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# Platelets accelerate endoplasmic reticulum stress and promote hepatic steatosis

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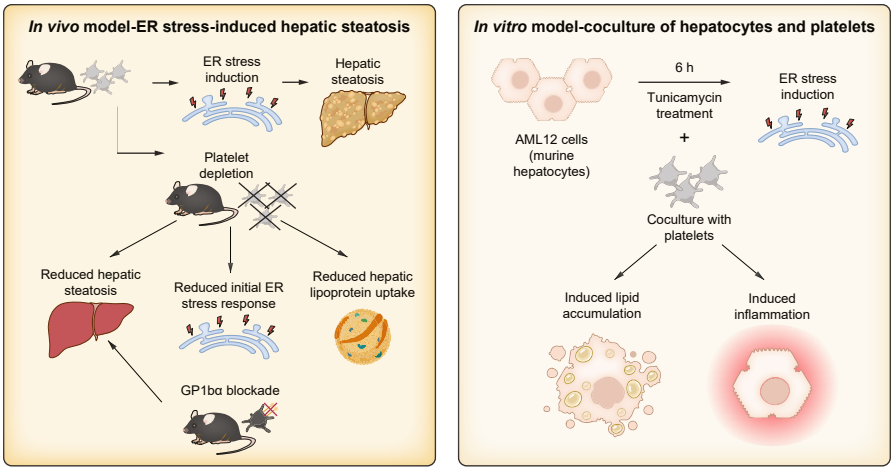
## Graphical abstract




## Highlights:

- Platelets are locally activated by ER stress, infiltrating the liver and increasing lipoprotein accumulation.
- Platelets enhance early unfolded protein responses and impact lipoprotein packaging.
- Platelets amplify ER stress signaling in the liver, exacerbating steatosis.
- Platelets regulate acute-phase response and immune cell influx into the liver.
- Blocking GPIbα impaired hepatic platelet recruitment and reduced lipoprotein accumulation.

## Impact and implications:

This paper shows that blood platelets are capable of regulating specific metabolic functions in liver cells (so-called ER stress) and thereby contribute to the development of hepatic steatosis.

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# Platelets accelerate endoplasmic reticulum stress and promote hepatic steatosis

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**Background & Aims:** Metabolic dysfunction-associated steatotic liver disease (MASLD) is the most common chronic liver disease, characterized by endoplasmic reticulum (ER) stress, which impairs hepatic lipoprotein metabolism. Blood platelets can modulate cellular responses to ER stress and accumulate in the liver during MASLD, contributing to disease development through interaction with liver-resident (immune) cells. Given the role of platelets in modulating hepatic ER stress *in vitro*, this study aims to investigate their effects on hepatic ER stress and steatosis.

**Methods:** C57BL/6 mice (n = 6–12) were depleted for platelets and injected with tunicamycin (1 μg/g) to induce ER stress-induced hepatic steatosis. Hepatic platelet infiltration and activation was investigated by flow cytometry, ELISA, and electron microscopy. Livers were analyzed for ER stress markers by quantitative PCR, Western blotting, proteomics, and flow cytometry. Thereafter, platelet–hepatocyte co-cultures (n=4) and *in vivo* blocking of GPIbα (n = 5–10) were used for further mechanistic investigations.

**Results:** Our study demonstrates that hepatic ER stress leads to 1.8-fold accumulation ($p$ = 0.007) of platelets. Depletion of platelets decreased ER stress-induced liver steatosis by 35% ($p$ <0.0001). Mechanistically, we found that platelet depletion ameliorated the initial ER stress response and increased the influx of neutrophils into the liver. Inhibiting the platelet-specific receptor glycoprotein 1bα (GPIbα) partly mimicked the effects of platelet depletion, leading to impaired hepatic platelet recruitment and reduced triglyceride accumulation.

**Conclusions:** This study reveals a new role of platelets in hepatic ER stress and the concomitant effect on hepatic steatosis.



## Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) is the most common chronic liver disease, with an estimated prevalence of 25% in the adult population worldwide. MASLD is often considered the hepatic manifestation of metabolic syndrome, as its major risk factors include obesity, type 2 diabetes, and inflammation.[1] Obesity and type 2 diabetes induce a stress response in the endoplasmic reticulum (ER),[2] which has been reported as an important driver of MASLD in both mice[3] and humans.[4]

Platelets are anucleated blood cells derived from megakaryocytes and are predominantly implicated in hemostasis. Over the past decade, platelets have been recognized for their integral role in modulating inflammatory processes in the liver (e.g. in viral hepatitis[5,6] and liver regeneration[7]). Additionally, a growing body of evidence emphasizes platelets as active players in metabolic diseases including obesity and fatty liver disease.[8] Studies using intravital microscopy have demonstrated that activated platelets infiltrate the liver during both the early and late stages of MASLD.[8] Hepatic platelet influx is primarily mediated via the platelet receptor glycoprotein Ibα (GPIbα) and is dependent on interaction with Kupffer cells, with hyaluronan-CD44 binding playing a crucial role.[8]

We have recently demonstrated that platelets induce ER stress and the unfolded protein response (UPR) in murine and human macrophages by activating the IRE1α and ATF6 pathways.[9] Macrophage ER stress was accompanied by intracellular lipid accumulation and increased release of proinflammatory cytokines, but not cell death. Platelet-induced ER stress could not be reversed by blocking inflammation and was dependent on the direct interaction between platelets and macrophages.[9] Indeed, a pilot case study in patients at risk for MASLD revealed that antiplatelet therapy, but not high-dose non-steroidal anti-inflammatory drugs, reduced MASLD progression.[8] However, antiplatelet therapies are associated with an increased risk of bleeding,[10] which can impact health-related quality of life. Thus, it is important to understand how ER stress affects platelet activation and how platelets modulate hepatic ER stress and subsequent lipid accumulation. To investigate the effect of platelets on hepatic ER stress *in vivo*

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without confounding influence of the metabolic syndrome, ER stress was selectively induced in the liver, and platelet(s) (function) was targeted by blocking and depletion antibodies.

## Materials and methods

### Mice treatment

Wildtype C57BL/6 mice (male, 10–12 weeks old) were i.p. injected with tunicamycin (1 μg/g bodyweight, Sigma, Darmstadt, Germany) or vehicle treatment. Platelets were depleted by i.v. injection of an anti-GPIbα-antibody (1 μg/g bodyweight, #R300, Emfret, Eibelstadt, Germany) 1 h before tunicamycin treatment. The ligand-binding site of platelet GPIbα was blocked by i.v. injection of 100 μg p0p/B antigen-binding fragment.[11] Isotype antibodies (#C301, Emfret, Eibelstadt, Germany) served as control. Mice were sacrificed and blood and tissue were collected 4 or 24 h after tunicamycin treatment. Further details about the mice can be found in the Supplementary Materials and methods.

### Cell culture

The hepatocyte cell line AML12 (ATCC, Manassas, Virginia, USA, CRL-2254™) was cultured in DMEM-F12 medium (Gibco, Waltham, Massachusetts, USA) containing 10% FBS (Sigma, Darmstadt, Germany) and treated with tunicamycin (0.5–1 μg/ml, Sigma, Darmstadt, Germany) ± isolated platelets (500,000 platelets/μl) for 6 h.

### Biochemical analyses and statistics

AML12 cells alongside mouse plasma and tissue were analyzed using proteomic and RNA analyses, microscopy, (immune) histochemistry, flow cytometry, and lipid and lipoprotein analyses. Additional details are described in the Supplementary Materials and methods.

## Results

### Hepatic ER stress induces local platelet activation and platelet recruitment into the liver

To investigate whether hepatic ER stress promotes platelet activation and recruitment into the liver, we analyzed blood and liver tissues at different time points following the induction of ER stress (Fig. 1A). Quantitative analysis of fluorescence immunohistochemistry of liver sections revealed an increase in CD41+ platelets 4 h post-induction of hepatic ER stress (Fig. 1B). In line, hepatic levels of the platelet-specific degranulation marker platelet factor 4 (PF4/CXCL4) were significantly increased in liver homogenates after 4 h (Fig. 1C) and remained elevated until 24 h (Fig. 1D), indicating both platelet recruitment and activation. Electron micrographs of liver sections confirmed the presence of platelets within the liver tissue (Fig. 1E). Platelets were surrounded by hepatocytes (Fig. 1F) and in close proximity to blood vessels as visualized in the tomograms (Fig. 1G).

