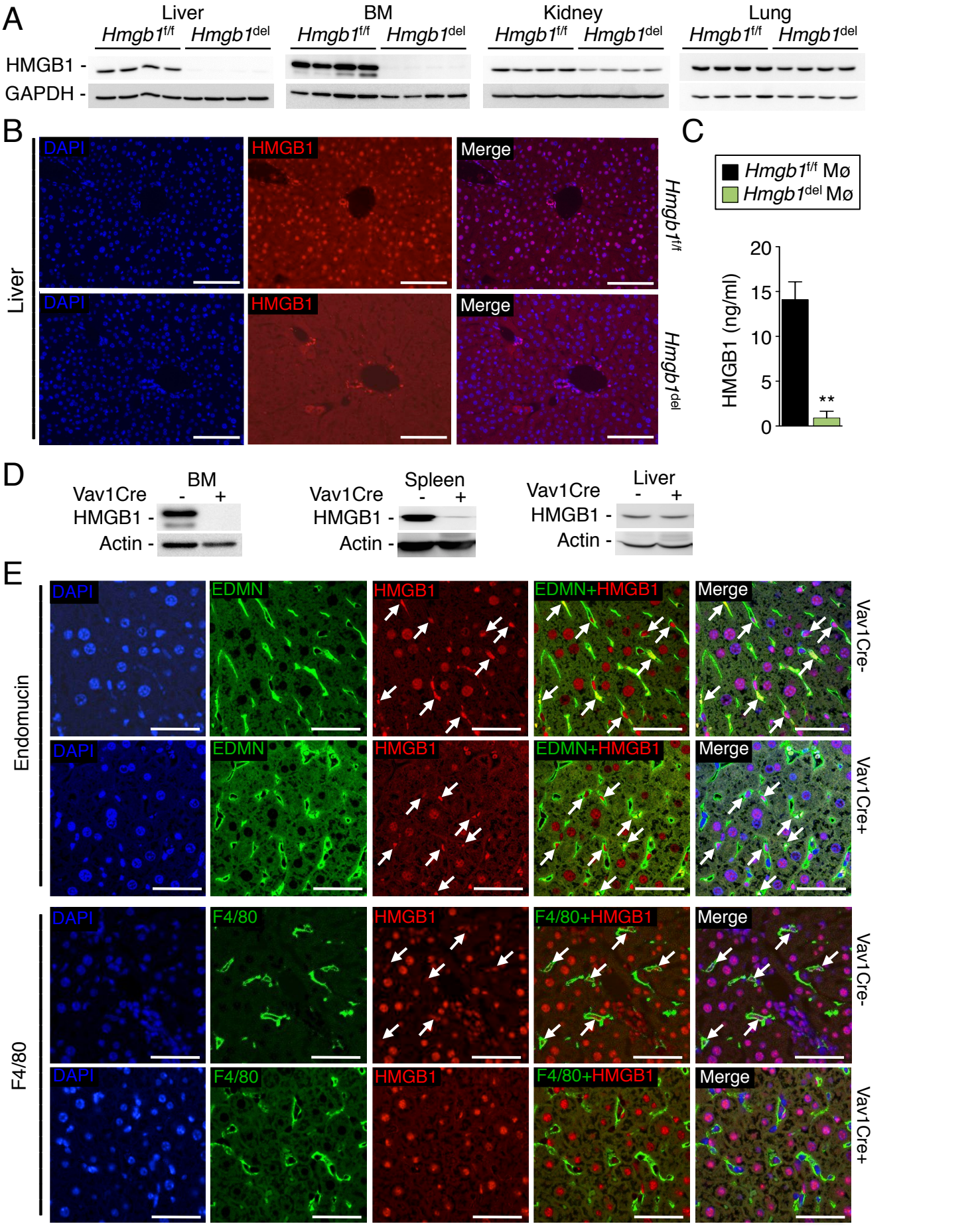
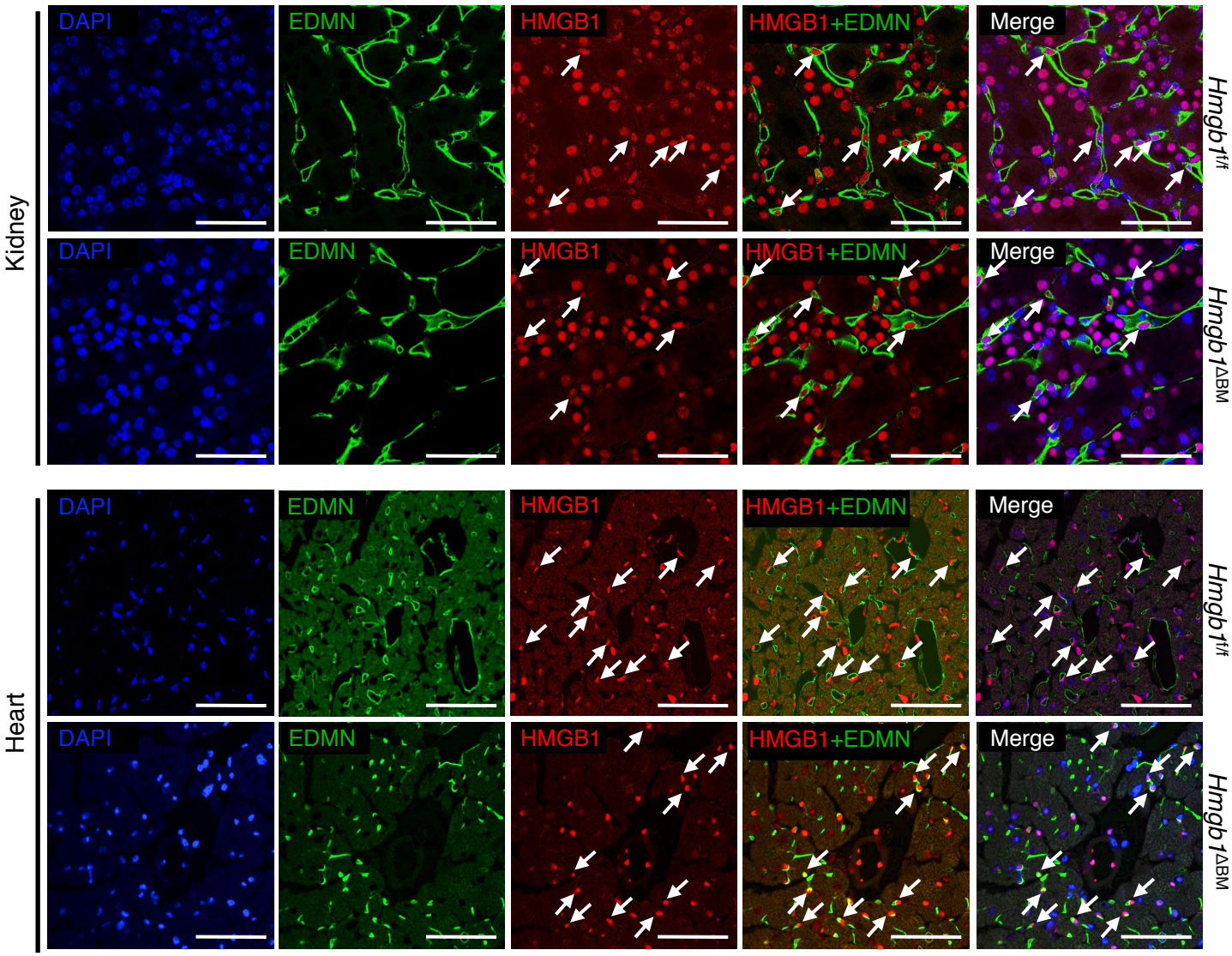
