Duffy-Positive Reticulocytes Generated *In Vitro* from Human Peripheral Blood CD34$^+$ Haematopoietic Stem Cells Are Susceptible To Invasion by *Plasmodium knowlesi*

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Abstract

Plasmodium knowlesi shows a predilection towards human reticulocytes that have the Duffy blood group antigens required for malaria parasite invasion. Low concentration of reticulocytes obtained from peripheral blood restricts the use of these cells for *P. knowlesi in vitro* culture. Therefore, the present study aimed to generate reticulocytes *in vitro* from human peripheral blood CD34$^+$ haematopoietic stem cells (PB-derived CD34$^+$ HSCs) and determine the presence of the Duffy blood group antigens on generated reticulocytes before invasion with *P. knowlesi*. Following expansion and differentiation of PB-derived CD34$^+$ HSCs to reticulocytes, the Duffy blood group of generated reticulocytes was determined by using a polymerase chain reaction-sequence specific primer (PCR-SSP) before inoculation with the malaria parasite. Results show that the generated reticulocytes had *FY*A/FY*B* genotype indicated by the amplification bands at 720 bp. These Duffy-positive reticulocytes as enucleated cells on day 14 of differentiation were also invaded by the malaria parasite. This study demonstrates the susceptibility of the reticulocytes generated from the HSCs with the Fy(a+b+) phenotype for *P. knowlesi* invasion, which may lead to the establishment of *in vitro* culture and research of this malaria parasite.

Keywords: Peripheral blood, CD34$^+$ haematopoietic stem cell, reticulocyte, Duffy blood group, polymerase chain reaction-sequence specific primer, *Plasmodium knowlesi, in vitro* culture

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Introduction

The zoonotic *P. knowlesi* is becoming a major cause of human malaria in Malaysia. This malaria parasite is widely distributed in Malaysian Borneo (Sabah and Sarawak) and Peninsular Malaysia (Kelantan, Pahang, Selangor, Johor and Negeri Sembilan) [1]. In 2018, the endemic cases of *P. knowlesi* malaria brought Southeast Asia in the second rank of malaria burden [2]. The current intervention seems to be successful in reducing transmission of human-only malaria parasites but is ineffective in eliminating the factor that leads to the increase in zoonotic malaria [3]. Deaths due to *P. knowlesi* prove that this zoonotic malaria parasite has the capability to cause severe malaria and fatal in humans [4].

Research on *P. knowlesi* in non-human primates has a great importance to understand the biology of this malaria parasite [5, 6]. The necessity to grow the malaria parasite in macaques however restricts research to laboratories with access to suitable primate facilities. The previous attempts to culture *P. knowlesi in vitro* using human erythrocytes showed lower rates of malaria parasite replication due to the decreased level of the Duffy blood group antigens (also known as the Duffy antigen receptor for chemokines, DARC) on the membrane of mature erythrocytes as compared with young erythrocytes called reticulocytes [7, 8].

The Duffy antigen (Fy) plays an important role as a receptor for the invasion of *P. knowlesi* [9]. This antigen is encoded by the *FY* gene, which has three main alleles, *FY*A*, *FY*B* and *FY*B*^{ES} [10]. *FY*A* and *FY*B* confer the common Duffy phenotypes Fy(a+b+), Fy(a+b−) and Fy(a−b+). Homozygozity of the *FY*B*^{ES} allele results in the phenotype Fy(a−b−) [11]. The *P. knowlesi* ligand (PkDBPαII) has been shown to bind to Duffy-positive human erythrocytes, while Duffy-negative human erythrocytes have been stated refractory to invasion by this malaria parasite [12].

The preference of *P. knowlesi* towards Duffy-positive reticulocytes has led to the collection of these cells from peripheral blood (PB) [13]. Reticulocytes however circulate in PB at a very low concentration (0.5-1% of total erythrocytes) and for a very short time (1 day) [14]. Therefore, the present study was aimed to generate reticulocytes from PB-derived $CD34^+$ HSCs and determine the presence of Duffy blood group on the generated reticulocytes prior to invasion with *P. knowlesi*.

