

Multi-nucleated giant cell formation from human cord blood monocytes *in vitro*, in comparison with adult peripheral blood monocytes

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Introduction

Human neonates are susceptible to infections [1,2], especially by microorganism pathogens, which are capable of surviving intracellularly in phagocytes (e.g. mycobacterium [3], listeria [4], herpes simplex virus [5], cytomegalovirus and toxoplasma [6]). Neonates sometimes develop a serious systemic infection with rapid progress from these pathogens. The immune reactions to these pathogens are depressed in neonates; in particular, poor mobilization of phagocytes (neutrophils and macrophages) and decreased production of interferon (IFN)- γ by CD4⁺ T lymphocytes have been well established [6,7].

Multi-nucleated giant cells (MGCs; Langhans-type cells), formed from macrophage fusion, are recognized widely as a cellular feature of chronic inflammatory disorder. MGCs were first described by Langhans in tuberculoid lesions, and are found to be present in a variety of granulomatous conditions including infection by the above-listed pathogens,

Summary

Multi-nucleated giant cells (MGCs; Langhans-type cell), formed from macrophage fusion, are recognized as a hallmark histological feature in chronic inflammation. However, their precise pathological role is still poorly understood, especially for microorganism pathogens in the neonatal immune system, which are capable of surviving intracellularly in phagocytes. To conduct a partial evaluation of the monocyte function of neonates, we investigated the ability of human cord blood monocytes to form MGCs *in vitro* by stimulating various cytokines and comparing them with adult peripheral blood monocytes. Monocytes from cord blood and adult peripheral blood were isolated and cultured for 14 days with cytokines known to induce MGC *in vitro*. The fusion index in experiments with a combination of interleukin (IL)-4 and macrophage colony-stimulating factor (M-CSF) and a combination of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) was significantly lower in cord blood than in adult blood monocytes ($P = 0.0018$ and $P = 0.0141$, respectively). The number of nuclei per MGC was significantly lower in cord blood than in adult blood monocytes in experiments with IL-4 alone, the combination of IL-4 and M-CSF, and the combination of IL-4 and GM-CSF ($P < 0.0001$). These results suggest the possibility that the susceptibility of newborns to mycobacterium infection is due partly to impaired MGC formation.

Keywords: cord blood, interleukin-4, M-CSF, monocyte, multi-nucleated giant cell (Langhans-type cell)

sarcoidosis, rheumatoid arthritis, neoplasm and the foreign body reaction by the host defence mechanism. *In vitro* MGCs formation was described first by Black in 1976 [8], and various cytokines including macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4 and interferon (IFN)- γ have been found to induce MGCs formation *in vitro* [9–13]. In addition, several molecules have been found to be related to MGC formation [14–27]. Although the molecular mechanism of MGC formation has been gradually elucidated, the pathophysiological actions of MGC and their precise roles in immune responses are still poorly understood. Furthermore, these findings are from experiments established in animals or human adults, and there is little information on MGC in human neonates [28,29]. The granuloma structure (including MGCs) and their protective function against mycobacterial infections have been discussed in current studies [30–33]; histopathological findings in congenital tuberculosis patients have been studied and few

granulomatous lesions or MGCs were shown in their lesions [34–36].

We speculated that the reason why human neonates are susceptible to the pathogens listed above is partly because of the decreased ability of MGCs formation. In this study, we investigated the ability of human cord blood monocytes to form MGCs *in vitro* by stimulation of the cytokines listed above. We attempted to evaluate partly the monocyte function of neonates and to compare the results with those obtained using adult peripheral monocytes.

Materials and methods

Reagents

RPMI-1640 and phosphate-buffered saline (PBS) were from GIBCO (Grand Island, NY, USA). The human cytokines (IL-4, M-CSF, GM-CSF, IFN- γ and TNF- α) were from Wako Pure Chemical Industries (Osaka, Japan).

Blood samples

Adult peripheral blood was obtained from healthy volunteers, in accordance with the principles of the Declaration of Helsinki. Umbilical cord blood was obtained from healthy term neonates immediately after delivery. Parental informed consent was obtained for every donor, and the examination protocol of peripheral blood samples was in accordance with the guidelines of the Institutional Review Board of Okayama University Hospital. Both types of blood samples were collected in heparinized syringes and processed within 3 h.

