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Journal Pre-proof

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Please cite this article as: Boudhabhay I, Poillerat V, Grunenwald A, Torset C, Leon J, Daugan MV, Lucibello F, El Karoui K, Ydee A, Chauvet S, Girardie P, Sacks S, Farrar CA, Garred P, Berthaud R, Le Quintrec M, Rabant M, de Lonlay P, Rambaud C, Gnemmi V, Fremeaux-Bacchi V, Frimat M, Roumenina LT, Complement activation is a crucial driver of acute kidney injury in rhabdomyolysis, *Kidney International* (2020), doi: https://doi.org/10.1016/j.kint.2020.09.033.

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Complement activation is a crucial driver of acute kidney injury in rhabdomyolysis

10 human RIAKI biopsies

Tubular C3d deposit

Vascular C5b-9 deposits

Glycerol induced rhabdomyolysis RIAKI mouse model

Tubular C3b/iC3b deposits

C5aR1 on tubular and interstitial cells

WT   C3-/-

PBS   GLY   GLY   PBS

C3 knockout reverses RIAKI transcriptomic signature

C3-/-

+ Hemopexin
WT

Kidney Protection

CONCLUSION: Complement system is activated in rhabdomyolysis induced acute kidney injury (RIAKI).

Complement blocking and heme scavenging are promising therapeutic strategies.

kidney
INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL SOCIETY OF NEPHROLOGY

Boudhabhay, 2020

[QUERY TO AUTHOR: title and abstract rewritten by Editorial Office – not subject to change]

Complement activation is a crucial driver of acute kidney injury in rhabdomyolysis

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Running title: Complement in RIAKI

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Abstract:

Rhabdomyolysis is a life-threatening condition caused by skeletal muscle damage with acute kidney injury being the main complication dramatically worsening the prognosis. Specific treatment for rhabdomyolysis-induced acute kidney injury is lacking and the mechanisms of the injury are unclear. To clarify this, we studied intra-kidney complement activation (C3d and C5b-9 deposits) in tubules and vessels of patients and mice with rhabdomyolysis-induced acute kidney injury. The lectin complement pathway was found to be activated in the kidney; likely via an abnormal pattern of Fut2-dependent cell fucosylation, recognized by the pattern recognition molecule collectin-11 and this proceeded in a C4-independent, bypass manner. Concomitantly, myoglobin-derived heme activated the alternative pathway. Complement deposition and acute kidney injury were attenuated by pre-treatment with the heme scavenger hemopexin. This indicates that complement was activated in a unique double-trigger mechanism, via the alternative and lectin pathways. The direct pathological role of complement was demonstrated by the preservation of kidney function in C3 knockout mice after the induction of rhabdomyolysis. The transcriptomic signature for rhabdomyolysis-induced acute kidney injury included a strong inflammatory and apoptotic component, which were C3/complement-dependent, as they were normalized in C3 knockout mice. The intra-kidney macrophage population expressed a complement-sensitive phenotype, overexpressing CD11b and C5aR1. Thus, our results demonstrate a direct pathological role of heme and complement in rhabdomyolysis-induced acute kidney injury. Hence, heme scavenging and complement inhibition represent promising therapeutic strategies.

Key words: C3 deposition/ complement/ heme/ hemopexin/ lectin pathway/ rhabdomyolysis-induced acute kidney injury/

Translational Statement

Rhabdomyolysis induced acute kidney injury (RIAKI), which is the main complication of this life-threatening condition is associated with a poor prognosis, mainly due to the lack of specific treatments. Until recently, understanding of its physiopathology didn't include any role for complement activation. We provide here new evidences for complement system activation in human kidney biopsies with rhabdomyolysis. We discovered that complement system activation is mainly induced by both the lectin and the alternative pathways in a mouse model of RIAKI, attenuated by pre-treatment with heme-scavenger, hemopexin. Finally, we demonstrate a direct causal role of the complement system, showing that mice lacking C3 are protected from RIAKI. Actual RIAKI treatment is limited to volume expansion, forced diuresis and renal replacement therapy. Therefore, heme scavenging and complement inhibition represent promising therapeutic strategies for these critically ill patients opening a new field in rhabdomyolysis treatment, to be explored in the future.

Introduction

Rhabdomyolysis is a life-threatening condition caused by skeletal muscle injury and subsequent release of breakdown products into the circulation. The causes are multiple, but dominated by trauma ("crush syndrome"), muscle hypoxia, genetic defects, medication or drug abuse.[1] Acute kidney injury (AKI) is the major complication of rhabdomyolysis, seen in up to 50% of the patients.[2, 3] Rhabdomyolysis-induced AKI (RIAKI) occurs in adults, due to trauma or intoxication, and in children due to inherited muscle disorders. There is no specific treatment for RIAKI and patients' management is mostly symptomatic. Myoglobin and its oxygen-carrying moiety heme play a key role in RIAKI through: (i) renal vasoconstriction;[4] (ii) direct injury of proximal tubular cells through oxidative stress, lipid peroxidation,[5] and macrophage activation;[6-8] (iii) myoglobin precipitation with uromodulin, forming pigmented casts in distal tubules. RIAKI was considered complement-independent, since no C3c-positive staining was detected in the renal biopsies in the few published case reports. Nevertheless, complement may be implicated in RIAKI, since C3 depletion improved renal function in a rat model.[9] Complement is an essential effector of innate immunity.[10] However, complement overactivation is implicated in diseases affecting kidneys and/or associated with heme release.[11, 12] Indeed, cell-free heme activates the alternative pathway.[13] Moreover, complement is pathogenic in ischemia-reperfusion injury (I/R).[14-17]

Here, we demonstrate that complement is activated in RIAKI in mice and patients and describe the mechanisms, by which it contributes to the AKI.

