Abstract

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 (KO, B10D2/oSn) and their corresponding wild-type mice (WT, B10D2/nSn) were exposed to 90-minute partial (70%) hepatic IR with either anti mouse-C5 monoclonal antibody (BB5.1) or corresponding control immunoglobulin (IgG) administration 30 min prior to ischemia. C5a-receptor 1 (C5aR1) 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 HMGB-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. C5aR1-antagonist was as effective as anti-C5-Ab for reducing transaminases.

Conclusion: 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.

Figure Legends

Figure 1

Schematic illustration of the experimental design. C5-knockout (KO, B10D2/oSn) and their corresponding wild-type mice (WT, B10D2/nSn) were exposed to partial (70%) hepatic ischemia of the left and median lobes for 90 minutes, followed by reperfusion. IgG1 isotype control (clone MOPC-21), anti-C5-Ab (BB5.1), or C5aR1-antagonist (PMX53) was administered 30 minutes before ischemia.

Abbreviations: WT, wild-type; IgG, KO, knockout; immunoglobulin; C5aR1-ant, C5a receptor 1 antagonist.

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 Day 9, and recovered by Day 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 hours after reperfusion, reaching up to 218% of the pre-ischemic value. It gradually decreased thereafter to Day 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 two different hemolytic assays.

(C) Representative liver sections of immunohistochemical staining for C5b-9 with the antibody recognizing mouse C5b-9 2 and 6 hours 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 hours after reperfusion, respectively. MAC deposition was not detected in the other groups, except for one slide in *WT+C5aR1-Ant*, 6 hours after reperfusion.

Abbreviations: WT, wild-type mice; KO, knockout; IgG, immunoglobulin; C5aR1-

ant, C5a receptor 1 antagonist; IRI, ischemia / reperfusion injury; MAC, membrane attack complex.

Figure 3

Improved hepatic IRI by C5 regulation

(A) Serum ALT levels, given as indices for hepatocellular damage, were significantly reduced by C5-knockout, anti-C5-Ab, and C5aR1-antagonist 2 and 6 hours after reperfusion. All data are presented as the mean ± SEM. Differences among groups were assessed via a one-way ANOVA (P <0.001, n =8 mice/group), followed by Bonferroni's post-test (‡: 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 (inter-group difference by a one-way

ANOVA, *P* < 0.001; time-point assessment by Bonferroni's post-test, †: *P* <0.01, ‡: *P* < 0.001 *vs*. *WT+Control-IgG*). These results were consistent with serum ALT release.

Abbreviations: WT, wild-type; KO, knockout; IgG, immunoglobulin; C5aR1-ant, C5a receptor 1 antagonist; IR, ischemia/reperfusion; ALT, alanine aminotransferase; SEM, standard error of the mean; ANOVA, analysis of variance.

Figure 4

Reductions in HMGB-1 release and proinflammatory cytokines/chemokines

(A) Serum HMGB-1 concentrations 2 and 6 hours after hepatic ischemia/reperfusion. They were significantly reduced by C5-knockout, anti-C5-Ab, and C5aR1-antagonist at both time points (*n* = 6 mice/group; inter-group 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

hours than at 6 hours.

(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 hours after reperfusion. Although no marked trends were observed 2 hours after reperfusion, C5-knockout, anti-C5-Ab, and C5aR1-antagonist all significantly decreased the expression of proinflammatory cytokines and chemokines from those in the WT control group 6 hours 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 one-way ANOVA, followed by Bonferroni's post-test (*: $P < 0.05$, †: $P < 0.01$, ‡: $P < 0.001$ *vs*. *WT+Control-IgG*).

Abbreviations: HMGB-1, high-mobility group box 1 protein; WT, wild-type; IgG, immunoglobulin; KO, knockout; C5aR1-ant, C5a receptor 1 antagonist; GAPDH, glyceraldehyde-3-phosphate; SEM, standard error of the mean; ANOVA, analysis of variance.

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 hours 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 hours after reperfusion. In contrast, F4/80+ cell numbers were significantly maintained by C5-knockout, anti-C5-Ab, and C5aR1-antagonist, particularly 2 hours after reperfusion. The recruitment of CD11b+ cells was significantly greater in *WT+Control-IgG* than in sham controls 6 hours after reperfusion. This distinctive alteration in liver macrophage subsets after IR was significantly alleviated by C5-knockout, anti-C5-Ab, and C5aR1-antagonist 6 hours after reperfusion.

