GMCSF modulates Myeloid derived suppressor cells and Tregs activity in decompensated cirrhotic patients with sepsis

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Abstract

Background

Decompensated cirrhosis patients are more prone to bacterial infections. Myeloid derived suppressor cells (MDSCs) expand in sepsis patients and disrupt immune cell functions. GM-CSF therapy helps in restoring immune cell functions and resolve infections. Its role in MDSCs modulation in cirrhotic with sepsis is not well understood.

Methods

164 decompensated cirrhotic; 62 without(w/o), 72 with sepsis and 30 with sepsis treated with GM-CSF and 15 healthy were studied. High-dimensional flow cytometry was performed to analyse MDSCs, monocytes, neutrophils, CD4 T-cells and Tregs at admission, day3 and 7. *Ex-vivo* co-cultured MDSCs with T-cells were assessed for proliferation and apoptosis of T-cells, differentiation to T-regs. Plasma factors and mRNA levels were analysed by cytokine-bead assay and qRT-PCR.

Results

Frequency of MDSCs and T-regs were significantly increased (p=0.011, and p=0.02) with decreased CD4 T-cells(p=0.01) in sepsis than without sepsis and HC (p=0.000, p=0.07 and p=0.01) at day0, and day7. In sepsis patients, MDSCs had increased IL-10, Arg1 and iNOS mRNA levels (p=0.016, p=0.049 and p=0.06). Ex-vivo co-cultured MDSCs with T-cells drove T-cell apoptosis (p=0.03, p=0.03) with decreased T-cell proliferation and enhanced $FOXP3^{+}$ expression (p=0.05 and p=0.05) in sepsis compared to no sepsis at day0. Moreover, blocking the MDSCs with inhibitors suppressed FOXP3 expression. GM-CSF treatment in sepsis patients significantly decreased MDSCs and $FOXP3^{+}$Tregs but increased CD4 T-cell functionality and improved survival.

Conclusion

MDSCs have immunosuppressive function by expanding $FOXP3^{+}$ Tregs and inhibiting $CD4^{+}$ T-cell proliferation in sepsis. GM-CSF treatment suppressed MDSCs, improved T-cell functionality and reduced Tregs in circulation.

Introduction

Sepsis ranges from any infection to septic shock and cirrhosis has been recognised as an independent mortality risk factor in septic shock patients[1]. Development of sepsis in cirrhosis patients considerably

increases both short and long-term mortality due to immunological changes and systemic hemodynamic. While in-hospital mortality of cirrhosis patients with septic shock is higher i.e. more than 70%[2].

Liver cirrhosis generally show cirrhosis-associated immune dysfunction(CAID), by altering both innate as well as adaptive immunity[3]. Impaired neutrophil's phagocytic ability decreased HLA-DR expression and phagocytic activity of monocytes is hall mark of cirrhosis patients with sepsis. This dysfunctional innate immunity leads to dysfunctional B and T-cells in alcoholic liver cirrhosis[4].

Defective myelopoiesis drives immature myeloid cells towards myeloid derived suppressor cells(MDSCs), instead of monocytes, dendritic cells(DCs) and neutrophils[5]. These MDSCs are heterogenous in nature and based on expression of CD14, CD15, CD11b, CD33 and HLADR, they are distinctly characterized as of monocytic ($CD14^{+ve}CD11b^{hi}CD33^{+ve}HLADR^{lo}$;M-MDSCs) and granulocytic ($CD14^{-ve}$ $CD15^{+ve}CD11b^{hi}CD33^{+ve}HLADR^{lo}$;G-MDSCs) lineage[6]. Both MDSCs and Tregs are suppressive in nature and support each other, while MDSCs help in Tregs expansion, Tregs in return control the differentiation and function of MDSCs[7–8].

M-MDSCs and G-MDSCs drive their suppressive activities in two different ways. M-MDSCs function either by antigen-specific and non-specific pathways and secrete low ARG1 but suppresses other cells by iNOS mediated STAT1 nitration[9]. While G-MDSCs function specifically through the antigen-specific pathways by hyper activation of ARG1, ROS and NO by superoxide producing peroxynitrite (PNT)[10].

Further, it has been shown that GM-CSF therapy reversed the monocytic deactivation by increasing HLA-DR and TLR4 expression in sepsis patients. Further, GM-CSF treatment was also correlated with increased anti-inflammatory cytokine production with less need of mechanical ventilation and longer hospital stay[11].

Adding GM-CSF to the standard care reduced the infectious complications and shorten the antibiotic therapy duration in abdominal sepsis patients[11–12]. Further, few groups have observed higher leukocyte counts, increased monocytic HLA-DR expression, and improvement from infection with GM-CSF therapy. Secreted GM-CSF in tumor microenvironment also recruits PD-L1 expressing MDSCs with profound ability of immune-suppression and differentiation. At the same time, knockdown or blocking of GM-CSF reduced the IDO and PD-L1 expression in liver-MDSCs[13].

There is limited knowledge about MDSCs and its functionality in liver cirrhosis patients. Therefore, the aim of the present study was to investigate the role of MDSCs in immune dysfunction in decompensated cirrhosis patients with sepsis and modulation of MDSCs, T-cells, Tregs with GM-CSF therapy which may have impact on disease pathogenesis and patient survival.

Methods

Study Groups and Blood Sampling

We enrolled 164 decompensated cirrhosis patients; without (w/o) sepsis (n=62), with sepsis (n=72), with sepsis and treated with GM-CSF (n=30), and healthy controls (HC, n=15) at Institute of Liver and Biliary Sciences(ILBS), New Delhi between 2017 and 2020[Supplementary Figure1]. In an on-going randomized controlled trial, DC patients with sepsis were given 250 mcg of GM-CSF intravenously for about 6 hours daily for 5 days. All the patients received standard medical treatment which included nutrition, antibiotics, and supportive care as part of standard medical treatment.

This study was approved by the Research and Institutional ethics committee with IEC No IEC/2016/45/NA/C2 and informed consent was obtained from all the subjects enrolled in the study. In this longitudinal study, patients were closely monitored from admission and studied at baseline, day3 and 7. Patients with history of any hepatitis infection (HBV, HCV etc.), with HCC or any other site malignancy or any other co-morbidities, and no consent given were excluded from the study. It was carried out in accordance with the ethical standards of the Helsinki declaration.

Details of patient's recruitment criteria and blood sampling is mentioned in the Supplementary methods.

Multi-parametric whole blood Immune phenotyping

MDSC, T-cells and Tregs were characterised in whole blood using antibodies against surface and intracellular markers labelled with different fluorochromes. Details are mentioned in the Supplementary methods.

