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Study of cord blood natural killer cell suppressor activity

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Abstract: We tested the immunosuppressive effect of cord blood (CB) natural killer (NK) cells using highly purified CB NK cells in mixed lymphocyte cultures (MLC) containing autologous CB T cells as responders. Control cultures were done without NK cells. Our findings revealed that CB NK cells induced a dose-dependent inhibition of T lymphocyte proliferation as evidenced by decreased ^{3}H-thymidine incorporation in MLC. The T cell alloproliferation was significantly decreased in the presence of an NK cell to responder cell ratio of 0.1, 0.2 or 0.4 compared with control cultures done without NK cells ($p = 0.02$, 0.003 and 0.0002, respectively). T lymphocyte inhibition was also achieved using irradiated CB NK cells and still demonstrable on addition of disparate CB NK and T cells to the MLC.
In agreement with previous reports, adult blood NK cells inhibited the alloreactive T cells in the MLC using adult T lymphocytes as responders. Compared to control cultures done without NK cells, statistically significant inhibition of ^{3}H-thymidine incorporation in MLC was observed at a ratio of NK cells to responder cells ratio of 0.2 or 0.4 ($p = 0.02$).
To investigate the mechanism whereby CB NK cells can interfere with the development of alloreactive T cells in MLC, we measured the tumour necrosis factor-α (TNF-α) concentrations in MLC supernatants using NK cell-depleted or unseparated CB mononuclear cells (MNC) as responders. The results revealed significantly high levels of TNF-α in the absence of NK cells ($p = 0.007$). We conclude that CB NK cells suppress alloreactive T lymphocytes as do their counterparts in adult blood. However, the high NK to T cell ratio in CB could contribute to a more marked suppressive potential compared to that in adult blood. The mechanism of NK-mediated inhibition is likely related to disruption of the TNF-α pathway of T-lymphocyte activation.

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Accumulating data indicate that the incidence and severity of graft-versus-host disease (GVHD) after matched (1, 2) and mismatched (3) cord blood (CB) transplantation are reduced compared with the results after bone marrow transplantation (4). The precise mechanisms accounting for this observation are currently under investigation. It is of interest that CB T lymphocytes respond normally to alloantigens as compared with similarly stimulated adult T cells (5–7). However, in contrast to adult T cells, restimulation of alloantigen-primed CB T cells results in hyporesponsiveness associated with defective T cell signalling (8, 9). Further studies have shown that reduced CB T lymphocyte alloreactivity is due to the action of multiple peripheral tolerance mechanisms. In view of this, the suppressor behaviour of CB $CD4^{+}$ $CD45RA^{+}$ cells, the ability of CB cells to mature into suppressor Th1 type cells and the absence of lymphocyte activating factor in CB sera were recently reviewed (10). Indeed, several experimental models of haematopoietic cell transplantation have

suggested that natural killer (NK) cells may enhance tolerance induction (11–13).

By definition, NK cells are large granular lymphocytes that comprise 10–15% of adult blood lymphocytes (14). They play a key role in immune surveillance and mediate major histocompatability complex (MHC) unrestricted cytotoxicity towards tumour and virally-infected cells (15–17). Their capacity to suppress antibody production (18, 19) and allo-CTL generation in mixed lymphocyte cultures (MLC) has previously been reported (20, 21).

In the CB, the percentage and absolute number of NK cells are significantly higher than that in adult blood (10, 22–25). Numerous studies have demonstrated that CB NK cytotoxicity is lower than that of adult blood (26–28) and the normal killing activity of these cells is induced by IL-2, IL-12 or IL-15 (26, 28). However, the suppressor effect of CB NK cells and their role in damping CB T cell alloreactivity are not clear.

In the present study, we examined the *in vitro* effect of CB NK cells on the alloreactivity of T cells in MLC. Parallel studies of adult blood counterparts were performed. We report here that CB NK cells inhibit T lymphocyte alloreactivity in MLC. Possible mechanisms that could explain NK-mediated suppression are discussed.

