Specific Macrophage Subtypes Influence the Progression of Rhabdomyolysis-Induced Kidney Injury

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ABSTRACT
Rhabdomyolysis can be life threatening if complicated by AKI. Macrophage infiltration has been observed in rat kidneys after glycerol-induced rhabdomyolysis, but the role of macrophages in rhabdomyolysis-induced AKI remains unknown. Here, in a patient diagnosed with rhabdomyolysis, we detected substantial macrophage infiltration in the kidney. In a mouse model of rhabdomyolysis-induced AKI, diverse renal macrophage phenotypes were observed depending on the stage of the disease. Two days after rhabdomyolysis, F4/80lowCD11bhighLy6bhighCD206low kidney macrophages were dominant, whereas by day 8, F4/80highCD11b+Ly6blowCD206high cells became the most abundant. Single-cell gene expression analyses of FACS-sorted macrophages revealed that these subpopulations were heterogeneous and that individual cells simultaneously expressed both M1 and M2 markers. Liposomal clodronate-mediated macrophage depletion significantly reduced the early infiltration of F4/80lowCD11bhighLy6bhighCD206low macrophages. Furthermore, transcriptionally regulated targets potentially involved in disease progression, including fibronectin, collagen III, and chemoattractants that were identified via single-cell analysis, were verified as macrophage-dependent in situ. In vitro, myoglobin treatment induced proximal tubular cells to secrete chemoattractants and macrophages to express proinflammatory markers. At day 30, liposomal clodronate-mediated macrophage depletion reduced fibrosis and improved both kidney repair and mouse survival. Seven months after rhabdomyolysis, histologic lesions were still present but were substantially reduced with prior depletion of macrophages. These results suggest an important role for macrophages in rhabdomyolysis-induced AKI progression and advocate the utility of long-term follow-up for patients with this disease.


Severe damage of skeletal muscle, or rhabdomyolysis, is the second most common cause of death seen after an earthquake. AKI is a life-threatening complication of rhabdomyolysis. Rhabdomyolysis-induced AKI represents 10% of all AKI cases. Histologic data in this context are extremely scarce. Experimental evidence indicates that myoglobin (released from injured muscles) exerts its deleterious effects in part through a direct mechanism on proximal tubular cells involving peroxidase-like activity and...
deleterious iron release, and by an indirect mechanism involving the obstruction of the distal tubule by casts resulting from the interaction between myoglobin and uromodulin. Furthermore, myoglobin disturbs renal microcirculation, which is decreased because of the hypovolemia. AKI is then further worsened via hypoxia-related mechanisms. It is now acknowledged that inflammation plays a major role in the development of ischemic AKI, but the possibility that rhabdomyolysis-induced AKI could be an inflammatory disease has not yet been proposed.

While macrophage infiltration has been reported in a rat glycerol-induced AKI model and in a kidney biopsy specimen from a patient with rhabdomyolysis, the role or phenotype of the macrophages remains to be determined in rhabdomyolysis-induced AKI. Macrophages have long been recognized to be heterogeneous. While M1 macrophages are proinflammatory, classically activated macrophages, M2 macrophages are associated with immunoregulatory and tissue-remodeling functions. In vivo studies suggest that the phenotype of macrophages is changing over time, in response to both the microenvironment and the stage of the disease. Mononuclear cell depletion was associated with the exacerbation of kidney lesions induced by cisplatin or acute GN, suggesting a protective role for these cells. Conversely, macrophage depletion was associated with the reduction of lesions in obstructive nephropathy and ischemic AKI, suggesting that macrophages exacerbate AKI. Taken together, depletion experiments in AKI have led to conflicting data and demonstrate the need of additional studies to define macrophage subtypes.

It is well recognized that AKI is a risk factor for the development of CKD in humans and that the damaged tubule plays a role in the pathogenesis of CKD in mice. The severity of AKI is correlated to the rate of progression to CKD. Furthermore, recent studies showed that the macrophage phenotype controls long-term AKI outcome. In rhabdomyolysis-induced AKI, while injury is transient and associated with a favorable outcome, the effect on long-term renal function has not yet been studied. Of note, relapsing rhabdomyolysis may lead to CKD. This study was undertaken to evaluate the role of macrophages in rhabdomyolysis-induced AKI and subsequent CKD.