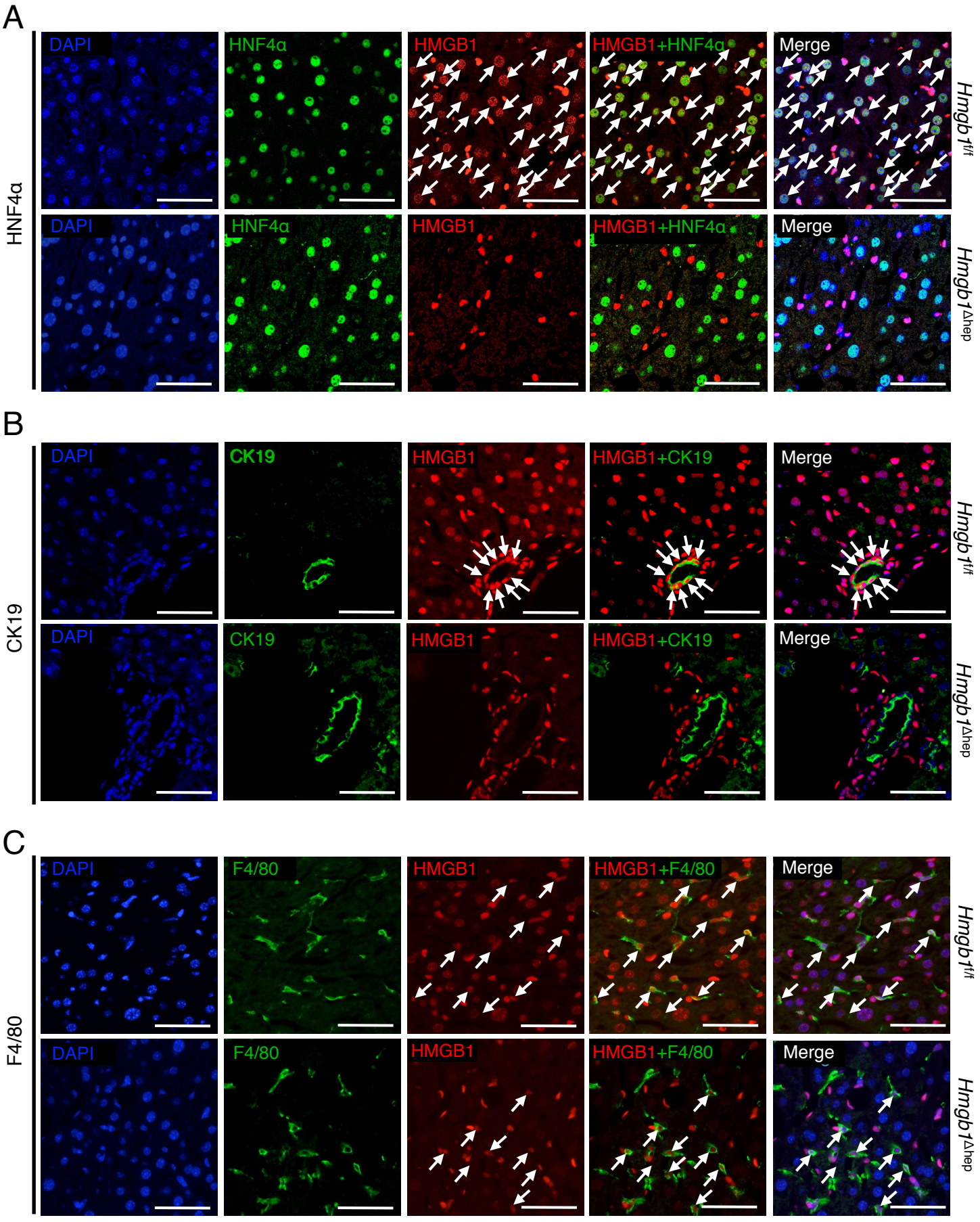


