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# Immune regulation of T lymphocyte by a newly characterized human umbilical cord blood stem cell

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**Abstract**

Previous work identified a novel type of stem cell from human umbilical cord blood, designated cord blood-stem cells (CB-SC). To further evaluate their immune characteristics, we cocultured CB-SC with allogeneic peripheral blood lymphocytes in the presence of phytohaemagglutinin (PHA) or interleukin-2 (IL-2). Results showed that CB-SC could significantly inhibit lymphocyte proliferation and reduce tyrosine phosphorylation of STAT5 in both PHA- and IL-2-stimulated lymphocytes, along with the regulation on the phenotypes of $CD4^+$ and $CD8^+$ T cells. Additionally, CB-SC also suppressed the proliferation of IL-2-stimulated $CD4^+CD25^+$ regulatory T cells. Mechanism studies revealed that programmed death receptor-1 ligand 1 (PD-L1) expressed on CB-SC membrane, together with a soluble factor nitric oxide (NO) released by PHA-stimulated CB-SC, not prostaglandin E2 (PGE2) and transforming growth factor-β1 (TGF-β1), mainly contributed to the T cell suppression induced by CB-SC, as demonstrated by blocking experiments with a nitric oxide synthase inhibitor ($N^{\omega}$-nitro-L-arginine, L-NNA) and a neutralizing antibody to PD-L1. Our findings may advance our understanding of the immunobiology of stem cells and facilitate the therapeutic application of cord blood stem cells.


*Keywords:* Stem cell; Umbilical cord blood; T lymphocyte; Nitric oxide; PD-L1



## 1. Introduction

Stem cell-based therapy, including embryonic and adult stem cells, may provide rational approaches for treating human dominant diseases, *e.g.*, diabetes, neuronal degenerative diseases, and cardiovascular diseases [1–5]. To date, clinical transplantation of tissues and organs between two unrelated individuals results almost invariably in graft rejection, unless immunosuppressive therapy is given to control the immune response. Similarly, immune rejection is also challenging stem cell-based therapy [6,7]. How to develop strategies to overcome the inevitable immunological barriers of host that will achieve long-term therapeutics post-transplantation of stem cells and/or stem cell-derived cells? This is an emerging area for stem cell-based therapy and must be addressed before clinical application can realize its full potential. To circumvent these barriers, several approaches [8–11] are being investigated including immunosuppressive drugs, encapsulation, manipulation of host immune responses, constitution of immune chimerism, *in vitro* fertilization (IVF), and altered nuclear transfer (ANT). Currently, no final solution has been achieved [9]. The use of human embryonic stem (ES) cells as an attractive mean to deal with immune rejection issues has been limited by ethical and safety issues (for example, formation of teratoma) [12]. The use of adult stem cells is limited by their shortage and high immunogenicity for allogenetic transplantation [3,13]. Therefore, new strategies are needed. Recently, umbilical cord blood has provided an alternative source for generation of stem cells [14–16]. We have identified a novel type of stem cell from human umbilical cord blood, designated cord blood-stem cells (CB-SC) [14]. CB-SC displayed unique characteristics including expression of embryonic markers, a high potential for expansion, and very low immunogenicity, along with multiple potential of differentiation. In this report, we demonstrated that CB-SC produced immune regulations of lymphocyte proliferation and T cell subsets. Thus, CB-SC may provide an ideal stem cell model for therapeutics.

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## 2. Materials and methods

### 2.1. Cell culture for CB-SC

Culture of CB-SC was preformed as previously described [14]. In brief, human umbilical cord blood samples (50–100 ml/unit) were obtained from healthy donors (Life-Source Blood Services, Glenview, IL). Mononuclear cells were isolated using Ficoll–Hypaque ($\gamma = 1.077$, Sigma), followed by removing red blood cells using Red Blood Cell Lysis buffer (eBioscience, San Diego, CA). Mononuclear cells were seeded into 8-Well Lab-Tek II Chamber Slides (Fisher Scientific) at $1–2 \times 10^5$ cells/ml, 0.5 ml/well, or into 150 mm × 15 mm Petri dishes (Becton Dickinson Labware, Franklin Lakes, NJ) at $1 \times 10^6$ cells/ml, 25 ml/dish in RPMI 1640 medium supplemented with 7% fetal bovine serum (Invitrogen, Carlsbad, CA), and incubated at 37 °C, 8% $CO_2$ conditions.

### 2.2. Coculture of CB-SC with PHA- or IL-2-stimulated allogeneic lymphocytes

CB-SC adhere very tightly to the culture dishes and display large rounded morphology, it is easy to distinguish between CB-SC and lymphocytes. CB-SC growing at 80% confluence were used for coculture with allogeneic lymphocytes. Allogeneic lymphocytes were collected from buffy coats of healthy donors (Life-Source Blood Services) after Ficoll–Hypaque separation followed by removing all attached cells and then cocultured with CB-SC at the different ratios of CB-SC:lymphocyte. For lymphocyte stimulation, lymphocyte suspensions ($1 \times 10^6$ cells/ml) with or without PHA (10 μg/ml, Sigma), or IL-2 (500 U/ml, eBioscience) were, respectively, seeded onto CB-SC cell cultures in regular culture medium. PHA- or IL-2-stimulated lymphocytes without CB-SC served as positive control; lymphocytes cultured only in regular medium served as negative control. Lymphocytes cocultured with CB-SC without PHA or IL-2 stimulation served as an additional negative control. After 4–5 days, the suspended lymphocytes were collected for cell count and/or flow analysis. The supernatant were collected and kept at −20 °C for following examination of soluble factors.

### 2.3. Western blot

Lymphocytes were collected after coculture with CB-SC for 40 h at 37 °C, 8% $CO_2$ conditions. Cells were solubilized with lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin), with a cocktail of protease inhibitors (Sigma). Cell samples (20 μg protein each) were mixed with a loading buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, 2 mg of Bromophenol Blue) in a volume ratio of 1:1, boiled, loaded, and separated by electrophoresis on 7.5% SDS gel. The separated proteins were then transferred to a nitrocellulose membrane, blocked with 5% non-fat dry milk in TBST for 1 h and incubated with different antibodies: including rabbit anti human total STAT5a/b and phospho-STAT5 (Tyr694) antibodies (Cell Signaling Technology, Danvers, MA) at 1:1000 dilution, diluted in PBST for 1 h at room temperature. After washing, the blot was exposed to a horseradish peroxidase-conjugated secondary antibody (1:2000; Pierce) in PBS-T. The immunocomplexes were visualized by the enhanced chemiluminescence (ECL, GE healthcare) method. β-Actin served as an internal loading control.

