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The Membrane Attack Complex (C5b-9) in Liver Cold Ischemia and Reperfusion Injury

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Abstract

Activation of the complement cascade represents an important event during ischemia/reperfusion injury (IRI). This work was designed to investigate the role of the membrane attack complex (MAC; C5b-9) in the pathogenesis of hepatic IRI. Livers from B&W/Stahl/rC6(+) and C6(−) rats were harvested, stored for 24 hours at 4°C, and then transplanted [orthotopic liver transplantation (OLT)] to syngeneic recipients. There were 4 experimental groups: (1) C6(+)→C6(+), (2) C6(+)→C6(−), (3) C6(−)→C6(+), and (4) C6(−)→C6(−). At day +1, C6(−) OLTs showed decreased vascular congestion/necrosis, contrasting with extensive necrosis in C6(+) livers, that was independent of the recipient C6 status (Suzuki score: 7.2 ± 0.9, 7.3 ± 1.3, 4.5 ± 0.6, and 4.8 ± 0.4 for groups 1-4, respectively, $P < 0.05$). The liver function improved in recipients of C6(−) grafts (serum glutamic oxaloacetic transaminase: 2573 ± 488, 1808 ± 302, 1170 ± 111, and 1188 ± 184 in groups 1-4, respectively, $P < 0.05$). Intragraft macrophage infiltration (ED-1 immunostaining) and neutrophil infiltration (myeloperoxidase activity) were reduced in C6(−) grafts versus C6(+) grafts ($P = 0.001$); these data were confirmed by esterase staining (naphthol). The expression of proinflammatory interferon-γ, interleukin-1β, and tumor necrosis factor messenger RNA/protein was also reduced in C6(−) OLTs in comparison with C6(+) OLTs. Western blot–assisted expression of proapoptotic caspase-3 was decreased in C6(−) OLTs versus C6(+) OLTs ($P = 0.006$), whereas antiapoptotic Bcl-2/Bag-1 was enhanced in C6(−) OLTs compared with C6(+) OLTs ($P = 0.001$). Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling staining of apoptotic cells was enhanced ($P < 0.05$) in C6(+) OLTs compared with C6(−) OLTs. Thus, the terminal products of the complement system are essential in the mechanism of hepatic IRI. This is the first report using a clinically relevant liver cold ischemia model to show that local MAC inhibition attenuates IRI cascade in OLT recipients.

Ischemia/reperfusion injury (IRI), the major component of organ procurement damage, affects liver function after transplantation and contributes to the shortage of organs available for transplantation.[1] Moreover, the expanded criteria livers, which are increasingly becoming a more frequent source of organs, are more susceptible to ischemic insult.[2] Indeed, IRI plays a central role in posttransplant complications, including primary nonfunction (PNF) and acute and chronic rejection.[3,4] Thus, prevention of IRI is important not only to reduce the incidence of PNF but also to diminish the likelihood of late sequels and suboptimal graft function. Our

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better appreciation of the mechanisms involved in IRI is essential for the design of much needed therapeutic strategies to improve the outcome of liver transplants.[5]

The activation of complement represents a critical event during hepatic IRI.[6] The complement cascade can be rapidly activated by the release of cellular proteins to activate polymorphonuclear cells (PMNs) and Kupffer cells, thereby leading to reactive oxygen species formation and promotion of the inflammation response.[7] The central step in complement activation, the cleavage of C3 into C3b and C3a, is achieved through the assembly of enzyme complexes initiated by the classic, alternative, and lectin pathways. The classic pathway is antibody-mediated, whereas the lectin and alternative pathways are triggered by distinct carbohydrate or lipid patterns on microbes or stressed cells.[8] The C3b deposition leads to C5 cleavage into C5a/C5b, the latter of which then serves as the anchor for the assembly of a single molecule each of C6, C7, and C8. The resulting C5b-6-7-8 complex guides the polymerization of C9 into a tube, which is inserted into the lipid bilayer of the plasma membrane. This tube allows the passage of ions and small molecules, effectively creating an osmotic gradient that ultimately induces the cellular lysis. The C5b-9 membrane attack complex (MAC) promotes pore formation in target cells, directly inducing cell injury and necrosis.[9] Deposition of MAC not only directly induces cellular injury but also may amplify the inflammatory response by promoting the expression of proinflammatory mediators.[10] Additionally, MAC can influence the recruitment of inflammatory cells and leukocyte adhesion to endothelium.[11,12] As a result, MAC promotes the release of cell stimulants, including hydrolytic enzymes, reactive oxygen species, and cytokines.[13,14]

As the role of MAC in liver IRI remains largely unknown, in this work we aimed to address whether the genetic deficiency of a terminal complement protein, C6, one of the initial proteins involved in the formation of MAC, could ameliorate the cold ischemia damage and prove beneficial in orthotopic liver transplantation (OLT) recipients. The advantage of intervening at this level of the terminal C pathway is that interference with putative immunomodulatory functions of the early C components should be avoided.

Materials and Methods

Animals

Female B&W/Stahl/rC6 control and C6-deficient rats weighing 200-250 g were obtained (Charles River Laboratories). Rats were housed in the University of California at Los Angeles animal facility under specific pathogen-free conditions. Animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (publication no. 86-23).

Syngeneic OLT

Rats underwent isoflurane anesthesia and systemic heparinization (2 mL intravenously). After skeletonization of the liver, the portal vein, bile duct, and inferior vena cava were cannulated, and the liver was flushed with 10 mL of University of Wisconsin solution. Livers were stored for 24 hours at 4°C before being transplanted to syngeneic rats with revascularization without hepatic artery reconstruction.[15] The role of systemic hepatic C6 production versus local hepatic C6 production was analyzed in 4 OLT groups: (1) C6(+)→C6(+) (C6+/+; n = 6), (2) C6(+)→C6(−) (C6+/−; n = 5), (3) C6(−)→C6(+) (C6−/+; n = 6), and (4) C6(−)→C6(−) (C6−/−; n = 6). Rat recipients were sacrificed at 24 hours post-OLT, and liver tissue was sampled for analyses.

