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Development of an Editable Approach to the Study of Parasite-Erythroid Interactions

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Submitted in total fulfilment of the requirements of Master Degree
July 2020

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Abstract

Malaria remains responsible for an enormous health burden worldwide; considerable research effort is being devoted to finding ways to combat the disease and its transmission. Malaria is caused by Plasmodium species, and *P. falciparum* causes the most serious disease. The blood stage of the *P. falciparum* remains critically important to understand for development of treatments and vaccines. To initiate invasion, the *P. falciparum* merozoite recognises specific proteins on the host red cell membrane, known as invasion receptors. In order to study parasite–host interactions, laboratory-adapted *P. falciparum* strains that invade mature human red cells have been used. Gene modification methods are well established for *P. falciparum*; however, genetic manipulation of the red cell has not been extensively applied because erythrocytes are not nucleated. The *in vitro* cultivation of erythroid cell lines facilitates both the scalable production of host cells to support *P. falciparum* invasion and editing of nucleated precursors that can be genetically modified in a precise manner.

In this project, two erythroid cell lines – the Human Umbilical cord blood Derived Erythroid Progenitors (HUDEP-2) and the Bristol Erythroid Line- Adult (BEL-A), both of which can differentiate to more mature forms *in vitro* – were studied as possible host models. A FACS antibody panel, based on the stage-specific profile of HUDEP-2 and BEL-A cells, provided the means to analyse host invasion receptors as well as erythroid maturation markers.

Band 3 is a red cell membrane protein with an uncertain role in merozoite invasion. A gene knockout was constructed in expansion stage BEL-A cells using the lentiviral CRISPR/Cas9 system, targeting band 3 which may be involved in merozoite invasion of human erythrocytes. Single-cell-derived clones were isolated and preliminary validation using PCR and flow cytometry was performed to verify disruption of band 3. Completion of work to validate and functionally characterise the band 3 knockout, and experiments to assess effects on invasion, were curtailed by COVID-19 stay-at-home orders issued to Melbourne between March and July 2020.

Abstract

In summary, a genetically editable *in vitro* erythroid model was defined to study the function of host invasion receptors for *P. falciparum* merozoite invasion. Clonal band 3-deficient BEL-A cells were generated, thus paving the way for studying their role as invasion receptors.

Key words: *P. falciparum*; erythroid cell lines; invasion; CRISPR knock out

Abbreviations

aa	amino acid
ABCB6	ATP Binding Cassette Subfamily B Member 6
AE1	anion exchanger 1
AGRF	Australian Genome Research Facility
Alb	albumax
APC	Allophycocyanin
BEL-A	Bristol Erythroid Line- Adult
BFP	blue fluorescence protein
BFU-Es	Burst-forming unit-erythroid cells
BLAST	basic local alignment search tool
BLAT	BLAST-like alignment tool
bp	base pairs
BSG	basigin
BCL-XL	B-cell lymphoma-extra large
CD	cluster of differentiation
CFU-Es	colony-forming unit-erythroid cells
CR1	complement receptor 1
CRISPR	clustered regularly interspaced short palindromic repeat
CRISPRa	CRISPR activation
Cas	CRISPR-associated
CyRPA	cysteine rich protective antigen
DAF	decay accelerating factor
DIDS	4,4'-diisothiocyano-2,2'-stilbene disulfonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EBA	erythrocyte binding antigen
EBL	erythrocyte binding-like antigens
EPO	erythropoietin
EPOR	erythropoietin receptor
ER	endoplasmic reticulum

Abbreviations

EtBr	Ethidium bromide
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
F/R	forward/reverse
GFP	green fluorescence protein
GYPA	glycophorin A
GYPB	glycophorin B
GYPC	glycophorin C
HDR	homologous directed repair
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS	hereditary spherocytosis
hSCF	human stem cell factor
HUDEP-2	Human Umbilical cord blood-Derived Erythroid Progenitors
HPV	human papilloma virus
IFA	immunofluorescence assay
IMDM	Iscoe's Modified Dulbecco's Medium
InDel	insertion or deletion
L	liter
MEPs	megakaryocyte erythroid progenitors
MFI	Median fluorescence intensity
MSP1	merozoite surface protein 1
NCBI	National Center for Biotechnology Information
NHEJ	non-homologous end joining
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PE	Phycoerythrin
PfEMP1	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
PfRh	<i>Plasmodium falciparum</i> reticulocyte-binding-like protein homolog
PfRipr	<i>P. falciparum</i> Rh5 interacting protein
PI	Propidium Iodide
pM	10 ⁻¹² mole/L
PU	10 ⁻¹² unit

Abbreviations

RBC	red blood cell
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute Medium
SAO	Southeast Asian ovalocytosis
SFEM	Serum-free medium for culture and expansion of hematopoietic cells
sgRNA	single-guide RNA
SLC4A1	solute carrier family 4-member 1
SSC-FSC	side scatter – forward scatter
STAT5	Signal transducer and activator of transcription 5
WEHI	the Walter and Eliza Hall Institute of Medical Research

Chapter 1: Introduction

1.1 Malaria remains a health burden in endemic areas

According to the latest world malaria report, malaria mortality and case incidence rates decreased from 2010 to 2018, but progress has lagged since 2014 (WHO, 2019). In endemic regions, *Plasmodium falciparum* and *P. vivax* were responsible for an estimated 405,000 deaths in 2018, with children under five years the most vulnerable population. *P. falciparum* is the most lethal of the six malaria species that infect humans and responsible for over 93% of total estimated cases. Africa continues to bear the heaviest health burden caused by malaria, followed by South-east Asia and the Eastern Mediterranean. Despite decades of strenuous effort, a preventive vaccine that is suitable for extensive use globally is not yet available. RTS, S/A01, the current most advanced malaria vaccine, which aims to target the establishment of a liver infection after a mosquito bite, still requires optimisation to increase its efficacy and feasibility (reviewed in (Healer, Cowman, Kaslow, & Birkett, 2017)). Despite steps in the development of new drugs and vaccines, it is important to understand more about the biology of the parasites that cause malaria so that we can develop better treatments to fulfil the ultimate goal of malaria elimination.

1.2 Malaria parasites cause disease

Malaria is caused by parasites belonging to the *Plasmodium* genus of the phylum Apicomplexa. Due to differences in parasite-infected individuals' immunity, outcomes can range from parasite clearance to death of the host. Characteristic symptoms include fever, chills and headache. Splenomegaly can be observed after the early stage of the disease. Anaemia is often observed in clinical cases. Therefore, anaemia rates, to some extent, indicate the level of malaria control in endemic areas (WHO, 2018). Erythrocyte rupture caused by egress of the parasite from schizont stages account for the febrile periodicity in typical malaria cases. However, unlike other species, fever caused by *P. falciparum* infection exhibits a more irregular pattern. *P. falciparum* can cause severe

malaria that leads to conditions such as cerebral malaria and severe anaemia, which have a poor prognosis and can lead to life-threatening consequences if lacking proper diagnosis and treatment.

1.3 *P. falciparum* lifecycle and malaria pathogenesis

This project focusses on *P. falciparum*, which is the causative agent of the form of malaria with the highest morbidity and mortality globally (WHO, 2018). As illustrated by Figure 1.1 (reviewed in (Cowman, Tonkin, Tham, & Duraisingh, 2017)), to complete the two-host lifecycle and transmission of *P. falciparum* in humans, three requirements need to be met: human reservoirs must carry the sexual forms of the parasite (gametocytes), female Anopheles mosquitoes (arthropod vectors) must take blood meals containing gametocytes from carriers, and must inject sporozoites into another host when taking a second blood meal. After the sporozoites enter the host, the lifecycle can be divided into two stages: the liver stage, which is also referred to as the pre-erythrocytic stage, and the blood stage. At the liver stage, sporozoites invade hepatocytes and develop into thousands of liver-stage merozoites that are released into the blood, where they can invade erythrocytes.

Erythrocytic parasites consume haemoglobin as a source of energy and produce haemozoin, an iron-containing pigment, as metabolic waste. Merozoites develop to ring forms, then trophozoites and schizonts as they mature. Schizonts have a segmented appearance, because they are multi-nuclear late stages of asexual replication. Several dozen daughter merozoites per infected erythrocyte are released from late schizonts during egress and invade new erythrocytes, thus activating a new round of multiplication. Gametocytes differentiate from blood-stage parasites and can be taken up by a feeding mosquito for transmission to the next host.

The erythrocytic parasite stages are responsible for the pathogenicity of *P. falciparum* due to complex reasons that include both parasite and host factors. The rupture of infected erythrocytes at the schizont stage release daughter merozoites, as well as pyrogenic material. Antigens from erythrocytic lysis, parasites and their metabolic waste can be recognised by the host immune system as pyrogens and result in febrile outcomes (Crutcher, J. M., & Hoffman, S. L. (1996). Malaria. In S. Baron

(Ed.), Medical Microbiology. (4th ed.). University of Texas Medical Branch at Galveston.). Additionally, malarial anaemia is an important complication of malaria, and can be caused by direct destruction of red blood cells when schizonts burst and release merozoites, clearance of uninfected erythrocytes by phagocytic cells, and impaired erythropoiesis (Crutcher, J. M., & Hoffman, S. L. (1996). Malaria. In S. Baron (Ed.), Medical Microbiology. (4th ed.). University of Texas Medical Branch at Galveston.). Overall, the severity of disease is determined by a balance of factors between the *P. falciparum* parasite and host immunity (Deroost, Pham, Opdenakker, & Van den Steen, 2016).

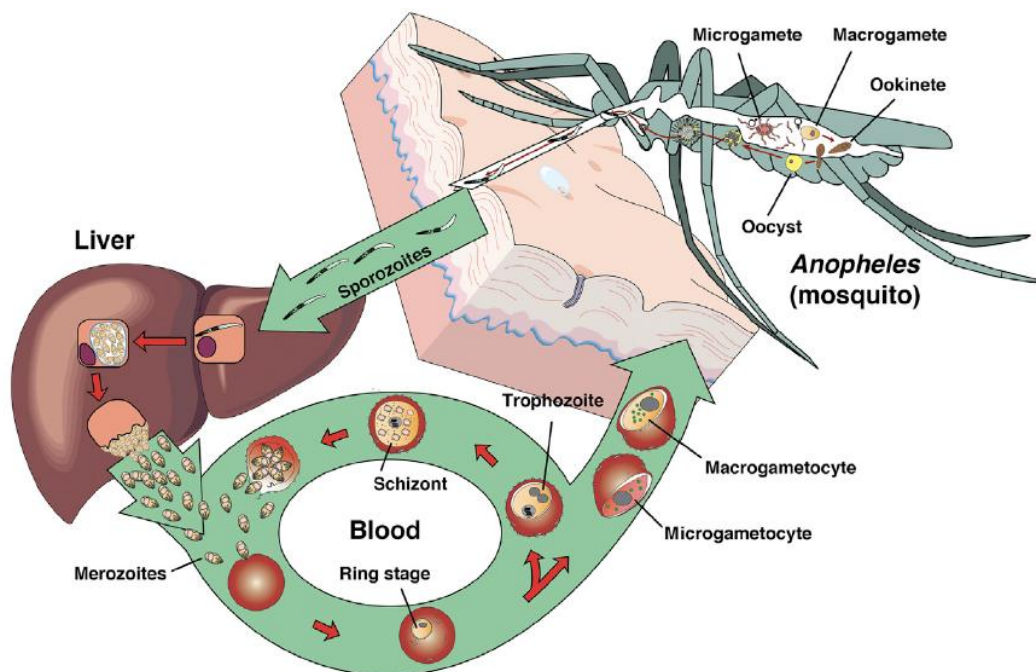


Figure 1.1 *P. falciparum* lifecycle (reviewed in (Cowman et al., 2017)).

1.4 Receptors on the host cell are required for invasion

Invasion of the merozoite form of *P. falciparum* into human erythrocytes can be divided into stages of attachment, reorientation, tight junction formation and internalisation (Fig. 1.2B). Merozoites attach to the erythrocyte membrane in a random orientation; a process of membrane wrapping, or reorientation then connects the apical end of the

parasite cell to the erythrocyte (reviewed in (Cowman et al., 2017)). Tight junction formation follows, allowing parasite penetration to occur through force generated by the acto-myosin motor, after which the host membrane reseals, forming a parasitophorous vacuole surrounding the internalised parasite (reviewed in (Cowman et al., 2017)). . Invasion is a highly coordinate event involving the actions of various proteins, with the interaction between host cells and parasites plays a crucial role in successful invasion (reviewed in (Cowman et al., 2017)). . To complete this process, parasites rely on the recognition of merozoite ligands by specific red blood cell surface receptors .

1.5 Known invasion host receptors and parasite ligands

Numerous receptors on the red blood cell membrane have been identified to be involved in *Plasmodium* spp. invasion. Importantly, each malaria species has preferred invasion receptors, which play key roles in host cell specificity with respect to the erythroid differentiation stage (reviewed in (Cowman et al., 2017)).

The classical sialic acid-dependent pathway is perhaps the most well studied and involves three important glycoproteins: glycoprotein A (GYPA, CD235a), glycoprotein B (GYPB) and glycoprotein C (GYPC). Glycoproteins are modified with carbohydrates, mostly sialic acid (Chasis & Mohandas, 1992). GYPA and GYPB carry the MNS blood group antigens and share high homology, whilst GYPC has little homology with them and carries the Gerbich blood group. Erythrocytes from individuals carrying mutations causing altered expression of either GYPA or GYPB can be more resistant to *P. falciparum* infection, whilst GYPA or GYPB-deficient individuals generally are resistant to severe malaria (Pasvol, Wainscoat, & Weatherall, 1982).

The glycoproteins on the surface of human erythrocytes act as receptors for the erythrocyte binding-like antigens (EBL) of *P. falciparum*, which bind in a sialic acid-dependent manner (reviewed in (Cowman et al., 2017)). The EBL family of *P. falciparum* ligands includes EBA175, EBA181, EBA140 and EBL1 (reviewed in (Cowman et al., 2017)). The EBA175 parasite ligand binds to GYPA (Orlandi, Klotz, & Haynes, 1992), causing its dimerization, and this may also result in downstream signaling in the merozoite for subsequent steps in invasion (Tolia, Enemark, Sim, & Joshua-Tor, 2005). GYPA is one of the most ubiquitous proteins expressed on the

erythrocyte surface, with approximately one million copies per cell (Cooling, 2015). EBL1 binds to GYPB (Mayer et al., 2009), whilst EBA140 binds to GYPC (Maier et al., 2003). EBA181 binds to an unknown receptor on the surface of the human erythrocyte (Gilberger et al., 2003). Removal of sialic acid using neuraminidase from the surface of the erythrocyte and the glycophorin proteins blocks binding of the EBL ligands to their cognate receptor (reviewed in (Cowman et al., 2017)).

Band 3 anion transport protein is a major integral red cell membrane protein and is part of a major complex in erythrocytes that includes the glycophorins (Cooling, 2015). Band 3 has been considered a potential receptor for *P. falciparum* invasion. The band 3-GYPE complex has been shown to bind to parasite MSP1 (Goel et al., 2003). More details regarding band 3 are covered in section 1.13.

Apart from the classical sialic acid-dependent pathway for invasion, some laboratory strains of *P. falciparum* do not require sialic acids on the surface of the erythrocyte for merozoite invasion (Dolan, Miller, & Wellems, 1990). For instance, the *P. falciparum* line Dd2 is able to efficiently invade erythrocytes treated with neuraminidase, host cells that have had the sialic acid moieties removed from the cell surface (Dolan et al., 1990). Additionally, some sialic acid-dependent strains such as Dd2 and W2mef have the ability to switch to alternative sialic acid-independent pathways, when sialic acids are removed from erythrocyte membrane by neuraminidase, demonstrating the plasticity of *P. falciparum* when facing selective pressure (Stubbs et al., 2005). The *P. falciparum* ligand responsible for this switch has been identified and is a member of the reticulocyte binding-like family (Stubbs et al., 2005).

The PfRh family of proteins from *P. falciparum* includes PfRh1, PfRh2a, PfRh2b and PfRh4 (reviewed in (Cowman et al., 2017)). PfRh4 has been shown to bind to complement receptor 1 (CR1, also referred to as CD35) on erythrocytes, and its expression was required for sialic acid-independent invasion of merozoites from Dd2 and W2mef *P. falciparum* lines (Tham et al., 2010). CR1 plays a complex role in the *P. falciparum* erythrocytic lifecycle by facilitating merozoite invasion through association with PfRh4 (Tham et al., 2010) but it also is able to bind PfEMP1, which is expressed on the surface of the *P. falciparum*-infected erythrocyte and responsible for

cytoadherence and consequently rosette formation (Rowe, Moulds, Newbold, & Miller, 1997). Collectively, CR1 is an important player in *P. falciparum* pathogenicity.

The host receptor basigin (BSG or CD147) is essential for merozoite invasion, by binding to PfRh5 (Crosnier et al., 2011), which is an important member of the PfRh family. BSG carries the Ok blood group antigen and has a complex set of functions that include being responsible for induction of the extracellular matrix metalloproteinase, stimulating collagenase, and serving as leukocyte activation antigen M6 (Muramatsu, 2012). Inhibition of PfRh5 interaction with BSG – using antibodies to PfRh5 or BSG as well as soluble BSG – blocks merozoite invasion, demonstrating its essential function in *P. falciparum* invasion (Crosnier et al., 2011). The binding of PfRh5 to BSG is an important factor in the tropism of *P. falciparum* for human erythrocytes (Wanaguru, Liu, Hahn, Rayner, & Wright, 2013). Indeed, it has been shown that the function of PfRh5 is distinct to that of other PfRh family members because of its smaller size and lack of a transmembrane sequence (Volz et al., 2016). PfRh5 forms a complex with PfRipr and CyRPA (Volz et al., 2016), and this complex is required for efficient binding to BSG and insertion of the complex in the host cell membrane (Wong et al., 2019). Additionally, it has been shown that interaction of this complex with BSG is required for a Ca²⁺ flux that moves into the erythrocyte (Volz et al., 2016; Weiss et al., 2015). This Ca²⁺ flux is necessary for the tight junction formation between the merozoite and the host cell membrane (Volz et al., 2016; Weiss et al., 2015).

The recent application of RNAi and CRISPR/Cas9 techniques to cell models that support *in vitro* erythropoiesis have identified new invasion receptors. A forward-pooled shRNA screen in *in vitro* cultured CD34⁺ erythroid progenitor cells showed that CD44 and CD55 are required for *P. falciparum* invasion (Egan et al., 2015). CD44 knockout JK-1 cells have been generated using CRISPR/Cas9 and a significant reduction in invasion was observed, suggesting CD44 contributes to merozoite invasion (Kanjee et al., 2017). In that work, the erythroleukemic cell line JK-1 was induced to differentiate using bromodomain inhibitors, thus initiating *in vitro* erythropoiesis. Kanjee et al. also showed that ABCB6 (ATP Binding Cassette Subfamily B Member 6)-null red blood cells are refractory to *P. falciparum* invasion with impaired merozoite attachment, consistent with previous work knocking down CD55 expression and

identifying ABCB6 as a hit in an RNAi (RNA interference) screen (Egan et al., 2018). However, these receptors may function as co-receptors and parasite ligands have yet to be identified. Furthermore, CD44 seems to contribute to an unknown complex together with BSG (Kanjee et al., 2017). Thus, as well as a possible role as a co-receptor facilitating parasite recognition, CD44 may have more importance than is generally recognised in the coordination of the host cell cytoskeleton for invasion.

The well-characterised invasion receptors for *P. falciparum* (Figure 1.2) are proteins with single-pass transmembrane domains that are responsible for carrying different blood groups (Satchwell, 2016). Multi-pass membrane-spanning proteins remain poorly investigated.

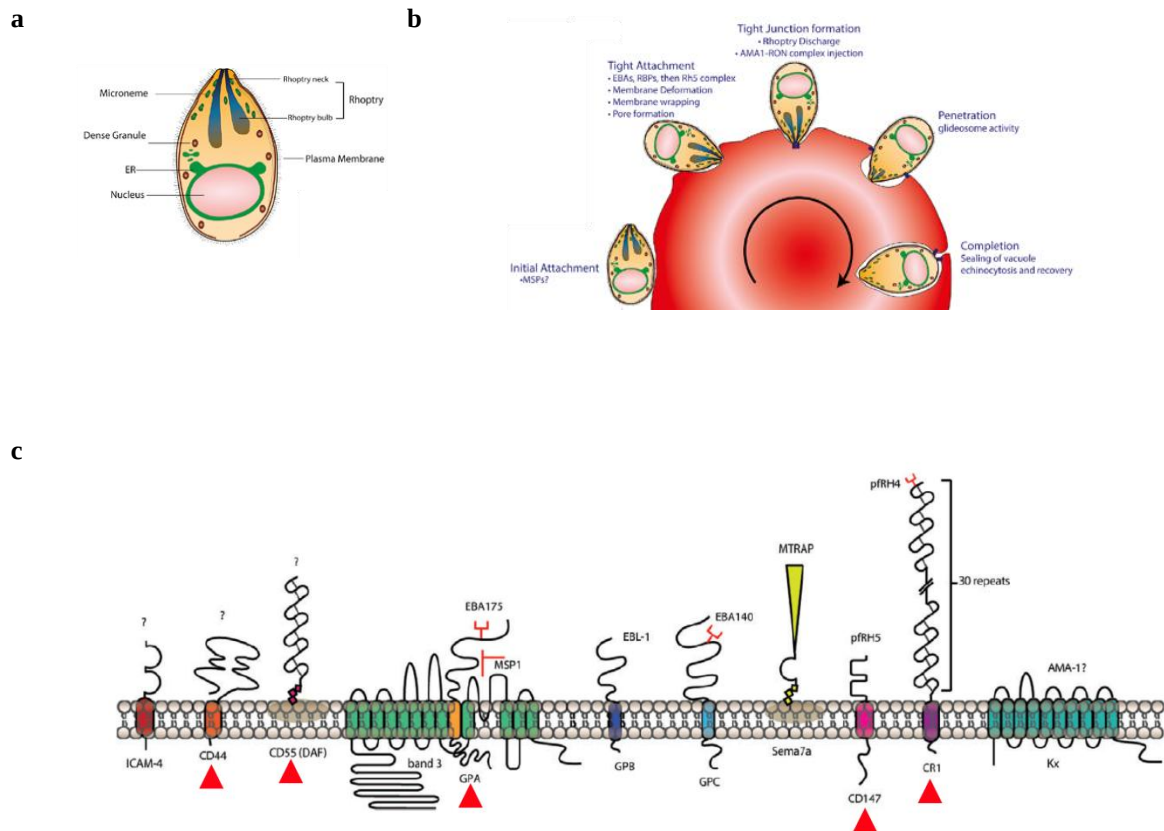


Figure 1.2 Invasion and invasion receptors

(a,b): Illustration of a merozoite and the invasion process (reviewed in (Cowman et al., 2017)). (c): Summary of identified invasion receptors to date (adapted from (Satchwell, 2016)). The five receptors marked by red arrowheads are included in the FACS panel described in chapter 3. ABCB6 is another strain-transcendent receptor described in a more recent study, thus was not included in the plot (Egan et al., 2018).

1.6 Previous models to study invasion receptors

The known parasite–host interactions during merozoite invasion have been identified using approaches including bait-prey assays with purified recombinant peptides, phage display, protein pull-downs and binding of parasite ligands from parasite supernatants to erythrocytes (reviewed in ((Cowman et al., 2017))). The ability to construct mutant human erythroid cells lacking expression of specific membrane proteins potentially

provides a way of identifying additional invasion receptors, as has been done previously (Egan, 2018). The fact that mature red blood cells lack a nucleus makes genomic editing impossible. Utilisation of natural mutations that occur in the human population has been a useful approach that has aided in the identification of receptors such as GYPC (Maier et al., 2003), CR1 (Tham et al., 2010) and ABCB6 (Egan et al., 2018). However, the ability to genetically modify and differentiate erythroid progenitors has been an important step forward, enabling greater understanding of *P. falciparum* invasion and identification of new receptors (Egan, 2018; Kanjee et al., 2017).

As the source of erythroid progenitors, either CD34⁺ cells isolated from donors or *in vitro* erythroid cell lines are predominantly used. Typical examples are hematopoietic progenitor cells (HPCs) and JK-1 (mentioned previously) (Egan, Jiang et al. 2015, Kanjee; Gruring et al. 2017). However, these models have important limitations. CD34⁺ cells are difficult to edit and have a limited propensity for *ex vivo* expansion. The JK-1 cell line is derived from a leukemia patient and it is not a true erythroblast, lacking a normal erythroid background. Another restriction of JK-1 cells is that many steps are required to induce erythroid-committed differentiation and filter specific populations (Kanjee et al., 2017). Therefore, developing improved erythroid models that can be edited to deliver desired profiles efficiently and abundantly *in vitro* is of critical importance.

1.7 Using HUDEP-2 cells as an *in vitro* erythropoiesis model

Human erythropoiesis begins when burst-forming unit-erythroid cells (BFU-Es) and colony-forming unit-erythroid cells (CFU-Es) are generated successively from megakaryocyte erythroid progenitors (MEPs). After that, pro-erythroblasts are produced and differentiate from basophilic erythroblasts to polychromatic and orthochromatic erythroblasts. Enucleation occurs when the orthochromatic erythroblast cytoplasm undergoes asymmetric division, yielding reticulocytes, which become mature erythrocytes (Dzierzak & Philipsen, 2013). *In vivo* erythropoiesis leads to formation of erythroblastic islands in bone marrow, providing a structural and functional unit which consists of central macrophages and surrounding erythroid cells (Dzierzak & Philipsen, 2013). Central macrophages, also referred to as nurse cells, phagocytose the extruded nucleus from erythroblasts. Other intercellular signaling and interactions, together with

the adhesion between cells and extracellular matrix, play crucial roles during *in vivo* erythropoiesis (Dzierzak & Philipsen, 2013). An issue with establishment of independent cultivation systems for *in vitro* erythropoiesis is the likely need for helper cells. Another barrier is that natural erythroblasts have an increasing apoptosis rate as they mature and differentiate. The explanation for this may be that, apart from the EPOR/STAT5 survival pathway, increased CD95/CD178 activation and failure to express BCL-X_L participate in regulation to maintain a homeostatic state of erythropoiesis (Liu et al., 2006; Socolovsky et al., 2001).