### 2.4. Flow analysis and cell sorting

Flow analysis was preformed as previously described [14]. In brief, cells were incubated with 2.5% horse serum (Vector Laboratories, Burlingame, CA) at room temperature to block non-specific staining. Cells were incubated with mouse anti-human primary antibodies (eBioscience), including CD44 (Clone IM7), CD62L (Clone DREG-56), CD69 (Clone FN50) for 45 min at 4 °C and then washed with cold PBS; consequently, cells were stained with FITC- or Cy™5-conjugated donkey anti-mouse second antibody (Jackson ImmunoResearch Laboratories) for another 45 min at 4 °C and followed by flow analysis. Isotype-matched mouse $IgG_{1\kappa}$ antibody (BD Pharmingen) served as negative control. To evaluate CD4/CD8 ratio, the treated or untreated lymphocytes were stained with FITC-conjugated mouse anti-human CD4 and PE-conjugated mouse anti-human CD8 antibodies. After staining, cells were analyzed using a CyAn ADP (DakoCytomation).

For cell sorting, freshly isolated peripheral blood mononuclear cells (PBMC) were stained with FITC-conjugated mouse anti-human CD4, PE-conjugated mouse anti-human CD8, and allophycocyanin (APC)-conjugated mouse anti-human CD25 (eBioscience) for 45 min at 4 °C and followed by three-way cell sorting, using MoFlo (DakoCytomation). The isotype-matched FITC-conjugated IgG served as negative control. After flow analysis and confirming high purity (>98%), $CD4^+CD25^-$, $CD4^+CD25^+$, and $CD8^+$ T cells were collected and then prepared for different experiments. To further evaluate effects of CB-SC on T cell subsets, the sorted $CD4^+CD25^-$, $CD4^+CD25^+$, and $CD8^+$ T cells ($2 \times 10^4$ cells/ml/well) were cocultured with CB-SC at a ratio 1:3 of CB-SC:T cells in 24-well plates, in the presence or absence of PHA or IL-2. After 2–5 days, the suspended lymphocytes were collected for cell count and/or flow analysis.

### 2.5. PGE2, TGF-β1, and nitric oxide (NO) assay

Levels of PGE2 and TGF-β1 in the supernatants were, respectively, examined using PGE2 and human TGF-β1 ELISA kits (R&D Systems, Minneapolis, MN) according to the recommended protocols. For TGF-β1 examination, samples were initially activated by 1N HCl and then neutralized with 1.2N NaOH/0.5 M Hepes. NO production was determined by using the Griess reagents [17] (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, 2.5% $H_3PO_4$, Sigma). To test the PHA dose responses, CB-SC were seeded at $1 \times 10^5$ cells/ml (0.5 ml/well) in 8-Well Lab-Tek II Chamber Slides. After attachment overnight, PHA was administrated

to cell culture at different doses: 0, 2.5, 5, 10, and 20 μg/ml in 0.5 ml culture medium/well. Supernatants were collected after treatment for 3–5 days for examination of NO production [17]. Absorbance was measured at 540 nm. Diluted sodium nitrite ($NaNO_2$, Sigma) solution was served as standard curve to calculate the amount of NO. The PHA-treated and untreated cells were used for iNOS immunostaining as described below.

### 2.6. Immunocytochemistry

Immunostaining was performed as previously described with minor modifications [14]. In brief for iNOS examination, PHA-treated and untreated cells were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.5% Triton X-100 (Sigma) for 5–6 min at room temperature. After blocking endogenous perioxidase activity and non-specific binding, cells were incubated with rabbit anti-inducible nitric oxide synthase (iNOS) polyclonal antibody (Biomol International, Plymouth Meeting, PA). Then, cells were stained with ABC kit (Vector Laboratories). Normal rabbit IgG (Santa Cruz) served as negative control. For PD-L1 staining, cell permeabilization was omitted and CB-SC were stained with mouse anti-human PD-L1 monoclonal antibody (eBioscience); mouse anti-human $IgG_{1\kappa}$ (BD Pharmingen) served as negative control; mouse anti-human CD45 (BD Pharmingen) served as positive control; mouse anti-human CD95 (eBioscience) staining served as additional control. Cells were viewed and photographed using a Zeiss Axiocam Color Camera with Zeiss Axioskop Histology/Digital Fluorescence microscope.



