Immunomodulatory receptor VSIG4 is released during spontaneous bacterial peritonitis and predicts short-term mortality

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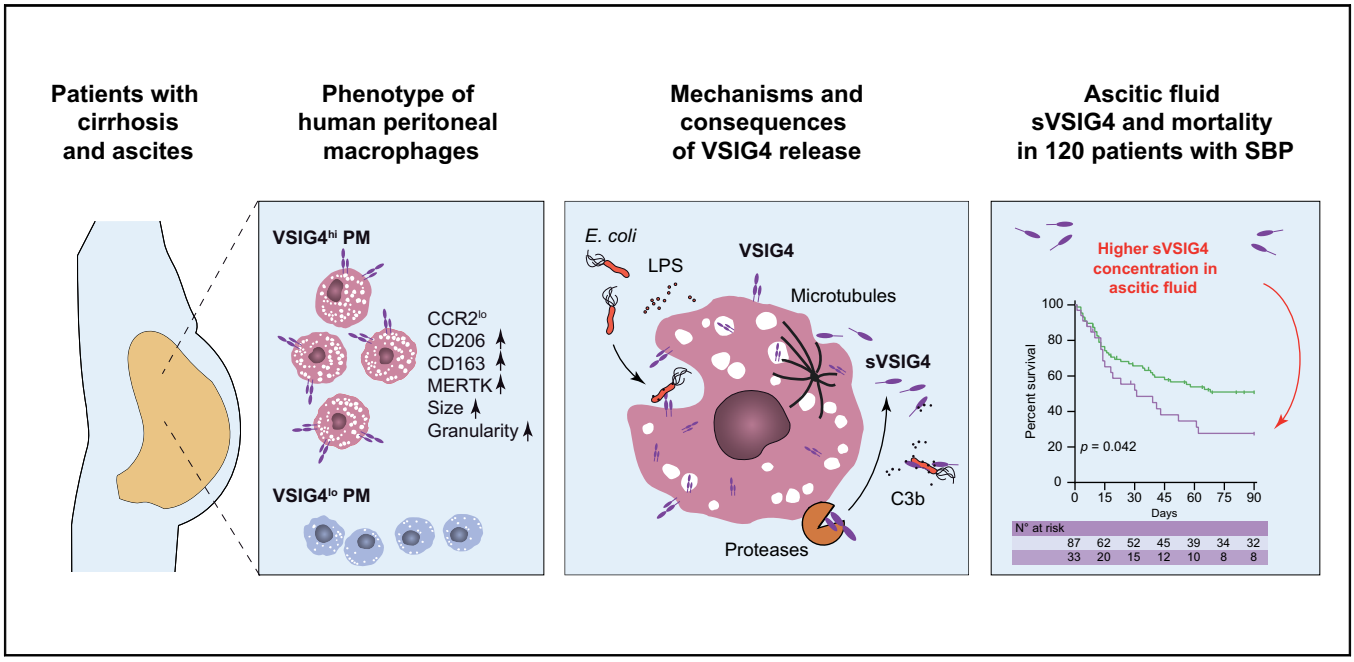
Graphical abstract



Highlights

- VSIG4 expression is high on human resting, large peritoneal macrophages (PMs) that co-express CD206, CD163, and MERTK.
- PM activation by TLR agonists or infection results in the loss of surface VSIG4 and release of soluble VSIG4 (sVSIG4).
- Ascites sVSIG4 correlates with organ dysfunction and inflammation during SBP.
- Higher ascitic fluid sVSIG4 concentrations indicated increased risk of 90-day mortality in 120 patients with SBP.
- Addition of an antibody binding to the extracellular domain of VSIG4 enhanced phagocytosis of bacteria *in vitro*.

Lay summary

Patients with liver cirrhosis who develop ascites have an increased risk of infection and mortality. Our study shows that in patients with infected ascites, the complement receptor VSIG4 is released by resident macrophages into the abdominal fluid where it can be measured. Patients with elevated levels of this protein in ascites are at high risk of dying within 90 days.

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Research article

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Immunomodulatory receptor VSIG4 is released during spontaneous bacterial peritonitis and predicts short-term mortality

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Background & Aims: V-set Ig-domain-containing 4 (VSIG4) is an immunomodulatory macrophage complement receptor modulating innate and adaptive immunity and affecting the resolution of bacterial infections. Given its expression on peritoneal macrophages (PMs), we hypothesised a prognostic role of peritoneal VSIG4 concentrations in patients with spontaneous bacterial peritonitis (SBP).

Methods: We isolated PMs from patients with cirrhosis and analysed VSIG4 expression and release by flow cytometry, quantitative real-time PCR, ELISA, and confocal microscopy. We measured soluble VSIG4 concentrations in ascites from 120 patients with SBP and 40 patients without SBP and investigated the association of soluble VSIG4 in ascites with 90-day survival after SBP using Kaplan–Meier statistics, Cox regression, and competing-risks regression analysis.

Results: VSIG4 expression was high on resting, large PMs, which co-expressed CD206, CD163, and tyrosine-protein kinase Mer (MERTK). *VSIG4* gene expression in PMs decreased in patients with SBP and normalised after resolution. During SBP, VSIG4^{hi} PMs were depleted (25% *vs.* 57%; p <0.001) and soluble VSIG4 in ascites were higher in patients with SBP than in patients without (0.73 *vs.* 0.35 μg/ml; p <0.0001). PM activation by Toll-like receptor (TLR) agonists or infection with live bacteria *in vitro* resulted in a loss of surface VSIG4 and the release of soluble VSIG4. Mechanistically, shedding of VSIG4 from PMs was protease-dependent and susceptible to microtubule transport inhibition. Soluble VSIG4 in ascites exceeded serum concentrations and correlated with serum creatinine, model for end-stage liver disease score and C-reactive protein during SBP. Concentrations of 1.0206 μg/ml or higher indicated increased 90-day mortality (hazard ratio 1.70; 95% CI 1.01–2.86; p = 0.046).

Conclusions: VSIG4 is released from activated PMs into ascites during SBP. Higher peritoneal VSIG4 levels indicate patients with organ failure and poor prognosis.

Lay summary: Patients with liver cirrhosis who develop ascites have an increased risk of infection and mortality. Our study shows that in patients with infected ascites, the complement receptor VSIG4 is released by resident macrophages into the abdominal fluid where it can be measured. Patients with elevated levels of this protein in ascites are at high risk of dying within 90 days.

Introduction

Spontaneous bacterial peritonitis (SBP) is a frequent precipitator of acute decompensation and acute-on-chronic liver failure in patients with cirrhosis.[1–3] Mortality after SBP ranges from 17 to 36% in the short term and reaches 60% after 1 year.[1,4,5] Factors that determine the individual mortality risk of SBP comprise organ failures and systemic inflammation at presentation, on the one hand,[2,5–9] and the adequate selection and timely administration of empirical antibiotic treatment, on the other hand.[1,5,10,11] Although there is some evidence that excessive peritoneal inflammation during SBP drives complicated disease courses, the peritoneal inflammatory state has rarely been incorporated into prognostic models of SBP. A few studies have identified elevated concentrations of inflammatory cytokines,[12–14] neutrophil markers,[15] and markers of macrophage activation[9,16] in ascitic fluid (AF) to be indicators of complications of SBP and poor prognosis.