One solution is to immortalize the erythroid progenitors, to establish a stable cell line, for example, via delivery of a tetracycline inducible HPV E6/E7 system into these cells (Kurita R, 2013). Two cell systems have been developed which display promise as an editable erythroid cell line: the Human Umbilical Cord Blood-Derived Erythroid Progenitors (HUDEP-2) and the BEL-A line. The HUDEP-2 line was established, which can successfully complete the differentiation from pro-erythroblasts to late orthochromatic erythroblasts (Kurita R, 2013). Notably, HUDEP-2 cells are better than JK-1 as an *in vitro* erythropoiesis model, because of their relatively normal karyotype and convenience that allows production of large amounts of erythroblasts. However, HUDEP-2 cells exhibit shortcomings that limit their utility as a host model for *in vitro* *P. falciparum* invasion. The low enucleation rate during tertiary differentiation poses a barrier to scalable production of enucleated host cells, although there has been an improvement using a murine stroma cell co-cultivation method (Divya S. Vinjamur, 2018). Another feature that makes HUDEP-2 cells less useful as a host for *P. falciparum* is the α , β , γ and ϵ haemoglobin composition of this cell line (Kurita R, 2013). The HUDEP-2 line is the only erythroid cell line that expresses α and β instead of γ and ϵ mRNAs (Kurita R, 2013).

1.8 Adult erythroid cell line BEL-A

The Bristol Erythroid Cell Line Adult (BEL-A) was established by immortalising adult erythroid progenitors using a tetracycline inducible HPV E6/E7 construct (Trakarnsanga et al., 2017). This cell line can produce enucleated reticulocytes which can be isolated using standard leukocyte reduction filters at large scale. The BEL-A cells share the cardinal features of *in vitro* erythropoiesis with other erythroid lines. However, they

have the advantage that they can undergo tertiary differentiation independently, reaching a 30% enucleation rate without being co-cultured with stroma cells, comparable to the 38% rate using the murine stroma cell method for HUDEP-2. Also α and β haemoglobin was detected without embryonal and fetal haemoglobin from BEL-A cell lysate (Trakarnsanga et al., 2017), suggesting differentiation of these cells mimics normal erythropoiesis *in vitro* better than other erythroid cells.

BEL-A cells can be genetically edited using CRISPR/Cas9 (Satchwell et al., 2019), and BSG expression has been removed from the cell surface by disruption of the corresponding gene, and this blocks *P. falciparum* merozoite invasion (Satchwell et al., 2019). Complementation of BSG expression restored the ability of *P. falciparum* to invade. Therefore, the BEL-A line is a proven system for the study of *P. falciparum* invasion of human erythroid cells (Satchwell et al., 2019).

1.9 Surface markers to distinguish erythroid differentiation stages

Morphological changes occur during terminal erythroid differentiation, with the cells becoming smaller in size and an increase in haemoglobin expression as they mature (Fig. 1.3). The nucleoli disappear when pro-erythroblasts develop to basophilic stage, as observed with Grünwald-Giemsa stained cells. The basophilic erythroblast has a relatively high ratio of nucleus to cytoplasm. The cytoplasm is basophilic due to the presence of ribosomes, and at later stages the developing cells appear eosinophilic owing to increased expression of haemoglobin from the polychromatic stage. Polychromatic erythroblasts have characteristic cart-wheel shaped nuclei which appear more condensed than earlier stages, and these subcellular organelles are expelled during polychromatic-orthochromatic-reticulocyte differentiation. Orthochromatic erythroblasts are the last nucleated stage, which undergo the nucleation process and become reticulocytes – the precursors of mature erythrocytes; however, reticulocytes have remnant ribosomes in the cytoplasm as detected by staining with methylene blue, and they are larger than mature erythrocytes (Thalia Papayannopoulou, 2018).

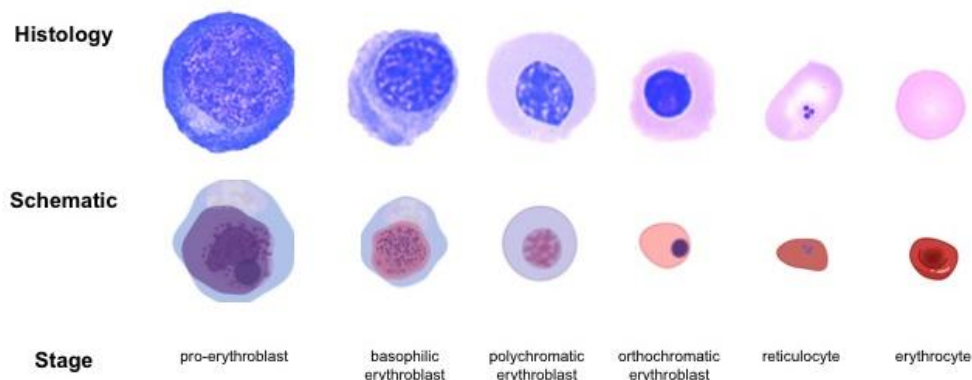


Figure 1.3 Morphology and staging of late erythropoiesis.

Histological images were taken from <http://medcell.med.yale.edu/histology/histology.php>, schematics were created with the biorender webtool (<https://biorender.com/>).

Whilst major morphological changes occur as erythroid cells differentiate, there are also extensive differences in the expression of specific surface markers which can be distinguished with specific antibody staining and fluorescence-activated cell sorting (FACS). CD49d (also dubbed $\alpha 4$ -integrin) and band 3 serve as useful cell surface markers that allow separation of specific stages (Hu et al., 2013). As erythroid cells develop (Figure 1.4), there is a decrease in CD49d expression and an increase in band 3 expression (Hu et al., 2013). CD44 expression reduces to some extent during terminal erythroid differentiation of both CD34⁺ human erythroid precursors (Hu et al., 2013) and murine erythroid cells (Chen et al., 2009). These results suggest that CD44 can serve as a maturation marker for human erythroid differentiation. Additionally, the combination of CD71 and GYPA has been used as an alternative for erythroid staging (Dzierzak & Philipsen, 2013).

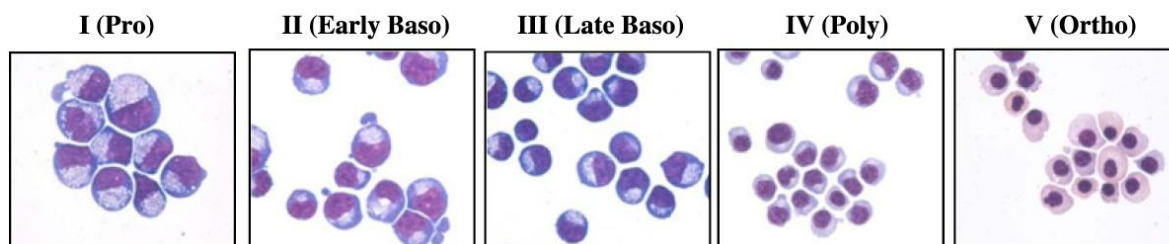


Figure 1.4 Morphology and staging of sorted CD34+ human erythroid cells using GYPA, integrin $\alpha 4$ and band 3 FACS panel (Hu et al., 2013).

1.10 Erythroblasts support *P. falciparum* invasion

In previous decades, the consensus was that reticulocytes and mature erythrocytes, which are enucleated, support the ability of *P. falciparum* parasites to invade, whilst erythroid precursors, that have nuclei, do not (Tamez, Liu, Fernandez-Pol, Haldar, & Wickrema, 2009). However, Tamez et al. investigated the stage-specific susceptibility of erythroid cells and revealed the earliest stage at which *P. falciparum* can invade successfully is the orthochromatic erythroblast (Tamez et al., 2009). Furthermore, late orthochromatic erythroblasts support subsequent development of the parasite after merozoite invasion, suggesting that parasite invasion and maturation can be segregated (Tamez et al., 2009). As mentioned previously, the work which identified CD44 as an invasion receptor used JK-1 cells as a host model (Kanjee et al., 2017). The same publication showed that impaired parasite development occurred inside the nucleated host cells after parasite entry. Interestingly, the earliest stage of JK-1 cells which supports *P. falciparum* invasion is the late polychromatic stage (Kanjee et al., 2017).

1.11 CRISPR/Cas9 system is a useful gene editing tool for parasite-erythroid interaction

The CRISPR sequence, which functions as a protective mechanism against phage infection, has provided an extremely useful tool for editing the genomes of many organisms (Jinek et al., 2012). Cas genes encode nucleases that constitute various Cas complexes, which function in recognizing and cutting foreign DNA of bacterial

invaders. Multiple homologs of Cas nucleases have been identified among different bacteria species, and the application of a CRISPR/Cas9 system in gene editing was first proposed in 2012 (Jinek et al., 2012). The technique is now well developed and used to edit genomes in mammalian and many other cell types, heralding a new era of efficient gene editing. Generally, there are two key components: a non-specific Cas9 nuclease or nickase, and a sequence-specific targeting single-guide RNA (sgRNA). Two different DNA editing consequences can be achieved by target cells: homologous directed repair (HDR) in a more precise manner when templates are provided, and non-homologous end joining (NHEJ). Exploiting its ability to bind to DNA, inactivated Cas9 can be used for CRISPR activation (CRISPRa) as well as bringing components of interest (e.g. transcription factors) to target sequences. Furthermore, sgRNA libraries make high-throughput screening feasible (Metzakopian et al., 2017).

This system has also been used to genetically edit mammalian cells to identify host receptors for invasion by *P. falciparum* (Kanjee et al., 2017; Satchwell et al., 2019). Indeed, it has also proven very efficient in enabling genetic editing of the genome of *Plasmodium*, including *P. falciparum* (Lee, Lindner, Lopez-Rubio, & Llinás, 2019). In conclusion, the CRISPR/Cas9 system provides the necessary versatility and flexibility for bidirectional studies of parasite-erythroid interaction.

1.12 Band 3 functions

Band 3 is encoded by the gene *SLC4A1* (solute carrier family 4-member 1), which maps to 17q21-qter. It is also referred to as CD233 and anion exchanger 1 (AE1). Band 3 is an abundant glycoprotein consisting of 911 amino acids and 14 transmembrane regions on erythrocytes (reviewed in (Reithmeier et al., 2016)). Band 3 also has a subtype in the kidney which lacks 64 residues at the N-terminus. The erythroid band 3 carries the Diego blood group, with the related antigens exhibited on the loops which link transmembrane segments. Two domains, which are structurally and functionally distinct, constitute Band 3. The first 403 residues make up the cytoplasmic part of band 3 at the N-terminus, which interacts with the protein ankyrin and the 4.2 protein complex, and thus is responsible for associating the red blood cell membrane with the underlying cytoskeleton fibres, including spectrin (Figure 1.5). Mutations in the erythroid subtype can cause hereditary spherocytosis, and so far, 17 related natural mutations have been

identified in patients (reviewed in (Reithmeier et al., 2016)). Band 3 plays a critical role in maintaining normal membrane tension and erythrocyte morphology; several case reports revealed that patients lacking Band 3 due to the Coimbra (V488M) mutation developed spherocytosis, with abnormal-shaped fragile red blood cells observed (Alloisio et al., 1997; Ribeiro et al., 2000). The N-terminus of band 3 is required for stabilisation of multiple proteins and complexes (Satchwell, Hawley, Bell, Ribeiro, & Toye, 2015). Drugs such as 4,4'-diisothiocyano-2,2'-stilbene disulfonate (DIDS) and H₂DIDS can block the Cl⁻/HCO₃⁻ anion exchanging gate of band 3 by bridging two lysine residues. This anion gate is required for cytoplasmic pH regulation and oxygen release from haemoglobin (reviewed in (Reithmeier et al., 2016)), because the C-terminus cytoplasmic tail interacts with type II carbonic anhydrase, which utilises CO₂ and water to produce bicarbonate ions and protons.

Much uncertainty still exists as to whether band 3 is an important receptor for *P. falciparum* merozoite invasion. Studies in genetically engineered mice suggested that band 3-null red blood cells did not support *P. falciparum* invasion (Baldwin, Li, Hanada, Liu, & Chishti, 2015). Also, some studies have suggested that the parasite protein MSP1 interacts with the GPA-band 3 complex (Baldwin et al., 2015). Overall, there is currently insufficient evidence to show that band 3 is important for *P. falciparum* invasion of human red blood cells.

Since band 3 is involved in at least two protein complexes associated with erythrocyte membrane, which involve GYPA and GYPC (reviewed in (Anstee, 2010; L. Bruce, 2006)), respectively, it is crucial to evaluate the potential influence of removing band 3 on expression of glycoporphins. Information regarding band 3-GYPA interaction, which is also known as the Wr^b blood group antigen, was provided by a holistic review (reviewed in (Reithmeier et al., 2016)). It is suggested that E658 of band 3 associates with R61 of GYPA extracellular moiety. E658K mutants appear to be Wr^b negative, adding to the evidence that the binding site is crucial to maintain band 3-GYPA interaction (reviewed in (Reithmeier et al., 2016)). E658 locates on the fourth extracellular loop of band 3, at a beginning site of the eighth transmembrane segment. Another piece of the puzzle comes from studies of the transport process, which produced experimental evidence about the assembly of band 3 and GYPA in ER and the

GYPA C-terminus's role in stabilising the COPII vesicle for successful delivery to the membrane. Band 3 is non-functional in GYPA-devoid cells, because it requires GYPA to fold correctly (reviewed in (Reithmeier et al., 2016)). Information of wild type GYPA organization is provided in Figure 1.6.

To date, band 3 deletion in *in vitro* human erythroid cultivation systems remains a theoretical possibility, which requires further work to determine the feasibility and dissect the functional consequences. There has been extensive research on the function of band 3 in erythropoiesis (Alloisio et al., 1997; L. Bruce, 2006; Chambers, Askin, Bloomberg, Ring, & Tanner, 1998; Kalli & Reithmeier, 2018; Ribeiro et al., 2000; Satchwell et al., 2015). Analysis of the effect of band 3 loss of expression has been used to determine its role in binding to glycoproteins, adducin and other proteins and thereby its function in the formation of macro-complexes that constitute the erythrocyte cytoskeletal meshwork. Work done by Ji and Lodish successfully disrupted the *band 3* gene in murine fetal liver cells, resulting in lack of expression of the protein (Ji & Lodish, 2012). Intriguingly, in these mutant cells there is a significant increase in enucleation rate compared with the wild type parents (Ji & Lodish, 2012). This raises the possibility that viable band 3 knockout human erythroid cells could be generated. Additionally, a study of the effect of ankyrin and band 3 on glycoprotein expression (Kalli & Reithmeier, 2018) revealed that neither is crucial for enucleation. Whilst generating band 3-null human late stage erythroblasts may be possible, another complicating factor is that band 3's anion exchanger function may cause pH abnormalities, which may greatly decrease cell viability. Therefore, generating a band 3 deficient model in the laboratory would be an interesting possibility.

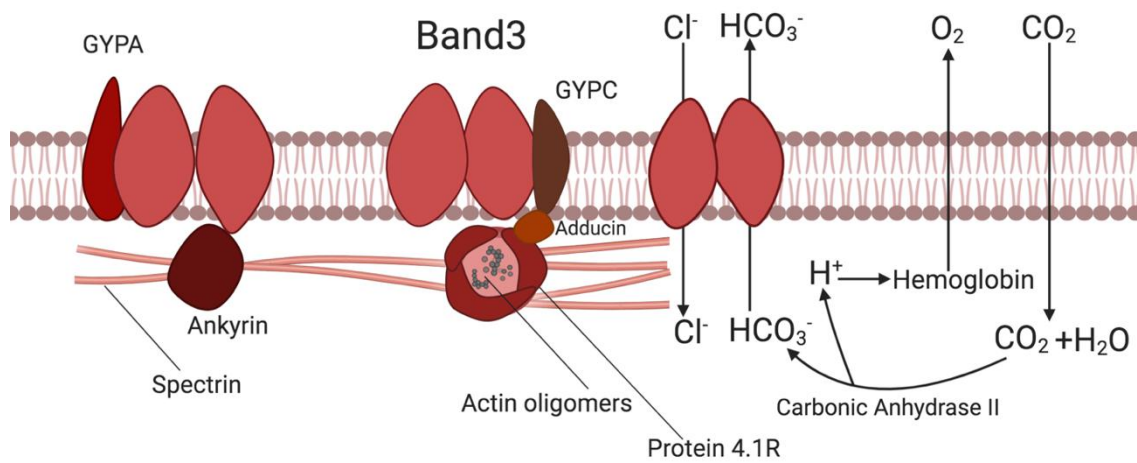


Figure 1.5. Summary of band 3 functions

Band 3 is involved in forming the ankyrin complex and the protein 4.1 complex. Its C-terminal cytoplasmic tail interacts with carbonic anhydrase II to allow the proton synthesis and liberation of oxygen from haemoglobin.

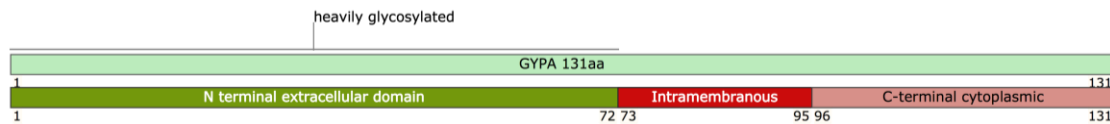


Figure 1.6. GYPA map

GYPA comprises 131 amino acids, with the first 72 aa heavily glycosylated as N terminal domain. AA73 to AA95 are buried as intramembranous domain, which is hydrophobic and may be involved in GYPA dimerisation. AA96 to AA131 make up the C-terminal cytoplasmic tail. The map was generated using SnapGene Viewer.

1.13 Project hypothesis and aims

Genetic manipulation of the host cell could be useful for the identification and discovery and validation of *P. falciparum* invasion receptors, and thus play an important role in dissecting host–parasite interaction. With the help of erythroid cultivation systems and given the need to validate putative invasion receptors and to explore the role played by band 3 in erythropoiesis, we carried out a project to address the following hypothesis:

Erythroid cell lines can be utilised for the development of editable models that may be useful in the future to study host–parasite interaction with *P. falciparum*.

The project had the following aims:

- A. To characterise erythroid models which may be used for *P. falciparum* invasion studies.
- B. To genetically edit erythroid cell lines using the CRISPR/Cas9 system to delete expression of possible red cell membrane proteins which may serve as host invasion receptors for *P. falciparum*.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell culture

BEL-A cell line

HUDEP-2 cells

293FT cells

DMEM

Stemspan SFEM 500 mL (STEMCELL Technologies, 9650)

IMDM + pen/strep-2ME (Walter and Eliza Hall Institute (WEHI) Media Kitchen)

Recombinant Human SCF Protein (R&D, 255-SC-200)

Human Holo-Transferrin Protein (R&D, 2914-HT-001G)

EPREX 1000 IU/0.5 mL PFS (Clifford Hallam, AN378145)

Human Insulin (Sigma-Aldrich, I2643-25mg)

Heparin Sodium Salt from Porcine Intestinal Mucosa (Sigma-Aldrich, H3393-50KU)

DEXAMETHASONE 4 mg/IML AMP (Clifford Hallam, 1045962)

Fetal Bovine Serum 500 mL (ThermoFisher, 26140079)

L-glutamine (ThermoFisher, 21051024)

2.1.2 Drugs and solutions

Doxycycline (Sigma-Aldrich, D3072)

Blasticidin (Sigma-Aldrich, D8537)

PI (Sigma, P4170-10MG)

Hoechst33258 (Biotium, JOMAR 40044)

Sodium bicarbonate (Sigma-Aldrich, S8761)

CaCl₂ (Sigma-Aldrich, C4901)

Na₂HPO₄ (Sigma-Aldrich, S3264)

HEPES (Sigma-Aldrich, H3375)

DPBS sterile filtered (Sigma-Aldrich, D8537)

2.1.3 Plasmids

FUCas9Cherry (Addgene#70182)

pMDL (Addgene#12251)

pRSV-rev (Addgene#12253)

pVSV-G (Addgene#138479)

2.1.4 Oligonucleotides and sgRNA PAM sites (5' to 3')

Table 2.1 Oligonucleotide sequences for PAM sites and PCR primers

Guide	Forward primer	Reverse primer
Band 3 site 1		
TGATGTGGTGTGGTAGT	AATATAAGGGGCTGA	CTTGTCAATCAGGTTA
CTGTGG	CCCAC	TCTCTG
Band 3 site 2		
CCAGCTCCTGCAGCTCC	CCTCTGCTTCCCACTC	CAGGGGCACCTGTGTT
ACATAG	CTTT	TAAG

2.1.5 Antibodies

FITC-antiCD233 (9439 BRIC 6, 9451 BRAC 17, 9452 BRAC 18, 9468 BRIC 200, 9472 BRIC 71, NHS BLOOD AND TRANSPLANT)

PE-antiCD49d (Clone ALC1/1, Abcam, product code ab91051)

APC-antiCD235a (Clone HI264, Abcam, product code ab91163)

BV421-antiCD235A (Clone GA-R2, BD, product code 562968)

APC-antiCD35 (Clone E11, ThermoFisher, product code 17-0359-42)

BV510-antiCD44 (Clone IM7, BioLegend, product code 103044)

PE/Cy7-antiCD55 (Clone JS11, BioLegend, product code 311314)

APC/H7-antiCD71 (Clone M-A712, BD Pharmingen, product code 563671)

APC-antiCD147 (Clone # TRA-1-85, R&D, product code FAB3195A)

APC-antiGYPC (9411 BRIC 10, 9417 BRIC 4, NHS BLOOD AND TRANSPLANT)

2.1.6 Equipment

BD LSRFortessa™

BD FACSAria™

2.2 Methods

2.2.1 Cell culture

The HUDEP-2 culture protocol was adapted from a previously established method (Kurita R 2013). HUDEP-2 cells grow in a 2-stage system: an expansion stage and a differentiation stage; the phase change is made possible by switching culturing media. During the expansion stage, the cells were kept at a density of $0.2\text{--}0.8 \times 10^6/\text{mL}$ (importantly, the cells start spontaneous differentiation if kept too dense). The expansion media formulation was as follows: Stemspan SFEM (STEMCELL technologies) supplied with 50 ng/mL recombinant human stem cell factor (hSCF, R&D systems), 4 µg/mL dexamethasone (Clifford Hallam), 3 IU/mL erythropoietin (EPPREX, Clifford Hallam) and 1 µg/mL doxycycline. Every 48 hours a full media change is performed with the density adjusted to 0.2×10^6 cells/mL.

Differentiation media components: IMDM supplied with 1% L-glutamine, 50 ng/mL (hSCF (R&D systems), 10 µg/mL human insulin, 330 µg/mL recombinant human holo-transferrin (R&D systems), 5% human AB plasma, 3 IU/mL heparin, 3 IU/mL EPO (EPPREX, Clifford Hallam). 1 µg/mL doxycycline was added to the media until the second half of the differentiation phase (day 6 and above). During differentiation, cells were kept at a density of $1\text{--}3 \times 10^6$ cells/mL. To trigger differentiation, expansion cells were spun down and washed with PBS once, then transferred to differentiation media. The day of switching cells to differentiation media is counted as differentiation day 0 (D0), and from day 6 (D6) doxycycline is removed from the media.

For BEL-A cell culturing, an adapted three-phase protocol was used (Trakarnsanga et al., 2017). For the initial expansion phase, cells were seeded at $0.1\text{--}0.15 \times 10^6$ cells/mL and density adjusted every other day. Full media change was performed when necessary. Formulation of the expansion media: Stemspan SFEM (STEMCELL technologies) supplied with 50 ng/mL hSCF (R&D systems), 4 µg/mL dexamethasone (Clifford Hallam), 3 IU/mL erythropoietin (EPPREX, Clifford Hallam) and 1 µg/mL doxycycline.

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For differentiation day 0 to day7 – the primary differentiation phase – IMDM supplied with additional 1% L-glutamine, 10 ng/mL hSCF (R&D systems), 10 µg/mL human insulin, 200 µg/mL hSCF(recombinant human holo-transferrin) (R&D systems), 3% human AB plasma, 3 IU/mL heparin, 3 IU/mL erythropoietin (EPPREX, Clifford Hallam), 2% FBS (ThermoFisher, 26140079) was used. From differentiation day 0 to day 3, 1µg/mL doxycycline was added to the media and cells kept at 0.2–0.35million/mL. From day 4 to day 7, the density was adjusted to 0.5×10^6 cells/mL. On day8, cells were transferred to tertiary differentiation media for terminal differentiation at 1×10^6 cells/mL, using IMDM supplied with additional 1% L-glutamine, 10µg/mL human insulin, 500µg/mL recombinant human holo-transferrin (R&D systems), 3% human AB plasma, 3 IU/mL heparin, 3 IU/mL erythropoietin (EPPREX, Clifford Hallam), 2% fetal bovine serum (FBS, ThermoFisher, 26140079). Endpoint was between day 13 and day 16, as severe loss of viable cells was observed then.

Centrifuging settings: 200g for the expansion stage, increasing to 250g as cells differentiated.

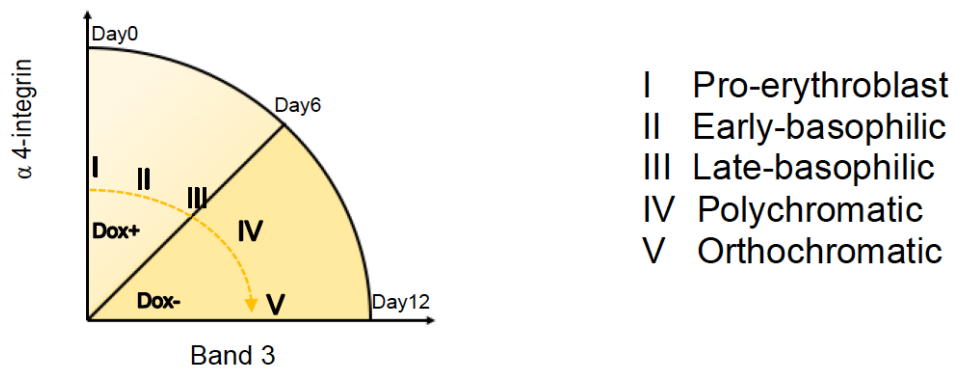
Freezing buffer consisted of 90% FBS and 10% dimethyl sulfoxide. The incubator was set at 37°C and supplied with 5% O₂.

