Complement 5 Inhibition Ameliorates Hepatic Ischemia/reperfusion Injury in Mice, Dominantly via the C5a-mediated Cascade

AUTHOR(S):

Kusakabe, Jiro; Hata, Koichiro; Tamaki, Ichiro; Tajima, Tetsuya; Miyauchi, Hidetaka; Wang, Yi; Nigmet, Yermek; ... Tanaka, Hirokazu; Tsuruyama, Tatsuaki; Uemoto, Shinji

CITATION:

Kusakabe, Jiro ...[et al]. Complement 5 Inhibition Ameliorates Hepatic Ischemia/reperfusion Injury in Mice, Dominantly via the C5a-mediated Cascade. Transplantation 2020, 104(10): 2065-2077

ISSUE DATE:

2020-10

URL:

http://hdl.handle.net/2433/281528

OPEN

Complement 5 Inhibition Ameliorates Hepatic Ischemia/reperfusion Injury in Mice, Dominantly via the C5a-mediated Cascade

Jiro Kusakabe, MD,[1] Koichiro Hata, MD, PhD,[1] Ichiro Tamaki, MD, PhD,[1] Tetsuya Tajima, MD,[1] Hidetaka Miyauchi, MD, PhD,[1] Yi Wang, PhD,[2] Yermek Nigmet, MD, PhD,[1] Yusuke Okamura, MD, PhD,[1] Toyonari Kubota, MD, PhD,[1] Hirokazu Tanaka, MD,[1] Tatsuaki Tsuruyama, MD, PhD,[3] and Shinji Uemoto, MD, PhD[1]

Background. Hepatic ischemia/reperfusion injury (IRI) is a serious complication in liver surgeries, including transplantation. Complement activation seems to be closely involved in hepatic IRI; however, no complement-targeted intervention has been clinically applied. We investigated the therapeutic potential of Complement 5 (C5)-targeted regulation in hepatic IRI. **Methods.** C5-knockout (B10D2/oSn) and their corresponding wild-type mice (WT, B10D2/nSn) were exposed to 90-minute partial (70%) hepatic ischemia/reperfusion with either anti-mouse-C5 monoclonal antibody (BB5.1) or corresponding control immunoglobulin administration 30 minutes before ischemia. C5a receptor 1 antagonist was also given to WT to identify which cascade, C5a or C5b-9, is dominant. **Results.** C5-knockout and anti-C5-Ab administration to WT both significantly reduced serum transaminase release and histopathological damages from 2 hours after reperfusion. This improvement was characterized by significantly reduced CD41+ platelet aggregation, maintained F4/80+ cells, and decreased high-mobility group box 1 release. After 6 hours of reperfusion, the infiltration of CD11+ and Ly6-G+ cells, cytokine/chemokine expression, single-stranded DNA+ cells, and cleaved caspase-3 expression were all significantly alleviated by anti-C5-Ab. C5a receptor 1 antagonist was as effective as anti-C5-Ab for reducing transaminases. **Conclusions.** Anti-C5 antibody significantly ameliorated hepatic IRI, predominantly via the C5a-mediated cascade, not only by inhibiting platelet aggregation during the early phase but also by attenuating the activation of infiltrating macrophages/neutrophils and hepatocyte apoptosis in the late phase of reperfusion. Given its efficacy, clinical availability, and controllability, C5-targeted intervention may provide a novel therapeutic strategy against hepatic IRI.

(*Transplantation* 2020;104: 2065–2077).

INTRODUCTION

Ischemia/reperfusion (IR) injury (IRI) is one of the major causes of liver failure in liver surgeries, including transplantation (liver transplantation [LT]). Systemic hypoxia or hypoperfusion in trauma, massive hemorrhage, sepsis, and congestive heart or respiratory failure also cause hepatic IRI. Hepatic IRI is a well-known deleterious factor that influences graft outcomes in LT, leading not only to

Received 25 February 2020. Revision received 16 April 2020.

Accepted 17 April 2020.

[1] Department of Surgery, Division of Hepato-Biliary-Pancreatic Surgery and Transplantation, Kyoto University Graduate School of Medicine, Kyoto, Japan.

[2] Alexion Pharmaceuticals Inc., New Haven, CT.

[3] Center for Anatomical, Pathological, and Forensic Medical Research, Kyoto University Graduate School of Medicine, Kyoto, Japan.

This study was supported by the Platform Project for Supporting Drug Discovery and Life Science Research (Platform for Drug Discovery, Informatics, and Structural Life Science, S.U. and K.H., No.15ak0101031h0002 and 16ak0101031h0003) from the Japan Agency for Medical Research and Development (AMED), and partly by Alexion Pharmaceuticals, Inc.

*The authors of this manuscript have conflicts of interest to disclose, as described by **Transplantation**.*

J.K., K.H., and S.U. designed the study. J.K., I.T., T.Ta., and H.M. participated in the performance of the research. J.K., K.H., I.T., T.Ta., H.M., Y.W., Y.N., Y.O., T.K., H.T., and T.Ts. participated in the analysis and interpretation of data. J.K. and K.H. wrote the article, and S.U. edited the final version of the draft. K.H. obtained the research grant. All the authors approved the final version of the article.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

Correspondence: Koichiro Hata, MD, PhD, Department of Surgery, Division of Hepato-Pancreato-Biliary Surgery and Transplantation, Kyoto University Graduate School of Medicine, Kawahara-cho 54, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. (khata@kuhp.kyoto-u.ac.jp).

ISSN: 0041-1337/20/10410-2065

DOI: 10.1097/TP.0000000000003302

<zdoi;10.1097/TP.0000000000003302>

delayed graft function (10%–25%) and primary nonfunction (5%–15%)[1] but also to late graft failure by causing ischemic cholangiopathy or enhancing immunological alloreactions through the activation of innate immunity.[1-3] Increasing clinical evidence also demonstrates that hepatic IRI may accelerate cancer recurrence after LT for hepatocellular carcinoma.[4] Furthermore, the current critical shortage of donor organs has expanded the use of extended-criteria donors from the elderly, those with steatosis, and donation after circulatory-death (DCD) donors; however, these "marginal" organs are particularly vulnerable to IRI. To improve the outcomes of LT, the mechanisms underlying hepatic IRI need to be elucidated and novel therapeutic approaches developed for deleterious conditions.

The complement system is closely involved in hepatic IRI.[5,6] Various means of complement inhibition have been investigated, and cobra venom factor, C1-inhibitor, and soluble complement receptor 1 were previously reported to effectively attenuate hepatic IRI.[5,7,8] However, complement-targeting interventions against hepatic IRI have not yet been applied in clinical practice. Eculizumab, a humanized monoclonal antibody to complement component-5 (C5), has been approved for diverse complement-mediated disorders, such as paroxysmal nocturnal hemoglobinuria, atypical hemolytic uremic syndrome, and refractory generalized myasthenia gravis, in many countries.[9] Eculizumab has yielded better therapeutic effects against these intractable diseases than ever before.[10,11] Its indication has been expanding to other complement-mediated refractory diseases, including Guillain-Barré syndrome and aquaporin-4 antibody-positive neuromyelitis optica spectrum disorder.[12,13] Eculizumab has also been described in kidney antibody–mediated rejection.[14-16] By binding directly to C5, eculizumab inhibits the enzymatic cleavage of C5 into C5a and C5b, thereby preventing not only the C5a-induced chemotaxis of proinflammatory immune cells but also the formation of C5b-9, also known as membrane attack complex (MAC).[17] Due to its dramatic efficacy and simultaneous safety achieved by inhibiting terminal complement activities without interfering with other host immunities, we hypothesized that C5-targeted regulation is a promising therapeutic option for hepatic IRI.

Besides evaluating the therapeutic effects of C5 inhibition, it is also essential to obtain a better understanding of the mechanisms underlying the C5 involvement in hepatic IRI in rodent models before its clinical application. Previous studies reported the impact of C5a or C5b blockade in hepatic,[1,3,18,19] as well as in renal IRI[20-22]; however, the dominant cascade, C5a or C5b, has yet to be identified. Hepatic IRI is a biphasic phenomenon.[23] Early-phase injury is initiated by the release of endogenous damage-associated molecular patterns and the activation of liver-resident macrophages and dendritic cells, followed by microcirculatory disturbance.[23] In the late phase, activated monocytes and neutrophils are recruited into hepatic parenchyma to amplify local immune responses.[24] However, previous studies mainly examined alterations in late-phase pathologies by complement modulation in hepatic IRI. Therefore, C5 involvement in hepatic IRI as well as the mechanisms by which C5 inhibition ameliorates liver damage throughout the dynamic process need to be clarified before proceeding to clinical applications.

Therefore, the present study investigated the therapeutic potential of C5 regulation in hepatic IRI and the mechanisms underlying C5 involvement by comparing the effects of total C5 blockade with C5a receptor 1 (C5aR1) inhibition in both the early and late phases.

MATERIALS AND METHODS

Animals

Male wild-type (WT), B10D2nSn-Slc (C5+/+, WT) and knockout (KO), B10D2oSn-J (C5–/–, KO) mice (8–10 wks, 20–25 g) were purchased from Japan SLC, Inc., (Hamamatsu, Japan) and the Jackson Laboratory (Bar Harbor, ME), respectively. All animals were kept in specific pathogen-free conditions in a temperature- and humidity-controlled environment with a 12-hour light-dark cycle and were allowed free access to tap water and standard chow pellets ad libitum. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory animals (National Institutes of Health Publication 86-23, 2011 revision). All experimental protocols were approved by the Animal Research Committee of Kyoto University (MedKyo-17546 and -18193).

