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# Cord Blood Nucleated Cells Induce Delayed T Cell Alloreactivity

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## ABSTRACT
Cord blood (CB) mononuclear cells (MNCs) can be transplanted in HLA mismatched recipients with limited graft rejection or graft-versus-host disease (GVHD). Previous studies have shown that naïve T cells and hyporesponsive dendritic cells are largely represented in CB. Data presented here demonstrate that CB MNCs are unable to stimulate allogeneic T cell proliferative or cytotoxic responses in standard in vitro assays. However, a suppressive effect of CB MNCs was ruled out because purified CD34$^+$ cells or CD14$^+$ monocytes stimulated T cell responses that were not inhibited by add-back of CB MNCs. The lack of antigen-presenting cell (APC) activity of CB MNCs in primary mixed lymphocyte culture (MLC) did not induce allogeneic T cell anergy. In fact, rechallenge of T cells with CB CD34$^+$ cells, or immature monocyte-derived dendritic cells (iMo-DCs) in secondary MLC induced potent T cell proliferative responses. A delayed APC activity of CB MNCs was observed after stimulation with irradiated allogeneic T cells for 6 days, likely because of the upregulation of CD86 and HLA-DR on CB cells. Cytotoxic lymphocytes (CTL) were generated after stimulation of blood T cells with CB MNCs for 4 weeks or CB-derived iMo-DCs for 1 week. Concomitant stimulation of T cells with CB iMo-DC obtained from 2 CB units resulted in the generation of CTLs specific for each CB, independently of the CB:CB cell ratio. These data suggest that the APC activity of CB cells possibly increases posttransplant, and may contribute to delayed graft rejection and/or acute and chronic GVHD.
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### KEYWORDS
Cord blood • APC • CTL • Mo-DC • CD34

## INTRODUCTION

Cord blood (CB) is a source of hematopoietic stem cells (HSCs) increasingly utilized in allogeneic HSC transplantation of patients with hematologic malignancies who do not have an HLA matched related or unrelated donor [1,2]. Because the number of nucleated cells available for HSC transplantation is smaller in CB than in marrow or mobilized peripheral blood, smaller recipients, such as pediatric patients, usually represent more suitable candidates for CB transplant. Besides a quantitative difference of number of cells, CB differs from other HSC sources also for the prevalence of naïve T cells and an overall immunologic immaturity and Th2 cytokine skewing of the T cells included in the graft [2-7]. In addition, studies performed on isolated monocytes, or in vitro-generated monocyte-derived dendritic cells, have also shown that CB antigen-presenting cells (APCs) are less susceptible to complete maturation and activation compared to their counterparts in adult peripheral blood [8,9]. These immunologic characteristics may explain why 1 to 2 HLA antigen-mismatched CB units can be transplanted with acceptable rates of severe acute graft-versus-host disease (aGVHD) or rejection. It is interesting, however, that the immune reconstitution observed after CB or bone marrow transplantation seems to be comparable [10,11].

Transplantation of 1 CB unit in adult patients often results in a more prolonged time to neutrophil and platelet engraftment compared to bone marrow or peripheral blood stem cell (PBSC) transplant because of the limited cell dose infused [12]. Recently,

it has been demonstrated that concomitant transplantation of 2 CB grafts is an effective method to increase the total cell dose transplanted in adult patients, and can shorten the time to hematopoietic engraftment without increasing the risk of GVHD or rejection significantly [13]. In addition, in approximately 1/3 of the patients receiving a double CB transplant the engraftment of cells derived by both CB units was demonstrated by chimerism analysis on day 21 post-transplant. However, following this initial phase of mixed chimerism, only the cells derived from 1 of the 2 CB will permanently engraft the patient, whereas cells derived from the other CB graft progressively disappear by means of mechanisms that are yet to be clarified.

Although the lower incidence of severe aGVHD in CB transplants compared to bone marrow/peripheral blood transplants may depend on the inefficient response of CB T cells, the development of chronic GVHD (cGVHD) may depend also on the antigen-presenting function of donor CB cells [14].

In this study, we analyzed the alloantigen-presenting function of cells present in CB grafts. Our results suggest that although CB cells have poor capacity of stimulating T cell alloresponses, they do not induce T cell anergy, but rather develop a delayed, but effective immunogenic activity.

## MATERIALS AND METHODS

### Cell Separation

The CB is obtained from discarded placentas from the New York Blood Bank. An institutional review board protocol has been approved for the procurement of CB.

