Adult and cord blood endothelial progenitor cells have different gene expression profiles and immunogenic potential

Eugenia R. Nuzzolo[1], Sara Capodimonti[1], Maurizio Martini[2], Maria G. Iachininoto[1], Maria Bianchi[1,3], Alessandra Cocomazzi[2], Gina Zini[1,3], Giuseppe Leone[1], Luigi M. Larocca[2], Luciana Teofili[1,3]

[1]Department of Haematology; [2]Department of Pathology; [3]UNICATT Cord Blood Bank, Università Cattolica del Sacro Cuore, Rome, Italy

Background. Endothelial colony-forming cells (ECFC) are endowed with vascular regenerative ability *in vivo* and *in vitro*. In this study we compared the genotypic profile and the immunogenic potential of adult and cord blood ECFC, in order to explore the feasibility of using them as a cell therapy product.

Materials and methods. ECFC were obtained from cord blood samples not suitable for haematopoietic stem cell transplantation and from adult healthy blood donors after informed consent. Genotypes were analysed by commercially available microarray assays and results were confirmed by real-time polymerase chain reaction analysis. HLA antigen expression was evaluated by flow-cytometry. Immunogenic capacity was investigated by evaluating the activation of allogeneic lymphocytes and monocytes in co-cultures with ECFC.

Results. Microarray assays revealed that the genetic profile of cord blood and adult ECFC differed in about 20% of examined genes. We found that cord blood ECFC were characterised by lower pro-inflammatory and pro-thrombotic gene expression as compared to adult ECFC. Furthermore, whereas cord blood and adult ECFCs expressed similar amount of HLA molecules both at baseline and after incubation with γ-interferon, cord blood ECFC elicited a weaker expression of pro-inflammatory cytokine genes. Finally, we observed no differences in the amount of HLA antigens expressed among cord blood ECFC, adult ECFC and mesenchymal cells.

Conclusions. Our observations suggest that cord blood ECFC have a lower pro-inflammatory and pro-thrombotic profile than adult ECFC. These preliminary data offer level-headed evidence to use cord blood ECFC as a cell therapy product in vascular diseases.

Keywords: endothelial progenitor cells, cord blood, vasculogenesis.

Introduction

Vasculogenesis is a complex process leading to *de novo* formation of vascular structures. It results from the orchestration of various signals tailored to coordinate migration, proliferation and differentiation of different cell types in order to enable the perfusion of specific organs and tissues by blood. Although vasculogenesis is fundamental during embryonic and foetal life, vascular repair is a physiological event also throughout adult life[1]. In 1997, Asahara and colleagues demonstrated that blood vessel fixation during adult life is ensured by circulating endothelial progenitor cells which are attracted to ischaemic tissues, where they proliferate and give rise to new endothelium[2]. This "putative endothelial stem cell" population includes a large spectrum of cells and a hierarchy of progenitors with different lineage derivations and functions[3]. Although the exact immune-phenotype of putative vascular stem cells is still elusive, from the functional standpoint endothelial colony-forming cells (ECFC), identified by Ingram in 2004, are considered the unique circulating progenitors endowed with vascular regenerative ability *in vivo* and *in vitro*[3,4]. The identification of putative vascular stem cells unveiled a new scenario for treating ischaemic diseases and several trials have been conducted to improve vascular function by transplanting autologous endothelial progenitors[5-7]. Since patients with vascular diseases may have a damaged or depleted vascular stem cell pool, transplantation of allogeneic endothelial progenitors could be much more efficacious[8]. In this perspective, cord blood is a highly attractive source of ECFC[9,10], particularly because cord blood ECFC have a greater proliferative potential than adult ECFC and can, therefore, be expanded for several times without exhibiting signs of senescence[4,11].

In this study we compared the genotypic profile of endothelial cells obtained from adult and cord blood ECFC in order to highlight any differences relevant

to a variety of endothelial functions. Furthermore, we evaluated the immunogenic potential of the ECFC progeny in order to explore the feasibility of using them as a cell therapy product.

Material and methods

Cell samples

Cord blood samples were collected at the Unicatt Cord Blood Bank of the Catholic University of Rome. All samples were obtained after normal deliveries between 37 and 42 weeks of pregnancy, according to the institutional guidelines, and after informed consent. Only those units not suitable for haematopoietic stem cell transplantation because of low nucleated cell content were used for the purpose of this study. Peripheral blood samples were obtained from adult healthy donors after informed consent; the leucocyte buffy coats discarded during whole blood fractioning were used to obtain ECFC and for co-cultures of ECFC and mononuclear cells.

