Red blood cells derived from peripheral blood and bone marrow CD34+ human haematopoietic stem cells are permissive to *Plasmodium* parasites infection

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The production of fully functional human red cells in vitro from haematopoietic stem cells (hHSCs) has been successfully achieved. Recently, the use of hHSCs from cord blood represented a major improvement to develop the continuous culture system for Plasmodium vivax. *Here, we demonstrated that CD34+ hHSCs from peripheral blood and bone marrow can be expanded and differentiated to reticulocytes using a novel stromal cell. Moreover, these reticulocytes and mature red blood cells express surface markers for entrance of malaria parasites contain adult haemoglobin and are also permissive to invasion by* P. vivax *and* Plasmodium falciparum *parasites.*

Key words: CD34+ human haematopoietic stem cells - NIH 3T3 cells - *Plasmodium*

The use of human haematopoietic stem cells (hHSCs) and specific factors to drive their differentiation into reticulocytes and mature red blood cells (RBCs) (Giarratana et al. 2005, 2011) represented a major improvement to develop a continuous in vitro culture system for blood stages of *Plasmodium vivax* as it provided a constant source of reticulocytes derived from progenitors cells isolated from cord blood (Panichakul et al. 2007). Unfortunately, this system was not highly reproducible and it yielded extremely low percentages of infected reticulocytes (< 0.0015%) presumably and partly due to the foetal haemoglobin content of reticulocytes from cord blood (Panichakul et al. 2007). More recently, however, Noulin et al. (2012) described a new method for the production and cryopreservation of cord blood HSC-derived reticulocytes to be used for both *Plasmodium falciparum* and *P. vivax* invasion tests, with substantially higher percentage of infected hHSC-derived reticulocytes (< 2.5%).

To determine whether peripheral blood (PB) (from buffy coats of healthy donors) and bone marrow (BM) (Lonza Group Ltd) CD34+ hHSCs could be used as another source of reticulocytes production for furthering development of an in vitro culture system for *P. vivax*, we set to demonstrate that RBCs differentiated from these progenitors cells express adult haemoglobin and that reticulocytes and erythrocytes derived from them are permissive to invasion, respectively, by *P. vivax* and *P. falciparum* parasites. To do so, we purified through Ficoll-Hypaque gradient centrifugation PB mononuclear cells from buffy coats of blood from healthy donors. Next, CD34+ hHSCs were isolated through magnetic beads couple to anti-CD34 antibodies (Miltenyi Biotec) according to the manufacturers' instructions. Using this procedure, we usually obtained 10^4-10^5 CD34+ hHSCs starting from approximately 1-3 x 10^8 cells from 50 mL of buffy coat. Cells were subsequently expanded and differentiated to reticulocytes in a three-step protocol using NIH 3T3 cells (ATCC) to sustain expansion and differentiation of CD34+ hHSCs to reticulocytes and mature RBCs. These cells had been previously shown to have the capacity of stimulating the generation of haematopoietic colony-forming cells (Bigas et al. 1995), but never used before as feeder stromal cells in this differentiation process. Results demonstrated that NIH 3T3 cells help to support differentiation, enucleation and cell culture viability of PB CD34+ hHSCs to reticulocytes and mature RBCs (A in Figure). In addition, corresponding cell expansion levels as those reported for MS5 stromal cells of around 16,000-fold were obtained with HIH 3T3 cells. As expected (Giarratana et al. 2005), at day 15 after differentiation started, we obtained the maximum percentage of reticulocytes (83.5 ± 12.1%). Of note, similar results were obtained from CD34+ hHSCs from BM (not shown).

Whereas adult haemoglobin is composed of two alpha and two beta subunits, foetal haemoglobin is composed of two alpha and two gamma subunits, commonly denoted as α2γ2. To determine the haemoglobin composition of reticulocytes derived from PB and BM CD34+ hHSCs, we used Maldi TOF Mass Spectrometry. As controls, we

