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Phenotypic Comparison of Regulatory T Cells: Mobilized Peripheral Blood Versus Umbilical Cord Blood

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ABSTRACT

Regulatory T cells (Tregs) play a central role in maintaining immune tolerance and modulating inflammatory responses. Different cellular sources may give rise to Tregs with distinct phenotypic properties, which could influence their clinical applicability in immune-mediated diseases.

In this exploratory comparative study, we performed a comparative flow cytometric analysis of Treg populations derived from mobilized peripheral blood (mPB) and umbilical cord blood (UCB). Tregs were identified based on a standard $CD4^+CD25^+CD127^{low/-}$ gating strategy, followed by phenotypic assessment using markers associated with differentiation (CD45RA/RO, CD27), activation (CD69), and immune checkpoint–related regulation (PD-1, TIM-3).

Our results demonstrate source-dependent differences in the distribution of naive and memory-like Treg subsets, as well as in the expression of activation and checkpoint-associated markers.

These preliminary findings suggest phenotypic heterogeneity between mPB- and UCB-derived Tregs and warrant validation in larger cohorts and functional studies.

Keywords: Flow cytometry; Immune checkpoint markers; Immunophenotyping; Mobilized peripheral blood; Regulatory T cells; Umbilical cord blood

INTRODUCTION

Regulatory T cells (Tregs) are a specialized subset of $CD4^+$ T lymphocytes that play a central role in the maintenance of immune homeostasis and peripheral tolerance.[1–3] By suppressing excessive immune activation, Tregs contribute to the prevention of autoimmune reactions, allergic inflammation, and chronic immune-mediated tissue damage.[4–6] Alterations in Treg frequency or phenotype have been reported in a wide range of inflammatory and allergic disorders, highlighting the importance of detailed phenotypic characterization of these cells.[7–9]

From a clinical and translational perspective, Tregs can be obtained from different cellular sources, each shaped by a distinct immunological environment. Mobilized peripheral blood (mPB), commonly used in hematopoietic stem cell transplantation, reflects an

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antigen-experienced immune system influenced by prior immune activation and cytokine exposure.[10,11] In contrast, umbilical cord blood (UCB) represents an immunologically immature source enriched in naive lymphocyte populations with unique regulatory properties.[12] These inherent differences suggest that Tregs derived from mPB and UCB may display distinct phenotypic characteristics.[13]

Flow cytometric analysis remains a fundamental approach for investigating Treg heterogeneity. Markers such as CD45RA and CD45RO are widely used to distinguish naive and memory-like Treg subsets,[14] while CD27 provides information related to differentiation status and cellular survival.[15] CD69 is an early activation marker that has also been associated with tissue retention and recent immune stimulation. Together, these markers allow assessment of Treg differentiation and activation states across different cellular sources.[16]

In addition to classical differentiation and activation markers, immune checkpoint receptors such as programmed cell death protein 1 (PD-1) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) were included in the phenotypic panel. These molecules function as inhibitory regulators of T-cell signaling and play essential roles in maintaining immune homeostasis and preventing excessive immune activation.[17,18] Although sustained expression of PD-1 and TIM-3 has been associated with T-cell exhaustion in chronic infections and malignancy, their expression on regulatory T cells may reflect regulatory activation, chronic antigen exposure, or advanced differentiation rather than functional impairment.[19–21] Evaluation of these checkpoint-associated markers therefore provides additional insight into the regulatory and activation landscape of Tregs derived from different cellular sources.

In the present study, we performed a comparative phenotypic analysis of regulatory T cells derived from mPB and UCB. By assessing markers related to differentiation, activation, and immune checkpoint–associated regulation, we aimed to identify source-dependent phenotypic differences. A clearer understanding of these intrinsic characteristics may contribute to the rational selection of Treg sources for adoptive regulatory T-cell–based therapies and other cellular immunotherapeutic approaches aimed at restoring immune tolerance in immune-mediated disorders.[22]

MATERIALS AND METHODS

Study Design and Sample Collection

This exploratory comparative experimental study was conducted on a total of 10 samples, including 5 mPB samples and 5 UCB units. Mobilized peripheral blood samples were obtained from healthy donors (n = 5; 3 males and 2 females; mean age, 33 years; range, 23–46 years) undergoing granulocyte colony-stimulating factor (G-CSF) mobilization at Shariati Hospital under the supervision of the Hematology, Oncology and Cell Therapy Research Institute. Peripheral blood stem cells were collected by leukapheresis according to institutional clinical protocols. Umbilical cord blood units were obtained through the Cord Blood Bank of the Iranian Blood Transfusion Organization at the time of full-term delivery. Collection was performed in sterile anticoagulant-containing collection bags according to standardized banking procedures. Written informed consent was obtained from all adult donors and from mothers prior to cord blood collection.

All samples were processed freshly within 24 hours of collection and were not cryopreserved prior to flow-cytometric analysis. To maintain cell viability and marker stability, UCB samples were kept at ambient temperature (20–25 °C), whereas mPB samples were transported at 4–8 °C, in accordance with standard clinical handling protocols for each sample type.