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a

	BEL-A cell	HUDEP-2
Expansion phase	SFEM 50ng/mL SCF 3U/mL EPO 1 μ M DEX 1 μ g/mL DOX 0.05-0.15 million/mL	SFEM 100ng/mL SCF 3U/mL EPO 1000U/mL DEX 1 μ g/mL DOX 0.2-0.8 million/mL
Phase-I differentiation	IMDM 1X p/s 3% AB serum 2% FBS 3U/mL heparin 3U/mL EPO 50ng/mL SCF 200 μ g/mL transferrin 10 μ g/mL insulin 1ng/mL IL-3 Day 0-3, 0.2-0.35 million/mL Day4-7, 0.5 million/mL	IMDM 1X L-glu 1X p/s 5% AB plasma 3U/mL heparin 3U/mL EPO 10 μ g/mL insulin 50ng/mL SCF 330 μ g/mL transferrin 1 μ g/mL DOX 1-3million/mL
Phase-II differentiation	IMDM 1X p/s 3% AB serum 2% FBS 3U/mL heparin 3U/mL EPO 500 μ g/mL transferrin 10 μ g/mL insulin 1 million/mL	From day 6 take DOX off 1-3million/mL

b



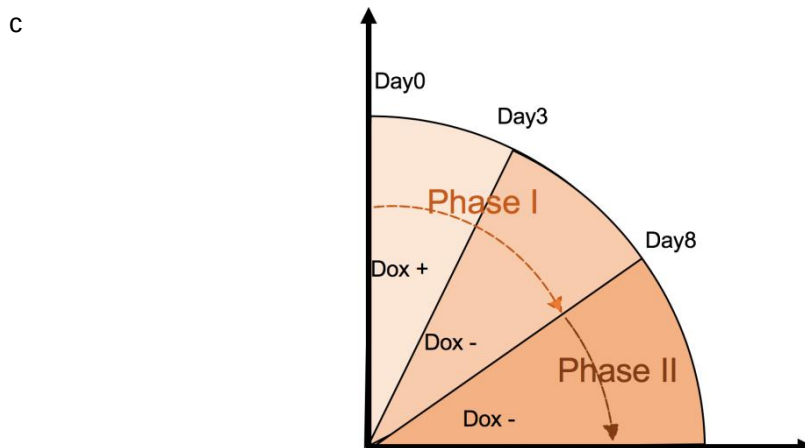


Figure 2.1. Cultivation system of the HUDEP-2 and BEL-A cells

(a). The media composition of BEL-A and HUDEP-2 cells. (b). A typical time course for HUDEP-2 differentiation. (c). A typical time course for BEL-A differentiation.

2.2.2 FACS and antibody titration

The FACS buffer consisted of 10% FBS in PBS. Cells were spun down and washed with the FACS buffer once before being filtered and distributed into FACS tubes. Typically, a 100 μ L staining buffer (FACS buffer) system can be applied to $1-5 \times 10^6$ cells; the volume of staining buffer was adjusted proportionally. After staining with antibodies for 30 minutes in the dark, cells were washed with FACS buffer once before being subjected to an analyzer or sorter. To reduce the error caused by pipetting, a cocktail containing antibodies and staining buffer was made before staining and distributed equally into tubes. The incubation was carried out on ice to preserve the activity of antibodies. Antibodies used: APC-antiGYPA (HI264, Abcam), PE-antiCD49d (ALC1/1, Abcam), FITC-antiCD233 (BRIC 6, NHS blood and transplant), BV421-AntiGYPA (HIR2, BD), APC-antiCD35 (E11, ThermoFisher), BV510-antiCD44 (IM7, BioLegend), PE/Cy7-antiCD55 (JS11, BioLegend), APC/H7-antiCD71 and APC-antiCD147 (TRA-1-85, R&D). BD LSRFortessaTM and BD FACSAriaTM were used for analysing and sorting. For the live/dead discrimination, PI was used. Data is analysed using software FlowJo v 7.6.5 (Tree Star Inc.). Antibody volumes used for each 100 μ L staining system were 10 μ L APC-antiGYPA, 30 μ L PE-antiCD49d, 1 μ L FITC-antiCD233, 5 μ L APC-antiCD35, 0.625 μ L BV421-AntiGYPA, 0.625 μ L BV510-antiCD44, 1.25 μ L PE/Cy7-antiCD55, 1 μ L APC/H7-antiCD71, 2.5 μ L APC-antiCD147

and 0.8 μ L Hoechst33258. The concentration of PI was 5 μ g/mL for live/dead discrimination. For the sake of simplicity, the term “bulk population” refers to all viable cells passing the size gate and the singlet gate when being analysed by FACS. “Gated population” denotes cells falling into gate A to E on CD49d-CD233 plots. The corresponding expansion subpopulations were recorded as “expansion ancestors” for each differentiation replicate.

The appropriate working volumes of APC-antiCD35, BV510-antiCD44, PE/Cy7-antiCD55, APC-antiCD147 and BV421-AntiGYPA were determined as minimum to give resolution between unstained negative control peaks and the peaks from stained cells.

2.2.3 Cell profiling workflow

For each biological replicate in the membrane profile tracking process, a new vial of 1-2 million cells from the same freezing batch was thawed on the first day. After being kept in expansion media for four days, the cells were switched to differentiation media. This day was recorded as day 0 for differentiation, and the cells were separated into equal technical duplicates for differentiation. For each technical duplicate, the initial cell quantity was around 5 million at a density of 1×10^6 cells/mL. A full media change was taken when the culturing system had accumulated sufficient metabolic waste, judging by an acidic state of cultivation and viable cell counting post-trypan blue staining, typically every two days to keep cells under optimal conditions. Samples were taken every three days and recorded as day 0, 3, 6, 9 and 12, respectively. For each replicate, after a 4-day-expansion, cells were divided into two parts, with one expanding and the other induced to differentiate. This day was recorded as day 0. For each checkpoint, both expansion cells and differentiation cells were subjected to FACS analysis. Cytospin slides were made on each specific day for morphological determines.

To show the fold change of relative abundance, relative expression was calculated using the equation “MFI sample/MFI reference cells”. Two red blood cell samples were also included to show the abundance of the markers, analysed on day 3 and day 12 to prevent error caused by individual technical operations. For gated populations, the normalisation used MFI from the corresponding pro-erythroblast stage.

To generate the curves for illustration of changing trends, Loess regression was employed in R as a smoother to give an overall, time-related changing view of each marker on either bulk population or gated population. Median fluorescence intensity (MFI) values were used for the comparison in this thesis rather than using mean fluorescence intensity, which might be skewed by the presence of outliers. Populations constituting less than 0.1% of the total were omitted from the data for generating the curves to prevent errors in MFI caused by minor populations.

2.2.4 Cytospin and staining

For morphology examination and invasion assays, typically $2 \sim 4 \times 10^5$ cells were spun down at 1000 rpm onto a glassware slide using a cytospin centrifuge (ThermoFisher), then the slide was fixed in methanol for 5~10 minutes, stained with 100% May-Grünwald stain for two minutes and 2% Giemsa stain for 10 minutes, and finally rinsed in water and air dried.

2.2.5 Plasmid preparation

SgRNA vectors targeting band 3 (Figure 2.2) were obtained from a commercially available arrayed CRISPR guide library delivered by Merck, which was originally developed by Wellcome Trust Sanger Institute (Metzakopian et al., 2017). Two guides for each gene were included in the arrayed library, and clones containing the four guides were obtained courtesy of the WEHI screening lab. The clones were amplified in L broth (WEHI media kitchen) supplemented with ampicillin at 37°C.

The mCherry-Cas9 plasmid was obtained from the Marco Herold Lab at WEHI.

Clones containing pMDL, Rev and VSV.g, the three plasmids for lentiviral packaging, were supplied courtesy of Miss Lisa Verzier and cultured at 32°C.

All plasmids were extracted using an illustra plasmidPrep Midi Flow Kit (28904268) from GE Healthcare, followed by an ethanol precipitation to concentrate.

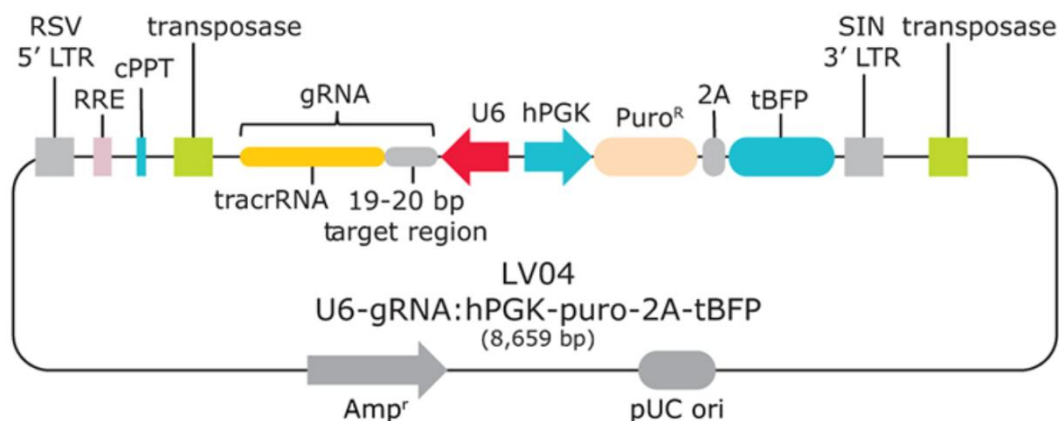


Figure 2.2 SgRNA plasmid map from Merck

From <https://www.sigmaaldrich.com/catalog/product/sigma/hsangerg?lang=en®ion=AU>.

2.2.6 Ethanol precipitation

A volume of 3M sodium acetate equivalent to 1/10 of midi-prep elution was added and mixed well, followed by adding 100% EtOH equivalent to twice the total volume. The mixture was incubated in a -20°C freezer overnight, then aliquoted into 1.5 mL tubes and spun at maximal speed in a microcentrifuge for 10 min. Supernatant was aspirated off and 1 mL pre-chilled 70% ethanol added to each tube. The tubes were spun at maximum speed for 2 min before being transferred into a laminar flow hood. Most of the supernatant was discarded and the tubes were left to air-dry for 10–15 minutes to evaporate most of the ethanol. Deionised water was used to dissolve the nucleic acid pellet. Concentration was measured by a NanoDrop 1000 spectrophotometer.

2.2.7 Transfection and transduction

For viral production, 293T cells were transfected using a calcium-phosphate co-precipitation method. The transfection cocktail was made by mixing nuclease-free water, lentivirus plasmids carrying Cas9 and/or sgRNA, packaging vector pMDL, RSV-REV, VSV, CaCl₂ and HeBS buffer (2X: 0.28 M NaCl, 0.05 M HEPES and 1.5 mM Na₂HPO₄, pH adjusted between 6.95 and 7.05). Then the mixture was immediately vortexed for 10 seconds and incubated for 10 min at room temperature before being added dropwise evenly onto 293FT cells. Target cell media was used to replace the supernatant one day

after transfection. After another 24-hour incubation, virus-containing supernatant was collected and passed through a 0.45 μm filter to remove cells. For a 10 cm Petri dish, 250 μL nuclease-free water, 250 μL 0.5 M CaCl_2 , 5 μg pMDL, 2.5 μg pRSV-rev, 3 μg pVSV-G, 10 μg constructed plasmid and 500 μL HeBS were needed to make the cocktail. Media formulation for 293T cell culture and lentiviral infection: DMEM supplied with 10% FBS, 100 U/mL pen/strep and 2 mM L-glutamax. The harvested supernatant was snap-frozen on dry ice and stored in a -80°C freezer for future use.

Transduction was carried out by spinning target cells in the viral supernatant at 2200 rpm for 1.5 hours with polybrene to increase the infection rate. After transduction, cells were either sorted using FACS to get populations expressing the desired fluorescence signals or selected using puromycin for two days at 2 days post infection (dpi).

2.2.8 Limiting dilution

A bulk population of sorted cells was serially diluted and seeded into 96-well plates at a density of 0.3 cells per well and one cell per well in conditioned media, made by filtering supernatant from a 24-hour culture of expanding cells with optimal states. For setting up, 50 μL conditioned media with doxycycline was used per well using a multipipette plus (Eppendorf). Every three days, doxycycline was added to stimulate the HPV E6/E7 construct. The growth rate was monitored to detect viable clones.

2.2.9 Genome DNA extraction and primer design

At least one million (typically two million) cells were spun down at 250 g for 5 min in a benchtop centrifuge to make the preparation for genome DNA extraction. Then the supernatant was aspirated, and the cells washed with PBS once. The final spin used 600g for 5 min, and the pellets were frozen at -20°C after discarding the supernatant. Genome DNA from wild type BEL-As and the Selected clones was extracted using a commercial kit from bioline (ISOLATE II Genomic DNA Kit) following the vendor's instructions.

Primers for both cutting sites of band 3 were designed using Primer3 and modified manually using Snapgene software, with the following selection criteria: GC content 40% to 60%, length ranging from 18 to 22 bases, T_m ranging from 60° to 62°C and the T_m difference within the same pair less than 5°C . Two pairs of forward and reverse primers

were designed for the two cutting sites of band 3. To facilitate the downstream Sanger sequencing, the amplicon flanking the first cutting site of band 3 had 750 base pairs (bp), and 679 bp for site2. The BLAT and *in silico* PCR functions from UCSC genome browser were exploited to validate the specificity of the primers within the GRCh38/hg38 database and the product sizes. All primer pairs went through the analysis to exclude potential homodimers, heterodimers, self-hairpins (etc.) using the OligoAnalyzer Tool from the IDT website. Annealing temperatures were calculated using the T_m calculator function from the ThermoFisher website.

2.2.10 Oligos and PCR conditions

To amplify the product flanking the sites, Tables 2.2.1-2.2.3 were used:

Typically, to make up a 20 µl system (all measurements in µl):

Table 2.2.1. PCR system composition

System	H ₂ O	5*HF Buffer	10 mM dNTP	Phusion	Template	10 pM F/R Primers
Total 20	(13.4-X)	4	0.4	0.2	X	1 each

As for the genome DNA, typically 10 ng to 20 ng template was used for a 20 µL system and other volumes were adjusted proportionally.

Table 2.2.2. Program settings to amplify products flanking band 3 site 1 or site 2

Step	Lid temp. 100 °C
1	98°C, 30s
2	98°C, 10s
3	70°C, 30s
4	72°C, 60s
5	GOTO Step2, 30X
6	72°C, hold 5min
7	12°C, ∞

Table 2.2.3. Program settings to amplify product flanking both band 3 sites

Step	Lid temp. 100 °C
1	98 °C, 30s
2	98 °C, 10s
3	68 °C, 30s
4	72 °C, 60s
5	GOTO Step2, 30X
6	72 °C, hold 5min
7	12 °C, ∞

2.2.11 PCR Cleanup and Sanger Sequencing

PCR products were purified using a commercial kit (Bioline ISOLATE II PCR and Gel Kit), following the manufacturer's instructions. Sanger sequencing was carried out by the AGRF PD service.

2.2.12 Parasite culture

Routine *P. falciparum* 3D7 parasite culture was RPMI-HEPES media supplied with 0.2% sodium bicarbonate, 10% Albumax, 4% hematocrit using O⁺ red blood cells, in a sealed box gased with 1% O₂, 5% CO₂ in nitrogen, at 37°C. Five per cent sorbitol was used to synchronise the parasites for the ring stage. O⁺ red blood cells from two individual donors were mixed to overcome the effects of personal difference. For the parasites routine examination, thin blood smeared slides were fixed in methanol for 1 min and stained with 10% Giemsa for 6 min.

2.2.13 Co-incubation optimization of FACS assay

RBCs (from RedCross) were used to determine both the optimal media condition and the ambient atmosphere for the co-incubation of parasites and erythroid cells invasion assays. Two 96-well plates were incubated in parallel under each atmosphere condition, while each media condition had triplicate wells. For quantification, average invasion rates were recorded from two wells in each triplicate, with the remaining well subjected for blood smear. The readout of this assay relied on FACS 28 hours after setting up.

Several different media conditions were tested in the assay, because preliminary trials produced low invasion rates, when a full differentiation medium for HUDEP-2 cells was used for the 48-hour co-incubation. 3 IU/mL heparin was supplied as the anti-coagulant for AB plasma in the HUDEP-2 cells medium. At a concentration of 50–100 µg/mL, heparin can block all parasite invasion events, and lower concentrations, for example 5 µg/mL, leads to a significant reduction of invasions (Kobayashi et al., 2013). The following conditions as alternative for AB plasma and heparin were then investigated: 5% FBS alone, 10% Alb alone, and a mixture of 5% FBS and 10% albumax(Alb). For comparison, a normal RPMI medium supplied with NaHCO₃ and 10% Alb was involved as a positive control, with two other conditions including total withdrawal of AB plasma and heparin from IMDM differentiation media and full differentiation media containing 5% AB plasma and 3IU/mL heparin. To investigate the ambient atmosphere and its influence on the parasites' ability to invade, two conditions – 5% CO₂, 1% O₂ in N₂ and 5% CO₂, 21% O₂ in N₂ – were tested for parasite cultivation and HUDEP-2 cultivation, respectively. Parasites from the same culture dish were sorbitol-synchronised a day before and diluted to 0.5% initial parasitemia with uninfected red blood cells and different media. 48 hours after, EtBr was used to stain infected RBCs and the EtBr positive rate was analysed by FACS, to indicate the infection rate. Each parallel plate had three additional wells containing normal parasite culturing media as positive controls, which were smeared to confirm invasion rate.

2.2.14 Data analysis and graphics

Except histograms and pseudocolour plots, all HUDEP-2 and BEL-A profiling plots in chapter 3 were generated in RStudio (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>). Packages used included ggplot2, tidyverse, ggmosaic, cowplot, ggsci, dplyr, gridExtra, readxl and all attaching packages. Antibiotics killing curves and the bar graph co-incubation conditions were analysed and created with the Prism v8 application. Illustrations of band 3 epitopes were created with the biorender webtool (<https://biorender.com/>). Alignment of sequences used either NCBI BLAST tool or UCSC BLAT function.

Chapter 3: The HUDEP-2 line as a host model for *P. falciparum* invasion assay

3.1 Introduction

Naturally occurring RBC mutants in the human population that lack or have altered forms of specific surface proteins are valuable tools to study the role of specific receptors used by *P. falciparum* for invasion (Egan et al., 2018; Maier et al., 2003). For example, melanesian populations with Gerbich blood group express an altered form of GYPC and this has been useful in identifying the *P. falciparum* invasion ligand EBA140 that specifically binds to this receptor (Maier et al., 2003). Additionally, ABCB6 deficient RBCs impaired *P. falciparum* invasion (Egan et al., 2018), providing strong evidence to validate its role as an invasion receptor. However, analyses such as this are dependent on identification of blood donors lacking specific markers due to congenital or acquired diseases, and not all invasion receptors can be studied in this manner. Mutation of specific surface proteins would ideally be introduced artificially using genetic editing technologies, but this is not possible for mature RBCs as they lack nuclei. The development of nucleated erythroid precursors that can be genetically edited and differentiate into more mature erythroid cells have become very useful in analysing the role of specific receptors for *P. falciparum* merozoite invasion (Egan et al., 2015; Kanjee et al., 2017). A forward RNAi screen identified CD55, using CD34+ erythroid precursors, as important host proteins required for *P. falciparum* merozoite invasion (Egan et al., 2015). Subsequently, both CD55 knock down cultured cells and natural CD55 deficient RBCs isolated from a patient showed reduced susceptibility to *P. falciparum* merozoites, thus validating CD55's crucial role to support invasion (Egan et al., 2015).

The availability of the genetically editable JK-1 erythroleukemic cell line (Kanjee et al., 2017) and the establishment of HUDEP-2 cells (Kurita R, 2013) has facilitated a broad

spectrum of studies (Grevet et al., 2018; Moir-Meyer et al., 2018). HUDEP-2 is a tractable immortalized erythroid cell line capable of recapitulating late erythropoiesis and promises scalable production of cells for various applications (Kurita R, 2013) . There has also been an elegant established workflow for CRISPR editing these cells (Moir-Meyer et al., 2018). Therefore, we explored the potential of HUDEP-2 cells as a host model for *P. falciparum* invasion assays.

In this chapter, HUDEP-2 cells have been analysed to determine the expression of potential *P. falciparum* invasion receptors on the surface using FACS during their differentiation. A robust workflow for erythroid membrane profiling using an extensive FACS panel was established. Development of a FACS size gate for isolation and optimization of *P. falciparum* invasion assays were carried out to determine its suitability for this analysis.

3.2 Results

3.2.1 Optimization of *in vitro* erythropoiesis using the HUDEP-2 line

To understand HUDEP-2 cells with respect to the expression of membrane surface and differentiation marker proteins fluorescence-activated cell sorting (FACS) was used to evaluate maturation status and distinguish between stages of erythropoiesis. As described previously, there is an established and validated FACS antibody panel to distinguish the maturation stages of normal erythroblasts, comprising of three surface markers: $\alpha 4$ -integrin (CD49d), band 3 (CD233) and GYPA (Hu et al., 2013). HUDEP-2 cells decrease CD49d and increase band 3 and GYPA expression as they differentiate, thus making it possible to sort the cells using five gates drawn on the CD49d versus CD233 plot (Hu, Liu et al. 2013). The same panel of antibodies was employed for FACS analysis of the HUDEP-2 cell line.

As HUDEP-2 cells differentiate, there was a shift from the top left quadrant to bottom right quadrant for the CD49d-CD233 plot, indicating their increasing maturity (Figure 3.1 a). As indicated in the cytopins in Figure 3.1 b, five distinct maturation stages can be identified from the differentiation bulk population by their CD49d-CD233 pattern,

recorded as pro-erythroblasts, early and late basophilic, polychromatic and orthochromatic erythroblast stage sequentially. Sorted cells from each individual stage exhibited corresponding morphological characteristics. As the cells progress through differentiation, both cell size and nucleus-to-cytoplasm ratio decreased, with pro-erythroblasts exhibiting the greatest values for both parameters. As the cells matured the cytosol became more eosinophilic.

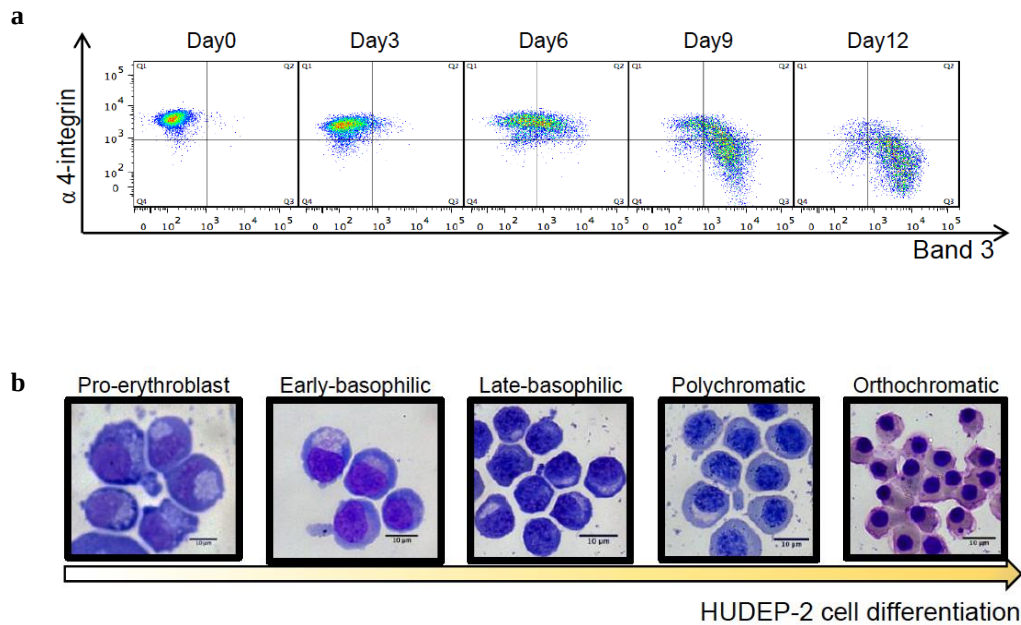


Figure 3.1 HUDEP-2 differentiation course

(a). $\alpha 4$ -integrin-band 3 FACS panel to distinguish the maturation stage of HUDEP-2 cells.
 (b). Morphological characteristics of HUDEP-2 cells from five maturation stages gated by $\alpha 4$ -integrin-band 3 pattern. Black bars denote 10 μm .

3.2.2 Characterisation of HUDEP-2 cell membrane surface proteins and differentiation state

To determine if the antibody panel could be used in the same FACS experiments to detect the different membrane surface proteins and maturation protein markers for HUDEP-2 cells titration experiments were performed as described previously (2.2.2 FACS and antibody titration, Chapter 2). As shown in Figure 3.2, overlapping histogram peaks from different combinations revealed the compatibility of the antibodies for CD35, CD44, CD55, CD71 and CD147 with the original three-colour

maturation FACS antibody panel. Therefore, the FACS experiments included antibody detection for both maturation protein markers and the invasion receptors simultaneously.

