

Suppressive effects of intravenous immunoglobulin (IVIG) on human umbilical cord blood immune cells

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We investigated *in vitro* immunomodulatory effects of intravenous immunoglobulin (IVIG) on cord blood mononuclear cells (CBMNC), macrophages, dendritic cells and CD3⁺ T cells were isolated from umbilical venous blood. Cell proliferation used ³H-TdR incorporation, culture supernatants were assayed for cytokines using ELISA, and surface marker expressions were determined by flow cytometry. IVIG suppressed CBMNCs and CD3⁺ T-cells proliferation, secretions of IL-10, INF- γ and TGF- β_1 , but not IL-4, and PHA-induced expressions of surface molecules (CD25, CD45RA and CD45RO), with more pronounced effects for CBMNCs. IVIG decreased cord blood (CB) macrophage phagocytosis and CD14, HLA-DR and CD86 expressions. IVIG increased CD14 expression and decreased MCH II expression for differentiation-stage CB dendritic cells (DCs) and increased CD14 expression and decreased CD80 and CD83 expressions of mature DCs, suggesting that IVIG intervention inhibited DC differentiation and maturation. In addition to T cells, IVIG immunomodulatory effects on CBMNCs involve a variety of cells and molecules. CB macrophages and CBMNC-DCs are targets of IVIG.

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Intravenous immunoglobulin (IVIG) is a blood product that is comprised of pooled IgG immunoglobulins extracted from plasma that was mainly used as replacement therapy for immune deficiencies in the late 1970s. Since the early 1980s, however, it has been widely used for treating various immune-related diseases, including acute infection, and inflammatory and autoimmune diseases. Thus, its immunomodulatory role has received increasing attention.

In recent years, IVIG has been used extensively during the perinatal and neonatal periods (1, 2). A number of studies have confirmed the immunomodulatory effects of IVIG on various segments of the immune system, including T cells, natural killer cells, dendritic cells, cytokine production by mononuclear cells, leukocyte

recruitment and complement activity (3–8). However, most studies have used adults' cells and reports on the effects of IVIG on immune functions in neonates are very limited. Neonates are a special population whose immune systems are not mature, and the phenotypes and functions of immune cells in neonates are different from those in adults. Therefore, it is necessary to study the effects of IVIG on immune functions and the corresponding mechanisms in neonates to guide the clinical applications for IVIG during the perinatal and neonatal periods.

The objectives of this study were to investigate the modulating effects of IVIG on cord blood mononuclear cells (CBMNCs), cord blood macrophages and cord blood dendritic cells to understand the effects of IVIG on different

cellular components of neonates' immune systems. To explore additional immunomodulatory mechanisms, the effects of IVIG on PHA-induced DNA synthesis by CBMNCs and CD3⁺ T cells were compared, including the effects of IL-2 and IL-4.

Methods

Cord blood samples

Cord blood samples were collected in heparinized tubes from the placental-end umbilical veins of eight randomly selected full-term newborns at the time of delivery that were born in the delivery room of our hospital. They had normal parturitions, an average fetal age of 40 wk and normal Apgar scores. The mothers had been healthy during pregnancy and had no history of specific diseases. This study was approved by the institutional review board of our hospital, and all the mothers of the participant newborns gave written informed consent.

Cord blood mononuclear cells (CBMNC) isolation and proliferation

CBMNCs were isolated by density gradient centrifugation using lymphocyte separation medium (8). Neonatal umbilical cord blood (30–50 ml with heparin anticoagulation) was collected and centrifuged at 3000 rev/min for 10 min. Plasma in the upper layer was collected and was inactivated in a 56°C water bath for 30 min. The sample was placed on ice for 10 min and was centrifuged at 3000 rev/min for 10 min. The fibrin deposits were discarded, and the supernatant was pipetted into a 1.5 ml Eppendorf tube. The sample was centrifuged at 13,200 rev/min for 2 min, and serum in the upper layer was collected for later use. After serum removal, the cells were diluted at a ratio of 1:4 in PBS. Ficoll-Hypaque (Histopaque-1077, Sigma, Chemical Co., St Louis, MO, USA) was used to separate the mononuclear cells using density gradient centrifugation. The cells were washed $\times 3$ with PBS, after which RPMI 1640 medium containing autologous serum was used to adjust the cell suspension to a concentration of 2×10^6 /ml. The cells were pipetted into a 6-well plastic culture plate and were cultured at 37°C in 5% CO₂ for 2 h. Finally, the samples were washed gently using PBS to remove the non-adherent cells. Cord blood monocytes (CBMC) were obtained for other experiments.

Intravenous immunoglobulin (IVIG) was prepared using human IVIG powder (Batch No.

2008060201-02; Shanghai Biological Product Institute, Shanghai, China). The purity of IVIG was determined using an acetate membrane electrophoresis method according to the 'Chinese Pharmacopoeia'; the purity of IVIG reached 100%. As a check on the toxicity of the IVIG preparation, a quantitative LAL assay was used to determine the endotoxin levels, which were 0.91 EU/ml. Mononuclear cells cultured with and without 0.91 EU/ml of endotoxin showed no differences in cytotoxic effects (data not shown). Freeze-dried IVIG powder was dissolved in RPMI 1640 before the experiments and filtered by positive-pressure filtration. The final concentration of IVIG was 10 mg/ml.

CBMNCs (2×10^6 /ml) were pipetted into wells of 96-well plates. An equal volume of either prepared human IVIG (experimental group) or RPMI 1640 (control group) was also added to the wells. To determine the effects of cytokines on cell proliferation, some cultures were supplemented with either IL-2 (100 U/ml, Takeda Co., Japan) or IL 4 (10 ng/ml, Gibco). Phytohemagglutinin (PHA, 25 μ g/ml, Sigma, USA) was used to stimulate cells for duplicate wells per experimental condition. The cells were cultured for 72 h. ³H-TdR (China Academy of Sciences, Shanghai Institute of Nuclear Research) was added 6 h before the end of the culture (1 μ Ci of 3.7×10^4 Bq/well). The cells were harvested, and a YJS-80 liquid scintillation counter (China Academy of Sciences, Shanghai Institute of Nuclear Research) was used to measure decay counts per minute (cpm).

