

# Bone Marrow and Peripheral Blood Mononuclear Cell Phenotype Changes after Cultivation and Autologous Infusion in Patients with Primary Biliary Cholangitis

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## Abstract

**Background:** Primary biliary cholangitis (PBC) is a condition affecting the liver and immune system. In this study, the impact of autologous bone marrow-derived mononuclear cell (BM-MNC) transplantation on PBC patients was investigated.

**Methods:** Sixteen eligible PBC patients participated at the National Scientific Medical Center in Astana, Kazakhstan, between 2017 and 2022, and BM-MNCs were harvested from their anterior iliac crest. After isolating and cultivating the BM-MNCs, they were infused back into the patient's peripheral veins. Changes in BM-MNC and peripheral blood mononuclear cell (PB-MNC) phenotypes were assessed before and after a 24-hour cultivation period and 72 hours post-transplantation. We monitored liver function parameters over 6-month intervals and conducted flow cytometry analysis to assess CD markers on BM-MNCs before and after cultivation and PB-MNCs before and after transplantation. Statistical analysis included the Friedman test for liver parameters and the Wilcoxon signed-rank test for BM-MNC and PB-MNC comparisons.

**Results:** Our findings revealed significant reductions in liver function tests after multiple transplantations. Flow cytometry analysis before and after a 24-hour culture and autologous BM-MNC infusion revealed the expansion of specific cell populations, with significant increases in CD3<sup>+</sup>, CD4<sup>+</sup>, CD16<sup>+</sup>, CD20<sup>+</sup>, CD25<sup>+</sup>, CD34<sup>+</sup>, CD105<sup>+</sup>, CD73<sup>+</sup>, CD117<sup>+</sup>, and CD34<sup>+</sup> populations, while CD4<sup>+</sup>25<sup>+</sup>, CD34<sup>+</sup>105<sup>+</sup>, and CD4<sup>+</sup>FOXP3<sup>+</sup> populations decreased. Interestingly, a contradictory finding was observed with a decrease in bone marrow CD34<sup>+</sup>105<sup>+</sup> cell lines (P=0.03) alongside an increase in peripheral CD34<sup>+</sup>105<sup>+</sup> population (P=0.03).

**Conclusion:** In summary, our study shows that BM-MNC transplantation in PBC patients leads to changes in immune cell populations and liver function. These findings suggest potential therapeutic applications of BM-MNC transplantation in managing PBC and offer insights into the dynamics of immune cells associated with this treatment approach.

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**Keywords** • Primary biliary cholangitis • Leukocytes, mononuclear • Bone marrow • Peripheral blood stem cells

## What's Known

- Prior studies have explored bone marrow-derived mononuclear cells and peripheral blood mononuclear cells in various diseases, as well as the potential of autologous transplantation.
- However, their specific impact on primary biliary cholangitis patients and the changes in immune cell populations, including T lymphocytes and regulatory T cells, remain insufficiently understood.

## What's New

- This study reveals significant changes in immune cell populations and liver function in primary biliary cholangitis patients following bone marrow-derived mononuclear cell transplantation, suggesting potential therapeutic benefits, which advances our understanding of the procedure's immunological impact.

## Introduction

The past decade has witnessed a surge in autoimmune disorders, notably impacting individuals in their forties and fifties, with primary biliary cholangitis (PBC) being a prime example. PBC, predominantly affecting women, is characterized by autoantibodies targeting mitochondrial (AMA) and nuclear (ANA) antigens in cholangiocytes.<sup>1</sup> Incidence rates range from 1.91 to 40.2 cases per 100,000, with onset typically between ages 50 and 60, and a 4:1 female-to-male ratio.<sup>2,3</sup>

Stem cell therapy, utilizing hematopoietic stem cells (HSCs) and mesenchymal stromal/stem cells (MSCs), has shown promise in treating liver cirrhosis.<sup>4,5</sup> These cells can differentiate into hepatocyte-like and cholangiocyte cells, with mechanisms involving autocrine and paracrine signaling.<sup>6</sup> Additionally, stem cells can mitigate fibrogenesis.<sup>7</sup> Mononuclear cells (MNCs), especially bone marrow-derived MNCs (BM-MNCs), have applications in addressing PBC due to their potential to differentiate into hepatocyte-like cells.<sup>8,9</sup>

MSCs are defined by the International Society for Cell & Gene Therapy (ISCT®) based on their adherence to plastic surfaces, specific surface antigen expression, and trilineage differentiation potential.<sup>10</sup> Clinical studies have supported the effectiveness of umbilical cord-derived MSCs and BM-MSCs in treating PBC patients.<sup>11</sup> HSCs, constituting approximately 1-2% of bone marrow cells, play a vital role in hematopoiesis.<sup>12</sup> CD34+ and CD117+ HSCs, along with various markers of mature cells, are involved in the process.<sup>13,14</sup>

Transplantation of HSCs has been shown to reduce hepatic cytolysis and alleviate cholestasis in PBC. A meta-analysis confirmed the benefits of autologous BM-MNC therapy, reducing cholestasis, and hepatocyte cytolysis, and improving patient scores, with effects lasting up to 24 weeks.<sup>15</sup>

This study investigates the therapeutic potential of autologous bone marrow

mononuclear cell (BM-MNC) transplantation for PBC. The study, conducted at the National Scientific Medical Center in Astana, Kazakhstan, focuses on 16 PBC patients who received BM-MNC transplantation at 6-month intervals between 2017 and 2020. It assesses the impact of this therapy on biochemical responses and BM-MNC populations during a single procedure, as well as the characteristics of post-infusion peripheral blood MNCs (PB-MNCs) and their implications for PBC pathogenesis. Despite global diversity in BM-MNC protocols, this research aims to enhance our understanding of their therapeutic effects on PBC.

## Patients and Methods

### *Patient Selection and Characteristics*

Ethical approval for the study was obtained from the Ethical Board at the National Scientific Medical Center on 7.02.2018 (No. 051/CT-35, Protocol No. 053/CT-47) in accordance with the Helsinki Declaration of 1964 (2013 edition). After a comprehensive discussion regarding the study objectives and procedures, informed consent was obtained from each participant for participating in the study and the bone marrow aspiration procedure.

The study enrolled a total of 16 patients diagnosed with PBC who met the inclusion criteria to receive autologous BM-MNCs at the National Scientific Medical Center in Astana, Kazakhstan, between 2017 and 2022.