Supplementary Figure 1.

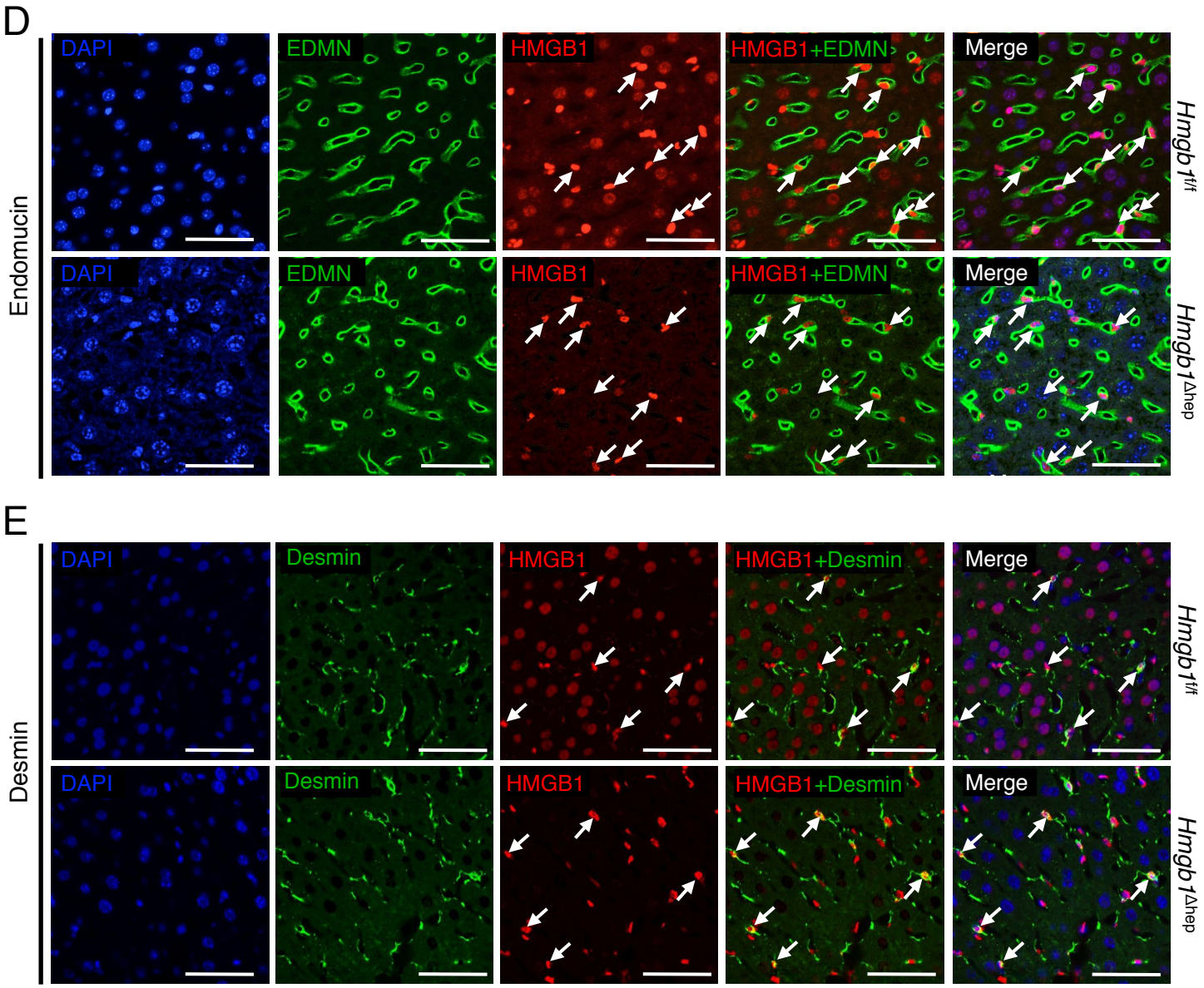
F

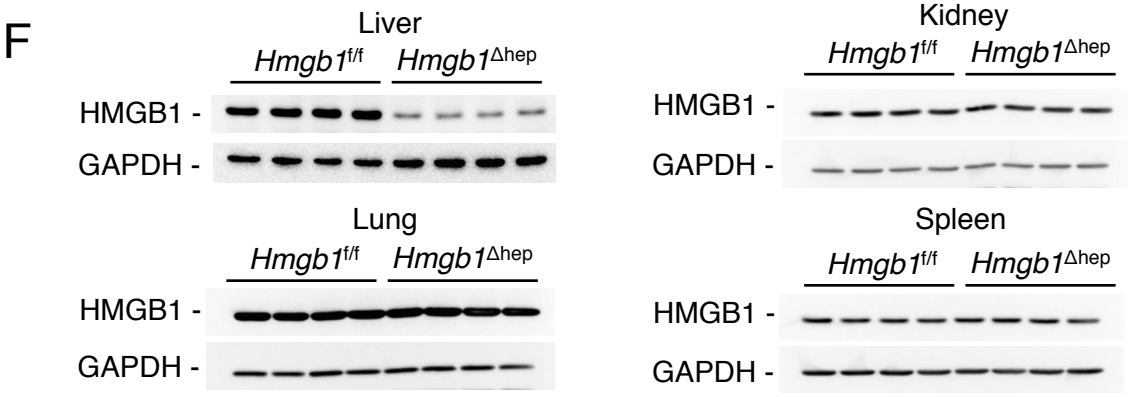


Supplemental Figure 1 (cont). Confirmation of HMGB1 deletion by Mx1Cre and Vav1Cre. A-C. Mx1Cre-mediated Hmgb1 deletion results in almost complete loss of HMGB1 protein in the liver and bone marrow, partial loss in other tissues and almost complete loss of HMGB1 secretion in LPS-stimulated macrophages (n=4 per group). **D**. Vav1Cre-mediated Hmgb1 deletion leads to strong reduction of protein levels in bone marrow and spleen but not liver (n=4 per group). **E.** Vav1Cre efficiently deletes HMGB1 in F4/80-positive macrophages in the liver but only to a small extent in endomucin-positive endothelial cells (arrows pointing to endothelial cells or macrophages expressing HMGB1). **F.** Similarly, most endothelial cells in the kidney and heart retain HMGB1 expression (arrows point towards endothelial cells with HMGB1 expression). Statistical significance assessed via two-tailed unpaired t-test (C). ** $p<0.01$. Scale bars 100 μm (B) and 50 μm (E, F).

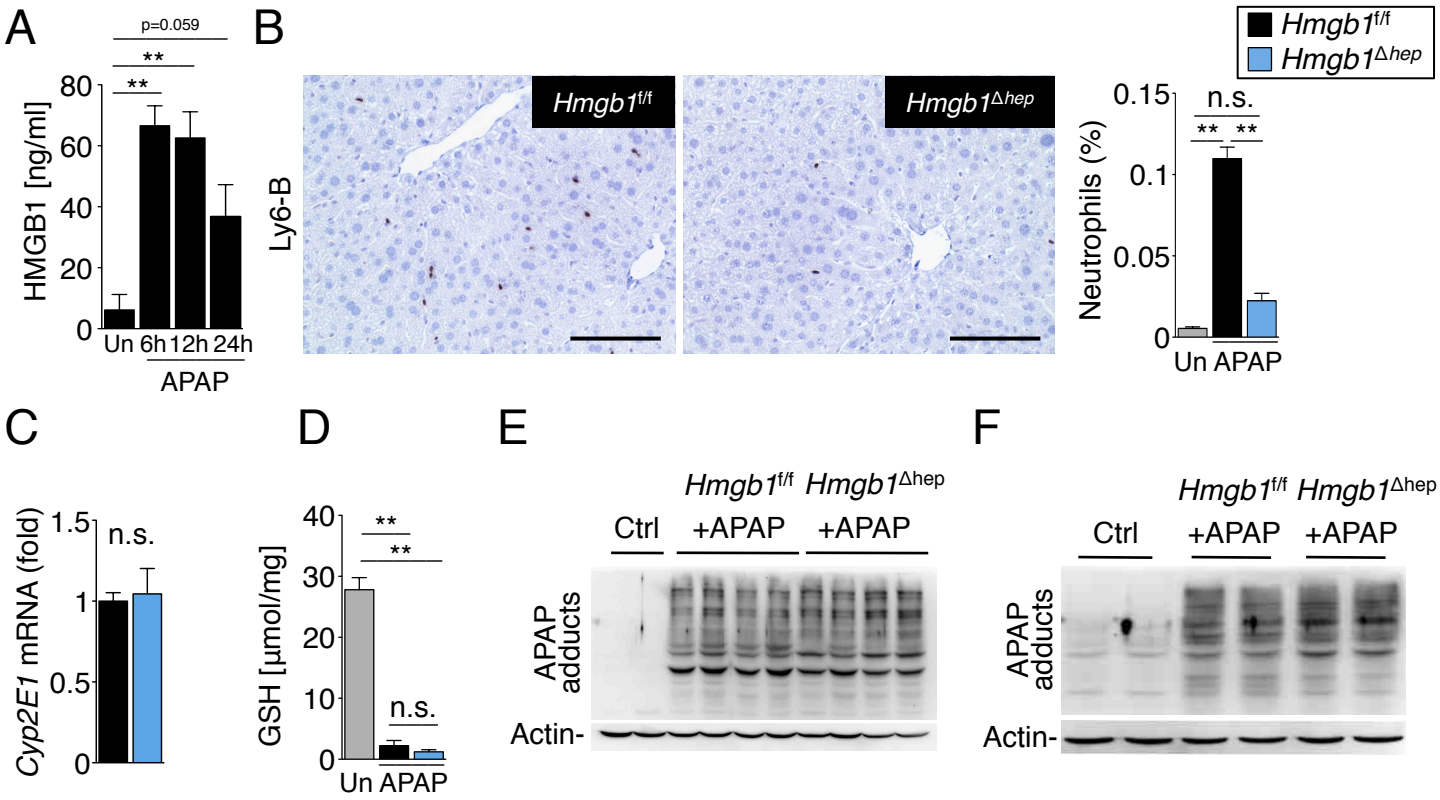


Supplementary Figure 2.