Keywords: Bacterial infection; Prognostic factor; Risk of death; Biomarker.
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We have recently shown that peritoneal macrophage (PM) subsets from patients with cirrhosis are primed by pathogen-associated molecular patterns (PAMPs) to release pro-inflammatory cytokines.[16] These tissue-resident large PMs express the mannose receptor CD206 on their surface and are characterised by higher gene expression of macrophage-specific scavenger receptors, Fc receptors, and alternative activation markers including the complement receptor V-set Ig-domain-containing 4 (*VSIG4*). VSIG4, also referred to as CRIg or Z39Ig, is expressed by human and murine PMs but absent from circulating immune cells and macrophages from lymphoid organs.[17–19] It is involved in the clearance of complement component 3b (C3b)-opsonised bacteria[20] and acts as a direct pattern recognition receptor for lipoteichoic acids from Gram-positive bacteria.[21] In addition to its role in bacterial clearance, VSIG4 limits excessive inflammation from classically activated macrophages[22] and suppresses cytokine production by T cells.[23] Soluble VSIG4 (sVSIG4), which is released under conditions of tissue macrophage activation,[24] inhibits complement activation through the alternative pathway.[25,26]

Given its restriction to tissue-resident macrophages, its role in bacterial clearance and the modulation of inflammation, we hypothesised that sVSIG4 is released from activated PMs during SBP and its peritoneal concentration indicates a high risk of short-term mortality.

Patients and methods

Patient inclusion and sample selection

Patients with decompensated cirrhosis who underwent diagnostic ascites tap or therapeutic paracentesis between November 2019 and September 2021 at the University Hospital RWTH Aachen were eligible for PM immune phenotyping and *in vitro* analyses. Exclusion criteria were evidence of secondary peritonitis, tuberculous peritonitis, peritoneal carcinomatosis, and HIV coinfection. Patients were stratified for the presence of SBP by using current diagnostic criteria.[27]

AF samples from 120 patients with SBP who were recruited for previous prospective studies, were analysed to assess the association of AF sVSIG4 with survival after SBP using biomaterial from patients with SBP recruited at the Jena University Hospital between May 2014 and September 2019 (cohort 1, n = 39), at the Jena University Hospital between October 2010 and January 2013 (cohort 2, n = 39), and at the Bonn University hospital between April 2012 and March 2018 (cohort 3, n = 42). Among those, 83 participants with SBP were previously reported.[16] AF samples from 40 patients without SBP recruited at the Jena University Hospital served as a control.

The study was approved by the Internal Review Board of the University Hospital RWTH Aachen (the Ethics Committee of the Medical Faculty of the RWTH Aachen, no. EK 327-19), the Internal Review Board of the Jena University Hospital (no. 2880-

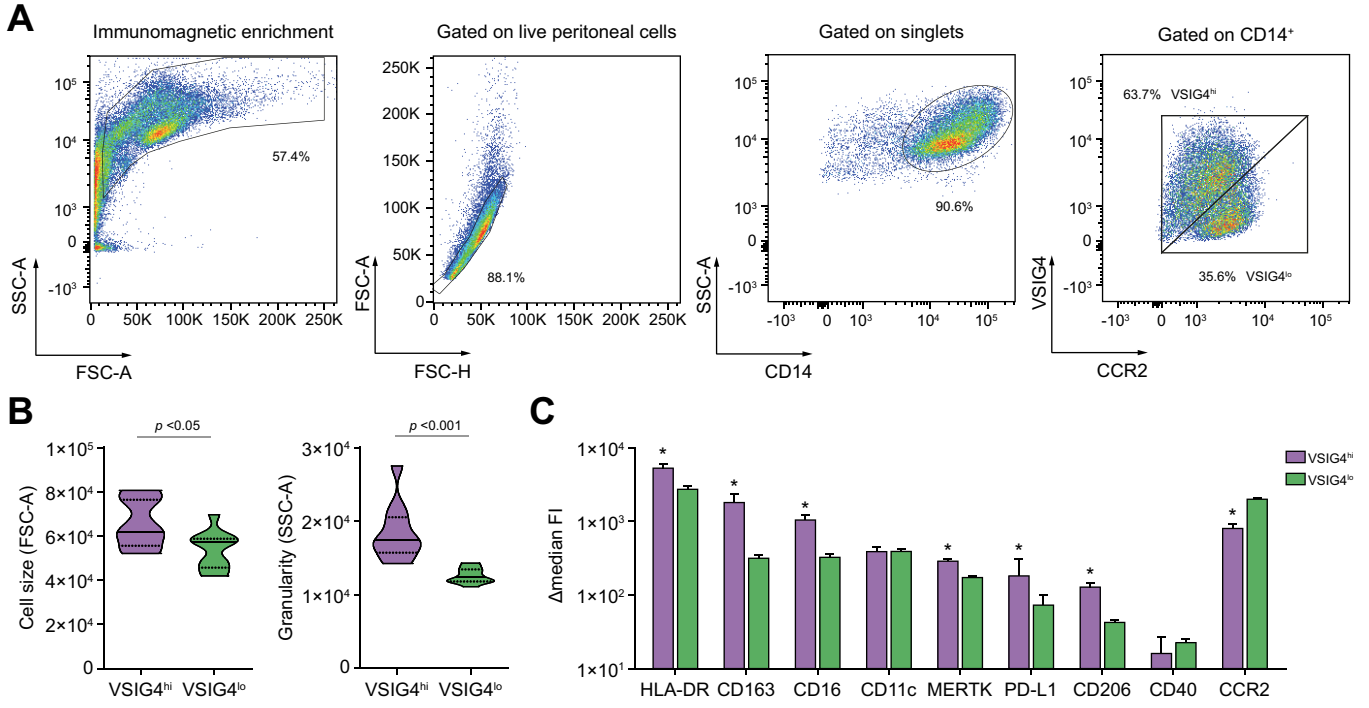

Fig. 1. Characterisation of VSIG4^{hi} human PMs. (A) Flow cytometry gating strategy to identify PM subsets. Representative plots from a patient with decompensated cirrhosis without SBP are shown. PMs were enriched by positive immunomagnetic cell separation and identified as viable CD14+ cells. VSIG4^{hi} PMs were identified by flow cytometry using co-staining with CCR2 as this resulted in a better discrimination of distinct cell populations. (B) Cell size (FSC-A, left) and granularity (SSC-A, right) differ between VSIG4^{hi} and VSIG4^{lo} PMs. Values of *p* from the Mann–Whitney *U* test are indicated. (C) Surface expression of macrophage surface markers differ between VSIG4^{hi} and VSIG4^{lo} PMs. Surface expression of HLA class II histocompatibility antigen (HLA-DR), scavenger receptor cysteine-rich type 1 protein M130 (CD163), low-affinity Ig gamma Fc region receptor III (CD16), integrin alpha-X (CD11c), MERTK, PD-L1/CD274, macrophage mannose receptor 1 (CD206), TNF receptor superfamily member 5 (CD40), and CCR2 was quantified in 9 patients with decompensated cirrhosis without SBP (delta median fluorescence intensity as compared with FMO controls) **p* <0.05 in the Wilcoxon matched-pairs signed rank test. CCR2, C-C chemokine receptor type 2; FSC, forward scatter; FMO, fluorescence minus one; HLA, human leucocyte antigen; HLA-DR, human leucocyte antigen-DR isotype; MERTK, tyrosine-protein kinase Mer; PD-L1, programmed cell death 1 ligand 1; PM, peritoneal macrophage; SBP, spontaneous bacterial peritonitis; SSC, side scatter; TNF, tumour necrosis factor; VSIG4, V-set Ig-domain-containing 4.