Liver IRI Model

We used the established mouse model of partial warm hepatic IRI (Figure 1).[25-27] Mice were anesthetized under isoflurane. An atraumatic clip (AS-1; Natsume Seisakujo Co., Ltd., Tokyo, Japan) was used to interrupt the artery and portal venous supply to the left and middle liver lobes, inducing segmental (70%) hepatic ischemia without intestinal congestion. After 90-minute ischemia, the clamp was removed and reperfusion was initiated. Mice were given an intravenous injection of anti-C5-Ab, BB5.1[28] provided by Alexion Pharmaceuticals (Cheshire, CT) at a dose of 40 mg/kg 30 minutes before ischemic insult.[19] They were then humanely sacrificed, and liver/blood samples were collected 2 and 6 hours after reperfusion. Portions of liver tissue were procured from ischemic lobes, and blood was collected from the vena cava. Control WT and KO mice were pretreated with the same dose of control immunoglobulin (IgG) (mouse IgG-1 isotype control, clone MOPC-21, BioXCell, NH). WT mice were also treated with C5aR1-antagonist (PMX53, Tocris Bioscience, Minneapolis, MO),[29] a cyclic hexapeptide AcF (OpdChaWR) at a dose of 1 mg/kg.[29-31] Sham-operated mice underwent the same procedure but without any vascular occlusion. We used OpdChaWR (PMX53) in our study because its pharmacokinetics has been thoroughly investigated,[29,32,33] and it was used in various complement-related rodent studies.[18,30,31]

Complement Hemolytic Assay

Terminal complement activity in mouse sera was measured by the standard method to assess its ability to lyse chicken erythrocytes, which had been presensitized with erythrocyte-specific Abs, as detailed elsewhere.[34] Normal B10D2nSn-Slc mouse serum diluted 1 of 10 in gelatin veronal-buffered saline (GVBS) (B100, CompTech, Tyler, TX) was used as the 100% lysis control. Experimental samples were prepared by diluting the test serum 1 of 10 in GVBS then mixing with human C5-deficient serum

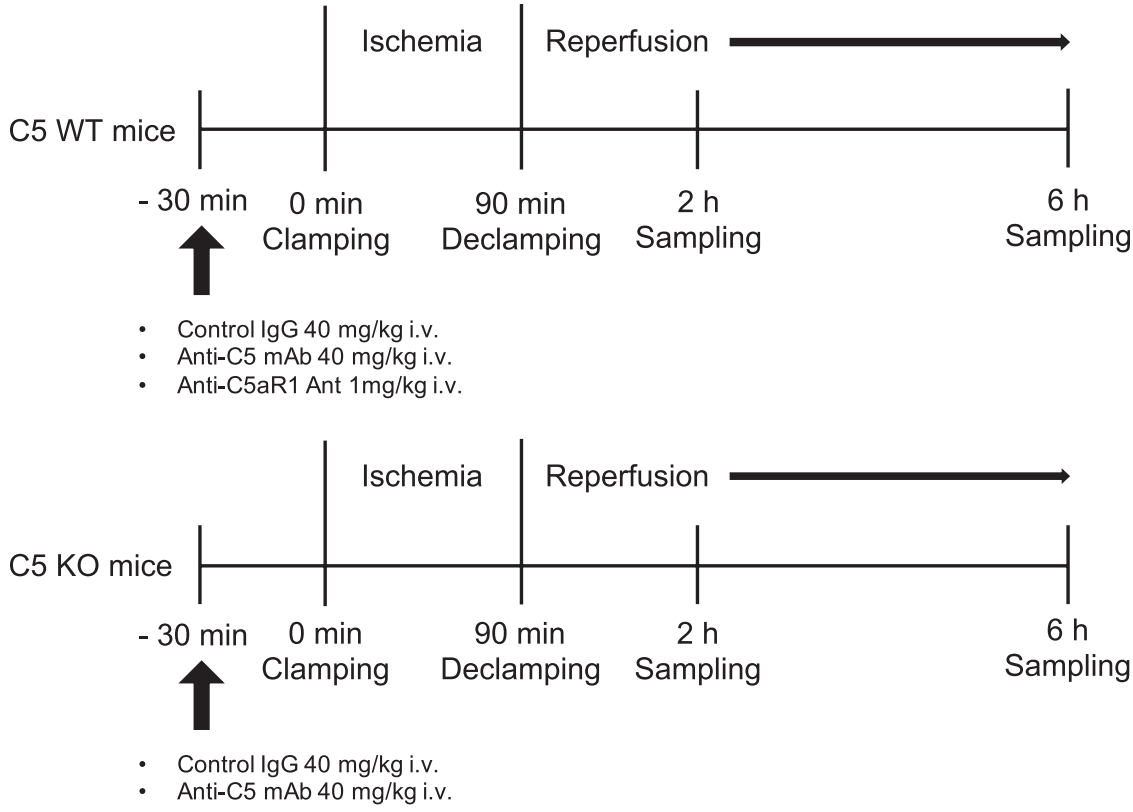

FIGURE 1. Schematic illustration of the experimental design. C5-KO (B10D2/oSn) and their corresponding WT mice (WT, B10D2/nSn) were exposed to partial (70%) hepatic ischemia of the left and median lobes for 90 min, followed by reperfusion. IgG-1 isotype control (clone MOPC-21), anti-C5-Ab (BB5.1), or C5aR1-antagonist (PMX53) was administered 30 min before ischemia. C5aR1-Ant, C5a receptor 1 antagonist; IgG, immunoglobulin; KO, knockout; WT, wild-type.

and GVBS. Each sample was triplicated, and 2 µL of 500 mmol/L EDTA was added to the third well to be used as "no hemolysis" color control standards. Chicken erythrocytes were presensitized with an antichicken red blood cell polyclonal Ab (103-4139, Rockland, PA) at 4°C for 15 minutes, added to the plate, and then incubated at 37°C for 30 minutes. After centrifugation, the supernatant was transferred to microtiter plates and then read at OD415 using a microplate reader. The degree of hemolysis was calculated using the following formula: % hemolysis = 100 × ([OD sample − OD sample color control]/[OD 100% lysis control − OD 100% lysis color control]). The results obtained were confirmed by another hemolytic assay (data not shown). Briefly, hemolytic activity was calculated based on the extent of hemolysis of unsensitized sheep erythrocytes after the incubation with mouse serum in the presence of zymosan. In this so-called reactive lysis, erythrocytes are lysed if the complement cascade is activated not on erythrocyte membranes but in the fluid phase.

Hepatocellular Damage

Serum alanine aminotransferase (ALT) levels in peripheral blood, an indicator of hepatocellular injury, were measured using a standard spectrophotometric method with an automated clinical analyzer (JCA-BM9030; JEOL, Ltd., Tokyo, Japan).

Histology

Liver paraffin sections (4-µm thickness) were stained with hematoxylin & eosin. The severity of hepatic IRI (necrosis, sinusoidal congestion, and centrilobular ballooning) was blindly graded by 2 independent pathologists, according to the modified Suzuki criteria on a scale of 0–4.[35]

ELISA

High-mobility group box 1 (HMGB-1) is passively released to the extracellular space from necrotic or apoptotic cells.[36] In hepatic IR, Toll-like receptor-4 (TLR-4) signaling drives HMGB-1 release, which, in turn, activates immune cells and inflammatory responses through its interaction with soluble molecules, such as TLR-4.[37] Serum HMGB-1 levels were quantified with HMGB-1 ELISA Kit II (Shino-Test, Tokyo, Japan) according to the manufacturer's protocol.

Quantitative Reverse-transcription Polymerase Chain Reaction

Proinflammatory cytokines play pivotal roles in hepatic IRI.[38] CXC chemokines, including CXCL-1 and CXCL-2, act as neutrophil chemoattractants and are also crucial in IRI.[39] Therefore, proinflammatory cytokines and chemokines in liver tissues with or without C5 regulation were analyzed by reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from liver tissues using an RNeasy Kit (Qiagen, Venlo, the Netherlands), and cDNA was prepared using an Omniscript RT kit (Qiagen). Quantitative RT-PCR was performed using the StepOnePlus Real-Time PCR System (Life Technologies, Tokyo, Japan). The primers used to amplify specific gene fragments are listed in Supplemental

Digital Content 1, SDC, http://links.lww.com/TP/B938. Target gene expression was calculated by the ratio to the housekeeping gene, GAPDH.

Immunohistochemistry

After the deparaffinization of liver sections, the antigen was retrieved with citrate buffer (10 mmol/L, pH 6.0). After blocking with Protein Block Serum-Free (X0909, DAKO, Tokyo, Japan), sections were incubated with the primary antibodies (Table S1, SDC, http://links.lww.com/TP/B938). In light microscopy, sections were treated with biotinylated rabbit anti-rat IgG and goat anti-rabbit IgG (1:300). After an incubation, immunoperoxidase (VECTASTAIN Elite ABC Kit, Vector Labs, Burlingame, CA) was applied and then visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution with hematoxylin counterstaining. In fluorescence microscopy, sections were treated with Alexa 488-conjugated goat anti-rat IgG (1:500) and covered with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Sections were observed with a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). Positive cells were counted blindly at 10 high-power fields/section (×400). Negative controls were prepared by an incubation with normal rat-IgG or rabbit-IgG (sc-2026 and -2027; Santa Cruz Biotechnology, Santa Cruz, CA) instead of the first Abs. The CD41-positive area was quantified using ImageJ Software (NIH, Bethesda, MD).

Western Blot

Liver tissues were homogenized in RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA), soluble protein lysates (30 μg/sample) were subjected to 12%-gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). After blocking with 5% skim milk, primary antibodies recognizing cleaved caspase-3 (Asp175; CST, Danvers, MA) and β-actin (PM053; MBL, Nagoya, Japan) were applied at dilutions of 1:500 and 1:2000, respectively, at 4°C overnight. After washing, the membranes were reacted with the horseradish peroxidase-conjugated secondary antibody (P0448; DAKO, Santa Clara, CA). Chemiluminescence was detected with ImmunoStar Zeta (WAKO Pure Chemical Industries, Ltd., Osaka, Japan) and visualized with a charge-coupled device camera (EZ-capture, Atto Corporation, Tokyo, Japan). Band intensities were quantified with ImageJ Software.

Statistical Analysis

All data are expressed as means ± SEM. Differences among experimental groups were analyzed using 1-way ANOVA for unpaired data, followed by post hoc tests to compare therapeutic effects with *WT+Control-IgG*, if appropriate. $P < 0.05$ was considered to be significant. All calculations were performed with Prism 7 (Graph Pad Software, Inc., San Diego, CA).

RESULTS

Chronological Alterations in Complement Hemolytic Activity

To investigate whether anti-C5-Ab sufficiently inhibited systemic terminal complement activities in our mouse model, serum hemolytic activity was assessed 30 minutes, 1, 3, 7, 9, and 10 days after an intravenous injection of anti-C5-Ab. As shown in Figure 2A, 1 dose of BB5.1 completely inhibited complement hemolytic activity until day 9, and this activity recovered on day 10.