Results

Complement activation in kidneys of RIAKI patients

Ten biopsies of RIAKI patients were collected. The main features of patients (RIAKI 1-RIAKI 10) are summarized in **Table 1.** Kidney biopsies revealed a typical pattern of RIAKI, characterized by acute tubular necrosis (ATN) associated with tubular casts (**Fig. 1A**). C3c staining was negative in the tubules in 8/10 RIAKI patients, and in 2 controls: a patient with AKI (Cr=420 μmol/L) caused by ATN without rhabdomyolysis and in an allograft kidney biopsy from a patient 2 days post-transplantation, used as example of I/R (**Fig. 1B**). Nevertheless, C3d staining, regardless of rhabdomyolysis etiology, revealed a strong cytoplasmic and granular staining within the tubular cells. C3d staining was tested in 4 patients with ATN. With the exception of one patient with hypovolemia-associated ATN, C3d staining was positive in ATN induced by ischemic causes especially in I/R but negative in other causes such Cisplatin toxicity (**Fig. 1C**). Vascular C5b-9 staining was detected in big vessels and on arterioles (**Fig S1A,B**) in 6 tested biopsies. We found evidence for localization of C5b-9 staining together with C3d in 3/6 cases (**Fig S1C**), although not in all vessels, as described in graft rejection.[18]

Systemic markers of complement activation were analyzed in the plasma of 13 patients with rhabdomyolysis (P1-P13) (**Table S1**). No patient had auto-immune disease. One patient (P2) had chronic kidney disease related to Bardet Biedl syndrome and type 2 diabetes mellitus was present in three patients (P2, P6 and P11). Soluble C5b-9 (sC5b-9) was increased in only 2 patients one of which, P2, developed septic shock early after admission which could explain this result. P7 also had inflammatory syndrome, but not so severe. In P12 the sC5b-9 was borderline. Plasma P13 corresponds to the patient with biopsy RIAKI 9.

Validation of the RIAKI mouse model

We studied the implication of complement in RIAKI using a mouse model in C57BL/6 mice (**Fig. S2A**). Immediately after glycerol injection (GLY), all mice presented with lameness of the injected limb (**Fig. S2B**). Rhabdomyolysis biomarkers, CK and ASAT, were elevated in GLY vs PBS mice (p=0.0042 and p<0.0001 respectively) (**Fig. S2C**). A dark discoloration of the urine of GLY mice at 6h was indicative of necrotic tubules and myoglobinuria (**Fig. S2D**). AKI was evident by the increase in both blood urea nitrogen (BUN) (**Fig. S2E**) and creatinine (Cr) (p<0,0001 and p=0,0006 respectively). Likewise, proteinuria increased in GLY mice (p=0,02), mainly composed of albumin (60kDa band) (**Fig. S2F**). Tubular damage/injury

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markers Lcn2 (Lipocalin 2, the mouse ortholog of human NGAL) and Havcr1 (KIM-1), and endothelial activation markers P-Selectin (p<0,0001), E-Selectin (p<0,0001), ICAM-1 (p<0,0001) and VCAM-1 (p<0,0001), (**Fig. S2G**) were upregulated in GLY mice. Lcn2/NGAL staining was intense on tubular cells of GLY mice (**Fig. S2H**). Likewise, VCAM-1 staining appeared positive in peri-tubular capillaries (**Fig. S2I**).

Complement activation occurs in the kidney and correlates with RIAKI in mice

Histopathological examination of GLY mice kidneys revealed the same lesions as in humans, with ATN and tubular casts (**Fig. 2A**). Staining with anti-C3b/iC3b antibody revealed the expected weak signal in PBS mice, on the tubular basement membrane and Bowman's capsule.[16] However, after glycerol injection, this staining increased dramatically in the tubular lumen and inside the cytoplasm of tubular cells and at the basolateral pole (**Fig. 2B, C**). No significant difference was found between CD31 staining in PBS, Glycerol and Cisplatin injected mice. The antibody does not recognize intact C3, indicating activation of complement in RIAKI. Conversely, cisplatin-injected mice did not show an increase in tubular C3b/iC3b deposits. Quantification of the staining revealed a linear correlation with AKI markers, assessed by BUN (r^2=0.64; p<0,0001) and by plasma creatinine (r^2=0,69; p<0,0001) in GLY mice (**Fig. 2D**).

Complement activation occurs locally in the kidney

We used a C3 western blot of plasma and urine to investigate complement system activation (**Fig. S3A**). In plasma, C3 western blot of GLY mice was similar to PBS mice, with no sign of C3 cleavage (**Fig. S3B** left), whereas in urine C3b (recognized by α' band) appeared at 2 hours following GLY injection and more markedly at 24 hours after GLY injection (right). The same pattern was observed in all 3 tested patients with RIAKI (R1:CK= 88 000 ui/L; Cr=141μmol/L, R2: CK=10000ui/L; Cr=1615μmol/L; R3: CK=17126ui/L; Cr=903μmol/L), with no cleavage in plasma but in urine (**Fig. S3C**). The urine R2 corresponds to biopsy RIAKI5. Moreover, among 12 tested patients with RIAKI, C3 was decreased for only one case (**Table S1**).

Systemic C3 mainly contributes to complement activation in the kidney

Further, we tested if plasma and/or local C3 was involved in RIAKI. C3 kidney mRNA was upregulated in GLY mice compared to PBS (p<0.0001), indicating renal production

during RIAKI (**Fig. S4A**). Moreover, C3d-staining in human and mouse kidneys appeared cytoplasmic and co-localized with LAMP1, indicative of intracellular C3, at least in part with lysosomal localization (**Fig. S4B, S4C**). To evaluate the role of systemic C3 in RIAKI, we injected glycerol in HepatoFH-/- mice, which have a low concentration of plasma C3 at steady state because of systemic FH deficiency and subsequent C3 consumption (**Fig. S4D**).[19] C3 kidney mRNA expression was similar in hepatoFH-/- GLY mice compared to WT-GLY mice (p=0.8), indicating persistent renal production. However, hepatoFH-/- GLY mice tended to be protected from RIAKI (**Fig. S4E**), with less tubular injury assessed by Lcn2 expression (**Fig. S4F**), and with less C3-deposits in their kidneys (**Fig. S4G**). Likewise, WT mice injected with cobra venom factor (CVF) prior to glycerol, displayed persistent plasma C3 consumption, 24h post-injection (**Fig. S4H)**, and nearly no C3b/iC3b deposits within the kidneys in comparison to mice injected with PBS prior to glycerol (**Fig. S4I).**