Materials and Methods

Expansion and differentiation of PB-derived $CD34^+$ HSCs to reticulocytes

Isolation of $CD34^+$ HSCs from peripheral blood mononuclear cells (PBMCs) of healthy donors (n=4) (Ethics approval ID: USM/JEPeM/15100345) was carried out by using MACS system (Miltenyi Biotec, USA). The cell purity was confirmed by FACS (BD Bioscience, USA) using CD34 and CD71 antibodies (BD Bioscience, USA). The cells (2×10^5 cells) were expanded in 4 mL serum-free media (Sigma-Aldrich, USA) consisting of 50 ng/mL FMS-like tyrosine kinase 3 (R&D Systems, USA), 50 ng/mL interleukin 6 (R&D Systems, USA), 50 ng/mL stem cell factor (Sigma-Aldrich, USA) and 50 ng/mL thrombopoietin (R&D Systems, USA) and cultured at 37°C in a 5% humidified CO_2 [14]. On day 5 of expansion, the cells were transferred into new 6-well plates for differentiation.

The expanded cells (2×10^5 cells) were cultured with 4 mL IMDM differentiation medium (Sigma-Aldrich, USA) consisting of 4 mM L-glutamine (Sigma-Aldrich, USA), 10

µg/mL folic acid (Sigma-Aldrich, USA), 10 µg/mL insulin (Sigma-Aldrich, USA), 40 µg/mL inositol (Sigma-Aldrich, USA), 1.6×10^{-4} M monothioglycerol (Sigma-Aldrich, USA), 120 µg/mL transferrin (Sigma-Aldrich, USA), 1% penicillin/streptomycin (Gibco, USA) and 1% foetal bovine serum (FBS) in IMDM (StemCell Technologies, USA) [14]. During the first stage of erythroid differentiation (day 0-8), the medium was supplemented with 10^{-6} M hydrocortisone (Sigma-Aldrich, USA), 5 ng/mL interleukin 3 (R&D Systems, USA), 100 ng/mL stem cell factor (Sigma-Aldrich, USA) and 25 ng/mL erythropoietin (R&D Systems, USA). An extra 3 mL medium was added after four days of culture. At day 8, the cells were pelleted and the medium consisting of erythropoietin was added before transferring into new small flasks for the second stage of differentiation (day 8-11). During the final stage of differentiation (day 11-20), the growth factor-free medium was added and refreshed every three days with the addition of 10% FBS.

Cresyl blue staining of reticulocytes

At day 14 of differentiation, generated reticulocytes (2×10^5 cells) were suspended in 100 µL PBS before adding 100 µL cresyl blue staining solution (0.3%) (Sigma-Aldrich, USA) [14]. After 30 minutes of incubation at room temperature, the cells were centrifuged by using a cytospin (Cyto-Tek 2500 Cytocentrifuge, Sakura Finetek, USA). The slides were air-dried and the cells were visualised by using a bright field microscope (Olympus BX41, Nishi Shinjuku-ku, Japan). Reticulocytes appeared as enucleated cells with at least three dots of cresyl blue RNA and counted against a total of 500 cells.

RNA and DNA extraction

A total RNA from generated reticulocytes was isolated by using Ambion TRIzol® reagent (Invitrogen, USA) and converted to genomic cDNA by using AMV Reverse Transcriptase (Promega, USA) according to the manufacturer's instructions. Meanwhile, DNA of the *P. knowlesi*-infected blood from patients (n=2) collected from Hospital Gua Musang, Kelantan (Ethics approval ID: NMRR-16-997-29069) was extracted by using Invisorb Spin Forensic kit (STRATEC Molecular GmbH, Germany).