Next, we investigated whether hepatic ER stress caused systemic platelet activation. We found that plasma levels of PF4 tended to be increased 4 h after the induction of ER stress (Fig. 1H). To exclude a direct stimulatory effect of ER stress on platelet reactivity, we performed *ex vivo* experiments in which we treated isolated naive platelets with different concentrations of tunicamycin (1–10 ng/ml) and measured platelet activation by P-selectin (CD62P) exposure using flow cytometry (Fig. 1I). Tunicamycin did not increase P-selectin surface expression under basal conditions and no additive effect of tunicamycin could be observed upon re-stimulation of platelets with different concentrations of ADP. Higher tunicamycin concentrations (10 ng/ml) showed a trend towards reduced platelet reactivity. Thus, a direct stimulatory effect of tunicamycin on platelet reactivity could be excluded. However, platelets could still be indirectly activated by ER stress *in vivo.* To address this question, we measured platelet activation in blood samples at different time points following induction of ER stress. Neither basal platelet activation, nor platelet reactivity, were affected by ER stress after 2 h (Fig. 1J–L) or after 24 h (Fig. S1A and B). These observations were independent of the stimuli and activated pathway as both, ADP, which mimics secondary platelet activation *via* P2Y receptors, and a PAR4 agonist, which initiates the thrombin signaling pathway, showed similar effects. Moreover, neither degranulation nor inside-out signaling in platelets was affected as both P-selectin (Fig. 1K and L; Fig. S1C) and GPIIb/IIIa activation (Fig. S1D) remained unchanged in response to tunicamycin. To investigate whether hepatic ER stress increased the interaction of platelets with immune cells, such as neutrophils and monocytes, we measured platelet–neutrophil (Ly6G+CD41+) (Fig. 1M) and platelet–monocyte aggregates (CD115+CD41+) (Fig. 1N) in the blood 2 h after the induction of hepatic ER stress. We observed no differences in the formation of platelet–leukocyte aggregates either under *in vivo* conditions (Fig. 1M and N) or upon stimulation of blood *ex vivo* with ADP or a PAR4-activating peptide (Fig. S1E and F). Taken together, these findings suggest that hepatic ER stress promoted local platelet activation and recruitment into the liver without affecting systemic platelet reactivity.

### Hepatic ER stress signatures are not affected by platelet depletion after 24 h

We next investigated whether platelets could modulate hepatic ER stress *in vivo*. To this end, we induced hepatic ER stress in control and platelet-depleted mice for 24 h (Fig. 2A). Platelet depletion was confirmed by flow cytometry with a >95% reduction in platelet counts 1 h after antibody injection (Fig. S2), which was maintained throughout the treatment period (Fig. 2B). Electron micrographs of liver sections showed that induction of ER stress caused enlargement of the rough ER in hepatocytes (Fig. 2C), which was accompanied by loss of cytoplasm. The livers of platelet-depleted mice showed fewer mitochondrial deformations (e.g. decreased cristae), smaller ER fragments, and decreased lipid droplets and glycogen (Fig. 2D), indicating that platelets aggravated intracellular processes during ER stress. To elucidate which branch of the UPR was activated by platelets, mRNA levels of the three transmembrane ER sensors were quantified, either directly or via their downstream targets, via qPCR (Fig. 2E). Tunicamycin led to a drastic increase in mRNA levels of the ER lumen-residing chaperon BIP as well as the transmembrane sensor IRE1α, which resulted in increased splicing of XBP-1. Gene expression levels of the downstream targets eIF2α and ATF4 were either not elevated or only mildly elevated by tunicamycin, respectively (data not shown). However, none of the

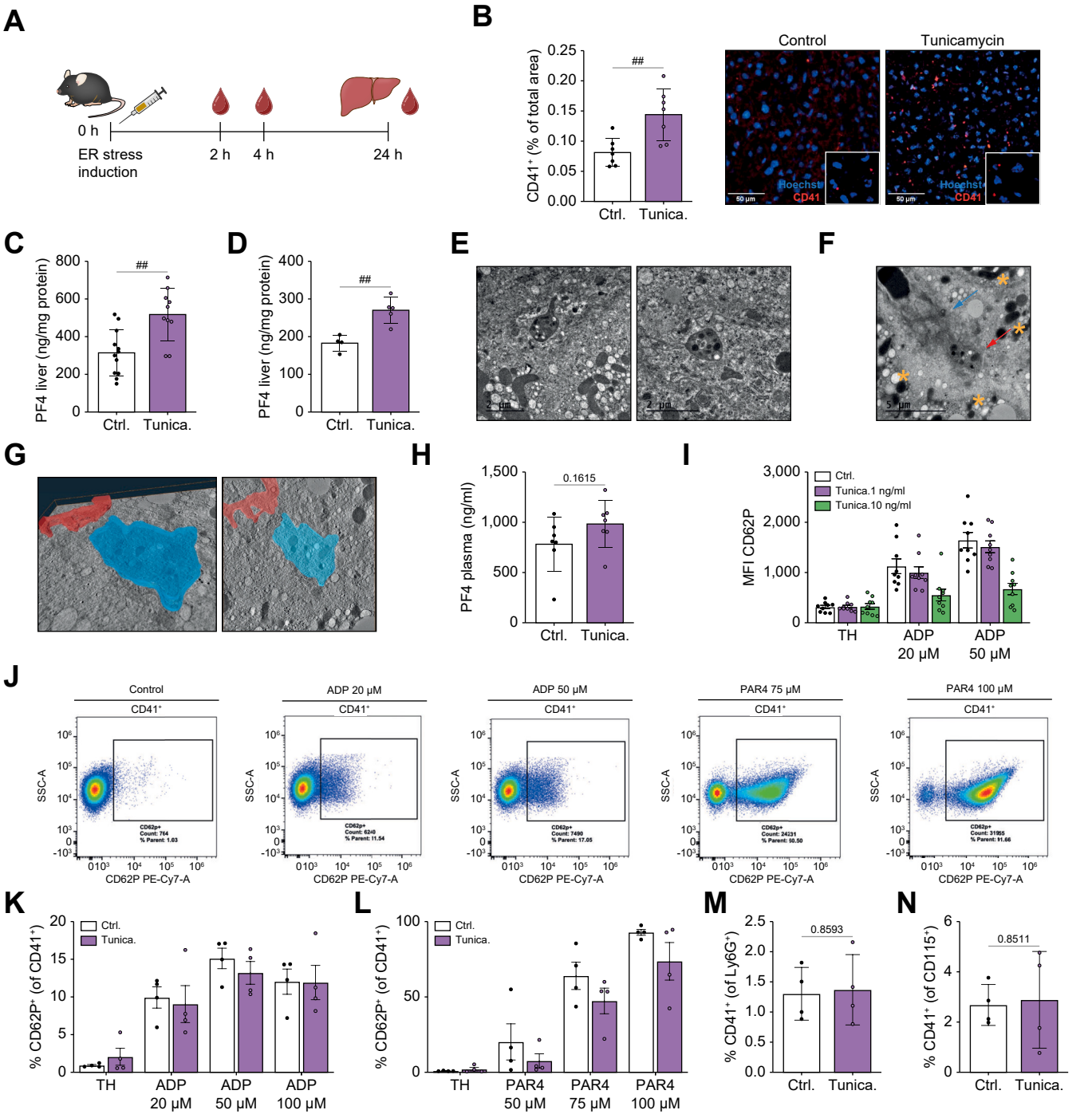


**Fig. 1. Hepatic ER stress promotes platelet recruitment and activation.** (A) Experimental design. (B) Liver sections (4 h): Hoechst (blue), anti-CD41 (red) (n = 4). Hepatic platelet factor 4 (PF4/CXCL4) levels:(C) 4 h (n = 10–12), (D) 24 h (n = 4–5). (E,F) Liver electron micrographs (24 h): (F) platelets (red arrow), blood vessels (blue arrow), hepatocytes (yellow stars). (G) 3D reconstruction: blood vessels (red), platelets (blue). (H) Plasma PF4 levels (4 h, n = 7). (I) Isolated platelets stimulated with buffer (TH) or ADP (n = 9). (J) Representative blots. (K,L) *In vivo* platelet activation (2 h) and re-stimulation (n = 4). (M) Platelet–neutrophil–aggregates and (N) platelet–monocyte–aggregates of tunicamycin-treated mice (2 h) (n = 4). Data represent mean ± SD. Two-way ANOVA or Mann-Whitney test; $^{\#\#}p$ <0.01.

