DOI: 10.1002/JLB.3A0820-527R

ARTICLE

JLB JOURNAL OF LEUKOCYTE BIOLOGY

Metallothioneins alter macrophage phenotype and represent novel therapeutic targets for acetaminophen-induced liver injury

Lindsey Devisscher[1] | Sanne Van Campenhout[1,2] | Sander Lefere[1,2] | Sarah Raevens[2] | Laurentijn Tilleman[3] | Filip Van Nieuwerburgh[3] | Hannelore P. Van Eeckhoutte[2] | Anne Hoorens[4] | Michael A. Lynes[5] | Anja Geerts[2] | Debby Laukens[2] | Hans Van Vlierberghe[2]

[1] Gut-Liver ImmunoPharmacology Unit, Department Basic and Applied Medical Sciences, Liver Research Center Ghent, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium

[2] Hepatology Research Unit, Department of Gastroenterology and Hepatology, Liver Research Center Ghent, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium

[3] Laboratory of Pharmaceutical Biotechnology, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

[4] Department of Pathology, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium

[5] Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut, USA

Correspondence
Prof. Dr. Lindsey Devisscher, Basic and Applied Medical Sciences, Gut-Liver ImmunoPharmacology Unit, Ghent University, Corneel Heymanslaan 10, B-9000 Ghent, Belgium
E-mail: lindsey.devisscher@ugent.be

Abstract

Acetaminophen (APAP) intoxication is the foremost cause of drug-induced liver failure in developed countries. The only pharmacologic treatment option, N-acetylcysteine (NAC), is not effective for patients who are admitted too late and/or who have excessive liver damage, emphasizing the need for alternative treatment options. APAP intoxication results in hepatocyte death and release of danger signals, which further contribute to liver injury, in part by hepatic monocyte/macrophage infiltration and activation. Metallothionein (MT) 1 and 2 have important danger signaling functions and might represent novel therapeutic targets in APAP overdose. Therefore, we evaluated hepatic MT expression and the effect of anti-MT antibodies on the transcriptional profile of the hepatic macrophage population and liver injury following APAP overdose in mice. Hepatic MT expression was significantly induced in APAP-intoxicated mice and abundantly present in human livers. APAP intoxication in mice resulted in increased serum transaminase levels, extended necrotic regions on liver histology and induced expression of proinflammatory markers, which was significantly less pronounced in mice treated with anti-MT antibodies. Anti-MT antibody therapy attenuated proinflammatory macrophage polarization, as demonstrated by RNA sequencing analyses of isolated liver macrophages and in LPS-stimulated bone marrow-derived macrophages. Importantly, NAC and anti-MT antibodies were equally effective whereas administration of anti-MT antibody in combination with NAC exceeded the efficiency of both monotherapies in APAP-induced liver injury (AILI). We conclude that the neutralization of secreted MTs using a monoclonal antibody is a novel therapeutic strategy as mono- or add-on therapy for AILI. In addition, we provide evidence suggesting that MTs in the extracellular environment are involved in macrophage polarization.

Abbreviations: AILI, acetaminophen-induced liver injury; ALT, serum alanine aminotransferase; APAP, acetaminophen; AST, aspartate aminotransferase; BMDM, bone marrow-derived macrophage; KC, Kupffer cell; MoMF, monocyte-derived macrophage; MTs, metallothioneins; NAC, N-acetylcysteine; NAPQI, N-acetyl-p-benzoquinone imine.

KEYWORDS
acetaminophen, acute liver injury, drug-induced liver failure, hepatic inflammation, Kupffer cells, liver immunity, macrophages, metallothioneins, mouse model, paracetamol

1 | INTRODUCTION

Acetaminophen (APAP), also known as paracetamol, is the most widely used antipyretic and analgesic over-the-counter drug and is also the leading cause of acute liver failure in the United States and Europe.[1] APAP is metabolized in hepatocytes by glucuronidation and sulfatation to nontoxic metabolites, but a small fraction of APAP is metabolized to the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is, at nontoxic doses, conjugated by endogenous glutathione and excreted via the bile.[2] However, when APAP is overdosed, glutathione concentrations are insufficient to convert the increasing amount of NAPQI. NAPQI binds to mitochondrial proteins and forms cytotoxic protein adducts, which cause oxidative stress, the production of reactive oxygen species and mitochondrial dysfunction.[2,3] Mitochondrial dysfunction results in hepatocyte necrosis and the release of danger signals (danger-associated molecular patterns, DAMPs) that are able to attract, activate, and/or alert nearby immune cells to "danger" for the host, resulting in an inflammatory response.[4,5] Activated immune cells, including resident liver macrophages (Kupffer cells, KCs), secrete chemokines, which further increase the influx of immune cells in areas of hepatocyte damage, and cytokines, enhancing inflammation and further tissue damage.[2] Infiltrating monocytes can differentiate into monocyte-derived macrophages (MoMFs) and it has been shown that KCs, MoMFs, and infiltrating monocytes have distinct roles in the pathogenesis of APAP-induced liver injury (AILI). Early infiltration of monocytes can aggravate liver injury whereas MoMFs are crucial for recovery from AILI.[6–10] Thus, liver infiltrating and resident macrophages represent attractive therapeutic targets in the context of liver injury. At present, N-acetylcysteine (NAC) is the only pharmacologic treatment option for patients suffering from AILI.[2] However, NAC is only effective when given within the first 12–24 h after APAP ingestion and its benefit decreases with increasing time between overdose and treatment.[3,11] For those patients who are admitted to the hospital too late, liver transplantation is the only curative and life-saving option. Consequently, there is an urgent need to find alternative treatment options for patients suffering from AILI to extend the available therapeutic window.

We have previously identified metallothioneins (MTs) as a new class of danger signals that are released from dying cells and are able to attract leukocytes both in vivo and in vitro.[12,16] MTs are highly conserved, cysteine-rich metal-binding proteins with a low molecular weight and the isoforms MT1 and MT2 are considered to be acute phase proteins due to their rapid induction upon inflammation.[13] Under physiologic conditions, cytosolic MTs are involved in metal homeostasis and transport,[14] and have anti-oxidative and anti-inflammatory properties.[12] Mice lacking MT1 and MT2 showed a higher disease activity in experimental AILI models, presumably as a consequence of the absence of redox management provided by MT and the lack of an important source of essential zinc and copper ions that are necessary for liver regeneration.[15] However, during necrotic cell death, MTs are released in the extracellular environment and function as danger signals by attracting other immune cells to the inflammatory site.[12] The MT1 and MT2 genes are located near other chemokine genes and MTs have been shown to drive the migration of leukocytes in a chemotactic gradient.[12,16] In addition, blocking extracellular MTs with monoclonal anti-MT antibody has been shown to reduce leukocyte infiltration and disease activity in experimental colitis.[12] Because the pathogenesis of AILI is driven by cell death, danger signal release, and monocyte/macrophage infiltration and activation,[10,17–19] extracellular MTs that function as danger signals might represent a novel therapeutic target in AILI. Indeed, NAC is effective by replenishing glutathione concentrations and prevents damage following hepatocyte necrosis but has little effect on the ensuing inflammatory response mediated by danger signal release, immune cell infiltration, and activation and subsequent bystander damage. As such, blocking MT danger signal functions and intervening downstream in the inflammatory cascade, might have an additive therapeutic value for patients who present with exacerbated liver inflammation. We hypothesize that blocking extracellular MTs may impact the inflammatory environment in experimental AILI and thus may represent a therapeutic target to be added to the standard treatment with NAC in experimental AILI.