All data are presented as the mean ± SEM (*n* = 6 mice/group). Differences

among the groups were assessed via a one-way ANOVA at each time point, followed by Bonferroni's post-test (*: $P < 0.05$, †: $P < 0.01$, ‡: $P < 0.001$ *vs*. *WT+Control-IgG*).

Abbreviations: WT, wild-type; IgG, immunoglobulin; KO, knockout; C5aR1-ant, C5a receptor 1 antagonist; SEM, standard error of the mean; ANOVA, analysis of variance.

Figure 6

Platelet aggregation

(A) Representative tissue sections of ischemic livers stained by CD41 2 and 6 hours 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-knockout, anti-C5-Ab, and C5aR1-antagonist from those in *WT+Control-IgG* 2 and 6 hours after reperfusion.

All data are presented as the mean ± SEM (n = 6 mice/group). Differences

among the groups were assessed via a one-way ANOVA at each time point, followed by Bonferroni's post-test (*: $P < 0.05$, †: $P < 0.01$, ‡: $P < 0.001$ *vs*. *WT+Control-IgG*).

Abbreviations: WT, wild-type; IgG, immunoglobulin; KO, knockout; C5aR1-ant, C5a receptor 1 antagonist; SEM, standard error of the mean; ANOVA, analysis of variance.

Figure 7

Neutrophil infiltration and oxidative stress

(A) Representative liver sections stained by Ly6-G 2 and 6 hours 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 Ly6G + cells became evident 6 hours after reperfusion in *WT+Control-IgG*, and was significantly decreased by C5-knockout, 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 hours 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 hours 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 hours, 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 hours 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 one-way ANOVA at each time point, followed by Bonferroni's post-test (*: $P < 0.05$, †: $P < 0.01$ *vs*. *WT+Control-IgG*).

Abbreviations: WT, wild-type; IgG, immunoglobulin; KO, knockout; C5aR1-ant, C5a receptor 1 antagonist; Ly6-G, lymphocyte antigen 6 complex locus G; SEM, standard error of the mean; ANOVA, analysis of variance; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

Figure 8

Apoptotic cell death

(A) Representative ssDNA (single-stranded DNA) staining of liver sections 2 and 6 hours 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 hours than at 2 hours after reperfusion. However, they were significantly decreased by C5-knockout, anti-C5-Ab, and C5aR1-antagonist.

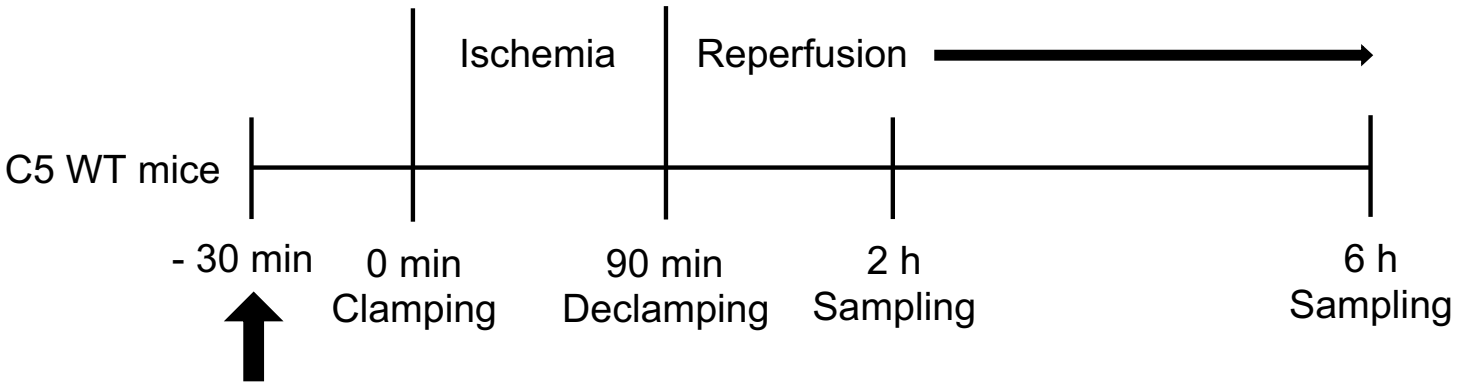
(C) Western blot analysis for cleaved caspase-3 expression 2 and 6 hours after reperfusion. Protein expression was quantified with ImageJ Software and

normalized to that of β-actin. Cleaved caspase-3 was strongly expressed in *WT+Control-IgG* 6 hours after reperfusion, and was significantly decreased by anti-C5-Ab and C5-knockout.