The study protocol was reviewed and approved by the institutional ethics committee (Approval No. IR.MODARES.REC.1403.051), and all procedures were conducted in accordance with the Declaration of Helsinki and national regulatory guidelines.

Mononuclear Cell Isolation

For UCB samples, red blood cells were first sedimented using 6% hydroxyethyl starch (HES; WACKER Chemie, Germany) according to standard cord blood processing protocols. Following erythrocyte sedimentation, the mononuclear cell (MNC) fraction was isolated by density gradient centrifugation using Lymphodex (Ficoll solution; Inno-Train Diagnostic GmbH, Germany). Briefly, samples were diluted 1:1 with phosphate-buffered saline (PBS) and carefully layered over Lymphodex, followed by centrifugation at 400*g* for 30 minutes at room temperature without brake. The MNC layer was collected and washed twice with PBS prior to downstream applications.

For mPB samples collected by apheresis, density gradient separation was not performed, as the apheresis procedure effectively reduces red blood cell content and provides a mononuclear cell–enriched fraction suitable for subsequent regulatory T-cell purification. Avoiding density gradient reagents in peripheral samples may facilitate future translational or good manufacturing practice (GMP)-compatible workflows.

Regulatory T-cell Purification

Regulatory T cells were isolated from the MNC fraction using a 2-step magnetic separation method (Miltenyi Biotec, Germany) according to the manufacturer's instructions. First, $CD4^+$ T cells were enriched via negative selection to deplete non-$CD4^+$ cells. Subsequently, $CD25^+$ cells were positively selected from the enriched $CD4^+$ fraction to obtain a purified $CD4^+CD25^+$ regulatory T-cell population. Purity of the isolated Tregs was confirmed by flow cytometry based on the phenotype $CD4^+CD25^+FOXP3^+CD127^{low/-}$.

Flow Cytometry and Viability Assessment

Phenotypic analysis of isolated regulatory T cells (Tregs) was performed using a Thermo Fisher Attune Flow Cytometer (Thermo Fisher Scientific, USA). Prior to Treg phenotyping and before fixation/permeabilization, cell viability was assessed on unfixed cells using 7-aminoactinomycin D (7-AAD; BD Biosciences, USA) as a quality-control step. Only samples showing viability greater than 90% were subsequently processed for Treg immunophenotyping.

Two separate staining panels were used: one for Treg purity and identity, and a second for phenotypic characterization. Detailed information on all antibodies, including their clones, fluorochromes, and manufacturers, is provided in Supplementary Table S1.

Although antibodies were obtained from multiple commercial suppliers, all clones and fluorochromes were validated for human Treg phenotyping, and proper compensation controls were applied to ensure accurate multicolor analysis. Manual color compensation was performed using single-stained cell preparations for each antibody–fluorochrome conjugate included in the panel. Each fluorochrome-conjugated antibody was acquired individually in its corresponding fluorescence channel, and compensation was manually adjusted to correct for spectral overlap and fluorescence spillover between detection channels, irrespective of the antibody manufacturer. Photomultiplier tube (PMT) voltages were adjusted to optimize signal detection and ensure clear separation and alignment of positive and negative populations. The finalized compensation settings were saved and applied consistently during sample acquisition.

Panel 1: Treg Purity and Identity Assessment

To confirm the identity and purity of isolated Tregs, cells were stained with CD4, CD25, CD127, and FOXP3. Intracellular FOXP3 staining was performed using the Beckman Coulter Intracellular Staining (Fixation/Permeabilization) Kit, according to the manufacturer's instructions.

The gating hierarchy used for analysis included initial lymphocyte selection based on forward scatter area (FSC-A)/side scatter area (SSC-A) properties to exclude debris, followed by doublet exclusion using forward scatter height (FSC-H) versus FSC-A plots. These gates were applied before downstream identification of $CD4^+CD25^+FOXP3^+CD127^{low/-}$ Tregs. Cell viability was assessed prior to staining using 7-AAD, and only samples with >90% viability were included in the analysis. Representative gating plots are provided in Supplementary Figure 1.

Panel 2: Phenotypic Characterization

To assess differentiation, activation, and immune checkpoint expression, viable gated Tregs were stained with CD45RA, CD45RO, CD27, CD69, PD-1, and TIM-3. Notes on PD-1 and TIM-3: Both antibodies were detected in the VL1 channel (violet laser, 405 nm). Because they were measured in the same channel, PD-1 and TIM-3 staining were performed in separate experiments.

Marker expression was reported as the percentage of positive cells. Fluorescence-minus-one (FMO) controls were used to define positivity thresholds for markers with low-density expression or continuum-like staining patterns, specifically for FOXP3, CD127, PD-1, and TIM-3. Representative gating strategies and FMO-based thresholding are shown in Supplementary Figure 1. Data acquisition and analysis were performed using Attune NxT Software (Thermo Fisher Scientific, USA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism. Data are presented as mean±standard deviation. Each group included 5 independent samples. Prior to

parametric testing, data normality was assessed for each marker and group using the Shapiro–Wilk test. Given the small sample size, the results of normality testing were interpreted cautiously. Comparisons between mPB and UCB groups were performed using 2-tailed unpaired Student *t* tests, with Welch correction applied when unequal variances were identified. Nonparametric Mann–Whitney *U* tests were additionally performed as sensitivity analyses to assess the robustness of the findings. Because this study was exploratory and hypothesis-generating, no formal correction for multiple comparisons was applied; therefore, all *p* values are reported as nominal and should be interpreted accordingly. A nominal *p* value < .05 was considered statistically significant.

