Original Article

CEACAM-1 Induced CSF3-receptor Downregulation in Bone Marrow Associated With Refractory Neutropenia in Advanced Cirrhosis

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Abstract

Background and Aims: Cirrhosis patients exhibit cytopenia, and, at times refractory neutropenia to granulocyte colony-stimulating factor (G-CSF), which acts through the CSF3-receptor (CSF3R), and changes in CSF3R can affect the response. We conducted this study to assess the CSF3R status and its relevance in cirrhotic patients. ***Methods:*** Cirrhotic patients (*n*=127) and controls (*n*=26) with clinically indicated bone marrow (BM) examination were studied. BM assessment was done by qRT-PCR and immunohistochemistry (IHC) for *CSF3R*. Circulating G-CSF, CSF3R, and carcinoembryonic antigen cell adhesion molecule-1 (CEACAM1) were measured. BM hematopoietic precursor cells and their alterations were examined by flow cytometry. The findings were validated in liver cirrhosis patients who received G-CSF for severe neutropenia. ***Results:*** The mean age was 48.6±13.4 years, and 80.3% were men. Circulatory CSF3R reduction was noted with the advancement of cirrhosis, and confirmed by qRT-PCR and IHC in BM. CSF3R decline was related to decreased hematopoietic stem cells (HSCs) and downregulation of CSF3R in the remaining HSCs. Cocultures confirmed that CEACAM1 led to CSF3R downregulation in BM cells by possible lysosomal degradation. Baseline low peripheral blood-(PB)-CSF3R also predisposed development of infections on follow-up. Decreased CSF3R was also associated with nonresponse to exogenous G-CSF treatment of neutropenia. ***Conclusions:*** Advanced liver cirrhosis was associated with low CSF3R and high CEACAM1 levels in the BM and circulation, making patients prone to infection and inadequate response to exogenous G-CSF.

Introduction

Bone marrow (BM) and the liver retain an evolutionarily conserved developmental relationship. BM responds favorably to peripheral tissue injury and various stress factors and augments tissue repair and innate immune response.[1,2] Granulocyte colony-stimulating factor (G-CSF), is produced by macrophages, endothelial cells, and bone marrow stromal cells.[2] It stimulates neutrophil production and hematopoietic stem cell (HSC) mobilization, both at basal and stress-induced granulopoiesis, which is mediated by granulocyte colony-stimulating factor receptor (G-CSFR), also known as CSF3R or CD114. CSF3R is primarily expressed in BM precursor cells, i.e., HSCs, and has intracellular, transmembrane, and extracellular domains. Although it is a cell surface receptor, extracellular domain shedding can be detected in the peripheral circulation.[3]

Several hematological abnormalities are associated with the progression of cirrhosis, including severe cytopenia, leukopenia, and neutropenia with a predisposition to infection. Neutropenia refractory to exogenous G-CSF treatment is occasionally seen in advanced liver cirrhosis.[4] Increased carcinoembryonic antigen cellular adhesion molecule-1 (CEACAM-1) can act inhibit CSF3R.[5] We wondered whether leukopenia and refractory neutropenia to G-CSF in patients with liver cirrhosis could be attributable to quantitative and qualitative variations in CSF3R. Alterations in CSF3R could be related to changes in the microenvironment or single

nucleotide polymorphisms in the CSF3R gene. This study aimed to assess the status and mechanism of CSF3R in liver cirrhosis and its association with leukopenia, refractory neutropenia, and naturally acquired infections.

Methods

Patients

This prospective cohort study included a patient baseline assessment and a 6 month follow-up for naturally acquired infections. The study included all 127 cirrhotic patients who underwent BM examinations between Jan 2017 and Jun 2019 at the Institute of Liver and Biliary Sciences, New Delhi. BM examination was performed as per the clinical indications, i.e., cytopenia, pyrexia of unknown origin, and dyserythropoiesis in refractory anemia as described in our previous study.[6] Clinical indications were similar across the Child-Turcott-Pugh (CTP) classes (Supplementary Table 1). In these cirrhosis patients, the major etiology was ethanol-related, followed by viral, nonalcoholic steatohepatitis, and cryptogenic. Cirrhotic patients with associated neoplastic diseases and suboptimal tissue (i.e., three marrow spaces) were excluded. The Institutional Ethics Committee of the Institute of Liver and Biliary Sciences, New Delhi, India, approved this study protocol (IEC/81/MA06). Informed consent was obtained from patients to use their leftover samples and clinical data after rendering the diagnosis. The leftover BM aspirate and PB, plasma, and cells were isolated and stored in the national liver disease biobank with the clinical annotation. Fresh samples (*n*=26) were used for flow cytometry assays. Individuals without liver diseases, chronic ailments, or neoplastic pathologies in whom BM examinations were done to investigate infiltrative or infective diseases were considered as controls. Those who did not have any systemic or BM pathology were eligible, as previously described.[7,8]

BM examination

BM aspirates and biopsies were examined for a differential myelogram, various hematopoietic cell morphologies, fibrosis, and iron stores. For quantitative reverse-transcription polymerase chain reaction (qPCR), total RNA was isolated with RNeasy Mini Kits (Qiagen, Limburg, Netherlands) from stored BM biopsy tissue, treated with DNase-I (Ambion, Life Technologies, Carlsbad, CA) following the manufacturer's instructions, and reverse transcribed to complementary (cDNA; Applied Biosystems, Life Technologies, Carlsbad, CA) following the manufacturer's instructions. cDNAs were augmented with SYBR green qPCR master mix (Thermo Fisher Scientific) for 40 cycles on a ViiA 7 PCR system (Applied Biosciences). Transcripts were progressively expanded for gene with gene-specific specific primers shown in Supplementary Table 2. Gene expression was normalized against the 18S ribosomal RNA housekeeping gene as a standard internal control and negative control.

