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ORIGINAL ARTICLE

NADPH oxidase depletion in neutrophils from patients with cirrhosis and restoration via toll-like receptor 7/8 activation

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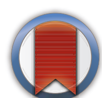
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ABSTRACT

Objective Cirrhosis downregulates phagocyte oxidant production via their antibacterial superoxide-generating system, NADPH oxidase (NOX2) and increases patients' susceptibility to infection and mortality rate. To explore novel biochemical parameters that explain susceptibility to infections, we investigated the expression of NOX2 and partners in neutrophils of patients with severe alcoholic cirrhosis and have provided a novel approach to restore superoxide production capacity in patients' neutrophils and blood.

Design Neutrophils were isolated from patients with decompensated alcoholic cirrhosis. NOX2 activity was assessed after stimulation of purified neutrophils or whole blood with the bacterial-derived peptide fMet-Leu-Phe. The expression of NOX2 and partners was studied by western blot analysis, flow cytometry and reverse transcription-PCR.

Results The impaired superoxide production by patients' neutrophils was associated with a severe deficient expression of the NADPH oxidase catalytic core flavocytochrome-b558 (gp91^{phox}/NOX2 and p22^{phox}), its cytosolic partner p47^{phox} but not p67^{phox}. NOX2 expression decreased rapidly by protein degradation involving elastase released during degranulation of healthy neutrophils stimulated with fMet-Leu-Phe, or highly present in patients' plasma. Interestingly, the deficient superoxide production was reversed by treatment of patients' neutrophils and whole blood with toll-like receptor 7/8 (TLR7/8) agonists. This treatment stimulated a rapid NOX2 transcription and translation through a process involving mammalian target of rapamycin (mTOR) whose expression was also deficient in patients' neutrophils. NOX2 expression was also increased by the TLR4 agonist lipopolysaccharide but with only a modest improvement of reactive oxygen species production.

Conclusion Impairment of neutrophil oxidants production in alcoholic cirrhosis is associated with NOX2 degradation and deficient mTOR-dependent translational machinery. The NOX2 depletion can be reversed via TRL7/8 activation and might be used to restore antimicrobial responses of immunocompromised patients.

INTRODUCTION

Cirrhosis is a major cause of morbidity and mortality worldwide with an estimated 2% of all global death.[1] Patients with cirrhosis exhibit an increased susceptibility towards infections, indicating impairment of host-defence systems.[2–5] A common complication of cirrhosis is the development of bacterial peritonitis, which is a major cause of short-term death.[5 6] Sepsis is also a major cause of mortality of patients with decompensated alcoholic cirrhosis and is associated with multiorgan failure and immune deficiencies.[7 8] Immune paralysis was also reported in non-alcoholic patients and persisted in patients surviving to sepsis.[9] Strategies aiming at restoring host immunity and antimicrobial mechanisms are thus of great interest.

Neutrophils represent the first line of cellular defence against bacterial infections[10] and play an important role in innate immunity and inflammation. Efficient neutrophil antibacterial function requires a massive production of reactive oxygen species (ROS) via their superoxide-generating NADPH oxidase 2 (NOX2), a phenomenon termed respiratory burst (RB). NOX2 activation is dependent on the formation of a multiprotein complex at the plasma membrane which comprises the catalytic core flavocytochrome b558 heterodimer consisting in two associated transmembrane proteins, gp91^{phox} (NOX2) and p22^{phox}, and cytosolic components, p67^{phox}, p47^{phox}, p40^{phox} and Rac1/2.[10–12] Efficient neutrophil superoxide production requires an optimal expression of NOX2 and partners, and signalling events for assembly of the motor system NADPH oxidase. Superoxide instantly dismutates forming hydrogen peroxide, a substrate used by myeloperoxidase to kill bacteria.[13] The biological importance of NOX2 is unfortunately illustrated by an inherited genetic disorder, chronic granulomatous disease (CGD), characterised by mutations in NOX2 or components, a severe deficient RB and increased patients' susceptibility to microbial infections.[10] In patients with alcoholic hepatitis, a defective ROS production was also predictive of infection.[14 15]

Neutrophils exert a dual role in alcohol liver diseases. They contribute to liver injury through oxidative stress induction in animal models[16 17] and patients with alcoholic steatohepatitis.[18] However, during disease progression to cirrhosis, neutrophils become altered with impairment of ROS production, phagocytosis and exocytosis,[14 19–27] which promotes microbial infection. Although the biochemical mechanisms

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Significance of this study

What is already known on this subject?

- Neutrophils represent the first line of cellular defence of the host against microbial infections.
- Patients with advanced alcoholic cirrhosis are more susceptible to infections due to deficient neutrophil antibacterial activities, particularly of the production of reactive oxygen species (ROS) via the superoxide-generating NADPH oxidase 2 (NOX2) system.
- Impairment of ROS production by patients' neutrophils is associated with a deficient signalling by surface receptors for bacterial formylated peptides such as fMet-Leu-Phe (fMLP).

What are the new findings?

- The impaired ROS production by neutrophils from patients with decompensated alcoholic cirrhosis is associated with a severe deficient expression of NADPH oxidase catalytic core flavocytochrome-b558 (gp91^{phox}/p22^{phox}), its cytosolic partner p47^{phox} but not p67^{phox}.
- Downregulation of gp91^{phox} expression occurs via two processes including a gp91^{phox} cleavage by elastase highly present in patients' plasma or released during neutrophil degranulation induced by fMLP, and a defective mammalian target of rapamycin (mTOR)-dependent translational machinery.
- The deficient gp91^{phox} expression and ROS production by patients' neutrophils is reversed by agonists of intracellular TRL7/8 receptors in both patients' neutrophils and whole blood.
- TRL7/8 activation induced transcription of gp91^{phox} and its synthesis through a process involving mTOR whose expression is also deficient in patients' neutrophils.

How might it impact on clinical practice in the foreseeable future?

- The deficient gp91^{phox} expression might be used as a novel factor increasing patients' susceptibility towards infection.
- TRL7/8 agonists might be used to restore gp91^{phox} expression and neutrophil antimicrobial responses of immunodepressed patients.
- Caution should be taken about the clinical use of mTOR antagonists in immunocompromised patients.

underlying these functional defects remain largely unknown, an impaired signalling was observed affecting phospholipase C,[21] mitogen-activated protein kinases (MAPK),[26 27] AKT[27] and a novel signalling axis, mTOR/p38-MAPK/p47$^{phox(ref26)}$ associated with a severe impaired ROS production in neutrophils from patients with alcoholic cirrhosis.[26] Whether impairment of neutrophil oxygen-dependent antimicrobial function is related to altered expression of NADPH oxidase remains unexplored. This aspect is strategically of interest in understanding patients' susceptibility to infections[10] given the important role of mTOR in protein biosynthesis and as therapeutic target.[28]

In this study, we took advantage of neutrophils from patients with decompensated alcoholic cirrhosis to investigate the expression of NADPH oxidase components and mTOR. A severe impaired expression of gp91^{phox}, p22^{phox}, p47^{phox} and mTOR was observed. Gp91^{phox} turned out to be depleted by degradation mediated by elastase in patients' plasma, and by a deficient mTOR-dependent translational machinery. Interestingly, the deficient ROS production was reversed through activation of intracellular TLR7/8 involving de novo gp91^{phox} synthesis via a mTOR-dependent process.