Hemolytic activity was then measured in hepatic IRI with or without anti-C5-Ab immediately after; 2 and 6 hours after; and 1, 3, and 4 days after reperfusion. As shown in Figure 2B, 70%-lobar hepatic IR showed peaked serum complement activity 2 hours after reperfusion (*WT+Control-IgG*). It gradually decreased thereafter until day 4. Of note, hemolytic activity was completely inhibited by anti-C5-Ab throughout. This inhibition was confirmed by 2 different hemolytic assays.

In liver tissues after hepatic IRI, MAC deposition was observed only in some hepatocytes around the central veins (zone 3) 2 and 6 hours after reperfusion, representing the "mosaic" pattern in *WT+Control-IgG* (Figure 2C). MAC deposition was rarely observed in the other groups, except for 1 slide-in *WT+C5aR1-Ant* 6 hours after reperfusion.

Transaminase Release and Histopathological Analyses

Serum ALT was significantly lower in *WT+Anti-C5-Ab* than in *WT+Control-IgG*, which was similar to those in KO mice. As shown in Figure 3A, C5aR1-antagonist also showed significant improvements 2 and 6 hours after reperfusion. Figure 3B shows consistent histopathological improvements in lobular edema, congestion, ballooning, and hepatocellular necrosis by C5/C5aR1 inhibition in the 4 treatment groups from *WT+Control-IgG* ($P < 0.001$). Suzuki's histological scoring showed significantly ameliorated liver tissue damage in all the treatment groups from that in *WT+Control-IgG* (Figure 3C), indicating the significant protective effects of C5-KO, anti-C5-Ab, and C5aR1-antagonist against hepatic IRI.

HMGB-1 Release

To evaluate comprehensive liver damage and assess the impact of HMGB-1 release on subsequent inflammatory responses, serum HMGB-1 was measured using ELISA. Serum HMGB-1 concentrations in *WT+Control-IgG* were higher 2 hours after reperfusion than at 6 hours (Figure 4A). C5-KO, anti-C5-Ab, and C5aR1-antagonist attenuated HMGB-1 release as early as 2 hours after reperfusion, resulting in significant reductions in subsequent inflammatory responses.

Proinflammatory Cytokines and Chemokines

Alterations in proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and chemokines (CXCL-1 and CXCL-2) in liver tissues with or without the C5 regulation were analyzed by RT-PCR. Although no marked trends were observed 2 hours after reperfusion, C5-KO, anti-C5-Ab, and C5aR1-antagonist reduced the expression levels of proinflammatory cytokines and chemokines induced by IR

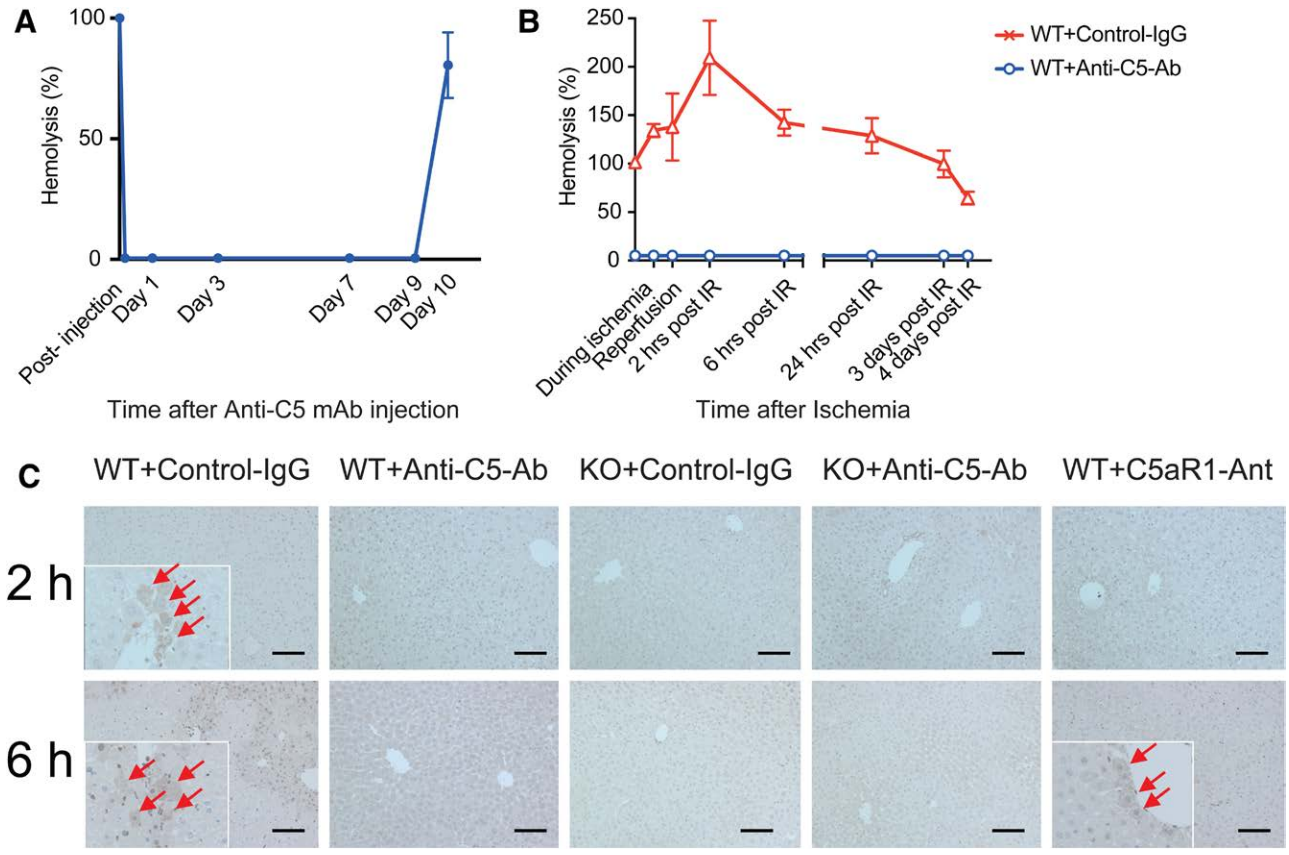

FIGURE 2. Chronological alterations in complement hemolytic activity. A, Transition of hemolytic activity in mouse sera after an intravenous injection of anti-C5-Ab. Hemolytic activity was completely inhibited immediately after the injection until d 9 and recovered by d 10 ($n=3$ mice/group, at each time point). B, Transition of hemolytic activity in mouse sera after an intravenous injection of anti-C5-Ab or control IgG in the hepatic IRI model. In *WT+Control-IgG* (the red line), hemolytic activity peaked 2 h after reperfusion, reaching up to 218% of the preischemic value. It gradually decreased thereafter to d 4. In *WT+Anti-C5-Ab* (the blue line), hemolytic activity was completely inhibited throughout the experimental period ($n=3$ mice/group, at each time point). This inhibition was confirmed by 2 different hemolytic assays. C, Representative liver sections of immunohistochemical staining for C5b-9 with the antibody recognizing mouse C5b-9 2 and 6 h after reperfusion ($n=6$ mice/group, magnification ×200). The scale bar in each panel indicates 100 μm. Arrows in enlarged images indicate hepatocytes with membrane attack complex (MAC) deposition. In *WT+Control-IgG*, MAC deposition was observed only around some central veins in 2 and 3 out of 6 slides 2 and 6 h after reperfusion, respectively. MAC deposition was not detected in the other groups, except for 1 slide-in *WT+C5aR1-Ant*, 6 h after reperfusion. C5aR1-Ant, C5a receptor 1 antagonist; IgG, immunoglobulin; IRI, ischemia/reperfusion injury; KO, knockout; MAC, membrane attack complex; WT, wild-type mice.

to less than those of the control 6 hours after reperfusion (Figure 4B).

Altered Populations in Liver-resident and Infiltrating Macrophages

Macrophages are well-known key players in the pathogenesis of hepatic IRI.[40-42] C5a has been identified as a potent macrophage chemoattractant.[43] As shown in Figure 5, F4/80+ cell numbers were significantly lower in *WT+Control-IgG* than in sham controls but were maintained in *WT+Anti-C5-Ab*, KO mice, and *WT+C5aR1-Ant* 2 hours after reperfusion. These alterations were also observed 6 hours after reperfusion. F4/80 is a representative marker for macrophages, mainly Kupffer cells (KC), and its decrease leads not only to the disruption of homeostatic balance in liver tissues but also to the activation of infiltrating macrophages/neutrophils in hepatic IR.[41]

The recruitment of CD11b+ cells in *WT+Control-IgG* did not significantly differ from that in sham controls 2 hours after reperfusion; however, they were recruited thereafter, and significantly more CD11b+ cells were evident in *WT+Control-IgG* than in sham controls in the late phase (6 h). This infiltration was significantly attenuated in *WT+Anti-C5-Ab*, KO mice, and *WT+C5aR1-Ant*. Liver macrophage subsets were previously reported to be characteristically altered by hepatic IR, that is, decreases in KCs and increases in infiltrating macrophages.[41] C5-KO, anti-C5-Ab, and C5aR1-antagonist significantly ameliorated decreases in macrophages, mainly KCs, represented by F4/80+ cells, and increases in infiltrating macrophages/neutrophils, represented by CD11b+ cells, 2 and 6 hours after reperfusion, respectively.

Platelet Aggregation in the Hepatic Microcirculation

Platelet aggregation and subsequent microcirculatory impairments are critical in the pathogenesis of hepatic IRI,[44] which is enhanced by complement activation.[45] Platelet thrombi, represented by CD41+ spots, were significantly fewer in *WT+Anti-C5-Ab*, KO mice, and *WT+C5aR1-Ant* than in *WT+Control-IgG* 2 and 6 hours after reperfusion (Figure 6). Thus, platelet aggregation occurred early after hepatic IR, and this deleterious phenomenon was significantly suppressed by C5-KO, anti-C5-Ab, and C5aR1-antagonist.

