Tolerance associated with cord blood transplantation may depend on the state of host dendritic cells

Running title: Dendritic cells and neonatal tolerance

ENMEI LIU[1,2], HELEN KW LAW[2], YU-LUNG LAU[2]

[1]Division of Respiratory Disorders, Children's Hospital, Chongqing University of Medical Sciences, Chongqing; [2]Department of Paediatrics & Adolescent Medicine, Faculty of Medicine, The University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong, China. [E.L., H.K.W.L., Y.L.L.]

Correspondence:

Dr. Yu-Lung Lau

Department of Pediatrics & Adolescent Medicine

Faculty of Medicine

The University of Hong Kong

Queen Mary Hospital

Pokfulam, Hong Kong, China

Tel: (852) 2855 4205

Fax: (852) 2855 1523

E-mail: lauylung@hkucc.hku.hk

Summary

Allogeneic cord blood (CB) transplantation is associated with less severe GvHD, thought to be due to the immaturity of CB T cells, but how T cells interact with host and donor-derived dendritic cells (DCs) to initiate GvHD has not been elucidated. We therefore investigated the responses of CB and adult blood $CD4^+$ T cells co-cultured with adult host DCs of different maturities. Primed by adult host DCs, CB and adult blood $CD4^+$ T cells underwent similar changes in the expression of CD45RA/45RO, CD25, CD40L and CTLA-4. However, CB $CD4^+$ T cells, when primed by either immature or BCG-treated adult host DCs, produced lower IFN-γ and higher IL-10, which is of regulatory T cell-like cytokine profile, as compared to adult blood $CD4^+$ T cells. On the contrary, LPS-treated adult host DCs significantly up-regulated IFN-γ and down-regulated IL-10 production levels from CB $CD4^+$ T cells to that from adult blood $CD4^+$ T cells. The sustained low IFN-γ and high IL-10 production from CB $CD4^+$ T cells co-cultured with adult blood DCs might account for the less severe GvHD occurrence after CB transplantation, which could be reversed by LPS-treated adult blood DCs.

Keywords: cord blood, dendritic cell, T regulatory cell, cytokine, tolerance

INTRODUCTION

Allogeneic bone marrow transplantation has been used as an effective treatment for various malignancies and genetic disorders (Tabbara, 1996; Ikehara, 1998), however, graft-versus-host disease (GvHD) remains a major complication despite significant advances have been made in histocompatability matching and conditioning regimens (Hows *et al*, 1986). Recently, allogeneic umbilical cord blood transplantation is increasingly being used for haematopoeitic reconstitution and it has a lower incidence of severe GvHD than bone marrow transplantation (Gluckman *et al*, 1997). The definitive mechanism for this advantage of cord blood is unknown. Since GvHD is initiated by the direct recognition of alloantigens on recipient antigen-presenting cells (APCs) by donor T cells, the most common explanation for the low incidence of GvHD after cord blood transplantation is the reduced and naïve T cell function in comparison with adult blood T cells. Cord blood contains higher percentage of the naïve $CD4^+CD45RA^+$ T cells; lower antigen and mitogen specific T cell proliferation; and lower cytokine production on stimulation (Cohen & Madrigal, 1998). Recently, the tolerant nature of cord blood T cells to produce high levels of IL-10 has been suggested as an underlying factor in the reduced incidence of GvHD associated with cord blood transplantation (Levings *et al*, 2001; Rainsford & Reen, 2002).

As the professional APCs, dendritic cells (DCs) play a critical role in initiating primary T cell response (Steinman, 1991). Direct presentation of host alloantigens by resident host DCs is likely to exert a dominant effect on the activation of donor T cells, resulting in both acute and chronic GvHD (Shlomchik *et al*, 1999). Through modulating cytokine profile of donor DCs or promoting Th2-inducing DCs, granulocyte colony-stimulating-factor decreases acute GvHD (Arpinati *et al*, 2000; Reddy *et al*, 2000). Matthews *et al* (2000) reported that when co-culturing cord blood T cells and adult blood T cells with allogeneic adult blood monocyte-

derived DCs, cord blood T cells have stronger proliferative responses to allogeneic DCs than adult blood T cells, sustained expression of CD40L and pro-inflammatory cytokine production in comparison with adult blood T cells, and speculated that reduced GvHD in cord blood transplantation could be the result of the failure of host APCs to strongly stimulate cord blood T cells rather than an innate inability of cord blood T cells to respond effectively to alloantigens.

In our previous studies, we not only demonstrated dysfunction of cord blood T cells (Tu *et al*, 1999; Tu *et al*, 2000), but also immaturity of cord blood monocyte and monocyte-derived DCs (Liu *et al*, 2001a; Liu *et al*, 2001b). Following these observations, we further investigated in this study the interaction of cord blood donor DCs/T cells with adult host DCs/T cells to test the hypothesis that the less severe GvHD with cord blood transplantation is related to the properties of both cord blood T cells and DCs. We compared the expansion, phenotypic expression and cytokine production between cord blood $CD4^+$ T cells and adult blood $CD4^+$ T cells primed by immature and mature adult DCs. In addition, we investigated the effects of cord blood DCs and adult blood DCs on adult blood $CD4^+$ T cells.

MATERIALS AND METHODS

Isolation of monocytes. Human umbilical cord blood was obtained from the placentae of normal, full-term infants, after the placentae were delivered and separated from the infants, with prior written informed consent of their mothers. The Ethics Committee of the University of Hong Kong approved the protocol. Normal adult blood came form blood donors of Hong Kong Red Cross. All samples were collected in heparinized flasks. Cord blood mononuclear cells (CBMC) and adult peripheral blood mononuclear cells (PBMC) were isolated from whole blood by centrifugation, using Ficoll-Hypaque gradients (Pharmacia Biotech, Uppsala, Sweden). Mononuclear cells at the interface were collected, washed threes times, and resuspended at $1x10^8$/ml pH 7.2 Hanks/Hepes buffer. Monocytes were isolated from mononuclear cells by positive selection using anti-CD14 conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated cells from the positive fraction were cultured at a density of $1x10^6$ cells/ml in RPMI-1640+10% FCS (Sigma Chemical Co., St.Louis, MO), supplemented with 50IU/ml penicillin and 50μg/ml streptomycin. Cell viability, as measured by trypan blue exclusion, was more than 95%. The purity of monocytes was measured by flow cytometry with 85% to 95% of the cells being CD14 positive.