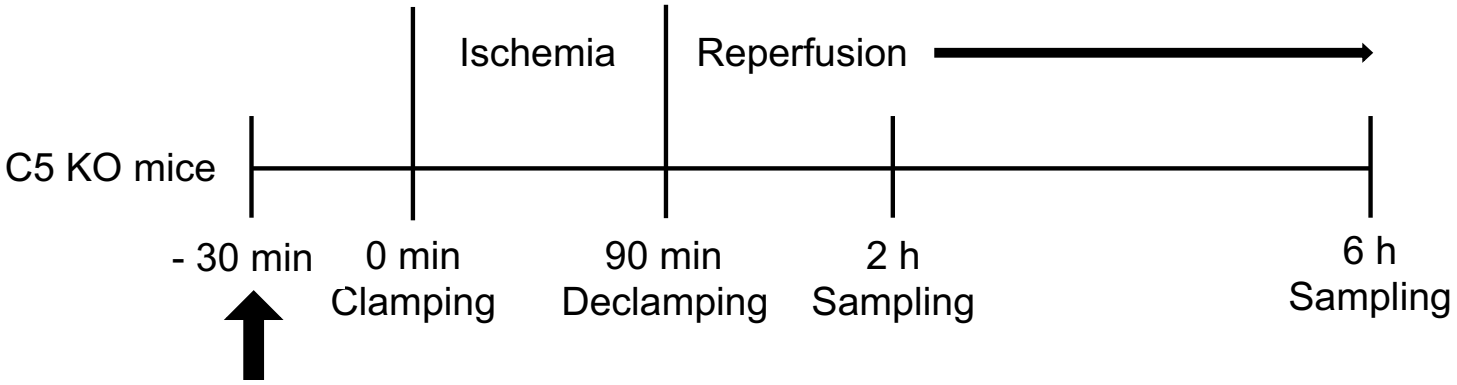
All data are presented as the mean ± SEM (*n* = 6 mice/group). Differences among groups were assessed via a one-way ANOVA, followed by Bonferroni's post-test (*: $P < 0.05$, ‡: $P < 0.001$ *vs*. *WT+Control-IgG*).

Abbreviations: ssDNA, single-stranded DNA; WT, wild-type; IgG, immunoglobulin; KO, knockout; C5aR1-ant, C5a receptor 1 antagonist; SEM, standard error of the mean; ANOVA, analysis of variance.

