

Sepsis impairs immunocompetent plasmacytoid dendritic cell reconstitution from hematopoietic stem/progenitor cell through altered bone marrow environment

Jie Lu^{a,1}, Zhuo Lv^{a,1}, Meizhu xue^b, Dan Fan^c, Huiping Yang^d, Yi Hong^e, Yichen Wang^a, Zhangqiang Guo^a, Jiajun Ma^a, Jie Huang^f, Lijun Meng^g, Shuiyan Wu^{a,h,*}, Zhenjiang Bai^{a,**}

^a Department of Pediatric Intensive Care Unit, Children Hospital of Soochow University, Suzhou, China

^b Department of Pediatrics, Changshu NO.1 People's Hospital, China

^c Department of Pediatrics, The First People's Hospital of Wuhu, China

^d Department of Pediatrics, Fuyang Traditional Chinese Medicine Hospital, China

^e Department of Pediatrics, Changshu Hospital Affiliated to Nanjing University Of Chinese Medicine, China

^f Department of Cardiovascular Medicine, Children Hospital of Soochow University, Suzhou, China

^g Advanced Molecular Pathology Institute of Soochow University and SANO and SANO Medical Laboratories, China

^h Pediatric Hematology & Oncology Key Laboratory of Higher Education Institutions in Jiangsu Province, China

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ABSTRACT

Plasmacytoid dendritic cells (pDCs) are crucial components of the immune response during viral infections, yet their function and development in the late phase of sepsis remain poorly understood. In this study, we investigated the impact of prolonged sepsis on pDCs and their progenitors in cecal ligation and puncture (CLP)-induced septic mice. We observed a significant reduction in both pDCs and their progenitors, alongside the presence of mature and regulatory pDCs. These mature and regulatory pDCs exhibited impaired type I interferon (IFN) secretion and antigen presentation capacity. In a Flt3L culture system of hematopoietic stem/progenitor cells (HSPCs) from CLP and Sham mice, we found that CLP-derived HSPCs exhibited an impaired ability to generate immunocompetent pDCs, as evidenced by lower IFN- α expression and reduced pDC recovery. Further investigation revealed downregulation of the key transcription factor TCF4 during pDC differentiation in these progenitor cells. Ectopic expression of TCF4 in these progenitors restored pDC generation. Additionally, we observed elevated levels of granulocyte colony-stimulating factor (G-CSF) in the bone marrow supernatant of septic mice. The addition of G-CSF to the culture system significantly impaired the generation of immunocompetent pDCs from HSPCs of normal mice. These findings suggest that sepsis may impair the production of immunocompetent pDCs from HSPCs by modulating key genes involved in pDC differentiation, potentially contributing to immune suppression and increased susceptibility to opportunistic infections in the later stages of sepsis.

List of abbreviations: Abs, Antibodies; APC, Antigen-presenting cell; BM, Bone marrow; cDNA, Complementary DNA; CDP, Common dendritic cell progenitor; cDC, Conventional dendritic cell; CFSE, Carboxyfluorescein succinimidyl ester; CLP, Cecal ligation and puncture; DC, Dendritic cell; FACS, Fluorescence-activated cell sorting; FBS, Fetal bovine serum; Flt3L, FMS-like tyrosine kinase 3 ligand; Foxp3, Forkhead box P3; G-CSF, Granulocyte colony-stimulating factor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HSC, Hematopoietic stem cell; HSPC, Hematopoietic stem/progenitor cell; IFN, Interferon; IL, Interleukin; IRF, Interferon regulatory factor; Lin, Lineage markers; MDP, Monocyte and dendritic cell progenitor; MFI, Mean fluorescence intensity; MPP, Multipotent progenitor; mregDC, Mature regulatory DC; PBMC, Peripheral blood mononuclear cell; pDC, Plasmacytoid dendritic cell; PD-L1, Programmed death-ligand 1; qPCR, Quantitative real-time PCR; R848, Resiquimod; RNA-seq, RNA sequencing; SCF, Stem cell factor; SpiB, Spi-B transcription factor; TCF4, Transcription factor 4; TF, Transcription factor; Th1, T helper 1 cell; TLR, Toll-like receptor; TNF, Tumor necrosis factor; Treg, Regulatory T cell.

* Corresponding author at: Department of Pediatric Intensive Care Unit, Children Hospital of Soochow University, Suzhou, China.

** Corresponding author.

E-mail addresses: wushuiyany@163.com (S. Wu), 18913510429@163.com (Z. Bai).

¹ J.L. and Z.L. contributed equally to this work.

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1. Introduction

Sepsis is a life-threatening organ dysfunction syndrome caused by a dysregulated host response to microbial infections, including bacteria, viruses, and fungi (Singer et al., 2016). In 2017, there were 48.9 million reported cases of sepsis globally, with a high mortality rate of 22.5 %, accounting for nearly 20 % of all deaths worldwide (Rudd et al., 2020). However, many patients who survive the initial septic shock succumb to secondary infections and reactivation of latent viruses due to prolonged immunosuppression (Hotchkiss et al., 2013; Seymour et al., 2019; Liu et al., 2022). Plasmacytoid dendritic cells (pDCs), as antigen-presenting cells that activate adaptive immunity, are well-known for producing large amounts of type I interferons (IFNs) in response to both viral and bacterial (e.g., *Staphylococcus aureus*) infections (Rahman et al., 2019). Depletion of pDCs and impaired type I IFN production have been observed, accompanied by viral load expansion and an increase in regulatory T cells (Tregs) during long-term infections in mice, highlighting the importance of immunocompetent pDCs (Swiecki et al., 2010; Lynch et al., 2018; Mitchell et al., 2020; Lee et al., 2009; Abbas et al., 2020). However, little is known about the specific functional changes of pDCs in the late phase of sepsis.

pDCs develop from hematopoietic stem and progenitor cells (HSPCs) in the bone marrow, differentiating hierarchically into multipotent progenitors (MPPs), monocyte and dendritic cell progenitors (MDPs), and common dendritic cell progenitors (CDPs) (Anderson et al., 2021). Recent studies have shown that during long-term infection or inflammation, HSPCs exhibit an imbalance with differentiation skewing towards the myeloid lineage at the expense of lymphopoiesis. This hematopoietic adaptation is associated with changes in transcription factors (TFs) and cytokines (Xu et al., 2022; Chavakis et al., 2019). We sought to address several questions: during sepsis, does the failure of pDC reconstitution result from hematopoietic abnormalities? Does long-term sepsis alter the BM microenvironment? What are the intrinsic (e.g., TFs) and extrinsic (e.g., cytokines) mechanisms mediating the impaired reconstitution of immunocompetent pDCs from HSPCs?

In this study, we demonstrated that septic pDCs exhibit a mature regulatory phenotype, resembling "mregDCs" (Kvedaraite and Ginhoux, 2022; Maier et al., 2020), and show impaired capacity to produce type I IFNs during the late phase of sepsis. These mature pDCs also suppress the differentiation of naïve T cells into Th1 cells while promoting the differentiation of Tregs in vitro. Moreover, BM HSPCs from septic mice failed to generate immunocompetent pDCs, accompanied by down-regulation of key pDC-specific transcription factors, TCF4 and SpiB. We further examined multiple cytokines in BM supernatants and serum, uncovering a critical role of G-CSF in the BM microenvironment during the late phase of sepsis in impairing the reconstitution of immunocompetent pDCs from their progenitors.

Taken together, these findings provide new insights into the impaired reconstitution of immunocompetent pDCs during the late phase of sepsis, which may contribute to increased susceptibility to opportunistic infections and the long-term mortality of sepsis patients.

2. Method

2.1. Clinical samples

This study was conducted in the pediatric intensive care unit (ICU) of the Children's Hospital of Soochow University. A total of 8 critically ill sepsis patients were enrolled between September 2021 and December 2021. The demographic information of the sepsis patients is provided in Table 1. The study was approved by the Medical Ethics Committee of the Children's Hospital of Soochow University (Suzhou, China), under license number 2021CS001. Written informed consent was obtained from the parents or guardians of the sepsis patients and from healthy volunteers upon their initial hospital admission.

Table 1

Characteristics of the 8 children with sepsis included in the study.

	Sepsis (n = 8)
Age(years)	2.33 [1.2275–2.6625]
Male gender [n (%)]	7 (12.5 %)
Principal diagnosis besides sepsis	
Encephalitis	1 (12.5)
encephalomyelitis	2 (25)
pneumonia	3 (37.5)
meningitis	2 (25)
Prism-III score	8 [2–10.5]
ICU-free days in 30 days	6.5 [5.5–8.5]

Values are expressed as median [interquartile range], or a number (percentage) PRISM-III, pediatric risk of mortality score-III.

2.2. Mice

All mouse studies were conducted in accordance with protocols approved by the Ethics Committee of Soochow University under license number SUDA20240119A06. C57BL/6 and Balb/c mice were purchased from SPF (Beijing) Biotechnology Co., Ltd. Male and female mice over 6 weeks of age were used in all experiments. All mice were bred and housed in a specific pathogen-free (SPF) facility in groups of 5 per ventilated cage, with a temperature of 21 ± 1 °C, room humidity ranging from 40 % to 70 %, and a 12-hour light/dark cycle (20:00–08:00) to ensure a gradual transition.

2.3. CLP model of polymicrobial sepsis

Cecal ligation and puncture (CLP) is the most clinically relevant experimental model of sepsis. The CLP model of polymicrobial sepsis was performed as previously described (Rittirsch et al., 2009). Mice were anesthetized with an intraperitoneal injection of chloral hydrate (4 %, 400 mg/kg body weight) and placed on the operating table. After disinfection of the skin, a 1-cm-long incision was made along the midline of the abdomen to expose the cecum. The cecum was ligated at the midpoint between the distal pole and the base, then punctured once with a 21-gauge needle. Approximately 1.0 mm of stool was extruded by gently compressing the ligated cecum. The incision was then closed in two layers: peritoneum and skin, to prevent fluid leakage after repositioning the cecum. All mice received fluid resuscitation by subcutaneously injecting 1 ml of 0.9 % normal saline. Sham-operated mice underwent the same procedure without ligation or puncture of the exposed cecum.

2.4. Abs, flow cytometry analysis, and cell sorting

All Abs used for immunofluorescence staining were purchased from BD Biosciences or Biolegend. Magnetic microbead-conjugated Abs and streptavidin from Miltenyi Biotec. The antibodies are listed in Table 2. Recombinant mouse IL-2, Flt3L, SCF, and G-CSF were purchased from Peprotech. Cells were stained with appropriate concentrations of mAbs as previously described (Meng et al., 2016). Dead cells were stained using the Live/Dead Fixable Aqua Dead Cell Stain (Invitrogen). Flow cytometry analyses were performed using the Attune NxT Flow Cytometer (Life Technologies). Mouse pDC subsets were sorted on a BD Influx or BD FACSAria II (BD Biosciences) as CD11c⁺ MHC-II⁺ B220⁺ cells. For HSPC enrichment, BM cell suspensions were obtained using Ficoll-Paque density gradient. Cells were isolated with CD117 microbeads (Miltenyi Biotec, 130–091–224) according to the manufacturer's instructions.