Histology

Liver specimens were fixed in a 10% buffered formalin solution and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin-eosin. The severity of liver IRI was graded with modified Suzuki criteria.[16] In this classification, sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration are graded from 0 to 4. The absence of necrosis, congestion, or centrilobular ballooning is given a score of 0, whereas severe congestion/ballooning degeneration, as well as >60% lobular necrosis, is given a value of 4.

Immunohistology

Liver samples were embedded in Tissue-Tek OCT compound (Miles), snap-frozen, and stored at −80°C. Five-micrometer-thick sections were fixed in cold acetone, inhibited with 0.3% H_2O_2 in phosphate-buffered saline, and blocked with 5% bovine serum albumin. The primary antibody against ED-1 (macrophage/monocyte; Chemicon International) was added at optimal dilution. Bound primary antibody was detected with a peroxidase-conjugated goat anti-mouse secondary antibody (Dako Envision kit/horseradish peroxidase). Negative controls included sections in which primary antibody was replaced with a dilution buffer. The sections were developed with 3,3′-diaminobenzidine tetrahydrochloride. A blinded evaluation of the samples was performed, and 3 separate fields were examined at 100× magnification, the number of labeled cells per field being quantified.

Liver Neutrophil Infiltration

We tested OLTs for the presence of myeloperoxidase (MPO), an enzyme specific for neutrophils.[17] The frozen tissue was thawed and placed in 4 mL of iced 0.5% hexadecyltrimethylammonium bromide and 50 mmol of a potassium phosphate buffer solution with the pH adjusted to 5. Each sample was homogenized for 30 s and centrifuged at 15,000 rpm for 15 minutes at 4°C. Supernatants were mixed with hydrogen peroxide–sodium acetate and tetramethylbenzidine solutions. The change in absorbance was measured spectrophotometrically at 655 nm. One unit of MPO activity was defined as the quantity of enzyme degrading 1 mol of peroxide/minute at 25°C/g of tissue.

In parallel, the accumulation of activated neutrophils in the livers was assessed by staining for chloroacetate esterase, a specific marker for neutrophils (naphthol ASD–chloroacetate esterase kit; Sigma). PMNs were identified by positive staining in 10 high-power fields and by morphology.

Western Blots

Liver tissue samples were homogenized in an ice-cold phosphate-buffered saline buffer with 1% Triton-100 (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, and 1% Triton-100, with 5 mg/mL each of leupeptin, aprotinin, pepstain, and chymostain). The homogenates were centrifuged at 10,000g at 4°C, and the resulting supernatants were mixed with the sample buffer. Samples containing 40 μg of protein were separated by sodium dodecyl sulfate electrophoresis on 10%-20% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated with specific primary antibodies against Bag-1, Bcl-2, caspase-3, and β-actin (Santa Cruz Biotechnology). After being washed, the membranes were incubated with horseradish peroxidase–conjugated donkey anti-rabbit secondary antibody (Santa Cruz Biotechnology). Immunoreactive bands were visualized with Super Signal West Pico chemiluminescent substrate (Pierce). Relative quantities of protein were determined with a densitometer (Kodak Digital Science 1D Analysis software) and presented in comparison to β-actin expression.

Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from livers by the guanidium isothiocyanate method, as described.[18] Selected genes were tested by quantitative polymerase chain reaction. Five micrograms of purified DNase I-treated RNA was converted into complementary DNA with oligo dT and Superscript III (Invitrogen, Carlsbad, CA), and this was followed by RNaseH digestion (Invitrogen). Primers were as follows: C6 sense (5′-GCTGCTGGTCTTCCTGGAG-3′) and C6 antisense (5′-ACAGTCTTCTTCTTGC-CAATGC-3′), interleukin-1β (IL-1β) sense (5′-GGAT-GATGACGACCTGCTAG-3′) and IL-1β antisense (5′-GGCTTATGTTCTGTCCATTGAG-3′), tumor necrosis factor alpha (TNF-α) sense (5′-ACTGAACTTCGGGGT-GATTGG-3′) and TNF-α antisense (5′-GTGGGC-TACGGGCTTGTC-3′), and interferon-γ (IFN-γ) sense (5′-TGAGCATCGCCAAGTTCG-3′) and IFN-γ antisense (5′-CCAGAATCAGCACCGACTC-3′). The SYBR Green DNA polymerase chain reaction kit (Applied Biosystems) and Platinum Taq (Invitrogen) were used for RT-PCR analysis. All polymerase chain reaction reactions were run in triplicate. The relative differences in expression between groups were expressed with cycle time values. Results were expressed as fold changes.

Cytokine Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA to detect TNF-α, IFN-γ, and IL-1β levels was performed with eBioscience (San Diego, CA) ELISA kits. Briefly, flat-bottom, 96-well microtiter plates (Costar, Corning, NY) were coated with capture antibodies (antirat TNF-α, IFN-γ, and IL-1β). Serum samples diluted at 1:5 were triplicated, and the standards were used at 1000 pg/mL; this was followed by 1-2 serial dilutions. Biotinylated detecting antibodies were added and were followed by avidin–horseradish peroxidase. Tetrameth-ylbenzidine was used as the substrate, and the color reaction was stopped with 1 M H_3PO_4. Plates were read at 405 nm in an ELISA reader. Serum concentrations of TNF-α, IFN-γ, and IL-1β were calculated from the standard curve.