RESULTS

Evidence of Macrophage Infiltration into the Kidney during Rhabdomyolysis-Induced AKI

In a 35-year-old patient, admitted for severe drug-induced rhabdomyolysis requiring dialysis (Figure 1A), a kidney biopsy revealed tubular necrosis and casts (Figure 1B). Compared with the limited presence of CD68+ macrophages in a control kidney (Figure 1C, left), macrophages were abundant in kidney tissue and within the tubular lumen (Figure 1C right, D). To better understand the role and nature of the infiltrate, we used a murine model of rhabdomyolysis-induced AKI relying upon intramuscular glycerol injection. Two days after glycerol administration, we observed high mortality (Figure 1E) and severe AKI as indicated by increased BUN concentrations (Figure 1F). We confirmed kidney macrophage infiltration in mice using immunohistochemistry and flow cytometry (Figure 1, G and H, Supplemental Figure 1A).

Proximal Tubular Cells Might Participate in Macrophage Recruitment after Exposure to Myoglobin

Filtrated myoglobin first contacts the renal proximal tubule. To evaluate potential involvement of the myoglobin-exposed proximal tubule in macrophage recruitment, we treated a mouse proximal tubule cell line (MCT) with myoglobin. Treatment induced the expression of the positive control heme oxygenase (Hmox1), a critical enzyme for myoglobin degradation and detoxification (Figure 2A). In the presence of myoglobin, MCT expressed significantly more of the proinflammatory cytokines Ccl2 and Cc17 (Chemokine [C-C motif] ligand) at mRNA level (Figure 2A) and secreted more CCL7 protein in the supernatant (Figure 2B). This suggested that proximal tubular cells participate in macrophage recruitment upon exposure to myoglobin.

Rhabdomyolysis Drastically Affects the Kidney Macrophage Phenotype

It is known that specific macrophage subtypes contribute to kidney injury (M1) and repair (M2) in ischemia–reperfusion. In our model, kidney function was altered at day 2 and not yet fully restored at day 8 in surviving animals (Figure 3A), suggesting that repair was still ongoing at this stage. We discriminated three monocyte/macrophage populations according to the expression levels of CD11b and F4/80 (Figure 3B): F4/80+CD11b+ (R0), F4/80lowCD11bhigh (R1), and F4/80highCD11b+ (R2). Ly6b is a marker known to be absent on resident macrophages. We analyzed Ly6b and F4/80 expression gradually from R0 to R2 and observed that their inverse expression pattern was uninterrupted and progressive (Supplemental Figure 1B), suggesting that R2 cells corresponded to mature resident macrophages.

Under normal conditions (day 0) and consistent with previous data, the R2 macrophage fraction represented the majority and encompassed an M2-like contingent (CD206high, CD36high) (Figure 3, D and F, Supplemental Figure 1, C–E). At day 2, R1 macrophages became the dominant subtype (Figure 3C), likely corresponding to inflammatory macrophages, derived from monocytes and possessing an M1-like contingent, without M2 markers (CD206low, CD36low, Ly6bhigh, Ly6glow, CD209high). The percentage of Ly6b+ cells were significantly increased when examined within each macrophage type individually (P<0.001) (data not shown).

By the repair phase (day 8), R2 macrophages accounted for the majority again. The M2 markers (CD206 and CD36) increased at this time point (Supplemental Figure 2, A and B). R0 cells seemed to correspond to other myeloid cells (CD206+, CD36+, Ly6bhigh, Ly6ghigh, CD209low).
Figure 1. Rhabdomyolysis leads to macrophages recruitment in both human and mouse kidneys. (A) A 35-year-old patient was admitted for severe rhabdomyolysis. BUN and creatine kinase (CK) serum levels are reported (normal BUN, 2.5–7 mmol/L; CK, <170 IU/L). After 6 days the patient was anuric and a kidney biopsy was performed. (B) Hematoxylin-eosin staining of a kidney section (original...
Myoglobin Polarizes Macrophages Simultaneously Toward M1 and M2 Phenotypes In Vitro

The modification of the macrophage phenotype during rhabdomyolysis-induced AKI raises the question as to whether myoglobin can polarize macrophages. Primary macrophages, FACS-sorted from control kidneys (CD11b+F4/80+ fraction), and peritoneal macrophages were stimulated with myoglobin (Figure 4). In both macrophage subtypes, myoglobin induced significant upregulation of Hmox1, Ccl2, M1 activation marker Il1b and M2 activation marker CD206. However, in peritoneal macrophages, the effects of myoglobin were clearly dose-dependent and included an increase in the expression of Ccl7 and Nlrp3 (inflammasome transcription factor). The specificity of myoglobin-induced changes in target expression was validated by treatment with IL-4 or IFNγ+LPS (Supplemental Figures 3 and 4). Our data clearly show the concomitant expression of both M1- and M2-type markers, suggesting the coexistence of different macrophage subsets or a high plasticity potential whereby macrophages can simultaneously express both types of markers. To solve this question we evaluated gene expression at the single-cell level in kidney macrophages.