Inclusion criteria and exclusion criteria are explained in table 1. Liver biopsy and histology were performed 3 to 8 months before admission. All patients received the standard therapy of ursodeoxycholic acid (UDCA, Ursosan®, PRO. MED.CS Praha a.s., Czech Republic) at a dose of 15 mg/Kg daily but were selected for BM-MNC therapy due to the ineffectiveness of the standard treatment.

### *Child-Pugh-Turcotte (CPT) Scoring*

In this study, the CPT scoring system<sup>16</sup> was

**Table 1:** Inclusion and exclusion criteria of patients with primary biliary cholangitis for evaluation of bone marrow and peripheral blood mononuclear cell phenotype changes after cultivation and autologous infusion

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"> <li>• Confirmation of ursodeoxycholic acid (UDCA)-resistant PBC through laboratory and morphological assessments for a minimum of 2 years, with alkaline phosphatase levels &gt;1.75 <math>\mu</math>kat/L and AMA titer &gt;1:40, despite daily intake of UDCA at a dose of 13-15 mg/Kg/day, along with persistent pruritus and fatigue</li> <li>• Morphological criteria of PBC, include non-purulent granulomatous lymphocytic cholangitis, cholestasis, ductopenia, and progressive fibrosis.</li> </ul>	<ul style="list-style-type: none"> <li>• Age over 75 years</li> <li>• Inability to comply with therapy or provide informed consent</li> <li>• History of cancer or positive cancer antigen markers such as CEA&gt;5 ng/mL, CA-15-3&gt;25 U/mL, CA125&gt;35 U/mL, CA19-9&gt;39 U/mL, AFP&gt;5.0 IU/mL</li> <li>• The presence of concomitant acute conditions, including mental disorders, that may pose a threat to the patient or negatively impact the outcomes</li> <li>• Pregnancy or breastfeeding</li> <li>• Hematological disorders such as anemia with hemoglobin levels below 90 g/L, leukocytes less than 3000/<math>\mu</math>L or above 10000/<math>\mu</math>L, coagulation disorders with platelet count less than 100000/<math>\mu</math>L, and abnormal APTT and INR levels above two times the upper normal limit</li> <li>• Creatinine levels above 100 <math>\mu</math>mol/L</li> <li>• Inflammatory or infectious skin lesions at the site of bone marrow aspiration (upper anterior iliac crests)</li> <li>• Positive HIV status or a history of Hepatitis B and C infections.</li> </ul>

employed to assess the severity of liver disease in patients with PBC undergoing autologous BM-MNC transplantation. The CPT score, a widely used clinical tool, provides valuable insights into the degree of liver dysfunction and patient prognosis in liver-related conditions.

The CPT score was calculated based on five clinical parameters: total bilirubin (TB), serum albumin (SA), prothrombin time/international normalized ratio (INR), ascites, and hepatic encephalopathy. Each parameter was assigned a score according to established criteria, and the total CPT score was determined by summing these values. The resulting CPT score placed patients into three classes: A (well-compensated), B (moderately decompensated), or C (severely decompensated), reflecting the severity of liver dysfunction.

To calculate the CPT score, the patients' TB levels, SA concentrations, and INR using standard laboratory assays were measured. The presence and severity of ascites and hepatic encephalopathy were assessed through clinical examination and relevant diagnostic methods. Regular monitoring of the CPT score was performed at multiple time points, including before and after autologous BM-MNC transplantation, to track changes in liver function and adapt treatment strategies as necessary.

#### *Model for End-Stage Liver Disease (MELD) Scoring*

The MELD score<sup>17</sup> was employed as a fundamental tool in this study to evaluate the severity of liver disease in patients with PBC who underwent autologous BM-MNC transplantation. The MELD score is a widely recognized and validated scoring system used to predict patient survival and prioritize organ allocation for liver

transplantation.

The MELD score is calculated using a formula that incorporates three key laboratory parameters: serum bilirubin, serum creatinine, and the INR for prothrombin time. Each of these parameters is assigned a specific weight in the MELD score calculation. The resulting MELD score provides an objective measure of liver function and is used to stratify patients into different risk categories, reflecting the severity of liver dysfunction.

In this study, serum bilirubin, serum creatinine, and INR values were measured using standard laboratory assays. The MELD score was calculated according to the prescribed formula, and the resulting score was used to assess and monitor the severity of liver disease in PBC patients before and after autologous BM-MNC transplantation.

#### *Anti-Mitochondrial Antibodies (AMA) Analysis*

In this study, the assessment of AMA played a pivotal role in the diagnosis and characterization of PBC in the patient cohort. Serum samples were collected and subjected to immunoassays, including enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IIF), to detect the presence of AMA, which are specific autoantibodies targeting mitochondrial antigens. Additionally, the quantification of AMA titers allowed for an in-depth understanding of the concentration of these autoantibodies in patient sera.

#### *Bone Marrow Aspiration (BMA)*

The BMA procedure was conducted under aseptic surgical conditions. A Jamshidi-type bone marrow aspiration/harvesting needle (8 G/10 cm, Sterylab, Italy) was used to obtain the bone marrow aspirate from the anterior iliac crest.

To prevent clotting, the aspirate was collected in 20 mL syringes, with each syringe containing 1 mL of a 1:1000 heparin solution. All subsequent handling of the autologous cells was performed with aseptic techniques, following the current guidelines of Good Manufacturing Practices, to mitigate the risk of microbial contamination.<sup>18</sup>

#### *Isolation and Cultivation of Autologous BM-MNCs*

In a sterile environment, bone marrow aspirates were processed, divided into five tubes with 20 mL each, and mixed with 3 mL of Dulbecco's PBS (Thermo Fisher Scientific, USA) to enhance mononuclear cell yield. This mixture was carefully layered over 10 mL of Ficoll-Paque™ (GE Healthcare, Sweden) and centrifuged, resulting in three layers: serum, a middle buffy coat rich in mononuclear cells, and a lower layer with granulocytes and erythrocytes. The middle buffy coat was transferred to a cultivation flask and combined with a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, USA) and DMEM/Nutrient Mixture F-12 (DMEM-F12, Thermo Fisher Scientific, USA), supplemented with 20% fetal bovine serum and antibiotics. The cell suspension was then incubated under controlled conditions.

To ensure sterility, Gram staining and polymerase chain reaction tests were conducted on the cell culture to detect various microorganisms. The process involves safeguarding against hepatitis C virus (HCV), hepatitis B virus (HBV), *Staphylococcus epidermidis*, *Staphylococcus aureus* (including Methicillin-resistant *Staphylococcus aureus*, MRSA), *Pseudomonas aeruginosa*, Group A  $\beta$ -hemolytic streptococcus, *Bacillus cereus*, *Mycoplasma* sp., and fungi. This procedure is essential for maintaining the purity and quality of the cultured cells for subsequent experiments or clinical applications.