Comparative effects of IVIG on CBMNCs and CD3⁺ T cells

After CBMNCs were isolated from the cord blood samples, CD3⁺ T lymphocytes in the CBMNCs were purified using a magnetized anti-CD3 antibody (Miltenyi Biotec, Germany). The CBMNC and CD3⁺ T lymphocytes were washed with RPMI-1640 culture medium and resuspended to 10^6 cells/ml. Purified CD3⁺ T lymphocytes were stained with trypan blue to ensure that cell viability was >95%.

The isolated CBMNCs or CD3⁺ T lymphocytes were pipetted into 6-well plates. Each cell type was divided into four groups for different combinations of PHA or freeze-dried human IVIG (10 mg/ml; Shanghai Biological Product Institute) as follows. (i) Control group: RPMI-1640 medium; (ii) PHA stimulation only group; (iii) Early activation group: PHA was added at first, and then IVIG was added after 24 h of culture; (iv) PHA + IVIG group: PHA and IVIG were added simultaneously. The cells were

cultured at 37°C in 5% CO₂ for 72 h, after which the cells were centrifuged and collected for evaluation of cell surface markers and supernatants were collected for the determinations of cytokines.

ELISA for cytokines in culture supernatants

Assays for cytokines secreted by CBMNCs and CD3⁺ T cells used commercial ELISA kits for IL-10, TGF-β₁, IL-4, and IFN-γ (PharMingen, San Diego, CA, USA) following the respective manufacturer's instructions.

Cell surface markers

CBMNCs and purified CD3⁺ T lymphocytes were suspended in PBS and their concentrations were adjusted to 5 × 10⁶/ml. Cell suspensions (100 μl) were mixed with 10 μl of the following conjugated monoclonal antibodies: (i) allophycocyanin (APC)-labeled mouse anti-human CD4 (Becton Dickinson, USA), (ii) phycoerythrin (PE)-labeled rat anti-human CD25 (Caltag Laboratories, USA), (iii) fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD45RA, (iv) phycoerythrin (PE)-labeled CD45RO (Immunotech, France), and (v) magnetized anti CD3⁺ antibody (Miltenyi Biotec, Germany). Samples were well mixed and incubated on ice for 30 min avoiding light, after which samples were washed ×3 with PBS. Control antibodies were fluorescence-labeled isotype-matched rat or rabbit immunoglobulins. Surface molecule analysis was performed for 10,000 cells per label using a flow cytometry (FACSCalibur, Becton Dickinson, USA). The proportions of positive cells were calculated using CellQuest software.

Cord blood macrophage phagocytosis and surface molecule expressions

An anticoagulant (EDTA-Na₂) was added to neonatal cord blood, and the sample was diluted by half using DMEM medium (Gibco, USA). Cells were washed ×3 using DMEM, and the concentration was adjusted to 2 × 10⁶ cells/ml. The cells were pipetted into 6-well culture plates and cultured at 37°C with 5% CO₂ for 2 h. The culture wells were rinsed ×3 with DMEM to eliminate non-adherent cells. The monocytes/macrophages that had adhered to the culture wells were scraped with a rubber scraper and were resuspended in DMEM. The cells were pipetted into 6-well culture plates at a concentration of 2 × 10⁶ cells/well and were

divided into a control group and an IVIG group. Only DMEM medium was added to the control group. Freeze-dried human IVIG (Shanghai Biological Product Institute; 10 mg/ml in DMEM) was added to the IVIG group. The cells were cultured at 37°C with 5% CO₂ for 72 h, and the cells were collected by centrifugation. The proportion of CD14⁺ cells was confirmed to be not less than 80% by flow cytometry.

The phagocytosis of the monocytes/macrophages was determined using the following methods. The treated monocytes/macrophages were rinsed with PBS and resuspended to a concentration of 1 × 10⁶/ml. From this, 150 μl of the cell suspension was added to 150 μl of fluorescein isothiocyanate-labeled dextran (FITC-dextran; molecular weight 40,000, concentration = 1 mg/ml; Sigma, USA). As a negative control, another 150 μl cell suspension was added to 150 μl PBS. These cell suspensions were incubated at 37°C with 5% CO₂ for 1 h, and then the cells were washed ×4 with PBS and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA). Ten thousand cells were evaluated for each sample for the proportion of FITC-dextran-positive cells and the relative fluorescence intensity (RFI). The proportion of FITC-dextran-positive cells and RFI were calculated using Cell Quest software.

For surface molecule analysis, the concentration of the cultured monocytes/macrophages was adjusted to 1 × 10⁶/ml, and then cultured under three different conditions: (i) Sensitized group, GM-CSF (50 ng/ml) was added and the samples were cultured for 96 h; (ii) Activation group, GM-CSF was added and the samples were cultured for 24 h before LPS (1 μg/ml) was added, and then the samples were cultured for an additional 72 h; (iii) Early inhibition group, GM-CSF was added and the samples were cultured for 24 h before IVIG was added, and then the samples were cultured for another 24 h before LPS (1 μg/ml) was added. These samples were cultured for an additional 48 h. Two 100 μl aliquots of each cell suspension were collected and mixed with either 10 μl of FITC-CD14 and PE-CD86 or 10 μl of PE-CD14 and FITC-HLA-DR. The samples were incubated on ice for 30 min avoiding light, and then washed ×3 using PBS. The surface molecules were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA). Ten thousand cells were evaluated for each sample for the proportion of positive cells, and RFI was determined using Cell Quest software. RFI was determined by: [MFI (mean fluorescence

intensity) of the stained group]/[MFI of the unstained group] $\times$ 100.

Induction culture for CBMNC-dendritic cells (DCs) and their surface molecule expressions

Isolated CBMNCs were cultured in RPMI-1640 with 10% calf serum in a plastic culture dish. Different combinations of rhGM-CSF (Pepro- tech, 50 ng/ml), rhIL-4 (Pepro- tech, 10 ng/ml), LPS (serum type 055:B5, 1 μ g/ml, Sigma, USA) and dissolved freeze-dried human IVIG (20 mg/ml; Shanghai Institute of Biological Products, Shanghai, China) were added for four different groups: (i) 4G group: rhGM-CSF and rhIL-4 were added on the same day of cell isolation; (ii) 4G + LPS group: rhGM-CSF and rhIL-4 were added on the same day of cell isolation, and LPS was added after 5 days of culture; (iii) 4G + IVIG group: rhGM-CSF, rhIL-4 and IVIG were added on the same day of cell isolation; (iv) 4G + IVIG + LPS group: rhGM-CSF, rhIL-4 and IVIG were added on the same day of cell isolation, and LPS was added after 5 days of culture. LPS was used to induce DCs to enter into the maturation stage. Cells were cultured at 37°C with 5% CO₂. Cell morphology was observed using an inverted microscope during culture, the medium was changed every 3 days, and the cells were collected for examination after 7 days.

