Research Article

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Impaired intracellular signaling, myeloperoxidase release and bactericidal activity of neutrophils from patients with alcoholic cirrhosis ☆

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Background & Aims: Myeloperoxidase exocytosis and production of hydrogen peroxide via the neutrophil superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase contribute to efficient elimination of bacteria. Cirrhosis impairs immune functions and increases susceptibility to bacterial infection. We recently showed that neutrophils from patients with decompensated alcoholic cirrhosis exhibit a severe impairment of formylpeptide receptor (fPR)-mediated intracellular signaling and superoxide production. Here, we performed *ex vivo* studies with these patients' neutrophils to further investigate myeloperoxidase release, bactericidal capacity and signaling events following fPR stimulation by the formylpeptide formyl-met-leu-phe (fMLP).

Methods: Myeloperoxidase release was studied by measuring extracellular myeloperoxidase activity. Activation of signaling effectors was studied by Western blot and their respective contribution to myeloperoxidase release studied using pharmacological antagonists.

Results: fMLP-induced myeloperoxidase release was strongly impaired in patients' neutrophils whereas the intracellular myeloperoxidase stock was unaltered. The fMLP-induced phosphorylation of major signaling effectors, AKT, ERK1/2 and p38-MAP-Kinases, was also strongly deficient despite a similar expression of signaling effectors or fPR. However, based on effector inhibition in healthy neutrophils, AKT and p38-MAPK but not ERK1/2 upregulated fMLP-induced myeloperoxidase exocytosis. Interestingly, patients' neutrophils exhibited a defective bactericidal capacity that was reversed *ex vivo* by the TLR7/8 agonist CL097, through potentiation of the fMLP-induced AKT/p38-MAPK signaling axis and myeloperoxidase release.

Conclusions: We provide first evidence that neutrophils from patients with decompensated alcoholic cirrhosis exhibit a deficient AKT/p38-MAPK signaling, myeloperoxidase release and bactericidal activity, which can be reversed via TLR7/8 activation. These defects, together with the previously described severe deficient superoxide production, may increase cirrhotic patients' susceptibility to bacterial infections.

Introduction

Neutrophils play a key role in the elimination of invading microorganisms [1]. This innate defence function requires a fine coordination of two major neutrophil activities; the release of myeloperoxidase (MPO) from azurophilic granules (exocytosis) and production of reactive oxygen species (ROS) by the superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a phenomenon termed respiratory burst. MPO utilizes hydrogen peroxide (H_2O_2) derived from superoxide dismutation, and chloride to form hypochlorite and chloramine, which are toxic agents for bacteria [2,3]. MPO biological importance is further illustrated in the findings in *MPO*-knockout mice of increased infections by *Klebsiella* and *Candida*, increased mortality [4] and prolonged inflammation [5]. MPO release and ROS production are triggered by various pro-inflammatory mediators amongst which bacterial formylated peptides which also act as chemoattractants, thus alerting neutrophils in case of infection through stimulation of formylpeptide receptor (fPR), a G-protein

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Abbreviations: MPO, myeloperoxidase; fMLP, formyl-met-leu-phe; MAPK, mitogen-activated protein kinases; ERK1/2, Extracellular Signal-Regulated Kinases1/2; ROS, reactive oxygen species; fPR, formylpeptide receptor; TLR, Toll-like receptors.

coupled receptor [11]. Neutrophil antibacterial activities are tightly regulated by signaling events triggered via fPR including phospholipases, G-proteins, protein kinases such as protein kinase C (PKC), mitogen-activated protein kinases (MAPK) and mammalian target of rapamycin (mTOR) [6–8]. Signaling impairment under pathological situations or by drugs leads to neutrophil dysfunctions, which are detrimental to host-defence [9–11].