Monocyte isolation and cell culture

Adult peripheral blood and cord blood mononuclear cells were isolated by Histopaque (Sigma, St Louis, MO, USA) density gradient centrifugation. The monocytes were isolated by negative selection with monoclonal antibody (mAb)-coated immunological magnetic beads and a cell sorter (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) after centrifugation. Purity, assessed by detection of reactive oxygen species with a fluorescence probe (hydroxyphenyl fluorescein) stimulated with 10 ng/ml phormol myristate acetate (PMA) and saturated with anti-CD14 mAb (IOM2; Immunotech; Marseille, France) by flow cytometer, were both more than 95%. The monocytes were cultured at 37°C and a 5% CO₂ atmosphere in condition medium consisting of RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin at 1×10^6 cells/ml/well in 24-well tissue culture plates (Sumitomo Bakelite, Tokyo, Japan) with various cytokines. The cells were maintained for 14 days without changing the medium. All culturing

procedures were performed under sterile conditions, and each experiment was performed with at least seven independent samples.

Cell staining and evaluation of MGCs formation

After 14 days' incubation, the culture plates were gently rinsed twice with PBS and dried well, and the cells then stained with a May – Grünwald–Giemsa stain (Merck Ltd, Darmstadt, Germany). Digital pictures at low power (10 \times objectives) were taken with a microscope (Keyence, Osaka, Japan) under low voltage (LV) light for the quantification of MGC. The fusion rate of monocytes was determined by counting the number of nuclei within MGC (>3 nuclei/cell) in randomly acquired areas per total number of nuclei in the same areas (at least 500 nuclei): fusion rate (%) = (number of nuclei within MGC/total number of nuclei \times 100). The number of nuclei per MGC (20 cells acquired randomly in each experiment) was also evaluated.

Collection of supernatants and analysis of cytokines

After 14 days' incubation without change of culture medium, the supernatants were collected and centrifuged at 500 g for 5 min to remove particulate debris, then stored at –30°C. IL-1 β was measured by a two-site-directed ELISA, with an exclusion limit of 10 pg/ml (Pierce Biotech, Rockford, IL, USA). Tumour necrosis factor (TNF)- α was assessed by a specific ELISA, with a sensitivity of 0.5 pg/ml (Pierce Biotech). IL-4 was also measured by an ELISA with a sensitivity of 2.0 pg/ml. IFN- γ was measured by an enzyme immunoassay (EIA) with a sensitivity of 0.1 pg/ml.

Superoxide anion (O₂⁻) production

The amount of superoxide released from isolated monocytes (1×10^6 cells/ml) was determined with a Hitachi spectrophotometer U 2000 (Tokyo, Japan) as the change in absorbance at 550 nm resulting from superoxide dismutase (SOD)-inhibitable cytochrome c reduction at 37°C [37]. The reaction was carried out for 5 min with PMA (10 ng/ml) with calcium chloride (1.5 mM) and magnesium sulphate (1.2 mM) in the presence of 1 mM NaN₃, after which the reaction was stopped by addition of 0.5 mM N-ethylmaleimide. The generation of superoxide was calculated by subtracting the change in absorbance in the presence of SOD (1 mM) from that in its absence, and then dividing this value by 21.1×10^3 M/cm for the molar extinction coefficient.

Statistical analysis

Data in the table and figures are shown as means \pm standard deviation (s.d.) unless indicated otherwise. Statistical

analysis was performed with unpaired *t*-test (GraphPad Prism version 5.00 for Mac OS X; GraphPad Software, San Diego, CA USA). Results with *P*-values less than 0.05 were considered statistically significant.

Results

IL-4 and IL-4 with M-CSF, IL-4 with GM-CSF-induced MGCs *in vitro*

In our culture system, many of the monocytes were degenerated, and only a small number of MGCs appeared spontaneously in the controls without cytokine (Fig. 1). These

MGCs were small, generally with three to five nuclei. In the experiments to evaluate the cytokines, which were described previously as inducing MGCs, M-CSF (20–100 ng/ml) alone, GM-CSF (20 ng/ml) alone, a combination of M-CSF (20 ng/ml) and TNF- α (20 ng/ml), a combination of GM-CSF (20 ng/ml) and TNF- α (20 ng/ml) and a combination of GM-CSF (20 ng/ml) and IFN- γ (1000 U/ml) maintained cell densities and promoted monocytes adhesion, but did not affect MGC formation significantly, from both adult blood and cord blood monocytes in comparison with the controls (data not shown). IL-4 (20 ng/ml), a combination of IL-4 (20 ng/ml) and M-CSF (100 ng/ml) and a combination of IL-4 (20 ng/ml) and GM-CSF (20 ng/ml) increased

