Supplementary material to :

Impaired AKT/MAP-Kinase signaling, myeloperoxidase release and bactericidal activity of neutrophils from patients with alcoholic cirrhosis

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Materials and Methods

Reagents

Antibodies used i.e. phospho-ERK1/2(Thr202/Tyr204), phospho-p38MAP-Kinases (Thr180/Tyr182), phospho-AKT(S473) were from Cell Signaling (Leiden, The Netherlands), anti-ERK1 and peroxidase-conjugated antibodies were from Santa Cruz (Heidelberg, German), anti-actin was from Millipore (Saint Quentin-en-Yvelines). The myeloperoxydase substrate ortho-dianisidine dichloride was from Sigma Aldrich (Saint Louis, MO, USA). Myeloperoxidase fluorescent substrate was from Biovision. The protease inhibitor cocktail was from Roche and the Restore western-blot stripping buffer was from Pierce. SDS-PAGE, western-blotting reagents were from BioRad (Marnes la Coquette, France) and Iblot nitrocellulose stacks from Life Technologies (Saint Aubin, France). CL097 was from InvivoGen (San Diego, USA). All other reagents were from Sigma Aldrich.

Purification of neutrophils

Peripheral blood from healthy volunteers or cirrhotic patients was collected in EDTA-containing tubes. Neutrophils were purified by a first step sedimentation of whole blood with 1% Dextran in saline followed by centrifugation on a cushion of Ficoll-Hypaque (400g-30 min) to remove mononuclear leukocytes. Contaminating red cells were lysed under hypotonic conditions (30sec) [1] and the purified neutrophils (95-98%) were washed twice and suspended in Hank's balanced salt solution (HBSS) at pH 7.4 and containing Mg++ and Ca++.

Degranulation assay

Neutrophils ($2x10^6$/400µl HBSS) were incubated in Eppendorf tubes at 37°C in the absence or presence of 0.5µg/ml cytochalasin B (CB) for 5 min prior stimulation with fMLP (1µM) for 2min. To stop degranulation, the tube bottom was incubated in ice-cold methanol (-80°C) for 5 sec and

stored in ice. Cells were pelleted at 4°C and supernatants were used immediately for enzyme assays. In some experiments, cell pellets were homogenized (10^7 cells/ml) in PBS containing a cocktail of antiproteases for total MPO activity, or in *Laemmli* lysis buffer [8] for western-blot analyses.

MPO activity was measured by a spectrophotometric method using its specific colorimetric substrate, O-dianisidine [2] adapted for 96-well microplate. Briefly, degranulation supernatants (50µl) were incubated with 200 µM of O-dianisidine in a final volume of 250µl HBSS and the reaction was triggered with 4.4 mM H_2O_2. MPO activity was continuously recorded at optical density (OD) of 460 nm using a thermostated microplate-reader spectrophotometer (THERMOmax™, Molecular device, California). MPO activity was calculated over a 3min period and expressed as DO/10^6 cells or as percentage of control values. In some experiments, MPO activity was measured using the fluorescent MPO peroxidation substrate (Biovision) according to manufacturer protocol.

Bactericidal activity

Neutrophils ($5x10^5$ cells) were incubated at 37°C in 200 µl HBSS containing $5x10^4$ viable opsonized E coli and 1% serum [1]. The PMN/bacterial suspension was then stimulatedwith 1µM fMLPfor 25 min under agitation (200 rpm) and tubes were stored in ice.Cells were lysed with 0.1% Triton X100 for 10min at room temperature, and aliquots of the bacterial suspensions were plated on LB agar and incubated overnight to quantify the number of colony forming unit.