ER sensors were affected by platelet depletion. Hepatic mRNA levels of CHOP showed the highest upregulation (up to 30-fold) caused by the induction of ER stress. This increase in mRNA expression was significantly lower in the absence of platelets (Fig. 2E). We subsequently performed Western blotting analyses to correlate the gene expression data of the ER stress targets to their protein expression (Fig. 2F and G). Protein expression of the UPR stress chaperon BIP could not be detected under basal conditions but showed a significant increase in response to ER stress. Protein levels of ER stress targets, including CHOP, were not affected by platelet depletion (Fig. 2F and G). These findings indicated that, despite

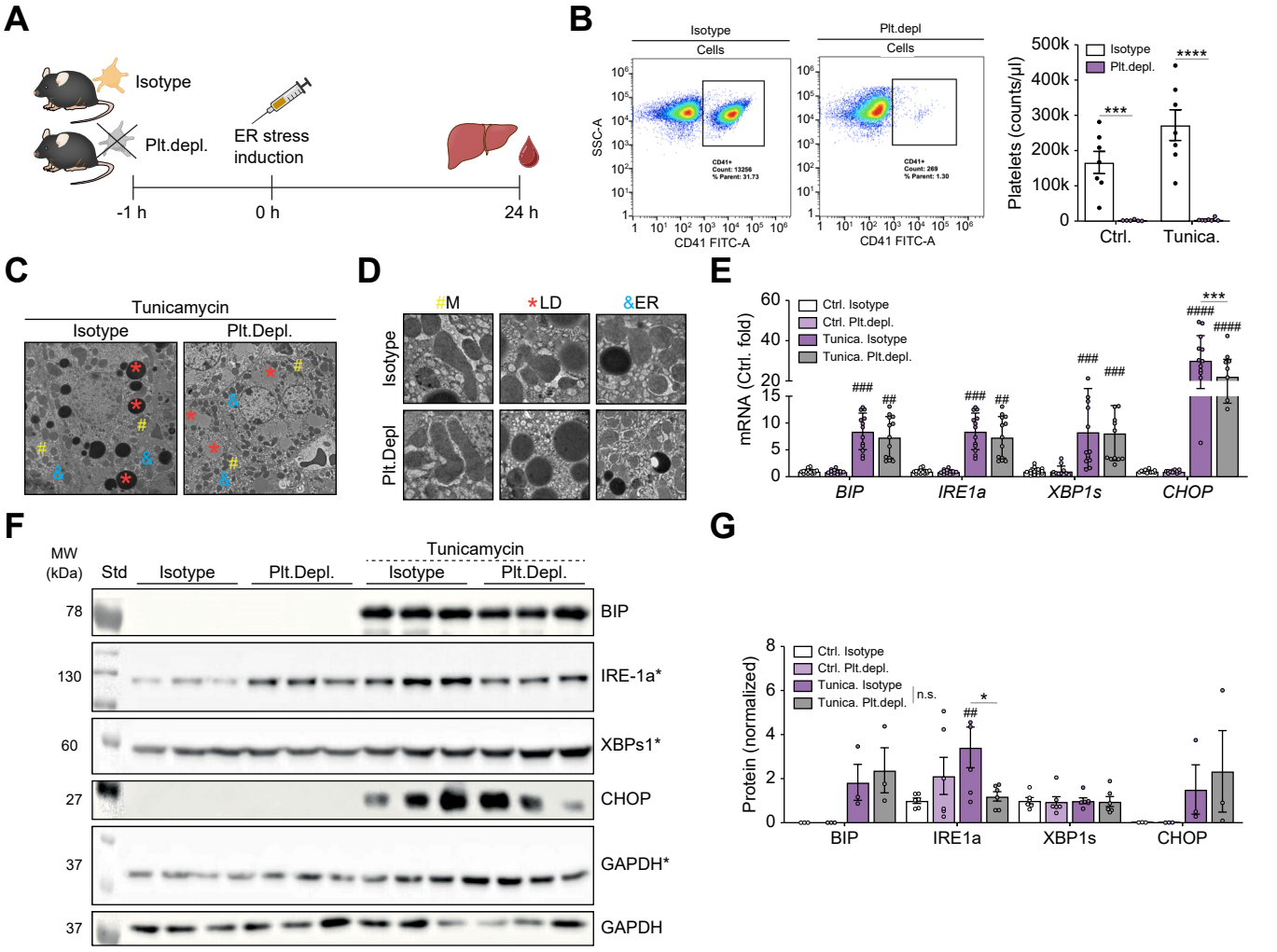


**Fig. 2. Platelet depletion alters hepatic morphology but does not affect ER stress.** (A) Experimental approach. (B) CD41+ cells in blood (n = 7). (C) Electron micrographs of liver sections (24 h). (D) Deformed mitochondria (M, #), lipid droplets (LD, *), and ER enlargement (ER, &). (E) Hepatic expression of 78-kDa glucose-regulated protein (BIP/GRP78), inositol-requiring enzyme 1 (IRE1α), spliced version of the X-box splicing protein (XBP1spl), and C/EBP homologous protein (CHOP) (n = 10–13). (F) Representative Western blots: corresponding membrane marked with star (*). (G) Relative quantification of protein levels relative to GAPDH (n = 3–8). Data represent mean ± SD. Two-way ANOVA; ***$p$ <0.001, ****$p$ <0.0001 (platelet depletion); $^{\#\#}p$ <0.01, $^{\#\#\#}p$ <0.001, $^{\#\#\#\#}p$ <0.0001 (tunicamycin).

changes in intracellular morphology, there were no major differences in gene and protein expression of ER stress markers.

### Platelet depletion dampens ER stress-induced hepatic steatosis

Next, we determined whether platelet depletion affected the development of hepatic steatosis induced by ER stress. ER stress led to a pronounced hepatic lipid accumulation (Fig. 3A), which was significantly decreased in the livers of platelet-depleted mice. Quantification of hepatic lipids revealed a significant reduction of triglyceride (Fig. 3B) and cholesterol content (Fig. 3C) in the absence of platelets, which confirmed the beneficial effect of platelet depletion on the development of ER stress-induced hepatic steatosis. In line with this, platelet depletion significantly reduced plasma activity levels of alanine aminotransferase (ALT, Fig. 3D), but not aspartate aminotransferase (AST, Fig. 3E) and lactate dehydrogenase (LDH, Fig. 3F). Liver weight was also unaffected by ER stress or by platelet depletion (Fig. 3G). Next, we investigated whether ER stress-induced changes in hepatic lipoprotein release by measuring plasma lipid levels. We found that plasma triglyceride (Fig. 3H), cholesterol (Fig. 3I), and non-esterified fatty acids (NEFA) (Fig. 3J) levels were drastically decreased by ER stress; however, this was not ameliorated by platelet depletion. In line, plasma levels of ApoB48 and ApoB100 proteins, markers of chylomicrons and VLDL/LDL, respectively, were reduced to undetectable levels because of ER stress-induced impairment of lipoprotein processing (Fig. S4A–C). Hepatic mRNA expression levels of genes involved in lipoprotein packaging and transport confirmed these findings (Fig. S4D). Hepatic expression of fatty acid oxidation and lipolysis genes

