Inflammation in ischemia and reperfusion: From mice to men
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IMMUNOGLOBULIN M, C-REACTIVE PROTEIN AND COMPLEMENT ACTIVATION IN RAT HEPATIC ISCHEMIA-REPERFUSION INJURY

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INTRODUCTION

Ischemia reperfusion (I/R) leads to tissue injury when tissue is subjected to temporary ischemia with subsequent restoration of blood supply. Paradoxically, this reflow triggers a complex inflammatory response which aggravates tissue damage. Hepatic I/R injury is responsible for a substantial part of damage in clinical settings such as in surgical resection with portal triad clamping followed by reperfusion, performed as an elective preplanned procedure or as an emergent maneuver to control excessive bleeding. Complement activation has been demonstrated to play an important role in liver I/R injury, as well as in the pathogenesis of a variety of liver disorders including liver fibrosis, steatosis, alcoholic liver disease and liver transplantation. The inflammatory response in liver I/R is caused by the activation of Kupffer cells, which leads to formation of reactive oxygen species as well as production of proinflammatory cytokines such as tumor necrosis factor and IL-6 as well as chemokines, which are mainly responsible for the induction of neutrophil sequestration in the liver.

The complement system is an important part of the innate defense against infection and is comprised of approximately 30 soluble proteins and membrane-bound receptors that are activated following invasion of foreign pathogens. The effector molecules that are generated have diverse biological activities, namely, defense against bacterial infection through opsonization; activation of leukocytes; removal of immune complexes and apoptotic cells; and the augmentation of B cell and T cell-mediated immunity. Three distinct pathways are known to activate the complement cascade, namely the classical, alternative, and the mannose-binding lectin pathways. The classical pathway is initiated by binding of antibody-antigen complexes to the subcomponent C1q, subsequently forming the C1 complex with the classical pathway-specific serine proteases C1r and C1s. The alternative pathway is activated by altered self molecules or distinct carbohydrate or lipid motifs on pathogens, leading to recruitment of C3 and factor B. Mannan-binding lectin (MBL) is initiated by one of five different lectin pathway-specific carbohydrate recognition molecules in man that form complexes with serine proteases. Once activated in I/R injury, complement leads to liver damage either by directly lysing liver cells through the formation of a membrane attack complex in the plasma membrane or by recruiting neutrophils and activating both neutrophils and Kupffer cells. All 3 pathways have been described to contribute to complement activation in liver I/R injury.

Besides in liver I/R injury, complement activation has been demonstrated to play an important role in a number of other organs, such as the lung, intestine, heart, and kidney. Key roles for the acute phase protein C-reactive protein (CRP) and immunoglobulin M (IgM) in the initiation of complement-mediated I/R injury has been established in various experimental I/R models. In intestinal and hind-limb models IgM has been shown to specifically initiate I/R injury via activation of the classical pathway of complement upon binding to ligands exposed in damaged tissue. These findings have been confirmed by studies with a monoclonal IgM antibody. Other studies have emphasized the interaction of IgM with...
we evaluated the time course of depositions and the relation to hepatocellular injury and the relative importance of IgM and CRP binding to ischemic tissues and complement deposition. Hepatic I/R there is no effective therapy at present. In order to gain more insight in the pathogenesis of hepatic I/R injury is limited. Consumption of complement by administration of cobra venom factor has been shown to reduce tissue damage in a rat model of liver I/R. Comparable results have been found in studies with complement inhibitors such as C5aR antagonist, sCR1 and C1 esterase inhibitor. We have previously demonstrated CRP to colocalize with both activated C3 and C5b9 in hepatocytes in ischaemically injured areas. Hence CRP is suggested to directly participate in local inflammatory processes, possibly via complement activation. In the setting of hepatic I/R, a possible role of CRP-mediated complement activation via the classical pathway has been suggested in immunohistochemical analysis of human livers after partial hepatectomy. After human liver transplantation, membrane attack complex (MAC) depositions - final products of complement activation - have been shown to be elevated in postoperative graft specimens and to correlate with leukocytes and platelet accumulation in these specimens, as well as with increased postoperative serum levels of aspartate aminotransferase.