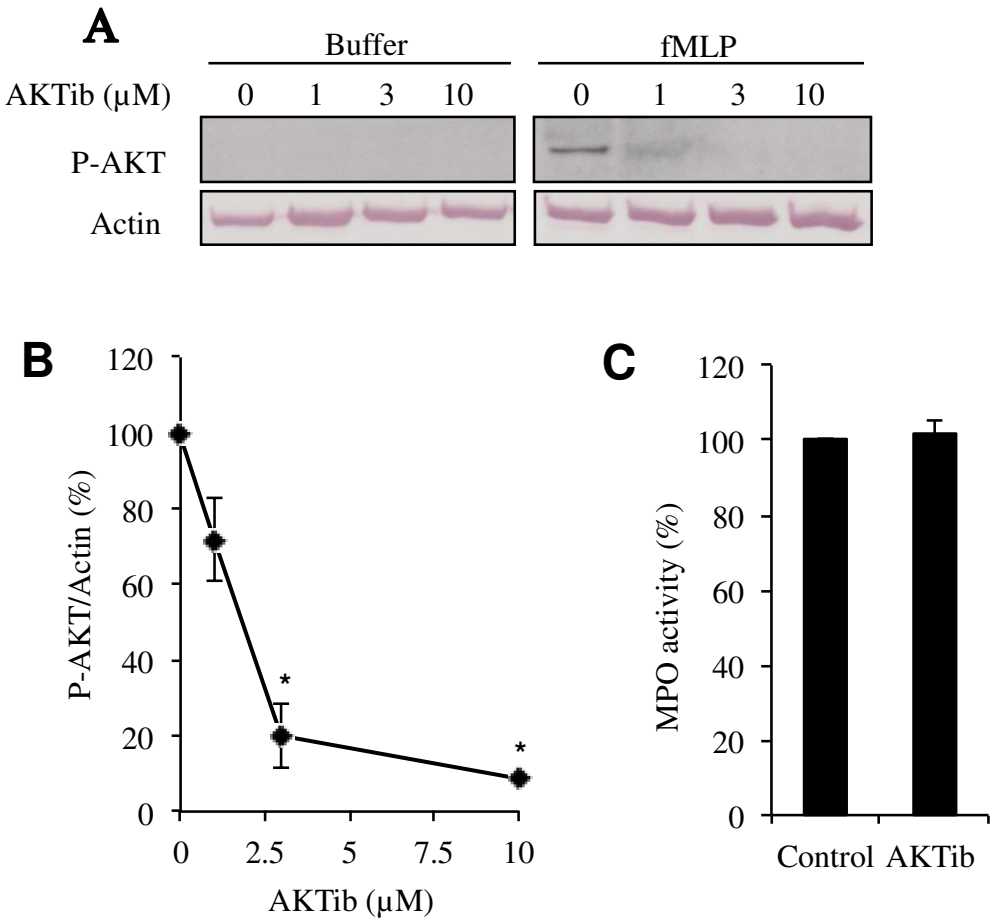
Western-blot analysis

Cells were lysed in 50 mM Tris-HCl, pH 6.8 containing 2.5 mM orthovanadate, 2.5 mM EDTA, 5 M urea, 1 mM DTT, a cocktail of antiproteases (Complete™, Roche) and 1X *Laemmli* sample

buffer, and subjected to western-blot experiments using standard protocols [1]. Enhanced chemiluminescence (ECL)was used for detection of HPO-conjugated secondary antibody. In some experiments, membranes were washed and subsequently reprobed with appropriate antibodies of interest, and detection with alkaline phosphatase-conjugated secondary antibodies was performed using nitroblue tetrazolium (NBT) as substrate. Protein phosphorylation was quantified with the NIH Image J 1.62 software and expressed as a percentage of actin expressionor of the protein of interest.