Material and methods

Collection of CB and adult peripheral blood lymphocytes

CB was obtained by venipuncture from the fetal side of full-term placenta immediately after delivery and collected on citrate–phosphate–dextrose (CPD). Heparinized peripheral blood was collected from healthy adult blood donors. Mononuclear cells (MNC) with densities less than 1.077 g/cm^3 were isolated by centrifugation using a Ficoll/Isopaque density gradient (Sigma Immuno Chemicals). Cells were washed twice and resuspended in RMPI-1640 medium (Sigma Immuno Chemicals) containing 10% heat inactivated pooled human serum, penicillin/streptomycin (50 units/ml and 50 µg/ml, respectively).

Monoclonal antibodies

BY55 mAb is produced in our laboratory as described elsewhere (29). BY55 mAb detects a cell surface antigen of 80 kDa expressed exclusively by cytotoxic lymphocytes. In cord blood, BY55 mAb stains only NK cells. The percentage of CB NK BY55$^+$ cells is 20–35%. In normal human peripheral blood, cytotoxic lymphocytes labelled with BY55 mAb correspond to 20–25% of MNC, including mostly CD3 negative lymphocytes, the majority of T-cell receptor (TCR) γδ positive T lymphocytes, and a minor subset of CD8$^+$ αβ with cytotoxic T-lymphocyte (CTL) activity (24, 29). FITC-conjugated goat anti-mouse IgM was purchased from Caltag Laboratories (South San Francisco, CA). CD14-FITC, CD56-PE and CD3-FITC antibodies were obtained from Immunotech (France).

Immunofluorescence assays

The number of CB T lymphocytes, NK cells, and monocytes was assessed using direct immunofluorescence staining as described elsewhere (30). Briefly, CB MNC were incubated with CD3-FITC, CD56-PE or CD14-FITC monoclonal antibodies for 30 min at 4 °C. After the final wash, cells were fixed in 1% formaldehyde. CD3$^+$ T lymphocytes, CD56$^+$ NK cells and CD14$^+$ monocytes were analysed using a FACS Vantage microfluorometer (Becton Dickinson).

Isolation of CB NK cells and T lymphocytes using BY55 mAb

This was performed using indirect immunofluorescence staining as previously described (29). Briefly, cells were first incubated with BY55 mAb, and then washed and incubated with FITC-conjugated anti-mouse IgM. After the final wash, BY55$^+$ and BY55$^-$ fractions were sorted in the lymphoid gate using a FACS Vantage microfluorometer (Becton Dickinson). Trypan blue exclusion was performed on sorted and unsorted cells, and the viability was $>90\%$. In some experiments, CD4$^+$ lymphocytes were isolated by an immunomagnetic selection procedure (M-450 CD4 Dynabeads Dynal, France) according to the manufacturer's instructions.

Isolation of adult NK and T lymphocytes

CD56 mAb was used because BY55 mAb stains both NK cells and a subpopulation of T cells in adult blood. Briefly, cells were incubated with CD56 mAb for 30 min at 4 °C. After the final wash, CD56$^+$ NK cells and CD56$^-$ enriched T-cells were sorted from the lymphocyte gate as described above.

Mixed lymphocyte culture (MLC)

The allogeneic response of cord blood cells was determined in the one-way MLC as detailed previously (31). Briefly, irradiated (30 Gy) allogeneic HLA non-identical peripheral blood mononuclear cells or irradiated (70 Gy) Epstein–Barr virus (EBV) transformed B cells were used as stimulators at a concentration of 5×10^4 cells per well. Responder CB or adult T cells were used at the same concentration. Purified CB or adult NK cells were added at day 0 to MLC at a concentration of

5×10^3, 1×10^4, or 2×10^4, giving an NK cell to responder cell ratio of 0.1, 0.2 or 0.4, respectively. In some assays, irradiated (30 Gy) CB NK cells were added to MLC using the above NK/T cell ratios. Cultures were performed in triplicate in 96-well plates and incubated at 37 °C in 5% CO_2. At the end of 6 d, the cultures were pulsed with ^{3}H-thymidine (1 μCi per well) (Amersham, France) and reincubated for 12 h. ^{3}H-Thymidine incorporation was determined using automated cell harvesting and scintillation counting (Packard).