CB or peripheral blood (PB) mononuclear cells (MNCs) were obtained by centrifugation over Ficoll/Hypaque (Amersham Biosciences, Piscataway, NJ) gradients. Light density cells were washed twice in phosphate-buffered saline (PBS; Cambrex, Walkersville, MD) with 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO), and $CD34^+$ (positive selection) or $CD14^+$ (negative selection) cells were purified by MidiMACS high-gradient magnetic separation columns (Miltenyi Biotec, Auburn, CA). To assess the purity, aliquots of isolated $CD34^+$, $CD34^-$, or $CD14^+$ cells were restained with anti-CD34 or anti-CD14 monoclonal antibodies (mAbs) FITC-conjugated (Becton-Dickinson [BD], San Jose, CA). PB T cells were immunomagnetically purified from MNCs on a MidiMACS column by negative selection of non-T cells by using a Pan T Cell Isolation Kit II (Miltenyi Biotec). This kit utilizes a cocktail of biotin-conjugated mAbs against CD14, CD16, CD19, CD36, CD56, CD123, and Glycophorin A as a primary labeling reagent, and antibiotin mAbs conjugated to microbeads as a secondary reagent. The unlabeled cells that passed through the column were then stained with an anti-CD3-FITC mAb (BD) as a check for purity. Isolated $CD34^+$, or $CD3^+$ T cell samples were acquired on a FACS Calibur™ (BD) and analyzed using the Cell Quest™ software (BD), and showed, on average, 95% cell purity. In selected experiments, CB $CD14^+$ monocytes were isolated and then monocyte-derived immature dendritic cells (iMoDCs) were generated [15]. Briefly, $CD14^+$ cells were cultured in RPMI-10% HS supplemented with 1000 U/mL granulocyte-macrophage colony stimulating factor (GM-CSF) (Amgen, Thousand Oaks, CA) and 1000 U/mL IL-4 (R&D Systems, Inc., Minneapolis, MN) for 6 days. Coexpression of HLA-DR, CD86, and CD1a was analyzed to check the differentiation of DCs.

### Flow Cytometry

Flow cytometric analysis was performed on samples of PB and CB products. In selected experiments CB MNCs were stained with mAbs prior to and after incubation with irradiated (30 Gy) T cells (1:2 ratio) for 6 days and reisolation over Ficoll/Hypaque. The following FITC, or PE, or PerCP-conjugated monoclonal antibodies (mAbs) were employed: CD34, HLA-DR, CD14, CD86, CD3, CD11c, and CD19 from Becton-Dickinson, and CD1c (BDCA-1) and BDCA-2 from Miltenyi Biotec. Appropriate isotype controls were also utilized.

### Primary MLC

Unfractionated CB MNCs, $CD34^+$, $CD34^-$, CD3-depleted (TCD), or $CD14^+$ cells were freshly isolated, washed twice, irradiated (30 Gy) and then tested as stimulators (S) in a primary MLC. Irradiated CB T cells and autologous PB MNCs were used as negative controls. Cells were resuspended in medium containing RPMI-1640 (Cambrex), 10 mM HEPES (Cambrex), 100 U/mL penicillin (Cambrex), 100 μg/mL streptomicin (Cambrex), and 10% AB human serum (HS, HyClone, Logan, UT) inactivated at 56°C for 30 min; $5 \times 10^4$ purified PB-derived T cell responders (R) were mixed with S at 1:2 S/R ratio in round-bottomed 96-well plates for 6 days at 37°C in a 5% $CO_2$ humidified atmosphere. In selected experiments where CB MNCs were used as S in a primary MLC, CB MNCs from the same CB were initially incubated with irradiated (30 Gy) allogeneic T cells. On day 6 of culture the CB MNCs were harvested, irradiated, and tested as stimulators in MLC (S:R = 1:2) with the same responders used in the initial MLR. Experiments with CB MNCs add-back were also performed to test if increasing doses of Ag-specific CB MNCs would suppress the immunostimulatory activity of $CD34^+$ cells. Cells were pulsed with 1 μCi/well $^3$H-thymidine for 18 hours before harvest on day 6. Stimulation index (SI) was calculated for

each individual experiment as: SI = cpm (T cell R + S)/ cpm (T cell R).