RESULTS

Initial Characteristics of Collected Samples

A total of 10 samples were analyzed, including 5 mPB and 5 UCB samples. All donors provided informed consent. To obtain at least 1 million regulatory T cells for flow cytometry, different sample volumes were used due to inherent differences in total nucleated cell (TNC) counts between sources. mPB samples required smaller volumes because of higher white blood cell counts, whereas UCB required larger volumes to achieve sufficient cell numbers.

Table -1 summarizes the mean and SD of sample volume, TNC, MNC, and $CD3^+$ cell counts for each group, illustrating differences in starting material and cell yield.

Characteristics of Isolated Regulatory T Cells

The characteristics of Tregs after magnetic separation are summarized in Figure 1. Treg yield was expressed as the total number of isolated cells. The mean total Treg yield was $0.6\pm0.2 \times 10^6$ cells from 1.4±0.4 mL of mPB and $1.1\pm0.5 \times 10^6$ cells from 30±6.1 mL of UCB. This reflects the higher total nucleated cell concentration in mPB, allowing sufficient Treg yield from a smaller sample volume. Viability, assessed by 7-AAD exclusion, was high in both sources but slightly lower in UCB-derived Tregs, likely due to additional manipulation and processing steps required for these samples.

Table -1. Characteristics of umbilical cord blood (UCB) and mobilized peripheral blood (mPB) samples used for Treg isolation

Group	Sample Volume, mL	TNC, $\times10^6$ cells	MNC, $\times10^6$ cells	$CD3^+$ Cells, %
mPB (n = 5)	1.4±0.4	187±52.4	112.3±45.0	47.9±8.5
UCB (n = 5)	30±6.1	9.0±1.5	4.4±1.0	3.0±0.4

CD3: cluster of differentiation 3; MNC: mononuclear cell; mPB: mobilized peripheral blood; TNC: total nucleated cell; Treg: regulatory T cell; UCB: umbilical cord blood.

A

	MPBSC		UCB		
	(Mean ± SD)	(Range)	(Mean ± SD)	(Range)	P-value
Total Treg yield ($\times10^3$ cells)	608±293	250-1000	1152±535	460-1800	0.0916
Treg of MNC (%)	0.6±0.2	0.2-1.0	1.2±0.5	0.4-1.8	0.0528
Viability (%)	94.3 ± 1.6	92.6-96.2	90.4 ± 1.5	88.8-92.2	0.0041
FOXP3 (%)	31.3±14.1	18.3-52.2	64.3±17.5	48.0-91.3	0.0118
Purity (%)	20.2±11.6	8.0–37.0	50.2±8.7	38.0–62.0	0.0021