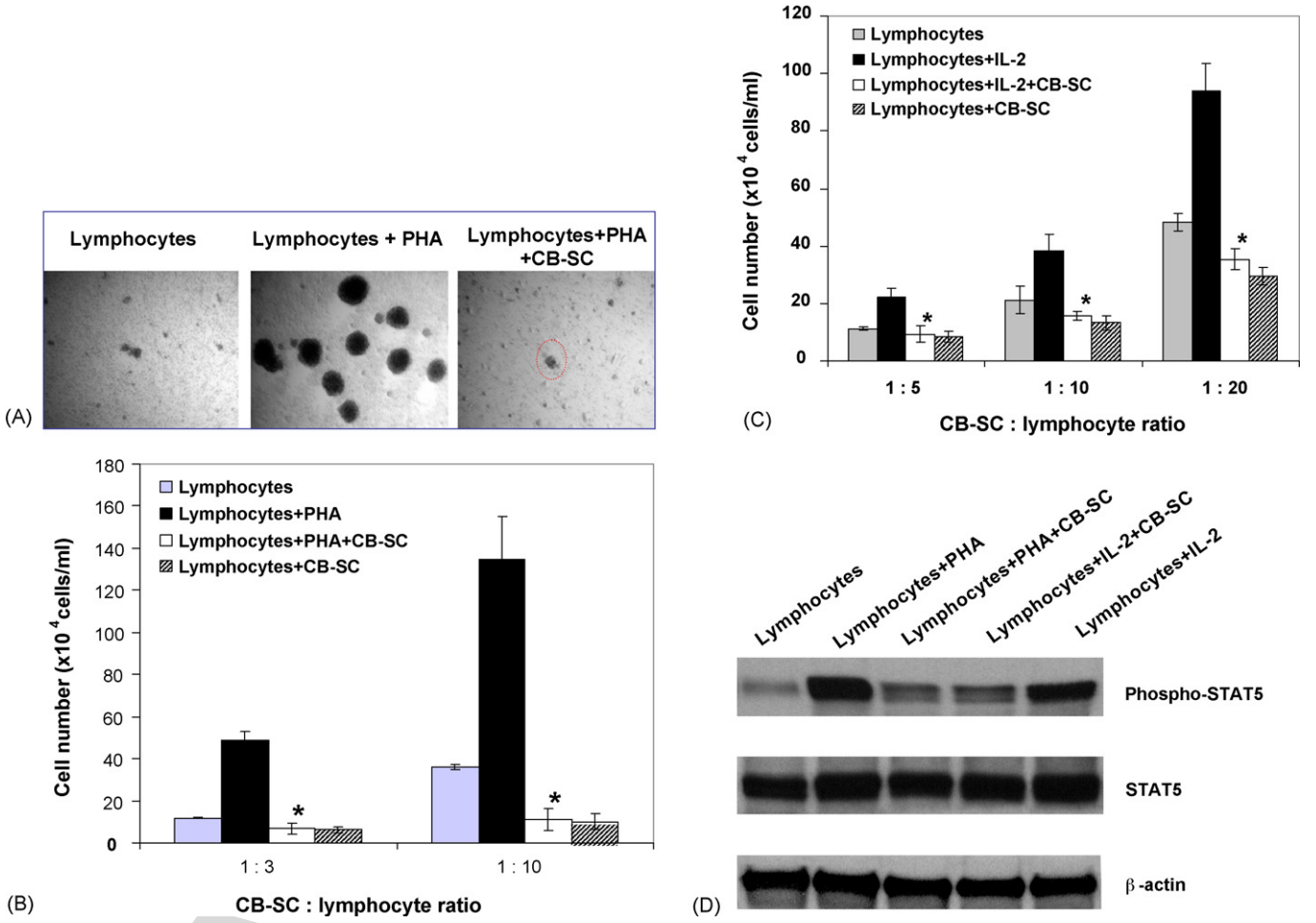


Fig. 1. Inhibitory effects of CB-SC on the lymphocyte proliferation. (A) CB-SC reduced the cell clump formation of PHA-stimulated lymphocytes, showed by phase contrast microscope. PHA-stimulated lymphocytes formed larger cell clumps (middle panel); CB-SC (attached cells) cocultured with PHA-stimulated lymphocytes formed smaller cell clump (marked in red circle, right panel), normal lymphocyte culture served as control (left panel). Original magnification, ×50. (B) CB-SC inhibited the proliferation of PHA-stimulated lymphocytes. Allogeneic lymphocytes were quantified after coculture with CB-SC for 4–5 days at ratios 1:3 and 1:10 of CB-SC:lymphocytes, in the presence or absence of mitogen 10 μg/ml PHA; $^{*}p < 0.01$. Data represent mean (±S.D.) of three experiments. (C) CB-SC inhibited the proliferation of IL-2-stimulated lymphocytes. Allogeneic lymphocytes were quantified after coculture with CB-SC for 4–5 days at ratios 1:5, 1:10, and 1:20 of CB-SC:lymphocytes, in the presence or absence of 500 U/ml IL-2; $^{*}p < 0.05$. Data represent mean (±S.D.) of three experiments. (D) CB-SC inhibited the STAT5 tyrosine phosphorylation in both PHA- and IL-2-stimulated lymphocytes. Allogeneic lymphocytes were collected and lysed for Western blotting after coculture with CB-SC for 40 h at a ratio 1:10 of CB-SC:lymphocytes, in the presence of 10 μg/ml PHA or 500 U/ml IL-2. Western blot was performed using rabbit polyclonal antibodies to phosphorylated STAT5 (phospho-STAT5) or total STAT5a/b. β-Actin served as an internal control. Data represent one of two experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### 2.7. Transwell culture system and blocking experiments

To evaluate the effect of cell–cell contact on the inhibition of lymphocyte proliferation, the sorted T cells were initially seeded in Culture Plate Inserts (0.4 μm pore size, Millipore Corporation, Bedford, MA) in 24-well plates. PHA- or IL-2-stimulated lymphocytes without in transwells served as controls.

To block the action of PD-L1, the functional grade purified anti-human PD-L1 monoclonal antibody was administrated at 20 μg/ml in 0.1% BSA/PBS buffer. The 0.1% BSA/PBS buffer-treated wells served as controls. After incubation with CB-SC at 37 °C for 2 h, cells were washed with PBS to remove the unused antibody. The sorted $CD8^+$ T cells ($1 \times 10^5$ cells/ml/well) were seeded onto the PD-L1 antibody-treated wells in duplicate. To block the action of nitric oxide (NO), $N^{\omega}$-nitro-L-arginine (L-NNA, Sigma) was administrated at a final concentration of 600 μM in culture medium. For double blocking on PD-L1 and nitric oxide, CB-SC were initially pretreated with PD-L1 antibody and then followed by administration of L-NNA (600 μM). After coculture for