Single-Cell Transcriptional Analysis of R1 and R2 Macrophages In Vivo

Cells were sorted according to F4/80 and CD11b expression and loaded into a C1 single-cell device, in order to obtain cDNA from a known single-cell. Next, quantitative PCR-based gene expression profiling was performed for 96 genes using 22–48 cells for each condition (Figure 5A, Supplemental Figure 5, A and B). For some genes, including housekeeping genes (Gapdh, Ppia), the distribution of expression (Log2Ex) values was unimodal. Conversely, most of the genes exhibited heterogeneous multimodal expression (e.g., F4/80 and CD11b in Figure 5B). Some genes were upregulated in the glycerol condition irrespective of R1/R2 status (Figure 5C). Violine plots
for 96 genes are available in Supplemental Figure 5C. All P values are available in the Supplemental Material. Several of these targets are involved in phagocytosis, danger signaling, lymphocyte co-stimulation, migration, proliferation, activation, and the inflammasome. Because it is known that the cytoskeleton can affect macrophage activation, it is interesting to point out that β-actin (Actb) was overexpressed in the glycerol condition. Chemokines known to be involved in macrophage recruitment, including Ccl2, Ccl7, and Ccl9, were also induced. The simultaneous overexpression of a chemokine (Ccl7) and its cognate receptor (Ccr1) suggest the induction of an autocrine process. The only monitored gene exhibiting decreased expression levels in the glycerol condition was H2-Aa (CMHII) consistent with previous data. Analyses of correlation values between expressed genes from R1-Gly and R2-Gly identified a group of three genes (CD11b, Itgb2, and Fcgr3) as a coexpression network, the upstream regulator of which could be Stat3 (Supplemental Figure 6, A and B).

Figure 3. The kidney macrophage subtype evolution mirrors kidney function. (A) The elevated BUN levels observed at day 2 (Figure 1F) decreased by day 8, indicating ongoing kidney repair (n=6–11). (B–D) Analysis of macrophages obtained from kidney cell suspensions. Three regions were discriminated according to the expression levels of CD11b and F4/80 as follows: F4/80−CD11b− (R0), F4/80−CD11b− (R1), and F4/80−CD11b− (R2). CD11b corresponds to R0+R1+R2 sum. Macrophage distribution among the three regions was affected by glycerol injection at day 2 and day 8. (B) Representative dotplot gated on 30,000 live CD45+ cells. (C) R0, R1, R2, and total CD11b+ cell counts in kidney samples (n=3–5; **P<0.01 and ***P<0.001 compared with day 0; P<0.05 and ####P<0.0001 compared with day 2). (D) Distribution of CD11b+ cells in the three regions (n=3–5). Macrophage subpopulations were characterized by expression of surface markers using flow cytometry. Mean fluorescence intensity (MFI) is reported on the y-axis for the following markers: (E) CD206 (M2 marker) and (F) CD36 (M2 marker). (n=3–9; A star above a line displays P<0.05; $displays significant difference between R2 at day 2 or day 8 compared with R2 at day 0 [p<0.05]; displays significant difference between R1 at day 8 compared with R1 at day 2 [p<0.05]. Lines display means±SEM for individual data).
Figure 4. Myoglobin polarizes macrophages simultaneously toward M1 and M2 phenotypes in vitro. Primary renal macrophages (left; R2 and R1 fractions sorted from control kidneys) and peritoneal macrophages (right) were treated with increasing doses of myoglobin for 4 hours. Relative mRNA expression (fold induction) is depicted for (A) Hmox1, (B and C) chemoattractants Ccl2 and Ccl7, (D) M1 marker (Il1b), (E) M2 marker (CD206), and (f) inflammasome transcription factor Nlrp3. n=pool of 11 mice for renal isolates, n=3–4 individual mice for peritoneal isolates. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.)
Figure 5. Rhabdomyolysis modifies individual macrophages transcriptional activity. (A) Study design. Kidney macrophages were FACS-sorted to obtain R1 (F4/80$^{\text{low}}$CD11$^{\text{b}^+}$) and R2 (F4/80$^{\text{high}}$CD11$^{\text{b}^+}$) macrophages from mice, 2 days after intramuscular injection of saline or glycerol (Gly). Cells were loaded onto a chip to obtain cDNA from individual, viable single cells. Ninety-six mRNA expression profiles were then monitored by quantitative PCR. Data were converted into Log2Ex, which gives a higher value for cells with better detection, and plotted as violin plots. (B) Controls. The housekeeper genes Gapdh and Ppia exhibited unimodal expression (bottom left). F4/80 and CD11b mRNAs expression were consistent with proteins used in sorting (bottom right). (C) Glycerol effect. Differential gene expression between the glycerol and saline conditions. H2-Aa (CMH II) is the only monitored gene with a decreased expression in glycerol compared to saline. (D) MDS/PCoA. The scatter plot shows the distribution of mRNA expression profiles for each condition.
Multidimensional scaled principal coordinate analysis (MDS/PCoA) with expression data from 89 detected genes shows that sorted macrophages have distinct expression profiles with the first coordinate separating cells derived from mice treated with glycerol from controls and the second further separating R1-Gly from R2-Gly (Figure 5D), cells with profiles clearly separable by hierarchical clustering (Supplemental Figure 6C). CD209 was only detected in the R1-saline group and appeared as a potent discriminator, consistent with the high protein level observed at day 0 (Supplemental Figures 1E and 5C). Next we analyzed whether classic M1 and M2 markers were segregated as specific R1/R2 genes. M2 markers were in fact found to be expressed in both R1-Gly (Fm1 and Chi3l3) and R2-Gly (CD36 or Mrc1/CD206) cells. However, R2-Gly cells differed from R1-Gly cells in their expression of M1 markers, such as Tif1, Tlr4, and the chemotaxis signaling molecules Cdh2 and Cd2, as well as the fibrotic genes Mmp13 and Cxcl16 (Figure 5, C, E, F).