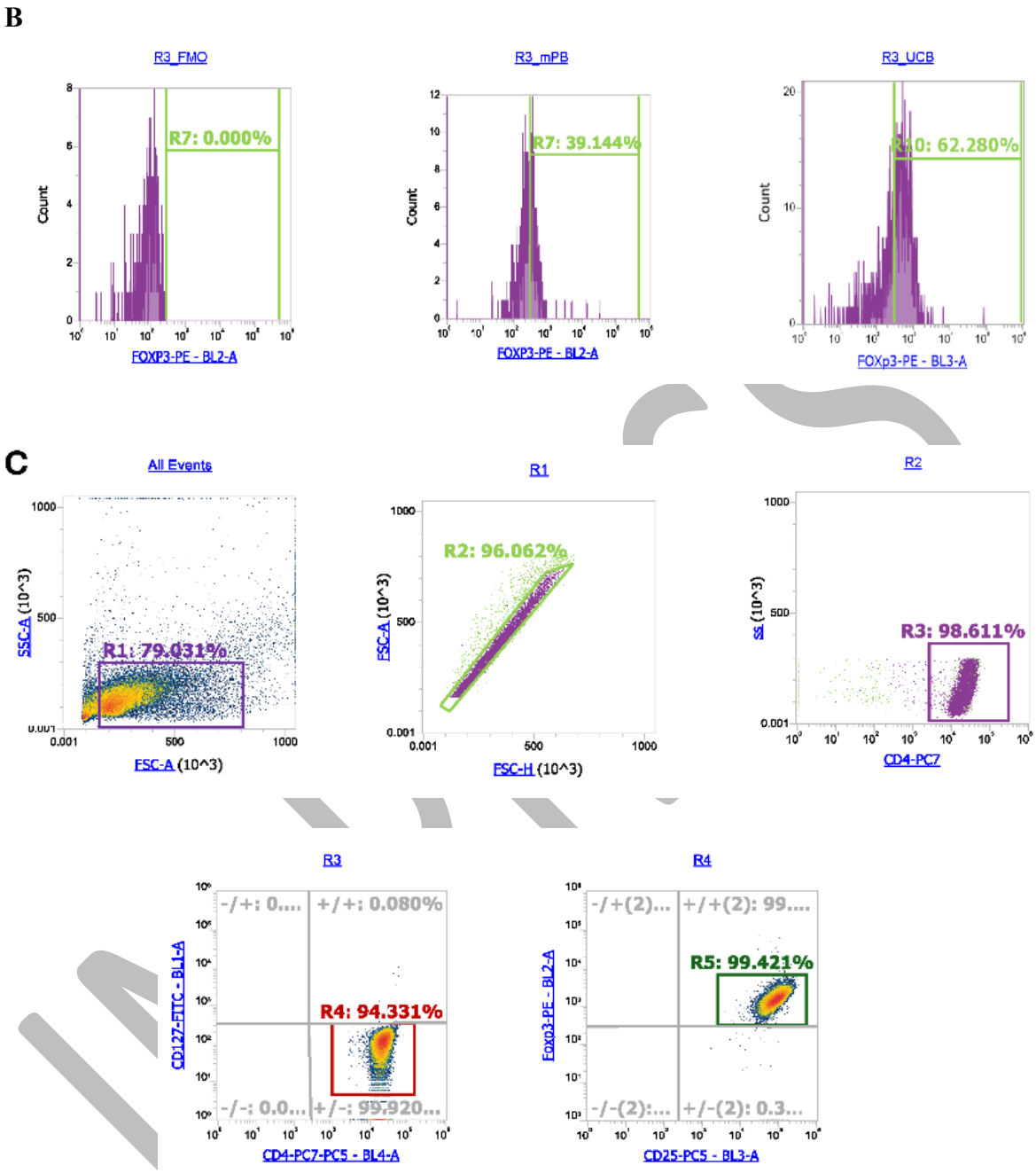

Figure 1. Characteristics and quality assessment of isolated regulatory T cells. A. Summary table showing the characteristics of Tregs isolated from mPB and UCB samples. Parameters include total isolated Treg yield (×10^3 cells), percentage of Tregs among MNCs, viability, FOXP3 expression, and purity, defined as $CD4^+CD127^{low/-}CD25^+FOXP3^+$ cells. Data are presented as mean±SD and range (n = 5 per group). P-values were calculated using Welch's t-test. Given the exploratory nature of the study and the small sample size, p-values should be interpreted cautiously. Nominal p-values are reported, and no formal correction for multiple comparisons was applied. B. Representative histogram showing intracellular FOXP3 expression within the gated $CD4^+CD25^+CD127^{low/-}$ population, compared with the fluorescence-minus-one (FMO) control. C. Representative flow cytometry gating strategy for Treg identification: lymphocytes (FSC-A/SSC-A) → singlets (FSC-H/FSC-A) → $CD4^+$ T cells → $CD127^{low/-}$ cells → Tregs ($CD25^+FoxP3^+$).

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Treg purity was defined as the proportion of $CD4^+CD127^{low/-}CD25^+FOXP3^+$ cells within the isolated cell preparations. Purity was higher in UCB-derived Treg preparations than in mPB-derived Treg preparations (50.2±8.7%, range 38.0–62.0% vs 20.2±11.6%, range 8.0–37.0%). This difference was nominally significant using an unpaired 2-tailed *t*-test with Welch correction (p=0.002). Treg purity was lower and more variable in mPB-derived preparations than in UCB-derived preparations, as shown by both the lower mean value and wider range of purity values. Despite the higher overall cell concentration in mPB samples, UCB samples provided a larger proportion of phenotypically defined $FOXP3^+$ Tregs. Given the limited sample size and exploratory nature of the study, these findings should be interpreted with caution.

Sequential gating based on lymphocytes (FSC-A/SSC-A) → singlets (FSC-H/FSC-A) → $CD4^+$ T cells → $CD127^{low/-}$ cells → $CD25^+FOXP3^+$ Tregs confirmed consistent Treg identification across all samples, supporting the reliability of the isolation procedure for downstream phenotypic analyses.

UCB-derived samples showed approximately 1.9-fold higher Treg frequency among MNCs and 1.9-fold higher Treg isolation yield compared with mPB-derived samples. Although these differences did not reach statistical significance, likely due to the limited sample size and inter-sample variability, they suggest a potential biological advantage of UCB as a Treg source that warrants further investigation in larger studies.

Phenotypic Characterization of Isolated Tregs

Phenotypic analysis revealed significant differences between Tregs derived from mPB and UCB. UCB-derived Tregs displayed a markedly naive phenotype, characterized by significantly higher CD45RA expression (94.28±4.38%) compared with mPB-derived Tregs (37.70±13.69%; p<0.001), and significantly lower CD45RO expression (0.90±0.65% vs 67.74±17.61%; p=0.001). CD27 expression was also significantly elevated in UCB-derived Tregs (80.78±14.43%) relative to mPB-derived cells (32.60±12.40%; p<0.001).