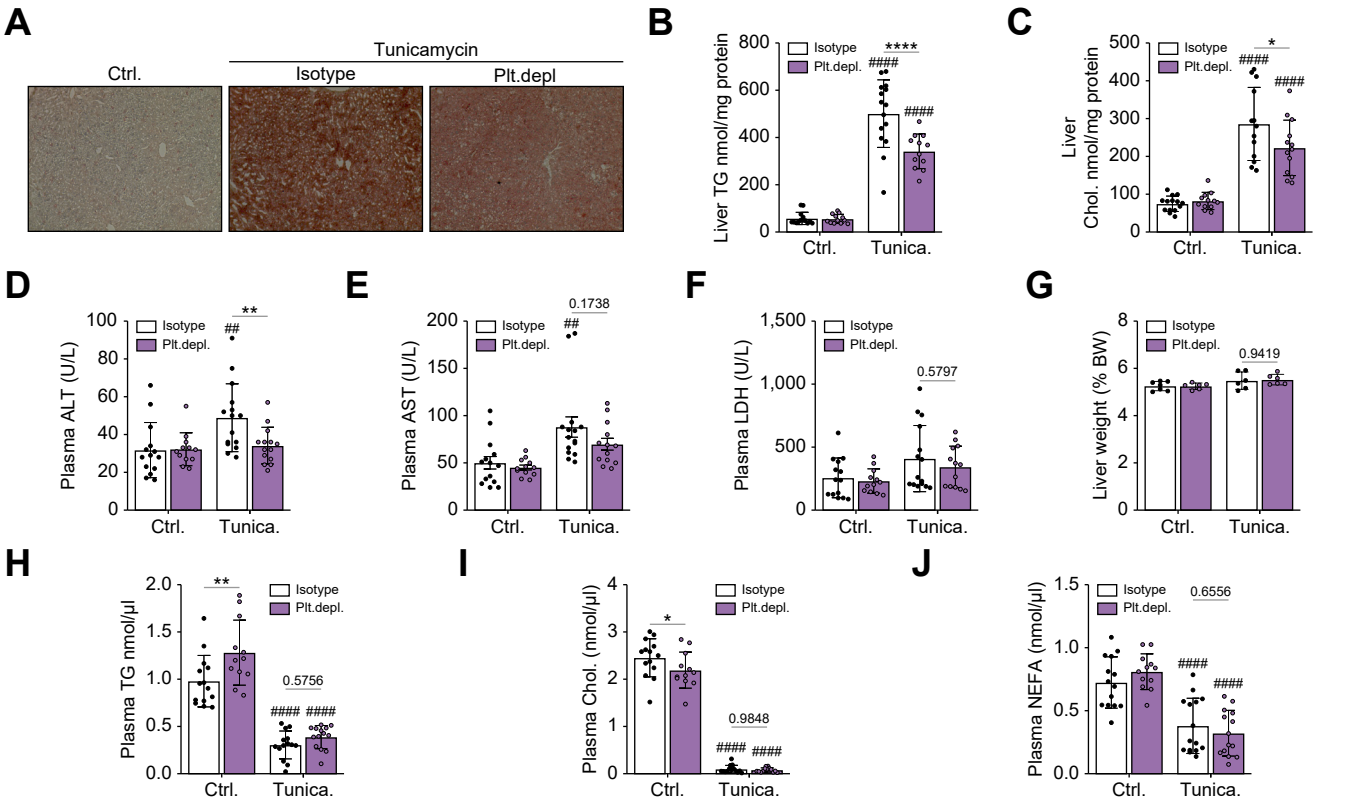


**Fig. 3. Platelet depletion ameliorates ER stress-induced liver steatosis after 24 h.** (A) Oil red O staining of liver sections. (B) Liver triglycerides (TG, n = 12–15) (C) and cholesterol (Chol., n = 12–15). (D–F) Plasma activity of alanine aminotransferase (ALT, n = 12–14), aspartate aminotransferase (AST, n = 12–14), and lactate dehydrogenase (LDH, n = 12–14). (G) Liver weight (n = 6–7). (H) Plasma triglycerides (TG, n = 12–14), (I) cholesterol (n = 12–14), and (J) non-esterified fatty acids (NEFA, n = 12–14). Data represent mean ± SD. Two-way ANOVA; **p* <0.05, ***p* <0.01, *****p* <0.0001 (platelet depletion); ##*p* <0.01, ###*p* <0.001, ####*p* <0.0001 (tunicamycin).

were not affected by platelet depletion (Fig. S4E). Together, these findings indicate that platelet depletion dampened ER stress-induced hepatic steatosis. However, after 24 h, the severity of the impairment in lipoprotein metabolism obscured platelet-dependent changes.

### Initial 4-h hepatic ER stress is decreased by platelet depletion

As we observed platelet activation early in the induction of hepatic ER stress, we hypothesized that platelet depletion modulated the initial – and not the adaptive – phases of hepatic ER stress to reduce hepatic steatosis. To test this hypothesis, we analyzed livers and plasma from control and platelet-depleted mice 4 h after the induction of ER stress (Fig. 4A). After this short treatment period, liver steatosis was already detectable, indicated by a 2.5-fold increase in hepatic triglycerides (Fig. 4B) and a 1.5-fold elevation of hepatic cholesterol content (Fig. 4C). In the absence of platelets, hepatic triglyceride levels increased only twofold, whereas hepatic cholesterol content remained at basal levels, underlining the effect of platelets on the early onset of ER stress-induced hepatic steatosis. Plasma activities of ALT (Fig. 4D), AST (Fig. S5A), and LDH (Fig. S5B) were not affected by ER stress at this time point, indicating the absence of liver damage. Hepatic mRNA and protein expression levels of ER stress significantly reduced BIP protein expression (Fig. 4F), IRE-1a phosphorylation (Fig. 4I), splicing of XBP-1 (Fig. 4J) as well as the mRNA expression of ATF4 (Fig. 4I), and CHOP (Fig. 4M). Plasma levels of NEFA were similar between isotype-treated and platelet-depleted mice (Fig. S5C), which was in line with comparable levels of ER stress markers in adipose tissue (Fig. S5D and E). Plasma triglycerides were significantly reduced by tunicamycin, and there was a trend towards this effect being ameliorated by platelet depletion (Fig. 4N). The same trend was observed for plasma protein levels of ApoB48 and ApoB100 (Fig. 4P) and hepatic mRNA expression of ApoB100 (Fig. S5F). In line with this, ER stress-induced upregulation of VLDLR mRNA levels trended to decrease with platelet depletion, suggesting reduced lipoprotein uptake (Fig. S5F). Collectively, we found that platelet depletion ameliorated the initial responses to ER stress leading to reduced hepatic steatosis by modulating lipoprotein metabolism.

### Co-culture of platelets and hepatocytes increases lipid accumulation

To gain further mechanistic insight into the crosstalk between platelets and hepatocytes, we developed an *in vitro* system in which we co-cultured hepatocytes with isolated platelets in either the presence or absence of ER stress (Fig. 5A). To determine protein levels and detect crosstalk-induced pathways, we performed proteomics analysis quantifying

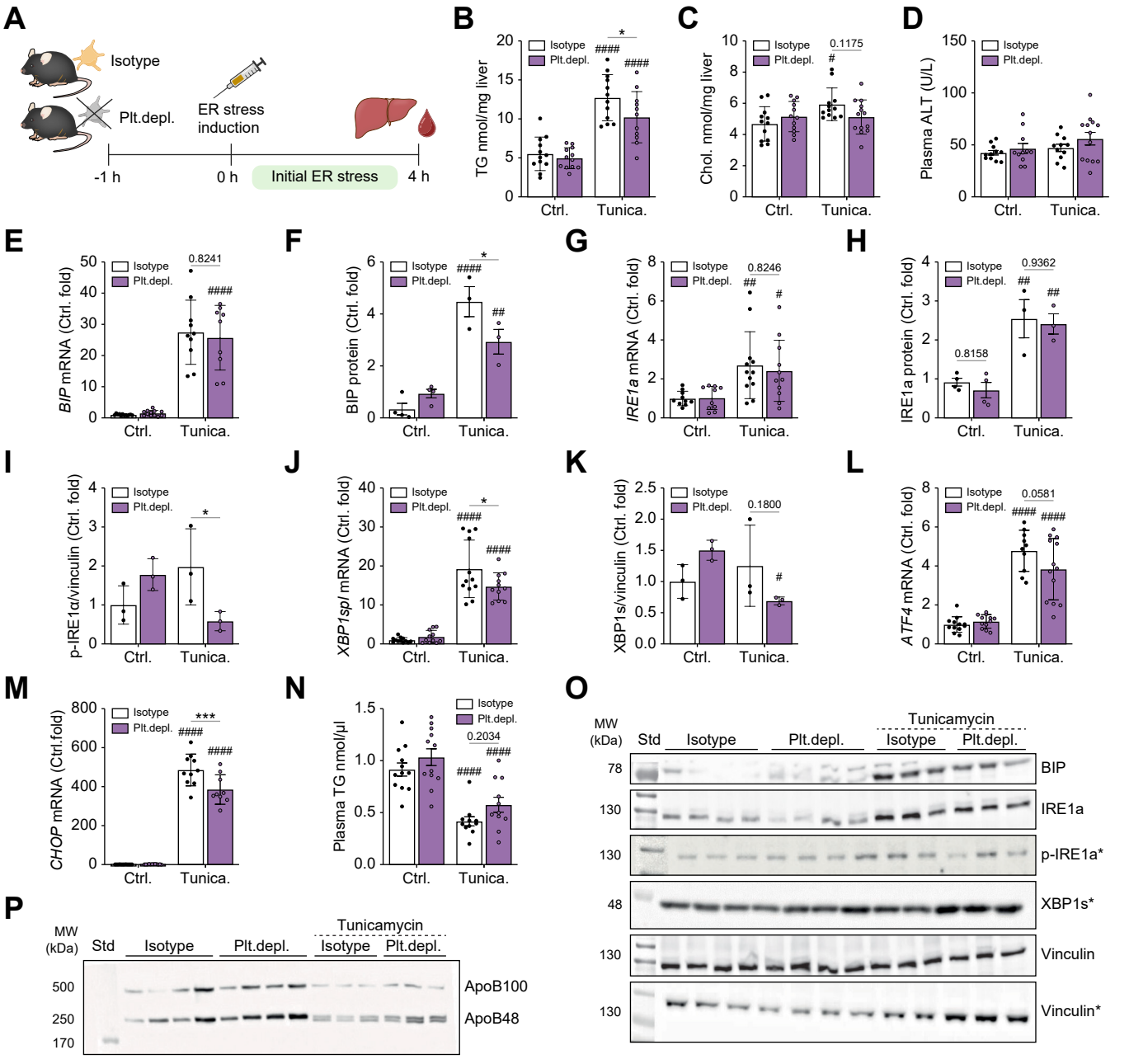


**Fig. 4. Platelet depletion reduces early hepatic ER stress.** (A) Experimental approach. (B) Hepatic triglyceride (TG, n = 11) and (C) cholesterol (Chol., n = 11) content. (D) Alanine aminotransferase (ALT, n = 11) activity. Hepatic gene and protein expression: (E,F) 78-kDa glucose-regulated protein (BIP/GRP78, n = 9–10, n = 3–4), (G,H) inositol-requiring enzyme 1a (IRE-1a, n = 10–12, n = 3–4), (I) phosphorylated IRE-1a (n = 3), (J,K) spliced X-box splicing protein (XBP1spl, n = 10–12, n = 3), (l) activating transcription factor 4 (ATF4, n = 10–13), and (M) C/EBP homologous protein (CHOP, n = 9–10). (O) Representative Western blots: same membranes marked with star (*). (N) Plasma triglyceride (TG, n = 10–12) levels. (P) Plasma levels of apolipoprotein (Apo) B48 and ApoB100. Data represent mean ± SD. Two-way ANOVA; *$p$ <0.05, ***$p$ <0.001 (platelet depletion); #$p$ <0.05, ##$p$ <0.01, ####$p$ <0.0001 (tunicamycin).