To confirm the role of R1-Gly in rhabdomyolysis-induced AKI and validate the results obtained with single-cell transcriptional analysis, we performed macrophage depletion experiments using liposomal clodronate.

**Liposomal Clodronate-Mediated Macrophage Depletion Protects against Glycerol-Induced AKI and Related Mortality**

Empty liposomes (EL) or liposomal clodronate (CL) were injected intraperitoneally as indicated in the experimental workflow (Figure 6A). Neither EL nor CL had an effect in saline-treated mice. Pretreatment with CL before rhabdomyolysis was renoprotective (Supplemental Figure 7A) and significantly improved animal survival (Figure 6B and Supplemental Figure 7B). We observed a renoprotective effect irrespective of the time of injection (Figure 6C), as attested by BUN monitoring 8 days after glycerol administration (Figure 6D). Gly-CL treatment significantly improved the GFR estimated at day 2 by inulin-FITC (3.5-fold increase; P<0.01, data not shown). CL administration systematically depleted macrophages and was still effective in the glycerol-condition (Figure 7A) at the protein level and the global mRNA level in kidneys. Results were similar with collagen III (Figure 7B). Consistent with *in vitro* (Figure 4) and *in vivo* analyses (Figure 5C), CL-mediated macrophage depletion decreased glycerol-induced Ccl2 and Ccl7 transcripts (Figure 7C), and the kidney structure was partially preserved from glycerol-induced lesions (Figure 7D). CL treatment reduced the glycerol-induced increase in kidney weight (from 339±9 mg in the GlyEL group to 300±9 mg in the GlyCL group; P<0.05, data not shown). To rule out influence of CL on the rhabdomyolysis response to glycerol injection, we assessed the intensity of rhabdomyolysis in CL-treated mice. CK systemic levels were similarly elevated in EL-treated and CL-treated mice (Figure 7E).

**Rhabdomyolysis Promotes Long-Term Fibrosis, and Renoprotective Effects of Clodronate Are Maintained over Time**

Recent evidence suggests that even “cured” AKI can progress to CKD.6 One month after rhabdomyolysis, BUN levels were still slightly elevated compared with levels in the control group (Figure 8A). The macrophage distribution between R0, R1, and R2 was similar in the control and the glycerol-treated mice with or without CL (data not shown). It has already been shown that the protective effects of CL involve CD206+ reparative macrophages.28 As shown in Figure 8B, CL treatment increased the CD206+ cell fraction, which is known to participate in matrix collagen endocytosis (Supplemental Figure 9).29 Patent fibrosis could be observed in the glycerol-treated mice, as assessed by collagen III deposits and Masson trichrome staining (Figure 8C). Consistent with the effect observed at day 2, CL led to a reduction of collagen III deposits (Figure 8, C and D). The experiment was repeated 7 months after rhabdomyolysis and showed long-term alterations in kidney function and structure (Figure 8, E–H). Kidneys from glycerol-treated mice were smaller (Figure 8F), with an uneven surface and higher collagen III deposits (Figure 8, G and H). These alterations were attenuated in CL-treated mice. Interestingly, the percentage of CD11b+ F4/80+ cells with respect to total live cells (obtained by FACS) were 2.2% (NaCl/EL), 3.7% (GlyEL), and 3.6% (GlyCL), suggesting a persistent low-grade inflammation (ANOVA P<0.03 for GlyEL versus NaCl/EL; n=6–10). Macrophage phenotyping showed no significant differences between GlyEL and GlyCL groups concerning the distribution of R1/R2 or the expression levels of Ly6-B or CD206.