2 | METHODS

2.1 | Human tissue

Liver tissue was obtained from three patients who underwent liver transplantation due to APAP-induced acute liver failure and liver tissue from three patients with colorectal cancer metastasis, sampled at a distance from a colorectal tumor, as control group. These samples were evaluated for MTs' immunoreactivity by immunohistochemistry. This study was approved by the local ethics committee of University Hospital Ghent (EC UZG 2015/1113 and 2017/0539).

2.2 | Animal experiments

Seven-week-old male C57BL/6 mice were purchased from Janvier (Le Genest-Saint-Isle, France). Mice were fasted for 20 h prior to receiving 500 μl PBS or 300 mg/kg APAP (Sigma, Diegem, Belgium), dissolved in 500 μl warm PBS, by i.p. injection. APAP-injected mice were treated after 2 h with NaCl (vehicle, control), 15 mg/kg anti-MT antibody (clone UC1MT)[20] or an equivalent dose of IgG (isotype-matched

control for antibody specificity), 200 mg/kg NAC (Sigma), or the combination of NAC and anti-MT antibodies i.p. All treatments were adjusted to equal volumes of 200 μl. Blood sampling was performed under anesthesia 24 h after APAP administration after which the mice were sacrificed and liver samples collected. Liver damage was assessed by analysis of serum aspartate and alanine aminotransferase (AST and ALT, respectively) levels, histology on H&E stained sections (semi-quantitative analysis of necrotic areas), expression of proinflammatory cytokines/chemokines by quantitative real-time PCR, and infiltration of leukocytes by flow cytometry. The animals were housed in the animal facility of the Faculty of Medicine and Health Sciences of Ghent University (Belgium) according to the national guidelines for animal protection. The ethical committee of animal experimentations at the Faculty of Medicine and Health Sciences of Ghent University (Belgium) approved the protocols (ECD15/20).

2.3 | Histology and immunohistochemistry

Paraffin-embedded mouse liver sections were rehydrated by serial immersion in xylene and ethanol and stained with H&E. Bright field images were captured using an Olympus (BX41, Antwerp, Belgium) light microscope. Necrotic areas were quantified in at least 10 fields per mouse using Cell^D software (Olympus). Results are expressed as the mean necrotic area per field (% ± SEM). The final score is represented as the mean of the scores determined by two independent researchers, who were blinded to the study identity.

Human liver tissue was formalin fixed, paraffin embedded, and evaluated for MTs' immunoreactivity by immunohistochemistry. MT staining was carried out using mouse monoclonal anti-MT clone E9 (Zymed Laboratories, San Francisco, CA, USA). An isotype-specific irrelevant antibody (mouse IgG1, Dako, Glostrup, Denmark) was used in a matched concentration to control for nonspecific binding of the primary antibody. MTs' immunoreactivity was evaluated by two independent observers.

2.4 | Bone marrow-derived macrophages (BMDMs)

Femoral and tibial bone-marrow canals of C57BL/6 mice were flushed with cold sterile PBS to obtain BMDMs. Cells were cultured in 10 × 15 mm petri-dishes in DMEM+Glutamax (Invitrogen, Merelbeke, Belgium) supplemented with 10% FBS, penicillin/streptomycin (Invitrogen), and recombinant murine M-CSF (PeproTech, London, United Kingdom). Medium was changed every other day and cells were seeded for experiments on day 7. Cells were stimulated with 100 ng/ml LPS (Ultrapure LPS from *E. coli* K, Invivogen, San Diego, CA, USA) with and without addition of 100 μg/ml UC1MT for 24 h. Cells were lysed for RNA extraction and supernatant was collected for cytokine analysis.

2.5 | Quantitative real-time PCR

Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Benelux, Venlo, Netherlands) with on-column DNase treatment. The concentration and purity of the total RNA were determined using a spectrophotometer (WPA Biowave II, Isogen Life Science, Netherlands). One microgram of total RNA was converted to single strand complementary DNA by reverse transcription (Superscript, Invitrogen) with oligo(dT) priming. The cDNA was diluted 1/8 and was used in real-time quantification with SYBR Green (Roche, Vilvoorde, Belgium) and 250 mM of each primer. A 2-step program was run on a LightCycler 480 (Roche). Cycling conditions were 95°C for 10 min and 45 cycles of 95°C for 10 s and 60°C for 1 min. A melting curve analysis confirmed primer specificity. All reactions were performed in duplicate and normalized to GAPDH and succinate dehydrogenase complex subunit A. The PCR efficiency of each primer pair was calculated using a standard curve from reference cDNA. The amplification efficiency (E) was determined using the formula 10−1/slope. The primer pairs used are listed in Table 1.

2.6 | Flow cytometry and fluorescence-activated cell sorting

The livers were flushed with PBS, isolated and chopped into small pieces and incubated for 20 min in 1 mg/kg collagenase A (Sigma) and 10 U/ml Dnase (Roche) in a heated bath (37°C). Suspensions were filtered and cells were stained with CD45-APC-Cy7, F4/80-FITC, Ly6G-PercCP-Cy5.5, Ly6C-V450, CD11b-PECy7, and Tim4-PE from BD Bioscience for 20 min at 4°C in the dark. Cells were sorted using an AriaII flow cytometer (BD Bioscience Headquarters Franklin Lakes, New Jersey, U.S.) based on the following gating strategy[21]:

live CD45+CD11b^{hi}Ly6G-Ly6C^{hi} monocytes, CD45+F4/80+CD 11b^{int}Ly6C-Ly6G-Tim4+ KCs, CD45+F4/80+CD11b+Ly6C-Ly6G-Tim4- MoMF; and analyzed using FlowJo software (TreeStar). Sorted cells were lysed in RNeasy Lysis Buffer (RLT) plus β-mercaptoethanol for RNeasy Lysis Buffer (RNA) extraction and expression analyses.

2.7 | RNA sequencing analysis of hepatic macrophages

After RNA extraction, the concentration and quality of the total extracted RNA were verified by using the "Quant-it ribogreen RNA assay" (Life Technologies, Grand Island, NY, USA) and the RNA 6000 Nano chip (Agilent Technologies, Santa Clara, CA, USA), respectively. Subsequently, 1 ng of RNA was used to perform an Illumina sequencing library preparation using the QuantSeq 3′ mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria) according to the manufacturer's protocol. During library preparation, 22 PCR cycles were used. Libraries were quantified by qPCR, according to Illumina's protocol "Sequencing Library qPCR Quantification protocol guide," version February 2011. A High Sensitivity DNA chip (Agilent Technologies) was used to evaluate the library's size distribution and quality. Sequencing was performed on a high throughput Illumina NextSeq 500 flow cell generating 75 bp single reads.

TABLE 1 Primer pairs

Gene symbol	Forward primer	Reverse primer
Mouse		
GAPDH	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA
SDHA	CTTGAATGAGGCTGACTGTG	ATCACATAAGCTGGTCCTGT
TNFα	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
IL-6	GCTGGTGACAACCACGGCCT	AGCCTCCGACTTGTGAAGTGGT
MCP-1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGAATTTACGGGT
CXCL2	GCGCCCAGACAGAAGTCATAG	AGCCTTGCCTTTGTTCAGTATC
NLRP3	ATCAACAGGCGAGACCTCTG	GTCCTCCTGGCATACCATAGA
NLRP6	TGACCAGAGCTTCCAGGAGT	TTTAGCAGGCCAAAGAGGAA

For these samples, an average of 9.9 ± 3.4 million reads were generated. These reads were first trimmed using Cutadapt software[22] version 1.18 to remove the "QuantSEQ FWD" adaptor sequence. The trimmed reads were mapped against the Mus musculus GRCh38.91 reference genome using STAR software[23] version 2.6.0c. The RSEM[24] software, version 1.3.1, was used to generate the count tables.