2.5. Cell surface marker

Mouse cDCs were defined as CD11c⁺ MHC-II⁺ CD11b⁺ cells and pDCs were defined as CD11c⁺ MHC-II⁺ B220⁺ cells (Cabeza-Cabrerizo

Table 2
Antibodies.

Antibodies	SOURCE	IDENTIFIER
Magnetic CD117 microbeads	Miltenyi Biotec	130–091–224
Streptavidin MicroBeads	Miltenyi Biotec	130–048–101
Biotin anti-mouse CD117 (c-Kit) Antibody	biolegend	105804
Magnetic CD4 microbeads	Miltenyi Biotec	480069
Live/Dead Fixable Aqua Dead Cell Stain	Invitrogen	L34957
Pacific Blue™ anti-mouse I-Ab Antibody	Biolegend	116422
APC anti-mouse CD11c Antibody	Biolegend	117310
APC/Cyanine7 anti-mouse/human CD45R/B220 Antibody	Biolegend	103224
Alexa Fluor® 700 anti-mouse/human CD11b Antibody	Biolegend	101222
PE anti-mouse/human CD11b Antibody	Biolegend	101208
PE/Cyanine7 anti-mouse CD40 Antibody	Biolegend	124621
Brilliant Violet 605™ anti-mouse CD80 Antibody	Biolegend	104729
FITC anti-mouse CD86 Antibody	Biolegend	105006
PE/Cyanine7 anti-mouse CD274 (B7-H1, PD-L1) Antibody	Biolegend	137908
Alexa Fluor® 488 anti-mouse Galectin–9 Antibody	Biolegend	137907
PE anti-mouse CD85k (gp49 Receptor) Antibody	Biolegend	144903
APC/Cyanine7 anti-mouse CD3 Antibody	Biolegend	100221
APC/Cyanine7 anti-mouse/human CD11b Antibody	Biolegend	101225
APC/Cyanine7 Ter119	Biolegend	116223
APC/Cyanine7 anti-mouse Ly-6G/Ly–6C (Gr–1) Antibody	Biolegend	108423
FITC anti-mouse Ly–6A/E (Sca–1) Antibody	Biolegend	108105
PE/Cyanine7 anti-mouse CD117 (c-Kit) Antibody	Biolegend	105814
PE anti-mouse CD117 (c-kit) Antibody	Biolegend	161503
APC anti-mouse CD135 Antibody	Biolegend	135309
Brilliant Violet 421™ anti-mouse CD4 Antibody	Biolegend	100438
APC anti-mouse CD25 Antibody	Biolegend	101910
PE anti-mouse/rat/human FOXP3 Antibody	Biolegend	320007
BD Pharmingen™ APC-Cy™7 Rat Anti-Mouse IFN-γ	BD	561479
FITC anti-human CD3 Antibody	Biolegend	344804
FITC anti-human CD14 Antibody	Biolegend	325604
FITC anti-human CD15 (SSEA–1) Antibody	Biolegend	301904
FITC anti-human CD19 Antibody	Biolegend	302206
FITC anti-human CD20 Antibody	Biolegend	302304
FITC anti-human CD56 (NCAM) Antibody	Biolegend	304604
APC/Cyanine7 anti-human HLA-DR Antibody	Biolegend	307618
Pacific Blue™ anti-human CD11c Antibody	Biolegend	337212
PE/Cyanine7 anti-human CD123 Antibody	Biolegend	306010
PE anti-human CD1c Antibody	Biolegend	331506

et al., 2021; Reizis, 2019). Mouse HSCs were characterized as lineage marker (CD3/CD11b/Ter119/B220/Gr1) negative(Lin[−] Sca1⁺ c-kit⁺ CD135[−] cells. MPPs were characterized as Lin[−] Sca1⁺ c-kit⁺ CD135⁺ cells. MDPs were characterized as Lin[−] Sca1⁺ CD135⁺ c-kit⁺ cells. CDPs were characterized as Lin[−] Sca1⁺ c-kit^{int} CD135⁺ cells (Ghosh et al., 2014; Fan et al., 2021). Human pDCs were characterized as Lin[−] HLA-DR⁺ CD11c⁺ CD1c[−] CD123⁺ cells.

2.6. In vitro induction of pDCs from HSPCs

DCs were generated from BM cells harvested from 8- to 10-week-old C57BL/6 normal, sham-operated and CLP surgery (septic) mice. BM cells were flushed from the femurs and tibias. After Ficoll gradient centrifugation, mouse BM mononuclear cells were sorted using CD117 microbeads (Miltenyi Biotec, 130–091–224) to isolate CD117⁺ hematopoietic stem/progenitor cells (HSPCs). HSPCs were then cultured in RPMI 1640 medium (Gibco) containing 10 % fetal bovine serum (Dongling Biotech), recombinant mouse Flt3L (50 ng/ml) and SCF (10 ng/ml) for 9 days, as described previously (Tian et al., 2021; Mochizuki et al., 2016). In some experiments, Total DCs were sorted to isolate CD11c⁺ B220⁺ pDCs using a flow cytometry cell sorter. To induce DC activation,

immature pDCs (defined as CD11c⁺ B220⁺) were incubated overnight with R848 (Resiquimod, 100 ng/ml) and CPG ODN (1 mM) (Invivogen), which activate Toll-like receptors 7/8 and 9, respectively. Cells were then harvested for experiments. In some experiments, 10 ng/ml G-CSF was added to the co-culture system at the beginning.

2.7. Retroviral transduction of HSPCs

To restore TCF4 expression in septic HSPCs, we generated a TCF4-GFP retroviral vector by cloning full-length murine TCF4 cDNA into the MIGR1-GFP backbone (Addgene #27490), with empty MIGR1-GFP vector serving as the control. Retroviral particles were produced by co-transfecting Platinum-E cells with pCL-Eco (4 μg, Addgene #12371) and either MIGR1-TCF4-GFP or control pMIG-GFP vector (20 μg) using Lipofectamine 3000 (Thermo Fisher). The culture medium was replaced 12 h post-transfection, and viral supernatant was collected after 36 h. Isolated c-kit⁺ HSPCs were pre-stimulated for 36 h in RPMI 1640 medium supplemented with 10 % FBS, recombinant mouse Flt3L (50 ng/ml), and SCF (10 ng/ml) to induce cell cycle entry. Cells were transduced via spinoculation (3000 rpm, 30°C, 180 min) using viral supernatant at an MOI of 5 with 8 μg/ml polybrene (Sigma-Aldrich), followed by overnight incubation. Complete medium replacement was performed 24 h post-transduction.

2.8. In vitro induction of cDCs from HSPCs

Sorted BM HSPCs (as described above) were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 10 % fetal bovine serum (FBS), GM-CSF (10 ng/ml), IL-4 (5 ng/ml) and SCF (10 ng/ml) for 6 days. On day 6, the medium was replaced with IMDM containing 10 % FBS, GM-CSF (10 ng/ml), IL-4 (5 ng/ml), and TNF-α (4 ng/ml), and cells were cultured for an additional 3 days as described previously (Zhang et al., n.d.).

2.9. Mixed lymphocyte reaction (MLR)

CD4⁺ T cells (from BALB/c mice) were isolated from the spleen and lymph nodes using CD4 microbeads (Miltenyi Biotec). FACS-sorted pDCs were co-cultured with CFSE (Life Technologies)-labeled CD4⁺ T cells at ratios of 1:10 and 1:20 in round-bottom 96-well plates in RPMI medium containing 10 % fetal bovine serum and 5 ng/ml recombinant mouse IL-2. Cells were collected on day 7 of co-culture and stained for surface markers CD4 and CD25, as well as for intracellular cytokine production, using an intracellular staining kit (BD Cytofix/Cytoperm™ Kit, BD Biosciences, CA). Foxp3 staining was performed using the Foxp3/Transcription Factor Staining Buffer Set (BD Biosciences) according to the manufacturer’s protocol.

2.10. Intracellular staining of IFN-α in pDCs

Peripheral blood from sepsis patients on day 14 (with day 1 defined as the day of diagnosis) and age-matched healthy donors was collected and processed promptly. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density gradient centrifugation. The PBMCs were then collected and stained for surface markers, including Lineage (CD3/CD14/CD15/CD16/CD19/CD20/CD56), HLA-DR, CD11c, CD123 and CD1c, as well as for intracellular IFN-α production, using an intracellular staining kit (BD Cytofix/Cytoperm™ Kit, BD Biosciences, CA) according to the manufacturer’s protocol.

2.11. Analysis of cytokines

To obtain BM supernatants, tibias and femurs were harvested from experimental mice. The metaphysis and diaphysis of the long bones were separated with scissors, and 80 μl of PBS was flushed through each diaphysis into an Eppendorf tube, collected, and re-flushed through the

bone. Cells were pelleted by centrifugation at 400 g for 5 min. The supernatant was collected, and any remaining cells were removed by further centrifugation at 500 g for 5 min. Serum was prepared by collecting blood via cardiac puncture after terminal anesthesia with pentobarbital. The blood was incubated at 4 °C for at least 3 h to clot, then centrifuged at 12,000 g for 10 min at 4 °C. The serum supernatant was transferred to an Eppendorf tube and immediately frozen at −80 °C. Cytokines were analyzed using a Luminex 200 apparatus (Luminex Corporation). The following cytokines were measured in BM supernatants and serum samples: IL-1 β , IL-4, IL-10, IL-6, granulocyte-colony-stimulating factor (G-CSF), IFN- γ , tumor necrosis factor- α (TNF- α) and granulocyte/macrophage-colony-stimulating factor (GM-CSF). BM supernatants and serum samples were diluted at ratios of 1:1 and 1:2, respectively.

2.12. Real-time qPCR

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed using a commercial kit with random primers (Takara). Complementary DNA (cDNA) was quantified through quantitative real-time polymerase chain reaction (qPCR) using a SYBR Green PCR mix (Takara) on a LightCycler 480 PCR System (Roche). The thermocycler conditions included an initial hold at 95°C for 10 min, followed by a three-step PCR program: 95°C for 20 s, 55°C for 30 s, and 72°C for 30 s, for a total of 40 cycles. Transcript abundance was calculated using the delta Ct method, normalized to 18S rRNA. All primer sequences are listed in Table 3.