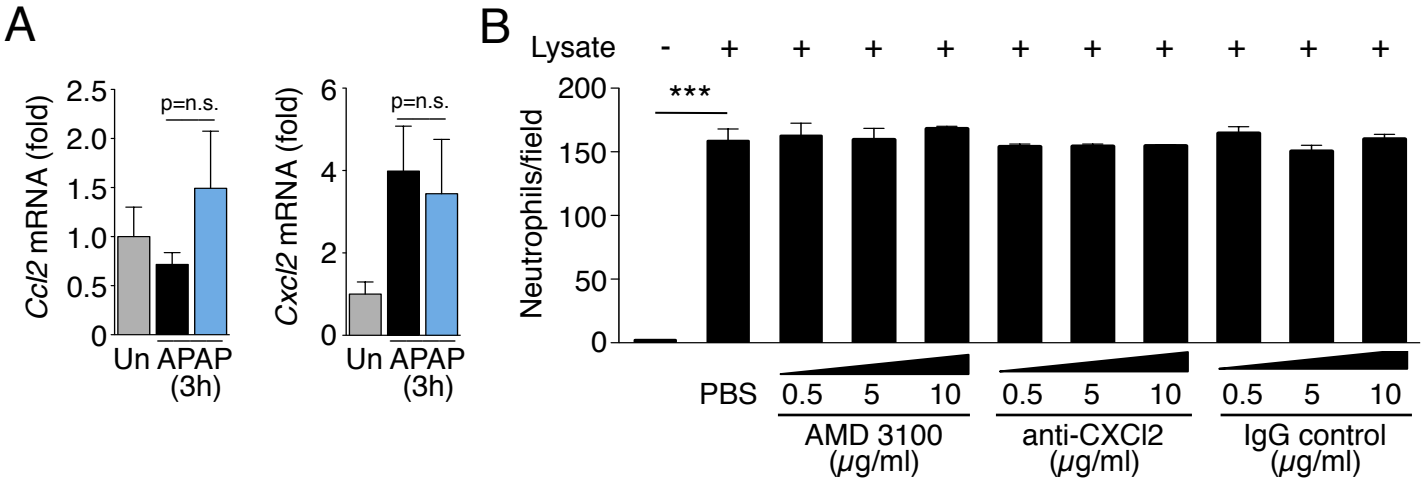




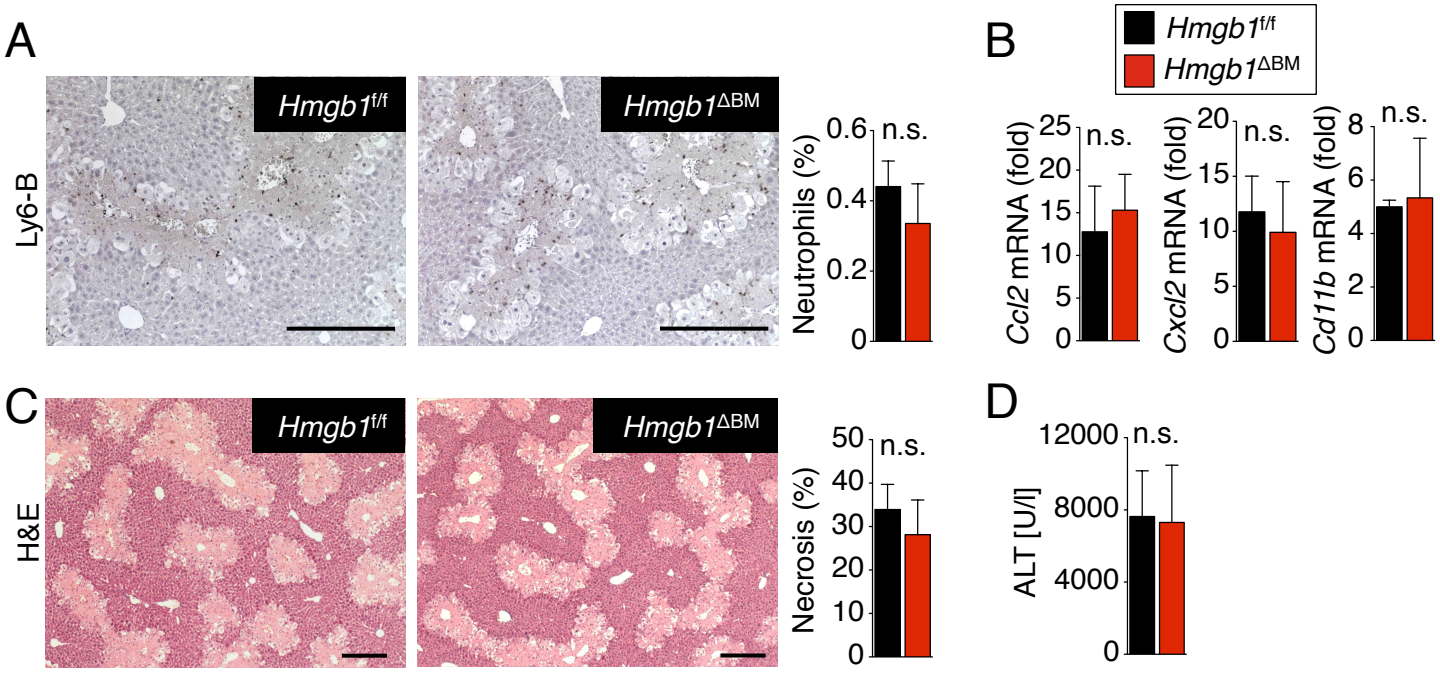
Supplementary Figure 2 (cont.). *Hmgb1*$^{\Delta hep}$ mice exhibit effective deletion of HMGB1 in hepatocytes and biliary epithelial cells but not in other hepatic cell populations. A-E. Confocal microscopy of liver sections co-stained for HMGB1 and markers for liver cell subpopulations including hepatocytes (HNF-4α, A), biliary epithelial cells (cytokeratin 19, B), Kupffer cells (F4/80, C), endothelial cells (endomucin, D) and hepatic stellate cells (desmin, E) in *Hmgb1*$^{f/f}$ and *Hmgb1*$^{\Delta hep}$ mice, showing deletion in hepatocytes and biliary epithelial cells, and preserved expression in Kupffer cells, endothelial cells and hepatic stellate cells of *Hmgb1*$^{\Delta hep}$ mice (arrows indicate HMGB1 expression in cells positive for each of these markers). F. Western blot analysis reveals strongly reduced HMGB1 expression in the liver, and preserved HMGB1 expression in kidneys, lungs and spleens of *Hmgb1*$^{\Delta hep}$ mice (n=4 per group). Scale bars 50 μm.



Supplemental Figure 3. HMGB1 status does not affect acetaminophen metabolization, but early neutrophil recruitment to the liver. A. Serum HMGB1 levels were determined after treating mice with 300 mg/kg i.p. acetaminophen ("APAP", n=4 per group). **B.** Hepatic neutrophil infiltration was assessed in *Hmgb1*$^{f/f}$ (n=7) and *Hmgb1*$^{\Delta hep}$ (n=7) mice 3h after treatment with acetaminophen (300 mg/kg) **C.** Hepatic *Cyp2e1* mRNA levels were determined in *Hmgb1*$^{f/f}$ and *Hmgb1*$^{\Delta hep}$ (n=5 per group) mice by qPCR. **D.** Early hepatic GSH depletion was determined in *Hmgb1*$^{f/f}$ and *Hmgb1*$^{\Delta hep}$ (n=3 per group) mice 60 minutes after treatment with acetaminophen (300mg/kg). **E.** Hepatic acetaminophen adducts were determined in untreated control mice (n=2) and *Hmgb1*$^{f/f}$ (n=4) and *Hmgb1*$^{\Delta hep}$ (n=4) mice treated with acetaminophen (300 mg/kg) for 3h. **F.** Acetaminophen adducts were determined in untreated primary hepatocytes (n=2) and primary hepatocytes isolated from *Hmgb1*$^{f/f}$ (n=2) and *Hmgb1*$^{\Delta hep}$ (n=2; representative results from three separate isolations), after treatment with acetaminophen (10 mM) for 3h. Statistical significance assessed via one-way ANOVA followed by Tukey´s Multiple Comparison Test (A, B, D), or two-tailed unpaired t-test (C), respectively. * $p<0.05$, ** $p<0.01$. n.s.= non-significant. Scale bars 200 μm (B).



Supplemental Figure 4. Chemokines are not regulated by HMGB1 status at early time points of hepatic injury, and do not regulate neutrophil migration towards necrotic liver tissue. A. Hepatic expression of *Ccl2* and *Cxcl2* mRNA was assessed in *Hmgb1*$^{f/f}$ (n=7) and *Hmgb1*$^{\Delta hep}$ (n=7) mice 3h after treatment with acetaminophen (300 mg/kg). **B**. Neutrophil migration towards necrotic liver extracts was determined in the presence or AMD3100, an inhibitor of CXCL2 receptor CXCR4, or vehicle (PBS), or a neutralizing antibody against CXCL2 or isotype-matched control antibody (n=3 per group, shown is a representative of three independent experiments, each performed in triplicate). Statistical significance assessed via one-way ANOVA followed by Tukey´s Multiple Comparison Test. *** $p<0.001$. n.s.= non-significant.