In Vivo Detection of Apoptosis

Apoptosis was detected with the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) method. A commercial *in situ* histochemical assay (Klenow-FragEL, Oncogene Research Products, Cambridge, MA) was performed to detect the DNA fragmentation in formalin-fixed, paraffin-embedded OLT sections. The results were scored semiquantitatively by the averaging of the number of apoptotic cells per high-power field at a magnification of 400×. Six fields were evaluated per tissue sample.

Statistical Analysis

Results are expressed as the mean ± the standard error of the mean (SEM). Differences between groups were analyzed with 1-way analysis of variance and the statistical package SPSS (SPSS, Inc., Chicago, IL). P values < 0.05 were considered significant.

Results

OLTs

The overall mortality in this study designed to analyze the role of systemic hepatic C6 production versus local hepatic C6 production in rat liver transplant recipients was 13%. Out of 23 syngeneic OLTs, 3 died in 24 hours after surgery (n = 1 for the C6+/+ group and n = 2 for the C6+/− group). The quantitative reverse-transcription polymerase chain reaction (qRT-PCR)-assisted evaluation of OLTs at day +1 revealed that C6-deficient OLTs consistently lacked C6 messenger RNA expression (Fig. 1).

C6(−) OLTs Show Preservation of the Hepatic Architecture and Improved Liver Function

We examined whether C6 deficiency confers protection against IRI in a well-established model of 24-hour liver cold ischemia followed by OLT. As shown in Fig. 2 and detailed in Table 1, OLTs from C6(−) donors demonstrated somewhat decreased signs of vascular congestion and significantly diminished necrosis. In marked contrast, extensive areas of necrosis were seen in C6(+) OLTs, regardless of the C6 recipient status (Suzuki score at day + 1:7.2 ± 0.9 and 7.3 ± 1.3 for C6 +/+ and C6 +/− versus 4.5 ±0.6 and 4.8 ± 0.4 for C6 −/+ and C6−/−, respectively, $P < 0.005$). The preservation of histological detail in C6(−) OLTs was associated with improved liver function. As shown in Fig. 3, serum aminotransferase levels were decreased in C6(−) OLTs in comparison with C6(+) OLTs [serum glutamic oxaloacetic transaminase (sGOT): 2573 ± 488 and 1808 ± 302 for C6 +/+ and C6+/−versus 1170 ± 111 and 1188 ± 184 for C6−/+ and C6−/−, respectively, $P < 0.05$; serum glutamic pyruvic transaminase (sGPT): 1616 ± 327 and 1213 ± 172 for C6+/+ and C6+/− versus 657 ± 82 and 647 ± 87 for C6−/+ and C6−/−, respectively, $P< 0.005$].

C6(−) OLTs Reveal Decreased PMN/Macrophage Infiltration and Proinflammatory Cytokine Expression

We employed MPO assay to determine whether MAC activation affected local PMN infiltration. MPO activity (U/g) in C6(−) OLTs was significantly reduced at 24 hours after transplantation in comparison with C6(+) grafts (Fig. 4, left panel; 3.9 ± 0.7 and 2.3 ± 0.2 for C6+/+ and C6+/− versus 0.9 ± 0.1 and 1.2 ± 0.3 for C6−/+ and C6−/−, respectively, $P = 0.001$). These MPO activity data were confirmed by neutrophil infiltration using esterase staining (Naphthol). Significantly decreased numbers of activated neutrophils (positive cells per high-power field) were detected in C6(−) OLTs in comparison with their C6(+) counterparts (Fig. 5, right panel; 4.2 ± 0.8 and 3.5 ± 1.3 for C6+/+ and C6+/−versus 1.9 ± 0.2 and 0.25 ± 0.3 for C6−/+ and C6−/−, respectively, $P = 0.04$).

In parallel, we used immunohistology to determine whether C6 expression affected local macrophage sequestration. Indeed, infiltration by ED-1+ macrophages at 24 hours post-transplant (positive cells per high-power field) was markedly decreased in C6(−) OLTs in comparison with C6(+) OLTs (148 ± 70 versus 262 ± 48, respectively, $P< 0.01$; Fig. 5). Macrophages localized predominantly around necrosis areas, which were more extensive in C6(+) OLTs than C6(−) OLTs.

As local inflammation represents the cardinal feature of organ IRI, we then assessed the expression of proinflammatory cytokines in OLT groups by qRT-PCR. Figure 6 shows that the expression of TNF-α, IFN-γ, and IL-1β in C6(−) transplanted livers was notably reduced in comparison with C6(+) OLTs (TNF-α: 1.8 ± 0.2, 1.4 ± 0.4, 2.7 ± 0.4, and 2.1 ± 0.2 fold changes for C6−/−, C6−/+, C6+/+, and C6 +/−, respectively, $P< 0.05$; similar results were obtained for IFN-γ and IL-1β). These results were confirmed by ELISAs performed in serum samples from the transplanted animals 24 hours after reperfusion. TNF-α serum values (pg/mL) were 321.4 ± 16.9, 290.5 ± 18.1, 668.2 ± 21.1, and 608.4 ± 24.3 for C6−/−, C6−/+, C6+/+, and C6 +/−, respectively ($P < 0.005$). Significant differences were encountered in the ELISAs for IFN-γ and IL-1β as well (IFN-γ: 284.4 ±20.1, 243.5 ± 18.9, 605.6 ± 25.9, and 552.7 ± 24.4 for C6−/−, C6−/+, C6+/+, and C6 +/−, respectively, $P < 0.005$; IL-1β: 301.4 ± 22.7, 275.8 ± 19.7, 631.4 ± 24.1, and 569.1 ± 26.4 for C6−/−, C6−/+, C6 +/+, and C6+/−, respectively, $P< 0.005$). These results suggest that the proinflammatory response in the ischemic livers depends, at least in part, on the activation/action of MAC.