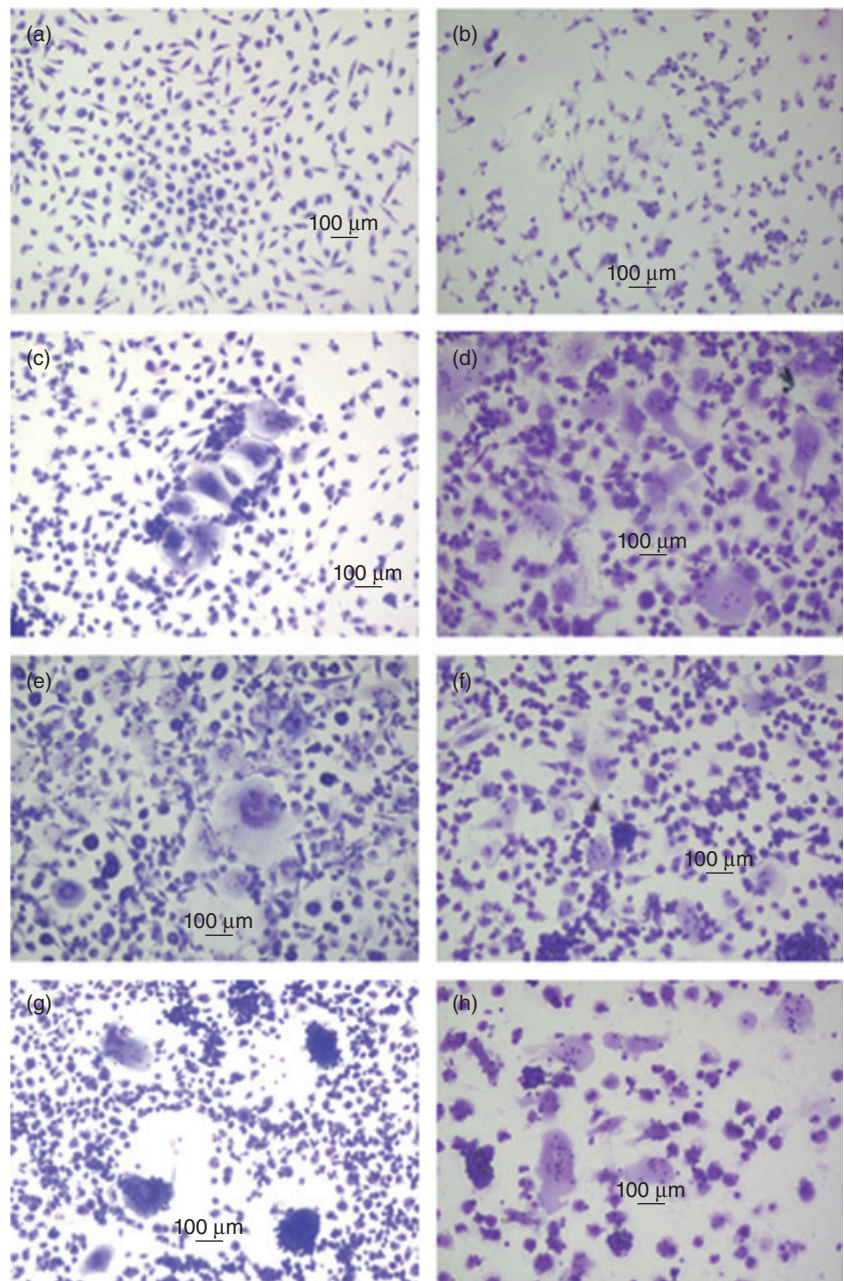


Fig. 1. Morphology of multi-nucleated giant cells (MGCs) induced by interleukin (IL)-4, IL-4 with macrophage colony-stimulating factor (M-CSF) and IL-4 with granulocyte-macrophage colony-stimulating factor (GM-CSF) *in vitro*. Adult peripheral blood and cord blood monocytes were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin, at 1×10^6 cells/ml/well in 24-well tissue culture plates with or without cytokines. After 14 days' incubation without changing the medium, the cells were stained with a May-Grünwald-Giemsa stain. (a) No added cytokine: adult blood; (b) no added cytokine: cord blood; (c) IL-4 (20 ng/ml) alone: adult blood; (d) IL-4 (20 ng/ml) alone: cord blood; (e) IL-4 (20 ng/ml)+M-CSF (100 ng/ml): adult blood; (f) IL-4 (20 ng/ml)+M-CSF (100 ng/ml): cord blood; (g) IL-4 (20 ng/ml)+GM-CSF (20 ng/ml): adult blood; (h) IL-4 (20 ng/ml)+GM-CSF (20 ng/ml): cord blood.

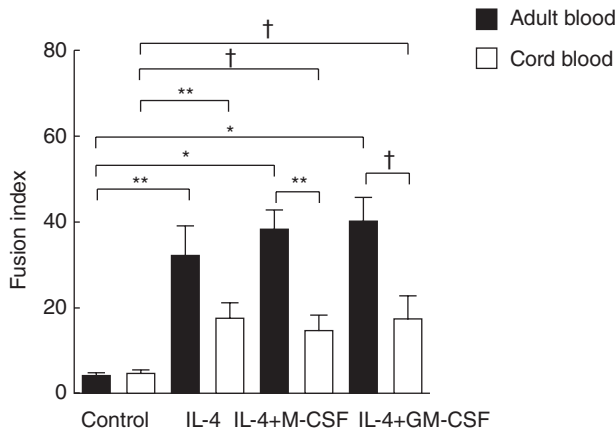


Fig. 2. Effect of cytokines on multi-nucleated giant cell (MGC) formation; adult blood *versus* cord blood monocytes. Adult peripheral blood (solid bar) and cord blood (open bar) monocytes were cultured as described in the legend to Fig. 1. The results were calculated from seven independent experiments as the fusion index. Significantly different from control or between cord blood and adult blood: * $P < 0.0001$, ** $P < 0.01$, † $P < 0.05$.

the fusion index significantly both from adult blood and cord blood monocytes, respectively (Fig. 2). In addition, these MGCs differentiated sometimes into large ones with 20 or more nuclei and/or large cytoplasmic spreading. There was no distinct difference in the characteristics of MGC among the different cytokines added (IL-4 alone, the combination of IL-4 and M-CSF and the combination of IL-4 and GM-CSF) (Fig. 1).