Figure 1

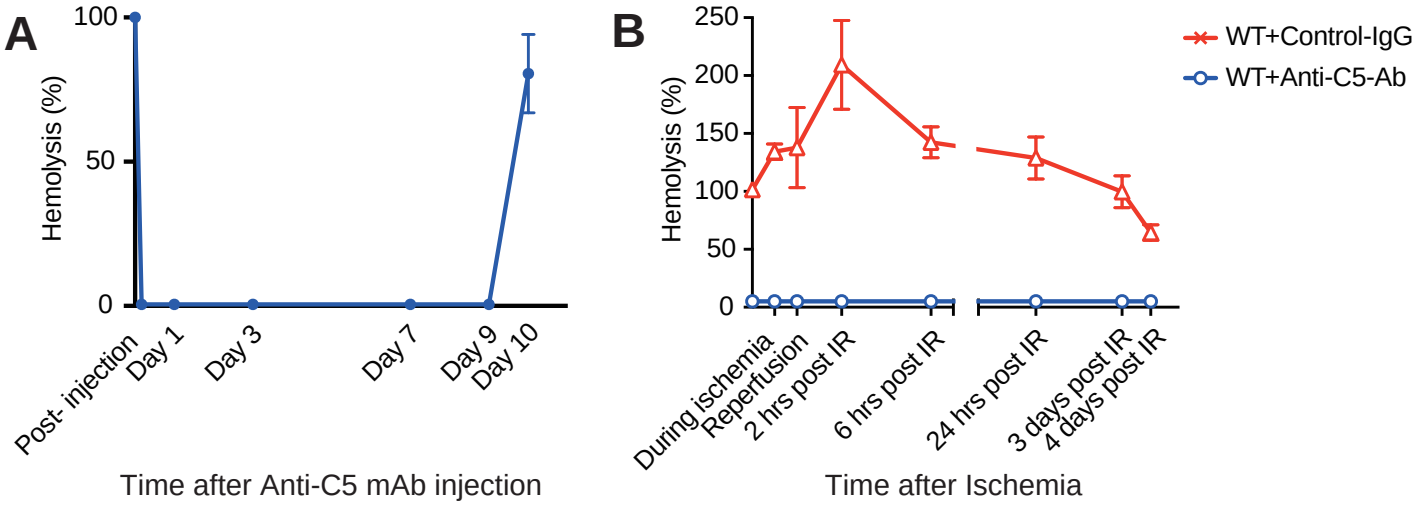


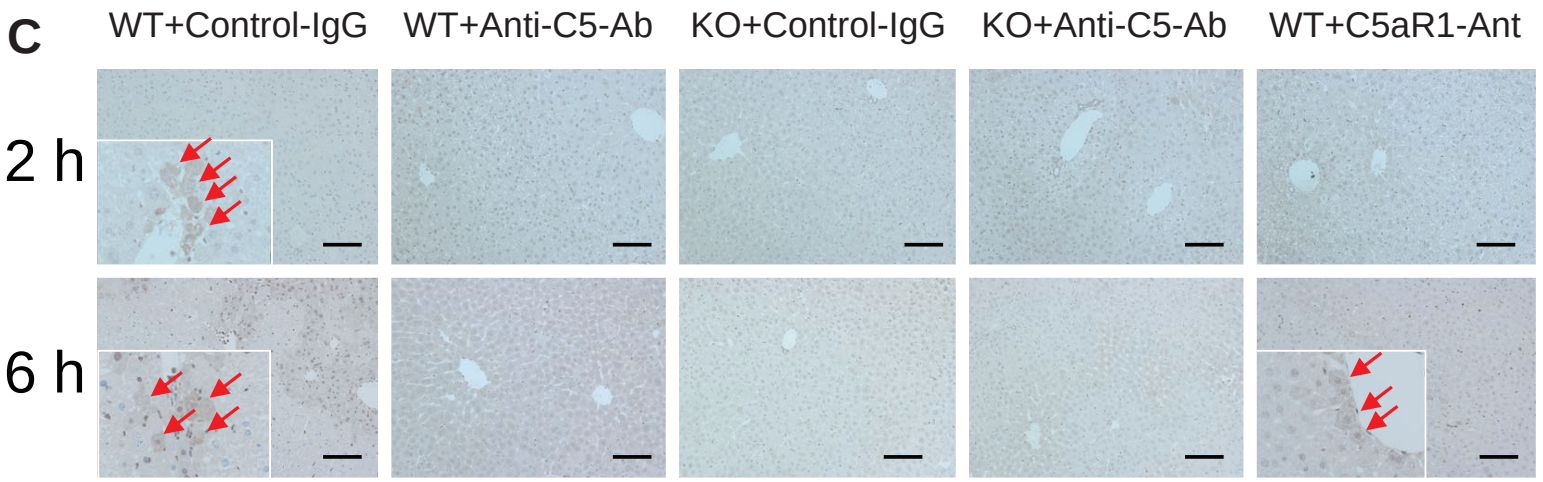
- Control IgG 40 mg/kg i.v.
- Anti-C5 mAb 40 mg/kg i.v.
- Anti-C5aR1 Ant 1mg/kg i.v.



- Control IgG 40 mg/kg i.v.
- Anti-C5 mAb 40 mg/kg i.v.