However, the number of studies exploring the role of CRP, IgM and the complement system in the pathogenesis of hepatic I/R injury is limited. Partly due to incomplete knowledge of the underlying pathophysiological mechanisms of hepatic I/R there is no effective therapy at present. In order to gain more insight in the relative importance of IgM and CRP binding to ischemic tissues and complement deposition we evaluated the time course of depositions and the relation to hepatocellular injury and inflammation in a hepatic I/R rat model.

MATERIALS AND METHODS

Animals and experimental groups
Male Wistar rats (250–300 g, n = 60; Harlan, Horst, the Netherlands) were acclimated for one week and housed under standardized laboratory conditions in a temperature-controlled room (22–24 °C), with an alternating 12 h-light/dark cycle. The rats had standard chow and water ad libitum throughout this period. Rats were fasted before the operation. This study was approved by Animal Experiments and Welfare Committee of the Academic Medical Center, University of Amsterdam. Sixty rats were randomly allocated to one of the eleven experimental groups: 60 minutes of partial (70%) liver ischemia followed by 0, 3, 6, 12 or 24 hours of reperfusion (each n=6). Sham-operated control groups with corresponding times of reperfusion were included, as well as a control group sacrificed before the time of ischemia (each n=5).

Operative procedure
All rats were anesthetized by inhalation with a mixture of air: O2 (1.5:0.5 V/V, 2 l/min) and isoflurane 2.0-2.5% (Florence, Abbott laboratories, Queensborough, UK). After endotracheal intubation (14G Venflon®, Becton Dickinson, Franklin Lakes, NJ), rats were ventilated (Zoovent ventilator, Instruvet, Amerrogen, the Netherlands); anaesthesia was maintained using the same mixture. Adequate ventilation was confirmed by continuous measurement of end-tidal CO2, assuring physiological pH during the entire procedure. Rectal temperature was maintained at 37.0 °C (±0.1 °C). A midline laparotomy was performed and partial (70%) liver ischemia was induced by clamping the vessels to the median and left lateral lobes with a microvascular clamp for 60 min, after which reperfusion was restored by clamp release. Sham groups underwent similar manipulations of the liver hilus except for the clamping, and were kept under anaesthesia for an equal time period. The abdomen was closed in two layers using a 4/0 Vicryl suture (Ethicon®) and the animals were allowed to wake up. Adequate post-operative analgesia was achieved by administering buprenorphine (0.16 ml/kg subcutaneous, Temgesic®, Schering-Plough, Utrecht, The Netherlands). After the indicated reperfusion time, rats were sacrificed under anesthesia. Blood samples were taken from the inferior caval vein in heparin or EDTA containing tubes (Becton Dickinson), centrifuged (1200 x g, 10 minutes, 4 °C) and subsequently plasma was stored at -80 °C. Livers were removed immediately after blood was drawn, frozen in liquid nitrogen and stored at -80 °C or fixed in 4% (w/v) formaldehyde for further analysis.

Hepatocellular injury

Histopathology
Multiple 4-μm sections of the median ischemic liver lobes of rats in ischemic and sham groups were stained with hematoxylin and eosin (H&E). Non-ischemic liver lobes were used as a control. For each rat, a liver section was scored blindly by two independent observers (GD and WG). Tissue damage and inflammation were assessed by scoring necrosis, hepatocellular vacuolization and inflammation. Necrosis and hepatocellular vacuolization were expressed as a percentage of cells per microscopic field (20x enlargements). A semi-quantitative score of 0 to 4 was used as follows: 0 = 0%, 1 = 1–9%, 2 = 10–19%, 3 = 20–29% 4 = 30–100%. Inflammation was graded (40x enlargement) as follows: 0 = no inflammatory cells, 1 = occasional presence of inflammatory cells, 2 = moderate; inflammatory cells in some areas, 3 = inflammatory cells in almost all areas, 4 = substantial number of inflammatory cells diffusely throughout the entire section. The total injury score for each rat was expressed as the sum of scores for necrosis, hepatocellular vacuolization and inflammation (maximum score = 12).

Plasma ALT, AST
Alanine aminotransferase (ALT), aspartate aminotransferase (AST) were assayed in plasma by routine clinical chemistry (General Clinical Chemistry Laboratory, AMC, The Netherlands) at all time points.

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**Inflammatory response**

**Neutrophil infiltration (MPO)**

Myeloperoxidase (MPO) activity, an index of neutrophil infiltration, was measured in liver homogenates of median lobes of ischemic and sham groups according to a method modified from Krawisz et al. with some modifications. Values were normalized to the total protein concentration and MPO activity expressed as U/mg protein.