### Secondary MLC

After 6-7 days of culture in a primary MLC, T lymphocytes were harvested, centrifuged over Ficoll/ Hypaque, and rested for 24 to 48 hours at 37°C. Then they were cocultured for 3 days at 1-2 $\times$ 10$^4$ cells/well with irradiated CB MNCs (5 $\times$ 10$^4$/well), or CB CD34$^+$ cells (2.5 $\times$ 10$^4$/well), or Mo-DCs (0.1 $\times$ 10$^4$/well) obtained from the original CB, or for 6 days with third-party PB MNCs (5 $\times$ 10$^4$/well). Cells were pulsed with 1 $\mu$Ci/well $^3$H-thymidine for 18 hours before harvest on day 3 or 6. The SI was calculated for each individual experiment as: SI = cpm (T cell R + S)/cpm (T cell R).

### CTL Assay

A standard $^{51}$Cr release assay was employed as previously described [16]. Briefly, lymphocytes that had been primed with irradiated CB MNCs or CB Mo-DCs for 1 to 4 weeks (effectors) were harvested and plated, at effector:target (E/T) ratios from 50:1 to 6.25:1, with 1-2 $\times$ 10$^3$ $^{51}$Cr-labeled target cells, in U-bottomed 96-well plates. After 4 to 6 hours of incubation at 37°C, 5% CO$_2$, culture supernatants were transferred into Luma PlatesTM, which were then counted on a microplate scintillation counter. PHA-lymphoblasts, generated by culturing CB MNCs for 6 days in the presence of 10 $\mu$g/mL PHA and 20 U/ ml IL-2 were employed as target cells. Target cells were labeled by an overnight culture at 37°C in the presence of $^{51}$Cr at 100 $\mu$Ci/mL. Results were expressed as % specific killing, calculated as follows: (CPM test − CPM spontaneous)/(CPM maximum − CPM spontaneous) $\times$ 100, where CPM spontaneous refers to values detected in wells containing target cells only, whereas CPM maximum refers to values detected in wells where target cells were incubated in the presence of lysing reagents, such as Triton X.

### Statistical Analysis

Statistical analysis was performed by using the $t$-test (2 tailed), or ANOVA test when more than 2 series of data were compared.

### RESULTS

### Phenotypic Characterization of Circulating CB APCs

Circulating APCs include monocytes, myeloid (mDC), and plasmacytoid (pDC) DC and B cells. To determine the phenotype of cells used for in vitro experiments, we initially measured the levels of these cell subsets and CD34$^+$ cells, as well as the expression of HLA-DR and costimulatory molecules such as CD40, CD80, and CD86, in CB samples prior to

(WHOLE CB) and following MNC separation over Ficoll-Hypaque gradient (MNC CB) (Figure 1). Results demonstrate that CB grafts contain different subsets of APCs encompassing CD14$^+$ monocytes, CD19$^+$ B cells, and small amounts of CD1c$^+$CD19$^-$ mDC and BDCA2$^+$ pDC. Flow cytometry analysis after separation of MNCs revealed an increased number of monocytes (CD14$^+$) ($P = .01$) and expression of CD80, CD86 ($P = .01$), and CD40 ($P = .02$) compared to the WHOLE CB, whereas the proportion of HLA-DR$^+$ cells was not statistically different. The proportion of CB CD34$^+$ or CD14$^+$ cells expressing HLA-DR was on average 51% $\pm$ 6% and 35% $\pm$ 19%, respectively, and the proportion expressing CD86 was 1.9% $\pm$ 0.8% and 82% $\pm$ 5%, respectively.

### Lack of T Cell Alloreactivity to CB MNCs

To test CB MNCs capacity of stimulating T cell responses in vitro, we initially irradiated CB MNCs and mixed them with allogeneic T cell responders (1:1 ratio) in MLC. In addition, irradiated T cell-depleted (TCD) CB MNCs were also used as stimulators to test the hypothesis of whether by removing CB T cells we could eliminate a subset of T cells with suppressive activity. CB T cells and autologous PB MNCs were utilized after irradiation as negative controls and third-party PB MNCs as positive control. CB MNCs did not stimulate any T cell response in primary MLC independently of the presence or absence of CB T cells (Figure 2A), suggesting that it is unlikely that regulatory T cells may inhibit CB APC activity. On the contrary, when we tested purified CB CD34$^+$ or CD14$^+$ monocytes as stimulators a clear T cell response was induced (Figure 2B). To assess if CB MNCs have a suppressive activity, CB MNCs and CD34$^+$ cells were isolated from the same CB, and increasing doses of irradiated CB MNCs were mixed together with a fixed dose of irradiated CD34$^+$ cells (ratio 0.25:1 to 1:1) in primary MLC. Add-back of CB MNC did not inhibit CD34$^+$ cell immunogenicity (Figure 2C). Similar results were also obtained mixing CB MNCs with CD14$^+$ cells (data not shown). In addition, supernatants of MLC with irradiated CB MNCs and allogeneic responders were tested for the presence of Th1/Th2 type cytokines by means of the Bio-Plex Cytokine immunoassay (Bio-Rad, Hercules, CA) and no detectable level of any of the cytokine analyzed was found (not shown). These findings demonstrate that CB MNCs do not stimulate allogeneic T cell proliferative responses. However, they do not have any suppressive function.

