ISOLATION AND CHARACTERIZATION OF RETICULOCYTES DIFFERENTIATED FROM HUMAN PERIPHERAL BLOOD-DERIVED CD34$^+$ HEMATOPOIETIC STEM CELLS AND THE POTENTIAL OF RETICULOCYTE INVASION BY *PLASMODIUM FALCIPARUM*

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Abstract. *In vitro* reticulocyte model may serve as a promising tool for understanding human erythropoiesis in health and disease. Yet, the availability of reticulocyte model is restricted by the limited number of these circulating cells in human peripheral blood (PB). Here, we described a method to isolate and characterize reticulocytes differentiated from human PB-derived CD34$^+$ hematopoietic stem cells (HSCs) and evaluate the potential of reticulocyte invasion by the malaria parasite, *Plasmodium falciparum*. PB-derived CD34$^+$ HSCs were isolated from a total of 200 mL PB of healthy volunteers (n=20), and were grown and expanded for five days in serum-free media supplemented with cytokines and growth factors followed by differentiation with erythroid-supporting cytokines. The expansion rate of PB-derived CD34$^+$ HSCs, the phenotype and morphology of generated reticulocytes were studied. The susceptibility of functional reticulocytes to invasion by *P. falciparum* was determined. During expansion, the total cell population increased approximately 2.11 ± 0.10-fold in an optimized culture initiated with PB-derived CD34$^+$ HSCs. The characteristics and morphology of reticulocytes were apparent on day 14 of differentiation. Moreover, the invasion assay also proved that *P. falciparum* invaded the HSC-derived reticulocytes generated in this study. The simplified and reproducible protocol using CD34$^+$ HSCs from PB makes generated reticulocytes beneficial for fundamental research in erythroid development and provides the *in vitro* reticulocyte model for studying malaria parasites that target erythroid cells.

Keywords: Reticulocyte, human peripheral blood, CD34$^+$ hematopoietic stem cell, erythropoiesis, malaria parasite.

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Introduction

Reticulocytes are immature erythrocytes that have lost the nucleus but retain residual RNA [1]. Reticulocytes are produced through a step-wise process of differentiation known as erythropoiesis. This process starts when multipotent hematopoietic stem cells (HSCs) develop into myeloid progenitor cells, followed by erythroid precursor cells [2]. The proerythroblast appearance indicates the most immature stage of erythroid precursor cells, followed by basophilic, polychromatophilic and orthochromatic normoblasts that give rise to reticulocytes. After a series of division and maturation steps in the bone marrow (BM), reticulocytes are released into the circulation where their maturation to erythrocytes is completed [3].

The *in vitro* generation of reticulocytes has recently been focused due to the renewed interest in the manufacturing of blood products for clinical application and the development of *in vitro* cultures of reticulocytes-invading species of human malaria parasites [4,5]. *P. falciparum* is responsible for the most severe form of malaria [6] because this parasite has the capability to invade erythrocytes of all ages, while the less virulent *P. vivax* and *P. knowlesi* show a predilection for reticulocytes [6,7]. Considering its susceptibility to be invaded by these malaria species, reticulocytes is particularly important as host for malaria study. There has been an interest in producing reticulocytes *in vitro* to establish *in vitro* cultures of malaria parasite-infected reticulocytes [8]. This, however, has been restricted by the limited number of reticulocytes obtained from sources such as peripheral blood (PB) from which the cells represent only 0.5–2 % of the circulating erythrocytes and have a short life cycle (~24 hours) [9,10]. Previous studies have demonstrated a technique to enrich reticulocytes from sources of high cell concentration such as haemochromatosis blood and umbilical cord blood (UCB) [11]; however, the reticulocytes enriched from these sources are not proliferative and may not be able to self-renew. Therefore, an attempt to produce sufficient numbers of reticulocytes *in vitro* by differentiation of HSCs into erythroid lineage is urgently needed.

HSCs isolated from peripheral blood mononuclear cells (PBMCs) have exhibited the ability to induce erythroid differentiation and subsequently generate reticulocytes *in vitro* [5,12]. The expansion potential of HSCs derived from PB is almost comparable to other stem cell sources such as UCB and BM [13]. Besides, PB-derived CD34$^+$ HSCs are readily accessible and can be collected at higher quantities than UCB- and BM-derived CD34$^+$ HSCs. In this study, we isolated and characterized reticulocytes differentiated from human PB-derived CD34$^+$ HSCs and determined the reticulocyte functionality by the ability of *P. falciparum* to proliferate in these cells.