Isolation of cord blood naïve $CD4^+$ T cells and adult $CD4^+$ T cells. Untouched cord blood $CD4^+$ T cells and adult blood $CD4^+$ T cells were isolated from CBMC and PBMC by negative immunomagnetic selection using the $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Generation of immature DCs in vitro. A total of $1x10^6$ monocytes were cultured in the presence of IL-4 (10ng/ml; R&D, Minneapolis, USA) and GM-CSF (50ng/ml; R&D,

Minneapolis, USA) for 7 days at 37ºC in a humidified atmosphere containing 5% CO_2 as in our previous study (Liu *et al*, 2003b). The cultures were fed with fresh RPMI-1640+10%FCS medium and cytokines every three days and cell differentiation was monitored by light microscopy.

Generation of mature DCs in vitro. Bacillus Calmette-Guerin mycobacteria (BCG) (50μl, Danish strain 1331, Statens Serum Institute, Denmark) and LPS (10μg/ml, Sigma, USA) which have been confirmed to promote DC maturation by up-regulation of MHC class II molecules and co-stimulatory molecules (Liu *et al*, 2003a) were added for the last 2 days of the immature DCs culture.

Co-cultures of different DCs with cord blood naïve $CD4^+$ T cells and adult $CD4^+$ T cells. Freshly isolated cord blood naïve $CD4^+$ T cells and adult $CD4^+$ T cells (1 x 10^6/ml) were co-cultured with immature, BCG-treated and LPS-treated adult blood DCs (DC:T cell = 1:10) for 7 days. After priming, absolute viable cell counts were obtained by Trypan blue exclusion to document T cell expansion. Cells were collected for phenotypic expression analysis and supernatants were stored at -20°C for cytokine assays.

Immunofluorescence staining. Cord blood naïve $CD4^+$ T cells and adult $CD4^+$ T cells primed with different DCs were transferred to microcentrifuge tubes, diluted with 1 ml PBS and centrifuged 13,000g for 10 seconds. The supernatant was removed, and the cell pellet was resuspended in the small amount of remaining PBS. Fluorochrome conjugated antibodies at concentrations of 4 μg/10^6 cells were added and then the cell suspension was incubated for 15 minutes at 20°C. After incubation, the cells were diluted in 1 ml of wash solution (PBS+0.1% fetal bovine serum +0.1% azide) and after centrifugation and removal of

supernatant, the cells were resuspended in 300 μl of the same solution for flow cytometric analysis. FITC, PE and PC5 conjugated isotype controls and CD45RA–FITC, CD45RO-FITC, CD4-PE antibodies were purchased from Coulter (Miama, Florida, USA). CD25-FITC antibody was purchased from PharMingen (San Diego, CA, USA). Flow cytometric analysis was performed with a COULTER EPICS ELITE Flow Cytometer (Coulter Corporation, Miami, Florida, USA). The machine was optimized daily using standard fluorescence bead (Coulter Corporation, Miami, Florida, USA). Ten thousand events per sample were collected into listmode files and analyzed by the WINMDI 2.8 analysis software.

Analysis of CD40L and CTLA-4 expression. After primed by DCs for 7 days, CD4+ T cells were resuspended in fresh RPMI-1640+10% FCS at $1x10^6$/ml and re-stimulated with PMA (25ng/ml, Sigma, Saint Louis, Missouri, USA) and ionomycin (1μg/ml, Sigma, Saint Louis, Missouri, USA) for 5 hours. Surface staining of CD40L-PE was performed before the intracellular staining (as described below) of CTLA-4-PC5. Both CD40L-PE and CTLA-4-PC5 antibodies were purchased from PharMingen (San Diego, CA, USA).

Analysis of IFN-γ expression. Briefly, $1x10^6$/ml fresh isolated CBMC/PBMC or live T cells after primed by DCs for 7 days were simulated or re-simulated by PMA+ionomycin for 5 hours. Brefeldin A (10μg/ml, Sigma, Saint Louis, Missouri, USA) was added after the first hour of culture to stop the golgi activities and then for further 4 hours culture. Intracellular staining was performed as described below and IFN-γ-FITC antibody was purchased from PharMingen (San Diego, CA, USA).

Intracellular immunofluorescence staining

In genereal, cells for intracelluar staining were washed with PBS, resuspended in 500μl of 1xFACS ™ Lysing Solution (BD immunocytometry systems, San Jose, CA, USA), then vortexed and incubated for 10 min at room temperature. Cells were washed with PBS and centrifuged at 500g for 5 min, then 500μl of 1xFACS Permeabilizing Solution was added. Cells were vortexed and incubated for 10 min at room temperature in the dark. Cells were washed by adding PBS containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3, and centrifuged for 5 min. Twenty microlitres of fluorencent-conjugated intracellular antibodies were added into cell suspension, vortexed and incubated for 30 min at room temperature in the dark.

Detection of IFN-γ, IL-10, IL-4 production by T cells primed by DCs. After 7 days culture, the supernatants of cord blood and adult blood T cells cocultured with DCs were harvested and stored at –20°C**.** The levels of IFN-γ, IL-10, IL-4and were measured by ELISA kits (R&D Systems Inc, Minneapolis, USA). The minimal detection levels of IFN-γ, IL-10, and IL-4 were 15.6pg/ml, 62.5pg/ml and 31.2pg/ml respectively.

Statisical Analysis

All samples were paired and differences between groups analysed by paired Student t test or the non-parametric equivalents. In most cases, data were expressed as mean±SEM.

RESULTS

The percentages of $CD4^+CD25^+$ cells were the same in cord blood $CD4^+$ T Cells and adult blood $CD4^+$ T cells

The percentages of $CD4^+CD25^+$ cells, which represent a population of regulatory T cells responsible for inducing tolerance (Sakaguchi *et al*, 1995; Takahashi *et al*, 1998; Itoh *et al*, 1999), was the same in purified cord blood $CD4^+$ T cells (6±0.8%, n=8) and adult blood $CD4^+$ T cells (7±0.6%, n=4) (Fig 1A). As in our previous report (Tu *et al*, 2000), more than 95% cord blood $CD4^+$ T cells were $CD45RA^+$ naïve T cells compared to 68% in adult T cells, while less than 5% cord blood $CD4^+$ T cells were $CD45RO^+$ memory T cells compared to 32% in adult $CD4^+$ T cells (Fig 1A).