Differential gene expression analysis between groups of samples was performed using DESeq2.[25] Genes having a false discovery rate (FDR) <0.05 and a fold change >2 were considered significantly distinctive between groups. Differentially expressed genes were used for gene set enrichment analyses (GO biologic process) using Cytoscape[26] and BiNGO plug-in.[27]

2.8 | Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., 2365 Northside Dr. Suite 560 San Diego, CA 92108, USA). Data are presented as mean ± SEM. Differences between two groups were calculated using an unpaired *t*-test in case of normality or the Mann-Whitney statistic if the data were not normally distributed. Multiple-group comparisons were performed using ANOVA followed by a Bonferroni posttest. A value of $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | MTs are highly expressed in experimental and human AILI

In AILI, the liver is the main source of MT1 and MT2 and it has been reported that both isoforms are up-regulated upon various inflammatory stimuli.[28,29] Indeed, APAP overdosing resulted in a significant increase in MT1/2 mRNA expression compared to control mice (Fig. 1A).

To confirm MT1/2 expression in human AILI, liver samples of patients who were transplanted for AILI and liver samples of patients without AILI were analyzed for MT1/2 immunoreactivity. Immunohistochemistry shows an abundant expression of MT1/2 in hepatocytes of liver sections of all patients with loss of cellular integrity in centrolobular necrotic areas of APAP-intoxicated livers (Fig. 1B). The latter suggests extracellular release of MT1/2 and indicates the potential of MT1/2 as extracellular contributors to AILI.

3.2 | The combinational therapy of anti-MT antibodies and NAC exceeds each monotherapy and represents a new therapeutic strategy in AILI

We next evaluated the therapeutic potential of blocking extracellular MTs by highly specific anti-MT antibodies (clone UC1MT), alone and in combination with the standard of care NAC, in APAP overdosed mice. APAP overdose resulted in a significant increase in serum transaminase levels that was significantly lower in both NAC and anti-MT treated mice. Moreover, the combination of NAC and anti-MT exceeded the attenuated induction of ALT levels after each monotherapy (Fig. 2A). This was also confirmed on histology, with smaller and fewer necrotic regions on liver histology after APAP overdose in NAC and anti-MT treated mice compared to untreated mice, which was again most pronounced in mice treated with the combination of NAC and anti-MT antibodies (Fig. 2B). Anti-MT antibody specificity was confirmed by therapy efficacy comparison against IgG control therapy in APAP intoxicated mice (Fig. 2C).

3.3 | Anti-MT antibody therapy attenuates APAP-induced liver inflammation

To further evaluate how anti-MT antibodies exert their effect in AILI, we further evaluated hepatic inflammatory markers and cell infiltration in mice after APAP overdose with and without anti-MT treatment. APAP-intoxicated mice showed up-regulated

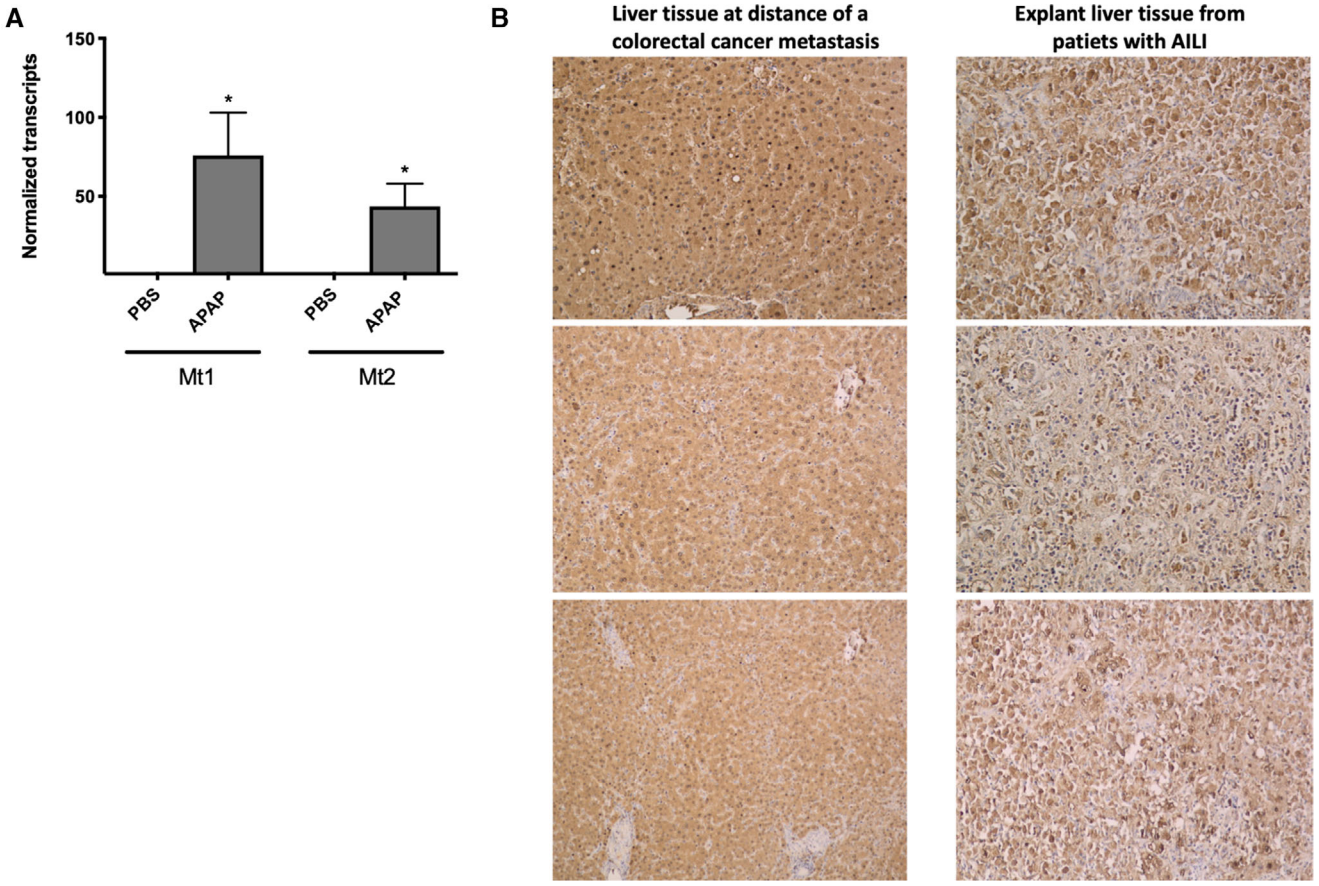

FIGURE 1 **Hepatic metallothionein (MT)1/2 expression in mouse and human acetaminophen-induced liver injury (AILI).** (**A**) Mouse hepatic MT1 and MT2 mRNA expression 24 h after an acetaminophen (APAP) overdose. $n = 5$, $^*P < 0.05$. (**B**) MT1/2 immunoreactivity in the liver of human patients with and without AILI. Magnification ×200

transcription of genes encoding the chemokines *Mcp1* and *Cxcl2*, responsible for monocyte and neutrophil attraction, respectively, the inflammasome *Nlrp3* and the proinflammatory cytokines *Tnfα*, *Il1β*, and *Il6*, which was less pronounced in mice treated with anti-MT antibodies compared to control treated mice with AILI (Fig. 3A). APAP overdose also resulted in a significant increase of live CD45+Ly6G+F4/80$^-$CD11b$^+$Ly6C$^+$ neutrophils and CD45+Ly6G-F4/80+CD11b^{hi}Ly6C^{hi} monocytes whereas CD45+Ly6G-F4/80+CD11b^{int}Ly6C$^-$Tim4$^+$ KCs were depleted, as previously described,[21] but no effect of anti-MT antibodies was observed (Fig. 3B).