MGC formation from cord blood monocytes induced by IL-4 and IL-4 with M-CSF, and IL-4 with GM-CSF, in comparison with adult peripheral blood monocytes

As described previously, the fusion index was low and there was no difference in the index between adult blood and cord blood monocytes in the control without cytokine (Fig. 2). The fusion index in the experiment with IL-4 alone was lower in cord blood than in adult blood monocytes, but not significantly different ($P = 0.0958$). In contrast, the fusion index in the experiment with the combination of IL-4 and M-CSF and that with the combination of IL-4 and GM-CSF was significantly lower in cord blood than in adult blood monocytes ($P = 0.0018$ and $P = 0.0141$, respectively) (Fig. 2). These findings resulted from the number of nuclei per MGCs rather than from the prevalence of MGCs. The number of nuclei per MGC was significantly higher in adult blood than in cord blood monocytes in all experiments with IL-4 alone, the combination of IL-4 and M-CSF and the combination of IL-4 and GM-CSF ($P < 0.0001$) (Fig. 3). The average number of nuclei per MGCs from cord blood monocytes was about half that from adult blood monocytes.

Cytokine production from monocytes after 14-d culture with IL-4 and IL-4 with M-CSF, IL-4 with GM-CSF

The production of several cytokines was evaluated in the supernatant of culture medium for 14 days. Three individual samples were analysed in each group. In both adult blood and cord blood, IL-4 alone, the combination of IL-4 and M-CSF and the combination of IL-4 and GM-CSF did not stimulate the production of TNF- α , IFN- γ and IL-1 β compared with the control (no stimulant). Differences between adult blood and cord blood in each group were not statistically significant (Table 1). Cytokine levels in the supernatant of culture medium at 5 days also showed the same results (data not shown).