Table 1 Patient characteristics

No. of patients	44
Age, years	59.1 (±1.5)
Female (%)	6 (13.6)
Ascites, n (%)	39 (89)
Hepatocellular carcinoma, n (%)	0 (0)
Encephalopathy, n (%)	7 (16)
Serum albumin, g/L	25.8 (±1.06)
Serum bilirubin, μmol/L	6.47 (±1.13)
White blood cell count, per mm^3	7.865 (±0,70)
International normalised ratio	1.61 (±0.07)
Serum creatinine, mg/dL	1.04 (±0.12)
C reactive protein, mg/L	27.63 (±9.91)
Child-Pugh score	10.1 (±0.3)
Child-Pugh class C, n (%)	30 (68)
MELD score	18.03 (±1.09)
Corticosteroids, n (%)	0 (0)
Treatment with β-blockers, n (%)	10 (23)
Acute alcoholic hepatitis, n (%)	12 (27)
Previous history	
N. of patients	44
Acute decompensation, n (%)	30 (68)
Bacterial infection, n (%)	16 (36)
Acute variceal bleeding, n (%)	14 (32)

Plus minus values are SE of the mean.
Non-inclusion criteria were: evidence of recent GI bleeding, current bacterial infections and treatment with corticosteroids, pentoxifylline and other immunosuppressive drugs in the past 30 days, and presence of hepatocellular carcinoma, other cancer or HIV infection. This study was approved by our Institutional Review Board, and written informed consent was obtained from all patients. Healthy subjects (controls) were obtained through an agreement with the Blood Bank (Établissement Français du Sang, EFS, n° 2015012778) in accordance with national regulations. The age of controls was 38.7±3.1 years (mean±SEM, n=38) with 56% females.
MELD, model of end-stage liver disease.

MATERIALS AND METHODS

See online supplementary material for detailed descriptions

Patients

Patients were hospitalised in the Liver Unit of the Beaujon Hospital (Clichy, France) for assessment before liver transplantation. Inclusion criteria were age over 18 years, biopsy-proven alcoholic cirrhosis, Child-Pugh class B or C cirrhosis. Patients were negative for HBV and HCV infections. At the time of blood collection, alcohol consumption was stopped for at least 1 week. The clinical characteristics of patients and previous history are provided in table 1.

Neutrophil isolation and respiratory burst

Neutrophils from venous blood collected in EDTA and acid-citrate dextrose were purified by standard methods using Ficoll-Hypaque and Dextran.[26 27] RB was studied using the

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superoxide-specific cytochrome c reduction assay and by chemiluminescence.[26]

Expression of NADPH oxidase components

NOX2 and partners were studied by various approaches including western blot analysis, flow cytometry and reverse transcription-PCR analyses.

Statistical analysis

Unless otherwise stated, data represent means±SEM. Significant differences were identified using the Student's paired t-test, or one-way analysis of variance followed by the Bonferroni post hoc test by use of GraphPad Prism V.6.0 (GraphPad San Diego, California, USA), and designated by *p<0.05; **p<0.01 and ***p<0.001.

RESULTS

The impaired NOX2 activity of neutrophils from patients with alcoholic cirrhosis is associated with a deficient expression of gp91^{phox}, p22^{phox}, p47^{phox} but not p67^{phox}

We previously reported that neutrophils from patients with decompensated alcoholic cirrhosis exhibited a depressed superoxide production,[26] as confirmed here (figure 1A) with a new cohort of patients (table 1). To further examine the molecular basis of this deficiency, the amount of NADPH oxidase components was determined by western blot analysis. In comparison to neutrophils from healthy volunteers, patients' neutrophils exhibited a severe impaired expression of gp91^{phox}, p22^{phox}, p47^{phox} but not p67^{phox} (Figure 1B-D). These pathological deficiencies occurred without apparent alteration of transcriptional activity since the mRNA levels remain unchanged (figure 1E).

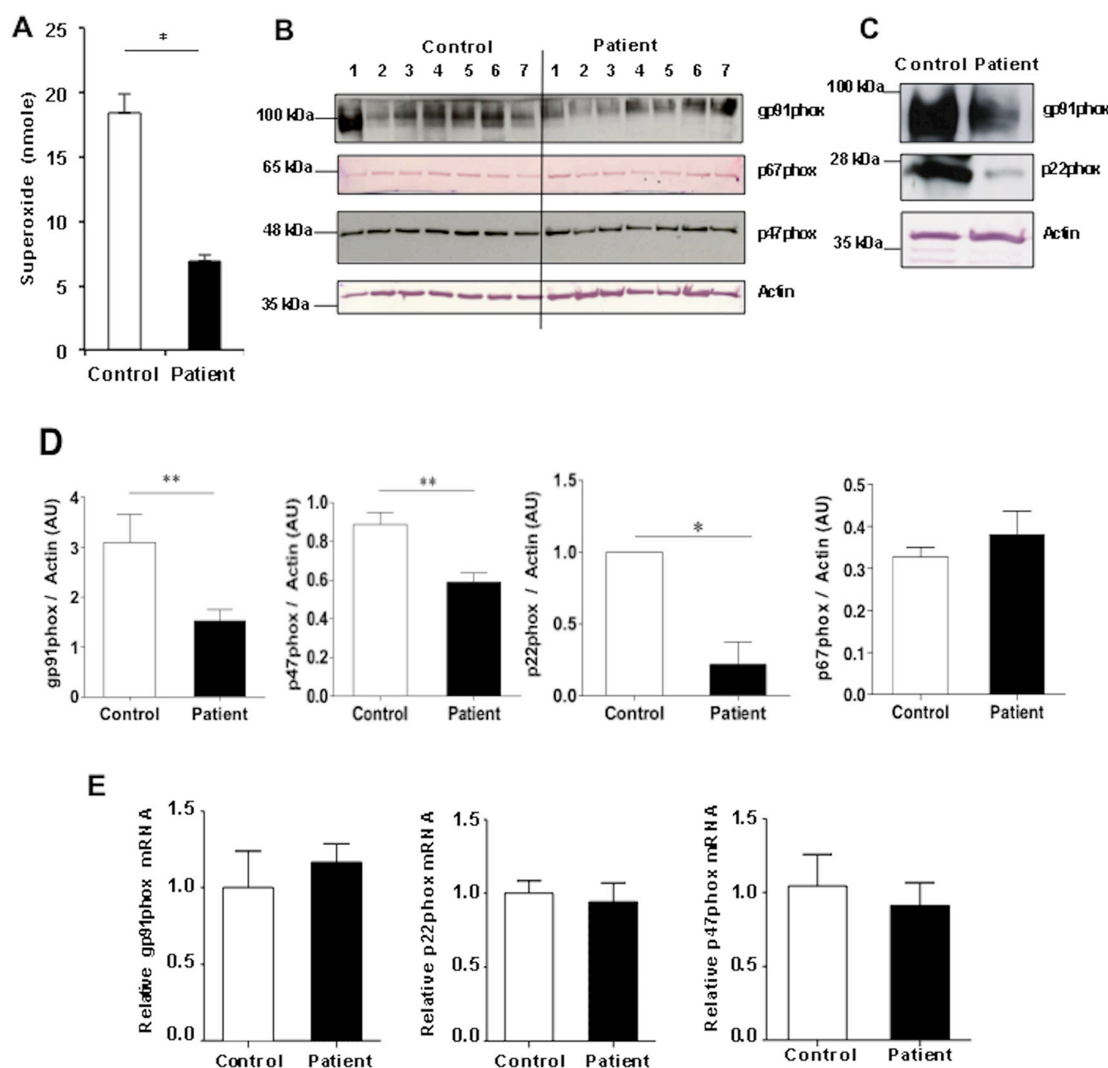

Figure 1 Neutrophils from patients with alcoholic cirrhosis exhibit a deficient expression of gp91^{phox}, p22^{phox}, p47^{phox} but not p67^{phox}, and superoxide production. (A) Total superoxide production by control and patients' neutrophils induced by formyl-Met-Leu-Phe (1 µM) and expressed in nmol/10^6 cells (n=44). (B, D) Western blot analysis of gp91^{phox}, p67^{phox}, p47^{phox} and actin of healthy (control) and patients' neutrophils and quantification relative to actin level (n=7). (C) A representative western blot analysis of p22^{phox} in control and patients' neutrophils and quantification relative to actin level (n=6). (E) RNA expression of gp91^{phox}, p22^{phox} and p47^{phox} relative to GAPDH (n=6). Mean values±SEM; *p<0.05 and **p<0.01 vs control were determined by the Bonferroni test.