The concentration of the CBMNC-DCs was adjusted to 1×10^6 /ml after PBS washing and re-suspension. Then, 100 μ l of cell suspension was added to 4 μ l of the following mAbs (BD PharMingen, CA, USA): differentiation markers

FITC-CD14, PE-CD11c, phycoerythrin cyanine5 conjugate-labeled CD1a (PE-Cy5, PC5-CD1a); co-stimulatory molecule markers FITC-CD80, FITC-CD86; DC maturation marker FITC-CD83; and FITC-MHC II (including HLA-DR, HLA-DP and HLA-DQ). The samples were mixed and incubated for 15 min avoiding light and then washed $\times 3$ using PBS. Control antibodies were FITC-, PE- and PC5-labeled isotype rat immunoglobulins. The surface molecules were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA). Ten thousand cells were evaluated for each sample.

Statistical analysis

Results are given as means $\pm$ s.d. Comparisons between two groups used independent two-sample *t*-tests. For more than two groups, one-way analysis of variance (ANOVA) was used. If a significant difference was detected among group means, multiple comparisons of means were performed using the Bonferroni procedure with type-I error adjustment. Statistical analyses used SPSS 15.0 statistics software (SPSS Inc, Chicago, IL, USA). All statistical assessments were two-sided and the significance level α was set at 0.05.

Results

IVIG suppresses CBMNC proliferation, with or without PHA stimulation

The effects of IVIG on the proliferation of CBMNCs are shown in Fig. 1. As indicated by the incorporation of ³H-TdR, the IVIG

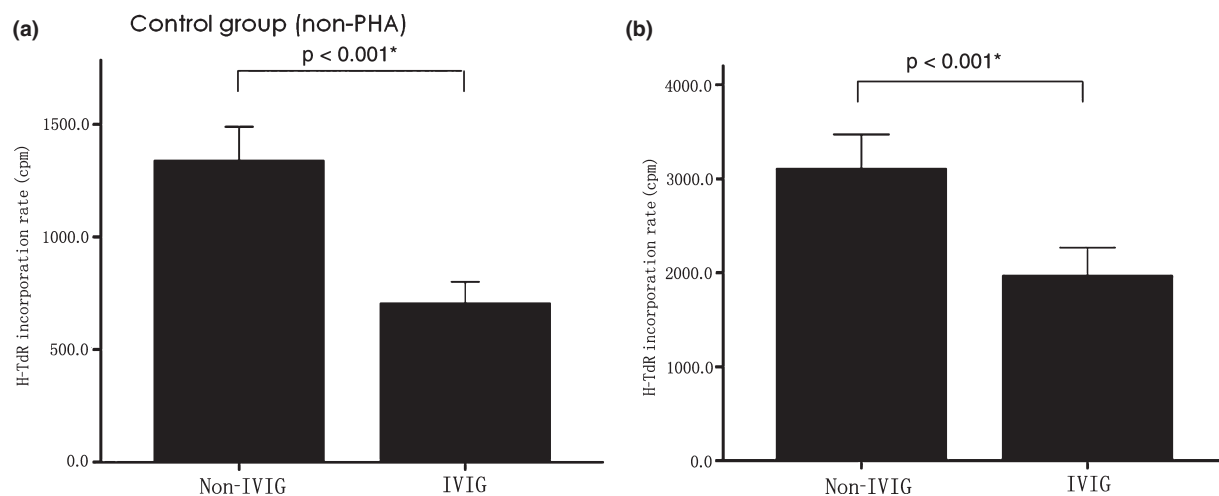
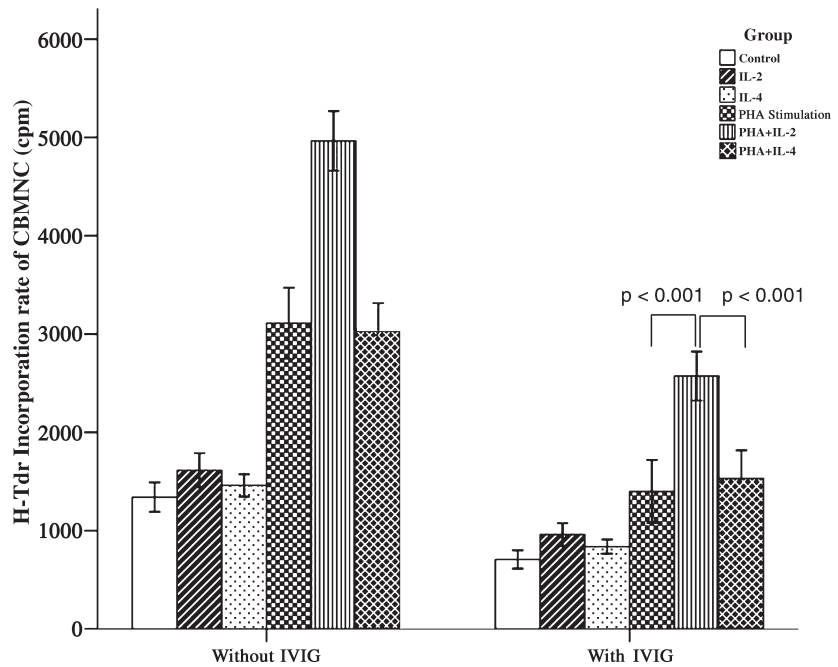


Fig. 1. Effects of IVIG on the proliferation of cord blood mononuclear cells (CBMNCs). CBMNCs were either cultured without (non-IVIG) or with IVIG. ³H-TdR uptake was determined (a) without PHA stimulation or (b) with PHA stimulation. Results are means $\pm$ s.d. Group comparisons were by *t*-test.

Fig. 2. Effects of IL-2 and IL-4 on IVIG-induced inhibition of DNA synthesis of cord blood mononuclear cells (CBMNCs). CBMNCs were cultured with or without IVIG and either no additional supplements (control), with IL-2 supplementation or IL-4 supplementation. ^3H -Tdr uptake was determined with or without PHA stimulation. Results are means $\pm$ s.d. Group comparisons were by one-way ANOVA, followed by pair-wise multiple comparisons between groups using Bonferroni's test with $\alpha = 0.001$ adjustment.



treatment significantly decreased the proliferation (DNA synthesis) of CBMNCs in conditions both with and without PHA stimulation (both $p < 0.001$).