The percentage of IFN-γ^+ cells from CBMC was reduced as compared to PBMC upon PMA plus ionomycin stimulation

Freshly isolated CBMC and adult PBMC were incubated in RPMI+10%FCS with PMA+ionomycin for 5 hours, then the percentages of IFN-γ^+ cells were detected by intracellular cytokine staining (Fig 1B). Remarkably higher percentage of IFN-γ^+ cells was detected in PBMC (33±3%, n=8) than in CBMC (4±0.8%, n=8, $p<0.001$) (Fig 1C).

Cord blood $CD4^+$ T cells had higher capacity to expand than adult blood $CD4^+$ T cells

After 7 days of co-culturing with adult blood immature DCs, the cell number did not increase significantly in both cord blood (n=10) and adult blood (n=4) $CD4^+$ T cells. However, when primed by BCG-treated and LPS-treated adult blood DCs, there was significantly higher cell number in cord blood T cells than that in adult blood T cells (Fig 2A).

CD45RA/RO expression on cord blood CD4$^+$ T cells and adult blood CD4$^+$ T cell became the same after priming with DCs

After co-culturing with immature and BCG-treated adult blood DCs for 7 days, the percentage of CD45RA$^+$ cells decreased and the percentage of CD45RO$^+$ cells increased in both cord blood CD4$^+$ T cell and adult blood CD4$^+$ T cells. BCG-treated DCs did not promote further differentiation of CD45RA$^+$ cells to CD45RO$^+$ cells in either cord blood or adult blood CD4$^+$ T cells as compared with immature DCs (Fig 2B). Hence both immature and BCG-treated mature DCs can promote maturation but only BCG-treated or LPS-treated mature DCs can promote expansion of CD4$^+$ T cells.

BCG-treated DCs induced higher percentage of CD25$^+$ cells in both cord blood and adult blood CD4$^+$ T cells

Similar percentages of CD25$^+$ cells were observed in cord blood CD4$^+$ T cells and adult CD4$^+$ T cells after priming with adult blood DCs. Compared with immature DCs, BCG-treated DCs induced significantly higher percentage of CD25$^+$ cells in both cord blood ($p=0.0072$) and adult blood ($p=0.0121$) CD4$^+$ T cells (Fig 2C).

High surface CD40L expression and intracellular CTLA-4 expression in cord blood naïve CD4$^+$ T cells compared with that in adult blood CD4$^+$ T cells after priming with DCs and re-stimulated by PMA plus ionomycin

Cord blood CD4$^+$ T cells were co-cultured with immature DCs, BCG-treated or LPS-treated DCs for 7 days, and re-stimulated by PMA+ionomycin for 5 hours before the surface staining and intracellular staining for CD40L and CTLA-4 respectively. Preliminary experiments showed little cytosolic expression of CD40L and surface expression of CTLA-4 (data not

shown). Hence, subsequent experiments were focused on surface expression of CD40L and cytosolic expression of CTLA-4.

After co-culturing with immature or BCG/LPS-treated adult blood DCs, cord blood T cells had higher percentage of $CD40L^+$ cells than on adult $CD4^+$ T cells, but did not reach statistical significance (Fig 3A). After priming with immature and BCG-treated adult blood DCs, relatively higher expression of CTLA-4 were found in cord blood $CD4^+$ T cells than that in adult blood $CD4^+$ T cells, but did not reach statistical significance (Fig 3B). Compared with immature DCs, BCG-treated DCs significantly down-regulated CTLA-4 expression in adult blood $CD4^+$ T cells ($p=0.0195$) but not in cord blood naïve $CD4^+$ T cells. Whereas, LPS-treated DCs significantly down-regulated the percentages of $CTLA\text{-}4^+$ cells both in cord blood $CD4^+$ T cells ($p=0.0134$) and adult blood $CD4^+$ T cells ($p=0.0042$).

Sustained lower IFN-γ and higher IL-10 production from cord blood $CD4^+$ T cells and as compared to adult blood $CD4^+$ T cells was reversed by LPS-treated DCs

The lower percentage of IFN-γ positive cells in cord blood than in adult blood T cells co-cultured with immature DCs became similar in both cord blood and adult blood T cells after co-cultured with LPS-treated DCs (Fig 4A). Immature and BCG-treated DCs induced significantly lower IFN-γ production (Fig 4B) and higher IL-10 (Fig 4C) from cord blood $CD4^+$ T cells than that of adult blood $CD4^+$ T cells. However, primed by LPS-treated DCs, similar levels of IFN-γ and IL-10 production were found in cord blood naïve $CD4^+$ T cells and adult $CD4^+$ T cells (Figs 4B & 4C). Our results showed both cord blood and adult blood $CD4^+$ T cells produced little IL-4 after primed by DCs (Fig 4D).

LPS-treated cord blood DCs induced adult blood CD4$^+$ T cells to produce lower level of IFN-γ and higher level of IL-10 than LPS-treated adult blood DCs

In allogeneic bone marrow transplantation the unusual situation arises in which both host and donor-derived DCs are present (Shlomchik *et al*, 1999). Our results showed LPS-treated cord blood DCs induced significantly lower level of IFN-γ (Fig 5A) and higher level of IL-10 (Fig 5B) than LPS-treated adult blood DCs from adult blood CD4$^+$ T cells. Adult blood CD4$^+$ T cells produced little IL-4 after primed by adult or cord blood DCs (Fig 5C).

DISCUSSION

Less severe GvHD with cord blood transplantation has made cord blood an attractive alternative to bone marrow for transplantation. It is generally accepted that the reduced and naïve T cells function of cord blood T cells in comparison with adult blood T cells result in the low incidence of GvHD after cord blood transplantation (Cohen & Madrigal, 1998). In freshly isolated $CD4^+$ T cells, we found that cord blood had significantly higher $CD45RA^+$ and significantly lower $CD45RO^+$ cells than adult blood (Fig 1A) and both had about 6% $CD4^+CD25^+$ T cells which represent a population of regulatory T cells (Sakaguchi *et al*, 1995; Takahashi *et al*, 1998; Itoh *et al*, 1999) that are distinct from recently activated $CD4^+$ T cells (Ng *et al*, 2001). There was no difference in the percentage of $CD25^+$ cells between freshly purified cord blood $CD4^+$ T cell and adult $CD4^+$ T cell as reported before (Dieckmann *et al*, 2001; Ng *et al*, 2001). Similar to the results of Chalmers *et al* (1998), we found that upon stimulation by PMA and ionomycin, cord blood mononuclear cells had lower percentage of IFN-γ^+ producing cells as compared to adult peripheral blood mononuclear cells (Fig 1**C**) and this is related to the more naïve T cells in cord blood than in adult blood (Chalmers *et al*, 1998).