BM Immunohistochemistry

BM biopsies were stained immunohistochemically for antibodies raised against CSF3R (PAB476Hu01), hematopoietic cell kinase (HCK, PAC522Hu01), splenic kinase (SYK, PAE-275Mu01), and carcinoembryonic antigen cellular adhesion molecule (CEACAM1, MAC977Hu22, (all from CloudClone, Katy, TX, USA). Cytoplasmic/surface positive cells were counted in 25 high-power fields (20×, 1.3 mm²), and aver-

age number of cells/field was reported. Positive cells were assessed by Image J software.

ELISA

ELSA of human G-CSF (E-EL-H0079) and CSF3R (E-EL-HO799, both from Elabscience, Hubei, China, and CEACAM-1 (CSB-EL005157HU, Cusabio, Hubei, China) were performed on BM and peripheral blood (PB) plasma from cirrhosis patients and control participants.

Flow cytometry

Immunostaining was done using the whole BM aspirate sample. RBCs were lysed in 1× RBC lysis buffer for 10 m, kept on ice followed by washing with 1× phosphate-buffered saline (PBS) and centrifuged at 4°C for 5 m at 2,000 rpm. Cells were immunostained by antibodies raised against CD45 (cat. 560975) CD34 (cat. 555824), CSF3R (cat. no. 554538), and TLR3 (cat. no. 565984, all from BD biosciences, San Diego, CA, USA). After washing with 1× PBS, cells were fixed with 0.1% paraformaldehyde and assayed by flow cytometry.

For granulocyte-macrophage colony-forming unit (CFU-GM) and burst forming unit-erythroid (BFU-E) cultures, BM mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation and then resuspended in RPMI 1640 (Sigma) containing 10% fetal bovine serum (Sigma). The BM mononuclear cells (BMMCs) were plated at 1×10^6 cells/mL duplicated in Iscove's media (Cellgro). One culture was supplemented with 2 U/mL recombinant human erythropoietin and 6 U/ml recombinant human interleukin-3 (Genetics Institute, Cambridge, MA) for burst forming units-erythroid (BFU-E). The other was cultured with 3 U/mL recombinant human granulocyte-macrophage stimulating factor (rhGM-CSF) for colony-forming unit granulocyte-macrophage (CFU-GM). Cultures were incubated at 37°C in 5% CO_2. Colonies were counted at the end of incubation, 10 days for CFU-GM and 14 days for BFU-E colonies.

In vitro treatment with CEACAM-1

BMMCs were isolated from CTP-A BM aspirates and (1×10^5 cells in 500 µL RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% antibiotic, 1% nonessential amino acids, and 10% glutamine were seeded in each well of 24-well culture plates. Cultured cells were treated with a CEACAM1 compound (Prospec, Pro-2352) at a concentration of 200 ng/ml and incubated at 37°C in 5% CO_2 for 24 h. Cell survivability was checked by trypan blue, and toxicity assays were done with tetrazolium. Cells were collected by fluorescence-activated cell sorting (FACS) analysis using CD34-FITC and CSF3R-APC antibodies. Sanger sequencing for CSF3R was carried out in PB samples of all 174 study participant to identify any single nucleotide polymorphisms (SNPs) in the *CSF3R* gene.

Statistical analysis

Statistical analysis was carried out with SPSS, version 22.0 (IBM Corp. Armonk, NY, USA) and MedCalc version 20.0 (https://www.medcalc.org/). Student's t-test or the Mann-Whitney test was used to evaluate between-group differences of data with parametric or nonparametric distributions. One-way analysis of variance for multiple-group comparisons of parametric data and the Kruskal-Wallis test

Table 1. Baseline characteristics of controls and cirrhosis patients

	Control (*n*=26)	Child-Turcott-Pugh class-A {CTP A (*n*=36)}	Child-Turcott-Pugh class-B {CTP- B (*n*=54)}	Child-Turcott-Pugh class-C {CTP-C (*n*=37)}
Age, years	37 (27–48)	54 (42–64)	48 (39–58)	49 (41–58.8)
Sex, male: female	21/5	27/9	43/11	32/5
Etiology, ethanol/viral/non-alcoholic steatohepatitis/cryptogenic	NA	14/11/9/2	27/14/5/8	19/9/4/5
Hemoglobin, g/dL	9.4 (8.4–11.7)	8.3 (7.3–9.5)	8.2 (7.3–10.8)	7.9 (7.8–9.6)
Total leukocyte count ×10^6/L	6 (4.9–10.7)	4.2 (3.2–9)	4.7 (2.9–7.9)	4.2 (2.7–8.6)
Platelet count ×10^9/L	150 (126–204)	52 (29–142)	51 (40–126)	42.5 (30–79)
International normalized ratio	1 (1.1–1.4)	1.43 (1.3–1.73)	1.54 (1.3–2)	1.7 (1.43–2.2)
Total bilirubin, mg/dL	1.45 (0.8–1.8)	1.9 (1–2.6)	2.2 (1.6–4.9)	2.2 (1.9–5)
Albumin, g/dL	3.8 (3.5–4.5)	2.9 (3–3.8)	2.7 (2.4–3.2)	2.4 (2.2–3)
Serum creatinine, mg/dL	0.7 (0.6–0.9)	0.8 (0.7–0.9)	1.2 (0.7–1.8)	1.8 (1.2–2.2)
Lactate dehydrogenase, IU/L	560 (413–740), n=15	575 (398–712), n=16	559 (434–759), n=43	550 (480–684), n=23
Serum ferritin, mg/dL	841 (215 1,457), n=8	111 (13.6–567), n=11	165 (45.9–458), n=15	138 (46.9–669.5), n=13
Bone marrow reticulin, 0/1/2/3	25/1/0/0	32/3/1/0	49/5/3/0	24/10/2/0
Bone marrow cellularity, %	60(45–80)	65 (45–80)	55 (50–75)	55 (50–80)
CD34 cells in bone marrow biopsy, 20×	20 (15–35)	22 (16–40)	14.5 (10–21)	10 (7–18)

Data are medians (interquartile range).

for multiple-group comparisons of nonparametric data. *P*-values <0.05 were considered significant.