Quantification of TLR7/8 mRNA

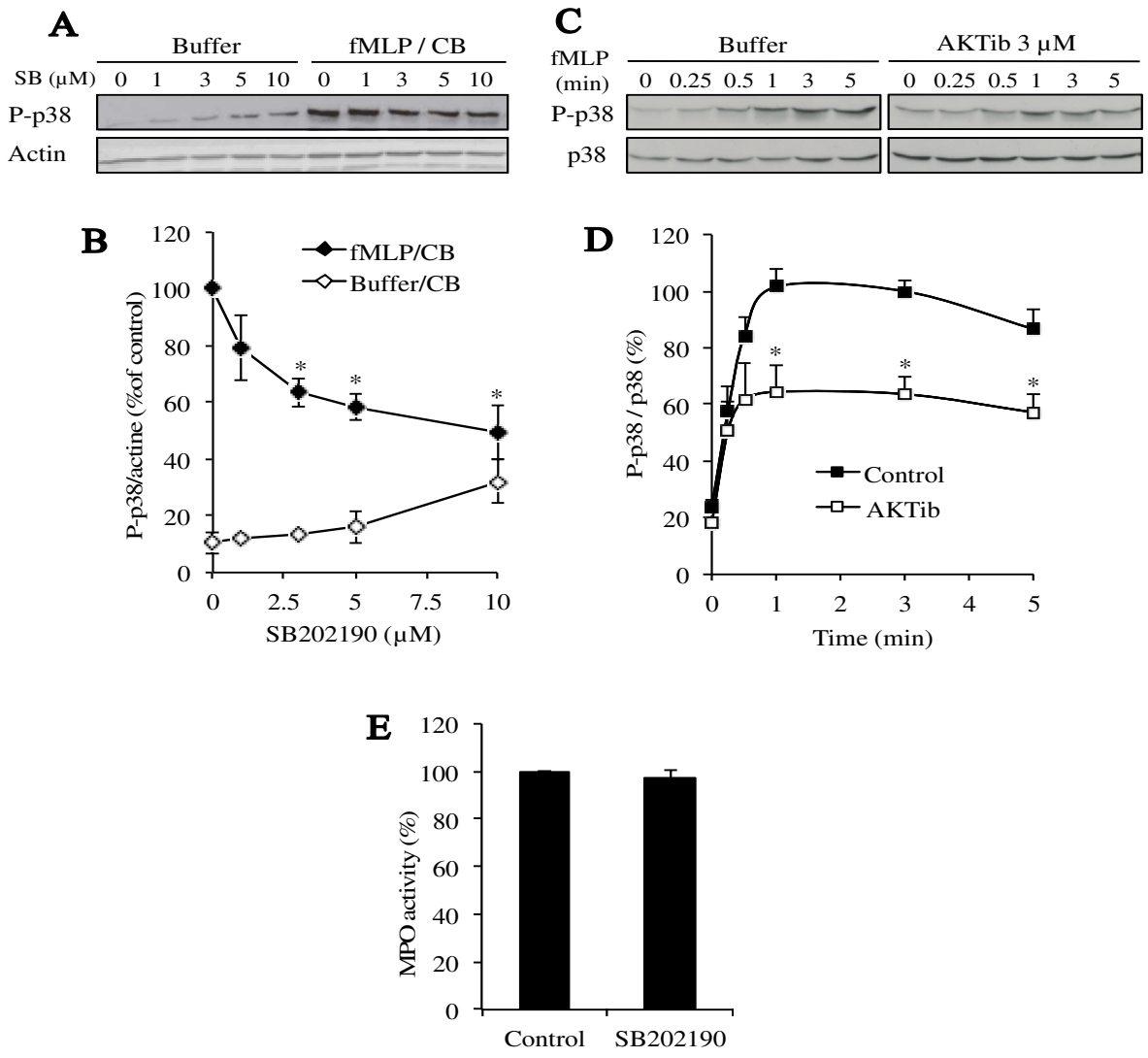
Total RNA was extracted from 5 millions neutrophils in 1 ml Trizol according to the manufacturer protocol and stored at -80°C until use. A quantitative real-time PCR (qPCR) was used to quantify relative messenger RNA(mRNA) of the genes of interest. Complementary cDNA was synthesized from 1µg mRNA using 10 mM OligodT and 200 units reverse transcriptase (Invitrogen, Cergy-Pontoise, France). The following sequence of DNA-oligos primers for qPCR were used : TLR8 Forward : GAGCCGAGACAAAAACGTTC, TLR8 Reverse : TGTCGATGATGGCCAATCC ;TRL7 Forward : TTACCTGGATGGAAACCAGCTAC, TLR7 Reverse : TCAAGGCTGAGAAGCTGTAAGCTA. All real-time PCR reactions were performed using the RocheLightCycler® 480 device and the CliniSciences Kapa Sybr Fast qPCR kit according to the manufacturer's protocol. Samples were run in duplicate, and the melting curve and melting peak were controlled for each primer pair. Relative expression levels for each gene were calculated using the $2^{-\Delta ct}$ method, with normalization to GAPDH. Results are expressed relative to the TLR8 mRNA of the control group of healthy volunteers (n= 12 in each group).



Supplementary Fig. 1.AKTib1/2 inhibited fMLP-induced phosphorylation of AKT(S473) in neutrophilswithout interfering with MPO catalytic activity.

(A, B) Neutrophils from healthy donors were pretreated without (control) or with AKTib1/2 (1-10µM; 15min). The fMLP (1µM, 1min) induced phosphorylation of AKT(S473) (A) was quantified and expressed as percentage of values obtained with control fMLP (n=4; *$p<0.05$ versus control. (C) MPO activity was quantified in cell-free lysates obtained from sonicated resting neutrophils. Lysates were pretreated in vitro in the absence (Control) or presence of AKTib1/2 (10µM) for 15 min. MPO activity is expressed as percentage of control values (n=3, 100% = 0.4 OD/3min).