Cell cultures

The ECFC colony assays were performed according to the method of Ingram *et al.*[4], as previously described[12,13]. Briefly, Ficoll density-gradient isolated mononuclear cells were suspended in EGM-2 media (EGM-2 Bulletkit, Lonza, Milan, Italy) on six-well dishes coated with human fibronectin. After 2 days non-adherent cells were discharged and residual adherent cells were grown in EGM-2 medium for 28 days, with medium replacement every 3 days. Well-circumscribed monolayers of cobblestone-appearing cells, growing from day 9 to day 28, were counted as ECFC. The endothelial nature of the ECFC was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR), assessing the expression of CD34, CD146, CD45 and vascular endothelial growth factor-receptor (Figure 1A). The ability of the ECFC to form capillary-like structures on Matrigel was evaluated as detailed elsewhere (Figure 1B)[12,13]. The colonies were counted blindly by two independent investigators (ERN and MGI), with irrelevant inter-observer variations. Confluent ECFC at passages IV to VI were detached by trypsin-EDTA solution (Lonza) pooled and gently washed; the cells were then used for genotype profiling and HLA evaluation. Furthermore, in order to evaluate the ability of ECFC to activate allogeneic monocytes and lymphocytes, mononuclear cell samples were co-cultured in parallel with both adult and cord blood ECFC or mesenchymal cells for 24 hours. Non-adherent cells were harvested and then evaluated in PCR array specific for lymphocyte and monocyte activation. Each experiment was carried out in triplicate. Mesenchymal stromal cells (MSC) used for HLA evaluation were obtained from bone marrow samples as previously described[14].

Microarray analysis

Total RNA was extracted using the RNeasy Mini Kit (Quiagen, Milan, Italy) and reverted with a RT First Strand cDNA Kit (SABiociences, Qiagen) according to the manufacturer's protocol. The "Human Endothelial Cell Biology" (PAHS-015) and the "Human T Cell & B Cell Activation" (PAHS-053Z) PCR arrays were used to analyse mRNA levels of 84 key genes involved in endothelial cell biology and in T- and B-cell activation,

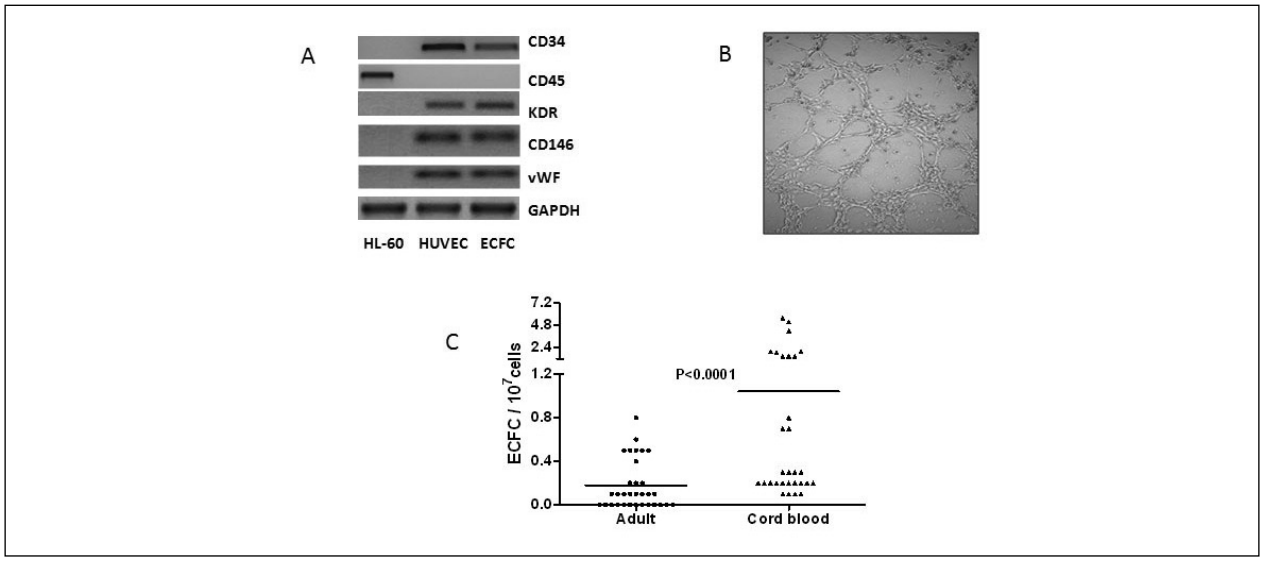

Figure 1 - Characterisation of cord blood ECFC. (A) Typical RNA transcripts assessing the endothelial origin of ECFC. Human umbilical vein endothelial cells (HUVEC) and HL-60 leukemic cells were used as endothelial and hematopoietic cell controls, respectively. (B) Capillary-like structures formed on Matrigel by ECFC. (C) The frequencies of ECFC differ significantly between adult healthy donors and cord blood samples (mean values±SEM on 107 seeded mononuclear cells). The frequencies were 1.04±0.27 for cord blood samples and 0.17±0.04 for adult samples, respectively.