Materials and Methods

Blood Collection and CD34$^+$ HSC Isolation

A total of 200 mL PB was collected from healthy volunteers (n = 20) with informed consent as approved by the Human Research Ethics Committee Universiti Sains Malaysia (USM/JEPeM/15100345). PBMCs were isolated by density gradient centrifugation (400 × *g*, 20 minutes) using lymphocyte separation medium (Corning, USA). Isolation of CD34$^+$ HSCs was carried out by using the magnetic assorting cell sorting method (MACS, Miltenyi Biotec, Germany) according to the manufacturer's protocol. The purity of isolated CD34$^+$ HSCs was determined by flow cytometry using the PerCP-Cy5.5-conjugated mouse anti-human CD34 monoclonal antibody (Clone 8G12; BD Bioscience, USA).

Expansion of PB-derived CD34$^+$ HSCs

Isolated CD34$^+$ HSCs were expanded as reported previously [10]. The cells at a density of 2×10^5 cells in 4 mL of serum-free media II (Sigma, USA) in 25 cm^2 flasks were cultured at 37 °C in a 5 % humidified CO_2. The medium was enriched with FMS-like tyrosine kinase 3 (50 ng/mL) (R&D system, USA), interleukin-6 (50 ng/mL) (R&D system, USA), stem cell factor (50 ng/mL) (Sigma, USA) and thrombopoietin (50 ng/mL) (R&D system, USA). On the fifth day of culture, the cells were counted using a hemocytometer and transferred into new 6-well plates for differentiation assay. Fold expansion was calculated by dividing total viable CD34$^+$ HSCs obtained on day 5 of culture by the initial seeding number on day 0.

Differentiation of PB-derived CD34$^+$ HSCs into Reticulocytes

Expanded CD34$^+$ HSCs were cultured in IMDM differentiation medium (Sigma, USA) [10] with slight modification. The medium was added with nutrition supplements such as L- glutamine (4 M) (Sigma, USA), folic acid (10 µg/mL) (Sigma, USA), insulin (10 µg/mL) (Sigma, USA), inositol (40 µg/mL) (Sigma, USA), monothioglycerol (1.6×10^{-4} M) (Sigma, USA), transferrin (120 µg/mL) (Sigma, USA), penicillin/streptomycin (1 %) (Invitrogen, USA) and fetal bovine serum (FBS) in IMDM (1 %) (StemCell Technologies, Canada). The cells (2×10^5 cells) were seeded before being incubated at 37 °C with 5 % CO_2. During early erythroid differentiation (day 0–8), 4 mL IMDM differentiation medium was supplemented with hydrocortisone (10^{-6} M) (Sigma, USA), interleukin-3 (5 ng/mL) (R&D system, USA), stem cell factor (100 ng/mL) (Sigma, USA) and erythropoietin (25 ng/mL) (R&D System, USA). An extra 3 mL of IMDM differentiation medium was added after four days of culture. The cells were washed at day 8 and the medium consisting of erythropoietin (25 ng/mL) (R&D System, USA) was added before cells were added in new 25 cm^2 flasks for the terminal erythroid differentiation (day 8–11). On day 11, cultured cells were washed and the fresh IMDM differentiation medium without growth factors was added and changed every three days with the addition of 10 % FBS during the third stage of differentiation, which was the maturation stage of reticulocytes (day 11–14). The culture was completed on day 14.

Characterization of PB-derived CD34$^+$ HSC Maturation by Flow Cytometry

Cell surface phenotype of CD34$^+$ HSCs was analyzed at day 0, 8, 11 and 14 of differentiation to monitor their maturation into reticulocytes. Briefly, 2×10^5 cells were pelleted and re-suspended in 50 µL FACS buffer (BD Bioscience, USA) before adding PerCP-Cy5.5-conjugated mouse anti-human CD34 monoclonal antibody (0.05 µg/mL; Clone 8G12; BD Bioscience, USA), PerCP-Cy5.5-conjugated mouse anti-human CD45 monoclonal antibody (0.024 µg/mL; Clone 2D1; BD Bioscience, USA), APC-conjugated mouse anti-human CD36 monoclonal antibody (4 µL; Clone CB38; BD Bioscience, USA) and FITC-conjugated mouse anti-human CD71 monoclonal antibody (0.20 µg/mL; Clone L01.1; BD Bioscience, USA). Stained cells were incubated at room temperature in the dark for 20 minutes. The following isotype controls were used as negative controls: PerCP-Cy5.5-conjugated mouse IgG1 monoclonal immunoglobulin (0.10 µg/mL; Clone X40; BD Bioscience, USA), APC-conjugated mouse IgM monoclonal immunoglobulin (4 µL; Clone G155-228; BD Bioscience, USA) and FITC-conjugated mouse IgG2a monoclonal immunoglobulin (0.05 µg/mL; Clone X39; BD Bioscience, USA). Stained cells were washed and re-suspended in 400 µL FACS buffer before being analyzed using FACS Canto II (BD Bioscience, USA) and FCS Express 5 (DeNovo, USA). CD36 and CD71 expressions were used to determine reticulocyte populations.

