Lipoapoptosis induced by saturated free fatty acids stimulates monocyte migration: a novel role for Pannexin1 in liver cells

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Abstract Recruitment of monocytes in the liver is a key pathogenic feature of hepatic inflammation in nonalcoholic steatohepatitis (NASH), but the mechanisms involved are poorly understood. Here, we studied migration of human monocytes in response to supernatants obtained from liver cells after inducing lipoapoptosis with saturated free fatty acids (FFA). Lipoapoptotic supernatants stimulated monocyte migration with the magnitude similar to a monocyte chemoattractant protein, CCL2 (MCP-1). Inhibition of c-Jun NH₂-terminal kinase (JNK) in liver cells with SP600125 blocked migration of monocytes in a dose-dependent manner, indicating that JNK stimulates release of chemoattractants in lipoapoptosis. Notably, treatment of supernatants with Apyrase to remove ATP potentially inhibited migration of THP-1 monocytes and partially blocked migration of primary human monocytes. Inhibition of the CCL2 receptor (CCR2) on THP-1 monocytes with RS102895, a specific CCR2 inhibitor, did not block migration induced by lipoapoptotic supernatants. Consistent with these findings, lipoapoptosis stimulated pathophysiological extracellular ATP (eATP) release that

increased supernatant eATP concentration from 5 to ~60 nM. Importantly, inhibition of Panx1 expression in liver cells with short hairpin RNA (shRNA) decreased supernatant eATP concentration and inhibited monocyte migration, indicating that monocyte migration is mediated in part by Panx1-dependent eATP release. Moreover, JNK inhibition decreased supernatant eATP concentration and inhibited Pannexin1 activation, as determined by YoPro-1 uptake in liver cells in a dose-dependent manner. These results suggest that JNK regulates activation of Panx1 channels, and provide evidence that Pannexin1-dependent pathophysiological eATP release in lipoapoptosis is capable of stimulating migration of human monocytes, and may participate in the recruitment of monocytes in chronic liver injury induced by saturated FFA.

Keywords Pannexin1 · Monocyte migration · JNK · Pathophysiological ATP release

Introduction

Nonalcoholic steatohepatitis (NASH) has emerged as the most common cause of chronic liver injury in developed countries [1]. Clinical studies have shown that hepatocyte lipoapoptosis represents a key morphologic and pathogenic feature of human NASH [1, 2]. Saturated free fatty acids (FFA) in the circulation stimulate hepatocyte lipoapoptosis, and elevated concentrations of saturated FFA have been found in patients with NASH [3–5]. Lipoapoptosis is characterized by a sustained activation of c-Jun NH₂-terminal kinase (JNK), and the magnitude of JNK-dependent lipoapoptosis correlates with the stage of hepatic inflammation and fibrosis in NASH [2]. These findings indicate that hepatocyte lipoapoptosis plays a key role in mediating hepatic inflammation in NASH

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[6, 7]. However, the cellular mechanisms responsible have not been defined.

Recent studies have shown that recruitment of monocytes in the liver is a key feature of chronic liver injury in mice and humans [8–10]. Blood monocytes in the circulation migrate in response to chemokines and damage-associated molecular pattern molecules (DAMPs) released from injured hepatocytes, hepatic stellate cells, and endothelial cells. Once in the intrahepatic tissue, monocytes differentiate into macrophages, and these recruited monocytes/macrophages stimulate hepatic inflammation and fibrosis by releasing proinflammatory cytokines [10–13]. Specifically, a chemokine (C-C motif) ligand 2 or CCL2, also called monocyte chemoattractant protein-1 (MCP-1), stimulates migration of monocytes in mouse models of NASH and in chronic liver injury induced by CCl₄ and bile duct ligation [8, 9, 11]. Interestingly, elevated levels of CCL2 have also been found in patients with NASH, both intrahepatically and systemically, suggesting that CCL2 may be relevant to human NASH [14].

In addition to CCL2, extracellular ATP (eATP) also stimulates monocyte migration by stimulating purinergic receptors on the plasma membrane of monocytes [15]. eATP functions as an endogenous find-me signal for the recruitment of monocytes and macrophages to the site of the dying cell [15, 16]. Moreover, eATP is a potent proinflammatory signal that stimulates secretion of IL-1 β and activation of the inflammasome in macrophages [17, 18]. These findings suggest that stimulation of ATP release in apoptosis contributes to the recruitment of inflammatory cells and tissue inflammation. We have recently shown that lipoapoptosis induced by saturated FFA stimulates Panx1-dependent pathophysiological ATP release in liver cells [19]. However, the concentration of eATP in lipoapoptosis has not been measured, and it is not known whether eATP and/or other DAMP molecules released from dying liver cells are capable of stimulating monocyte migration.

Panx1 is an integral membrane protein and mediates ATP release under physiological conditions in many different cells [20–24]. Panx1-dependent ATP release is implicated in the regulation of vascular tone, mucociliary lung clearance, and taste-bud function [25]. In addition to physiological ATP release, Panx1 also mediates ATP release under pathophysiological conditions. Recent studies have shown that Panx1 contributes to neuron death and seizures under ischemic or epileptic conditions in the brain [26, 27]. Panx1 has been reported to mediate cardiac fibrosis in response to pressure overload [28]. Several recent studies indicate that Panx1 also mediates pathophysiological ATP release during apoptosis [16, 19, 29].

The purpose of these studies was to assess the role of lipoapoptosis in mediating release of eATP from liver cells, and to assess whether these molecules are capable of stimulating monocyte migration. We found that lipoapoptosis induced by saturated FFA stimulates migration of human

monocytes in part by stimulating Panx1-dependent pathophysiological ATP release. These results suggest that one potential function of Panx1-dependent pathophysiological ATP release is to stimulate the recruitment of monocytes.