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NOX2 is rapidly depleted following neutrophil stimulation by the bacterial-derived synthetic peptide formyl-Met-Leu-Phe

To explore the mechanisms of NOX2 depletion, we examined whether the expression of NADPH oxidase components is subjected to temporal modulation on cell stimulation, as this is the case for many transmembrane proteins. To this end, healthy neutrophils were stimulated with formyl-Met-Leu-Phe (fMLP), a potent RB activator (figure 1A). This treatment time-dependently decreased NOX2 expression up to 60% within 30–45 min (figure 2A,B). Concomitantly, the expression of immune-reactive

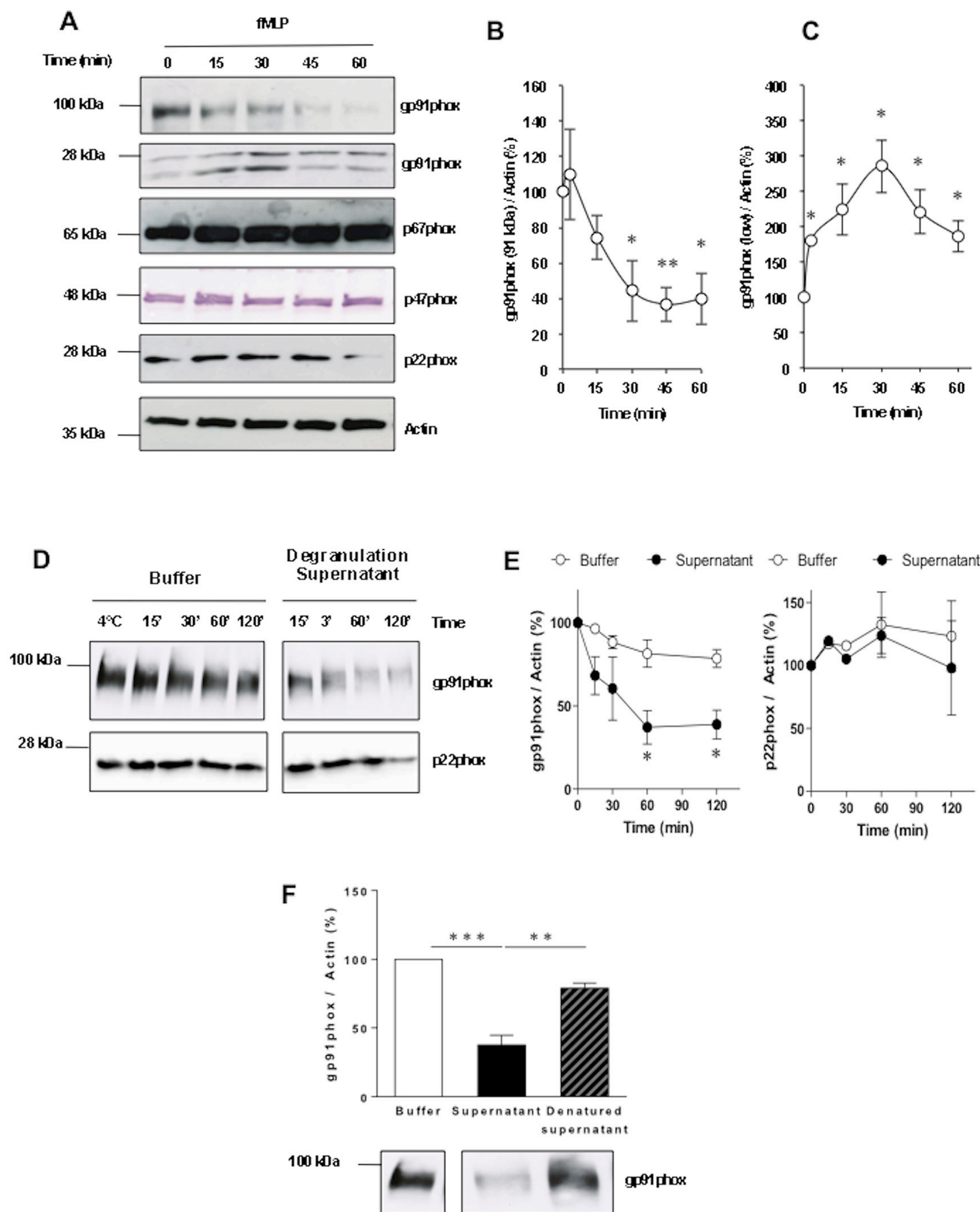

Figure 2 Stimulation of neutrophils by formyl-Met-Leu-Phe (fMLP) induces degradation of gp91^{phox} and p22^{phox} but not p67^{phox} and p47^{phox}. (A) A representative western blot analysis of gp91^{phox}, p67^{phox}, p47^{phox}, p22^{phox} and actin in healthy neutrophils, after stimulation with fMLP (1 μM) for various periods. (B) Amounts of gp91^{phox} and (C) its small fragment (22–28 kDa) expressed relative to actin and presented as percentage of control values taken at time zero (n=5). The quantification of p22^{phox}, p67^{phox} and p47^{phox} is provided in online supplementary figure 1. (D, E) Expression of gp91^{phox} and p22^{phox} in membrane fractions prepared from healthy neutrophils and treated in vitro without (Buffer) or with a cell-free degranulation supernatant obtained from fMLP-stimulated neutrophils. (E) Quantification of gp91^{phox} and p22^{phox} relative to actin level (n=4). (F) Western blot analysis of gp91^{phox} in a membrane fraction of healthy neutrophils, after in vitro treatment without (Buffer) and with denatured and non-denatured degranulation supernatants (n=3). *p<0.05, **p<0.01 and ***p<0.001 vs control values, were calculated by the Student's t-test (B and D) and by the Bonferroni test (D–F).

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gp91^{phox} fragments of approximately 22–26 kDa increased (figure 2C), consistent with a proteolytic degradation of gp91^{phox}. The expression of p22^{phox} also decreased but at later times and lower extent, while p47^{phox} and p67^{phox} remained unaltered (see online supplementary figure 1).

The observation that transmembrane but not cytosolic components of the NADPH oxidase were degraded strongly suggested that degradation may be initiated extracellularly, likely by exocytosis of granular proteases. To explore this hypothesis, cell-free degranulation supernatants were prepared from stimulated neutrophils, then incubated with membrane fractions obtained from resting neutrophils, which provides the advantage to minimise possible degradation of gp91^{phox} by cytosolic proteases. This treatment confirmed that gp91^{phox} is rapidly degraded (figure 2D,E), while p22^{phox} was not significantly altered. A role of proteases in the depletion of gp91^{phox} was further suggested by the observation that heat-denatured degranulation supernatants were less active (figure 2F).

A major role of elastase in the degradation of NOX2

To identify proteases potentially involved in NOX2 degradation, in silico analysis was performed with the gp91^{phox} sequence using the MEROPS database[29] and computer tools to predict proteases that can cleave selected substrates.[30]Among five candidates detected (see online supplementary table 1), elastase was the unique protease from neutrophils. To examine its potential contribution to gp91^{phox} degradation, a first approach was used by treating healthy neutrophils with purified elastase at 0.1 and 0.5U, which provided a proteolytic activity similar to that obtained with supernatants from degranulating neutrophils.[27] This treatment also led to gp91^{phox} cleavage, as shown by the concomitant increase of immune-reactive gp91^{phox} fragments (figure 3A) and the dramatic decreased surface expression of gp91^{phox} (see online supplementary figure 2). A degradation of p22^{phox} was observed with 0.5 unit of elastase (figure 3B). Elastase released during physiological stimulation of neutrophils by fMLP also contributed to gp91^{phox} and p22^{phox} degradation, as supported by the preventing effect of the neutrophil elastase inhibitor (NEI, MeOSuc-AAPV-CMK) (figure 3B). To examine whether this gp91^{phox} degradation was initiated extracellularly, a cell-free degranulation supernatant was first pretreated with NEI to block elastase activity, then incubated with intact neutrophils. This treatment prevented the depletion of gp91^{phox} (figure 3C), supporting a role of degranulating elastase in the gp91^{phox} cleavage.