Morphological Observation of Enucleated and Nucleated Cells

Morphological examination of differentiated cells towards erythroid lineage was performed on cytospin slide preparation stained with cresyl blue solution for determination of enucleated cells. The cells (2×10^5 cells) at day 0, 8, 11 and 14 of differentiation were pelleted (300 × *g*, 5 minutes) and suspended in 50 µL PBS before adding 50 µL cresyl blue staining solution (0.3 %, Sigma, USA) [10]. After 30 minutes of incubation at room temperature, the cells were centrifuged at 58 × *g* for 3 minutes by using a cytospin (Cyto-Tek 2500 Cytocentrifuge, Sakura Finetek, Japan). The slides were air-dried and cells were visualized by using bright field microscopy (Olympus BX41, Japan) under 100× magnification with oil immersion. Reticulocytes appearing as enucleated cells with at least three dots of cresyl blue RNA were counted from 500 total cells and multiplied by 100 %. The slides were then stained with 10 % Giemsa for determination of nucleated cells.

Parasite Invasion Assay

P. falciparum (3D7 strain) was cultured in RPMI 1640 medium containing GlutaMAX I and 25 mM HEPES (GIBCO, Invitrogen, USA), 0.25 % Albumax II, 0.05 mg/mL hypoxanthine (Sigma, USA), 0.2 % glucose (Sigma, USA) and 0.025 mg/mL gentamicin (Duopharma, Malaysia) [14]. Synchronized young ring stage parasites were allowed to mature into schizonts after synchronization with 5 % sorbitol. Synchronized matured stage parasites were harvested by using the MACS system (Miltenyi Biotec, Germany) and adjusted to 2 % parasitemia (2 % hematocrit) before being mixed with HSC-derived reticulocytes and mature erythrocytes enriched from whole blood (positive control) in 24-well plates. The parasites were incubated at 37 °C for 48 hours with 5 % CO_2. The parasitemia of the control group at 48–hour post-inoculation was determined by Giemsa-stained thin blood smears, whereas infected reticulocytes were cytospun before being stained with Giemsa.

Data Analysis

Data analysis was performed by using GraphPad Prism software version 8.0.1 (San Diego, California, USA). All data were expressed as mean ± standard error mean (SEM). For $CD34^+$ HSC expansion, comparison between two sets of parametric data was analyzed by using Student's t-test and considered significant when $P < 0.05$. As for the percentage of expression of CD34, CD45 and CD36/CD71 on PB-derived $CD34^+$ HSCs on day 0, 8, 11 and 14, the data were analyzed using one-way ANOVA and considered significant when $P < 0.05$. All experiments were performed at least three times.

Results and Discussion

Isolation and Expansion of PB-derived CD34$^+$ HSCs

The CD34$^+$ HSCs were isolated from PBMCs by MACS procedure using magnetic beads coupled with anti-CD34 antibodies. As determined by flow cytometry the purity of CD34$^+$ HSCs before and after isolation was 0.98 % (Figure 1(A)) and 49.21 % (Figure 1(B)), respectively. Next, the isolated CD34$^+$ HSCs were allowed to expand until day 5 in serum-free expansion medium in the presence of FMS-like tyrosine kinase 3, interleukin-6, stem cell factor and thrombopoietin. After day 5, the total number of CD34$^+$ HSCs significantly increased by 2.11 ± 0.10-fold in the culture (Figure 1(C)) as compared to day 0 ($P = 0.0021$).