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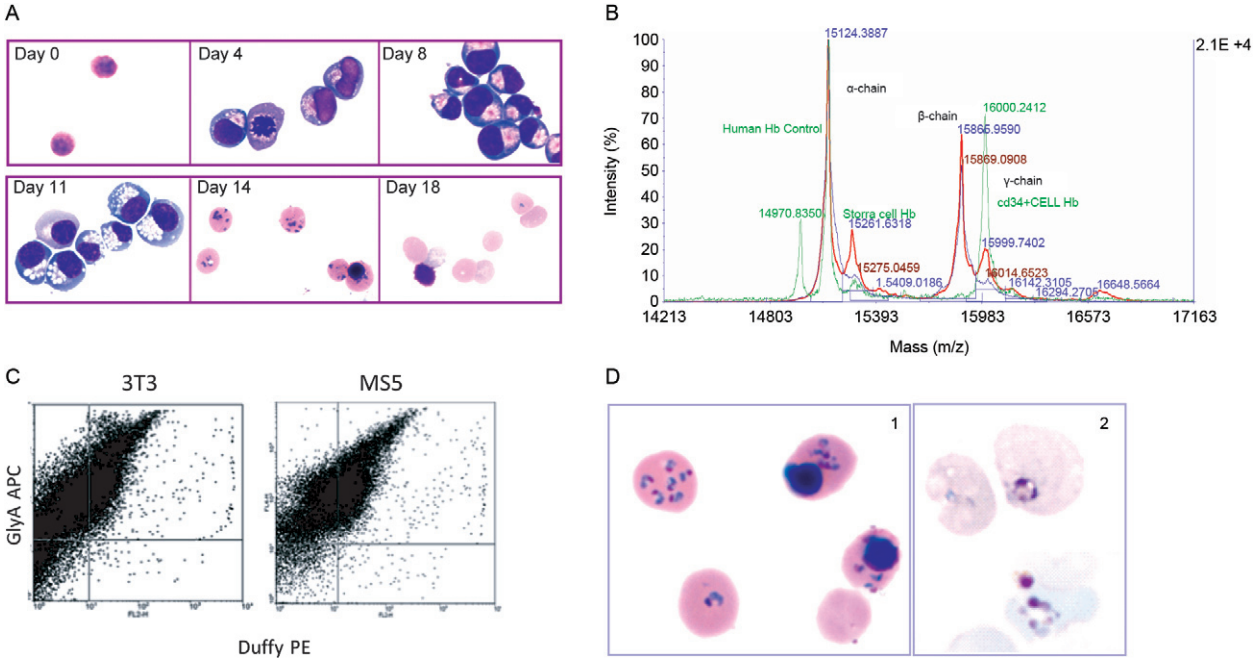
used reticulocytes derived from cord blood. Samples were analysed in a linear positive mid mass mode to determine the molecular mass of the intact chains. Three signals corresponding to the globin chains α, β and γ (around 15,130, 15,870 and 16,000 Da respectively) were detected (B in Figure). As expected, PB and BM CD34⁺ hHSCs synthesised adult haemoglobin (with α and β chains) whereas reticulocytes derived from cord blood CD34⁺ hHSCs synthesised foetal haemoglobin (with α and γ chains).

Invasion experiments of ex-vivo generated RBCs by *P. falciparum* (3D7 strain) and *P.* vivax (wild isolates from the Brazilian Amazon Region) were then assayed as previously described (Trager & Jensen 1976, Costa et al. 2011, Russell et al. 2011) to demonstrate that these cells can be used for in vitro culture. To do so, we first demonstrated by flow cytometry that day 14 growing RBCs from CD34⁺ hHSCs from PB (C in Figure) and BM (not shown) expressed surface markers required for invasion of malaria parasites, namely the Duffy blood group and the glycophorin A antigen. Next, we performed invasion assays using parasites from the 3D7 *P. falciparum* strain and *P. vivax* isolates directly obtained from patients at the Tropical Medicine Foundation Dr Heitor Vieira Dourado (FMT-HVD) [all patients were assisted by the Ethical Review Board from FMT-HVD (approval 257.815)]. Results demonstrated that erythrocytes and reticulocytes differentiated from PB CD34⁺ hHSCs were permissive for invasion of *P. falciparum* and *P. vivax* (D1, 2 in Figure, respectively). Similar results were obtained with CD34⁺ hHSCs from BM (not shown). Moreover, as previously reported in RBCs obtained from CD34⁺ hHSCs from cord blood (Panichakul et al. 2007) or PB (Tamez et al. 2011), invasion was also observed in nucleated cells (erythroblasts).

Here, we show for what we believe is the first time, the permissiveness of RBCs produced from human PB and BM CD34⁺ hHSCs to be invaded by *P. falciparum* and *P. vivax*. Moreover, these cells express adult haemoglobin and the Duffy receptor, which is the main receptor for entrance of *P. vivax* (Chitnis & Miller 1994). To facilitate implementation of this method, we have used buffy coats from blood of healthy donors. Furthermore, in addition to MS5 feeder supporting cells (Giarratana et al. 2005), we also tested fibroblast NIH 3T3 cells (ATCC) for expansion and differentiation of CD34⁺ hHSCs. These cells seem to stimulate haematopoietic proliferation and differentiation through soluble molecule(s) (Bigas et al. 1995) suggesting the possibility of production of reticulocytes with no stromal support as has been recently shown with other factors (Timmins et al. 2011). Of notice, a method for cryopreservation of cord blood hHSC-derived reticulocytes that can be successfully invaded by both cryo-



Phenotypic and functional analyses of red blood cells (RBCs) derived from CD34⁺ human haematopoietic stem cells (hHSCs) of peripheral blood (PB) and bone marrow (BM). A: differentiation of hHSCs observed on bright field after cytospin and staining with Giemsa and Brilliant cresyl blue at the indicated days. Magnitude 1,000X; B: analysis of haemoglobin by Maldi TOF Mass Spectrometry [human control RBCs (red), reticulocytes from BM CD34⁺ cells (blue), reticulocytes from human umbilical cord CD34⁺ cells (green)]; C: flow cytometry analysis of day 14 reticulocytes derived from PB CD34⁺ hHSCs and differentiated either with 3T3 or MS5 feeder stromal cells. Cells were double labelled with antibodies against glycophorin A (GlyA-APC) and Duffy blood receptor (Duffy-PE); D: Giemsa stained smears of RBCs derived from PB CD34⁺ hHSCs infected with *Plasmodium falciparum* (1) and *Plasmodium vivax* (2) after 24 h post infection.

preserved *P. falciparum* and *P. vivax* isolates has been recently described (Noulin et al. 2012). A combination of these methods and reticulocytes from PB and BM $CD34^+$ hHSCs is therefore amenable for testing whether they allow exponential growth of *P. vivax* parasites.

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