Figure 2





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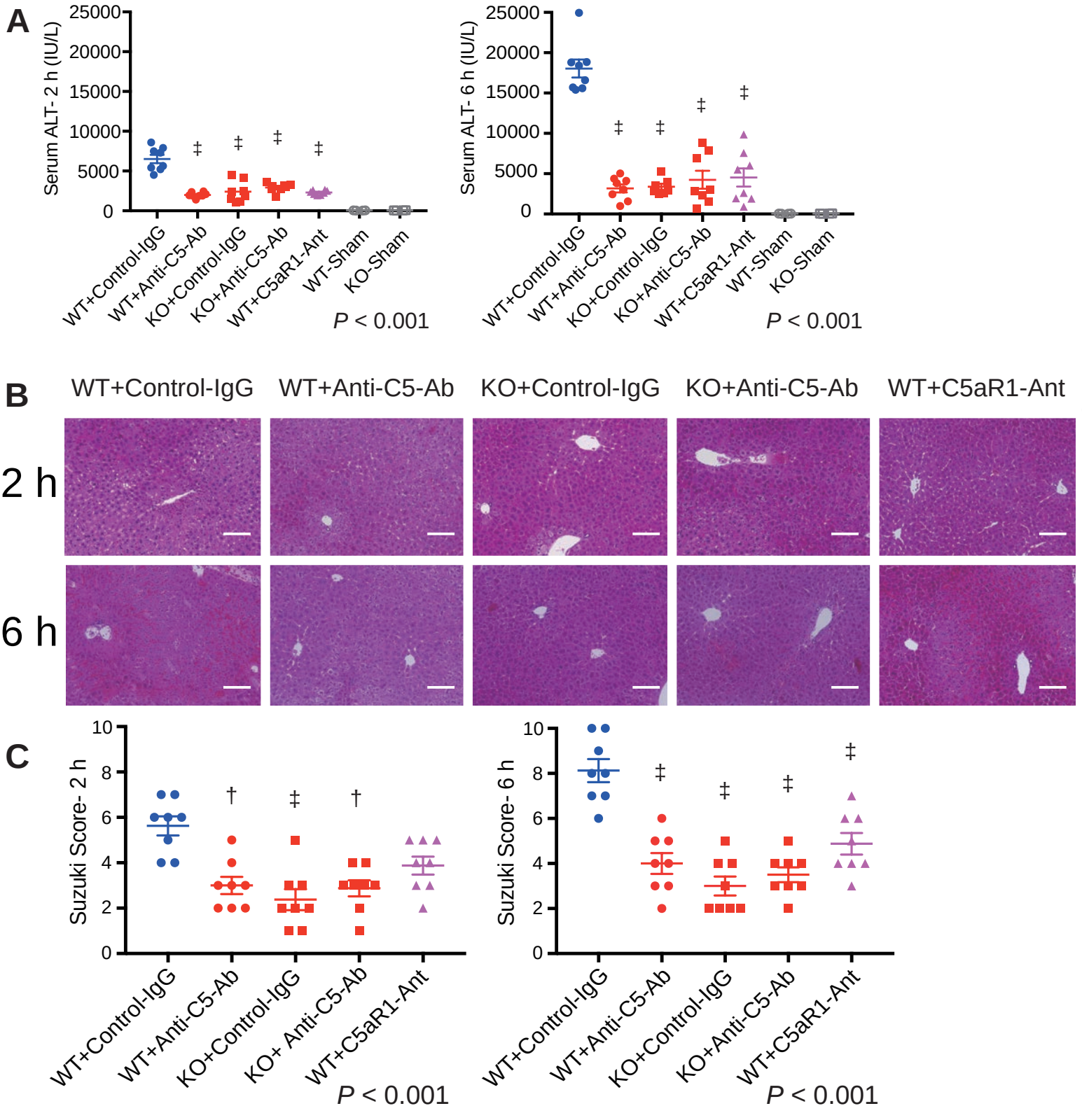

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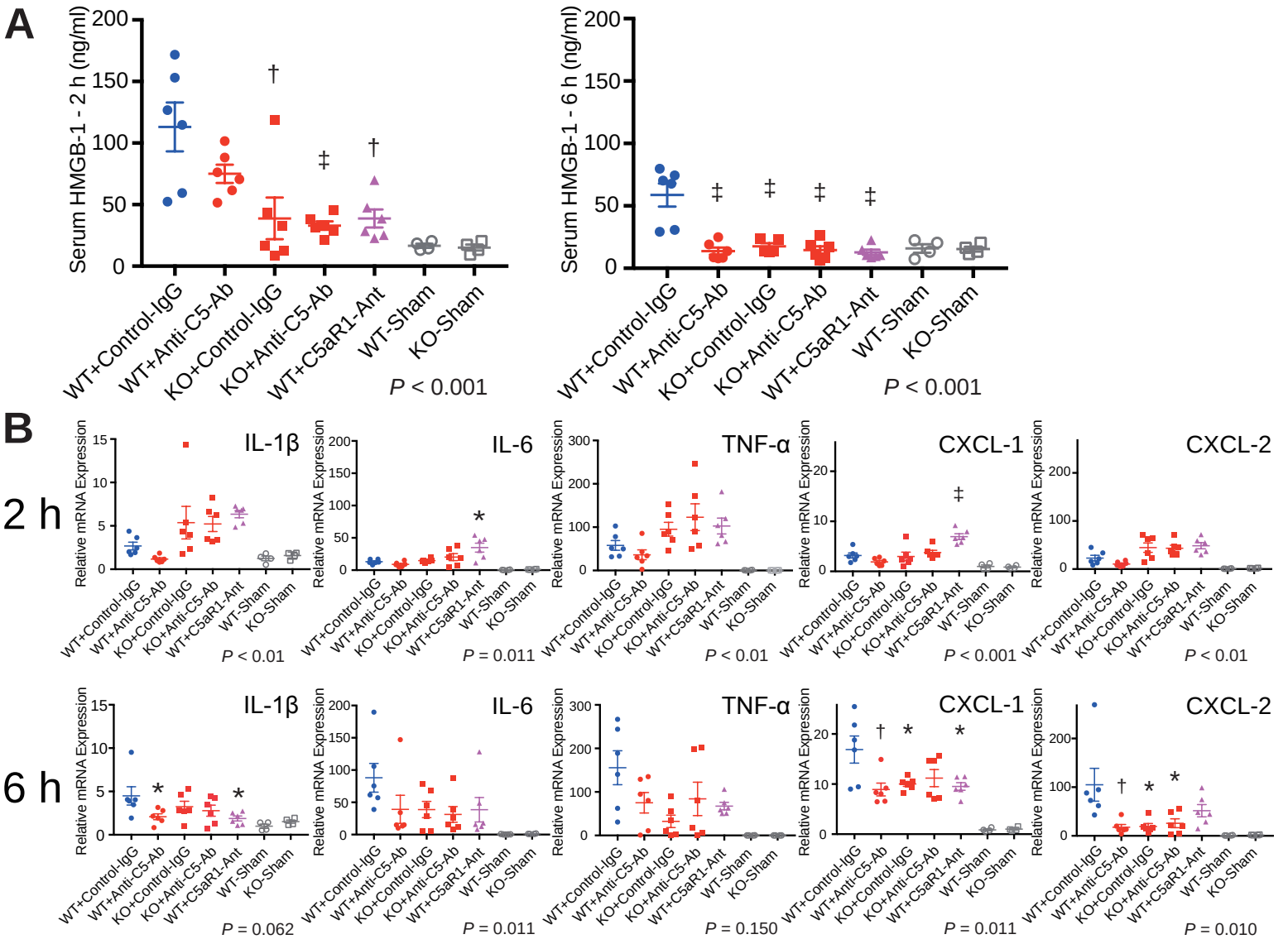