#### *Infusion of Autologous BM-MNCs*

In our study, after a 24-hour incubation period, a BM-MNC suspension containing  $1.25 \times 10^7$  cells was diluted with 200 mL of 0.9% normal saline and infused into patients' peripheral veins over 3.5 hours. Dexamethasone was administered 30 min before infusion. All 16 patients received seven BM-MNC transplantations at six-month intervals. We assessed the impact of cultivation on BM-MNC properties during the first transplantation. The phenotypical properties of PB-MNCs were examined before and after the initial BM-MNC infusion. Liver function tests were conducted before each infusion, generating

seven panels of biochemical response data at 6-month intervals. Liver parameters, including alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (AP), TB, and direct bilirubin (DB), were evaluated to assess liver function before and after the BM-MNC transplantation procedure.

#### *Preparation of BM-MNCs for flow Cytometry*

In preparation for flow cytometry analysis, BM-MNC samples were collected before and after a 24-hour cultivation period. Initially, 0.1 mL samples were collected and placed in 5 mL tubes. Before adding the cultivation media, a 0.1 mL sample with a target amount of  $10^6$  BM-MNCs was transferred into a specialized tube for CD-mapping analysis using flow cytometry.

After the 24-hour cultivation, the cell suspension underwent centrifugation, and the supernatant was carefully removed. Another sample of the cell mass was collected for flow cytometry analysis, specifically assessing cell viability and CD-mapping. Thus, two sets of bone marrow samples for flow cytometry were obtained - one before cultivation and one after.

These samples were suspended in 2 mL of cold PharmingenStain BSA buffer, maintaining a minimum concentration of  $10^6$  cells/mL. All procedures occurred at 4 °C and in a dark environment. Monoclonal antibody reagent solutions for various CD markers were prepared and added to the sample tubes, followed by a 20-min incubation at room temperature. The cells underwent three washes and were resuspended in a cold PBS/FBS solution with sodium azide. Finally, the samples were kept on ice, in the dark, and analyzed within an hour after lysis.

#### *Preparation of Whole Blood Samples for Flow Cytometry Analysis of PB-MNCs*

Peripheral blood samples were collected before and 72 hours after BM-MNC autotransplantation for flow cytometry analysis. Citrate-stabilized whole blood was processed immediately at 18 °C to 20 °C, with 0.1 mL transferred into Falcon test tubes. Monoclonal antibody reagents were added and incubated in the dark at 4 °C. Cells were washed, lysed, and washed again before resuspension in cold PBS for flow cytometry within 24 hours. This method ensures the analysis of peripheral blood samples before and after autotransplantation to monitor changes in cell populations and surface markers, facilitating the assessment of treatment outcomes.



**Table 2:** The list of CD-antigens analyzed in the present study and their brief description

CD marker	Function	Cells	Reference values (%); mean±SD or median (range)	Reference values (cells/μL); mean±SD or median (range)
D3	TCR, T cell receptor	Thymocytes, mature T-lymphocytes	73 (61-85) 72.80±1.90	1.40 (1.10- 1.70)×10 <sup>6</sup> 655-2.823
CD4	Co-receptor for MCH-II	T-helper lymphocyte (CD3+CD4+)	45 (35-55) 46.30±1.30 36.46±0.24	0.80 (0.70-1.10)×10 <sup>6</sup> 321-1.38
CD8	Co-receptor for MCH-I	T-cytotoxic lymphocyte (CD3+CD8+)	27 (19-35) 22.40±2.40 25.32±0.20	0.70 (0.50-0.90)×10 <sup>6</sup> 220-1.66
CD16/56	Low-affinity Fc receptor for IgG. Mediates phagocytosis and ADCC (Antibody-dependent cellular cytotoxicity)	Monocytes, macrophages, NK cells (CD3–CD16+CD56+). Neutrophils and stimulated eosinophils.	15 (12-18) 9.80±1.50 9.48±0.22 5.35-30.93	0.3 (0.20-0.40)×10 <sup>6</sup> 0.05-0.70×10 <sup>6</sup> 77-427
CD19	Regulates B cell development, activation, and differentiation. Signal transduction	B-lymph (CD19+) mature and immature	12 (7-17) 9.66±1.0 4.70-19.13	0.30 (0.20- 0.40)×10 <sup>6</sup> 72-460
CD20	Ca-channel. Regulator of B-lymph. activation	Mature B-lymphocytes	(6-18) 9.30±0.25 4.63-21.0	150-400 66-529
CD25	Activation marker, α-chain for IL-2	T- and B-activated lymphocytes, monocytes, macrophages	0.80-2.25 0.33-2.80	7-52
CD3/25	TCR+α-chain of IL-2. Early activation	T-activated	3 (0.50-6.0) 0.50-5.95	7-94
CD4/25	TCR+α-chain of IL-2. Early activation	Effector T cells	7.90±1.10 0.33-2.80	7-52
CD34	Adhesion molecule and regulator of migration. Early hematopoiesis, mediates stem cells adhesion to the BM ECM or stromal cells	Marker of human HSC, their progenitors, and capillary endothelial cells.	0.04 (0.01-0.14)	1.85 (0.90-4.69)×10 <sup>6</sup>
CD45	Critical for B- and T-cell receptor-mediated activation. PTPRC thymic selection	Hematopoietic cells	No data	4000-10000
CD73	Lymphocyte differentiation.	B- and T cell subsets, dendritic reticulum cells, epithelial cells, MSC	79.31±7.88	No data
CD105	Accessory receptor for TGF-β, angiogenesis, and neovascularization	Activated endothelial cells, MSC	2.44±0.93	0.09±0.07
CD34/105	Angiogenesis. Regulatory component of TGF-β-receptor complex. Modulates cellular response to TGF-β Regeneration and development	Primitive circulating hematopoietic progenitors Activated circulating endothelial cells, monocytes, and macrophages, MSC, erythroid precursors	No data	No data
CD117	Mast/stem cell growth factor receptor (SCFR) proto-oncogene. important hematopoietic regulator; cell survival, proliferation, differentiation, mobilization.	Mast cells, melanocytes, germ cells, HSC, and precursors in the bone marrow	0.21±0.27 (0.001-1.70)	No data
CD4/FOXP3	Immune tolerance	Natural Tregs, adaptive/ induced Tregs	0.59-0.79	12.79-17.41