C6 Deficiency Modulates Proapoptotic and Antiapoptotic Pathways After OLT

We employed Western blots to assess the relative intragraft expression levels of proapoptotic (caspase 3) and antiapoptotic (Bcl-2/Bag-l) gene products. Interestingly, the expression of

caspase-3 was decreased in C6(−) OLTs in comparison with C6(+) OLTs (0.75 ± 0.002, 0.67 ± 0.06, 0.51 ± 0.02, and 0.59 ± 0.04 fold change for C6+/+, C6+/−, C6−/+, and C6−/−, respectively, $P = 0.006$). In contrast, Bag-1 protein levels were enhanced in C6(−) OLTs in comparison with C6(+) OLTs (0.35 ± 0.01, 0.38 ± 0.02, 0.47 ± 0.03, and 0.69 ± 0.02 fold change for C6+/+, C6+/−, C6−/+, and C6−/−, respectively, $P = 0.001$) as well as Bcl-2 (0.54 ± 0.03, 0.68 ± 0.05, 0.79 ± 0.03, and 0.82 ± 0.04 fold change for C6+/+, C6+/−, C6−/+, and C6−/−, respectively, $P = 0.001$; Fig. 7). These data indicate that the MAC deposition in the liver tissue stimulates the apoptotic cascade, with resultant susceptibility of OLTs to caspase-dependent death. To confirm these findings, liver samples were evaluated according to the TUNEL method. Grafts from C6+ donors consistently showed significant hepatocellular apoptosis, as characterized by dense nuclear margination at 24 hours after transplant (25 ± 8 and 22 ± 8 TUNEL+ cells per field for C6+/+ and C6 +/− grafts, respectively; Fig. 8A,B). In contrast, the number of apoptotic cells in C6 − grafts was significantly reduced (7 ± 5 and 8 ± 2 TUNEL+ cells per field for C6−/+ and C6−/− grafts, respectively, $P < 0.05$ among groups; Fig. 8C,D).

Discussion

This study was designed to address the role that the terminal complement cascade, C5b-9, plays in the pathophysiology of liver IRI. Rat syngeneic OLTs were performed in C6(+) and C6(−) respective donor-recipient pairs following 24 hours of cold storage. Such an experimental design is consistent with the documented systemic and local C6 production in the transplanted tissue.[19] Hepatic biosynthesis is responsible for the majority of circulating C6,[20] although human endothelial cells, monocytes, and macrophages may also contribute to its production.[21] In fact, macrophage-derived C6 can be a significant factor in tissue injury, as exemplified by graft rejection,[22] whereas both extrahepatic production and intrahepatic production of C6 are equally effective in the generation of renal C5b-9 complexes.[23]

We found that local ablation of the C5b-9 complex in C6(−) grafts ameliorated hepatocellular injury independently of the recipient C6 status. Indeed, 1 day after transplant, both sGOT and sGPT were significantly lower in the recipients of C6(−) OLTs in comparison with those expressing C6. Furthermore, the hepatic architecture was largely preserved in C6(−) OLTs. The hepatic microvasculature was somewhat less congested, with significantly fewer zones of frank necrosis, in comparison with C6(+) OLTs. Other authors have reported that blocking the entire complement cascade beyond C3 can improve microvascular perfusion and decrease hepatocellular injury.[24,25] By selectively blocking the terminal components of the complement cascade (C5b-9) solely, while leaving C3-5a expression intact, we have achieved a significant decrease in the extent of hepatocellular necrosis. In doing so, we demonstrate, for the first time, that MAC arising from the grafted liver does play an important role in IRI and does so independently of the expression of earlier complement components.

The deposition of MAC not only directly induces the cellular injury but also is the key in amplifying the inflammation response. It promotes the expression of proinflammatory mediators[10] and secretion of soluble chemotactic agents that recruit inflammatory cells.[26] Accordingly, we have demonstrated that OLTs lacking C6 were characterized by significantly lower levels of key proinflammatory cytokines IL-1β, TNF-α, and IFN-γ.

C5a is a known chemotactic agent that up-regulates the Mac-1 receptor on circulating PMNs, causing their recruitment into the hepatic sinusoids.[7] Because the C5a levels were comparable in all recipient groups, we anticipated the same leukocyte infiltration in all the grafts. However, we found significantly lower PMN and macrophage infiltration in C6(−) OLTs in comparison with C6(+) OLTs. This could be due, at least in part, to decreased levels of IL-1β and TNF-α in C6(−) grafts. These 2 factors are known to up-regulate the expression of adhesion molecules,

favoring the recruitment of PMNs/macrophages into the liver parenchyma. In a model of antigen-induced arthritis using C6(−) rabbits, it was also demonstrated that MAC up-regulated leukocyte recruitment. However, a substantial number of PMNs and macrophages remained in the synovium and synovial fluid, and this indicated that other proinflammatory pathways, in addition to MAC formation, may contribute to the influx of inflammatory cells.[27] In a model of allergic encephalomyelitis in C6(−) rats, MAC induced the expression of P-selectin and intercellular cell adhesion molecule-1 on vascular endothelium, promoting T-cell/macrophage tissue infiltration.[28] Finally, in a rabbit model of myocardial infarction and IRI, MAC was demonstrated to mediate PMN recruitment to the reperfused myocardium through the local induction of IL-8 expression.[29]

Possibly the most convincing evidence to date on the role of MAC in cellular recruitment, independent of C5a and the rest of the complement system, comes from a mouse model of renal warm IRI.[30] In that study, C6(−) mice exhibited a degree of protection similar to that of those with more proximal interruption of the complement cascade (C3). Moreover, although C6(−) animals were unable to generate functional MAC, they could form C5a. However, adjunctive prevention of C5a formation in these mice failed to produce further benefit, as evidenced by renal function and local inflammation response. On the basis of these findings, it appears that MAC is the principal element of the complement system responsible for renal injury and inflammatory cell recruitment after ischemia and reperfusion. Perhaps this might be the case in the liver as well, although further studies blocking earlier steps in the complement cascade are needed before any firm conclusions can be reached.