In contrast, mPB-derived Tregs demonstrated significantly increased expression of activation and exhaustion–associated markers. CD69 expression was markedly higher in mPB samples (66.10±18.23%) compared with UCB samples (2.76±1.34%; p=0.001). Similarly, PD-1 and TIM-3 expression were significantly elevated in mPB-derived Tregs (62.40±14.15% and 69.20±8.11%, respectively) compared with UCB-derived Tregs (28.30±13.28% and 23.30±9.40%, respectively; p=0.004 for PD-1 and p<0.001 for TIM-3).

Collectively, these results demonstrate that UCB-derived Tregs retain a significantly more naive phenotype, whereas mPB-derived Tregs exhibit a more activated and differentiated immunophenotypic profile. These phenotypic differences are illustrated in Figures 2 and 3, which show representative flow cytometry plots and quantitative comparisons of marker expression between the two sources.

Comparative Statistical Analysis of Phenotypic Markers

Using Welch's t-test, nominally significant differences were observed across all evaluated phenotypic markers between mPB- and UCB-derived Tregs. Treg purity differed significantly between groups, with higher purity observed in UCB-derived Tregs compared with mPB-derived Tregs (p=0.002). UCB-derived Tregs exhibited higher CD45RA expression (p<0.001), whereas mPB-derived Tregs showed higher CD45RO expression (p=0.001). Additional nominally significant differences were observed in CD27 expression, which was higher in UCB-derived Tregs (p<0.001), and in CD69 expression, which was higher in mPB-derived Tregs (p=0.001). Furthermore, mPB-derived Tregs showed higher expression of PD-1 (p=0.004) and TIM-3 (p<0.001). Because no formal correction for multiple comparisons was applied, these results should be interpreted cautiously as exploratory and hypothesis-generating.

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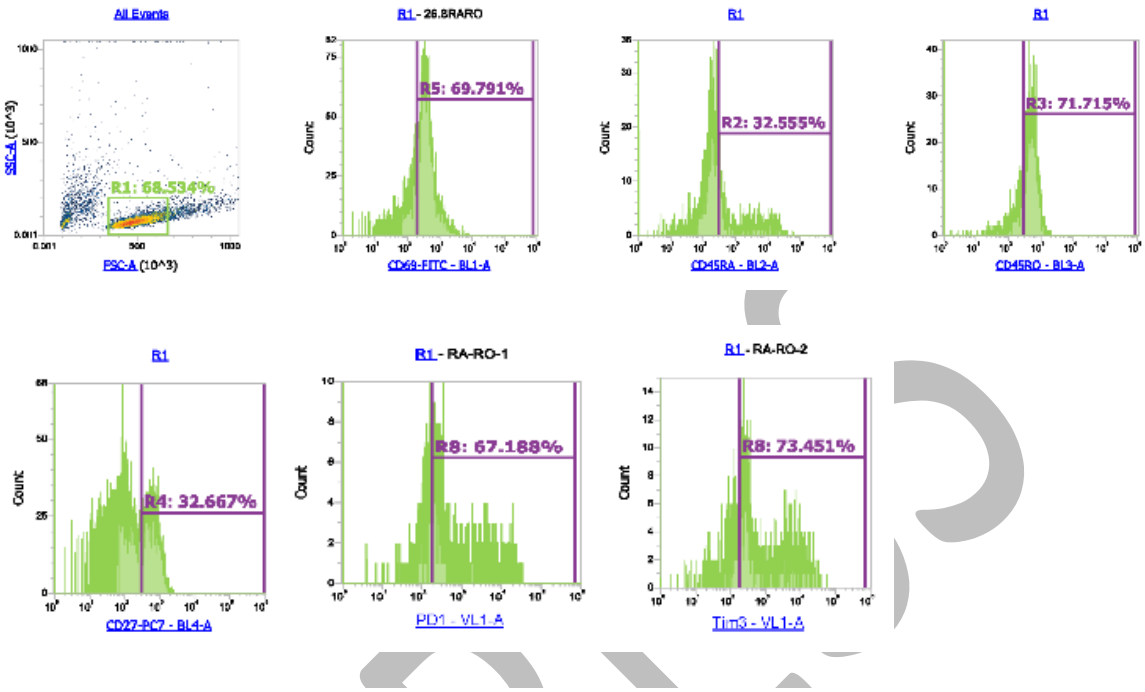