Analysis of plasma analytes using Cytokine Multiplex Bead Array Assay

To understand the significance of various cytokines and growth factors linked to sepsis as well as MDSC, we investigated the concentrations of forty-one plasma cytokines, chemokine and growth factors. Detail mentioned in the Supplementary methods.

Ex- vivo MDSCs generation and characterization

PBMCs were isolated by the Ficoll-hypaque density gradient centrifugation method. Freshly isolated PBMCs were used for generation of MDSCs and characterized using Giemsa stain. Detail is in the Supplementary methods.

Preparation of MDSCs, T-cells and adherent Monocytes

Detail for preparation of MDSC, T-cells and adherent monocytes are mentioned in the Supplementary methods.

MDSC Functionality, T-cells Apoptosis, T-cells proliferation, Generation of Tregs under Th0 and Th17 condition, inhibition of MDSCs via Inhibitors and quantitative RT-PCR analysis all are detailed in the Supplementary methods.

Statistical Analysis

Data were analyzed using the statistical software Prism (version 6; GraphPad Software, San Diego, CA) and SPSS version 22 (IBM Corp Ltd. Armonk NY. USA). The comparison for continuous data is carried by using Onaway ANOVA/ Kruskal–Wallis test followed by probability adjustment by the Mann–Whitney test or by Bonferroni test post-hoc comparison as appropriate and it is represented as mean ± standard deviation (SD). The *p*-values < 0.05 were considered as statistically significant. Data with unequal distribution were used as medians. Besides this multinomial logistics regression was also applied along with diagnostic tests (ROC Curve).

Results

Baseline characteristics of 164 DC patients, 62 patient's w/o sepsis (age 48 ± 5 years, 87% males) and 72 patients with sepsis (42 ± 9, 97% males) and 15 age matched healthy controls were analysed at the time of admission and enrolment in the study. Alcohol was the predominant etiology (70%) in DC patients. Sepsis patients showed significant increase in total bilirubin, AST levels, INR, PCT, lactate, MELD Na and creatinine compared to w/o sepsis in Table 1. Whole blood immune scan revealed lymphopenia but increased neutrophils in sepsis patients [Supplementary Figure 2A-B].

Table 1
Baseline clinical as well as biochemical characteristics of study groups

MEDIAN and RANGE	Healthy Control (N = 15)	DC w/o Sepsis (N = 62)	DC with Sepsis (N = 72)	P Value in between w/o and with sepsis
Age	32 (20-40)	48 (22-62)	44 (29-60)	0.07
Male: Female	11:4	54:8	70:2	-
Total Bilirubin (mg/dl)	1 (0.3-1.5)	4.5 (1.6-24)	14.5 (2.2-31.7)	0.00
AST (IU/ml)	20 (5-40)	59.75 (31-510)	114 (31-1037)	0.05
ALT (IU/ml)	25 (10-40)	34.5 (20-634)	41.5 (11-233)	1.00
INR (sec)	1 (0.8-1.2)	1.66 (1.1-3.3)	2.58 (1.58-6.75)	0.00
PCT (ng/ml)	0.8 (0.2-2)	0.42 (0.04-3.25)	8.4 (0.07-88.4)	0.03
Lactate (mmol/l)	1.5 (1-2)	1.4 (0.6-5.2)	2.1 (0.2-13.3)	0.02
Sodium (mmol/l)	140 (136-145)	133 (124.3-142.7)	131 (113.2-148.4)	1.00
Creatinine (mg/ml)	0.6 (0.2-1)	0.86 (0.3-2.9)	1.3 (0.3-5.18)	0.04
MELD Na	8 (6-10)	23 (10-37)	32.5 (14-40)	0.00
SIRS criteria				
TLC (10^9 L)	6 (4-11)	6.3 (3.1-19.8)	12.65 (2.7-43.6)	0.00
PULSE (/minute)	70 (60-100)	84 (60-110)	94 (62-132)	0.00
RR (/minute)	14 (12-16)	20 (16-24)	22 (14-34)	0.02
Temperature (F)	98 (97-99)	98.2 (97-98.9)	98.4 (96-100)	1.00
Differential Leukocyte count				
Neutrophils (%)	60 (40-75)	70 (59-89)	81 (36-95)	0.00
Lymphocytes (%)	30 (20-45)	16 (3.4-36)	8 (1-29)	0.00
Monocytes (%)	5 (2-10)	11 (2-18)	8 (2-30)	0.03

Increase in MDSCs in Sepsis patients

At the time of admission(Day0):

Based on the gating strategy, expression of total MDSCs ($CD11b^{+}CD33^{+}$ $HLADR^{-ve}$) and G-MDSCs ($CD11b^{+}CD33^{+}HLADR^{-ve}CD14^{-ve}$), were significantly increased (p=0.011 and p=0.005) in sepsis patients compared to w/o sepsis. But M-MDSCs showed no significant difference between the groups[Figure 1A-B]. Logistic regression model positively predicted increase in total MDSCs and G-MDSCs with high sensitivity and specificity (0.732,p=0.003 and 0.744,p=0.002) in both groups[Figure 1C]. However, G-MDSCs were found to be positively correlated with increased bilirubin and MELD-Na (p=0.007,p=0.049) in sepsis patients[Figure 1D].

At follow-up time points:

On day3 and 7, there was significant decrease in T-MDSC and G-MDSC in sepsis patients at day7 (p=0.04, p=0.01) compared to day0, but no difference in M-MDSCs [Supplementary Figure 3].

Decrease in $CD4^{+}$ T-cells, its subsets while increase in Tregs in sepsis patients

At the time of admission(Day0):

Presence of MDSCs modulate T-cell differentiation [10], therefore to analyse the impact of MDSCs on CD4 T-cells and T-cell differentiation, we have used CD45RA and CCR7 markers to evaluate the presence of naïve, T_{CM}, T_{EM} and T_{EMRA} in circulation. Sepsis patients showed significant decrease in %CD4 T-cells compared to w/o sepsis and HC (p=0.000 and p=0.01)[Figure 2A]. Although, naïve T-cells were not found significantly different between the groups, but T_{CM} was decreased in sepsis patients compared to w/o sepsis (p=0.009)[Figure 2B]. In fact, T_{EM} and T_{EMRA} populations were also decreased in sepsis group, but this difference was observed compared to HC only (p=0.000 and p=0.001)[Figure 2B]. Decrease in T_{CM} in sepsis was positively correlated with increased total bilirubin levels (p=0.038)[Figure 2C].