To further examine the gp91^{phox} external portions potentially targeted by elastase, we took profit of the gp91^{phox} antibody 7D5,[31] which recognises external gp91^{phox} loop 2 and 3, and the Biacore surface plasmon resonance technology to monitor real-time neutrophil interaction with the 7D5 antibody-coated sensor chip. Untreated neutrophils (control) showed a time-dependent specific binding to the 7D5 antibody (figure 3D). This binding was reduced in time and intensity with neutrophils pretreated with elastase and washed. A reduced interaction was also detected with neutrophils pretreated with a supernatant from degranulated neutrophils. These data are consistent with alterations of external epitopes and/or expression of gp91^{phox} at the surface of neutrophils.

Plasma from patients with alcoholic cirrhosis inhibits NOX2 expression and superoxide production by neutrophils

Elastase has been proposed as a marker of severity in alcohol-induced chronic liver damage.[32] However, the relevance of elastase in regulating neutrophil NOX2 activity is unknown. Patients' plasma contained a greater amount of elastase in comparison to plasma of healthy subjects (see online supplementary figure 3), in agreement with previous works.[32] To examine whether elastase regulates gp91^{phox} expression/activity, healthy neutrophils were treated without (control) or with plasma from patients or healthy volunteers and washed before measurement of gp91^{phox} expression by FACS (see online supplementary figures 4,5). Patients' plasma but not control decreased gp91^{phox} expression (figure 3E) and superoxide production (figure 3F). Moreover, impairment of ROS production was prevented by NEI (figure 3E,F), strongly suggesting a damaging effect of patients' plasma elastase.

NOX2 impairment was reversed on TLR7/8 activation in patients' neutrophils and whole blood

We previously reported that the defective ROS production by neutrophils from patients with cirrhosis was associated with a deficient phosphorylation of p47^{phox} by MAP kinases.[26] MAP kinases are also important signalling effectors of receptors of the innate immunity such as TLR.[33] We previously showed that activation of intracellular TRL7/8 by CL097 greatly potentiated AKT and MAP kinase signalling and reversed the deficient degranulation and bactericidal activity of patients' neutrophils.[27] Based on these observations, we examined whether NOX2 expression/activity was improved. Treatment of patients' neutrophils with CL097 for 30 min increased gp91^{phox} transcripts (figure 4A) and expression (figure 4B). The expression of p22^{phox} also increased but not that of p67^{phox} and p47^{phox}. CL097 by itself stimulated a weak production of superoxide (figure 4C) and strongly potentiated the fMLP-induced superoxide production in patients' neutrophils to a level closed to that of healthy neutrophils (figure 4C). Interestingly, the beneficial effect of CL097 was also observed in whole patients' blood (figure 4D), and was reproduced with another TLR7/8 agonist, R848 (figure 4E) but not with its inactive analogue CL264 (data not shown) supporting specific effects of CL097 and R848. Treatment of whole blood with CL097 also increased gp91^{phox} transcripts, and expression determined in permeabilised neutrophils by FACS analyses (see online supplementary figure 6A–C). The increased gp91^{phox} expression was associated with de novo protein synthesis as suggested by the cycloheximide inhibitory effect (see online supplementary figure 6C).

A role of mTOR in the restoration of NOX2 expression induced by CL097

mTOR is a key signalling effector that regulates vital cellular functions and synthesis of various proteins.[28] We recently showed that mTOR is rapidly activated in fMLP-stimulated neutrophils and upregulates superoxide production through activation of the p38-MAP kinase/p47^{phox} axis.[26] This original signalling pathway was deficient in neutrophils from patients with cirrhosis.[26] We show here with the new cohort of patients that mTOR expression was also impaired (figure 5A). To examine whether mTOR regulates NOX2 expression, healthy neutrophils were treated with rapamycin to block mTORC1 activation.[26] Rapamycin prevented the CL097-induced increased NOX2 expression (figure 5B), consistent with a major contribution of mTORC1. Furthermore, basal and CL097-mediated NOX2 expression may mobilise the translational machinery as supported by the cycloheximide inhibitory effects, which further suggests that NOX2 synthesis is constitutive (figure 5C). Finally, both rapamycin and cycloheximide reduced CL097-mediated potentiation of superoxide production by fMLP-stimulated neutrophils (figure 5D),

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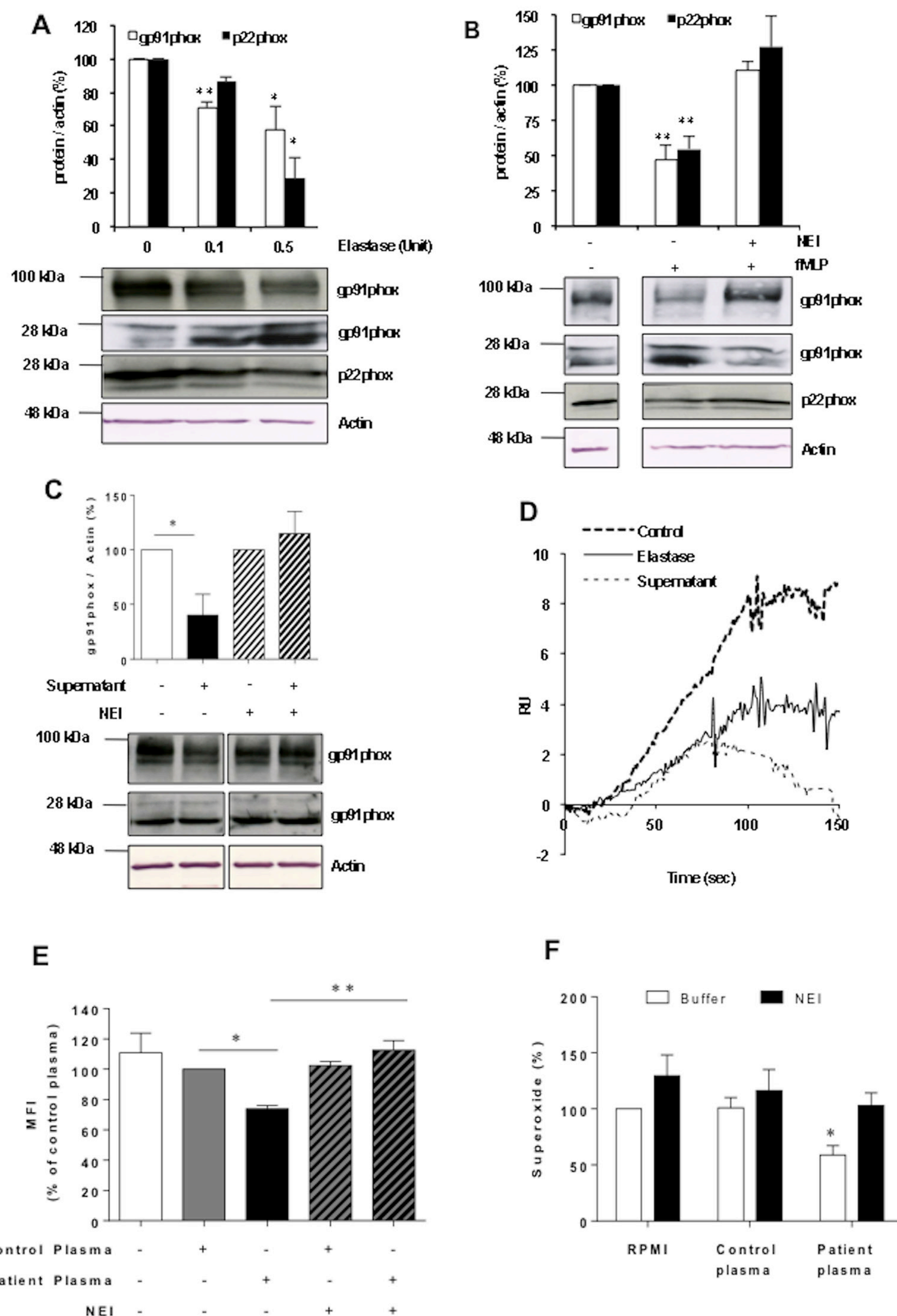