respectively, in 96-well plates on CFX96ô Real-Time PCR Detection Systems (Bio-Rad, Rome, Italy). For real-time PCR, first-strand cDNA were added to the quantitative RT-PCR Master Mix (SuperArray Bioscience). Samples were heated for 10 minutes at 95 C and then subjected to 40 cycles of denaturation at 95 C for 15 seconds and annealing and elongation at 60 C for 1 minute. Data were analysed using the RT^2 Profiler PCR array data analysis template v3.0 (SABiociences). Relative changes in gene expression were calculated using the ΔΔCt (cycle threshold) method. Gene expression was analysed twice.

Real-time polymerase chain reaction analysis

The most significant results obtained in the RT^2 PCR array were validated by real-time PCR using the KAPA SYBR FAST One-Step qRT-PCR Kit (KAPABIOSYSTEMS, Boston, MA, USA), following the manufacturer's protocol, in CFX96ô Real-Time PCR Detection Systems (Bio-Rad). In detail, among the genes included in the "Human Endothelial Cell Biology" array, results were validated by RT-PCR for intercellular cell adhesion molecule-I (*ICAM1),* nitric oxide synthase (*NOS3*), tissue factor pathway inhibitor (*TFPI*), occludin (*OCLN*), von Willebrand factor (*vWF*), interleukin 1β *(IL1β*) and CASP2 and RIPK1 domain containing adaptor with death domain (*CRADD*) genes. Among the genes included in the "Human T Cell & B Cell Activation" array, results obtained for inducible costimulator-ligand (*ICOSL)*, interleukin 6 (*IL6*), interleukin 8 (*IL8)* and colony-stimulating factor -2 (*CSF2*) were validated by RT-PCR. In the former case we selected at least one gene for each cell pathway involved (adhesion, coagulation, vessel tone and permeability, inflammation, apoptosis). In the latter case, we evaluated all genes with a significant difference of expression at arrays. The forward and reverse primers used were: *OCLN*, forward 5'-AAG AGT TGA CAG TCC CAT GGC ATA C-3' and reverse 5'-ATC CAC AGG CGA AGT TAA TGG AAG-3'; *NOS3* forward 5'- TGG TAC ATG AGC ACT GAG ATC G-3' and reverse 5'-CCA CGT TGA TTT CCA CTG CTG-3'; *vWF* forward 5'-AGC CCA TTT GCT GAG CCT TG-3' and reverse 5'-CCT GGC ACC ATG CAT TTC TG-3'; *ICAM1* forward 5'- GGC CGG CCA GCT TAT ACA C-3' and reverse 5'-TAG ACA CTT GAG CTC GGG CA-3'; *CRADD* forward 5'- CCC AAA GAT ACG TGG TTG CAG-3' and reverse 5'- TGG TTT TCC GTC AAG ATT CCT-3'; *TFPI* forward 5'- ATT TCA CGG TCC CTC ATG GTG TCT-3' and reverse 5'-GGC GGC ATT TCC CAA TGA CTG AAT-3'; *IL1β* forward 5'- AAT CTG TAC CTG TCC TGC GTG TT-3' and reverse 5'-TGG GTA ATT TTT GGG ATC TAC ACT CT-3'; *ICOSL* forward 5′-CGT GTA CTG GAT CAA TAA GAC GG-3' and reverse 5′-TGA GCT CCG GTC AAA CGT GGC C-3'; *IL6* forward 5′-AGC CCT GAG AAA GGA GAC ATG TA-3' and reverse 5′-TCT GCC AGT GCC TCT TTG C-3'; *CSF-2* forward 5′- TGG CCT GGG CTT CCT CAT-3' and reversev5′-GGA TGA CAT GCC TGT CAC-3'; *IL8* forward 5′- CTC TTG GCA GCC TTC CTG ATT-3' and reverse 5′- TAT GCA CTG ACA TCT AAG TTC TTT AGC A-3'. Each analysis was performed in triplicate and *β-actin* (forward 5'-GGC GGC ACC ACC ATG TAC CCT-3' and reverse 5'-AGG GGC CGG ACT CGT CAT ACT-3') was used to normalise the gene expression in investigated samples (relative gene expression).