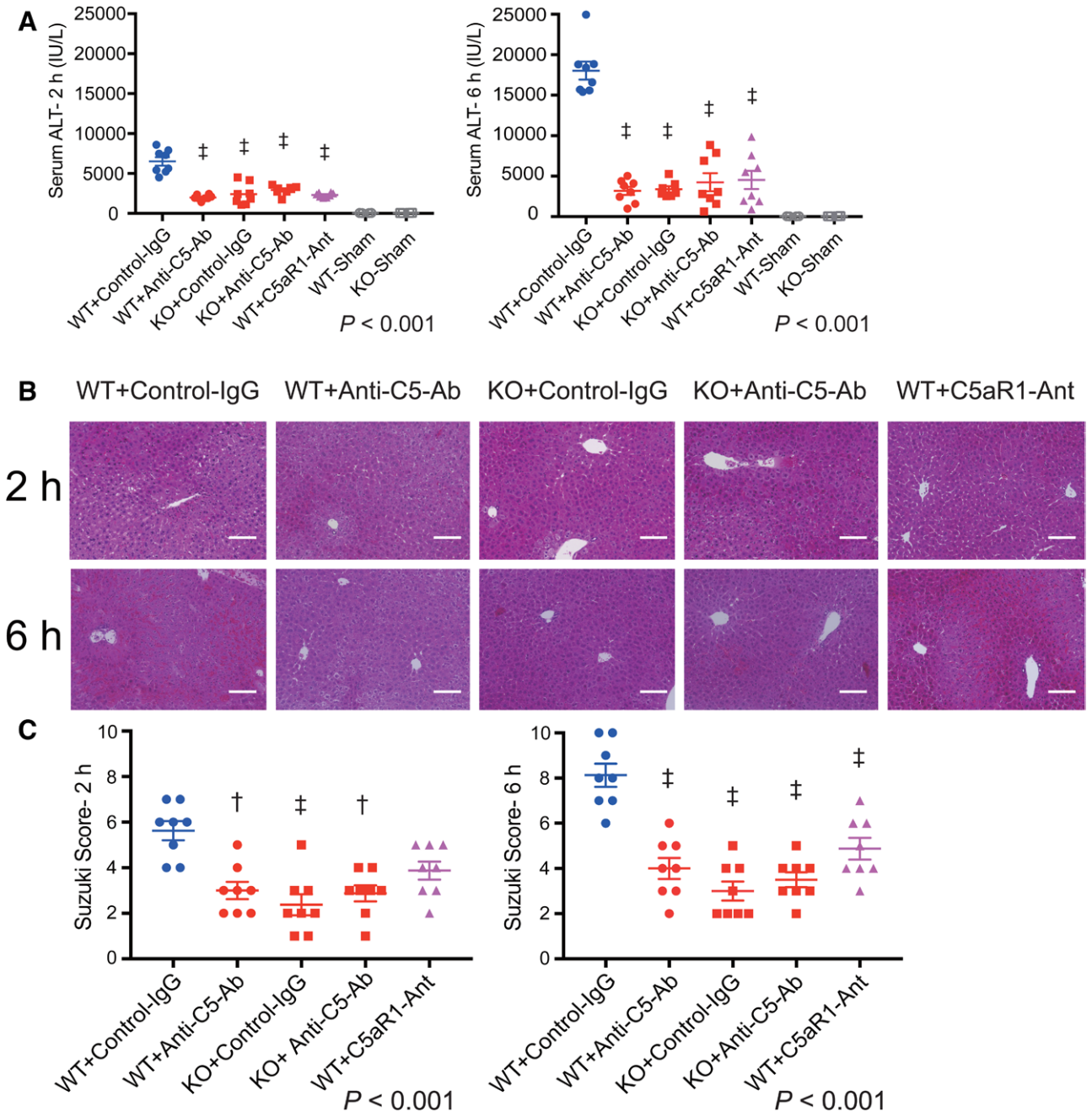

FIGURE 3. Improved hepatic IRI by C5 regulation. A, Serum ALT levels, given as indices for hepatocellular damage, were significantly reduced by C5-KO, anti-C5-Ab, and C5aR1-antagonist 2 and 6 h after reperfusion. All data are presented as the mean ± SEM. Differences among groups were assessed via a 1-way ANOVA ($P<0.001$, $n=8$ mice/group), followed by Bonferroni's posttest (‡: $P<0.001$ *vs WT+Control-IgG*), unless otherwise indicated. Graphs are invisible due to small values in sham-operated groups. B, Representative tissue sections (hematoxylin & eosin staining) of ischemic livers after reperfusion (magnification ×200). The scale bar in each panel indicates 100 μm. C, Suzuki's histological grading of IRI (intergroup difference by a 1-way ANOVA, $P<0.001$; time-point assessment by Bonferroni's posttest, †: $P<0.01$, ‡: $P<0.001$ *vs WT+Control-IgG*). These results were consistent with serum ALT release. ALT, alanine aminotransferase; C5aR1-Ant, C5a receptor 1 antagonist; IgG, immunoglobulin; IR, ischemia/reperfusion; KO, knockout; WT, wild-type.

Neutrophil Infiltration

C5a is a well-known neutrophil chemoattractant,[43] and neutrophils play a pivotal role in inflammatory responses and resultant tissue injury during hepatic IR.[46] Ly6-G+ cells, that is, activated neutrophils, were not yet recruited 2 hours after reperfusion in all groups, including *WT+Control-IgG*. The number of Ly6-G+ cells was significantly lower in *WT+Anti-C5-Ab*, KO mice, and *WT+C5aR1-Ant* than in *WT+Control-IgG* 6 hours after reperfusion (Figure 7A and B). Thus, neutrophil infiltration was significantly attenuated by total C5 inhibition and C5aR1-antagonist, although the effects of the latter were not similar to those of the former at the peak of hepatic IRI.

Oxidative Tissue Damage

The generation of reactive oxygen species (ROS) and resulting oxidative stress is central pathologies in hepatic IRI. 8-OHdG is a sensitive marker for oxidatively damaged

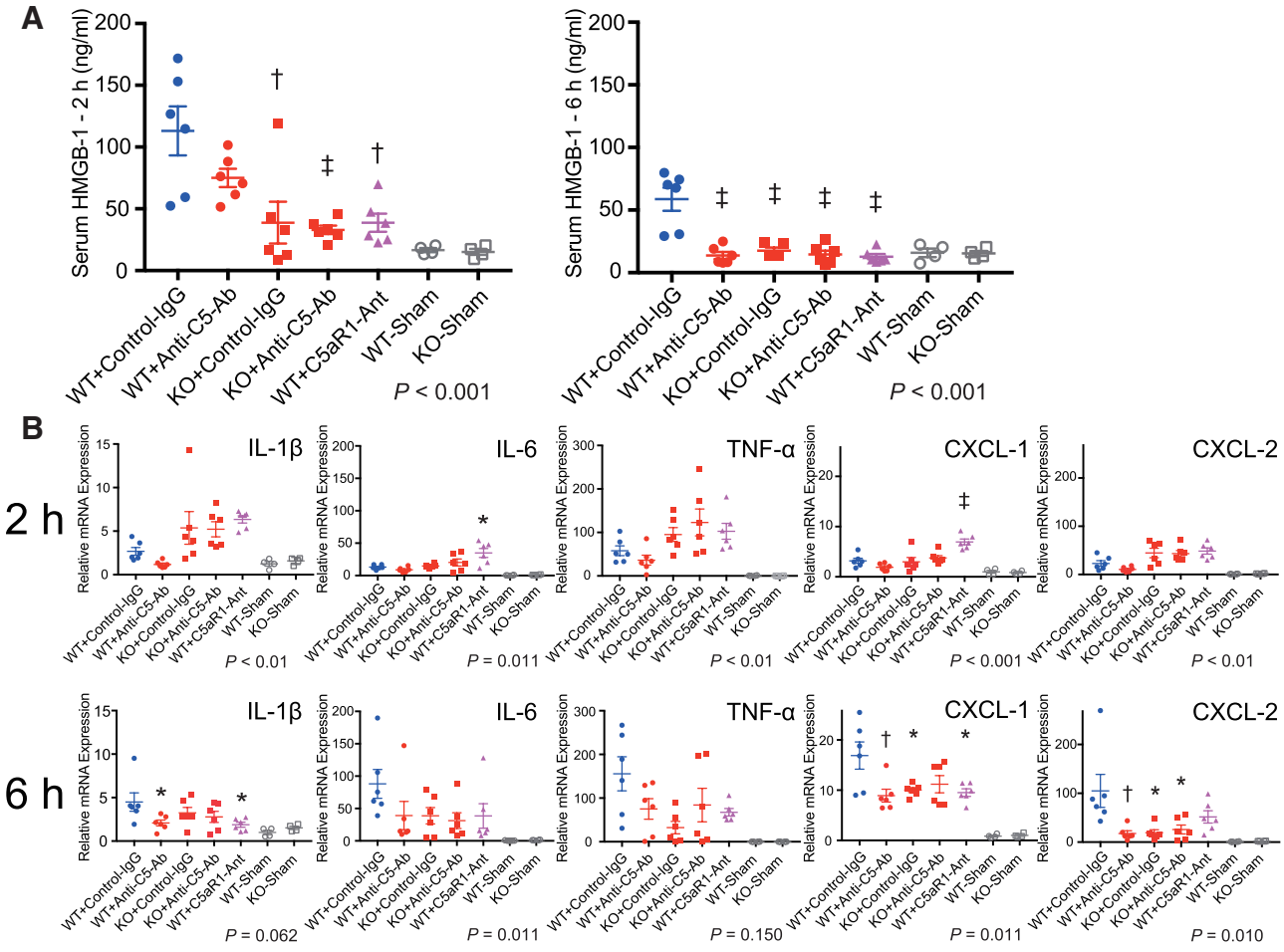

FIGURE 4. Reductions in HMGB-1 release and proinflammatory cytokines/chemokines. A, Serum HMGB-1 concentrations 2 and 6 h after hepatic ischemia/reperfusion. They were significantly reduced by C5-KO, anti-C5-Ab, and C5aR1-antagonist at both time points ($n = 6$ mice/group; intergroup difference: $P < 0.001$; time-point assessment: †: $P < 0.01$, ‡: $P < 0.001$ *vs WT+Control-IgG*). Of note, serum HMGB-1 concentrations were higher at 2 h than at 6 h. B, Quantitative reverse transcription-polymerase chain reaction analysis of proinflammatory cytokines and chemokines (IL-1β, IL-6, TNF-α, CXCL-1, and CXCL-2) 2 and 6 h after reperfusion. Although no marked trends were observed 2 h after reperfusion, C5-KO, anti-C5-Ab, and C5aR1-antagonist all significantly decreased the expression of proinflammatory cytokines and chemokines from those in the WT control group 6 h after reperfusion. Data were normalized to GAPDH gene expression ($n = 6$ mice/group). All data are presented as the mean ± SEM. Differences among the groups were assessed via a 1-way ANOVA, followed by Bonferroni's posttest (*: $P < 0.05$, †: $P < 0.01$, ‡: $P < 0.001$ *vs WT+Control-IgG*). C5aR1-Ant, C5a receptor 1 antagonist; GAPDH, glyceraldehyde-3-phosphate; HMGB-1, high-mobility group box 1 protein; IgG, immunoglobulin; KO, knockout; WT, wild-type.