The complement system is over-activated through a C4-bypass pathway

In vitro, a dose-dependent increase in C3 activation fragments was observed on renal proximal tubular epithelial cells (RPTEC), incubated with increasing dose of myoglobin and serum (**Fig. 3A** left). No C4 deposits were detected (**Fig. 3A** right). Moreover, C4 was negative in 9 RIAKI biopsies tested (**Fig. 3B**). C1q staining was mostly negative in tubules of 6/8 RIAKI patients. However, 2 patients had a granular staining in scarce tubules (RIAKI 1 and 7), 1 had focal and segmental deposits in glomeruli (RIAKI 4) and 2 had positive staining of peritubular capillaries (RIAKI 8 and 9) (**Fig. S5A**). C1s staining was positive in vessels and capillaries including glomerular ones with very weak tubular staining in 2/2 RIAKI patients. (**Fig. S5B**). In mice, Glycerol injection induced an increased glomerular C1q staining (**Fig. S5C**). Gene expression revealed a decreased C1s1 expression of -0.54 log2 fold-change (p=0.02) without significant difference for C1r (-0.38 log2 fold-change (p=0.19)). Furthermore, glycerol injection in C4-/- mice revealed no protection from RIAKI (**Fig. 3C**) and even enhanced vascular aggression (up-regulation of P-Selectin (p<0,001), E-Selectin (p<0,001), VCAM-1 (p<0,001), and ICAM-1 (p<0,001) (**Fig. 3D**). Tubular injury markers, Lcn2 and Havcr1 were not different in C4-/- GLY mice, compared to WT-GLY but significantly upregulated compared to WT-PBS (Lcn2: p=0,02 Havcr1: p=0,04) (**Fig. 3E**). Finally, C3b/iC3b staining revealed intense tubular deposits in C4-/- mice, similar to WT-GLY mice, suggesting that complement activation is independent from C4 in this model (**Fig. 3F).**

A C4 by-pass lectin pathway operates in ischemic kidneys through collectin-11/L-fucose interaction.[20] Collectin-11 (CL-11) staining increased in mouse RIAKI kidney tubules. Moreover, CL-11 and C3b/iC3b showed partial colocalization, indicating that complement was largely deposited at the site of CL-11 binding (**Fig. 3G**). Human renal biopsies, including those of RIAKI patients, showed CL-11 overexpression on tubular cells, suggesting that this protein is readily available in the kidney (**Fig. S6A**). CL-11 staining was positive mainly on proximal tubules identified by their auto-fluorescent brush border. (**Fig. S6B**)

Complement dysregulation contributes to C3 deposits

Complement receptor 1–related protein y (Crry) is the only complement regulator on mouse tubular epithelial cells.[16] Staining of Crry revealed a linear repartition at the basolateral pole of tubular cells in PBS mice, but decreased and lost polarity in GLY mice. This could amplify complement activation and explain the basolateral localization of C3 deposits (**Fig. S7A**). Complement Factor H (FH) is a plasma regulator, which can bind to the cell membrane to control alternative pathway. Cfh gene expression was downregulated in GLY kidneys (**Fig. S7B**). Kidney tissue from a patient with idiopathic nephrotic syndrome caused by minimal change disease was FH-low/negative whereas liver sections, a biopsy from a patient with lupus nephritis and a biopsy from a patient with ATN without rhabdomyolysis were positive (**Fig. S7C**). In human RIAKI tissue sections, FH staining was positive in a small portion of the proximal tubules, showing an intracellular granular pattern. Compared to C3d-staining, only a few of the C3d-positive tubules appeared FH-positive (**Fig. S7D**).

Heme overload promotes complement activation.

Numerous C3 deposits did not colocalize with CL-11. Therefore, alternative pathway could be triggered independently. As cell-free heme is a well-known alternative pathway activator in hemolysis, we studied if myoglobin-released free heme, reflected by HO-1 up-regulation in GLY mice (**Fig. 4A**), contribute to complement activation and kidney injury. In vitro, incubation of renal proximal tubular epithelial cells (RPTEC) with oxidized heme and serum, increased C3 deposits (**Fig. 4B**). In vivo, we evaluated the contribution of heme by a preventive injection of its natural scavenger, hemopexin. The injected hemopexin dose was the maximal well-tolerated in heme-overload.[21] The induction of rhabdomyolysis was efficient since CK and ASAT were not different in GLY mice +/- hemopexin. However, renal function (BUN) improved in hemopexin injected mice (p=0,01) (**Fig. 4C**). No change in tubular injury markers was observed, but vascular injury markers were markedly

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decreased (P-selectin: p=0,0095; E-Selectin: p=0,014; VCAM-1: p=0,03 ICAM-1: p=0,06) (**Fig. 4D-E**). Finally, C3 deposits significantly decreased (p=0,02) (**Fig. 4F-G)** whereas renal C3 gene expression was not different from GLY mice (p=0,5; not shown).

Tubular cell and macrophage C5aR1 is upregulated in mouse RIAKI

Renal C3aR1 mRNA expression in GLY-injected mice was not different from PBS mice (p=0,89) (**Fig. 5A**). However, C5aR1 mRNA was markedly upregulated in GLY mice (p<0,0001) (**Fig. 5A**). Strong staining for C5aR1 was detected in the tubules of GLY mice and in interstitial cells (**Fig. 5B**). To identify those cells, C5aR1 was measured on myeloid populations from kidneys by flow cytometry. Eight hours after glycerol injection, there was an increase in macrophage and neutrophil infiltration within GLY kidney compared to PBS (p=0,0152 and p=0,0022 respectively) (**Fig. 5C**). At 24 hours, there was no quantitative difference in neutrophil and macrophage infiltration, as described.[22] No difference was detected for C5aR1 expression on neutrophils in both groups (p=0,18), but in GLY mice both M1 (CCL2, TNFα) and M2 (Arginase 1) markers were co-expressed (**Fig. S8**). Indeed, intrarenal macrophages showed phenotypic alteration. Expression of C5aR1 was not different among macrophages expressing class II HLA (CD45$^+$Ly6G$^-$CD68$^+$IAb$^+$; p=0,55) and their proportion did not differ between PBS and GLY mice. However, CD45$^+$Ly6G$^-$CD68$^+$IAb$^-$C5aR1$^+$ macrophages increased in GLY mice (p=0,02) mostly because of the emergence of a peculiar sub-population of CD45$^+$Ly6G$^-$CD68$^+$IAb$^-$C5aR1$^+$CD11b^{hi} cells (**Fig. 5D**). CD11b/CD18 is a receptor for C3b/iC3b. Interestingly, immunofluorescence on frozen kidneys revealed a close relationship between CD11b$^+$ cells and C3b/iC3b deposits (**Fig. 5E**).

