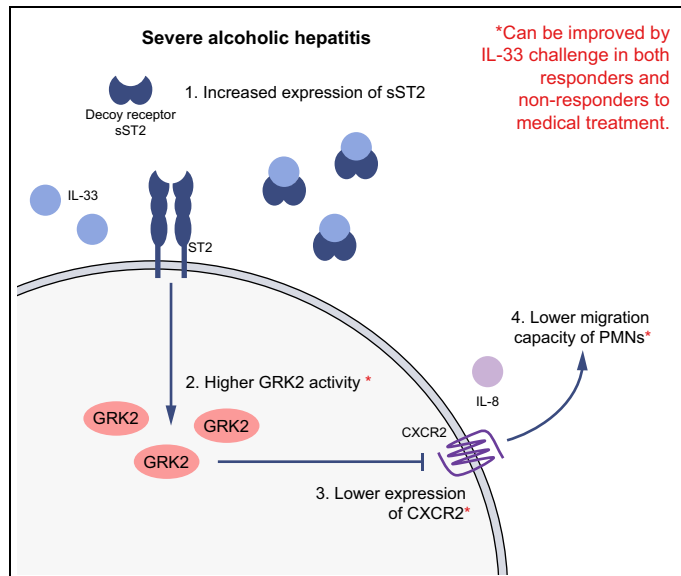# IL-33/ST2 pathway regulates neutrophil migration and predicts outcome in patients with severe alcoholic hepatitis

Graphical abstract

Authors

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Lay summary

The neutrophils of patients with severe alcoholic hepatitis are associated with a defect in the IL-33/ST2 pathway. This defect is associated with lower migration capacities in neutrophils and a higher probability of getting infected. Administration of IL-33 to the neutrophils at least partly restores this defect and may be effective at reducing the risk of infection in patients with severe alcoholic hepatitis.

Highlights

- The IL-33/ST2 pathway is defective in the neutrophils of patients with severe alcoholic hepatitis.
- This defect is associated with a higher risk of developing infection.
- Dosage of the decoy receptor sST2 helps identify patients at risk of death and/or infection.
- The defect in IL-33/ST2 in neutrophils is responsible for lower migration capacities.
- Treating neutrophils with recombinant IL-33 restores, at least in part, their migration capacities.

IL-33/ST2 pathway regulates neutrophil migration and predicts outcome in patients with severe alcoholic hepatitis

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Background & Aims: Severe alcoholic hepatitis (SAH) is associated with a high risk of infection. The IL-33/ST2 pathway is involved in sepsis control but data regarding its role in alcohol-related liver disease (ALD) are lacking. We aimed to characterize the role of IL-33/ST2 in the polymorphonuclear neutrophils (PMNs) of patients with ALD and SAH.
Methods: Serum and circulating neutrophils were collected from patients with SAH, alcoholic cirrhosis and healthy controls. We quantified IL-33/ST2 pathway activity and CXCR2 at baseline and after exposure to IL-33. We also determined the migration capacity of PMNs.
Results: The decoy receptor of IL-33 (soluble ST2 [sST2]) was increased in SAH vs. cirrhosis and controls, demonstrating the defect in this pathway during ALD. The sST2 level was associated with response to treatment, 2-month survival, infection-free survival and probability of infection in SAH. Endotoxemia was weakly correlated with sST2. GRK2, a negative regulator of CXCR2, was overexpressed in PMNs of patients with SAH and cirrhosis and was decreased by IL-33. CXCR2 levels on PMNs were lower in SAH vs. cirrhosis and controls. Treatment with IL-33 partially restored CXCR2 expression in SAH and cirrhosis. PMN migration upon IL-8 was lower in patients with SAH and cirrhosis vs. controls. Treatment with IL-33 partially restored migration in those with SAH and cirrhosis. Interestingly, the migration capacity of PMNs and the response to IL-33 were enhanced in responders to corticosteroids (Lille <0.45) compared to non-responders.
Conclusion: The IL33/ST2 pathway is defective in SAH and predicts outcome. This defect is associated with decreased CXCR2 expression on the surface of PMNs and lower migration capacity, which can be corrected by IL-33, especially in patients responding to steroids. These results suggest that IL-33 has therapeutic potential for SAH and its infectious complications.
Lay summary: The neutrophils of patients with severe alcoholic hepatitis are associated with a defect in the IL-33/ST2 pathway. This defect is associated with lower migration capacities in neutrophils and a higher probability of getting infected. Administration of IL-33 to the neutrophils at least partly restores this defect and may be effective at reducing the risk of infection in patients with severe alcoholic hepatitis.
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Introduction

Severe alcoholic hepatitis (SAH) is a life-threatening condition associated with short-term liver failure and a high risk of death. SAH is mainly defined by a Maddrey's discriminant function ≥32[1] and cirrhosis is observed in more than 90% of cases.[2,3] The only treatment that has been shown to be effective in reducing short-term mortality (i.e. at 28 days) is prednisolone, 40 mg/day for 1 month.[4–6] In case of severe alcoholic hepatitis, the estimated short- and medium-term probability of death is around 20–30%. The Lille model is a helpful prognostic score which identifies patients that do not respond to medical treatment and who are at high risk of death.[7] This model ranges from 0 to 1 and patients with a score >0.45 are classified as non-responders. There is no alternative pharmaceutical treatment for these patients to date and their outcome is poor.

Bacterial infections are one of the main drivers of the increased risk of mortality in severe AH. An estimated 25% of patients with SAH are admitted with infection at diagnosis and

another 25% will develop infection during treatment with corticosteroids.[8] Thus, targeting infection in patients with SAH is an attractive area of drug development.

There are multiple mechanisms associated with the increased risk of infection during alcohol-related liver disease (ALD), which involve different levels of the innate and adaptive immune system. For example, the antigen presentation function of antigen presenting cells is downregulated, monocyte and macrophage bacterial killing is reduced and T lymphocyte interferon (IFN) production in response to lipopolysaccharide (LPS) is reduced.[9] The presence of a profound immune dysfunction associated with a high level of circulating neutrophils in AH may seem paradoxical. Some studies have reported an increased resting burst in circulating polymorphonuclear neutrophils (PMNs) which is in contrast with a reduced oxidative burst induced by *E. coli* and a decrease in phagocytosis capacities which has been suggested by some authors.[10–12] Another key function of PMNs is the migration to the site of infection, which seems to be impaired in decompensated alcohol-related cirrhosis.[13] When considering the crucial role of PMNs in the resolution of sepsis, studies to understand which pathways are involved in impairment of PMN function, especially migration, are essential. The IL-33/ST2 pathway is a potential target that could play a role in the high rate of infections in alcoholic liver disease, in particular in severe AH.