2.13. mRNA sequencing analysis

BM CD117⁺ (c-kit⁺) HSPCs were sorted from CLP-treated mice (n = 2) and Sham mice (n = 2) on day 14 post-sepsis. Total RNA was extracted using TRIzol and subjected to RNA-seq analysis for each group. RNA was used to construct Illumina TruSeq paired-end sequencing libraries following the manufacturer’s protocols at Gene-wiz. Sequencing was performed on the Illumina HiSeq 2500 platform, generating approximately 50 million clean reads per sample. Reads were mapped to the mouse genome (mm10), and gene fragments were quantified using HTSeq. Trimmed mean of M value (TMM) normalization was applied to evaluate gene expression in normalized Reads Per Kilobase of exon per Megabase of library size (RPKM). Differentially expressed genes between CLP-treated and Sham-treated HSPCs were identified with a fold-change ≥ 2 and a false discovery rate (FDR) ≤ 0.05 using edgeR software.

3. Statistics

Survival rates between the Sham group and the CLP group were compared using the log-rank (Mantel-Cox) test. For data with a normal distribution, Student’s *t*-test (two-tailed) was used for two-group comparisons, and one-way ANOVA with Bonferroni’s multiple comparison test was applied for multiple group comparisons. For data that did not follow a normal distribution, the Kruskal-Wallis test with Dunn’s multiple comparison test was used for multiple group comparisons, or

Mann-Whitney U tests (two-tailed) were used for two-group comparisons. Samples that experienced technical failure during processing were excluded from the analyses. P-values ≤ 0.05 were considered statistically significant.

4. Results

4.1. Sepsis induces sustained depletion of pDCs in the spleen and bone marrow

We previously reported that two major subsets of dendritic cells (DCs)—conventional DCs (cDCs) and plasmacytoid DCs (pDCs)—show impaired numbers and functions in the peripheral blood of patients during the acute phase of sepsis (Lu et al., 2021). Similarly, in the acute phase of the cecal ligation and puncture (CLP) model, a decrease in both cDC and pDC populations was observed (Strother et al., 2016). To further investigate whether changes in DC subsets persist during the immunosuppressive phase of sepsis, we established a polymicrobial CLP mouse model to examine these alterations in the late phase of the disease (days 14 and 28). The 28-day mortality rate of the CLP group was 55 %, while no mice in the Sham group died (Fig. 1A). Enhanced loss of body weight and a higher clinical score (Including: appearance, level of consciousness, activity, response to stimuli and eye discharge) in the CLP group confirmed the successful establishment of the sepsis model (data not shown).

DCs can be broadly categorized into two groups: cDCs and pDCs. We assessed the proportion and number of cDCs (defined as CD11c⁺ MHC-II⁺ CD11b⁺ cells) and pDCs (defined as CD11c⁺ MHC-II⁺ B220⁺ cells) in the spleen and bone marrow (BM) of three groups of mice: normal, sham and CLP, at days 14 and 28 post-infection (Fig. 1B, C) (Cabeza-Cabrerizo et al., 2021; Reizis, 2019). Importantly, the percentage and number of cDCs and pDCs were reduced four- and seven-fold, respectively, in the spleen of CLP-treated mice at day 14 post-infection compared to their Sham counterparts (Fig. 1D). The frequency of pDCs was significantly reduced in the BM of CLP-treated mice at day 14, with this reduction lasting up to 28 days after infection in both the BM and the peripheral spleen (Fig. 1D, E). In contrast, compared to normal or Sham mice, the production of cDCs in the BM of CLP-treated mice increased at day 14 and returned to normal by day 28 (Fig. 1E). We also observed a decreased percentage and number of cDCs in the spleen at day 14, while no difference was found for cDCs at day 28, indicating recovery of the cDC subset (Fig. 1D). These findings suggest that sepsis may have a profound and sustained impact on pDC reconstitution.

4.2. pDCs show phenotype of activation, but the capacity to produce IFN- α and IFN- β is impaired

One characteristic of pDCs is their ability to produce type I interferons (IFNs) when stimulated by viruses or self-DNA (Guilliams et al., 2016). To assess the functional response of pDCs to viral stimulation in the late phase of sepsis, we measured IFN- α and IFN- β production after stimulating splenic pDCs with R848 and CPG ODN. Our results showed that splenic pDCs from Sham mice produced significantly increased IFN- α and IFN- β in response to these agonists compared to unstimulated pDCs (Fig. 2A). In contrast, splenic pDCs from CLP-treated mice produced lower levels of IFN- α and IFN- β after stimulation with R848 and CPG ODN at day 14 post-infection compared to Sham mice (Fig. 2A). A parallel assay with human pDCs also demonstrated a lack of increased IFN- α production in response to TLR7 and TLR9 agonists in peripheral pDCs from septic patients on the 14th day of hospitalization (Fig. 2B), consistent with the findings in septic mice. These data suggest that pDCs acquire functional defects in the late phase of sepsis.

In addition to type I IFN production, pDCs play a crucial role as antigen-presenting cells (APCs) that activate adaptive immune responses (Di Pucchio et al., 2008; Tel et al., 2013; Swiecki and Colonna, 2015). To examine the activation status of pDCs in the peripheral spleen,

Table 3
Primer for real-time RT-PCR.

Primer	5'→3'(Forward)	5'→3'(Reverse)
18 s	CGGCTACCACATCCAAGG	GCTGCTGGCACCAGACTT
Mouse IFN- α	AAGCCATCCTTGTGCTAAGAGA	AGCAAGTTGGTTGAGGAAGAG
Mouse IFN- β	TGGGTGGAATGAGACTATTGTT	CTCCACGTCATCTTTCTCTC
Mouse SPIB	GAACCACCATGCTTGCTCTG	TCCAGCCCATGTAGAGTCA
Mouse TCF4	TTTGCCGTCTTCAGTCTACG	GCATGAAGAAGGAGCTAGGG
Mouse Flt3	CCCTACTTTCCAGGCACATT	CATTGAACCTGAGAGCTGA
Mouse IRF8	TACAATCAGAGGTGGATGC	TTCAGAGCAGACGGTAACCT
Mouse PU.1	CGAAGAAGATGACCTACCA	ACTTTCTTCACCTCGCCTGT

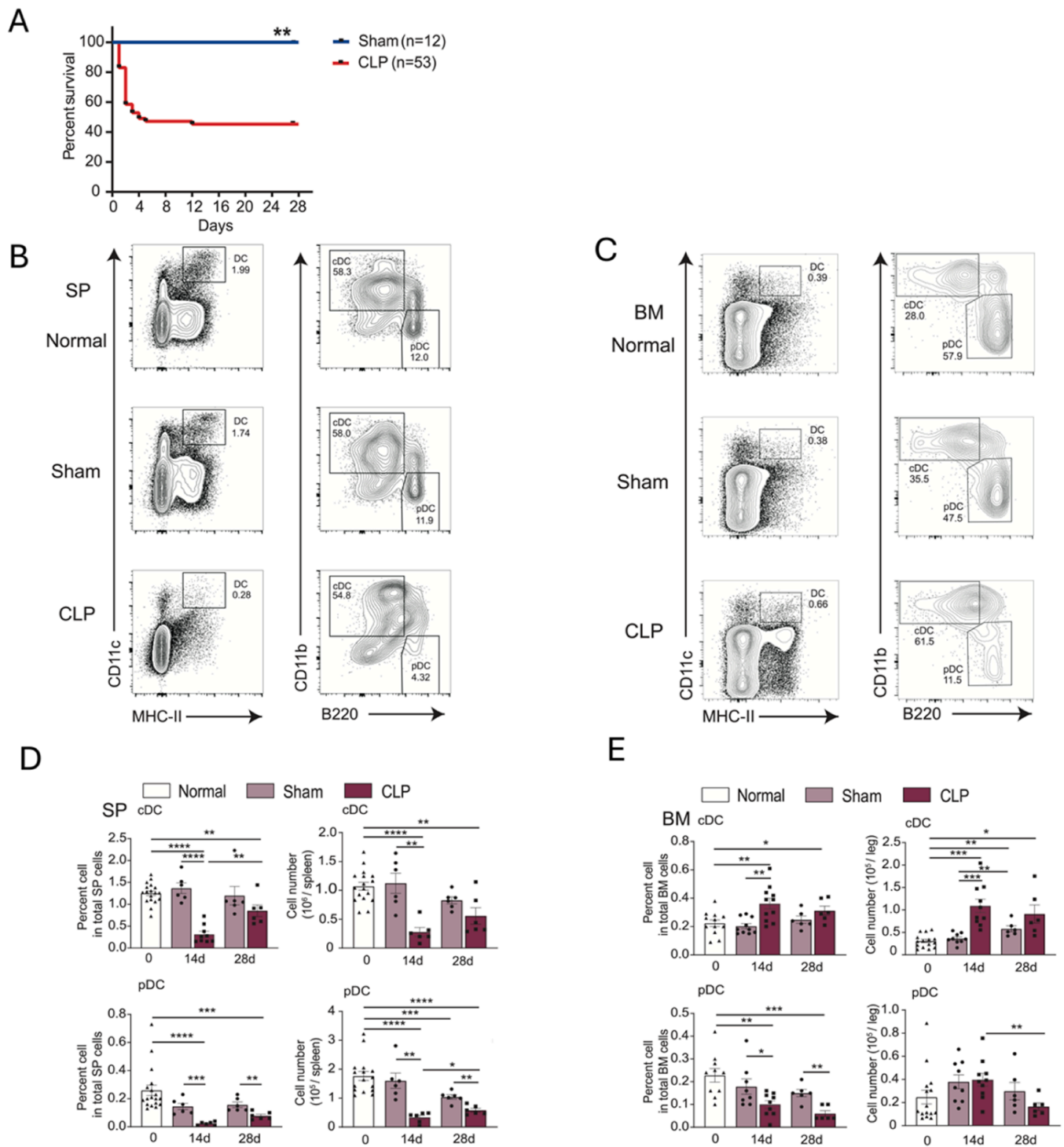


Fig. 1. cDCs recover to normal levels by 28 days post-infection, whereas the overall population of pDCs decreases and remains reduced through day 28. Sham and CLP surgeries are performed on B6 mice, and spleen and bone marrow are analyzed at 14 and 28 days post-surgery. B6 mice without surgery serve as the Normal group (day 0). (A) The 28-day survival rates of CLP and Sham mice were observed. (B) Representative flow cytometry plots from the spleen of Normal, Sham, CLP groups of mice at day 14 showing the gating strategy of cDC and pDC populations. (C) Representative flow cytometry plots from the bone marrow of Normal, Sham, CLP mice at day 14 showing the gating strategy of cDC and pDC populations. (D) Graphs show the percentage and cell number of cDCs and pDCs in the spleen of Normal, Sham, CLP groups of mice. (E) Graphs show the percentage and cell number of cDCs and pDCs in the bone marrow of Normal, Sham, CLP groups of mice. Error bars indicate means $\pm$ SEM. Data conforming to normal distribution were compared using two-tailed t test, while those with non-normally distributed were tested using Mann-Whitney U-test. Log-rank (Mantel-Cox) test was performed for comparing mouse survival curves. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