**Plasma IL-6**

Plasma IL-6 levels were determined in ischemic and sham groups with a hybridoma growth factor assay at all time points as described. IL-6 values were expressed as units per ml (U/ml), 1 unit being approximately 1 pg/mL.

**Complement activation**

**Immunohistochemical deposition of CRP, IgM and C3c**

Four µm thick frozen sections of median ischemic lobes of ischemic and sham groups were processed and prepared for immunohistochemistry. Non-ischemic lobes were used as controls. The primary antibodies (Abs) used were rabbit anti-rat Ab against CRP, goat anti-rat IgM (µ-chain specific, Bethyl Laboratories, Montgomery, TX) and polyclonal goat anti-rat C3c (Nordic Immunology, Eindhoven, The Netherlands). Frozen sections were mounted onto glass slides, dried for one hour by exposure to air, and fixed in acetone (VWR international, Leuven, Belgium). After rinsing in Phosphate Buffered Saline (PBS), the slides were incubated at room temperature for 10 minutes with normal swine serum (for CRP) or normal rabbit serum (for C3 and IgM) (both DAKO, Copenhagen, Denmark), diluted 1/10 (normal swine serum) or 1/50 (normal rabbit serum) in PBS containing 1% (w/v) bovine serum albumin (BSA) (PBS-BSA). Incubation of the slides with specific Ab solutions (diluted in PBS-BSA) was performed for 1 hour (goat anti-rat IgM antibody diluted 1/50; rabbit anti-rat CRP diluted 1/100 and goat anti-rat C3c diluted 1/1500). The slides were washed three times in PBS and subsequently incubated with horseradish peroxidase conjugated immunoglobulins (DAKO) in PBS-BSA (rabbit anti-goat-HRP 1:100 for IgM, swine anti-rabbit-HRP 1:50 for CRP and rabbit anti-mouse-HRP 1:25 for C3c). The specificity of the staining procedure was confirmed by replacing each primary Ab with PBS. Thereafter, the slides were washed again in PBS and incubated for five minutes using EnVision DAB (DAKO) diluted 1:50. Subsequently slides were washed extensively in tap water, counterstained with haematoxylin for thirty seconds, dehydrated, cleared, and finally mounted. Two investigators (GD and HN) assessed the percentage of positive surface area for immunohistochemical staining of CRP, IgM and C3c in one section per experimental group. Tissue sections of insufficient quality considered inappropriate for scoring were not included in the analysis. The percentage of positive surface area was determined by subdividing the total area of the slide into equal parts and then estimating the percentage of positive area in each visual field by two independent observers (GD and HN). Finally, an average percentage of positive surface area was calculated from these subdivisions. For the final scoring results, consensus was achieved by the two investigators.

**Plasma rat IgM and CRP**

Plasma concentrations of CRP and IgM were determined during 24 hours of reperfusion. Rat CRP was assessed with an ELISA as described previously. In brief, purified rabbit Abs against rat CRP (1 µg/ml) were used as catching Abs. The same biotinylated Abs according to established procedures were used as a detecting Ab. Samples were serially diluted in PTG. Plates were developed with strept-PO. Purified rat CRP was used as a standard. Results were expressed as a percentage of rat CRP in a control pool. Abs and purified rat CRP were a kind gift from Dr. N Diaz Padilla (Sanquin). Levels of rat IgM were measured in plasma as described previously. Briefly, goat anti-rat IgM (Bethyl Laboratories, Montgomery, TX) was used as catching Abs. Goat anti-rat IgM-HRP (µ-chain specific) (SouthernBiotech, Birmingham, USA) was used as a detecting Ab. As a standard a pool of normal rat plasma with known rat IgM content was used and results were expressed as a percentage of this control pool. All assays were performed in duplicate and results were averaged.