IL-2, but not IL-4, ameliorates IVIG-induced DNA synthesis inhibition of CBMNCs

Fig. 2 shows the effects of co-stimulation with either IL-2 or IL-4 on the IVIG-induced DNA synthesis inhibition of CBMNCs for non-PHA and PHA conditions. Without PHA stimulation, neither IL-2 nor IL-4 caused any significant differences in the IVIG-induced DNA synthesis inhibition of CBMNCs. In contrast, with PHA stimulation, IL-2, but not IL-4, significantly enhanced the proliferation of CBMNCs in the presence of IVIG-induced suppression ($p < 0.001$).

Differential effects of IVIG on cytokine secretion by CBMNCs

Fig. 3 shows the effects of IVIG on the secretions of IL-4, IL-10, IFN- γ and TGF- β_1 by CBMNCs for four different culture conditions (see Methods). There was no significant difference in IL-4 secretion among the four groups (Fig. 3a). However, there were significant differences in the secretions of IL-10 (Fig. 3b), IFN- γ (Fig. 3c) and TGF- β_1 (Fig. 3d) by CBMNCs among the four groups: the control group, the PHA-induced group, the early activation group and the PHA + IVIG group ($p < 0.05$).

For the PHA-induced group (PHA stimulation only), significantly increased levels of IL-10,

INF- γ and TGF- β_1 were found compared to the control group (all $p < 0.001$). For the early activation group, the addition of IVIG after 24 h of PHA stimulation significantly decreased the IL-10, INF- γ and TGF- β_1 secretions by CBMNCs (all $p < 0.001$). For the PHA + IVIG group (PHA and IVIG added simultaneously), IVIG also markedly reduced IL-10, INF- γ and TGF- β_1 secretions of CBMNCs after PHA stimulation (all $p < 0.001$). The time after PHA stimulation that IVIG was added (either simultaneously with PHA or 24 h later) did not affect its suppressive effects on the PHA-induced IL-10, INF- γ and TGF- β_1 secretions by CBMNCs.

Differential effects of IVIG on CBMNC expressions of CD25, CD45RA and CD45RO

Fig. 4 shows the effects of IVIG on CBMNC cell surface expressions of CD25 (Fig. 4a), CD45RA (Fig. 4b) and CD45RO (Fig. 4c) for the four groups with or without PHA and IVIG treatments. For the control group, the percentages of CD25 $^+$, CD45RA $^+$ and CD45RO $^+$ expressions were $6.68 \pm 0.81\%$, $88.70 \pm 2.86\%$ and $13.96 \pm 0.56\%$, respectively. After PHA stimulation for 72 h, CD25 $^+$ increased to $77.51 \pm 2.54\%$, CD45RA $^+$ was $96.90 \pm 1.91\%$ and CD45RO $^+$ expression increased to $74.04 \pm 2.15\%$. The percentages of CD25 $^+$, CD45RA $^+$ and CD45RO $^+$ expressions after PHA stimulation were significantly higher than for the control group ($p < 0.001$).

Compared with the PHA-inducing group, decreased expressions of CD25 $^+$ ($7.79 \pm 1.14\%$),