Polymerase chain reaction-sequence specific primer (PCR-SSP)

The PCR reaction was carried out by using 12.5 µL of 2×EasyTaq® PCR SuperMix (+dye) (TansGen Biotech, China), 0.25 µL of each forward and reverse primer for control (human growth hormone) (1st Base, Singapore), 0.5 µL of each forward and reverse primer for Duffy A or B (1st Base, Singapore), 2 µL of each DNA sample (20 ng/mL) (STRATEC Molecular GmbH, Germany) and 9 µL ddH_2O. Primers were designed as previously described [15] and listed in Table 1. The two forward primers are highly specific and targeted single nucleotide polymorphism that differentiate the two Duffy (A and B) alleles [15]. In contrast, a pair of control primers was used to amplify human growth hormone and added into each PCR reaction mixture [15]. PCR condition was started with initial denaturation step of 5 minutes at 94°C followed by 35 incubation cycles for 30 seconds at 94°C, 60 seconds at 61°C and 30 seconds at 72°C. Then, 7 minutes at 72°C followed with hold at 4°C. Positive control (erythrocyte of known Duffy presence detected using serological technique described elsewhere [16]) and negative control (no DNA template) were also included to validate the adopted PCR-SSP typing of Duffy blood group. The PCR product was separated in 2% agarose gel containing 5 µL SYBR safe DNA gel stain (Invitrogen, USA) and visualised by using a molecular imager® Gel Doc™ XR with Image Lab™ Software (Bio-Rad, USA).

Table 1: Primers for nested PCR-SSP

Primer	Primer sequence	Product size (bp)
FY*A-F	5´-CAGCTGCTTCCAGGTTGCCAC	720
FY*B-F	5´-CAGCTGCTTCCAGGTTGGTAT	
FY-R	5´-GCCCTCATTAGTCCTTGGCTCTCAT	

Collection of P. knowlesi isolates

P. knowlesi samples were collected from patients (n=2) admitted to Hospital Gua Musang, Kelantan. Patients were recruited based on inclusion and exclusion criteria approved by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (MOH) (NMRR-16-997-29069) as well as by the Human Research Ethics Committee USM (USM/JEPeM/15100345). Blood samples (10 mL) of the selected patients were withdrawn and transferred in heparin anticoagulant tubes. Diagnosis of *P. knowlesi* infection was determined by microscopic examination of Giemsa-stained thick and thin blood smears. Confirmation of *P. knowlesi* infection was conducted by using a species-specific nested-PCR assay [9].

Parasite invasion assay

Cryovials containing *P. knowlesi*-infected blood were taken out from the liquid nitrogen storage and allowed to warm for 1 minute at 37°C until completely thawed [9]. The parasitaemia and asexual stage of the malaria parasite after thawing were determined. The parasite was cultured for at least 1 day before being used for invasion assay. The parasite was cultured with the Duffy-positive generated reticulocytes in 25 mM RPMI 1640 medium containing GlutaMAX I and HEPES (Gibco, USA), 0.25% Albumax II, 0.05 mg/mL hypoxanthine (Sigma-Aldrich, USA), 0.2%glucose (Sigma-Aldrich, USA) and 0.025 mg/mL gentamicin (Duopharma, USA) [17, 18]. The malaria parasite was incubated at 37ºC for 24 hours in 5% CO_2. Susceptibility of the generated reticulocytes to invasion by the malaria parasites was determined by Giemsa-stained thin blood smears [19, 20].

Results and Discussion

Characterisation of reticulocytes differentiated from PB-derived $CD34^+$ HSCs

Reticulocytes can be collected from different sources in the human body [21, 22], however, they are not extensively disseminated in the blood circulation [23] and often technically difficult to isolate using percoll density centrifugation [24]. PB-derived $CD34^+$ HSCs have advantages in term of the accessibility of PB that can be acquired with minimal invasiveness as compared with other sources such as umbilical cord blood and bone marrow [13, 25].

In the present study, the $CD34^+$ cell-enriched population was isolated from PBMCs and allowed to expand until day 5 in serum-free expansion medium in the presence of growth factors and cytokines such as FMS-like tyrosine kinase 3, interleukin 6, stem cell factor and thrombopoietin. The total number of $CD34^+$ HSCs increased by ~2-fold in the culture. The flow cytometry analysis also revealed an increase in the $CD34^+/CD71^+$ population between day 0 and day 5 (Figure 1). The expansion fold obtained from this study exceeded the number reported by the previous study [10] in which they obtained only 1-fold for PB-derived $CD34^+$ HSPCs by using the same technique. The difference in the expansion fold obtained might be

due to the difference in the volume of the PB-derived CD34$^+$ HSPCs used in the experiment, thus might result in the different number of the cells obtained.