### CB MNC Do Not Induce T Cell Anergy

We then tested if CB MNCs would induce Ag-specific T cell unresponsiveness. T cell R that did not proliferate in primary MLC with CB MNCs were
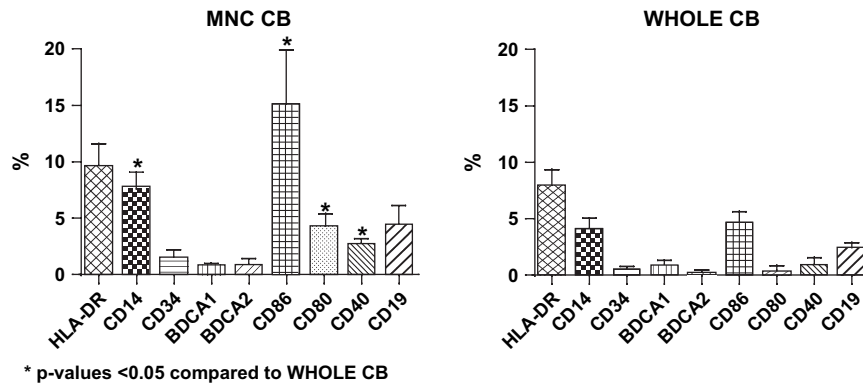
**Figure 1.** Flow cytometry analysis of APCs in CB. Phenotypic expression of markers of monocytes (CD14), mDC (CD1c), pDC (BDCA2), HSC (CD34), B cells (CD19), costimulatory molecules (CD80, CD86, CD40), and HLA-DR was analyzed in unseparated CB cells (WHOLE CB) (right side) or MNC CB (left side) (n = 7 experiments). In MNC CB it was observed that a significantly greater percentage of cells expressing CD80 ($P = .01$), CD86 ($P = .01$), CD14 ($P = .01$), and CD40 ($P = .02$) compared to WHOLE CB, whereas differences for the other markers were not statistically significant.

rechallenged in secondary MLC with irradiation CB MNCs at 1:2 ratio, or CD34$^+$ cells at a 1:1 ratio, or Mo-DCs at a 10:1 ratio, or third-party PB MNCs at a 1:2 ratio. Despite the fact that T cells were still unresponsive to CB MNC in secondary MLC, they did respond to CD34$^+$ cells and at a greater extent to Mo-DCs from the same CB (Figure 3). These results demonstrate that CB MNCs inefficient APC activity does not result in antigen-specific T cell anergy. In addition, these findings point out that, in CB, CD34$^+$ cells have greater APC activity than MNCs, but more limited than iMo-DCs generated with GM-CSF and IL4.

### Stimulation with Allogeneic T Cells Increases CB MNC Immunogenicity

Freshly isolated CD34$^+$ cells can stimulate allogeneic T cell responses efficiently [17,18]. In addition, we have recently demonstrated that CB CD34$^+$ cells can mature into CD11c$^+$CD86$^+$ APC upon stimulation with allogeneic T cells that produce cytokines

such as tumor necrosis factor (TNF)$\alpha$ and GM-CSF [15], and become more potent APCs within a few days of culture. Therefore, we tested if allogeneic T cells can also enhance the APC activity of unselected CB MNCs. Fresh CB MNC cells did not coexpress HLA-DR and CD86. Interestingly, coexpression of HLA-DR and CD86 costimulatory molecule increased in a small fraction of CB MNC after a 6-day stimulation with T cells (Figure 4A).

More importantly, CB MNCs stimulated by T cells became also capable of inducing T cell alloresponses in MLC (Figure 4B). These results suggest that a cell:cell contact with allogeneic T cells may rapidly increase the allostimulatory effect of CB MNCs.