IL-33 is a member of the IL-1 family that is mainly located in the nucleus of barrier cells, such as epithelial cells and endothelial cells, but also in several organs, such as the lungs, skin, spleen and liver.[14–17] IL-33 participates in the regulation of gene expression but is also considered a damage-associated molecular pattern (DAMP) released after cell injury and/or necrosis especially in tissue macrophages.[18] Binding of IL-33 to the transmembrane form of ST2 (suppression of tumorigenicity 2) (ST2L) leads to subsequent activation of multiple intracellular signaling pathways which depend on cell type and location. In addition to IL-33/ST2 signaling on the cell surface, the *ST2* gene also encodes a soluble form of the protein (sST2) lacking the transmembrane domain and acting as a decoy receptor for IL-33, inhibiting signal transduction of this pathway. In liver diseases, the IL-33/ST2 pathway has been involved in the pathogenesis of liver injury related to viral hepatitis, fatty liver disease, acute hepatitis and has been suggested to play a role in fibrogenesis.[19] It has been shown that the IL-33/ST2 pathway attenuates sepsis in the specific setting of infection without liver impairment by enhancing neutrophil influx to the site of infection (*i.e.* migration) via increased expression of chemokine receptor CXCR2 on circulating PMNs in animal models and *ex vivo* experiments.[20] Based on the relationship between IL-33/ST2 pathway activation and neutrophil migration we hypothesize that this pathway could play a role in the PMN immune dysfunction observed in alcoholic liver disease and severe AH.

The aims of our study were i) to characterize the role of the IL-33/ST2 pathway in the PMN of patients with ALD and severe AH; ii) to evaluate the impact of IL-33/ST2 pathway modulation on circulating PMN function *ex vivo*, especially migration capacities.

Patients and methods

Patients

This study included patients with severe alcoholic hepatitis (SAH), decompensated cirrhosis and healthy controls. Individuals with SAH were included from a prospective cohort of consecutive patients (n = 161), managed in the hepatology department of Hôpital Claude-Huriez, Lille, France. Alcoholic hepatitis was clinically suspected using the classical diagnostic criteria, *i.e.* recent onset of jaundice (less than 3 months), heavy alcohol consumption, clinical signs of hepatic decompensation (ascites, encephalopathy), elevated aminotransferases (less than 300 IU/L with aspartate aminotransferase >alanine aminotransferase) and high serum bilirubin levels (greater than 3.0 mg/dl) with no other identifiable cause of liver disease.[21] Alcoholic hepatitis was confirmed by liver biopsy in 148 patients (91.9% of cases) and diagnosis was based on the presence of the following criteria: hepatocellular damage (presence of Mallory bodies and hepatocellular ballooning), PMN infiltrate and steatosis associated with fibrosis.[21] In the remaining cases, liver histology was not available in 13 patients for the following reasons: 4 patients due to severe alterations in hemostasis contraindicating liver biopsy, 2 patients due to the presence of a transjugular intrahepatic portosystemic shunt and 7 patients for a procedure failure. Liver biopsy was performed according to standard clinical practice in France and not for research purposes. SAH was defined as a Maddrey's discriminant function ≥32 and the severity of liver injury was also assessed by the model for end-stage liver disease (MELD) and Child-Pugh scores. After liver biopsy, 154 (95.6%) patients were treated with prednisolone (40 mg/day) for 7 days and therapeutic response was assessed by the Lille model.[7] Seven patients were not treated with prednisolone because of very severe liver dysfunction which is associated with a very low probability of response to steroids. A non-response to medical treatment was defined by a Lille model >0.45 according to the ideal cut-off published in the original paper.[7] The response to medical management in non-treated patients was also assessed by the Lille model as this model has also been shown to be effective in placebo-treated patients.[3–5] Systematic screening of infection was performed at admission and consisted of chest X-ray and blood, urine, and ascites cultures as published by our group.[8] During the follow-up, the same infection screening as that of admission was renewed in case of clinical or biologic signs of infection. Serum and plasma samples were collected before the initiation of treatment and at day 7 in patients treated with prednisolone. Serum and plasma samples were collected at diagnosis of SAH and 7 days thereafter in non-treated patients. It should be noted that patients were not included in the study if they developed sepsis between 2 blood samples.

Clinical outcome

Patients were followed-up for at least 2 months. Survival in patients with SAH was calculated from the first day of treatment with prednisolone to the last follow-up or liver transplantation (n = 4). Survival in patients with decompensated cirrhosis was calculated from the time of blood sample collection. The status (alive or dead) of lost to follow-up patients was assessed by telephoning a family member, general practitioner, or both, or by contacting the death registry at the patient's birthplace. We prospectively recorded all infectious events to evaluate the incidence of infection in patients with SAH.

Analyses performed

Two types of analyses were performed in patients with SAH: cellular analyses on fresh neutrophils obtained from 2012 to 2019 (see below) and serum analyses on frozen samples

collected from 2006 to 2012 before the initiation of the study and then prospectively from 2012 to 2019 to increase the sample size. Diagnostic criteria and patient management were the same in the 2 cohorts of patients.

Patients with SAH were compared to patients with decompensated alcoholic cirrhosis but without superimposed SAH admitted to our unit during the study period. The diagnosis of cirrhosis was based on either liver biopsy, clinical, laboratory and/or imaging features. We also included healthy controls during the same study period.

The procedure for blood sample collection (cells, serum, plasma) was the same in patients with SAH and cirrhosis, and healthy controls. To collect plasma and serum, blood samples were centrifuged at 1,500 rpm for 10 min at 4°C. We used the MACSxpress Neutrophil isolation human kit (ref. 130104434, Miltenyi Biotec, Germany) to isolate PMNs. Briefly, 4.5 ml of whole blood were incubated with 2 ml of manufacturer's buffer in Falcon 15 ml tubes for 5 min on the Macsmix rotator at 12 rpm. The tube was then placed on the MACSxpress Separator for 15 min at room temperature. The plasma supernatant was then collected with high purity PMNs. PMNs were incubated in the presence or absence of 50 ng/ml of recombinant human IL-33 50 ng/ml (ref. 3625-IL, Bio-Techne USA) for 30 min at 37°C. Blood samples were collected when prednisolone was initiated (day 0 - D0) and 7 days thereafter (day 7 - D7) in the SAH group and at admission to the cirrhosis group.

All patients agreed to participate in the study and written consent was obtained before analyses were performed. The study was registered under the number CPP14/67 at the "Comité de Protection des Personnes" of the Hauts de France région.

IL-33 and sST2 level
We assessed IL-33 and sST2 serum concentrations by ELISA using human IL-33 ELISA kit (ref. ADI-900-201, ENZO Life Sciences, USA) and ST2 human kit (ref. DY523B-05, Bio-Techne USA). Duplicate measurements were performed for each sample.

Quantification of endotoxemia
Endotoxemia was assessed using HPLC-MS/MS (high-performance liquid chromatography coupled with mass spectrometry) to detect 3-hydroxymyristate (3-HM), a lipid component of LPS, in the serum of controls, patients with cirrhosis and patients with SAH. Methods are described in detail in Weil *et al.* and Pais de Barros *et al.*[22,23]

Immunocytochemistry for GRK2
Two-hundred thousand PMNs were fixed with cold methanol for 30 s on Cytospin slides. Slides were incubated (or not as negative controls) overnight at 4°C with primary anti-GRK2 polyclonal rabbit antibody (ref. 137666, Abcam, U.K) and stained with secondary FITC–conjugated goat antibody to rabbit (ref. A11034 Life technologies, USA). We captured images with a DM5500B microscope (Leica, Germany) equipped with DFC310 FX camera (Leica, Germany) and analyzed them by Leica Application Suite (LAS, Leica, Germany). All the cells from at least 5 randomly chosen fields in each slide performed in duplicate, were analyzed from at least 2 individual experiments. GRK2 expression was quantified in parallel for the entire set after defining thresholds using ImageJ software.[24] Correction for uneven illumination in fluorescence images was performed at the same time. Results are expressed as mean intensity with relative units (MIRU).