Figure 3 Degradation of gp91^{phox} and p22^{phox} in healthy neutrophils treated with elastase or patients' plasma. (A) Western blot analysis of gp91^{phox} and p22^{phox} in healthy neutrophils treated without (control) or with elastase for 1 hour. (B) Western blot analysis of gp91^{phox} and p22^{phox} in healthy neutrophils treated without (control) or with the elastase inhibitor (NEI, 100 μM, 15 min) before stimulation with 1 μM fMet-Leu-Phe (fMLP) for 45 min (n=4). (C) Western blot analysis and quantification of gp91^{phox} in healthy neutrophils pretreated without (control) or with 100 μM NEI for 15 min before stimulation with a cell-free degranulation supernatant for 45 min (n=4). (D) Sensorgrams of the time course interaction of neutrophils with the anti-gp91^{phox} antibody (7D5)-coated CM3 sensorchip, analysed with the Biacore X100. Data are expressed as resonance unit (RU) and are representative of three independent experiments with healthy neutrophils treated with buffer (control), elastase (0.5 U) or a cell-free degranulation supernatant. (E) FACscan analysis of gp91^{phox} expression at the surface of healthy neutrophils pretreated for 12 hours at 37°C with RPMI (control) or with plasma of healthy volunteers or patients with cirrhosis, either without or with 100 μM NEI (n=5). (F) Production of superoxide by phorbol myristate acetate (PMA)-stimulated healthy neutrophils which were pretreated for 12 hours in the absence (RPMI) or presence of plasma from healthy volunteers (control) and patients with cirrhosis, either with or without 100 μM NEI. Superoxide production is expressed as percentage of control values (n=5). Mean±SEM, *p<0.05 and **p<0.01 vs control were calculated by the Bonferroni test.

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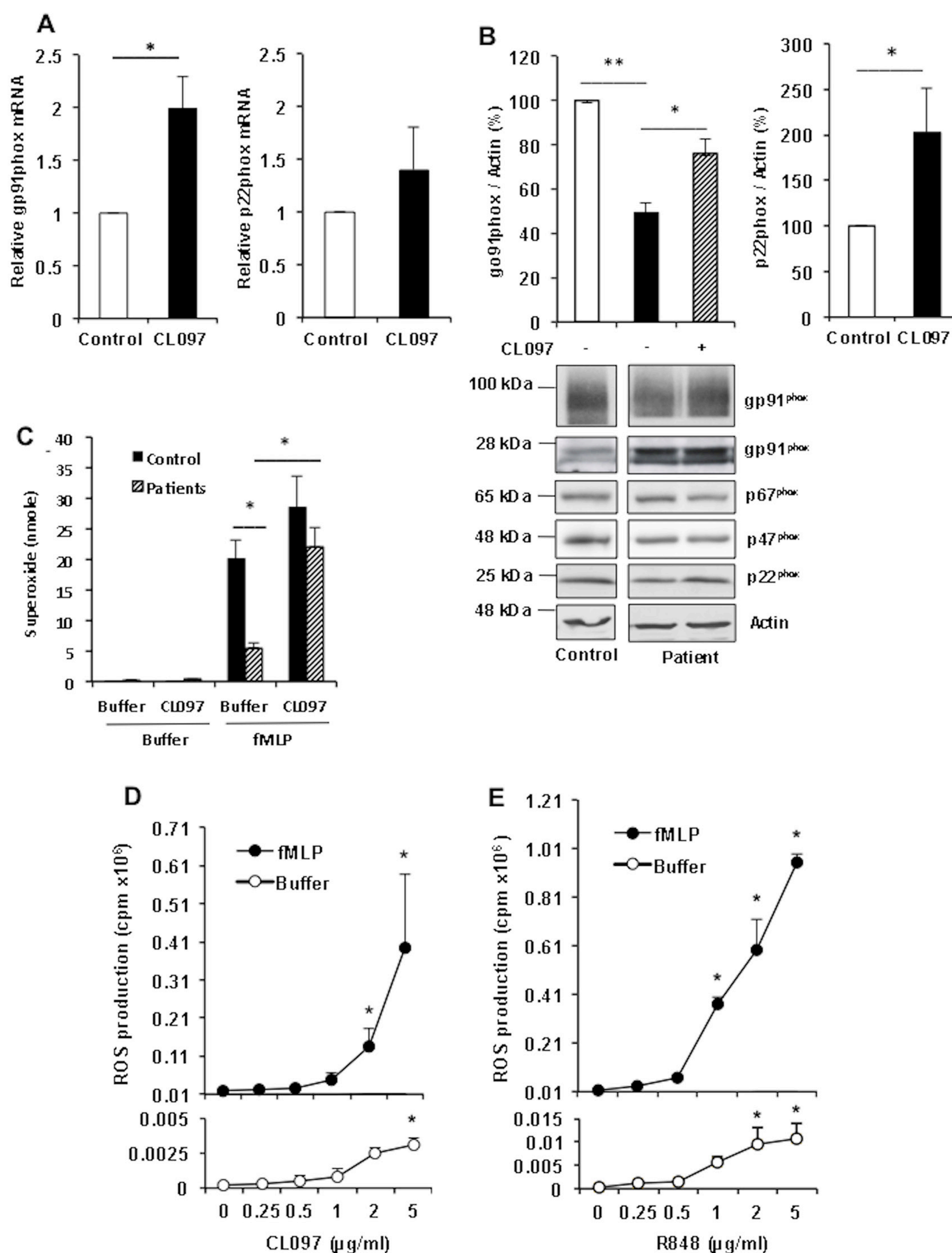

Figure 4 The toll-like receptor 7/8 agonist CL097 restores NADPH oxidase (NOX2) expression and activity in patients' neutrophils. (A) Expression of gp91^{phox} and p22^{phox} mRNA relative to GAPDH (n=6). (B) Western blot analysis and quantification of gp91^{phox} and partners in patients' neutrophils treated without (buffer) or with CL097 (2.5 µg/mL) for 30 min at 37°C (n=5–6 in each group). (C) fMet-Leu-Phe (fMLP)-induced superoxide production by healthy and patients' neutrophils which were pretreated with CL097 (2.5 µg/mL) for 15 min. Mean values±SEM are expressed in nmol/10^6 cells (n=8). (D and E) Reactive oxygen species (ROS) production measured by chemiluminescence (CL) in patients' whole blood treated first without (control) or with CL097 or R848 for 15 min, then with 1 µM fMLP. Results represent the peak of CL response expressed in cpm (n=6). Mean values, *p<0.05 and **p<0.01 *vs* control, were calculated by the Bonferroni test.

suggesting a contribution of newly synthesised NOX2 in ROS production.