### Delayed Cytotoxic T Lymphocyte (CTL) Activity Induced by CB MNCs

We next evaluated whether allogeneic PB CTLs can be generated by stimulation with CB MNCs. Purified PB T cells were cultured with allogeneic CB
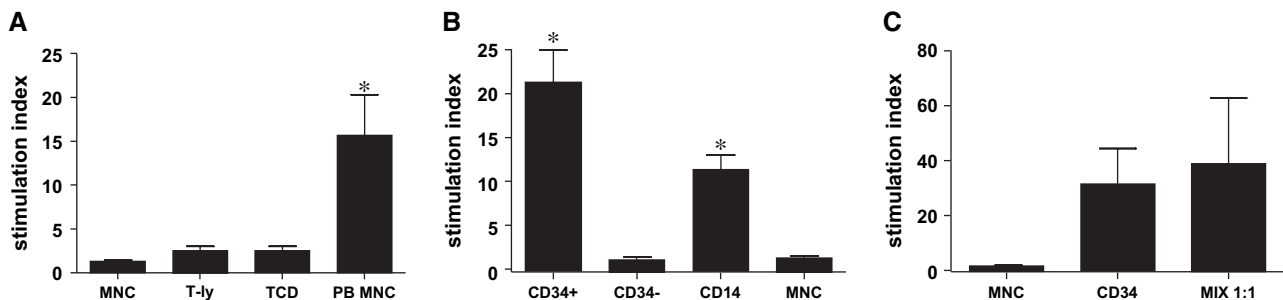


**Figure 2.** Impaired allo-antigen APC activity of CB MNC. (A) CB MNC, or CB T cells (T-ly), or T cell-depleted CB MNC (TCD), or allogeneic PB MNC as positive control, were irradiated and mixed with allogeneic T cells in a primary MLC (n = 5 experiments). PB MNC stimulated a significantly greater T cell response than all the other stimulator cells ($P < .05$). (B) Immunomagnetically purified CD34$^+$ cells, or CD34$^-$ cells, or CD14$^+$ monocytes were compared to MNC from the same CB as stimulators of T cell alloreactivity (n = 3 experiments) CD34$^+$ and CD14$^+$ cells stimulated a significantly greater T cell response than CD34$^-$ cells or CB MNCs ($P = .03$). (C) Irradiated CB MNCs, or CD34$^+$ cells, or the combination of CB MNC and CD34$^+$ cells at a 1:1 ratio were mixed with allogeneic T cell responders in MLC (n = 3 experiments). T cell alloreactivity was measured by $^3$H-Thymidine incorporation assay after 6 days of culture. Results are shown as mean SI ± SD.
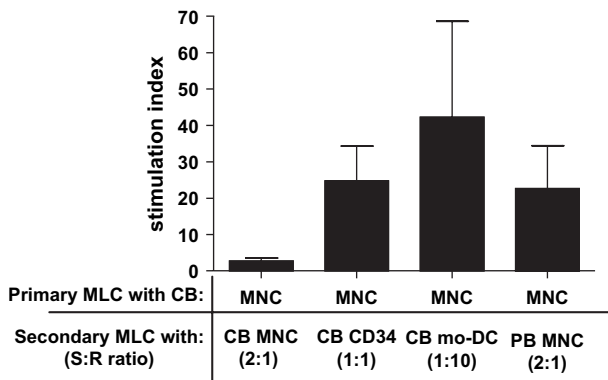
**Figure 3.** CB MNC do not induce T cell unresponsiveness. Allogeneic T cells were stimulated in primary MLC with irradiated CB MNC for 6 days and were harvested and rested for 1 to 2 days. Then they were restimulated in a secondary MLC with irradiated MNC at an S:R ratio of 2:1, or with purified CD34$^+$ cells at a 1:1 ratio, or iMo-DC at a 1:10 ratio, all derived from the same CB, for 3 days or with third-party PB MNC for 6 days. Allogeneic T cells were unresponsive to CB MNC but responded to more potent APC such as CD34$^+$ cells or iMo-DC. Results are shown as mean S.I. ± SD of 3 separate experiments.