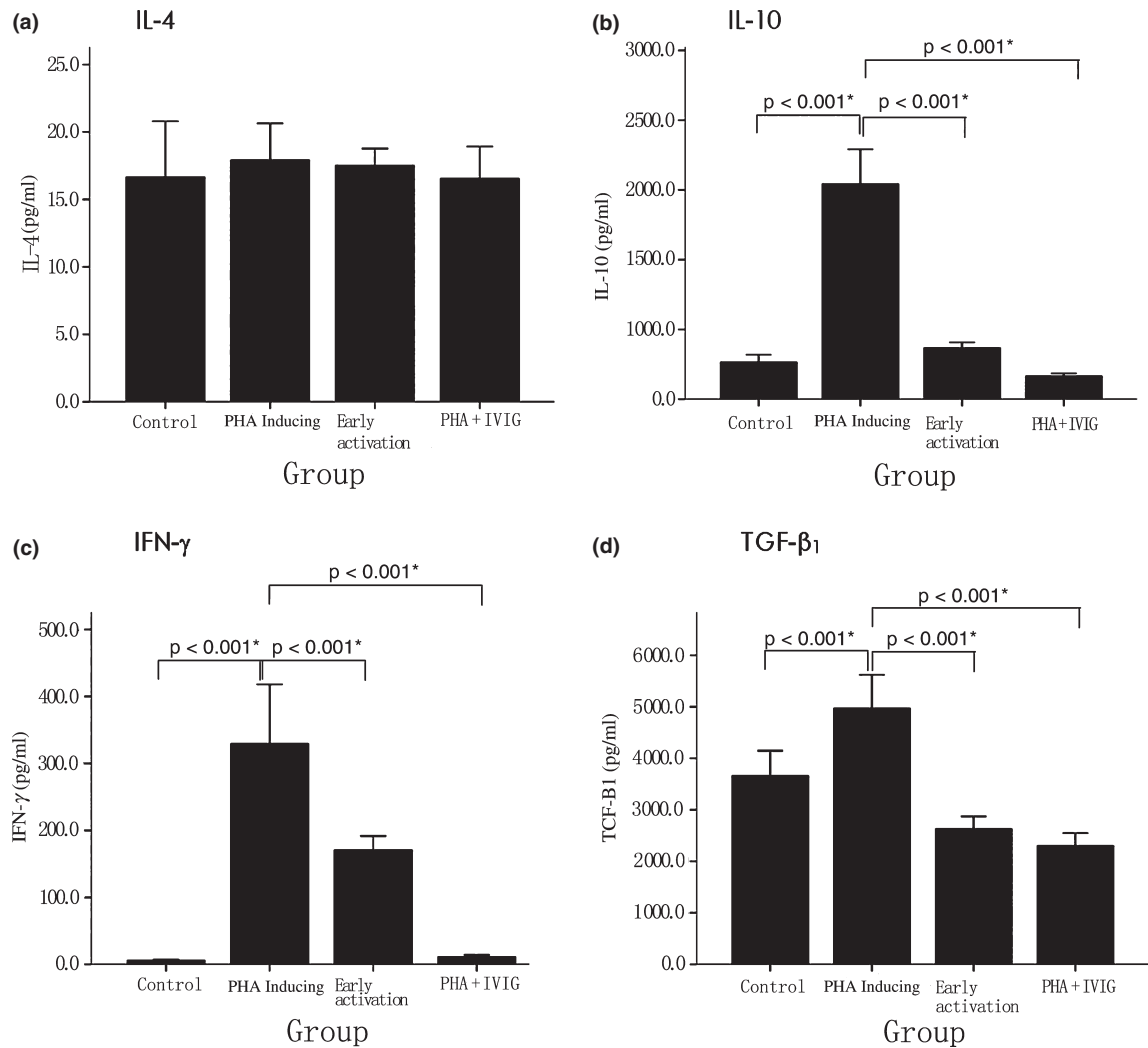


Fig. 3. Effects of IVIG on cytokine secretions by cord blood mononuclear cells (CBMNCs). CBMNCs were cultured using four different conditions (see Methods) followed by ELISA of culture supernatants for (a) IL-4, (b) IL-10, (c) IFN- γ and (d) TGF- β . Results are means $\pm$ s.d. Group comparisons were by one-way ANOVA, followed by pair-wise multiple comparisons between groups using Bonferroni's test with $\alpha = 0.017$ adjustment.

CD45RA⁺ ($84.74 \pm 2.45\%$) and CD45RO⁺ ($19.74 \pm 3.59\%$) were observed in the IVIG + PHA group (all $p < 0.001$). This suggested that the addition of IVIG inhibited the PHA-induced expressions of these cell surface markers on CBMNCs. However, for the early activation group (IVIG 24 h after PHA initiation), only decreased expressions of CD45RA⁺ ($70.65 \pm 2.83\%$) and CD45RO⁺ ($63.72 \pm 4.44\%$) were observed (both $p < 0.001$). IVIG may not suppress the CD25 expression of activated CBMNCs.

Inhibitory effects of IVIG are more pronounced for CBMNCs than for CD3⁺T cells

The CBMNC isolates were comprised of a number of immune cell subpopulations, and

IVIG appears to suppress some of their functions, possibly T-cell-specific functions. Table 1 compares CBMNCs and CD3⁺ T cells isolated from the CBMNCs for IVIG-induced effects on PHA-induced cytokine secretions and expressions of CD25⁺, CD45RA⁺ and CD45RO⁺ (results in Table 1 are %inhibitory effects; % inhibition is the IVIG-induced percentage change in the cytokine secretion or marker expression). There were significant differences in the inhibitory effects of IVIG on the PHA-induced secretions of IL-10, IFN- γ and TGF- β ₁ between CBMNCs and CD3⁺T cells (all $p < 0.001$). For the PHA-induced expressions of CD25⁺, CD45RA⁺ and CD45RO⁺, the inhibitory effects of IVIG on the CBMNCs and CD3⁺T cells were statistically different. With IVIG, the %inhibition of CD25⁺, CD45RA⁺ and CD45RO⁺