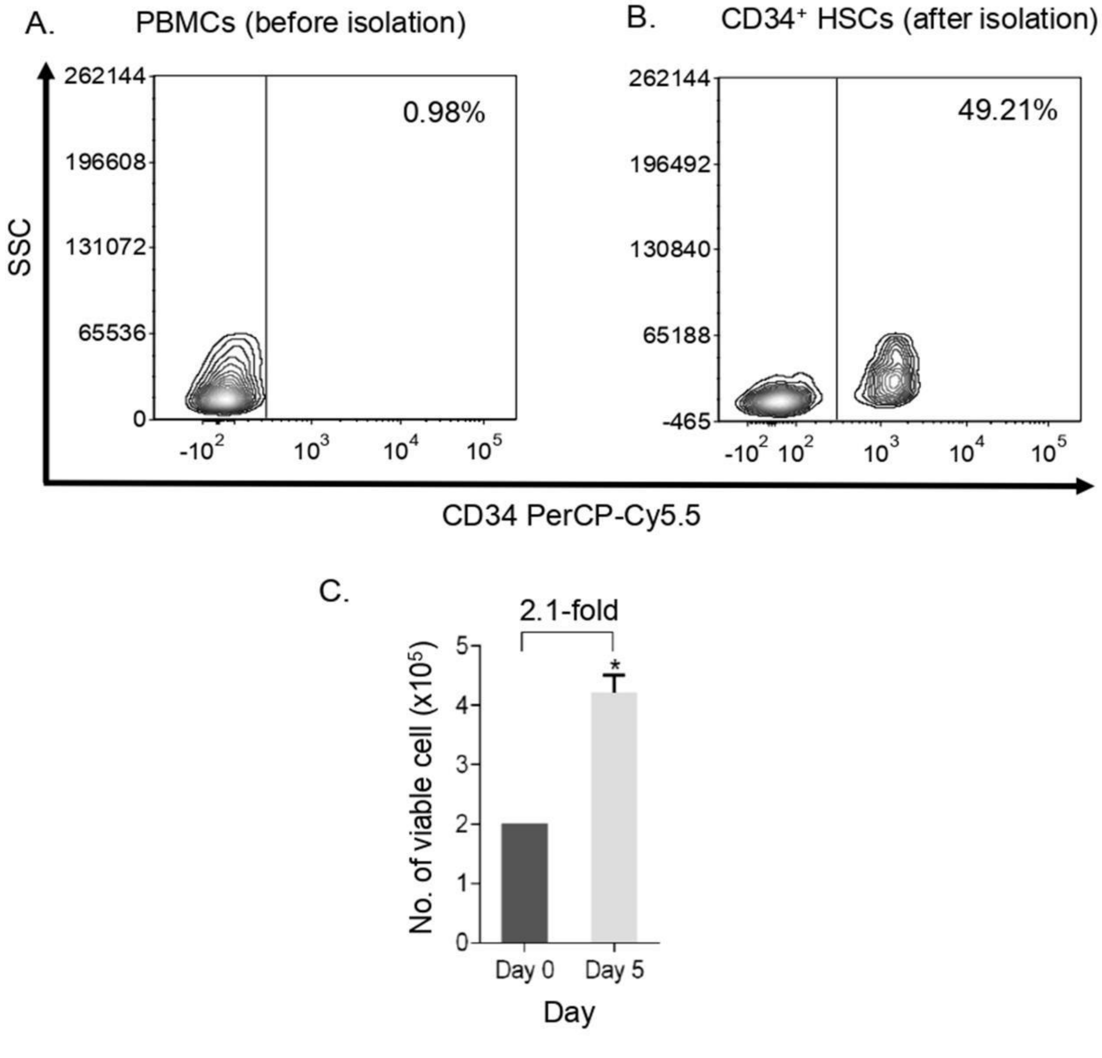

Figure 1: Expansion of PB-derived CD34$^+$ HSCs. The representative contour plots showing PBMC population (A) before and (B) after CD34$^+$ HSC isolation and (C) Graph bar depicting cells number (fold) during PB-derived CD34$^+$ HSC expansion. The results are expressed as mean ± SEM (n=3). * $P < 0.05$.

Phenotypic Characterization of Reticulocytes Differentiated from PB-derived CD34$^+$ HSCs

Following expansion, the differentiation of expanded PB-derived CD34$^+$ HSCs to reticulocytes was performed by using three stages of erythropoiesis assay. The proportion of differentiating cells expressing different surface antigens (CD34, CD45 and CD36/CD71) was determined at different time points (day 0, 8, 11 and 14) (Figure 2 and Table 1). Before differentiation, most cells expressed a high level of CD34 (66.42 ± 3.52 %) and CD45 (51.18 ± 4.71 %), a marker of HSCs.