C3-/- mice are protected from RIAKI

To explore the causal link between complement and RIAKI, we injected glycerol in C3-/- mice. C3-/- GLY mice presented the same lameness of the injected limb and increase in CK and ASAT, compared to PBS WT mice (p=0,03 and p=0,0043 respectively) reaching the same level as WT-GLY mice (p=0,2 and p=0,5 for CK and ASAT). In addition, HO-1 was equally upregulated in WT-GLY and C3-/- GLY mice (**Fig. S9A**). However, BUN was significantly lower in C3-/- GLY compared to WT-GLY mice (p=0,002) (**Fig. 6A**), disrupting the correlation between kidney injury and muscle injury (**Fig. 6B**). In addition, C3-/- GLY mice kidneys displayed very light ATN and rare tubular casts (not shown) following hematoxylin/eosin staining. Even sensitive markers of tubular injury such as

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Lcn2 and Havcr1 were reduced in C3-/- mice, (p=0.001 and p=0.0005 respectively) (**Fig. S9B**), as well as endothelial activation markers (P-Selectin: p=0,001; E-selectin: p=0.01; ICAM-1: p=0;0005; VCAM: p=0,0005) (**Fig. S9C**).

RIAKI in mice is associated with a pro-inflammatory and apoptotic transcriptomic signature.

Bulk RNA sequencing analysis on kidneys from PBS versus GLY mice revealed 2056 significantly upregulated genes, 1786 significantly downregulated genes and 16683 genes without significant difference (difference was considered significant when p<0,05 and fold change at least superior to 2) (**Fig. 6C**). Analysis of the 25 most up-regulated and the 25 most down-regulated genes, allowed definition of a rhabdomyolysis transcriptomic signature (**Fig.6D**). Interestingly, one of the most upregulated genes was Fucosyltransferase 2 (Fut2) enzyme, responsible for tagging stressed cells with fucosyl residues, recognized by the lectin pathway.[23] Performing gene ontology on all significantly upregulated genes showed the most up-regulated pathways involved the inflammatory response (cytokine and chemokines such as Cxcl1, Cxcl2, Cxcl3, Cxcl5, Cxcl14, Cxcl17, Ccl2, Ccl3, Ccl4, Ccl7, Ccl9, Ccl11, Ccl12, Ccl17, Ccl20, Il6, Il11, Il24, TNFα, members of the IL1 family, etc), and the cell response to inflammatory processes (response to biotic stimulus such as Glutathione S-transferase A-1 gene, protein phosphorylation and regulation of intracellular transduction), as well as apoptosis (e.g Trib1 and Myc). Classical tubular injury markers Lcn2 (NGAL) and Havcr1 (Kim1) were dramatically upregulated. The most downregulated pathways affected renal metabolism (drug metabolic processing, small molecule processing and lipid metabolism), and intracellular transports (ion transport and trans-membrane transport such as SLC34a3 coding for Sodium-dependant phosphate transport protein 2C and SLC12A1 gene, coding for the Na$^+$/K$^+$/2Cl$^-$ cotransporter).

C3 deficiency attenuates the RIAKI signature in mice

C3-/- mice injected with PBS presented a transcriptomic profile very close to the PBS WT mice. Strikingly, C3-/- mice injected with glycerol (C3-/- GLY) lost the majority of the RIAKI signature, compared to the WT-GLY mice (**Fig. 6E**). Indeed, inflammatory pathways were drastically less upregulated than in WT-GLY mice and, cellular metabolism or ion transport were less affected. Lcn2 and Havcr1 were significantly reduced in C3-/-mice, confirming the data from the RTqPCR (**Fig. 6E**, **Fig. S9B**). Comparison of RIAKI

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signature genes between WT and C3-/- PBS (WT/C3-/- PBS), or glycerol (WT/C3-/- GLY) allowed us to classify them into 3 groups: 1) genes, which are completely normalized in the C3-/- GLY mice (p-value WT-GLY vs C3-/-GLY<0.05 and p-value C3-/- PBS vs WT-GLY>0.05); 2) genes which are partially normalized in the C3-/- GLY mice (p-value 'WT-GLY vs C3-/-GLY'<0.05 and p-value 'C3-/- PBS vs WT-GLY'<0.05); 3) genes which are not affected by C3 deficiency (p-value 'WT-GLY vs C3-/-GLY'>0.05 and p-value 'C3-/-PBS vs WT-GLY'>0.05). The completely normalized genes included Fut2 among the upregulated and a set of down-regulated genes, involved in pathways related to potassium ion transport and protein dephosphorylation. Partial correction by C3 deficiency was achieved for upregulated genes involved in the cellular response to cytokines (such as: Ccl12, Cxcl1, Fgb, Lcn2, Socs3, Sox9, Spp1, Timp1) and apoptosis (the same set except Cxcl1 but including Fgg and Sfn). Among them Ccl12, Fgb, Lcn2 and Sox9 belong to the cellular response to IL-1 pathway. The set of downregulated genes, attenuated in C3-/-mice, were not enriched in any particular pathway. A set of genes, including such involved in the cellular response to hypoxia (Eif4ebp1 and Myc) were not affected by C3 deficiency.