Flow cytometry analysis
CXCR2 expression
After isolation, we pretreated PMNs with IL-33 (50 ng/ml) or HBSS as described above for 30 min at 37°C. Surface staining of circulating PMNs with antibodies to CXCR2 (12-1829-42, eBiosciences) for 30 min at 4°C was performed and cells were then analyzed by a Cyan ADP (Beckman Coulter, USA). For each condition, an isotype control tube (with mouse IgG1 k ISO control, eBiosciences) was used for non-specific signal subtraction.

SIGIRR expression
Surface staining of circulating PMNs was performed using APC-labeled anti-SIGIRR (FAB990A, R&D Systems) for 15 min at room temperature. Cytometry analyses were performed on a Navios flow cytometer using the Navios software (Beckman Coulter).

Apoptosis assessment
After isolation, we performed pretreatment of PMNs by IL-33 (50 ng/ml) or HBSS as described above for 30 min at 37°C. According to manufacturer's instructions, cells were washed with PBS and suspended in Annexin V Binding Buffer (FITC Annexin V Apoptosis Detection Kit with 7 AAD, 640922, Biolegend). Cell staining was performed using FITC labeled Annexin V and 7-AAD viability staining solution. After 15 min of incubation at room temperature, Annexin V Binding Buffer was added prior to cell analysis on a Navios flow cytometer. Intact cells were lacking both expression of Annexin V and 7-AAD and apoptotic cells were considered as cells expressing Annexin V but with low expression of 7-AAD.

Neutrophil functional tests
Migration. The migration capacity of PMN was assessed *ex vivo*.[25–27] Briefly, after isolation of PMNs 1×10^6 cells were deposited in the insert (polycarbonate filter 3 μm) of transwell kits (ref. 3415, Corning, USA). Five nM of recombinant human IL-8 (ref. 208-IL, Bio-Techne USA) or 5 nM of recombinant human IL-8 plus IL-33 50 ng/ml in HBSS (red phenol, calcium and magnesium free) were added to the lower well. PMNs were then incubated for 1 h at 37°C, 5% CO_2. The insert was removed, the culture medium containing cells was collected and the number of cells were counted by FACS (LSR FORTESSA X20 cytometer (BD Biosciences, USA) using Flow-Count Fluorospheres (ref. 7547053, Beckman Coulter, USA).

Phagocytosis. The Phagotest (ref. 341060, BD Bioscience) was used to measure phagocytosis using FITC-labeled opsonized *E. coli* bacteria. The mean fluorescence intensity of the respective antibodies on neutrophils was analyzed by FACS analysis with LSR FORTESSA X20 cytometer (BD Biosciences, USA).

Statistical analysis
Quantitative variables were expressed as medians with interquartile ranges. Categorical variables were expressed in numbers and percentages. Comparisons between quantitative variables were performed by the Mann-Whitney *U* test, paired-sample Student's *t* test or Kruskal-Wallis, as appropriate. Correlations between quantitative variables were performed by calculating the Spearman's rank correlation coefficient. Strength of correlation was defined using the Spearman' coefficient (r): strong correlation if r >0.75, moderate correlation if r >0.5 and weak if r <0.5.[28] Two-month overall survival was calculated by the

Table 1. Baseline characteristics of patients with severe alcoholic hepatitis and cirrhosis included in prognostic analyses.

	SAH	Cirrhosis	*p* value
Number	119	24	
Male/Female, n (%)	82/37 (68.9/31.1)	19/5 (79.2/27.8)	0.3
Age, years	50.4 (41.8–58.1)	60.5 (52.7–66.9)	0.0001
Ascites, n (%)	71 (59.7)	21 (87.5)	0.05
Hepatic encephalopathy, n (%)	19 (16.2)	10 (41.6)	0.01
Leukocytes, G/L	11.2 (8.6–14.5)	9.1 (6.1–11.7)	0.09
CRP, mg/L	28.5 (20.5–43)	13.5 (6–17.3)	0.03
Albumin, g/L	27 (24–31)	33.5 (30.5–38.7)	0.002
Bilirubin, mg/dl	17 (11.3–26.9)	4.4 (1.7–6.6)	<0.0001
Creatinine, mg/dl	0.8 (0.6–1.1)	1.4 (1–1.5)	0.001
Prothrombin rate, %	40 (33–51)	47 (34–60)	0.06
MELD score	25 (22–31)	19 (16–23)	0.001
Child-Pugh score	11 (9–12)	9 (7–11)	0.01
Lille model	0.25 (0.08–0.58)	n.a.	
Responders according to 0.45, N (%)	75 (63)	n.a.	
2-month survival, %	75.5 (67.3–83.7)	65.3 (45.8–84.8)	0.12

3-HM, 3-hydroxymyristate; MELD, model for end-stage liver disease; SAH, severe alcoholic hepatitis; sST2, soluble ST2.
The characteristics detailed below are those of patients included in the serum analyses (IL-33, sST2 and 3-HM) performed to determine the relationship between the IL33-ST2 pathway with prognosis and infection. Data are presented as median (IQR). Comparisons between quantitative variables were performed by the Mann-Whitney *U* test or Kruskal-Wallis, as appropriate.

Kaplan-Meier method and intergroup comparison according to sST2 tertiles was done using the log-rank test. We assessed the association between sST2 (treated as a continuous variable) and endpoints (2-month mortality or a composite of mortality and infection within the first 2 months) using Cox's proportional hazard regression model. The association between sST2 and infection at 2 months was assessed using Fine and Gray's model, treating death as a competing risk. We derived effect sizes from Cox's and Fine and Gray's models by calculating hazard ratio (HR) per 1 SD increase in sST2, as well as discrimination values by calculating the Harrell's c-index of agreement (using the approach of Wolbers and colleagues in the presence of competing risks for infection outcome[29]). Finally, we drew ROC curves for 2-month prediction of overall and infection-free survival analyses. The significance level was set at 0.05 with a 2-sided test. All statistical analyses were performed using NCSS 2011 or SAS (version 9.4, SAS Institute, Cary, NC) software.

Results

A total of 161 patients with SAH, 72 with severe cirrhosis and 28 healthy controls were included in the different analyses in the study. The main patient characteristics at inclusion are reported in Table S1. As expected, compared to patients with cirrhosis, patients with SAH were younger (52.4 [42.6–58.6] *vs.* 58.2 [51.5–63.7] years, *p* <0.0001), and more frequently had parameters indicative of systemic inflammatory response syndrome (leukocyte count at 11.7 [8.7–15.1] *vs.* 8.6 [5.1–11.1] G/L, *p* = 0.0005 and CRP at 29 [21–39] *vs.* 12 [9–17] mg/L, *p* = 0.0006). Bilirubin and MELD score were higher in the SAH group compared to the cirrhosis group (17.4 [10.9–29.1] *vs.* 5 [3.2–7.7] mg/dl, *p* <0.0001; and 25 [22–31] *vs.* 20 [17–26], *p* <0.0001, respectively); however, Child-Pugh score (11 [10-12] *vs.* 10 [8-13], *p* = 0.3) and 2-month survival (71.9 [65–78.8] *vs.* 66.6 [55.5–77.8], *p* = 0.22) were not different between the 2 groups.