Experimental procedures

Cell cultures The hepatocyte model, HTC cell line, was derived from a rat hepatoma. Huh7 and HepG2 cells are human hepatoma cell lines. HTC, Huh-7, and HepG2 cells were cultured in a high-glucose Dulbecco's modified Eagle's medium (DMEM) containing penicillin/streptomycin and 10 % fetal calf serum as previously described [4, 19].

THP-1 monocytes and human peripheral blood mononuclear cells (PBMC) were used to study monocyte migration. THP-1 monocytes cells were cultured in RPMI-1640 (Gibco) supplemented with 10 mM Hepes and 10 % FCS. All cells were used within 24 h after plating. PBMC were obtained from fresh human blood of healthy volunteers as leukopaks using a Ficoll-Hypaque density gradient as recently described [30] and in accordance with our Institutional Review Board guidelines.

Reagents Palmitic acid (16:0), stearic acid (18:0), palmitoleic acid (16:1), Apyrase (catalog number A6535), and RS102895 were obtained from Sigma-Aldrich (St Louis, MO). The JNK inhibitor SP600125 and the pan-caspase inhibitor zVAD(OMe)-FMK (zVAD-fmk) were from Calbiochem (La Jolla, CA).

Induction of lipoapoptosis and supernatant collection FFA were used to induce lipoapoptosis in liver cells. FFA were dissolved in isopropanol, and added to the culture media that contained DMEM and 1 % bovine serum albumin (BSA). The concentration of isopropanol in vehicle was 0.5 % in final incubations. The concentration of FFA was from 400 to 800 μ M, and was similar to the fasting total FFA plasma concentrations observed in human NASH [31, 32]. Liver cells were incubated with FFA for ~16 h. After overnight incubation, cells were washed twice with a FFA-free RPMI media that contained RPMI-1640 (Gibco), 10 mM Hepes, and 1 % BSA. Cells were incubated in 1 ml of RPMI media for 2 h. After incubation, supernatants were collected and centrifuged at 425g for 2 min at room temperature. Supernatants were transferred to a fresh tube, and used for the migration assay and measurement of ATP concentration.

JNK activation Activation of JNK in HTC cells was assessed using Western blot analysis of JNK phosphorylation. Cells were washed with cold PBS twice, and lysed in buffer (150 mM NaCl, 1 mM EDTA, 1.0 % NP-40, 1 % deoxycholic acid, 0.1 % SDS, 50 mM Tris, pH 7.4, 1 mM sodium

orthovanadate, 1 mM NaF, 1 mM PMSF) containing protease inhibitors (Roche, IN) and Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific, IL). Cells were then scraped off the dish and transferred into a microcentrifuge tube, incubating at 4 °C for 30 min. Subsequently, cells were centrifuged at 12,000 rpm for 20 min, and protein supernatants were stored at –80 °C. For analysis, 20 mg of protein was separated on 12 % SDS-PAGE, and transferred to a PVDF membrane (Millipore, MA). The membrane was blocked in a TBST buffer (25 mM Tris-HCl, 150 mM NaCl, 0.1 % Tween-20, pH 7.4) with 5 % BSA (Jackson ImmunoResearch Laboratories Inc., PA), and incubated overnight at 4 °C with a rabbit anti-SAPK/JNK antibody and a rabbit anti-Phospho-SAPK/JNK (Thr183/Tyr185) monoclonal antibody at 1:1000 dilutions, respectively (Cell Signaling, MA). Endogenous control antibody was chicken anti-GAPDH polyclonal antibody at 1:10,000 dilutions (Millipore, MA). Secondary antibodies were HRP-conjugated Donkey anti-rabbit IgG and Donkey anti-chicken IgY (both from Jackson ImmunoResearch Laboratories Inc., PA) for anti-JNK/Phospho-JNK and anti-GAPDH, respectively. Bands were visualized using RapidStep ECL reagent (Calbiochem, CA). Blots were scanned, and the band intensities were measured using ImageJ 1.45 software (NIH Bethesda, MD) as shown in Figure 1.

Caspase3/7 activity Lipoapoptosis was confirmed by measuring activation of caspase3/7 using a Caspase-Glo 3/7 assay according to the manufacturer's description (Promega Corp. Madison, WI) as described [4]. Luminescence of caspase-glo is directly proportional to caspase3/7 activation, and was measured with a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA).

XBP-1 splicing Detection of XBP-1 splicing was measured using RT-PCR. The total RNA was extracted as previously described [19]. RT-PCR was performed to amplify rat *Xbp-1* cDNA using the primers forward 5'-AAA CAG AGT AGC AGC ACA GAC TGC-3' and reverse 5'-TCC TTC TGG GTA GAC CTC TGG GAG-3'. The PCR products were then digested with *Pst*I-HF™ restriction enzyme (NEB, Ipswich, MA, catalog number R3140S) for 2 h at 37 °C, and then analyzed on 1.5 % agarose gel electrophoresis. The XBP-1 spliced form shows a single band at 480 bp, while unspliced XBP-1 were cut by *Pst*I, producing two bands of 287 and 193 bp. Densitometry was performed to quantitate XBP-1 bands, and the data are expressed as the relative intensity of the ratio of spliced to unspliced bands.

Migration of monocytes Human THP-1 monocytes and PBMC were used in a migration assay. Prior to migration, monocytes (2×10^6 cells/ml) were placed in the top chamber of 24 well Transwell plates (Corning, NY) in RPMI media that contained RPMI-1640 (Gibco), 10 mM Hepes, and 1 % BSA.