3.4 | Differential gene expression of hepatic macrophages in AILI and after anti-MT antibody therapy

To characterize the different hepatic macrophage populations in AILI and the effect of anti-MT antibody therapy on liver macrophages in vivo, we isolated KCs, infiltrated monocytes and MoMF by FACS from the livers of healthy mice and mice with AILI, and performed mRNA sequencing analysis of these hepatic macrophage populations. APAP overdose resulted in differential gene expression (FDR < 0.05) of 651 genes in KCs, 795 genes in MoMF, and 917 genes in MO. In the principal component analyses, the different cell populations in steady state, after APAP and after anti-MT antibody treatment, are separated by the first two principal components (Supporting Information Fig. S1). Gene enrichment mapping showed that genes involved in chemotaxis (including *S100a8*, *Ccl9*, *Cxcr2*, *Ccl2*, *Ccl4*, *Cxcl3*), cytokine/chemokine production (including *Il6*, *Tnfrsf11a*, *Lbp*, *Prry2*, *Smad3*, *Mif*), inflammation and immune responses (including *Il1rl1*, *Il1a*, *Arg1*, *Tlr1*, *Tlr13*, *Nfkb1a*, *Jun*), and leukocyte differentiation and activation (including *Bcl6*, *Hps1*, *Cd1d1*, *Il7r*, *Il6*, *Jak3*) were mainly up-regulated in liver infiltrated monocytes and, with respect to genes involved in chemotaxis and inflammatory response, to a lesser extent in MoMFs (Fig. 4). In KCs, APAP resulted in the up-regulation of genes involved in lipid catabolism (such as *Gba2*, *Gla*), cell cycle (such as *Haus2*, *Smc3*, *Ccn1*), and angiogenesis (*Il1a*, *C5ar1*, *C3ar1*, *Ptgs2n serpine1*, *Angpt2*) (Fig. 4).

Anti-MT treatment in mice with AILI clearly resulted in differential gene expression in the three analyzed cell populations (Fig. 5A, B). Anti-MT treatment down-regulated 166 genes in KCs (>2-fold down-regulated, FDR < 0.05) including genes involved in proinflammatory processes such as *Spata2* (*tnfr1-sc*), which is involved in TNF-induced necroptosis[30] (*Traf5* [TNF receptor associated factor 5]) involved in

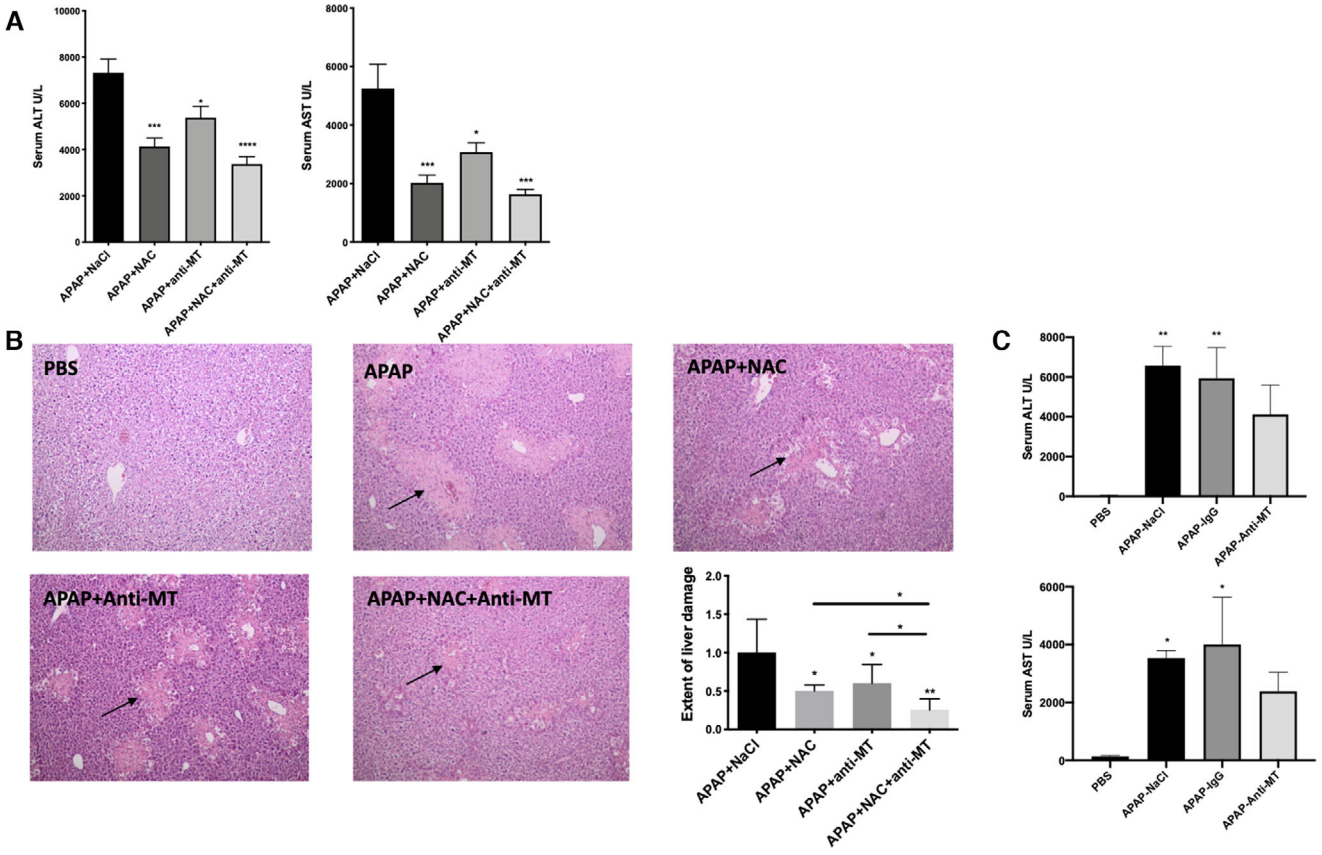

FIGURE 2 **Anti-MT (metallothionein) antibodies are equally effective as N-acetylcysteine (NAC) and combinational therapy exceeds each monotherapy**. (**A**) Serum alanine transaminase (ALT) and aspartate transaminase (AST) levels in acetaminophen (APAP) overdosed mice treated with NaCl, NAC anti-MT antibodies, or the combination of NAC and anti-MT antibodies. Data are pooled from two independent experiments, $n = 5–10$, $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (**B**) Representative images of H&E stained liver sections and corresponding score of the extent of liver damage in APAP-overdosed mice treated with indicated treatments. Data are pooled from two independent experiments, $n = 5–10$, $^{*}P < 0.05$, $^{**}P < 0.01$. Magnification ×100. (**C**) Serum ALT and AST levels in APAP overdosed mice treated with NaCl, anti-MT antibodies, or isotype IgG control antibodies 24 h following APAP overdosing or PBS (control group), $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to PBS