Dendritic cells play a critical role in initiating primary T cell response (Steinman, 1991). Co-culturing allogeneic host DCs with donor T cells is a physiological model to understand how cord blood T cells might respond to alloantigens in the initiation of GvHD (Matthew *et al*, 2000). Consistent with previous study (Matthew *et al*, 2000), we found that no matter primed with immature or BCG-treated/LPS-treated adult DCs, cord blood $CD4^+$ T cells had a higher capacity to expand than adult blood $CD4^+$ T cells (Fig 2A) and expressed similar level of CD40L (Fig 3A). Moreover, the percentages of $CD45RA^+$ T cells decreased and $CD45RO^+$ T cells increased to similar levels in both cord blood and adult blood $CD4^+$ T cells (Fig 2B).

The expression of CD25 and CTLA-4 on cord blood and adult blood $CD4^+$ T cells had no difference as well (Fig 2C). These results suggested that cord blood T cells had stronger proliferative response than adult blood T cells to mature allogeneic DCs (Matthews *et al*, 2000), and had similar potential as adult blood T cells to undergo maturation and activation process. These results also suggested that although immature and mature DCs had similar effect on CD4+ T cell maturation process, mature DCs had higher potential than immature DCs to induce T cell proliferation and activation in both cord blood and adult blood.

The relative amount of T helper (Th) 1-type versus Th2-type cytokines appears to be important in determining the extent of GvHD (Ferrara *et al*, 1996; Blazar *et al*, 1997). Increased expression of IFN-γ is associated with acute GvHD in both human and experimental animal models (Troutt & Kelso, 1993; Dickinson *et al*, 1994; Szebeni *et al*, 1994). However, as a member of the Th2 cytokine family, IL-10 is the most potent anti-inflammatory cytokine to inhibit the production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α that are thought to contribute to the development of GvHD (D'Andrea *et al*, 1993; Moore *et al*, 1993). The relationship between IL-10 production and reduced GvHD is evidenced by several publications. In murine model, IL-10 might prevent the induction of GvHD (de La Selle *et al*, 1998). Inhibition of GvHD reactivity has also been associated with high level of IL-10 production in vivo (Krenger *et al*, 1994, Rainsford & Reen 2002) postulated that the production of IL-10 in abundance maybe the regulator of increased tolerance associated with cord blood transplantation. With this physiological model, we further confirmed after priming by immature or BCG-treated adult blood DCs, cord blood $CD4^+$ T cells produced not only lower level of IFN-γ (Fig 3B), but higher level of IL-10 (Fig 3C) than adult blood $CD4^+$ T cells, whereas IL-4 production levels remained very low in both cord blood and adult blood $CD4^+$ T cells (Fig 3D). This cytokine profile from cord blood

$CD4^+$ T cells was similar to that of the regulatory T cell (Tr1) cytokine profile. Tr1 cells are distinct from classical Th1 or Th2 cells in that they produce high levels of IL-10, moderate amounts of TGF-β, IFN-γ and IL-5, low IL-2 and no IL-4. More importantly, Tr1 cells suppress immune responses in vitro and in vivo via a mechanism that is partially dependent on the production of immunoregulatory cytokines IL-10 and TGF-β (Groux *et al*, 1997; Cottrez *et al*, 2000). Since IL-10 is clearly an important regulatory cytokine and has been implicated in some models of transferable T cell tolerance (Groux *et al*, 1996), sustained higher level of IL-10 production by cord blood $CD4^+$ T cells might induce other donor T cells such as cytotoxic T cells to be tolerant, resulting in reduced severe GvHD occurrence after cord blood transplantation. Based on our results, we postulated that, after priming by host DCs, cord blood $CD4^+$ T cells were skewed to a Tr1-like profile with high IL-10, medium IFN-γ and no IL-4 production, while adult $CD4^+$ T cells, however, were prone to polarize towards Th1 with low IL-10 and high IFN-γ production.

The direction of T cell polariztion induced by DCs may depend not only on DC lineages, but also their method of isolation and maturation (Cella *et al*, 1999), the ratio of DCs to T cells (Tanaka *et al*, 2000), maturation stage (Kadowaki *et al*, 2000) or the duration of DC activation (Langenkamp *et al*, 2000). In humans, monocyte-derived $CD11c^+$ DCs polarize naïve T cells predominantly towards a Th1 profile, whereas the $CD11c^-$ DC subset induced T cells produce Th2 response (Rissoan *et al*, 1999). Jonuleit *et al* (2000) and Dhodapkar *et al* (2001) strongly suggested that immature DCs may control peripheral tolerance by inducing the differentiation of human Tr1. Based on our previous experience (Liu *et al* 2001b; 2003a; 2003b), we selected monocyte-derived DCs to T cells at a ratio of 1:10 to get high response in the co-culture. Primed by immature DCs, cord blood $CD4^+$ T cells produced higher level of IL-10 and lower level of IFN-γ than adult blood $CD4^+$ T cells. BCG-treated DCs up-regulated

IFN-γ and down-regulated IL-10 production and CTLA-4 expression from cord blood $CD4^+$ T cells but did not reach adult levels. Although BCG can promote DCs maturation (Thurnher *et al*, 1997; Kim et al, 1999), we documented BCG was not as efficient as LPS to up-regulate CD86 and Fas expression, and induce IL-12 production from DCs (Liu *et al*, unpublished data). We then showed in this present study that LPS-treated adult blood DCs could skew cord blood $CD4^+$ T cells to Th1 polarization as their sustained low IFN-γ and high IL-10 production was reversed. LPS has been implicated in the pathogenesis of GvHD (Tutschka, 1988) and toll-like receptor 4, a major receptor for LPS, is associated to risk of GvHD occurrence (Lorenz *et al*, 2001). We therefore speculate that LPS might, through activating host DCs to stimulate donor T cells, increase the GvHD risk in the clinical setting of gram negative bacterial sepsis. Further elucidation of this pathway may lead to novel strategy to decrease the risk of GvHD.