Results

Participant characteristics

From January 2017 to June 2019, 268 BM examinations were done at the Institute of Liver and Biliary Sciences for various clinical indications. One hundred forty-five (145) were in cirrhotic patients, and 29 were in patients without liver disease or pathologies who were considered controls. The other 94 patients had other diseases and were excluded. The BM of six cirrhotic patients and three controls was suboptimal, and 12 cirrhotic patients had associated neoplastic pathology and were excluded from the study. The selection of study participants is summarized in Supplementary Figure 1. In the cirrhosis group, 36 patients (28.3%) were class CTP A. 54 (42.6%) were CTP B, and 37 (29.1%) were CTP-C. The most frequent etiology was alcohol-associated cirrhosis (41.1%). The bassline characteristics of the CTP classes and controls are summarized in Table 1.

CSF3R in cirrhosis

We assessed the ligand (G-CSF) and receptor (CSF3R) in the BM and PB plasma. The plasma levels of G-CSF were increased (Fig. 1A), whereas the receptor levels were decreased (Fig. 1B) with the advancing stages of cirrhosis. The G-CSF levels were confirmed at the transcriptional level by qRT-PCR of the BM biopsies (Fig. 1C). Immunohistochemis-try also showed reduced numbers of CSF3R+ cells/20× field with cirrhosis progression. (Fig. 1D). The results indicate reduced CSF3R expression in the BM precursor cells. The differences in CSF3R in BM (*p*=0.439) and PB (0.850) of patients with various cirrhosis etiologies were not significant. The BM- and PB-CSF3R levels of male and female cirrhosis patients were comparable (*p*=0.539 and *p*=0.131).

During a follow-up of 6 months, 26 patients had naturally acquired infections, seven had pneumonia, six had urinary tract infection, 10 had spontaneous bacterial peritonitis, and three had other infections. The patients who developed infections had lower BM-CSF3R (*p*=0.056) and PB-CSF3R (*p*=0.022) levels than those who did not develop infections (Fig. 1E). Patients with PB leukopenia (total leukocyte count <4×10^6/L, *n*=31) also lower levels of CSF3R in the BM (*p*=0.001) and PB (*p*=0.217, Fig. 1F).

Low CSF3R and neutropenia in cirrhosis

PB-CSF3R correlated with the total leukocyte count (ρ=0.603, *p*=0.022) and absolute neutrophil count (ρ=0.731, *p*=0.01). Baseline PB-CSF3R sensitivity was 86.96% (95% CI: 66.4%-97.2%) and specificity was 84.62% (77.2%-90.3%) for severe neutropenia (neutrophils 1,000/mm) with an area under the curve (AUC) =0.907, *p*<0.001) and a cutoff value of 36.8 pg/mL (Fig. 2A). It showed moderate 61% sensitivity and 59% specificity for the occurrence of infection, with an AUC=0.677, *p*=0.004 (Fig. 2B), with a cutoff value ≤55 pg/mL.