Assay of TNF-α in MLC supernatants

The TNF-α concentration was measured in the supernatant of CB MLC before and after NK cell depletion. Depletion of NK cells was achieved by cell sorting as described above. The total MNC culture media without stimulating cells and media of purified NK cells and donor cells were used as control cultures. After 6 d incubation at 37 °C in 5% CO_2, the MLC supernatants were collected, separated in aliquots and stored at −80 °C until the cytokine assay. The TNF-α concentration of was determined using Medgenix TNF-α EASIA (Fleurus, Belgium) according to the manufacturer's instructions. This technique consists of a sandwich enzyme immunoassay in which several monoclonal capture antibodies and horseradish peroxidase-labelled polyclonal antibodies are used. The minimum detectable concentration is estimated to be 3 pg/ml. The inter- and intra-assay variations are less than 10%. Samples were tested in duplicate.

Statistical analysis

The Mann–Whitney test was used to examine the significance of differences between test and control samples. $p < 0.05$ was considered statistically significant.

Results

CB and adult T lymphocytes have a similar proliferative response toward allogeneic stimuli

As shown in Table 1, the proliferative response of CB T cells to allogeneic MNC was greater than that of adult blood T lymphocytes. However, the difference was not statistically significant ($p = 0.05$). Similarly, the proliferative response of T lymphocytes to EBV-transformed B cells was comparable in the tested CB and adult blood samples.

Inhibition of T-cell proliferative response by autologous NK cells

In this approach, the NK cells were added to autologous T cell responders. As shown in Fig. 1A, inhibition of the T cell proliferative response was significant using an NK cell to responder cell ratio of 0.1, 0.2, and 0.4 ($p = 0.02$, 0.003, and 0.0002, respectively). Figure 1B shows that inhibition of T cell alloproliferation was significant using an adult NK cell to responder cell ratio of 0.2 and 0.4 ($p = 0.02$). As shown in Table 2, the alloproliferative response of the control cultures was inhibited by 39, 48 and 83% using an irradiated (30 Gy) CB NK cell to responder cell ratio of 0.1, 0.2, and 0.4 respectively. Using an irradiated (30 Gy) CB $CD4^+$ cell to responder cell ratio of 0.1, 0.2, and 0.4, a stimulation of the control alloreactive T cell response of the order of 52, 66 and 88% was achieved.

NK-mediated suppression across the MHC barrier

This was investigated by incubating CB NK $BY55^+$ cells with HLA-disparate CB T lymphocytes in MLC. As shown in Fig. 2, T cell proliferation was significantly depressed when the NK cell to responder cell ratio was 0.2 or 0.4 ($p = 0.04$ and 0.03, respectively).

Enhanced TNF-α production after NK cell depletion

TNF-α was measured in MLC supernatants using NK-depleted or unseparated CB MNC as responders. The concentration of TNF-α before NK cell depletion was 215 ± 62 pg/ml. After NK cell depletion, the concentration of this cytokine was 355 ± 59 pg/ml. As shown in Fig. 3, NK cell depletion resulted in a significant increase in TNF-α ($p = 0.007$). In the control cultures, containing MNC in the culture medium, the concentration of TNF-α was 67 ± 22 pg/ml. In those containing

Table 1. *In vitro* responses of CB and adult T lymphocytes cells to different stimuli

	Cellular proliferation (cpm × 10^3)		
T-cells	Medium	Irradiated MNC	EBV
Cord blood	0.225 ± 0.05	68.21 ± 19.26	122.10 ± 3.32
Adult blood	0.212 ± 0.02	59.95 ± 13.52	118.60 ± 30.54

Mean ± SD of ^{3}H thymidine incorporation in five different experiments. CB or adult T cells were incubated with culture medium. Irradiated adult MNC. Epstein–Barr virus transformed B-cell line.

Table 2. Modification of CB T cell proliferative response in the presence of irradiated CB NK or $CD4^+$ lymphocytes

Irradiated cell type	Controls	Ratio of irradiated cells to responder cells		
		0.1	0.2	0.4
NK	90.6 ± 7.3	55.1 ± 2.1[a]	46.6 ± 5.4[a]	15 ± 1.4[a]
$CD4^+$	30.9 ± 6.2	47.1 ± 4.5[b]	51.4 ± 11.2[b]	58 ± 8.7[b]

Data are expressed as mean cpm $\times 10^3$ of three different experiments with standard deviations.
[a] Significant inhibition and [b] significant stimulation relative to corresponding control cultures.