IL-33/ST2 pathway is impaired in patients with SAH

A total of 119 patients with SAH, 24 with severe cirrhosis and 12 healthy controls were included in the serum analysis part of the study. The main characteristics at inclusion are reported in

Table 1 and are very close to the characteristics of patients with SAH described above.

Circulating IL-33 levels were relatively low in the 3 groups and we observed no significant difference between IL-33 levels of controls (91.7 pg/ml; IQR 80.5–96.5), or patients with cirrhosis (86 pg/ml; IQR 77.1–90.8) or SAH (107.7 pg/ml; IQR 80–147), *p* = 0.12 for comparison between control and SAH and *p* = 0.11 between cirrhosis and SAH (Fig. 1A). In contrast, there were marked differences in blood levels of the IL-33 decoy receptor (sST2), known to be the regulator of this pathway, between the 3 groups. The level of sST2 was increased in SAH (415,062 pg/ml, 231,461–691,729) compared to cirrhosis (191,511 pg/ml, 126,316–316,040, *p* <0.0001) and control (16,545 pg/ml, 11,645–22,162, *p* <0.0001). These results strongly suggest an attenuated IL-33/ST2 pathway in SAH compared to the other groups (Fig. 1B). We then assessed the relationship between IL-33 and sST2 level and the response to steroid treatment according to the 0.45 cut-off of the Lille model. At baseline (initiation of prednisolone), serum levels of IL-33 were not different between responders (*i.e.* Lille <0.45) and non-responders (*i.e.* Lille ≥0.45): 104.1 pg/ml (81.2–127.8) *vs.* 119.2 pg/ml (78.2–164.2), *p* = 0.3 (Fig. 1C). In contrast, responders had lower sST2 levels at baseline than non-responders: 336,775 pg/ml (205,896–636,541) *vs.* 598,788 pg/ml (270,260–708,915), *p* = 0.01 (Fig. 1D). These important results indicate that serum sST2 levels could be useful to predict response to prednisolone.

In SAH group, IL-33 levels were not different between D0 and D7 either on overall patients (107.7 *vs.* 109.2 pg/ml, *p* = 0.2), or in responders (104.1 *vs.* 106.6 pg/ml, *p* = 0.5) or non-responders (119.2 *vs.* 109.3 pg/ml, *p* = 0.2). sST2 levels increased between D0 and D7 in the SAH group from 415,062 to 656,775 pg/ml, *p* <0.0001. Such increases were observed in both groups of responders and non-responders (336,775 *vs.* 589,500, *p* <0.0001 and 598,788 *vs.* 761,656, *p* <0.0001 pg/ml, respectively).

sST2 predicts survival in patients with SAH and cirrhosis

The overall 2-month survival in patients with SAH and cirrhosis, included in serum analysis of the IL-33/ST2 pathway, was 76.1%
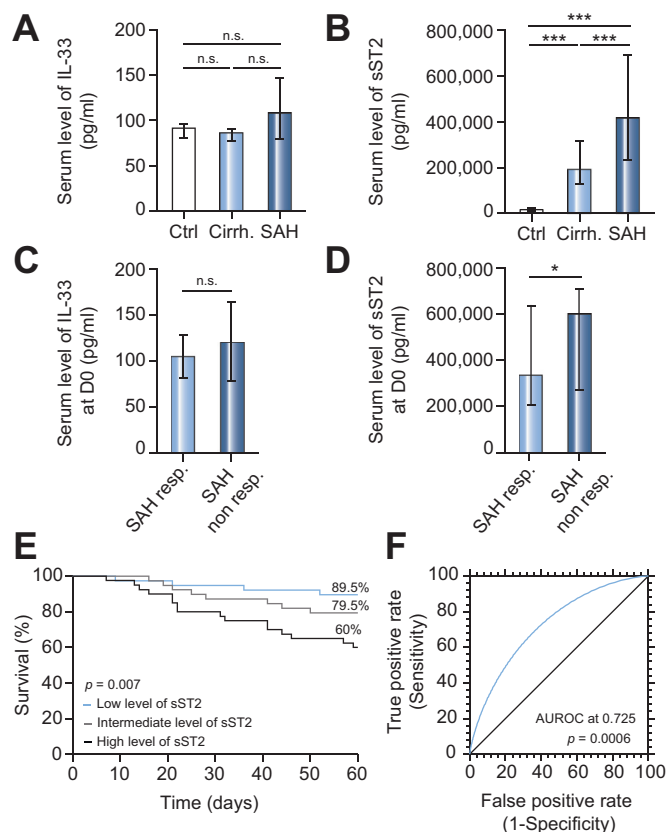
Fig. 1. **IL-33/ST2 pathway expression and association with survival.** (A, B) Serum levels of IL-33 (A) and sST2 (B) from healthy controls (n = 12), patients with cirrhosis (n = 24) and patients with SAH (n = 119). Results are expressed as median in pg/ml with IQR. (C, D) IL-33/ST2 pathway expression according to response to medical treatment assessed by the Lille score with responders (n = 75) defined by a score <0.45 and non-responders (n = 44) by a score ≥0.45. (E) 2-month survival according to percentile distribution (≤33th [n = 39], between 33th and 66th [n = 40], ≥66th [n = 40]) level of sST2 at D0 in patients with SAH. (F) ROC curve in binormal representation of the capacity of sST2 to predict 2-month survival in patients with SAH (n = 119). Comparisons between quantitative variables were performed by the Mann-Whitney U test or Kurskall-Wallis, as appropriate. Two-month overall survival was calculated by the Kaplan-Meier method and intergroup comparison according to sST2 tertiles was done by the log-rank test. n.s., non significant, *p ≤0.05, **p <0.01 and ***p <0.001. Cirrh, cirrhosis; Ctrl, healthy controls; SAH, severe alcoholic hepatitis; sST2, soluble ST2.

and 65.3%, p = 0.12, respectively. Twenty out of the 28 deaths (71.4%) in the SAH group and 6 out of the 8 deaths (75%) in the cirrhosis group were related to infection. In patients with SAH, 2-month survival was higher in those with low concentrations of sST2 (*i.e.* those with serum sST2 lower than 285,355 pg/ml) according to the percentile distribution of sST2 at D0 (≤33th percentile, 33–66th percentile and ≥66th percentile) than in those with intermediate levels (between 285,355 and 625,756 pg/ml) or high levels of sST2 (above 625,756 pg/ml): 89.5% (79.8–99.2) *vs.* 79.5% (66.8–92.2) *vs.* 60% (44.8–64.2), respectively, p = 0.007 (Fig. 1E).