Further, Tregs was increased in sepsis patients (p=0.02, p=0.01) compared to w/o sepsis and HC[Figure 2D-E]. Logistic regression model positively predicted Tregs with high sensitivity and specificity (0.769, p=0.003) in both sepsis and w/o sepsis patients. But Tregs found positively correlated with increase in MELD score in sepsis patients (p=0.015)[Figure 2F].

At follow-up time points:

No difference in percentages of total CD4 T-cells, T_{NAIVE} T_{CM}, T_{EMRA} and T_{EM} was observed in follow-up between the groups. But somehow percentage frequencies of Tregs were significantly decreased in sepsis patients on day3 and day7 (p=0.002 and p=0.008) compared to day0[Supplementary Figure 4A-D].

MDSCs express more IL-10, Arg1 and iNOS than monocytes

Sorted MDSCs from sepsis patients showed increased IL-10, ARG1 and iNOS expression, however significant increase was observed in IL-10 compared to HC (p=0.016). Expression of ARG1 in G-MDSC

was increased in sepsis patients compared to w/o sepsis and HC while no difference observed in M-MDSC[Figure 3A]. When IL-10, ARG1 and iNOS expression in MDSCs was compared with monocytes, fold change expression of ARG1 and iNOS was found significantly increased in sepsis MDSCs (p=0.045, and p=0.049) compared to sepsis monocytes but no difference in IL-10 expression[Figure 3B]. Though plasma levels of IL-10, IL-6 and IL-8 were significantly increased in sepsis compared to HC and w/o sepsis. Further, MIP-3α, and IL-1β was decreased in sepsis, and MIP-3α was negatively correlated with T-MDSC and G-MDSC while IL-1β and IP-10 positively correlated with M-MDSCs in sepsis patients [Supplementary Figure 5A-B].

MDSCs suppressed T-cells functionality

To check the suppressive effect of MDSCs on T-cells, FACS sorted MDSCs and CD4 T-cells were ex-vivo co-cultured and analysed for CD4 T-cells apoptosis and proliferation[Supplementary Figure 6]. To know whether MDSCs and monocytes have similar effect on T-cells apoptosis and proliferation, we have additionally co-cultured T-cells with monocytes.

At the time of admission(Day0):

We have observed increase in apoptosis of T-cells and decrease in T-cell proliferation in ex-vivo cultured MDSC+T-cells in sepsis patients compared to w/o sepsis and HC[Figure 3C-D]. But monocytes did not show suppressive ability when co-cultured with T-cells, suggesting MDSCs have immunosuppressive ability in sepsis but not monocytes.

At follow-up time points:

We found no difference on follow-up between the groups in T-cell apoptosis and proliferation[Supplementary Figure 7A-B].

MDSCs induces FOXP3$^+$ expression on T-cells

Ex-vivo cultured T-cells with MDSCs in TH0 condition (without the presence of any T-cell stimulant) showed increased expression of $CD4^+FOXP3^+$ (p=0.05) in sepsis patients but no such increased expression was observed in HC and w/o sepsis patients[Figure 3E].

Further, it was observed that in the presence of Th17 proliferating conditions [in presence of recombinant TGF-β (5 ng/ml) and IL-6 (20 ng/ml)], MDSCs induce more $CD4^+FOXP3^+$ expression on T-cells (p=0.05) in sepsis patients, while IL-17 producing T-cells were minimal in disease condition compared to HC (p=0.031)[Figure 3F].

At follow-up time points:

T-cells cultured with MDSCs in TH0 condition showed no difference in expression of $CD4^+FOXP3^+$ but expression of $CD4^+FOXP3^+$ cells in TH17 condition significantly decreased on day3 compared to day0 in

w/o sepsis patient[Supplementary Figure 8A-B].

Blocking the MDSCs suppresses the expression of FOXP3 $^{+}$ Tregs.

As MDSCs suppresses via Arginase1 and iNOS, therefore we have further explored role of MDSCs blockers; L-NMMA (iNOS inhibitor), and nor-NOHA (Arg1 inhibitor) on T-cells functionality. By blocking MDSCs activity with nor-NOHA and L-NMMA, there was significant decrease in $CD4^{+}FOXP3^{+}$ expressing T-cells (p=0.014, and p=0.05) in sepsis patients but no such significant difference was observed in w/o sepsis patients and HC[Figure 3G].

At follow-up time points:

While MDSC inhibitors shows significant decrease in the expression of $CD4^{+}FOXP3^{+}$ at day3 in sepsis patient compared to day0[Supplementary Figure 8C].

GM-CSF treatment suppresses MDSC and Tregs in Sepsis patients.

GM-CSF known as stimulant for bone marrow to produce myeloid cells and helps in their proliferation myeloid cells but also helps in their proliferation[11–12]. However, effect of exogenous treatment of GM-CSF in modulation of MDSCs and Tregs was not explored in sepsis patients. We have analysed, 30 sepsis patients which was given 250 mcg of GM-CSF intravenously over 6 hours for 5 days along with the standard care. Blood samples were collected post 12 hour after GM-CSF administration (day1). Baseline as well as follow-up clinical and biochemical characteristics of sepsis patients with and without GM-CSF treatment was analysed[Supplementary Table 4].

After day1 of GM-CSF therapy, HLA-DR expression on monocytes were increased and CXCR1 on $CD11b^{+ve}$ neutrophils was decreased in compared to without GM-CSF [Supplementary Figure 9]. Further, after GM-CSF therapy at day1 and 3, T-MDSCs and G-MDSCs were decreased in sepsis patients. But no significant effect on M-MDSC even after GM-CSF treatment [Figure 4A].

GM-CSF therapy also showed its impact on $CD4^{+ve}$ T-cells and its subsets. We found significant increase in CD4 expression at day1 after GM-CSF therapy (p=0.09) while both T_{NAIVE} and T_{CM} were found to be significantly increased in GM-CSF group (p=0.04, p=0.003) compared to without GM-CSF[Figure 4B-C].

Further, percentage frequency of Tregs were significantly decreased in GM-CSF group (p=0.003) compared to without GM-CSF[Figure 4D].

GM-CSF treatment reverses the effect of MDSC on T-cells and Tregs.

In ex-vivo co-cultured MDSC+T-cells, percentage apoptosis in T-cells was significantly decreased (p=0.005) after day1 GM-CSF therapy compared to without GM-CSF. But this effect was not observed at day3 on follow-up[Figure 5A]. Similarly, T-cells proliferation was significantly increased in GM-CSF group (p=0.023) compared to without GM-CSF[Figure 5B].

In TH0 condition, expression of $CD4^+FOXP3^+$ on T-cells (p=0.004) was significantly decreased after GM-CSF day1 compared to without GM-CSF. At follow-up of day3, $CD4^+FOXP3$ expressing T-cells were constantly found decreased in GM-CSF group compared to without GM-CSF[Figure 5C].

