THE ROLE OF ROSETTE FORMATION IN PATHOGENESIS OF VIVAX MALARIA

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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KUALA LUMPUR

2014

ABSTRACT

Rosette formation is one of the unique biological phenomena that have been linked to pathobiology of malaria. It is believe to be associated with the severe outcomes of falciparum malaria. Most of the knowledge about rosetting is obtained from in-depth studies conducted on *Plasmodium falciparum*. However, the rosetting phenomenon and pathobiology of vivax malaria is not well studied. This research project aimed at deciphering the unknown aspects behind rosetting phenomenon of *P. vivax*, and investigating the role of rosette formation in pathobiology of vivax malaria. In total, 121 fresh *P. vivax* isolates, 48 cryopreserved *P. vivax* isolates, 122 fresh *P. falciparum* isolates and 5 cryopreserved *P. falciparum* isolates were recruited into this research project. A novel technique suitable for reticulocyte characterization and rosetting assay in field setting was developed from this research project. Based on the field studies conducted, rosette formation is common in *P. vivax*. However, rosetting is not significantly correlated to clinical parameters such as reticulocyte count and parasitaemia. Besides, cryopreservation and thawing processes affect the rosetting capability of *P. vivax* isolates. Rosette formation was found to be initiated at the early trophozoite stage of *P. vivax* and the rosetting development reached plateau at the end of the erythrocytic maturation. Giant rosettes were found more frequently in *P. vivax* than *P. falciparum*. In addition, gametocytes were found to be involved in rosette formation. Unlike *P. falciparum*, the rosetting phenomenon of *P. vivax* is independent of human ABO blood groups and complement receptor 1 (CR1/CD35). However, rosetting phenomena of *P. vivax* and *P. falciparum* are dependent on the BRIC4 region of human glycophorin C (CD236R), strongly indicating the BRIC4 region of CD236R as another rosetting coreceptor for *P. vivax* and *P. falciparum*. On elucidating the roles of rosette formation in pathobiology of malaria, the significantly high preference for normocytes instead of reticulocytes in rosette formation clearly shows that rosetting is

unlikely to assist merozoite reinvasion in vivax malaria. Furthermore, increased rosetting rates upon exposure to anti-malaria drug compounds and human white blood cells suggest that the rosetting phenomenon may serve as an intrinsic protective mechanism of the malaria parasites against their environmental threats.

ABSTRAK

Pembentukan rosette merupakan salah satu fenomena biologikal yang telah dikaitkan dengan patobiologi malaria. Fenomena ini dipercayai berkaitan dengan keparahan penyakit malaria. Kebanyakan ilmu pengetahuan tentang pembentukan rosette telah diperoleh dari kajian-kajian yang mendalam terhadap *Plasmodium falciparum*. Namun demikian, fenomena pembentukan rosette and patobiologi vivax malaria kurang dikaji and diketahui. Project ini berobjektif untuk menyiasat soal-soal tentang pembentukan rosette oleh *P. vivax*, dan peranan yang dimain oleh rosette dalam patobiologi vivax malaria. Secare keseluruhannya, 121 sampel segar *P. vivax*, 48 sampel pepbekuan awet *P. vivax*, 122 sampel segar *P. falciparum* dan 5 sampel pembekuan awet *P. falciparum* telah digunakan dalam projek kajian ini. Sebuah teknik baru untuk pengiraan korpuskal merah muda (reticulocyte) dan eksperimen rosette yang sesuai digunakan dalam kerja lapangan telah dihasilkan menerusi projek ini. Berdasarkan projek ini, fenomena pembentukan rosette adalah biasa di kalangan *P. vivax*. Namun begitu, pembentukan rosette tidak berkaitan langsung dengan parameter klinikal seperti kiraan reticulocyte and parasitemia. Selain itu, proses pembekuan awet and pemanasan sel menjejaskan keupayaan *P. vivax* untuk membentuk rosette. Kejadian pembentukan rosette bermula pada tahap trophozoite awal dan berkembang sehingga mencecah tahap tepu pada tahap akhir proses permatangan dalam darah. Rosette gergasi telah ditemui dengan lebih banyak di *P. vivax* berbanding dengan *P. falciparum*. Selain itu, gametocyte juga terlibat dengan proses pembentukan rosette ini. *P. vivax* didapati berbeza dengan *P. falciparum* di mana pembentukan rosettenya tidak bergantung pada kumpulan ABO darah manusia dan reseptor complement 1 (CR1/CD35). Pembentukan rosette *P. vivax* dan *P. falciparum* didapati bergantung pada bahagian BRIC4 glycophorin C manusia (CD236R). Dengan ini, bahagian CD236R dipercayai memainkan peranan penting sebagai satu lagi reseptor sampingan pembentukan rosette untuk *P. falciparum* dan *P.*

vivax. Bagi kajian mencari peranan rosette dalam malaria, adalah didapti bahawa korpuskal merah dewasa (normocytes) lebih dipilih untuk pembentukan rosette berbanding dengan reticulocyte. Dengan ini, jelaslah bahawa pembentukan rosette tidak membantu penjangkitan baru merozoite *P. vivax*. Lebih-lebih lagi, peningkatan tahap pembentukan rosette semasa pertembungan dengan ubat anti-malaria dan korpuskal putih manusia mencadangkan bahawa pembentukan rosette merupakan satu tindakan pertahanan diri oleh parasite daripada unsur-unsur merbahaya di keliling mereka.

ACKNOWLEDGEMENTS

After two and a half years of learning and studying, with so many fieldtrips and travelling, coupled with countless challenges, falls and obstacles, finally, this work is approaching its finishing line. This project would never be successfully accomplished without the help, guidance and support from various people, whom I ought to acknowledge and express my gratitude.

Firstly, the project would never be initiated without the effort, supervision, support and assistance provided by my supervisors, Assoc. Prof. Dr. Lau Yee Ling and Prof. Dr. Fong Mun Yik. Their motivating advises and encouragement had driven me to take on this project and endure the challenges. I am so grateful to have them supporting me, guiding me and helping me throughout this journey. Without them, this PhD project would be unbelievably difficult, if not impossible. Thank you for your help, support and trust. I would like to express my gratitude to my foreigner advisors, Prof. Dr. Laurent Rénia and Asst. Prof. Dr. Bruce Russell for their guidance and supervision during the course of this research project. Many thanks to them for offering such an interesting research topic and encouraging me to take on this particular research project that was deemed “weird” and “too challenging for a PhD student” by many parties at the very first place. Without their guidance, the work and goals in this project would never be accomplished and I would never have the opportunity to learn, see and experience so much in the field of medical sciences. Thanks for taking me under your wings, and many thanks to the knowledge, advises, experience and of course inspiring and downright funny stories that you guys shared with me, and thank you for offering me this wonderful opportunity to learn with you. Once again, to these four incredible scientists from different parts of the world: A very big thank you!