In order to get more insight on the prognostic performance of sST2 in predicting outcome of patients with SAH, we estimated the effect size of the association between sST2 and outcome by calculating HR per 1 SD increase and Harrell's c discrimination index. We also drew ROC curves. sST2 was significantly associated with mortality (HR 1.87; 95% CI 1.3–2.67; p <0.0001). Area
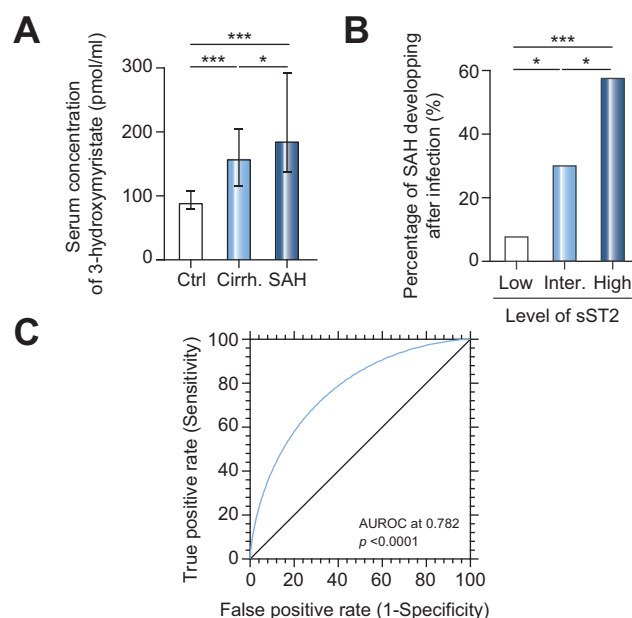

Fig. 2. **sST2, endotoxemia and infection risk.** (A) Levels of circulating 3-HM in the 3 groups (Ctrl n = 12, Cirrh n = 24, SAH n = 119). Results are expressed in pmol/ml. (B) Percentage of patients with SAH developing infection after initiation of medical treatment according the percentile distribution of sST2 at baseline. (C) ROC curve in binormal representation of the capacity of sST2 to predict 2-month infection-free survival in patients with SAH (n = 119). Comparisons between quantitative variables were performed by the Mann-Whitney U test or Kurskall-Wallis, as appropriate. n.s., non significant, *p ≤0.05, **p <0.01 and ***p <0.001. 3-HM, 3-hydroxymyristate; Cirrh, cirrhosis; Ctrl, healthy controls; SAH, severe alcoholic hepatitis; sST2, soluble ST2.

under the ROC curve to predict mortality at 2 months was good: 0.725 (Fig. 1F), as well as Harrell's c-index: 0.7, p <0.0001. sST2 also predicted 2-month mortality in the subgroup of patients with cirrhosis: HR 1.85, 95% CI 1.07–3.21, p = 0.03 and area under the ROC curve was at 0.778.

sST2 levels correlate with endotoxemia and predict the risk of infection in SAH and in cirrhosis.

In patients with SAH, infection occurred after a median time of 21 (8–39) days following corticosteroid initiation. As expected, quantification of endotoxemia using HPLC-MS/MS showed that patients with SAH had higher levels of 3-HM than patients with cirrhosis and controls: 184.03 *vs.* 156.5 pmol/ml, p = 0.05, and *vs.* 87.8 pmol/ml, p <0.0001, respectively (Fig. 2A).

Importantly, the probability of developing infection at 2 months increased along with the level of sST2 according to the percentile distribution of sST2 at D0 in SAH: 7.7% in low level *vs.* 30% in intermediate level (p = 0.01) *vs.* 57.7% in high level p <0.0001. The probability of developing an infection was different in those with intermediate *vs.* high levels of sST2 (p = 0.01). (Fig. 2B). A good performance of sST2 was also found to predict death or infection at 2 months (composite endpoint): HR 1.89, 95% CI 1.46–2.44, p <0.0001. The subsequent area under the ROC curve was 0.782 (Fig. 2C) and Harrell's c-index was 0.72, p <0.0001. Finally, when considering the high probability of death in the group of patients with SAH, we determined the Harrell's c-index to predict infection at 2 months, taking death as a competitive event. Performance of sST2 was confirmed with a c-index at 0.73, p <0.0001. Interestingly, the prognostic value of sST2 to predict death or infection

(composite endpoint) was confirmed in the subgroup of patients with cirrhosis: HR 1.66, 95% CI 1.02–2.71, p = 0.04 and area under the ROC curve was 0.784.

Correlation between sST2, endotoxemia and liver function

We found a weak but significant correlation between sST2 levels and MELD score on the overall cohort of patients with cirrhosis and SAH: Spearman's r = 0.43, p <0.0001. This weak correlation was confirmed in the subgroup of patients with SAH: Spearman's r = 0.397, p <0.0001. Endotoxemia assessed by serum 3-HM measurement was also weakly associated with sST2 levels on the overall cohort of patients with cirrhosis and SAH: Spearman's r = 0.335, p <0.0001. The weak correlation between sST2 and endotoxemia was confirmed in the subgroup of patients with SAH: Spearman's r = 0.331, p = 0.0006.

Downstream effectors of IL-33/ST2 (GRK2 and CXCR2) are affected in SAH and their expression is targeted by IL-33 challenge

The kinase GRK2 is a negative regulator of membranous CXCR2 expression and its activity is decreased by the IL-33/ST2 pathway. Immunocytochemistry staining showed increased expression at baseline of GRK2 in circulating PMNs of patients with SAH compared to patients with cirrhosis (p = 0.001) and in patients with cirrhosis compared to controls (p = 0.04): MIRU at 19,527 *vs.* 12,415 *vs.* 8,887. In the presence of IL-33, PMNs showed a marked decrease in GRK2 expression (MIRU) in patients with SAH or with cirrhosis, from 19,527 to 14,500, p = 0.009 and from 12,415 to 9,744, p = 0.06, respectively, but not in controls: from 8,887 to 9,632, p = 0.2 (Fig. 3A and 3B).

We then evaluated the expression of the chemokine receptor CXCR2 on the surface of circulating PMNs. CXCR2 is known to be downregulated by GRK2. Consistent with data on GRK2, CXCR2 expression was decreased in the SAH (mean fluorescence index 33.1) and cirrhosis (49.6) groups compared to control (109.3) groups, p <0.0001 (Fig. 3B). The difference between cirrhosis and SAH was also significant (p = 0.01). Although there was no effect in controls (109.3 *vs.* 106.3, p = 0.87), treatment with IL-33 partially restored CXCR2 expression in the SAH (33.1 *vs.* 50.8, p <0.001) and cirrhosis (49.6 *vs.* 66.5, p = 0.007) groups (Fig. 3C). These results show that CXCR2 expression, a key receptor for neutrophil migration, is downregulated in advanced stages of ALD, especially in SAH. A challenge with IL-33 can restore its expression by decreasing GRK2 activity.

SIGIRR expression using the FACS analysis was not different between controls, patients with cirrhosis or those with SAH, with mean fluorescent intensities (MFIs) of 0.97 *vs.* 0.99 *vs.* 1.07, p = 0.8, respectively. SIGIRR expression was also not modified by IL-33 challenge (Fig. S2).

Of note, we did not observe any significant apoptosis upon IL-33 treatment in any group of patients (controls, patients with cirrhosis or with SAH): 0.6% *vs.* 0.79%, p = 0.6 in controls (n = 4), 1.01% *vs.* 1.49%, p = 0.11 in cirrhosis (n = 6) and 0.98% *vs.* 0.83%, p = 0.5 in SAH (n = 5).