Further, in IL-17 proliferating conditions, MDSCs did not show ability to induce the expression of $CD4^+FOXP3^+$ on T-cells in GM-CSF group (p=0.000) compared to without GM-CSF. Similarly, till day3 MDSCs were unable to induce $CD4^+FOXP3^+$ expression in GM-CSF group compared to without GM-CSF. But percentage frequency of IL-17 expressing T-cells was significantly increased in GM-CSF group (p=0.027) compared to without GM-CSF at day 1 and day3[Figure 5D].

GM-CSF treatment reversed MDSC expression on Tregs.

GM-CSF therapy in sepsis patients leads to significant decrease in $CD4^+FOXP3^+$ Tregs. In vitro co-cultured assay of CD4 T-cells and MDSCs with L-NMMA (iNOS inhibitor), and nor-NOHA (Arg1 inhibitor) inhibitors, decreased in $CD4^+FOXP3^+$ Tregs was observed after GM-CSF treatment at day1 and day3[Figure 5E].

GM-CSF treatment improves survival of sepsis patients

Survival in decompensated cirrhosis patients with sepsis is mostly compromised. Kaplan–Meier survival curves evidently proved that GM-CSF therapy along with standard care improved survival in sepsis patients compared to patients with only standard care [Figure 5F].

Discussion

Our study shows that MDSCs are significantly increased in sepsis patients which help in the expansion of Tregs and suppresses CD4 T-cells. Administration of GM-CSF in sepsis patients reverses the immune paralysis due to sepsis which involves suppression of MDSCs and Tregs and enhances the CD4 T-cells.

Sepsis is described as organ dysfunction due to bacterial infections and induced by dysregulated host immune response resultant longer stay in hospitals affecting mortality rate[15]. Immune dysfunction in liver cirrhosis patients is common which enhances bacterial translocation from gut to liver resultant endotoxemia, systemic inflammation and septic shock[16]. Decompensated cirrhosis patients show leukopenia which affects both T helper (Th) and cytotoxic T-cells (Tc), monocytosis with altered function, neutrophils with impaired phagocytosis, B-cells with memory B-cell dysfunction and defective NK cells with reduced response to cytokine stimulation [4]. Our study shows decrease in both CD4 and CD8 T-cells, monocytes but increase in circulating neutrophils, MDSC, and Tregs in decompensated cirrhosis patients with sepsis.

MDSCs are heterogenous population of cells, and their origin is either monocytic or granulocytic[6]. Elevated levels of MDSCs have been positively correlated with severe sepsis or septic shock and longer stay of patients in ICU[17]. As MDSCs known to have immunosuppressive activity via Arginase-1, iNOS or ROS for inhibiting the functionality of immune cells especially T-cells. Increase in MDSCs acts as a potent

inhibitor of T-cell-mediated immunity in autoimmune hepatitis and cancer, which is attributed to the production of Arginase1, ROS, iNOS, and IL-10[18–19]. Increase in hepatic $CD11b^{+}CD33^{+}$ MDSCs positively correlated with liver fibrosis stages while MDSC number linearly correlated with the tumor volume[20]. Further, M-MDSCs were strongly correlated with raised ALT, AST, and decreased T-cell proliferation[21]. It was earlier reported that M-MDSCs suppressed T-cell functions and antimicrobial innate immune responses in ACLF patients[22]. In this study, in vitro co-cultured MDSCs with CD4T-cells suppressed the proliferation and enhanced apoptosis of CD4T cells in decompensated sepsis patients.

It is known that in tumor microenvironment, many known circulating factors including IL-6, IFN-γ, TGF-β, VEGF, G-CSF, GM-CSF, M-CSF and SCF induce the recruitment, accumulation, and activation of MDSCs[23], and also modulate MDSCs to produce more of NO and ROS[19]. Our study revealed positive correlation of new molecules IL-1β and IP-10 with M-MDSC but negative correlation of MIP-3α with T-MDSC and G-MDSC. Reduced IL-1β receptor binding ability or IL-1β levels, reduces the accumulation and suppressive activity of MDSC resultant augmentation of anti-tumor immunity and delayed tumor growth[24]. Further chemokine CXCL10/IP-10 significantly increased in mice model of septic shock as they cause the activation of chemokine receptor CXCR3, an important regulator of lymphocyte trafficking and activation[25]. MIP-3α (macrophage inflammatory protein-3)/CCL20 generally expressed on several immune cells but with stronger chemotactic effect and interaction with chemokine receptor CCR6on lymphocytes. CCL20/CCR6 axis regulates the activation and suppression of immune cells[26].

Both MDSC and Tregs are known suppressor cells and help each other i.e Tregs regulate the differentiation and function of MDSC via TGF-β while MDSC helps in the expansion of Tregs in colitis mice model[7]. Rheumatoid arthritis mice model, MDSCs derived IL-10 help in the generation of Tregs but attenuate inflammation. It clearly states that MDSCs regulate Th17/Treg cells and control inflammation[8]. We have also showed that in decompensated cirrhosis patients with sepsis, MDSCs significantly enhanced the expression of FOXP3 on $CD4^{+}$ T-cells and behaved as Tregs. While stimulation with L-NMMA (iNOS inhibitor) and nor-NOHA (inhibitor of arginase 1) significantly suppresses the expansion of Tregs in sepsis patients. It clearly concludes that suppression of immunosuppressive activity of MDSCs will decrease the expansion of $CD4^{+}FOXP3^{+}$ cells in sepsis.

Administration of GM-CSF in sepsis patients reversed the monocytic deactivation by increasing HLA-DR and TLR4 induced cytokine production, as well as decreases the time of mechanical ventilation and length of hospital[27]. *In vitro* mice model showed that the combination of GM-CSF signaling blockade and gemcitabine suppressed the MDSC phenotype and functionality [19, 32]. In another study, both GM-CSF and G-CSF prevented diabetes by reducing MDSCs and Treg cells[28]. Our results clearly suggested the benefit of GM-CSF therapy in sepsis patients, as it decreases the total MDSCs and G-MDSCs percentage but showed no change in M-MDSCs. GM-CSF administration reversed the immune response by increasing the CD4 T-cells and decreasing MDSCs as well as FOXP3 expression on $CD4^{+}$ T-cells. Although earlier it was documented that in ACLF patients, G-CSF therapy helped in mobilization of bone

marrow–derived $CD34^+$ cells for hepatic regeneration as well as improves survival[29]. G-CSF reduces the disease severity and delays the mortality of severe alcoholic hepatitis patients[30].