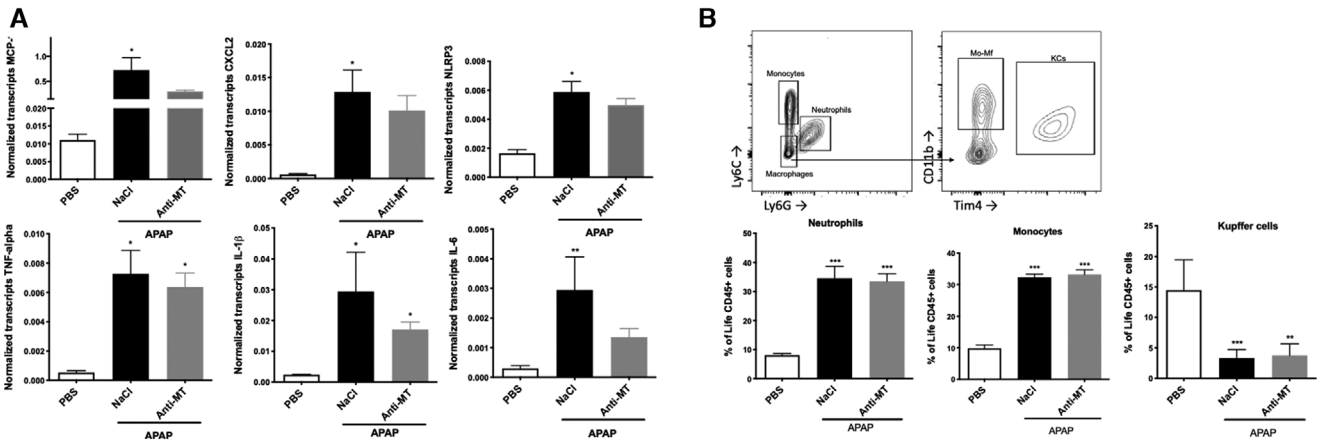

FIGURE 3 **Anti-MT (metallothionein) antibody treatment dampens hepatic inflammation in mice with acetaminophen-induced liver injury (AILI)**. (**A**) Expression of proinflammatory markers in PBS control and acetaminophen (APAP) intoxicated mice treated with anti-MT antibodies or vehicle (NaCl). $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to PBS. (**B**) Gating strategy for hepatic monocytes and macrophages. Flow cytometric analyses of infiltrated CD45+Ly6G+Ly6C^{int}TIM4-neutrophils, CD45+Ly6G-F4/80+CD11b^{hi}Ly6C^{hi} monocytes and CD45+Ly6G-F4/80+CD11b^{int}Ly6C^{lo}TIM4+ resident Kupffer cells. $n = 5$, $^{*}P < 0.05$, $^{***}P < 0.001$ compared to PBS

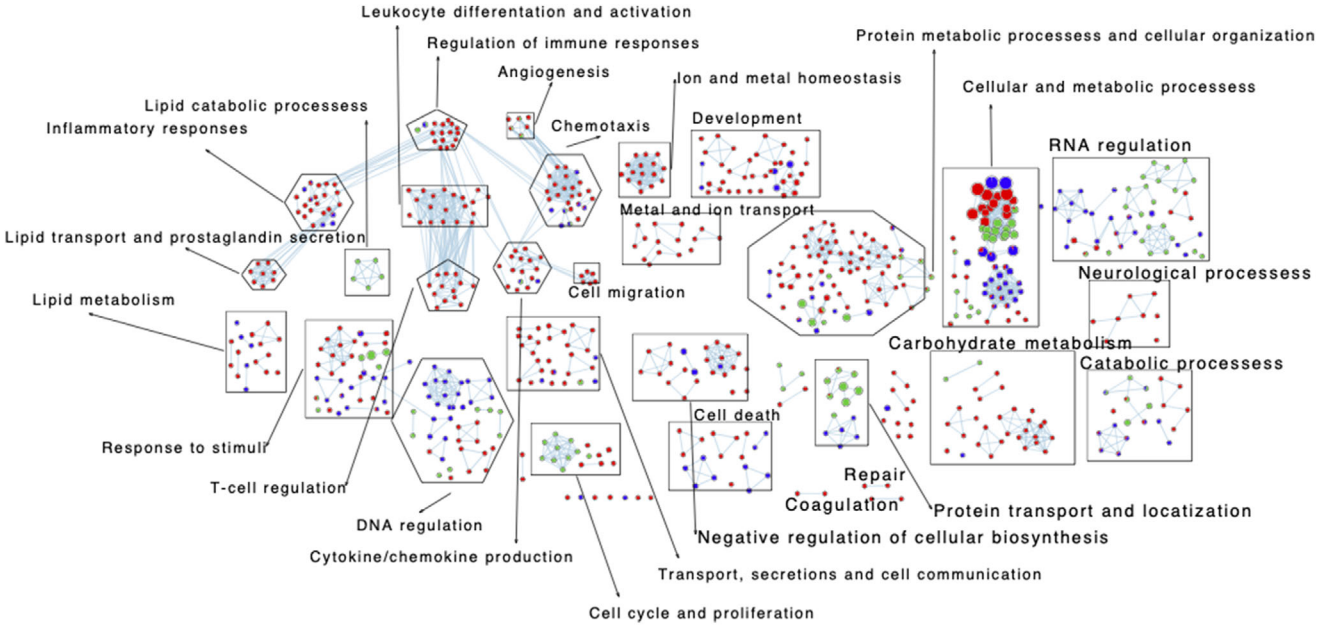

FIGURE 4 **Enrichment map of significantly up-regulated GO pathways in Kupffer cells (KCs), monocyte-derived macrophages (MoMFs) and monocytes isolated from the livers of mice 24 h after acetaminophen intoxication.** Green = KCs, blue = MoMFs, red = monocytes. *n* = 3–6

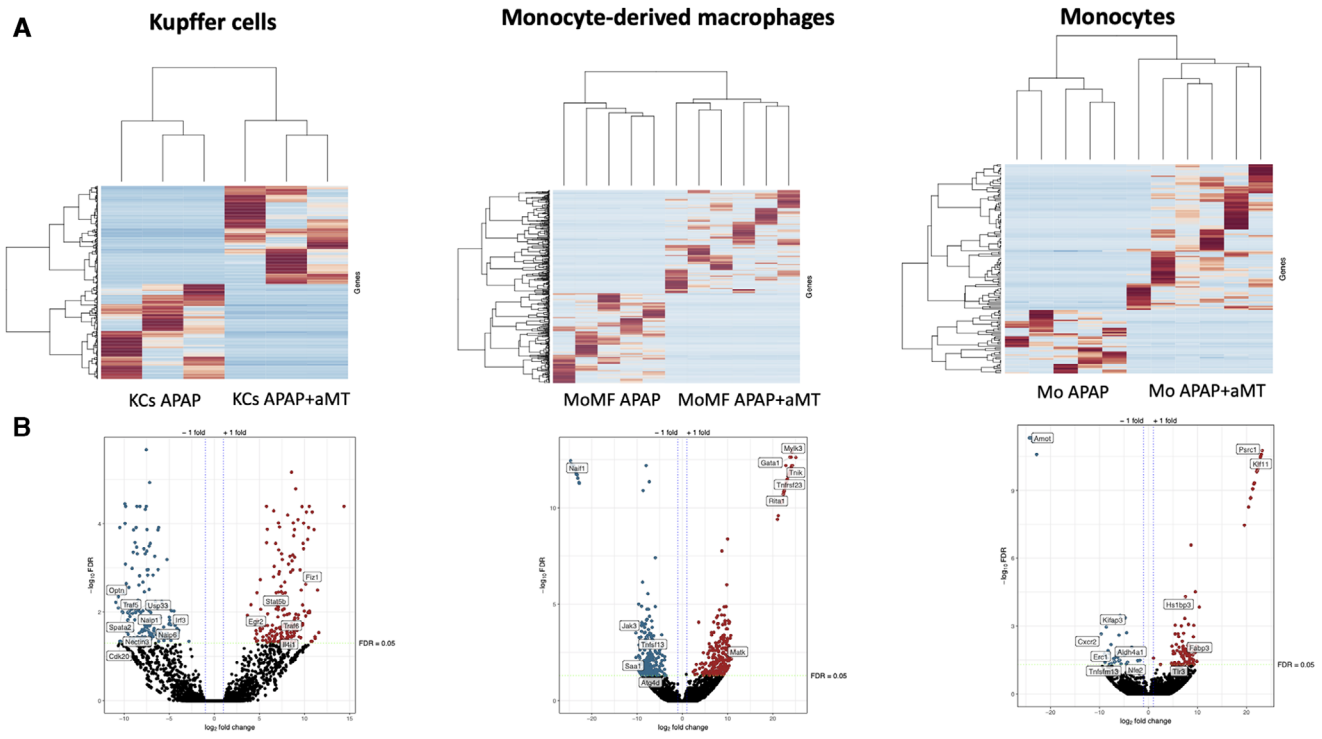

FIGURE 5 **(A) Clusters and heatmap of differentially expressed genes and (B) volcano plots showing the log fold change and false discovery rate (FDR) of the genes in Kupffer cells, monocyte-derived macrophages and monocytes after anti-MT (metallothionein) antibody treatment compared to control treatment in mice with an acetaminophen overdose.** *n* = 3–6