I am grateful to have the opportunity to visit and work with the staff in Shoklo Malaria Research Unit, Mae Sot, Thailand. I am grateful to Prof. Dr. Francois Nosten, the director of the institute, for his warm welcome whenever I visited the institute. Thank you for providing me the space and opportunity to conduct my experiments. And of course, to all the staff of SMRU, especially Madam Kanlaya Sriprawat (P'Poo), Mr. Chirapat Wansing (P'Neng), Miss Wiriya Simmawong (P'Sye), Dr. Cindy Chu, Dr. Rose McGready, Dr. Chiara Andolina, Dr. Germana Bancone, Dr. Stephane Proux, and Mr. Peter Christensen for their help and support throughout this research project. I am so happy to befriend and work with all of you. Thank you so much. I also owe a big thank you to Dr. Benoit Malleret, Dr. Marjorie Mauduit and Dr. Rossarin Suwanarusk for their help and professional advice during the course of this research project.

I would like to express my gratitude to Prof. Dr. Rohela Mahmud, the head of Department of Parasitology, Faculty of Medicine, University of Malaya for her continuous support for this project. Besides, I would like to take the opportunity to address my gratitude to the staff of Laurent Rénia Laboratory, Bruce Russell's Vivax Malaria Laboratory, and Department of Parasitology. Special thanks to the following people for their help and support during my stay in Singapore: Mr. Lee Giap Chin, Mr. Sum Jia Siang, Miss Claudia Anthony, Miss Sharrada Subramaniam, Dr. Ramapraba Appanna, Miss Thavamalar Balakrishnan, and Madam Teung Sue Hwa. Last but not least, to my beloved family: without you, I wouldn't be standing at where I am today. You are my inspiration to move further and higher every day. Besides, I would like to thank my religion for giving me strength and showing me directions whenever I feel lost or clueless. To whomever that reads this thesis: believe in yourself, and never get intimidated and beaten by failure, challenges, insults or even discrimination. You will never know how much you can overcome until you try. Cheers.

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LIST OF ABBREVIATIONS

Abbreviation	Indication
°C	Degree of Celcius
µl	Microlitre
µg	Microgram
ng	Nanogram
WBC	White blood cells
ex vivo	Experiment on living tissues outside the organism under artificial condition that mimics natural condition.
in vivo	Biological interactions or experiments that happen within a living organism.
in vitro	Experiment on extracted living tissues outside the living organism.
NMB	New methylene blue
Ab	Antibody
Fab	Fragment antigen-binding
CD	Cluster of differentiation
CR	Complement receptor
BRIC	Bristol Immunochemistry
MOI	Multiplicity of infection
SCF	Stem cell factor
shRNA	Small hairpin RNA
GFP	Green fluorescent protein
Epo	Erythropoietin
CF	Fibrous cellulose

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LIST OF PUBLICATIONS

Publications from this research project

Lee, W. C., Russell, B., Lau, Y. L., Fong, M. Y., Chu, C., Sriprawat, K., Suwanarusk, R., Nosten, F. & Rénia, L. (2013). Giemsa-stained wet mount based method for reticulocyte quantification: A viable alternative in resource limited or malaria endemic settings. *PLoS ONE,* 8, e60303.

Lee, W. C., Malleret, B., Lau, Y. L., Mauduit, M., McGready, R., Fong, M. Y., Cho, J. S., Suwanarusk, R., Zhang, R., Albrecht, L., Costa, F. T. M., Preiser, P., Rénia, L., Nosten, F. & Russell, B. (2014). Glycophorin C (CD236R) mediates vivax malaria parasite rosetting to normocytes. *Blood,* 123, e100-e109.

Other publications related to malaria research

Lee, W. C., Chin, P.W., Lau, Y.L., Chin, L.C., Fong, M.Y., Yap, C.J., Supramaniam, R. R. & Mahmud, R. (2013). Hyperparasitaemic human *Plasmodium knowlesi* infection with atypical morphology in peninsular Malaysia. *Malaria Journal,* 12: 88.

Lau, Y. L., Lee, W. C., Tan, L. H., Kamarulzaman, A., Syed Omar, S. F., Fong, M. Y., Cheong, F. W. & Mahmud, R. (2013). Acute respiratory distress syndrome and acute renal failure from *Plasmodium ovale* infection with fatal outcome. *Malaria Journal,* 12: 389.

Braima, K. A., Sum, J. S., Ghazali, A. M., Muslimin, M., Jeffery, J., Lee, W. C., Shaker, M. R., Elamin, A. M., Jamaiah, I., Lau, Y. L., Mahmud, R., Kamarulzaman, A., Sitam, F., Mohd-Noh, R. & Abdul-Aziz, N. M. (2013). Is there a risk of suburban transmission of malaria in Selangor, Malaysia? *PLoS ONE,* 8: e77924.

Sum, J. S., Lee, W.C., Amir, A., Braima, K. A., Jeffery, J., Abdul-Aziz, N. M., Fong, M. Y. Lau, Y. L. (2014). Phylogenetic study of six species of *Anopheles* mosquitoes in Peninsular Malaysia based on inter-transcribed spacer region 2 (ITS2) of ribosomal DNA. *Parasites & Vectors,* 7: 309.

INTRODUCTION

1.1 History of malaria

Malaria is one of the oldest infections that have been plaguing mankind for centuries. Records about malaria can be traced back to thousands of years ago in medical writings, such as the Yellow Emperor's Inner Classic (Huang Di Nei Jing) from ancient China (CDC, 2010a). Malaria is believed to be responsible for the deterioration, and hence the fall of the Roman Empire (Thompson, 2011). This infection was also one of the major life-threatening challenges encountered during the construction of the Panama Canal (Darling, 1910; Chamberlain, 1929). Malaria has been successfully eradicated from many parts of the world, especially the developed countries of the temperate region. Nevertheless, the loss brought by this disease, be it lives or socio-economical progress, is still catastrophic in many developing countries, especially those in Africa (Sicuri *et al*., 2013).