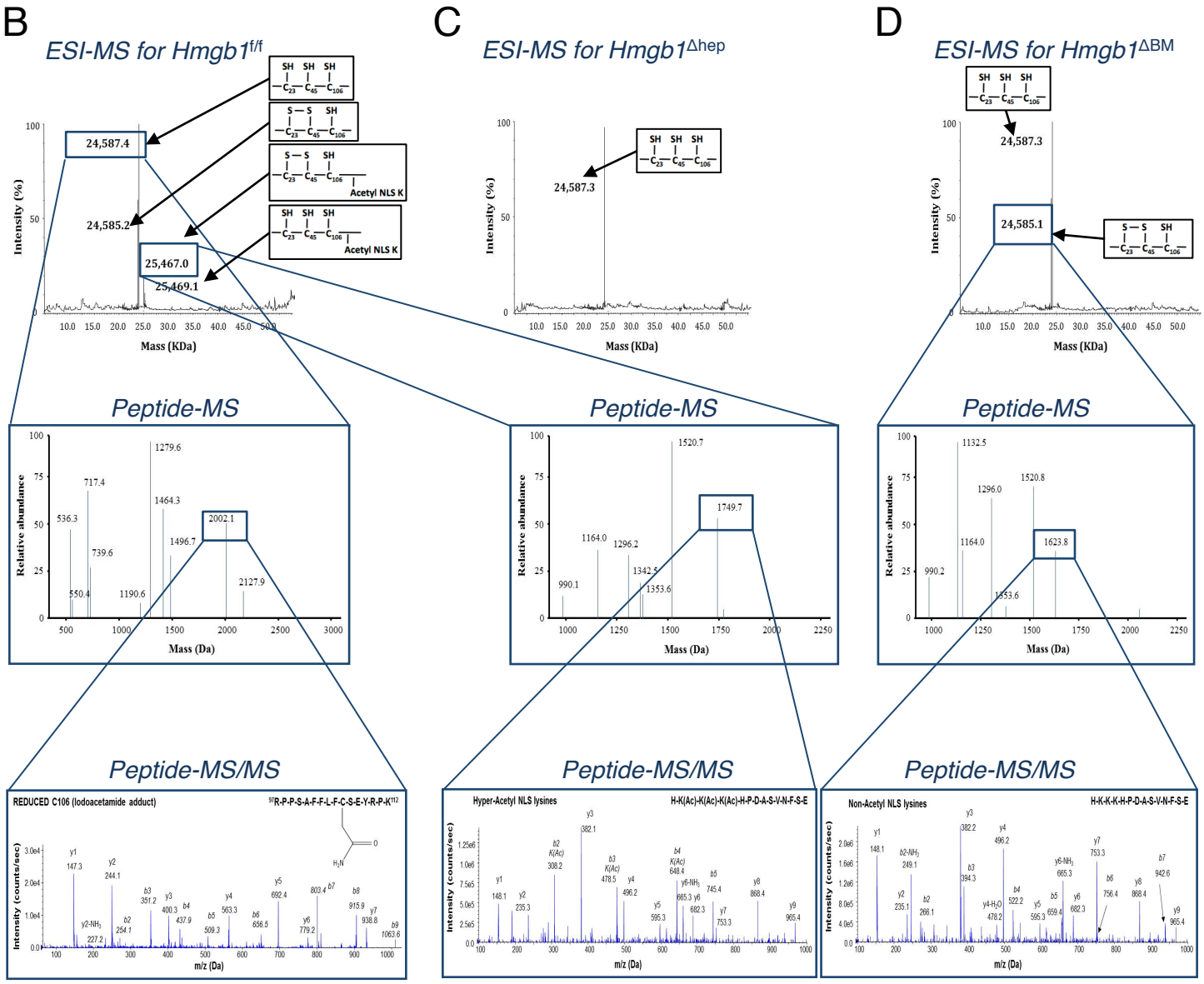


Supplemental Figure 5. HMGB1 in bone marrow-derived cells does not alter acetaminophen-induced inflammation and injury. A-D. Vav1Cre-negative *Hmgb1*$^{f/f}$ *mice* (n=5) and Vav1Cre-positive *Hmgb1*$^{f/f}$ *mice* (*Hmgb1*$^{\Delta BM}$, n=7) were injected with acetaminophen (300 mg/kg), followed by determination of hepatic neutrophil infiltration (A), hepatic inflammatory gene expression (B), hepatic H&E staining and quantification of necrosis (C) and serum ALT levels (D), 24h later. Statistical significance assessed via two-tailed unpaired t-test (A-D). n.s.= non-significant. Scale bars 200 μm (A, C).

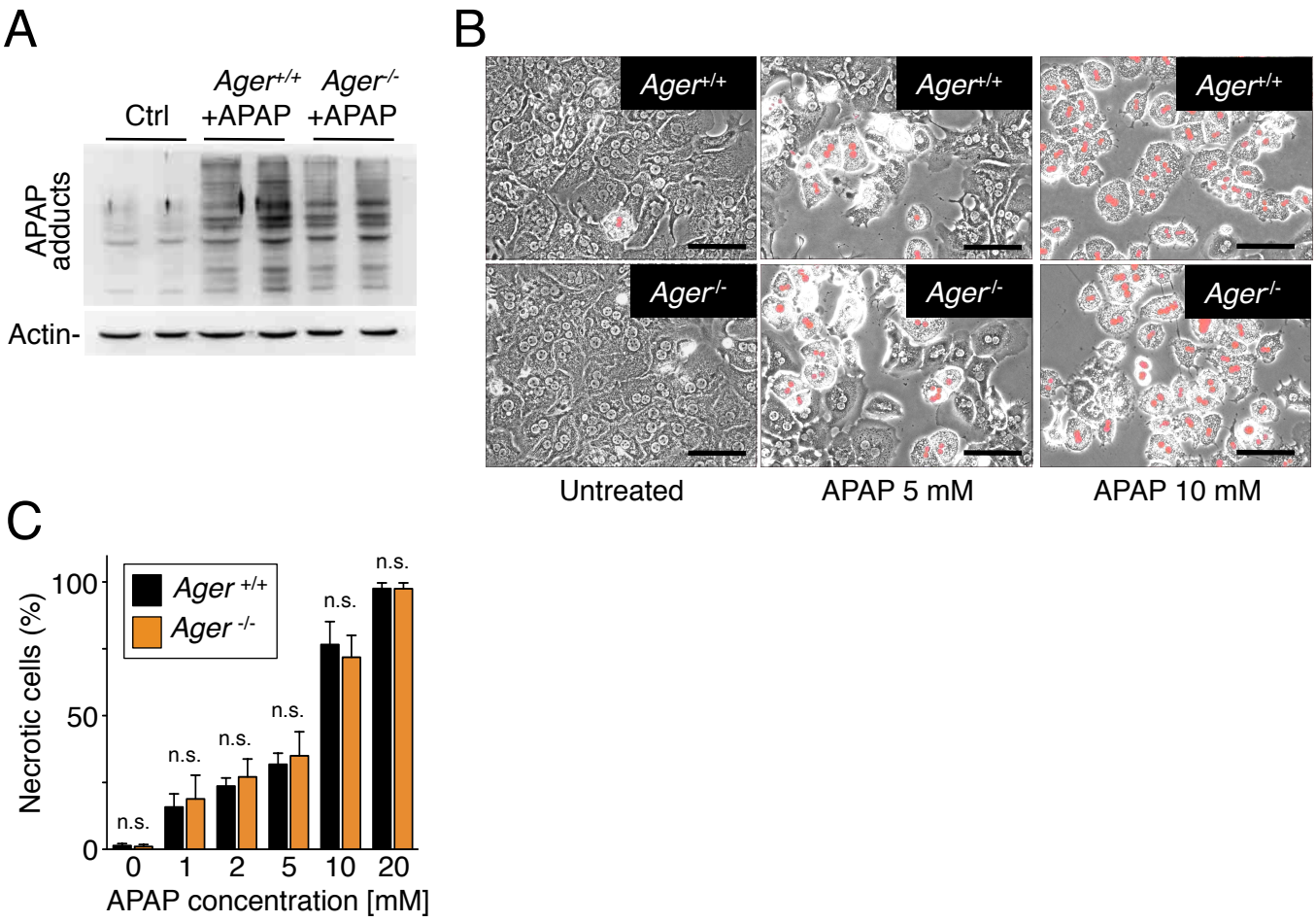
A

Detected
Not detected

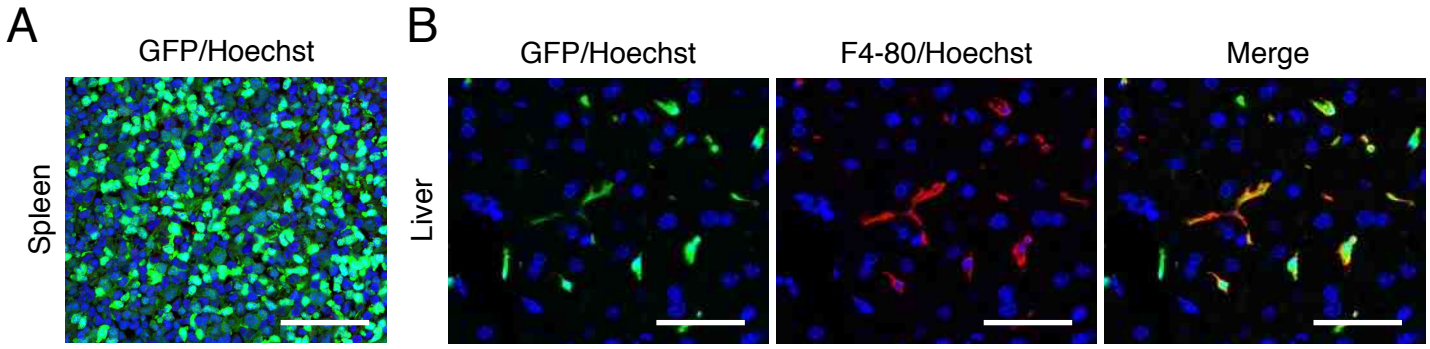
Genotype	Redox			Acetylation		None-acetylation		Hyper-Phos Ser34	None -Phos Ser34	Relative HMGB1 level
	Disulfide HMGB1	All thiol HMGB1	Sulfonic acid HMGB1	NLS1	NLS2	NLS1	NLS2			
Hmgb1$^{\Delta hep}$		■				■	■		■	+
Hmgb1$^{f/f}$	■	■		■	■	■	■		■	++++
Hmgb1$^{\Delta BM}$	■	■				■	■		■	++
Hmgb1$^{f/f}$	■	■		■	■	■	■		■	++