The proapoptotic caspase 3 and antiapoptotic Bag-1/Bcl-2 gene products were measured in C6 (+) and C6(−) grafts. Interestingly, the expression of caspase 3 was consistently significantly lower and the expression of Bag-1/Bcl-2 was consistently significantly higher in MAC-deficient OLTs. These results were confirmed by the TUNEL assay, which demonstrated a significantly increased frequency of apoptotic cells in C6(+) grafts versus MAC-deficient livers. Although this is the first time that the link between MAC and apoptosis has been observed in a hepatic IRI model, these findings are not totally unexpected. Indeed, C5b-9 was first described to mediate apoptosis in a C6-deficient rat model of glomerulonephritis.[31] The link between MAC and apoptotic pathways was subsequently confirmed in a model of renal injury.[32] The exact mechanisms by which MAC may induce apoptosis have not been defined, although recent evidence lends support to the hypothesis that the influx of extracellular calcium, which regulates the lytic activity of MAC,[33,34] may also induce the opening of mitochondrial pores, allowing the release of the stored proapoptotic factors.[35]

In summary, terminal C5b-9 products of the complement system are essential in the mechanism of hepatic IRI. The C6(−) OLTs were relatively resistant to cold IRI, as evidenced by better preservation of hepatic architecture, improved liver function, decreased PMN/macrophage infiltration, and amelioration of the local proinflammatory response in association with selective modulation of proapoptotic and antiapoptotic pathways. To the best of our knowledge, this is the first report that shows that inhibition of MAC complement formation by the use of C6-deficient liver grafts attenuates early IRI in OLT recipients.

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Abbreviations

C6+/+	transplant of a C6-positive graft to a C6-positive recipient
C6+/−	transplant of a C6-positive graft to a C6-negative recipient
C6−/+	transplant of a C6-negative graft to a C6-positive recipient
C6−/−	transplant of a C6-negative graft to a C6-negative recipient
ELISA	enzyme-linked immunosorbent assay

IFN-γ	interferon-γ
IL	interleukin
IRI	ischemia/reperfusion injury
MAC	membrane attack complex
MPO	myeloperoxidase
OLT	orthotopic liver transplantation
PMN	polymorphonuclear cell
PNF	primary nonfunction
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RT-PCR	reverse-transcription polymerase chain reaction
SEM	standard error of the mean
sGOT	serum glutamic oxaloacetic transaminase
sGPT	serum glutamic pyruvic transaminase
TNF-α	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase–mediated dUTP nick endlabeling

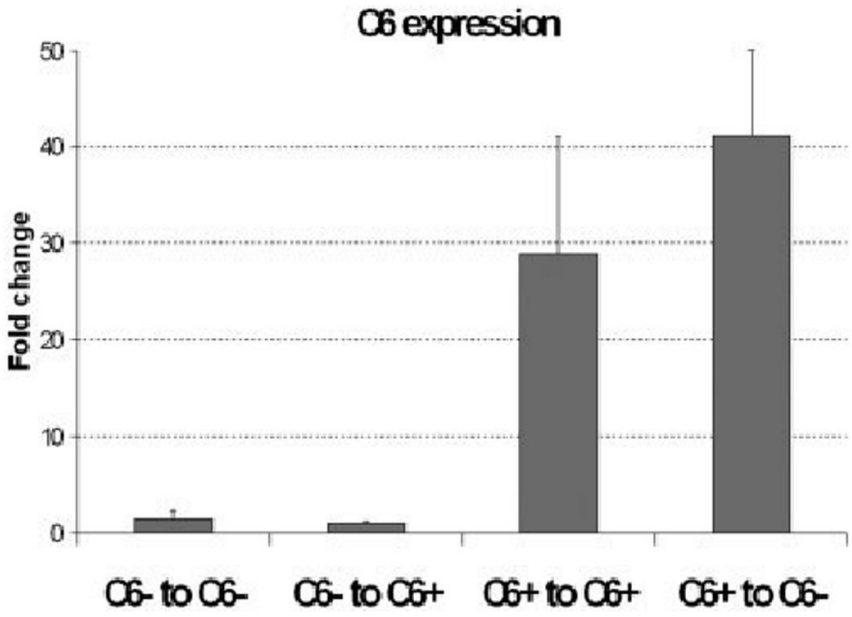

Figure 1.
Differential C6 gene expression in rat OLTs. Livers from C6(+) or C6(−) rats were harvested, stored for 24 hours at 4°C, and then transplanted orthotopically to respective C6(+) and C6(−) recipients. OLTs were harvested 24 hours later, and C6 expression was analyzed by qRT-PCR. There was an absence of C6 expression in livers from C6(−) animals in contrast to the C6-competent grafts (n = 6 per group).