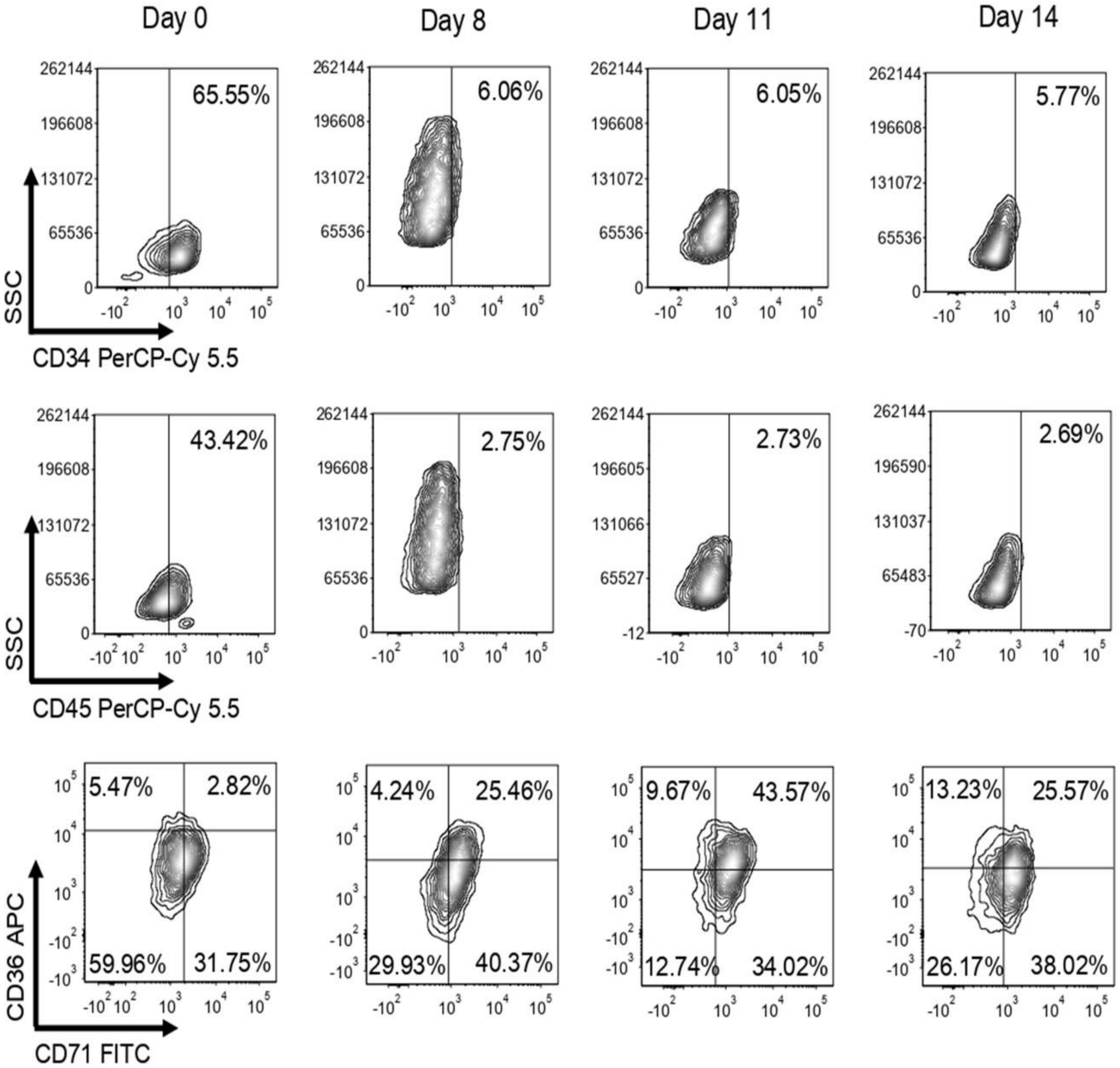

Figure 2: The percentage of expression of CD34, CD45 and CD36/CD71 during reticulocyte differentiation assay. The representative contour plots show the percentage of positive cells expressed on day 0, 8, 11 and 14.

By day 8, the percentage of CD34 and CD45 was decreased significantly to 6.62 ± 4.12 % ($P < 0.001$) and 4.32 ± 1.22 % ($P < 0.001$), respectively (Table 1). On the other hand, the frequency of cells expressing CD36/CD71, a marker of erythroid differentiation, was low at day 0 (2.59 ± 0.37 %) (Table 1). By day 8, the CD36/CD71 expression increased significantly to 25.59 ± 0.85 % ($P < 0.001$) and continued to express on day 11 (42.81 ± 1.23

%) ($P < 0.001$), before decreasing to 20.65 ± 1.22 % ($P < 0.001$) by day 14 as the maturation progressed (Table 1).

Table 1: Summary of the percentage of expression of CD34, CD45 and CD36/CD71 on PB-derived CD34$^+$ HSCs

Day of differentiation	**Markers**		
	SSC/CD34	**SSC/CD45**	**CD36/CD71**
0	66.42 ± 3.53%	51.18 ± 4.71%	2.59 ± 0.37%
8	6.62 ± 4.12%	4.32 ± 1.22%	25.59 ± 0.85%
11	4.68 ± 2.33%	4.85 ± 3.79%	42.81 ± 1.23%
14	2.82 ± 1.57%	4.57 ± 1.06%	20.65 ± 1.22%

The data are presented as mean ± SEM obtained from three independent experiments demonstrating the expression of CD34, CD45 and CD36/CD71 on day 0, 8, 11 and 14 of CD34$^+$ HSCs differentiation (Table 1).