We conclude that MDSCs have immunosuppressive function in decompensated cirrhosis with sepsis which inhibits $CD4^+$ T-cell numbers as well as their functionality and expanded $CD4^+FOXP3^+$ Tregs. GM-CSF therapy decreased MDSCs and improved T-cells functionality while decreasing Tregs and undoubtedly increases the chances of survival of sepsis patients.

Abbreviations

Myeloid derived suppressor cells (MDSCs), cirrhosis-associated immune dysfunction (CAID), Decompensated cirrhosis (DC), intensive care unit (ICU), immature myeloid cells (IMCs), Dendritic cells (DCs), Granulocytic MDSCs (G-MDSCs), Monocytic MDSCs (M-MDSCs), Inducible nitric oxide synthase (iNOS), Reactive oxygen species (ROS), peroxynitrite (PNT), Transforming growth factor beta (TGF-β), Interleukin (IL), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Granulocyte colony-stimulating factor (G-CSF), M-CSF (macrophage colony-stimulating factor), Vascular endothelial growth factor (VEGF), Stem cell factor (SCF), Alanine Aminotransferase (ALT), Aspartate aminotransferase (AST), Acute on chronic liver failure (ACLF), Forkhead box P3 (FOXP3), indoleamine 2, 3-dioxygenase (IDO), programmed cell death ligand 1 (PD-L1), Healthy control (HC), Randomized controlled trial (RCT), Hepatocellular carcinoma (HCC), Systemic inflammatory response syndrome (SIRS), Total leukocyte count (TLC), Respiratory rate (RR), The international normalized ratio (INR), Ethylenediaminetetraacetic acid (EDTA), Peripheral blood mononuclear cells (PBMCs), Phosphate-buffered saline (PBS), Fetal bovine serum (FBS), Roswell Park Memorial Institute Medium (RPMI), Lipopolysaccharide (LPS), Room temperature (RT), Red blood cells (RBC), propidium iodide (PI), Carboxyfluorescein succinimidyl ester (CFSE), Fluorescence activated cells sorting (FACS), Nω-Methyl-L-arginine acetate salt (L-NMMA), Nω-Hydroxy-nor-L-arginine diacetate Salt (nor-NOHA), Statistical Package for the Social Sciences (SPSS), standard deviation (SD), differential leukocyte count (DLC), Procalcitonin (PCT), Model for end stage liver diseases (MELD Na) naïve T-cells ($CD4^+CD45RA^+CCR7^+$), central memory T-cells (TCM,; $CD4^+CCR7^+CD45RA^-$), effector memory (TEM; $CD4^+CD45RA^-CCR7^-$) and terminally differentiated T-cells (TEMRA; $CD4^+CD45RA^+CCR7^-$).

Declarations

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Consent to participate: The study was conducted in accordance with the principles enshrined in the Declaration of Helsinki and approved by institutional ethics committee of Institute of liver and biliary sciences, INDIA.

Consent to publish: All authors have reviewed and approved the final manuscript.

Availability of data and material: The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Code availability: Not applicable

Authors contribution: RS collected clinical samples, performed all experiments after inputs from NTP and did initial analysis; RM, VR and SKS helped in recruitment and characterization of patients in each group. MI helped in performing experiments and GK helped in statistical analysis. Initial draft of manuscript was written by RS. SKS, RM, SB, NK and GR have provided inputs in the manuscript. NTP revised, corrected and finalised the manuscript.