### Flow Cytometry

Flow cytometry analysis was conducted using the BD FACS Calibur analyzer (Becton Dickinson, USA) to assess the BM and PB-MNC pools based on their CD-markers and combinations, including CD3+, CD4+, CD8+, CD16+, CD20+, CD25+, CD34+, CD45+, CD73+, CD105+, CD117+, CD34+45+, CD4+25+, CD34+105+, and CD4+FOXP3+. The values were recorded as the number of cells expressing a specific phenotype per microliter of blood, which were then converted into a percentage of cells with that particular phenotype out of a total of  $10^7$  cells. Data collection and processing were performed using the BD FACSDiva™ software (version 6.1.3, BD Biosciences, Australia), with software parameters adjusted to optimize the voltage at the cytometer photomultiplier. The viability of the harvested cells was determined using flow cytometry (FACS Calibur, Becton Dickinson, USA) and ranged from 94% to 97%. To ensure quality control of the flow cytometry procedures, daily calibration of the flow cytometer was conducted using BD CaliBRITE™ beads (BD Biosciences, Australia) to set fluorescence compensation and check instrument sensitivity. Additionally, an isotype control was performed using Mouse BALB/c IgG1- $\kappa$  reagent solution (BD Biosciences, Australia). Moreover, a brief description of the CD-antigens used in this study is provided in table 2.

### Statistical Analysis

The statistical analysis was conducted using IBM SPSS Statistics (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0, USA). The series of seven measurements for each liver parameter (ALT, AST, GGT, AP, TB, DB) were assessed using the Friedman test, which is a non-parametric test for matched

groups. *Post-hoc* analysis was performed using Dunn's test to assess pairwise differences among multiple groups for each liver parameter, helping to identify which specific groups exhibited significant differences in the median values.

To compare the proportions of BM-MNC populations before and after culturing, as well as PB-MNCs before and after infusion of cultured BM-MNCs, the Wilcoxon signed-rank test was employed for matched sample groups. Results were presented as mean $\pm$ SD for normally distributed values, where  $m$  represents the mean and  $\sigma$  represents the standard deviation. Non-parametric data were expressed as Me [LQ; UQ] (min-max), where Me denotes the median, LQ represents the lower quartile or Q1 (the lowest 25% of values), UQ indicates the upper quartile or Q3 (the highest 75% of values), and min and max correspond to the minimum and maximum values, respectively. A P value below 0.05 was considered statistically significant.

## Results

### Patient Data and Characteristics

Based on the inclusion and exclusion criteria, a total of 16 female patients diagnosed with PBC and aged between 29 and 60 years old (median [LQ; UQ] age 50 [39.8; 55]) were selected for the study. These patients had shown resistance to standard UDCA treatment for a minimum of two years. They underwent a total of seven repeated transplantations of autologous BM-MNCs at 6-month intervals. The average duration of the disease was 6.81 $\pm$ 3.03 years, ranging from 3 to 15 years. All patients, except one, had other concurrent medical conditions. Among the patients, 12 had developed portal hypertension, while one had encephalopathy.

**Table 3:** Demographic data of primary biliary cholangitis (PBC) patients before transplantation of autologous bone marrow-derived mononuclear cells (BM-MNCs) (n=16)

Indices	Values
Age, years; median (range)	50 (29-60)
Disease duration, years; mean $\pm$ SD (range)	6.81 $\pm$ 3.03 (3-15)
Child-Pugh-Turcotte (CPT) score; mean $\pm$ SD	5.56 $\pm$ 0.77
Model for End-Stage Liver Disease (MELD); mean $\pm$ SD	7.63 $\pm$ 1.16
Concomitant diseases; n (%)	15 (93.75)
Portal hypertension; n (%)	12 (75)
Encephalopathy; n (%)	1 (6.25)
Alanine aminotransferase (ALT), $\mu$ kat/L; median (LQ; UQ)	1.95 (1.38; 2.72)
Aspartate aminotransferase (AST), $\mu$ kat/L; median (LQ; UQ)	1.51 (1.20; 2.02)
Alkaline phosphatase (AP), $\mu$ kat/L; median (LQ; UQ)	15.69 (10.96; 19.89)
Gamma-glutamyl transpeptidase (GGTP), $\mu$ kat/L; median (LQ; UQ)	5.84 (3.45; 8.10)
Total bilirubin (TB), mmol/L; median (LQ; UQ)	19.05 (14.28; 29.63)
Direct bilirubin (DB), mmol/L; median (LQ; UQ)	20.70 (11.13; 28.12)
AMA+, IU/mL; median (range)	90 (10.2-267.15)
Anti-nuclear antibodies (ANA)+; n (%)	2 (12.50)
Anti-mitochondrial antibodies (AMA-)/ANA-; n (%)	3 (18.75)

**Table 4:** Baseline data of primary biliary cholangitis (PBC) patients before transplantation of autologous bone marrow-derived mononuclear cells (BM-MNCs) (n=16)

No.	Age	Duration, years	Child-Pugh-Turcotte (CPT) score	Model for End-Stage Liver Disease (MELD)	Anti-mitochondrial antibodies (AMA)
1	49	3	5	7	171.36
2	49	9	6	10	79.0
3	50	10	5	7	236.47
4	39	7	5	6	Negative
5	32	3	5	10	Negative
6	55	3	6	9	95.70
7	53	6	5	7	22.78
8	60	3	5	7	47.56
9	29	3	5	6	43.57
10	50	6	5	6	151
11	55	6	5	7	Negative
12	60	10	6	8	Negative
13	55	15	9	9	0.76
14	40	10	5	7	12.40
15	54	11	5	7	174.20
16	34	4	7	9	Negative

The baseline means CPT score was  $5.56 \pm 0.77$ , and the mean MELD score was  $7.60 \pm 1.16$ . Tables 3 and 4 present the baseline data of the PBC patients before the transplantation of autologous BM-MNCs.

#### *BM-MNCs Aspiration, Isolation, and Cultivation*

The BMA procedure yielded an approximate total volume of  $80.50 \pm 23.2$  mL. The average cell count in the aspirate was estimated to be  $27.52 \pm 2.39 \times 10^6$  cells/mL. After the isolation process, the average concentration of BM-MNCs reached  $3.43 \pm 0.56 \times 10^6$  cells/mL, which were subsequently cultivated in the incubation box. Following a 24-hour incubation period, the target quantity of total BM-MNCs in the suspension was set at  $1.25 \times 10^7$  cells. The sterility tests conducted on the BM-MNC cultures, including bacterial growth, hepatitis, HIV, fungi, and mycoplasma, were all negative as determined by Gram staining and polymerase chain reaction tests.