TNF-induced NF-κB activation,[31] *Naip1* and *Naip6* (NLR = Nod-like receptor (NLR) family apoptosis inhibitory proteins), which are innate immune sensors belonging to the NLR Family Apoptosis Inhibitory Protein (NAIP) family,[32] and *Irf3* (IFN regulatory factor 3), which induces the transcription of type I IFN genes[33] (Fig. 5B). Among the 171 up-regulated genes in KCs (>2-fold up-regulated), we identified regulators of alternative activated macrophage polarization, *Il4i1* (IL4 induced 1), *Erg2* (early growth response gene-2), and *Stat5b* (signal transducer

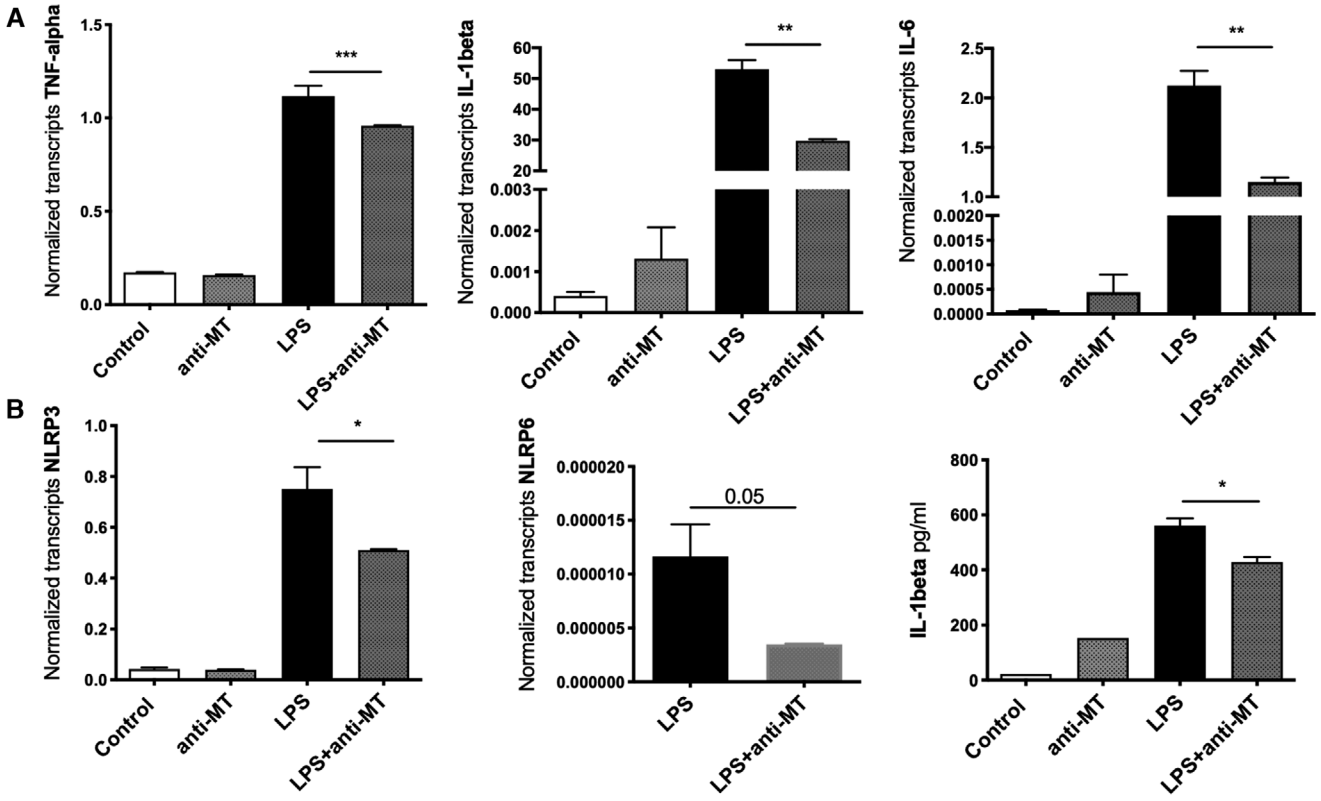

FIGURE 6 **Anti-MT (metallothionein) antibodies attenuate induced proinflammatory macrophage polarization**. (**A**) Expression of proinflammatory cytokines in bone-marrow derived macrophages (BMDMs) following incubation with 100 ng/ml LPS, 130 μg/ml anti-MT antibody (UC1MT), the combination of LPS and anti-MT antibodies or control medium for 12 h. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**B**) Expression of NLRP3 and NLRP6 and supernatant IL-1β levels after incubation of BMDMs with 100 ng/ml LPS, 130 μg/ml anti-MT antibody (UC1MT), the combination of LPS and anti-MT antibodies or control medium for 12 h . $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

and activator of transcription 5B) (Fig. 5B). In MoMF and monocytes, individual genes linked to proinflammatory processes were down- and up-regulated after anti-MT treatment. In MoMF, 242 genes were >2-fold down-regulated, including *Naif1* (nuclear apoptosis inducing factor 1), a negative regulator of cell growth, *Jak3* (Janus kinase 3), which is involved in macrophage differentiation,[34] *Saa1* (serum amyloid A 1), expressed in response to an inflammatory stimulus,[35] *Atg4d* (autophagy related 4D cysteine peptidase, an IRF8-dependent autophagy gene[36]), and *Tnfsf13* (TNF ligand superfamily member 13) (Fig. 5B). Highly up-regulated genes in infiltrated MoMF from anti-MT treated mice with AILI included *Mylk3* (myosin light chain kinase 3, encodes for MLCK which is involved in macrophage motility[37]), *Tnik* (TRAF2 and NCK interacting kinase), which regulates the c-Jun N-terminal kinase pathways, *Gata1*, a transcription factor that blocks IL-6-induced macrophage differentiation and apoptosis,[38] and *Tnfrsf23* (TNF receptor superfamily member 23, a trail decoy receptor) (Fig. 4). Of the 46 down-regulated genes in monocytes from anti-MT treated mice, we identified *Amot*, an angiogenesis related gene, the chemokine *Cxcr2*, *Tnfsfm13* (tumor necrosis factor [ligand] superfamily, membrane-bound member 13), *Aldh4a1*, a p53 target, and *Nfe2*, a transcription factor that interacts with antioxidant responsive elements. In contrast, *Klf11*, which is involved in IL-12p40 production,[39] and *Tlr3* were up-regulated in monocytes from anti-MT treated mice following APAP overdose, together with, among others, *Psrc1* and *Fabp3*, genes encoding for proteins involved in cholesterol transport (Fig. 5).