G-CSF was given to 15 patients with severe neutropenia. Six did not have an improvement in leukocyte count. It was noted that nonresponders had lower blood CSF3R levels, and of those, baseline CSF3R was almost undetectable in three (Fig. 2C).
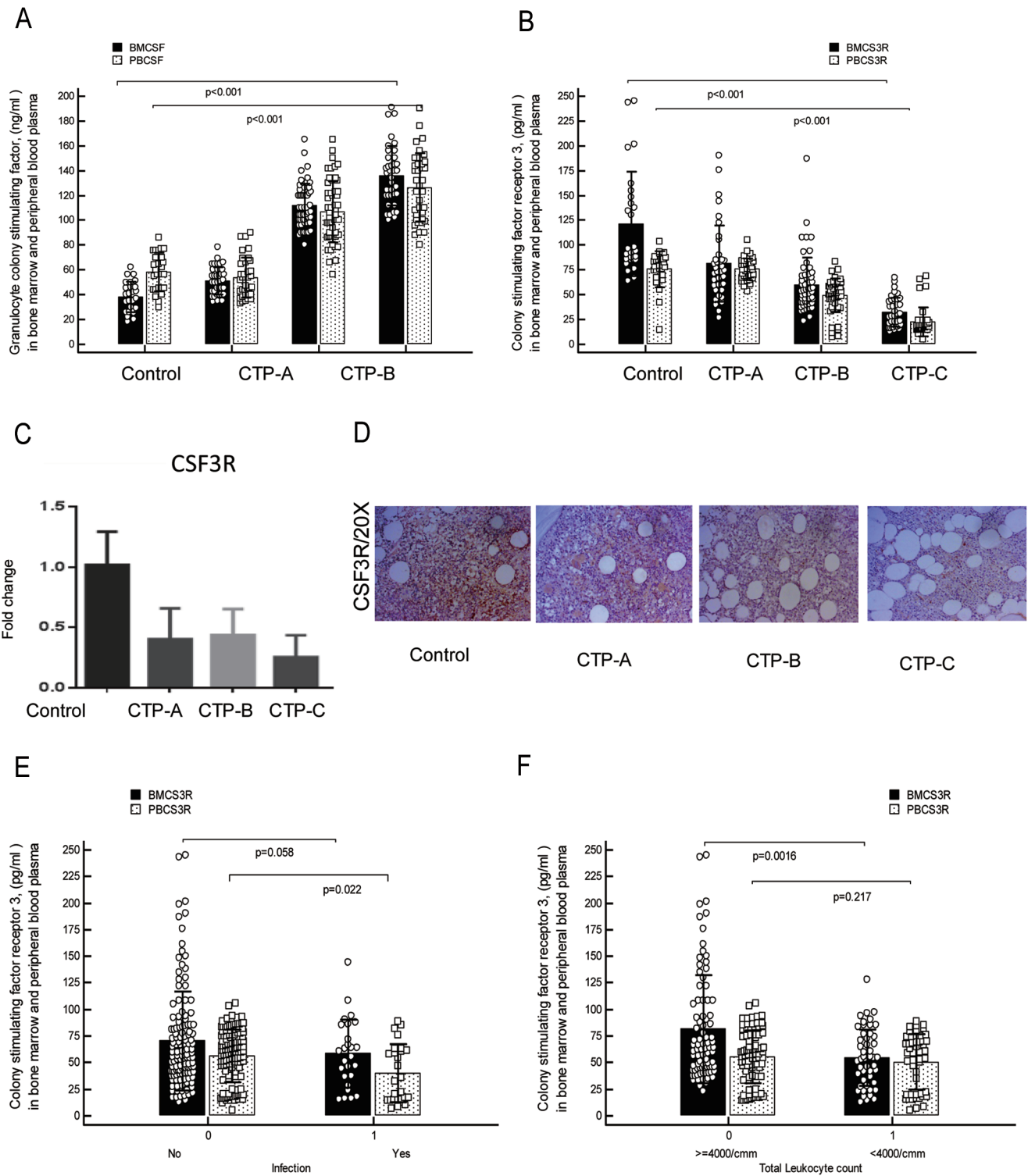
Fig. 1. GSCF and CSF3R in controls and CTP class A, B, and C cirrhosis patients. (A) Granulocyte colony-stimulating factor (G-CSF) levels in the bone marrow plasma and peripheral blood plasma of controls and (mean±SD). (B) Colony-stimulating factor receptor (CSF3R) levels in the bone marrow plasma and peripheral blood plasma of control and cirrhosis cases by CTP-A, -B, and -C (mean±SD). (C) Quantitative RT-PCR of colony-stimulating factor receptor 3 (*CSF3R*) mRNA in bone marrow biopsies (mean±SD), *n*=5 each. (D) Representative images of Immunohistochemistry (IHC) expression of *CSF3R* in bone marrow biopsies of control and Child-Turcott-Pugh (CTP)-A, B-, C- classes of cirrhosis, all cases. (E) Comparison of baseline colony-stimulating factor receptor (CSFR) levels in bone marrow plasma and peripheral blood plasma of cirrhotic patients with naturally acquired infection during follow-up, and (F) cirrhotic patients who had leukopenia and with normal total leukocyte count, all cases. **p<0.001, *p=0.001 to <0.05; ns, not-significant.
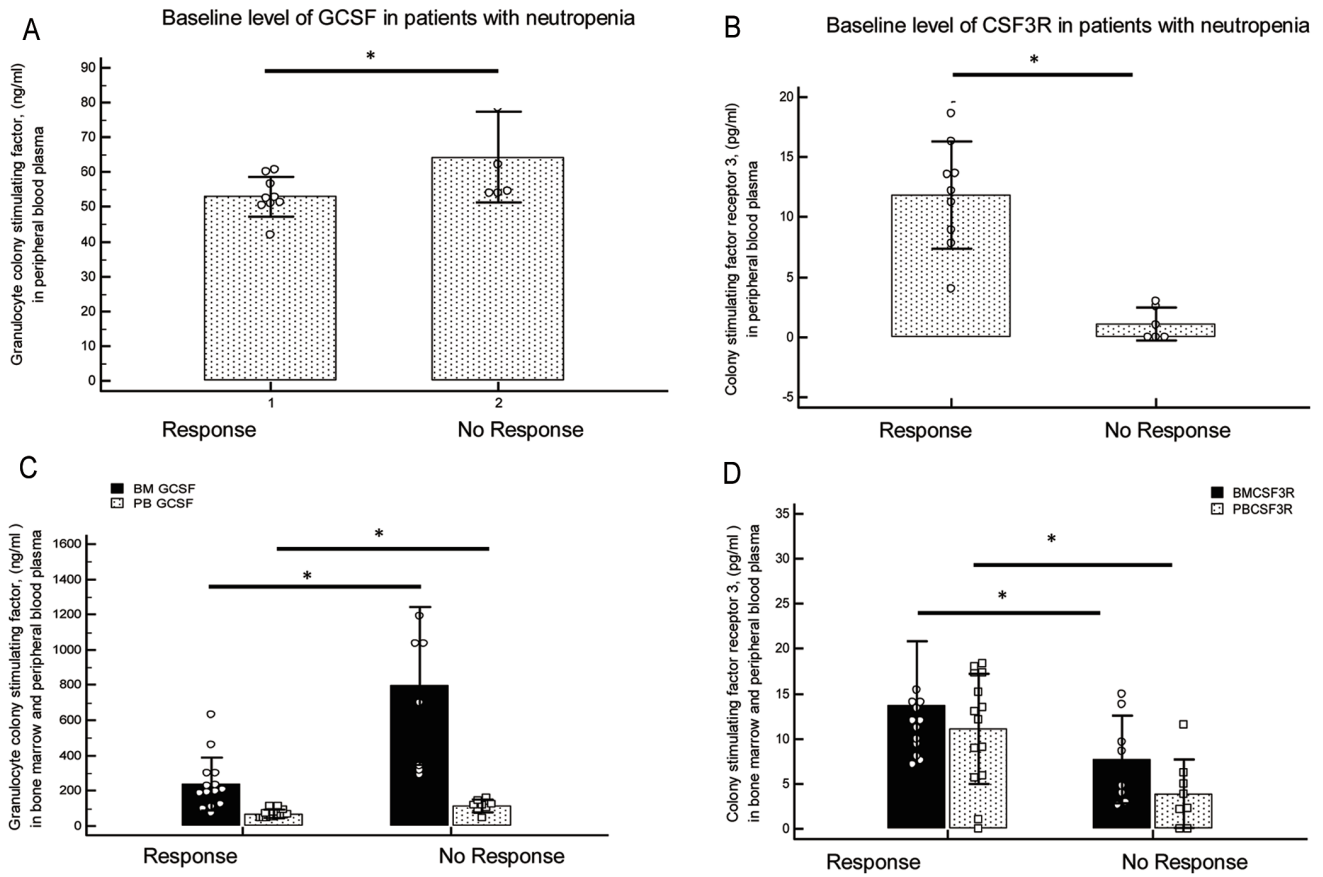
Fig. 2. Association of Colony-stimulating factor receptor (CSF3R) with neutropenia and infection. (A) Area under the curve of circulatory colony-stimulating factor receptor (CSF3R) level in peripheral blood for neutropenia and (B) infection. (C) Baseline Colony-stimulating factor receptor (CSF3R) in peripheral blood plasma from the original cohort (*n*=15) and (D) Validation cohort (*n*=22) of cirrhosis patients who responded to granulocyte- colony stimulating factor (GCSF) treatment for neutropenia vs. nonresponders {median, interquartile range (IQR) }. **$p<0.001$, *$p=0.001$ to <0.05; ns, not significant.