**Data analysis**

Statistical Package for the Social Sciences (SPSS 12.0.1, SPSS Inc, Chicago, IL) for Windows was used for data analysis. Data are expressed as mean ±SEM. The sample size of study groups was considered appropriate based on earlier studies in which similar differences in immunohistochemical staining of C3 between groups were demonstrated. To evaluate whether observed differences at specific time points were significant between sham and ischemia groups, Mann-Whitney tests were performed. The correlation of immunohistological staining percentages of CRP, IgM and C3c were determined using Spearman non-parametric correlation coefficients (Rs). To evaluate the correlation of the time course of CRP, IgM and C3 depositions throughout reperfusion a non-parametric ANOVA (Kruskall-Wallis) with rank transformed measurements was performed. Significance was set at a value of P < 0.05.

**RESULTS**

**Hepatocellular injury**

**Histopathology**

Injury to the liver upon I/R was assessed by histopathological scoring of necrosis, hepatocellular vacuolization and inflammation. Results were expressed as a composite total score for damage as explained in the methods section. The course of this total score demonstrated an increase after reperfusion in the ischemia group with a peak at 12 hours of reperfusion (P<0.05 vs. 3 hours) (Figure 1). Injury scores were significantly higher in the ischemia group than in the sham-operated group at all time points except just after ischemia, which is at 0 hours of reperfusion immediately after releasing the clamp (P<0.05) (Mann-Whitney). The injury scores were significantly higher after 12 hours of reperfusion when compared to 6 hours of reperfusion. Histological examination revealed no pathologic changes in the liver of the non-ischemic lobes 24 hours after reperfusion.
Plasma ALT, AST
Hepatocellular injury following hepatic I/R was assessed by measuring plasma levels of AST and ALT. Plasma ALT and AST activities in rats sacrificed before ischemia were within normal range (< 35 U/L and < 105 U/L, respectively, data not shown). Upon one hour of ischemia, levels remained within normal range. However, reperfusion resulted in an increase in AST and ALT values in the ischemic groups compared to the sham groups, with highest values after 6 hours of reperfusion (5300 ± 1000 U/L and 3100 ± 800 U/L, respectively) (P<0.05 versus sham at all time points). Scores were significantly higher in the ischemic groups when compared to the corresponding sham groups at all time points (P<0.05) (Mann-Whitney).

Inflammatory response
Neutrophil infiltration (MPO)
Neutrophil infiltration in the liver following I/R injury was assessed by measuring MPO activity in homogenates of liver tissue samples, as demonstrated in Figure 2. Hepatic MPO activity, corrected for total protein content of the sample, significantly increased from 3 to 6 hours of reperfusion (P <0.05), showing highest levels after 12 hours of reperfusion (1.1 ± 0.2 U/mg protein; P<0.05 compared to 6 hours). MPO activity in the ischemic liver was significantly higher after 0, 6 and 12 hours of reperfusion when compared to sham-operated animals (P<0.05). MPO activity in non-ischemic liver tissue homogenates was < 0.1 U/mg protein (data not shown).

Plasma IL-6
Plasma levels of IL-6 were determined at all time points and normalized to levels in pooled plasma of control animals, as shown in Figure 3. Reperfusion resulted in an increase in IL-6 levels in the ischemic groups compared to the sham groups, demonstrating highest levels after 6 hours of reperfusion. At 6 hours of reperfusion IL-6 levels demonstrated a significant decrease compared to levels at 12 hours and a further significant decrease compared to levels at 24 hours (both P<0.05) (Mann-Whitney). At 0, 12 and 24 hours of reperfusion IL-6 values were significantly higher in the ischemic groups when compared to the corresponding sham groups (P<0.05) (Mann-Whitney).
Complement activation

**Immunohistochemical deposition of CRP, IgM and C3c**

CRP and IgM as triggers of complement activation as well as complement activation product C3c depositions in the liver were determined using immunohistological staining as described. Intense brown positivity of hepatocytes was observed in hepatocytes stained for CRP, IgM and C3c. Figure 4 shows immunohistochomal staining of CRP, IgM and C3c in representative frozen sections after 12 hours of reperfusion, in sham-operated animals and in non-ischemic liver tissue. Figure 5 demonstrates immunohistochemical staining of CRP, IgM and complement C3c expressed as percentage of depositions on a series of frozen sections of liver tissue after 60 minutes of ischemia and 0, 3, 6, 12 and 24 hours of reperfusion. Deposition of each parameter increased from 3 to 6 hours of reperfusion in the ischemia group, reaching highest levels levels after 12 hours to subsequently decline to levels at 24 hours of reperfusion. In sham groups CRP, IgM and C3 depositions demonstrated no increase at all. The contribution of CRP and IgM in complement-mediated hepatic IR was assessed by comparing the percentage of positive area surface of staining at each reperfusion time. Correlations were examined between the percentages of CRP, IgM and C3c depositions (Spearman rank-correlation test). A significant correlation was observed between the percentages of depositions of CRP and IgM (r(S)=0.569; P<0.001). No significant correlation between staining percentages of C3c and IgM and C3c and CRP was found. When analysing specifically the time course of depositions of C3c, CRP and IgM throughout reperfusion, C3c and CRP staining and C3c and IgM staining demonstrated a significantly similar time course (r(S) = 0.797 and r(S) =0.656 respectively) (P< 0.0001) (non-parametric ANOVA with rank transformed measurements). No significant correlation between the time course of CRP and IgM staining percentages was found.