Discussion

In addition to myoglobin casts and acute tubular necrosis which are common histology features for rhabdomyolysis,[24] complement deposits occurred in kidneys of mice and patients with RIAKI, independent of the rhabdomyolysis cause. Complement activation occurred intrarenally most likely by the lectin pathway via a C4-bypassing mechanism, and by the alternative pathway via heme. Intrarenal macrophages in RIAKI became particularly sensitive to complement, overexpressing C5aR and CD11b. C3-/- mice were protected from kidney injury, demonstrating a direct pathophysiological link and placing complement as a potential therapeutic target in RIAKI.

We discovered C3d-deposits in the kidneys of patients with RIAKI, regardless of the cause of rhabdomyolysis, demonstrating that complement activation is a consequence of a common factor, present during muscle injury. C3d staining was negative in most of the tested ATN without rhabdomyolysis biopsies, suggesting that complement activation is not a consequence of all tubular injuries. Interestingly, C3c staining was negative/weak in RIAKI biopsies. This can be explained by the rapid cleavage of iC3b to C3c then to C3d, since all biopsies were performed more than 24 hours after the initiation of rhabdomyolysis.[25] In lupus nephropathy (LN) C3c vs C3d staining allows the

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differentiation between active and not-active LN.[26] Noteworthy, C3c but not C3d-immunostaining is performed as routine work-up on kidney biopsies and might explain why C3-deposits have not been described until now in RIAKI.

Furthermore, we detected massive C3-deposits in the mouse model. C3 cleavage or consumption did not occur in plasma from the patients or mice. Thus, complement activation occurs locally within the kidney, and we detected C3 fragments in urine of patients and mice. This intrarenal complement activation could be due to the local overexpression of C3, as described in lupus nephritis[27] and in graft rejection,[28, 29] and/or mediated by the systemic C3 as in I/R[30] or hemolysis-induced renal injury.[12] Since glycerol-injected mice with plasma C3 consumption (FHhepato-/- and CVF-treated mice) were protected from complement activation, the major role was played by the systemic C3.

Complement could be activated in RIAKI by different pathways. In rat RIAKI, enhanced C1q staining in tubules was detected[9], whereas here C1q was mainly negative in RIAKI biopsies and enhanced only in some glomeruli in the mouse model. Renal fibrosis models revealed increased expression of C1q, C1r, and C1s and classical pathway activation in the interstitial compartment.[31, 32] We detected a decrease of C1s and C1r expression at 24h. Nevertheless, we cannot exclude an involvement of the classical pathway in RIAKI, potentially later in time, with the progression of the fibrotic process as previously suggested.[32] However, C4-/- mice were not protected from RIAKI and C3 deposits, suggesting that classical pathway and C4 have a minor role in RIAKI's complement activation. The alternative pathway is responsible, at least in part, for the observed deposits. Indeed, heme binds C3, activates the alternative pathway[13] and it induced complement activation on primary tubular cells. This process could be mediated also by properdin.[33, 34] Moreover, the injection of heme-scavenger hemopexin, which can be filtered through the glomerular basement membrane, partially improved kidney function and vascular aggression, as described.[7] Hemopexin decreased C3-deposits, showing a direct role of heme in the intrarenal complement activation.

Conventional lectin pathway proceeds through C4,[35] but a C4-bypass pathway was found in post-ischemic tissue, where CL-11 binds abnormally fucosylating cells and its associated protease MASP-2 cleaves directly C3 to C3b and C3a.[36, 37] Fucosyltransferase 2 (Fut2) is the enzyme which fucosylates epithelium in case of enhanced inflammation.[23] In RIAKI, we detected CL-11 staining, partially colocalizing with C3b/iC3b and Fut2 was one of the most up-regulated genes in the transcriptomic signature. Moreover, Fut2 is strongly dependent on complement, as it was completely normalized in the C3-/- mice,

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suggesting that complement activation, potentially triggered by heme, creates an environment that promotes a vicious cycle of further lectin pathway activation. The RIAKI is therefore a particular situation, where both the alternative and the lectin pathways may be triggered in the kidney and the alternative pathway is not a mere amplification loop. Moreover, the reduced regulatory capacity of the stressed tubular cells in RIAKI that we found amplifies the complement activation.

To better understand the molecular mechanisms of RIAKI and the role of complement, we defined a "RIAKI transcriptomic signature" in the mouse model. A clear signature of apoptosis was detected, consistent with the tubular injury. If there was no evidence for increase of TNF alpha expression in kidney,[38] a strong pro-inflammatory signature was observed with a high involvement of genes for response to cytokines and cytokine production. This is concordant with the hypothesis that tubular injury observed in rhabdomyolysis is an inflammatory disease,[38-40] involving infiltrating immune cells, especially macrophages in glycerol induced RIAKI.[6, 39, 41, 42] Myoglobin was shown to modulate the phenotype of the macrophages.[8] Additionally, myoglobin-activated tubular cells in culture express Ccl2 and Ccl7, potent macrophages-recruiting factors.[6] Indeed, significant upregulation of these and other chemokines and cytokines were detected, which could explain the enhanced infiltration of macrophages and neutrophils. Markers of both M1 and M2 macrophages were detected as described.[6]

We identified a RIAKI-associated sub-population of macrophages, negative for HLA-II but expressing strongly C5aR1 and CD11b. This population will be highly susceptible to complement overactivation, being responsive to contact with C3b/iC3b as well as with C5a. Macrophage CD11b/CD18 engagement by direct contact with heme-activated platelets triggered macrophage extracellular trap formation and kidney injury.[7] C3b/iC3b is a ligand of CD11b/CD18 and since we detected CD11b+ cells in close contact with C3b/iC3b deposits on affected proximal tubules in situ, we hypothesize that macrophage activation and kidney damage could be mediated due to this direct contact. Moreover, the overexpression of C5aR1 on tubular cells, together with its increase on macrophages could contribute to the AKI, as described in renal I/R injury[43, 44] and hemolysis.[12] Evaluation of C5a in urine of human and murine RIAKI could be a potential future biomarker, as urine C5a has been associated with AKI.[45] Interestingly, we found no tubular C5b-9 deposits in mice or in humans, contrary to previous report in rat RIAKI. [9] This might reflect an efficient regulation of C5b-9 at this site, likely due to CD59 overexpression [9]. A low

sensitivity of the C5b-9 staining cannot be excluded, despite the clear detection of C5b-9 in blood vessels.