To date, six distinct *Plasmodium* species are responsible for human malaria transmission, namely *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi*, *P. ovale wallikeri*, and *P. ovale curtisi*. All these medically important malaria parasites are transmitted to humans by female *Anopheles* mosquitoes. In general, these parasites have similar life cycle (Figure 1.1), except for the development of the dormant liver stage called hypnozoites (Figure 1.2). Only *P. vivax* and *P. ovale* spp. can form this dormant stage within the human host, which is responsible for the relapses of malaria (Wahlgren & Perlmann, 1999; Garcia, 2001; Mueller *et al*., 2009 Nolder *et al*., 2013). Humans serve as the intermediate hosts of the parasites while the *Anopheles* mosquitoes serve as the definitive hosts. As *P. knowlesi* is a zoonotic parasite, long-tailed macaque monkeys and pig-tailed macaque monkeys are the natural intermediate hosts.

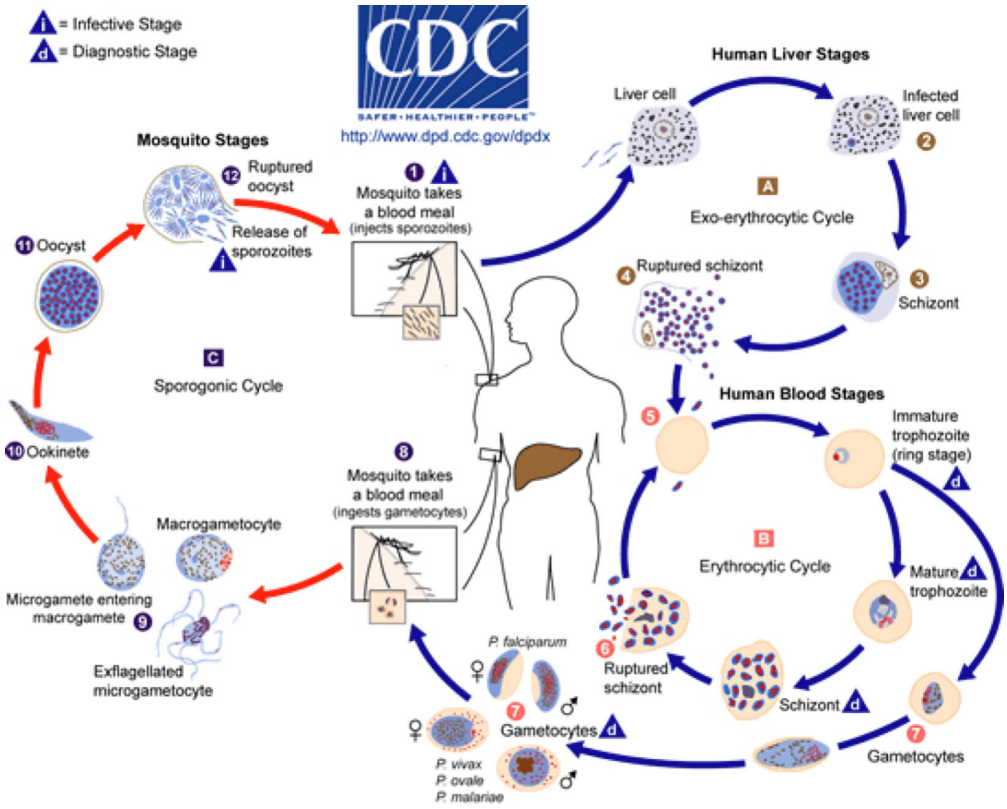

Figure 1.1 Life cycle of *Plasmodium* spp. (Diagram source: CDC, 2010b)

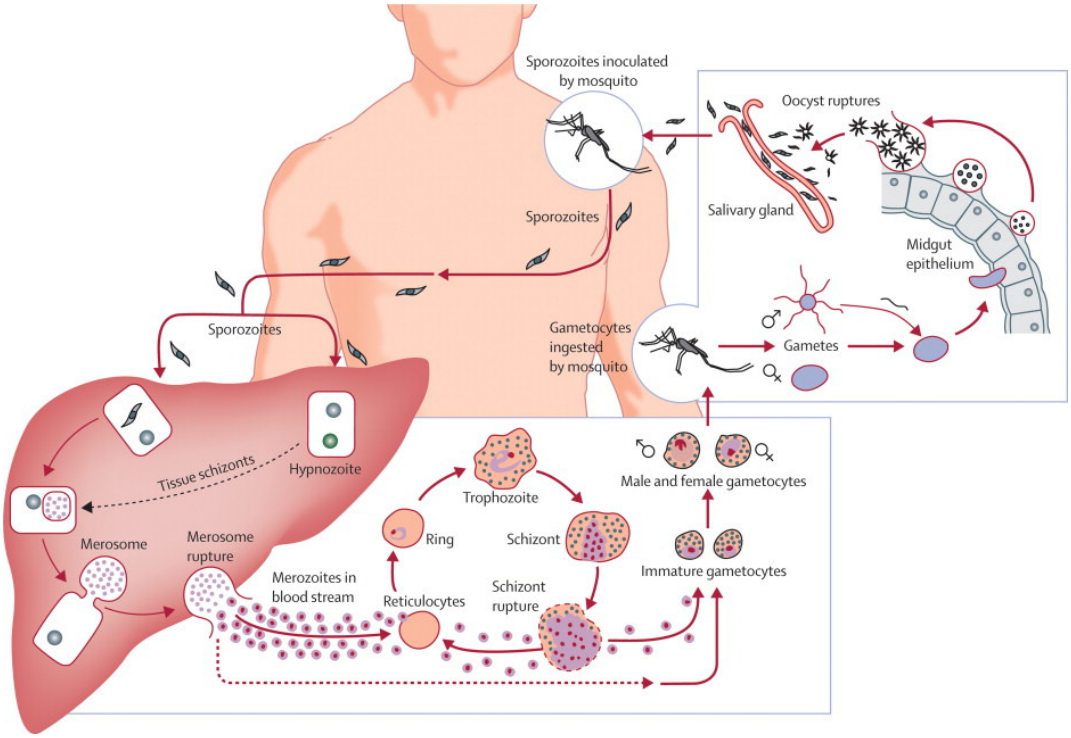

Figure 1.2 Life cycle of *P. vivax* and *P. ovale* spp., highlighting hypnozoite development in human liver. (Diagram source: Mueller *et al*., 2009)

1.2 Clinical complications of malaria

Individuals with malaria usually show signs and symptoms that include fever paroxysms, rigors, myalgia, arthralgia, sweating, loss of appetite, diarrhea, fatigue, hepato-splenomegaly, and coughing. Some of the clinical presentations of malaria are similar to many other tropical infections. Therefore, malaria may be misdiagnosed upon admission, especially in non-malaria endemic regions.