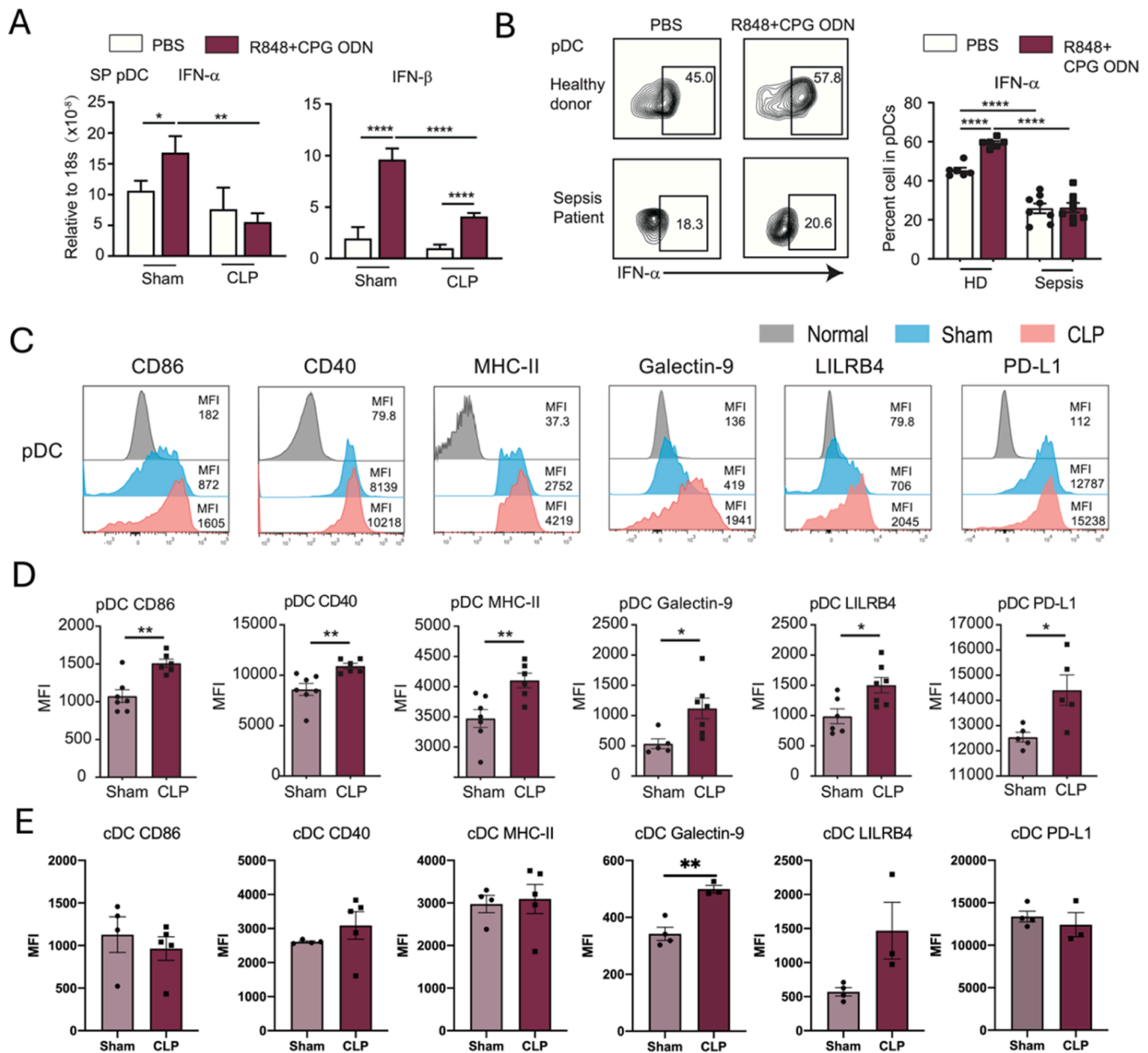


Fig. 2. pDCs show phenotype of activation, but the capacity to produce IFN- α and IFN- β is impaired. (A) Real-time qPCR analysis of genes in FACS-sorted pDCs (defined as MHC-II $^{+}$ CD11c $^{+}$ B220 $^{+}$) in spleen after stimulating with or without R848 (100 ng/ml) and CPG ODN (1 μ M) for 3 hr between Sham mice and CLP-treated mice. (B) The pDCs in the peripheral blood of sepsis patients on the 14th day of hospitalization and healthy donors (HD) were evaluated for intracellular IFN- α by flow cytometry after being stimulated or left unstimulated with R848 (100 ng/ml) and CpG ODN (1 mM) overnight. (C) Flow cytometry histograms showing the mean fluorescence intensity (MFI) of CD86, CD40, MHC-II, Galectin-9, LILRB4 and PD-L1 expression on splenic pDCs (CD11c $^{+}$ B220 $^{+}$ CD11b $^{-}$) at day 14 among the normal group, Sham group and CLP group. (D) Statistical plots of the mean fluorescence intensity (MFI) of CD86, CD40, MHC-II, Galectin-9, LILRB4 and PD-L1 on splenic pDCs between Sham mice and CLP-treated mice. (E) Statistical plots of the mean fluorescence intensity (MFI) of CD86, CD40, MHC-II, Galectin-9, LILRB4 and PD-L1 on splenic cDCs between Sham mice and CLP-treated mice. Error bars indicate means $\pm$ SEM. Data conforming to normal distribution were compared using two-tailed t test, while those with non-normally distributed were tested using Mann-Whitney U-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. HD.

we analyzed the expression of various markers by flow cytometry (Fig. 2C). At day 14 of CLP-treated mice, splenic pDCs showed signs of activation, as indicated by increased expression of co-stimulatory molecules (CD40 and CD86) and MHC class II (MHC-II) molecules (Fig. 2D). In cancer studies, activation of mature pDCs has been shown to induce high levels of immunosuppressive molecules (Kojima et al., 2011; Tofalori et al., 2019; Deng et al., 2018). Similarly, we observed upregulation of immunosuppressive molecules (PD-L1, Galectin-9, and LILRB4) on splenic pDCs in CLP-treated mice at day 14 post-sepsis compared to Sham mice (Fig. 2D). Parallel analysis of cDCs revealed no significant differences in the expression of these activation markers between CLP

and Sham groups, except for a modest increase in Galectin-9, suggesting a partial functional recovery of cDCs (Fig. 2E). Thus, sepsis induces an activated phenotype in pDCs, but impairs their ability to produce type I IFN.

4.3. pDCs from septic mice suppress differentiation of T cells into Th1 while enhancing Treg differentiation in vitro

Dysfunction of CD4 $^{+}$ T cells and an imbalance between T helper (Th) and regulatory T (Treg) cell populations contribute to the immunosuppression observed in septic patients, making them more susceptible to

secondary infections (Yeh et al., 2020; Bolognese et al., 2018). However, the precise role of pDCs as antigen-presenting cells (APCs) in driving $CD4^+$ T cell-mediated immune responses during sepsis remains poorly understood. To investigate the ability of activated splenic pDCs from CLP-treated mice and Sham mice to prime naïve $CD4^+$ T cells, we co-cultured pDCs with allogeneic $CD4^+$ T cells in vitro (Fig. 3A). The purity staining of sorted pDCs is shown in Fig. 3B. Splenic pDCs from CLP-treated mice were more efficient at inducing $CD4^+$ T cell proliferation compared to those from Sham mice, as indicated by a higher percentage of proliferating $CD4^+$ T cells (Fig. 3C). This suggests the involvement of activation markers on septic pDCs. Moreover, these

activated pDCs promoted the differentiation of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells while reducing the production of $IFN-\gamma$ by T cells, leading to a decreased Th1/Treg ratio (Fig. 3D, E). These findings provide further evidence that pDCs play a crucial role in the immunosuppressive response during sepsis: splenic pDCs in the late phase of sepsis exhibit an immunosuppressive phenotype with upregulated expression of immunosuppressive molecules (PD-L1, LILRB4 and Galectin-9). Furthermore, these activated pDCs induce Treg differentiation, which may exacerbate sepsis-induced immunosuppression.