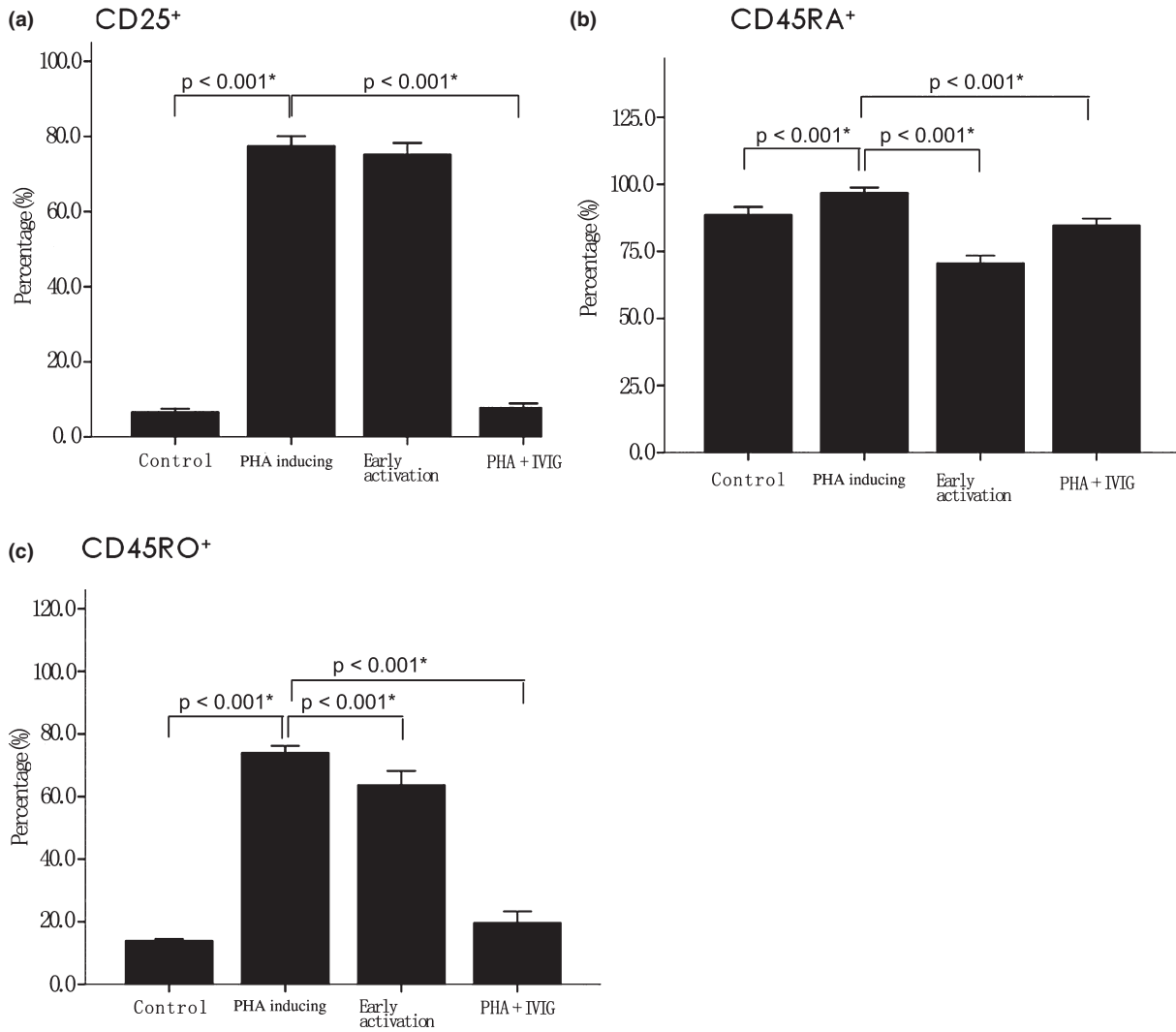


Fig. 4. Effects of IVIG on cell surface marker expressions on cord blood mononuclear cells (CBMNCs). CBMNCs were cultured using four different conditions (see Methods) followed by flow cytometry analysis for (a) CD25⁺, (b) CD45RA⁺ and (c) CD45RO⁺. Results are means $\pm$ s.d. Group comparisons were by one-way ANOVA, followed by pair-wise multiple comparisons between groups using Bonferroni's test with $\alpha = 0.017$ adjustment.

Table 1. Comparisons of IVIG inhibitory effects on PHA-induced cytokine secretions and marker expressions for cord blood mononuclear cells (CBMNCs) and CD3⁺ T cells

% Inhibitory effects of IVIG	CBMNCs (n = 8)	CD3 ⁺ T cells (n = 8)	p-values§
Cytokine secretions			
IL-4 (%)	7.03 $\pm$ 4.04	5.33 $\pm$ 3.45	0.754
IL-10 (%)	89.11 $\pm$ 0.56	77.78 $\pm$ 1.01	<0.001
IFN- γ (%)	96.56 $\pm$ 0.09	75.95 $\pm$ 1.21	<0.001
TGF- β ₁ (%)	53.52 $\pm$ 1.05	17.99 $\pm$ 0.98	<0.001
Expressions			
CD25 ⁺ (%)	89.98 $\pm$ 0.43	35.90 $\pm$ 0.36	<0.001
CD45RA ⁺ (%)	12.55 $\pm$ 0.60	1.18 $\pm$ 0.52	<0.001
CD45RO ⁺ (%)	73.27 $\pm$ 1.84	54.44 $\pm$ 1.93	<0.001

§p-values from independent two-sample *t*-tests comparing CBMNCs and CD3⁺T cells.

expressions for CD3⁺ T cells were lower than those in CBMNCs (all $p < 0.001$).

Variable effects of IVIG on surface molecule expressions for CBMNC-DCs (dendritic cells)

Effects of IVIG on expressions of surface molecules for CBMNC-DCs are shown in Table 2. Using different induction conditions (see Methods), there were significant differences in the expressions of CD14, CD1a, CD80, CD86, CD83 and MHCII among the 4G, 4G + LPS, 4G + IVIG and 4G + IVIG + LPS groups ($p < 0.05$). Significant differences for CD80 and CD83 were found between the 4G and 4G + LPS groups ($p < 0.017$). Significant

Table 2. Effects of IVIG on the expressions of surface molecules by cord blood mononuclear cells (CBMNC)-DCs

Expression (%)	Groups†				p-value*
	4G (n = 8)	4G + LPS (n = 8)	4G + IVIG (n = 8)	4G + IVIG + LPS (n = 8)	
CD14	9.28 ± 0.72	8.89 ± 0.87	21.46 ± 2.97 ^c	16.90 ± 0.77 ^d	<0.001 ^a
CD11c	91.58 ± 2.32	92.23 ± 3.11	90.64 ± 3.43	88.74 ± 1.90	0.823
CD1a	18.88 ± 1.56	17.43 ± 1.78	11.66 ± 1.39	14.76 ± 1.67	0.019 ^a
CD80	11.48 ± 1.14	19.59 ± 1.47 ^b	6.86 ± 0.85	8.54 ± 1.06 ^d	<0.001 ^a
CD86	19.53 ± 2.08	32.90 ± 4.04	23.05 ± 3.59	28.61 ± 3.19	0.038 ^a
CD83	17.15 ± 1.17	28.40 ± 2.29 ^b	9.49 ± 0.83	17.11 ± 1.62 ^d	<0.001 ^a
MHCII	88.35 ± 3.50	92.53 ± 1.67	70.55 ± 2.89 ^c	75.29 ± 2.55 ^d	<0.001 ^a

*p-values from one-way anova among the four groups.

^aSignificant difference among the four groups, $p < 0.05$.

^bSignificant difference between 4G and 4G + LPS, $p < 0.017$.

^cSignificant difference between 4G and 4G + IVIG, $p < 0.017$.

^dSignificant difference between 4G + LPS and 4G + IVIG + LPS, $p < 0.017$.

Pair-wise multiple comparisons between groups were determined using Bonferroni's test with $\alpha = 0.017$.

†4G group: rhGM-CSF and rhIL-4 were added on the same day of cell isolation; 4G + LPS group: rhGM-CSF and rhIL-4 were added on the same day of cell isolation, and LPS was added after 5 days of culture; 4G + IVIG group: rhGM-CSF, rhIL-4 and IVIG were added on the same day of cell isolation; 4G + IVIG + LPS group: rhGM-CSF, rhIL-4 and IVIG were added on the same day of cell isolation and LPS was added after 5 days of culture.

differences for CD14 and MHCII were found between the 4G and 4G + IVIG groups ($p < 0.017$), suggesting that IVIG up-regulated the CD14 expression and decreased the MHCII expression in CBMNC-DCs during the differentiation stage (without LPS). When the CBMCs were cultured into immature DC, CD14 expression on the cell surface will be decreased significantly. IVIG intervention up-regulated the CD14 expression of the immature DC, suggesting that IVIG intervention inhibited the DC differentiation. There were also significant differences for the expressions of CD14, CD80, CD83 and MHCII between the 4G + LPS and 4G + IVIG + LPS groups ($p < 0.017$). These results suggest that large-dose IVIG increased the CD14 expression in the mature DCs and altered the responses of mononuclear cells to IL-4 and GM-CSF by partially inhibiting the differentiation of CBMCs into DCs and the maturation of DCs. In addition, IVIG only inhibited the expressions of CD80 and CD83 for matured CBMNC-DCs, but not for CBMNC-DCs during the differentiation stage.