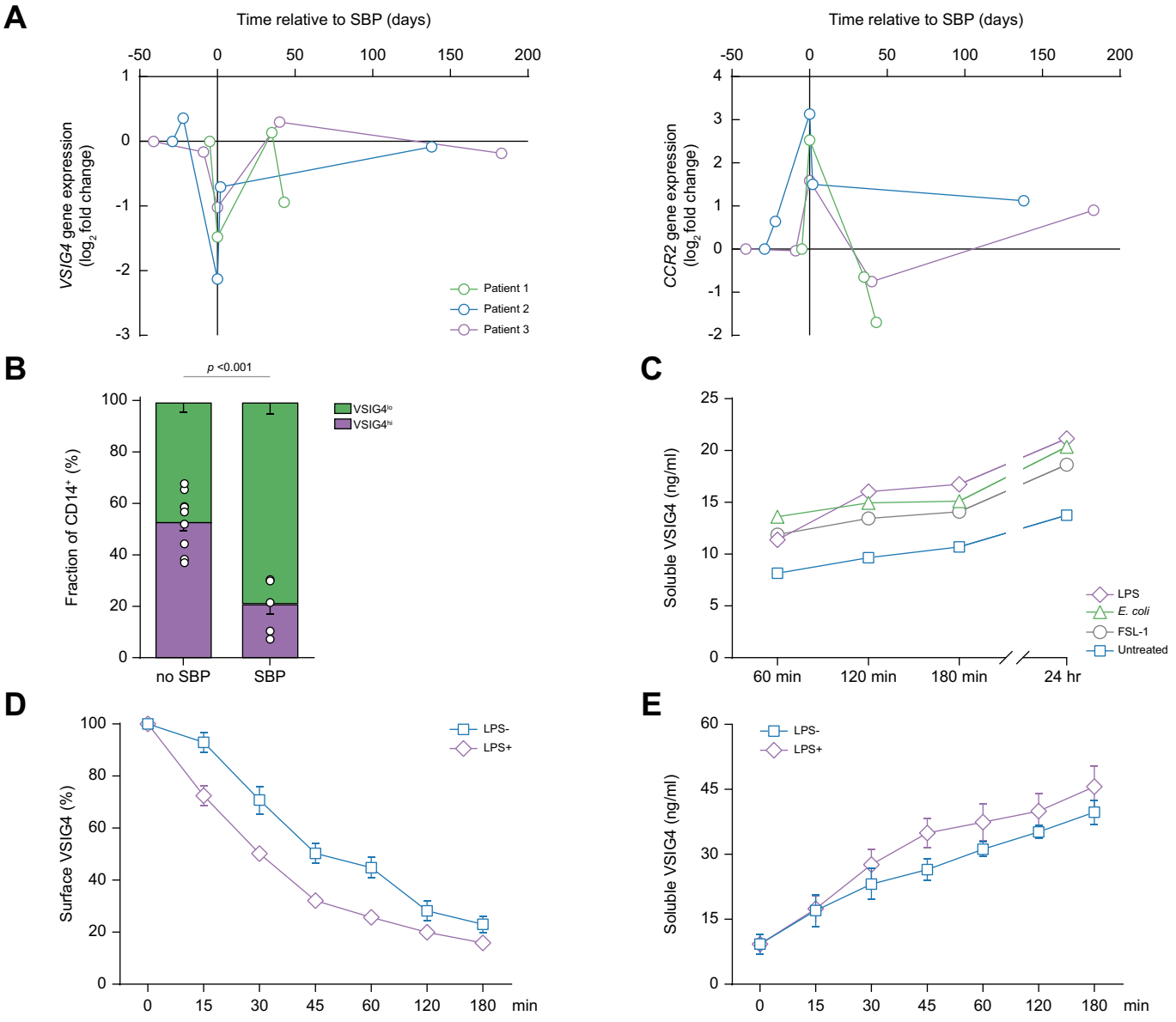

Fig. 2. Changes in VSIG4 expression and VSIG4 release in response to inflammatory stimuli. (A) Expression of *VSIG4* and *CCR2* mRNA in immunomagnetically enriched CD14+ cells from 3 patients before, during, and after SBP (Day 0 = SBP). Expression levels were normalised to *GUSB* and *ACTB* as housekeeping genes. (B) Fraction of VSIG4^{hi} PMs in the presence and absence of SBP. Mean and SEM and individual values are shown. Value of *p* from the Mann–Whitney *U* test. (C) Immunomagnetically enriched CD14+ PMs were stimulated with 10 ng/ml LPS, 10 ng/ml FSL-1, or live *E. coli* K12 at an MOI of 30 *in vitro*. Cell culture supernatant was collected at the indicated time points and sVSIG4 was measured by ELISA. (D and E) Immunomagnetically enriched CD14+ PMs were cultured in the absence or presence of LPS at 1000 ng/ml for 15–180 min. Cell culture supernatant was collected at the indicated time points and sVSIG4 was measured by ELISA and VSIG4 surface expression was analysed by flow cytometry. Delta median fluorescence intensity as compared with FMO controls were normalised to uncultured PMs. Means and SEMs are shown. CCR2, C-C chemokine receptor type 2; FMO, fluorescence minus one; MOI, multiplicity of infection; PM, peritoneal macrophage; SBP, spontaneous bacterial peritonitis; sVSIG4, soluble V-set Ig-domain-containing 4; VSIG4, V-set Ig-domain-containing 4.

08/10, 3683-02/3), and the local ethics committee of Bonn University Medical Centre (no. 130/18). Patients gave written informed consent before inclusion.

Isolation of PMs

Up to 500ml AF was collected from patients. Peritoneal cells were enriched by centrifugation and mononuclear cells were isolated from AF using Lympholyte-H separation media (Cedarlane, Burlington, Ontario, Canada). CD14+ cells were enriched by magnetic cell separation using positive selection for human CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany) for downstream analysis and *in vitro* experiments.

Cell culture

CD14+ PMs were cultured in 96-well plates (3×10^5/well) using RPMI-1640 medium (PAN-Biotech, Aidenbach, Germany) supplemented with 10% pooled heat-inactivated FBS (PAN-Biotech) and 100 IU/ml penicillin/streptomycin (PAN-Biotech) at 37°C and