**DISCUSSION**

It is extremely rare to find a thorough description of human kidney specimens in rhabdomyolysis. Recent reviews do not
describe histologic findings. The potential clinical relevance of the present work stems from the similarities between the macrophage infiltrate observed in our patient and a reported patient with infection-induced rhabdomyolysis.

In a rat model, macrophages, defined according to F4/80 detection, have been observed 2 days after rhabdomyolysis; a
Figure 7. CL-mediated macrophage depletion attenuates kidney lesions 2 days after glycerol injection. Mice received EL or ICL in the saline (NaCl) or the glycerol (Gly) condition according to the protocol shown in Figure 6E. (A and B) CL reduced glycerol-induced fibronectin (Original magnification, ×400 in A) and collagen III (Original magnification, ×200 in B) accumulation at the protein level (left panel; n=3–9) and at the mRNA level in the whole kidney (right panel; n=5–6). (C) Hmox1 was upregulated by glycerol treatment and not modified by CL. CL reduced glycerol-induced Ccl2 and Ccl7 mRNA expression (n=5–6). (Versus NaCl EL: ***P<0.001 and ****P<0.0001. Versus glycerol EL: #P<0.05; ##P<0.01; ###P<0.001; ####P<0.0001.) (D) CL reduced glycerol-induced lesions on periodic acid-Schiff staining (Original magnification, ×200 in representative examples). (E) CL did not affect rhabdomyolysis intensity (n=4–8).
Figure 8. CL-mediated macrophage depletion attenuates kidney fibrosis. (A–D) One month after rhabdomyolysis. CTL, age-matched control group; Gly, glycerol-treated group; Gly CL, CL-treated mice according to the pre, post, and glycerol CL protocol depicted in Figure 6C. (A) BUN levels (n=2–6). (B) CD206+ expression was significantly increased in the glycerol CL condition among CD11b+ cells (R0, R1, and R2) (n=5–8). (C) Collagen III and Masson trichrome staining (representative examples are shown). (D) Collagen III deposit


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detailed description of macrophage origin, infiltration, and function was lacking. Here, we identified a subset of mononuclear cells (either macrophages or dendritic cells) involved in the development of rhabdomyolysis-induced AKI.

As expected,24,25 R2 cells, which express CD36 and CD206, were predominant in the normal kidney. These R2 macrophages exhibited an M2-like resident phenotype. Two days after the glycerol injection, the R1 macrophage subset (F4/80low CD11bhigh) dramatically increased, as described in immune (lupus)31 and nonimmune renal diseases (unilateral ureteral obstruction32 and ischemia33). Substantial infiltration of proinflammatory mononuclear cells in the early phase of rhabdomyolysis-induced injury is based on our data, potentially explained by the following mechanisms (Figure 9).

First, myoglobin exerts a direct toxic effect on the proximal tubule, prompting proximal epithelial cells to secrete macrophage-recruiting chemokines such as Ccl2 and Ccl7, as previously shown in the distal tubule33 and substantiated by data obtained in vitro.

Second, since R1 macrophages were found to express Ccl2 and Ccl7 receptors (Ccr1, Ccr2, Ccr5), they migrate from blood to the kidney interstitium. The high level of Ly6b expression suggests that R1-Gly macrophages predominately originate from diapedesis of mononuclear precursors and not from a phenotypic switch of resident macrophages.