#### *Infusion of BM-MNCs to PBC Patients and Dynamic Changes in Liver Function Parameters*

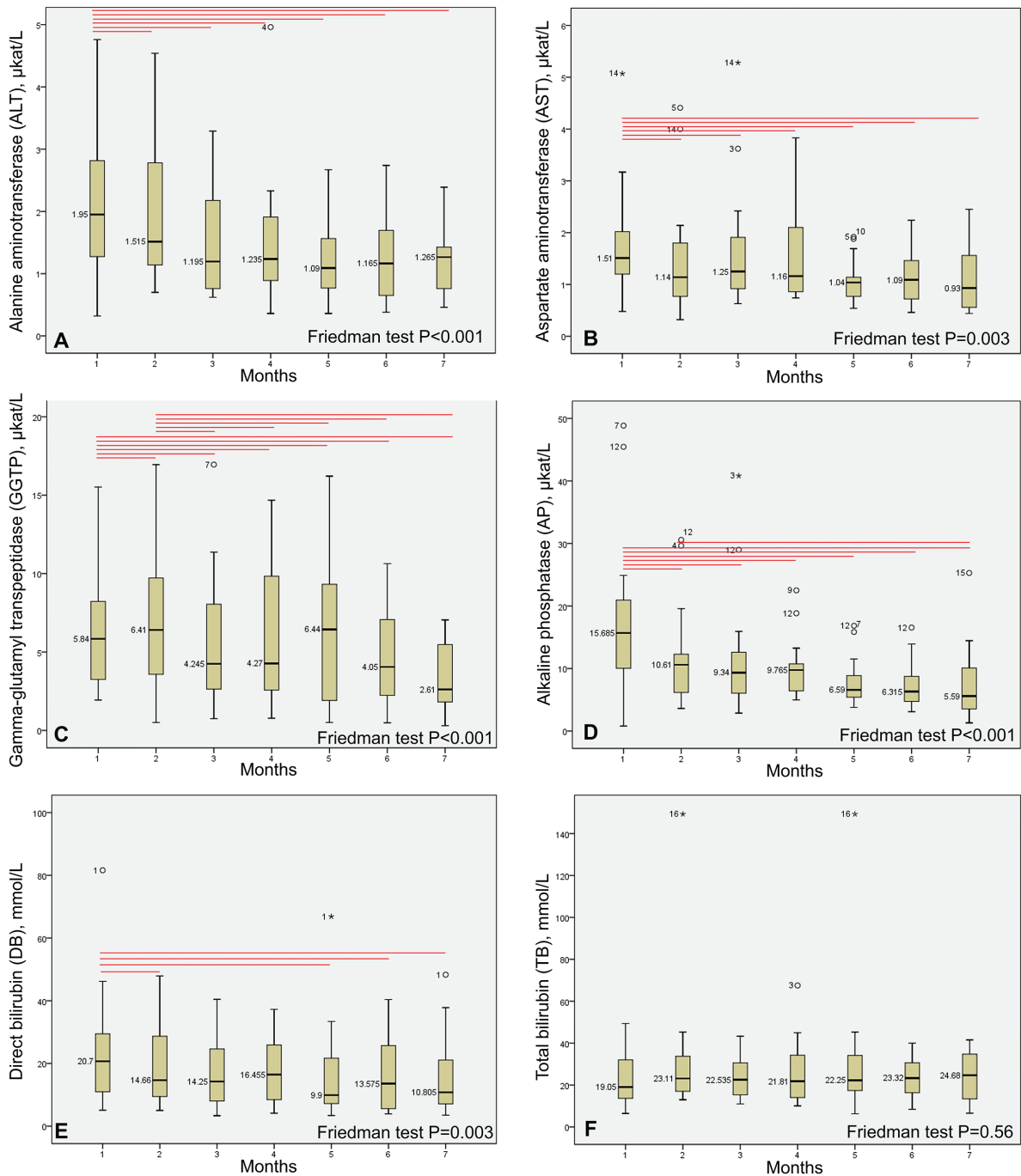
While undergoing infusion, three patients experienced brief and manageable symptoms, including mild shivering, chills, and a fever reaching up to  $37.3$  °C. These temporary side effects were effectively alleviated through the administration of 400 mL of normal saline and an additional intravenous injection containing 4 mg/mL of dexamethasone. Two patients developed superficial hematomas (bruising) at the site of BMA, measuring 2 and 4 cm in diameter, respectively, on the second day after the procedure. These hematomas resolved on their own within 3-4 days without requiring any medical intervention.

Following the infusion of autologous BM-MNCs, a significant increase in white blood cell (WBC) count was observed in all 16 patients, from  $5.04 \pm 0.20$  to  $9.80 \pm 2.90$  ( $P < 0.001$ ) on the third day. Subsequent monitoring of the patients for 18-20 months revealed no long-term complications associated with the BMA and BM-MNC infusion.

The liver function tests (ALT, AST, GGT, AP, TB, DB) were measured before each BM-MNC transplantation (at six-month intervals) in all patients. Statistically significant reductions were observed in all parameters; TB: ALT ( $P < 0.001$ ), AST ( $P = 0.003$ ), GGT ( $P < 0.001$ ), AP ( $P < 0.001$ ), and DB ( $P = 0.003$ ). However, there was no statistically significant change in TB ( $P = 0.560$ ). In fact, a mild increase in TB levels was observed by the 7<sup>th</sup> transplantation. The dynamic changes in the liver function test measurements at 6-month intervals are presented in figure 1.

#### *Flow Cytometry Analysis of BM-MNCs Before and After Culturing*

Flow cytometry analysis of CD characteristics, conducted before and after the 24-hour cultivation and autologous BM-MNC infusion, revealed the expansion of specific cell populations. The following cell populations showed significant increases after the procedure: CD3<sup>+</sup> cells (total lymphocyte count), CD4<sup>+</sup> (T cells), CD16<sup>+</sup> (NK or natural killer cells), CD20<sup>+</sup> (mature B-lymphocytes), CD25<sup>+</sup> (T- and B-activated lymphocytes, monocytes, and macrophages), CD34<sup>+</sup> (HSCs population), CD105<sup>+</sup> and CD73<sup>+</sup> (MSC population), CD117<sup>+</sup> and CD34<sup>+</sup> (precursors, erythroid, granule-monocytic, megakaryocytic, and endothelial progenitors, mast cells, and CD3/CD4/CD8