MNCs at 2:1 ratio for 1 week, or were restimulated with CB MNCs weekly for 4 weeks, prior to assessing their CTL activity against CB PHA blasts at effector: targets ratios ranging from 6:1 to 50:1, in a standard 51Chromium release assay. CB MNCs failed to elicit any CTL activity after a standard 7-day priming of allogeneic T cell effectors. However, after 4 rounds of weekly stimulation with CB MNCs, allogeneic CTLs capable of lysing antigen-specific targets were generated (Figure 5A). In parallel experiments iMo-DC, generated by culturing CB CD14$^+$ cells with GM-CSF and IL-4 for 6 days were utilized to stimulate allogeneic T cells for 1 week prior to test them in a CTL assay. In these experiments CB iMo-DC, induced the generation of CTLs after only 1 week of culture (Figure 4B). These findings confirm that CB MNC, are weak APCs but can induce CTL delayed responses upon repeated stimulation of T cells, comparable to CTL responses induced by CB iMo-DC, in 1 week.

**Simultaneous Induction of CTL Activity by APCs Derived from 2 CB Grafts**

Because allogeneic transplantation of 2 mismatched CB units results in long-term engraftment of only 1 CB unit, we tested whether the separate or simultaneous stimulation of allogeneic T cells with 2 distinct mismatched CB Mo-DCs would result in comparable generation of PB CTLs. CD14$^+$ cells were purified from 2 CB units and iMo-DCs were generated with GM-CSF and IL-4 from each unit (CB1 and CB2) as described in the Materials and Methods section. Then, iMo-DCs were irradiated and mixed (1-3 × 10$^4$ cells/well) with allogeneic 5 × 10$^4$ T
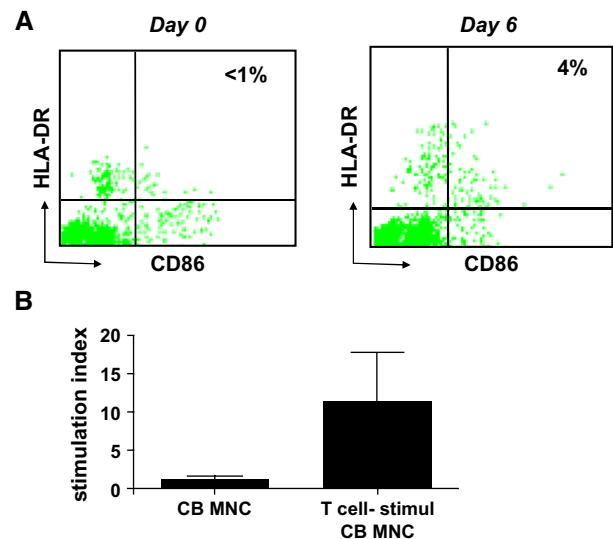


**Figure 4.** Increased APC activity of CB MNC after stimulation with allogeneic T cells. (A) Coexpression of HLA-DR and CD86 costimulatory molecule was tested by flow cytometry in CB MNC cells freshly isolated (day 0). The same staining was repeated after culturing CB MNC that had been initially stimulated with irradiated allogeneic PB T cells for 6 days and then reseparated over Fycoll/Hypaque gradients to eliminate the dead T cells (day 6) (see Materials and Methods). (B) CB MNC freshly isolated or reseparated after stimulation with T cells (T cell-stimul CB MNC) were irradiated and mixed with allogeneic PB T cell responders at a 1:1 ratio in MLC. T cell alloreactivity was measured by $^3$H-Thymidine incorporation assay after 6 days of culture. Results are the mean SI ± SD of 3 separate experiments.

cells/well for 7 days. To test whether CTL activity was induced by both CB units and if it was Ag-specific and dose-dependent, irradiated CB1 and CB2 cells were mixed at 0:1, 1:0, 1:1, 1:2, 1:3, 2:1, and 3:1 ratio prior to plating them with T cells. In 3 separate experiments, Ag-specific CTL activity was generated with both CB cells and the CB1:CB2 ratio did not seem to influence the grade of CTL activity observed. The percentage of lysis of PHA targets at 50:1 E:T ratio in 1 representative experiment is shown in Figure 6. These results suggest that Ag-specific allogeneic CTL activity can be efficiently induced by 2 distinct CB-derived APCs simultaneously.

**DISCUSSION**

In this study we demonstrate that although CB nucleated cells have a poor alloantigen presenting activity in vitro, they do not induce T cell unresponsiveness and can stimulate delayed T cell alloproliferative responses after being activated by T cells, or CTL responses after multiple stimulation of T cell effectors.