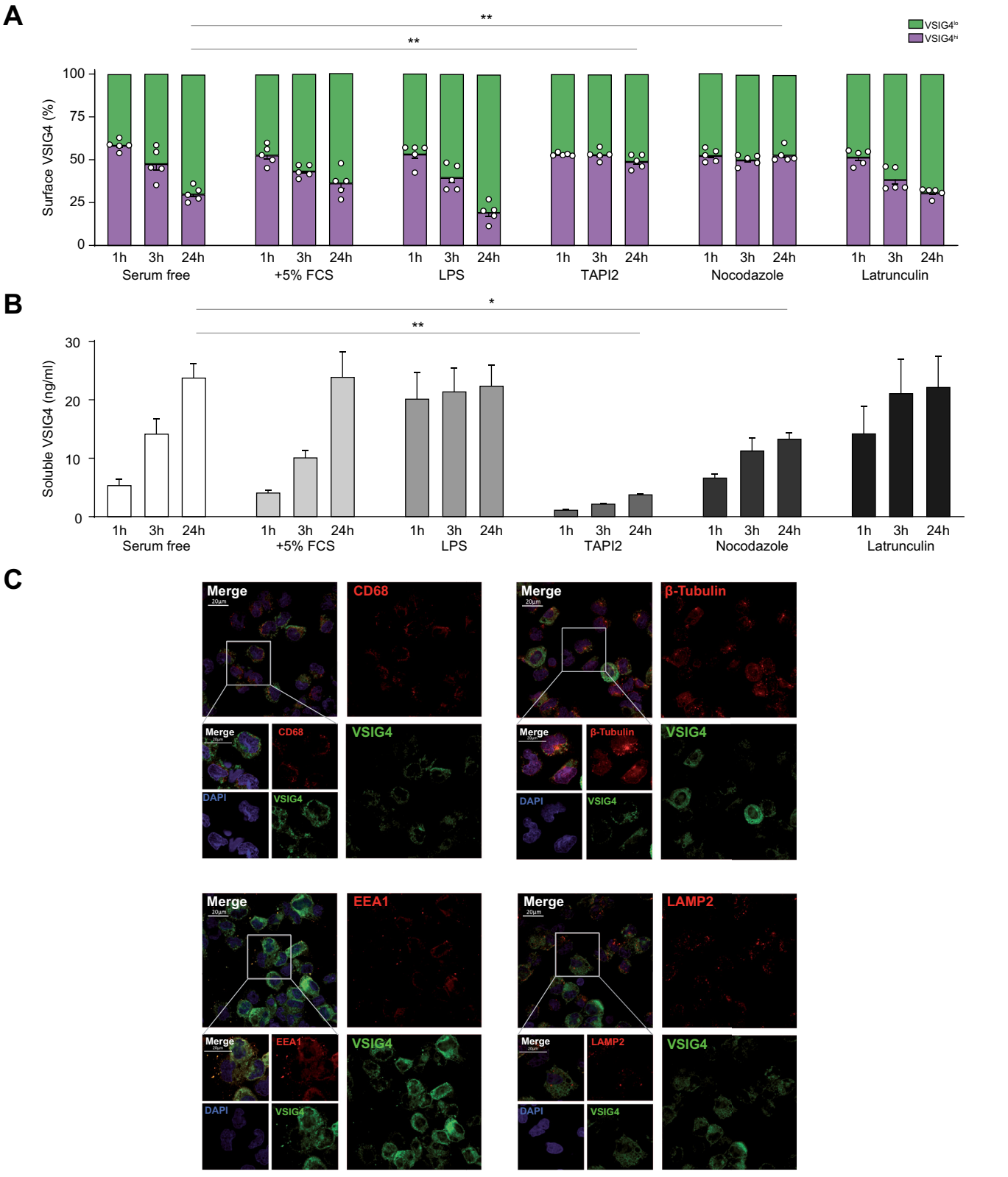

Fig. 3. VSIG4 is released from large human PMs in a protease- and microtubule-dependent fashion. (A and B) Mononuclear cells were isolated by density gradient centrifugation from AF in patients without SBP. CD14+ PMs were enriched by MACS, seeded in cell culture plates, and cultured in the presence of 0.1% BSA, 5% heat-inactivated FCS, 10 ng/ml LPS, 2 mg/ml TAPI-2, 50 ng/ml nocodazole, or 1 μg/ml latrunculin B for the indicated times. (A) Percentage of VSIG4^{hi} and VSIG4^{lo} CD14+ PMs as determined by flow cytometry and (B) sVSIG4 concentrations in cell culture supernatant as determined by ELISA are indicated. Means and SEMs, and *p* values from the unadjusted non-parametric Mann–Whitney *U* test are shown (**p <0.01; *p <0.05). (C) Cellular distribution of VSIG4 in human PMs were analysed by confocal microscopy. Mononuclear ascites cells from AF from patients with cirrhosis without SBP were isolated by density gradient

Table 1. Baseline characteristics of patients with decompensated cirrhosis and SBP stratified for outcome at 90 days.

	Total (N = 120)	Survivors* (n = 58)	Non-survivors (n = 62)	*p* value
Age (years)	59 (52–67)	59 (51–65)	61 (52–77)	0.21
Male sex (%)	89 (74)	42 (72)	47 (76)	0.68
Alcohol-related cirrhosis (%)	87 (73)	44 (76)	43 (69)	0.54
Child–Pugh C (%)	80 (67)	33 (57)	47 (76)	0.034
MELD score	21 (16–28)	18 (13–23)	24 (20–31)	<0.001
Bilirubin (μmol/L)	54 (23–144)	46 (20–76)	67 (31–185)	0.018
INR	1.5 (1.3–1.9)	1.4 (1.3–1.7)	1.5 (1.3–2.1)	0.038
Platelets (/nl)	112 (66–203)	119 (78–202)	100 (58–221)	0.38
Creatinine (μmol/L)	142 (90–248)	123 (82–162)	186 (113–297)	0.001
White blood cells (/nl)	10.5 (7.3–15.8)	9.4 (6.7–14.0)	12.0 (8.0–17.8)	0.08
C-reactive protein (mg/L)	77 (45–125)	71 (35–115)	80 (46–131)	0.24
Albumin (g/L)	26 (22–30)	27 (23–31)	25 (22–30)	0.44
AF protein (g/L)	14 (9–20)	14 (9–22)	13 (8–19)	0.15
AF cell count (/μl)	2,260 (1,045–5,048)	2,255 (988–4,478)	2,260 (1,093–6,065)	0.41
AF neutrophils (/μl)	1,390 (590–3,678)	1,235 (515–3,403)	1,580 (590–5,060)	0.24
Culture-positive SBP	46 (39)	20 (35)	46 (39)	0.46

Baseline characteristics are shown as frequencies or medians with IQRs. Values of p are based on the Wilcoxon–Mann–Whitney test for continuous and Fisher's exact test for categorical variables. *Including 10 patients receiving a liver transplant. AF, ascitic fluid; INR, international normalised ratio; MELD, model for end-stage liver disease; SBP, spontaneous bacterial peritonitis.

5% CO_2. Cells were stimulated with 1,000 ng/ml lipopolysaccharide (LPS) for 15 min to 3 h or with 10 ng/ml LPS, 10 ng/ml FSL-1 (InvivoGen, San Diego, CA, USA), 10 ng/ml synthetic monophosphoryl lipid A (MPLA) (InvivoGen), or *Escherichia coli* K12 at a multiplicity of infection (MOI) of 3–30. Incubation with sVSIG4 (Fc Chimera Protein, CF R&D Systems, Minneapolis, MN, USA), anti-VSIG4 monoclonal antibody (13100-1-2/C217_130529) (Abmart, Shanghai, China), or mouse IgG control (Abmart) was performed for 24 h before stimulation with LPS or incubation with bacteria. Phagocytosis was analysed using opsonised fluorescently labelled inactivated *E. coli* (Invitrogen, Carlsbad, CA, USA) at an MOI of 5 for 2 h.