Third, once in the renal interstitium, myoglobin could directly activate macrophages. Exposure to myoglobin led to overexpression of the inflammasome component (Nlrp3) and proinflammatory factors (Il1b, Ccl2). This is consistent with (1) a recently published study showing that heme (a component of myoglobin) plays an essential role in Il1b secretion by macrophages during kidney inflammation34 and (2) our data showing that macrophages exhibited high levels of Nlrp3 (in vitro and in vivo for R2-Gly) and also expressed P2rx7 (Supplemental Figure 5C). It was previously shown that myoglobin, through NO scavenging, reduced the immunosuppressive activity of IFN–γ–activated macrophages.35

Our data showed simultaneous expression of M1 and M2 markers. R1-Gly macrophages display a differentiation potential toward M2 in addition to their inflammatory activity. R2-Gly cells most likely correspond to M2b macrophages, according to the classic definition of Mantovani.36 Eight days and one month after the glycerol injection, the return to an R2-dominant macrophage fraction suggested a reversion from proinflammatory to a reparative state of activation, as previously proposed.7 R2 macrophages could contribute to renal repair by increasing the expression of enzymes involved in extracellular matrix degradation (e.g., MMP-13 in single cell analysis) and CD206. After 1 month, CL-treated mice showed reduced collagen III deposits accompanied by CD206 enrichment, which has been identified as a determinant actor in intracellular collagen capture.29

To provide evidence for a role of macrophage in rhabdomyolysis-AKI, we depleted macrophages using CL, even though CL also depletes dendritic cells,37 which are also present in the CD11b‘F4/80‘ fraction. CL was protective in terms of kidney structure and function and mice survival. CL treatment led to an increase of the CD206‘ cell fraction in glycerol-treated mice, an effect previously described after ischemia.28 We suggest that R1 macrophage depletion could be protective via two additive mechanisms: (1) a general reduction in the accumulation of ECM components such as collagen III and fibronectin (it is known that fibronectin deposits in the kidney interstitium can alter its structure and dampen kidney function,38 and it has been previously reported that treatment with CL reduced fibronectin deposits in a limb-regeneration model39) and (2) a decrease in the inflammatory response, especially auto- and paracrine chemotactant loops involving Ccl2, Ccl7, and Il1b.

The short-term renal prognosis of rhabdomyolysis-induced AKI is relatively benign. However, recent studies increasingly emphasize the link between AKI and CKD.16 We observed a minor decrease in renal function, but this was accompanied by severe structural alterations of the kidney, including the presence of interstitial fibrosis, suggesting a shift from AKI to CKD. Beneficial effects have been reported for many chemical compounds or strategies in the context of experimental rhabdomyolysis, but none have specifically targeted subpopulations of immune cells. While macrophage depletion treatments performed within the right time frame displayed powerful effects, delayed treatments were ineffective. This indicates that any intervention aimed at R1 macrophages just after the initiation of the aggression could efficiently attenuate the development of AKI and subsequent CKD. In the more general context of the increase of incidence of AKI,40,41 our data have provided evidence that long-term follow-up and estimation of GFR after rhabdomyolysis could play an important role in the detection of silent fibrosis and permit patients to undergo appropriate nephroprotective measures.

CONCISE METHODS

Animal Model

C57BL/6J mice (male, 8–10 weeks old) were purchased from Janvier (Le Genest Saint Isle, France) and housed in a pathogen-free, quantification (n=5–7). (Versus control: **P<0.01 and ****P<0.0001. Versus Gly: #P<0.05 and ##P<0.01.) (E–H) Seven months after rhabdomyolysis. NaCl EL, glycerol EL, and pre-glycerol early CL refer to mice treated according to the protocol depicted in Figure 6A. (E) BUN levels (n=6–14). (F) Kidney mass indicated global atrophy after glycerol injection. (n=6–14) (G) Collagen III and Masson trichrome staining (representative examples are shown). (H) Collagen III deposits were still significantly increased in the glycerol condition, which was partially prevented by CL (n=6–14). (Versus NaCl EL: *P<0.05 and ****P<0.0001. Versus glycerol: #P<0.05, ##P<0.01, ####P<0.0001.)
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with anti-CD16/32 (Biolegend) and stained with anti-CD45-brilliant violet 510 (Biolegend), anti-CD11b-PE/Cy7 (Biolegend), anti-F4/80-AF647 (Biolegend), anti-Ly6b-AF700 (AbD serotec), anti-CD206-PE (Biolegend), anti-CD209-PE (Lifespan Biosciences), anti-CD36-AF488 (Biolegend), anti-Ly6g-AF700 (Biolegend), and anti-dectin1 PerCP-eFluor710 (eBioscience). A known quantity of Countbright beads (Molecular Probes) was added. Acquisition was performed on a BD LSR-Fortessa cytometer. Standard analyses were performed on FACS Diva Software (Becton Dickinson, Franklin Lakes, NJ). Maturation pathway analysis was performed on INFINICYT software (Cytognos SL, Salamanca, Spain).