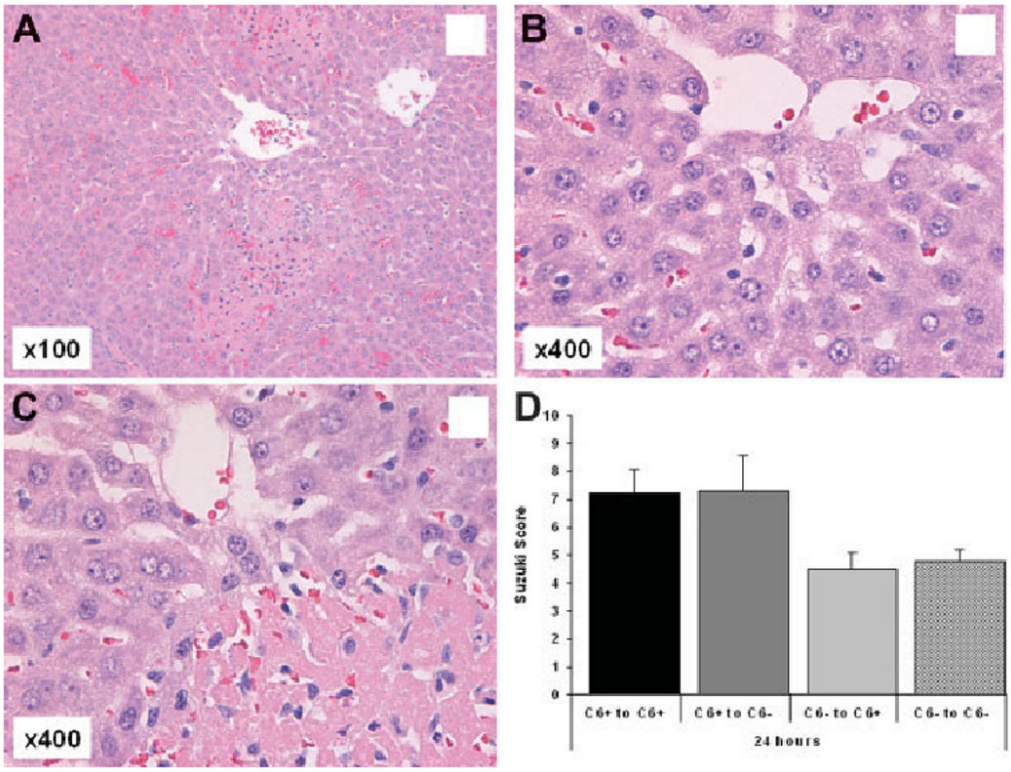

Figure 2.

Representative photomicrographs of livers after 24 hours of cold ischemia followed by OLT (day +1). (A) At a lower magnification, areas of congestion and necrosis in close proximity to the centrilobular vein can be observed. At a higher magnification, (B) hepatocellular ballooning degeneration and vacuolization and (C) coagulative necrosis and PMN infiltration are evident. (D) These changes were present to a certain extent in all hepatic grafts 24 hours after reperfusion but were significantly more pronounced in the livers from C6(+) donors, as reflected in the Suzuki score. The Suzuki score is presented as the mean ± SEM of 3-6 OLTs per group ($P < 0.05$).

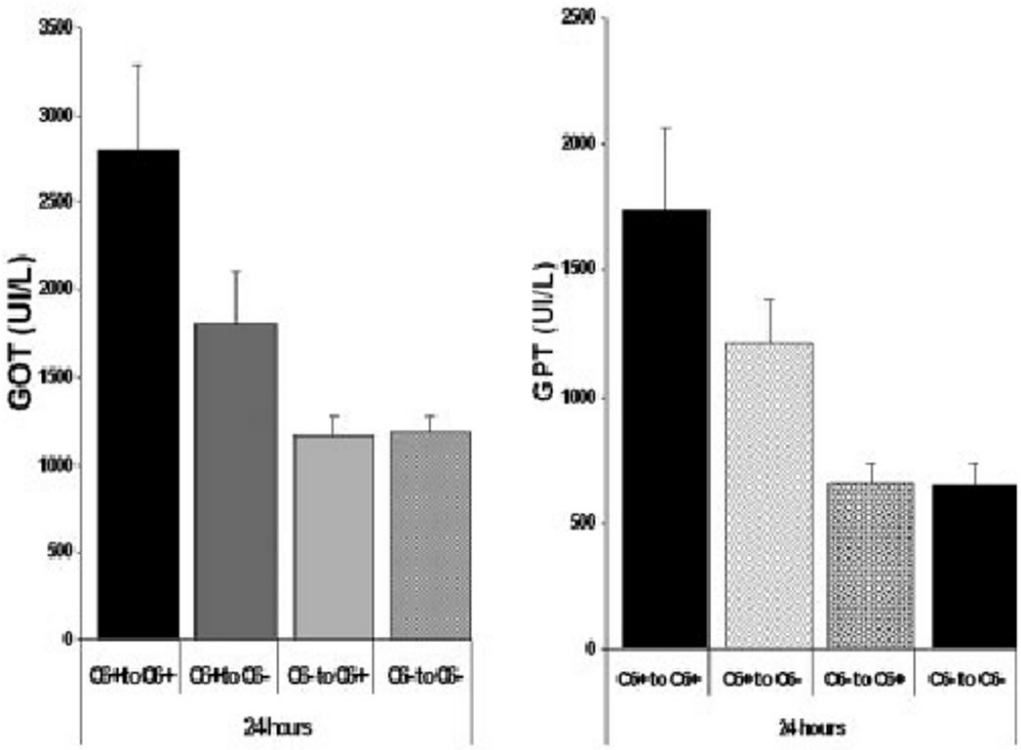

Figure 3.
Serum aminotransferase levels in rats transplanted with C6 (+) or C6(−) livers that were subjected to 24 hours of cold ischemia. The sGOT and sGPT levels were lower in C6(−) OLT recipients. The data are presented as the mean ± SEM of 3-6 OLTs per group ($P < 0.005$ for both).