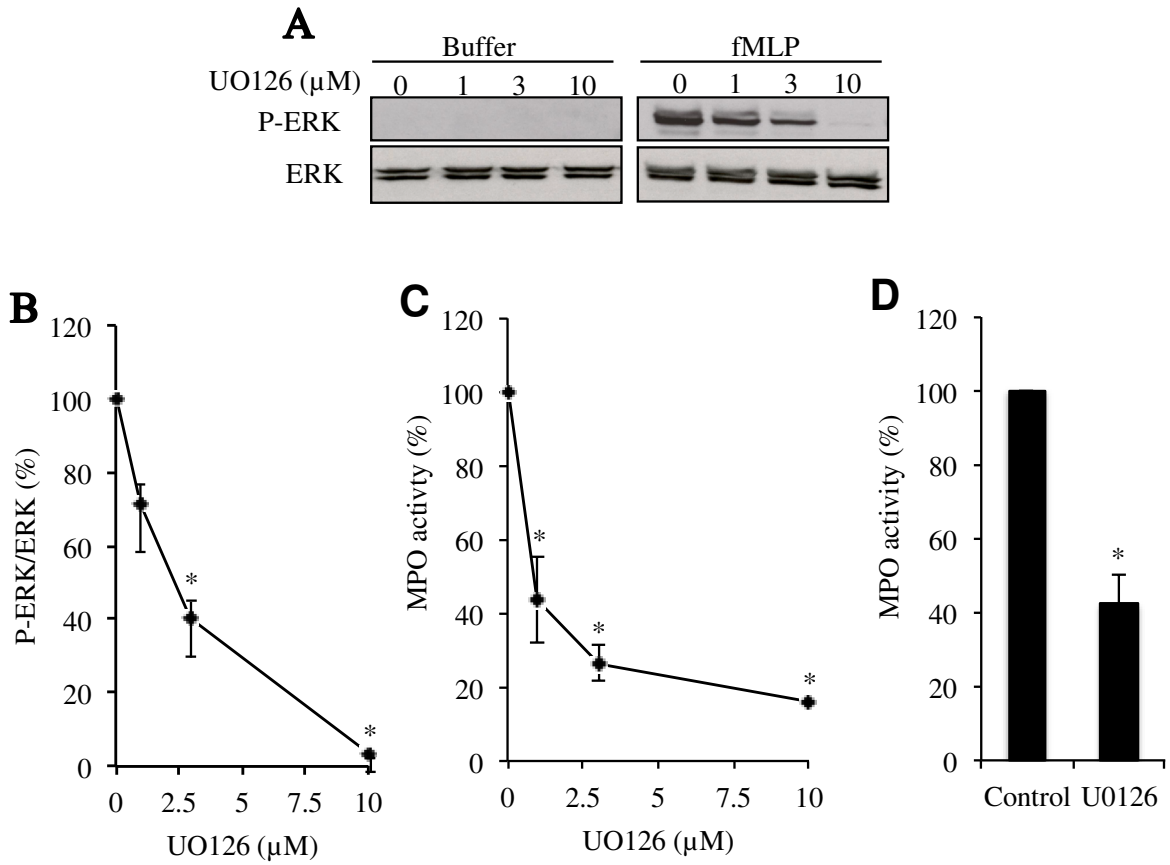
Supplementary Fig.2



Supplementary Fig. 2. <u>SB202190 and AKTib1/2 inhibited</u> fMLP-induced phosphorylation of p38-MAPK in neutrophils, <u>without interfering with MPO catalyticactivity.</u>

Neutrophils from healthy volunteers were pretreated without (control) or with the p38-MAPK inhibitor SB202190 (1-10µM) or AKTib1/2 (3µM) for 15 min, then stimulated with 1µM fMLP for 1min (A) or for various times (C). Phosphorylation of p38-MAPK was quantified (B, D) and expressed as percentage of values obtained with fMLP control (B) (n=6; *$p<0.05$ versus control). (E) <u>MPO activity was quantified in cell-free lysates obtained from sonicated resting neutrophils. Lysates were treated in vitro in the absence (Control) or presence of SB202190(10µM) for 15 min, and MPO activity is expressed as percentage of control values (n=3, 100% = 0.4 OD/3min).</u>

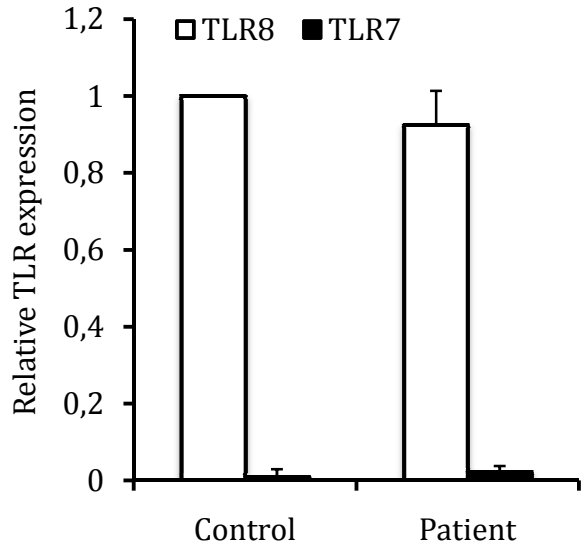
Supplementary Fig.3



Supplementary Fig. 3.The MEK1/2 antagonist U0126 inhibitedfMLP-induced phosphorylation of ERK1/2 in neutrophils and interfered with the MPO catalytic activity.