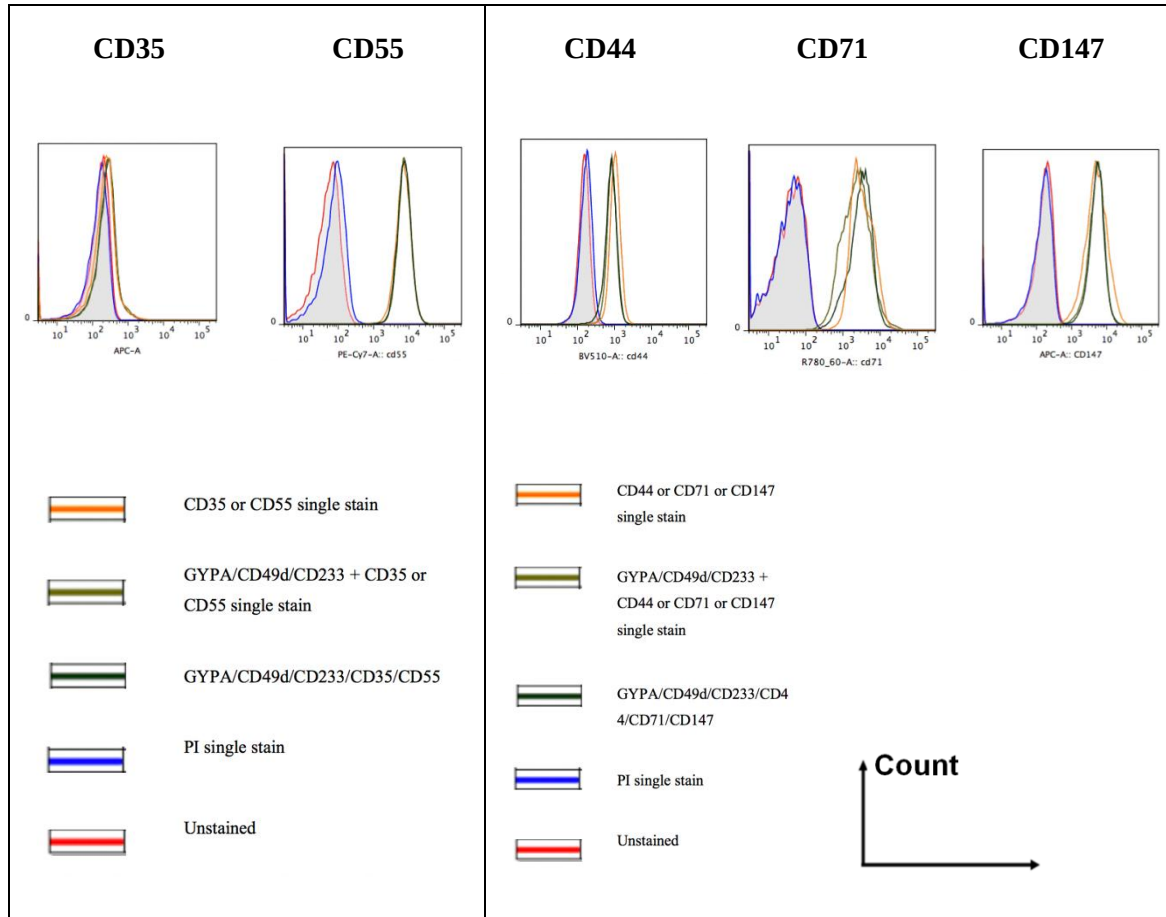


Figure 3.2 Overlaid histogram peaks to test the combinations

A surface membrane protein profiling experiment was performed using the expanded FACS panel during differentiation of HUDEP-2 cells (2.2.3 Cell profiling workflow, Chapter 2). The CD49d-CD233-GYPA expression pattern showed the differentiating cells shifting from top left quadrant to bottom right quadrant on the CD49d versus CD233 plot indicating decreasing expression of $\alpha 4$ -integrin as CD233 increased (Figures 3.3 and 3.4). For further analysis, cells were gated into five populations: population A was regarded as equivalent to pro-erythroblasts, early basophilic stage cells were recorded as population B, late basophilic stage cells as C, polychromatic erythroblasts as D and orthochromatic erythroblasts as E, to count the proportions of each differentiation stage in bulk population on day 0, 3, 6, 9 and 12 (Figure 3.5). As

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cells differentiated, pro-erythroblasts decreased gradually, and were replaced with more mature stages as the cells differentiated. The culture became quite heterogenous and mixed from day 6, with all five stages present simultaneously.

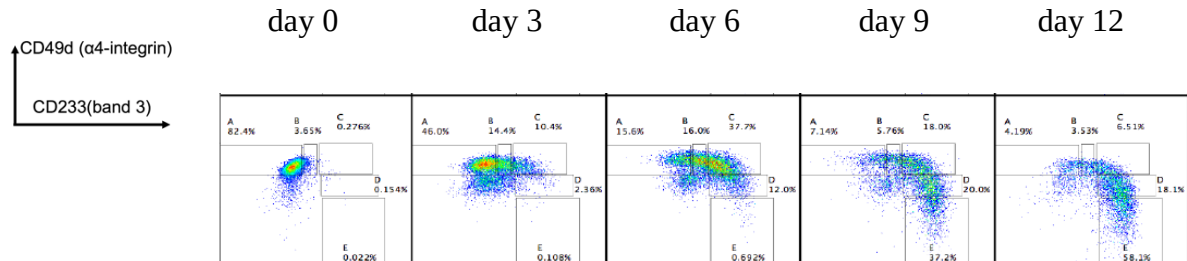


Figure 3.3. Representative CD49d versus CD233 results from CD49d/CD233/CD235a/CD35/CD55 tube

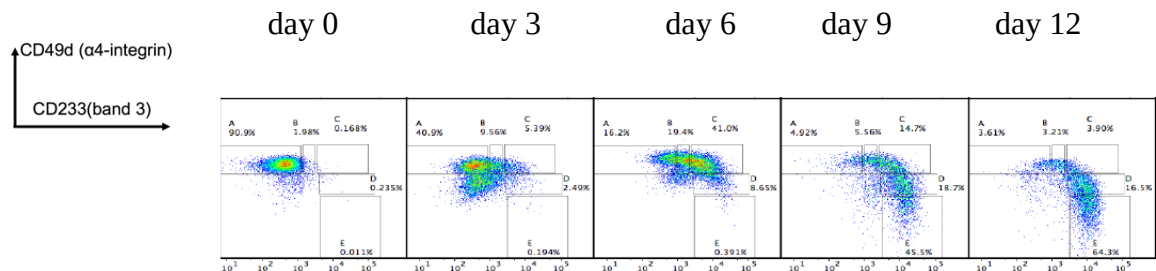


Figure 3.4. Representative CD49d versus CD233 results from CD49d/CD233/CD235a/CD44/CD71/CD147 tube

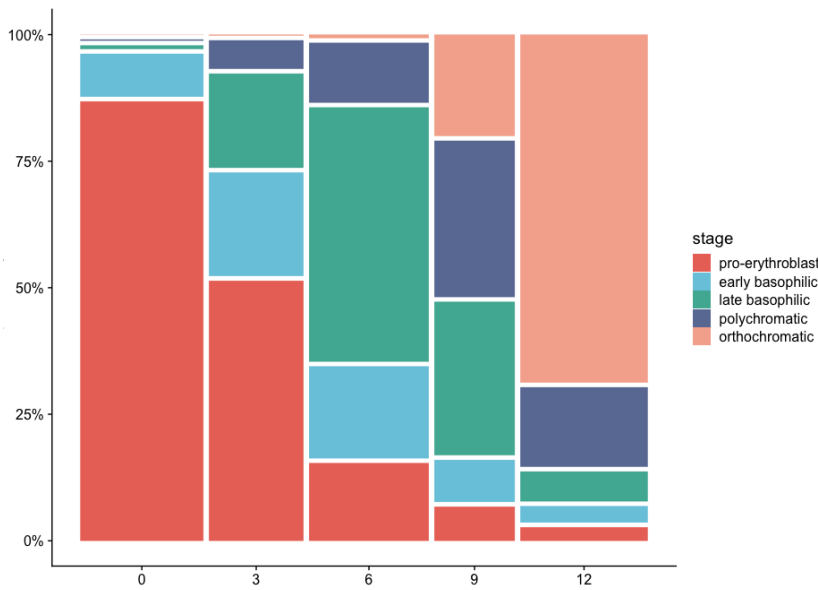


Figure 3.5. Percentage contribution from 5 stages to the bulk population

Results averaged from six differentiation experiments.

As indicated in Figure 3.6, the expression of CD44 decreased substantially during erythropoiesis as cells became more mature and was brighter than the signal from both RBC-positive controls stained under the same conditions. However, the reduction trend was not as prominent as with CD49d. CD55 signals remained bright across the whole course. CD71 expression showed a trend to increase as cells differentiated from day 6 to day 9, followed by a reduction to day 12. CD35 signals remained dim and changed little. A reduction in CD147 signal was also observed in the bulk population from day 9 to day 12. CD71 and CD49d, which were not expressed by mature red blood cells, signals from HUDEP-2 differentiation cells were comparable with or brighter than red blood samples stained under the same conditions using CD35, CD44, CD55 and CD147. Day 9 and day 12 HUDEP-2 cells exhibited high surface abundance of CD233 and CD235a, at a similar amplitude as red blood cell samples.

Chapter 3: The HUDEP-2 line as a host model for *P. falciparum* invasion assay

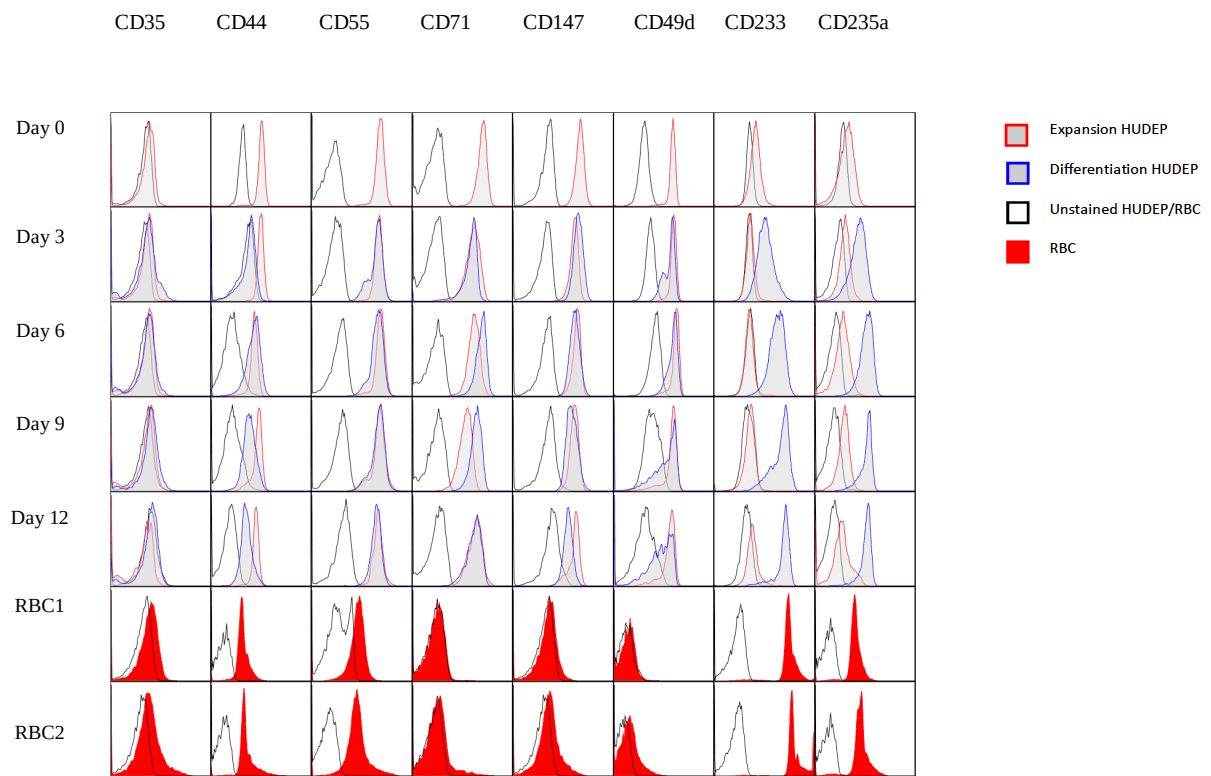


Figure 3.6. Representative histograms for HUDEP-2 bulk population

As indicated by Figure 3.7, on bulk population, there was a decrease of CD71 surface abundance on day 3, while a recovery and increase were observed afterwards, from day 6 to day 9, followed by a reduction of CD71 signal from day 9 to day 12. Collectively, decreasing trends were observed for CD44, CD49d, CD147 and CD49d from day 0 to day 12. CD233 and CD235 expression increased across the differentiation time-course.

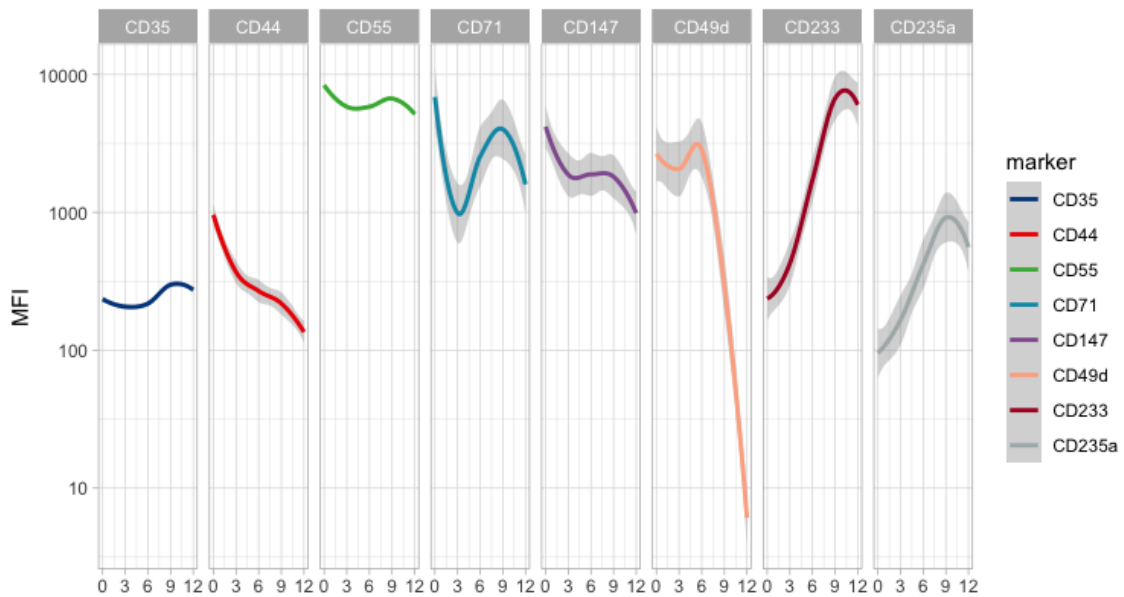


Figure 3.7. Membrane profile during differentiation on bulk population

Results averaged from six differentiations.

Similar trends were reflected in the curves for the gated population (Figure 3.8), except CD71, which increased as cells matured to polychromatic erythroblasts and peaked at this stage, followed by a reduction when cells developed to orthochromatic stage.

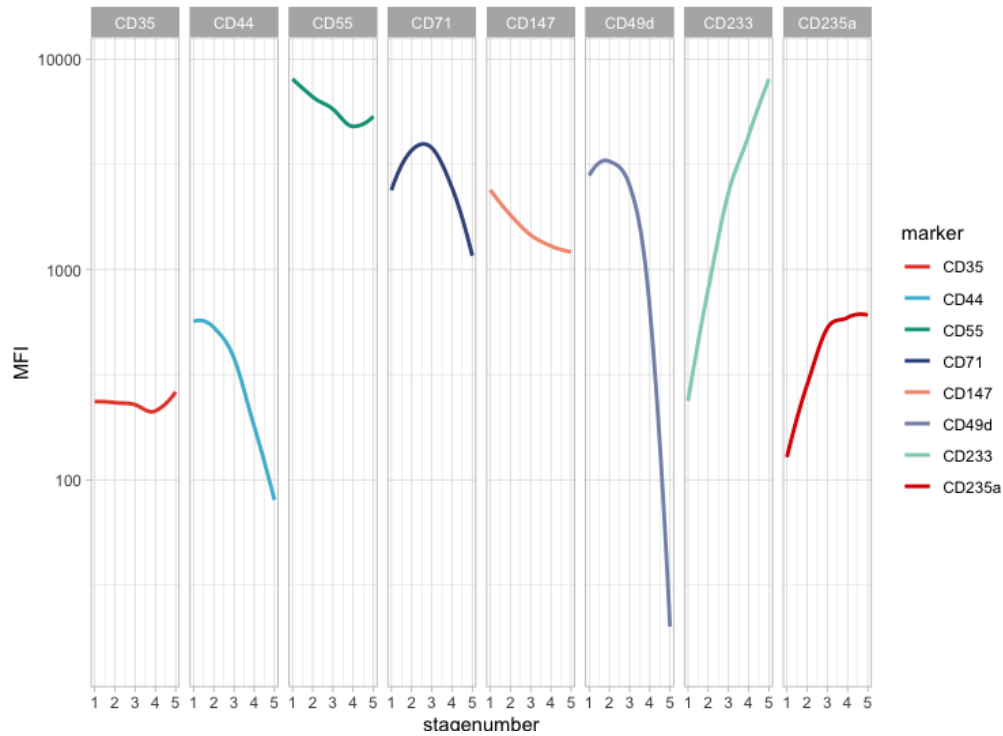


Figure 3.8. Absolute MFI values from gated populations

Results averaged from six differentiations.

Morphological results from the cytopspin slides were analysed to ensure that cells were showing normal differentiation (Figure 3.9 bottom panel). In comparison to the white pellet from expansion stage cells, pellets became redder as cells differentiated, indicating that they were accumulating haemoglobin (Figure 3.9 top panel).

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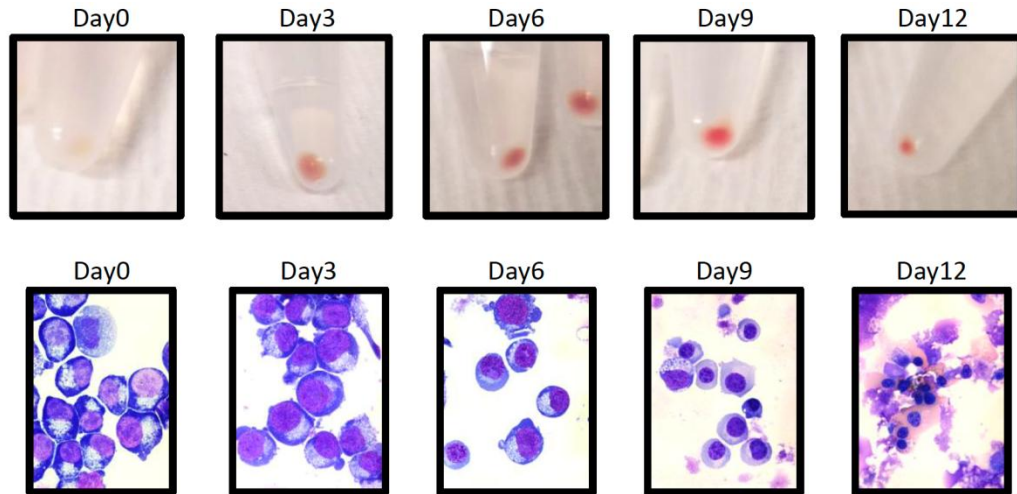


Figure 3.9. Representative morphological changes during differentiation.

Top: Cell pellets across differentiation. Bottom: Cytopsin images from corresponding cell pellets across differentiation.

For quantification, heatmaps in Figure 3.10 shows the comparison between bulk differentiating cells and their corresponding expansion ancestors.

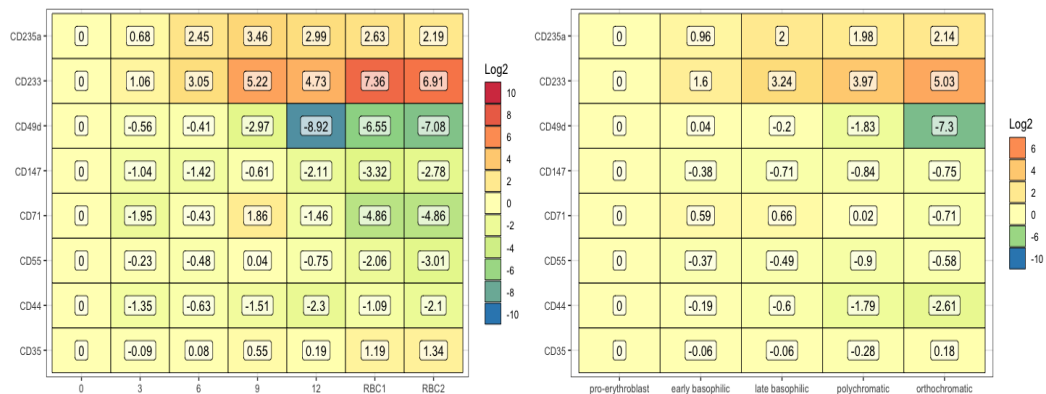


Figure 3.10. Heatmaps showing fold change in relative expression

Left panel: Results from bulk population. Right panel: Results from gated population.

3.2.3 FACS sorting of differentiated HUDEP-2 cells for *P. falciparum* merozoite invasion

For the isolation of a homogeneous population of late-stage differentiated HUDEP-2 cells, there was a need to develop an antibody-independent method, thus avoiding the potential of antibody binding blocking access for binding by *P. falciparum* merozoites in invasion assays. As Figure 3.11 indicates, there was a decrease in cell size when HUDEP-2 cells differentiated, providing a means to purify host cells for parasite invasion assays using size-based methods.

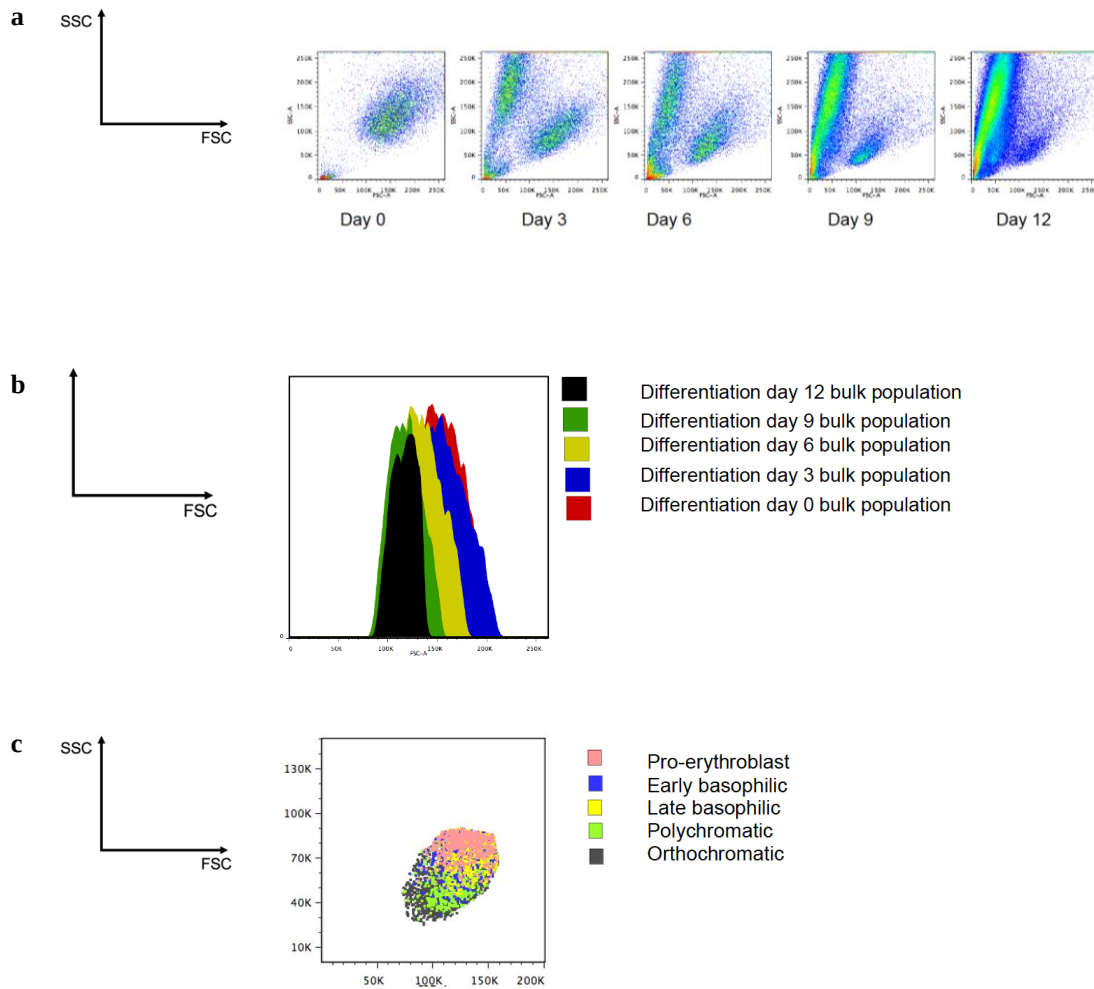


Figure 3. 11. HUDEP-2 cells decrease in size as they differentiate

(a). FSC-SSC plots from the HUDEP-2 differentiation course, and an accumulation of debris was observed. (bc). FSC parameters from both bulk and gated populations.

A FACS gate allowing identification of a specific subpopulation of smaller cells was established (Figure 3.12). As a sorting reference, a subpopulation of the cultivation was labelled with APC-antiGYPA, PE-antiCD49d and FITC-antiCD233, to gate polychromatic and orthochromatic stage cells. Then cells at both stages were back-gated onto the SSC-FSC plot to outline the borders of size and granularity, to sort the

remaining unstained cells. Sorted cells differentiated normally during the 48-hour recovery period between sorting on day 6 and maturation day 8. FACS analysis and morphological observation (Figures 3.13, 3.14 and 3.15) show that the sorted cells matured in a more synchronised manner than unsorted cells.