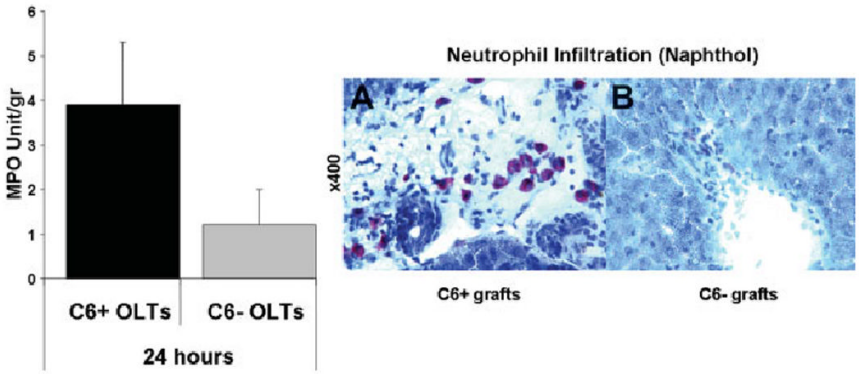

Figure 4.

Neutrophil accumulation in OLTs (day +1) was decreased in C6(−) livers in comparison with C6(+) OLTs as analyzed with an MPO assay (left panel; $P < 0.001$, n = 3-4 per group) or a naphthol ASD-chloroacetate esterase kit (right panel; $P < 0.05$). Representatives of 3 experiments are shown (400 × magnification).

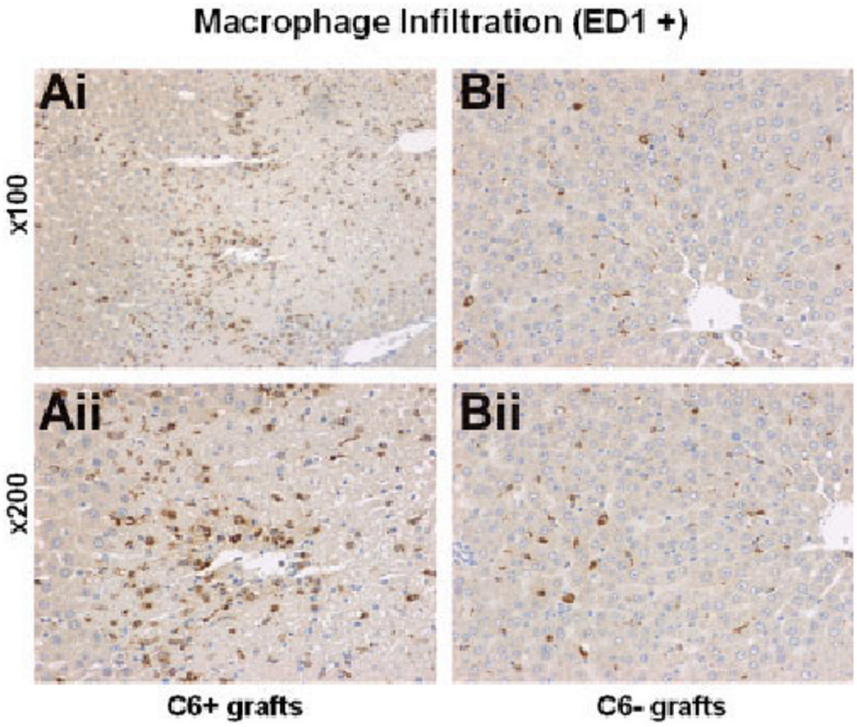

Figure 5.
Immunohistochemical staining of OLTs after 24 hours of cold ischemia followed by reperfusion (day +1) at magnifications of 100 × and 200 ×. Macrophage infiltration was dense and severe in C6 (+) OLTs in comparison with C6(−) OLTs (262 ± 48 and 148 ± 70 labeled cells per high-power field at 100 × magnification, respectively, $P < 0.01$).

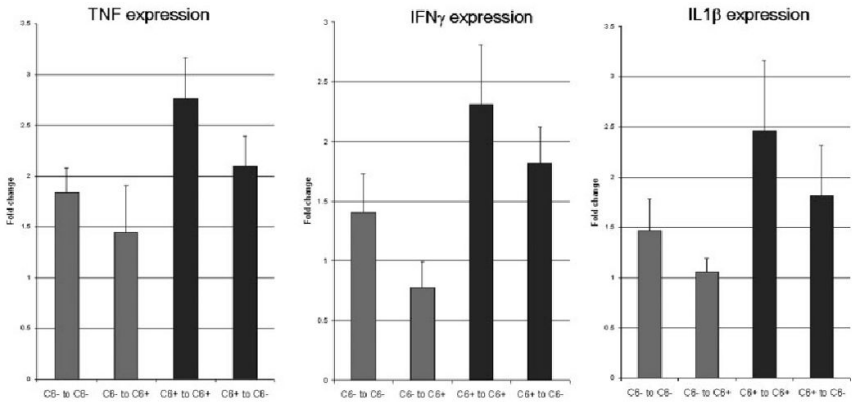

Figure 6.

Differential expression of TNF-α, IFN-γ, and IL-1 β in liver grafts at day 1 post-transplant as determined by qRT-PCR. There was increased cytokine expression in C6(+) OLTs versus C6 (−) OLTs. Controls consisted of the mean of 3 donor livers (n = 3-6 per group, $P < 0.05$ for all 3 cytokines).