The role of donor DCs on GvHD occurrence has not been elucidated. G-CSF modulates cytokine profile of dendritic cells and decreases GvHD through effect on the donor DCs (Reddy *et al*, 2000). It is conceivable that transplantation of G-CSF-stimulated peripheral blood stem cells does not result in overwhelming acute GvHD because the graft contains predominantly Th2-inducing DCs (Arpinati *et al*, 2000). Our results indicated that adult $CD4^+$ T cells produced similar level of IL-10 but lower level of IFN-γ after priming by immature cord blood DCs as compared by adult blood DCs. However, LPS-treated cord blood DCs, as immature cord blood DCs, induced similarly production low level of IFN-γ and high level of IL-10 production by adult blood $CD4^+$ T cells. Hence, besides the intrinsic skewing of cord blood T cells to produce low level of IFN-γ and high level of IL-10, cord blood DCs could also skew adult blood $CD4^+$ T cells to produce low IFN-γ and high IL-10.

The mechanism of how cord blood DCs might impact on less severe GvHD occurrence after cord blood transplantation needs to be studied further.

We concluded that the sustained low IFN-γ and high IL-10 production from cord blood $CD4^+$ T cells might account for the less severe GvHD occurrence after cord blood transplantation but this could be reversed by LPS-treated adult blood DCs. Clinical use of immature DCs to promote Tr1 cell development and strategy to avoid activation of host DCs could have high impact on the reduction of GvHD.

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LEGENDS

Fig 1. Comparison of the percentages of subsets of purified $CD4^+$ T cells **(A)**, IFN-γ^+ cells in CBMC and PBMC stimulated by PMA+ionomycin **(B,C)** between cord blood and adult blood. **(A)** In freshly isolated cord blood (n=8) and adult blood $CD4^+$ T cells (n=4), the percentage of $CD4^+CD25^+$ cells which represent regulatory T cells had no significant difference ($p>0.05$). **(B)** Stimulated by PMA (25ng/ml) plus ionomycin (1μg/ml) for 5 hours, the IFN-γ^+ CBMC can be detected by intracellular cytokine staining. The figure shows a representative flow cytometry analysis of 8 experiments. **(C) T**he percentages of IFN-γ^+ cells in CBMC (n=8) was significantly higher than that in PBMC (n=8, $p<0.001$).

Fig 2. Comparison of absolute count of live T cells **(A)**, the percentages of $CD45RA^+$ and $CD45RO^+$ cells **(B)** and the percentages of $CD25^+$ cells in cord blood (CB) and adult blood (AB) $CD4^+$ T cells **(C).** **(A)** Primed by immature or BCG/LPS-treated mature DCs for 7 days and stained by trypan blue to exclude dead cells, the live cells were counted under microscope. BCG-treated and LPS-treated mature DCs significantly increased cell number of T cells. Cord blood $CD4^+$ T cells (n=10) had higher potential to proliferate compared to adult blood T cells (n=4) after primed by BCG-treated ($p=0.0032$) or LPS-treated DCs ($p=0.0129$). **(B)** Primed by immature or BCG-treated mature DCs for 7 days, the percentage of $CD45RA^+$ cells down-regulated and $CD45RO^+$ cells up-regulated in cord blood (n=9) and adult blood $CD4^+$ T cells (n=7) to similar levels. The effects of immature and BCG-treated DCs on phenotype conversion of cord blood and blood T cells had no difference. **(C)** Primed by immature or BCG-treated mature DCs, the percentage of $CD25^+$ cells in cord blood (n=8) and adult $CD4^+$ T cells (n=5) had no significant difference. BCG-treated mature

DCs had higher capacity than immature DCs to induce $CD25^+$ cell production both in cord blood (p=0.0072) and adult blood $CD4^+$ T cells (p=0.0121).

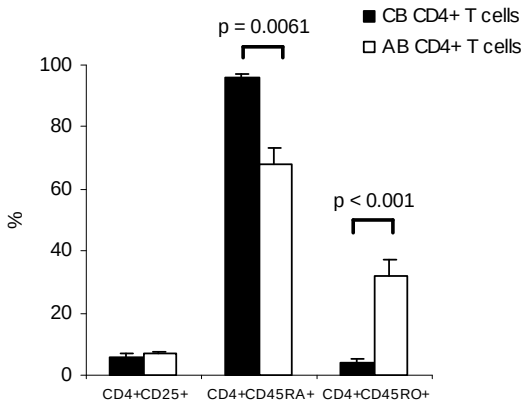
Fig 3. Comparison of the percentage of $CD40L^+$ cells **(A),** CTLA-4^+ cells **(B)** and IFN-γ^+ cells **(C)** in cord blood and adult blood $CD4^+$ T cells primed by DCs for 7days and then re-stimulated by PMA (25ng/ml) plus ionomycin (1μg/ml) for 5 hours. **(A)** No matter primed by immature or mature DCs, the percentage of $CD40L^+$ cells in cord blood $CD4^+$ T cells (n=5) was higher than that in adult $CD4^+$ T cells (n=5), but did not reach statistical significance. Compared to immature DCs, mature DCs induced by BCG and LPS did not enhance the percentages of $CD40L^+$ cells both in cord blood and adult blood $CD4^+$ T cells. **(B)** CTLA-4 expression was similar in both cord blood and adult $CD4^+$ T cells primed by either immature or LPS-treated DCs. Compared to immature DCs, BCG-treated DCs significantly down-regulated CTLA-4 expression in adult blood $CD4^+$ T cells (p=0.0195), while LPS-treated DCs significantly down-regulated the percentages of CTLA-4^+ cells in both cord blood (p=0.0134) and adult blood $CD4^+$ T cells (p=0.0042).