Animal Research (Ethics): Not applicable

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Figures

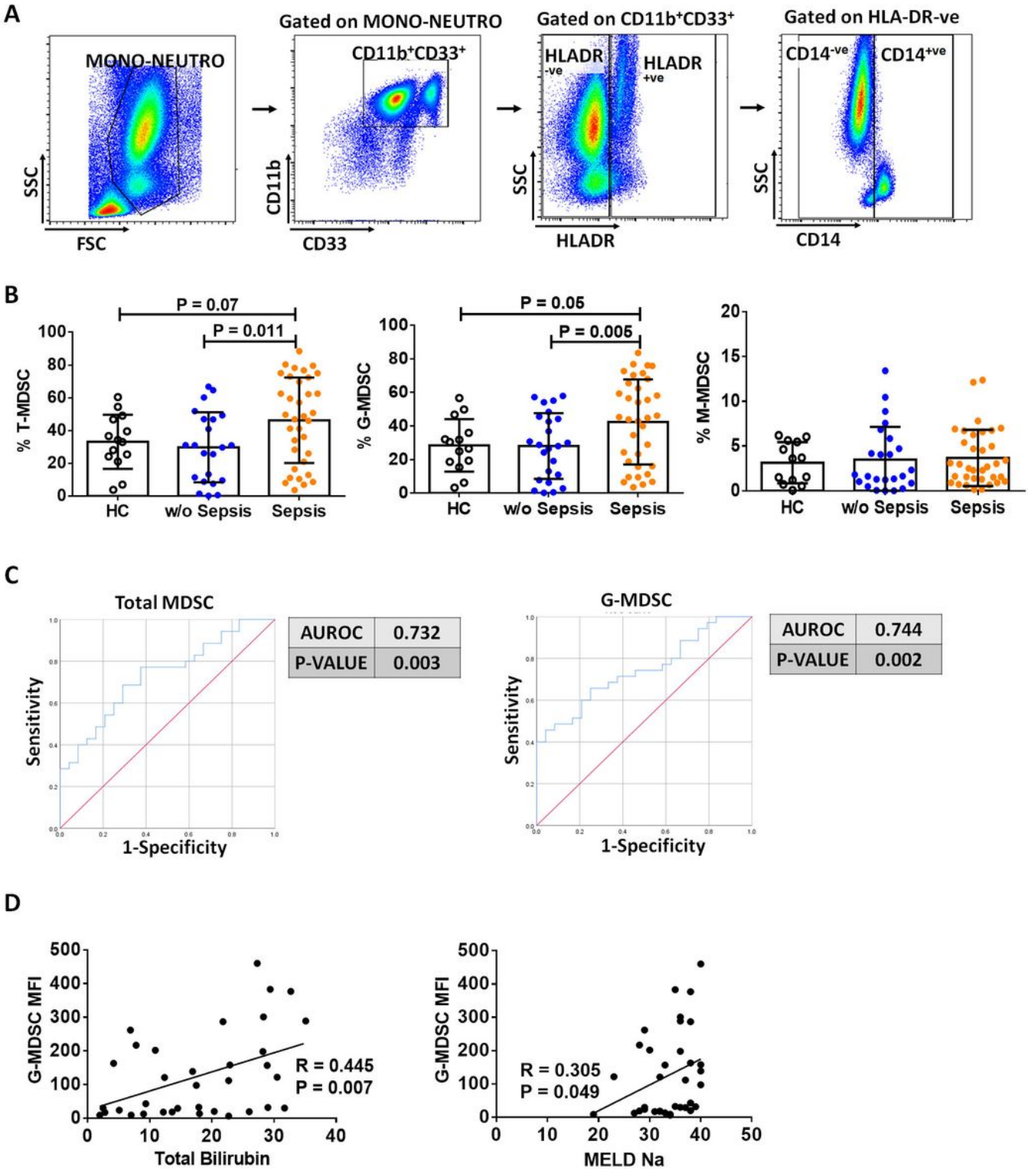

Figure 1

Identification of MDSCs in patient groups. A) Sequential gating strategy for identification of MDSC and its subsets using flow cytometry. MDSC characterized as CD11b+CD33+HLA-DR-ve, G-MDSC as CD11b+CD33+HLA-DR-veCD14-ve and M-MDSC as CD11b+CD33+HLA-DR-veCD14+. B) Scatter dot plot shows %frequency of T-MDSC, G-MDSC and M-MDSC between the groups. C) ROC curve shows high specificity and sensitivity of T-MDSC and G-MDSC in sepsis patients compared to w/o sepsis. D)

Correlation of G-MDSC MFI (median fluorescence intensity) with total bilirubin and MELD Na in sepsis patients. Results are expressed as the mean ± SD.

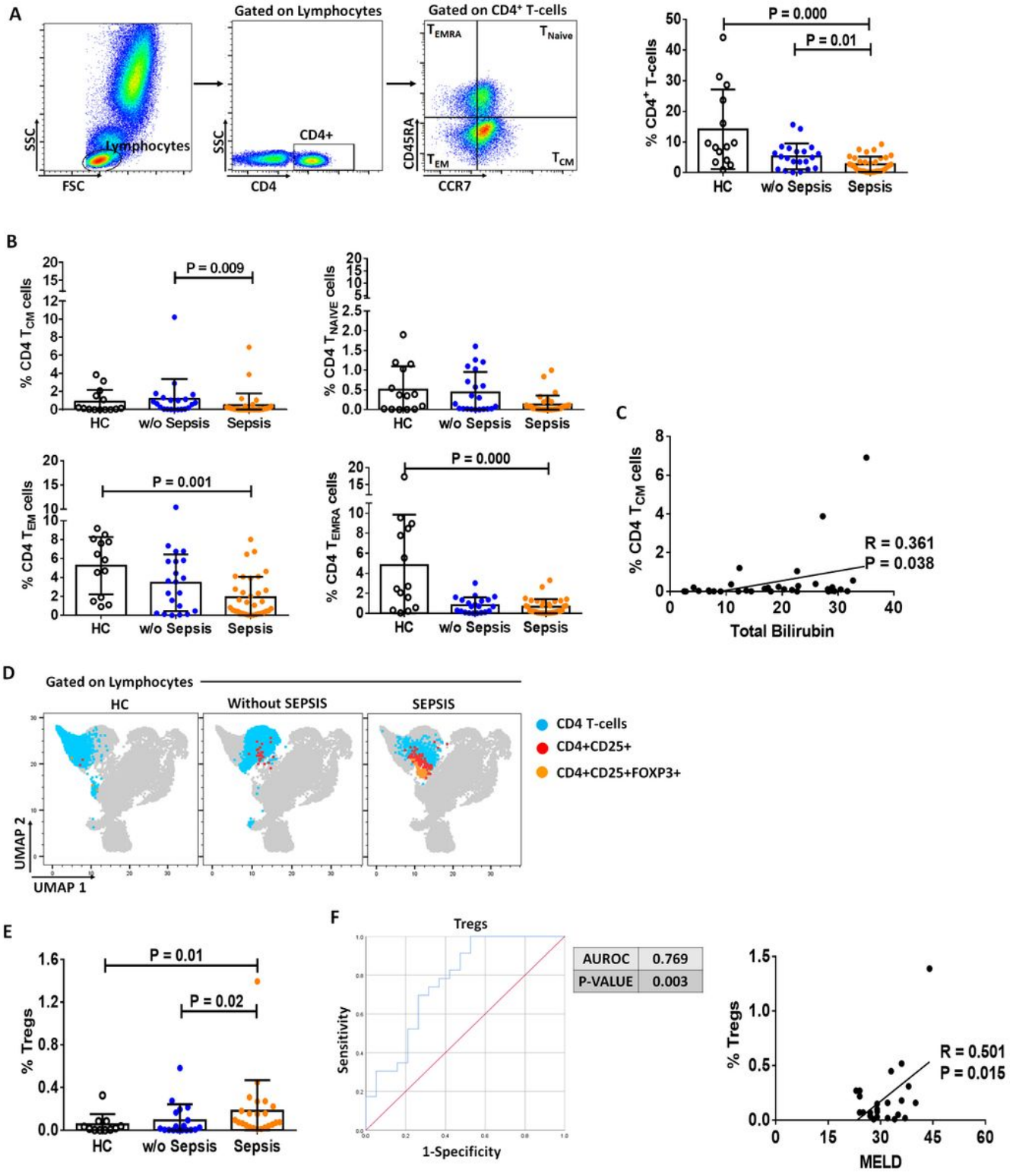

Figure 2

Identification of CD4 T-cells, its subsets and Tregs in patient groups. A) Sequential gating strategy for identification of CD4 and its subsets using CCR7 and CD45RA i.e TCM, TNAIVE, TEM and TEMRA. Scatter dot plot shows %frequency of CD4 T-cells in patient groups. B) Scatter dot plot shows %frequency of

TCM, TNAIVE, TEM and TEMRA. C) Correlation between %CD4 TCM and total bilirubin in sepsis group. D) UMAP visualization of pooled lymphocytes of HC, w/o and with sepsis for characterization of Tregs. E) Scatter dot plots shows %frequency of Tregs. F) ROC curve shows high specificity and sensitivity of Tregs in sepsis group compared to w/o sepsis. Also, correlation of %Tregs with MELD in sepsis group. Results are expressed as the mean ± SD.