As an attempt to examine the relevance of NOX2 cleavage and the mTOR-mediated reduced NOX2 synthesis in the defect of ROS production, this latter response was studied in the presence of NEI, the elastase antagonist. NEI did not reverse the deficient fMLP-induced ROS production, although a weak trend for improvement was noted (see online supplementary figure 7). Furthermore, NEI did not alter the CL097-mediated restoration of ROS production. Thus, elastase released by exocytosis

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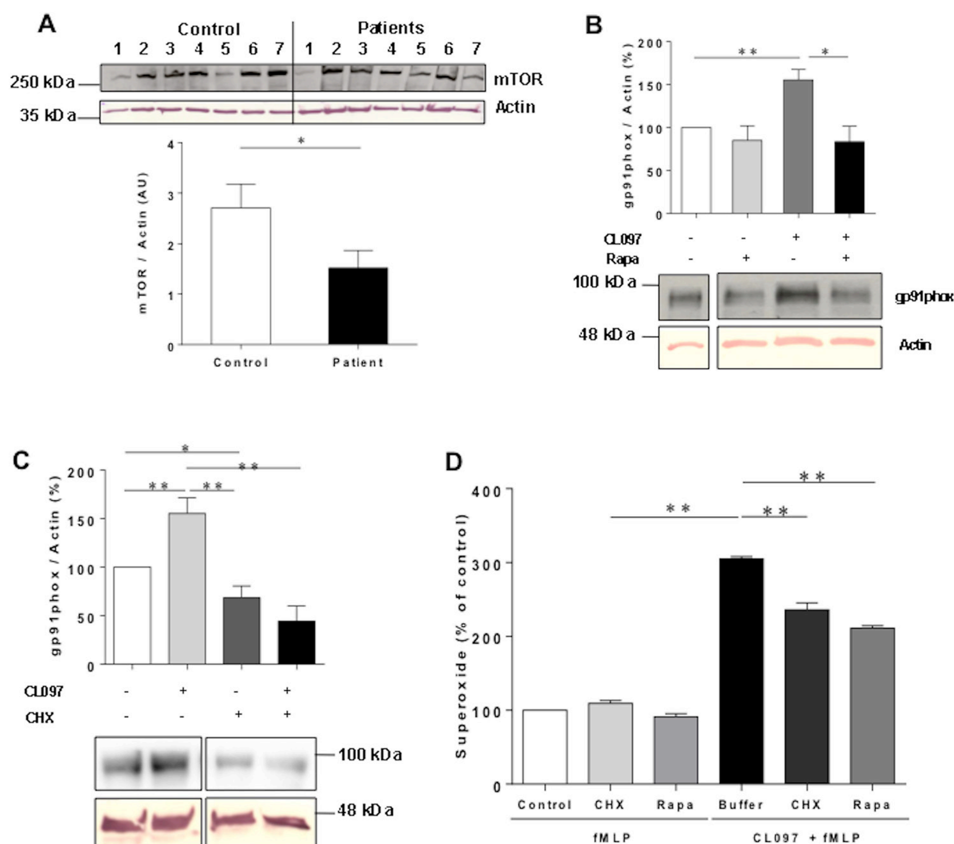

Figure 5 Toll-like receptor 7/8 (TRL7/8) activation induces NADPH oxidase (NOX2) synthesis through mammalian target of rapamycin (mTOR)-dependent process. (A) A representative western blot analysis and quantification of mTOR and actin in neutrophils from healthy donors (control) and patients with cirrhosis (n=14 in each group). (B and C) Western blot analysis and quantification of gp91^{phox} in healthy neutrophils which were pretreated respectively with the mTOR antagonist rapamycin (10 nM) or 100 μM cycloheximide (CHX) for 15 min before stimulation with CL097 (2.5 μg/mL) for 1 hour at 37°C. (D) Total superoxide production of healthy neutrophils pretreated with 10 nM rapamycin or 100 μM CHX for 10 min, then with 2.5 μg/mL CL097 for 10 min before stimulation with 1 μM fMLP (n=5). Means(±SEM), *p<0.05 and **p<0.01 vs control, were calculated by the Student's t-test.

concomitantly to ROS production did not induce early detrimental degradation of NOX2. Consistent with this observation, significant NOX2 cleavage was observed after 20–30 min of neutrophil stimulation with fMLP (figure 2B). Interestingly, in CL097-treated cells, NOX2 was not degraded (see online supplementary figure 7), an effect associated with a rapid rise of basal NOX2 level due to de novo synthesis (figure 5C). These data suggest that dysregulation of mTOR-dependent NOX2 synthesis in patients' neutrophils may predominantly contribute to the impaired NOX2 expression and ROS production.

Lipopolysaccharide induces expression of NOX2 and partners in patients' neutrophils with a modest improvement of oxidative capacity

To examine whether other TLR agonists upregulate NOX2 expression, we studied the effects of lipopolysaccharide (LPS), a TLR4 agonist found to be increased in alcohol liver diseases. LPS greatly enhanced the expression of NOX2 which was detectable after 3 hours of cell treatment (figure 6A,B) but not after 1 hour (data not shown). The expression of its partners, p22^{phox}, p67^{phox} and p47^{phox} also increased but at lower extent. However, LPS neither triggered ROS production nor potentiated the fMLP-induced ROS production by patients neutrophils, unlike CL097 (figure 6C,D). By contrast, with healthy neutrophils, LPS both triggered a weak ROS production and potentiated fMLP-induced ROS production with a lower efficiency than CL097. Interestingly, with patients' neutrophils treated for 3 hours with LPS, basal ROS production increased weakly and the fMLP-induced ROS production was moderately potentiated (figure 6E,F).

DISCUSSION

This study provides insights into mechanisms of patients' susceptibility to infection and suggests a novel approach to reverse the deficient antimicrobial activity of patients' neutrophils. Our data provide first evidence that neutrophils from patients with advanced alcoholic cirrhosis exhibit a severe deficient expression of the flavocytochrome b558 (gp91^{phox}/NOX2 and p22^{phox}), p47^{phox} but not p67^{phox}, thus providing explanations to their impaired ROS production induced by various stimuli[21 26] and deficient bactericidal activity.[27] Impairment of gp91^{phox} involved two processes; a proteolytic degradation via elastase present in patients' plasma or released during neutrophil exocytosis, and a defective gp91^{phox} translational machinery, both leading to gp91^{phox} depletion. The deficient NOX2 expression was reversed via TRL7/8 activation, which restored ROS production, as illustrated in figure 7.

A degradation of gp91^{phox} was previously reported in other cell types through the proteasome via its negative regulator of ROS[34]