approximately 7,600 proteins from the hepatocyte lysates. We applied modeling accounting for gene-wise dispersion in the data using DESeq2[12] and assessed differential protein expression in response to either tunicamycin treatment (Fig. 5B) or co-culture with platelets (Fig. 5C). The most upregulated proteins under tunicamycin treatment were known ER stress marker proteins including Ddit3/CHOP, Hspa5/BIP, Xbp1, and Hsp90b1/GRP94 (Fig. 5D–G). Similarly, co-culture with platelets modulated hepatocyte protein expression resulting in the differential expression of 27 proteins. Although a subset of the upregulated proteins may be result from traces of highly expressed platelet proteins detected after removal of the platelets (Serpine2, Myh9, Pf4, Abcb9, Itgb3, Gp1bb, Rab27b), other differentially expressed proteins indicate activation of the lipid metabolism pathway (Plin2, Apob; Fig. 5H and I) and the cell adhesion/inflammatory signaling pathway (Icam1, Vcam1; Fig. 5J and K) in AML12 cells following co-culture with platelets. To corroborate the findings from the

proteomics analysis, we performed gene expression analysis of the selected ER stress markers BIP (Fig. 5L), IRE1a (Fig. 5M), sXBP1 (Fig. 5N), and CHOP (Fig. 5O). All markers were significantly increased by the induction of ER stress in a concentration-dependent manner; however, not affected by co-culture with platelets. Next, we wanted to ascertain whether platelets affected lipid accumulation in hepatocytes as suggested by both the proteomics (e.g. upregulation of Plin2, downregulation of ApoB) and *in vivo* data. We found that platelet–hepatocyte co-culture significantly increased the hepatocyte lipid content, which was measured as an increase in the mean fluorescence intensity of the neutral lipid dye Bodipy® (Fig. 5P and Q). Moreover, we detected an ER stress-dependent increase in VLDLR mRNA levels (Fig. 5R). To test whether this increase in lipids was associated with inflammation, as indicated by the proteomics data, we measured inflammatory gene expression in hepatocytes. We found that platelet co-culture led to a significant upregulation of IL-6 (Fig. 5S) and iNOS (Fig. 5T) mRNA levels, which increased in an ER stress-dependent manner. Co-culture experiments with platelets releasates revealed that these effects were mainly mediated by cell-to-cell contact and could not be phenocopied by soluble factors released into the media (Fig. S6A–F). In summary, we found that platelets modulated lipid metabolism and inflammation in hepatocytes upon ER stress.

### Hepatic ER stress leads to an acute immune cell response that is aggravated by platelet depletion

To investigate which proteins were regulated by platelets during hepatic ER stress *in vivo*, we performed proteomic analysis of liver lysates from platelet-depleted and isotype-injected mice exposed to ER stress. Differential protein expression (Fig. 6A) revealed a significant upregulation of the inflammatory proteins 12-lipoxygenase (Alox12, Fig. 6B) and interleukin 1 receptor antagonist (Il1rn, Fig. 6C) as well as the acute-phase proteins haptoglobin (Hp, Fig. 6D) and serum amyloid A1 (Fig. 6E) and A2 (Fig. 6F) in the livers of platelet-depleted mice. To test whether the observed inflammatory protein profile was associated with increased immune cell recruitment, we analyzed hepatic immune cell influx by flow cytometry. We found an increase of neutrophils in hepatic homogenates after 24 h of ER stress, which further increased upon platelet depletion (Fig. 6G and H). Relative distributions of hepatic immune cell populations are shown in Fig. 6I. Immunofluorescence staining of liver sections for the neutrophil marker myeloperoxidase (MPO) revealed that platelet depletion significantly accelerated the recruitment of neutrophils already after 4 h of ER stress (Fig. 6J and Fig. S7A) and confirmed the flow cytometry data at 24 h (Fig. S7B). This effect was specific for neutrophils as the percentage of CD68+ cells (macrophages, Kupffer cells) was neither affected by tunicamycin nor by platelet depletion after 4 h (Fig. 7K and Fig. S7C) and 24 h (Fig. S7D). In line with this, mRNA expression levels of *Ccr2*, *Itgax*, *Clec4f*, and *Emr1* did not increase with ER stress (Fig. S7E–H). To investigate whether ER stress affected the inflammatory activation of hepatic cells, we analyzed markers of macrophage activation and polarization via qPCR (Fig. 6K and L, Fig. S7I–L). We found that *Arg1* (Fig. S7I), *Ym1* (Fig. S7J), *Fizz1* (Fig. S7K), *Il6* (Fig. S7L), and *Il1b* (Fig. 6K) mRNA levels were significantly increased by ER stress. However, only expression levels of CD206 (Fig. 6L) and Il1b (Fig. 6K) were significantly decreased by platelet depletion. For IL-1β, we confirmed qPCR results by ELISA (Fig. 6M and N) and found that ER stress-induced IL-1β induction was decreased by platelet depletion both in the early (Fig. 6M) and adaptive (Fig. 6N) phases of ER stress. In summary, we found that platelet depletion accelerated ER stress-induced infiltration of neutrophils into hepatic tissue while dampening the activation of liver-resident macrophages.

### Blocking of GPIbα decreases hepatic platelet influx and ameliorates hepatic steatosis

Platelet-derived GPIbα has been identified as an important driver of thrombo-inflammatory disease models.[13] Furthermore, it is considered crucial for the recruitment of platelets into the liver and plays an important role in the development of metabolic dysfunction-associated steatohepatitis (MASH) and subsequent hepatocarcinogenesis.[8] Thus, we wanted to test whether GPIbα is also involved in platelet trafficking upon induction of hepatic ER stress. For this purpose, we blocked GPIbα with an antigen-binding fragment (Fab) 1 h before the induction of ER stress (Fig. 7A). Successful Fab binding to platelets was confirmed by flow cytometry (Fig. 7B), resulting in GPIbα blockage in over 80% of platelets (Fig. 7C). Total platelet counts were not affected (Fig. S8A). Blocking of GPIbα significantly reduced hepatic triglyceride accumulation (Fig. 7S), indicating a protective role in the development of hepatic steatosis. Hepatic cholesterol content (Fig. S9A), as well as plasma ALT (Fig. S9B) and AST levels (Fig. S9C), were not affected by GPIbα blocking. Next, we tested whether GPIbα is involved in the recruitment of platelets into the liver. We found that GPIbα blocking reduced ER stress-induced influx of platelets into the liver (Fig. 7E and F) as both CD41+ tissue area (Fig. S9D) and cellular fraction (Fig. 7E) were reduced. The reduction of hepatic platelets was associated with decreased numbers of CD68+ macrophages (Fig. S8B–D) but did not affect hepatic levels of PF4 (Fig. 7G), indicating that GPIbα blockade reduces platelet-induced immune cell recruitment rather than platelet activation. Hepatic expression of the ER stress sensors BIP showed a trend towards reductions when GPIbα was blocked on platelets (Fig. 7H). In contrast, hepatic mRNA levels of XBP1spl (Fig. 7i), ATF4 (Fig. 7J), and eIF2α (Fig. 7K) were not affected by GPIbα blockade at this time point. In line with the results obtained with platelet depletion, we observed a trend towards reduced hepatocyte cell death indicated by decreased mRNA levels of CHOP (Fig. 7L), DERL1 (Fig. 7M), and GADD34 (Fig. 7N). Plasma triglyceride and cholesterol levels showed a minor trend towards being less decreased upon GPIbα blocking, pointing to potentially decreased lipoprotein uptake (Fig. S9A and B). This was supported by a GPIbα-dependent regulation of hepatic VLDLR mRNA levels, while expression levels of the ligand ApoB100 were unaffected (Fig. S9C and D). Collectively, we found that blocking GPIbα decreases platelet recruitment upon ER stress and partially prevented the detrimental effects of platelets in the development of hepatic steatosis.