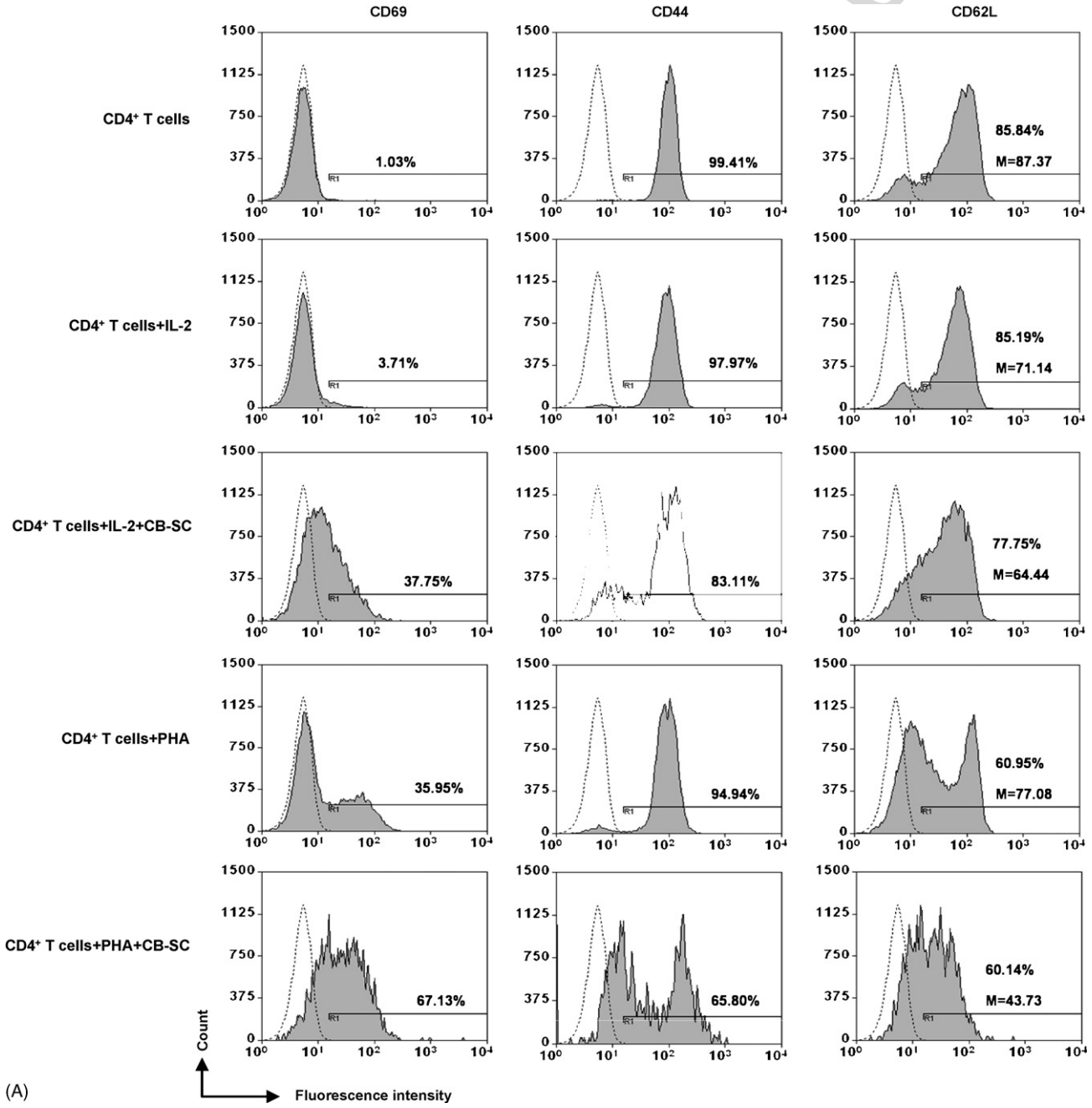


Fig. 2. (A and B) Effects of CB-SC on the sorted $CD4^+$ and $CD8^+$ T cells. The $CD4^+$ and $CD8^+$ T cells were sorted from human peripheral blood mononuclear cells and, respectively, cocultured with CB-SC at a ratio 1:10 of CB-SC:T cells in 24-well plates, in the presence of 500 U/ml IL-2 or 10 μg/ml PHA. After coculture for 2 days, floated T cells were collected for flow analysis using mouse anti-human monoclonal antibodies to CD44, CD62L, and CD69. The isotype-matched IgG served as negative control for quantification of positive cell percentage. Both positive cell percentage and mean fluorescence intensity (M) were displayed to show the difference of CD62L expression among different treatments. Data represent one of three experiments with similar results.

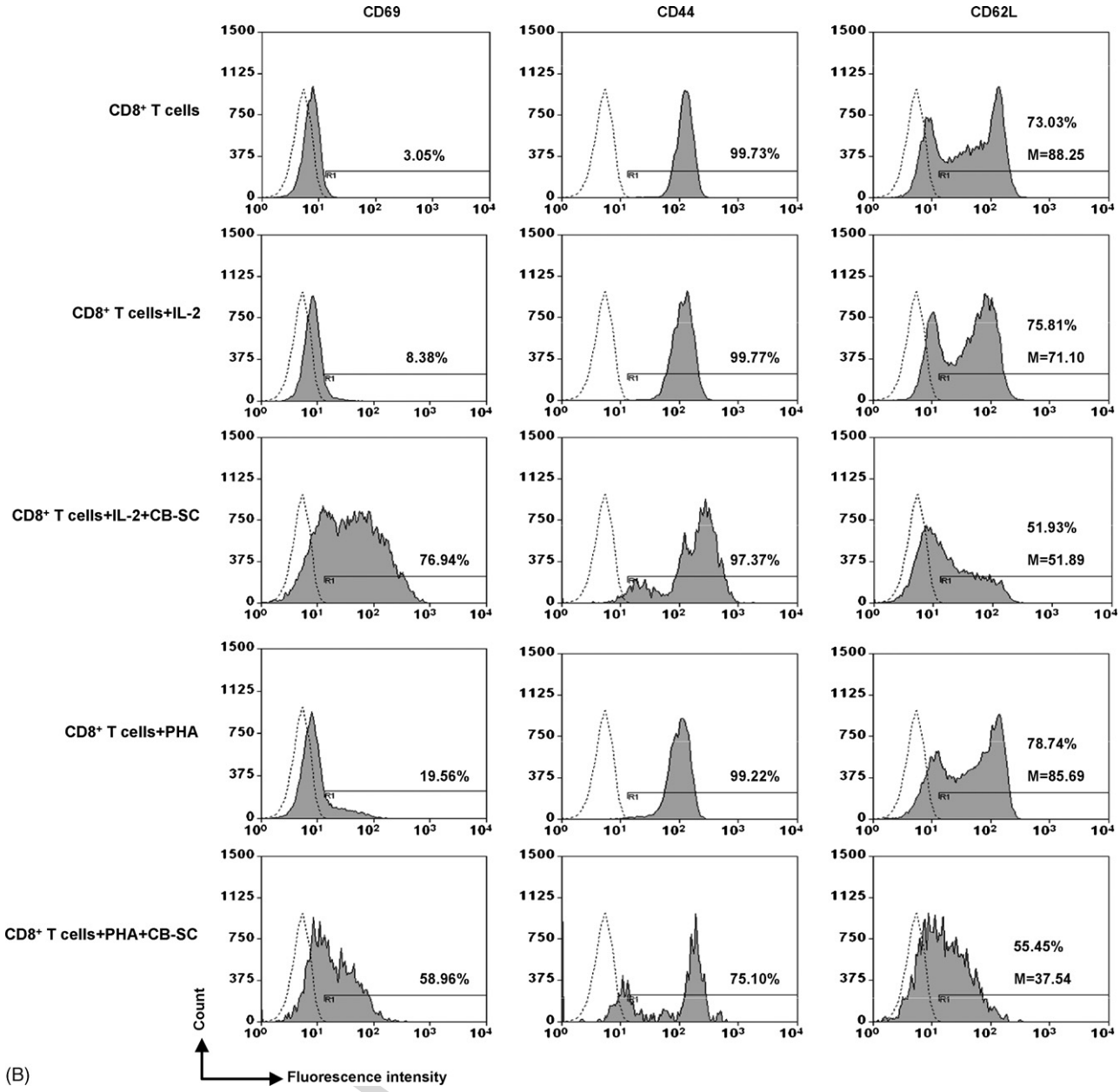


Fig. 2. (*Continued*).

4–5 days, the suspended lymphocytes were collected for cell count.

### 2.8. Statistics

Statistical analyses of data were performed by the Student's *t*-test to determine statistical significance. Values are given as mean ± S.D. (standard deviation).

## 3. Results

### 3.1. Inhibitory effects of CB-SC on lymphocyte proliferation

CB-SC were initially cocultured with mitogen PHA-stimulated lymphocytes at a 1:10 ratio of CB-SC:lymphocytes. Using phase-contrast microscopy, we observed that PHA-stimulated lymphocytes formed numbers of cell clumps of different size in the absence of CB-SC (Fig. 1A, middle panel). However, the number of cell clumps was significantly reduced after coculture with CB-SC; most of lymphocytes were individually distributed in the culture medium and only a few cell clumps of very small size were observed in the supernatant of coculture (Fig. 1A, right panel). We quantified cell number in different groups. Results showed a significant decrease in the PHA-stimulated lymphocyte proliferation by coculture with CB-SC (Fig. 1B, $p < 0.01$).