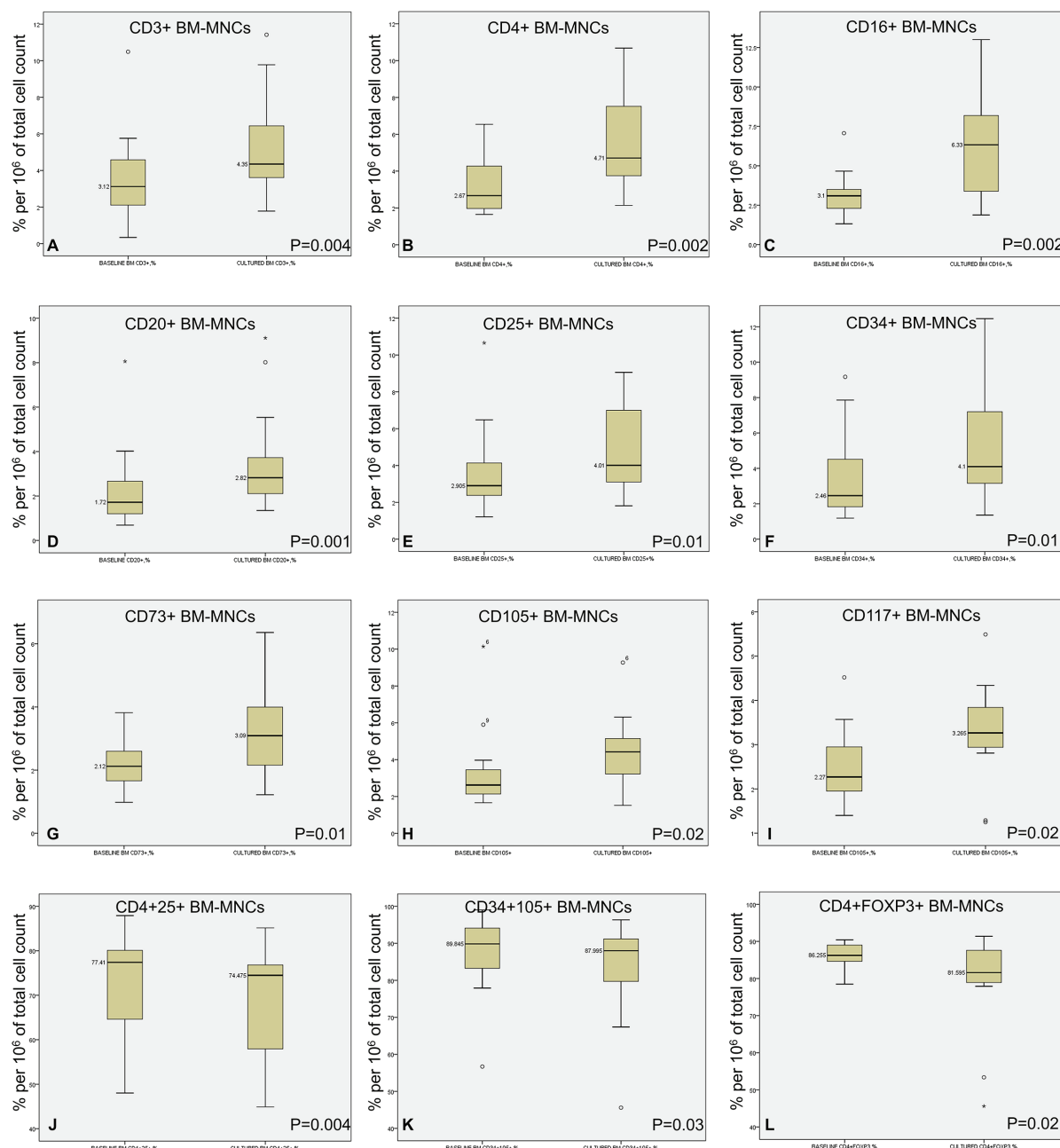
**Figure 1:** Dynamic changes of liver function parameters (Median, LQ; UQ; min-max) in primary biliary cholangitis (PBC) patients at six-month intervals during transplantation of autologous bone marrow-derived mononuclear cells (BM-MNCs) are presented with a sample size of  $n=16$ . The red lines above the boxes show significant differences between months ( $P<0.05$ ).

prothymocytes) ( $P<0.05$ ). On the other hand, the following populations decreased after the procedure:  $\text{CD4}^+\text{CD25}^+$  (activated effector T cells, Tregs),  $\text{CD34}^+\text{CD105}^+$  (Monocytes, Macrophages), and  $\text{CD4}^+\text{FOXP3}^+$  (BM Tregs) ( $P<0.05$ ). No significant changes were observed in the CD-markers of  $\text{CD8}^+$ ,  $\text{CD45}^+\text{CD34}^+\text{CD45}^+$  BM-MNCs pools. The expansion of BM-MNC populations (% per  $10^6$  of total cell count) after 24-hour cultivation and autologous BM-MNC infusion is presented in figure 2.

#### Flow Cytometry Analysis of PB-MNCs Before and After Autologous BM-MNCs Infusion

The examination of PB-MNCs and their corresponding CD-antigens before and 72 hours after transplantation revealed the expansion of various cell populations. The following pools showed significant increases:  $\text{CD3}^+$ ,  $\text{CD4}^+$ ,  $\text{CD8}^+$ ,  $\text{CD16}^+$ ,  $\text{CD20}^+$ ,  $\text{CD25}^+$ ,  $\text{CD34}^+$ ,  $\text{CD45}^+$ ,  $\text{CD73}^+$ ,  $\text{CD105}^+$ ,  $\text{CD117}^+$ ,  $\text{CD3}^+\text{CD73}^+$ ,  $\text{CD34}^+\text{CD45}^+$ ,  $\text{CD4}^+\text{CD25}^+$ ,  $\text{CD34}^+\text{CD105}^+$ , and  $\text{CD4}^+\text{FOXP3}^+$ .