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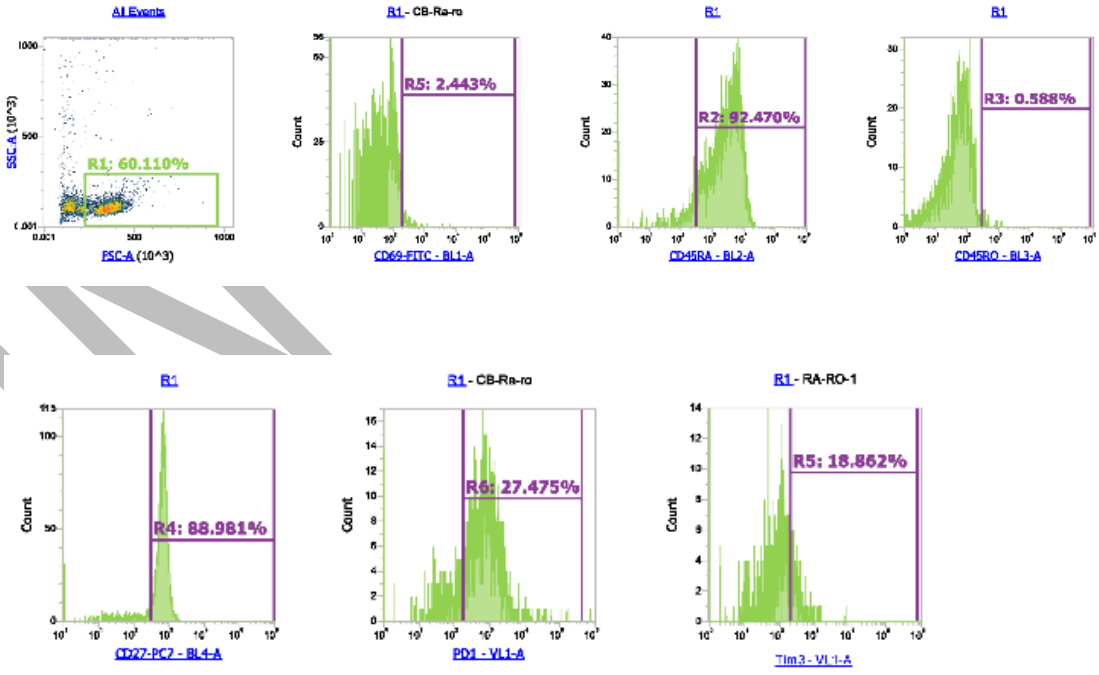

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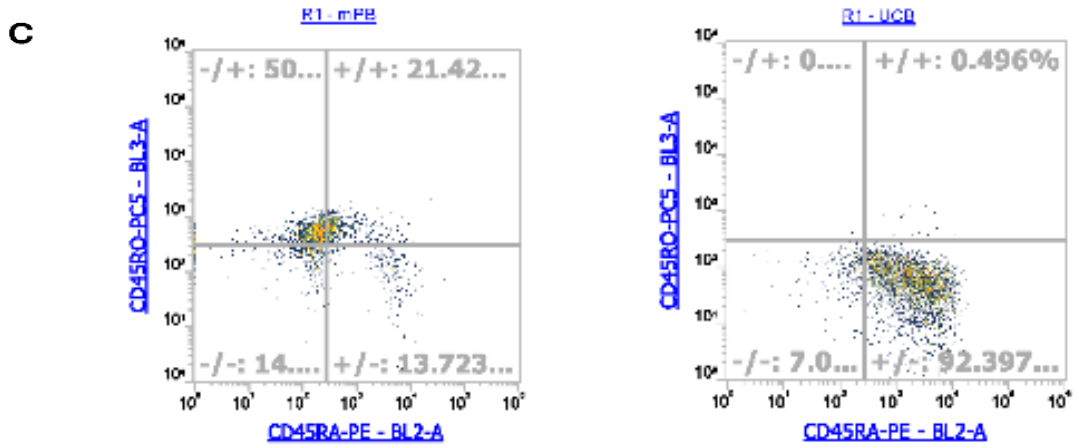

Figure 2. Representative phenotypic comparison of Tregs derived from mPB and UCB. A., B. Representative flow cytometry histograms showing the expression of CD69, CD45RA, CD45RO, CD27, PD-1, and TIM-3 in Tregs derived from (A.) mPB and (B.) UCB samples. Percentages indicate marker-positive cells in the representative samples shown. C. Representative CD45RA versus CD45RO dot plots illustrating the distribution of $CD45RA^+$ and $CD45RO^+$ subsets. Across the analyzed samples, significant differences were observed for CD45RA (p<0.001), CD45RO (p=0.001), CD27 (p<0.001), CD69 (p=0.001), PD-1 (p=0.004), and TIM-3 (p<0.001). No formal correction for multiple comparisons was applied; p values are nominal.