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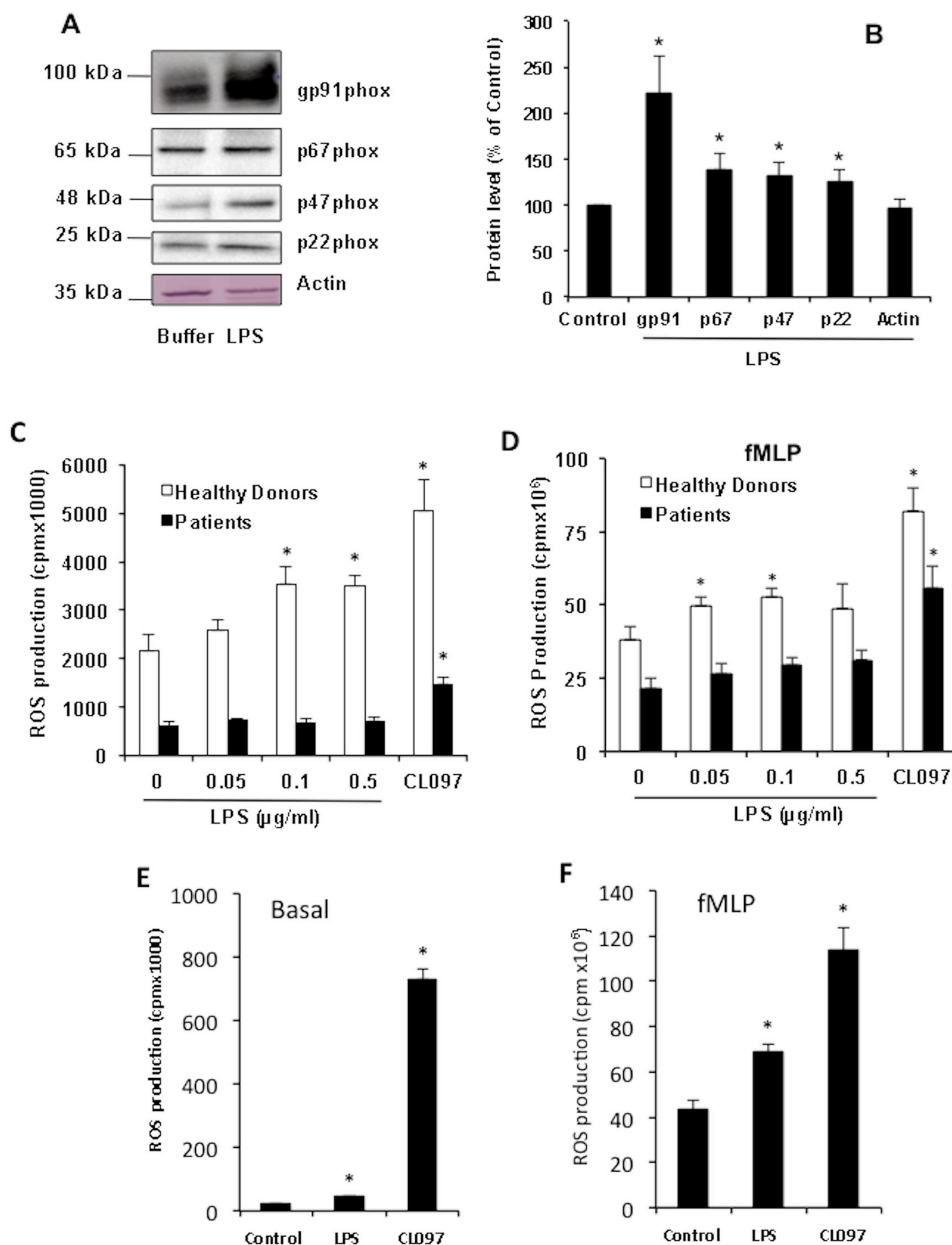

Figure 6 Lipopolysaccharide (LPS) stimulates the expression of NADPH oxidase (NOX2) and partners, with a moderate improvement of reactive oxygen species (ROS) production in patients' neutrophils. (A, B) A representative western blot analysis and quantification of NOX2 and partners in patients' neutrophils treated in the absence and presence of LPS (100 ng/mL) for 3 hours (n=5). (B and D) Neutrophils from patients or healthy volunteers were incubated in the absence (control) or presence of indicated amount of LPS or CL097 (2.5 µg/mL) and their production of ROS was assessed by luminescence. Data are expressed as percentage of respective control values (n=6–8). Means(±SEM), *p<0.05 vs untreated control, were calculated by the Student's t-test.

or inhibition of Hsp90.[35] Although a role of proteasome cannot be excluded in the impaired expression of gp91^{phox}, p22^{phox} or p47^{phox} in patients' neutrophils, the gp91^{phox} depletion induced here in neutrophils by patients' plasma or fMLP, was initiated extracellularly mainly by elastase. The cleavage sites of gp91^{phox} by proteases remain unknown. In silico analysis of gp91^{phox} sequence identifies three potential elastase sites (rq/LRI (V/t) n/rw)[29] located around V90, Ile160 and Thr269. Ile160, located in the second gp91^{phox} external loop, is part of the 7D5 epitope, which explains the altered 7D5 binding to elastase-pretreated neutrophils. Moreover, the cleavage site around V90 and T270 would generate a small gp91^{phox} fragment (20–22 kDa) similar

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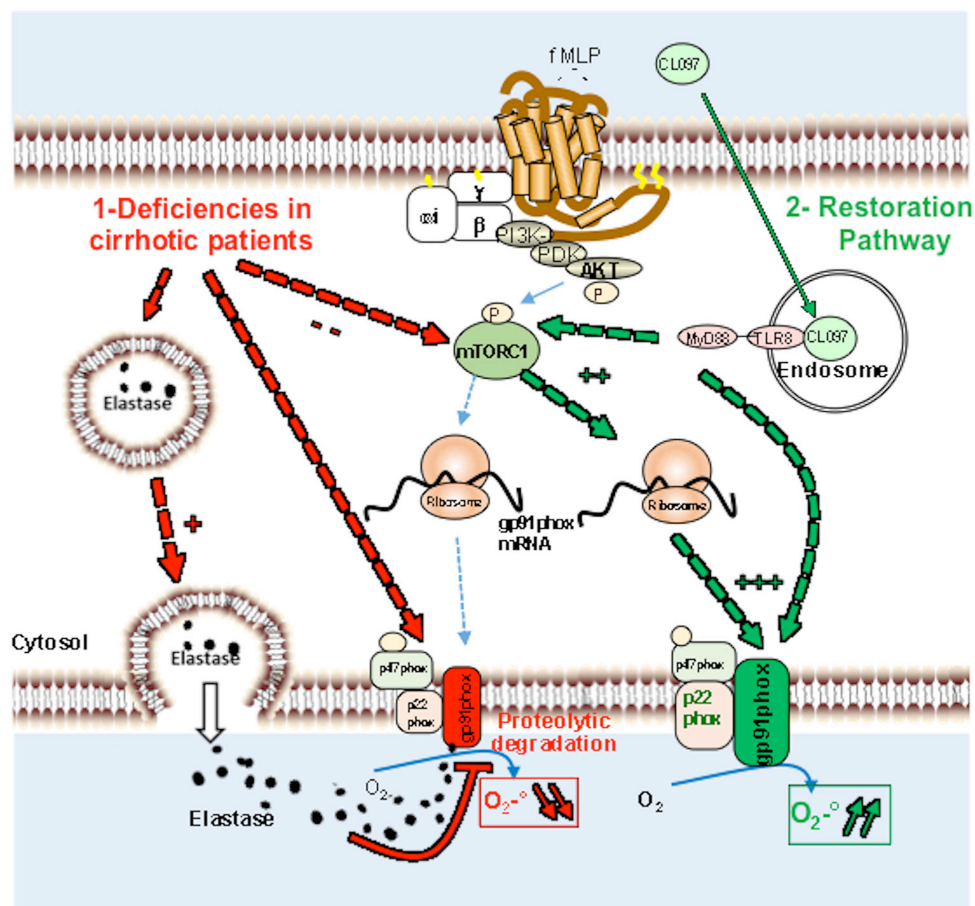

Figure 7 Proposed model for the deficient activation of NADPH oxidase (NOX2) in neutrophils from patients with cirrhosis and its restoration via toll-like receptor 7/8 (LR7/8) activation. In normal individuals, stimulation of neutrophils by the bacterial fMet-Leu-Phe (fMLP) via its G-protein-coupled receptor (fPR) induces a rapid activation of the AKT/mTOR/p38-MAPK signalling axis. These kinases contribute to phosphorylation of p47^{phox}, a major component of the antibacterial superoxide-generating transmembrane flavocytochrome b558 composed of gp91^{phox} (NOX2) and p22^{phox}. Following neutrophil stimulation, NOX2 is rapidly and transiently activated, and then cleaved by extracellular elastase released during degranulation, thus limiting local inflammation. In neutrophils from patients with alcoholic cirrhosis, superoxide production is severely impaired due to a deficient expression of gp91^{phox}, p22^{phox}, p47^{phox} and mTOR, thus increasing patients' susceptibility to infection. Activation of intracellular TLR7/8 byCL097 in patients' neutrophils and blood ex vivo reverses the deficient expression of gp91^{phox}, and restores its antibacterial oxidant capacity.

to that previously observed after treatment of cytochrome b558 or neutrophil membranes with the staphylococcal V8 protease,[36] and observed here in stimulated neutrophils. In vivo evidence of a gp91^{phox} cleavage was reported with sera of patients with cirrhosis which contained materials detected with the 7D5 antibody,[37] presumably corresponding to small gp91^{phox} fragments.