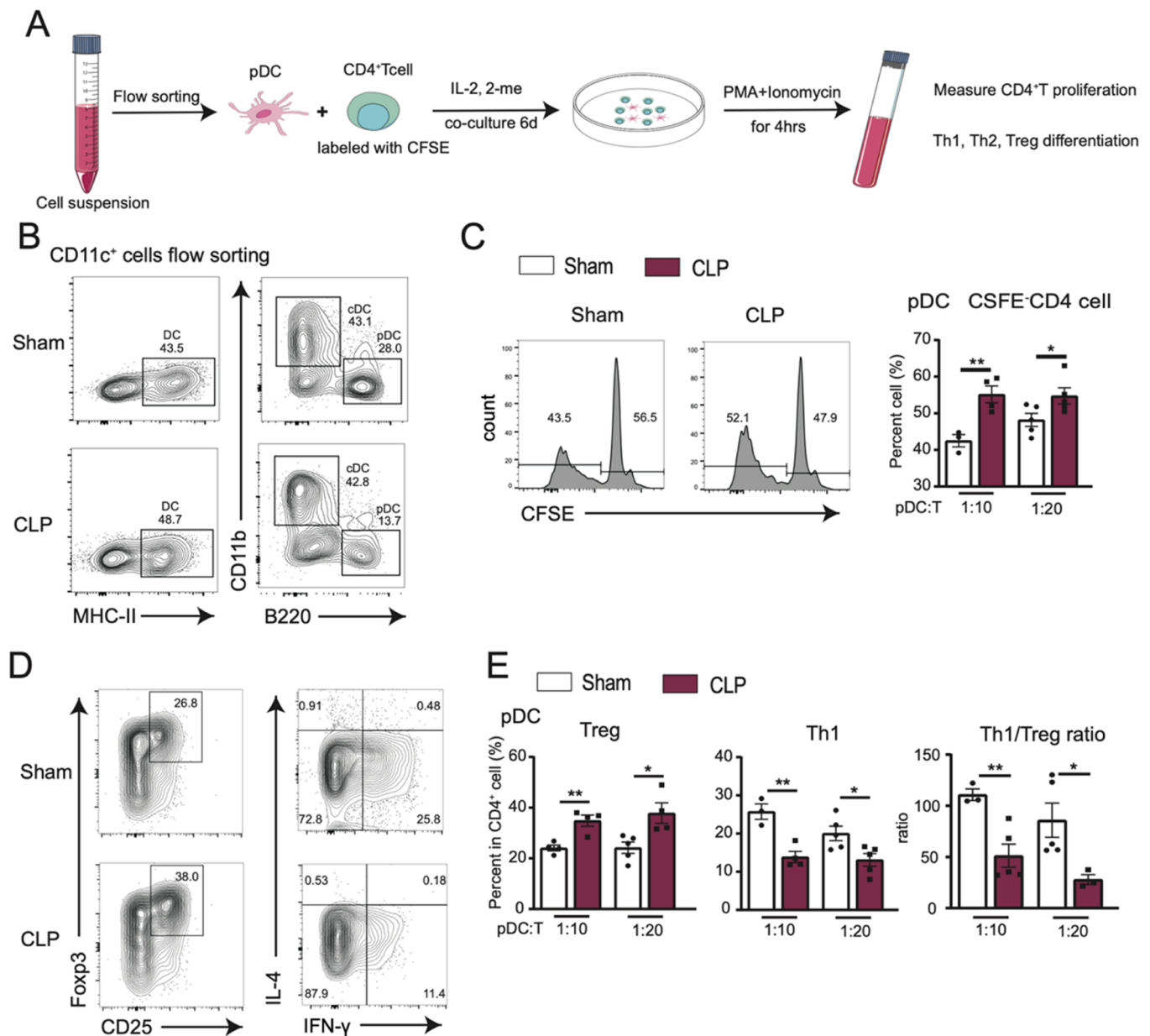


Fig. 3. pDCs suppress differentiation of naïve T cells into Th1 while inducing Treg differentiation in vitro. (A) The procedure for co-culturing pDCs ($CD11c^+$ $MHC-II^+$ $B220^+$) with $CD4^+$ T cells. $CD4^+$ T cells were isolated from BALB/c mice, labeled with CFSE, and incubated with B6 FACS-sorted splenic pDCs from CLP-treated mice and Sham mice at day 14 post-sepsis at a certain ratio. After 6 days of co-culture, $CD4^+$ T cells were stimulated with PMA and ionomycin for 4 h and then tested for their proliferation and differentiation. (B) Representative flow cytometry plots showing the gating strategy for FACS-sorted splenic pDCs (defined as $CD11c^+$ $MHC-II^+$ $B220^+$ cells). (C) Proliferation of $CD4^+$ T cells induced by splenic pDCs from CLP-treated mice and Sham mice. (D) Representative flow cytometry plots of $IFN-\gamma^+$ $CD4^+$ T cells (defined as Th1 cells) and $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (defined as Treg cells). (E) Statistical plots showed the percentage of $IFN-\gamma^+$ $CD4^+$ T cells (defined as Th1 cells) and $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (defined as Treg cells), and Th1/Treg ratio. Error bars indicate means $\pm$ SEM. Data conforming to normal distribution were compared using two-tailed t test, while those with non-normally distributed were tested using Mann-Whitney U-test. * $P < 0.05$, ** $P < 0.01$.

4.4. BM HSPCs in the late phase of sepsis show reduced DC progenitors and impaired reconstitution of immunocompetent pDCs *in vitro*

Given that pDCs are short-lived (Lutz et al., 2022; Zhan et al., 2016), we investigated whether the prolonged reduction of pDCs in the late phase of sepsis was linked to long-term adaptations of pDC precursor and progenitor cells. To test this hypothesis, we assessed the effect of the late phase of sepsis on the generation of BM pDC progenitors and precursors, including hematopoietic stem cells (HSCs), multipotent progenitors (MPPs), monocyte and dendritic cell progenitors (MDPs) and common dendritic cell progenitors (CDPs) (Anderson et al., 2021) (Fig. 4A). As shown in Fig. 4B and Fig. 4C, the percentage and number of CDPs, which directly differentiate into pDCs, were reduced at day 14 in CLP-treated mice, and this reduction persisted at day 28, with CDP numbers remaining significantly decreased compared to Sham controls. Similarly, the number and percentage of MDPs, which give rise to pDCs via CDPs, were also reduced at day 14 in CLP-treated mice, with this reduction sustained through day 28 (Fig. 4B, C). In contrast, the number of MPPs remained unchanged throughout the late phase of sepsis (Fig. 4B, C). Interestingly, the number of HSCs increased at day 14 in CLP-treated mice and returned to normal by day 28 (Fig. 4B, C). Since the HSC population includes both short-term and long-term HSCs, the increase at day 14 may reflect the expansion of short-term HSCs induced by emergency hematopoiesis during sepsis. These findings suggest that the reduced generation of pDC progenitors and precursors contributes to the impaired reconstitution of pDCs in the late phase of sepsis.

We then explored whether the intrinsic capacity of hematopoietic stem and progenitor cells (HSPCs) to reconstitute pDCs was altered in the late phase of sepsis. When we cultured sorted BM progenitor cells, with purity shown in Fig. 5B, in the presence of Flt3 ligand and stem cell factor (SCF) to generate pDCs *in vitro* (Fig. 5A), pDC generation was significantly compromised in cultures derived from septic mice at both day 14 and 28 after infection (Fig. 5C, D), indicating an intrinsic defect in HSPCs to reconstitute pDCs during the late phase of sepsis. We further examined the function of BM-derived pDCs from these cultures, comparing those from CLP and Sham mice at day 14. BM-derived pDCs from CLP-treated mice showed diminished IFN- α production in response to stimulation with TLR7 and TLR9 agonists (Fig. 5E), although no substantial difference in IFN- β production was observed between the two groups. Moreover, BM-derived pDCs from CLP-treated mice exhibited slightly higher expression of co-stimulatory molecules (CD40 and CD86), antigen-presentation molecules (MHC-II) and immunosuppressive molecules (Galectin-9 and LILRB4) following TLR agonist stimulation (Fig. 5F), consistent with the *in vivo* findings in septic mice (Fig. 2D). These results reveal that the late phase of sepsis impairs the ability of BM progenitors to generate immunocompetent pDCs. Taken together, our findings indicate that the sustained reduction in MDPs and CDPs contributes to the persistent decrease in pDC populations during the late phase of sepsis. Additionally, the intrinsic capacity of septic HSPCs to reconstitute immunocompetent pDCs is also impaired, as evidenced by reduced IFN- α production and an increased Treg/Th1 ratio in mixed lymphocyte reactions (MLRs).

4.5. The key transcription factors TCF4 and SpiB are downregulated in pDCs and their progenitors from septic mice

A small number of transcription factors are known to be required for the specification of pDCs from hematopoietic progenitors (Nutt and Chopin, 2020). We sought to determine whether key transcription factors were involved in impaired pDC reconstitution in the late phase of sepsis. To investigate this, we performed mRNA sequencing analysis of bone marrow HSPCs enriched using CD117 microbeads from cecal ligation and puncture (CLP) and Sham mice at day 14 post-operation. HSPCs from septic mice showed reduced expression of SpiB and TCF4 (Fig. 6A, B), which are master regulators of pDC development and function (Fan et al., 2021; Lutz et al., 2022). There were no differences in

the expression of Flt3 and IRF8 between the two groups (Fig. 6A, B), both of which regulate the differentiation of both pDCs and cDCs from HSPCs (Nutt and Chopin, 2020; Sichien et al., 2016).

To further assess the role of TCF4, we ectopically expressed TCF4 using a retroviral system in CD117⁺ HSPCs isolated from CLP mice at day 14 post-operation. Ectopic expression of TCF4 via retroviral transduction partially rescued pDC generation compared to the control group (Fig. 6C, D), suggesting that TCF4 plays a critical role in pDC reconstitution from HSPCs under septic conditions.

A key observation in septic mice was the specific impairment of pDCs, while the percentage and number of cDCs remained comparable to Sham mice in the spleen and were even higher in the bone marrow. This led us to hypothesize that the cDC generation pathway remained intact in septic mice. To test this, we cultured HSPCs from septic mice in an *in vitro* GM-CSF system, which selectively promotes the generation of cDCs (Fig. 6E). As expected, recovery of cDCs from septic mice was similar to that of Sham mice (Fig. 6F, G).

Thus, these findings demonstrate that the late phase of sepsis selectively impairs pDC reconstitution from HSPCs by downregulating the key transcription factors TCF4 and SpiB, while preserving the cDC differentiation pathway.

4.6. G-CSF remains high in bone marrow in septic mice and impairs the reconstitution of pDC progenitors and pDCs *in vitro*

Recent studies have revealed that long-term infection remodels the BM microenvironment through systemic cytokine secretion, leading to adaptations in the lineage-specific proliferation, differentiation and function of HSPCs (Xu et al., 2022; Chavakis et al., 2019; Johnson et al., 2020). To investigate the role of cytokines in the BM microenvironment during the late phase of sepsis, we measured a series of cytokines, including pro-inflammatory factors (IL-1 β , IL-6, TNF- α), anti-inflammatory factors (IL-4, IL-10) and growth factors (G-CSF and GM-CSF), in both BM supernatants and serum of mice at day 14 post-infection. We observed a significant increase in G-CSF levels in both the serum and BM supernatant of CLP-treated mice at day 14 post-operation (Fig. 7A). In comparison to Sham mice, levels of IL-1 β , TNF- α , and IL-6 were also elevated in the BM supernatant of CLP-treated mice, although to a lesser extent than G-CSF, with no significant difference observed in the serum (Fig. 7A). This finding is consistent with G-CSF being widely associated with post-infection responses in sepsis patients (Lee et al., 2016).

Previous studies have suggested that G-CSF, a novel immune tolerance mediator, exerts immunoregulatory effects on DCs and T cells (Yang et al., 2016). We found that the addition of G-CSF induced pDC generation with a phenotype similar to that of mreg pDCs from septic mice, characterized by increased expression of immune co-activation and co-inhibitory molecules (MHC-II, CD86, PD-L1 and LILRB4) and decreased production of type I interferons (IFNs) following TLR agonist stimulation (Fig. 7F, G). Notably, G-CSF also led to a reduced generation of DC progenitors and pDCs (Fig. 7B–E). Moreover, we observed a downregulation of pan-DC transcription factors, including IRF8 and FLT3, in addition to pDC-specific transcription factors TCF4 and SpiB (Fig. 7H, I). This findings contrast with the *in vivo* data from septic mice, implying that the regulation of DC reconstitution is influenced by multiple factors beyond G-CSF.

Taken together, our results suggest that G-CSF plays a critical role in remodeling the BM microenvironment during the late phase of sepsis, impairing the reconstitution of pDC precursors and immunocompetent pDCs.