The plate inserts (Costar catalog number 3421, Corning, NY) had a polycarbonate membrane with a pore size of 5 µm. Migration of monocytes was induced by adding 600 µl of a test solution to the bottom chamber. The plates were incubated for 2 h, and monocytes in the bottom chamber were counted using an automated Scepter cell counter controlled by Scepter Pro software (EMD Millipore, Hayward, CA). The Scepter counter uses a Coulter principle to generate a histogram of the cell size. The size of THP-1 monocytes was from 9 to 17 µm (Fig. 2a), and the size of migrating PBMC ranged from 8 to 11 µm (not shown). The migration of lymphocytes and monocytes in PBMC experiments were also monitored by flow cytometry using a FACSCalibur (BD Biosciences). Note that monocytes, but not lymphocytes, from PBMC migrated to the monocyte chemokine CCL2 and eATP in the Transwell assays. Migration to eATP was compared to CCL2 (20 ng/ml) as a positive control. The percent of migration was calculated relative to the migration induced in the presence of CCL2 (100 %).

ATP quantitation The concentration of ATP in supernatants was quantified by measuring luminescence as previously described [15, 16]. Briefly, the samples of supernatants (5 µl) were added to a clear tube with 200 µl of the optimal measuring solution that contained OptiMEM (Gibco), 12 mM Hepes, and 0.1 % BSA. Subsequently, 4 µl of firefly luciferin-luciferase (Sigma) was added to the tube and gently mixed. The final concentration of luciferin-luciferase was 2 mg/ml, and luminescence of the tube was measured using a TD 20/20 luminometer. Luminescence of ATP standard solution was used to determine the concentration of ATP in supernatants.

Imaging and analysis Cells were plated on coverslips in the recording chambers. Prior to imaging, cells were washed with a solution that contained 142 mM NaCl, 4 mM KCl, 1 mM KH₂PO₄, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes, and 10 mM D-glucose (pH=7.25, osmolarity ~300 mosM). Cells were visualized through a ×40 oil immersion objective (NA=1.35) and an Olympus IX71 microscope. The fluorescent images from randomly selected cells were acquired with a Sensicam QE camera controlled by a SlideBook 3.0 software (Intelligent Imaging Innovations, Denver, CO). The fluorescence was measured by drawing a region of interest over the entire cell, and subtracting background fluorescence. Background fluorescence was measured from the regions with no cells.

Activation of Panx1 channels by YoPro-1 uptake YoPro-1 has molecular weight ~600 Da similar to ATP, and does not permeate biological membranes. YoPro-1 is not fluorescent in a solution, but becomes fluorescent upon binding to nucleic acids. Activation of Panx1 channels in the plasma membrane mediates YoPro-1 uptake, and YoPro-1 fluorescence has been

Fig. 1 Palmitic acid stimulates JNK-dependent lipopapoptosis in hepatocytes. **a** JNK activation was measured using Western blot analysis. Western blots for phosphorylated JNK (*P-JNK*), and the total JNK were obtained from hepatocytes (HTC cells) after overnight incubation with 0.5 % isopropanol (*Vehicle*), 500 μ M palmitic acid (*PA*), and different doses of SP600125 (*SP*) as indicated. *PA* treatment stimulated JNK phosphorylation, and *SP* inhibited this effect in a concentration-dependent manner. **b** *Graph* illustrates the ratio of phosphorylated JNK and the total JNK intensities from the blots shown in **a**. **c** Caspase3/7 activity was measured after overnight treatment of HTC cells with 0.5 % isopropanol

(*Vehicle*), *PA* (500 μ M), and addition of zVAD-FMK or *SP* (both at 100 μ M). zVAD-FMK was added 1 h before addition of *PA*, and *SP* was added at the same time with *PA*. Note that *SP* potently inhibited caspase3/7 activation in HTC cells (* P <0.001). The number of dishes analyzed was from 4 to 6. **d** Splicing of XBP1 was measured after overnight incubation with 0.5 % isopropanol (*Vehicle*) or 500 μ M *PA*, and in the absence or presence of 100 μ M *SP*. *PA* induced XBP1 splicing and *SP* did not inhibit this effect. **e** Quantification of XBP1 bands from **d** showing relative spliced/unspliced XBP1 band intensity in the presence or absence of JNK inhibitor, *SP*

used to quantify Panx1 activation in apoptosis [16, 19, 29]. For these experiments, cells were stained with 1 μ M YoPro-1 for 15 min at room temperature.

Panx1 shRNA transfection Rat Panx1 gene expression was knocked down using a short hairpin RNA (shRNA) purchased from SABiosciences, MD (catalog number KR49872P). Briefly, 500 ng of Panx1 shRNA expression plasmids containing puromycin resistance were reverse transfected into HTC cells in a 24-well plate using Lipofectamine LTX (Life Technologies, NY). Control cells were obtained by transfection with a control plasmid that contained scrambled sequence. After incubation at 37 °C for 48 h, cells were re-plated and grown in a selection media containing DMEM supplemented with 10 % FBS, 1 % L-glutamine, and 3 μ g/ml puromycin. The selection was continued for 2 weeks, and a stock of the stably transfected cells was collected and stored in liquid nitrogen for further use. The

validation of Panx1 knock down in each clone was performed by a real-time PCR.