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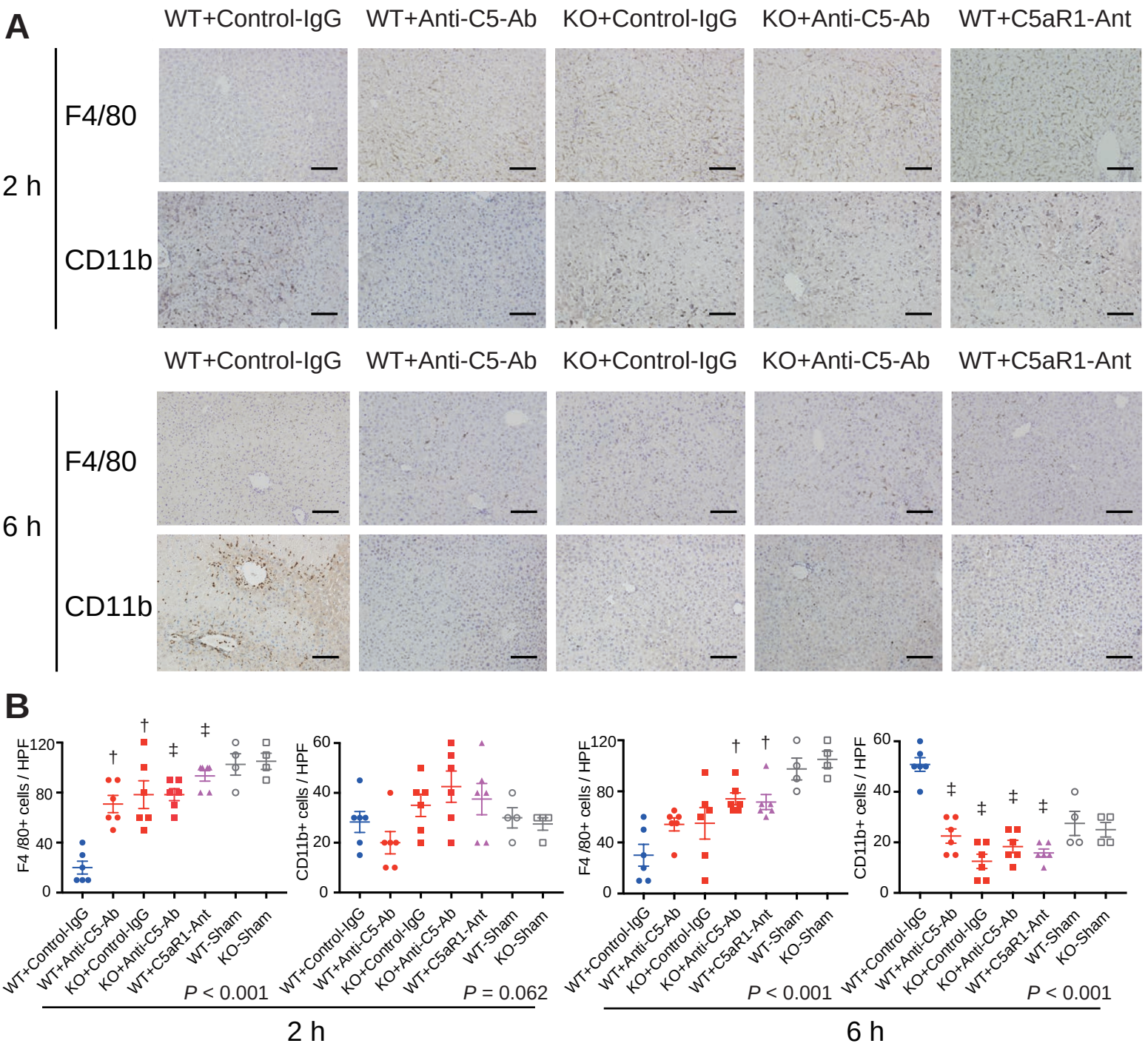