IVIG inhibits phagocytosis and surface marker expressions of cord blood macrophages

The effects of IVIG on the phagocytosis capability of cord blood macrophages are shown in Fig. 5. Compared to the control group, the addition of IVIG inhibited the phagocytosis of cord blood macrophages as shown by FITC-dextran ingestion by macrophages. Effects of IVIG on the expressions of CD14, HLA-DR and CD86 for the cord blood macrophages are shown

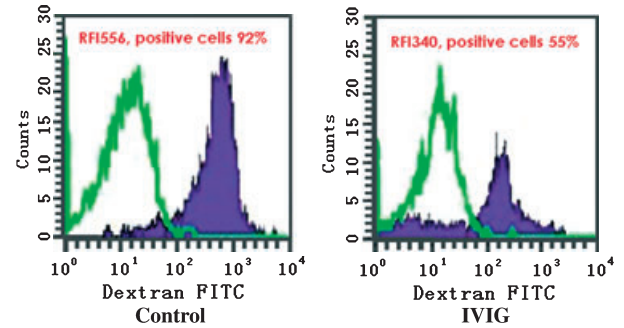


Fig. 5. Effects of IVIG on the phagocytosis of cord blood macrophages. Cord blood macrophages were cultured either with IVIG or without IVIG (control) along with fluorescein isothiocyanate (FITC)-dextran (see Methods). Flow cytometry was used to determine the amount of phagocytosed fluorescein isothiocyanate (FITC)-dextran. Relative fluorescence intensity (RFI) is described in Methods.

in Fig. 6. Compared with the sensitized group and the activation group, addition of IVIG in the early inhibition group inhibited the expressions of CD14, HLA-DR and CD86 on the surfaces of the cord blood macrophages.

Discussion

The effects of IVIG on immune cells from adults have been studied extensively, but research on the effects of IVIG on immune cells during the perinatal and neonatal periods is very limited. Our study found that IVIG treatment resulted in a number of in vitro suppressive effects on CBMNCs in general, including inhibition of proliferation, decreased secretions of IL-10, INF- γ and TGF- β_1 , but not IL-4, and decreased

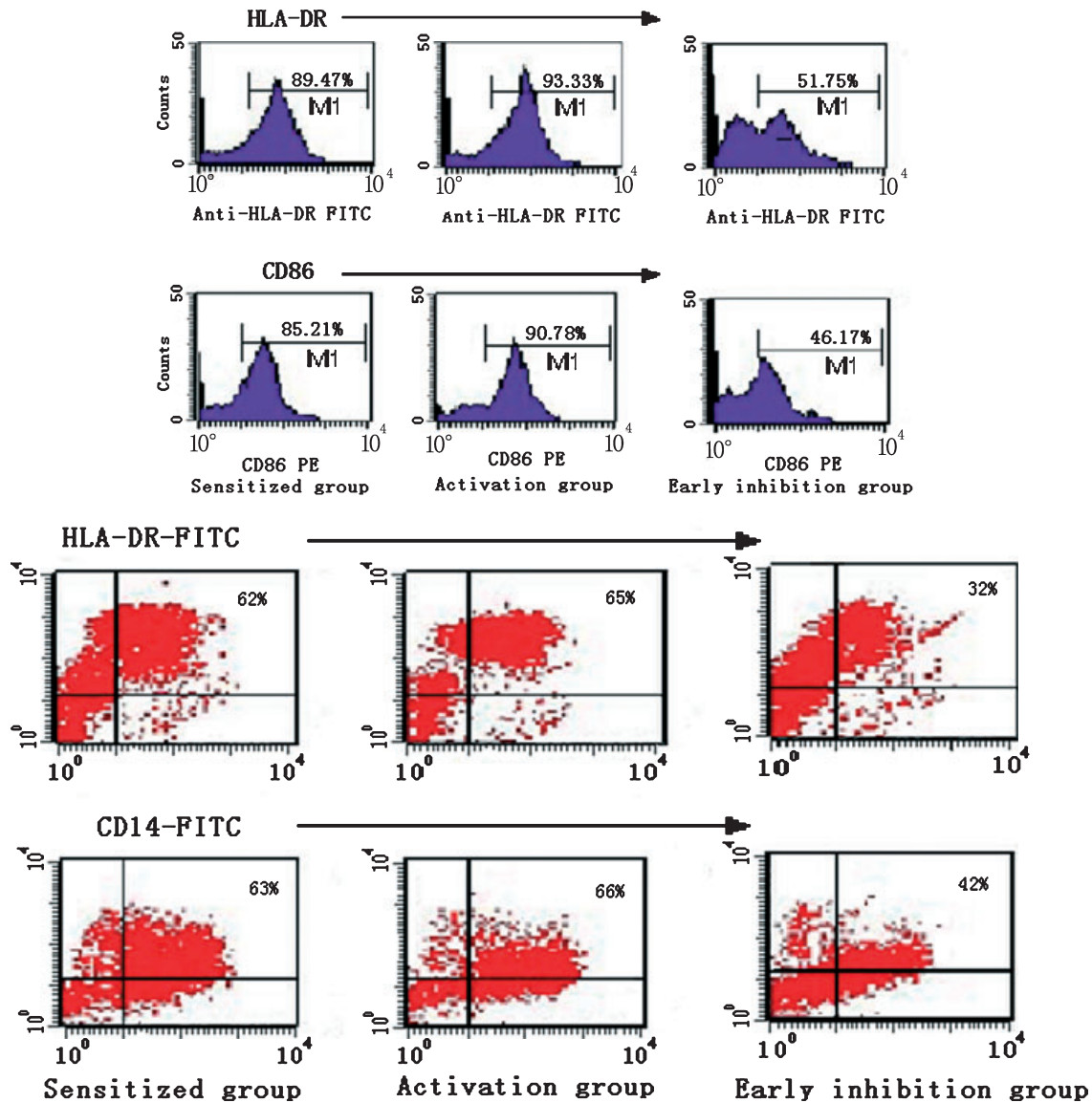


Fig. 6. Effects of IVIG on the expressions of CD14, HLA-DR and CD86 on the surfaces of cord blood macrophages. Sensitized group: GM-CSF 50 ng/ml 96 h; Activation: GM-CSF 24 h and LPS 1 μ g/ml 72 h; Early inhibition group: GM-CSF 24 h, IVIG 24 h and LPS 48 h. Relative fluorescence intensity (RFI) is described in Methods.