DNA. As demonstrated in Figure 7C and D, 8-OHdG+ cells were partially observed at 2 hours after reperfusion. At 6 hours, *WT+Control-IgG* showed diffusely scattered positive hepatocytes panlobularly. In the other treatment groups, however, oxidative damage was significantly alleviated.

Cell Apoptosis

Since apoptosis is one of the main forms of cell death in IRI,[47] we examined whether C5 regulation affects hepatocyte apoptosis induced by IR. As shown in Figure 8A and B, ssDNA-positive cell numbers were significantly lower in *WT+Anti-C5-Ab*, KO mice, and *WT+C5aR1-Ant* than in *WT+Control-IgG* 6 hours after reperfusion ($P < 0.001$). Cleaved caspase-3 expression was also significantly lowered in *WT+Anti-C5-Ab* and *KO+Control-IgG* than in *WT+Control-IgG* at 6 hours after reperfusion (Figure 8C and D, $P < 0.001$). Collectively, these results indicate that apoptotic cell death markedly increased between 2 and 6 hours after reperfusion, which was more-significantly ameliorated by total C5 inhibition than by C5aR1 blockade at 6 hours.

DISCUSSION

In the present study, we demonstrated the therapeutic impacts of C5 and C5aR1 inhibition on hepatic IRI and elucidated the underlying mechanisms. Most previous reports investigating hepatic IRI in rodent models have focused on biological reactions at 6 hours or later after reperfusion. One novelty in the current study was, therefore, detailed assessments for acute-phase reactions, that is, just 2 hours after reperfusion. Total C5 and C5aR1 inhibition both significantly ameliorated hepatic IRI, which was characterized by the suppression of (1) platelet aggregation in the hepatic microcirculation; (2) HMGB-1 release in the early phase of reperfusion, as well as by the down-regulation of (1) infiltrating macrophages and neutrophils, (2) cytokine and chemokine release, (3) ROS generation, and (4) hepatocyte apoptosis in the late phase.

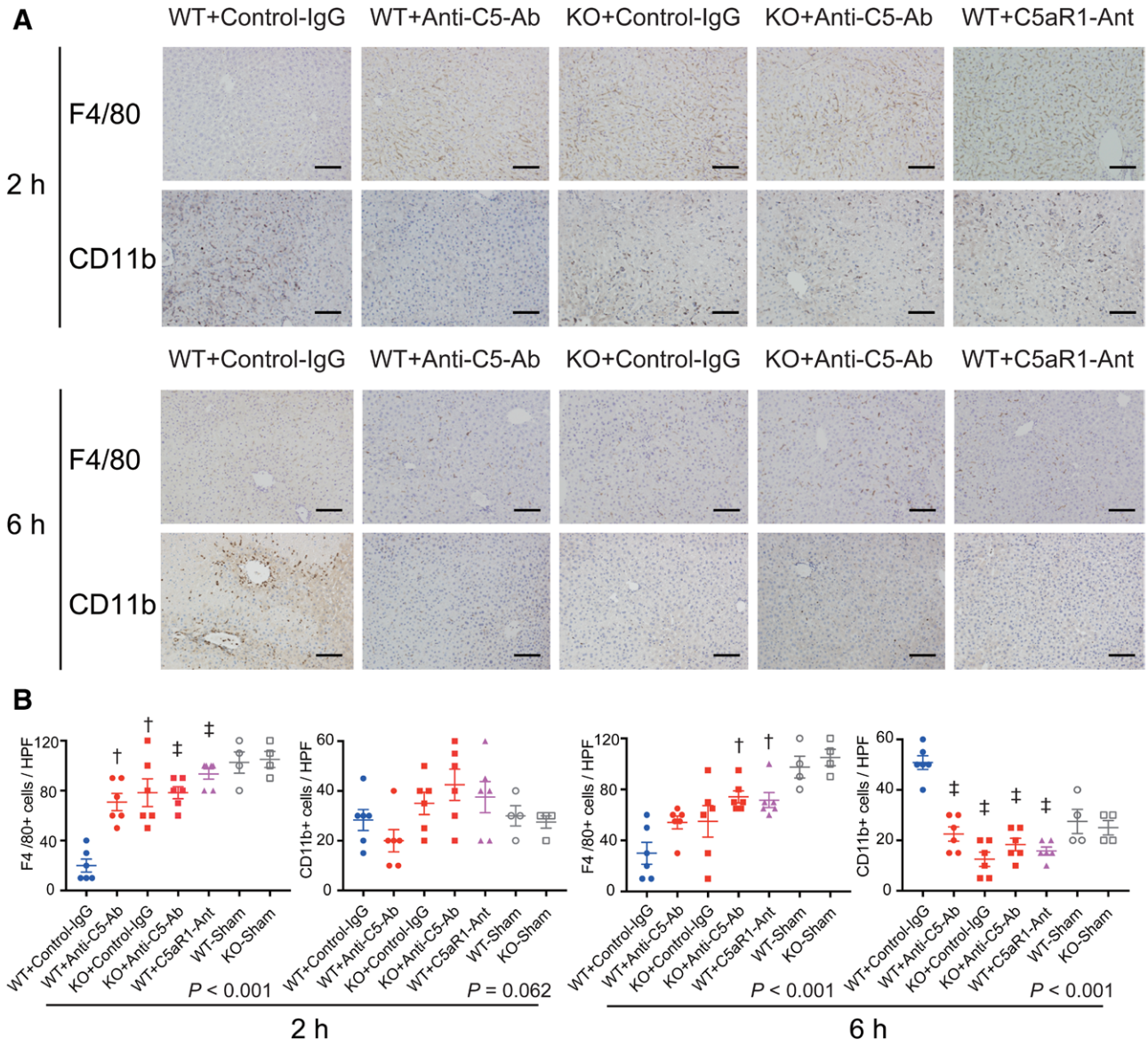

FIGURE 5. Alterations in liver macrophage populations after hepatic IRI. A, Representative tissue sections of ischemic livers stained by F4/80 and CD11b 2 and 6 h after reperfusion (magnification ×200). The scale bar in each panel indicates 100 μm. B, Quantification of hepatic F4/80+ and CD11b+ cell accumulation. The number of F4/80+ cells in *WT+Control-IgG* was lower than that in sham controls 2 and 6 h after reperfusion. In contrast, F4/80+ cell numbers were significantly maintained by C5-KO, anti-C5-Ab, and C5aR1-antagonist, particularly 2 h after reperfusion. The recruitment of CD11b+ cells was significantly greater in *WT+Control-IgG* than in sham controls 6 h after reperfusion. This distinctive alteration in liver macrophage subsets after IR was significantly alleviated by C5-KO, anti-C5-Ab, and C5aR1-antagonist 6 h after reperfusion. All data are presented as the mean ± SEM ($n = 6$ mice/group). Differences among the groups were assessed via a 1-way ANOVA at each time point, followed by Bonferroni's posttest (*: $P < 0.05$, †: $P < 0.01$, ‡: $P < 0.001$ *vs* *WT+Control-IgG*). C5aR1-Ant, C5a receptor 1 antagonist; IgG, immunoglobulin; KO, knockout; WT, wild-type.

C5a is a well-known anaphylatoxin that amplifies the activation and transmigration of leukocytes, causes basophil/mast cell degranulation, enhances vascular permeability, and promotes vasoconstriction.[48] On the other hand, C5b constitutes MAC formation that triggers inflammation, apoptosis, and cell lysis.[49] By using C5-KO animals (the complete and permanent inhibition of total C5 activity), anti-C5-Ab (complete, but transient inhibition of total C5 activity), and C5aR1-antagonist (interrupting the C5a-C5aR1 interaction only without any interference with C5b and its downstream cascades), we demonstrated that the interruption of the C5a-mediated cascade was the main mechanism underlying the protection by C5 regulation in hepatic IRI. In fact, C5aR1-antagonist was as effective as anti-C5-Ab for reducing hepatocellular damage, represented by transaminase release. In hepatic IRI, C5a mediates leukocyte activation and transmigration, resulting in hepatocyte necrosis and apoptosis. C5b and MAC then directly lyse target cells, promoting the release of damage-associated molecular patterns, cytokines, chemokines, and additional anaphylatoxins.[1,3,19] MAC deposition was not obvious, even in WT controls exposed to IRI in our model. Collectively, the present results suggest that the C5a-mediated cascade functions dominantly in the complement-mediated pathogenesis of hepatic IRI.