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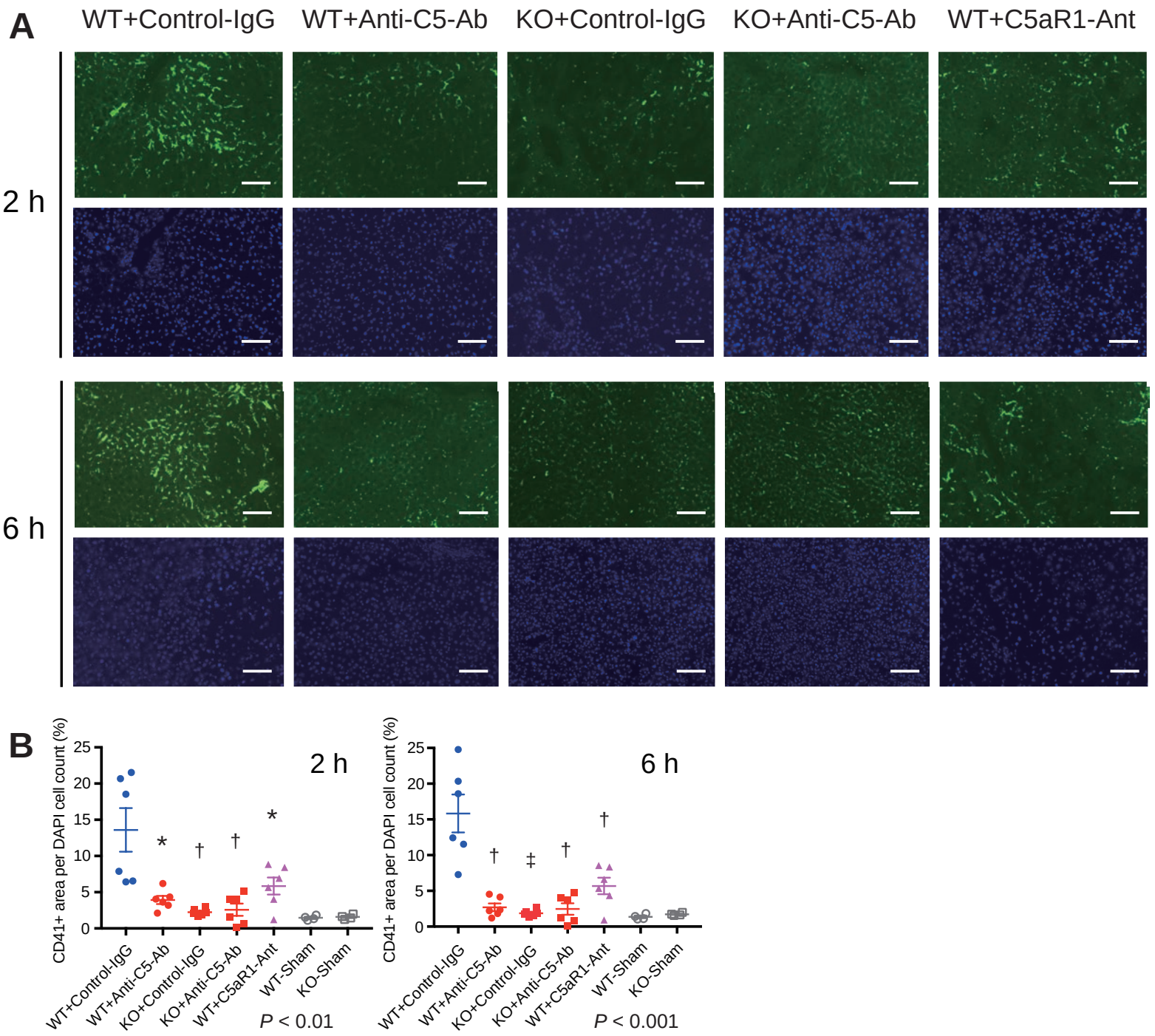