Validation

We took a separate cohort where G-CSF therapy was given to 22 patients with severe neutropenia from July 2019 to December 2020. Fourteen were responsive and eight were nonresponsive to treatment (Supplementary Table 3). Baseline PB-CSF3R levels were lower in BM and PB in patients who eventually did not respond to the therapy (Fig. 2D).

Low CSF3R levels relate to both decreased stem cell population and downregulation of surface receptors

Because the decrease in CSF3R receptors could result from a decrease of BM progenitor cells, counted those cells in the various classes of cirrhosis patients. As expected,[7] we found lower CD34+ cells in CTP class B and class C than in class A patients and in controls (Fig. 3A, B). Further assessment revealed that the percentage population of CSF3R+ CD34+ cells of the remaining total CD34+ cells also showed a declining trend with advancing cirrhosis (Fig. 3A, C). The reduced stable pool of CD34+ cells could have been caused by an increase of circulating G-CSF. The mobilized HSCs led to the formation of CFU and BFU in BM. We found that the CFU-GM and BFU-E were more pronounced in CTP A or B patients. (Fig. 3D). The data indicate that the low CSF3R in advanced cirrhosis was

related to decreased CD34 cells in the BM and downregulation of CSF3R in the remaining CD34 cells in the BM.

Carcinoembryonic antigen cell adhesion molecule-1(CEACAM1) downregulates CSF3R in cirrhosis