Defective PMN migration in SAH: improvement by IL-33

Phagocytosis capacity

The phagocytic capacity of circulating PMNs was evaluated in the 3 groups of patients by a well-validated test (*i.e.* Phagotest). No difference was observed in phagocytic capacity among the 3 groups (MFIs of 63.7, 67.5, and 70.1, respectively, p = 0.8). IL-33
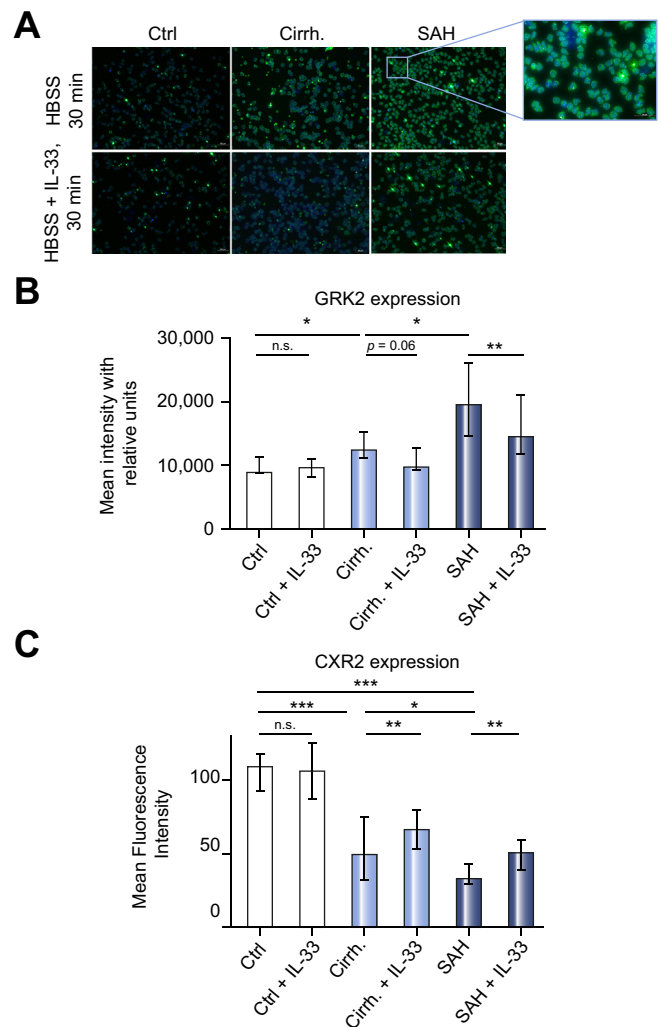

Fig. 3. Expression of molecular effectors of the IL33/sST2 pathway in circulating PMNs. (A) Expression of molecular drivers of the IL33-sST2 pathway in circulating PMNs. Representative immunocytochemistry staining (×200) of intracytoplasmic GRK2 expression in PMNs from Ctrl, Cirrh and SAH patients, with and without pretreatment by IL-33. A picture showing the cytosolic staining of GRK2 in a patient with SAH is provided at a greater magnitude (×400). (B) Quantification of immunocytochemistry staining expressed in MIRUs in the 3 groups (Ctrl n = 7, Cirrh n = 8, SAH n = 7). (C) CXCR2 expression by FACS. Expression of transmembranous chemokine receptor CXCR2 on circulating PMNs of Ctrl (n = 10), Cirrh (n = 15) and SAH (n = 22) patients with or without pretreatment by IL-33. Results are expressed as median MFIs with IQR. Comparisons between quantitative variables were performed by the Mann-Whitney U test, paired-sample Student's t test or Kruskall-Wallis, as appropriate. n.s., non significant, $^*p$ ≤0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001. Between 0.05 and 0.1 the p value is indicated. Cirrh, cirrhosis; Ctrl, healthy controls; MFI, mean fluorescent intensity; MIRU, mean intensity with relative units; PMNs, polymorphonuclear neutrophils; SAH, severe alcoholic hepatitis; sST2, soluble ST2. (This figure appears in color on the web.)

pretreatment did not significantly change the phagocytic capacity in the 3 groups (Fig. 4A).

Migration capacity

The migration capacity of PMNs driven by IL-8 was assessed using transwell assay experiments, in the presence or absence of IL-33. Spontaneous migration (in the absence of cytokines) was low at baseline and no difference was observed between the SAH,
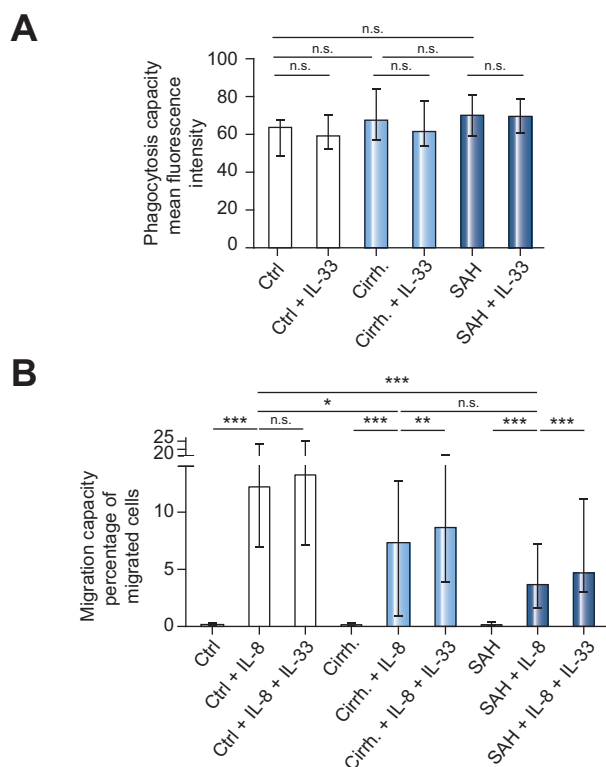
A

B

Fig. 4. Evaluation of migration function and phagocytosis of circulating PMNs during ALD. (A) Migration capacity of circulating PMNs of Ctrl (n = 10), Cirrh (n = 15) and SAH (n = 22) patients has been evaluated by transwell assays in the presence or absence of IL-33. Results are expressed by median percentage (and IQR) of migrated cells after standard deposition in the upper well of 1×10^6 cells. (B) Phagocytic capacity was evaluated by standard test with FACS. Results are expressed as median in MFIs with IQR in Ctrl (n = 3), Cirrh (n = 10) and SAH (n = 3) patients. Comparisons between quantitative variables were performed by the Mann-Whitney U test, paired-sample Student's t test or Kruskall-Wallis, as appropriate. n.s., non significant, $^*p$ ≤0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001. Cirrh, cirrhosis; Ctrl, healthy controls; MFI, mean fluorescent intensity; PMNs, polymorphonuclear neutrophils; SAH, severe alcoholic hepatitis; sST2, soluble ST2.
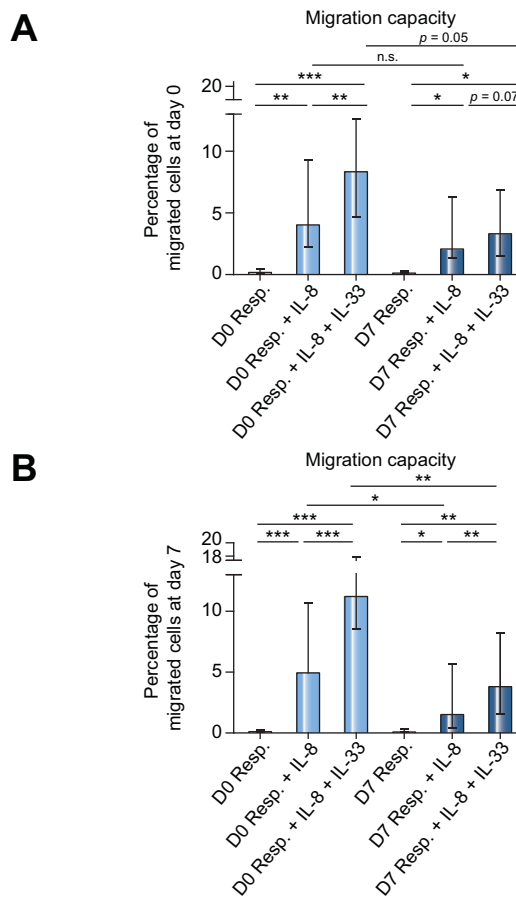
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Fig. 5. Evaluation of migration capacity of PMNs in SAH according to response to treatment. Migration capacity of circulating PMNs of patients with SAH (n = 22) has been performed by transwell assays in the presence or absence of IL-33. Results are expressed as median percentage (and IQR) of migrated cells after standard deposition in the upper well of 1×10^6 cells. (A) Evolution of migration capacity of circulating PMNs at D0 according to response to treatment (responders n = 11, non-responders n = 11). (B) Evolution of migration capacity of circulating PMNs at D7 according to response to treatment. Comparisons between quantitative variables were performed by the Mann-Whitney U test, paired-sample Student's t test or Kruskall-Wallis, as appropriate. n.s., non significant, $^*p$ ≤0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001. Between 0.05 and 0.1 the p value is indicated. PMNs, polymorphonuclear neutrophils; SAH, severe alcoholic hepatitis.