Malaria may lead to various forms of clinical complications. The most noteworthy complication is cerebral malaria. This form of severe malaria is caused almost exclusively by *P. falciparum* (Trampuz *et al*., 2003). Consquently, *P. falciparum* was wrongly perceived as the only malaria parasite that can cause potentially fatal "malignant malaria" (Galinski, 2008; Mueller *et al*., 2009). Severe complications, sometimes with fatal outcome, can be caused by other malaria parasites as well. Severe malaria can manifest in the form of acute respiratory distress syndrome (ARDS), acute kidney injury (AKI), splenic rupture, hypoglycaemia, placental malaria, hypotension and shock, thrombocytopaenia, hyperparastiaemia, as well as severe anaemia (Martell *et al*., 1979; Lee *et al*., 1999; Tanois *et al*., 2001; Prakash *et al*., 2003; Trampuz *et al*., 2003; Lomar *et al*., 2005; Anstey *et al*., 2007; Uneke *et al*., 2007; Cox-Singh *et al*., 2008; Das, 2008; Mohan *et al*., 2008; Rojo-Marcus *et al*., 2008; Thapa *et al*., 2009; Lacerda *et al*., 2011; Deroost *et al*., 2013; Hachimi *et al*., 2013; Lau *et al*., 2013; Lee *et al*., 2013a).

1.3 Rosetting phenomenon in malaria

Malaria causes substantial changes to the host's infected erythrocytes. Rosette formation is one of such well-known phenomena (Roberts & Janovy, 1999; Garcia, 2001). Rosetting in malaria is defined as a phenomenon where an erythrocyte infected

with *Plasmodium* spp. binds to at least two uninfected erythrocytes (David *et al.*, 1988) (Figure 1.3). This phenomenon is observed in various species such as *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* (David *et al.*, 1988; Udomsangpetch *et al*., 1995; Angus *et al*., 1996; Lowe *et al*., 1998; Rahman *et al*., 2010). Rosetting phenomenon, along with deep vein sequestration, places malaria as a disease unique from other infections that show similar clinical presentations.

Since its discovery in the late 1980s, rosette formation has been associated with clinical complications seen in falciparum malaria (David *et al*., 1988; Udomsangpetch *et al*., 1989; Hasler *et al.*, 1990; Udomsangpetch *et al*., 1992; Wahlgren *et al*., 1992; Breman, 2001; Sherman, 2005). Rosetting is believed to play important roles in the pathogenesis of malaria (Wahlgren *et al*., 1989; Sherman, 2005). This phenomenon is postulated to assist deep vein sequestration in inducing mechanical occlusion of vasculature. Besides, rosetting is believed to play roles in immuno-evasion mechanisms of malaria parasites. In addition, this biological event is postulated to enhance merozoites reinvasion upon the rupture of schizont.

Until now, these postulated roles have not been validated. Furthermore, the postulated roles of rosetting werre put forward based on the falciparum malaria model. Therefore, these roles may not be applicable to malaria caused by other species of malaria parasites.

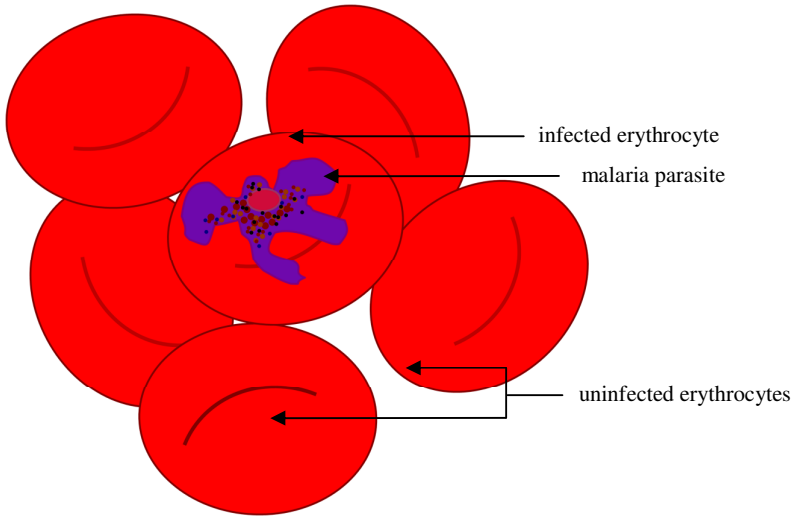

Figure 1.3 A schematic diagram showing a rosette formed by a *Plasmodium*-infected erythrocyte.

1.4 Rosetting phenomenon in vivax malaria

In contrast to the numerous observations of rosetting in *P. falciparum*, information on the basic properties of rosettes formed by *P. vivax*-infected erythrocytes is scarce. *P. vivax* has been overshadowed by *P. falciparum* for a very long time on the arena of malaria research, creating a huge knowledge gap between the two species (Galinski & Barnwell, 2008; Mueller, 2009). As a result, many properties of this second most prevalent malaria pathogen are predicted by extrapolation from findings in *P. falciparum*. However, different species of parasites may behave differently, making deductions of biological properties based on findings from another species difficult and sometimes, of limited significance.

A better understanding on the biological properties of a parasite contributes to the deciphering of puzzles behind the parasite's pathogenesis. Hence, there is a need to devote greater attention to rosetting properties of *P. vivax*. In addition, the postulated roles of rosetting must be tested to elucidate its actual role(s) played in malaria. A better characterization and comparison of this unique phenomenon in both *P. falciparum* and *P. vivax* will be beneficial for the understanding of the pathobiology of these parasites.

1.5 Objectives

Based on the goal of deciphering unknown aspects behind rosetting phenomenon of *P. vivax*, this research project carried four objectives as elaborated below.

1.5.1 Development of a better technique for haematological investigations

This research project aimed at developing an improved, cheaper and more convenient method for visualization and characterization of rosetting events. There is an

obvious need for an improved method to compensate the flaws or disadvantages posed by the current routine techniques. At the same time, this research project also aimed at developing an easier and more accurate alternative for reticulocyte profiling on malaria-infected samples, which can be used in parallel with the rosetting characterization.

1.5.2 Characterization of rosetting properties of *P. vivax*

The basic biological properties of rosette formation in *P. vivax* were investigated. These encompassed time course rosetting development of the parasite along its erythrocytic stage maturation, effect of cryopreservation on rosetting capability of the vivax malaria isolates, rosetting prevalence, and relationship of rosetting event with different clinical parameters of the vivax malaria isolates. Besides, this research project also aimed at identifying the receptors needed by *P. vivax* to form rosettes.

1.5.3 Comparison of rosetting properties between *P. vivax* and *P. falciparum*.

The data collected from the experiments conducted during this research project were also used to compare and contrast the rosetting properties of *P. vivax* and *P. falciparum*. The similarities and differences of rosetting events between the two species were highlighted. Such comparison would serve as a better reference for predicting and extrapolating rosetting properties of other medically important malaria parasites.