## Discussion

Platelets are increasingly recognized as active contributors to metabolic diseases associated with low-grade inflammation,

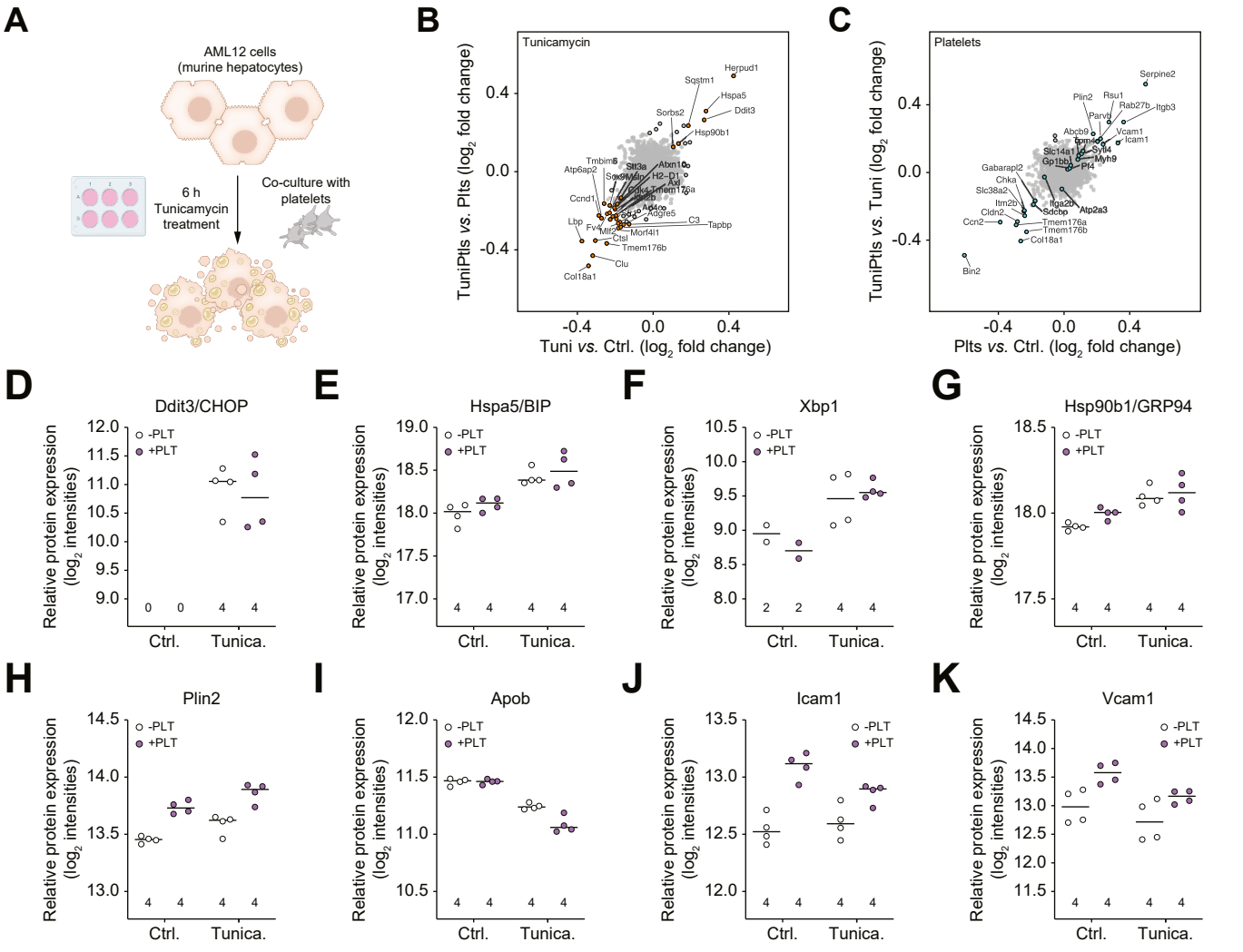


**Fig. 5. Co-incubation of platelets and hepatocytes increases hepatocyte lipid.** (A) Schematic overview (B–K) Differential protein expression analysis of AML12 lysates (Ctrl): tunicamycin-treated (Tuni), platelet co-culture (Plts), combination (TuniPlts). $Log_2$-transformed DEseq normalized differential average protein expression (~7,600 proteins, n = 4) (B) comparing tunicamycin treatment of Ctrl and Plts samples or (C) platelet co-culture of Ctrl and Tuni-treated cells. Fifty-two (tunicamycin) and 27 (platelets) significantly changed proteins (padj <0.05). (D–K) Relative protein expression (normalized log2-intensities): (D) C/EBP homologous protein (CHOP, n = 4), (E) 78-kDa glucose-regulated protein (BIP/GRP78, n = 4), (F) X-box splicing protein (XBP1, n = 2–4), (G) 94 kDa glucose-regulated protein (GRP94, n = 4) and (H) perilipin 2 (Plin2, n = 4), (L) apolipoprotein b (Apob, n = 4), (J) intracellular adhesion molecule 1 (Icam1, n = 4), and (K) vascular cell adhesion molecule 1 (vcam1, n = 4). Gene expression of (L) BIP/GRP78 (n = 4–5), (M) IRE1α (n = 4–5), (N) spliced XBP1 (XBP1spl, n = 4–5), (O) CHOP (n = 4–5). (P,Q) Hepatocyte lipid content (n = 3). (R) Gene expression of very low-density lipoprotein receptor (VLDLR, n = 5), (S) interleukin-6 (IL-6, n = 3–4), (T) inducible nitric oxide synthase (iNOS, n = 3–4). Data represent mean ± SD. Two-way ANOVA; $^{*}p$ <0.05, $^{**}p$ <0.01 (platelets); $^{\#}p$ <0.05, $^{\#\#}p$ <0.01, $^{\#\#\#\#}p$ <0.0001 (tunicamycin).

such as atherosclerosis[14] or MASH.[15] Earlier studies showed that platelets are recruited into MASH livers by interacting with Kupffer cells *via* hyaluronan-CD44 binding, where they promote inflammation and subsequent hepatocarcinogenesis.[8] Nevertheless, it is still unclear how platelets respond to metabolic stresses and contribute to disease development. We have previously shown that platelets induce ER stress and metabolic alterations in macrophages *in vitro*.[9] As ER stress is a hallmark of hepatic steatosis, our current study aimed to investigate how hepatic ER stress affects platelet activation and recruitment *in vivo,* and to ascertain how platelets affect ER stress-induced hepatic steatosis. We found that hepatic ER stress promoted the influx of platelets into the liver *via* GPIbα. Depletion of platelets or blocking GPIbα -mediated platelet recruitment ameliorated ER stress-induced hepatic steatosis. Mechanistically, we demonstrated that the depletion of platelets decreased early hepatic ER stress responses and lipoprotein accumulation.

In our model, platelet recruitment was an early event in the hepatic response to ER stress. Platelets were localized in close proximity to blood vessels, where they had direct contact with hepatocytes for cellular crosstalk. Elevated levels of the platelet degranulation marker PF4 indicated that platelets became activated by ER stress and locally released their granule content. *Ex vivo* induction of ER stress in isolated platelets did not affect platelet reactivity, indicating that platelets become indirectly activated by the ER stress response of liver-resident cells *in vivo*. Moreover, we did not