5. Discussion

In this study, we identified that sepsis has a profound and sustained impact on the reconstitution of dendritic cell (DC) progenitors and immunocompetent plasmacytoid dendritic cells (pDCs). Sepsis leads to

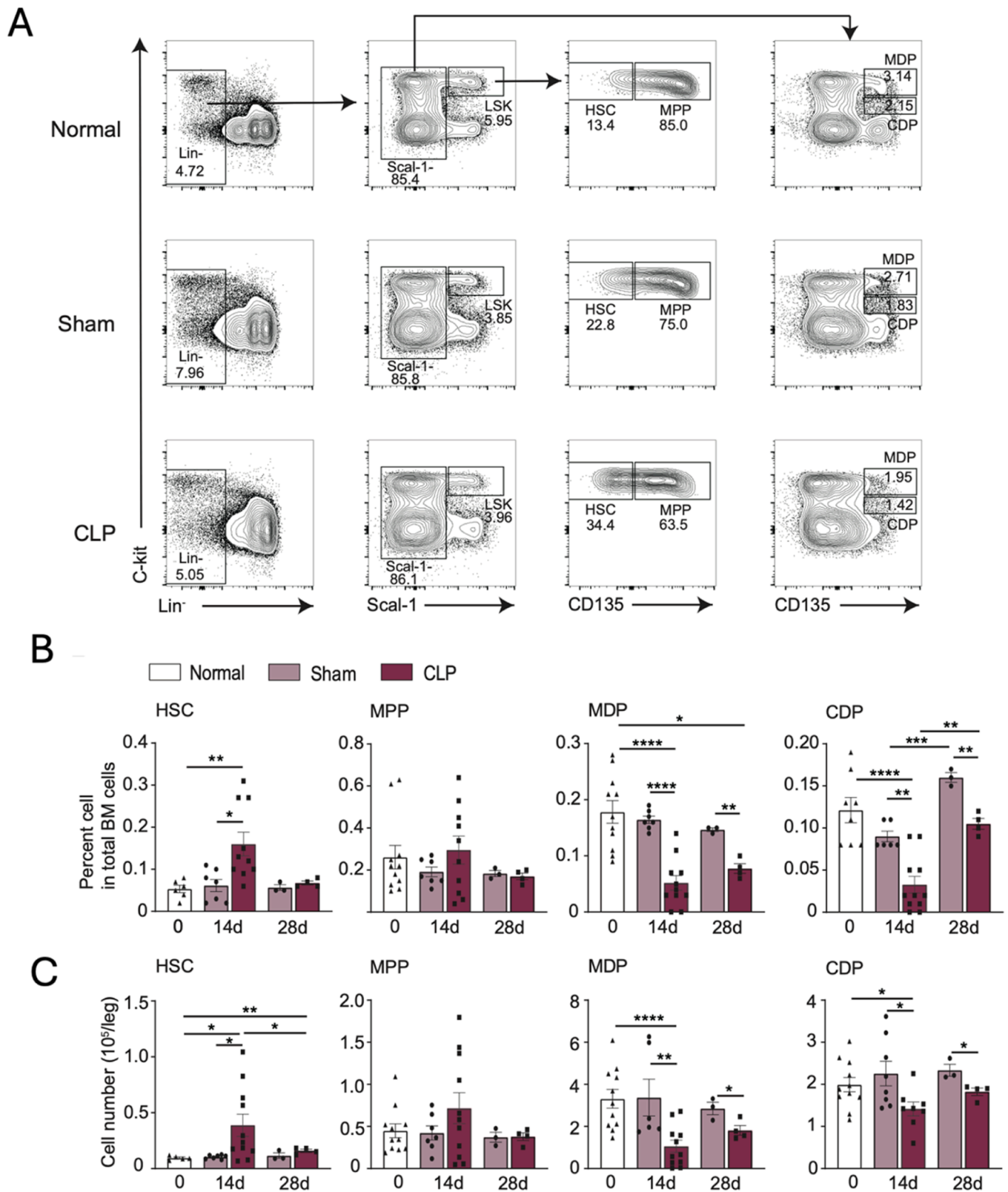


Fig. 4. BM pDC progenitors are reduced at the late phase of sepsis. (A) Representative flow cytometry plots of BM pDC progenitors. Mouse HSCs were characterized as Lin⁻ Sca1⁺ CD135⁺ cells, MPPs as Lin⁻ Sca1⁺ CD135⁺ cells, MDPs as Lin⁻ Sca1⁻ CD135⁺ c-kit^{int} cells and CDPs as Lin⁻ Sca1⁻ c-kit^{int} CD135⁺ cells. 'Lin' includes markers for CD3, CD11b, Ter119, B220, and Gr1(B ~ C) Graphs depict the percentage (upper) and the absolute number (lower) of indicated progenitors in BM at day 14 and 28 following Sham or CLP operations. Error bars indicate means $\pm$ SEM. Data conforming to normal distribution were compared using two-tailed t test, while those with non-normally distributed were tested using Mann-Whitney U-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

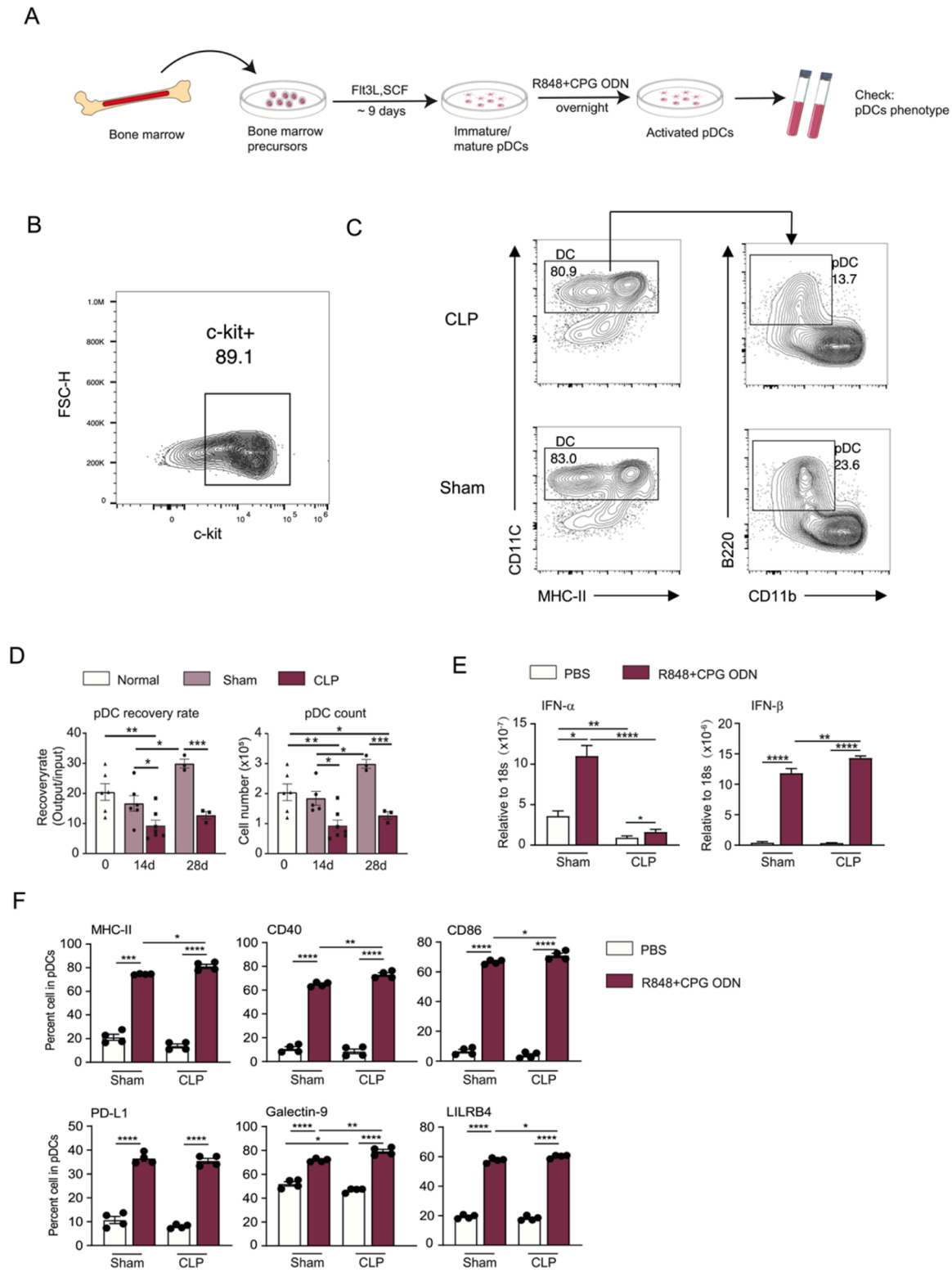
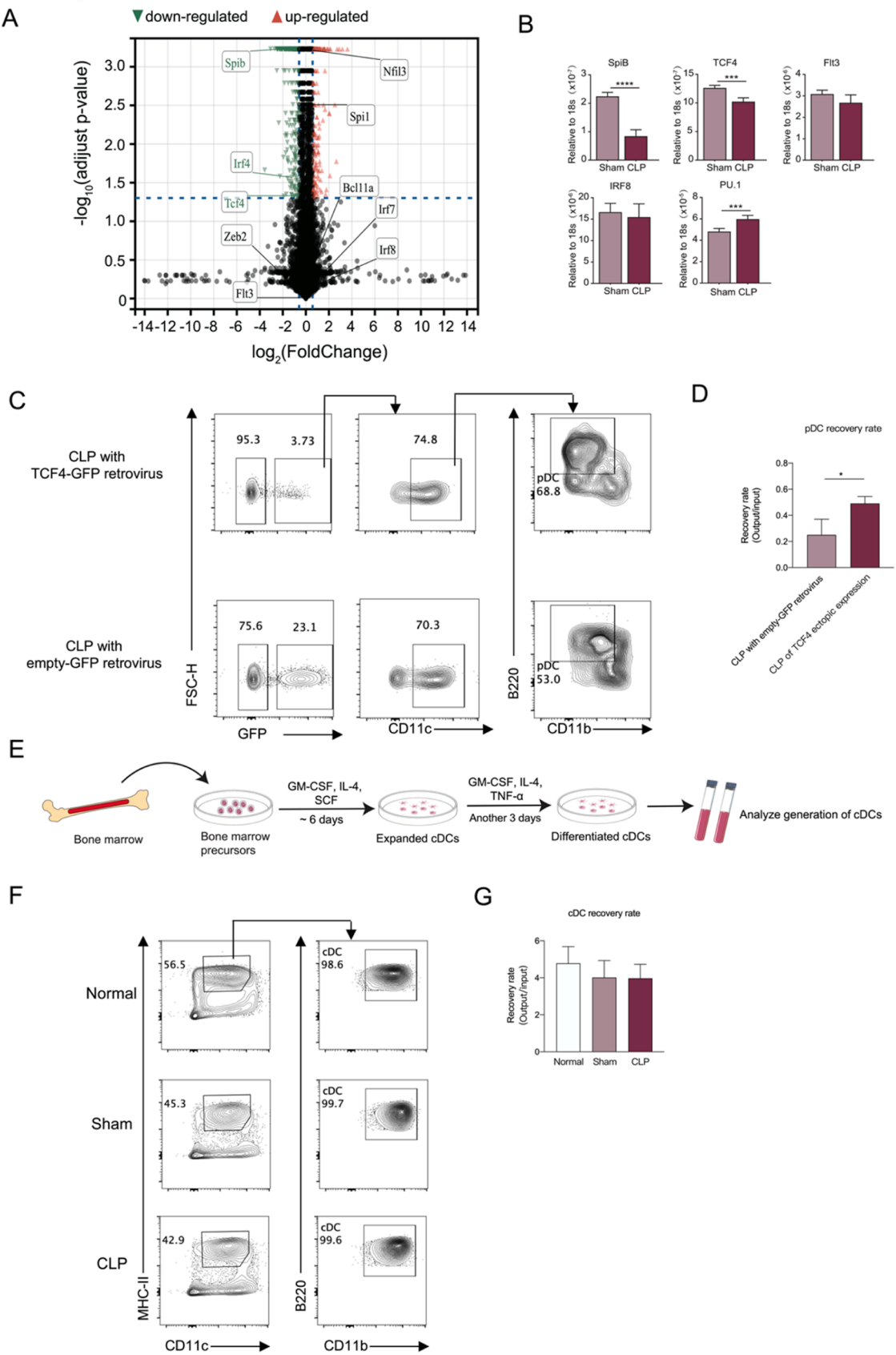