Human leucocyte antigen analysis

The expression of class I and II HLA was evaluated by anti-human fluorescein thiocyanate (FITC)-conjugated mouse monoclonal antibodies to HLA-ABC and HLA-DR, using an appropriate isotype control (FITC mouse IgG1k), all purchased from Becton-Dickinson (Franklin Lakes, New Jersey, USA). At baseline and after 24 hours of incubation with 10 ng/mL of γ-interferon, endothelial cells were detached from culture dishes by trypsin-EDTA solution (Lonza); cells were then washed twice in phosphate-buffered saline, incubated with specific or isotype-matched antibodies and passed through a FACScan flow cytometer (Becton-Dickinson) equipped with a 488 nm excitation light source. Results were expressed as mean fluorescence intensity (MFI) ratio, obtained normalising the value of MFI of stained cells to that of cells incubated with the isotype control antibody.

Results

Thirty cord blood samples and 32 adult blood samples were cultured to obtain ECFC. The frequency of ECFC (mean value±SEM on 10^7 plated cells) was 1.04±0.27 for cord blood samples and 0.17±0.04 for adult samples ($P < 0.0001$, Figure 1C). The ECFC frequency was significantly higher in cord blood than in adult samples, in accordance with previous observations[4,11]. Overall, the ECFC content in cord blood appeared to be more than five times higher than in adult blood. Cord blood ECFC could be propagated in culture significantly longer than adult ECFC. In accordance with previous observations[4], the proliferative ability of all samples of adult ECFC was exhausted after 4-7 passages, while most cord blood ECFC were able to produce secondary colonies even after 12 passages (*data not shown*).

Endothelial cells originating from adult and cord blood endothelial colony-forming cells have different gene expression profiles

Microarray experiments were carried out pooling RNA extracted from 22 samples of cord blood ECFC and 11 samples of adult ECFC. The 84 genes analysed in microarray experiments are involved in a wide range of endothelial

cell pathways, including permissibility and vessel tone, angiogenesis, cell activation, injury response, cell survival and apoptosis. The comparison between matched adult and cord blood ECFC showed significant differences for 17 out of the 84 probe sets (20.2% of investigated genes). Of the 17 differentially expressed genes, 7 were up-regulated and 10 were down-regulated. Tables I and II illustrate the genes selectively up- or down-regulated and their respective fold-change of expression. As compared to adult cells, cells derived from cord blood ECFC had higher expression of *ICAM-1*, tumour necrosis factor-related apoptosis inducing ligand (*TRAIL*), *NOS3*, L-selectin (*SELL*), *TFPI*, *OCLN* and *vWF* (Table I). In contrast, the following genes were expressed at lower levels in endothelial cells obtained from cord blood ECFC than in those derived from ECFC in adult blood: matrix metalloprotease-1 and -9 (*MMP1* and *MMP9*, respectively), arachidonate 5-lipo-oxygenase (*ALOX5*), tumour necrosis factor-alpha (*TNFα*), colony-stimulating factor-2 (*CSF2;* also known as *GM-CSF*), BCL2-like 1 (*BCL-L1*), placental growth factor (*PGF*), integrin beta-3 (*ITGB3*), *IL1β* and *CRADD* (Table II). These results were validated by RT-PCR in at least one gene for each cell pathway involved (Figure 2). Overall, we found differentially expressed genes involved several pathways, including cell adhesion (*ICAM1, vWF, OCLN, SELL, ITGB3*), leucocyte recruitment (*CSF2, PGF*), coagulation (*vWF, TFPI*), vessel tone and microvascular permeability (*NOS3*), endothelial cell sprouting and angiogenesis (*vWF, NOS3, MMP1, MMP9*), inflammatory responses (*CSF2, ALOX5, TNFα*, *IL1β*) and cell survival and apoptosis (*TRAIL, BCL2L1, CRADD*).

Table I - Differential gene expression of matched cord blood and adult ECFC.

Gene	Fold regulation	ΔCt Adult	ΔCt Cord blood	P
ICAM1	84.2	0.6±0.2	1.2±0.1	0.002
TRAIL	21.0	2.9±0.6	7.3±1.4	0.007
NOS3	13.9	0.5±0.1	2.8±0.1	0.004
SELL	7.8	7.5±0.2	10.5±1.9	0.002
TFPI	6.6	3.2±0.5	9.3±0.4	<0.001
OCLN	6.1	0.5±0.6	5.9±1.7	<0.001
VWF	5.0	3.0±0.5	4.9±1.4	0.015

Genes up-regulated in cord blood samples. *ICAM1*: intercellular cell adhesion molecule-I; *TRAIL*: TNF related apoptosis inducing ligand; NOS3: constitutive nitric oxide synthase 3; *SELL*: L-selectin; *TFPI*: tissue factor pathway inhibitor; *OCLN*: occludin; *vWF*: von Willebrand factor.

Table II - Differential gene expression of matched cord blood and adult ECFC.