**Plasma rat IgM and CRP**

To investigate the relation between local and systemic processes, plasma concentrations of CRP and IgM were determined during 24 hours of reperfusion. Plasma levels of rat CRP demonstrated a significant decrease from 3 to 6 hours of reperfusion (mean 91 ± 5 and 73 ± 3 of control pool) and from 12 to 24 hours of reperfusion (73 ± 2 and 58 ± 3 % of control pool) (P<0.05) (Mann-Whitney). Rat IgM concentrations in plasma did not significantly change throughout reperfusion time (data not shown).
DISCUSSION

In the present study we evaluated complement-mediated tissue damage in a hepatic I/R model in rats. The time course of IgM and CRP binding to ischemic tissues and complement deposition were studied as well as the relation between local and systemic processes to gain insight in the relative importance of these molecules in I/R-injury. Complement activation is an early event in I/R injury, and IgM and CRP are known complement activators via the classical route. The role of IgM and CRP as triggers for complement activation has been established in various experimental I/R models (intestinal, hindlimb, renal, cardiac)\textsuperscript{12-15}. In a rat hepatic I/R model, CRP has been demonstrated to colocalize with C3c and the membrane attack complex C5b9, suggesting classic pathway-mediated complement activation\textsuperscript{37}. However, literature about the relationship between CRP and IgM is scarce. IgM activates the classical route of complement by binding to C1q, whilst CRP is known to interact with complement proteins such as C1q, complement factor H, and C4b-binding protein. Whereas colocalisation of CRP and IgM has been demonstrated in intestinal and myocardial I/R models\textsuperscript{36,37}, it is unclear whether both molecules fulfill a competitive or synergistic role in complement activation. Whereas literature has demonstrated rat CRP and IgM to activate the endogenous complement system in rats, both CRP and IgM are not considered acute phase proteins in rats\textsuperscript{31,38}. To our knowledge, a role for the lectin or alternative pathway-mediated hepatic I/R injury has not been described in literature to date.

The liver comprises parenchymal cells (hepatocytes) and nonparenchymal cells, such as endothelial cells, stellate cells, Kupffer cells, macrophages, and lymphocytes\textsuperscript{39}. Hepatocytes are responsible for biosynthesis of about 80\% to 90\% of plasma complement components and their soluble regulators\textsuperscript{40}. Hepatocytes are known to have a low expression of complement-regulatory proteins and might therefore be at risk for complement-mediated injury. As both IgM and CRP are known complement activators, we attempted to assess the contribution CRP, IgM and C3c in complement-mediated hepatic I/R injury by comparing the relative intensities of staining at each reperfusion time with tissue injury and local inflammation. Hepatocellular injury assessment of ischemic tissue demonstrated an increase of histopathology scores after reperfusion in the ischemic group with a peak at 12 hours of reperfusion. Similarly, plasma AST and ALT values showed peak values after 6 hours of reperfusion, representing systemic hepatocellular damage as these enzymes leak from hepatocytes. The local inflammatory response in ischemic liver tissue as assessed by hepatic MPO activity significantly increased from 3 to 6 hours of reperfusion, peaking after 12 hours of reperfusion. In summary, the time sequence of IgM, CRP and activated complement deposits in ischemic liver tissue demonstrate a similar relationship in time as hepatocellular damage and inflammatory responses in this rat hepatic I/R model.