To provide direct proof that complement is a mediator of injury in RIAKI, we induced RIAKI in C3-/- mice. C3-/- mice have already been described as protected in a model of mechanical ischemia/reperfusion.[15] Remarkably, C3-/- mice were also protected from RIAKI, and mostly lost the RIAKI transcriptomic signature, demonstrating the pathogenic role of C3 and/or downstream complement activation in this model. In particular, the cellular response to cytokines and apoptosis pathways appeared to be C3-dependent. Renal tubular injury and dysfunction were markedly attenuated in glycerol-injected mice, deficient in interleukin (IL)-1β or NLRP3 inflammasome.[22] Indeed, the cellular response to the IL1 pathway genes were attenuated in C3-/- mice. Complement overactivation and C5b-9 induce macrophages to release IL-1β,[46, 47] providing a potential mechanistic link between complement and the IL-1/cytokine response pathway. C3-/- mice were also protected from fibrosis and peritubular rarefaction in a model of unilateral ureteral obstruction and authors proposed that macrophages polarization could be involved with C3a induced M1 macrophages responsible in vitro for angiogenesis suppression.[48] Therefore, complement C3 and/or downstream components are directly pathogenic for tubular cells and act in cooperation with macrophages to promote renal injury. However, specific cell types where inflammatory and apoptotic pathways are activated cannot be defined by the bulk RNA sequencing approach. Finally, C5b-9 deposits were detected in vessels of patients, suggesting that endothelial activation could contribute to the vascular aggression in RIAKI. Consistent with this hypothesis, the vascular aggression observed in the mouse model was attenuated in the C3-/- injected with glycerol.

These results suggest a benefit for complement inhibition in RIAKI. C3b is the central protein of the complement cascade, allowing its own amplification through C3 convertase formation. C3-/- mice protection and loss of RIAKI transcriptomic signature suggest the therapeutic potential of C3 inhibition. Indeed, if C5 inhibition is the gold standard for complement inhibition, there is growing interest for C3 targeted therapies in diseases such as Paroxysmal Nocturnal Hemoglobinuria and many other conditions.[49]

In conclusion, our study demonstrates that complement activation, promoted by renal ischemia and heme overload, plays a key role in RIAKI in both mice and humans, independent from the initial cause of muscle damage. Therefore, drugs targeting complement activation and/or heme overload would represent promising therapeutics in critically ill patients.

Materials and Methods

The complete Materials and Methods are given in Supplement.

Collection of human kidney biopsies, urines and sera.

Patients. Kidney biopsies and clinical data from 10 patients with RIAKI were retrieved. All biopsies were performed to eliminate an alternative cause of AKI, or as part of the autopsy in one patient who died from rhabdomyolysis (RIAKI 6). All patients gave informed consent for the use of part of the biopsy for scientific purposes. Urine was collected from 3 and plasma from 11 RIAKI patients and leftover samples from the routine diagnostics were used for this study. All procedures were performed according to national ethical guidelines and were in accordance with the Declaration of Helsinki. Staining was performed for C3c, C3d, Lamp1, C4d, C1q, C5b-9, Collectin-11,[50] Factor H and CD31 on the kidney material (**Table S2**); C3 and sC5b-9 were measured in plasma and western blot for C3 was performed in plasma and urine.

Animal experimentation

C57BL/6 WT, C3-/- and C4-/- and hepatoFH-/- mice[19] at the same genetic background were used for this study. Experimental protocols were approved by the Charles Darwin ethical committee (Paris, France) and by the French Ministry of Agriculture (Paris, France), APAFIS#2148 2019091015099240v1. Rhabdomyolysis was induced by intramuscular injection of 7.5ml/kg 50% glycerol.

Transcriptomic analysis.

mRNA was extracted and retrotranscribed in cDNA. Gene markers of kidney injury (**Table S3**) were analyzed by RTqPCR and RNAseq.

Immunofluorescence (IF) and immunochemistry (IHC) in mice

Frozen kidney sections were used for IF and IHC are summarized in **Table S1**.

Complement activation on proximal tubular cells in vitro.

RPTEC were exposed to skeletal muscle myoglobin or oxidized species of heme (heme-ox), followed by human serum. Staining for C3 fragments was analyzed by flow cytometry.

Evaluation of the myeloid infiltrate in mouse kidneys

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Eight or 24h after injection of PBS or glycerol, mice were sacrificed, and the kidneys were dilacerated, and cell suspension was stained as described in **Table S3**.

Author contributions: Designed the research: LR, IB, MF. Performed the research: IB, VP, AG, CT, JL, MVD, FL. Provided patient samples and take care for the patients: KEK, AY, SC, PG, MLQ, RB, MLQ, MR, PL, CR, VG. Analyzed the data: LR, IB, VP, AG, MVD, SS, CF, VFB, MF. Provided vital reagents: PG. Wrote the manuscript: IB, VP, AG, MF, LR.

Conflict of interest: Dr. Roumenina reports grants from CSL Behring, outside the submitted work. The other authors have declared that no conflict of interest exists.