Gene	Fold Regulation	ΔCt Adult	ΔCt Cord blood	P
MMP1	−90.1	4.1±1.1	2.5±0.5	<0.001
MMP9	−81.7	13.9±0.9	7.5±0.1	0.028
TNFα	−55.8	13.9±3.9	8.1±0.2	<0.001
ALOX5	−26.7	12.3±0.2	7.6±0.2	0.015
CSF2	−16.3	12.3±0.2	8.3±0.6	0.004
BCL2L1	−8.7	2.2±1.0	1.3±0.3	<0.001
PGF	−8.30	2.6±0.9	1.4±0.2	<0.001
ITGB3	−5.1	1.3±0.2	1.4±0.1	0.007
IL1β	−5.2	12.4±0.2	10.0±2.1	0.048
CRADD	−4.3	2.6±0.4	1.8±0.5	0.028

Genes down-regulated in cord blood samples. *MMP1*: matrix metalloprotease-1, *MMP9*: matrix metalloprotease-9, *ALOX5:* arachidonate 5-lipo-oxygenase, *TNFα*: tumour necrosis factor , *CSF2*: colony-stimulating factor 2, *BCL-L1*: BCL2-like 1, *PGF*: placental growth factor, *ITGB3*: integrin beta 3, *IL1β*: interleukin1β and *CRADD*: CASP2 and RIPK1 domain containing adaptor with death domain.

Endothelial cells originating from adult and cord blood endothelial colony-forming cells have different capacities to activate allogeneic mononuclear cells

In order to evaluate the competence of cells derived from ECFC to evoke an immune response, we incubated allogeneic mononuclear cells with confluent adult ECFC, cord blood ECFC and mesenchymal cells, which are acknowledged as non-immunogenic cells[14]. Six experiments were carried out for adult and cord blood

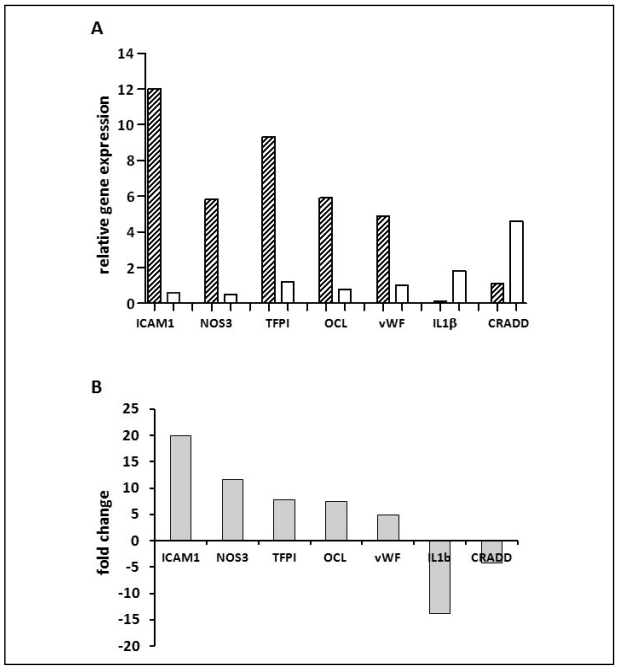

Figure 2 - Real time PCR of selected genes which were found to be differentially expressed in cord blood ECFC (dashed columns) and adult ECFC (white columns) at microarray assay. (A) Mean values of relative gene expression (i.e., the ratio between the investigated gene expression and the β-actin gene expression). SEM values are lower than 5% and have been omitted. (B) Mean gene expression fold change in cord blood ECFC in comparison with adult ECFCs. Other abbreviations as in the text.

endothelial cells and three experiments were performed for mesenchymal cells. Gene expression arrays for T-cell and B-cell activation indicated that mononuclear cells expressed significantly less mRNA for *ICOS-L, IL8, IL6* and *CSF-2* when they were incubated with cord blood ECFC than when they were incubated with adult ECFC (Figure 3A). These data were confirmed by RT-PCR. In addition, by comparing these results with those obtained in lymphocytes incubated with mesenchymal cells, we found that cord blood ECFC induced weaker expression of *IL6*, *IL8*, *CSF2* and *ICOSL* than that triggered by either adult ECFC or mesenchymal cells (Figure 3B).