The mechanism of VSIG4 release from PMs was investigated using different blocking agents. Proteases were inhibited using 2 mg/ml tumour necrosis factor (TNF) protease inhibitor 2 (TAPI-2) (Santa Cruz Biotechnology, Dallas, TX, USA), and intracellular transport was studied disrupting microtubules with nocodazole (50 ng/ml) or inhibiting actin polymerisation with latrunculin B (1 μg/ml, Merck, Darmstadt, Germany). Furthermore, the influence of serum proteases was investigated using serum-free medium containing 0.1% bovine serum albumin (BSA) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cells and supernatant were collected at the indicated time points.

Immunoassays

VSIG4 was quantified by immunoassay (RayBiotech, Norcross, GA, USA) according to the manufacturer's manual using appropriate dilutions of 1:100 for serum, 1:3,000 for AF, and 1:20 for cell culture supernatant. Given the high dilution, means of at least 3 independent measurements in AF were used for analysis. AF levels of C3b and C3a were measured by immunoassays (LifeSpan BioScience, Seattle, WA, USA, and Hycult Biotech, Wayne, PA, USA) in dilutions of 1:10,000 and 1:2,000, respectively, according to the manufacturers' recommendations. TNF was quantified in cell culture supernatant by immunoassay (Invitrogen) according to the manufacturer's recommendations using 1:20 dilutions.

Confocal microscopy

For immunostaining, CD14+ PMs were immunomagnetically enriched and transferred on a slide (800×*g* for 5 min) using the Cytospin 4 centrifuge (Thermo Fisher Scientific). Cells were fixed for 10 min with 4% paraformaldehyde (PFA) (Merck) and permeabilised for 30 min in PBS containing 0.3% Triton X-100 (Merck). Unspecific binding was blocked with PBS containing 5% BSA (Merck) for 30 min. Primary antibodies against VSIG4 (EPR22576-70) in combination with β-tubulin (1E1-E8-H4), lysosome-associated membrane protein 2 (LAMP2) (H4B4) or early endosome antigen 1 (EEA1) (ab206860) or CD68 (KP1) (Abcam, Cambridge, UK) were applied 1:100 in PBS + 0.05% Tween 20 (Merck) over night at 4°C. Appropriate fluorescent conjugate secondary antibodies (AF488 or AF546, Invitrogen) were applied at a 1:1,000 dilution for 2.5 h at room temperature. Slides were sealed using mounting medium containing DAPI (Vectrashield, Burlingame, USA). The LMS 710 confocal laser scanning microscope (Zeiss, Jena, Germany), Nikon A1 Ti2 N STORM (Nikon, Tokyo, Japan), and ZEN2 Blue edition (Carl Zeiss Microscopy, Jena, Germany) were used for imaging.

Flow cytometry

For the analysis of surface marker expression, PMs were incubated with fluorochrome-conjugated primary antibodies at optimal dilutions at 4°C for 30 min in PBS containing 0.5% foetal calf serum and 2 mmol/L EDTA. Single-colour staining, isotype-matched controls (IMCs), and fluorescence minus one (FMO) controls were performed. The following antibodies were used:

centrifugation and CD14+ cells were enriched by MACS, transferred to glass slides, and fixed with PFA. Cells were permeabilised with Triton X-100 and co-stained for VSIG4 in combination with the macrophage marker CD68 (top left), the structural component of microtubules β-tubulin (top right), the early endosome-associated protein EEA1 (bottom left), and the LAMP2 (bottom right). Nuclei were stained with DAPI. Representative images with scale bars and inserts are shown. AF, ascitic fluid; BSA, bovine serum albumin; EEA1, early endosome antigen 1; FCS, foetal calf serum; LAMP2, lysosome-associated membrane protein 2; MACS, magnet-activated cell sorting; PFA, paraformaldehyde; PM, peritoneal macrophage; SBP, spontaneous bacterial peritonitis; TAPI-2, tumour necrosis factor protease inhibitor 2; VSIG4, V-set Ig-domain-containing 4.