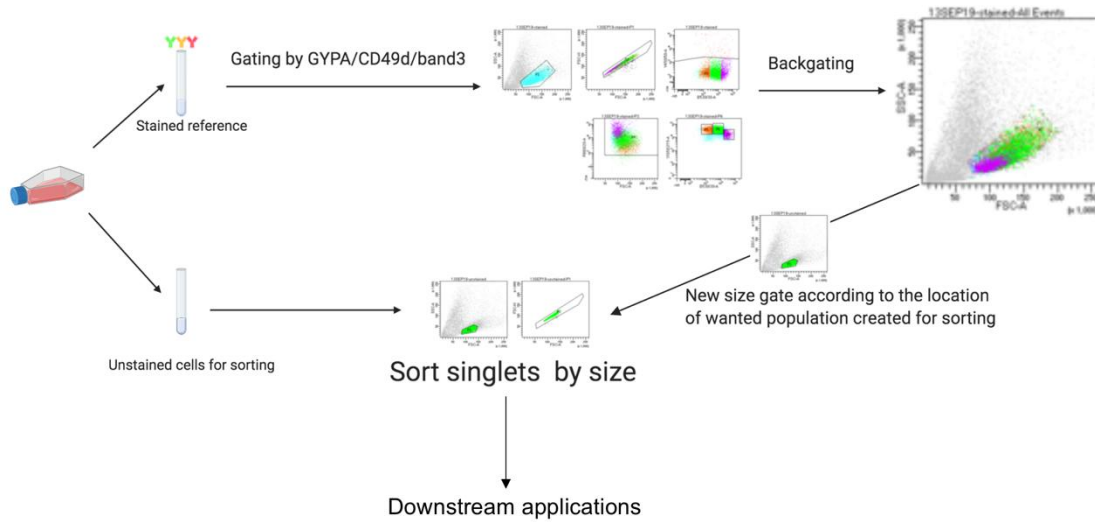


Figure 3.12. FACS size gate for isolation of late stage HUDEP-2 cells

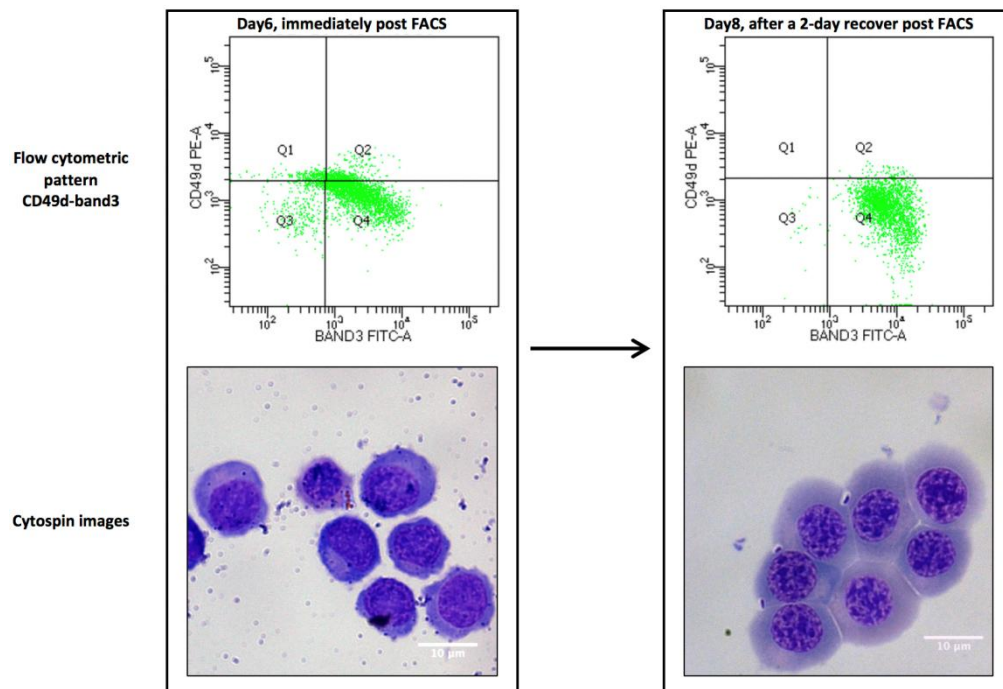


Figure 3.13. Schematic view of the size gate

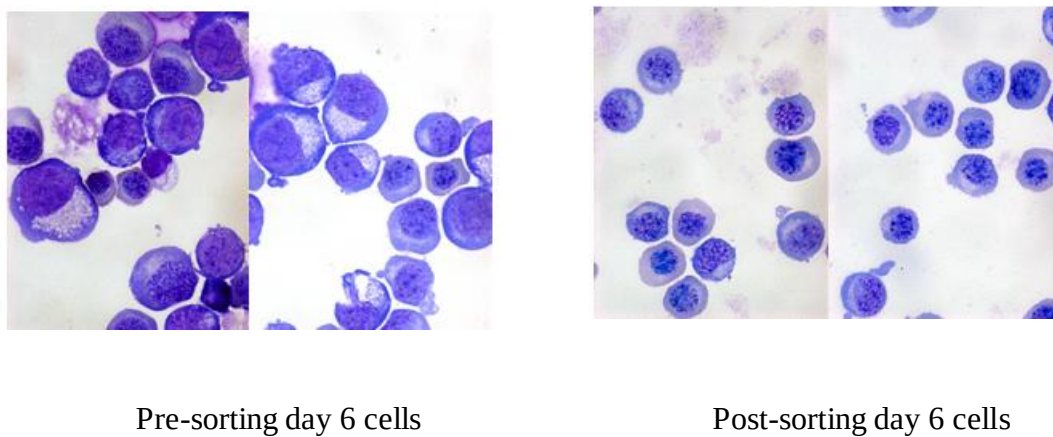
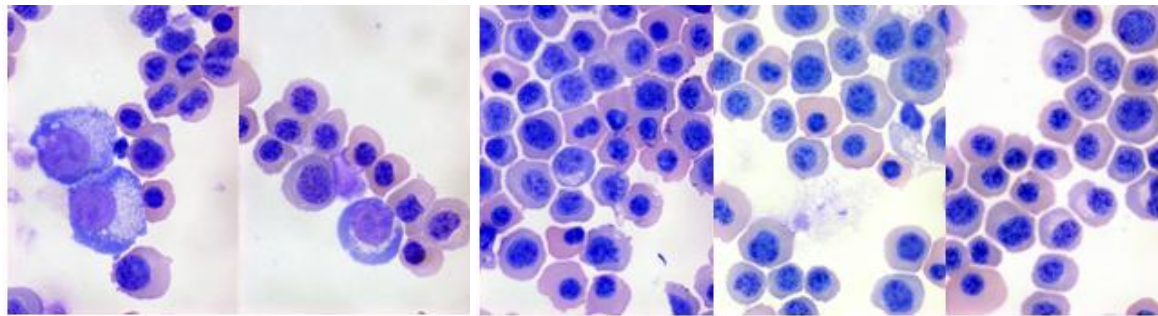


Figure 3.14. Morphology of pre-sorting cells and post-sorting cells on day 6



Pre-sorting day8 cells

Post-sorting day8 cells

Figure 3.15. Morphology of pre-sorting cells and post-sorting cells on day8

3.2.4 Optimisation of invasion conditions

Typical parasite invasion assays using RBCs require different culture conditions compared with HUDEP-2 cell culture (Trager & Jensen, 1976). Thus, assessing whether parasite invasion is possible in conditions conducive to HUDEP-2 culture is essential if the two systems are to be combined. Parasites invasion experiments are typically undertaken in an atmosphere compositing of 1% O₂, 5%CO₂ in N₂, using RPMI medium supplied with 10% Alb. Conversely, HUDEP-2 cultivation s undertaken at 5% CO₂ in humidified air at 37 °C.

To determine appropriate ambient atmosphere and media conditions that allow survival and growth of HUDEP-2 cells and the *P. falciparum* parasites for future merozoite invasion of late nucleated erythroid cells, a FACS experiment (2.2.13 Co-incubation optimization FACS assay, Chapter2) using RBCs was conducted, as RBCs support *P.falciparum* invasion well. Microscopic evaluation and FACS results (Figure 3.16) produced similar estimated invasion rates. No significant differences in parasitemia were detected between the two ambient atmospheres for the same medium condition using multiple comparisons. However, significant reductions in parasitemia were

observed for the full differentiation medium and AB plasma withdrawal wells (marked as IMDM+ cytokines in Figure 3.16), compared with positive controls. The addition of either FBS or Alb produced the comparable parasitemia after 28 hours. Parasitaemia from the combination of 5% FBS and 10% Alb was highest, therefore this condition was considered suitable for invasion assays using parasites and HUDEP-2 cells.

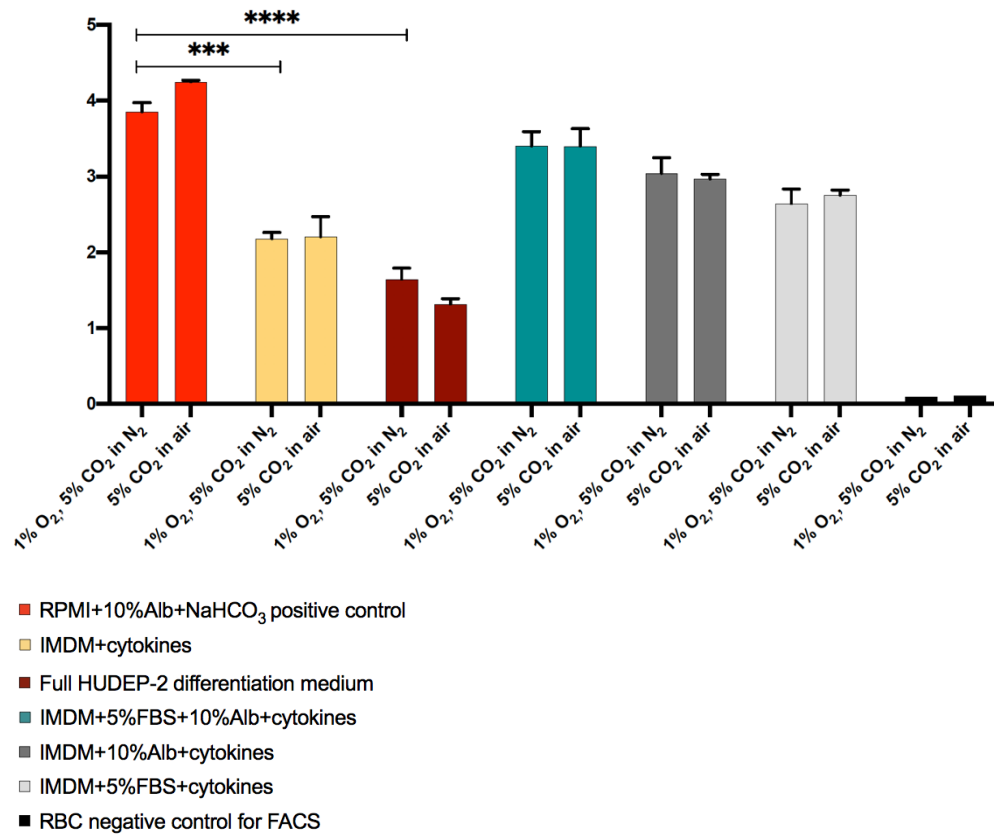


Figure 3.16. Ambient gas and media influence on 28 hr parasitemia using RBCs

3.3 Discussion

The results presented above demonstrate the successful establishment of a workflow for immunophenotyping and morphological staging using Giemsa-Grünwald staining to

track the progression of an *in vitro* erythroid cell line (the HUDEP-2 line). Time-course surface abundance of various invasion receptors – CR1/CD35, CD44, DAF/CD55, basigin (BSG/CD147) and glycophorin A (GYPA/CD235a) was revealed across HUDEP-2 cell differentiation. Given that all critical ligands are present on HUDEP2 cells at later stages of differentiation, and that orthochromatic HUDEP-2 cells have highest amount of accumulated hemoglobin in cytosol, together with the established FACS size gate to isolate those cells, orthochromatic stage HUDEP-2 cells promise to have the suitability of being hosts for *P. falciparum*.

For future work aiming to establish the platform of late HUDEP-2 cell invasion assays, the optimization of co-incubation conditions (i.e., a combination of 5% FBS and 10% Alb and carrying out the invasion assay in 5% CO₂, 21% O₂ in N₂) presented above is crucial, to avoid impeding parasite invasion while still keeping host cells at an optimal state. Further optimizations, for instance, MOI may be required to improve the performance of this platform. However, the system is now ready for piloting and optimisation of HUDEP-2 invasion experiments.

Previous work using nucleated models, i.e., erythroblasts (Tamez et al., 2009) and JK-1 cells (Egan et al., 2015), was dependent on light microscopy for readout, so was the pilot invasion trials using late HUDEP-2 cells, bringing a limitation that it is expertise-requiring and artifacts could occur during sample preparation. Therefore, it is crucial to involve more techniques for evaluating the invasion consequence, for instance, fluorescent parasites, e.g. D10 PHG, could be utilized in quantifying invasion rate. Additionally, specific medium instead of parasite culture medium could be required to maintain an optimal state of those nucleated cells, adding to the inconvenience when setting up the system.

Unlike nucleated host models which have several limitations, enucleated cells generated from edited precursors promise to be a more appropriate model for *P.falciparum*. Additionally, it is easier to do infected and non-infected discrimination and carry out quantification in enucleated cells, as they lack most of the subcellular structures. Furthermore, the same conditions for parasite cultivation should be amenable for enucleated cell invasion assays, thus easing the whole process. Whilst the HUDEP-2

cell line is tractable, an intrinsic limitation comes from its low enucleation rate. Thus, HUDEP-2 cells may not be the most appropriate host model, and it is appropriate to explore potential invasion properties in a second *ex vivo* erythroid cell line, for instance, the BEL-A cell line (Trakarnsanga et al., 2017).

Chapter 4: Establishing an editable erythroid model using the BEL-A line

4.1 Introduction

The current low enucleation rate hinders the potential of HUDEP-2 cells to support *P. falciparum* invasion assays, thus making it crucial to evaluate the performance of alternative cell lines. The BEL-A cell line (Trakarnsanga et al., 2017) can produce enucleated cells and is likely a suitable model for studying parasite *P. falciparum* – erythrocyte interactions.

Recently, a BSG knockout BEL-A cell was generated, and used to show that this receptor was essential for invasion by *P. falciparum* (Satchwell et al., 2019). Additionally, complementation of the *bsg* gene into the knockout cell line rescued *P. falciparum* merozoite invasion (Satchwell et al., 2019). Collectively, this work has shown that the BEL-A cell line can be used to generate reticulocytes and that genetic editing of the genome enables analysis of host cell receptors for *P. falciparum* merozoite invasion.

This chapter describes characterisation of the surface membrane proteins of BEL-A cells during differentiation to more mature erythroid cells using the same FACS antibody panel as outlined in Chapter 3. To explore the function of band 3, as a putative *P. falciparum* invasion receptor, CRISPR-Cas9 was used to introduce a deletion into the *band 3* gene, enabling characterisation of clonal band 3 deficient BEL-A cells.

4.2 Results

4.2.1 The BEL-A line shares cardinal *in vitro* erythropoietic features with HUDEP-2 cells

To study the tractability of the BEL-A cell line, a robust cultivation system was required, and the surface membrane protein profile of this cell line was characterised to understand their expression through erythroid development. This was important to determine the best differentiation state of the BEL-A cells to be used for testing the efficiency of *P. falciparum* merozoite invasion. This was performed using FACS analysis with the same antibody panel as used for the analysis of the HUDEP-2 cell line in Chapter 3.

Although both HUDEP-2 and BEL-A cells exhibited the characteristic $\alpha 4$ integrin-band 3 patterns when differentiating the latter cells tended to differentiate in a faster and more synchronised manner (Figure 4.1). The differentiation patterns for both the HUDEP-2 and BEL-A cells were compared from day 0 to day 9 using FACS and expressed as the percentage of the five maturation stages contributing to the bulk population (Figure 4.2). For example, on day 9 more than 70% of the BEL-A cells had reached the orthochromatic stage, comparable to the differentiation state of day 12 HUDEP-2 cells (Figure 3.7 (a)).

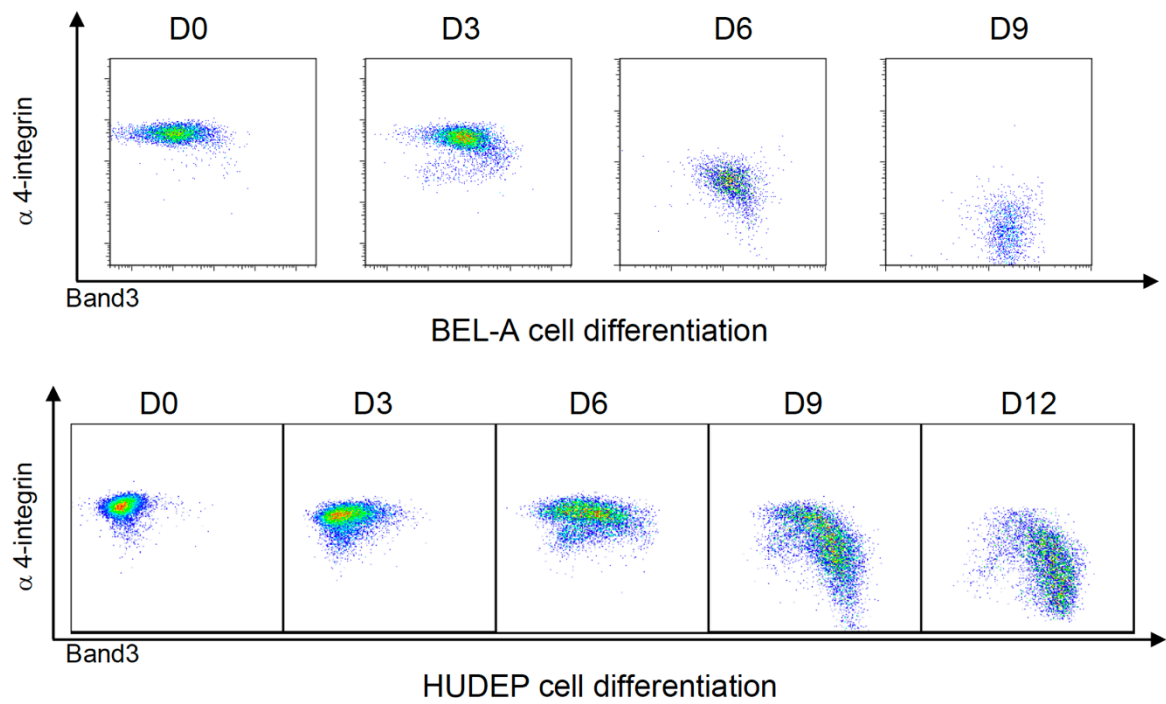


Figure 4.1. BEL-A cells differentiate faster than HUDEP-2 cells

Top panel: Representative α 4-integrin-band 3 plots across the course of BEL-A cell differentiation.
Bottom panel: Representative α 4-integrin-band 3 plots of HUDEP-2 cell differentiation.

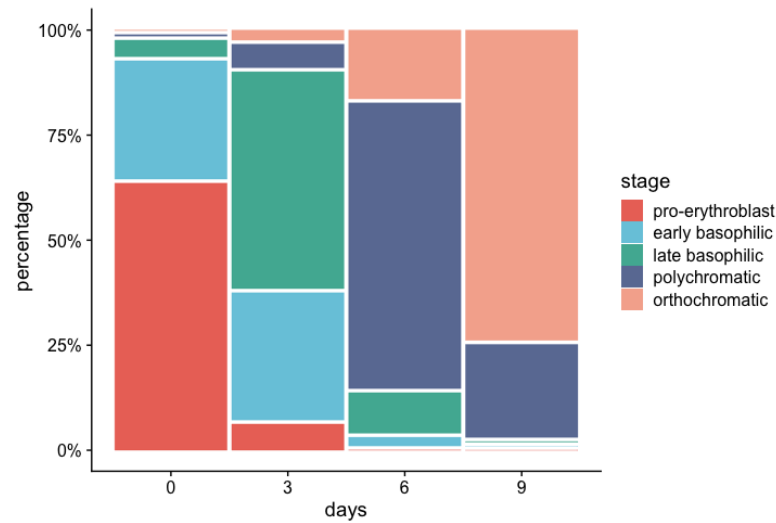


Figure 4.2. Contribution of each differentiation stage to the whole population in percentage form.

Results analysed on differentiation day 0, 3, 6 and 9. Averaged from triplicates.

The surface expression of invasion receptors CD35, CD44, CD55, CD71 and CD147, showed similar expression patterns for both HUDEP-2 and BEL-A cell lines (Figures 4.3, 4.4 and 4.5). Corresponding expansion stage cells for each differentiation were used as a reference to indicate the starting position of the peaks in Figure 4.4, and for normalization of fold changes in Figure 4.5. Consistent with the HUDEP-2 cells, surface CD44 and CD147 expression for BEL-A cells decreased as they became more mature during erythropoiesis. CD35 signals remained dim from day 0 to day 9 within all three differentiations. The CD71 pattern from the bulk BEL-A population MFI curve (Figure 4.3) exhibited a reduction from day 6 to day 9. Overall, this has shown that BEL-A cells express a wide array of surface proteins used by *P. falciparum* as host receptors for merozoite invasion.

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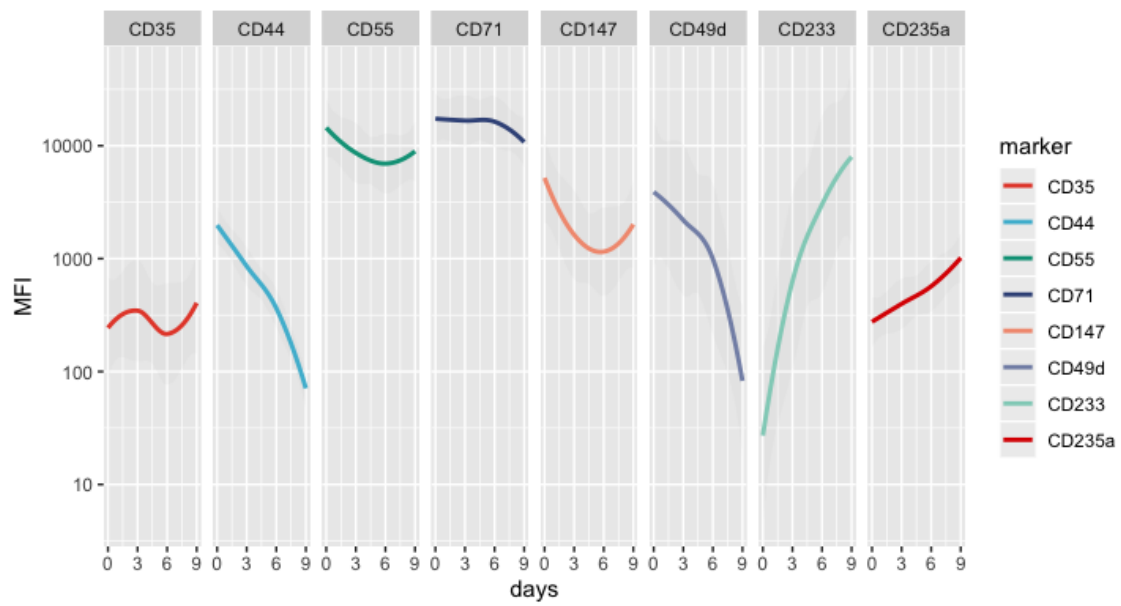


Figure 4.3. Membrane profile during differentiation in bulk BEL-A population.

Results averaged from triplicates.

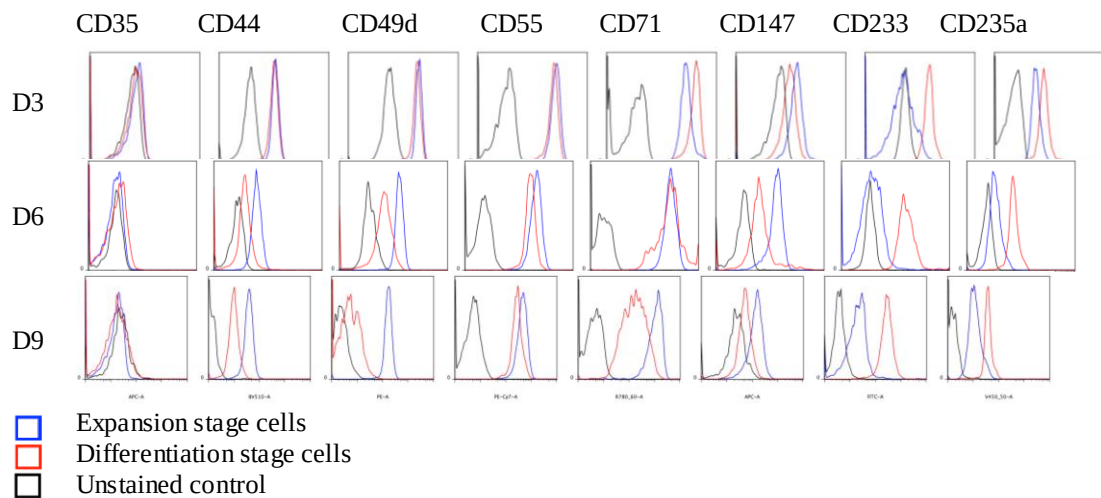


Figure 4.4. Representative FACS results from the bulk population of BEL-A cells.