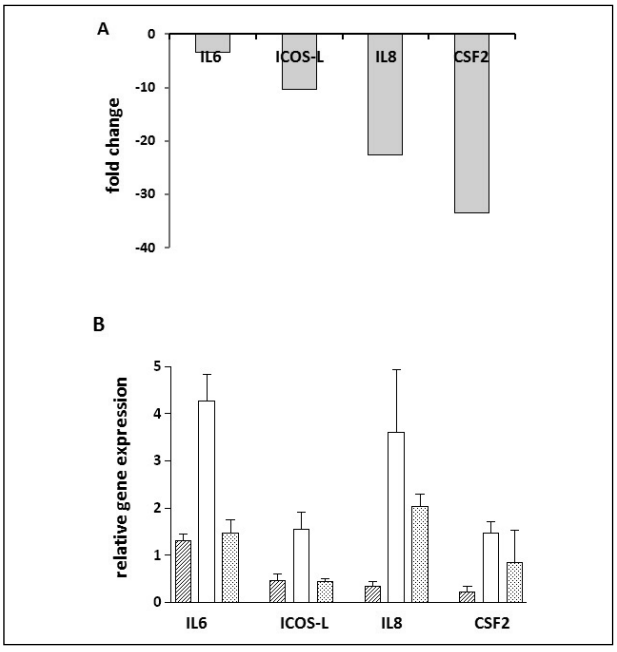

Figure 3 - Real time PCR evaluation of relative gene expression in allogeneic mononuclear cells co-cultured with ECFC and MSC. (A) Mean fold change in gene expression in mononuclear cells co-cultured with cord blood ECFC in comparison with the expression in cells from the same donor co-cultured with adult ECFC. Data obtained by gene expression arrays. (B) Mean values ± SEM of relative gene expression (i.e., the ratio between the investigated gene expression and the β-actin gene expression) in mononuclear cells incubated with cord blood ECFC (dashed columns), adult ECFC (white columns) and MSC (dotted columns).

Human leucocyte antigens are expressed at the same level on adult and cord blood endothelial colony-forming cells and mesenchymal cells

It has been previously reported that cells originating from adult ECFC express much less HLA than do mature endothelial cells[15]. We compared HLA expression of cord blood ECFC to that of adult ECFC and bone marrow MSC, which are acknowledged to be non-immunogenic *in vivo*[14]. Figure 4 illustrates the expression of HLA-ABC and HLA-DR in different cell samples at baseline and after incubation with γ-interferon. We found that all cell populations expressed similar amounts of HLA-ABC at baseline; this expression was further enforced after γ-interferon incubation (Figure 4A). In contrast, HLA-DR molecules were not detectable at baseline, whereas they became appreciable in all samples after incubation with γ-interferon, without their being significant differences among cord blood, adult cells and MSC (Figure 4B).

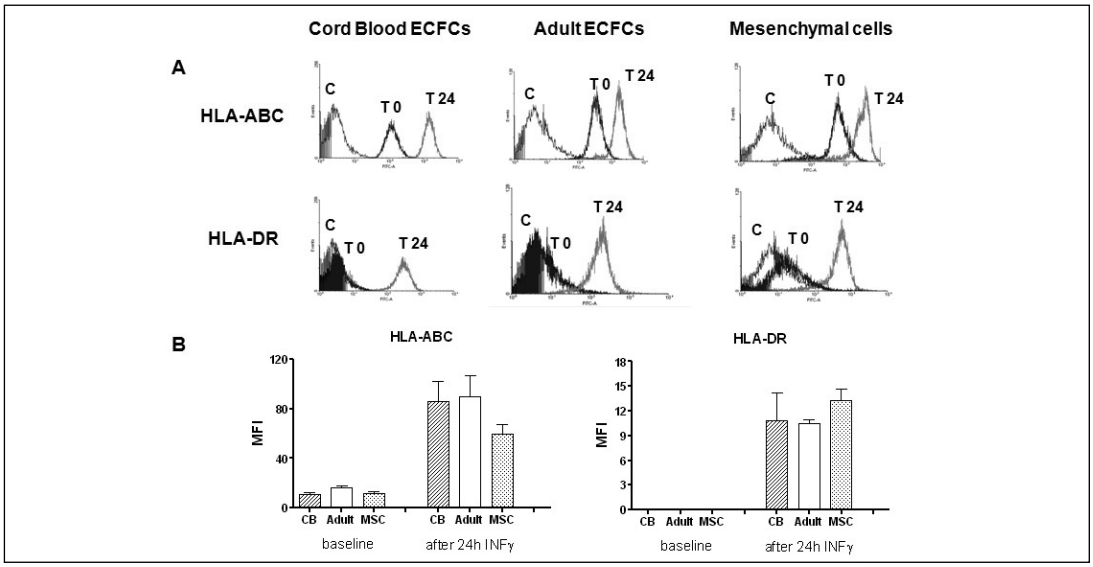

Figure 4 - Flow cytometry HLA-ABC and HLA-DR antigen expression in cord blood ECFC, adult ECFC and MSC. (A) Representative experiments. C represents the isotype controls; T0 indicates baseline HLA expression and T24 indicates the HLA expression after 24 hours of incubation with γ-interferon. (B) Mean values±SEM of HLA expression obtained in six experiments, expressed as mean fluorescence intensity ratio (MFI).