Morphological Characterization of Reticulocytes Differentiated from PB-derived CD34$^+$ HSCs

During early erythroid differentiation, the proerythroblast cells with a large round nucleus and minimal cytoplasm were observed on day 8 (Figure 3(A)). The cytoplasm of the proerythroblasts was stained in blue and there was a pinkish hue. During terminal erythroid differentiation, morphological changes of proerythroblasts to normoblasts were observed on day 11 where the nuclear size was reduced and the cytoplasm compartment was increased (Figure 3(A)). The cytoplasm of the normoblasts was stained in pink. During reticulocyte maturation, the morphological changes of normoblasts to reticulocytes were observed at day 14 where the nucleus was absent and the cresyl blue-stained RNA granules were present (Figure 3(A)). The reticulocyte count on day 14 was 30.00 ± 1.76% (Figure 3(B)). Besides, an active cell proliferation was achieved after day 8 of early erythroid differentiation, which produced approximately $2.1 \times 10^6 \pm 0.53 \times 10^4$ total viable cells (Figure 3(C)). On day 11, cells underwent terminal erythroid differentiation process and approximately $8.18 \times 10^5 \pm 0.94 \times 10^4$ cells were generated (Figure 3(C)). While at day 14 of reticulocyte maturation, less cell proliferation was achieved ($7.30 \times 10^5 \pm 0.95 \times 10^4$ cells), this is mainly due to the maturation of cells (Figure 3(C)).

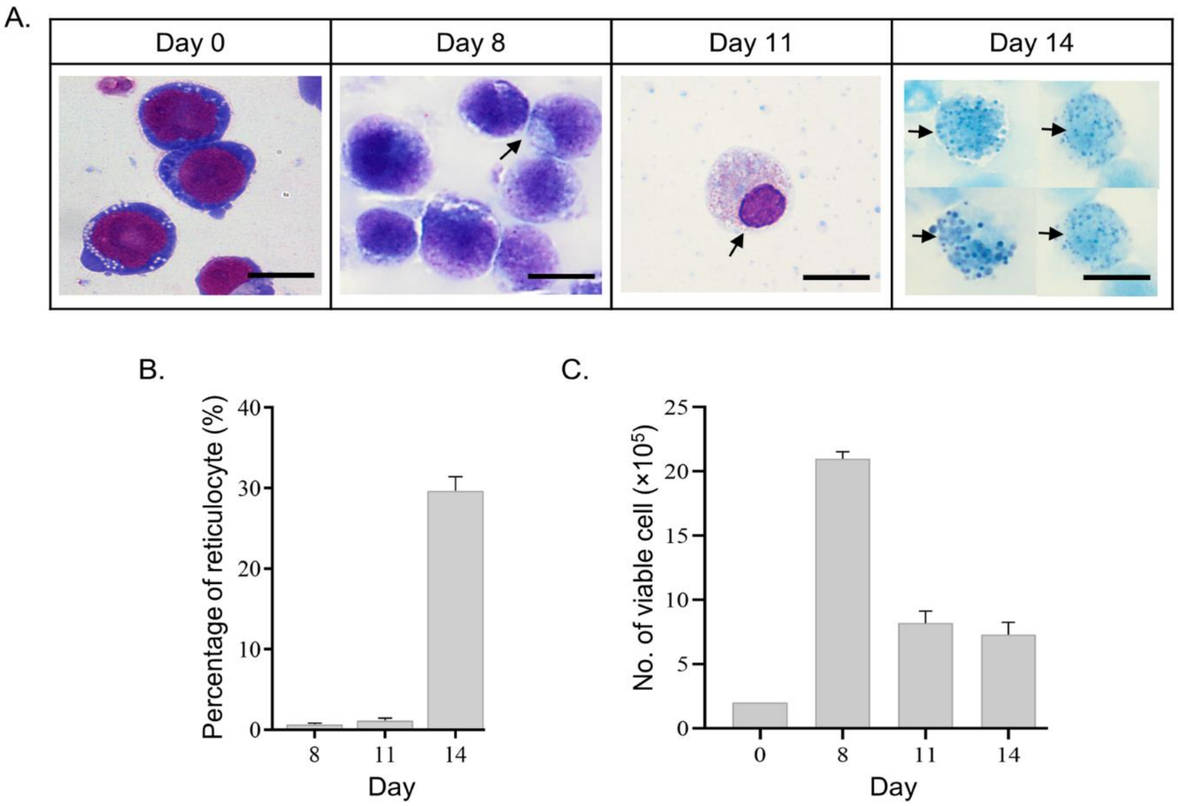

Figure 3: The morphology and number of differentiated PB-derived CD34$^+$ HSCs. (A) The representative images of PB-derived CD34$^+$ HSC maturation (day 0) to proerythroblast (day 8), normoblasts (day 11) and reticulocytes (day 14). Black arrows indicate the representative cells on that day. (B) The percentage of reticulocytes was calculated by dividing reticulocytes with a total of 500 cells and multiplied by 100% as determined by cresyl blue staining and (C) Number of viable cells from day 0 to day 14 (mean ± SEM) (n=3). Scale bars:10 µm.