Figure 9. Proposed early mechanisms involved in rhabdomyolysis-induced kidney injury. (1) In response to myoglobin, tubular cells secrete macrophage chemoattractants (Ccl2, Ccl7). (2) In response to these chemoattractants blood monocytes migrate to the renal interstitium. (3) In addition to its effect on tubular cells, myoglobin polarizes these macrophages toward a proinflammatory phenotype. (4) CD11b<sup>hi</sup>F4/80<sup>low</sup>Ly6b<sup>hi</sup> subtype macrophages enhance renal injury by secreting extracellular matrix compounds (fibronectin, collagen III), proinflammatory cytokines (Il1b, Il12p40) and by increasing recruitment of newly generated macrophages (secretion of Ccl2 and Ccl7).

Renal Function
Blood was drawn from the mouse tail vein and serum was collected to avoid dehydration-related hypovolemia. All reported experiments were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and were approved by a local animal care and use committee. To induce rhabdomyolysis, the animals were intramuscularly injected in each thigh with 7.5 ml/kg 50% glycerol (99.5% m/v AnalaR Normapur; VWR International S.A.S., Fontenay sous Bois, France) or saline as a control. To study the effect of clodronate, a macrophage-depleting drug, liposomal clodronate (Clodrosome) or the same volume of control liposome suspension (Encapsome; Encapsula Nanosciences LLC, Nashville, TN), was administered intraperitoneally to the animals as a full dose (40 mg/kg) the day before the glycerol injection or a half-dose (20 mg/kg) the day after the glycerol injection or a half-dose (20 mg/kg) the day after the glycerol injection, according to previously published protocols.

Characterization of Kidney Macrophages
Kidneys were decapsulated, minced, and incubated with collagenase (2 mg/ml, Sigma-Aldrich) and DNase 1KU/ml (Qiagen). After red blood cell lysis, cells were passed through a 40-μm mesh, incubated with anti-CD16/32 (Biolegend) and stained with anti-CD45-brilliant violet 510 (Biolegend), anti-CD11b-PE/Cy7 (Biolegend), anti-F4/80-AF647 (Biolegend), anti-Ly6b-AF700 (AbD serotec), anti-CD206-PE (Biolegend), anti-CD209-PE (Lifespan Biosciences), anti-CD36-AF488 (Biolegend), anti-Ly6g-AF700 (Biolegend), and anti-dectin1 PerCP-eFluor710 (eBioscience). A known quantity of Countbright beads (Molecular Probes) was added. Acquisition was performed on a BD LSR-Fortessa cytometer. Standard analyses were performed on FACS Diva Software (Becton Dickinson, Franklin Lakes, NJ). Maturation pathway analysis was performed on INFINICYT software (Cytognos SL, Salamanca, Spain).

Sorting of Macrophages Subsets
After the isolation of renal cells, as previously described, samples were stained with anti-CD45-BV510 (Biolegend), anti-CD11b-PE (Biolegend), and anti-F4/80-AF647 (Biolegend) after incubating with anti-CD16/32 (Biolegend). After one wash with PBS, the cells were sorted using the BD Influx cell sorter. Purity of cell-sorting is available in Supplemental Figure 10A.

Cell Model of Rhabdomyolysis
Myoglobin Solutions
Horse heart myoglobin (M1882; Sigma-Aldrich, Saint-Quentin Fallavier, France) was used. Myoglobin solutions were freshly prepared in PBS, filtered through 0.22-μm filters, and diluted in culture medium to a final concentration of 50, 250, or 500 μM according to experimental design. Absence of ferrous-oxymyoglobin was assessed by 409-nm absorbance.

Renal Cell Stimulation with Myoglobin
MCTs were carried in Ham’s F12/DMEM with 10% FCS, 1% penicillin/streptomycin, 10 ng/ml epidermal growth factor, 4 pg/ml triiodothyronine, 5 μg/ml insulin, and 36 ng/ml hydrocortisone. Cells were seeded in six-well plates, cultured for 48 hours, washed once with PBS, and deprived of FCS for 24 hours before treatment with myoglobin.

Macrophage Stimulation
IL-4 (20 ng/ml; Shenandoah Biotechnology) and IFN-γ (20 ng/ml, Shenandoah Biotechnology)+LPS (1 μg/ml; Sigma-Aldrich) were used as the positive controls for M2 and M1 polarization, respectively.