Discussion

Cord blood contains considerable amounts of endothelial progenitor cells and it is regarded with increasing interest as a feasible source of stem cells for the purposes of regenerative medicine[9,10]. Greater understanding of the genetic characteristics of these cells could help to decipher their functional properties. ECFC are endowed with endothelial proliferative potential[3,4] and are, therefore, potentially transplantable to treat vascular diseases. The efficacy of ECFC transplantation was definitely established in a recent study by Schwarz *et al.*, who demonstrated, in an animal model of hind limb ischaemia, that injection of ECFC and MSC reverted the effects of the induced ischaemia[16]. Importantly, the leading mechanism for the functional improvement was related to the physical incorporation of ECFC in the vasculature, while MSC had only a paracrine effect[16].

In this study we compared the genetic profile and immunogenic properties between ECFC obtained from cord blood and those obtained from healthy adults. Although our *in vitro* observations need to be confirmed *in vivo,* collectively they suggest that cord blood ECFC, both directly, through their own gene expression, and indirectly, through mononuclear cell activation, elicit a weaker immune response than adult ECFC (Figure 5). Through genotypic profiling we found that about 20% of investigated genes were significantly up- or down- regulated in cord blood samples. Interestingly, all of the genes that were differentially expressed belong to the family of molecules that are modulated *in vivo* when terminally differentiated endothelial cells cell are activated in response to an injury[17]. Endothelial activation represents the crossroad of several pathways, including thrombosis, angiogenesis and inflammation. It is, therefore, rather challenging to determine the exact role of each gene in the *in vitro* setting of proliferating endothelial progenitors. The most striking difference observed between cord blood and adult ECFC was the higher expression of *ICAM1* in cord blood cells. ICAM-1 is the ligand of the β2 integrin lymphocyte function-associated antigen-1[18]. The signalling triggered by the engagement of leucocytes with ICAM-1 converge to cause junctional disruption favouring cell diapedesis[18]. This observation is apparently in contrast with both the low expression of *ITGB3* (which also participates in cell adhesion and cell-surface mediated signalling[19]) and with the strong expression of *OCLN*, one of the main constituents of tight junctions[20]. Importantly, the response of ICAM-1 bound cells depends on the surrounding cytokine environment: in this regard, cord blood-derived endothelial progenitors appear to be much less reactive than adult ones. In fact, they express very low levels of pro-inflammatory and chemoattractant cytokines, including *PGF* (a member of the vascular endothelial growth factor family, which acts via the flt-1 receptor and promotes endothelial activation and macrophage recruitment into atherosclerotic lesions[21]), *IL1β*, *TNF*α and *ALOX5,* which catalyses the first two steps in the biosynthesis of leukotrienes, a group of pro-inflammatory lipid mediators derived from arachidonic acid[21-24]. It should be noted that the endothelial cell populations used in these experiments were not terminally differentiated cells, but circulating progenitors destined to be attracted into poorly perfused tissues in order to generate new vessels. Our data do, therefore, suggest that *ICAM-1* might contribute to cord blood progenitor cell recruitment and extravasation: once these cells are entrapped within ischaemic tissues, several local stimuli, including nitric oxide and von Willebrand factor production stimulate both endothelial progenitors and smooth muscle cells to proliferate and form new vessels[25-27]. The unexpected finding of L-selectin expression on endothelial progenitors further supports this hypothesis. Basically, the selectin family includes a group of cell adhesion molecules fundamental for recruiting leucocytes to the vessel wall[28]. E-selectin is expressed mostly on endothelial cells, P-selectin on platelets, endothelial cells and macrophages and L-selectin on leucocytes[28]. Surprisingly, we found that endothelial progenitors, particularly from cord blood, express variable amounts of L-selectin RNA. Since L-selectin binds to endothelial E-selectin, its expression on circulating progenitors could allow these cells to be recruited into ischaemic sites and undergo extravasation. Interestingly, the transient expression of L-selectin in mesangioblasts increases, by two to three-times, the transmigration and homing of these cells to the damaged heart in both human and mouse[29]. In this regard, the higher expression of L-selectin in cord