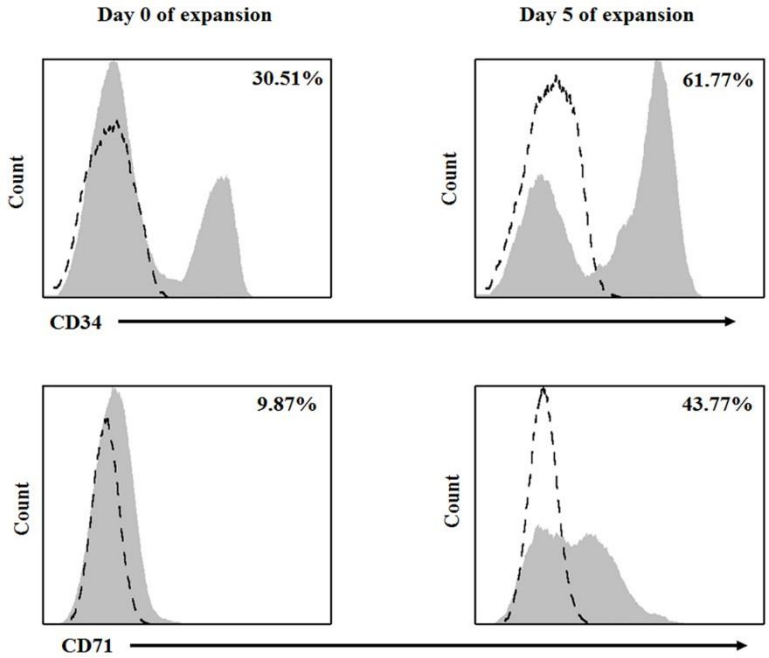

Figure 1. The expansion of PB-derived CD34$^+$ HSCs. Histograms show the expression of CD34 and CD71 on day 0 and day 5 of expansion. The grey-filled curve represents the marker and the dashed line curve represents the isotype control.

Following expansion, the differentiation of expanded PB-derived CD34$^+$ HSCs to reticulocytes was performed under three differentiation stages (stage 1 from day 0-8; stage 2 from day 8-11; stage 3 from day 11-20). After day 8 of stage 1 at which hydrocortisone, interleukin 3, stem cell factor and erythropoietin were added in the medium, the cells proliferated up to ~10-fold. This exceeds expansion fold previously reported (3.4-fold) [14]. In the addition of erythropoietin only in the medium, an early commitment of the CD34$^+$ HSCs into erythroid progenitors was observed by the presence of proerythroblast cells with a highly basophilic cytoplasm and a large nucleus on day 8 of stage 2 (Figure 2). After day 11 of stage 3 without erythropoietin, reticulocytes with cresyl blue-stained RNA granules were started to observe and reached the maximum count on day 14 (Figure 2, arrows). In this study, the expansion and differentiation of PB-derived CD34$^+$ HSCs to reticulocytes were achieved without the use of stromal cells. Stromal cells have been used to retain nucleated cells as well as free nuclei and debris so that the culture would appear clean [26]. The stromal cell co-culture is, however, a tedious method although it can maintain the quality and stability of the co-cultured cell population.