Superoxide anion production by monocytes

Isolated monocytes (1×10^6 cells/ml) in PBS were stimulated with PMA. Control O $_2^-$ production stimulated with PMA (10 ng/ml) ranged from 112 to 220 nmol cytochrome c reduced/ 10^6 cells for 5 min (171 ± 35 nmol; mean \pm s.d. of five individual adult volunteers), and ranged from 120 to 198 nmol cytochrome c reduced/ 10^6 cells for 5 min (170 ± 30 nmol; mean \pm s.d. of four individual cord blood samples).

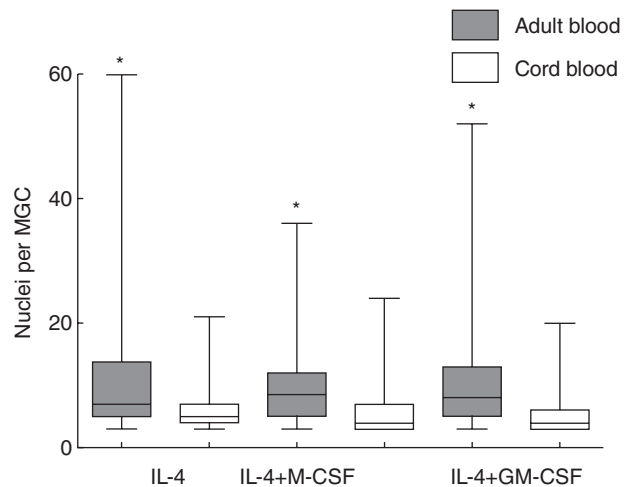


Fig. 3. Nuclei per multi-nucleated giant cells (MGCs) induced by cytokines; adult blood *versus* cord blood monocytes. Adult peripheral blood (solid box) and cord blood (open box) monocytes were cultured as described in the legend to Fig. 1. The number of nuclei per MGC (20 cells acquired randomly in each experiment) was counted. Results from seven independent experiments are shown in the box-and-whisker plots. Line in the box shows median point, box shows the point from first to third quartile, whiskers show the point from smallest to largest number. Significantly different between cord blood and adult blood: * $P < 0.0001$.

Table 1. Concentration of interferon (IFN)- γ , interleukin (IL)-1 β , IL-4 and tumour necrosis factor (TNF)- α in the supernatants of adult peripheral blood and cord blood monocytes after 14 days' culture with the cytokines listed below.

		IFN- γ (IU/ml)	IL-1 β (pg/ml)	IL-4 (pg/ml)	TNF- α (pg/ml)
Adult blood ($n = 3$)	Control	0.9 \pm 0.9	393 \pm 202	<2.0	49 \pm 5
	IL-4+M-CSF	1.0 \pm 0.8	159 \pm 180	3007 \pm 1074	36 \pm 20
	IL-4+GM-CSF	0.5 \pm 0.2	147 \pm 175	2633 \pm 421	35 \pm 13
	IL-4	0.7 \pm 0.4	217 \pm 162	4740 \pm 1935	44 \pm 23
Cord blood ($n = 3$)	Control	1.1 \pm 1.2	765 \pm 412	<2.0	51 \pm 9
	IL-4+M-CSF	0.1 \pm 0.1	583 \pm 761	3737 \pm 1004	28 \pm 10
	IL-4+GM-CSF	0.2 \pm 0.2	473 \pm 708	1960 \pm 1196	27 \pm 6
	IL-4	0.7 \pm 0.6	479 \pm 351	4580 \pm 1403	40 \pm 17

Three individual samples were analysed in each group. Differences between adult blood and cord blood in each group were not statistically significant; difference among controls and each group were also not statistically significant. M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor.

Discussion

MGC formation from umbilical cord blood mononuclear cells has rarely been studied, and was reported first by Zur Hausen *et al.* [28]. They found that mononuclear cells from human umbilical cord were fused more frequently into MGCs than those from adult peripheral blood. Because of the morphological appearance of the MGCs they attempted, but failed, to prove that a viral agent induced macrophage fusion. The first comparative analysis of experimentally induced MGCs from mononuclear phagocytes isolated from human cord blood and adult peripheral blood was reported by Gerberding *et al.* [29]. They found that spontaneous MGC formation occurred in both cord and adult blood mononuclear cell cultures by 7 days of incubation, although significantly fewer MGCs formed in cord blood cultures. PMA treatment of adult blood mononuclear cells resulted in a significant increase in MGC formation after 7, 14 or 21 days of culture, but PMA did not increase MGC formation significantly in cord blood cultures until 14 or 21 days of culture.