1.5.4 Investigation of the roles played by rosetting event in malaria

Another goal of this research project was to decipher the actual role(s) played by rosette formation in the course of *P. falciparum* and *P. vivax* infection. Instead of creating new hypotheses, the current postulated roles of rosetting were tested, in the hope of finding the actual role(s) that this biological phenomenon has in the pathogenesis of malaria.

LITERATURE REVIEW

2.1 *Plasmodium vivax*

Plasmodium vivax is the aetiological agent of the so-called "benign tertian malaria". *P. vivax* has the widest geographical distribution among the human malaria parasites (Roberts & Janovy, 1999; Garcia, 2001; Mendis *et al*., 2001; Mueller *et al*., 2009; MAP, 2012). It is also the most prevalent aetiological agent of malaria in temperate regions (Mendis *et al*., 2001) (Figures 2.1 & 2.2). Usually, *P. vivax* infections are accompanied with parasitaemias that are relatively lower than those of *P. falciparum* infection, due to the parasites' high preference to invade reticulocytes (Hegner, 1938; Kitchen, 1938; Russell *et al*., 2011). Besides, *P. vivax* is believed to show selective invasion of Duffy-positive reticulocytes (Miller *et al.*, 1976; Ranjan & Chitnis, 1999; Roberts & Janovy, 1999; Garcia, 2001). However, this species seems to have evolved and adapted to alternative invasion mechanisms. Recently, Duffy-negative patients in Africa and South America with mono-infection of *P. vivax* were reported (Cavasini *et al*., 2007; Ménard *et al*., 2010, Mendes *et al*., 2011).

Until recently, *P. vivax* was presumed by many as non-fatal or "benign" (Price *et al*., 2007; Galinski & Barnwell, 2008; Mueller, 2009; Gething *et al*., 2010). However, severe vivax malaria complications in the forms of ARDS, severe anaemia, AKI, and placental dysfunction have been documented (Yale *et al*., 1993; Islam *et al*., 1995; Torres *et al*., 1997; Pukrittayakamee *et al*., 1998; Curlin *et al*., 1999; Lomar *et al*., 2005; Anstey *et al*., 2009; Anstey *et al*., 2012; Marín-Menéndez *et al*., 2013; Patel *et al*., 2013). Besides, deaths from vivax malaria have been reported from time to time (Jayavanth & Bock, 2007; Anstey *et al*., 2009; Patel *et al*., 2013). Furthermore, there are many unreported cases in malaria endemic regions. Therefore, one can confidently state

that the mortality and fatality of *P. vivax* infection has been overlooked and underestimated.

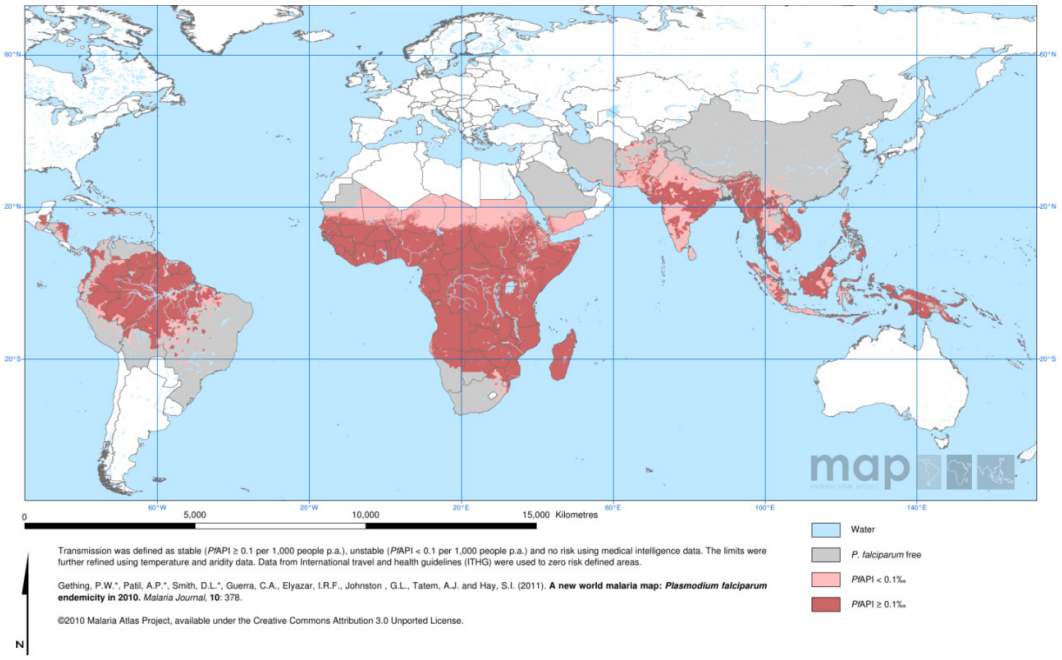

Figure 2.1 The spatial limit of global *P. falciparum* transmission in 2010 (diagram source: MAP, 2012).

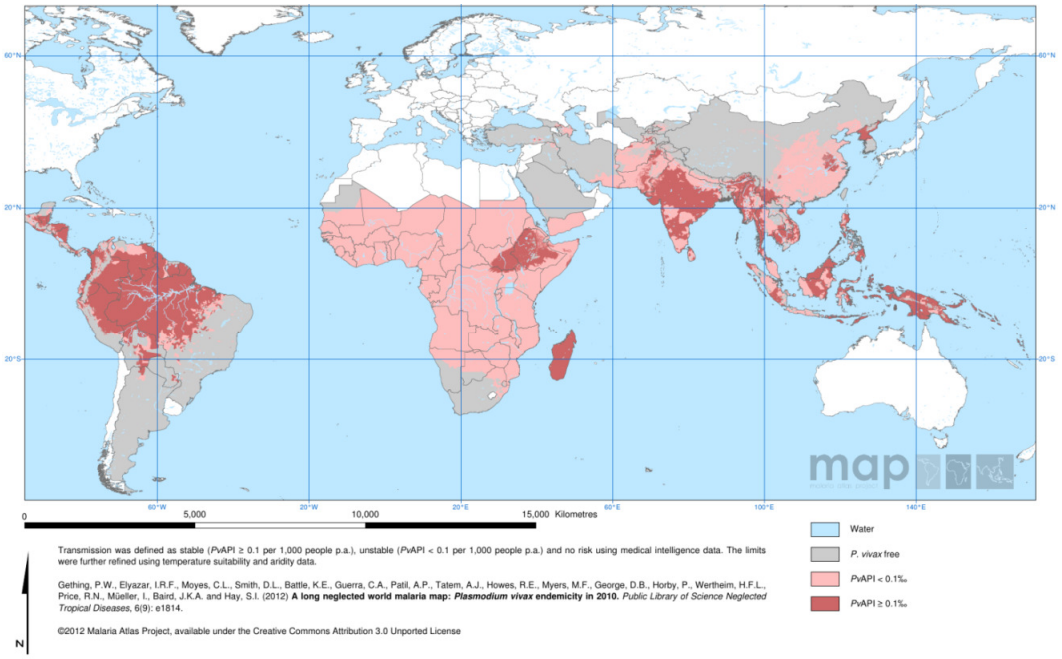

Figure 2.2 The spatial limit of global *P. vivax* transmission in 2010 (diagram source: MAP, 2012).