Supplementary information is available on Kidney International's website:

Complete Materials and Methods; Supplementary references; Supplementary Tables
Supplementary Figures

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Figure Captions

Figure 1. C3d staining is positive in tubular cells of rhabdomyolysis (RIAKI) kidneys, independent of its cause. A) Masson Trichrome (left) and periodic acid Schiff stain (right) of a kidney section showing casts in the tubular lumen (*) and acute tubular necrosis with loss of the proximal tubule brush border (arrows). **B)** C3c staining in kidney sections from patients with acute tubular necrosis (ATN) caused by hypovolemia (left), rhabdomyolysis (RIAKI, middle) or from a renal allograft (48 hours post-transplant; ischemia-reperfusion, right) **C)** C3d staining kidney sections of patients with ATN caused by hypovolemia (left), Cisplatin toxicity (middle) and kidney allograft at 48 hours post-transplant (ischemia-reperfusion, right). RIAKI caused by autoimmune myositis: (RIAKI 1), crush syndrome (RIAKI 2), strenuous exercise (RIAKI 3), unknown cause (RIAKI 4), suspicion of Mac Ardle syndrome (RIAKI 5), LIPN1 deficiency (RIAKI 6), muscle compression (RIAKI 7-8), Dengue fever (RIAKI 9) and sepsis (RIAKI 10). *Original magnification x40.*

Figure 2. C3b/iC3b tubular deposits correlate with RIAKI severity. A) Hematoxylin-Eosin staining of paraffin embedded kidneys from PBS mice (left) and GLY mice (right), *original magnification x40.* **B)** CD31 (red) and C3b/iC3b (green) immuno-fluorescence on frozen kidneys from PBS mice(left), glycerol-treated mice (middle) and Cisplatin- injected mice (CIS, right), *original magnification x20.* **C)** Quantification of C3b/iC3b tubular deposits using Halo© Software (left, ****p< 0,0001, Mann-Whitney test) (left). **D)** Correlation between C3b/iC3b tubular deposits and blood urea nitrogen (BUN) ($r^2 = 0,64$; p<0,0001) (left) and creatinine (r^2=0,69; p<0,0001) (right). *BUN: blood urea nitrogen.*

Figure 3. Complement system activation in human and murine RIAKI: implication of classical and lectin pathway. A) Flow cytometry analysis of C3 deposits (left) and C4 deposits (right) on renal proximal tubular epithelial cells (RPTEC) incubated with increasing doses of myoglobin prior to addition of normal healthy serum. **B)** C4 staining on paraffin-embedded kidney in RIAKI (left) compared to lupus nephropathy (right) patients. **C)** Kidney injury measured by blood urea nitrogen (BUN) in WT mice injected with PBS (blue) or glycerol (GLY) (red) and C4-/- (purple) mice injected with glycerol (C4-/- GLY). **D)** Vascular aggression measured by mRNA expression of P-Selectin and VCAM-1 in WT-PBS (blue), WT-GLY (red) and C4-/- GLY (purple) mice. **E)** Tubular aggression measured by Lcn2 (NGAL) and Havcr1 (KIM-1) mRNA expression in WT-PBS (blue), WT-GLY (red) and C4-/- GLY (purple) mice. **F)** CD31 (red) and C3b/iC3b (green) immunofluorescence on frozen kidneys from C4-/- mice injected with PBS (C4-/- PBS) (left) and C4-/- mice injected with glycerol (C4-/- GLY) (right), *original magnification x20.* **G)** Representative immunofluorescence of renal cortex showing C3b/iC3b (green), CL-11 (purple), a merged image of C3b/iC3b and CL-11 in WT-PBS (up) and WT-GLY (down) mice. Kruskall Wallis test with Dunn's correction: *p<0,05; **p<0,01; ***p<0,001; ****p<0.0001. *BUN: blood urea nitrogen*

Figure 4. Hemopexin improves kidney function through vascular protection and decreases complement activation in murine RIAKI. A) Kidney mRNA expression of Heme Oxygenase-1 (HO-1) in GLY and PBS mice. **B)** FACS analysis of C3d deposits on RPTEC with increasing concentration of Heme (Fe3+). **C)** Kidney injury measured by BUN in GLY mice (red) and WT mice injected with Hemopexin (IV) prior to glycerol (black). **D)**

Tubular aggression measured by Lcn2 (NGAL) and Havcr (KIM-1) mRNA expression in GLY mice (red) and WT mice injected with Hemopexin (IV) prior to glycerol (black). **E)** Vascular aggression measured by mRNA expression in kidneys of P-Selectin, E-Selectin, VCAM-1 and ICAM-1 from GLY mice (red) and WT mice injected with Hemopexin (IV) prior to glycerol injection (Hx/GLY) (black). **F)** Immunofluorescence on frozen kidneys: C3b/iC3b (green) and CD31 (red) in PBS mice (left) GLY mice (middle) and Hemopexin-injected GLY mice (Hx/GLY) (right). **G)** Quantification of tubular C3 deposits by HALO Software from GLY (red) and WT mice injected with Hemopexin (IV) prior to glycerol (black). (right). *Dotted line represents the mean value of each marker for PBS mice. BUN: Blood urea nitrogen. RPTEC: renal proximal tubular epithelial cells.* Mann-Whitney test*: *p<0,05; **p<0,01; ***p<0,001; ****p<0.0001*

Figure 5. C5a Receptor 1 is upregulated on tubular cells and on macrophages in kidneys after glycerol injection. A) Kidney mRNA expression for C3aR1 and C5aR1 in PBS (blue) and GLY-treated mice (red). **B)** C5aR1 (red) immunofluorescence on frozen kidneys from PBS (left) and GLY (right) mice, white arrows are indicating interstitial staining, *original magnification x20.* **C)** Cell count for macrophages and neutrophils 8 hours (left) and 24 hours (right) after PBS (blue) or glycerol (GLY) (red) injection. **D)** Flow cytometry on whole kidneys 24 hours after PBS (up) and glycerol (down) injection. Gating on macrophages CD45+ Ly6G- CD68+. Analysis of HLA class II molecule (Iab), C5aR1 and Cd11b reveals the emergence of macrophages CD68$^+$Iab$^-$C5aR1^{hi}Cd11b^{hi} **E)** C3b/iC3b (green) and Cd11b (red) immunofluorescence on frozen kidneys in GLY mice, *original magnification x20.*