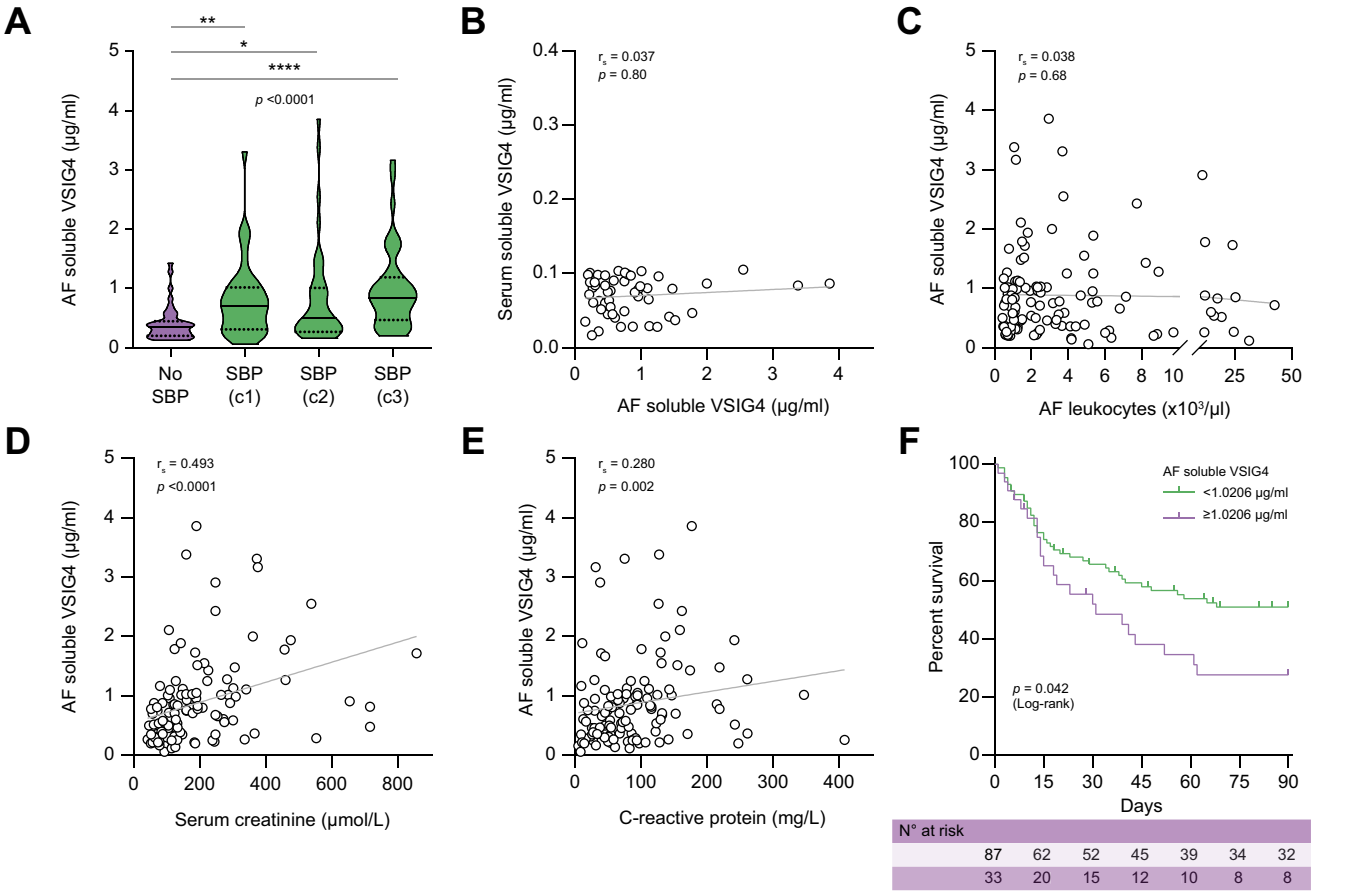

Fig. 4. sVSIG4 in AF identifies patients with SBP at risk for 90-day mortality. (A) Concentrations of sVSIG4 in cell-free AF from 120 patients with SBP as compared with those from 40 patients without SBP. sVSIG4 was measured by ELISA. Violin plots with medians and IQRs are shown. Value of *p* from the Kruskal–Wallis test and significance levels from *post hoc* Dunn's test (**p* <0.05; ***p* <0.01; *****p* <0.0001) are shown. Dot plots and linear regression indicating correlation between the concentration of AF sVSIG4 with (B) serum sVSIG4, (C) AF leucocyte count, (D) serum creatinine concentrations, and (E) serum C-reactive protein in patients with SBP. Spearman's rho (r_s) and *p* values are indicated. (F) Kaplan–Meier curves of 90-day mortality in 120 patients with SBP stratified for AF sVSIG4. Data were right-censored at loss to follow-up or liver transplantation. Value of *p* is from the log–rank test and patients at risk are indicated. AF, ascitic fluid; SBP, spontaneous bacterial peritonitis; sVSIG4, soluble V-set Ig-domain-containing 4; VSIG4, V-set Ig-domain-containing 4.

CD14 (clone HCD14), C-C chemokine receptor type 2 (CCR2) (K036C2), and tyrosine-protein kinase Mer (MERTK) (590H11G1E3) from BioLegend (San Diego, CA, USA); VSIG4 (JAV4) from eBioscience (San Diego, CA, USA); human leucocyte antigen-DR (HLA-DR) (G46-6), CD40 (5C3), CD206 (19.2), CD11c (B-ly6), CD16 (3G8), and programmed cell death 1 ligand 1 (PD-L1) (MIH1) from BD Bioscience (Franklin Lakes, NJ, USA); and CD163 (GHI/61.1) from Miltenyi Biotec. LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit from Thermo Fisher and IMC mouse IgG1 κ and IgG2a κ isotype controls from BD Pharmingen (BD Biosciences, Franklin Lakes, NJ, USA) were used. Analysis was performed using a FACSCanto II flow cytometer and FlowJo (v10) software (BD Biosciences, Franklin Lakes, NJ, USA).

Gene expression analysis

Total RNA was isolated from immunomagnetically enriched PMs using NucleoSpin RNA Kits (Macherey-Nagel, Düren, Germany). RNA concentration and purity were determined by spectrophotometry (DS-11 FX, DeNovix, Wilmington, DE, USA). Total RNA was reverse-transcribed in complementary DNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) in accordance with the protocol provided by the manufacturer. Quantitative real-time PCR (qRT-PCR) was performed using the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) using the following primers: ACCAAGACTGAGGCACCTAC and TCCAAGGTAGCCATCCATGT (VSIG-4), GTGGATTGAACAAGGACGCA and ACTTCTTCACCGCTCTCGTT (CCR2), CATGTACGTTGCTATCCAGGC and CTCCTTAATGTCACGCACGAT (ACTB), and AAACGATTGCAGGGTTTCACC and GCGTTTTTGATCCAGACCCA (GUSB). qRT-PCR was performed using the Rotor-Gene Q (Qiagen, Hilden, Germany) with a 3-step cycling program, with 1 cycle of 95°C for 10 min for initial denaturation, followed by 45 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. SYBR green fluorescence was recorded during the elongation step of each cycle. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative fold change in comparison with house-keeping genes ACTB and GUSB, and melting curves were generated for quality control.

Statistical analysis

Unless otherwise indicated, 2-sided non-parametric tests were used to avoid the assumption of a normal distribution. To perform between-group comparisons, we used Wilcoxon–

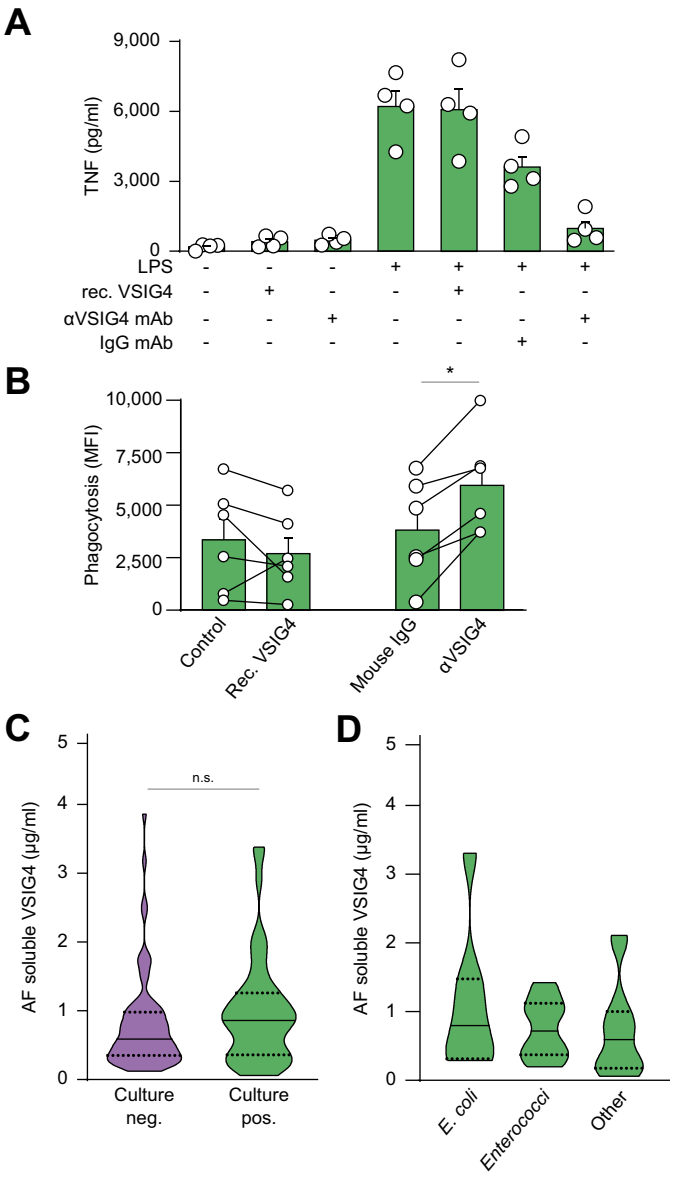

Fig. 5. Assessment of PM functions in the presence of recombinant VSIG4 and anti-VSIG4 antibodies. (A and B) Immunomagnetically enriched PM from patients with cirrhosis without SBP seeded in cell culture plates and incubated with 1 μg/ml of recombinant (rec.) VSIG4, monoclonal antibody targeting the extracellular domain of VSIG4 (αVSIG4), or mouse IgG for 24 h. Subsequently, either (A) cells were stimulated with 10 ng/ml LPS over 4 h and cell-free cell culture supernatant was analysed for TNF using ELISA or (B) cells were incubated with fluorescently labelled opsonised *E. coli* at an MOI of 5 over 2 h and phagocytosis was analysed using flow cytometry. **p* <0.05 in the Wilcoxon matched-pairs signed rank test. (C and D) Concentrations of sVSIG4 in cell-free AF from patients with SBP stratified for AF microbial culture results as using bed-side inoculation of blood culture bottles as per clinical routine. AF, ascitic fluid; MFI, median fluorescence intensity; MOI, multiplicity of infection; n.s., not significant; PM, peritoneal macrophage; SBP, spontaneous bacterial peritonitis; sVSIG4, soluble V-set Ig-domain-containing 4; TNF, tumour necrosis factor; VSIG4, V-set immunoglobulin-domain-containing 4.