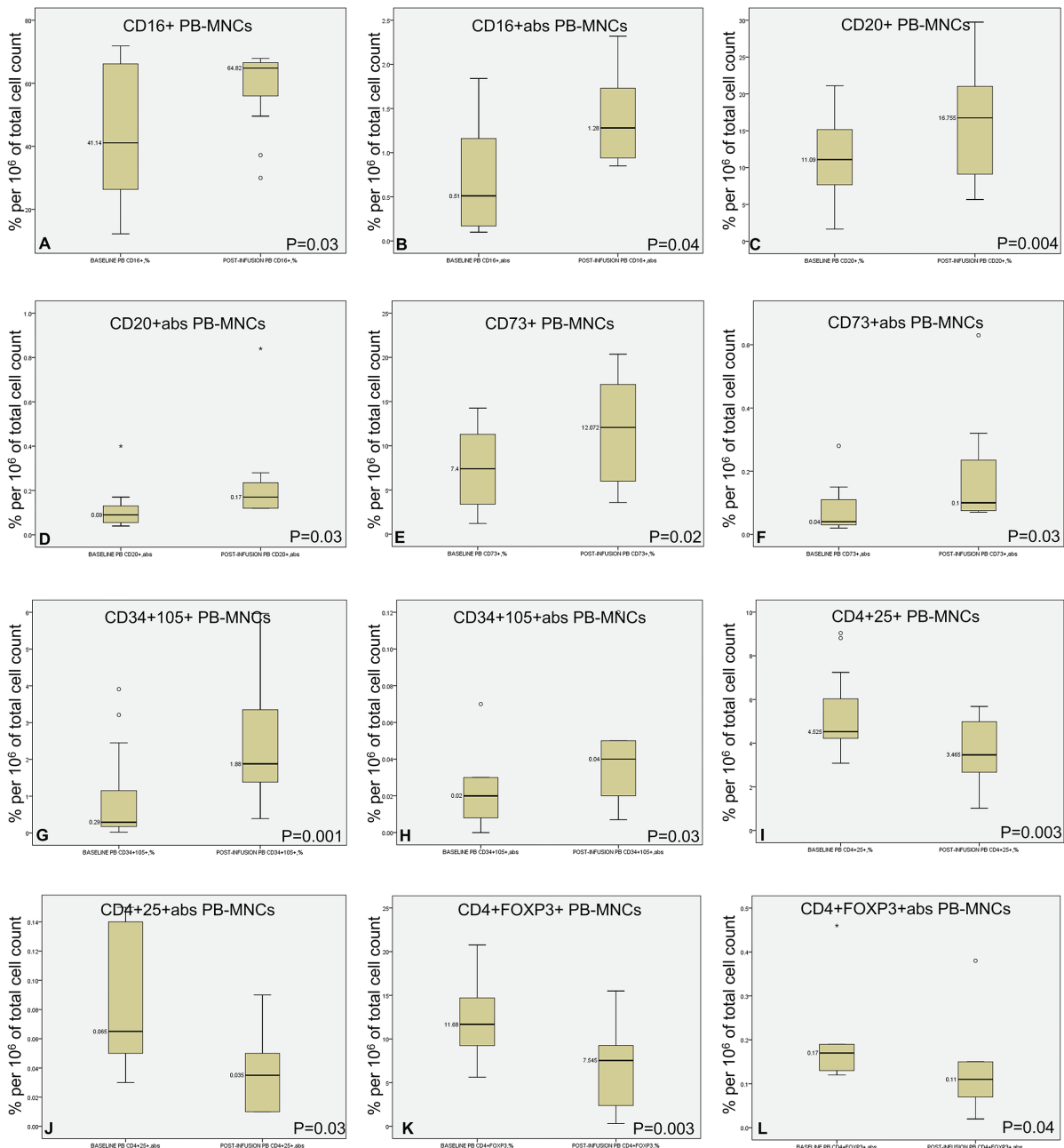
**Figure 2:** Median, LQ; UQ, min-max of bone marrow-derived mononuclear cells (BM-MNCs; % per 10<sup>6</sup> of total cell count) markers before and after 24-hour cultivation are represented with a sample size of n=16.

The infusion of autologous BM-MNCs into the peripheral blood resulted in a substantial increase in the circulating leukocyte population, as well as in CD16+ (NK cells), CD20+ (B-lymphocytes), and CD73+ (MSC). However, similar to the bone marrow pattern, the populations of CD4+25+ (effector T cells) and CD4+FOXP3+ (peripheral Tregs) cells decreased after the infusion of BM-MNCs.

Interestingly, a contradictory finding was observed, where the bone marrow CD34+105+ cell lines decreased (P=0.03), while the population of peripheral CD34+105+ increased (P=0.03). This suggests an increase in activated

endothelial cells, monocytes, macrophages, and erythroid precursors in the peripheral circulation. Only changes in both the percentage and absolute numbers of population counts were considered significant.

No significant changes were detected in the following PB-MNCs pools and their CD-markers: CD3+, CD4+, CD8+, CD25+, CD34+, CD45+, CD105+, CD117+, CD3+73+, and CD34+45+. The populations of PB-MNCs pools (% per 10<sup>6</sup> of total cell count and absolute values) that increased after the autotransplantation of BM-MNCs are presented in figure 3.



**Figure 3:** Median, LQ; UQ, min-max of peripheral blood mononuclear cells (PB-MNCs) markers before and 72 hours after transplantation of autologous bone marrow-derived mononuclear cells (BM-MNCs) are represented with a sample size of n=16.

### Discussion

The overall results of this study demonstrate the positive effects of autologous BM-MNC transplantation on biochemical responses in patients with PBC. Specifically, a significant reduction in transaminases, GGT, alkaline phosphatase, and direct bilirubin levels by the 7<sup>th</sup> procedure at the end of the study period was observed. However, it is important to note that the levels of TB did not exhibit a significant decrease, and no statistically significant influence of BM-MNCs on TB levels was observed in this study. Despite a mild elevation of TB slightly

above the normal limits, the substantial reduction in direct bilirubin levels provides compelling evidence of improved liver function, considering the role of the liver in bilirubin conjugation.

Furthermore, an expected increase was observed in the total lymphocyte count within specific populations, including T cells, natural killer cells, B cells, HSCs, monocytes, and endothelial cells, following the cultivation process. Similar patterns were seen in PB-MNCs after the infusion of BM-MNCs. To understand the underlying causes of this population expansion, we explored the concept of the stem cell niche. When BM-MNCs are

transferred from the hypoxic environment of the niche, where they exist in a quiescent and low-proliferative state, to a nutrient and oxygen-rich culture media, they start to proliferate. A study by Seyfried and colleagues suggests that macrophages residing in the bone marrow niche regulate HSC quiescence, and deficiency in macrophages can stimulate HSC mobilization.<sup>19</sup> Other researchers have reported that the osteoblast niche plays a role in maintaining stem cell quiescence through interactions involving N-cadherin, angiopoietin-1, thrombopoietin, and HSC receptors.<sup>20</sup> The extracellular matrix (ECM) acts as a scaffold that retains stem cells, regulates oxygen levels, and influences chemotaxis. Signaling molecules within the ECM govern cell shape, which in turn affects cell differentiation, chemotaxis, and apoptosis.<sup>21</sup>