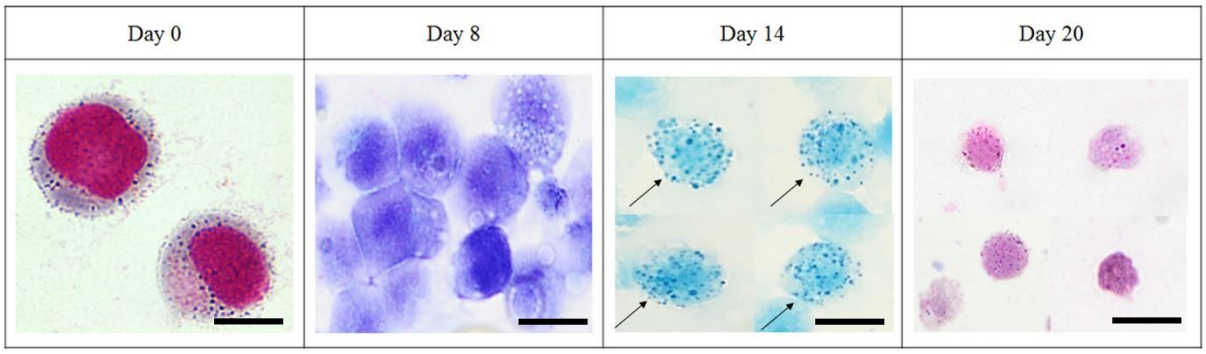

Figure 2. The morphology of differentiated PB-derived CD34^{+} HSCs on day 0, 8, 14 and 20 of differentiation shown by Giemsa staining. Black arrows indicate reticulocytes, which peak at day 14 of differentiation. Scale bar: 10 µM

Before differentiation, most cells expressed a high level of CD34, a marker of HSCs (Figure 3). By day 8, phenotypically the percentage of CD34 was decreased. A higher level of CD71, a marker of immature RBCs (reticulocytes) was expressed on day 14 and started to decrease until day 20. CD71 is the transferrin receptor known to be released during erythrocyte maturation [27]. Immature reticulocytes highly expressing CD71 are the predominant target cells for invasion by the malaria parasites including *P. knowlesi* [28]. This is in line with a recently published report [29] showing the malaria parasite preferentially invaded young reticulocytes (CD71^{high}) compared with mature erythrocytes.

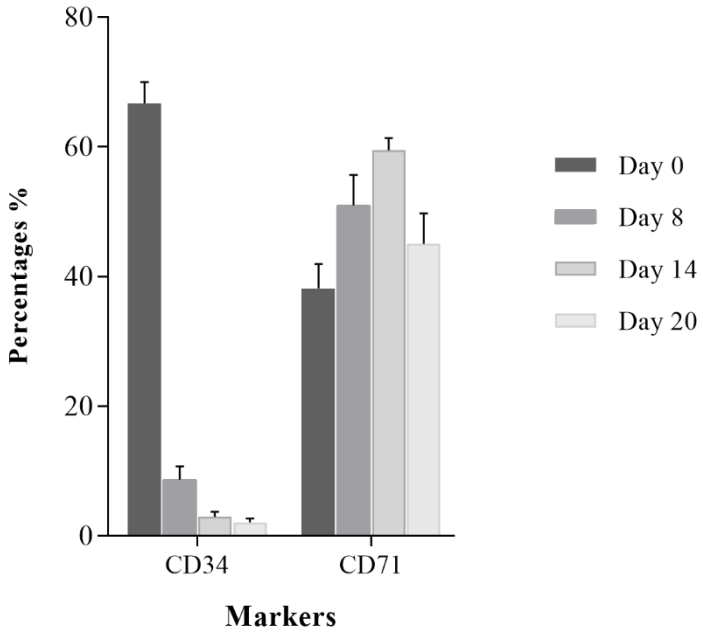

Figure 3. The expression of cell surface markers, CD34 and CD71 on erythroid stages. The graphs show the mean percentage of positive cells expressed on day 0 until day 20 of differentiation (mean ± SEM) (n=3).

The presence of the Duffy blood group on generated reticulocytes

In addition to incompatibilities in haemolytic disease of newborns and transfusion, the antigens of the Duffy blood group are of great interest in the invasion of erythrocytes by *P. knowlesi* and *P. vivax* [12]. The negative homozygous condition for the Duffy blood group Fy(a−b−) confers natural resistance to both parasite infections [12, 30]. Prior to invasion assay with *P. knowlesi*, the presence of the Duffy blood group on the generated reticulocytes from the PB-derived $CD34^+$ HSCs was determined by using PCR-SSP. The amplification products of the Duffy allele A and B were observed by the presence of a band at 720 bp (Figure 4). The presence of sharp, intact and thick 720 bp bands was observed for the positive control (CP) (erythrocytes of known Duffy presence), generated reticulocytes (R1 and R2) and *P. knowlesi*-infected blood (PK) samples. Samples of the generated reticulocytes and *P. knowlesi*-infected blood were genotyped as *FY*A/FY*B* (phenotypically Fy(a+b+), which is line with the previous studies [31, 32].