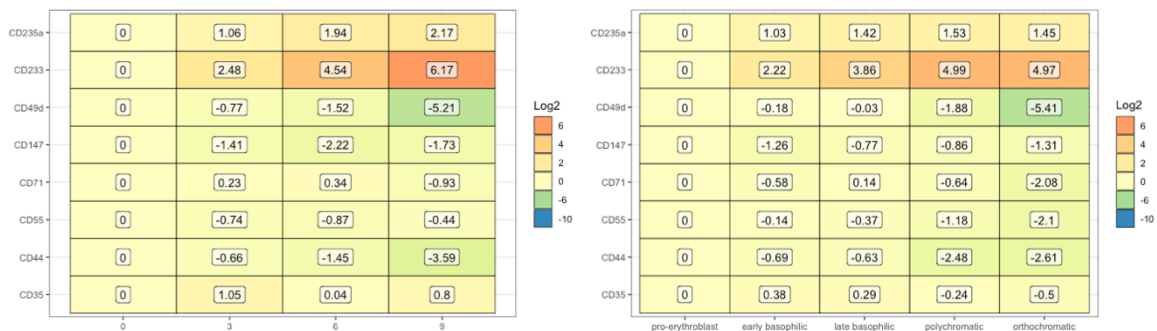


Figure 4.5. Heatmaps showing fold change in relative expression

Left panel: Results from bulk population. Right panel: Results from gated population. Results averaged from triplicates.

4.2.2 Generating clonal band 3 deficient BEL-A cells

The time-course BEL-A membrane profiling experiment provided information to understand the expression of crucial invasion receptors across erythroid differentiation. The presence of these surface markers, especially during late BEL-A differentiation, could be regarded as a prerequisite prior to deciding specific gene targets for genetic manipulation to construct loss-of-function mutants regarding specific surface proteins. Using the information generated with respect to the expression of specific proteins on the surface of BEL-A cells, it was decided to construct a cell line in which the *slc4a1*(band 3) gene had been disrupted using the CRISPR/Cas9 system.

In order to provide a BEL-A cell line in which we could perform genetic editing it was transduced with a construct that allow constitutive expression of mCherry-Cas9 (Aubrey et al., 2015). The transduced line was analysed by FACS and cells sorted to obtain those expressing the brightest mCherry signal (Supplementary Figure S1). These cells were put through two rounds of limiting dilution and FACS analysis to obtain the final line used for subsequent gene knockout experiments.

It was decided to target the *band 3* gene in BEL-A cells because of the evidence that it acts as a receptor for *P. falciparum* invasion (Baldwin et al., 2015). To disrupt the *band 3* gene, two sgRNAs targeting exon 4 and exon 5 (Table 2.1, Figure 2.2) were transduced into the Cas9-expressing BEL-A cells using lentivirus (Supplementary Figure S2). After treating with puromycin, 3 clones of putative *band 3* disrupted BEL-A cells were isolated. The puromycin killing concentration was determined by a pilot experiment (Figure S3).

4.2.3 Validation of *SLC4A1* (*band 3*) gene knockouts in selected clones

In order to analyse the gene-editing consequences after the transduction of both Cas9 and sgRNAs, PCR was used to amplify and sequence regions flanking each cutting site under the optimised conditions (Table 2.2.1 to Table 2.2.3, Chapter 2). According to the agarose gel electrophoresis results (Figure 4.6), for the *SLC4A1* gene, the wild type amplicon had an apparent size of 1703 bp, as shown by the band in the “WT” lane (Figure 4.6). Amplicons from the three selected knockout clones ranged between 800 bp and 1000 bp suggesting that regions of the *band 3* gene had been deleted in these cell lines.

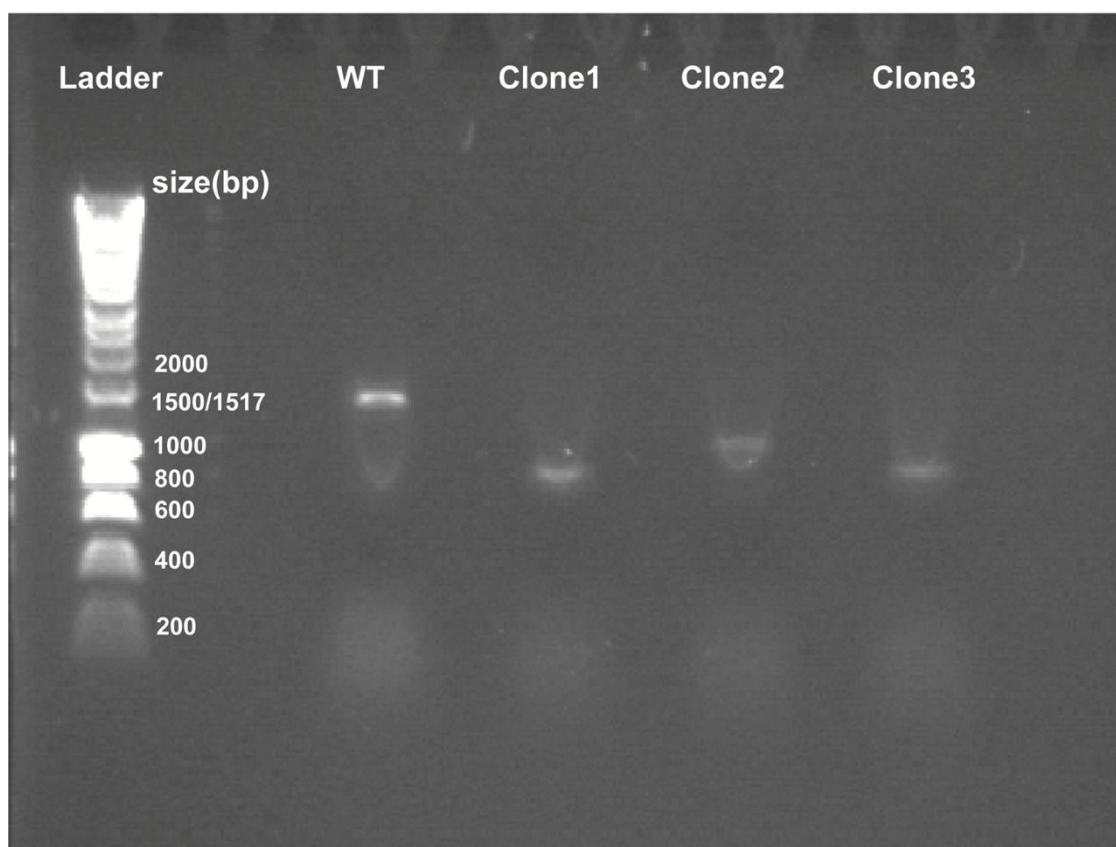


Figure 4.6. Agarose gel analysis of amplicons flanking both *band 3* cutting sites

Furthermore, it was not possible to amplify either cutting site with appropriate primers using gDNA extracted from the three clones, as opposed to the control suggesting they

had been deleted (Figure 4.7). Collectively, the PCR evidence suggested that in the three cloned lines at least part of the *band 3* gene had been deleted.



Figure 4.7. Agarose gel analysis of amplicons flanking band 3 cutting sites respectively

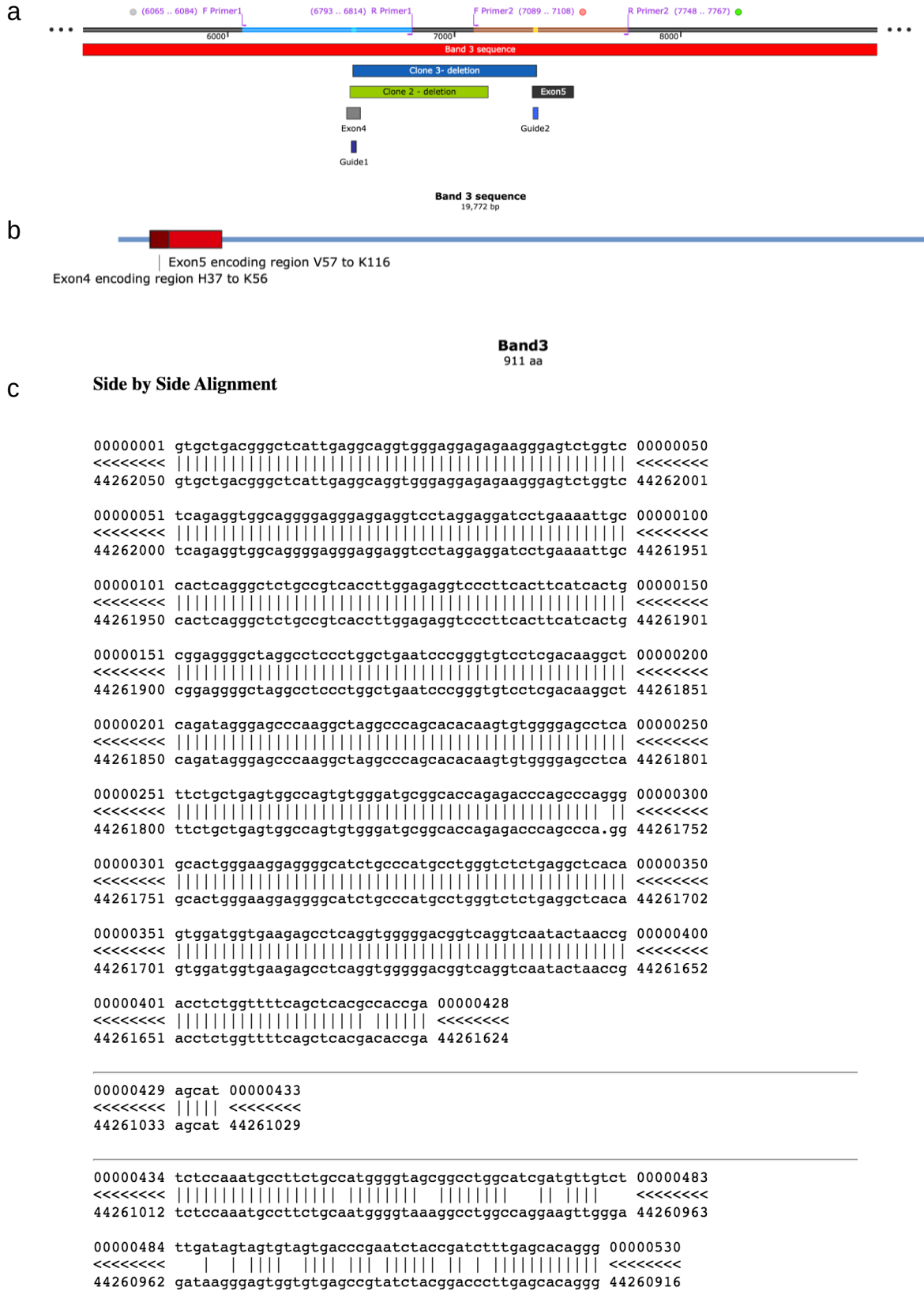
4.2.4 Sanger sequencing validates *band 3* knockout clones

To confirm that the *band 3* gene in the three cloned lines had been disrupted purified PCR amplicons flanking both Cas9 cutting sites were used as templates for Sanger sequencing to identify the disrupted regions (Figure 4.11). Both clone 2 and clone 3 had an intermediate interruption, with the preceding and the following sequences aligned with GRCh38/hg38 *SLC4A1*(band 3) sequences, respectively. The deleted sequences showed coverage of the binding sites for both site1 reverse primer and site2 forward primer. The length of the deleted sequences was consistent with the decreased sizes of

amplicon size as shown by PCR and agarose gel electrophoresis (Figure 4.6). Clone 2 had a 609 bp deletion whilst clone 3 had an 812 bp deletion. When aligned to the hg38 database, the deletion fragment of clone 2 covered the majority of exon 4 and ended before exon 5, whilst clone 3 had a deletion containing most of exon 4 and the initial part of exon 5 (Figure 4.11). Exon 4 and exon 5 encode amino acid H37 to K116 which is located in the N-cytoplasmic tail of the band 3 erythrocyte isoform. These gene disruptions also have a high possibility of causing nonsense mediated decay of mRNA (reviewed in (Baker & Parker, 2004; Hilleren & Parker, 1999)), therefore precluding the normal translation of functional band 3 (reviewed in (Baker & Parker, 2004; Hilleren & Parker, 1999)).

The gel electrophoresis results showed the amplicon from clone 1 was of a similar size to that from clone 3, indicating a similar size of deletion. However, due to insufficient time to obtain high-quality sequencing results through the unexpected cessation of laboratory work in late March 2020 following COVID-19 restrictions the deletion event has not yet been fully confirmed at the genomic DNA level. However, the deletion of part the *band 3* gene has been confirmed by PCR and DNA sequencing.

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d

Score	Expect	Identities	Gaps	Strand	
813 bits(440)	0.0	442/443(99%)	0/443(0%)	Plus/Plus	
Query	1	TGTGCTGACGGGCTCATTgaggcaggtgggaggagagaagggagtctggtctcagaggtg	60		
Sbjct	1		60		
Query	61	gcaggggagggagaggtcctaggaggaTCCTGAAAATTGCCACTCAGGGCTCTGCCGTC	120		
Sbjct	61	A.....	120		
Query	121	ACCTTGAGAGGTCCCTTCACTTCATCACTGCGGAGGGGCTAGGCCCTCCCTGGCTGAATC	180		
Sbjct	121		180		
Query	181	CCGGGTGTCCTCGACAAGGCTCAGATAGGGAGCCCAAGGCTAGGCCCAGCACACAAGTGT	240		
Sbjct	181		240		
Query	241	GGGGAGCCTCATTCTGCTGAGTGGCCAGTGTGGGATGCGGCACCAGAGACCCAGCCAGG	300		
Sbjct	241		300		
Query	301	GCACTGGGAAGGAGGGGACATCGCCATGCCTGGGTCTCTGAGGCTCACAGTGGATGGTG	360		
Sbjct	301		360		
Query	361	AAGAGCCTCAGGTGGGGACGGTCAGGTCAATACTAACCAGCCTCTGGTTTTCAGCTCAC	420		
Sbjct	361		420		
Query	421	GACACCGAGGCAACAGCCACAGA	443		
Sbjct	421		443		

Score	Expect	Identities	Gaps	Strand	
592 bits(320)	7e-173	322/323(99%)	0/323(0%)	Plus/Plus	
Query	1252	CAGGAGCTGGTGATGGACGAAAAGAACCAGGAGCTGAGATGGATGGAGGCGCGCGCTGG	1311		
Sbjct	440	...A.....	499		
Query	1312	GTGCAACTGGAGGAGAACCTGGGGGAGAATGGGGCCTGGGGCCGCCCGCACCTCTCTCAC	1371		
Sbjct	500		559		
Query	1372	CTCACCTTCTGGAGCCTCCTAGAGCTGCGTAGAGTCTTCACCAAGGGTGAGCCTCCTGGG	1431		
Sbjct	560		619		
Query	1432	CCCTTCTGGACCCCTGCAGATGAGGATTGTTGTGGGGTGCTGTGGCCAGCCTACCAGC	1491		
Sbjct	620		679		
Query	1492	TCACTCCTCCACTGCCTGCAGGTACTGTCTCCTAGACCTGCAAGAGACCTCCCTGGCTG	1551		
Sbjct	680		739		
Query	1552	GAGTGGCCAACCAACTGCTAGAC	1574		
Sbjct	740		762		

Figure 4.8. Sanger sequencing results for clone 2 and clone 3

(a): Alignment map of band 3. As shown in the map, the location of amplified regions flanking site1 and site2 are marked in blue and brown on black background. The cyan and yellow segment represent the sequences of guide1 and guide2 respectively. The relationship of guide1 and guide2 with exon4 and exon5 are shown as labelled rectangles below. Deletion of clone 2 and clone 3 from sequencing were also aligned. (b): Alignment map of AA encoded by exon4 and exon5 to full-length band 3. Information comes from corresponding datasheets and the NCBI database. Map generated using SnapGene Viewer. (c): Alignment of clone 2 sequencing results with GRCh38/hg38 sequence. (d): Alignment of clone 3 sequencing results with GRCh38/hg38 sequence.

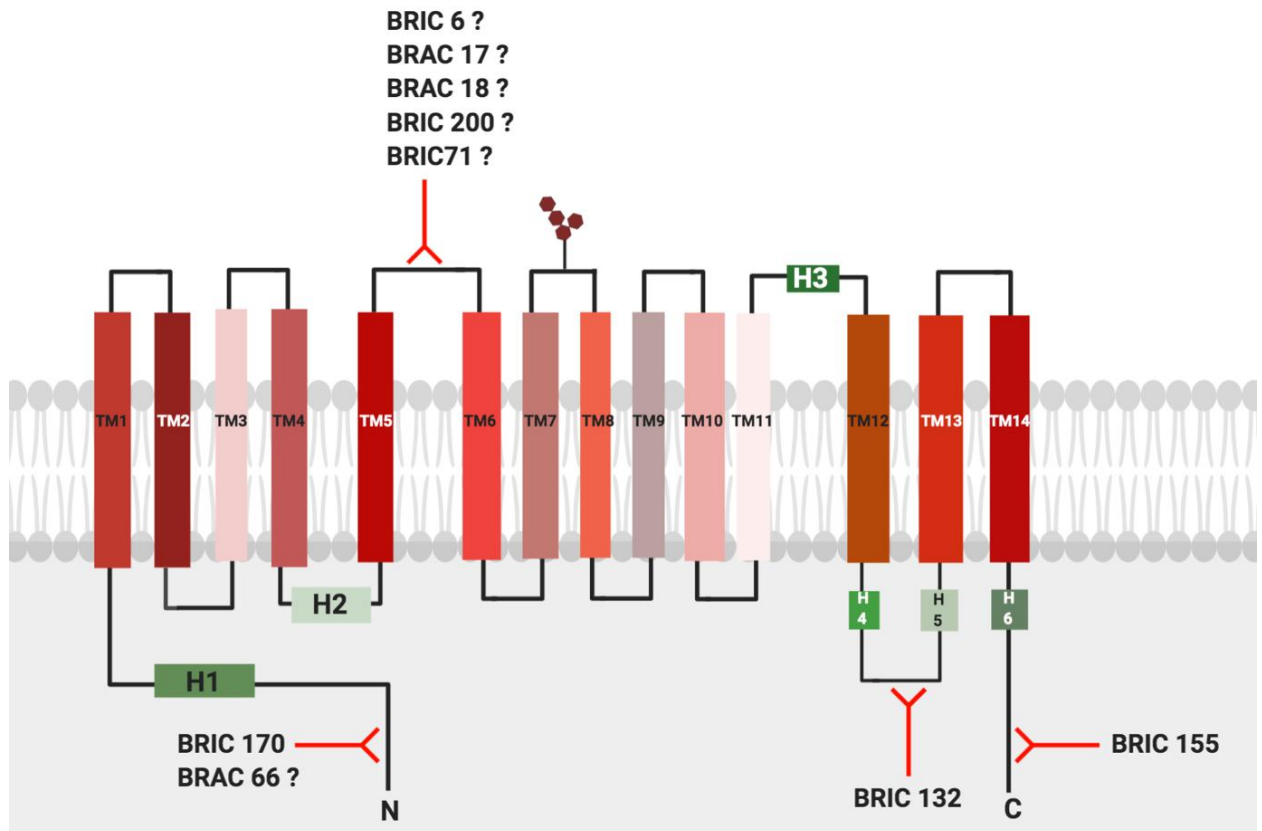
4.2.5 Profiling the three band 3 knockout clones

PCR amplification and Sanger sequencing provided genetic evidence that the BEL-A cloned lines contained deletion of at least part of the *band 3* gene. Next, it was important to confirm that these deletion events had resulted in the loss of band 3 protein

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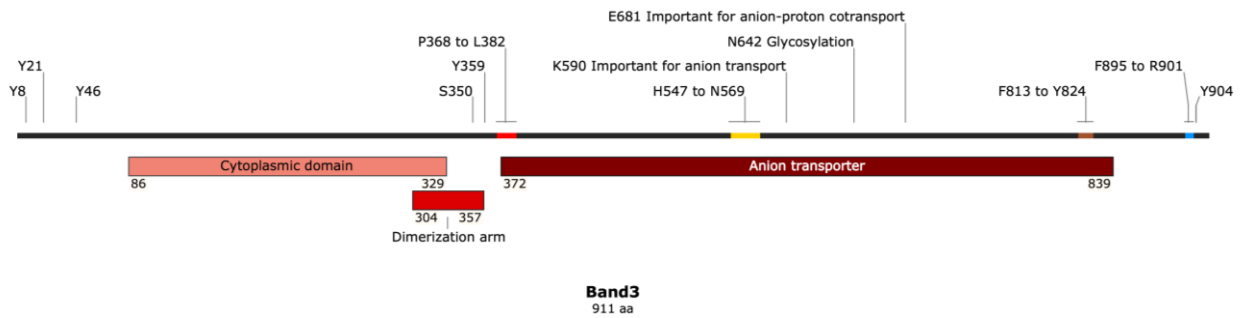
expression in the corresponding BEL-A cell lines. FACS analysis was used to detect the existence and membrane abundance of band 3 and other surface markers on wild type BEL-A cells and the three selected cloned lines. As summarized in Figure 4.9 and Table 4.1, there are several commercially available FACS antibodies against the extracellular moiety of band 3 which all target the third extracellular loop of this protein.

a



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b



c

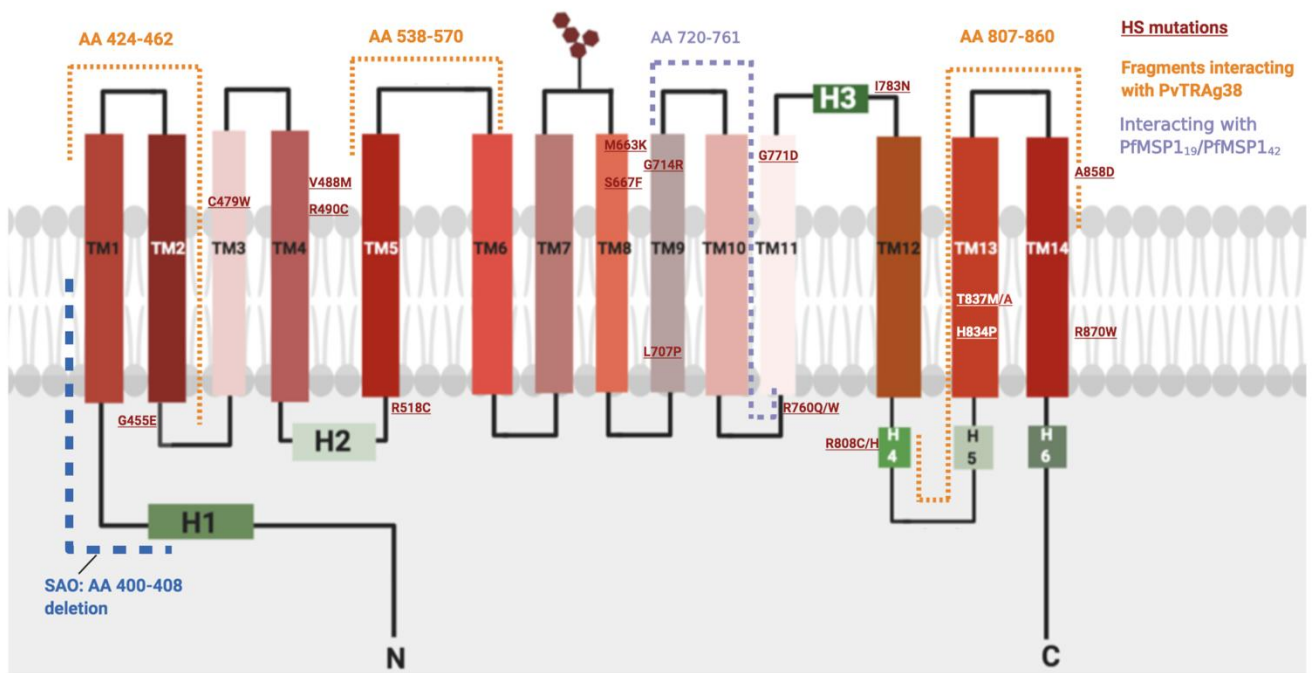


Figure 4.9 Band 3 antibody map and important band 3 sites

(a): Topology map of band 3 with different clones aligned. The topology graph is adapted from a published review (Reithmeier, Casey et al., 2016). (b): Sequential alignment of the for epitopes. Epitopes marked with red, yellow, brown and blue segments on black background. Y8, Y21, Y46, S350, Y359 and Y904 are phosphorylation sites suggested by experimental evidence. As denoted in the map, N642 is an important glycosylation site on the fourth extracellular loop, K590 is important for anion transport and E681 is important for anion-proton cotransport, according to experimental evidence. All information comes from corresponding datasheets and the NCBI protein database. The map was generated using SnapGene Viewer. (c). Clinically crucial sites on band 3 topology map. Underlined characters denote hereditary spherocytosis (HS)-related band 3 mutations. The eight amino acid deletions causing Southeast Asian ovalocytosis (SAO) are shown by the blue dashed line near the N terminus. Three orange dashed lines representing the band fragments associated with *P. vivax* tryptophan-rich antigen 38 (PvTRAg38) are marked on the map as well. Information for the band 3 topology graph and HS-related mutations came from a published review (Reithmeier, Casey et al., 2016). PvTRAg38-interacting fragment sequences come from published work (Alam, Choudhary et al., 2015). Purple dashed lines mark the sequence identified for the 19-kD domain of *P. falciparum* merozoite surface protein 1 (Goel, Li et al., 2003).