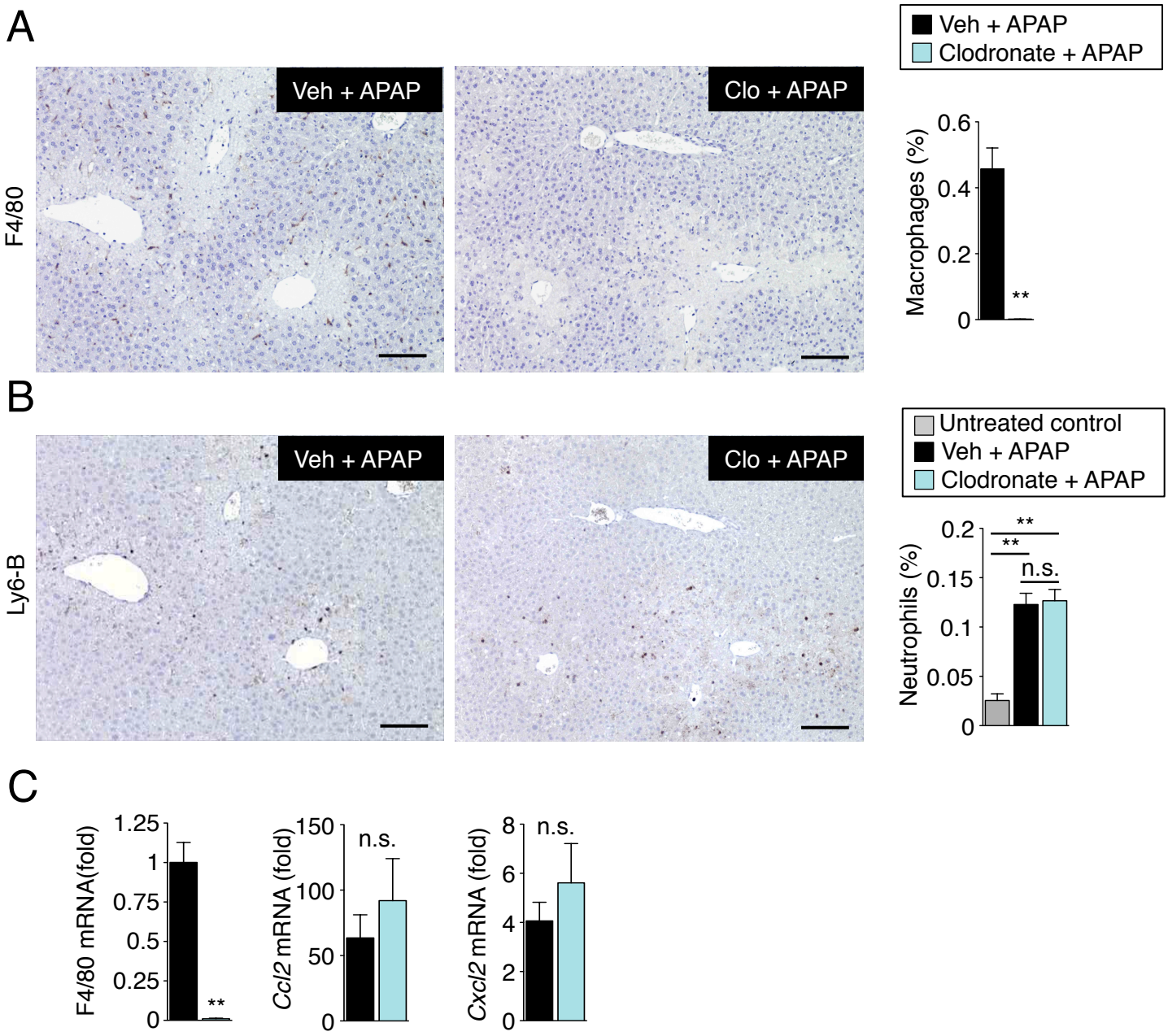
Supplemental Figure 6. Determination of cell compartment-specific HMGB1 posttranslational modifications following acetaminophen-induced liver injury. A. Molecular weights and a schematic representation of each HMGB1 isoform showing identified post-translational modifications are indicated on each spectra where required. Blue: Present. White: Not detected. **B-D**. Representative spectra of whole protein ESI-MS of HMGB1 isoforms isolated from serum of *Hmgb1*$^{f/f}$ (B) and *Hmgb1*$^{\Delta hep}$ mice (C), and *Hmgb1*$^{\Delta BM}$ (D) mice. Spectra are representative of at least 6 mice in each group treated with acetaminophen for 6 hours.



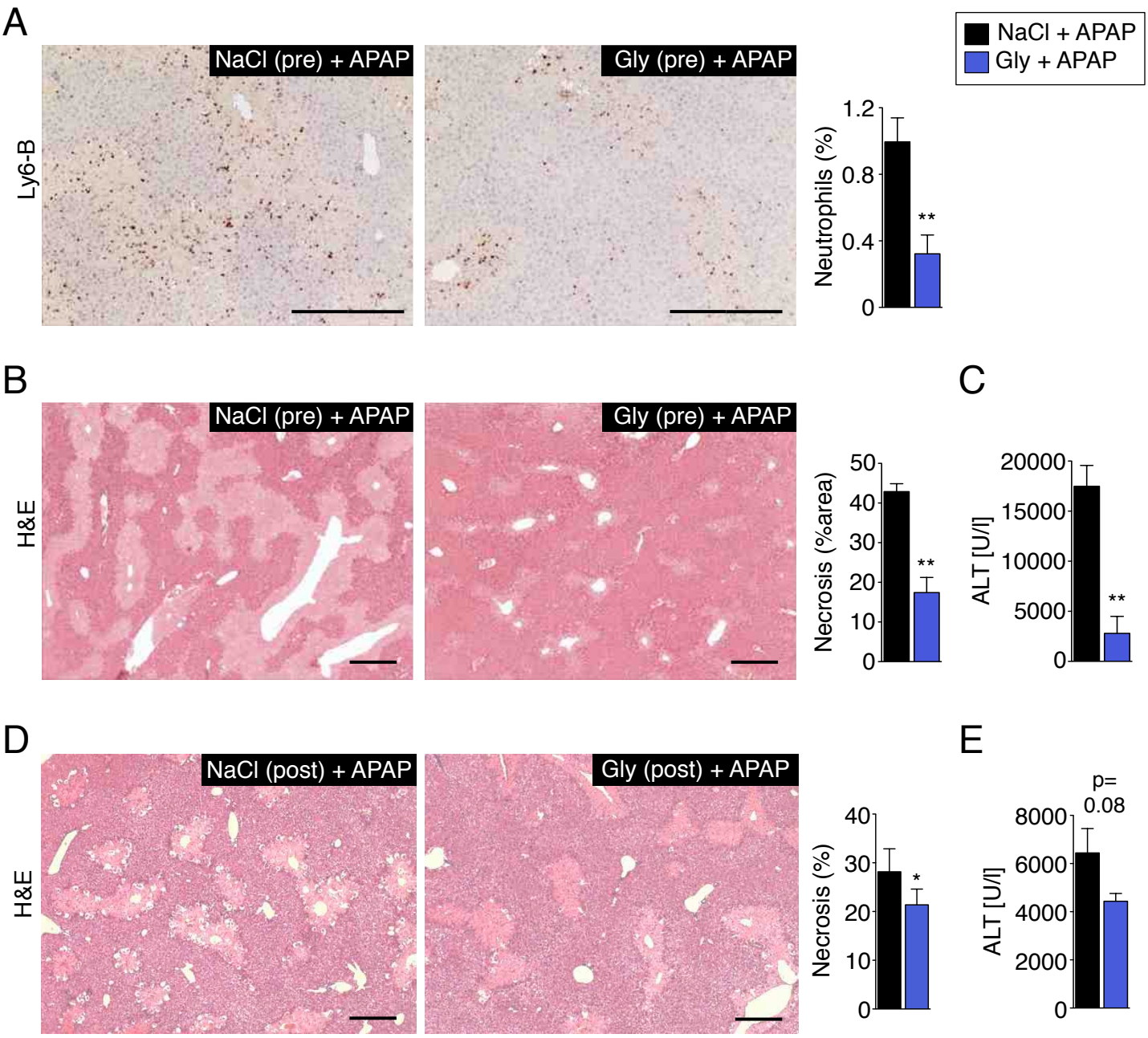
Supplemental Figure 7. RAGE status does not affect acetaminophen metabolization or acetaminophen-induced injury in primary hepatocytes. A. Acetaminophen adducts were determined in untreated primary hepatocytes (n=2) and primary hepatocytes isolated from *Ager* $^{+/+}$ (n=2) or *Ager* $^{-/-}$ (n=2, representative result from three separate isolations) mice, after treatment with acetaminophen (10 mM) for 3h. **B-C**. Primary hepatocytes, isolated from *wt* or *Ager*$^{-/-}$ mice (n=2 per group), were treated with various concentrations of acetaminophen for 24h and stained with propidium iodide (red) for cell death determination. Shown are representative images (B) and quantification of acetaminophen-induced cell death (C), representative results from three separate isolations. Statistical significance assessed via two-tailed unpaired t-test (C). n.s.= non-significant. Scale bars 100 μm (B).