CB NK cells and stimulators, TNF-α was 0 ± 0. It is worth noting that the immunophenotyping studies of CB samples used in this approach revealed that the percentage of $CD3^+$ T lymphocytes was 39 ± 14%, that of NK $BY55^+$ was 20 ± 5%, and that of monocyte $CD14^+$ was 26 ± 3%.

Discussion

The purpose of this study was to assess the contribution of CB NK cells to T cell alloreactivity on the supposition that the NK cells could constitute an important factor in the peripheral tolerance mechanisms.

In accordance with other studies (5–9), we demonstrated that the alloreactivity of CB T cells and of adult blood was comparable. Furthermore, our observation that immobilised anti-CD3 mAb resulted in a normal T cell proliferative response is in agreement with other studies (6) suggesting that the signal transduction via the TCR/CD3 complex is not defective.

To gain additional information about the mechanisms that explain the reduced T cell alloreactivity seen in CB transplanted individuals, we investigated the regulatory role of CB NK cells on the T cell responses in MLC. We demonstrate that CB NK cells induce a dose-dependent suppression of T lymphocyte proliferation in MLC as do their conterparts in adult blood. Interestingly, we observed a significant inhibition of T cell proliferation in CB samples compared to that of adult blood when the NK to T cell ratio was 0.1. This observation could be attributed to quantitative differences in the secretion of inhibitory mediators between CB and adult blood and/or an enhanced

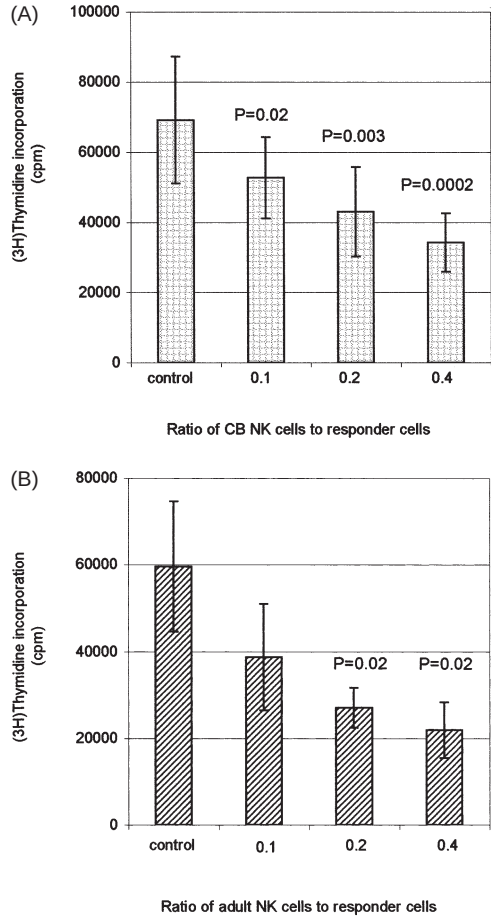

Fig. 1. T lymphocyte proliferative response in the presence of autologous NK cells. Autologous models of MLC were set up where NK and T cells were derived from either the same CB ($n = 5$) (A) or adult blood ($n = 3$) (B). NK cells were added to MLC at the concentrations shown. In control cultures, NK cells were not added. Cultures were treated as described in Material and Methods.

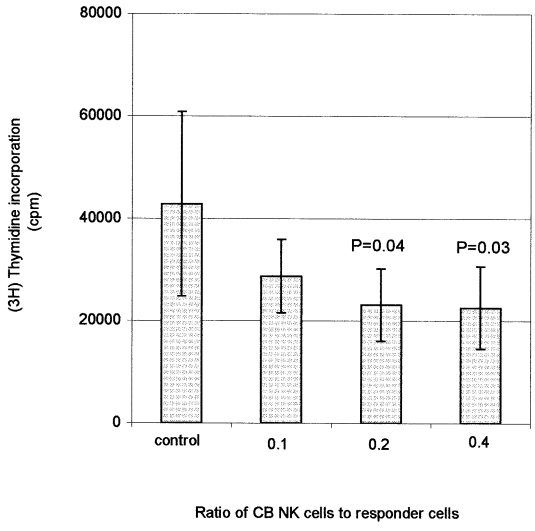

Fig. 2. CB NK-mediated inhibition across the MHC barrier. MLC ($n = 3$) were set up using irradiated (30 Gy) adult MNC as stimulators and enriched CB T-cells as responders. Different concentrations of allogeneic CB NK cells were added to MLC except those used as controls.