Figure 6. C3-/- mice are protected from RIAKI. A) Kidney injury (renal function) measured by BUN in WT-PBS mice (PBS) (blue), WT-GLY mice (GLY) (red) and C3-/- GLY mice (left) (green). **B)** Correlation between BUN and Creatine Kinase in WT-GLY mice (r^2=0,56, p=0,0329) and C3-/- GLY mice (non-significant) (right). **C)** Volcano plot of WT-GLY versus WT-PBS mice. Genes with a fold change >2 and an adjusted p-value of less than 0,05 (red) are differentially expressed between the two groups. **D)** Heatmap of normalized counts (RSEM) in each mouse for the top 50 most differentially expressed genes (25 upregulated and 25 downregulated), defining the RIAKI signature. A row normalization is applied. (upper panel). Gene ontology analysis of biological pathways modulated in WT-GLY versus WT-PBS mice (lower panel). **E)** Heat map of normalized counts (RSEM) in each mouse (n=4 mice/group), for the top 50 most differentially expressed genes (25 upregulated and 25 downregulated) in the WT-PBS, WT-GLY and C3-/- GLY mice. A row normalization is applied. (upper panel). on the right column, in red are the genes of the RIAKI signature, which are completely normalized in the C3-/- (p value for the comparison of WT-GLY vs C3-/- GLY < 0.05 + a p value for C3-/- PBS vs WT-GLY > 0.05). In yellow are the genes which are partially normalized in the C3-/- mice (p value for the comparison of WT-GLY vs C3-/- GLY < 0.05 + a p value for C3-/- PBS vs WT-GLY < 0.05). In green are the genes which are not affected by C3 deficiency (p value for the comparison of WT-GLY vs C3-/- GLY > 0.05 + a p value for C3-/- PBS vs WT-GLY > 0.05). Gene ontology analysis of biological pathways modulated in WT-GLY versus C3-/- GLY mice. (lower panel). *BUN: blood urea nitrogen*

"	Age	Etiology	Other medical conditions	Treatments	Genetic exploration	Maximum CK level during hospitalization (U/L)	Hemo-dialysis at diagnostic	Creatinine at the biopsy (µmole/L)	Delay between diagnosis-biopsy	Basal blood creatinine (µmole/L)	Optic microscopy	Immuno-fluorescence	C3c deposits
1	69	Auto-immune myositis	No	*na*	No	461400	No	412	<1 week	88	ATN/ATIN	No deposits	No
2	38	Car accident (crush syndrome)	No	No	No	2500	Yes	800	<1 week	*na*	*na*	*na*	*na*
3	21	Strenuous Exercise	No	No	yes, negative	10100	No	132	<1 week	96	two cylinders	No deposits	No
4	13	Unknown	Atopic dermatitis	*na*	yes, negative	20 000	No	373	> 1 month	40.4	Tip lesion	Focal IgM, C1q and C3	Yes
5	41	Suspicion of Mac Ardle disease	Appendicectomy, proteinuria	No	yes, ongoing	10 000	Yes	1615 (on dialysis)	<1 week	70	ATN, eosinophilic cylinder, mesangial thickening, no fibrosis	IgA and C3 +++, IgM+, kappa+, lambda+ in mesangium	Yes
6	5	Lpin 1 deficiency	No	No	yes, heterozygous LPIN 1 mutation c1011C-G et c.1230C-T	20 000	No	anuria (previous episode with 373 µmol/L requiring dialysis)	<1 week	*na*	ATN	*na*	*na*
7	62	Muscle compression	D2 hypertension	No	No	1200	No	238	< 1 month	70.4	ATN+chronic injuries	No deposits	No
8	88	Muscle compression	*na*	No	No	1200	No	150	< 1 month	*na*	ATIN	No deposits	No
9	63	Sepsis	No	No	No	100 000	Yes	363	< 1 month	*na*	ATIN/ATN with cylinder	No deposits	No
10	14	Dengue fever	No	no	No	>100 000	Yes	674 (on dialysis)	<1 week	na	ATN and cylinder	No deposits	No

Table 1. Clinical and biological characteristics (ATN: acute tubular necrosis, ATIN: acute tubulo-interstitial nephritis, D2: Type 2 Diabetes Mellitus, na: not available)

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A) RIAKI 1 · RIAKI 1

B) RIAKI 1 · I/R — **C3c** staining

C) ATN · Cisplatine · I/R — **C3d** staining

RIAKI 1 · RIAKI 2 · RIAKI 3 · RIAKI 4

RIAKI 5 · RIAKI 6 · RIAKI 7 · RIAKI 8

RIAKI 9 · RIAKI 10

A)

B)

C)

C3b/iC3b deposits

D)

A)

Cell count

C3 deposits

Cell count

C4 deposits

[Myoglobin]
- isotype
- 0 μg/mL
- 500 μg/mL
- 1000 μg/mL

B)

RIAKI   Lupus

C) BUN

mmol/L

**
*

PBS   GLY   C4-/- GLY

D) P-Selectin

Fold change 2^{-DCt}

***

PBS   GLY   C4-/- GLY

VCAM-1

Fold change 2^{-DCt}

***

PBS   GLY   C4-/- GLY

E) Lcn2

Fold change 2^{-DCt}

*
**

PBS   GLY   C4-/- GLY

Havcr1

Fold change 2^{-DCt}

*
***

PBS   GLY   C4-/- GLY

F)

C4-/- PBS   C4-/- GLY

G)

C3b/iC3b   CL-11   Merge

PBS   PBS   PBS

100 μm   100 μm   100 μm

GLY   GLY   GLY

100 μm   100 μm   100 μm

A)

C3aR1 — Fold change $2^{-\Delta Ct}$ — PBS, GLY

C5aR1 — Fold change $2^{-\Delta Ct}$ — PBS, GLY — ****

B) GLY — 100 µm

C)

8h — Macrophages — CD68+ cells among CD45+ Ly6G- — PBS, GLY — *

8h — Neutrophils — Ly6G + cells among CD45+ — PBS, GLY — **

24h — Macrophages — CD68+ cells among CD45+ Ly6G- — PBS, GLY — NS

24h — Neutrophils — Ly6G + cells among CD45+ — PBS, GLY — NS

D)

Gated on CD45+Ly6G-

PBS — FSC-A / CD68 — 71% — IA-IE / C5aR1 — 65% — 3 % — CD11b / C5aR1 — 94 % — 0%

Gated on CD45+Ly6G-

GLY — FSC-A / CD68 — 74 % — IA-IE / C5aR1 — 65% — 11% — CD11b / C5aR1 — 54% — 40 %

E) GLY — 10 µm