Alcoholic cirrhosis is a consequence of excessive alcohol consumption and represents a major cause of mortality worldwide with an estimated 3.8% of all global death [12]. This pathology combines different features of the liver disease including steatosis, inflammation, necrosis and fibrosis [13,14]. Cirrhosis is associated with immune dysfunctions and inability of host-defence systems to protect against infections [15]. Neutrophils contribute to the pathogenesis of cirrhosis through induction of liver injury in animal models (reviewed in [16]) as well in patients with alcoholic steatohepatitis [17]. Direct evidence for a role of neutrophils inducing liver injury was proposed by the observation of an intracellular oxidant stress in hepatocytes during neutrophil attack [18]. However, the view that neutrophils are systematically deleterious is not universally recognized. Indeed, Altamirano *et al.* showed that the higher neutrophil liver infiltration, the better the prognosis [19]. Neutrophils may exert their beneficial effects through production of hepatocyte growth factor [20], collagen degradation [21] or through granulopoiesis following G-CSF treatment [22]. Moreover, at least one study shows that G-CSF therapy is beneficial in patients with severe alcoholic hepatitis [23]. Finally, G-CSF therapy was found to improve survival in patients with acute-on-chronic liver failure, the most severe complication of cirrhosis [24]. Another common complication of cirrhosis is the development of sepsis, a major cause of death [12,25]. Although medical treatments exist to improve survival, about 35% of patients die within six months [26]. Of the numerous circulating host-defence mechanisms available, neutrophil production of ROS, microbicidal activity and phagocytosis are impaired in cirrhotic patients [27–35]. An impaired ROS production was also observed in liver transplanted recipients suffering from post-hepatitic cirrhosis [36]. However, the impact of alcoholic cirrhosis on receptor-mediated signaling events underlying neutrophil antibacterial activities remains largely unknown. We recently showed that neutrophils from patients with decompensated alcoholic cirrhosis, exhibit impaired signaling and ROS production induced by the bacterial tripeptide formyl-met-leu-phe (fMLP), which was further aggravated by the mTOR antagonist rapamycin [8]. This dysfunction was associated with a defective MAPK-dependent phosphorylation of p47phox, a major component of NADPH oxidase. Whether the defective signaling impacts other neutrophil defence activities is unknown.

In this study, we took advantage of these neutrophils from these cirrhotic patients to investigate possible alterations of MPO exocytosis induced by fMLP, and bactericidal activity. The state of activation of three major signaling effectors, AKT, p38-MAP-kinases and p44/42 MAP-kinases (ERK1/2), was also determined, and their respective contribution in MPO release was studied in healthy neutrophils using selective antagonists. Data reveal a severe deficient activation of the three signaling effectors in patients' neutrophils, impaired MPO release and bactericidal activity. Interestingly, these deficiencies can be reversed by a TLR7/8 agonist.

Patients and methods

Patients

Blood was obtained from patients hospitalized in the Liver Unit of Beaujon Hospital (Clichy, France). Inclusion criteria were age over 18 years, biopsy-proven cirrhosis, and Child-Pugh class B or C cirrhosis. Patients had a history of excessive alcohol ingestion (50 g/day), but no other causes of liver disease. Viral serologies for hepatitis B and C virus were negative. Alcohol consumption was stopped for at least 3 days. Clinical characteristics of patients are shown in Table 1. Patients with untreated or recently treated (less than one week) bacterial infection or gastrointestinal haemorrhage were not included. Cultures of ascites, urine and blood performed at the time of inclusion were all negative. Other exclusion criteria were treatment with corticosteroids, pentoxifylline or other immunosuppressive drugs in the past 30 days, and presence of hepatocellular carcinoma (HCC), other cancer, or human immunodeficiency virus infection. Healthy subjects (controls) were hospital employee volunteers or obtained from the blood bank (EFS, Paris, France). This study was approved by our institutional review board, and written informed consent was obtained from patients.

Materials and methods

Please see the Supplementary materials for details regarding neutrophil isolation [8], MPO exocytosis and assay [37], bactericidal activity [8], Western blot analyses of protein phosphorylation [8] and RNA quantification.

Statistical analysis

Unless otherwise stated, data represent means ± SEM. Statistically significant differences between means were identified using the Student's paired *t* test or Mann-Whitney *U* test, with a threshold of $p < 0.05$ and designated by *.

Results

Severe impairment of fMLP-induced MPO release and signaling in neutrophils from patients with advanced alcoholic cirrhosis

Stimulation of healthy neutrophils under optimal conditions by fMLP (1 μM) induced a weak release of MPO of approximately 10% of the total MPO content (Fig. 1A, C). Under these conditions, neutrophils from cirrhotic patients exhibited an impaired MPO release compared to that of healthy neutrophils. Optimizing the degranulation process by pretreating cells with the microtubule-disrupting agent cytochalasin B [37], potentiated fMLP-induced MPO release with the same efficacy, i.e. about 3–4 fold in both healthy and patients' neutrophils (Fig. 1B), suggesting no major alteration of the cytoskeleton mobilization efficiency. However, the fMLP-induced MPO exocytosis of neutrophils from cirrhotic patients, remained impaired relative to controls, while basal degranulation was not altered (Fig. 1B). To examine whether this deficient induction of MPO release was related to alteration of MPO intracellular stock, resting and stimulated cells were lysed, and MPO was quantified by measuring both MPO activity and expression by Western blot. In lysates of resting cells, the MPO activity was similar in both control and patients' neutrophils (Fig. 1C). In fMLP-stimulated cells, MPO activity decreased significantly in control neutrophils due to their MPO exocytosis (Fig. 1) but not in patients' neutrophils. MPO expression analyzed by Western blot in resting cells was not altered. Thus, the MPO intracellular pool was not impaired, which suggests that the deficient MPO release induced by fMLP in patients' neutrophils (Fig. 1A, B) may likely

Table 1. Characteristics of patients.