Fig 4. Comparison of percentages of IFN-γ^+ cells **(A)** and IFN-γ **(B),** IL-10 **(C),** IL-4 **(D)** and production in the supernatant of cord blood and adult blood T cells primed by adult DCs for 7 days and detected by ELISA. **(A)** Immature DCs induced significantly lower IFN-γ^+ cells in cord blood $CD4^+$ T cells (n=14) compared to adult blood $CD4^+$ T cells (n=7, p<0.0001), however, BCG-treated and LPS-treated mature DCs induced similar level of IFN-γ^+ cells in cord blood and adult blood $CD4^+$ T cells. **(B)** When primed by immature or BCG-treated DCs, cord blood $CD4^+$ T cells (n=10) produced significantly lower level of IFN-γ than adult blood $CD4^+$ T cells (n=6, p=0.0005 and p=0.007 respectively). LPS-treated DCs up-regulated IFN-γ production by cord blood

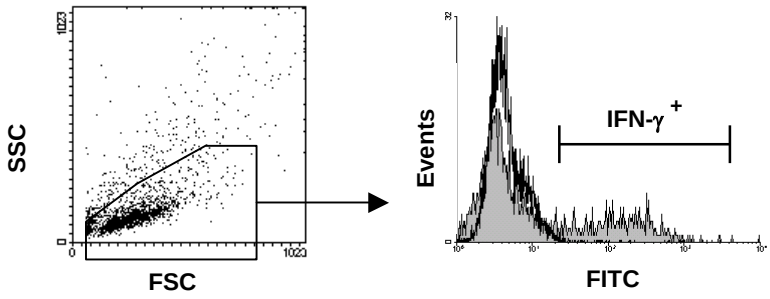
$CD4^+$ T cells (n=2) to the level by adult $CD4^+$ T cells (n=6). **(C)** Contrary to IL-10 production, significantly higher level of IL-10 production was found in cord blood $CD4^+$ T cells (n=7) compared to adult $CD4^+$ T cells (n=6, p=0.0262 and p=0.0088) when primed by adult immature and BCG-treated DCs respectively. LPS-treated DCs induced similar level of IL-10 production from cord blood (n=6) and adult blood $CD4^+$ T cells (n=6). **(D)** Whether primed by immature or BCG/LPS-treated mature DCs, little IL-4 was produced from both cord blood (n=6) and adult blood T cells (n=6).

Fig 5. Comparison of effects of cord blood and adult blood DCs on adult blood $CD4^+$ T cells. **(A)** Adult blood $CD4^+$ T cells produced significantly higher level of IFN-γ when primed by immature or LPS-treated mature adult blood DCs (n=6) than when primed by the corresponding cord blood DCs (n=6). **(B)** Primed by immature cord blood DCs (n=7) or adult blood DCs (n=7), adult blood $CD4^+$ T cells produced similar level of IL-10. Compared to adult blood DCs, both BCG-treated and LPS-treated cord blood DCs induced significantly higher levels of IL-10 production from adult blood $CD4^+$ T cells. **(C)** Whether primed by immature or BCG/LPS-treated mature cord blood or adult blood DCs, adult blood $CD4^+$ T cells produced little IL-4.

A


CB CD4+ T cells
AB CD4+ T cells
p = 0.0061
p < 0.001
%
100
80
60
40
20
0
CD4+CD25+
CD4+CD45RA+
CD4+CD45RO+

B


SSC
FSC
Events
IFN-γ +
FITC

C

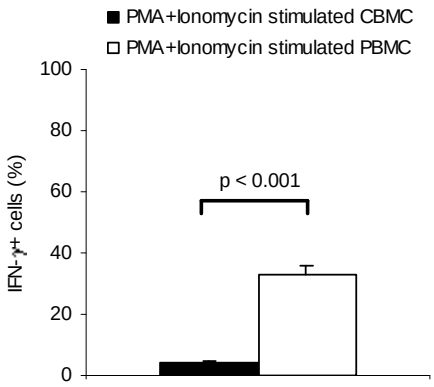

PMA+Ionomycin stimulated CBMC
PMA+Ionomycin stimulated PBMC
p < 0.001
IFN-γ+ cells (%)
100
80
60
40
20
0

Fig 1

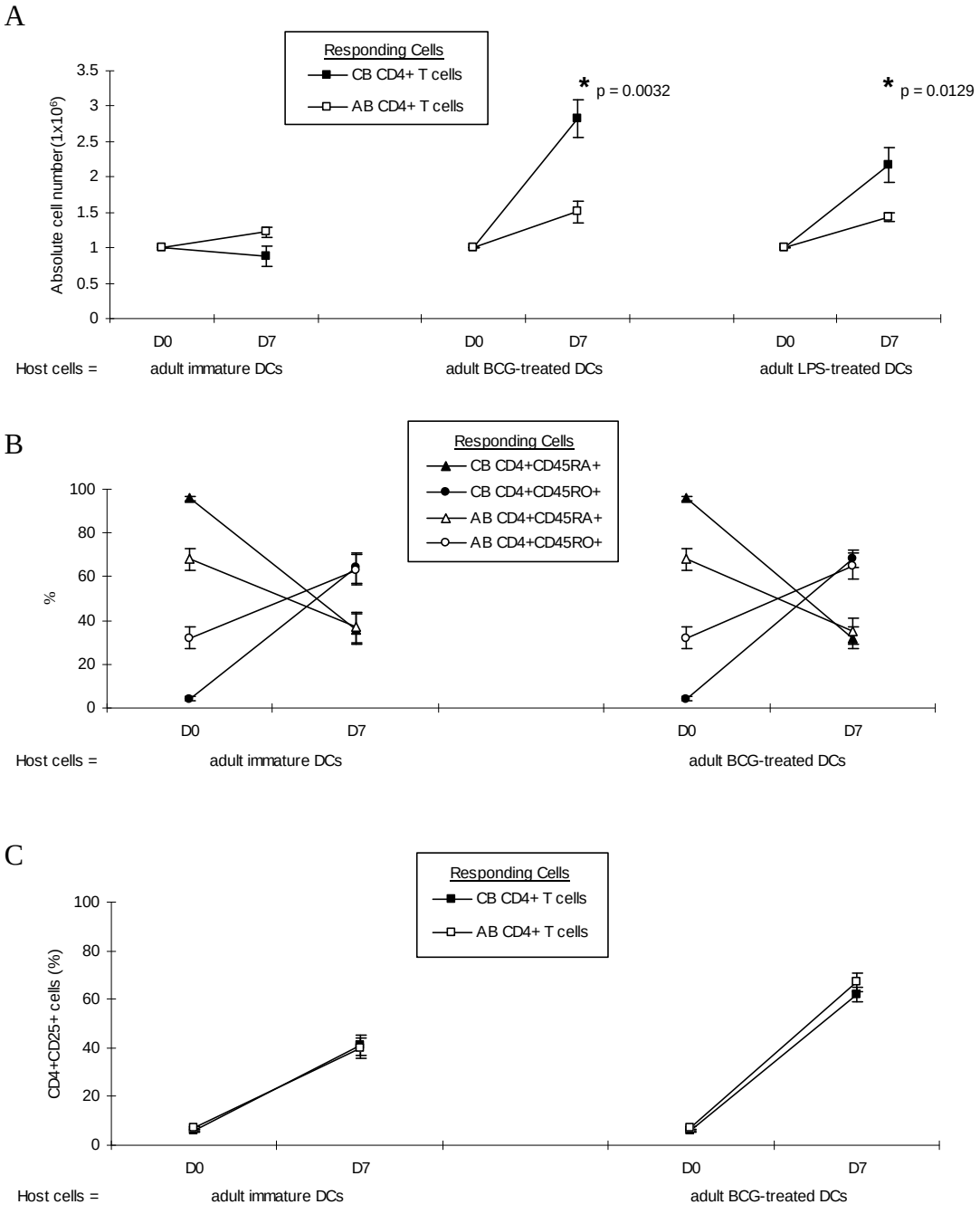
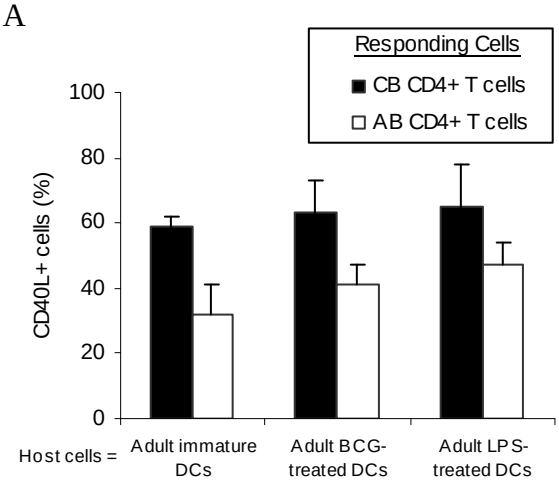