Next, we evaluated effects of CB-SC on physiological factor IL-2-stimulated lymphocyte proliferation. IL-2 (500 U/ml) as potent stimulator could significantly stimulate lymphocyte proliferation (Fig. 1C); however, this action was also significantly

inhibited after coculture with CB-SC at different ratios (Fig. 1C, $p < 0.05$). It suggests that CB-SC could inhibit the proliferation of both PHA- and IL-2-stimulated lymphocytes. To provide more evidence, we examined the STAT5 phosphorylation, which plays a crucial role in mediating the signal transduction of lymphocyte proliferation [18,19]. After coculture with CB-SC for 40 h, Western blot demonstrated that the tyrosine phosphorylation of STAT5 was significantly reduced in both PHA- and IL-2-stimulated lymphocytes (Fig. 1D). Thus, these results confirmed that CB-SC could inhibit lymphocyte proliferation.

### 3.2. Regulation of CB-SC on $CD4^+$, $CD8^+$ T lymphocytes and CD4/CD8 ratio

To evaluate immune regulation of CB-SC on T cell subsets, CB-SC were initially cocultured with unsorted lymphocytes in the presence of IL-2 or PHA stimulation. Compared with unstimulated lymphocytes, flow analysis showed that PHA stimulation could significantly increase the percentage of $CD8^+$ T cells and decrease $CD4^+$ T cell percentage by more than three-fold, respectively ($p < 0.05$), therefore CD4/CD8 ratio was inverted, but without changing the percentage of $CD4^-CD8^-$ T cells (Table 1); IL-2 stimulation could increase the percentage of $CD4^-CD8^-$ T cells, but failed to affect the percentage of $CD4^+$ and $CD8^+$ T cells, along with CD4/CD8 ratio (Table 1). After coculture with CB-SC, the percentage of $CD4^-CD8^-$ T cells was significantly raised in both IL-2- and PHA-stimulated lymphocytes (Table 1, $p < 0.05$ and $p < 0.01$, respectively); the percentage of $CD8^+$ T-cell in PHA stimulation was reduced to control level by coculture with CB-SC, the decreasing of $CD4^+$ T cell percentage was significantly reversed, and therefore CD4/CD8 ratio was significantly upregulated ($p < 0.05$) (Table 1).

Above results suggest that CB-SC may display regulation on PHA- or IL-2-activated T cell subsets. To further evaluate effects of CB-SC on a single T cell subset, we performed cell sorting and examined effects of CB-SC on the sorted $CD4^+$ (Fig. 2A) and $CD8^+$ (Fig. 2B) T cells, including expression of lymphocyte migration and activation-associated molecules CD44, CD62L, and CD69 [20–22]. Flow analysis showed that both untreated $CD4^+$ and $CD8^+$ T cells displayed negative or background level

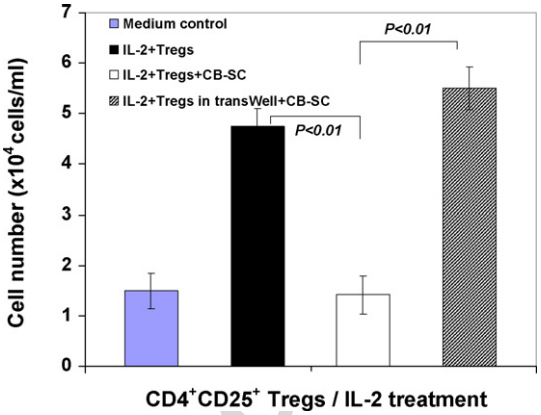


Fig. 3. Inhibitory effects of CB-SC on the sorted $CD4^+CD25^+$ regulatory T cells. The $CD4^+CD25^+$ regulatory T cells were sorted from allogeneic human peripheral blood mononuclear cells and cocultured with CB-SC at a ratio 1:3 of CB-SC:T cells in 24-well plates, in the presence of 500 U/ml IL-2. The $CD4^+CD25^+$ T cells at the same ratio were planted in transwells. For cell sorting, cells were doubly immunostained with FITC-conjugated mouse anti-human CD4 and allophycocyanin (APC)-conjugated mouse anti-human CD25. After coculture for 4–5 days, floated T cells were collected for cell count. Data represent mean (±S.D.) of three experiments.

of CD69, but high levels of CD44 and CD62L (Fig. 2A and B); compared with PHA or IL-2 treatment alone, expression of CD69 was significantly increased after coculture with CB-SC, however, levels of CD44 and CD62L were decreased on both PHA- or IL-2-treated $CD4^+$ and/or $CD8^+$ T cells; these changes were more significant on the PHA-treated T cells than those on the IL-2-treated T cells (Fig. 2A and B). These results indicate that CB-SC display immune regulation on both $CD4^+$ and $CD8^+$ T cell subsets.

### 3.3. Regulation of CB-SC on $CD4^+CD25^+$ regulatory T lymphocytes

Increasing evidence shows $CD4^+CD25^+$ regulatory (Tregs) T cells that play a critical role in regulation of immune responses and homeostasis [23–25]. To evaluate effects of CB-SC on $CD4^+CD25^+$ Tregs, we performed cell sorting and employed the sorted $CD4^+CD25^+$ Tregs from human peripheral blood. Results showed that IL-2 (500 U/ml) could significantly

Table 1
Regulation of CB-SC on CD4 and CD8 T cell subsets

| Treatments | $CD4^-CD8^-$ T cells (%) | $CD4^+$ T cells (%) | $CD8^+$ T cells (%) | CD4/CD8 ratio |
|---|---|---|---|---|
| Lymphocytes | 8.11 ± 3.54 | 65.65 ± 5.26 | 22.85 ± 4.54 | 2.87 ± 0.64 |
| Lymphocytes + IL-2 | 19.09 ± 3.9* | 60.13 ± 7.02 | 20.30 ± 2.85 | 2.96 ± 0.71 |
| Lymphocytes + IL-2 + CB-SC | 41.01 ± 5.3* | 43.02 ± 11.04 | 15.03 ± 5.80 | 2.92 ± 0.28 |
| Lymphocytes + PHA | 6.34 ± 1.42** | 18.35 ± 5.03* | 75.12 ± 2.8** | 0.24 ± 0.08* |
| Lymphocytes + PHA + CB-SC | 30.96 ± 9.67** | 38.76 ± 7.94* | 27.18 ± 2.30** | 1.42 ± 0.48* |
| Lymphocytes + CB-SC | 34.13 ± 15.25 | 45.22 ± 3.64 | 20.19 ± 10.9 | 2.23 ± 0.94 |

After 5-day coculture at a 1:10 ratio of CB-SC:lymphocytes in the presence or absence of stimulators (500 U/ml IL-2 or 10 μg/ml PHA), the suspended lymphocytes were collected for flow analysis using CyAn ADP (DakoCytomation). Lymphocytes only cultured in regular culture medium served as negative control (top row). Lymphocytes cocultured with CB-SC in the absence of PHA served as additional negative control (bottom row). Cells were double stained with FITC-conjugated mouse anti-human CD4 and PE-conjugated mouse anti-human CD8 antibodies. Isotype-matched $IgG_{1\kappa}$ served as negative control. Data represent mean (±S.D.) from three experiments.