Table 4.1. Anti-band 3 provided by NHS and corresponding AA sequence of the targets

LOT-Clone	Binding	Host	Epitope	Corresponding AA Sequence
9439 BRIC 6	Extracellular	Mouse IgG3	Unknown, guess: on the extracellular loop between amino acids 545 and 567	QHPLQKTYNYNVL VPKPQGGL
9451 BRAC 17	Extracellular	Rat IgG2b	Unknown, guess: on the extracellular loop between amino acids 545 and 567	QDHPLQKTYNYNVL MVPKPQGGL
9452 BRAC 18	Extracellular	Rat IgG2b	Unknown, guess: on the extracellular loop between amino acids 545 and 567	QDHPLQKTYNYNVL MVPKPQGGL
9468 BRIC 200	Extracellular	Mouse IgG1	Unknown, guess: on extracellular loop between amino acids 545 and 567	QDHPLQKTYNYNVL MVPKPQGGL
9472 BRIC 71	Extracellular	Mouse IgG1	Third extracellular loop, slightly different from BRIC 6/90/200 and BRAC 14/18/21/25	DHPLQKTYNYNVL VPKPQGGLPN
9449 BRIC 155	Cytoplasmic	Mouse IgG2b	amino acids 895-901 located at the cytoplasmic tail	FDEEEGR
9494 BRAC 66	N- Cytoplasmic	Rat IgG2	Unknown, near N-cytoplasmic tail	N.A.
9450 BRIC 170	N- Cytoplasmic	Mouse IgG1	AA 368-382 located near the N-terminal cytoplasmic tail	PDDPLQQTGQLFGGL
9458 BRIC 132	Cytoplasmic	Mouse IgG1	813-824 located at the cytoplasmic tail	FKPPKYHPDVPY

As a starting point, the $\alpha 4$ -integrin-GYPA-band 3 FACS panel was used to examine the clones and compare the expression of these proteins with wild type cells. The anti-band 3 antibody BRIC 6 was used to determine the membrane surface abundance of band 3 on wild type BEL-A cells and the three clones. Figure 4.10 shows that the three clones had constant dim signals from day 0 to day 9, and clone 1 and clone 2 appear band 3 negative, judging from the distance between their peaks and the unstained peak. This was particularly evident for the day 6 and 9 differentiated cells with the wild type cells consistently expressing high levels of surface detectable band 3 protein whilst showed essentially background levels of detection. These experiments suggest that the three cloned lines do not express detectable levels of band 3 on the membrane surface as detected using the BRIC-6 antibody.

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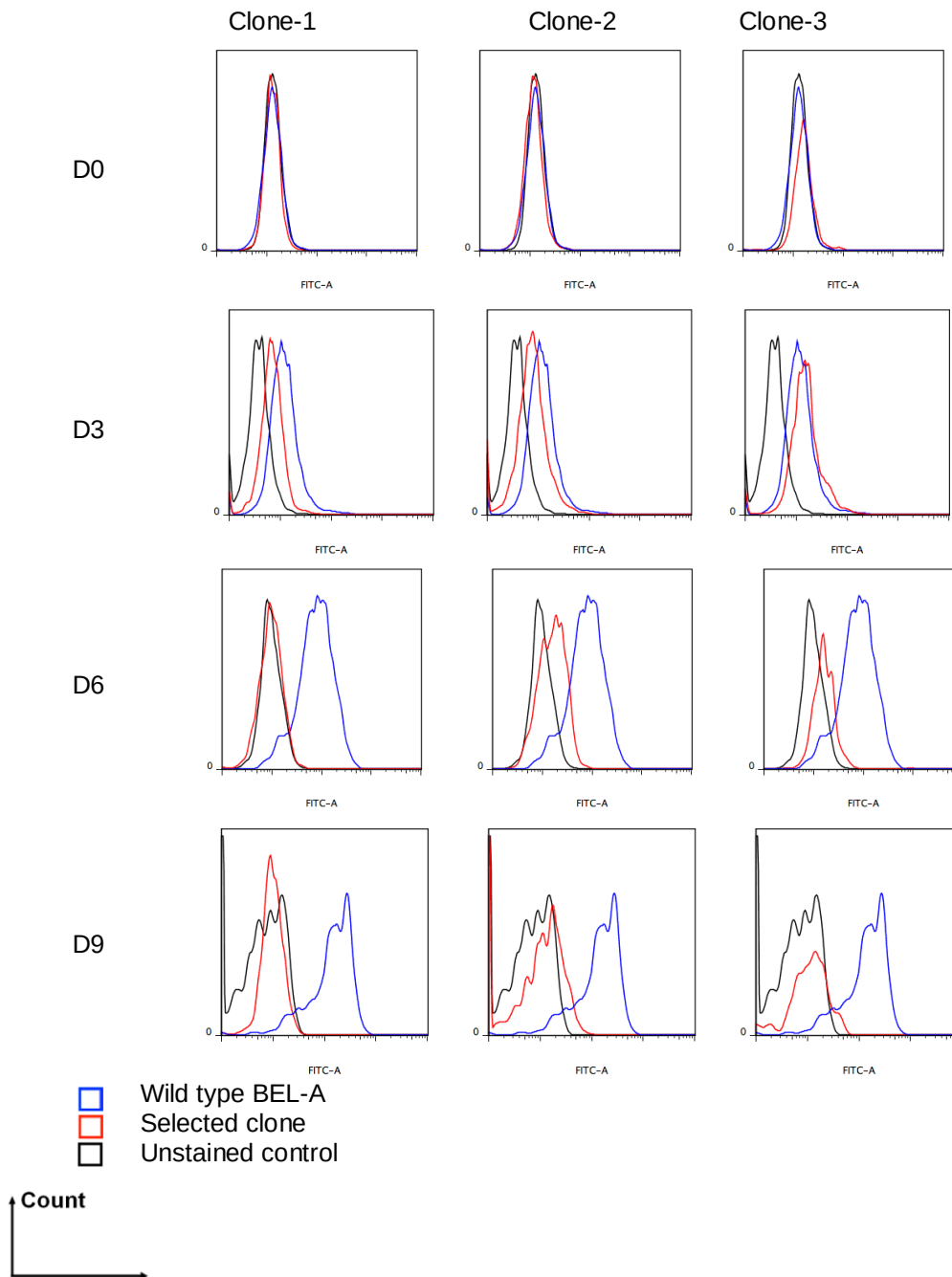


Figure 4.10. Representative Band 3 (BRIC 6) patterns of the three clones across erythroid differentiation.

Due to the severe cell loss from day 6 to day 9 observed in clone 3 differentiations and the limited opportunities to perform experiments (due to the COVID-19 pandemic), only clone 1 and clone 2 were used for further analysis. To provide additional evidence that band 3 was not expressed on the surface of clone 1 and 2 BEL-A cells additional antibodies to this protein were used for further FACS analysis (Figure 4.11). Clone 1 and 2 were tracked from differentiation day 0 to 9 for their surface expression of epitopes for BRIC 71, BRIC 200, BRAC 17 and BRAC 18 (Figure 4.11). None detected an obvious increase of band 3 on clone 1 or clone 2, whilst for both wild type and CRISPR control cells band 3 expression was detectable. Whilst these independent anti-band 3 antibodies provide additional evidence that band 3 was not expressed on the cell surface of clone 1 and 2, they all detect the third extracellular loop of the protein. Consequently, additional analysis of band 3 expression in these cell lines was planned to use immuno-fluorescence and western blots, however, circumstances with the pandemic did not allow this to be performed.

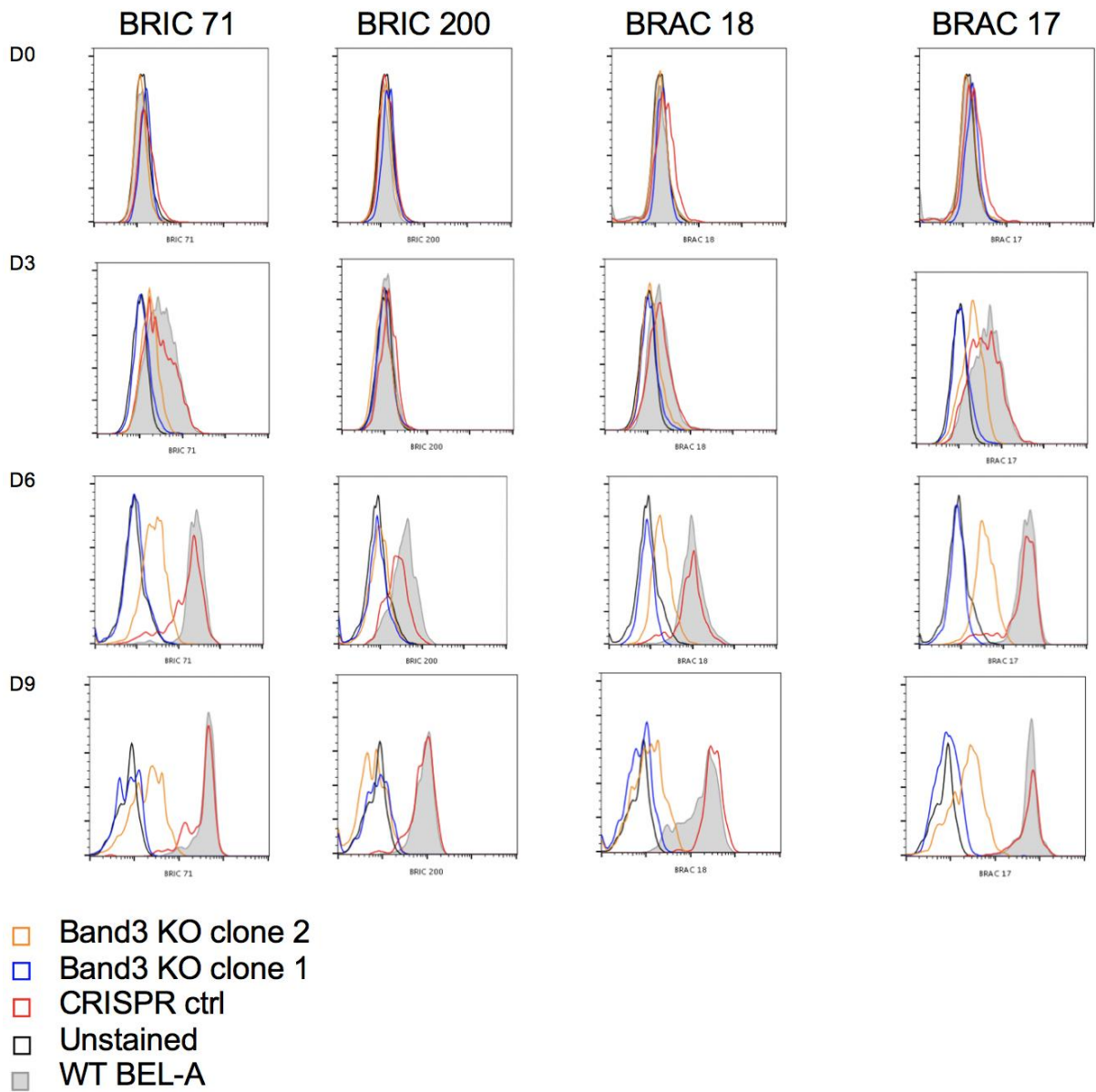


Figure 4.11. Representative time course band 3 surface expression of clone 1 and 2 cells

Similar results were obtained from duplicates.

Similarly, BSG expression was also examined using the antibody TRA-1-85 and FACS analysis on expansion stage cells taken from cultivation of wild type, clone 1, clone 2 and CRISPR control. As shown in Figure 4.13, clone 1 and clone 2 had comparable BSG expression compared to both wild type and CRISPR controls during expansion stage.

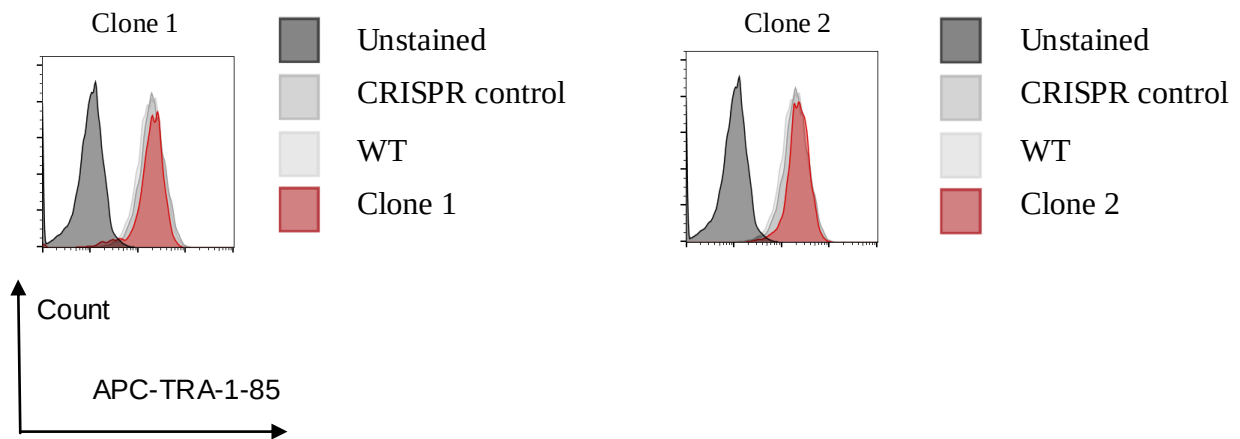


Figure 4.13. Representative BSG expression of expansion stage clone 1 and 2 cells

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As shown in Figure 4.14, GYPA expression for both clone 1 and clone 2 remained at the same level as the wild type control. Consistent with wild type BEL-A cells, the GYPA signal became brighter when both clones differentiated. This suggests that surface membrane expression of GYPA has not been affected by alterations in band 3 for both clone 1 and 2.

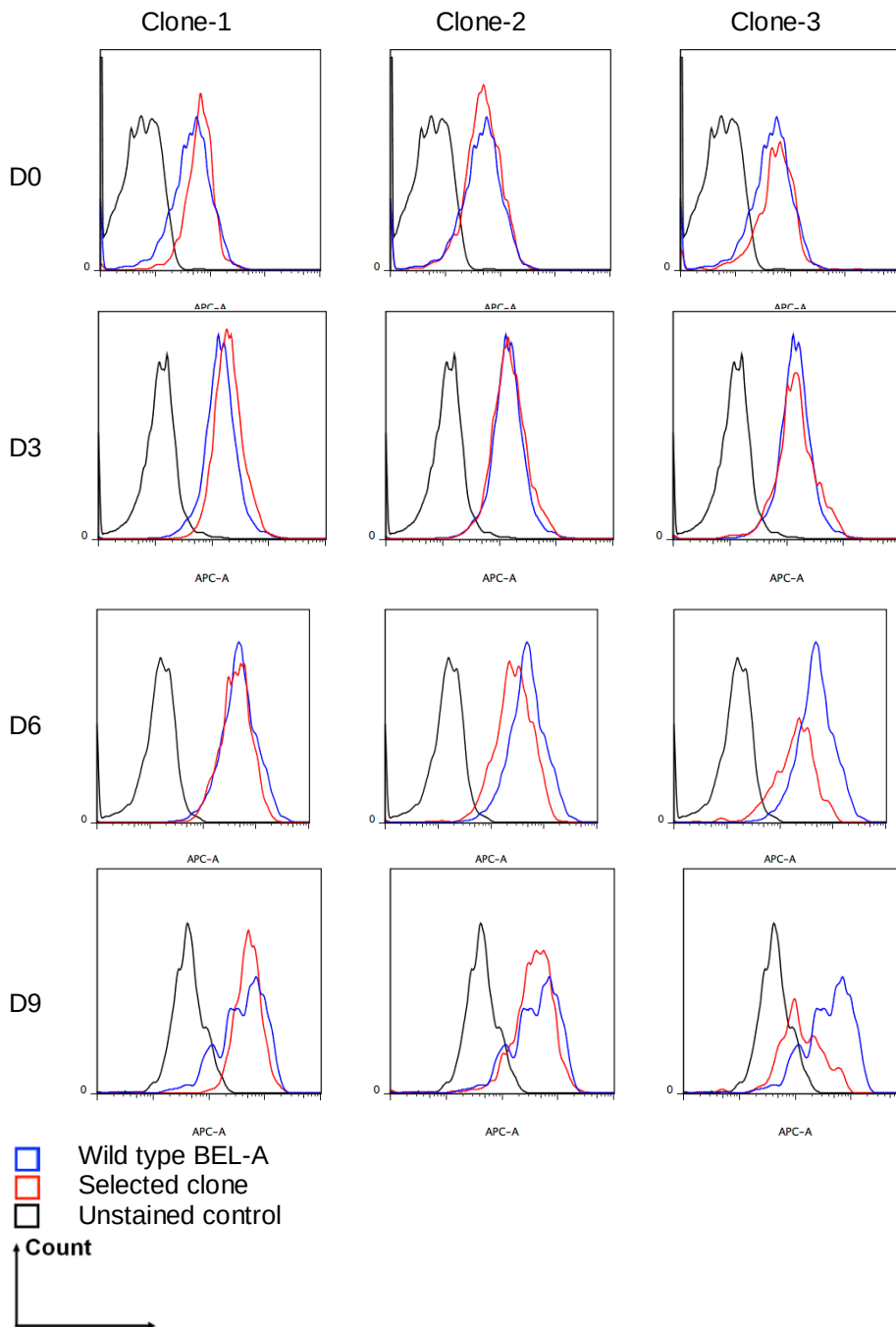


Figure 4.14. Representative GYPA patterns of the three clones across differentiation

Integrin $\alpha 4$ (CD49a) is an important erythroid differentiation marker and its expression was analysed for both BEL-A cline 1 and 2 and compared directly to band 3 and GYPA surface expression. When overlapping the $\alpha 4$ integrin-band 3 plots of bulk populations from day 3 to day 9, a similar reduction of $\alpha 4$ integrin signal was observed for both wild type clone 1 and clone 2 cells during differentiation. As expected, only wild type cells exhibited an increase in the band 3 signal compared to that of clone 1 and clone 2 (Figure 4.15).

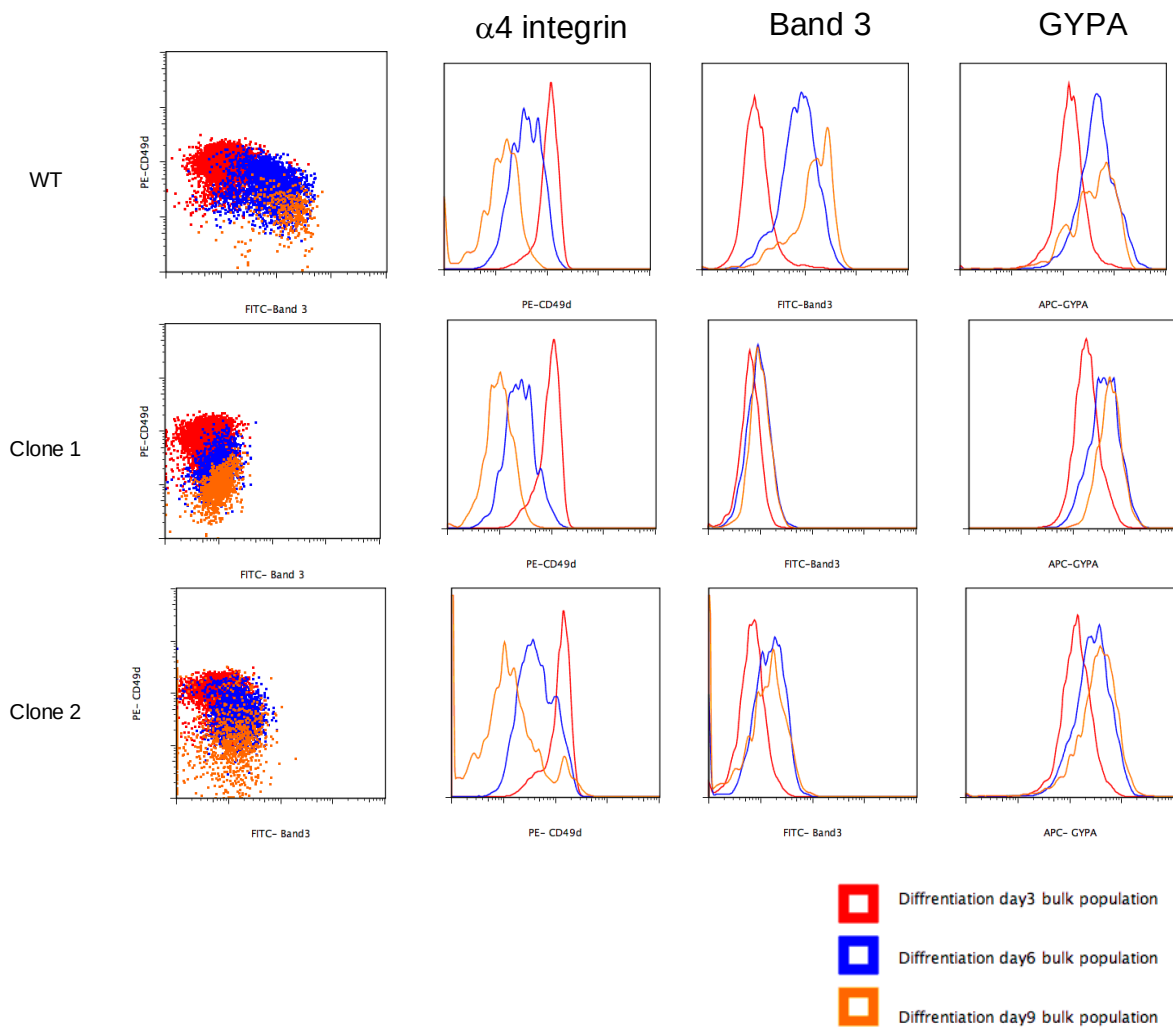


Figure 4.15. Representative $\alpha 4$ -integrin-band 3 expression of wild type BEL-A cells, clone 1 and clone 2

To examine membrane surface expression of the *P. falciparum* invasion receptors CD35, CD44, CD55 and CD71, BEL-A expansion stage cells taken from wild type control and the band 3 deficient clones were analysed by FACS using a specific antibody to each protein. The expression of CD35, CD44, CD55 and CD71 looked similar on both the wild type BEL-A cells and the three clones (Figure 4.16). The FACS analysis of surface proteins on BEL-A clone 1 and 2 cell lines has shown that whilst band 3 surface expression was essentially undetectable with multiple antibodies all of the other proteins tested appeared to be present at normal levels. This suggests that disruption of band 3 protein expression in clone 1 and 2 has not affected the level of expression of multiple surface proteins even though some of these are in major complexes, especially the glycophorins, as GYPA participates in the ankyrin complex (reviewed in (L. J. Bruce et al., 2003)) and GYPC is involved in the 4.1R core complex (Han, Nunomura, Takakuwa, Mohandas, & Jap, 2000; Marfatia, Leu, Branton, & Chishti, 1995).

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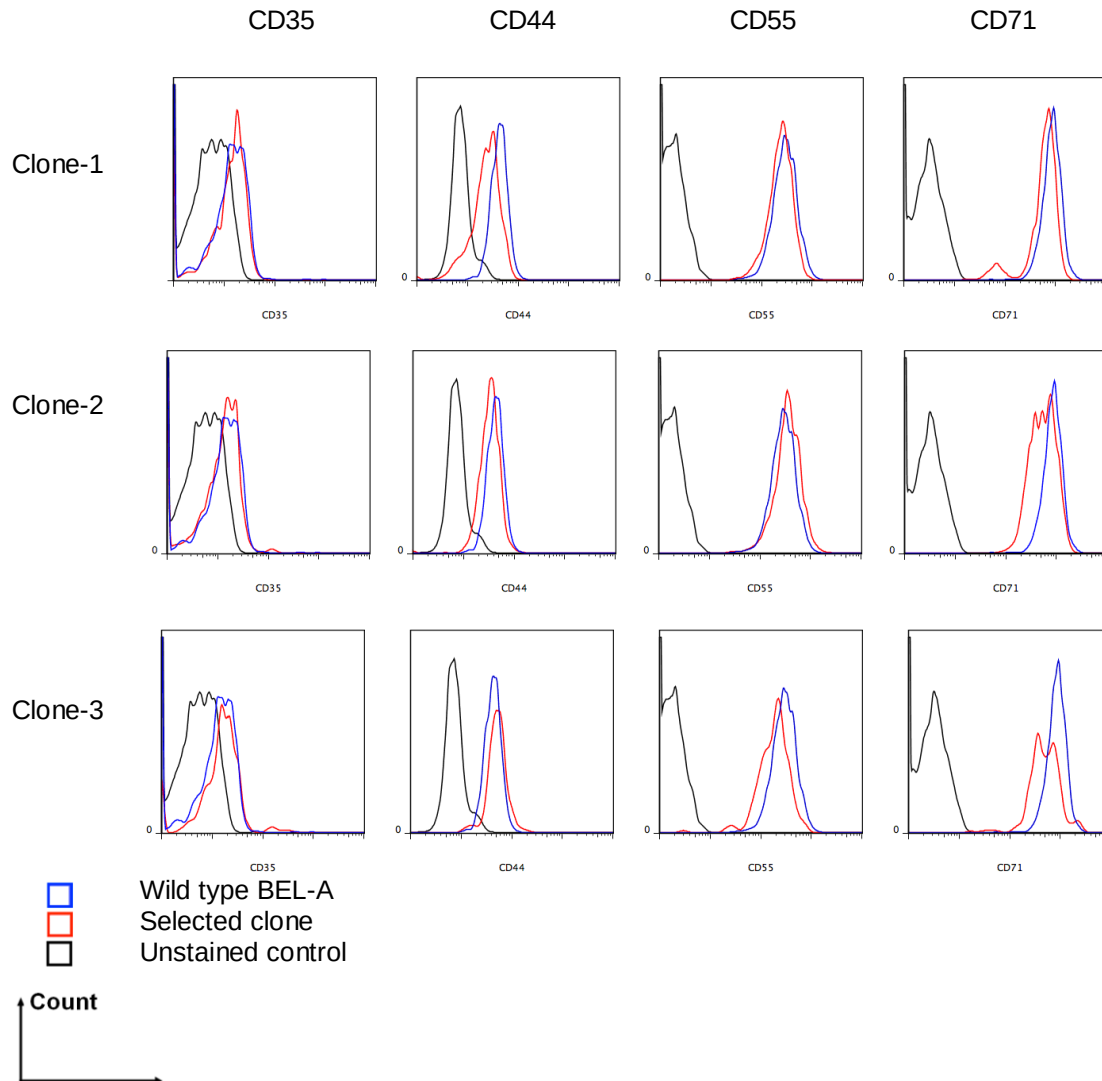


Figure 4.16. Representative CD35/CD44/CD55/CD71 profiles of expansion cells from the three clones

In order to determine if BEL-A clone 1 and 2 cells showed any differences in their ability to differentiate their morphological features were monitored compared to wild type cells using microscopy (Figure 4.17). Clone 1 and clone 2 exhibited comparable ability to differentiate from the pro-erythroblast stage to orthochromatic erythroblasts compared to wild type cells. Therefore, the alterations in expression of band 3 in BEL-A clone 1 and 2 has not affected the ability of these cell lines to undergo normal erythroid differentiation.