Mann–Whitney tests for continuous data or Fisher's exact test for discrete data. Bivariate non-parametric correlation analysis (Spearman) was performed to identify correlations between continuous variables. Continuous variables were dichotomised according to the maximum Youden index in receiver operating characteristics analysis. Time-to-event variables were displayed using the Kaplan–Meier method, and groups were contrasted using log-rank tests. Data were right-censored at loss to follow-up, at liver transplantation, or at 90 days. Univariate and multivariable Cox regression analysis was performed to identify risk factors for short-term mortality using SPSS version 27 (IBM, Armonk, NY, USA). Competing risk analysis was performed using Fine and Gray's proportional sub-hazards model using Stata version 16.1 (StataCorp, College Station, TX, USA). Data were presented using Prism 8 (GraphPad, La Jolla, CA, USA). Owing to the exploratory nature of the study, we applied a 2-sided significance level of $p = 0.05$ for tests without correction for multiple testing. Systematic randomisation and blinding were not performed.

Results

VSIG4^{hi} PMs were identified by flow cytometry using co-staining with CCR2 as this resulted in a better discrimination of distinct cell populations (Fig. 1A) as previously reported.[18] VSIG4^{hi} CCR2^{lo} PMs were larger and more granular than their VSIG4^{lo} CCR2^{hi} counterparts (Fig. 1B) and had significantly higher surface levels of HLA-DR, CD163, CD16, MERTK, PD-L1, and CD206 in the absence of SBP (Fig. 1C).

To investigate whether *VSIG4* expression in PMs was differentially regulated during SBP, we analysed 3 patients with decompensated alcoholic cirrhosis who presented without SBP at presentation and developed SBP during follow-up (Table S1 for baseline characteristics). *VSIG4* gene expression in CD14+ peritoneal immune cells decreased during SBP and normalised to baseline levels thereafter, whereas *CCR2* expression increased during SBP (Fig. 2A). In an independent sample set (Table S2), the median fraction of VSIG4^{hi} cells was 57% in the absence of SBP as compared with 25% in presence of SBP as determined by flow cytometry ($p = 0.0004$, Fig. 2B).

Having shown that *VSIG4* expression and VSIG4 positive PMs are reduced during peritonitis, we went on to investigate which bacterial products induce the release of sVSIG4 from PMs. Untreated PMs that were cultured in plastic cell culture dishes showed a time-dependent release of sVSIG4 into supernatant, which was lower than that in response to the TLR4 agonist LPS at 10 ng/ml, the TLR2/TLR6 agonist FSL-1 at 10 ng/ml, or infection with *E. coli* (Fig. 2C). There were no significant differences in release kinetics after infections with *E. coli* K12 at different MOIs between 3 and 30 (Fig. S1A).

Paired analysis of surface VSIG4 and sVSIG4 revealed that the release of VSIG4 into supernatant was accompanied by an early downregulation of membrane-bound surface VSIG4 after exposure to LPS (Fig. 2D and E). Surface shedding after LPS was similar to the synthetic TLR4 agonist MPLA (Fig. S1B and C).

The mechanisms underlying the release of VSIG4 from human PMs were further investigated. A time-dependent reduction of VSIG4 on the surface (Fig. 3A) along with an increase of sVSIG4 in supernatant (Fig. 3B) was observed under serum-free conditions. Actin polymerisation inhibition with latrunculin B did not affect VSIG4 release from PMs. In contrast, inhibiting microtubule polymerisation with nocodazole preserved VSIG4 surface expression and decreased sVSIG4 in cell culture supernatant (Fig. 3A and B). Involvement of microtubule in VSIG4 transport was further corroborated by immunostaining, and confocal microscopy showed β-tubulin to CD68-positive PMs to

colocalise with VSIG4 (Fig. 3C). Consistent with Helmy *et al.*,[20] we detected VSIG4 colocalising with EAA1-positive early endosomes to some degree but not with LAMP2-positive lysosomes (Fig. 3C). Incubation with the protease inhibitor TAPI-2 diminished sVSIG4 release under serum-free conditions (Fig. 3A and B), suggesting proteolytic cleavage by matrix metalloproteinases (MMPs), TNF alpha-converting enzyme (TACE), or disintegrin and metalloproteinases as 1 main mechanism for sVSIG4 shedding.

We went on to analyse the concentration of sVSIG4 in AF from 120 patients with SBP from 3 cohorts (Table 1) and compared it with that of 40 patients without SBP (Table S3). The median sVSIG4 concentration in AF was 0.73 μg/ml (IQR 0.36–1.05) in patients with SBP as compared with 0.35 μg/ml (IQR 0.20–0.45) in patients without SBP (p <0.0001). This difference was significant across the 3 cohorts (Fig. 4A). During SBP, sVSIG4 concentrations were markedly higher in AF than in serum without significant correlation between VSIG4 concentrations in these 2 compartments (Fig. 4B). We did not observe a correlation of AF sVSIG4 with AF leucocyte count but a moderate correlation with serum creatinine (r_s = 0.493; p <0.0001) and a weak correlation with C-reactive protein (r_s = 0.280; p = 0.002) (Fig. 4C–E). Higher AF sVSIG4 concentration did not correlate with lower concentrations of complement C3b, white blood cell count, total serum bilirubin, international normalised ratio (INR), AF cell count, AF protein, serum albumin, or platelets on a statistically significant level (Fig. S2A, B, and G).

According to Youden's index the best cut-off to discriminate high and low risk of 90-day mortality in cohort 1 was 1.0206 μg/ml with a sensitivity of 48% and a specificity of 76.2% (Fig. S2C). Applying this cut-off resulted in a successful discrimination of patients with poor 90-day outcome in 2 of 3 cohorts of patients with SBP (Fig. S2D–F). In the overall cohort, the cumulative estimate of 90-day survival was 27.7 ± 8.2% SE in patients with AF sVSIG4 of 1.0206 μg/ml or higher as compared with 51.0 ± 5.6% SE in patients with lower concentrations (p = 0.042 in the log-rank test; Fig. 4F).

AF sVSIG4 of 1.0206 μg/ml or higher was associated with higher hazard of death (hazard ratio 1.70; 95% CI 1.01–2.86; p = 0.046) in univariate Cox regression analysis.

In addition, we analysed 90-day mortality defining liver transplantation as a competing risk. In a Fine and Gray regression model, AF sVSIG4 concentrations of 1.0206 μg/ml or higher (subdistribution hazard ratio 1.73; 95% CI 1.05–2.85; p = 0.032) remained a significant predictor of 90-day mortality. Given the relevant correlation of VSIG4 with the model for end-stage liver disease (MELD) score (r_s = 0.350), AF sVSIG4 did not significantly contribute to 90-day mortality prediction, when adjusted for the MELD score in a multivariable model.