Real-time PCR Total RNA was extracted using Direct-zol RNA MiniPrep Kits (ZYMO Research, Irvine, CA) according to the manufacturer's instructions, and quantified using spectrophotometry. Two micrograms of total RNA was reverse-transcribed using oligo(dt) primer and Superscript RNase H-reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The real-time PCR reactions were then set up using RT² qPCR Primer Assay (Qiagen, Valencia, CA) specific for Panx1 with hypoxanthine phosphoribosyltransferase 1 (*Hprt* 1) as an endogenous control. The real-time PCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Briefly, 4 μ l of diluted cDNA samples (1:10 dilutions) was used for quantitative two-step PCR. The first step was performed at 95 °C for 10 min. The second step consisted

Fig. 2 Lipopoptosis stimulates release of chemoattractants in a JNK-dependent manner. **a** Monocyte migration was measured with Transwell plates using the human monocyte THP-1 cell line. Hepatocytes (HTC) treated overnight with palmitic acid (PA, 500 μM) or vehicle (0.5 % isopropanol), and in the presence or absence of the JNK inhibitor, 100 μM SP600125 (SP), were washed and incubated with fresh PA-free media for 2 h. After 2 h, supernatants were collected and loaded into the bottom chamber of the Transwell plates. The top chamber contained monocytes. After incubation, monocytes that migrated into the lower

chamber were counted using a Coulter cell counter. Histograms show the cell count versus the cell size. PA induced monocyte migration, and SP inhibited PA-induced migration. **b** The percent of migration was measured in response to supernatants obtained from hepatocytes treated overnight with vehicle, PA, or different doses of the JNK inhibitor (SP). The response to CCL2 was used as a positive control for maximum response (not shown). PA stimulated monocyte migration, and the JNK inhibitor (SP) prevented migration in a concentration-dependent manner ($n=6$ for all conditions). * $P<0.001$; ** $P<0.03$

of 50 cycles, and was performed in the presence of 200 nM specific forward and reverse primers for 15 s at 95 °C and 1 min at 65 °C, respectively. Nuclease-free water was used as a template for negative controls.

Statistics Data were expressed as mean±S.E.M. Results were compared using a two-tailed Student's *t* test on paired and unpaired data, and *P* values <0.05 were considered to be statistically significant.

Results

JNK activation mediates lipopoptosis in hepatocytes

Palmitic acid has been reported to stimulate JNK-dependent lipopoptosis in liver cells [4]. To assess whether palmitic acid activated JNK in a hepatocyte model, JNK phosphorylation was examined after overnight incubation with palmitic acid with the HTC cell line. Representative Western blots in Fig. 1a show that palmitic acid increased JNK phosphorylation, and a JNK inhibitor, SP600125, blocked this effect in a dose-dependent manner (Fig. 1a, b). To assess whether JNK mediates lipopoptosis in hepatocytes, caspase3/7 activity was measured after incubation with palmitic acid. Figure 1c shows that the JNK inhibitor SP600125 potently inhibited caspase3/7 activity. Similar results were obtained with a pan-caspase inhibitor zVAD-fmk (Fig. 1c).

To determine whether endoplasmic reticulum (ER) stress plays a role in lipopoptosis in hepatocytes, splicing of XBP1, an indicator of ER stress, was measured after incubation with palmitic acid. Figure 1d shows that palmitic acid increased the amount of spliced XBP1, and SP600125 partially inhibited XBP1 splicing. Figure 1e shows quantitative data by densitometry expressed as the spliced to unspliced ratios of XBP1 bands. Collectively, these data indicate that lipopoptosis in hepatocytes is mediated by ER stress and subsequent JNK activation.

Palmitic acid mediates release of chemoattractants from hepatocytes

To assess whether lipopoptosis stimulates release of eATP or other DAMPs capable of inducing migration of monocytes, supernatants from hepatocytes (HTC) treated with palmitic acid were tested for monocyte migration using a Transwell migration assay. The data in Fig. 2a illustrate that lipopoptotic supernatants stimulated migration of human monocytes with a magnitude similar to CCL2. When palmitic acid was dissolved in ethanol instead of isopropanol, lipopoptotic supernatants also stimulated THP-1 monocyte migration ($P<0.001$, $n=3$, data not shown). Figure 2b shows that inhibition of hepatocyte JNK activation with the specific JNK inhibitor SP600125 blocked monocyte migration induced by lipopoptotic supernatants. Notably, SP600125 inhibited lipopoptotic supernatant-induced THP-1 monocyte migration in a dose-dependent manner (Fig. 2b). These results indicate that activation of the JNK pathway in hepatocytes

mediates release of DAMPs capable of stimulating migration of monocytes.

eATP and CCL2 stimulate monocyte migration Previous studies have shown that monocytes migrate in response to extracellular nucleotides and the chemokine CCL2 [15]. To assess whether eATP modulates migration of THP-1

monocytes, the magnitude of migration was measured in response to different doses of purified eATP. Figure 3a shows that eATP stimulated THP-1 monocyte migration at concentrations greater than 10 nM, and a maximal response was observed at 1 μ M. Higher doses of eATP decreased monocyte migration. A similar dose-response relationship was obtained with CCL2 (Fig. 3b). These data confirmed that both eATP

Fig. 3 Monocyte migration induced by lipoapoptotic supernatants is mediated by ATP and CCL2. **a** Migration of human monocytes (THP-1) was measured in response to eATP using Transwell plates. The number of experiments was from 3 to 11. **b** Migration of human monocytes (THP-1) was measured in response to different doses of CCL2. The migration induced by CCL2 also exhibited a dose-response curve with higher doses inhibiting responses. **c** Monocyte migration (THP-1) was measured in response to supernatants obtained from hepatocytes (HTC) treated overnight with 0.5 % isopropanol (Veh) or 500 μ M palmitic acid (PA, $n=9$ for both). Supernatants from PA-treated hepatocytes stimulated migration of monocytes (THP-1), and the treatment of supernatants with Apyrase (Apy, 2 units/ml, $n=6$) potentially inhibited migration. **d** Migration of THP-1 monocytes (THP-1) and primary monocytes (PBMC) was measured in response to CCL2 (20 ng/ml) in the presence or absence of