N. of patients	32
Age, years	57.4 (±1.4)
Female (%)	6 (19)
Ascites, n (%)	28 (88)
Hepatocellular carcinoma (HCC), n (%)	0
Acute alcoholic hepatitis n (%)	7 (22)
Encephalopathy, n (%)	8 (25)
Serum albumin, g/L	24.7 (±1.1)
Serum bilirubin, μmol/L	116.2 (±21.2)
Prothrombin time, %	45.7 (±2.6)
International normalized ratio	1.8 (±0.1)
Serum creatinine, μmol/L	94.6 (±11.1)
C-reactive protein (CRP), mg/L	17,3 (±3.5)
Child-Pugh score	10.4 (±0.3)
Child-Pugh class C, n (%)	21 (66)
MELD score	19.1 (±1.1)
White blood cell count, per mm^3	7.650 (±0.645)
Treatment with β-blockers, n (%)	9 (28)
Antibiotics, n (%)	15 (47)
Corticosteroids, n (%)	0 (0)
Previous history	
Acute decompensation, n (%)	28 (88)
Bacterial infection, n (%)	18 (56)
Acute variceal bleeding, n (%)	12 (37)
Acute alcoholic hepatitis, n (%)	13 (40)

± values indicate standard error of the mean.
MELD, Model of End-Stage Liver Disease.

due to altered fPR-mediated signaling. To further explore this hypothesis, neutrophils were stimulated under optimal conditions with fMLP, and the activated phosphorylated forms of major signaling effectors were detected using Western blot [8,38,39]. Stimulation of healthy neutrophils induced a rapid phosphorylation of AKT, p38-MAPK and p44/42-MAPK (ERK1/2) (Fig. 2A). By contrast, in patients' neutrophils, the phosphorylation of the three effectors was dramatically impaired (Fig. 2B–D), whereas no alteration was observed in the expression of the three effectors (Fig. 3A, B) and the fMLP receptor fPR (Fig. 3C, D).

A major role of AKT, p38-MAPK but not ERK1/2 in fMLP-induced neutrophil MPO release

The signaling mechanisms by which bacterial peptides stimulate neutrophil MPO release remain largely unknown. To examine the respective contribution of AKT, p38-MAPK and ERK1/2 to MPO release, selective antagonists of these effectors were used in healthy neutrophils to prevent their activation. Treatment of neutrophils with AKTib1/2 (1–10 μM) to block AKT activation [38], as confirmed here (Supplementary Fig. 1A, B), reduced fMLP-induced MPO release by approximately 40–50%, without altering basal MPO release (Fig. 4A) or MPO catalytic activity measured in cell-free homogenates (Supplementary Fig. 1C). The inhibition of fMLP-induced p38-MAPK activation by SB202190 (2–10 μM) (Supplementary Fig. 2A, B), also reduced fMLP-induced MPO release by approximately 50% without altering basal MPO release (Fig. 4B) or MPO catalytic activity (Supplementary Fig. 2E). Since both antagonists reduced MPO release with similar efficiency, we examined whether p38-MAPK activation occurred downstream of AKT. Interestingly, p38-MAPK activation was reduced by about 50% in AKTib1/2-pretreated neutrophils (Supplementary Fig. 2C, D), suggesting that p38-MAPK is activated downstream of AKT. To examine the contribution of ERK1/2 to MPO release, cells were pretreated with U0126 (1–10 μM) to prevent fMLP-induced ERK1/2 phosphorylation [38], as confirmed here (Supplementary Fig. 3A, B). Surprisingly, the fMLP-induced MPO release was also blocked (Supplementary Fig. 3C). However, this complete inhibition was artefactual due in part to a direct interference effect of U0126 with MPO activity measured in neutrophil degranulation supernatants (Supplementary Fig. 3D). To bypass this pharmacological interference and examine whether ERK1/2 regulate exocytosis, the effect of U0126 was examined on the release of elastase, another marker of primary granules. Blocking ERK1/2 activation with U0126 did not alter fMLP-induced elastase release while superoxide production was impaired (Fig. 4C). These results suggest that ERK1/2 may not regulate MPO degranulation, in agreement with other works [40].