3.5 | Pharmacologic inhibition of extracellular MT1/2 dampens proinflammatory macrophage polarization in vitro

To further evaluate the effect of anti-MT antibodies on the phenotype of macrophages, we cultivated BMDMs and treated the cells with LPS, to induce polarization toward a proinflammatory phenotype, with and without co-incubation of anti-MT antibodies overnight. LPS significantly induced the expression of proinflammatory cytokines, which was attenuated in cells incubated with LPS+anti-MT antibodies (Fig. 6A). Because DAMP release and subsequent inflammasome activation is involved in the activation of leukocytes,[19] we additionally analyzed the expression of two important inflammasomes *Nlrp3* and *Nlrp6* and the outcome of inflammasome activation being IL1β release.[40] Indeed, LPS resulted in inflammasome activation and IL1β release, which was abrogated in case of co-incubation with anti-MT antibodies (Fig. 6B).

4 | DISCUSSION

APAP overdose-induced liver injury is the most frequent cause of acute liver failure in developed countries. For early presenting patients, NAC serves as effective antidote but its efficacy decreases with increasing time between overdose and hospital admission.

When excessive hepatotoxicity occurs, there is a lack of an effective therapy for the sequence of events that can progress to acute liver failure with the attendant need for emergency liver transplantation. Although controversial, the possibility exists that hepatocyte necrosis and the ensuing inflammatory response determines the severity of AILI and this possibility opens new avenues for potential therapeutic treatment options. NAC treatment is highly effective as an early treatment and other agents that interfere at the level of immune infiltration and/or activation may serve as add-on therapy for late-presenting patients. Here, we show that MT can act as proinflammatory mediator and represents an interesting therapeutic target to block the ensuing inflammatory response following AILI. Recently, the involvement of the gut microbiome in the pathogenesis of AILI has also been suggested, either by the direct action of APAP on the microbiome or indirectly by APAP metabolites produced by microbial species.[41–44] Depletion of gut microbiota or altering the microbial composition has shown to be effective in dampening or abrogating AILI.[41,45] Whether this could be an alternative strategy in patients presenting with an APAP overdose needs further investigation.

AILI in mice is a useful model to study the pathogenesis and to identify potential therapeutic strategies. Mice overdosed with APAP develop severe liver injury, as indicated by increased serum transaminase levels and extensive necrosis on liver histology 24 h post-overdose. Here, we show that this is accompanied by significant induction of MT1 and MT2, which is consistent with their characterization as acute phase proteins. This was confirmed in human liver tissue of patients with an APAP overdose and indicates their potential as therapeutic target. Proinflammatory cytokines and chemokines were also significantly induced following APAP overdose in mice. Interestingly, APAP-overdosed mice treated with anti-MT antibodies showed remarkably lower levels of serum transaminases and liver necrosis compared to untreated mice with AILI. Moreover, the expression of *Mcp1*, *Cxcl2*, *Il6*, and *Nlrp3* was not significantly induced in mice treated with anti-MT antibodies after APAP overdose. Although it has been shown that mice lacking MT1 and 2 show a higher disease activity in experimental AILI models,[15] here, we only and specifically target extracellular MTs because antibodies do not enter the cell and only exert their effect extracellularly. Our results point to the therapeutic potential of targeting extracellular MTs to dampen liver injury and inflammation following an APAP intoxication.

During necrotic cell death, MTs are released in the extracellular environment and function as danger signals by attracting other immune cells to the inflammatory site. We and others have previously reported on the chemotactic properties of extracellular MTs. Therefore, we additionally investigated liver immune cells that are implicated in the pathogenesis of AILI.[10,46–48] Although the percentage of neutrophils and monocytes was significantly increased and KCs decreased following APAP overdosing, we did not observe any effect on the proportion of these immune cells by anti-MT antibody treatment. Therefore, we further focused on the effect of anti-MT antibodies on the phenotype of liver monocytes and macrophages. Our data of whole genome sequencing analysis extend previous reports using microarray analysis[9] and Nanostring assays[10] to characterize liver macrophages in AILI. We confirm a distinct transcriptional profile of the three cell subsets with monocytes, and to a lesser extent MoMF, as the main proinflammatory macrophage subset during the acute phase of AILI. Interestingly, anti-MT antibody therapy resulted in a differential gene expression of the liver macrophage subsets with prominent results observed in KCs. KCs down-regulated important proinflammatory genes including *Naip1* and *Naip6*, inflammasome receptors that sense inflammasome activators and activate inflammasome assembly, typically upon bacterial infection.[32] In contrast, markers involved in alternative macrophage polarization and repair were up-regulated. Along same lines, MoMF showed a differential expression of markers involved in macrophage polarization and function after anti-MT treatment. Less effect on infiltrating monocytes, with respect to inflammatory phenotype polarization, was observed. Because classically activated proinflammatory monocytes/macrophages contribute to inflammatory injury and macrophage inflammasome activation has been implicated in the pathogenesis of APAP intoxication, we further investigated this in vitro. LPS stimulation of BMDMs increased the expression of proinflammatory cytokines, *Nlrp3*, *Nlrp6*, and IL1β release, all of which were attenuated in LPS+anti-MT treated cells suggesting that extracellular MTs are involved in macrophage inflammasome activation and proinflammatory polarization. An excessive inflammatory response and late-presenting AILI are associated with adverse outcomes, and usually in those patients NAC is less effective or ineffective. In those late-presenting patients, strategies that are targeted to the ongoing inflammation and the associated bystander damage may provide an effective supplemental therapy. Here, we show that anti-MT antibodies alter macrophage phenotype and are an effective treatment strategy in experimental AILI. Moreover, we show that anti-MT antibodies are equal to the standard of care NAC and addition of anti-MT antibodies to NAC exceeds the efficacy of either monotherapy in mice overdosed with APAP. These results suggest that anti-MT antibodies may represent a new therapeutic opportunity for AILI.

ACKNOWLEDGMENTS

The authors thank Inge Van Colen, Els Van Deynse, and Petra Van Wassenhove for their technical assistance. H.V. and A.G. are senior clinical investigators of the Research Foundation Flanders. S.R. and S.L. are sponsored by the Research Foundation Flanders.

AUTHORSHIP

L.D. and H.V. conceptualized the study and designed the experiments. L.D., S.C., and H.P.E. conducted the experimental work. L.T. and F.N. performed the RNA sequencing analyses. All authors contributed to

critical revision of the manuscript and data interpretation. All authors also read and approved the final manuscript.

DISCLOSURES

L.D., D.L., and M.A.L. are listed as co-inventors on a patent protecting the use of MT antagonists to treat intestinal inflammation (WO2013007678). All other authors declare no conflicts of interest.