Apyrase. Apyrase inhibited the migration induced by CCL2 in monocytes from PBMC by 50 % but not migration of THP-1 monocytes. The number of experiments was from 3 to 10. **e** To inhibit CCL2 receptors, monocytes were incubated with 20 μ M RS102895 (RS) for 2 h prior to migration assay. The migration was measured in response to supernatants obtained from hepatocytes (HTC) treated overnight with 0.5 % isopropanol (Veh) or 500 μ M PA. Note that RS did not significantly inhibit the migration induced by supernatants from PA-treated cells ($n=3$ for all conditions). **f** The migration of monocytes (THP-1) and PBMC was measured in response to CCL2 (20 ng/ml) after preincubation with 20 μ M RS for 2 h. RS inhibited migration induced by CCL2 ($n=3$ for both). * $P<0.001$; ** $P<0.003$. NS not significant. Note that monocytes, but not lymphocytes from PBMC cultures migrated to CCL2 and eATP as confirmed by flow cytometry (see “Experimental procedures”)

and CCL2 stimulated migration of THP-1 monocytes in a dose-dependent manner.

Pathophysiological eATP release contributes to monocyte migration We have recently shown that lipoapoptosis stimulates pathophysiological ATP release in hepatocytes [19]. Moreover, it has been reported that CCL2 expression is markedly increased in hepatocytes from *ob/ob* mice [13]. Thus, to assess whether eATP and/or CCL2 stimulate monocyte migration, supernatants from lipoapoptotic cells were treated with Apyrase to remove eATP. Notably, Apyrase potently inhibited migration of THP-1 monocytes induced by lipoapoptotic supernatants (Fig. 3c). Apyrase had no effect on THP-1 monocyte migration induced by CCL2, whereas it reduced the control primary monocyte (PBMC) migration by half (Fig. 3d). We also measured monocyte migration after treatment of THP-1 monocytes with a blocker of CCL2 receptors (CCR2), RS102895. Interestingly, RS102895 only had a modest effect on THP-1 monocyte migration in response to lipoapoptotic supernatants (Fig. 3e), but potently inhibited migration induced by CCL2 (Fig. 3f). Migration of primary monocytes (PBMC) in response to CCL2 was inhibited by

Apyrase and RS102895 (Fig. 3d, f). Addition of ATP did not significantly enhance migration of primary monocytes (PBMC) over vehicle (Supplementary Figure S1). Collectively, these findings provide support for the hypothesis that pathophysiological eATP release in lipoapoptosis results in increases in eATP concentration, and eATP may be a dominant DAMP responsible for stimulation of migration of THP-1 monocytes.

To further address this hypothesis, supernatant eATP concentrations were measured after overnight incubation of HTC cells with different FFA. We have recently shown that saturated but not unsaturated FFA stimulate pathophysiological ATP release [19]. Consistent with these results, we found that palmitic and stearic acids but not palmitoleic acid increased supernatant eATP concentration (Fig. 4a). Figure 4b shows that saturated FFA were more potent than unsaturated FFA in stimulating monocyte migration. When palmitic acid was dissolved in ethanol instead of isopropanol, supernatant eATP concentration increased by ~5-fold ($P<0.001$, $n=3$, data not shown). Palmitic acid also stimulated pathophysiological ATP release in other liver cells. Lipoapoptosis induced by palmitic acid in HepG2 cells increased supernatant eATP

Fig. 4 Pathophysiological ATP release in lipoapoptosis mediates monocyte migration. **a** ATP concentration was measured in supernatants obtained from HTC cells treated overnight with 0.5 % isopropanol (*Vehicle*), 500 μM palmitic acid (*PA*), stearic acid (*SA*), or palmitoleic acid (*PO*). **b** Migration of THP-1 monocytes was measured in response to supernatants from HTC cells treated overnight with different FFA (500 μM). Saturated FFA were more potent than unsaturated FFA in increasing ATP concentration and stimulating monocyte migration

(* $P<0.001$, NS not significant). The number of experiments was from 6 to 9. **c** Supernatant ATP concentration was measured from dishes with HepG2 cells treated overnight with different concentration of PA. **d** THP-1 monocyte migration was measured in response to supernatants obtained from HepG2 cells treated with different PA concentration. Note that PA increased ATP concentration and stimulated monocyte migration in a dose-dependent manner. Experiments were done in triplicate. * $P<0.001$; ** $P<0.03$

concentrations in a dose-dependent manner (Fig. 4c). Similar results were obtained in Huh7 cells (data not shown). Furthermore, lipoapoptotic supernatants obtained from HepG2 cells stimulated monocyte migration in a concentration-dependent manner (Fig. 4d). Collectively, these results indicate that lipoapoptosis in liver cells increases eATP concentrations, and these increased eATP levels likely induced the migration of THP-1 monocytes.

Panx1 mediates increases in extracellular ATP concentration We have recently shown that Panx1 mediates pathophysiological ATP release in lipoapoptosis [19]. To assess whether activation of Panx1 channels increases eATP concentrations, supernatant eATP concentrations were

measured after inhibition of Panx1 expression with shRNA. Figure 5a shows that two Panx1 clones exhibited decreased expression of Panx1 in HTC cells (~50 %). Because Panx1 antibodies were not reliable in detecting Panx1 in HTC cells as previously described [19], we could not confirm these findings using Western blot analysis. Notably, Panx1 shRNA inhibited an increase in supernatant eATP concentrations evoked by palmitic acid (Fig. 5b). We also measured YoPro-1 uptake to assess Panx1 channel activation in liver cells. Representative images in Fig. 5c show that lipoapoptosis evoked by palmitic acid potentially increased YoPro-1 uptake. Interestingly, Panx1 shRNA did not inhibit palmitic acid-evoked YoPro-1 uptake, and additional treatment with SP600125 inhibited this response (Fig. 5d). Collectively, these