A TLR7/8 agonist restored MPO release from neutrophils of cirrhotic patients

The above data strongly suggest that the impaired activation of AKT/p38-MAPK observed in patients' neutrophils (Fig. 2A, C)

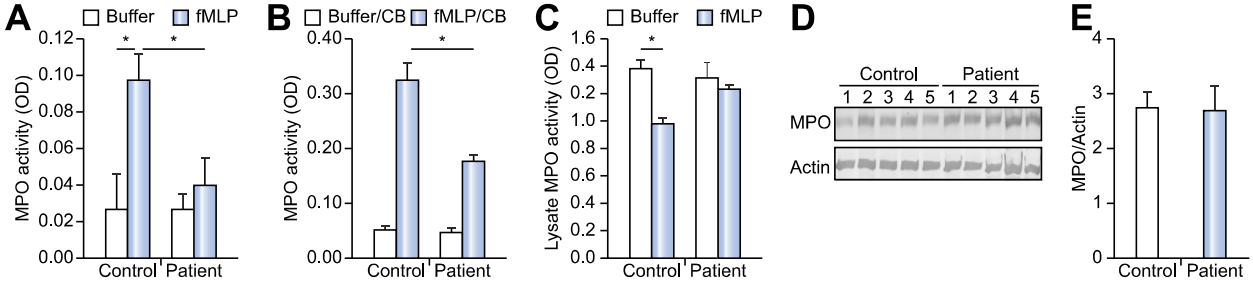

Fig. 1. Impaired fMLP-induced MPO release in neutrophils from patients with alcoholic cirrhosis. Neutrophils from healthy donors (control) and cirrhotic patients were pretreated without (A) or with cytochalasin B (5 μg/ml; 5 min) (B) prior stimulation with fMLP (1 μM; 2 min). MPO activity in degranulation supernatants is expressed as OD over 3 min (n = 14 in each group; *p <0.05 *vs.* control). (C) MPO activity in homogenates of resting and fMLP-stimulated neutrophils, expressed as OD per 3 min (n = 7, *p <0.05). (D) A representative Western blot of MPO of neutrophils from control and cirrhotic patients, and MPO quantification (E) expressed as MPO/actin ratio (n = 13 in each group).

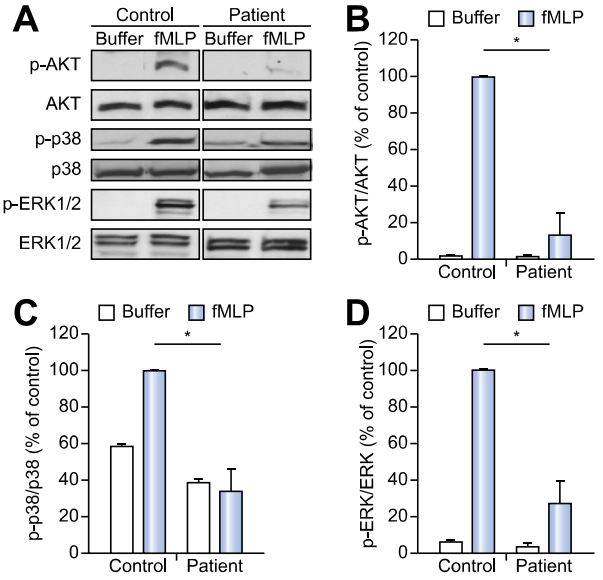

Fig. 2. Impaired fMLP-induced activation of signaling effectors in neutrophils from patients with alcoholic cirrhosis. (A) Neutrophils from control and patients with cirrhosis were stimulated with fMLP (1 μM; 1 min). (B–D) The phosphorylation of AKT (S473), p38-MAP-Kinases and p44/42-MAP-kinases (ERK1/2) is expressed as the percentage of control values obtained with fMLP (n = 4–6 in each group; $^{*}p$ <0.05 *vs.* control).

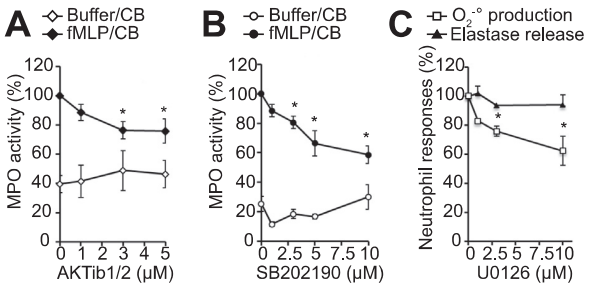

Fig. 4. A role of AKT and p38-MAPK but not ERK1/2 in fMLP-induced release of MPO from neutrophils. (A–C) Healthy neutrophils were pretreated for 15 min without (control) or with the antagonist of AKT (AKTib1/2), p38-MAPK (SB202190) or ERK1/2 (U0126), then with cytochalasin B for 5 min before stimulation with fMLP (1 μM, 2 min). MPO release is expressed as the percentage of control fMLP values (n = 6; $^{*}p$ <0.05 *vs.* control).