Although the efficacy of the C5aR1 blockade was similar to that by total C5 inhibition in the present study, previous studies demonstrated the significance of C5b-9 (MAC)

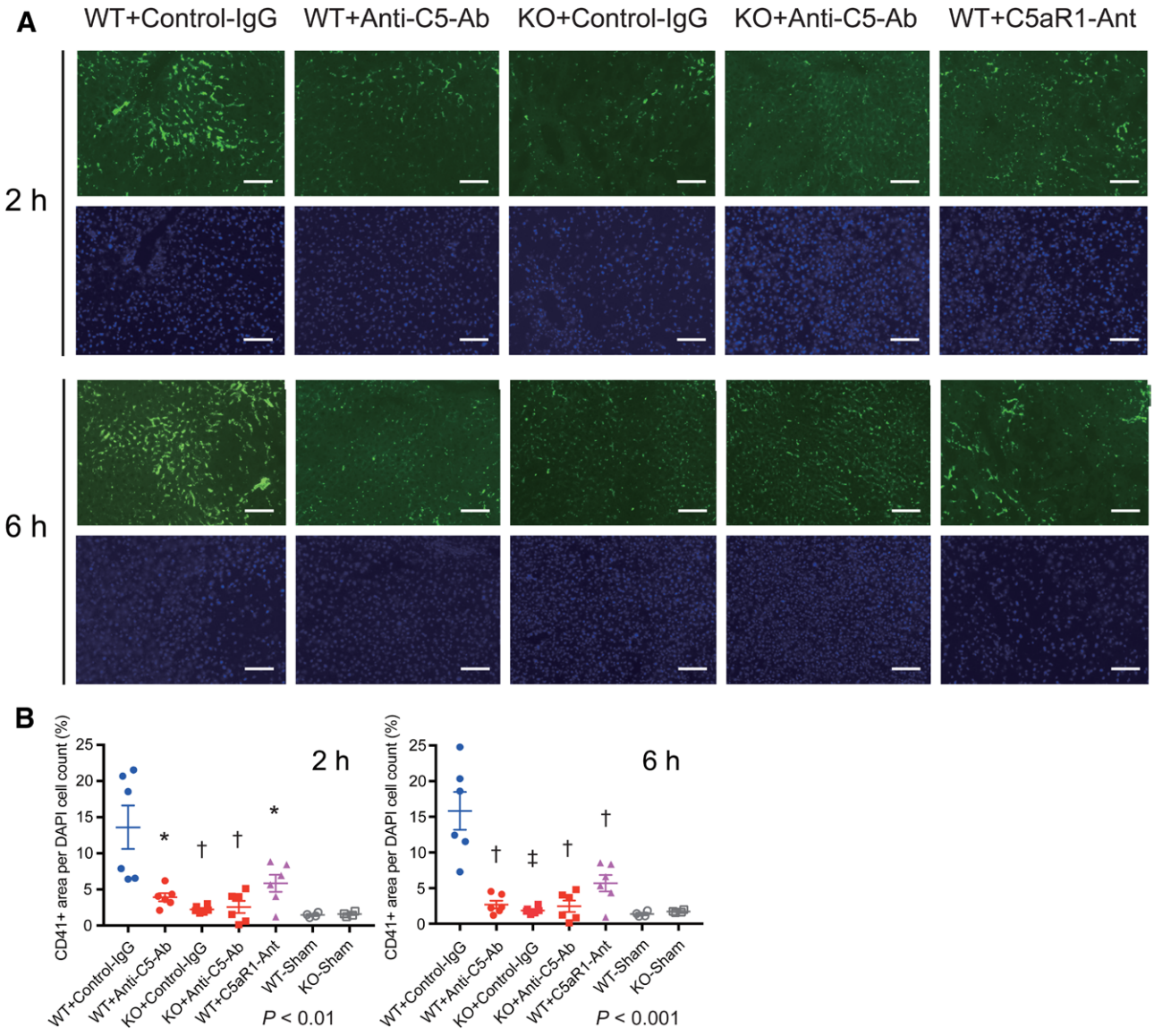

FIGURE 6. Platelet aggregation. A, Representative tissue sections of ischemic livers stained by CD41 2 and 6 h after reperfusion (magnification ×200). The scale bar in each panel indicates 100 μm. B, Quantification of intrahepatic platelet thrombi. CD41+ particles were significantly decreased by C5-KO, anti-C5-Ab, and C5aR1-antagonist from those in *WT+Control-IgG* 2 and 6 h after reperfusion. All data are presented as the mean ± SEM ($n=6$ mice/group). Differences among the groups were assessed via a 1-way ANOVA at each time point, followed by Bonferroni's posttest (*: $P<0.05$, †: $P<0.01$, ‡: $P<0.001$ *vs WT+Control-IgG*). C5aR1-Ant, C5a receptor 1 antagonist; IgG, immunoglobulin; KO, knockout; WT, wild-type.

in the pathogenesis of hepatic IRI.[1,3,19] MAC appears to trigger Nod-like Receptor Protein 3 (NLRP3) inflammasome activation, thereby promoting neutrophil recruitment,[50] IL-1β secretion, and caspase activation, which results in apoptosis.[51] In fact, the present results showed that decreased neutrophil infiltration and reduced hepatocyte apoptosis were more clearly observed following total C5 inhibition than with C5aR1 blockade, even though no significant difference existed in many parameters including transaminases. These highly suggest that C5b-9 (MAC) also plays some role in neutrophil infiltration and hepatocyte apoptosis in hepatic IRI. In comparison with anti-C5-Ab, the long-term efficacy and adverse effects of the C5aR1 blockade remain unclear. In contrast, not only short-term but also long-term efficacy as well as adverse events by humanized anti-C5-Ab, eculizumab, have been accumulated in clinical practice over the last 2 decades.

The liver is a characteristic immune organ that includes nearly 90% of tissue-resident macrophages in the whole body, well-known as KCs.[52] Therefore, KCs have been extensively investigated in diverse liver pathologies because of their significance in innate and adaptive immunities. However, the roles of KCs in hepatic IRI remain controversial.[42,53,54] A recent study reported that hepatic IR resulted in the necrotic depletion/reduction of KCs (F4/80+, CD11b–), whereas monocyte-derived macrophages (F4/80+, CD11b+), in turn, transmigrated to the liver.[41] These findings are consistent with the present results obtained from WT livers exposed to IR. The present study provided direct evidence to show that these distinctive alterations in liver macrophage subsets were significantly alleviated by total C5 and C5aR1 inhibition. Of note, C5-KO, anti-C5-Ab, and C5aR1-antagonist all significantly maintained F4/80+ cell numbers in ischemic

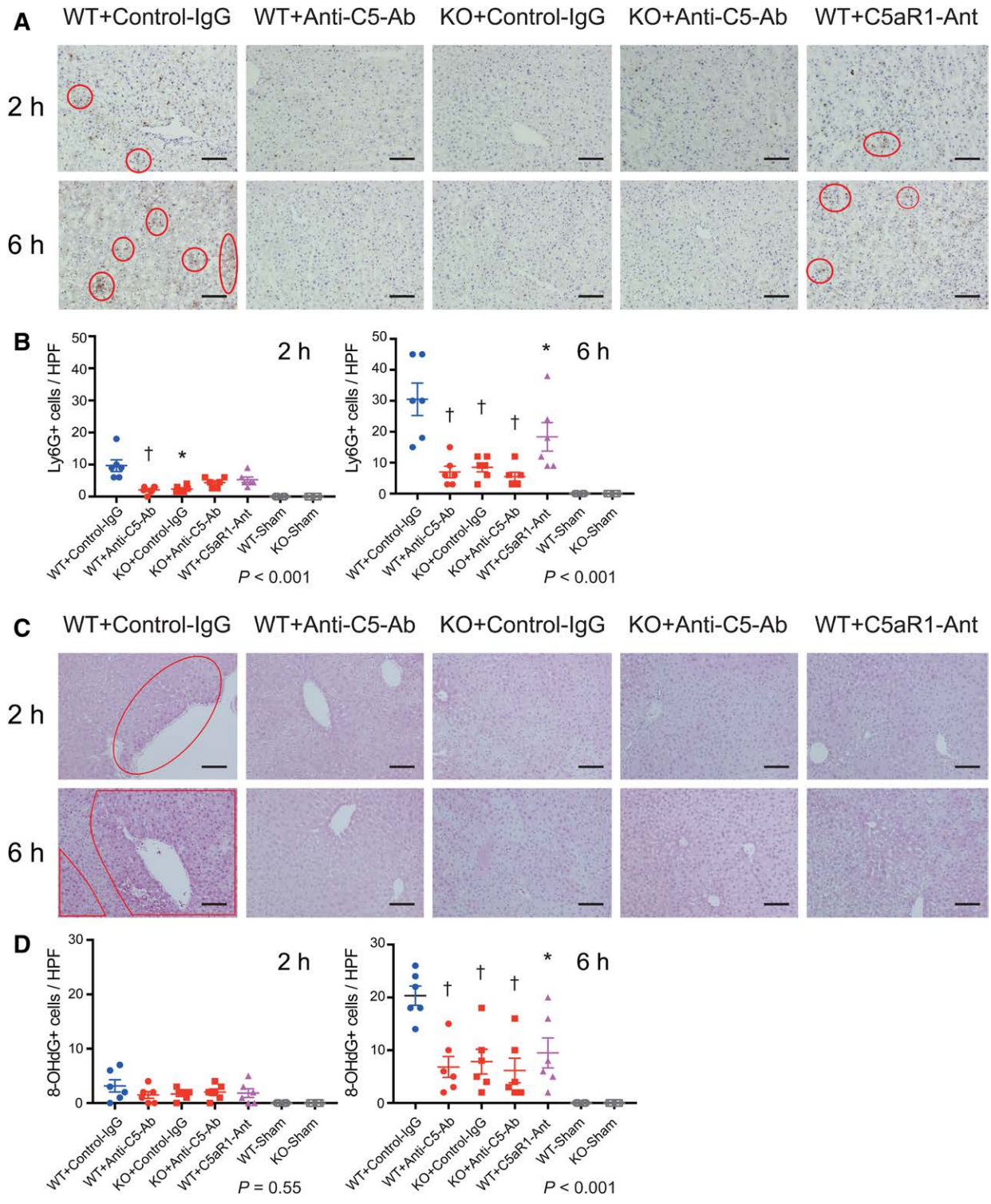

FIGURE 7. Neutrophil infiltration and oxidative stress. A, Representative liver sections stained by Ly6-G 2 and 6 h after reperfusion (magnification ×200). Aggregation of Ly6-G + cells was highlighted in red circles. The scale bar in each panel indicates 100 μm. B, Quantification of Ly6-G accumulation into the hepatic parenchyma. The infiltration of Ly6-G + cells became evident 6 h after reperfusion in *WT+Control-IgG* and was significantly decreased by C5-KO, anti-C5-Ab, and C5aR1-antagonist from that in *WT+Control-IgG*. C, Representative liver sections stained by the oxidative stress marker, 8-OHdG 2 and 6 h after reperfusion ($n = 6$ mice/group; magnification ×200). The scale bar in each panel indicates 100 μm. The staining intensity of 8-OHdG was high in nuclei with severe DNA damage. Two h after reperfusion, 8-OHdG+ cells were partially scattered around the portal (zone-1) or central venules (zone 3) in 2 out of 6 slides in *WT+Control-IgG*, as highlighted by red circle. This result was only observed in 0-1 out of 6 slides in the other groups. At 6 h, all slides in *WT+Control-IgG* exhibited diffusely scattered positive hepatocytes panlobularly, as outlined by red lines. In the other groups, this change was only observed in 0-2 out of 6 slides. D, Quantification of oxidative stress. The 8-OHdG+ hepatocytes became evident 6 h after reperfusion in *WT+Control-IgG*, which were significantly higher than those in the other groups. All data are presented as the mean ± SEM ($n = 6$ mice/group). Differences among the groups were assessed via a 1-way ANOVA at each time point, followed by Bonferroni's posttest (*: $P < 0.05$, †: $P < 0.01$ *vs WT+Control-IgG*). C5aR1-Ant, C5a receptor 1 antagonist; IgG, immunoglobulin; KO, knockout; Ly6-G, lymphocyte antigen 6 complex locus G; WT, wild-type; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