Fig. 5 Panx1 mediates pathophysiological ATP release in lipoapoptosis. **a** HTC cells were transfected with a scrambled or two-Panx1 shRNA. Both Panx1 shRNA decreased Panx1 expression in HTC cells by ~50 % (* P <0.001, n =4). **b** Supernatant ATP concentration was measured from dishes with HTC cells transfected with scrambled or Panx1 shRNAs, and after overnight treatment with 0.5 % isopropanol (Vehicle, n =6) or palmitic acid (PA 500 μM, n =6). PA treatment increased supernatant ATP concentration in cells transfected with a scrambled shRNA (* P <0.001). Panx1 shRNA inhibited PA-evoked increase in supernatant ATP

concentration (* P <0.02). **c** Fluorescent images of HTC cells incubated with 1 μM YoPro-1 in Vehicle and after overnight treatment with PA (500 μM). **c** YoPro-1 fluorescence was measured in cells transfected with a scrambled or two-Panx1 shRNA. Cells were treated overnight with PA (500 μM) and in the absence or presence of SP600125 (SP, 100 μM). PA treatment increased YoPro-1 uptake in cells transfected with a scrambled shRNA. Panx1 shRNAs did not inhibit YoPro-1 uptake induced by PA, and SP inhibited this response by ~50 %. The number of cells was from 51 to 158. * P <0.001; ** P <0.02

data indicate that activation of Panx1 channels in lipoapoptosis mediates an increase in eATP concentration.

Panx1 activation mediates monocyte migration The data presented above provide support for the hypothesis that stimulation of Panx1-dependent pathophysiological ATP release in lipoapoptosis may mediate migration of THP-1 monocytes. To further address this hypothesis, monocyte migration was measured after inhibition of Panx1 expression in HTC cells. Figure 6 shows that Panx1 shRNA inhibited migration of THP-1 monocytes induced by lipoapoptotic supernatants indicating that activation of Panx1 channels in lipoapoptosis may contribute to monocyte migration.

Panx1 activation in lipoapoptosis is regulated by JNK The data in Figs. 1 and 2 indicate that JNK regulates release of DAMP molecules capable of stimulating monocyte migration. To assess whether JNK regulates activation of Panx1 channels in lipoapoptosis, supernatant ATP concentration was measured after overnight treatment of HTC cells with palmitic acid and SP600125. Palmitic acid increased ATP concentration, and SP600125 decreased ATP concentration in a dose-dependent manner (Fig. 7a). We also measured YoPro-1 uptake after overnight treatment with palmitic acid and SP600125. Figure 7b shows that palmitic acid potently stimulated YoPro-1 uptake, which was inhibited by SP600125 in a dose-dependent manner. These results indicate that JNK regulates activation of Panx1 channels in lipoapoptosis.

Fig. 6 Panx1 mediates monocyte migration in lipoapoptosis. HTC cells were transfected with a scrambled or two-Panx1 shRNA. Cells were treated overnight with 0.5 % isopropanol (*Vehicle*, $n=6$) or palmitic acid (*PA* 500 μM , $n=6$), and migration of THP-1 monocytes was measured in response to supernatants using Transwell plates. PA treatment of cells transfected with a scrambled shRNA stimulated monocyte migration ($*P<0.001$). Note that PA-evoked monocyte migration was inhibited in cells transfected with Panx1 shRNA ($**P<0.02$)

Discussion

Panx1 mediates ATP release and extracellular ATP functions as a potent signal for the recruitment and activation of inflammatory cells. There are two principal findings of these studies. First, JNK is a novel regulator of Panx1 channels. Second, Panx1-dependent pathophysiological ATP release in lipoapoptosis mediates in part migration of human THP-1 monocytes with the magnitude similar to CCL2. Because recruitment of monocytes in the liver is a key pathogenic feature of hepatic inflammation in NASH, these findings suggest that one potential physiological function of Panx1-dependent pathophysiological ATP release may be to participate in the recruitment of monocytes.

Fig. 7 JNK regulates Panx1 activation in lipoapoptosis. **a** Supernatant ATP concentration was measured from dishes with HTC cells treated overnight with 0.5 % isopropanol (*Vehicle*) or 500 μM palmitic acid (*PA*), and in the presence of different concentration of SP600125 (*SP*). Lipoapoptosis evoked by PA increased ATP concentration in *Vehicle* ($n=6$). SP inhibited PA-evoked increase in ATP concentration in a concentration-dependent manner ($n=6$). $*P<0.001$; $**P<0.03$. **b** YoPro-1 fluorescence was measured in HTC cells treated with *Vehicle* or 500 μM PA, and in the presence of different SP concentrations. Lipoapoptosis evoked by PA stimulated YoPro-1 uptake, and SP inhibited this response in a concentration-dependent manner ($*P<0.001$). The number of cells analyzed was from 69 to 261

We have recently shown that Panx1 is expressed in primary hepatocytes and multiple liver cell lines [19]. Panx1 channels are not activated under basal conditions, and become activated in lipoapoptosis induced by saturated FFA. Here, we show that the inhibition of Panx1 expression in HTC cells by Panx1 shRNA decreases ATP concentration in lipoapoptotic supernatants, suggesting that Panx1 mediates in part an increase in extracellular ATP (eATP) concentration. We also found that lipoapoptosis increased supernatant ATP concentration in HepG2 and Huh7 cells. Thus, pathophysiological ATP release appears to be a common pathogenic feature of lipoapoptosis in liver cells, and Panx1 may be a potential mediator of this response.