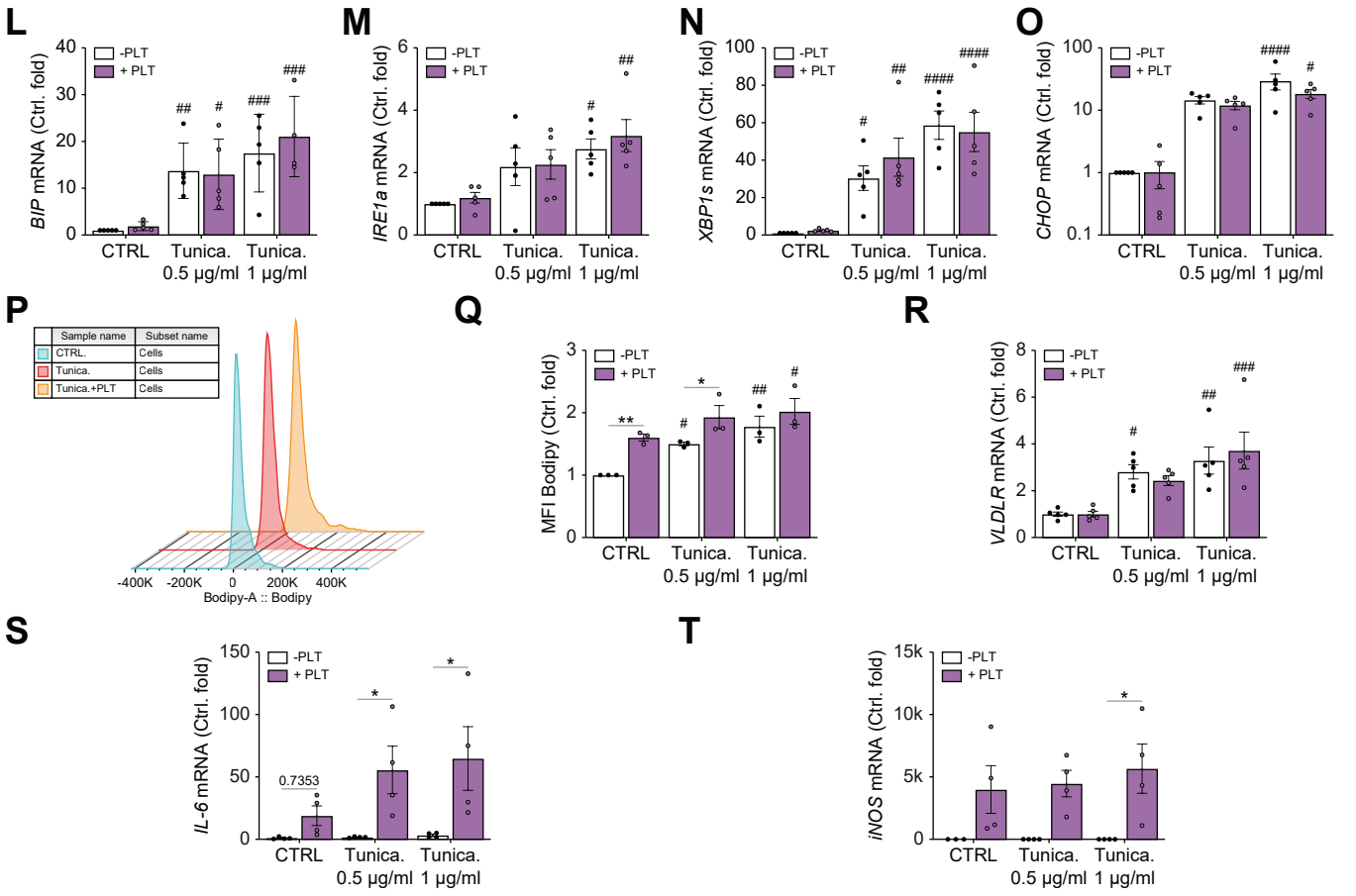


**Fig. 5.** (*continued*).

observe any signs of injured or compromised endothelium or intravascular thrombi (or clots) (data not shown) as reported in thrombo-inflammatory diseases such as ischemic stroke and myocardial infarction.[16] Rather, we observed a very mild inflammatory liver phenotype: only mRNA levels of IL-6 and IL-1β were significantly upregulated by ER stress. Protein levels of TNFα, IL-4, MCP-1, and IL-6 could be detected in liver homogenates but were not regulated by ER stress (Fig. S10). In line with this, ER stress induced a mild, late influx of monocytes and neutrophils, and platelet recruitment preceded hepatocyte damage. We have previously shown that neutrophils clear apoptotic extracellular vesicles by efferocytosis, and that efferocytosing neutrophils secrete factors to promote hepatocyte growth.[17] Thus, a beneficial effect of the secondary increase in neutrophils, potentially contributing to the restoration of liver homeostasis, is possible and is supported by the absence of neutrophil extracellular traps (data not shown). Overall, these findings indicate that metabolic, rather than inflammatory mediators, lead to the recruitment of platelets and the increase in hepatic steatosis.

ER stress leads to the accumulation of lipoproteins in hepatocytes, which results in decreased lipoprotein export into the plasma.[18] We observed a drastic reduction in plasma triglycerides and cholesterol levels after 24 h of ER stress, which was accompanied by decreased expression of the genes associated with lipoprotein packaging (ApoB, MTTP). In addition, we detected a significant (>10-fold) upregulation of the hepatic VLDL receptor, which has been reported to promote ER stress-induced hepatic steatosis upon high-fat diet feeding.[19] After 4 h of ER stress, triglyceride levels were already decreased in plasma, whereas we could not observe any effects on plasma cholesterol. As VLDL contains more triglycerides than LDL, we speculate that ER stress impacts VLDL rather than LDL uptake. Platelet depletion led to a significant reduction of early ER stress, and we observed a trend towards increased plasma triglyceride and ApoB100 and AboB48 lipoprotein levels, indicating that the protective effect of platelet depletion on hepatic steatosis was mediated via reduced VLDL uptake. Expression levels of VLDLR were indeed lower in the livers of platelet-depleted mice in the early phase of ER stress. VLDLR mediates hepatic lipid entry through receptor-mediated endocytosis or by LPL-dependent lipolysis,[20] and its expression is regulated by the transcription factor ATF4 during ER stress. Hepatocyte-specific knockout of ATF4 was reported to prevent VLDLR and CHOP induction by ER stress.[19] Indeed, our data revealed ATF4, VLDLR, and CHOP induction by ER stress was less pronounced following platelet depletion. Thus, our data indicate that platelets modulate hepatic steatosis by interfering with the ATF4 branch of ER stress, resulting in increased VLDL uptake.

*In vitro* data on the co-culture of platelets and hepatocytes revealed a significant increase in hepatocyte lipids. This

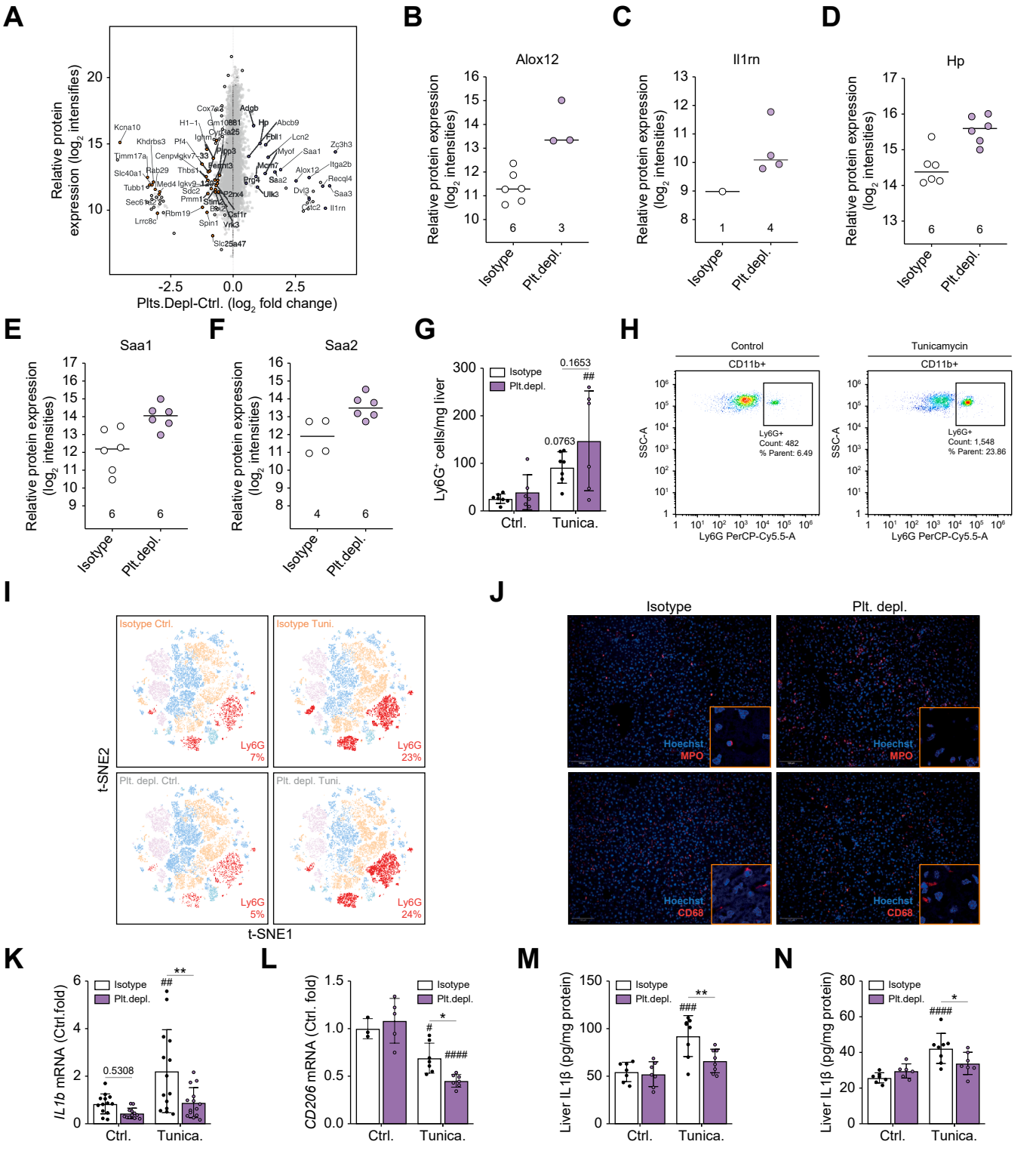


**Fig. 6. Hepatic ER stress leads to an acute immune cell response that is modulated by platelets.** (A–F) Differential protein expression of liver lysates (A) $Log_2$-transformed levels (~7,900 proteins, n = 6). (B–F) Normalized $log_2$-intensities: (B) 12-lipoxygenase (Alox12, n = 3–6), (C) interleukin-1 receptor antagonist (Il1rn, n = 1–4), (D) haptoglobin (Hp, n = 6), (E) serum amyloid A1 (n = 6) and (F) A2 (n = 4–6). (G) Liver Ly6G+ neutrophils (24 h, n = 6–7). (H) Representative flow blots. (I) t-SNARE blot (% of CD45+): Ly6G (red), cyan (CD8+), pink (CD4+), orange (CD11b+), blue (CD11b-). (j) Representative MPO (neutrophils, red) or CD68 (macrophages, red) and Hoechst (nuclei, blue) staining of liver sections (4 h). Hepatic expression of (K) IL1b (n = 14–16), (L) mannose receptor C type 1 (CD206, n = 3–6) (24 h). Protein levels of IL-1β after (M, n = 7) 4 h and (N) 24 h (n = 6–7). Two-way ANOVA; Data represent mean ± SD. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{****}p$ <0.0001 (platelet depletion) $^{\#\#}p$ <0.01, $^{\#\#\#}p$ <0.001, $^{\#\#\#\#}p$ <0.0001 (tunicamycin).