Through the years, malaria was perceived by many as "a single infection". Regardless of the aetiological agents, the parasite's cellular biology, clinical spectrum, host immunological responses, and the epidemiology of malaria were believed to be the same. It was then assumed that by focusing research on *P. falciparum* and extrapollating the knowledge to other species of human malaria parasites, would help malaria control; with successful malaria treatment and control strategies leading to eradication. However, it it now clear that such assumptions were erroneous (Bockaire *et al*., 2006; Andrade *et al*., 2010; Baird, 2010; Gething *et al*., 2010).

A good portrayal of the significant differences between *P. falciparum* and *P. vivax* is seen in the effort to eradicate malaria. There was a dogma that a single plan of malaria eradication would be suitable for all species of malaria parasites (Baird, 2010). Most of the malaria control and eradication programs are crafted based on knowledge gained from studies on *P. falciparum* alone. Following the implementation of such programs, the number of falciparum malaria cases decreased drastically. However, vivax malaria cases in these areas have been noted to oscillate at a relatively stable level throughout the implementation of malaria control program (Gething *et al*., 2010). Such a trend has been noted in Myanmar, Cambodia, Laos, Bangladesh, Nepal, Vietnam and Malaysia (WHO, 2012) (Figure 2.3). Obviously, the anti-malaria strategy for *P. falciparum* does not fit the war against vivax malaria. Hence, it would be more appropriate to re-define "malaria" as "a collection of pathological changes caused by different parasites" (Coggeshall, 1952; Baird, 2010).

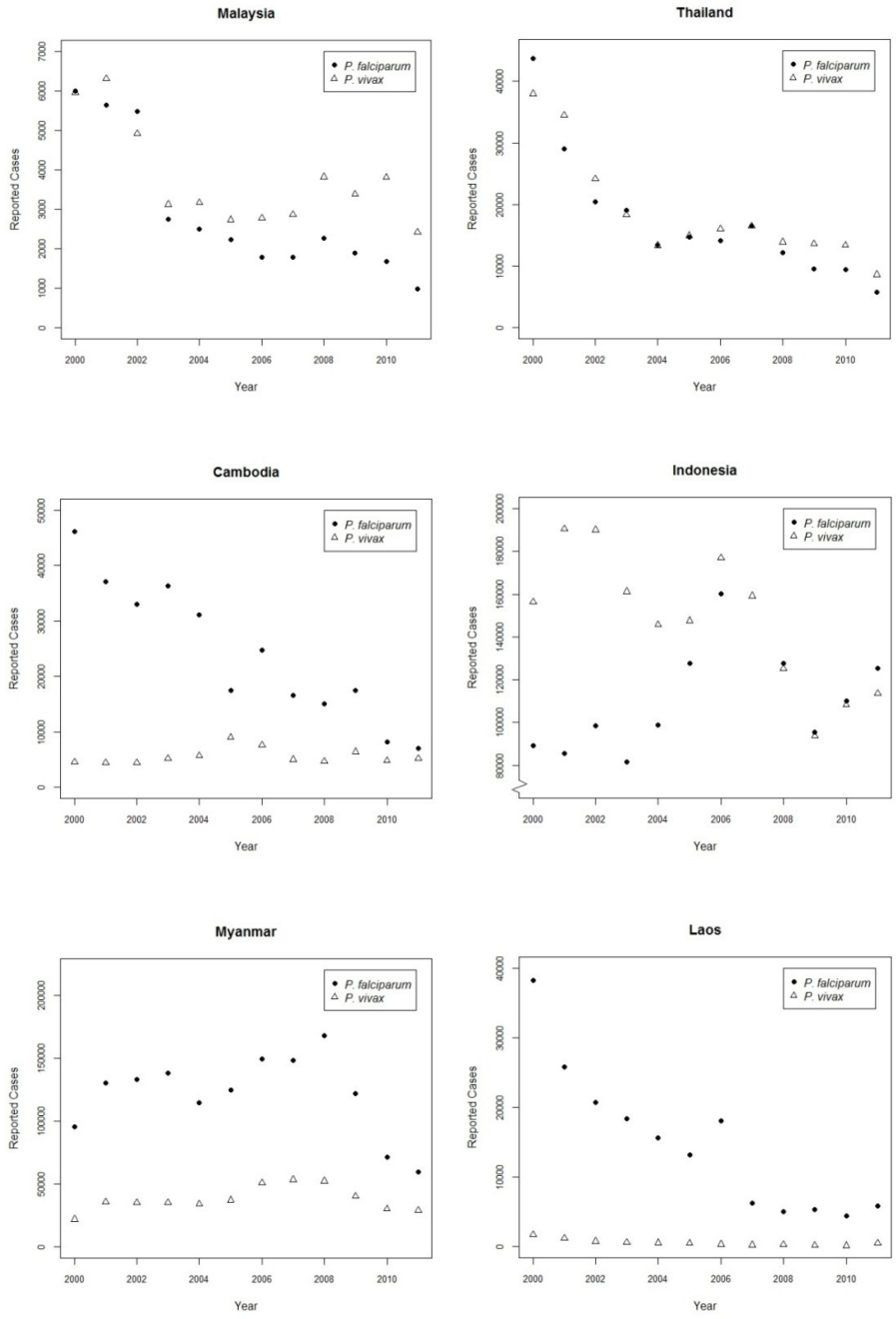

Figure 2.3 Plots showing annual report of malaria cases from selected countries of Southeast Asia and South Asia region since year 2000 (crude data sourced from World Malaria Report 2012).