* $p$-Value < 0.05.

** $p$-Value < 0.01.

stimulate the proliferation of $CD4^+CD25^+$ Tregs, but was inhibited after coculture with CB-SC (Fig. 3). To evaluate action of cell–cell contacting, CB-SC were cocultured with IL-2-stimulated $CD4^+CD25^+$ Tregs presented in transwell culture system. Results showed that the proliferation of IL-2-stimulated $CD4^+CD25^+$ Tregs was completely reversed after $CD4^+CD25^+$ Tregs were separated from CB-SC (Fig. 3). It suggests that cell–cell contacting plays an important role in the inhibition of proliferation of IL-2-stimulated $CD4^+CD25^+$ Tregs.

### 3.4. Mechanism studies on the inhibitory effects of CB-SC on T lymphocytes

During coculture of CB-SC with IL-2- or PHA-stimulated lymphocytes, cell–cell contacting and/or soluble factors released by CB-SC may contribute to the above inhibitory effects. To further evaluate the action of cell–cell contacting, we initially employed transwell culture system and compared the IL-2-stimulated with PHA-stimulated $CD8^+$ T cells after coculture with CB-SC. Results showed that, without transwell culture systems, both PHA- and IL-2-stimulated T cell proliferations were significantly inhibited after coculture with CB-SC (Fig. 4A); with transwell culture systems, the inhibition on the IL-2-stimulated T cell proliferation was completely overturned, however PHA-stimulated lymphocyte proliferation was only partially reverted (Fig. 4A, $p < 0.05$). These results suggest that cell–cell contacting play a critical role in mediating the inhibitory effects, especially in IL-2 stimulation; there may be other soluble factors involved in PHA stimulation.

Next, to find which potential inhibitors on CB-SC, we examined the expression of surface molecules such as the apoptosis-related CD95 and programmed death receptor-1 ligand 1 (PD-L1) [26–28], along with using human leukocyte common antigen CD45 as positive control [14]. Immunostaining