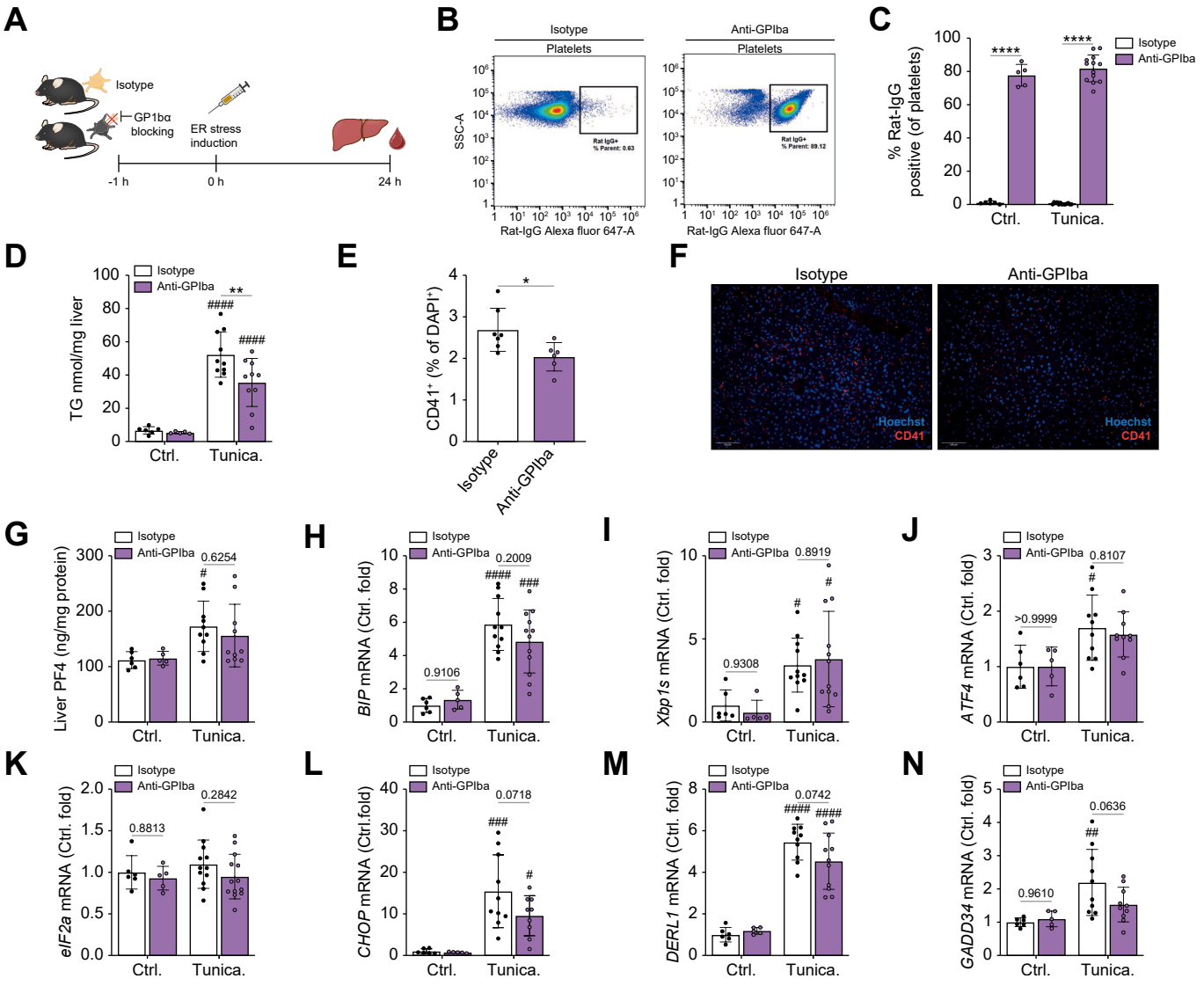


**Fig. 7. Blockade of GPIbα reduces platelet infiltration and hepatic steatosis.** (A) Experimental approach. (B,C) Anti-GPIbα Fab-fragment binding (n = 5–14). (D) Hepatic triglyceride (TG) content (n = 5–10). (E,F) Hepatic platelet infiltration: CD41 (platelets, red), nuclei (blue) (n = 6–7). (G) Hepatic platelet factor 4 (PF4) levels (n = 5–10). Hepatic gene expression (n = 5–12): (H) 78-kDa glucose-regulated protein (BIP/GRP78), (i) spliced X-box-binding protein (XBP1spl), (J) activating transcription factor 4 (ATF4), (K) eukaryotic translation initiation factor (eIF2α), (l) C/EBP homologous protein (CHOP), (M) Derlin 1 (DERL1), (N) growth arrest and DNA damage-inducible protein (GADD34). Data represent mean ± SD. Two-way ANOVA; *$p$ <0.05, **$p$ <0.01, ****$p$ <0.0001 (Anti-GPIba); $^{\#}p$ <0.05, $^{\#\#}p$ <0.01, $^{\#\#\#}p$ <0.001, $^{\#\#\#\#}p$ <0.0001 (tunicamycin). 'Two-way ANOVA' add 'or Mann-Whitney Test'.

platelet-specific effect decreased in a dose-dependent manner with increasing ER stress. Hepatocytes[21] and Kupffer cells[22] phagocytose platelets following stimulation by cytokines.[23] Thus, basal changes in lipid accumulation could be caused by increased phagocytosis of platelets and may not reflect the *in vivo* situation. To correct for residual platelet proteins or uptake of platelet material in the proteomics analysis, we separately measured the proteome of isolated platelets and subtracted the highest hits (e.g. Serpine2, Myh9, Pf4, Abcb9, Itgb3, Gp1bb, Rab27b) from our proteomic analysis. Nevertheless, this confounding factor cannot be completely excluded and remains a limitation of our study. Moreover, the lack of platelet-mediated VLDLR regulation in hepatocytes indicates the involvement of other cell types in the *in vivo* ER stress model. Indeed, VLDLR was strongly induced in bone marrow-derived macrophages upon platelet co-incubation (Fig. S11), suggesting an important role of Kupffer cells or monocyte-derived macrophages in hepatic lipoprotein uptake. In line with this, we found that blocking GPIbα not only decreased the hepatic infiltration of platelets, but also reduced the total number of macrophages. Moreover, platelet GPIbα blockade mimicked the effect of platelet depletion on hepatic triglyceride accumulation after 24 h. Hepatic gene expression levels of ER stress markers and VLDLR showed a trend towards downregulation by GPIbα blockade, indicating that hepatic steatosis is prevented by the same platelet-dependent mechanism.

MASLD is strongly linked to hepatic ER stress, a process that was recently denoted as a key mechanistic regulator that influences disease progression from simple steatosis to

inflammation-driven MASH.[24] Moreover, human MASLD samples show clear activation and dysregulation of major ER stress pathways[4] and platelets accumulate in the livers of patients with MASH.[8] Taken together, our data provide new mechanistic insights that may be highly relevant for MASLD in humans. Further studies are necessary to determine the extent to which these mechanisms contribute to the development of chronic MASLD. Identifying key mechanisms of platelet–hepatocyte crosstalk will be essential for developing novel therapeutics that selectively target metabolic, but not the hemostatic, functions of platelets, thereby preventing MASLD progression while preserving hemostasis and protection against infections.

In summary, this study uncovers a previously unrecognized role of platelets in modulating hepatic ER stress and liver steatosis *in vivo*.

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## Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ER, endoplasmic reticulum; GPIbα, glycoprotein Ibα; LDH, lactate dehydrogenase; NEFA, non-esterified fatty acids; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; MPO, myeloperoxidase; PF4/CXCL4 platelet factor 4; UPR, unfolded protein response.

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## Conflict of interest

There are no competing interests to disclose.
Please refer to the accompanying ICMJE disclosure forms for further details.

## Authors' contributions

Designed and supervised the study: MM. Contributed to designing experiments: JS, AA. Performed *in vivo* and *in vitro* experiments: MD. Performed experiments during the revision: TD, EA. Helped with *in vivo* experiments: WCS and MS. Provided help with *in vivo* Anti-GP1bα experiments: SB, BN. Performed ELMI: DK. Performed fluorescence microscopy: AMDC, HE. Performed and analyzed proteomics data: EA, NK, US. Analyzed data: MD, MM. Wrote the manuscript: MD, MM.

## Data availability

MS proteomics raw data together with the processing log files were deposited to the ProteomeXchange Consortium using the PRIDE partner repository (https://www.ebi.ac.uk(pride/) with the dataset identifier PXD060292. Other data are availabe from the corresponding author upon request.

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## Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2026.101767.

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*Author names in bold designate shared co-first authorship*

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