CEACAM1 (biliary glycoprotein) acts as a coinhibitory molecule for CSF3R and downregulates the CSF3R-STAT3 pathway.[4] qPCR showed that *CEACAM-1* was upregulated in cirrhosis BM compared with controls (Fig. 4A). IHC revealed that CEACAM1+ BM precursor cells were more pronounced in CTP-C and B than in CTP A patients and controls (Fig. 4B). Circulating CEACAM1 in BM (Fig. 4C) and PB plasma (Fig. 4D) showed a similar trend (both $p<0.001$), with the highest level in the CTP-C class. CEACAM1 levels in BM and PB plasma were negatively correlated with the CSF3R ($\rho=-0.668$, $p<0.001$ and $\rho=-0.729$, $p<0.001$, respectively). In contrast, BM-G-CSF and PB-G-CSF levels correlated positively with BM-CEACAM-1 ($\rho=0.726$, $p<0.001$) and PB-CEACAM-1 ($\rho=0.719$, $p<0.001$), respectively. CEACAM-1 is expressed mainly in the biliary and colonic epithelium. So we did IHC in this cohort's available liver biopsies (*n*=14, with six controls, three CTP-A, three CTP-B, and two CTP-C patients. Interestingly, we noted that in cirrhosis, especially in advanced cirrhosis, there was an increased hepatic expression of CEACAM-1 (Supplementary Fig. 2). In-vitro, cultured BMMCs from controls without cirrhosis and treated with CEACAM-1 for 24 h had a significant
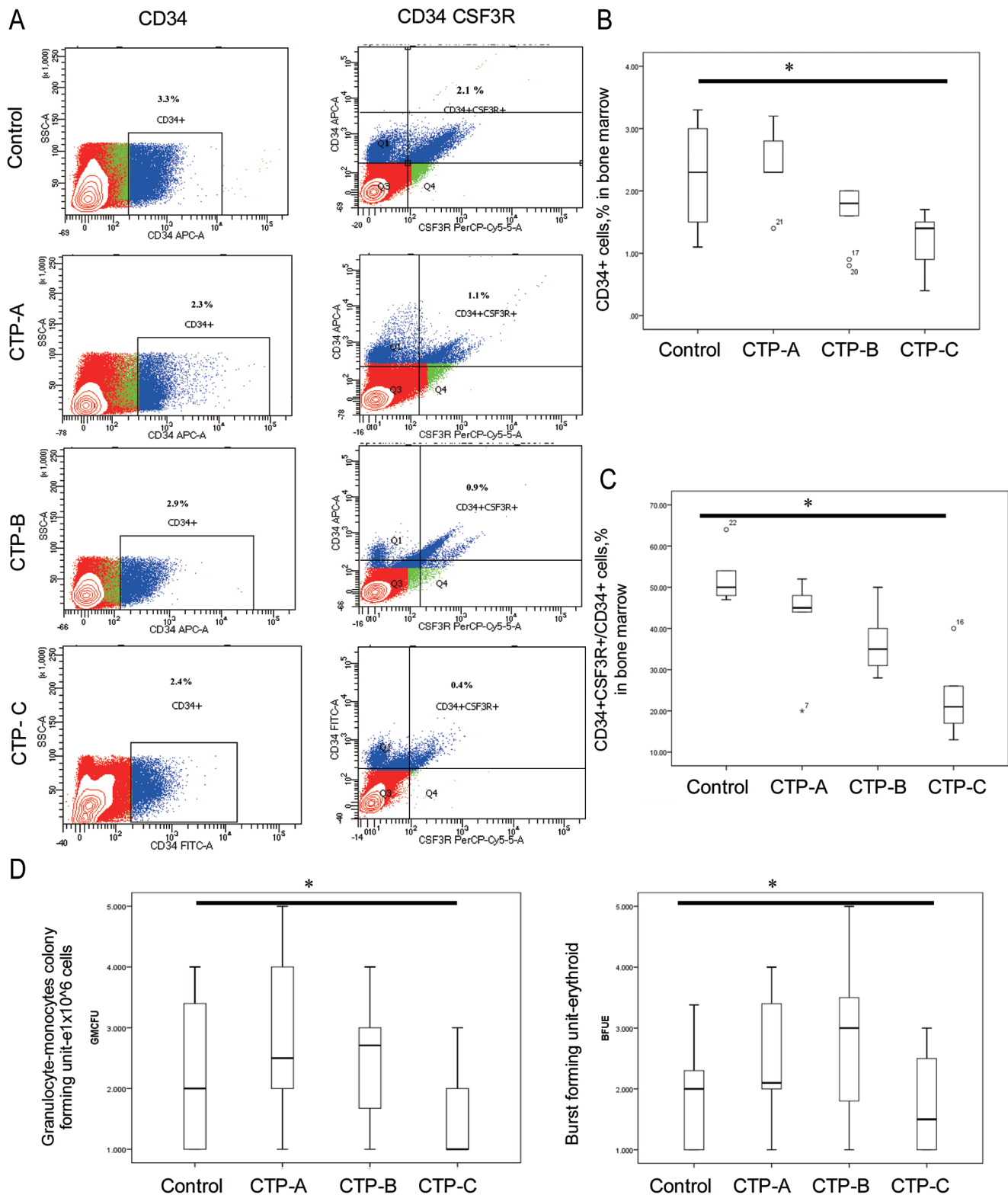
Fig. 3. Status of CSF3R in BM precursor cells. (A) Representative images of flow cytometry of bone marrow aspirates with acquired CD34+ cells (%) and CSF3R+CD34+ cells (%) in control and cirrhosis Child-Turcott-Pugh (CTP) classes A,B,C), *n*=25 each. (B) Distribution of CD34+ cells % in control and cirrhosis (CTP classes A, B, C), median, interquartile range (IQR), *n*=25 each. (C) Distribution of CSF3R+CD34+ (%) of total bone marrow C34+ cells, median (interquartile range), *n*=25 each. (D) Distribution of granulocyte monocyte-colony forming unit (GM-CFU) and burst forming unit-erythroid (BFU-E) across the study groups, median (inter-quartile range), *n*=10 each. **p<0.001, *p=0.001 to <0.05; ns, not significant.
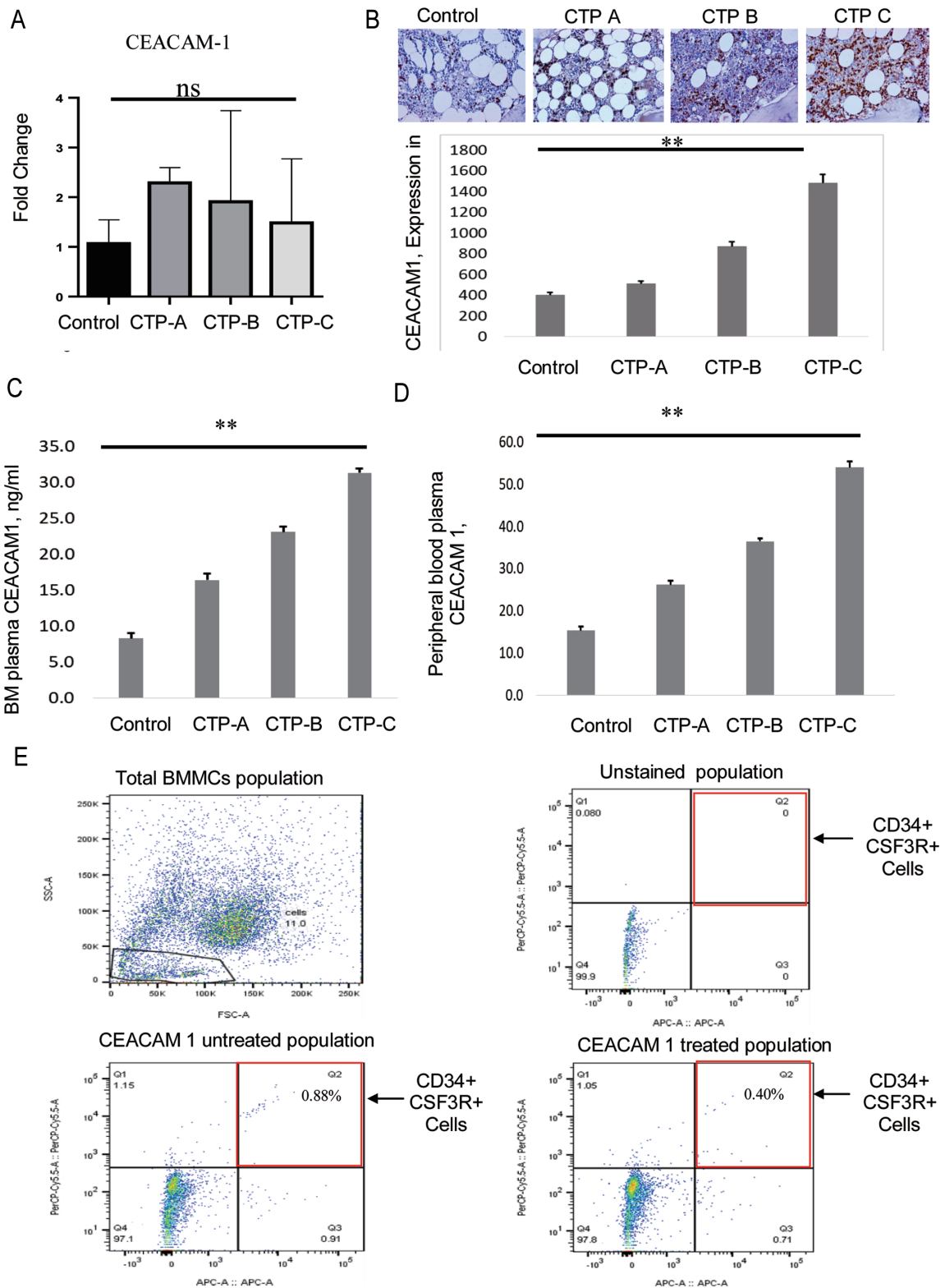
Fig. 4. CEACAM-1 in cirrhosis and its association with CSF3R. (A) mRNA expression of, carcinoembryonic antigen cell adhesion molecule-1 (*CEACAM-1*) gene in the bone marrow biopsies of control and cirrhosis classes (mean±SD), *n*=5. (B) Representative images and graphical representation of CEACAM-1+ bone marrow cells in control and cirrhosis classes (20×). (C) Graphical representation of CEACAM-1 in bone marrow plasma (mean±SD). (D) CEACAM-1 levels in the peripheral blood plasma of control and cirrhosis classes (mean±SD), and (E) representative images of flow cytometry of bone marrow mononuclear cultured cells display the difference in CD34+CSF3R+ cells without CEACAM-1 and with CEACAM-1 treatment for 24 h, *n*=5. **p<0.001, *p=0.001 to <0.05; ns, not significant.

mean reduction (−21.8%, *p*=0.031) of CD34+CSF3R+ cells compared with untreated cells (Fig. 4E).