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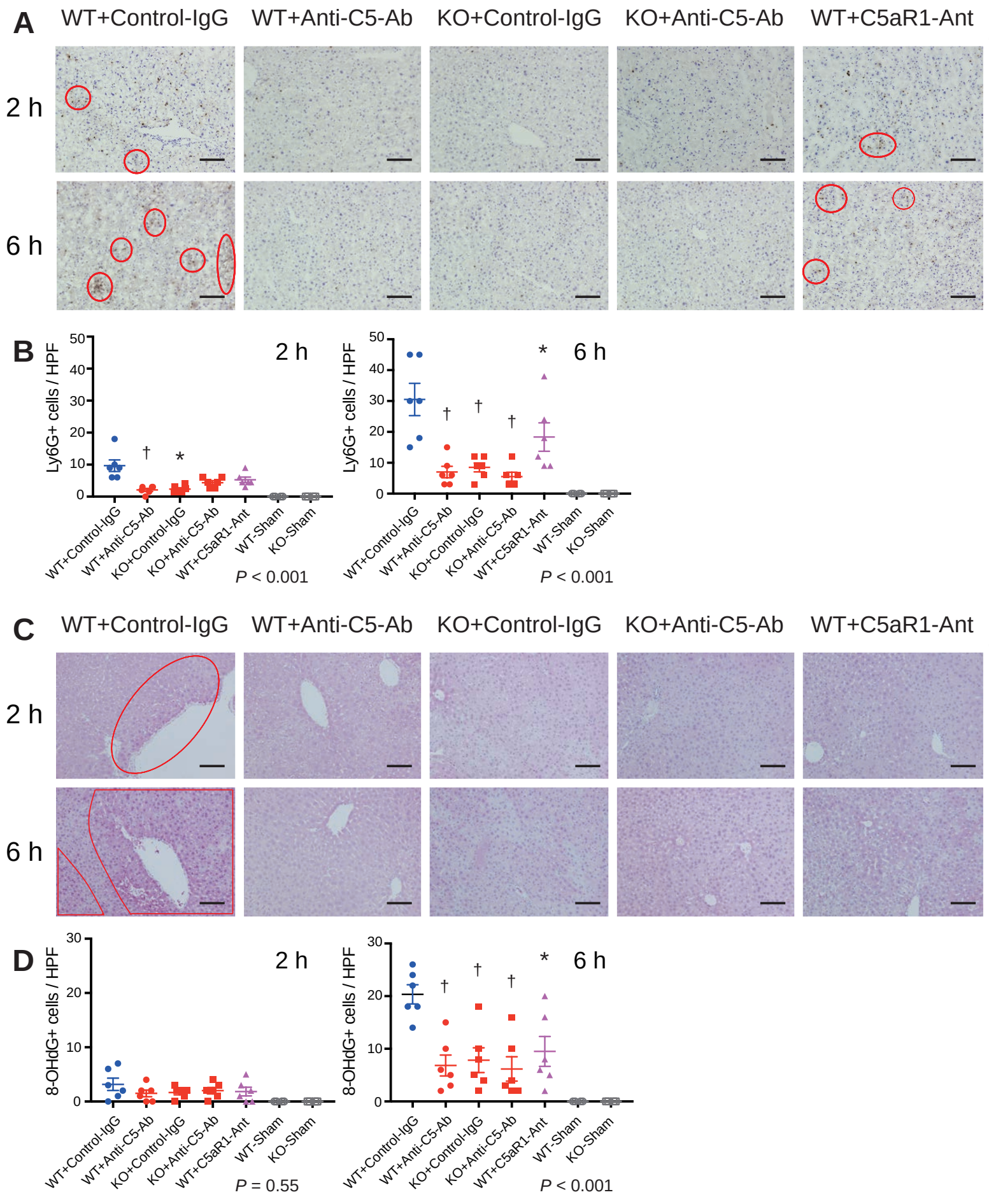

Figure 8