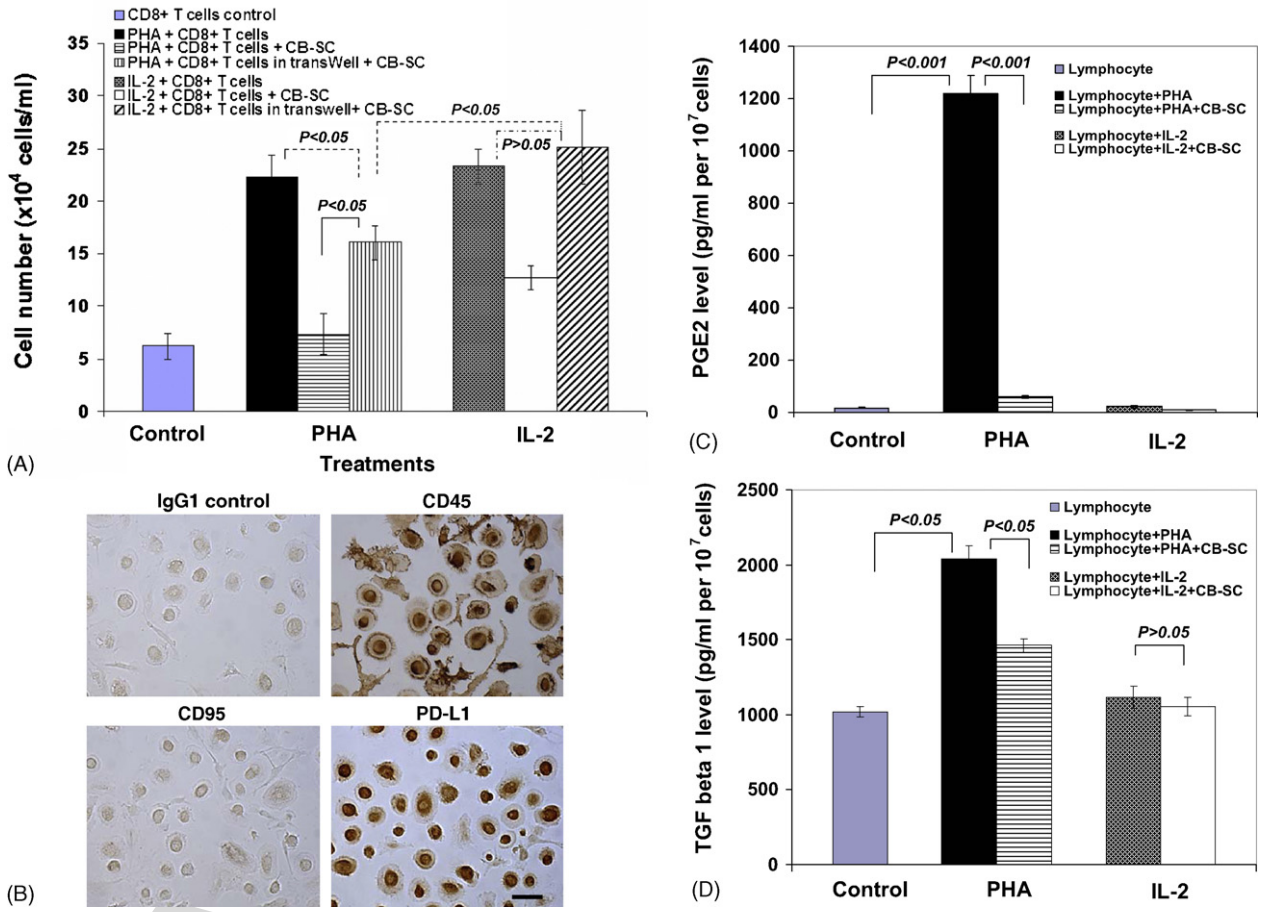


Fig. 4. Mechanism studies for the inhibitory effects of CB-SC on lymphocytes. (A) Comparison of IL-2-treated and PHA-treated $CD8^+$ T cells in transwell culture system. The sorted $CD8^+$ T cells were cocultured with CB-SC at a ratio 1:10 of CB-SC:T cells in 24-well plates with or without transwells, in the presence of 500 U/ml IL-2 or 10 μg/ml PHA. After coculture for 4–5 days, floated T cells were collected for cell count. Data represent mean (±S.D.) of three experiments. (B) Immunostaining for PD-L1 and CD95. CB-SC cultured in 24-well plates were used for immunostaining with mouse anti-human monoclonal antibodies to PD-L1 and CD95. Human leukocyte common antigen CD45 staining served as positive control [14]; isotype-matched $IgG_{1\kappa}$ served as negative control. Scale bar, 35 μm. (C) PGE2 assay in the cocultured supernatants. PGE2 was examined using ELISA kit. (D) TGF-β1 assay in the cocultured supernatants. The activated TGF-β1 level was examined using ELISA kit according the recommended protocol. (E) Nitric oxide (NO) assay in the cocultured supernatants. NO production was determined by using the Griess reaction. (F) Immunostaining for iNOS expression in the PHA-treated CB-SC. CB-SC were treated with 10 μg/ml PHA for 2–3 days and used for immunostaining. Normal rabbit IgG (insert, top left) served as negative control for rabbit anti-iNOS polyclonal antibody. Immunostaining results were obtained from three cord blood preparations and yielded the similar results. Scale bar, 47 μm. (G) Assay for NO production in PHA-treated CB-SC. CB-SC were treated with PHA at different doses for 3–5 days. The supernatants were collected for NO examination using Griess reaction. Results represent mean (±S.D.) of three experiments.

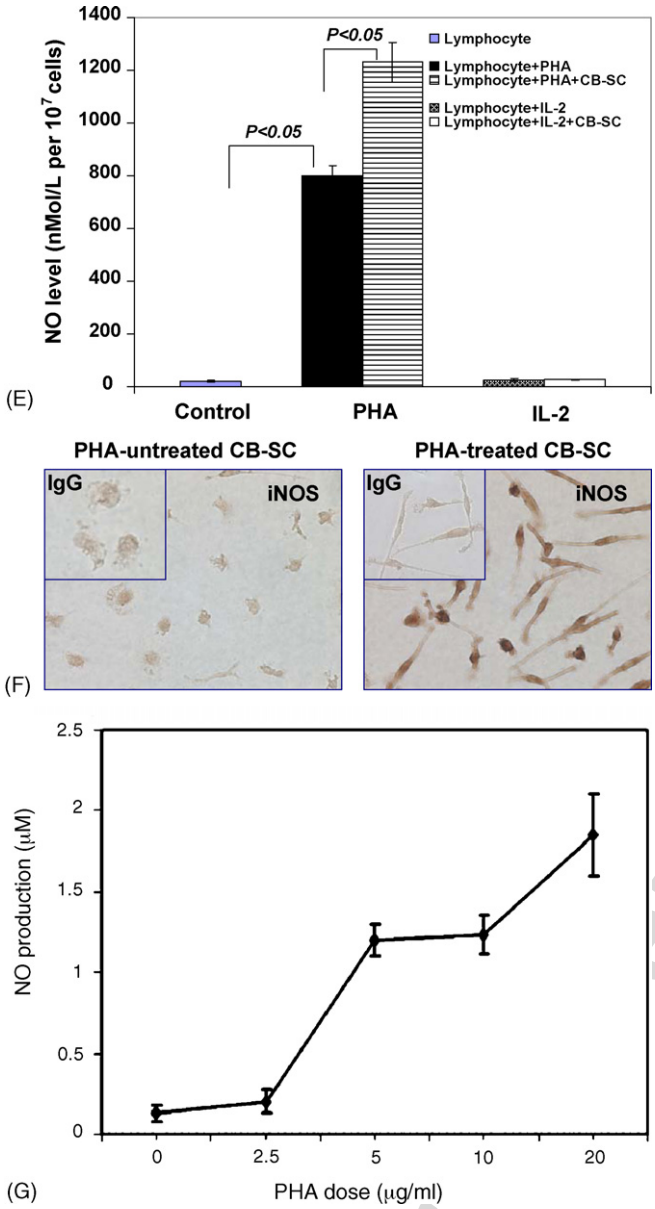


Fig. 4. (*Continued*).

showed that around 70% of CB-SC displayed PD-L1, but lacked expression of CD95 (Fig. 4B). It suggests that PD-L1 may contribute to the inhibitory action of CB-SC on lymphocytes.

To find which soluble factors contributed to the inhibitory effects, we examined levels of potential candidates [29–32] such as prostaglandin E2 (PGE2), transforming growth factor-β1 (TGF-β1), and nitric oxide (NO) in the supernatant of IL-2- or PHA-stimulated lymphocytes after coculture with CB-SC. Results showed that PHA significantly stimulated the production of PGE2 (Fig. 4C), TGF-β1 (Fig. 4D), and nitric oxide (Fig. 4E) ($p < 0.001$ for PGE2 and $p < 0.05$ for the latter two); while IL-2 stimulation only showed background levels as untreated lymphocyte controls (Fig. 4C–E) ($p > 0.05$). These results, together with above transwell cultures, suggest that these soluble factors may not contribute to the CB-SC-induced suppression on IL-2-stimulated lymphocyte proliferation; therefore, we focused on

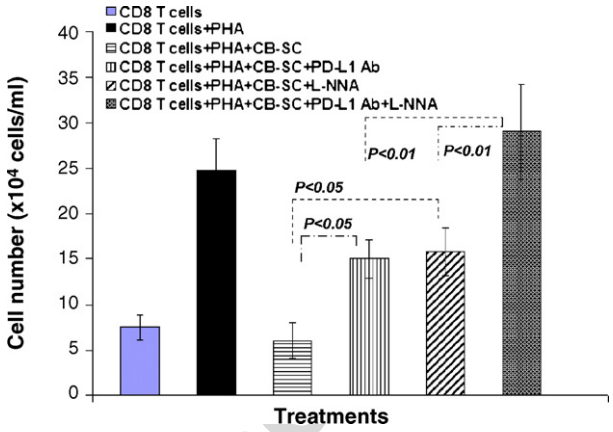


Fig. 5. Blocking experiments with an iNOS inhibitor L-NNA and a neutralizing antibody for PD-L1. Prior to administration of 600 μM L-NNA, CB-SC were incubated with an antibody to PD-L1 (20 μg/ml) for 2 h at 37 °C to neutralize the PD-L1. The sorted $CD8^+$ T cells were cocultured with CB-SC at a ratio 1:10 of CB-SC:T cells in 24-well plates, in the presence of 10 μg/ml PHA. After coculture for 4–5 days, floated T cells were collected for cell count. Data represent mean (±S.D.) of three experiments.