Panx1-dependent ATP release in apoptosis in Jurkat cells and thymocytes is mediated by a caspase-dependent cleavage of Panx1 at the C-terminus, and functions as a find-me signal for migration of monocytes to the site of the dying cell [16, 33]. Our recent studies have shown that Panx1-dependent eATP release in HTC cells is not dependent on the caspase3/7 activation [19]. Thus, it is likely that activation of Panx1 channels in liver cells is mediated through the mechanisms that are different than those described in Jurkat cells and thymocytes. We identified JNK as a novel regulator of Panx1 channels in HTC cells. Two important findings provide support for this hypothesis. First, the inhibition of JNK with SP600125 markedly decreased ATP concentration in lipoapoptotic supernatants, and inhibited YoPro-1 uptake in lipoapoptotic liver cells. Second, SP600125 inhibited migration of THP-1 monocytes evoked by lipoapoptotic supernatants. JNK lies upstream of caspase3/7 in a lipoapoptotic pathway, and plays a key role in mediating lipoapoptosis induced by saturated FFA [4, 6]. Interestingly, more than 40 proteins have been identified as JNK substrates in the liver, and many of these proteins are involved in stimulation of cell death and inhibition of insulin signaling [7]. While the present study provides evidence that Panx1 may be a novel target of hepatic JNK, it is not known whether JNK directly interacts with Panx1 and/or through a participation of other JNK substrates.

We used migration of human THP-1 monocytes as an immunological tool to assess the role of Panx1 in inflammation. These cells were selected because they migrate in response to DAMPs found in NASH livers as well as CCL2 and RANTES (CCL5) [15, 16, 34, 35]. Thus, it is likely that the chemokine receptors CCR2, CCR1, and CCR5 play a central role in monocyte migration, although almost 20 have been implicated in chronic liver diseases [36]. We also used PBMC as a control for primary human leukocytes. We found that monocytes from the PBMC, as judged by forward versus side scatter, also migrate in response to eATP; however, it is the combination of extracellular nucleotides and chemokine that is required for optimal migration. In the presence of eATP alone, concentrations from 1 to 100 nM in the absence of chemokine increased

primary monocyte migration by approximately 10 % (see supplementary data). This low level of activity is likely the result CCL2 secreted by monocytes in response to eATP [37]. In the presence of CCL2, Apyrase blocked migration of the control primary monocytes by 50 %, indicating endogenous production and release of eATP occurred in the Transwell assay, possibly by CCL2 activation of eATP release from migrating monocytes or as a result of eATP release from dying cells in the freshly isolated PBMC preparation. This was not the case for the THP-1 cell line which was completely blocked by each inhibitor in response to eATP or CCL2. Thus, THP-1 did not appear to produce levels of CCL2 or eATP that were sufficient to be detected in these functional assays. Taken together, these studies suggest that the well characterized THP-1 monocyte cell line migrate to eATP in lipoapoptotic supernatants.

Recent reports examined the eATP receptors involved in myeloid cell migration by employing selective agonist or antagonist of these receptors. For example, the P2Y2 receptor was identified as the nucleotide receptor for eATP and eUTP which induced CCL2 secretion from CD14⁺ monocytes and the THP-1 myeloid cell line. These researchers found that the selective antagonist, suramin, did not block receptor activity reproducibly at the higher concentrations of nucleotides often used for in vitro studies [37]. By contrast, Pam₃CSK₄-induced chemokine production by human monocytes was reported to be dependent on synergism between TLR2 and nucleotides, as the addition of Apyrase abrogated the response. Both TLR2 and nucleotides were required for neutrophil migration that was found to be dependent on P2Y2 and P2Y6 in a murine air pouch model [38]. These studies suggest that the P2Y2 receptor and possibly other receptors participate in myeloid cell migration to sites of injury.

Two key findings in this work provide support for the concept that one potential function of Panx1-dependent pathophysiological eATP release in lipoapoptosis is to stimulate migration of monocytes. First, removal of eATP by Apyrase markedly inhibited monocyte migration. Second, inhibition of Panx1 expression by Panx1 shRNA partially blocked monocyte migration in response to lipoapoptotic supernatants. Moreover, inhibition of CCL2 receptors in THP-1 monocytes with RS102895 failed to inhibit monocyte migration induced by eATP. These findings suggest a model for the role of Panx1 in lipoapoptosis shown in Fig. 8. eATP released from liver cells through Panx1 channels may function as a dominant DAMP that mediates monocyte migration. Since eATP is also a potent signal for stimulation of a proinflammatory IL-1 β from macrophages, elevated eATP concentration in lipoapoptosis induced by saturated FFA may significantly contribute to inflammation. In addition to eATP, recent studies have shown that hepatocytes from leptin-deficient mice or alcohol-fed mice have increased expression of CCL2 [13, 39]. Although the authors have not determined whether hepatocytes release CCL2 under these conditions, these studies

Fig. 8 Proposed model for the role of Panx1 in liver cells. Palmitic acid (PA) induces lipoapoptosis by stimulating ER stress which results in JNK. JNK stimulates pathophysiological ATP release by activation of Panx1 channels in the plasma membrane. Panx1 channels may be associated with the P2X7 receptor or they may function in a receptor-independent manner. The resulting increase in extracellular ATP concentration mediates migration of monocytes, and may contribute to hepatic inflammation by promoting recruitment of monocytes to the site of injury. CCL2 is produced by hepatocytes, liver myofibroblasts, and leukocytes during an inflammatory response

suggest that CCL2 may participate in the recruitment of monocytes in chronic liver injury induced by leptin deficiency and alcohol.