may contribute to the defective MPO release (Fig. 1A, B). It has been shown that p38-MAPK can be also activated upon stimulation of intracellular Toll-like receptors (TLR) 7/8 by the cell-permeable agonist CL097 leading to potentiation of neutrophil stimulation, as detected by superoxide production [41]. To determine whether the deficient MPO release from patients' neutrophils can be improved following TLR7/8 activation, the effect of CL097 was examined on MPO exocytosis and signaling. Pretreatment of patients' neutrophils in the presence of CL097 (0.5–2 μg/ml) for 10 min, induced a concentration-dependent potentiation of fMLP-induced MPO release, reaching physiological levels (Fig. 5A). This CL097 potentiation property was also observed on MPO release by neutrophils from healthy donors. Interestingly, CL097 also potentiated fMLP-induced phosphorylation of AKT in neutrophils from both control and cirrhotic patients and maintained the level of activated AKT up to 30 min, while in the absence of CL097, AKT phosphorylation returned to basal level, more rapidly in patients' neutrophils (Fig. 5B–E). More strikingly, CL097 potentiated both basal and fMLP-induced phosphorylation of p38-MAPK maintaining a high level of activated enzyme for at least 30 min in neutrophils of both control and cirrhotic patients (Fig. 5B–D and E–G). By contrast, in cells not treated with CL097, p38-MAPK phosphorylation was transient returning to baseline levels within 5 min. These CL097 potentiating effects may be mediated mainly through TLR8 since TLR7 was barely detectable in neutrophils (Supplementary Fig. 4), in agreement with other studies [42,43]. TLR8 was similarly expressed in both control and patients neutrophils,

Impaired bactericidal activity of patients' neutrophils and beneficial CL097 effects

The deficient signaling and MPO exocytosis of patients' neutrophils suggest that alterations may impact neutrophil bactericidal activities. We explored this hypothesis using a model of bacterial killing induced by fMLP [8]. Control neutrophils incubated with *E. Coli* induced significant bacterial killing (25–30%) upon stimulation with fMLP, in contrast to patients' neutrophils which were not responsive (Fig. 6A). Interestingly, CL097 alone induced significant bacterial killing by neutrophils from both controls and cirrhotic patients (Fig. 6B and C). CL097 also strongly potentiated fMLP-induced bacterial killing by patients' neutrophils whereas a non-significant increase was observed with control neutrophils. Thus, TLR8 activation may provide beneficial

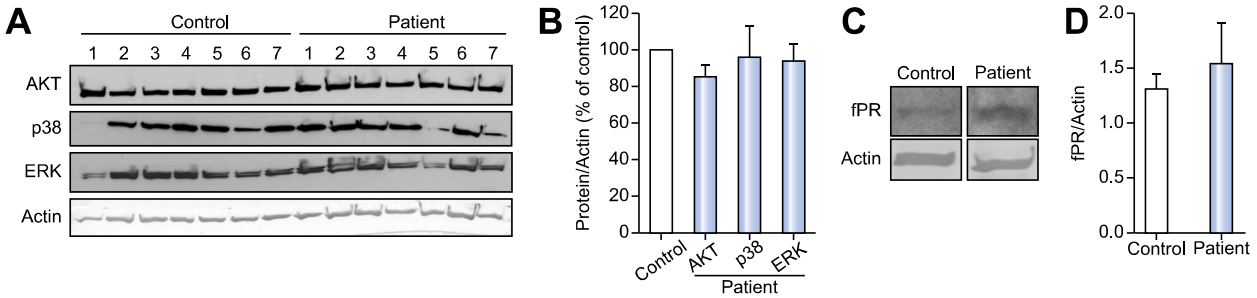

Fig. 3. Expression of signaling effectors and fPR in neutrophils of healthy donors and patients. Neutrophils from control and cirrhotic patients were disrupted in Trizol buffer for protein extraction. Proteins of interest were detected using Western blot (A, C), quantified (B, D) and expressed as percentage of control (n = 7 in each group).