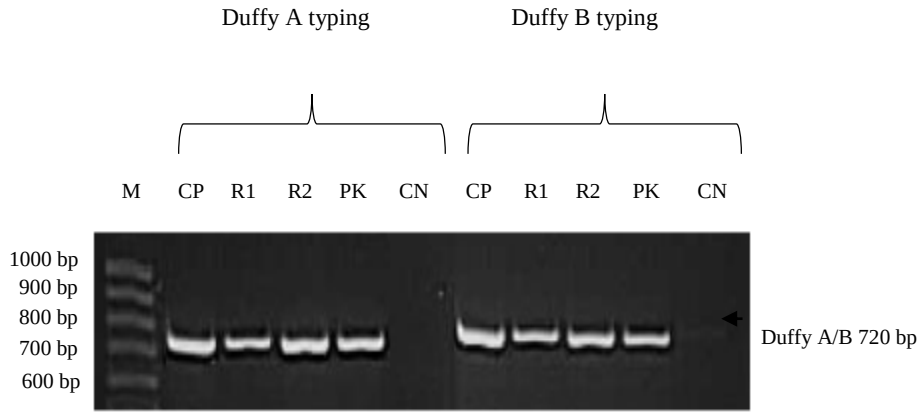

Figure 4: Agarose gel electrophoresis of PCR-SSP products of Duffy A and B alleles of a sample known with the presence of the Duffy blood group (positive control, CP), a sample without DNA template (negative control, CN), generated reticulocytes (R1 and R2) and *P. knowlesi*-infected blood (PK).

Invasion of generated reticulocytes by P. knowlesi

The results show that the $CD34^+$ HSC-derived reticulocytes with the Fy(a+b+) were invaded by *P. knowlesi* (Figure 5). The requirement for the Duffy receptor for human erythrocyte invasion by the malaria parasite has been reported [33], while Duffy-negative human erythrocytes have been demonstrated refractory to invasion by *P. knowlesi* [12]. This is confirmed by the DARC-dependence of the invasion of human erythrocytes by both the *P. knowlesi* A1-H.1 and UM01 lines [34]. Therefore, the present study demonstrates that the generated reticulocytes are functioned and potentially could serve as the target host cells for *P. knowlesi in vitro* culture.

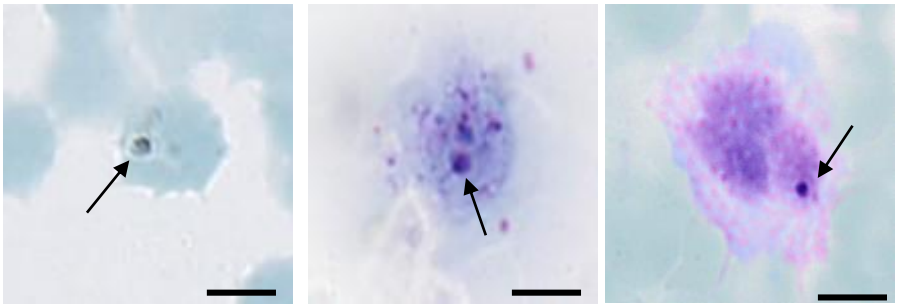

Figure 5. The invasion of generated reticulocytes derived from PB-CD34$^+$ HSCs with *P. knowlesi* (arrows). Scale bar: 10 µM

Conclusion

The susceptibility of the reticulocytes generated from the PB-derived CD34$^+$ HSCs to invasion by *P. knowlesi* was attributed to the Duffy blood group antigens. The Duffy blood group antigens are important for *P. knowlesi* infection as they provide a route for the entry of the malaria parasite into the generated reticulocytes. Nonetheless, the results need to be corroborated by further evaluation with any Duffy negative samples in order to compare the susceptibility of Duffy-positive and Duffy-negative reticulocytes to *P. knowlesi* infection.

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Author Contributions

All authors contributed towards 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.

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