Peritoneal Macrophage Isolation
The peritoneal cavity of C57BL/6J mice was gently washed with 5 ml PBS. The recovered buffer was collected by centrifugation (400 g, 10 min) and plated in 24-well plates at a density of 10<sup>6</sup> cells/well in GluTaMAX-I (Invitrogen, Saint Aubin, France) supplemented with 5% FCS, 1% P/S. Cells were allowed to adhere for 2 hours (37°C, 5% CO<sub>2</sub>) and were washed with PBS before incubation with fresh serum-free medium containing myoglobin or reference polarization reagents for 4 hours.
Renal Macrophage Isolation

Samples obtained from the cell sorter (CD11b⁺F4/80⁺ fraction) were plated in 96-well plates at a density of 50,000 cells/well in GlutaMAX-I supplemented with 5% FCS, 1% P/S. Cells were allowed to adhere for 2 hours (37°C, 5% CO₂) and were washed with PBS before incubation with fresh serum-free medium containing myoglobin or reference polarization reagents for 4 hours.

CCL2 and CCL7 Detection

Culture supernatants were obtained 48 hours after the myoglobin treatment, centrifuged at 400 g for 10 minutes, and stored at −80°C. ELISA was performed using Peprotech kits 900-K123 and 900-M126 (Peprotech, Neuilly sur Seine, France) according to the manufacturer’s protocol.

Single-Cell Gene Expression

Samples obtained from the cell sorter were subjected to C1 Single-Cell Auto Prep System according to the manufacturer’s protocol (PN 100–4904 A1, Fluidigm). Biomark quantitative PCR was performed according to the manufacturer’s protocol (ADP37, Fluidigm). Primers were DEAGene Assays (ASY-GE, Fluidigm) and had previously been tested to assure efficiency and specificity (absence of primer dimers) and to rule out genomic DNA cross-reactivity (see sequences in Supplemental Figure 10B). Data were exported in heatmap (csv) format for analyses in SINGLEAR according to the manufacturer’s protocol and in R for MDS/PCA and hierarchical clustering analyses.

Gene Expression Quantification

Total RNA was isolated from mouse kidneys or macrophages using Qiagen RNEasy Plus Mini kit (Qiagen, Valencia, CA). Reverse transcription was performed on a FlexCycler2 (Analytik Jena AG, Jena, Germany). Quantitative PCR amplification was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). Primer sequences are listed in Supplemental Figure 11. The integrity of RNA was assessed by the 3′:5′ amplicon ratio.

Histologic Analysis

We retrospectively analyzed paraffin sections of kidney biopsy specimens from patients referred for diagnostic evaluation in the Department of Nephrology and Organ Transplantation at Rangueil University Hospital, Toulouse, France. All patients gave informed consent for the use of part of the biopsy for scientific purposes. All procedures were performed according to national ethical guidelines and were in accordance with the Declaration of Helsinki. Cell components revelation method and anti-CD68 detection were performed at the Department of Pathology. Regarding the murine model of rhabdomyolysis, kidney, liver, and spleen were fixed in Carnoy solution. Routine histology and immunohistological staining and analysis were performed. Briefly, 2–4 μm paraffin-embedded tissue sections were cut and used for immunohistochemistry. Periodic Acid–Schiff coloration was performed using Hematoxylin (S3309; Dako, Trappes, France), Periodic acid (19840100; Thermo Fisher Scientific, Geel, Belgium), and Schiff reagent (109033; EMD Millipore, Darmstadt, Germany). Mouse tissues were first de-waxed in toluene and rehydrated through a series of graded ethanol washes before endogenous peroxidase blockage. Specific primary antibodies were incubated on mouse tissue sections for the detection of F4/80 (rat anti-mouse, 1/100, clone BM8, MF48000; Invitrogen, Saint Aubin, France), collagen III (rabbit anti-mouse, BP8014; Acris Antibodies GmbH, Herford, Germany) or fibronectin (rabbit anti-human/mouse, F3648; Sigma-Aldrich, Saint-Quentin Fallavier, France). Streptavidin-HRP revelation was performed using a biotinylated anti-rat IgG (BA-4001; Vector Laboratories, Burlingame, Canada). For the visualization of rabbit antibodies we used the Dako Envision system (K4010; Dako, les Ulis, France). Finally, sections were counterstained with hematoxylin. Negative controls for the immunohistochemical procedures included substitution of the primary antibody with non-immune sera. Sections were scanned using a Nanozoomer 2.0 RS (Hamamatsu Photonics SARL, Massy, France) and treated with the commercially available Morpho-expert image-analysis software (version 1.00; Explora Nova, La Rochelle, France) for morphometric analyses.

Statistical Analyses

For statistical comparisons involving more than two experimental groups, one-way ANOVA was used. For comparisons between two data sets, unpaired t tests were used.

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DISCLOSURES

None.

REFERENCES


15. Grif


37. Dong X, Swaminathan S, Bachman LA, Croatt AJ, Nath KA, Grif


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