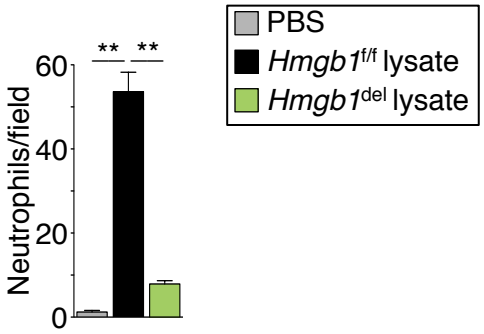
Supplemental Figure 8. Reconstitution of hepatic macrophages and spleen by transplantation of *Ager*$^{-/-}$ bone marrow. Reconstitution of mice with *Ager*$^{-/-}$ BM was demonstrated by GFP fluorescence (expressed in *Ager*$^{-/-}$ mice) which overlapped with F4/80 staining in the liver, and GFP fluorescence in the spleen. Scale bars 50 μm.



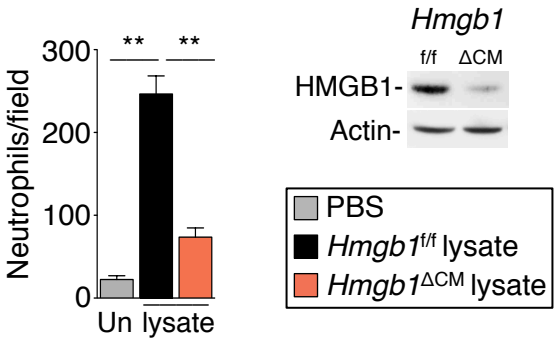
Supplemental Figure 9. Acetaminophen-induced neutrophil recruitment into the liver does not require macrophages. Mice were treated with vehicle liposomes (n=7) or liposomal clodronate (n=8), followed by injection of acetaminophen (300 mg/kg) three days later. **A-B.** Livers were stained with F4/80 (A) and Ly6B (B), and the numbers of F4/80-positive macrophages and Ly6B-positive neutrophils, respectively, was quantified. **C.** Hepatic expression of inflammatory genes was determined by qPCR and normalized to untreated control livers. Statistical significance assessed via one-way ANOVA followed by Tukey´s Multiple Comparison Test (B), or via two-tailed unpaired t-test (C), respectively. ** $p<0.01$, n.s.=non-significant. Scale bars 200 μm (A, B).



Supplemental Figure 10. Pharmacologic HMGB1 inhibition ameliorates acetaminophen-induced liver injury. A-C. Mice were pretreated with glycyrrhizin (50 mg/kg, n=9) or vehicle (n=8) followed by injection of acetaminophen (300 mg/kg) 2h later. Mice were sacrificed 24h later, followed by determination of hepatic neutrophil infiltration (A), hepatic H&E staining and quantification of necrosis (B) and serum ALT levels (C). **D-E.** Mice were injected with acetaminophen (300 mg/kg), followed by treatment with either glycyrrhizin (50 mg/kg, n=8) or vehicle (NaCl, n=7) 2h later. Mice were sacrificed 24h after acetaminophen injection, followed by hepatic H&E staining and quantification of necrosis (D) and determination of serum ALT levels (E). Statistical significance assessed via two-tailed unpaired t-test (A-E). *p<0.05, ** p<0.01, n.s.=non-significant. Scale bars 200 μm (A, B, D).



Supplemental Figure 11. Human neutrophils require HMGB1 for migration towards tissue lysates. Migration of human neutrophils, isolated from peripheral blood, towards towards *Hmgb1*-floxed and *Hmgb1*-deleted (*Hmgb1*^{del}) liver extracts (induced by Mx1Cre), was determined in Boyden chambers. Statistical significance assessed via one-way ANOVA followed by Tukey´s Multiple Comparison Test. ** $p<0.01$.



Supplemental Figure 12. HMGB1 promotes neutrophil migration towards cardiac tissue extracts. Neutrophil migration towards *Hmgb1*-floxed and *Hmgb1*-deleted (*Hmgb1*^{del}) cardiac tissue extracts (achieved by MHC-Cre-mediated deletion; insert showing immunoblot confirming HMGB1 deletion) was determined in a Boyden chamber. Statistical significance assessed via one-way ANOVA followed by Tukey´s Multiple Comparison Test. ** $p<0.01$.

All Western blot exposed on Kodak Image station (digital)

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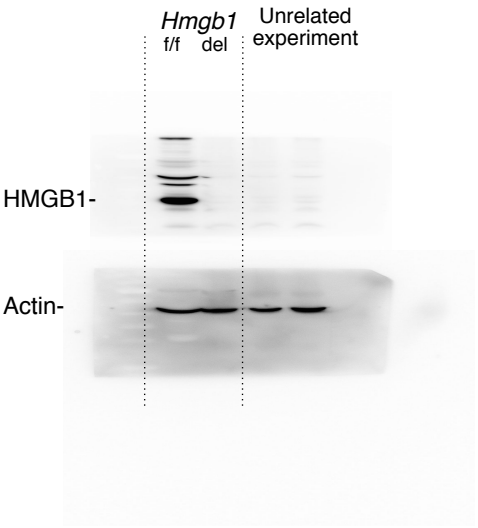

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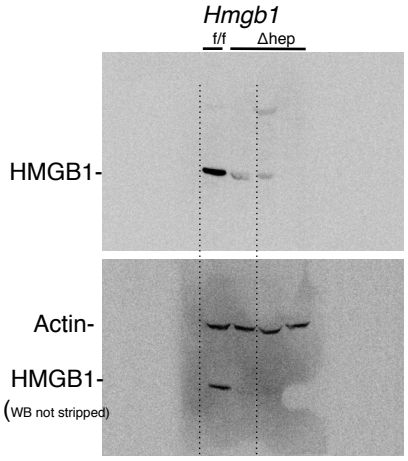

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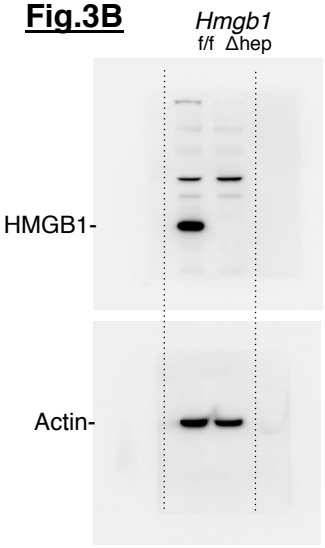