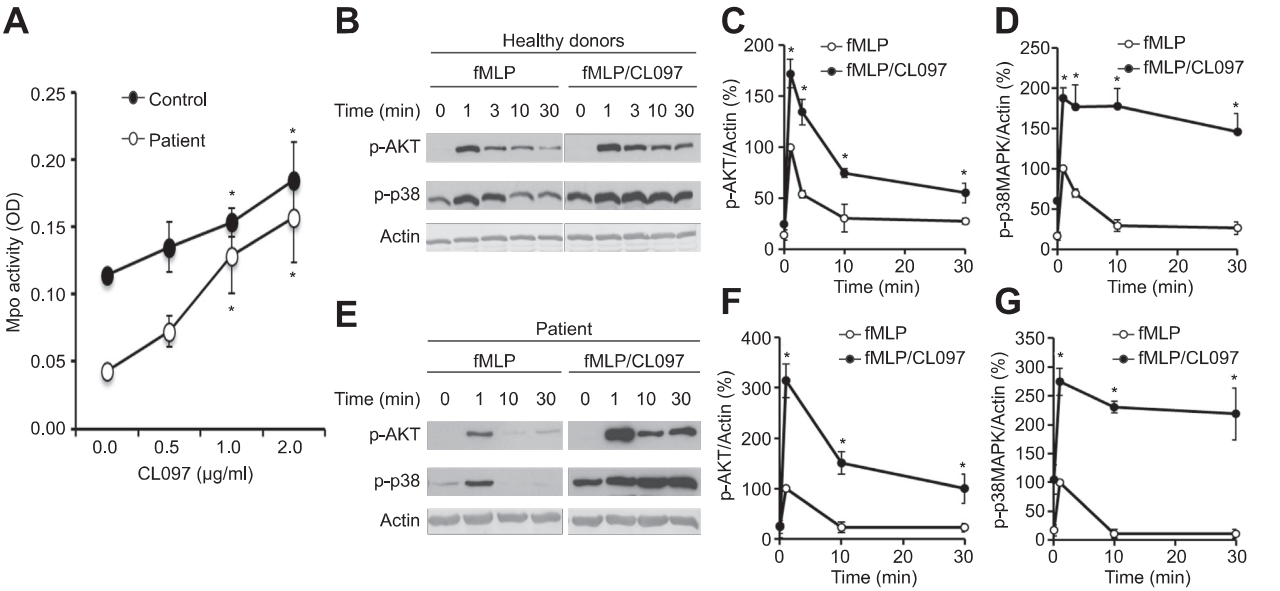

Fig. 5. The TLR7/8 agonist CL097 potentiated fMLP-induced MPO release and AKT/p38-MAPK signaling in neutrophils. (A) Neutrophils from healthy donors (control) and cirrhotic patients were pretreated for 15 min without or with CL097 (0.5–2 µg/ml), then stimulated with fMLP (1 µM) for 2 min. MPO release is expressed as OD × 10^6 cells/3 min (n = 5; *p <0.05 *vs.* control). (B–G) Neutrophils were pretreated in the absence or presence of CL097 (1 µg/ml) for 10 min before stimulation with fMLP (1 µM) for various times. Phosphorylated AKT and p38-MAP kinase are expressed relative to actin and the ratio represented as percentage of maximal control values obtained with fMLP (n = 4–5 experiments); *p <0.05 *vs.* control.

effects on neutrophil host-defence activities in patients with cirrhosis.

Discussion

Decompensated alcoholic cirrhosis is a severe liver disease with about 26% of patients having bacterial infection on hospital admission [17]. Approximately one third of patients die within the first month due to the development of multiorgan failure [44]. Neutrophils, the first cellular line of defence against microorganisms, become altered during cirrhosis progression with impaired phagocytosis, ROS production, exocytosis or chemotaxis [27–34,45]. Deficiencies were more or less marked as a function of the disease severity and occurred in both alcoholic and non-alcoholic cirrhosis [28–30,35] indicating that deficiencies may not result from direct effects of alcohol. The biochemical mechanisms underlying these neutrophil dysfunctions remain largely unknown.

This study provides first evidence that decompensated alcoholic cirrhosis severely impaired the activation of AKT, p38-MAPK and ERK1/2 induced by fMLP in human neutrophils without alteration of overall protein expression of signaling effectors, MPO (Fig. 3A, B) or the fMLP receptor fPR (Fig. 3C, D; Fig. 7). These data indicate that the molecular origins of such deficiencies may take place upstream of the three effectors, and at early steps of fPR signaling cascade (Fig. 7). Amongst potential upstream candidates, inositol-specific phospholipase C (InsPLC) was also impaired in fMLP-stimulated patients' neutrophils [30]. InsPLC generates diacylglycerol (DAG) and inositol trisphosphate known to increase cytosolic calcium concentration [46], a major effector of exocytosis [47]. DAG and calcium activate several PKC, which in turn stimulate AKT and MAP-kinases [48,49]. In neutrophils,