CEACAM-1 reduced CSF3R expression, possibly by lysosomal degradation

Because of elevated CEACAM1 levels, it is likely that the CSF3R receptor may be degraded in lysosomes and cannot be recycled. To test that hypothesis, we assessed the lysosomal contents in sorted BM CD34 cells by CXCR4 immunocytochemistry. We noted that the lysosome contents increased in cirrhosis patients compared with controls. Moreover, incremental changes were noted with the progression of cirrhosis (Fig. 5A, B). Lysosomes were assessed by flow cytometry of TLR3 in CD34 cells. The number of CD34+ CSF3R− TLR3+ cells were increased (*p*<0.001, Fig. 5C). CD34+CSF3R+TLR3− declined (*p*=0.001) with the severity of liver cirrhosis (Fig. 5D). Likewise, BM precursor cells showed incremental ubiquitin expression with cirrhosis progression (*p*=0.007, Fig. 5E, F). CD34+ CSF3R−TLR3+ and ubiquitin+ cells were found to be associated with BM CEACAM-1+ cells (ρ=0.642, *p*<0.001 and ρ=0.459, *p*<0.001, respectively).Thus, the increasing lysosomal content in CD34+ cells and increased ubiquitin in BM indirectly suggest that the CSF3R was degraded, possibly because of elevated CEACAM1.

CSF3R SNPs

As variation in the G-CSF response could also be associated with the genetic polymorphism of *CSF3R*, we tested for SNPs in the *CSF3R* gene. No SNPs were detected in this study population.

Discussion

This study found that the CSF3R G-CSF receptor levels were downregulated in liver cirrhosis, more so with the advancement of liver disease severity. The reduction in CSF3R was related to hematopoietic niche alterations, decreased HSC counts in BM, and reduced expression of CSF3R in remaining HSCs. It was related to elevated CEACAM-1 in circulation as well as in the BM. On validation, it was noted that patients with severe neutropenia who did not respond to G-CSF therapy had decreased CSF3R at baseline. This is the first human study to explore CSF3R in liver cirrhosis to the best of our knowledge. We argue that the lowered CSF3R in cirrhosis could determine the response to G-CSF treatment in neutropenic cirrhotics.

G-CSF has been a great success in the setting of chemotherapy-induced leukopenia and as a CD34+ mobilizing agent in BM transplants. Nevertheless, there have been concerns related to G-CSF-induced mobilization. The concerns are related to HSC niche disruption,[9] underlying chronic disease,[10] and genetic polymorphisms of hematopoietic niche molecules (CSF3R, VCAM).[11,12] The disruption of the HSC niche and the underlying chronic ailment issues are linked. We showed that HSCs and their niche components are adversely affected in advanced cirrhosis.[7,8] Impaired glucose tolerance in diabetes can cause an inadequate response to G-CSF. Likewise, another study from our group revealed that BM-MSCs have insulin resistance and impaired bioenergetics in cirrhotic patients.[13] Genetic polymorphisms of CSF3R were not identified in this study, which indicates a limited role of CSF3R polymorphisms and G-CSF therapy-related responses in cirrhosis patients.

No etiological differences were noted in the BM-CSF3R, PB-CSF3R, and CD34 cells in the study population (*p*=0.122),

which is consistent with the findings of our previous studies.[7,8] Thus, in advanced cirrhosis patients, primarily the disruption of the stem cell niche and possibly impaired glucose tolerance at the cellular level could induce a suboptimal response to GCSF. The disruption of the stem cell niche is reflected in this study by the decline of CD34 cells and the reduction of CSF3R in remaining CD34 cells. BM-CD34 cells were also lower in those who acquired infections than in those who did not, which indicates that quantitative and qualitative changes occur in the BM progenitor cells and cause hematological and immunological dysfunction in cirrhotic patients.

Downregulation of CSF3R was inversely correlated with CEACAM-1 in BM and circulation. CEACAM-1 is a CSF3R-inhibitory molecule.[14] Physiological CEACAM-1 is expressed mainly in the colonic and biliary epithelium,[15] and increased ductular reactions and metaplastic hepatocytes in cirrhosis can explain the excess of CEACAM-1 in circulation. CEACAM-1 act by contact with the cell surface and is a receptor/ligand.[16] So, CEACAM-1 is not produced by the BM, but instead is secreted by the diseased liver and possibly by the affected colonic epithelium in cirrhosis, and it is released into the circulation. Excessive CEACAM-1 acts as a coinhibitory receptor for CSF3R,[16,17] and after it is phosphorylated, it recruits SHP-1. SHP-1 downregulates G-CSFR by decreasing its phosphorylation, attenuating its downstream Stat3 activation, and the expression of mitogenic proteins Cyclin D1 and C-Myc. Thus, CEACAM-1 controls the proliferation and differentiation of myeloid progenitors.[18] Excess CEACAM-1 downregulates CSF3R, as we showed in the in-vitro experiment. CSF3R is degraded in the lysosomes by ubiquitination[3,19] and increased expression of TLR3 and ubiquitin and their inverse correlation with CSF3R indirectly supports possible lysosomal degradation of CSF3R under the influence of excess CEACAM-1. Elevated G-CSF could be harmful, especially if it occurs rapidly (20-50 times control, as in severe trauma).[20,21] Patients with advanced liver cirrhosis had G-CSF levels 2–4 times higher than controls. Persistently increased GSCF may induce HSCs to enter into refractory colony formation stages and lead to further decline in the BM pool of HSCs.