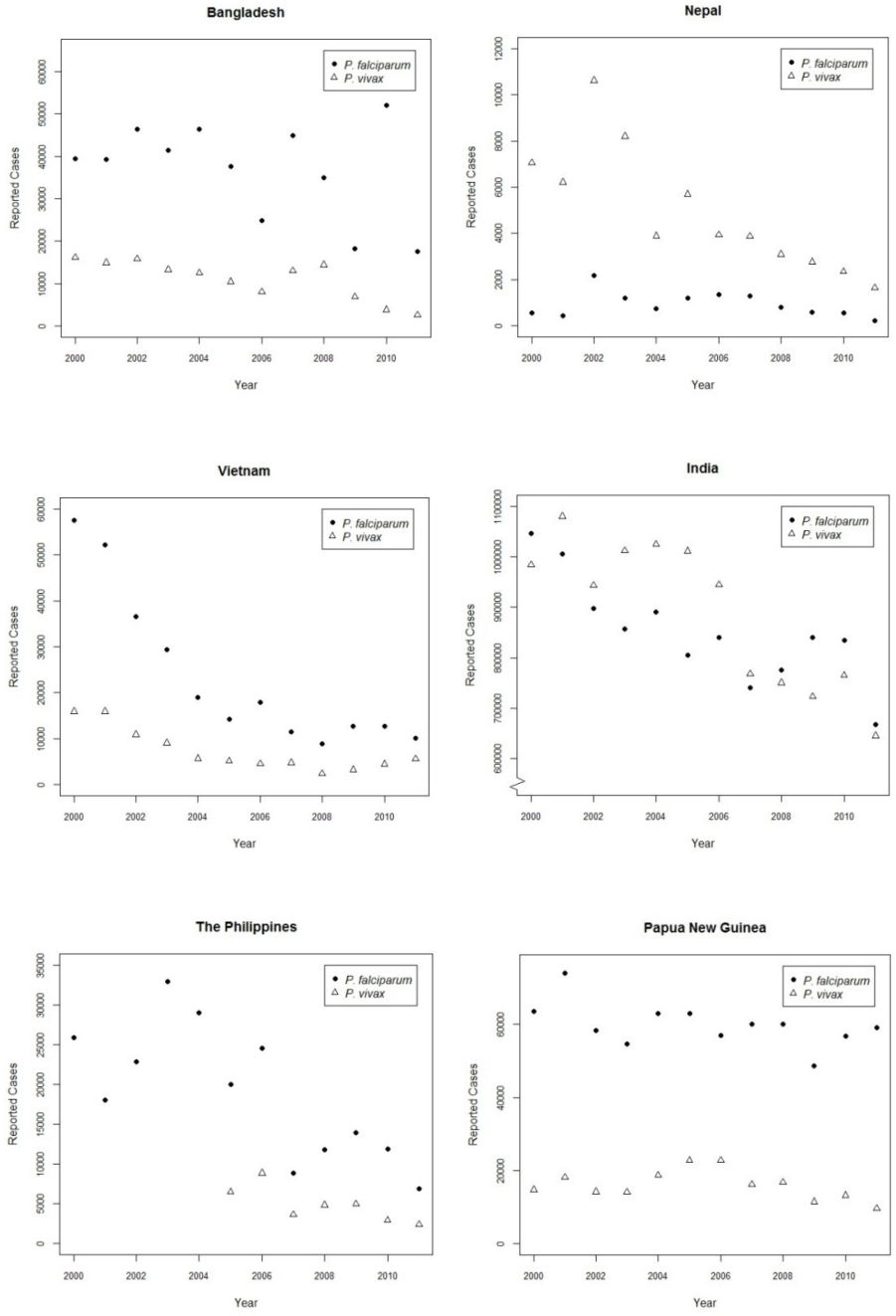

Figure 2.3, continued Plots showing annual report of malaria cases from selected countries of Southeast Asia and South Asia region since year 2000 (crude data sourced from World Malaria Report 2012). Twelve countries were selected for analysis based on their relative proximity to Malaysia and relative socioeconomic interaction with Malaysia.

It is understandable that *P. falciparum* was given more attention and research priority decades ago due to the higher fatality it causes. Owing to the hard work and efforts invested, the transmission of falciparum malaria has been successfully controlled and eradicated from many parts of the world. Since *P. vivax* has been shown to be a parasite that behaves differently from *P. falciparum*, coupled with its wide geographical distribution and prevalence, *P. vivax* should no longer be under-appreciated. More in-depth knowledge about the parasite is required to devise and carry out efficient plans for the eradication of vivax malaria, as well as malaria caused by other *Plasmodium* species. Therefore, more attention should be allocated to cellular biological studies of malaria parasites other than *P. falciparum*, though many puzzling biological aspects of *P. falciparum* remain unsolved after being studied intensively for so many years (Dondorp *et al*., 2000). Needless to say, such situation is even worse for *P. vivax*, especially with respect to certain specific biological properties such as the rosetting phenomenon.

2.2 Rosetting phenomenon and malaria severity

The rosetting phenomenon has been suggested as a marker of severe malaria development (Carlson *et al*., 1990a, Ringwald *et al*., 1993; Newbold *et al*., 1997; Rowe *et al*., 2007). A few studies were conducted to determine relationship between rosette formation of *P. falciparum* and cerebral malaria. However, these studies gave conflicting results and conclusions (Carlson *et al*., 1990a; Treutiger et al., 1992; al-Yaman *et al*., 1995; Rogerson *et al.*, 1996; Sherman, 2005), suggesting that rosetting is not the sole key factor responsible for severe symptom presentation in falciparum malaria. Nevertheless, the different experimental techniques and settings applied in these studies may lead to variations in the findings. More importantly, the correlations in these investigations were derived using cross sectional study design, which provides data and information in a "snapshot" mode. Such a model is good for deducing

correlation between a well-defined parameter and a well-defined disease outcome. However, it is difficult to set a non-subjective, "clear-cut" standard in defining disease severity. In addition, the results obtained from such a study design may not reveal the actual time-course development of an infection's pathogenesis, such as severe outcomes of the infection. Besides, any correlation found from such a study may not reflect causal relationship. These considerations may account for the differences of findings among these studies.

2.3 Roles of rosette formation in vasculature occlusion of falciparum malaria

As mentioned previously, the binding of parasitized erythrocytes to vasculature endothelial cells (sequestration) and the non-infected erythrocytes (rosette formation) have long been associated with severe outcomes of malaria (David *et al*., 1988; Udomsangpetch *et al*., 1992). Both these events are believed to be responsible for the deep vasculature occlusion seen in falciparum malaria (Chen *et al*., 2000; Adams *et al*., 2014) (Figure 2.4). Vasculature occlusion can be of mechanical and immunological origin. Sequestration and rosetting are believed to be responsible for the mechanical occlusion of deep vasculature.