Healthy neutrophils were pretreated without (control) or with the ERK1/2 inhibitor UO126 (1-10µM; 25min) then stimulated by fMLP (1µM, 1min). (A and B): ERK phosphorylation analyzed by western-blot and quantification (n=5, *$p<0.05$* versus control).(C)Fluorometric quantification of MPO activity in the degranulating supernatant of neutrophils pretreated or not (Control) with U0126 for 15 min (n=3).(D) MPO activity in cell-free lysates of resting neutrophils, which was pretreated in the absence (Control) or presence of U0126 (10 µM) for 15 min. MPO activity is expressed as percentage of control values (n=3, 100% = 0.4 OD/3min).

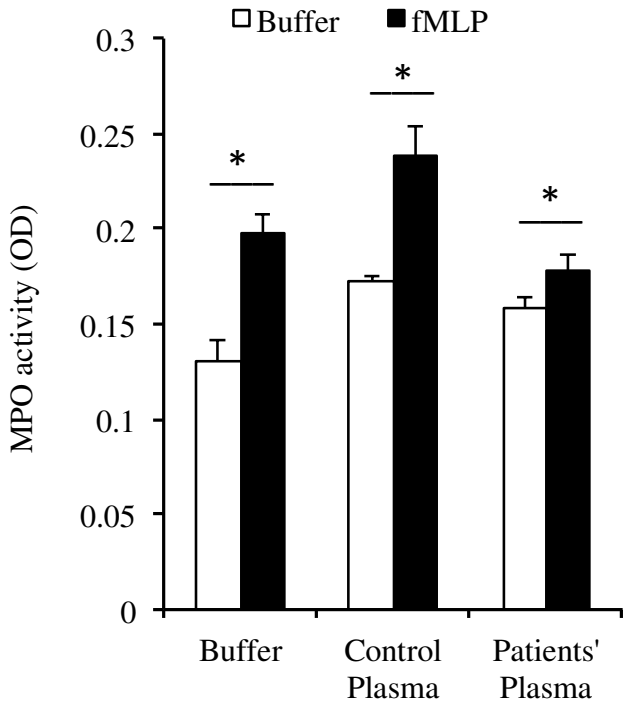
Supplementary Fig. 4



Supplementary Fig.4. RNA expression of TLR7/8 in neutrophils from control and cirrhotic patients.

RNA from $5x10^6$ neutrophils from control and cirrhotic patients was extracted and RT-qPCR ofTLR7 and TLR8 was performed in duplicate. The mean (± SEM) of 12 different healthy donors and patients is shown and expressed using the TRL8 values of healthy donors as reference.

Supplementary Fig. 5



Supplementary Fig. 5. Plasmas from cirrhotic patients impaired fMLP-induced MPO release of neutrophils from healthy donors.

Neutrophils from healthy donors were incubated at 37°C in RPMI in a 5% CO_2humidified atmosphere for 12H in the absence (Buffer) or presence of 25% plasma from cirrhotic patients or healthy donors (n=6 in each group). Cells were washed, resuspended in HBSS containing calcium and magnesium. The viability of collected cells assessed by the Blue Trypan exclusion test was approximately 92-95%. Degranulation of neutrophils ($0.5x10^6$ cells/400µL) was induced by fMLP (1µM) for 2 min and MPO activity was quantified in the extracellular medium and expressed as OD per 3min/10^6 cells (n=6, *$p < 0.05$).

References

[1] Rolas L, Makhezer N, Hadjoudj S, El-Benna J, Djerdjouri B, Elkrief L, et al. Inhibition of mammalian target of rapamycin aggravates the respiratory burst defect of neutrophils from decompensated patients with cirrhosis. Hepatology 2013;57:1163-1171.

[2] Nowak D. Hydrogen peroxide release from human polymorphonuclear leukocytes measured with horseradish peroxidase and o-dianisidine. Effect of various stimulators and cytochalasin B. Biomed Biochim Acta. 1990; 49:353-62