Finally, we investigated whether sVSIG4 levels could influence human PM function, suggesting a role beyond that of a mere surrogate marker for activation of large inflammatory PMs during SBP. Incubation with recombinant VSIG4 did not affect LPS-induced TNF release by PMs (Fig. 5A) or phagocytosis of opsonised *E. coli in vitro* (Fig. 5B). In contrast, incubation with a monoclonal antibody binding to the extracellular domain of VSIG4 (KTPESVTGTWKG) unexpectedly reduced LPS-induced TNF release and improved phagocytosis (Fig. 5A). In addition to changes in macrophage differentiation by targeting membrane-bound VSIG4, phagocytosis may also be affected by the availability of C3b for opsonisation and the compensation by other complement receptors. However, clinical data showed no correlation between high AF sVSIG4 levels and bacterial culture results or impaired ability to eliminate certain bacterial pathogens (Fig. 5C and D).

Discussion

Peritoneal inflammation during SBP is a well-regulated event involving the recruitment and activation of neutrophils, macrophages, and inflammatory T-cell subsets.[16,28,29] PMs comprise a key population of resident immune cells fulfilling specialised functions regarding the recognition and phagocytosis of microbial pathogens, the induction and resolution of inflammation, and the activation of resident and recruited immune cells. Accumulating evidence from mouse experimental models indicates a potential role for the immunomodulatory macrophage marker VSIG4 in protecting against bacterial infection, chronic inflammatory diseases, and immune-mediated liver injury (reviewed by Small *et al.*[30]). Here we show that it is released from human PMs after activation and that the concentration of sVSIG4 at SBP diagnosis predicts outcome in patients with SBP.

VSIG4^{hi} PMs from patients with cirrhosis show higher phagocytotic activity and bacterial killing than VSIG4^{lo} PMs alongside features of glucocorticoid-, IL-4/IL-13-, and lipid-activated macrophage signatures, consistent with an alternatively activated macrophage phenotype.[18] Accordingly, the expression of VSIG4 attenuates LPS-induced macrophage activation and cytokine release.[22] However, surface VSIG4 expression is restricted to resting macrophages, and it is lost upon activation.[23] Consistently, the expression of VSIG4 in PMs and the fraction of VSIG4^{hi} PMs was strongly reduced in patients with SBP in our study. This is in agreement with Irvine *et al.*,[18] who observed the lowest VSIG4 expression on PMs from 2 patients with SBP in their study. Our long-term observations suggest that the downregulation of *VSIG4* mRNA in PMs is transient upon inflammatory stimulation and returns to baseline after resolution of inflammation.

The disappearance of VSIG4^{hi} macrophages during SBP may be the result of dilution by a peritoneal influx of VSIG4^{lo} CCR2+ circulating monocytes, a net efflux of VSIG4^{hi} PMs to the omentum,[31] release of VSIG4 from the surface or membrane vesicles,[20] cell death of tissue-resident macrophages, or a combination of the above. In human large PMs, VSIG4 could be localised to the cell membrane and the cytosol and associated to EEA1-positive early endosomes but not LAMP2-positive lysosomes consistent with previous reports.[20] Release of sVSIG4 from PM into cell culture supernatants was dependent on protease-mediated truncation and microtubular transport. Further evidence that the sVSIG4 in AF was of predominantly peritoneal origin was provided as the serum concentrations were significantly lower and did not correlate with peritoneal concentrations.

In addition to VSIG4, researchers have suggested distinguishing human PM subsets by employing differences in the surface expression of CD206 or CD163.[16,18,32,33] Although we could confirm co-expression of these maturity markers on VSIG4^{hi} PMs, the release of surface VSIG4 into cell culture supernatant followed a different kinetic than other soluble macrophage activation markers, such as the mannose receptor CD206, the haemoglobin scavenger receptor CD163, and the urokinase plasminogen activator surface receptor CD87.[16] In contrast to other complement receptors, VSIG4 is localised on

constitutively recycling endosomes and is potentially involved in the constitutive removal of C3-opsonised apoptotic cells and cell debris, thereby preventing peritoneal inflammation.[20] The release of sVSIG4 into cell culture supernatant was largely independent of the inflammatory stimulus applied, and even untreated PMs that were cultured in plastic cell culture dishes showed a lower but constitutive time-dependent release of sVSIG4.

Besides being a marker of PM activation, sVSIG4 might also exert direct immunosuppressive properties by inhibiting complement activation via the alternative pathway[25,26] and suppressing CD8 and CD4 T-cell responses.[23] Given the low concentrations of classical and alternative pathway components in AF from patients with cirrhosis who develop SBP,[34] patients with cirrhosis may be particularly vulnerable to higher peritoneal concentrations of sVSIG4 affecting bacterial clearance. Our data, however, provide no evidence that the peritoneal concentrations of complement C3b are altered in patients with higher AF sVSIG4 concentrations during SBP. Despite the association of AF sVSIG4 with short-term mortality, we did not observe evidence that sVSIG4 is a surrogate marker of the severity of peritoneal inflammation. However, there was a consistent moderate association of AF sVSIG4 with renal function, and thus the MELD score, and a weak association with C-reactive protein.

Our observations suggest a link between peritoneal immune activation, systemic inflammation, and organ failure during SBP and support the use of peritoneal inflammation markers for risk stratification in SBP. In addition to being a surrogate marker for inflammatory macrophage activation during SBP, VSIG4 expression and release may also become a promising therapeutic target for patients at risk for a complicated course of SBP given its role in modulating innate and adaptive immunity. Future studies need to demonstrate whether the depletion of inflammatory PM subsets identified by VSIG4 and strategies improving bacterial phagocytosis by targeting VSIG4 may become promising non-antibiotic strategies for patients with cirrhosis and SBP.

Abbreviations

AF, ascitic fluid; BSA, bovine serum albumin; C3, complement component 3; CCR2, C-C chemokine receptor type 2; EEA1, early endosome antigen 1; FCS, foetal calf serum; FMO, fluorescence minus one; HLA-DR, human leucocyte antigen-DR isotype; IMC, isotype-matched control; INR, international normalised ratio; LAMP2, lysosome-associated membrane protein 2; LPS, lipopolysaccharide; MACS, magnet-activated cell sorting; MELD, model for end-stage liver disease; MERTK, tyrosine-protein kinase Mer; MFI, median fluorescence intensity; MMP, matrix metalloproteinase; MOI, multiplicity of infection; MPLA, monophosphoryl lipid A; PAMP, pathogen-associated molecular pattern; PD-L1, programmed cell death 1 ligand 1; PFA, paraformaldehyde; PM, peritoneal macrophage; qRT-PCR, quantitative real-time PCR; SBP, spontaneous bacterial peritonitis; sVSIG4, soluble V-set Ig-domain-containing 4; TAPI-2, tumour necrosis factor protease inhibitor 2; TLR, Toll-like receptor; TNF, tumour necrosis factor; VSIG4, V-set Ig-domain-containing 4.

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Conflicts of interest

All authors declare no conflict of interest with regard to this manuscript.

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

Authors' contributions

Performed experiments and analyses: JR, MF, OI, SS, SQ, MR, KG, TB. Recruited patients: PL, PAR, TB. Assisted with the experimental design and provided samples and clinical data: PAR, KG, HWZ. Performed statistical analyses: JR, MF, THW, TB. Assisted with the experimental design and the writing of the manuscript: CT, AS. Conceived of and supervised the study, obtained funding, and provided guidance with experimental design and writing of the manuscript: TB. Critically revised the manuscript for important intellectual content: All authors.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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