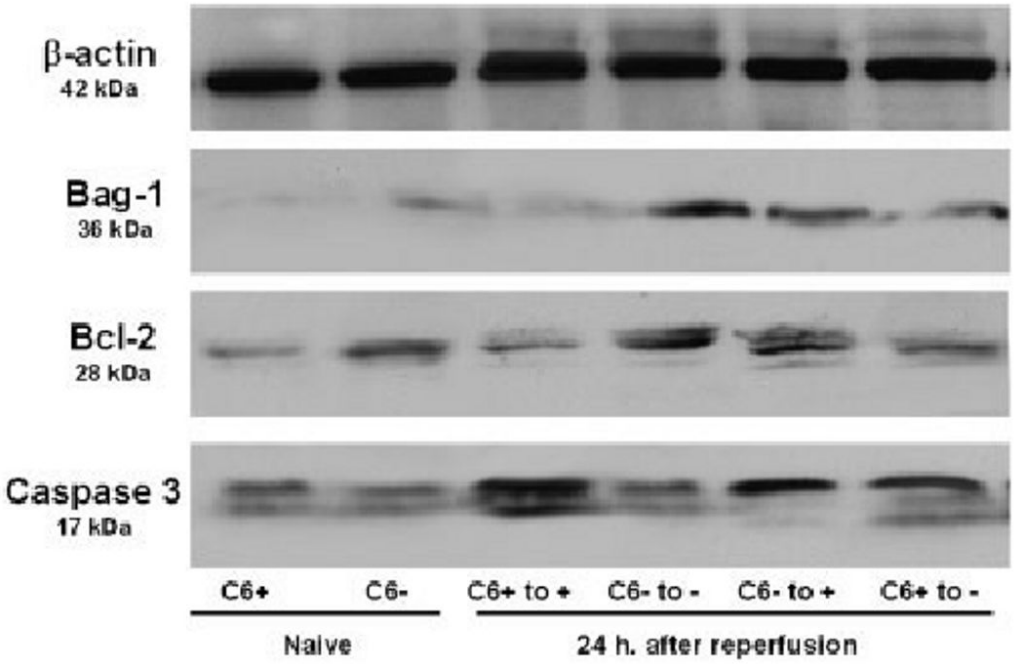

Figure 7.

Representative Western blot analysis of proapoptotic/antiapoptotic proteins in rat OLTs. Livers were stored for 24 hours at 4°C, transplanted into syngeneic rats, and then harvested at 24 hours. The protein expression was detected by polyclonal rabbit anti-rat antibodies. Antibody against actin was used as an internal control. Antiapoptotic (Bcl-2/Bag-l) molecules were found in higher levels in C6(−) livers in comparison with C6(+) livers. In contrast, proapoptotic caspase-3 was diminished in C6(−) liver grafts.

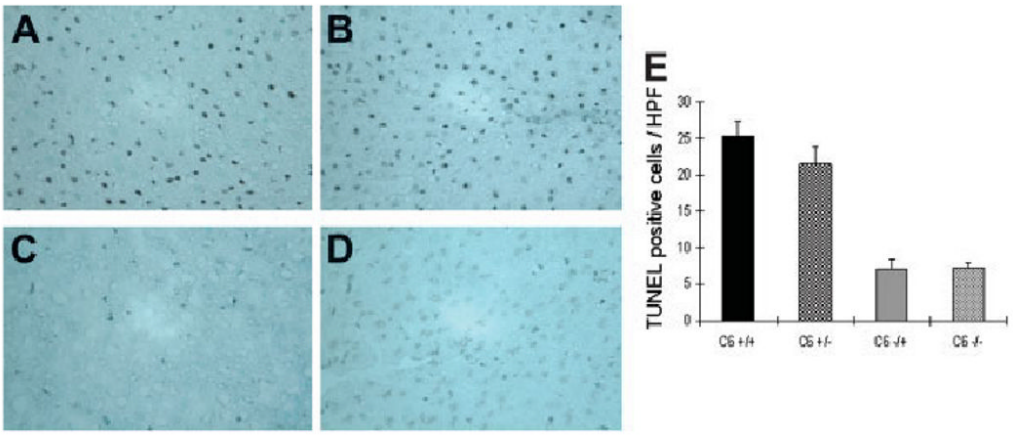

Figure 8.

TUNEL-assisted detection of apoptotic cells in rat OLTs 24 hours after transplant: (A) C6+/+, (B) C6+/−, (C) C6−/+, and (D) C6−/−. C6-positive grafts were characterized by extensive areas of TUNEL+ cells (brown spots), in contrast to the limited hepatocellular apoptosis observed in C6-deficient grafts. (E) The results were scored by the averaging of the number of TUNEL+ cells (mean ± standard error) per high-power field at 400 × magnification. A minimum of 6 fields were evaluated per sample.

Table 1

Ischemia/Reperfusion Injury Evaluation According to Suzuki Score Criteria

	Group	n	Mean	Standard Error	Range	*P*
Congestion	C6 +/+	5	2.00	0.31	1-3	0.120
	C6 +/−	3	2.33	0.33	2-3	
	C6−/+	6	1.33	0.21	1-2	
	C6−/−	6	2.00	0.25	1-3	
Ballooning	C6 +/+	5	2.20	0.49	1-3	0.415
	C6 +/−	3	2.33	0.88	1-4	
	C6−/+	6	1.50	0.34	1-3	
	C6−/−	6	1.50	0.22	1-2	
Necrosis	C6 +/+	5	3.00	0.31	2-4	0.003
	C6 +/−	3	2.67	0.33	2-3	
	C6−/+	6	1.67	0.33	1-3	
	C6−/−	6	1.33	0.21	1-2	
Suzuki score	C6 +/+	5	7.20	0.97	4-9	0.036
	C6 +/−	3	7.33	1.33	6-10	
	C6−/+	6	4.50	0.67	3-7	
	C6−/−	6	4.83	0.40	4-6	

Abbreviations: C6 +/+, transplant of a C6-positive graft to a C6-positive recipient; C6+/−, transplant of a C6-positive graft to a C6-negative recipient; C6 −/+, transplant of a C6-negative graft to a C6-positive recipient; C6 −/−, transplant of a C6-negative graft to a C6-negative recipient.