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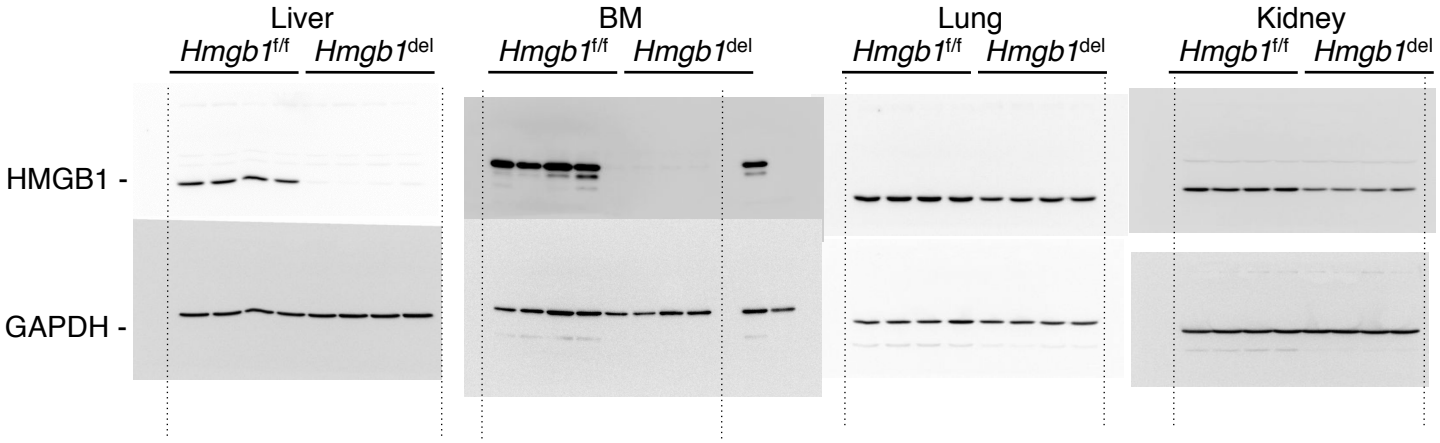

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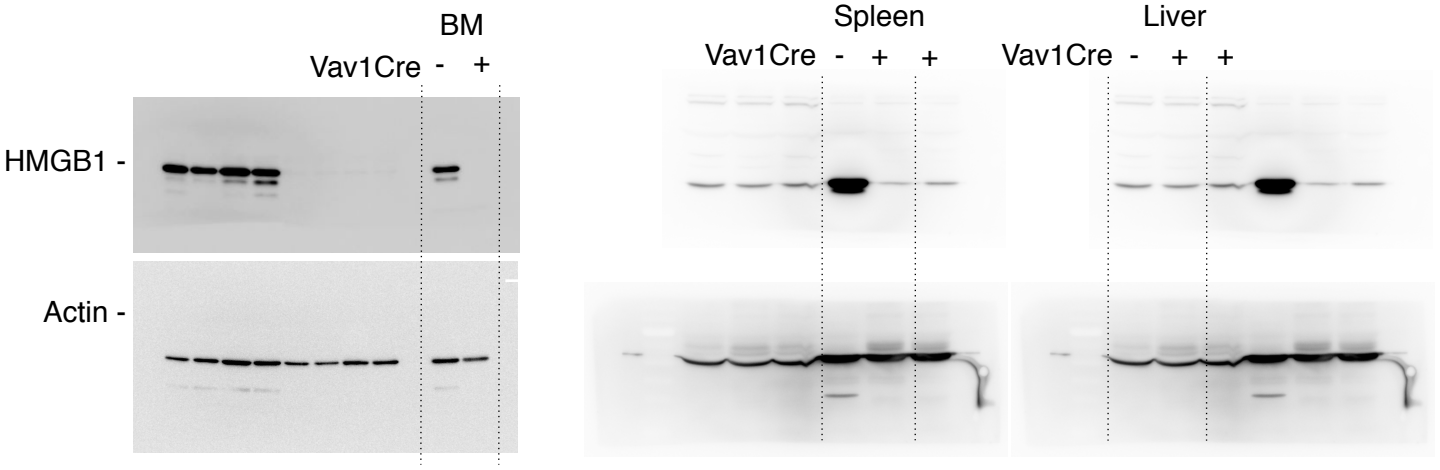

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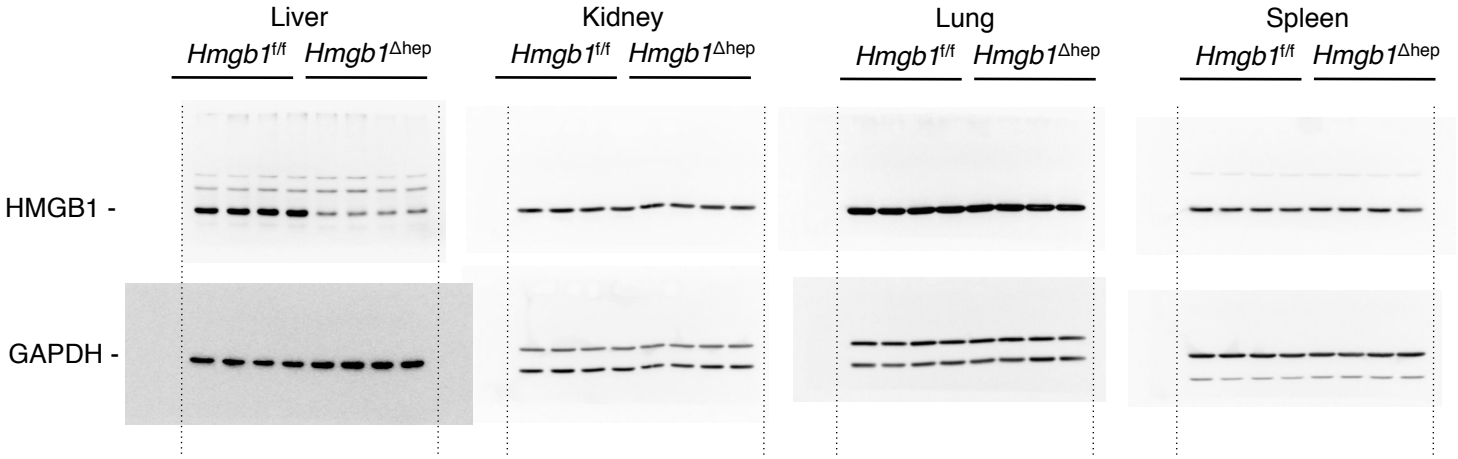

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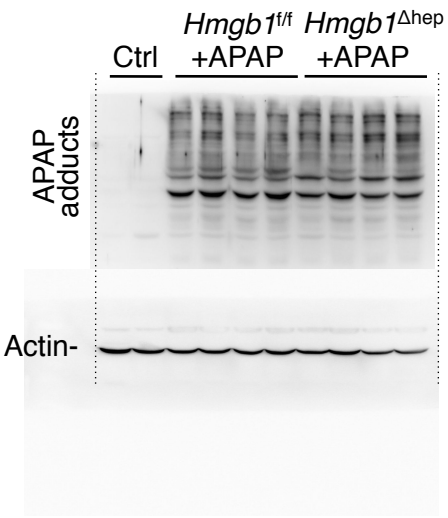

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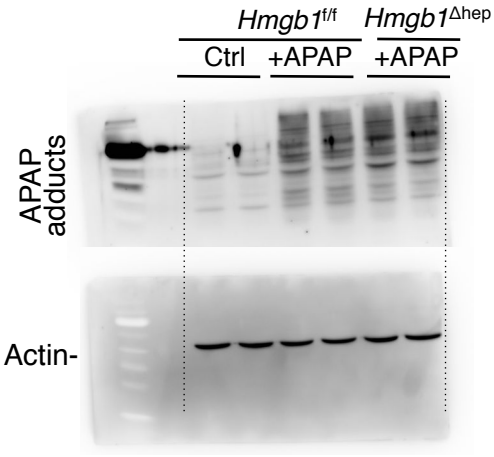

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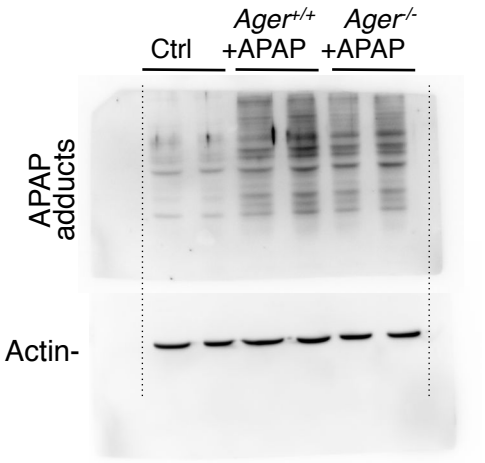

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