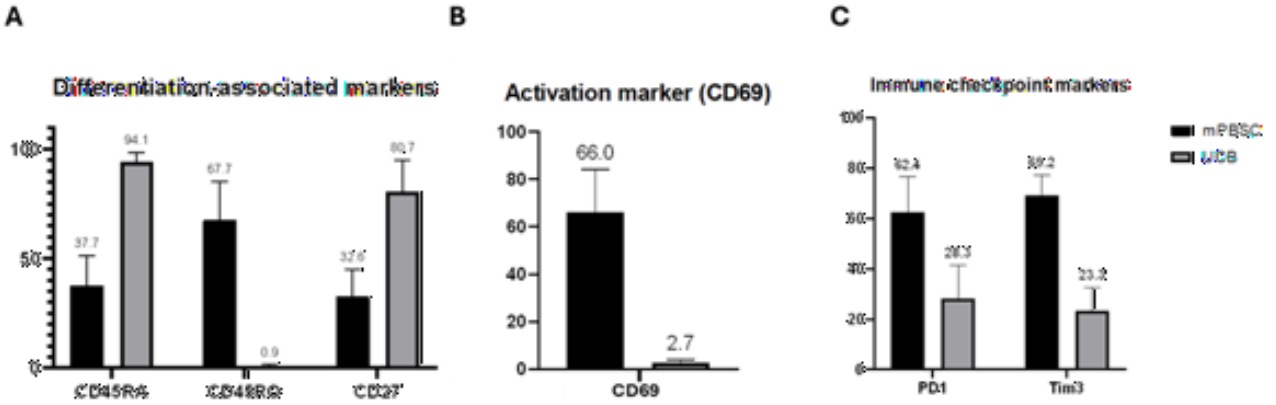

Figure 3. Phenotypic characterization of Tregs derived from mPB and UCB. A. UCB-derived Tregs showed nominally higher CD45RA expression, whereas mPB-derived Tregs showed nominally higher CD45RO expression. B. CD69 was nominally higher in mPB, whereas CD27 was higher in UCB. C. PD-1 and TIM-3 were nominally higher in mPB-derived Tregs. Data are mean±SD from 5 independent samples per group. Nominal p values: CD45RA, p<0.001; CD45RO, p=0.001; CD27, p<0.001; CD69, p=0.001; PD-1, p=0.004; TIM-3, p<0.001. No formal correction for multiple comparisons was applied.

DISCUSSION

Given their central role in immune regulation, Tregs remain a promising platform for adoptive cellular therapies.[22–24] In the present exploratory study, we performed a comparative phenotypic characterization of Tregs derived from mobilized peripheral blood (mPB) and umbilical cord blood (UCB), aiming to identify source-dependent differences that may be relevant to their potential therapeutic application.[25]

The selection of these 2 sources was based on their established use in hematopoietic stem cell transplantation. mPB offers high total nucleated cell counts, enabling Treg isolation from relatively small volumes, and is efficiently collected via leukapheresis with reduced red blood cell contamination, thereby

minimizing downstream processing steps.[10,26] In contrast, UCB has become increasingly accessible through the expansion of cord blood banking worldwide. Its extended storage capacity, relative tolerance to human leukocyte antigen (HLA) mismatch, and availability as a cryopreserved product make it a practical candidate for allogeneic and potentially off-the-shelf cellular approaches.[27,28]

Our findings suggest a clear divergence in the differentiation status of Tregs derived from these sources. UCB-derived Tregs exhibited a phenotype characterized by high CD45RA and CD27 expression and minimal CD45RO expression. In contrast, mPB-derived Tregs showed a phenotype enriched in CD45RO expression.[14,29–31] These observations are in line with established immunological principles, as UCB is enriched in naive T cells due to limited antigen exposure, whereas mobilized peripheral blood reflects not only cumulative immune experience but also the immunomodulatory effects of G-CSF administration, which has been reported to influence T-cell phenotype and activation status.[32,33] Because G-CSF is a cytokine-based mobilizing agent that can reshape the bone marrow immune microenvironment and modulate T-cell activation and trafficking, it may also influence the phenotype and activation status of circulating regulatory T cells.[34–36] Therefore, the checkpoint- and activation-associated features observed in mPB-derived Treg preparations could partly reflect G-CSF-induced immune modulation rather than source-dependent biology alone. In the present study, mobilized peripheral blood samples were collected after G-CSF administration and no non-mobilized peripheral blood comparator was available. Consequently, the specific contribution of G-CSF exposure cannot be separated from source-dependent characteristics or prior antigen experience. These findings should therefore be interpreted cautiously and considered exploratory, highlighting the need for future studies directly comparing mobilized and non-mobilized peripheral blood Tregs to better define the impact of G-CSF on Treg phenotype.

In addition to differentiation markers, differences were observed in activation- and checkpoint-associated molecules. mPB-derived Tregs displayed higher expression of CD69, PD-1, and TIM-3 compared with UCB-derived Tregs. CD69 is an early activation marker that may reflect recent immune stimulation, while PD-1 and TIM-3 are inhibitory receptors that are typically upregulated following repeated antigen exposure.[16,20,21] Increased expression of PD-1 and TIM-3 in mPB-derived Tregs should be interpreted in a context-dependent manner. TIM-3 has been associated with functionally specialized Treg subsets with enhanced suppressive features in some contexts,[37] whereas PD-1 expression has also been linked to dysfunctional or exhaustion-like regulatory phenotypes.[38] Importantly, mPB samples are typically collected after G-CSF administration, which is known to remodel the bone marrow microenvironment and peripheral immune compartment by altering cytokine networks, myeloid cell populations, and T-cell regulatory pathways.[36] Therefore, the elevated expression of PD-1 and TIM-3 may partly reflect G-CSF-associated mobilization imprinting rather than intrinsic superiority of mPB-derived Tregs.[34] These cells may be antigen-experienced and immunoregulatory, but they may also exhibit altered functional competence, reduced stability, or exhaustion-like features.[39] Consequently, in the absence of direct suppression assays and assessment of phenotypic stability over time, our findings should be considered phenotypic evidence of source-dependent Treg differences rather than proof of enhanced suppressive function.

Conversely, the phenotype observed in UCB-derived Tregs, characterized by $CD45RA^{+}CD27^{+}$ expression with low levels of activation and checkpoint markers, is consistent with a less differentiated and potentially more stable regulatory population. CD27 expression has been associated with survival and long-term persistence in T cells, and its higher expression in UCB-derived Tregs may be relevant for applications requiring *in vitro* expansion.[31] Nevertheless, these interpretations remain inferential and require functional validation.