A
Responding Cells
CB CD4+ T cells
AB CD4+ T cells
Absolute cell number(1x10⁶)
3.5
3
2.5
2
1.5
1
0.5
0
* p = 0.0032
* p = 0.0129
D0
D7
Host cells =
adult immature DCs
adult BCG-treated DCs
adult LPS-treated DCs
B
Responding Cells
CB CD4+CD45RA+
CB CD4+CD45RO+
AB CD4+CD45RA+
AB CD4+CD45RO+
%
100
80
60
40
20
0
Host cells =
adult immature DCs
adult BCG-treated DCs
C
Responding Cells
CB CD4+ T cells
AB CD4+ T cells
CD4+CD25+ cells (%)
Host cells =
adult immature DCs
adult BCG-treated DCs

Fig 2

A


Responding Cells
CB CD4+ T cells
AB CD4+ T cells
CD40L+ cells (%)
100
80
60
40
20
0
Host cells =
Adult immature DCs
Adult BCG-treated DCs
Adult LPS-treated DCs

B

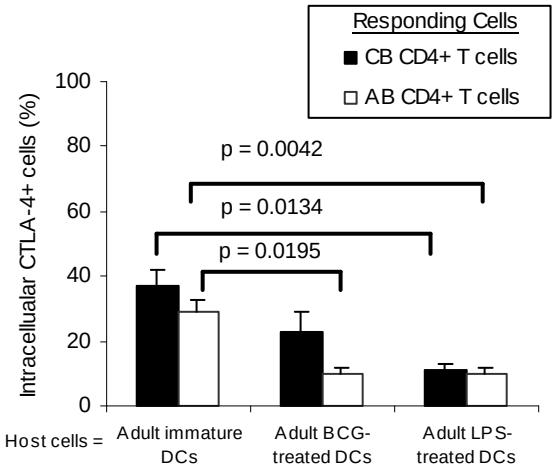

Responding Cells
CB CD4+ T cells
AB CD4+ T cells
Intracellualar CTLA-4+ cells (%)
100
80
60
40
20
0
p = 0.0042
p = 0.0134
p = 0.0195
Host cells =
Adult immature DCs
Adult BCG-treated DCs
Adult LPS-treated DCs