Invasion of Reticulocytes by P. falciparum

The results show that HSC-derived reticulocytes were successfully invaded by the malaria parasite. The detection of trophozoites was observed at 48–hour post-inoculation (Figure 4(A)). Mature erythrocytes, which were used as a positive control, were also successfully invaded by *P. falciparum* as determined by the appearance of trophozoite stage parasites at 48–hour post-inoculation (Figure 4(B)). The parasitemia of infected HSC-derived reticulocytes and infected mature erythrocytes was 5.20 ± 0.11% and 6.66 ± 0.26%, respectively. However, the invasion rate of infected HSC-derived reticulocytes was slightly lower than that of infected matured erythrocytes.

Reticulocytes can be collected from different locations in the human body such as UCB and PB [15]. However, they are not extensively disseminated in the blood circulation [8, 10] and often technically difficult to isolate using percoll density centrifugation [16]. Many studies have used HSCs derived from UCB to produce reticulocytes *in vitro* due to its high expansion potential [5, 10]. Although UCB is considered as a good source for CD34$^+$ HSC, it has been shown to comprise a high percentage of fetal hemoglobin that could affect proliferation and invasion efficiency of the malaria parasite [10, 17]. Meanwhile, BM is the second source that has high concentration of CD34$^+$ HSC. However, the collection from this source consumes a lot of time due to required surgical operation for donors, involvement of a strict schedule for collecting BM, and one may not be able to collect it at high density (volume). Thus, sources other than UCB and BM should be considered for CD34^{+} HSC isolation. PB-derived HSCs have advantages in term of the accessibility of PB that can be collected at higher densities (the volume of blood) compared to UCB and BM. Besides, the collection of PB-derived HSCs involves a safe and donor friendly collection procedure since no surgical operation is required for the donor. Therefore, our aim was to isolate and characterize reticulocytes differentiated from PB-derived CD34^{+} HSCs and evaluate the potential of reticulocyte invasion by *P. falciparum*.

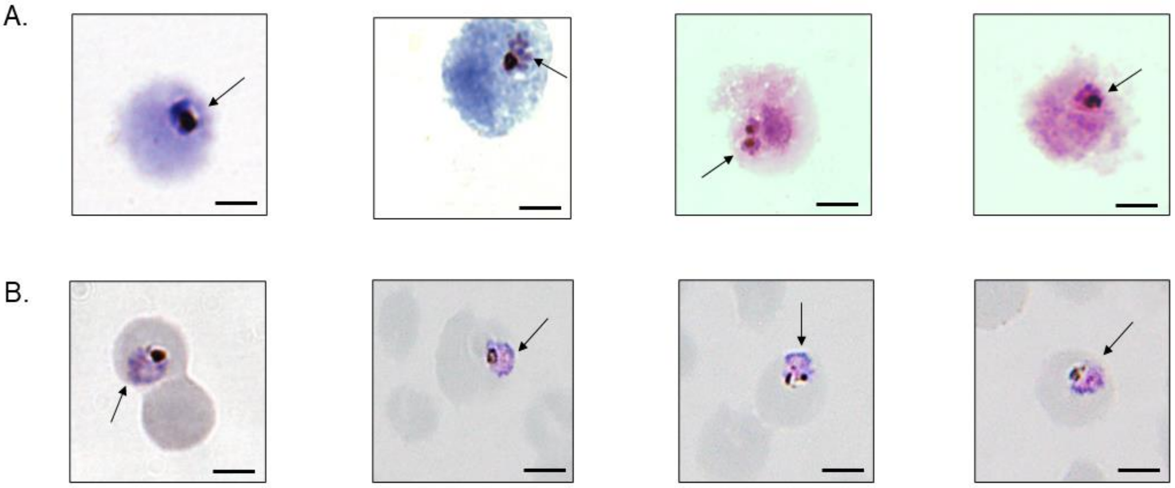

Figure 4: The invasion of *P. falciparum* into generated reticulocytes derived from PB-CD34^{+} HSCs. The representative images show (A) infected HSC-derived reticulocytes by *P. falciparum* at 48–hour post-inoculation (as indicated by arrows) and (B) mature erythrocytes enriched from whole blood by *P. falciparum* at 48–hour post-inoculation (as indicated by arrows). Scale bars:10 µm.