Fig. 5. BM HSPCs at the late phase of sepsis fail to generate immunocompetent pDCs in vitro. (A) The procedure of the culture of BM-derived pDCs: 14 or 28 days after Sham or CLP operation, sorted BM HSPCs were cultured with Flt3L (50 ng/ml) and SCF (10 ng/ml). pDCs were identified as CD11c⁺ B220⁺ CD11b⁻ on day 9 of culture. (B) Representative flow cytometry plots showing the purity of sorted CD117⁺ (c-kit⁺) HSPCs. (C) Representative flow cytometry plots showing BM-derived pDCs (CD11c⁺ B220⁺ CD11b⁻) on day 9 of culture. (D) The graphs show the recovery rate ($\text{recovery rate} = \frac{\text{percentage of pDCs in total cells} \times \text{output total cell number}}{\text{input total cell number}} \times 100\%$) and the absolute number of BM-derived pDCs on day 9 of culture. (E) Real-time qPCR analysis of gene expression of IFN- α and IFN- β in FACS-purified BM-derived pDCs (MHC-II⁺ CD11c⁺ B220⁺) stimulated with R848 (100 ng/ml) and CpG ODN (1 mM) for 3 h. (F) To induce pDC activation, BM-derived pDCs (CD11c⁺ B220⁺ CD11b⁻) were stimulated or not with R848 (100 ng/ml) and CpG ODN (1 mM) overnight and then harvested for FACS analysis to evaluate the expression of co-activatory and co-inhibitory molecules. Error bars indicate means $\pm$ SEM. Data conforming to normal distribution were compared using two-tailed t test, while those with non-normally distributed were tested using Mann-Whitney U-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.



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Fig. 6. The pDC reconstitution pathway is specifically impaired through TCF4 downregulation in HSPCs from septic mice. (A) Volcano plot showing fold changes in HSPCs from CLP and Sham mice, with significantly upregulated (red) and downregulated (green) genes highlighted. (B) Real-time qPCR analysis of transcription factor gene expression (e.g., TCF4, SpiB, Flt3, IRF8, and PU.1) in HSPCs from CLP and Sham mice on day 14. (C) Representative flow cytometry plots of BM-derived pDCs (CD11c⁺ B220⁺ CD11b⁺) differentiated from HSPCs after TCF4-GFP retrovirus transducing in vitro. (D) Graphs showing the recovery rate ($\text{recovery rate} = \frac{\text{percentage of pDCs in total cells} \times \text{output total cell number}}{\text{input total cell number}} \times 100\%$) of differentiated pDCs on day 3 after TCF4-GFP retroviral transduction. (E) The procedure of the culture of BM-derived cDCs: 14 days after Sham or CLP operation, sorted BM HSPCs were cultured with GM-CSF, IL-4, SCF for 6 days. The medium was then replaced with GM-CSF, IL-4, TNF- α and cultured until day 9. (F) Representative flow cytometry plots of BM-derived cDCs (CD11c⁺ MHC-II⁺ CD11b⁺) differentiated from HSPCs in vitro. (G) Graphs showing the recovery rate ($\text{recovery rate} = \frac{\text{percentage of cDCs in total cells} \times \text{output total cell number}}{\text{input total cell number}} \times 100\%$) of BM-derived cDCs on day 3 of culture. Error bars indicate means $\pm$ SEM. Data conforming to normal distribution were compared using two-tailed t test or Pearson's chi-squared test, while those with non-normally distributed were tested using Mann-Whitney U-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

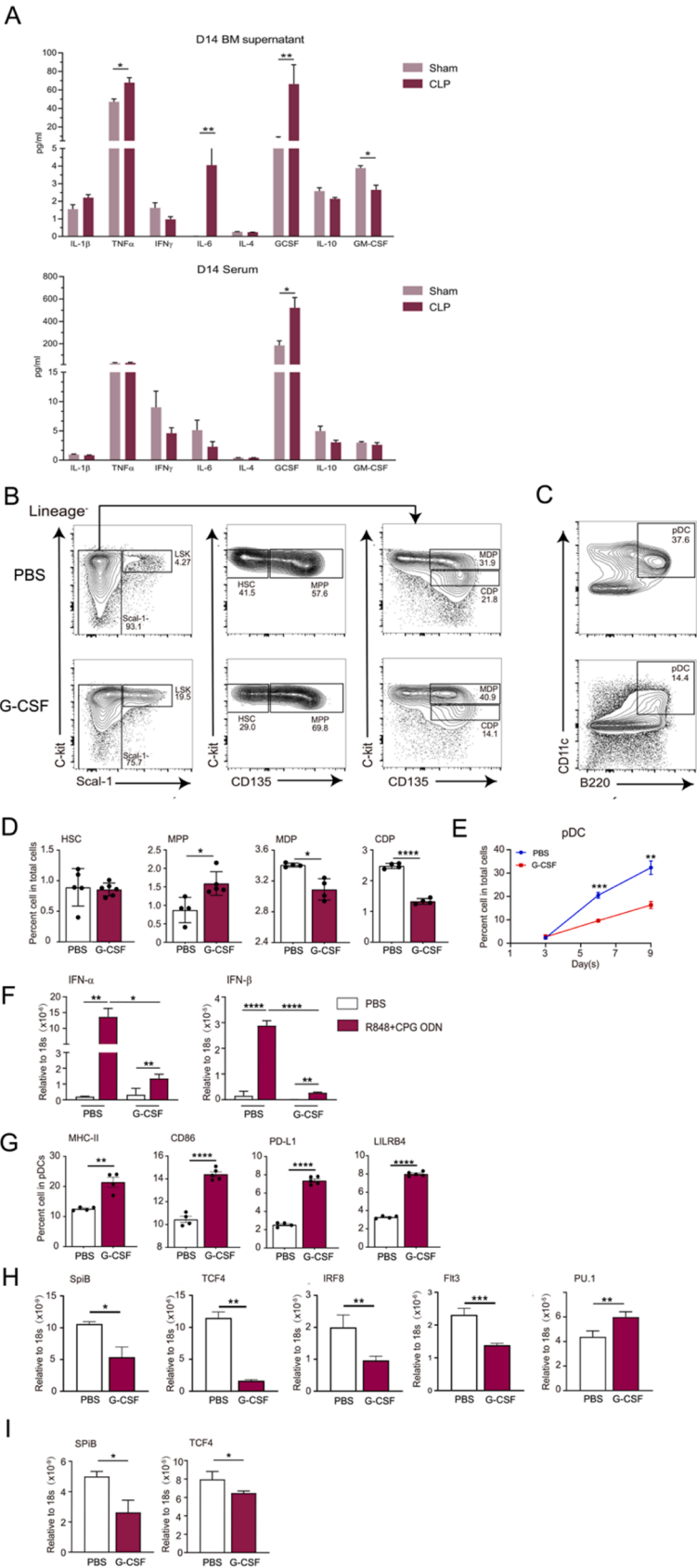
the downregulation of key transcription factors, TCF4 and SpiB, in bone marrow (BM) hematopoietic stem and progenitor cells (HSPCs), which are essential for pDC differentiation and function. Notably, we found that pDCs from septic mice exhibited a mature yet regulatory phenotype, characterized by high expression of co-stimulatory and co-inhibitory molecules, a reduced capacity to produce interferons (IFN- α and IFN- β), and an impaired ability to polarize CD4⁺ T cells into Th1 cells while promoting Treg polarization in vitro. Moreover, our study uncovered a critical role for G-CSF in the BM microenvironment of sepsis in regulating the reconstitution of pDCs and their progenitors. These findings provide valuable insights into the alterations in pDC reconstitution following acute septic shock and highlight the underlying cellular and molecular mechanisms.

In our previous study, we demonstrated that sepsis induces significant apoptosis of pDCs during the acute phase, which likely contributes to their initial depletion (Lu et al., 2021). The current study extends these findings by showing that sepsis also impairs the generation of new immunocompetent pDCs from HSPCs in the late phase, creating a "double hit" on the pDC compartment. The most significant functional consequence appears to be the loss of type I interferon production, which is critical for antiviral immunity. While apoptosis reduces pDC numbers, the surviving pDCs exhibit altered functionality, including impaired antigen-presenting capacity and a tendency to promote Treg differentiation. This combination of quantitative and qualitative defects in pDCs may significantly contribute to the immune suppression observed in late-stage sepsis. We observed that pDCs in the late phase of sepsis exhibited a highly activated status but paradoxically lost the capacity to produce normal amounts of IFN- α and IFN- β . More importantly, these pDCs acquired dual regulatory functions - suppressing Th1 differentiation while enhancing Treg polarization. This unique phenotype closely resembled "mature DCs enriched in immunoregulatory molecules" (mregDCs), a specialized cell state recently described in cancer (Kvedaraitė and Ginhoux, 2022; Maier et al., 2020). Notably, Yao et al. reported similar mregDCs derived from conventional DCs (cDCs) during acute sepsis (Yao et al., 2022), suggesting this may represent a conserved immunoregulatory program across DC subsets in sepsis. Mechanistically, this functional switch appears to be controlled through three synergistic pathways: First, the profound downregulation of type I interferon production (IFN- α/β) removes critical signals for Th1 polarization, consistent with the established role of pDC-derived IFN- α in promoting T-bet expression and Th1 development (Colonna et al., 2004; Lazarevic et al., 2013). Second, upregulated expression of co-inhibitory molecules (PD-L1) engages corresponding receptors on T cells to actively drive Treg differentiation, mirroring mechanisms observed in cancer-associated mregDCs. Third, alterations in antigen presentation machinery, evidenced by reduced MHC class II expression, may preferentially favor regulatory over effector T cell responses. The co-expression of co-stimulatory (CD80/CD86) and co-inhibitory (PD-L1) molecules creates a "balanced signal" microenvironment analogous to that described in sepsis-induced cDC-derived mregDCs (Yao et al., 2022), where initial T cell activation is followed by progressive exhaustion and Treg induction. This immunoregulatory reprogramming has significant clinical implications. One of the leading causes of death in sepsis patients is opportunistic infection-induced mortality, which can

occur long after the acute phase when immune suppression predominates. This clinical trajectory mirrors observations in viral infections: in dengue, reduced interferon production by pDCs directly correlated with disease severity (Pichyangkul et al., 2003), while in HIV, impaired pDC IFN- α responses preceded detectable viremia and predicted viral rebound (Mitchell et al., 2020). These parallels across infectious models underscore that pDC dysfunction—particularly IFN deficiency—represents a conserved pathway to adverse outcomes. However, sepsis uniquely induces irreversible transcriptional reprogramming of HSPCs via G-CSF-mediated TCF4/SpiB downregulation, distinguishing it from the transient suppression seen in viral infections.

