-stathmin (Calbiochem, Darmstadt, Germany) and anti-PCNA antibody (Santa Cruz, Santa Cruz, CA), followed by incubation with secondary antibodies (Molecular Probes, Eugene, OR) for 2 h. Sections were viewed using a Leica TCS NT laser microscope (Leica, Wetzlar, Germany).

Immunohistochemistry ED1/Lcn-2
Slides were incubated with anti-ED1 (Serotec, Oxford, UK) and anti-Lcn-2 (R&D). Sections were stained with secondary antibodies

(Biotinylated anti-rat antibody for Lcn-2 (R&D) and alkaline phosphatase–conjugated antibody for ED1 (Abcam, Cambridge, UK)). Samples were incubated with avidin–biotin horseradish peroxidase complex and visualized by incubating the sections with diamino-benzidine (Sigma) and hydrogen peroxides. For ED1 detection, slides were developed with 2-nitro-5-thiocyanobenzoic acid/5-bromo-4-chloro-3-indolyl phosphate and counterstained with hematoxylin.

Protein concentration

Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA).

ELISA

Supernatants were collected from cells and tissue was homogenized and clarified. A volume of 100 µl of each sample was applied to an ELISA well plate previously covered with the anti-Lcn-2 (R&D) and blocked for 1 h. After sample incubation, the detection anti-Lcn-2 antibody (R&D) was added. HRP-conjugated avidin (Invitrogen) was incubated for 1 h, the color reagent (OPD tablets; Dako, Glostrup, Denmark) was added, and color development was assessed.

Real-time reverse transcription PCR

Cellular RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany), and kidney RNA was extracted with TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized by using the iScript cDNA synthesis Kit (Bio-Rad), and quantitative reverse transcription PCRs were performed using the SYBR Green RT-PCR detection Kit (Bio-Rad). A measure of 1 µg of cDNA was used for the detection of PCNA, Ki-67, Lcn-2, IL-10, IL-1β, and mannose receptor expression. Primers to detect Lcn-2-receptor and megalin were purchased as validated primer sets from Qiagen. The sequences, amplicon length, and annealing temperature for each primer are given in Table 4. Results were normalized to glycer-aldehyde 3-phosphate dehydrogenase as an internal control for stable expression.

Statistical analysis

Data were analyzed using one-way analysis of variance, followed by Newman–Keuls test analyses. Data are presented as the means ± s.e.m. with *P* values < 0.05 considered significant.

DISCLOSURE

All authors declared no competing interests.

SUPPLEMENTARY MATERIAL

Figure S1. IL-10-transduced macrophages exhibit a predominant anti-inflammatory phenotype.
Figure S2. AdIL-10-transduced macrophages localize efficiently to inflamed/injured kidneys.
Figure S3. Macrophage phenotype is modulated by the IL-10/iron/lipocalin-2 axis.
Figure S4. IL-10 rescues HEK cell viability from anoxic injury *in vitro* via an iron-dependent mechanism.
Figure S5. Functional study of the expression of lipocalin-2 from IL-10-transduced bone marrow-derived macrophages.

Figure S6. Cell viability and function after DFO treatment.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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