Fig 3

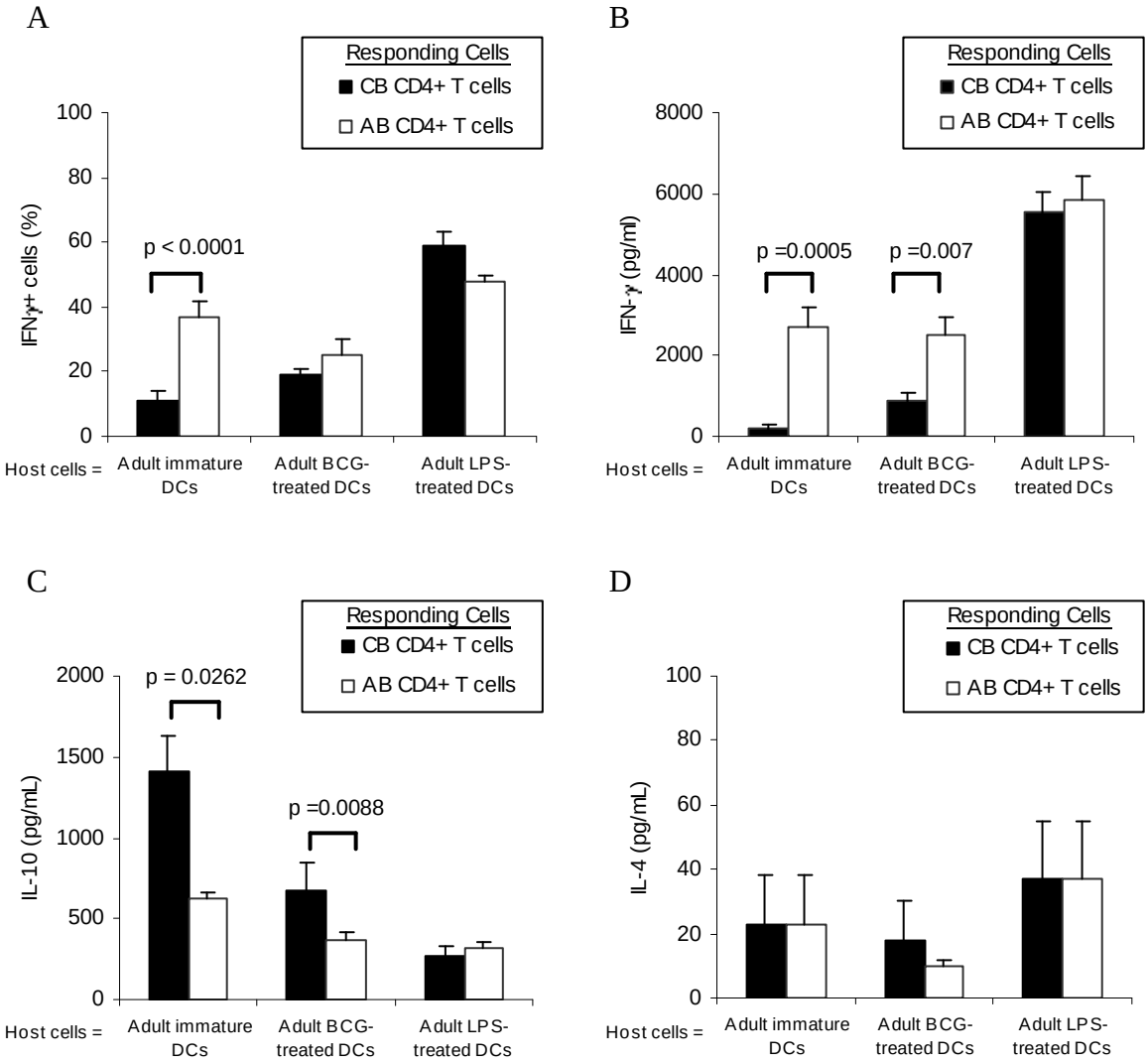
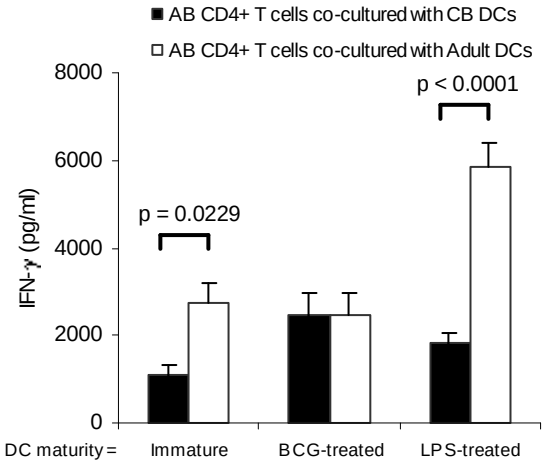
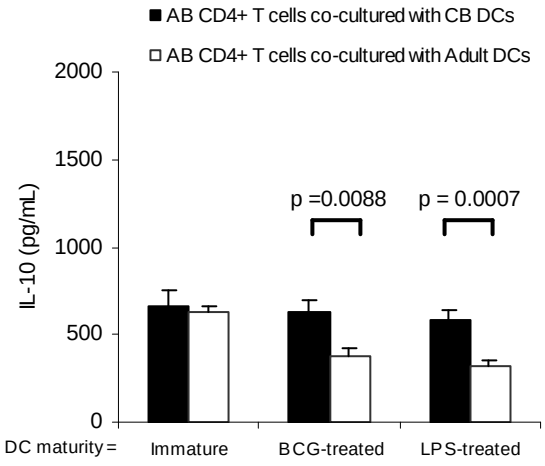

A
Responding Cells
CB CD4+ T cells
AB CD4+ T cells
IFN-γ+ cells (%)
100
80
60
40
20
0
p < 0.0001
Host cells =
Adult immature DCs
Adult BCG-treated DCs
Adult LPS-treated DCs
B
Responding Cells
CB CD4+ T cells
AB CD4+ T cells
IFN-γ (pg/ml)
8000
6000
4000
2000
0
p =0.0005
p =0.007
Host cells =
Adult immature DCs
Adult BCG-treated DCs
Adult LPS-treated DCs
C
Responding Cells
CB CD4+ T cells
AB CD4+ T cells
IL-10 (pg/mL)
2000
1500
1000
500
0
p = 0.0262
p =0.0088
Host cells =
Adult immature DCs
Adult BCG-treated DCs
Adult LPS-treated DCs
D
Responding Cells
CB CD4+ T cells
AB CD4+ T cells
IL-4 (pg/mL)
100
80
60
40
20
0
Host cells =
Adult immature DCs
Adult BCG-treated DCs
Adult LPS-treated DCs

Fig 4

A


AB CD4+ T cells co-cultured with CB DCs
AB CD4+ T cells co-cultured with Adult DCs
8000
6000
4000
2000
0
IFN-γ (pg/ml)
p < 0.0001
p = 0.0229
DC maturity =
Immature
BCG-treated
LPS-treated

B


AB CD4+ T cells co-cultured with CB DCs
AB CD4+ T cells co-cultured with Adult DCs
2000
1500
1000
500
0
IL-10 (pg/mL)
p =0.0088
p = 0.0007
DC maturity =
Immature
BCG-treated
LPS-treated

C

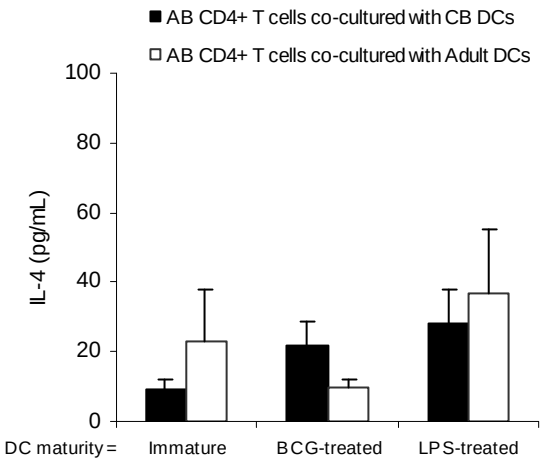

AB CD4+ T cells co-cultured with CB DCs
AB CD4+ T cells co-cultured with Adult DCs
100
80
60
40
20
0
IL-4 (pg/mL)
DC maturity =
Immature
BCG-treated
LPS-treated

Fig 5