One potential explanation for these discrepancies may be that hepatocytes *in vitro* lose their epithelial polarity, which may substantially influence both constitutive and lipoapoptosis-evoked release of ATP and other chemoattractants. However, it appears that these changes have no effect on the intracellular lipoapoptotic pathways, since there is a nice correlation between the studies of lipoapoptotic mechanisms in liver cell lines and primary mouse hepatocytes [4, 19, 30]. Consequently, other more *in vivo* models of NASH that mimic hepatocyte pathophysiology in the liver should be used in future studies to assess whether lipoapoptosis stimulates ATP release in dying hepatocytes, and whether this release is capable of stimulating migration of PBMC.

Assuming that Panx1-dependent pathophysiological ATP release mediates monocyte migration in lipoapoptosis, several additional points merit emphasis. First, recent studies have shown that apoptosis induced by anti-Fas in Jurkat cells and thymocytes stimulates release of ATP and UTP, and both nucleotides are capable of stimulating monocyte migration [15, 16]. Thus, in addition to ATP, liver cells may release UTP and other nucleotides that could contribute to monocyte migration. For example, if monocyte migration was mediated in a large part by nucleotides other than ATP, and their release was more

sensitive to JNK, this could explain the finding that SP600125 was more potent in inhibiting monocyte migration than decreasing ATP concentration in lipoapoptotic supernatants (compare Figs. 2b and 7a). Second, while Panx1-dependent pathophysiological ATP release may be beneficial in removing the dead cells by monocytes and other inflammatory cells as previously described [29, 40–42], chronic lipoapoptosis may result in a failure to rapidly remove dead cells which may promote infiltration of monocytes. Notably, hepatocyte lipoapoptosis and massive accumulation of monocytes have been found in mice and patients with chronic liver injury [8, 10, 11, 43, 44]. Of note, CCL2 is produced by hepatocytes and liver myofibroblasts in addition to Kupffer cells and infiltrating leukocytes and CCL2 are key to inflammatory cell infiltration resulting in fibrosis in chronic liver diseases [36, 45, 46]. Thus, if hepatocytes exhibit pathophysiological ATP release, it is attractive to speculate that in addition to CCL2 and other chemokines, increased concentration of extracellular ATP in chronic liver injury induced by saturated FFA may contribute to the accumulation of monocytes in the liver.

Third, millimolar concentrations of extracellular ATP have been reported to stimulate hepatic inflammation in a model of acute liver injury induced by acetaminophen [47]. In this model, hepatocytes undergo necrosis and apoptosis and release large amounts of ATP. These high ATP

concentrations (3–5 mM) mediate hepatic inflammation by activating Kupffer cells to stimulate production of a proinflammatory cytokine IL-1 β . Interestingly, millimolar concentration of ATP does not stimulate migration of monocytes (see Fig. 3a). We have recently shown that lipoapoptosis evoked by saturated FFA does not compromise cell membrane, thus arguing against a passive leakage of intracellular ATP from necrotic cells [19]. Moreover, here, we show that lipoapoptosis increases extracellular ATP concentration to the values that are much lower than those observed in acute liver injury (<100 nM), and these ATP concentrations are capable of stimulating migration of human monocytes with the magnitude similar to CCL2.

Finally, we have shown that Panx1 shRNA can inhibit both eATP release and Panx1 activation [19]. Here, Panx1 shRNAs blocked eATP release but Panx1 activation as determined by YoPro-1 uptake was still detected. One explanation for this discrepancy may be that Panx1 shRNAs or YoPro-1 concentrations used here were not optimal. Based on our recent work and the literature, there are several mechanisms for eATP release and YoPro-1 uptake in liver cells [48–50]. Although Panx1 can act in a receptor-independent fashion, it is equally possible that Panx1 pore formation is operating by a P2X7 receptor-driven mechanism [51–53]. P2X7 receptors are highly expressed on myeloid lineage cells including Kupffer cells, and P2X7 receptors are readily observed on hepatocytes and these purinergic receptors physically associate with Panx1 [52–54]. Moreover, it has been established that stimulation of P2X7 receptor induces Panx1-mediated pore formation and dye uptake [52]. We conclude that Panx1 shRNAs levels were sufficient to inhibit eATP release but not dye uptake. P2X7 receptor activation can activate dye uptake through other channels, for example intracellular Ca²⁺-dependent channels [55]. However, the dissociation of eATP release and dye uptake has also been observed in 1321N1 astrocytes [56]. The authors have shown that CBX-sensitive ATP release is not correlated with ethidium bromide uptake. Moreover, there are other channels that might potentially mediate eATP release and YoPro-1 uptake. In addition to Panx1, YoPro-1 uptake is mediated by connexin (Cx) hemichannels Cx32 and/or Cx26, two isoforms expressed in hepatocytes. This explanation is unlikely since our studies show that JNK modulates YoPro-1 uptake, and there is no evidence that hepatic JNK regulates the expression or function of Cx32 and Cx26.

Activation of JNK has been implicated in liver injury induced by saturated FFA, ischemia/reperfusion, acetaminophen, and in hepatocellular carcinoma [7]. Moreover, recent studies indicate that eATP and purinergic signaling contributes importantly to the pathogenesis of many liver diseases [57, 58]. Thus, the findings of the present study that hepatic JNK regulates pathophysiological eATP release and monocyte migration may have implications for understanding the mechanisms of liver injury in chronic disease states.

In summary, we found that lipoapoptosis induced by saturated FFA stimulates ER stress-mediated JNK activation and pathophysiological eATP release from liver cells via activation of Panx1 channels. Importantly, eATP stimulates migration of human primary monocytes and THP-1 monocytes with a magnitude similar to CCL2. These findings suggest that pathophysiological eATP release during lipoapoptosis is capable of stimulating migration of human monocytes.

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Author contributions FX, SLW, LD, and SFB performed the experiments. GJG assisted in manuscript preparation. GK designed the experiments; GK, FX, and LD prepared and approved the manuscript for submission.

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