In the present study, we used a pure population of monocytes from cord blood and adult peripheral blood with stimulation of various monoclonal cytokines (IL-4, M-CSF, GM-CSF, TNF- α , IFN- γ) that are known to induce the formation of MGCs *in vitro*. Among these cytokines IL-4 alone, a combination of IL-4 and M-CSF and a combination of IL-4 and GM-CSF could increase MGC formation significantly in both cord blood and adult peripheral blood culture. In the experiment with the combinations of IL-4 and M-CSF or IL-4 and GM-CSF, the fusion index was significantly lower in cord blood than in adult blood monocytes. In addition, we notably demonstrated that the number of nuclei per MGCs was significantly lower in cord blood than in adult blood monocytes in all experiments with IL-4 alone, the combination of IL-4 and M-CSF and the combination of IL-4 and GM-CSF. In these experiments we used a pure population of monocytes from cord blood and adult peripheral blood and evaluated the production of cytokines from cultured cells. TNF- α , IFN- γ and IL-1 β were detected, but differences

between adult blood and cord blood in each group were not statistically significant, so the difference in MGC formation seemed to be dependent upon the character of the monocyte itself but not the produced cytokines (autocrined monokines). Previous studies have also reported that the ability of neonatal monocytes and macrophages to produce several monokines (including TNF- α and IL-1 β) is similar or reduced modestly compared with adult cells [3,38]. Additionally, neonatal monocytes/macrophages produce superoxide anion as much as adult cells.

Knowledge of the functions of MGCs is still limited, but several recent studies have revealed their pivotal functions, particularly the function played in mycobacterial infection. In studies utilizing an *in-vitro* model of human tuberculous granulomas, Lay *et al.* have shown that the high-virulence mycobacterium, *Mycobacterium tuberculosis*, induces large MGCs with more than 15 nuclei per cell, whereas with low-virulence mycobacterium species, *M. avium* and *M. smegmatis* induce MGCs with a low number of nuclei per cell, fewer than seven [30]. The high-virulence mycobacterium species resulted in large granulomas where the MGCs are incapable of phagocytosis but still retain a strong antigen-presentation capability, thus appearing to be devoted to the destruction of bacilli. In a report of idiopathic disseminated bacille Calmette-Guérin (BCG) infection, Emile *et al.* found two types of granuloma [31]. The first type (tuberculoid type) consisted of well-circumscribed and well-differentiated granulomas, with epithelioid and MGCs containing very few acid-fast rods, surrounded by lymphocytes and fibrosis and occasionally with central caseous necrosis. The second (lepromatous) type consisted of ill-defined and poorly differentiated granulomas, with few if any giant cells and lymphocytes but widespread macrophages loaded with acid-fast bacilli. There was a strong correlation between the type of granuloma and clinical outcome. The tuberculoid type was associated with patients' survival, while the lepromatous type was associated with their poor outcome and death [32,33]. Furthermore, the histopathological appearances of congenital tuberculosis in the literature are similar to the lepromatous type, and are described

as being composed of central caseous necrosis with surrounding scanty epithelioid cells and lymphocytes and few or no MGCs [34–36].

These findings suggest the importance of the function of MGCs in preventing critical infection with mycobacterium. Our results showed that MGCs from cord blood monocytes had only half the number of nuclei in each cell than MGCs from adult peripheral blood. The histopathological appearances of congenital tuberculosis can be explained partly as the result of poor mobilization of phagocytes, decreased production of cytokines (especially IFN- γ) by T lymphocytes [3] and impaired formation of MGCs in the neonates.

Additional investigations are needed to confirm the decreased ability of cord blood monocytes to fuse into MGCs in other experimental conditions, and to determine what mechanism or molecule (cell surface fusion molecules) underlies the decreased ability of cord blood monocytes to fuse into MGCs. Additional research regarding the pathophysiology of MGC in neonates might be helpful for the appropriate treatment of infection with microorganisms which are capable of surviving intracellularly.

Disclosure

None.

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