Professional APCs that circulate in CB encompass monocytes and DCs. Circulating DCs include myeloid and plasmacytoid subtypes: mDC are identified as
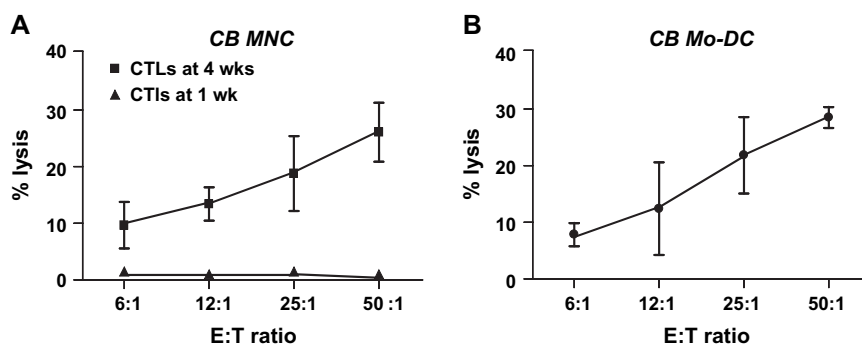
**Figure 5.** Allogeneic CTL activity induced by multiple restimulations with CB MNC. After priming allogeneic blood T cells with irradiated CB MNC for 1 or 4 weeks (A), or CB iMo-DC for 1 week (B), the cytotoxic effect against allo-antigen-specific PHA-CB blasts at an effector:target (E:T) ratio ranging from 6:1 to 50:1 was assessed in a standard $^{51}$Cr release assay. CTL generation was observed after stimulation of effectors with CB MNC for 4 weeks and with CB iMo-DC for 1 week. Autologous control effectors always showed <10% lytic activity. The results are shown as mean lytic activity ± SD of 3 separate experiments.

HLA-DR$^+$ CD123$^-$CD11c$^+$ and stimulate Th1 responses, and pDC are identified as HLA-DR$^+$ CD123$^+$CD11c$^-$ and more often induce Th2 responses [19,20]. Then, mDC can be further dissected in CD16$^+$ and CD16$^-$ fractions [21]. In CB, it has been previously shown that the percentage and absolute number of CD16$^+$ and CD16$^-$ mDC are smaller than in PB, whereas CD123$^+$ pDC are increased [22]. Nevertheless, an impaired function of pDC in newborns was demonstrated in vitro [23]. Previous experiments on myeloid DCs have shown that after activation of DCs generated from monocytes (mo-DC) with LPS, greater amounts of TNFα are produced by PB than CB DC [8]. Similarly, CB CD14$^+$ monocytes, which express less HLA class II molecules than PB counterparts, show a reduced upregulation of

HLA-DR and CD40 compared to PB monocytes upon stimulation with LPS [8] or Interferon γ (IFNγ) [24]. Because the number of mDCs is reduced in maternal blood [25] at the time of delivery, it has been speculated that the APC activity of both CB and mother PB DCs is impaired to facilitate immune tolerance during pregnancy.

In this study, we analyzed whether CB nucleated cells that are commonly utilized in HSC transplantation may stimulate T cells responses or favor allogeneic T cell unresponsiveness. Because our initial findings showed that CB MNCs do not stimulate any allogeneic T cell response in vitro, we utilized T cell-depleted MNCs to test whether the suppressive activity could be because of T cell suppressors or regulatory T cells present in the CB graft. To this purpose we utilized 2 separate methods of T cell depletion. Immunomagnetic negative selection of CD3$^+$ cells resulted in a CD3 negative cell population that showed no allostimulatory effect, comparable to the whole CB MNCs. This finding ruled out the effect of CB regulatory T cells suppressing alloreactivity, and also the hypothesis that CB MNCs may be not allostimulatory because of a greater content of resting/naïve T cells compared to PB MNCs. In contrast, after T cell depletion by selecting CD34$^+$ cells we obtained a cell population with increased APC activity. The allostimulatory effect was similar to what previously observed using marrow or blood CD34$^+$ cells [17,18]. Therefore, we hypothesize that in case T cell depletion is pursued in CB transplant, different methods may affect the immunogenic properties of the graft substantially. The different allostimulatory effect of CD34$^+$ cells and MNC within the same CB allowed us to perform add-back experiments that showed that CD34$^+$ cells maintained their immunogenic effect despite the presence of MNC at a 1:1 ratio, and therefore ruled out an inhibitory effect of CB MNCs. The next question was whether the inability of CB MNC to deliver
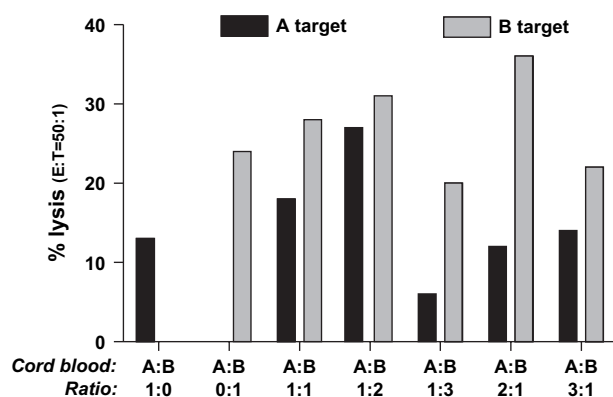