PHA stimulation for further mechanism studies. Compared with PHA stimulation alone, the levels of PGE2 and TGF-β1 were significantly decreased (Fig. 4C and D) ($p < 0.001$ and $p < 0.05$, respectively), but NO production was considerably increased after coculture with CB-SC (Fig. 4E) ($p < 0.05$). The data suggest that NO may represent an important soluble factor contributing to the inhibitory effects of CB-SC on lymphocyte proliferation.

To test the action of NO, we examined whether CB-SC expressed inducible nitric oxide synthase (iNOS) and produced NO in the presence of PHA. Immunostaining results demonstrated that CB-SC increased the level of iNOS expression after treatment with PHA (Fig. 4F, right panel); PHA-untreated CB-SC showed background level of iNOS (Fig. 4F, left panel). Using Griess reaction [17], we examined NO production. Results showed that PHA-treated CB-SC produced NO in a dose-dependent manner (Fig. 4G).

Finally, to evaluate actions of NO and surface molecule PD-L1 during coculture inhibition, we performed blocking experiments by administrating a specific iNOS inhibitor L-NNA [17] for NO production and a neutralizing antibody for PD-L1 [26,33]. Using the sorted $CD8^+$ T cells as example, results showed that single blocking with LNNA or PD-L1 neutralizing antibody could partially (about 30%) reverse the inhibition of CB-SC on the proliferation of $CD8^+$ T cells (Fig. 5); notably, L-NNA in combination with PD-L1 neutralizing antibody could completely block the inhibition of CB-SC on the proliferation of $CD8^+$ T cells (Fig. 5, $p < 0.05$). The data demonstrated that soluble factor NO and membrane molecule PD-L1 mainly contributed to the inhibitory effects of CB-SC on lymphocytes.

## 4. Discussion

Human autoimmune disease, for example, type 1 diabetes is a disease in which the pancreatic islet beta-cells are selectively destroyed by auto-reactive T lymphocytes [2]. Stem cell-based therapy may provide a potential approach for beta

cell-replacement therapy [1,2]. However, these T cells may also attack the stem cell-derived insulin-producing cells. Therefore, it is important to protect the stem cells and/or stem cell-derived cells from immune attack post-transplantation, without need for long-term immunosuppressive drug therapies. How to develop a strategy to control T lymphocytes and induce a state of immune tolerance? Firstly, we need to realize that their common pathophysiological mechanisms include: the recruitment of lymphocytes to grafted cells and followed by expansion of lymphocytes. Current works showed that CB-SC could significantly inhibit both PHA- and IL-2-stimulated lymphocyte proliferations, as further confirmed by reduction of phosphorylation of STAT5. Importantly, flow analysis demonstrated that lymphocyte migration-associated adhesion molecules CD44 and CD62L [20,21] were also decreased after coculture with CB-SC. These results suggest that CB-SC has potential to suppress the lymphocyte-caused inflammation. In addition, we evaluated CD69 expression and found its significant upregulation on both PHA- and IL-2-stimulated $CD4^+$ and $CD8^+$ T cells after coculture with CB-SC. It is important to note that CD69 has been previously regarded as an activation marker of lymphocytes [34,35]; until recently, studies in CD69-deficient mice have revealed that CD69 might act as negative regulator and self-control molecule during lymphocyte activation [22]. Therefore, coculture with CB-SC may promote the self-limitation of activated T cells through upregulation of CD69 expression.

As an important effector cell, $CD8^+$ T cell plays a critical role in immune surveillance of human body [36]. However, increasing evidence demonstrates that $CD8^+$ T cells also contribute to the initiation and progression of several autoimmune diseases, such as type 1 diabetes, systemic lupus erythematosus (SLE), multiple sclerosis (MS), and aplastic anemia [37,38]. These $CD8^+$ T cells may attack stem cells or stem cell-derived cells post-transplantation. Therefore, targeting on $CD8^+$ T cells may prevent autoimmune diseases. Current studies demonstrated that percentage of $CD8^+$ T cells have been significantly reduced in PHA-simulated lymphocytes after coculture with CB-SC; CD69 expression have been significantly increased on both PHA- and IL-2-activated $CD8^+$ T cells; additionally, the proliferation of both PHA- and/or IL-2-activated $CD8^+$ T cells were inhibited after coculture with CB-SC. These results prove that CB-SC can display the negative regulation on $CD8^+$ T cells. In addition to $CD8^+$ T cell subset, current studies demonstrate that CB-SC also inhibited the proliferation of IL-2-stimulated $CD4^+CD25^+$ regulatory T cells and regulated the phenotypes of $CD4^+$ T cells.

Mesenchymal stem cells (MSC) are a rare population residing in postnatal tissues and organs, such as the bone marrow, adipose tissue, fetal liver or spleen. They are negative for leukocyte common antigen CD45 and therefore different from CB-SC [14]. Recently, MSC have shown unique immunological properties, including failure to stimulate alloreactivity and suppress activation of T cells both *in vitro* and *in vivo* [31,39–43]. Mechanism studies reveal that TGF-β1 and PGE2 may partially contribute to the MSC-mediated suppression on lymphocytes [31,39]. Differently, current works showed that levels of TGF-β1 and PGE2 produced by PHA-stimulated lymphocytes were remarkably reduced after coculture with CB-SC, but NO production was increased. For IL-2 stimulation, these three soluble factors showed no significant differences in comparison with control cell cultures. Therefore, we have focused on PHA stimulation and further investigated the mechanism. To evaluate NO action, NO production in CB-SC was confirmed by expression of iNOS and PHA dose response. Importantly, blocking NO production alone with a specific inducible nitric oxide synthase (iNOS) inhibitor L-NNA could significantly restore the CB-SC-induced suppression of T cell proliferation, which is consistent with previous reports about the inhibitory effect of NO on lymphocyte proliferation [32,44–46]. Additionally, immunostaining showed that CB-SC expressed programmed death receptor-1 ligand 1 (PD-L1), which may be ligated by programmed death receptor-1 (PD-1) and decrease TCR-mediated lymphocyte proliferation [28]. To test this action, we performed blocking experiments by neutralization of PD-L1 with its antibody. The data showed that neutralization of PD-L1 could partially restore the CB-SC-induced suppression of T cell proliferation; notably, combination of L-NNA with a neutralizing antibody to PD-L1 could abrogate the CB-SC-induced suppression of T cell proliferation. These results indicate that NO and PD-L1 mainly contribute to the CB-SC-induced suppression.

In summary, cord blood-derived stem cells CB-SC produced immune suppressions on lymphocyte proliferation and T cell subsets. CB-SC may function not only as stem cells giving rise to different cell lineages, but also as immunomodulatory cells protecting themselves. These findings may lead to develop new therapeutics to treat human disease.

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