REFERENCES

1. Andrade RJ, Aithal GP, Björnsson ES, et al. EASL Clinical Practice Guidelines: drug-induced liver injury. *J Hepatol*. 2019;70:1222-1261.
2. Krenkel O, Mossanen JC, Tacke F. Immune mechanisms in acetaminophen-induced acute liver failure. *Hepatobiliary Surg Nutr*. 2014;3:331-343.
3. Paridaens A, Raevens S, Colle I, et al. Combination of tauroursodeoxycholic acid and acetylcysteine exceeds standard treatment for acetaminophen intoxication. *Liver Int*. 2017;37:748-756.
4. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol*. 2008;8:279-289.
5. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol*. 1994;12:991-1045.
6. Holt MP, Cheng L, Ju C. Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. *J Leukoc Biol*. 2008;84:1410-1421.
7. You Q, Holt M, Yin H, Li G, Hu C-J, Ju C. Role of hepatic resident and infiltrating macrophages in liver repair after acute injury. *Biochem Pharmacol*. 2013;86:836-843.
8. Holt MP, Yin H, Ju C. Exacerbation of acetaminophen-induced disturbances of liver sinusoidal endothelial cells in the absence of Kupffer cells in mice. *Toxicol Lett*. 2010;194:34-41.
9. Zigmond E, Samia-Grinberg S, Pasmanik-Chor M, et al. Infiltrating monocyte-derived macrophages and resident Kupffer cells display different ontogeny and functions in acute liver injury. *J Immunol*. 2014;193:344-353.
10. Mossanen JC, Krenkel O, Ergen C, et al. Chemokine (C-C motif) receptor 2-positive monocytes aggravate the early phase of acetaminophen-induced acute liver injury. *Hepatology*. 2016;64:1667-1682.
11. Raevens S, Van Campenhout S, Debacker PJ, et al. Combination of sivelestat and N-acetylcysteine alleviates the inflammatory response and exceeds standard treatment for acetaminophen-induced liver injury. *J Leukoc Biol*. 2020;107:341-355.
12. Devisscher L, Hindryckx P, Lynes MA, et al. Role of metallothioneins as danger signals in the pathogenesis of colitis. *J Pathol*. 2014;233:89-100.
13. Inoue K, Takano H, Shimada A, Satoh M. Metallothionein as an anti-inflammatory mediator. *Mediators Inflamm*. 2009;2009:1-7.
14. Borghesi LA, Lynes MA. Stress proteins as agents of immunological change: some lessons from metallothionein. *Cell Stress Chaperones*. 1996;1:99-108.
15. Liu J, Liu Y, Hartley D, et al. Metallothionein-I/II knockout mice are sensitive to acetaminophen-induced hepatotoxicity. *J Pharmacol Exp Ther*. 1999;289:580-586.
16. Yin X, Knecht DA, Lynes MA. Metallothionein mediates leukocyte chemotaxis. *BMC Immunol*. 2005;6:21.
17. Mcgill MR, Jaeschke H. Mechanistic biomarkers in acetaminophen-induced hepatotoxicity and acute liver failure: from preclinical models to patients. *Expert Opin drug Metab Toxicol*. 2014;10:1005-1017.
18. Woolbright BL, Jaeschke H, City K. The impact of sterile inflammation in acute liver injury. *J Clin T ransl Res*. 2017;3:170-188.
19. Woolbright BL, Jaeschke H. Role of the inflammasome in acetaminophen-induced liver injury and acute liver failure. *J Hepatol*. 2017;66:836-848.
20. Canpolat E, Lynes MA. In vivo manipulation of endogenous metallothionein with a monoclonal antibody enhances a T-dependent humoral immune response. *Toxicol Sci*. 2001;62:61-70.
21. Lefere S, Degroote H, Van Vlierberghe H, Devisscher L. Unveiling the depletion of Kupffer cells in experimental hepatocarcinogenesis through liver macrophage subtype-specific markers. *J Hepatol*. 2019;71:631-633.
22. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal*. 2011;17(1).
23. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29:15-21.Jan.
24. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 2011;12:323.
25. Love MI, Anders S, Hu- W, Differential analysis of count data—the DESeq2 package. 2016. 1-62 p.
26. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003;13:2498-2504.
27. Maere S, Heymans K, Kuiper M. BiNGO: a cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics*. 2005;21:3448-3449.
28. Min KS, Terano Y, Onosaka S, Tanaka K. Induction of hepatic metallothionein by nonmetallic compounds associated with acute-phase response in inflammation. *Toxicol Appl Pharmacol*. 1991;111:152-162.
29. Coyle P, Philcox JC, Carey LC, Rofe AM. Metallothionein: the multipurpose protein. *Cell Mol Life Sci*. 2002;59:627-647.
30. Feltham R, Webb AI, Silke J. SPATA 2—keeping the TNF signal short and sweet. *EMBO J*. 2016;35:1848-1850.
31. Tada K, Okazaki T, Sakon S, et al. Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. *J Biol Chem*. 2001;276:36530-36534.
32. Zhao Y, Shi J, Shi X, Wang Y, Wang F, Shao F. Genetic functions of the NAIP family of inflammasome receptors for bacterial ligands in mice. *J Exp Med*. 2016;213:647-656.
33. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol*. 2006;6:644-658.
34. Mangan JK, Rane SG, Kang AD, Amanullah A, Wong BC, Reddy EP. Mechanisms associated with IL-6-induced up-regulation of Jak3 and its role in monocytic differentiation. *Blood*. 2004;103:4093-4101.
35. Jumeau C, Awad F, Assrawi E, et al. Expression of SAA1, SAA2 and SAA4 genes in human primary monocytes and monocyte-derived macrophages. *PLoS One*. 2019;14:1-19.
36. Gupta M, Shin D-M, Ramakrishna L, et al. IRF8 directs stress-induced autophagy in macrophages and promotes clearance of Listeria monocytogenes. *Nat Commun*. 2015;6:6379.
37. Wilson AK, Gorgas G, Claypool WD, de Lanerolle P. An increase or a decrease in myosin II phosphorylation inhibits macrophage motility. *J Cell Biol*. 1991;114:277-283.
38. Tanaka H, Matsumura I, Nakajima K, et al. GATA-1 blocks IL-6-induced macrophage differentiation and apoptosis through the sustained expression of cyclin D1 and bcl-2 in a murine myeloid cell line M1. *Blood*. 2000;95:1264-1273.
39. Zhang W, Wang X, Xia X, et al. Klf10 inhibits IL-12p40 production in macrophage colony-stimulating factor-induced mouse bone marrow-derived macrophages. *Eur J Immunol*. 2013;43:258-269.
40. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell*. 2002;10:417-426.
41. Gong S, Lan T, Zeng L, et al. Gut microbiota mediates diurnal variation of acetaminophen induced acute liver injury in mice. *J Hepatol*. 2018;69:51-59.
42. Dey P. The role of gut microbiome in chemical-induced metabolic and toxicological murine disease models. *Life Sci*. 2020;258:118172.

43. Clayton TA, Baker D, Lindon JC, Everett JR, Nicholson JK. Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. *Proc Natl Acad Sci U S A*. 2009;106:14728-14733.
44. Mikov M, Caldwell J, Dolphin CT, Smith RL. The role of intestinal microflora in the formation of the methylthio adduct metabolites of paracetamol. Studies in neomycin-pretreated and germ-free mice. *Biochem Pharmacol*. 1988;37:1445-1449.
45. Cho S, Won K-J, Yang X, et al. Phenylpropionc acid produced by gut microbiota alleviates acetaminophen-induced hepatotoxicity. *bioRxiv*. 2019;811984.
46. Liu Z-X, Han D, Gunawan B, Kaplowitz N. Neutrophil depletion protects against murine acetaminophen hepatotoxicity. *Hepatology*. 2006;43:1220-1230.
47. Laskin DL, Gardner CR, Price VF, Jollow DJ. Modulation of macrophage functioning abrogates the acute hepatotoxicity of acetaminophen. *Hepatology*. 1995;21:1045-1050.
48. Ishida Y, Kondo T, Kimura A, Tsuneyama K, Takayasu T, Mukaida N. Opposite roles of neutrophils and macrophages in the pathogenesis of acetaminophen-induced acute liver injury. *Eur J Immunol*. 2006;36:1028-1038.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Devisscher L, Van Campenhout S, Lefere S, et al. Metallothioneins alter macrophage phenotype and represent novel therapeutic targets for acetaminophen-induced liver injury. *J Leukoc Biol*. 2022;111:123–133.
https://doi.org/10.1002/JLB.3A0820-527R