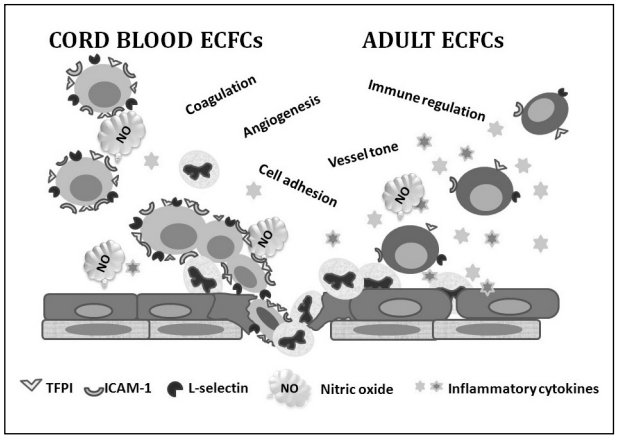

Figure 5 - Diagram illustrating the main differences between cord blood and adult ECFC as evidenced at microarray assays and in allogeneic mononuclear cell co-cultures.

blood could render these cells more suitable than adult progenitors for the purposes of regenerative medicine. We also found that cord blood endothelial progenitors express more TFPI RNA than do adult ones. Basically, TFPI antagonises the action of tissue factor, but it also has strong anti-inflammatory, anti-coagulant and lipid-lowering activities[30]. Similarly, matrix metalloproteases are implicated in a variety of biological processes, including thrombosis, fibrinolysis, inflammation, vascular remodelling and atherosclerosis[31,32]. We found that the expression of two widely acting metalloproteases, *MMP1* and *MMP9,* was lower in cord blood ECFC than in adult ones. Even though this finding could suggest the capacity of cord blood ECFC is inferior to that of adult ECFC at disintegrating the extracellular matrix and sprouting in surrounding tissues, it could also underlie a lower potential to cleave and activate several coagulation factors[31,32].

MSC are routinely administered *in vivo* in the absence of HLA matching without evidence of rejection[14,33]. In this study we show that expanded ECFC from both healthy adults and cord blood samples have comparable levels of HLA (class I and class II) as those on mesenchymal cells, both before and after γ-interferon stimulation. However, when co-cultured with allogeneic lymphocytes and monocytes, cord blood ECFC induced less co-stimulatory molecule expression and elicited a weaker pro-inflammatory cytokine response than both adult ECFC and MSC. We hypothesise that the eventual contemporary expression of immunoregulatory molecules, such as HLA-G, typical of cord blood cells[34], might explain this low immunogenicity. Additional future studies specifically tailored to clarify these complex networks are required.

Immunological studies on endothelial cells in the context of organ transplantation showed that the endothelium should be regarded both as a stimulator and as a target of the immune reaction[35]. In particular, endothelial cells can act as a perpetrator of the local immune response[35]. This study shows that cord blood endothelial progenitors, in comparison with their adult counterparts, elicit *in vitro*, directly or indirectly, a significantly weaker gene expression of a variety of mediators promoting inflammation and atherosclerosis, *i.e.* IL1β, IL8, PGF, ALOX-5, TNFα, CSF-2, MMP1 and MMP9 (Figure 5).

Certainly, our study has several limitations: firstly, only some of the markers implicated in immune recognition and immunogenicity were investigated. Moreover, our observations should be confirmed through additional methodological approaches, such as a one-way mixed lymphocyte reaction or cytokine assays in culture media. Furthermore, the gene expression arrays used in this study could have missed other important gene expression pathways. Finally, in order to draw definite conclusions on the potential use of cord blood ECFC, all these observations made *in vitro* should be validated *in vivo* in experimental models such as the immuno-competent hind limb ischaemia mouse model. However, despite all these limitations, our findings offer important grounds for reflection. Thanks to their anti-inflammatory and anti-thrombotic profile, ECFC might represent a potential cell therapy for patients with peripheral arterial diseases. Recently, allogeneic cardiac stem cell transplantation has been emerging as a possible therapeutic tool in the setting of cardiovascular diseases[36,37]. Considering the enormous numbers of patients with peripheral arterial diseases, pre-clinical studies designed to explore the feasibility of allogeneic cord blood ECFC transplant are urgently necessary.

Authors' contributions

Eugenia R. Nuzzolo and Maria G. Iachininoto: conception and design of the study, performance of experiments (cell cultures and flow cytometry), data analysis and interpretation, manuscript writing; Sara Capodimonti, Maurizio Martini and Alessandra Cocomazzi: performance of experiments (microarray and real time PCR), analysis of data, manuscript writing, final approval of the manuscript; Maria Bianchi and Gina Zini: sample contribution; Giuseppe Leone: conception and design of the study; Luigi M. Larocca: conception and design of the study, data analysis and interpretation; Luciana Teofili: conception and design of the study, sample contribution, data analysis and interpretation, manuscript writing. All Authors approved the final version of the manuscript.

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The Authors declare no conflicts of interest.

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Correspondence: Luciana Teofili
Istituto di Ematologia
Universit‡ Cattolica del Sacro Cuore
Largo Gemelli 8
00168 Roma, Italy
e-mail: lteofili@rm.unicatt.it