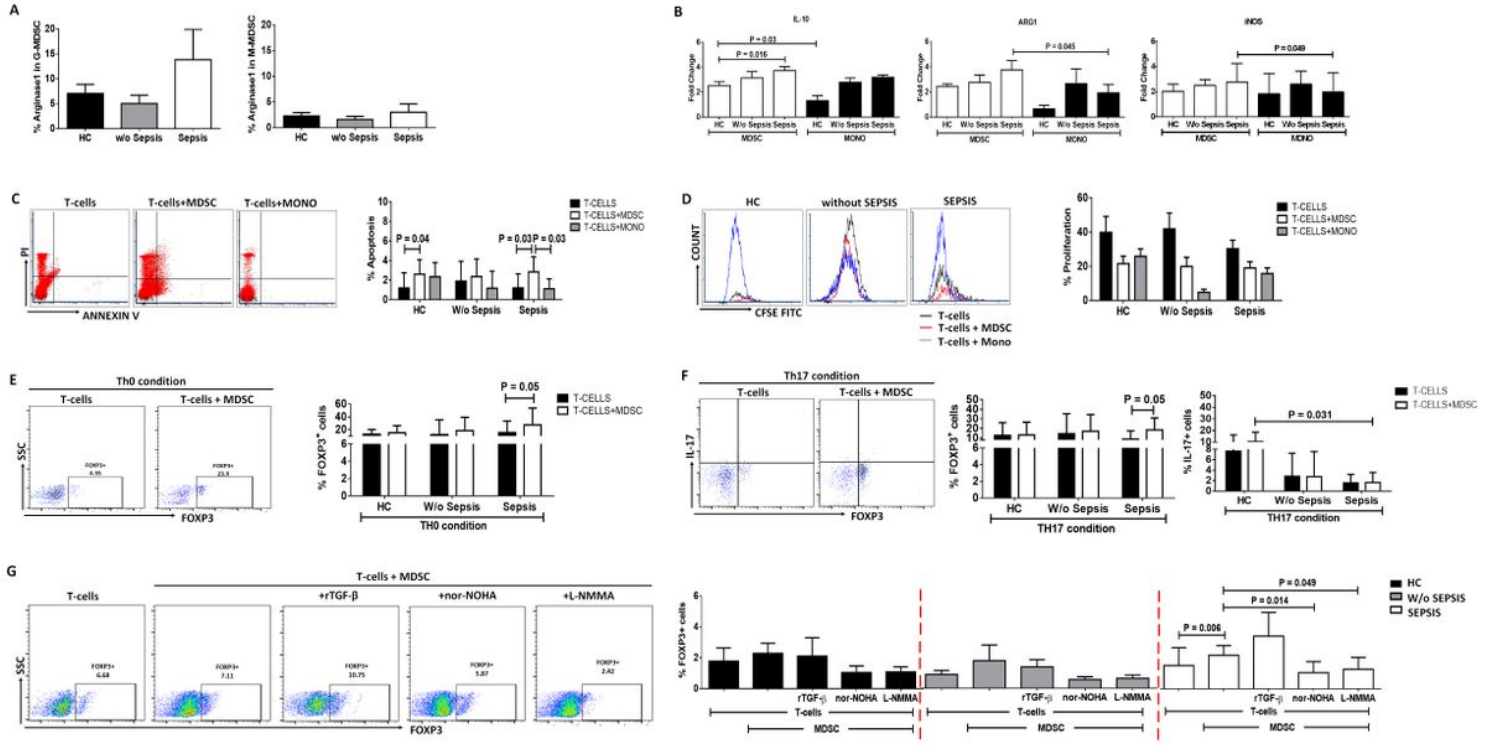

Figure 3

MDSC action on T-cell functionality and Tregs. A) Expression of ARG1 in G-MDSC and M-MDSC in patient groups. B) Fold change expression of IL-10, Arg1 and iNOS in sorted MDSC (white color) and monocytes (black color) in the patient groups through qRT-PCR. C) %Apoptosis using PI-ANNEXIN V and D) %Proliferation using CFSE through flow cytometry in T-cells cultured alone (black color), with MDSC (white color) and monocytes (grey color) in the patient groups. Expression of %FOXP3+ on CD4+ T-cells E) under TH0 condition and F) under TH17 proliferating condition was observed in the patient groups when T-cells cultured alone (black color) and with MDSC (white color). G) Expression of %FOXP3+ on CD4+ T-cells in T-cells cultured with MDSC along with stimulations i.e rTGF-β, L-NMMA and nor-NOHA in the patient groups. HC (black color), without sepsis (grey color) and sepsis (white color). Results are expressed as the mean ± SD.