PHA-induced expressions of surface molecules. We also evaluated IVIG effects on selected cord blood cellular components, including CD3⁺T cells, macrophages and dendritic cells that were both in a differentiation stage and a mature stage. The major suppressive effects noted for IVIG appeared to have been because of the 'activation state' of the particular cell components. In terms of the IVIG dose, our study showed that a physiological dose of IVIG (10 mg/ml) caused suppressive effects on CBMNC proliferation, cytokine secretions of CBMNCs, expressions of CD25, CD45RA, CD45RO and CD3⁺T cells and macrophages. However, a physiological dose of IVIG (10 mg/

ml) did not cause any significant suppressive effects on DC (data not shown). The suppressive effects of IVIG on DC appeared when a large dose (20 mg/ml) was used. We found for CD3⁺T cells that IVIG inhibited the PHA-induced secretions of IL-10, IFN- γ , TGF- β ₁, but not IL-4, and the expressions of CD25⁺, CD45RA⁺ and CD45RO⁺ for these cells. However, the degree of IVIG inhibition was significantly less for CD3⁺T cells than for CBMNCs in general for both cytokine secretion and surface marker expression. These results do suggest that IVIG induced suppressive effects on the cytokine secretions of other non-T lymphocytes among CBMNCs, possibly monocytes or dendritic cells.

These results might also indicate that the inhibitory effects of IVIG on the activation of cord blood T lymphocytes could be mediated by other non-T immune cells or immune molecules, as the degrees of inhibition for CD25⁺, CD45RA⁺ and CD45RO⁺ on CD3⁺T cells were significantly lower than for CBMNCs.

Increased CD25 expression (α chain of the IL-2 receptor) on the surfaces of T lymphocytes is a hallmark of T-lymphocyte activation. The IVIG-induced decrease in CD25 molecules indicates that IVIG could cause suppression of immune function by inhibiting the activation of cord blood T lymphocytes. As the receptor for IL-2, bound CD25 can transfer activation signals into cells, which results in their activation and proliferation (3). Thus, we can speculate that the inhibitory effects of IVIG on CBMNC proliferation are related to the inhibited activation of CBMNCs.

Along with the inhibition of CD25 expression, the expression of CD45RO⁺ on the cell surface was also inhibited, and the conversion of CD4⁺CD45RA⁺ cells to CD4⁺CD45RO⁺ cells among the CBMNCs was prevented; this may suppress the release of immunoglobulins by B lymphocytes (9, 10). It is noteworthy that IVIG did not induce suppressive effects on the CD25 expressions of activated CBMNCs. Rather, only the expressions of CD45RO⁺ and CD45RA⁺ were inhibited for CBMNCs that had already been activated.

It has been reported that IVIG may inhibit the phagocytosis of adults' monocytes/macrophages (11). Our study results on cord blood cells are consistent with these findings for cells from adults. We found that IVIG decreased the phagocytosis of cord blood macrophages and reduced the expressions of CD14, HLA-DR and CD86 on the surfaces of cord blood macrophages. Again, IVIG effects were noted for an 'activated' cell component.

It has also been shown in previous studies that IVIG can inhibit the differentiation, maturation and secretions of cytokines by dendritic cells from adults (12), and thereby indirectly inhibit the activation and proliferation of T lymphocytes (13). Our results also showed alterations of dendritic cells' cell surface marker expressions by IVIG, although the results were dependent upon the maturation state of DCs. In immature CBMNC-DCs, IVIG intervention up-regulated the expression of the differentiation marker CD14. When the CBMCs were cultured into immature DC, CD14 expression on the cell surface will be decreased significantly. Therefore, this result suggests that IVIG intervention

inhibited the DC differentiation. For mature (LPS-induced) CBMNC-DCs, IVIG treatment resulted in significantly up-regulated cell surface expressions of the CD14 differentiation marker. For two other CBMNC-DC differentiation-related surface molecules, there was a suppressive effect on the CD1a (MHC I-like) differentiation marker, but no apparent effects on CD11c expression in both mature and immature CBMNC-DCs. By comparison, the suppressive effects of IVIG on the expressions of co-stimulatory molecules, CD80 and CD83, were only observed for matured CBMNC-DCs, but not for CBMNC-DCs at the differentiation stage. These results suggest that IVIG can interfere with the differentiation of CBMC into DC, the maturation of DC and the co-stimulatory functions of mature DCs.

This is given further support by our observations that IVIG suppressed the expressions of the DC maturation marker, CD83 and MHC II, which is intimately involved with antigen-presentation functions to T cells. Dendritic cells are professional antigen-presenting cells that provide potent stimulation of naïve T cells. Thus, they play a central role for the initiation of primary immune responses. Our results demonstrated that CBMNC-DCs are one of the targets for the immunomodulatory effects of IVIG.

Besides a direct inhibition on cord blood T lymphocytes, IVIG may also inhibit the expressions of surface molecules and antigen-presentation functions of cord blood monocytes/macrophages and the cytokine secretions induced by monocyte/macrophage LPS activation. This could result in inhibiting the activation of and decreased killing ability of T cells. The immunomodulatory effects of IVIG on the immune system are very complex. The research on the effects of IVIG for adults' immune cells is more extensive, and the mechanisms have been more thoroughly studied. The immune systems of neonates are immature, and their immune responses may be different from those of adults. The inhibitory effects of IVIG on the immune cells of neonates are of clinical significance, which further clarifies the mechanisms of the effects of IVIG on neonatal immune-related disease. IVIG plays an important role for maintaining immune homeostasis, and clinicians should pay close attention to its immunosuppressive effects for non-immune-associated neonatal diseases.

The pros and cons for IVIG applications in neonates should be weighed after taking into account the therapeutic effects of IVIG and the immunosuppressive effects of IVIG. Therefore,

an in-depth study on the mechanisms of the immunomodulatory effects of IVIG during the neonatal period may provide a useful guideline for the clinical application of IVIG in neonates. However, this study is an *in vitro* study only, and cord blood rather than peripheral blood cells of neonates were used. This limits any generalizations of these research findings for neonates *in vivo*, and warrants further clinical research.

In conclusion, IVIG inhibits the proliferation, the secretions of IL-10, INF- γ and TGF- β_1 , and the PHA-induced expressions of surface molecules by CBMNCs *in vitro*. The immunomodulatory effects of IVIG on CBMNCs involve a variety of immune system cells and molecules; the effects on T cells may arise indirectly from the inhibitory effects on non-T cells. It is also confirmed that cord blood macrophages and CBMNC-DCs are targets of IVIG.

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