**Figure 6.** Concomitant induction of separate CTL clones induced by stimulation with APC from double CB units. Allogeneic blood T cell effectors were mixed with irradiated CB iMo-DC from 2 separate CB (CB A and B) at different CB:CB ratios ranging from 1:0 to 3:1 (CB:T cell ratio from 1:1 to 3:1) for 7 days. Then cytotoxic activity of effector cells against PHA blasts obtained from each CB (target A and target B) was measured in a standard $^{51}$Cr release assay at a 50:1 E:T ratio. This result is representative of 3 separate experiments.

adequate signaling to allogeneic T cells would result in T cell anergy. Rechallenge of T cells with CB MNCs indeed resulted in unresponsiveness. However, rechallenge of T cells initially stimulated by CB MNCs with CD34$^+$ cells or iMo-DC carrying the same alloantigen obtained potent secondary proliferative responses, ruling out the capacity of CB MNC to induce T cell anergy. A possible explanation for the inability of CB MNCs to trigger T cell responses may be because of the low expression of HLA-DR in CB MNCs, as opposed to CD34$^+$ cells, that prevents enough signaling to T cell receptor on T cells. Interestingly, after coculture of CB MNCs with irradiated allogeneic T cells for 6 days, an increase of cells coexpressing HLA-DR and CD86 was detected and CB MNCs that had been stimulated with T cells could then become capable of inducing allogeneic T cell responses in vitro. It is conceivable that this subset of CB MNCs upregulating the CD86 costimulatory molecule includes also CD34$^+$ cells, which can be rapidly induced to upregulate costimulatory molecules [26] and differentiate into APC upon contact with alloreactive T cells [15]. As expected, the weak APC activity of CB MNCs did not elicit allogeneic CTL responses in a standard 1-week assay. Nevertheless, 4 weekly restimulations of allogeneic T cells with CB MNCs resulted in the generation of CTLs capable of lysing CB targets. Altogether, these results may suggest that CB MNCs are unable to induce an immediate T cell alloreactivity. However, activation/differentiation of APC in the graft upon contact with T cells, and prolonged stimulation of allogeneic effectors by CB cells will result in both proliferative and cytotoxic responses.

These in vitro results allow us to speculate on possible in vivo effects. In fact, the scarce initial immunogenic function of CB APCs may prevent the initial rejection of the graft in recipients of HLA mismatch grafts and may contribute to reducing the development of aGVHD by the lack of indirect antigen presentation. Nevertheless, our findings suggest that CB MNCs include cells that can stimulate delayed alloreactivity. This could result in delayed graft rejection or failure of engraftment because of residual host T cells. In alternative, a delayed activation of CB-derived APCs could also contribute to the development of cGVHD by means of indirect presentation of alloantigens [14].

We also show here that CTL responses obtained after 4 weeks of restimulation of T cell effectors with CB MNCs were similar to what observed in 1 week with CB iMo-DCs. Then, in our experiments stimulating T cells with CB iMo-DCs from 2 CB units concomitantly, we could generate antigen-specific CTLs against each CB unit independently of the CB:CB ratio. We expect that a few weeks after transplant CB MNCs will be able to activate alloresponses of T cells of the host, and/or of the second CB unit in case of double transplant. In vivo experiments in nonobese diabetic(NOD)/SCID/γc$^{null}$ mice have previously shown that both CB CD4$^+$ and CD8$^+$ cells are necessary to reproduce the skewed chimerism observed in human recipients [27]. Despite the mechanism(s) for the competitive long-term engraftment of only 1 CB unit observed in recipients of double CB transplant still remains unclear, our data may suggest that changes in the phenotype and function of CB cell subsets after transplants should be further investigated as possible mediators of selective T cell alloreactivity or in vivo peripheral tolerance.

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