The study has some limitations. It may have been subject to selection bias because we only included cirrhotic patients who had BM tests. However, the clinical indications for undergoing bone marrow biopsy across the CTP classes were similar, and we consecutively enrolled all cirrhotic patients who needed BM examinations as per clinical indication. Hence, we expect the potential selection bias to be minimal.

The study has important clinical significance. It shed light on a few mechanistic aspects with important therapeutic implications. CSF3R assessment could be helpful for those patients who could would benefit from G-CSF therapy. CS3R improvement may be achieved by the effects of humanized anti-CEACAM-1 IgG4 antibody-like MK-6018[16,22] in the remaining HSCs of the BM of cirrhotic patients.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

CB, SMS and SKS conceptualized the study protocol; CB and SB designed the experiments; CB, SB, PN, DL performed
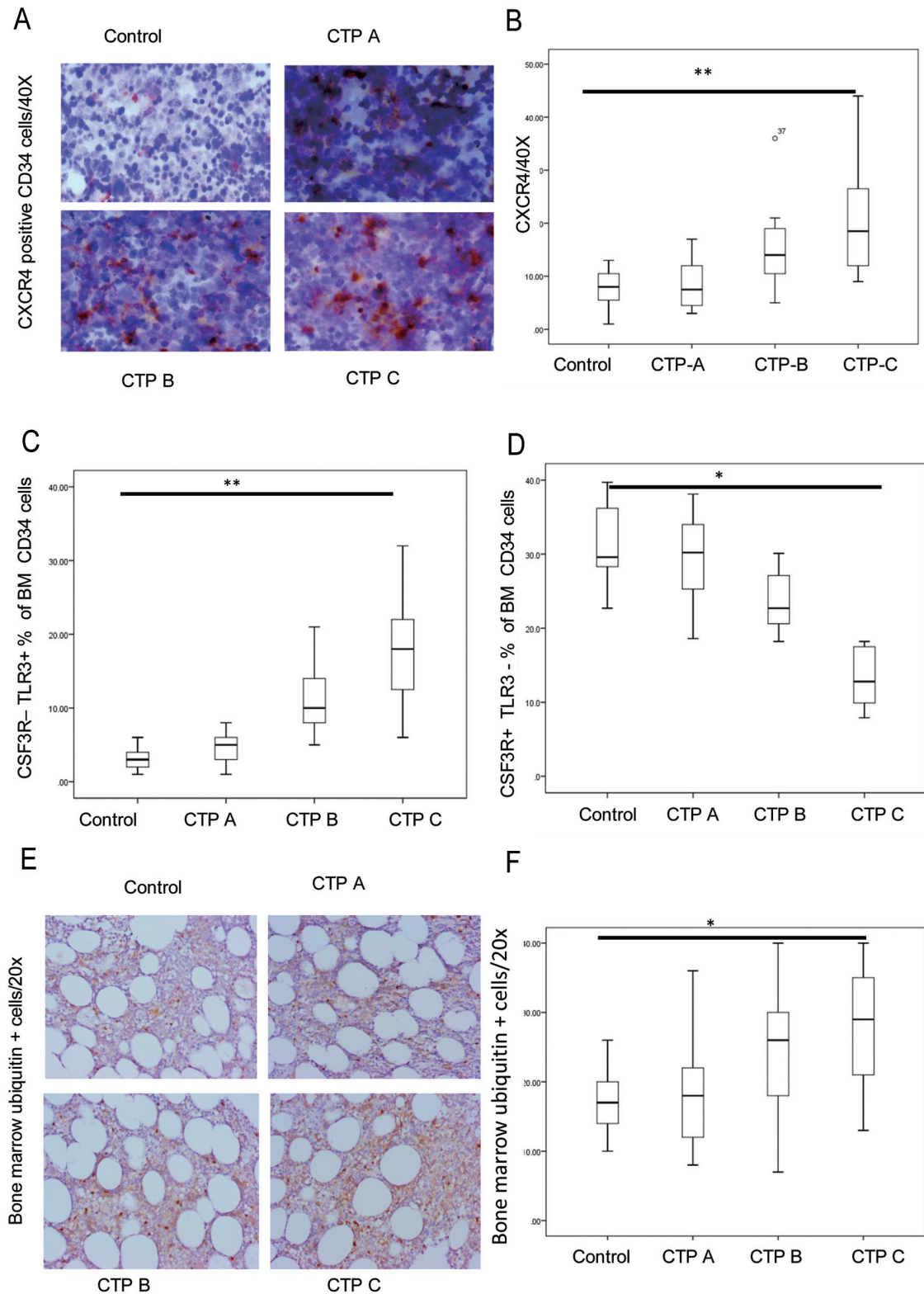
Fig. 5. Increased lysosomes in the bone marrow cells of cirrhosis patients. (A) C-X-C chemokine receptor type 4 (CXCR4+) cells in cytospin smears of sorted CD34 cells from bone marrow in the control and cirrhosis classes (Giemsa, 20×), and (B) Graph of distribution, median (interquartile range), *n*=10 each. (C) Distribution of CSF3R−TLR3+ CD34 cells, and (D) CSF3R+TLR3− CD34 cells in control and cirrhotic classes, median (interquartile range), *n*=25 each. (E) Representative images of ubiquitin-positive cells/20 fields in the bone marrow of study groups (20×) and (F) graphical representation of the cells across the study groups, median (interquartile range), all cases. **$p<0.001$, *$p=0.001$ to <0.05; ns, not significant.

the experiments; CB did the pathological analysis; SB did the experimental data analysis. SMS, SKS, BN, VP provided clinical data and inputs; CB drafted the manuscript; SKS revised it. SKS provided technical and administrative support.

Data sharing statement

Data used for this manuscript are available on request.

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