In individuals with chronic autoimmune inflammation, BM-MNCs endure prolonged exposure to high inflammatory levels, leading to a depletion of their proliferative capacity. However, when BM-MNCs are removed from this suppressive environment, they exhibit increased expansion potential. Both IFN $\gamma$  and IFN $\alpha$  have been identified as activators of HSCs, promoting their proliferation both *in vitro* and *in vivo*, particularly during infections.<sup>22</sup> Elevated IFN $\gamma$  levels have been detected in the serum of patients with prolonged bone marrow mobilization.<sup>22</sup> Persistent low-grade inflammation triggers continuous cytokine onslaught, activating leukocyte recruitment and damage repair mechanisms within the stem cell niche.<sup>23</sup> This persistent immune activation and chronic inflammation disrupt the quiescent state of HSCs, causing excessive proliferation, impaired self-renewal, and eventual exhaustion of the bone marrow HSC population.<sup>24</sup>

The constant exposure of niches to inflammatory cytokines, such as IFN $\gamma$  and IFN $\alpha$ , in patients with chronic autoimmune conditions creates a perpetual demand for proliferation and mobilization,<sup>25</sup> ultimately leading to BM-MNC exhaustion and suppression. Isolating BM-MNCs from inhibitory influences within the niche can initiate their proliferation and differentiation.<sup>26</sup> Furthermore, removing BM-MNCs from continuous exposure to inflammatory mediators and immune cells may activate normal stem cell repair mechanisms and restore their innate proliferative response.<sup>27</sup> This liberation from the niche's inhibitory effects, constantly bombarded by inflammatory cytokines such as IFN $\gamma$  and IFN $\alpha$ , offers the potential for rejuvenating BM-MNC proliferation and differentiation.<sup>28</sup>

Traditionally, certain cells were considered adversaries in the development of PBC,

but recent reevaluations have revised their roles and significance. Hydes and others demonstrated elevated levels of highly cytotoxic NK cells predominantly around intralobular bile ducts in PBC livers.<sup>29</sup> The apoptotic process plays a crucial role in maintaining a "clean" environment for highly active cholangiocytes.<sup>30</sup> Thus, expanding the culture of NK cells and macrophages to restore their circulating levels may improve defective phagocytosis of opsonized apoptotic BEC cells.

Cultivation also leads to the augmentation of B cells, which are believed to play a role in the production of AMA and ANA, key factors in the development of chronic non-suppurative destructive cholangitis in PBC. It may be reasonable to assume that depleting B cells could be an effective therapeutic approach for PBC patients. Rituximab, an FDA-approved mouse-human chimeric anti-CD20 monoclonal antibody, has been proposed as a potent option for B cell depletion in PBC patients due to its ability to reduce serum levels of AP, AMA, IgA, IgM, and IgG.<sup>31</sup> However, in murine models of PBC, B cell depletion exacerbated the disease and resulted in advanced biliary destruction.<sup>32</sup> The effectiveness of rituximab has recently been reassessed, with some studies failing to demonstrate statistically significant improvements in serum biochemical response markers after rituximab therapy in UDCA-resistant PBC patients. One study even reported the rapid progression of fatal cirrhosis following rituximab administration.<sup>33</sup> B cells can modulate T cells through antigen presentation, inhibiting their self-immune responses and potentially playing an anti-inflammatory or protective role.<sup>33</sup>

The reduction of bone marrow and circulating effector T-lymphocytes (CD4+25+) and Tregs (CD4+FOXP3+) following cultivation and infusion is of particular interest. The precise mechanism behind this reduction requires further investigation, as there may be other less obvious factors contributing to this depletion. However, based on the available published data, the previously established role of Tregs in the pathogenesis of PBC is now being questioned, and their depletion may even have potential benefits in reducing chronic inflammation associated with PBC. Contradictory findings exist regarding the behavior of Tregs in autoimmune liver diseases, including PBC.

The amounts of circulating and liver-resident CD4+FOXP3+Tregs, which infiltrate the portal tracts and hepatic bile ducts causing inflammatory necrosis of cholangiocytes, were significantly higher than the control group, as was the ratio of CD4+FOXP3+ to CD4+ T cells.<sup>34</sup>

Therefore, the authors suggested that impaired Treg functions may not play a significant role in the development of CNSDC in PBC, contrary to previous beliefs. Moreover, in the liver portal tracts, FOXP3+Treg infiltration directly correlated with the extent of chronic inflammation, indicating that FOXP3+Tregs might be responsible for sustaining chronic inflammation and CNSDC in PBC, although not being a primary link in the pathogenesis of PBC.

Our study's limitations include a small sample size of 16 PBC patients, potentially limiting the generalizability of our findings. The retrospective design and absence of a control group made it difficult to establish causal links between BM-MNC transplantation and observed changes in biochemical responses and immune cells. Future prospective studies with larger cohorts and controlled designs are needed to validate and expand upon our results. Furthermore, due to the intricate nature of immune responses in PBC, more extensive research is necessary to comprehensively explore the roles of specific immune cell populations in disease development and their reactions to BM-MNC transplantation.

### Conclusion

Our study observed increased populations of various T cells, including natural killer cells, B cells, HSCs, monocytes, and endotheliocytes after cultivation and BM-MNC infusion. Conversely, effector T lymphocytes (CD4+25+) and Tregs (CD4+FOXP3+) decreased, possibly due to the removal of suppressive BM-MNC populations from their niche. These findings challenge conventional beliefs about natural killer cells and Treg lymphocytes in PBC progression. Our data suggest that expanding natural killer cells and reducing Tregs could benefit PBC patients, as recent insights into their roles in autoimmune conditions warrant reconsideration of their therapeutic potential.

### Authors' Contribution

Conceptualization: D.S, M.A, and A.T; Data curation: N.J. and A.T; Funding acquisition: D.S; Investigation: D.S, M.A, N.J, R.Z, L.K, A.B, A.O, N.I, and T.T; Methodology: D.S, M.A, A.B, A.O, N.I, and T.T; Resources: P.H, N.M, A.K, Y.S, M.M, Sh.B, R.Sh, and N.T; Supervision: M.A, and A.T; Visualization: P.H, F.R, and R.Sh; Writing – original draft: D.S, N.J, and R.Z; Writing – review & editing: M.A, P.H, L.K, A.B, A.O, N.I, T.T, N.M, A.K, Y.S, F.R, M.M, Sh.B, R.Sh, N.T, and A.T. All authors have read and approved the final manuscript and agree to be accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Conflict of Interest

Authors Nazanin Jafari, Nader Tanideh and Amin Tamadon are employed by PerciaVista R&D Co. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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