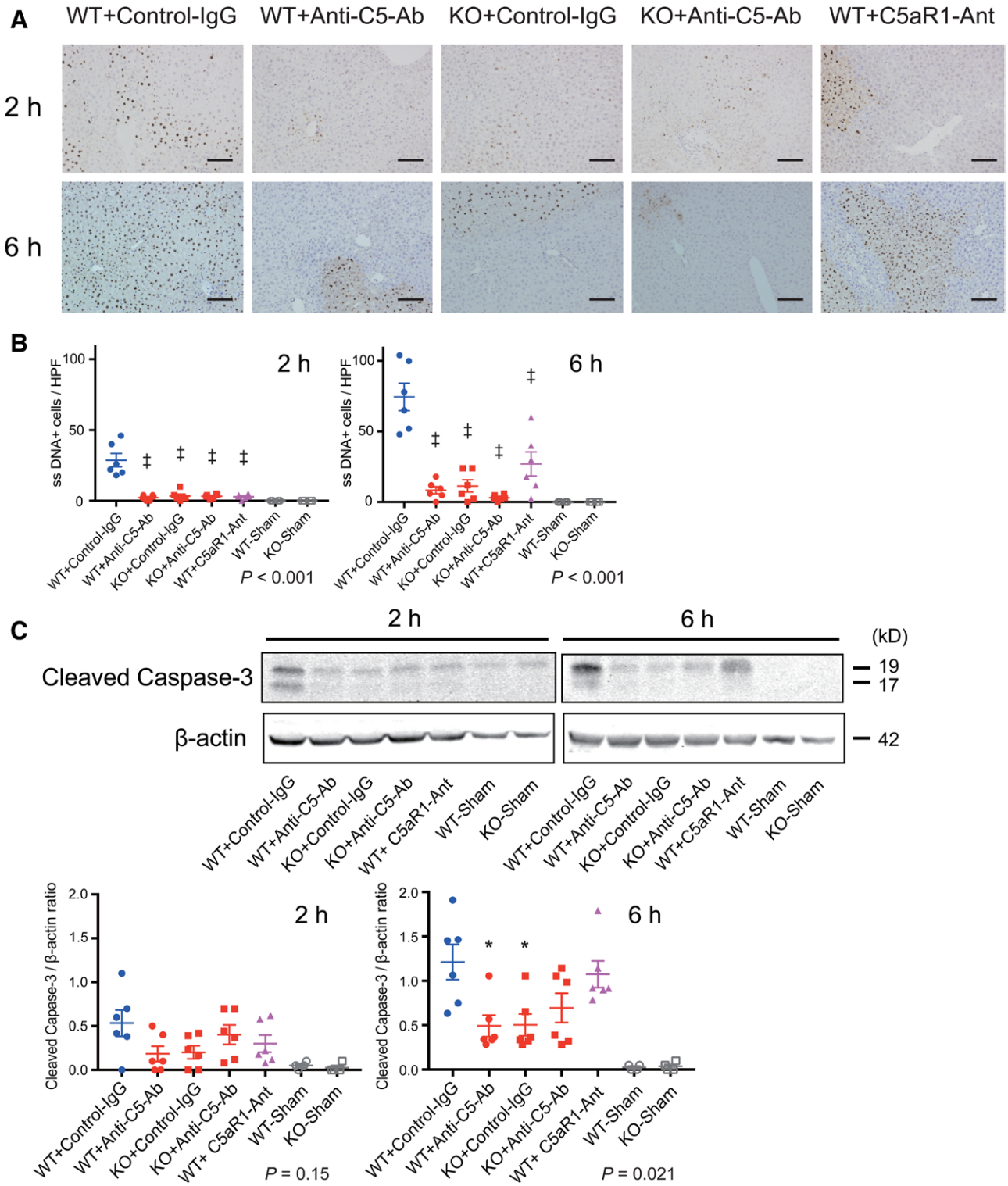

FIGURE 8. Apoptotic cell death. A, Representative ssDNA (single-stranded DNA) staining of liver sections 2 and 6 h after reperfusion (magnification ×200). The scale bar in each panel indicates 100 μm. B, Quantification of intrahepatic apoptosis. ssDNA+ cell numbers were higher in *WT+Control-IgG* at 6 h than at 2 h after reperfusion. However, they were significantly decreased by C5-KO, anti-C5-Ab, and C5aR1-antagonist. C, Western blot analysis for cleaved caspase-3 expression 2 and 6 h after reperfusion. Protein expression was quantified with ImageJ Software and normalized to that of β-actin. Cleaved caspase-3 was strongly expressed in *WT+Control-IgG* 6 h after reperfusion and was significantly decreased by anti-C5-Ab and C5-KO. All data are presented as the mean ± SEM ($n = 6$ mice/group). Differences among groups were assessed via a 1-way ANOVA, followed by Bonferroni's posttest (*: $P < 0.05$, ‡: $P < 0.001$ *vs WT+Control-IgG*). C5aR1-Ant, C5a receptor 1 antagonist; IgG, immunoglobulin; KO, knockout; ssDNA, single-stranded DNA; WT, wild-type.

livers and significantly attenuated CD11b+ cell infiltration after reperfusion. Based on the similar impact of all 3 C5 and C5a interventions on macrophage alterations, it is plausible that C5a-mediated signals play pivotal roles in shifting macrophage populations after hepatic IRI. Although a "chicken-or-the-egg" argument remains as to whether the altered macrophage response was the cause or merely a result of improved hepatic IRI, C5/C5aR1 inhibition significantly diminished overall hepatic IRI with the significant maintenance of the physiological population in liver macrophages from the early phase.

Microcirculatory disturbance is a detrimental phenomenon in hepatic IRI, resulting in an insufficient energy supply and impaired ATP restoration upon reperfusion.[47] Ischemia induces the expression of tissue factors on monocytes and vascular endothelia, which increases thrombin generation. Fibrin and platelet thrombosis subsequently form early in hepatic IRI.[55] Moreover, increasing attention is being paid to "signal crosstalk" between the complement and coagulation systems[55]: platelet activation is enhanced by several complement factors.[45] The initiator of the classical pathway, C1q, is involved in the regulation of platelet activation by upregulating P-selectin, and C3a and C5a also activate platelets through their receptors on the platelet surface.[56] C5b-9 induces the release of α-granules and microparticles from platelets and stimulates their procoagulant activities by augmenting prothrombinase.[57] Recent clinical trials demonstrated that C5 inhibition markedly attenuated thrombotic microangiopathy (TMA)-related symptoms in patients with paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome.[10,11] These functions of C5a and C5b-9 on platelets explain, at least in part, our result showing that C5 and C5aR1 inhibition significantly suppressed the formation of platelet thrombi from the early to late stages of reperfusion.

One of the limitations of the present study is that C5aR1-antagonist, PMX53, does not block a minor type of C5aR, known as C5L2.[48,58] C5L2 is expressed on similar cells that express the major type of C5aR, CD88, and a functional link between the 2 receptors has been suggested. However, C5L2 expression levels were markedly lower than those of CD88,[58] and C5L2 may merely function as a decoy receptor. Thus, it is generally accepted that CD88 primarily exerts effector functions, and clinical trials for C5a inhibition have evaluated the therapeutic antagonism of CD88 only.[59] Therefore, it appears to be reasonable to focus on CD88 inhibition. Another limitation may be the lack of a group with MAC inhibition only because a specific inhibitor of MAC was not commercially available. We herein analyzed MAC formation using immunohistochemistry and found that MAC deposition was minimal, even in WT livers after IR. These results suggest that the C5b-mediated cascade plays a less important role than the C5a-mediated cascade in hepatic IRI.

In conclusion, the anti-C5 antibody significantly attenuated hepatic IRI, predominantly via the C5a-mediated cascade. However, no complement-targeting intervention has yet been applied in clinical practice. Based on its efficacy, clinical availability, and controllability, the anti-C5 antibody and C5aR1-antagonist may provide a novel therapeutic strategy for various pathologies, including liver surgeries.

ACKNOWLEDGMENTS

The authors thank Hajime Kitamura and Etsuko Kitano, Department of Clinical Laboratory Science, Kobe Tokiwa University, and the Center for Anatomical, Pathological, and Forensic Medical Research, Kyoto University Graduate School of Medicine, for their skillful technical assistance.