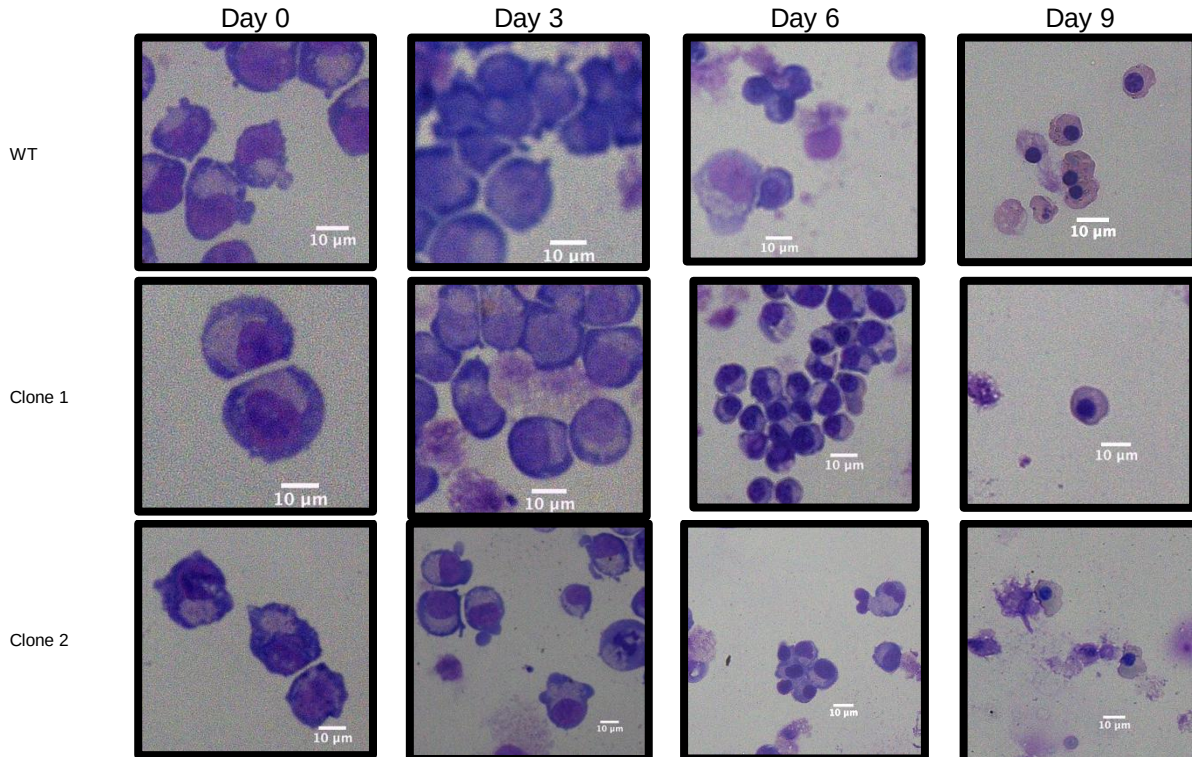


Figure 4.17. Morphology of the corresponding cells along a differentiation course

Magnification: 10X40. White bars represent 10 µm.

4.3. Discussion

The work described in this chapter revealed the dynamics regarding the surface abundance of several known *P. falciparum* invasion receptors on BEL-A cells during erythroid differentiation and confirmed this line appropriately expresses these host receptors. The aim was to identify a nucleated erythroid line that could be used for genetic editing as a model to study the function of host receptors required for *P. falciparum* merozoite invasion (Satchwell et al., 2019). The BEL-A cell line has been used previously to study the function of BSG in *P. falciparum* invasion (Satchwell et al., 2019) and this work has confirmed it as a useful model for this purpose. Additionally, this work has used the BEL-A cell line to disrupt expression of band 3 on the membrane surface. Band 3 is potentially an essential receptor for *P. falciparum* invasion (Baldwin et al., 2015; Goel et al., 2003) and these mutant lines provide the tools to test this hypothesis.

The BEL-A and the HUDEP-2 cells showed a similar pattern of expression for surface membrane proteins, as revealed by the extensive panel across the differentiation course, which was not done on the two cell lines by previous work. However, the ability of BEL-A cells to differentiate more quickly and homogeneously, together with the capability of producing nucleated cells, suggest the BEL-A line will be superior to the HUDEP-2 line as a host for *P. falciparum* invasion assays. This work is consistent with previous use of BEL-A cells for analysis of BSG and its role in *P. falciparum* invasion (Satchwell et al., 2019).

The generation of BEL-A lines in which band 3 expression was disrupted has provided the tools needed to dissect its potential role in *P. falciparum* merozoite invasion (Goel et al., 2003). Whilst lack of expression of band 3 on the BEL-A membrane surface has been suggested using a number of monoclonal antibodies, they all bind to the same extracellular loop. Consequently, it is important that this is further validated by western blots and IFA. It is likely that expression of the full band 3 protein has been blocked in both clone 1 and 2 has been fully disrupted because of the likelihood of nonsense mediated decay of mRNA that has been shown previously (Figure 4.6 to 4.8). It is interesting that altered band 3 expression has not affected the level of expression of other proteins in the major complexes including GYPA and GYPC (reviewed in (Anstee, 2010; L. Bruce, 2006)). It will be of interest to analyse the surface membrane localisation of these and other proteins that normally reside in the band 3 complex to determine the effect of loss of band 3 to the organisation of the complex.

Regarding the band 3-GYPA interaction, some clues are provided by previous literature. Using transfected K562 cells, evidence was provided that both band 3 and GYPA had the capacity of independently trafficking to membrane (Pang & Reithmeier, 2009), whilst the two proteins did interact in the ER (Pang & Reithmeier, 2009). Additionally, this work also investigated the influence of knocking down GYPA to band 3 expression on K562 cells, and no obvious reduction of band 3 was detected (Pang & Reithmeier, 2009). This finding was consistent of the finding that GYPA knock down CD34⁺ erythroid cells had normal level of band 3 (Bei, Brugnara, & Duraisingh, 2010). More recently, there has been a previous publication exploring the interaction between band 3

and GYPA using *in silico* methods (Kalli & Reithmeier, 2018). Their finding suggested that apart from the well-known ankyrin macro complex, band 3 could also associate with GYPA outside the complex (Kalli & Reithmeier, 2018). Collectively, band 3-GYPA interaction outside the ankyrin macro complex remains less explored, compared with the well-established knowledge of the Wr^b antigen (direct association of band 3 and GYPA). There is still uncertainty to fully dissect the whole dynamic process of band 3/GYPA trafficking to the membrane, their dependence on each other to fold, how these two proteins get trafficked and properly assembled into the complex, and so on.

Intriguingly, there do exist band 3 ^{-/-} mice models (Hassoun et al., 1998; Ji & Lodish, 2012; Peters et al., 1996). The animals had neonatal anemia and their RBCs exhibited spontaneous shedding of the membrane (Peters et al., 1996), whilst the organization of major cytoskeleton components and spectrin synthesis remained at a relatively normal level (Peters et al., 1996), raising the postulation that under normal circumstances band 3 is important to stabilize RBC membrane to avoid HS (Peters et al., 1996). As mentioned previously, loss of band 3 could lead to severe loss of glycoporphins, however, there exists discrepancy when analyzing RBCs from band 3 deficient mice. Complete loss of GYPA and band 4.2, but not components from the GYPC-4.1 R complex, was reported in 1998 (Hassoun et al., 1998), suggesting the chaperone-like role of band 3 for normal GYPA trafficking to RBC membrane (Hassoun et al., 1998). As contrast, another work revealed decreased TER 119 (a murine GYPA-associated protein) expression from either *in vivo* samples or *in vitro* cultured cells, instead of complete GYPA loss (Ji & Lodish, 2012). As presented by the work above, the GYPA and GYPC expression detected by FACS antibody HI264 remained comparable from clone 1, clone 2 and wild type BEL-A cells (Figure 4.12 & 4.14). Therefore, for future work, validating and quantifying GYPA expression on those clonal cells is of merit. If confirmed, our models would provide a new option for band 3- GYPA interaction study.

To summarize, BEL-A cells express parasite invasion ligands, and band 3 gene disrupted lines have been generated. To our knowledge, this may be the first to disrupt expression of band 3 on human erythroid cells. These BEL-A band 3 disrupted lines will be valuable tools, to study both *P. falciparum* invasion (especially, to validate whether band 3 is a receptor for MSP1). More generally, the models will be useful to

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define the role for band 3 in erythropoiesis (e.g., the impact of losing band 3 to cellular health and enucleation) and erythroid function and metabolism, since band 3 is so versatile and crucial.

Chapter 5: Discussion and future plan

5.1 Summary of results

In this work, the HUDEP-2 cell line was initially used as a novel host cell model for differentiation and invasion by *P. falciparum*. This erythroid cell line is likely better than either JK-1 or CD34+ cells, which have been used previously (Egan et al., 2015; Kanjee et al., 2017) HUDEP-2 cells are genetically more normal than leukemia cell-derived JK-1 cells, and they can also be genetically edited, and it is possible to generate large amounts of erythroid progenitors *in vitro*, promising a stable source of cells. An extensive FACS antibody panel that detected three classical maturation markers (CD49d, band 3 and GYPA) (Hu et al., 2013) as well as a spectrum of invasion receptors (CD35, CD44, CD55 and CD147), used by *P. falciparum* for merozoite invasion (Crosnier et al., 2011; Egan et al., 2015; Kanjee et al., 2017; Tham et al., 2010), was established to profile the HUDEP-2 cells across their differentiation. Whilst these studies showed that the HUDEP-2 line was able to differentiate into more mature erythroid cells that expressed host receptors required for *P. falciparum*, however, the ability of the merozoite-stage parasite to invade these cells was not efficient. Consequently, it was decided to test the ability of other cell lines to provide a differentiation model for *P. falciparum* parasite invasion.

The BEL-A erythroid cell line was available (Trakarnsanga et al., 2017) and it had been used previously to analyse *P. falciparum* invasion by deletion of the CD147 (basigin) receptor (Satchwell et al., 2019). This work established a FACS antibody panel that enabled profiling of the differentiation of the BEL-A cell line. This showed that BEL-A cells could be induced to differentiate to cells that express host cell receptors required for *P. falciparum* invasion.

In order to enable genetic editing of the BEL-A cells a line that constitutively expressed Cas9 was developed. This was tested by constructing three clonal BEL-A cell lines in which the band3 gene had been disrupted. This has provided *in vitro* models to study the effect of band 3 deficiency with respect to erythropoiesis, erythroid function and ultimately, *P. falciparum* invasion.

5.2 Understanding the functional role of band 3

Three BEL-A cloned cell lines that were band 3 deficient, as defined by PCR analysis and FACS antibody detection were isolated. This has provided cell lines to analyse the function of band 3 in erythroid differentiation and function as well as its role as a host receptor for *P. falciparum* merozoite invasion. However, due to the emergence of the COVID-19 pandemic it was not possible to take the analyses of these cells further. Outlined below are the studies required to utilise the band 3 deficient cell lines, constructed in this work, to increase our understanding of the role of this important protein.

Band 3 is known to have two primary functions that include anion exchange and maintenance of protein-protein interactions in the erythroid lineage (reviewed in (Reithmeier et al., 2016)). This protein is a $\text{Cl}^-/\text{HCO}_3^-$ exchanger (Kopito & Lodish, 1985) and plays an important part in pH regulation of the cell (Kopito & Lodish, 1985). Band 3 also provides binding sites for haemoglobin, and glycolytic enzymes as well as skeletal proteins such as ankyrin, protein 4.1 and protein 4.2 (reviewed in (Anstee, 2010; L. Bruce, 2006)). Therefore, lack of band 3 may have an effect on cellular pH, glycolytic metabolism and the structural integrity of the cytoskeleton in the host cell and these are all features that should be analysed to determine the role of this protein in these functions.

Lack of band 3 function is likely to have an effect on the morphology and elasticity of the erythroid cell as it differentiates. To analyse the morphology of these band 3-deficient erythroid progenitors, the IncuCyte platform can be used for real time imaging,

as it was proven to be a powerful tool for capturing cell dynamics like macrophage phagocytosis (Kapellos et al., 2016). This would enable the recording of the cells across their differentiation, especially during the tertiary differentiation stage when enucleation occurs. Also, it would be possible to determine the cellular localisation of specific proteins such as ankyrin, protein 4.1, spectrin and protein 4.2 to determine the effect on the integrity of the cytoskeleton. Additionally, changes in the deformability of the mutant cells caused by cytoskeletal changes can be determined by measuring the viscoelastic properties compared to wild type cells (Hochmuth & Waugh, 1987). This can be done using advanced analyses with atomic force microscopy (reviewed in (Shi, Cai, Zhou, & Wang, 2018)), optical tweezers (reviewed in (Nussenzveig, 2018)) and quantitative phase imaging (Popescu et al., 2005).

Band 3 acts as an important binding site for specific glycolytic enzymes (reviewed in (Reithmeier et al., 2016)), and this may have an effect on their function and regulation. The localisation of these glycolytic enzymes in band 3 deficient BEL-A cells could be analysed by immuno-fluorescence experiments to determine the differences in assembly of these important regulatory complexes. Additionally, it would be of interest to analyse the metabolome of the band 3 deficient BEL-A cells in order to understand the role of band 3 in glycolysis of these cells. Using cytosolic pH indicators, e.g., pHrodo Green AM Intracellular pH Indicator (ThermoFisher P35373), the intracellular pH status could be reflected and visualized in live cell imaging (Hwang, Kang, Kim, Shin, & Hong, 2019).

It will be of interest to determine if lack of band 3 function has an effect on cell amplification and enucleation rate across erythroid differentiation. As suggested by previous studies (Ji & Lodish, 2012), enucleation may happen earlier and at a more abundant level in the cultivation of the band 3 deficient clones (Ji & Lodish, 2012).

Additionally, live cell imaging experiments of the band 3 knockout BEL-A clones using dyes targeting different subcellular components can be utilised to identify differences. Cholesterol is an interesting candidate because it is related to the rigidity and deformability of the cells. RBCs are known to harbor high levels of cholesterol in the membrane (Conrard et al., 2018; Gregg & Reithmeier, 1983; Leonard et al., 2017), and clues hint that there exists interaction between band 3 and the surrounding lipids,

especially cholesterol (Gregg & Reithmeier, 1983; Mühlebach & Cherry, 1982; Schubert & Boss, 1982). Cholesterol/ phosphatidyl serine content could have a negative impact on band 3's anion transport activity (Schubert & Boss, 1982), which provides an interesting perspective for future work.

Apart from characterising the clones by themselves, recording the invasion attempts when the clones are used as hosts for parasites will be another aspect. As mentioned in the literature review, it is suggested that MSP1 interacts with the band 3-GYPA complex to allow invasion (Baldwin et al., 2015). Using different synthetic peptides, an interaction between PfMSP1 19kD-domain and band 3 was also hinted (Goel et al., 2003). Putatively the invasion into these clones will be altered at a specific point. Since the invasion process requires the concerted cooperation of multiple cellular components, use of a basic membrane dye, cytoskeleton dyes, dyes for indispensable invasion receptors like BSG, and in particular, the surface markers which interact with band 3 and play a role in invasion, namely GYPA (Groves & Tanner, 1992; Pang & Reithmeier, 2009) and GYPC (Marfatia et al., 1995). The co-localisation of dyes can hint at the relationship of corresponding proteins. With the help of microscopes like Zeiss Lattice and the developed algorithms, it will be possible to make high-resolution recordings of these events, track receptors at single molecule level and potentially construct a 3D-recapitulation of the whole process.

To understand the role of band 3 in the invasion of *P. falciparum* merozoites it will be possible to perform a number of studies using the band 3 deficient BEL-A cells that may identify new parasite ligands that bind to this potential host receptor. This would include testing of the ability of *P. falciparum* ligands to bind to the mutant BEL-A cells and detection using specific antibodies. Previously, supernatants from *P. falciparum* cultures have been generated that contain all parasite ligands have been used to test their ability to bind to receptors on mutant red cells, neuraminidase/trypsin/chymotrypsin-treated red cells and this has provided important information and identification of ligands and receptors (Gilberger et al., 2003). Using the band 3 deficient BEL-A cells provides an important tool to utilise this approach. Previously, MSP1 has been identified as a potential ligand for invasion (Baldwin et al., 2015; Goel et al., 2003) and either band 3 alone or the band 3-GYPA complex has been implicated as the receptor. It

will be possible to specifically test this hypothesis. Additionally, other parasite ligands such as EBA181 and other PfRh proteins may bind band 3. The band 3 deficient BEL-A cells will be an important tool to identify unknown ligand-receptor interactions.

Finally, the ability of *P. falciparum* to invade the band 3 deficient BEL-A cells will be an important question to be addressed. This can be done by live cell imaging using *P. falciparum* parasites that express fluorescent proteins such as green fluorescent protein (GFP) (Wilson, Crabb, & Beeson, 2010). For example, the D10 strain, which has steady and abundant expression of GFP signal can be utilised (Wilson, Crabb, & Beeson, 2010). Additionally, labelling of the host cells with fluorescent probes would be useful (Lukinavičius et al., 2014). This could include membrane binding dyes as well as actin probes (e.g., SPY555-actin) (Lukinavičius et al., 2014). As indicated by Figure S4 live cell imaging using a combination of SPY555-actin and SiR-DNA to stain expansion stage wild type and band 3 deficient BEL-A cells gave a promising resolution.

Once an understanding of the functional effects for lack of band 3 function on the BEL-A cells is obtained it will be important to complement the mutants with a functional band 3 gene. Complementation of the phenotypes observed will provide conclusive evidence of band 3 function in the BEL-A cells as well as its role in *P. falciparum* merozoite invasion.

To conclude, work presented above to generate clonal band 3 deficient BEL-A cells promises to provide interesting materials for both erythroid study and parasite invasion assays in the future.

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Supplementary data

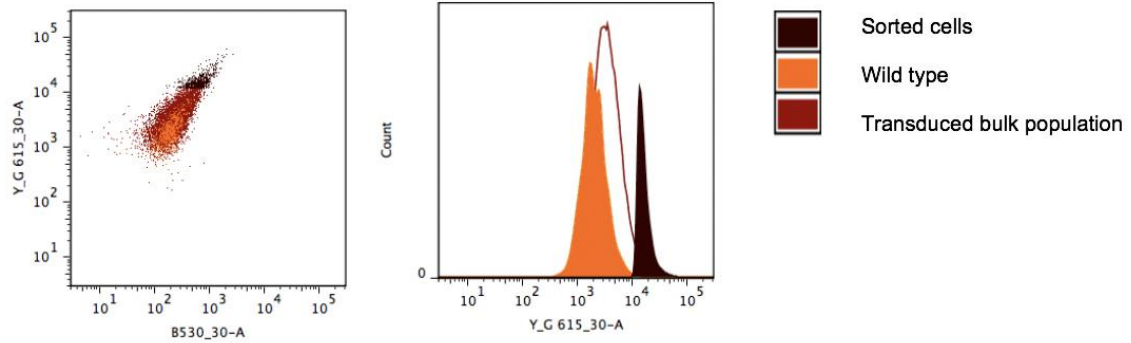


Figure S1. FACS sorting strategy for isolation of cells expressing brightest mCherry signal.

After transduction, cells carrying high mCherry signals were sorted as a bulk population, which constitutively expressed Cas9. Limiting dilution using bulk population of sorted cells was carried out to isolate monoclonal Cas9-expressing BEL-A cells. The brightest mCherry clone was picked after a FACS analysis.

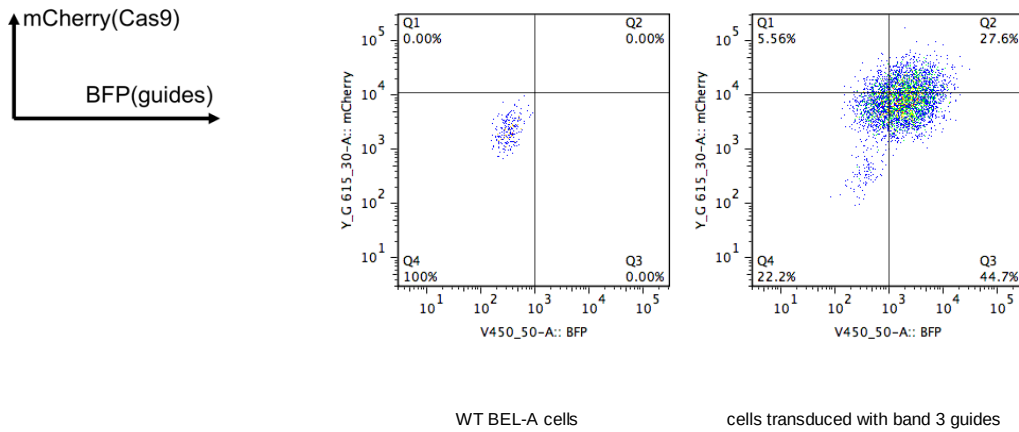


Figure S2. FACS analysis of BFP-mCherry pattern from double-transduced cells.

Approximately a quarter of total viable cells were BFP and mCherry double positive four days after transduction and were considered to carry both Cas9 and the corresponding guides, using wild type BEL-A cells as background.

Supplementary data

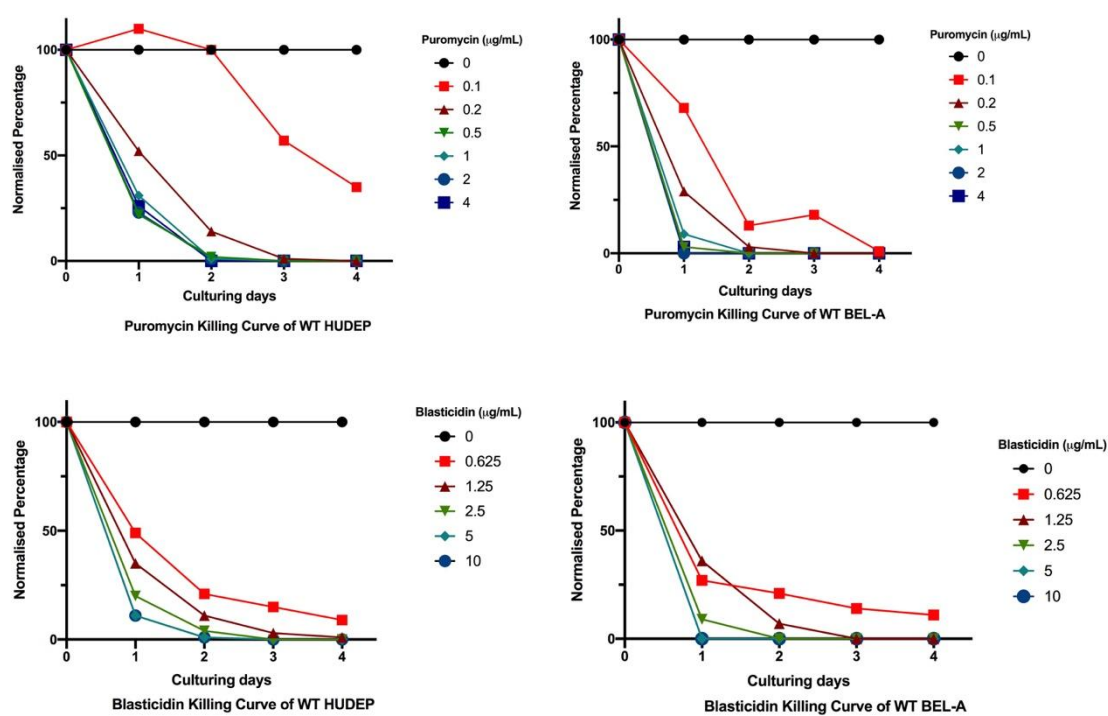
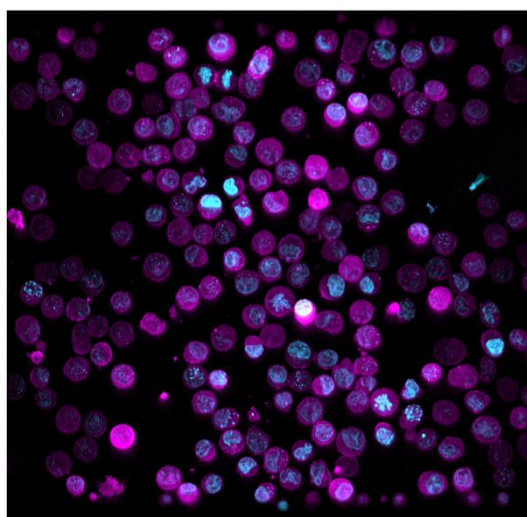
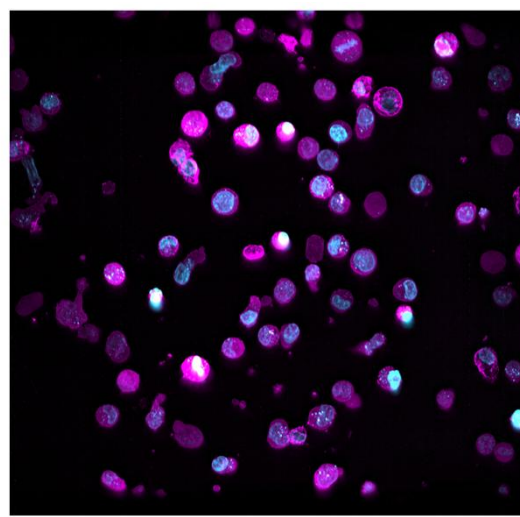


Figure S3. Killing curves of puromycin and blasticidin

Results averaged from duplicates. The gradients were obtained by serial dilution.



WT expansion cells



Clone 1 expansion cells

Figure S4. Pilot live cell imaging stained with SPY555-actin and SiR-DNA