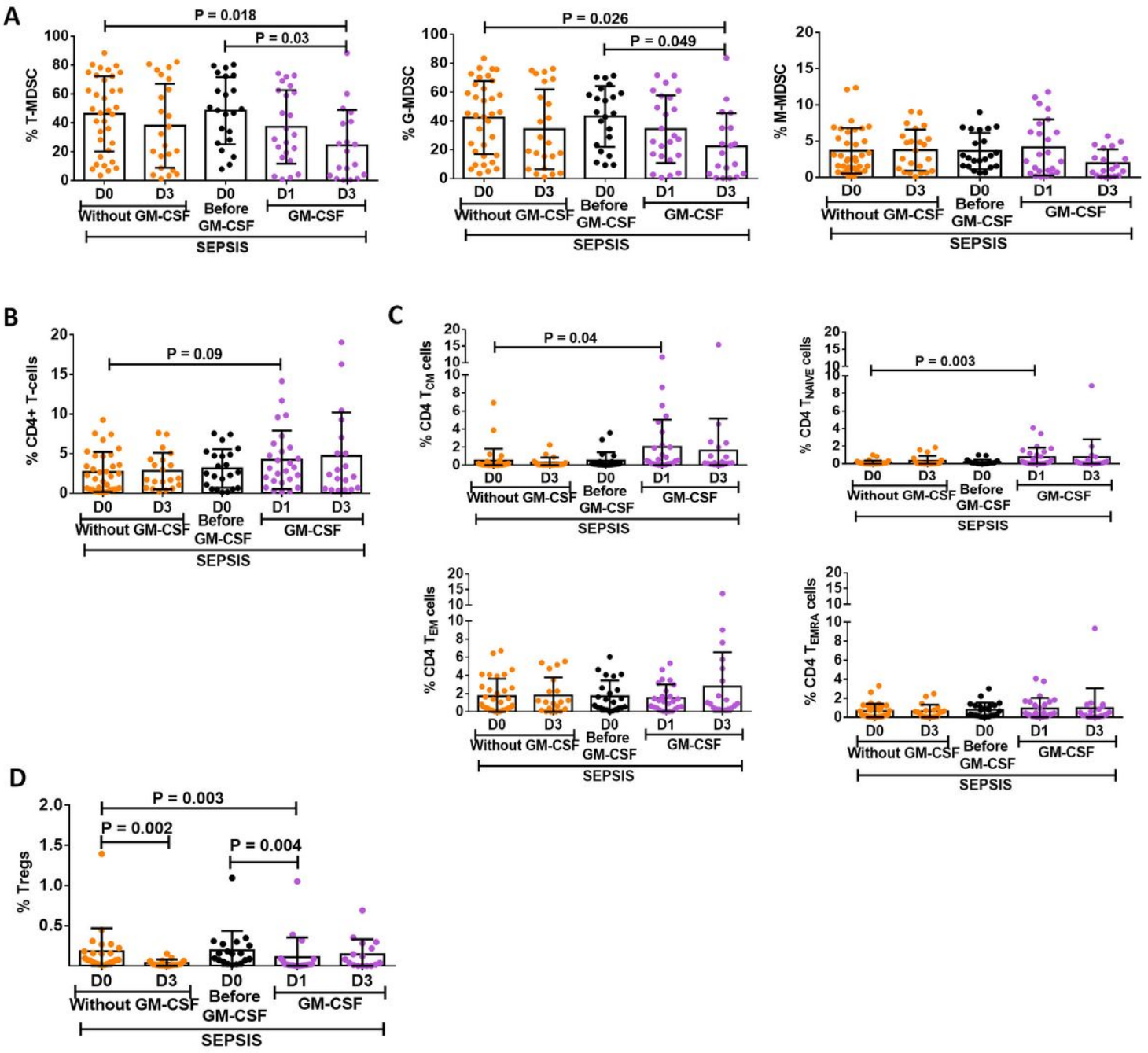

Figure 4

Effect of GM-CSF treatment on the expression of MDSC, CD4 T-cells, its subsets and Tregs. Scatter dot plot shows expression of A) %T-MDSC, G-MDSC and M-MDSC B) %CD4 T-cells C) CD4 T-cells subsets i.e TCM, TNAIVE, TEM and TEMRA and D) %Tregs in with and without GM-CSF group at different time points. Results are expressed as the mean ± SD.

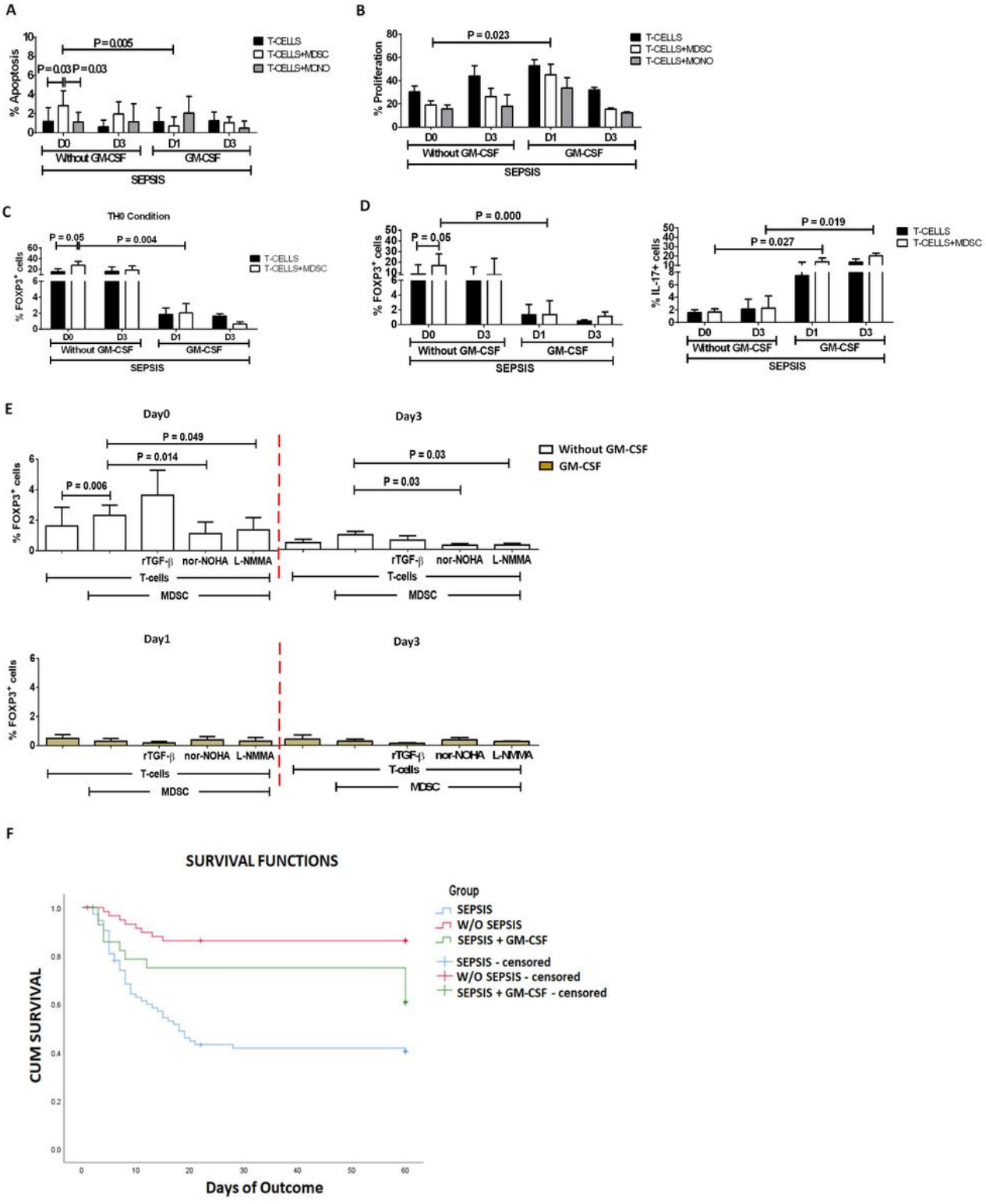

Figure 5

Effect of GM-CSF treatment on T-cell functionality and Tregs expression. Bar diagrams shows A) %Apoptosis using PI-ANNEXIN V and B) %Proliferation using CFSE in sepsis patients with and without GM-CSF in T-cells cultured alone (black color), with MDSC (white color) and with monocytes (grey color). Bar diagrams shows expression of %FOXP3+ on CD4+ T-cells C) under TH0 condition and D) under TH17 proliferating condition in T-cells cultured alone (black color) and with MDSC (white color). E) Expression

of %FOXP3+ on CD4+ T-cells in T-cells cultured with MDSC along with stimulations with rTGF-β, L-NMMA and nor-NOHA and %FOXP3+ Tregs expression was observed in with (light brown) and without GM-CSF (white color) group at different time points. Results are expressed as the mean ± SD. F) Survival in patient groups observed using Kaplan-Meier survival curves via SPSS.

Supplementary Files

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