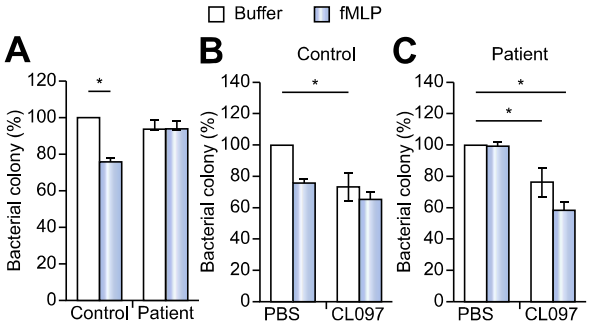

Fig. 6. Impaired bactericidal activity of neutrophils from cirrhotic patients and beneficial effects of the TLR7/8 agonist CL097. (A) Neutrophils from healthy volunteers (control) and cirrhotic patients were incubated with *E. coli* for 5 min, then treated or not (Buffer) with fMLP (1 µM) for 25 min. (B, C): Neutrophils incubated with bacteria were treated without (PBS) and with CL097 (1 µg/ml) for 2 min before stimulation with fMLP for 23 min. Cells were lysed and the number of bacterial colony is expressed as percentage of that of control resting cells (A, n = 6), or% of that of resting cells (PBS) in both cell populations (B, C, n = 6); *p <0.05.

fMLP-induced PLC (PLCβ2) is directly activated by the βγ subunits of a trimeric G-protein (Gi) [50] coupled to fPR, which suggests potential biochemical alterations at the G-protein level. Consistent with this hypothesis, ROS production induced by direct activators of G-proteins such as NaF or ALF4, was also impaired in neutrophils from cirrhotic patients [30]. Other early signaling events impaired by cirrhosis include the PI3-kinase/AKT signaling pathway observed in monocytes [51].

Neutrophil dysfunctions induced by cirrhosis result from intrinsic cellular alterations as they persisted after cell washing [27–30]. However, reversible dysfunctions were reported after

removal of endotoxins from patients' plasma [32]. In this study, patients' but not control plasmas also decreased fMLP-induced MPO exocytosis of neutrophils from healthy volunteers (Supplementary Fig. 5), which reveals the presence of diffusible inhibitors. Lipopolysaccaride (LPS) has been proposed as a candidate for this interference effect [32]. However, its mechanism of action may be indirect. Indeed, low LPS concentrations stimulate exocytosis [52] and prime neutrophils, leading to enhanced superoxide production induced by various agents including fMLP [53], whereas high LPS concentrations stimulate a weak superoxide production [54]. We thus speculate that this neutrophil stimulation and ROS oxidant effect may desensitize early signaling effectors leading to functional deficiencies upon subsequent stimulation of neutrophils. Other agents present in patients' plasma at high concentrations such as ammonia, may also provide deleterious effects, since it decreased neutrophil phagocytic capacity but simulated spontaneous production of ROS [55].

Of the three major signaling effectors studied here, only AKT and p38-MAPK regulate MPO release, as supported by inhibitory effects of their antagonists (Fig. 4A, B), their deficient activation in patients' neutrophils (Fig. 2) and the fact that p38-MAPK is activated downstream of AKT (Supplementary Fig. 2C, D). The involvement of p38-MAPK in exocytosis of primary granules confirms other works with neutrophils from *Src* knock-out mice, which identify p38-MAPK downstream of Src tyrosine kinase [40]. However, the mechanism by which AKT regulates p38-MAPK activity in neutrophils remains not known. Other protein kinases are suspected to regulate the release of MPO, including PKC based on the observation that phorbol myristate acetate (PMA) stimulates MPO release [28]. The PMA-induced degranulation was also impaired in neutrophils from cirrhotic patients [28], as well in our study (data not shown). Unexpectedly, ERK1/2, whose activation was strongly impaired in patients' neutrophils, did not regulate MPO/elastase release (Fig. 4C), in agreement with other studies [40]. However, a role of ERK1/2 in neutrophil defence functions is not excluded, since ERK1/2 regulated approximately 30% of ROS production of healthy neutrophils (Fig. 4C), thus providing the MPO substrate H_2O_2. The deficient ERK1/2 activation observed here in patients' neutrophils may contribute to their defective ROS production we previously reported [8]. In this study, 90% of the patients which were defective for MPO release, also exhibited a strongly deficient superoxide production (30–35% of control values), in agreement with our previous works [8]. The defective NADPH oxidase activity may be related to the deficient activation of AKT and p38-MAPK since both protein kinases phosphorylate a main component of NADPH oxidase, $p47^{phox}$, on respectively S304/S328 [56] and S345 [57]. The impairment of both MPO release and ROS production in patients' neutrophils may explain their deficient neutrophil antibacterial function (Fig. 6), in agreement with previous studies [27–33].