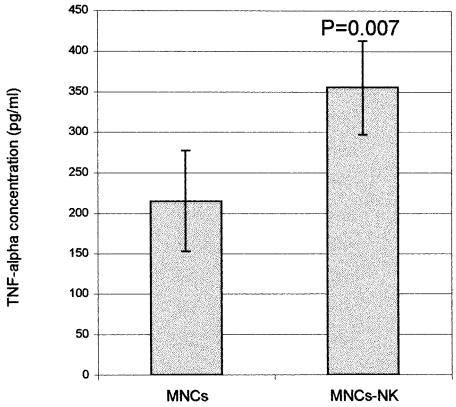

Fig. 3. TNF-α production after NK cell depletion. CB MNCs and CB MNC-NK depleted cell populations were cultured in the presence of irradiated (30 Gy) adult MNCs as stimulators. TNF-α concentrations (pg/ml) in the supernatants of five different experiments were measured after 6 d incubation at 37°C in 5% CO_2.

susceptibility of CB T cells to secreted suppressor factors. Bearing in mind that the NK to T cell ratio is considerably higher in CB than in adult blood, based on the significant difference in immunophenotypic profile between CB and adult blood lymphocytes (10, 22–25), our findings suggest that the CB suppressive potential might be higher than that of adult blood.

We also demonstrated that CB NK-mediated suppression could be achieved across the MHC barrier, and we showed that the NK regulatory effect is radioresistant. In our hands, cell overcrowding was ruled out as a possible cause of the depressed T cell proliferation. Recently, studies of the pathogenesis of GVHD have focused on the dysregulation of cytokine production, where high levels of TNF-α play a crucial role in T cell activation, and in the pathogenesis of acute GVHD (32–37). Previous results have shown the involvement of TNF-α in the IL-2-induced acquisition of optimal lytic competence by cytotoxic T cells (38). In addition, TNF-α has been found to up-regulate MHC class II expression on antigen-presenting cells (39, 40), and raised levels of this cytokine have been found in the sera of patients with GVHD (41).

The involvement of NK cells in the pathogenesis of GVHD is controversial. The capacity of NK cells to secrete cytokines such as tumour necrosis factor-α (TNF-α), interferon-γ (INF-γ) and IL-1β (42, 43) has suggested a role for these cells in the exacerbation of ongoing GVHD. In line with this hypothesis is the finding that NK cell depletion is beneficial in the prevention of GVHD (44). In contrast, other studies have shown that TGF-β plays an important role in protection against GVHD by NK cells (11).

In this study we investigated the effect of CB NK cells on the secretion of TNF-α by other MNC. Our results revealed inhibition of TNF-α secretion by CB NK cells. This observation suggests a new mechanism for NK-mediated suppression that may involve a blockade of the TNF-α pathway of T cell activation. Indeed, inhibition of endogenously generated TNF-α by human monocytes is associated with down-regulation of DR but not DP or DQ expression (40). Such modification of cell surface antigen expression on the antigen-presenting cells might lead to the expansion of HLA-DQ restricted suppressor T lymphocytes and inhibition of allospecific HLA-DR helper T cells (45).

In conclusion, CB NK cells induce a dose-dependent suppression of the alloreactive T lymphocyte response as do adult blood NK cells. The higher NK/T cell ratio in CB compared to adult blood provides evidence that the CB suppressive potential may be greater than that of adult blood. Finally, the mechanism of CB NK-mediated suppression might implicate regulation of TNF-α secretion. Further understanding of this phenomenon may be essential for the prevention and management of GVHD after hematopoietic stem cell transplantation.

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