In the present study, the high purity of PB-derived CD34^{+} HSCs was successfully obtained by using MACS microbeads. It has been previously reported that HSCs have the capability to continuously self-renew to produce various cell populations assisted *in vivo* by interactions between cytokines and their receptors [17]. Therefore, several combinations of cytokines known to act on HSCs have been employed *in vitro* in an attempt to produce culture conditions that are suitable for HSC expansion. In this study, SCF, TPO, FLT-3 and IL-6 cytokine cocktails were used to support HSC proliferation and expansion [18]. These cytokines play an important role in order to promote cell proliferation, cell division, chemotaxis and adhesion, cell survival and transformation of haematopoietic progenitors derived from HSCs in the blood lineages [19]. The result shows that the isolated PB-derived CD34^{+} HSCs expanded to 2.11 ± 0.10-fold under this culture condition. This exceeds expansion fold previously reported by Noulin et al. [10] which only obtained 1.30 ± 0.20-fold for PB-derived CD34^{+} HSCs.

Reticulocyte differentiation and maturation were characterized by the phenotypic profile of cell surface antigens and morphological analysis. The CD34 and CD45 were used to characterize early stages of culture in which the expression of both antigens was high on day 0 of culture. However, after cells became committed to the erythroid lineage, the expression of both CD34 and CD45 was significantly decreased [20]. The CD36/CD71 are important markers for characterization of erythroid differentiation [5, 10, 21]. CD71 is highly expressed during hemoglobinization of erythroid cells especially at normoblast stage after which expression decreases through the reticulocyte stage [5, 22]. Our data show that CD34, CD45 and CD36/CD71 expression were consistent with the previous studies on reticulocyte induction mechanism [5, 10, 20]. In correlation with morphological analysis, these data suggest that a progressive maturation of PB-derived CD34$^+$ HSCs to reticulocytes was achieved at day 14.

It is well known that erythropoietin is a crucial regulator of erythroid differentiation [21]. Yet, erythropoietin alone is inadequate to generate a sufficient number of reticulocytes. In addition to erythropoietin, other cytokines such as hydrocortisone, interleukin-3 and stem cell factor were utilized for *in vitro* reticulocyte differentiation in order to promote the proliferation of erythroid progenitors and direct the lineage specification to ensure full maturation into reticulocytes [23]. Moreover, Zhang et al. [21] reported that other cytokines were sequentially omitted in the last step of maturation due to their multilineage effects. This is consistent with the result of our study where during early erythroid differentiation, the cells were actively proliferated, followed by the decrease in the proliferation capacity in the terminal erythroid differentiation and reticulocyte maturation process. Nonetheless, the sufficient number of reticulocytes was obtained and the cells were functionally active as determined by the ability of *P. falciparum* to grow within the cells.

The infection of reticulocytes by the malaria parasites is an essential indicator of their robustness and biological function because the parasite growth needs the membrane integrity for efficient absorption of nutrients from the medium as well as metabolites available directly from infected reticulocytes [9]. Our data showed that reticulocytes collected on day 14 were successfully invaded by *P. falciparum*. Due to some difficulties to observe the parasite growth in reticulocytes within the 24–hour life cycle, we took an alternative to monitor the parasite growth over the 48–hour life cycle [18]. The previous study suggested that reticulocytes, which are marked by the surface transferrin receptor (CD71) are the predominant target cells for the invasion by the malaria parasite [23]. Thus, our data suggested that the generated reticulocytes derived from PB-derived CD34$^+$ HSCs promoted the expression of the surface marker, which would enable the invasion of *P. falciparum* into the cells. Therefore, we confirmed that the differentiation of PB-derived CD34$^+$ HSCs into reticulocytes as successful. The malaria parasite invaded both HSC-derived reticulocytes and mature erythrocytes, suggesting that the generated reticulocytes are functionally active. However, further in-depth investigations are needed in order to determine the precise mechanism of *P. falciparum* invasion into reticulocytes such as investigation on reticulocyte-specific receptor. The finding of this study would also open doors to future studies of other malaria parasites such as *P. knowlesi* and *P. vivax*.

Conclusions

CD34$^+$ HSCs derived from PB could be considered as a potential source of reticulocytes, which can be generated in sufficient quantities for experiments. The availability of this *in vitro* reticulocyte model will allow more details analysis to be done such as for fundamental research in erythroid development as well as the *in vitro* reticulocyte model for malaria research. Besides, further efforts should be directed towards cryopreservation of reticulocytes for providing a continuous source to be used for *in vitro* culture of the malaria parasite especially for *P. knowlesi* or *P. vivax* that lacks a convenient *in vitro* culture system. In the future, it would be judicious to isolate *P. knowlesi* and *P. vivax* from human patients and culture with generated reticulocytes derived from PB-derived CD34$^+$ HSCs for use in future experimental investigations of these malaria parasite species.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards as approved by the Human Research Ethics Committee Universiti Sains Malaysia (USM/JEPeM/15100345).

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