The apoptosis of pDCs during acute sepsis and their impaired regeneration in the late phase collectively contribute to severe immune dysfunction. While multiple aspects of pDC biology are compromised - including apoptosis, antigen presentation, T cell stimulation, and differentiation - the relative contributions of apoptosis versus differentiation blockade to overall pDC deficiency require further clarification. Our findings indicate that both mechanisms play significant but temporally distinct roles during different phases of sepsis. Since DCs are pivotal in pathogen sensing and initiating adaptive immune responses, the altered pDC phenotype and function in septic patients and mice may contribute to overall immune suppression and increased susceptibility to opportunistic infections. Notably, the paradoxical capacity of septic pDCs to simultaneously enhance CD4⁺ T cell proliferation while promoting Treg differentiation in MLR assays mirrors recent findings in cancer immunology. Wang et al. demonstrated that regulatory DC subsets (particularly cDC2-like mregDCs) can exhibit this dual functionality through co-expression of co-stimulatory (CD86/CD40) and inhibitory molecules (PD-L1/Galectin-9) - a phenotype strikingly similar to our septic pDCs (Wang et al., 2024). In cancer, such mregDCs initially prime T cell responses but subsequently drive exhaustion through sustained immune checkpoint signaling, ultimately creating an immunosuppressive microenvironment. This parallel suggests that pDC-mediated 'exhaustion priming' may be a conserved mechanism across chronic inflammatory conditions, including sepsis-induced immune paralysis. Nevertheless, our results underscore the importance of competent pDC reconstitution in sepsis patients in the long term.

We found that not only the number of DC progenitors (MDPs and cDPs) were reduced in septic mice, but also the intrinsic capacity of HSPCs to generate immunocompetent pDCs was impaired. In our previous study on sepsis patients, we demonstrated that DC progenitors in peripheral blood significantly decreased within seven days of hospitalization (Lu et al., 2021). However, clinical studies on intrinsic changes in HSPCs are limited due to the low abundance of HSPCs in peripheral blood and the restricted availability of bone marrow samples from septic patients. Here, using the CLP-induced septic mouse model, we were able to study both the quantitative and qualitative alterations of HSPCs in the bone marrow. Since TCF4 and SpiB are critical transcription factors for pDC specification from HSPCs, their decreased expression in HSPCs from septic mice may be a major cause of impaired pDC progenitor and pDC reconstitution. Ectopic expression of TCF4 in sepsis HSPCs rescued pDC generation in vitro, further validating our hypothesis. Notably, the observed impairment in IFN- α production may also involve dysregulation of IRF7, the master transcriptional activator of type I interferon



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Fig. 7. G-CSF impairs the development and function of pDC progenitors in vitro. BM hematopoietic progenitor cells (HSPCs) were isolated with CD117 microbeads from the BM of normal mice and then cultured in the presence of Flt3L and SCF, with or without the addition of G-CSF. (A) BM supernatants and serum samples from CLP and Sham mice were collected at day 14 post-operation and analyzed using a Luminex assay to quantify cytokines (IL-1 β , IL-4, IL-10, IL-6, G-CSF, IFN- γ , TNF- α , GM-CSF). (B) Representative flow cytometry plots show the gating strategy for pDC progenitors on day 3 of culture. (C) Representative flow cytometry plots show the gating strategy for pDCs on day 9 of culture. (D) The percentage of pDC progenitors (e.g., HSCs, MPPs, MDPs and CDPs) in total cells on day 3 of culture. (E) The percentage of pDCs in total cells on specific days of culture. (F) pDCs were cultured with or without G-CSF for 9 days, then FACS-purified (defined as CD11c⁺ B220⁺ CD11b⁻) and subsequently stimulated with R848 (100 ng/ml) and CpG ODN (1 mM) for 3 h. Real-time qPCR analysis of gene expression of IFN- α and IFN- β in G-CSF-treated and untreated cultures. (G) The graphs show the percentage of surface functional molecules (e.g., MHC-II, CD86, LILRB4, and PD-L1) in BM-derived pDCs (CD11c⁺ B220⁺ CD11b⁻) after stimulation with or without G-CSF on day 9 of culture. (H) Real-time qPCR analysis of pDC differentiation-related genes in G-CSF-stimulated HSPCs at day 3 of culture. (I) Real-time qPCR analysis of gene expression in FACS-purified pDCs on day 9 of culture after stimulation with or without G-CSF. Error bars indicate means $\pm$ SEM. Data conforming to normal distribution were compared using two-tailed t test, while those with non-normally distributed were tested using Mann-Whitney U-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

genes (Cisse et al., 2008). While our current data demonstrate that TCF4 knockdown directly compromises pDC development, and TCF4 is known to regulate IRF7 expression in pDCs (Cisse et al., 2008), the precise relationship between sepsis-induced TCF4 suppression and IRF7 activity requires further investigation. While our surface marker analysis revealed a regulatory phenotype in septic pDCs, their cytokine secretion profile likely reflects broader transcriptional reprogramming. The downregulation of TCF4—a known regulator of IRF7-dependent IFN production—suggests a fundamental defect in TLR-responsive cytokine secretion. This aligns with clinical observations that septic patients exhibit impaired IFN responses but elevated immunosuppressive mediators like IL-10 (Barrios et al., 2024). Future single-cell analyses will precisely delineate how sepsis alters the pDC secretome. Notably, we observed that the number and percentage of cDCs in septic mice returned to normal during the late phase after CLP. Through an in vitro GM-CSF culture system, we validated that GM-CSF-induced cDC generation was unaffected in sepsis HSPCs, which may partially explain the recovery of cDCs observed in septic mice. In contrast, pDC development primarily relies on FLT3L and downstream signaling pathways like TCF4 and SpiB, which were specifically impaired in HSPCs during sepsis. Notably, while TLR9-activated pDC precursors maintain TCF4 expression and acquire transient immunosuppressive properties (Duramad et al., 2005; Letscher et al., 2021), sepsis induces irreversible pDC dysfunction through G-CSF-mediated downregulation of both TCF4 and SpiB, highlighting how the septic microenvironment fundamentally alters pDC development. However, the underlying mechanisms responsible for the impairment of TCF4 and SpiB in sepsis HSPCs remain to be explored in future studies.

We also explored why acute septic shock has a prolonged effect on HSPCs in vivo. The BM microenvironment plays a crucial role in HSPC generation and lineage specification, with immune subsets and cytokines both contributing significantly to the construction of this environment. Previous reports have shown that immune subsets and their transcriptional profiles change dramatically shortly after septic shock, with these changes persisting for up to a month post-infection (Tao et al., 2024). We focused on the cytokines within the septic BM and found that several cytokines were significantly altered 14 days post-infection in septic mice. Among these, G-CSF levels were dramatically increased in both serum and BM supernatants, suggesting a critical role for G-CSF in HSPC adaptation. G-CSF is highly expressed during infection and drives emergency granulopoiesis by regulating myeloid lineage-specific transcription factors (Martin et al., 2021; Manz and Boettcher, 2014), consistent with our findings that G-CSF stimulation upregulates PU.1 in HSPCs ex vivo. Previous studies have shown that G-CSF induces HSC mobilization but impairs macrophages and osteoblasts, which leads to significant impairment in HSC reconstitution (Christopher et al., 2011; Winkler et al., 2012). Additionally, G-CSF has been reported to prevent the expansion and development of FLT3L-derived cDCs by downregulating IRF8 (Waight et al., 2013). In our study, we observed that G-CSF also suppressed pDC development by targeting MDPs and CDPs, accompanied by the downregulation of key transcription factors such as SpiB, TCF4, FLT3 and IRF8, leading to a loss of pDC generation ex vivo. In contrast to the inhibitory effects of G-CSF, recent studies have

identified interleukin-3 (IL-3) as a critical regulator of pDC function during viral infections in sepsis (Bénard et al., 2023). Specifically, IL-3 promotes pDC-mediated antiviral immunity through two key mechanisms: (1) By enhancing CXCL12-dependent recruitment of pDCs to infected tissues, thereby boosting local interferon production; and (2) Through synergistic action with TLR signals to upregulate co-stimulatory molecules (CD80/CD40/CD86) on human pDCs, improving their T cell-priming capacity without altering IFN- α secretion. Notably, low plasma IL-3 levels in septic patients correlate with increased HSV-1/CMV reactivation and worse outcomes (Bénard et al., 2023), suggesting that IL-3 may compensate for the pDC defects induced by G-CSF-mediated TCF4/SpiB suppression. Finally, while G-CSF treatment of HSPCs partially recapitulated the altered pDC phenotype and molecular changes observed in sepsis, it suggests that other factors also co-regulate pDC reconstitution in vivo. Therefore, Future studies, including gene editing mice, will be needed to further elucidate the role of specific cytokines in the BM microenvironment in regulating pDC reconstitution from HSPCs during sepsis.

In conclusion, our study highlights the long-term effects of sepsis on pDCs and reveals the underlying cellular and molecular mechanisms. We found that pDC reconstitution is impaired at the hematopoietic progenitor cell level, with both cell-intrinsic and extrinsic factors contributing to the impaired reconstitution of immunocompetent pDCs from HSPCs in sepsis. Further studies are required to systematically elucidate the molecular mechanisms behind altered reconstitution of specific immune subsets in sepsis over the long term. While this study establishes the hematopoietic defects underlying pDC dysfunction in sepsis, future studies employing adoptive transfer models of CLP-induced pDC precursors or mregDCs will be critical to determine their causal impact on sepsis mortality and the specific phases of sepsis pathogenesis they regulate.

CRedit authorship contribution statement

Yichen Wang: Project administration, Investigation, Conceptualization. **Yi Hong:** Project administration, Investigation. **Jiajun Ma:** Project administration, Investigation. **Zhangqiang Guo:** Project administration, Investigation. **Xue Meizhu:** Project administration, Investigation. **Zhenjiang Bai:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Zhuo Lv:** Writing – review & editing, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Huiping Yang:** Project administration, Investigation. **Dan Fan:** Project administration, Investigation. **Shuiyan Wu:** Resources. **Jie Lu:** Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lijun Meng:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Conceptualization. **Jie Huang:** Resources.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: nothing If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

No data was used for the research described in the article.

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