REFERENCES

1. Zhang J, Hu W, Xing W, et al. The protective role of CD59 and pathogenic role of complement in hepatic ischemia and reperfusion injury. *Am J Pathol.* 2011;179:2876–2884.
2. Buis CI, Hoekstra H, Verdonk RC, et al. Causes and consequences of ischemic-type biliary lesions after liver transplantation. *J Hepatobiliary Pancreat Surg.* 2006;13:517–524.
3. Fondevila C, Shen XD, Tsuchihashi S, et al. The membrane attack complex (C5b-9) in liver cold ischemia and reperfusion injury. *Liver Transpl.* 2008;14:1133–1141.
4. Nagai S, Yoshida A, Facciuto M, et al. Ischemia time impacts recurrence of hepatocellular carcinoma after liver transplantation. *Hepatology.* 2015;61:895–904.
5. Jaeschke H, Farhood A, Bautista AP, et al. Complement activates Kupffer cells and neutrophils during reperfusion after hepatic ischemia. *Am J Physiol.* 1993;264(4 Pt 1):G801–G809.
6. Diepenhorst GM, van Gulik TM, Hack CE. Complement-mediated ischemia-reperfusion injury: lessons learned from animal and clinical studies. *Ann Surg.* 2009;249:889–899.
7. Heijnen BH, Straatsburg IH, Padilla ND, et al. Inhibition of classical complement activation attenuates liver ischaemia and reperfusion injury in a rat model. *Clin Exp Immunol.* 2006;143:15–23.
8. Lehmann TG, Koeppel TA, Münch S, et al. Impact of inhibition of complement by sCR1 on hepatic microcirculation after warm ischemia. *Microvasc Res.* 2001;62:284–292.
9. Howard JF Jr, Utsugisawa K, Benatar M, et al; REGAIN Study Group. Safety and efficacy of eculizumab in anti-acetylcholine receptor antibody-positive refractory generalised myasthenia gravis (REGAIN): a phase 3, randomised, double-blind, placebo-controlled, multicentre study. *Lancet Neurol.* 2017;16:976–986.
10. Hillmen P, Young NS, Schubert J, et al. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med.* 2006;355:1233–1243.
11. Legendre CM, Licht C, Muus P, et al. Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *N Engl J Med.* 2013;368:2169–2181.
12. Misawa S, Kuwabara S, Sato Y, et al. Safety and efficacy of eculizumab in Guillain-Barré syndrome: a multicentre, double-blind, randomised phase 2 trial. *Lancet Neurol*. 2018;4422:1–11.
13. Pittock SJ, Berthele A, Fujihara K, et al. Eculizumab in aquaporin-4-positive neuromyelitis optica spectrum disorder. *N Engl J Med.* 2019;381:614–625.
14. Charlton M, Levitsky J, Aqel B, et al. International liver transplantation society consensus statement on immunosuppression in liver transplant recipients. *Transplantation.* 2018;102:727–743.
15. Böhmig GA, Wahrmann M, Eskandary F, et al. Novel approaches to block complement. *Transplantation.* 2018;102:1837–1843.
16. Berger M, Lefaucheur C, Jordan SC. Update on C1 esterase inhibitor in human solid organ transplantation. *Transplantation.* 2019;103:1763–1775.
17. Thomas TC, Rollins SA, Rother RP, et al. Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv. *Mol Immunol*. 1996;33:1389–1401.
18. Arumugam TV, Woodruff TM, Stocks SZ, et al. Protective effect of a human C5a receptor antagonist against hepatic ischaemia-reperfusion injury in rats. *J Hepatol.* 2004;40:934–941.
19. Marshall KM, He S, Zhong Z, et al. Dissecting the complement pathway in hepatic injury and regeneration with a novel protective strategy. *J Exp Med.* 2014;211:1793–1805.
20. Zhou W, Farrar CA, Abe K, et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. *J Clin Invest.* 2000;105:1363–1371.
21. De Vries B, Matthijsen RA, Wolfs TG, et al. Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition of late apoptosis and inflammation. *Transplantation.* 2003;75:375–382.

22. Peng Q, Wu W, Wu KY, et al. The C5a/C5aR1 axis promotes progression of renal tubulointerstitial fibrosis in a mouse model of renal ischemia/reperfusion injury. *Kidney Int.* 2019;96:117–128.
23. Datta G, Fuller BJ, Davidson BR. Molecular mechanisms of liver ischemia reperfusion injury: insights from transgenic knockout models. *World J Gastroenterol.* 2013;19:1683–1698.
24. Zhai Y, Busuttil RW, Kupiec-Weglinski JW. Liver ischemia and reperfusion injury: new insights into mechanisms of innate-adaptive immune-mediated tissue inflammation. *Am J Transplant.* 2011;11:1563–1569.
25. Hirao H, Uchida Y, Kadono K, et al. The protective function of galectin-9 in liver ischemia and reperfusion injury in mice. *Liver Transpl.* 2015;21:969–981.
26. He S, Atkinson C, Evans Z, et al. A role for complement in the enhanced susceptibility of steatotic livers to ischemia and reperfusion injury. *J Immunol.* 2009;183:4764–4772.
27. Uchida Y, Ke B, Freitas MC, et al. T-cell immunoglobulin mucin-3 determines severity of liver ischemia/reperfusion injury in mice in a TLR4-dependent manner. *Gastroenterology.* 2010;139:2195–2206.
28. Wang Y, Rollins SA, Madri JA, et al. Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. *Proc Natl Acad Sci U S A.* 1995;92:8955–8959.
29. Morgan M, Bulmer AC, Woodruff TM, et al. Pharmacokinetics of a C5a receptor antagonist in the rat after different sites of enteral administration. *Eur J Pharm Sci.* 2008;33:390–398.
30. Xu GL, Chen J, Yang F, et al. C5a/C5aR pathway is essential for the pathogenesis of murine viral fulminant hepatitis by way of potentiating Fgl2/fibroleukin expression. *Hepatology.* 2014;60:114–124.
31. Strey CW, Markiewski M, Mastellos D, et al. The proinflammatory mediators C3a and C5a are essential for liver regeneration. *J Exp Med.* 2003;198:913–923.
32. Strachan AJ, Shiels IA, Reid RC, et al. Inhibition of immune-complex mediated dermal inflammation in rats following either oral or topical administration of a small molecule C5a receptor antagonist. *Br J Pharmacol.* 2001;134:1778–1786.
33. Kumar V, Mahato S, Munshi A, et al. PPInS: a repository of protein-protein interaction sitesbase. *Sci Rep.* 2018;8:12453.
34. Wang HB, Ghiran I, Matthaei K, et al. Airway eosinophils: allergic inflammation recruited professional antigen-presenting cells. *J Immunol.* 2007;179:7585–7592.
35. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, et al. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. *Transplantation.* 1993;55:1265–1272.
36. Tsung A, Tohme S, Billiar TR. High-mobility group box-1 in sterile inflammation. *J Intern Med.* 2014;276:425–443.
37. Tsung A, Klune JR, Zhang X, et al. HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. *J Exp Med.* 2007;204:2913–2923.
38. Colletti LM, Kunkel SL, Walz A, et al. The role of cytokine networks in the local liver injury following hepatic ischemia/reperfusion in the rat. *Hepatology.* 1996;23:506–514.
39. Lentsch AB, Yoshidome H, Cheadle WG, et al. Chemokine involvement in hepatic ischemia/reperfusion injury in mice: roles for macrophage inflammatory protein-2 and KC. *Hepatology.* 1998;27:1172–1177.
40. Tejima K, Arai M, Ikeda H, et al. Ischemic preconditioning protects hepatocytes via reactive oxygen species derived from Kupffer cells in rats. *Gastroenterology.* 2004;127:1488–1496.
41. Yue S, Zhou H, Wang X, et al. Prolonged ischemia triggers necrotic depletion of tissue-resident macrophages to facilitate inflammatory immune activation in liver ischemia reperfusion injury. *J Immunol.* 2017;198:3588–3595.
42. Raptis DA, Limani P, Jang JH, et al. GPR120 on Kupffer cells mediates hepatoprotective effects of ω3-fatty acids. *J Hepatol.* 2014;60:625–632.
43. Monk PN, Scola AM, Madala P, et al. Function, structure and therapeutic potential of complement C5a receptors. *Br J Pharmacol.* 2007;152:429–448.
44. Yadav SS, Howell DN, Steeber DA, et al. P-selectin mediates reperfusion injury through neutrophil and platelet sequestration in the warm ischemic mouse liver. *Hepatology.* 1999;29:1494–1502.
45. Speth C, Rambach G, Würzner R, et al. Complement and platelets: mutual interference in the immune network. *Mol Immunol.* 2015;67:108–118.
46. Tan Z, Jiang R, Wang X, et al. RORγt+IL-17+ neutrophils play a critical role in hepatic ischemia-reperfusion injury. *J Mol Cell Biol.* 2013;5:143–146.
47. Zhai Y, Petrowsky H, Hong JC, et al. Ischaemia-reperfusion injury in liver transplantation–from bench to bedside. *Nat Rev Gastroenterol Hepatol.* 2013;10:79–89.
48. Sarma JV, Ward PA. New developments in C5a receptor signaling. *Cell Health Cytoskelet.* 2012;4:73–82.
49. Horiuchi T, Tsukamoto H. Complement-targeted therapy: development of C5- and C5a-targeted inhibition. *Inflamm Regen.* 2016;36:11.
50. Inoue Y, Shirasuna K, Kimura H, et al. NLRP3 regulates neutrophil functions and contributes to hepatic ischemia-reperfusion injury independently of inflammasomes. *J Immunol.* 2014;192:4342–4351.
51. Triantafilou K, Hughes TR, Triantafilou M, et al. The complement membrane attack complex triggers intracellular Ca2+ fluxes leading to NLRP3 inflammasome activation. *J Cell Sci.* 2013;126(Pt 13):2903–2913.
52. Li P, He K, Li J, et al. The role of Kupffer cells in hepatic diseases. *Mol Immunol.* 2017;85:222–229.
53. Devey L, Ferenbach D, Mohr E, et al. Tissue-resident macrophages protect the liver from ischemia reperfusion injury via a heme oxygenase-1-dependent mechanism. *Mol Ther.* 2009;17:65–72.
54. Ellett JD, Atkinson C, Evans ZP, et al. Murine Kupffer cells are protective in total hepatic ischemia/reperfusion injury with bowel congestion through IL-10. *J Immunol.* 2010;184:5849–5858.
55. Ekdahl KN, Teramura Y, Hamad OA, et al. Dangerous liaisons: complement, coagulation, and kallikrein/kinin cross-talk act as a linchpin in the events leading to thromboinflammation. *Immunol Rev.* 2016;274:245–269.
56. Martel CJ, Agger EM, Poulsen JJ, et al. CAF01 potentiates immune responses and efficacy of an inactivated influenza vaccine in ferrets. *PLoS One.* 2011;6:e22891.
57. Wiedmer T, Sims PJ. Participation of protein kinases in complement C5b-9-induced shedding of platelet plasma membrane vesicles. *Blood.* 1991;78:2880–2886.
58. Woodruff TM, Nandakumar KS, Tedesco F. Inhibiting the C5-C5a receptor axis. *Mol Immunol.* 2011;48:1631–1642.
59. Ricklin D, Mastellos DC, Reis ES, et al. The renaissance of complement therapeutics. *Nat Rev Nephrol.* 2018;14:26–47.