cirrhosis and control groups: 0.14%, 0.15%, 0.17%, respectively (p = 0.52) (Fig. 4B). Treatment with IL-8 increased the percentage of migrating PMNs across the 3 groups, but the effect of IL-8 was less marked in the SAH group (3.7%, p = 0.0001) than in cirrhosis (7.3%, p = 0.0003) or control (12.2%, p = 0.04) groups (Fig. 4B).

Treatment with IL-33 had no effect on the percentage of migrating cells in controls (12.2% *vs.* 13.1%, p = 0.7). In contrast, treatment with IL-33 increased the percentage of migrating PMNs compared to IL-8 treatment alone in the SAH and cirrhosis groups, showing partial restoration of the migration defect: SAH (3.7% *vs.* 4.7%, p = 0.0009) and cirrhosis (7.3% *vs.* 8.7%, p = 0.006) (Fig. 4B).

We finally analyzed the impact of response to treatment on the changes in PMN migration between the initiation of prednisolone (D0) and day 7 of therapy (D7) in SAH. IL-8 increased the percentage of migrating PMNs on D0 in responders (*i.e.* Lille <0.45): 0.19% *vs.* 4.02%, p = 0.002. This effect was further improved by IL-33: 4.02% *vs.* 8.33%, p = 0.006. Similarly, in non-responders, IL-8 increased the percentage of migrating PMNs on D0 compared to baseline (0.13% *vs.* 2.08%, p = 0.03) and the addition of IL-33 further increased this percentage (2.08% *vs.*

3.31%, p = 0.07). The percentage of migrated cells at D0 following treatment with IL-8 was similar in responders and non-responders (p = 0.3) but IL-33 induced greater migration of PMNs in responders than in non-responders (p = 0.05) (Fig. 5A).

This difference in migration between responders and non-responders was greater after 7 days of treatment with steroids. Indeed, at D7, treatment with IL-8 increased the percentage of migrating PMNs (0.11% *vs.* 4.93%, p = 0.0003) in responders and this percentage increased again with the addition of IL-33 (4.93% *vs.* 11.22% p = 0.0005). At D7, IL-8 and IL-33 also increased PMN migration in non-responders: (addition of IL-8: 0.08% *vs.* 1.51%, p = 0.03; addition of IL-33: 1.51% *vs.* 3.8%, p = 0.005). After 7 days of steroids, the difference became significant between responders and non-responders after challenge with IL-8 (4.93% *vs.* 1.51%, p = 0.04). Furthermore, treatment with IL-33 was more effective in PMNs from responders than in those from non-responders (11.22% *vs.* 3.8%, p = 0.005) (Fig. 5B). In order to

assess if larger amounts of IL-33 could overcome the less good response to IL-33 in non-responders, we performed migration assays with increased doses of IL-33 (50, 200, 500 and 1,000 ng/ml) in both responders and non-responders at baseline and after 7 days of prednisolone course. There was no improvement in migration capacity in both groups with the increased doses compared to the standard dose of 50 ng/ml (data not shown).

Discussion

Alcoholic hepatitis is associated with a high incidence of infection, a major driver of short-term mortality. While several pathways have been investigated in decompensated cirrhosis[9] there are fewer data in alcoholic hepatitis. This study shows that the IL-33/ST2 pathway is altered in PMNs from patients with SAH and that this defect can be restored, at least in part, by IL-33. Indeed, the levels of circulating sST2, a biomarker indicative of IL-33/ST2 pathway activity, are associated with short-term mortality, development of infection and response to steroids. We also observed that different downstream effectors of the IL33/ST2 pathway were altered in SAH. Indeed, SAH is associated with an increased expression of GRK2 and decreased expression of CXCR2, which can be restored by challenging PMNs with IL-33. Decreased CXCR2 expression can be linked to a reduced migration capacity induced by IL-8, which can be improved by challenge with IL-33. The main study findings are shown in Fig. 6.

The issue of controls is critical in translational research for alcoholic hepatitis. Indeed, most patients admitted with SAH (*i.e.* with a Maddrey discriminant function >32) have underlying cirrhosis. Thus, we used 2 different control groups (*i.e.* patients with cirrhosis and healthy controls) to prove that changes in the IL-33/ST2 pathway in SAH are related to alcoholic hepatitis rather than decompensated cirrhosis. Comparison between cirrhosis and controls also shows the defect in this pathway during liver decompensation without any inflammatory process.

Endotoxemia was quantified using the HPLC-MS/MS method to determine serum concentrations of 3-HM. We chose this technique because HPLC-MS/MS can more accurately identify patients with low-grade endotoxemia, compared to the classical LAL (limulus amebocyte lysate) test, due to its high specificity and low limits of detection and quantification. Moreover, the measurement is not affected by the lipid content of samples, thus avoiding bias due to lipid-rich particles observed with the LAL test.[23] Such a technique has been validated in patients with cirrhosis.[22]

Herein, we confirm that patients with SAH have higher endotoxemia than patients with cirrhosis and controls.[30] The weak although significant correlation between sST2 levels and MELD score or endotoxemia suggests that liver failure and latent infection do not play an exclusive role to drive sST2 up in patients with SAH. Compared to the study by Alves-Filho *et al.*[20] we think that it is not only infection or liver insufficiency that inhibits the IL33-ST2 pathway but rather that this defect is a characteristic of SAH. Future studies are required to determine if systemic inflammation associated with SAH plays a role in the defect in the IL-33/ST2 pathway.