Like gp91^{phox}, p22^{phox} was severely impaired in patients with cirrhosis, but was not degraded by elastase but weakly degraded in intact neutrophils stimulated with fMLP. These data are consistent with the notion that gp91^{phox} may initially undergo proteolysis, which would render p22^{phox} unstable[36 38] and degradable by intracellular proteases. Like p22^{phox}, p47^{phox} was deficient in patients' neutrophils but resisted to degradation in fMLP-stimulated neutrophils despite its high phosphorylation state.[12] Moreover, p47^{phox} impairment in patients' neutrophils occurred without apparent alteration of transcriptional activity (figure 1E), suggesting a possible defective translational machinery. Supports for a defective protein synthesis come from the impaired expression of mTOR and that mTOR regulates gp91^{phox} synthesis (figure 5D). A deficient gp91^{phox} expression and ROS production, but not p47^{phox}, was also observed in monocytes from patients with acute alcoholic hepatitis (AAH) and was predictive of infection.[15] These impairments were associated with impaired STAT-1 signalling and accumulation of its negative regulator, SOCS-1, as a consequence of alcohol consumption.[15] Among our patients, 27% also developed AAH and their deficient neutrophil ROS production tended to increase non-significantly (8.2±1.8, n=12 vs 11.6±1.3 nmol 02-°, n=32, p<0.17), suggesting an aggravating alcohol effect. However, in both groups, these impairments did not correlate with the disease severity (data not shown) unlike in other studies.[7] This difference may be related to the fact that most of patients (68%) are severely affected and by the low number of patients (table 1). Furthermore, these impairments were not due to a direct effect of alcohol per se since patients were abstinent, but may presumably involve persistent biochemical alterations previously initiated by alcohol. Among deleterious alcohol effects, transphosphatidylation is a major mechanism by which primary alcohols disrupt neutrophil oxidative burst. Transphosphatidylation is a reaction specific of phospholipase D, which generates inactive phosphatidylethanol in the presence of primary alcohols, instead of phosphatidic acid (PA). Since PA directly activates mTOR,[39] transphosphatidylation may impair both synthesis of proteins among which gp91^{phox}, and also NOX2 activation via

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the impairment of p47*phox* phosphorylation.[26] Moreover, alcohol metabolism generates ROS known to induce non-specific deleterious effects among which the inactivation of elastase antagonists,[40] which would explain the striking high elastase effect in patients' plasma (figure 3E, F).

To improve defence activities of patients' neutrophils, we recently used the TLR7/8 agonist CL097, which successfully reversed the deficient bactericidal activity and myeloperoxidase exocytosis of patients' neutrophils.[27] Consistent with this beneficial property, CL097 also reversed the deficient ROS production in both patients' neutrophils and whole blood (figure 4C–E). This restoration involved de novo synthesis of the flavocytochrome b558 (NOX2/gp91*phox* and p22*phox*) (figure 5B,D) but not its cytosolic partners p47*phox* and p67*phox* (figure 4A), suggesting that these latter components may be expressed in excess relative to NOX2. The synthesis of NOX2 was dependent on mTORC1 activation based on inhibitory effects of its specific inhibitor, rapamycin (figure 5B) and the presence of 5'-terminal oligopyrimidine (TOP) motifs, or related TOP-like motifs in gp91*phox* transcript (see online supplementary figure 6) previously shown to confer mTORC1-dependent translation control.[41] In patients' neutrophils, mTOR impairment may contribute to decrease gp91*phox* expression in addition to the elastase-dependent degradation process described here. However, the former process may be more relevant to explain the defect in ROS production. Indeed, NOX2 synthesis is constitutive and rapidly activatable (figure 5C), which in healthy neutrophils may compensate for NOX2 degradation by proteases released physiologically. In patients' neutrophils, the deficient protein synthesis machinery probably no longer makes it possible to compensate for the loss of NOX2 due to elastase highly present in patients' plasma or released by bacterial formylated peptides. The involvement of mTOR in NOX2 expression further raises concerns about potential aggravating effects of mTOR antagonists[26] used in clinics particularly with immune-compromised patients.

The mechanisms by which TLR7/8 activation up-regulates gp91*phox* synthesis in patients' neutrophils is not known. In addition to the major contribution of mTOR (figure 5B,D), we previously showed a prolonged activation of AKT,[27] the upstream mTOR activator and of p38-MAPK which was activated downstream mTOR.[26] While a potential contribution of these two effectors in regulating translation of gp91*phox* remains to consider, our results indicate that the deficient gp91*phox* expression by patients' neutrophils is reversible, which opens novel insights into treatments that aim to improve defence activities of immune-compromised patients. In addition to restoring neutrophil antimicrobial activities, R848 and CL097 also improve the production of cytokines[42 43] and lipid mediator biosynthesis.[44] Both agonists have been used in clinical trials for their immunostimulatory activity[45] or as adjuvants for the treatment of viral infections.[46 47] Thus, TLR7/8 agonists may constitute promising candidates to improve host-defence mechanisms of immunosuppressed patients, in addition to other strategies based on blockade of PD-1 and TIM3,[25] interferon[15] or the G-CSF therapy to improve granulopoiesis, cytokine production[48] and survival of patients with acute-on-chronic failure.[49]

In this study, the TLR4 agonist LPS also restored the expression of NOX2 but more slowly than TLR7/8 agonists, with a modest improvement of ROS production, in comparison to CL097. This discrepancy is possibly related to the phenomenon of homologous or heterologous desensitisation of membrane receptors of patients' neutrophils due to their exposure to blood LPS, as suggested by the inability of LPS and fMLP to stimulate ROS production in patients' neutrophils, unlike healthy neutrophils (figure 6C,D). This phenomenon may also explain the fact that LPS induces neutrophil dysfunction associated with organ failure and mortality.[50] Thus, strategies based on the activation of intracellular TLR7/8 would be advantageous, compared with those targeting surface receptors. Supporting this concept, the TLR7/8 agonist R848 reduced mortality in mice with sepsis,[51] unlike LPS which provides adverse effects.[50]

In conclusion, this study provides evidence that major components of the neutrophil antibacterial superoxide-generating system, NADPH oxidase, become deficient in patients with alcoholic cirrhosis, particularly its catalytic core flavocytochrome b558 (gp91*phox*, p22*phox*) and p47*phox*, which may be considered as novel factors of patients' susceptibility towards infection. Depletion of gp91*phox* involves two processes; by degradation mediated by elastase highly present in patients' plasma or released during neutrophil stimulation, and a deficient mTOR-dependent translational machinery. The deficient gp91*phox* expression and activity can be reversed via activation of TRL7/8 in patients' neutrophils and whole blood, which opens perspectives to restore neutrophil antimicrobial activity in immune-compromised patients.

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Contributors LR, RM, PER and AP designed the experiments. LR, AB, OH, JEB, PL and AP performed the experiments and analysed the data. EW and RM selected patients and provided blood samples. All authors discussed the results and commented on the manuscript. LR and AP wrote the manuscript.

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Competing interests None declared.

Patient consent Medical and biochemical informations of the study about patients are sufficiently anonymised and do not allow identification of patients.

Ethics approval The Inserm ethic commitee ref 2015012778.

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NADPH oxidase depletion in neutrophils from patients with cirrhosis and restoration via toll-like receptor 7/8 activation

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