Attempts to improve neutrophil functions in cirrhosis have been undertaken both *in vitro* based on removal of endotoxins in patients plasmas [32] or *in vivo* with patients' treated with G-CSF [45]. Our observations that cirrhosis impaired fPR signaling, prompts us to explore a novel strategy based on activation of endosomal TLR7/8 receptors by CL097, a compound shown to stimulate MAPK-dependent phosphorylation of $p47^{phox}$ and to increase ROS production in healthy neutrophils [41]. Interestingly, CL097 restored fMLP-induced MPO release and bactericidal activity of patients' neutrophils. This original and promising

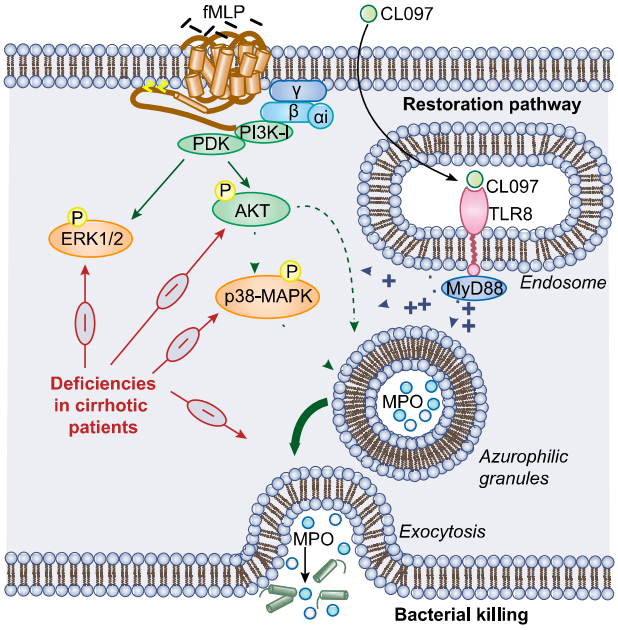

Fig. 7. Proposed model for fMLP-induced MPO release via the AKT/MAPK signaling in neutrophils from healthy donors and patients with alcoholic cirrhosis. Stimulation of neutrophils by the bacterial formylpeptide fMLP via its G-protein-coupled receptor (fPR) induces a rapid activation of AKT and p38-MAPK signalling, which contributes to MPO release. In cirrhotic patients, signaling, MPO release and bactericidal activity were impaired. Stimulation of the intracellular Toll-like receptor TLR8 potentiates AKT/p38-MAPK signaling and restores neutrophil MPO release and antimicrobial function of neutrophils.

CL097 property may be related to its ability to potentiate AKT and p38-MAPK activation and to maintain their activation state, since in the absence of CL097, the activation of these two kinases in patients' neurophils was weak and transient, and not sufficient to trigger physiological defence activities (Fig. 5). Since TLR7 was hardly detectable in human neutrophils, in contrast to TLR8 [42,43] as confirmed here (Supplementary Fig. 4), these data strongly suggest that TLR8 agonists may constitute promising candidates to improve neutrophil defence activities of immunodepressed patients. Furthermore, a number of TRL7/8 agonist immuno-stimulatory activities have been reported on neutrophil production of cytokines [58] and lipid mediators [59] and in other leukocytes including monocytes, antigen-presenting cells and Tregs cells, with a particular protective immunity of new born leukocytes [60]. It is worth noting that CL097 restored the production of G-CSF that was previously inhibited in monocytes pretreated with IFNα, a cytokine that causes neutropenia [61]. CL097 is also used as an adjuvant for the induction of hepatitis B antigen-specific Th1 responses in an immune tolerant state [62]. Finally, translational developments of TRL7/8 agonists with significant ameliorations were obtained with the TLR7 agonist Imiquimod in the treatment of genital and perianal warts caused by certain strains of human papillomvirus and genital herpes (reviewed [60]).

In conclusion, this study provides evidence that advanced alcoholic cirrhosis severely impaired the activation of AKT, p38-MAP-kinases, ERK1/2, and MPO release which was related to

the two deficient former signaling effectors. These deficiencies, together with our previously reported impaired ROS production led to a deficient bactericidal activity of patients' neutrophils, which may contribute to increase patient' susceptibility to bacterial infection. Interestingly, the defective signaling and defence activities of patients' neutrophils can be reversed upon TLR7/8 activation, which raises perspectives to improve host-defence mechanisms in immuno-depressed patients.

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Conflict of interest

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Authors' contributions

A.B., R.M. and A.P. designed experiments; A.B., L.R. and A.P. performed the experiments; E.W. and R.M. selected patients and analysed characteristics. All authors discussed and interpreted the results, and A.P. wrote the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2015.12.005.

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