SIGIRR has been suggested to be a downregulator of the Toll-like receptor pathways implicated in sepsis in murine models, especially in neutrophils, with conflicting results in terms of functional consequences.[31,32] In the present study, we did not observe any significant difference in SIGIRR expression between
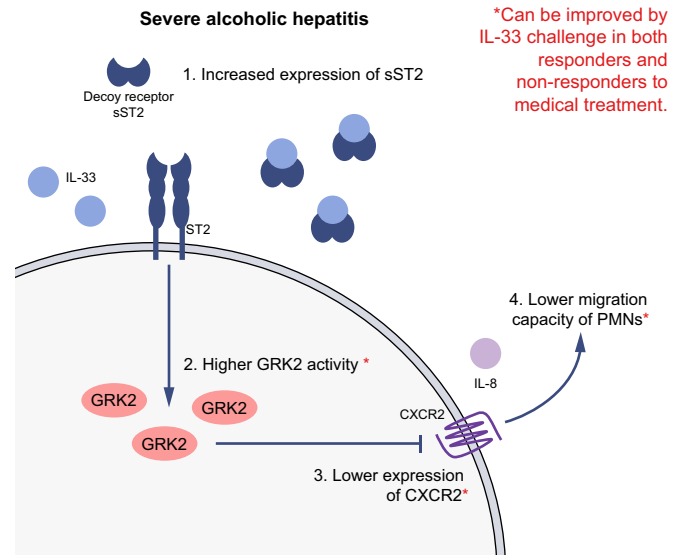

Fig. 6. Main study findings. (This figure appears in color on the web.)

controls, patients with cirrhosis and those with SAH, in the presence or the absence of IL-33.

Because the incidence of infection is a major driver of mortality in SAH,[5,8,33] there is an urgent need for reliable markers able to identify patients at a greater risk of becoming infected. Although baseline severity scores have been combined with scores evaluating medical management response, the percentage of well-classified patients is still around 80%. Thus, the outcome of some patients is not properly predicted in close to 20% of cases. Moreover, in patients initially classified as having good prognosis and who respond to therapy, the occurrence of an infection is responsible for a major drop in survival.[8] Circulating sST2 has been proposed as a biomarker in several diseases[34–36] and adding this marker to available scoring systems could optimize the prediction of clinical events in SAH. We show here using a large sample size and a robust methodology that sST2 not only predicts mortality but also the probability of getting infected in SAH and in cirrhosis, even though circulating levels of sST2 are different between the 2 conditions. Such results are promising because there is a large unmet need for biomarkers to predict the development of infection in both SAH and cirrhosis. Since elevated baseline sST2 values are associated with the risk of development of infection after treatment, patients with high sST2 might be candidates for trials testing strategies against infection. We observed higher levels of sST2 after 7 days of prednisolone exposure in both responders and non-responders to corticosteroids. It has already been reported that sST2 levels are increased by steroid treatment in ulcerative colitis and in systemic lupus erythematosus[37–39] and we feel that its evolution under prednisolone is not indicative of the global activity of the IL-33/ST2 pathway.

There has been no significant improvement in the pharmaceutical management of SAH in the past few years. Based on the knowledge that infection is associated with a high short-term probability of death, results in trials testing antibiotic prophylaxis are expected (Rifaximin: NCT02116556; Ciprofloxacin: NCT02326103; Amoxicillin and Clavulanate: NCT02281929). While strategies with antibiotics are promising, they do not restore the immune defect associated with SAH. Stimulating

PMN migration to the site of infection with IL-33 might represent an appealing option. Indeed, alterations in migration capacity have been associated with an increased risk of infection in humans.[40] Interestingly an increased migration capacity of circulating PMNs with IL-33 was found in both responders and non-responders to treatment in patients with SAH. We previously showed that non-responders to steroids are more likely to develop infection than responders.[8] The present study shows that PMNs from non-responders have a lower migration capacity following a challenge with IL-8 than those from responders and that IL-33 leads to an improvement in migration capacity in both groups. Although PMNs from non-responders are less responsive to IL-33, this cytokine improves migration capacity. This is particularly important because of the high rate of infection in these patients. Furthermore, PMNs from non-responders are less responsive to IL-33 at D0, suggesting that the defect in migration in these patients is already present in part before steroid exposure. The defect in the IL-33/ST2 pathway in the neutrophils of non-responders to corticosteroids seems to be related to higher levels of the decoy receptor sST2 but also to a less good response to IL-33 than responders that cannot be improved with higher doses of IL-33.

While neutrophils were treated with a dose of 50 ng/ml in the *ex vivo* experiments, the detected dose in the serum was around 100 pg/ml. Such a difference can be viewed as a supra-physiological stimulus. Other authors have also used similar doses.[20,41–44] In addition, we cannot exclude that the detection of IL-33 in the serum of patients lacks sensitivity. Based on our results, we propose that in patients with SAH, restoration of the IL-33/ST2 pathway may help prevent and treat infection in both responders and non-responders.

In the present study, the IL-33/ST2 pathway did not modulate the phagocytic capacity of circulating PMNs suggesting that this pathway only improves PMNs influx at the site of infection. We would like to underline that impairment in neutrophil function during ALD is not restricted to decreased phagocytosis but also includes defects in microbial killing with several pathways involved such as myeloperoxidase, TIM-3, PD-1, *etc.*[9] Thus, future studies targeting pathways involved in phagocytic capacity in circulating PMNs are needed.

Alcoholic hepatitis is a complex disease and the role of PMNs is not fully understood. Indeed, there is a paradox between the liver infiltration by neutrophils (suggesting that activation of these cells is harmful to the liver) and a defect in neutrophil migration capacity (suggesting that a defect in PMN function is harmful to infection). In addition, IL-8 is the most upregulated cytokine in patients with acute drinking/alcoholic hepatitis *vs.* patients without SAH in the CANONIC study evaluating the profile of patients with acute-on-chronic liver failure.[45] IL-8 is also elevated in the blood and in the liver of patients with SAH in the study by Taïeb *et al.*[46] Based on our results on neutrophil migration upon IL-8 and IL-33, we cannot exclude that promoting the IL-33/ST2 pathway in patients with ALD could result in a higher recruitment of neutrophils in the liver. Although the present study does not address this issue, it can be hypothesized that neutrophil migration triggers are different for the liver and the site of infection. The discrepancies between migration in the liver and to the site of infection could be related to different patterns of chemokine expression. However, IL-8 is not the only signal leading to neutrophil recruitment, as reviewed by Gustot *et al.*[9] Specific studies testing chemotaxis targets are needed for alcoholic hepatitis.

In conclusion, this study shows that circulating neutrophil migration is altered in SAH and that this defect is reversible by restoring the IL-33/ST2 pathway. Circulating sST2 levels are associated with survival and the probability of infection. Our results suggest that treatment with IL-33 represents a new potential therapeutic strategy to decrease the risk of infection in SAH.

Abbreviations

3-HM, 3-hydroxymyristate; ALD, alcohol-related liver disease; AH, alcoholic hepatitis; Cirrh, cirrhosis; Ctrls, controls; DAMP, damage-associated molecular pattern; HR, hazard ratio; IFN, interferon; LPS, lipopolysaccharide; MELD, model for end-stage liver disease; MFI, mean fluorescent intensity; MIRU, mean intensity with relative units; PMN, polymorphonuclear neutrophils; SAH, severe alcoholic hepatitis; sST2, soluble ST2.

Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Design of the study: FA, PM, LD, AL. Acquisition of data: FA, MBS, FM, GL, MN, JD, LCNW, JPPDB, DGH, SD, EG, AP, SCM, PM, LD, AL. Statistical analysis: FA, JL, ED, AL. Drafting of the manuscript and critical review: FA, RB, PM, LD, AL.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.12.017.

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