From a practical perspective, notable differences were observed in isolation characteristics between the 2 Treg sources. Although mPB samples yielded sufficient Treg numbers from smaller starting volumes, UCB-derived preparations showed a higher proportion of phenotypically defined Tregs after isolation. In contrast, mPB-derived preparations showed lower and more variable Treg purity. This difference represents an important potential confounding factor in the interpretation of the phenotypic profiles. The lower and more variable purity observed in mPB-derived preparations may be related to the greater cellular heterogeneity of mobilized peripheral blood, inter-donor variability, and the immunological effects of mobilization, which may alter leukocyte composition

and activation status.[36] In particular, mobilized peripheral blood may contain higher proportions of activated conventional $CD4^+$ T cells or other non-regulatory T-cell populations that partially overlap with Treg-associated phenotypes during enrichment and analysis.[40] These factors may reduce the specificity of Treg isolation and contribute to variability in post-isolation purity.

In this study, Treg purity was assessed using the main Treg-identification panel, whereas differentiation, activation, and checkpoint-associated markers, including CD27, CD45RA/CD45RO, CD69, PD-1, and TIM-3, were evaluated in separate tubes because of technical limitations in multicolor panel design. Therefore, lower purity in mPB-derived preparations may have increased the contribution of contaminating non-regulatory or conventional T cells to the observed phenotypic profiles. This is particularly relevant for activation- and checkpoint-associated markers, as contaminating activated conventional T cells may have influenced the measured expression of CD69, PD-1, and TIM-3, potentially exaggerating or distorting apparent source-related differences.[37,40]

Importantly, a consistent gating strategy was applied across all samples, supporting the reliability of Treg identification and reducing technical variability. However, because post-isolation purity differed between sources and phenotypic markers were assessed in separate tubes, the observed phenotypic profiles should not be attributed exclusively to intrinsic biological differences between Treg sources. Rather, they may reflect a combination of source-related Treg characteristics, prior antigen exposure, mobilization-associated changes, and differences in cellular composition after isolation. Accordingly, these phenotypic differences should be interpreted cautiously as exploratory observations. Future studies using more stringent purification strategies, integrated multicolor panels, larger sample sizes, and functional validation are required to confirm whether these differences are truly source-specific.

This study has several limitations. The relatively small sample size ($n = 5$ per group) limits statistical power and reflects restricted access to clinical-grade samples. In addition, methodological constraints related to the availability of flow cytometry antibodies and magnetic isolation reagents influenced the scope of phenotypic and functional analyses. As a result, the marker panel was limited to selected differentiation, activation, and checkpoint-associated molecules.

While the $CD4^+CD25^+FOXP3^+CD127^{low/-}$ phenotype is a widely used strategy for identifying human Tregs, it is important to acknowledge that certain activated conventional T cells can transiently express FOXP3. To minimize this overlap, we incorporated CD127 as a negative selection marker, which enhances the specificity of Treg identification.[41] However, the absence of additional markers such as Helios or cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) means that our study does not distinguish thymic-derived from peripherally induced Tregs,[40,42,43] nor does it allow a more detailed assessment of their subset identity, activation-associated phenotype, or suppressive potential. This remains a limitation that should be addressed in future larger-scale studies.

Importantly, this study was limited to phenotypic characterization, and suppressive function, lineage stability, and *ex vivo* expansion potential were not assessed. Therefore, any conclusions regarding the therapeutic suitability of mPB- or UCB-derived Tregs remain speculative and require validation in dedicated functional studies. Despite these limitations, the observed phenotypic differences were consistent across samples and aligned with established immunological principles.

Given the limited sample size and exploratory design of this study, these findings should be considered preliminary and hypothesis–generating. Future studies incorporating larger cohorts, expanded marker panels, and functional assays will be essential to validate and extend these observations. Therefore, the functional implications of the observed phenotypic differences should be interpreted with caution.

In conclusion, our results indicate that Tregs derived from mPB and UCB exhibit distinct phenotypic profiles across axes of differentiation, activation, and immune regulation. UCB-derived Tregs were enriched for a naive phenotype, whereas mPB-derived Tregs showed features consistent with antigen experience and activation. However, because this study did not include suppressive functional assays, cytokine profiling, or expansion experiments, these observations should be interpreted as phenotypic differences rather than evidence of therapeutic superiority of either source. Additional studies are needed to determine whether and how these differences affect functional performance and clinical utility in adoptive Treg therapies.

STATEMENT OF ETHICS

All procedures performed in this study involving human participants were conducted in accordance with the ethical standards of the institutional and national research committees and with the principles of the Declaration of Helsinki. The study protocol was reviewed and approved by the Ethics Committee of Tarbiat Modares University (Approval No. IR.MODARES.REC.1403.051). Written informed consent was obtained from all adult donors and from mothers prior to cord blood collection.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

AI ASSISTANCE DISCLOSURE

AI tools were used to assist with language polishing and rephrasing of the manuscript. No AI was used for data analysis, interpretation, or drafting scientific conclusions.

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