Brown granular cytoplasmic positivity of hepatocytes was observed in hepatocytes stained for CRP, IgM and C3c. In our study, liver sections of ischemia groups generally demonstrated a substantial amount of tissue damage. Staining areas demonstrated marked heterogeneity, which may be a result of variability in sensitivity to complement activation. Cytoplasmic deposits did not demonstrate a clear preference in zone localisation in liver tissue. Earlier studies have demonstrated more cytoplasmic C3 in pericentral and midzonal hepatocytes, where periportal areas have been claimed to be most resistant to ischemic injury\textsuperscript{37}. However, this C3 had not been identified as native or activated C3, whereas our study demonstrates deposition of the activation fragment C3c. Whereas earlier studies have demonstrated intense staining of the hepatocyte membranes in non-ischemic tissue suggested to result from the synthesis of native C3 by these cells\textsuperscript{42}, sham and non-ischemic tissue did not demonstrate such membrane staining in our study. We made no attempt to quantify the amount of staining in different tissue areas, considering the substantial variation in liver sections. Assessment of immunohistochemical staining in liver sections is known to demonstrate substantial variation. To ensure adequate scoring of CRP, IgM and C3c depositions, the percentage of positive surface area was quantified in median lobes in ischemic and sham groups as well as in non-ischemic lobes by two blinded investigators. Tissue from sham groups and non-ischemic lobes in our study demonstrated low background staining. Furthermore, no positive staining was detectable with the use of isotype controls, suggesting that the reactivity seen with anti CRP, IgM and C3c antibodies was not the result of nonspecific adsorption of serum proteins by damaged tissue.

To investigate whether CRP and IgM binding, local complement activation in the liver and activation of the cytokine IL-6 were reflected in the circulation as systemic processes, corresponding plasma concentrations were determined. One may hypothesize that tissue binding leads to depletion of activator molecules from plasma. Consequently, plasma levels of such molecules would be expected to be inversely related to hepatic staining. The time pattern of CRP staining indeed demonstrates such an inverse relationship with the time course of CRP plasma concentrations. Where CRP depositions increased from 3 to 12 hours of reperfusion, plasma levels of rat CRP significantly decreased from 3 to 6 hours of reperfusion and from 12 to 24 hours of reperfusion (P<0.05). Hence, decreasing levels of plasma CRP may signify consumption in damaged hepatic tissue. Alternatively however, decreasing levels of plasma CRP may result from a synthesis defect in damaged hepatic tissue. Hence, plasma CRP levels may represent hepatic function rather than local binding. Rat IgM concentrations in plasma did not significantly change throughout reperfusion time. Plasma IL-6 values reflecting the systemic inflammatory response, demonstrated peak levels after 6 hours of reperfusion, significantly decreasing thereafter to levels at 24 hours. The current study demonstrated that endogenous CRP, IgM and C3c localized in ischemic tissue in a rat model of acute hepatic I/R. Deposition of CRP, IgM and C3 in the ischemia groups increased from 3 to 6 hours of reperfusion, reaching peak levels after 12 hours. A significant correlation between staining of C3c and both CRP and IgM was observed (r(S) 0.656 and r(S) 0.796, respectively) (both P< 0.0001). Staining patterns of CRP and IgM were not significantly correlated. The similar staining pattern in time of IgM and CRP when compared to C3c suggests a role for both molecules in the activation of complement in the setting of hepatic I/R. The contribution of each protein to complement activation may be dependent on its relative concentration. As mentioned earlier, both CRP and IgM are not considered acute phase proteins in rats. Furthermore, both demonstrate comparable levels in rat plasma. CRP levels in rats are about 300-600 mg/L under basal conditions, increasing at
most 2-fold during acute-phase response. IgM is typically present in rat plasma at concentrations of approximately 400 mg/L.

In this study we have specifically examined the role of IgM and CRP-mediated complement activation in hepatic I/R injury. The time sequence of IgM, CRP and activated complement depositions in ischemic liver tissue demonstrate a similar relationship in time as hepatocellular damage and inflammatory response. Furthermore, CRP and IgM depositions in liver tissue show a parallel time course and correlated with the course of activated complement deposition. These data suggest that both IgM and CRP are mediators of hepatic I/R-induced complement activation in rats, in which CRP and IgM-mediated activation appear to occur simultaneously. Future co-localisation experiments may be needed to further determine the exact relationship between CRP and IgM in hepatic I/R injury.
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