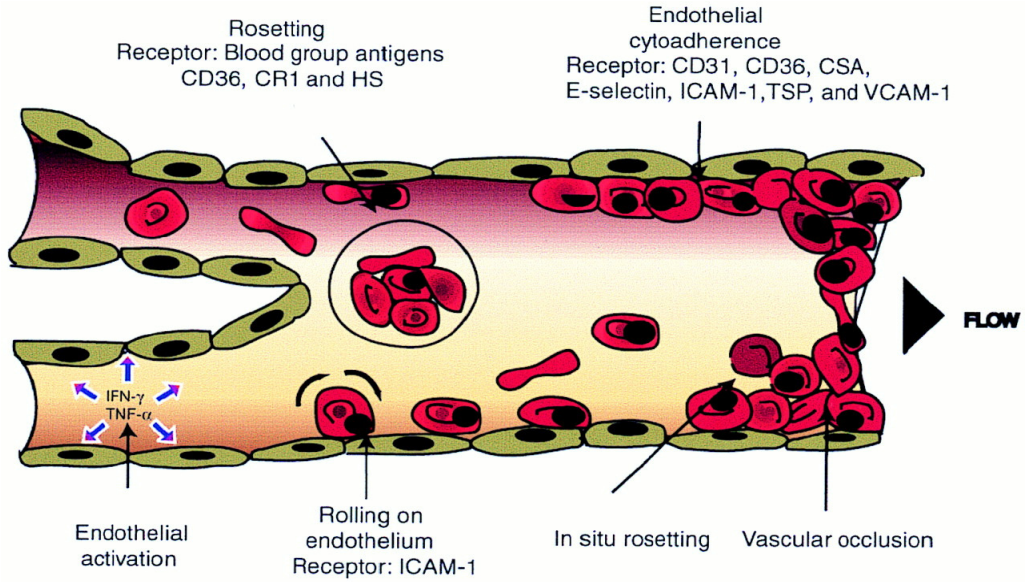

Figure 2.4 A schematic diagram showing the dynamics of cytoadhesion properties of *P. falciparum*, which include rosette formation and sequestration. (Diagram source: Chen *et al*., 2000).

Sequestration of infected erythrocytes decreases the lumen diameter of the affected vasculature, causing interruption of blood flow. Still, the blood flow may not be completely blocked. However, with rosette formation, the effect of blood flow disruption and blockade may be exaggerated. Rosettes can withstand a certain degree of shear force (Nash *et al*., 1992), resulting in the disruption of the erythrocyte aggregates in the artery, but not in the veins, venules or capillaries that have much lower flow rates and shear forces. Hence, the presence of rosettes in a vasculature that is already narrowed by layers of sequestered parasitized erythrocytes will further impede the circulation; subsequently increasing the risk of vasculature occlusion. In addition, the blockade of blood flow in small capillaries is further augmented by the decreased membrane deformability of the patient's infected erythrocytes, as well as the non-infected erythrocytes (Cranston *et al*., 1984; Nash *et al*., 1989; Dondorp *et al*., 1997; Paulitschke & Nash, 1993). Erythrocytes with decreased deformability will not be able to move through narrowed capillaries as easily as the erythrocytes with normal deformability. In short, rosetting and sequestration phenomena are believed to work synergistically in causing vasculature occlusion in falciparum malaria patients.

Among the severe complications that are linked to vasculature occlusion, cerebral malaria is definitely one of the most important. When the mechanical blockade occurs in the small capillaries of brain, it results in highly fatal cerebral malaria (Planche *et al*., 2005). Due to vasculature blockade, oxygen supply to the affected brain areas is compromised, causing immediate necrosis of the oxygen-sensitive brain cells. Apart from cerebral malaria, placental malaria and alveolar injury are other complications related to sequestration and rosetting. These events result in hampered utero-placental circulation and pulmonary function, respectively (Vásquez & Tobón, 2012; de Moraes *et al*., 2013; Rashidi & Roullet, 2013). Different strains of *P.*

falciparum show variable tendency of developing different complications. It has been shown that the strains of *P. falciparum* isolated from pregnant women and those isolated from children show different cytoadhesion affinities to different receptors (Beeson *et al*., 1999). Clearly, different strains of *P. falciparum* vary in their preferred site of sequestration (Dondorp *et al*., 2000).

2.4 Roles of rosette formation in immuno-evasion of *P. falciparum*

Rosette formation in malaria is also hypothesized to play a role in immuno-evasion of the malaria parasites (Sherman, 2005). Various parasite neo-antigens are expressed on the surface of infected erythrocytes, making these infected erythrocytes targets for antigen presentation and recognition. Rosettes may act as a "shield of host's antigens", protecting the parasite's antigens on the infected erythrocytes from being recognized by the antigen presenting cells. Theoretically, rosette formation may hinder phagocytosis as well. Such protective mechanism will assist malaria parasites to survive, develop and replicate within the host. There is no supportive evidence for this particular hypothesis up till now. Nevertheless, in Parasitology, masking host's immuno-recognition with host's own antigens has already been demonstrated, exemplified by *Schistosoma mansoni* that acquires human blood group antigens and human low density lipoprotein (LDL) as camouflage (Goldring *et al*., 1976; Chiang & Caulfield, 1989).

It is interesting to note that apart from contributing to vasculature occlusion and deep vein sequestration, the rosetting-related phenomenon is also viewed as an immuno-evasion strategy for *P. falciparum* to avoid splenic clearance of the human hosts (Miller, 1969). Sequestration of *P. falciparum* is mediated by the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) (Baruch *et al*., 1995; Su *et al*., 1995; Adams *et al*., 2014), which is the key player in rosette formation as well. Various membranous

changes happen on the infected erythrocyte with the addition of parasite's antigens onto the host erythrocyte membrane. Such antigens of parasitic origin include the ring-infected erythrocyte surface antigen (RESA) and the *Pf*EMP1 (Chotivanich *et al*., 2002). Hence, the erythrocytes infected with *P. falciparum* will show significant decrease in deformability as the parasites develop into mature stages (Cranston *et al*., 1984; Nash *et al*., 1989; Paulitschke *et al*., 1993). Consequently, these relatively rigid erythrocytes will not be able to pass through the spleen's cords of Billroth during splenic filtration, and will be retained from re-entering circulation and subsequently destroyed by the effector cells (Klausner *et al*., 1975). By staying within the deep vasculature, owing to the parasite's sequestration and rosetting ability, erythrocytes with mature parasites will not be available in peripheral circulation, thus escaping splenic clearance.

2.5 Roles of rosette formation in enhancing *P. falciparum* merozoite reinvasion

In addition, rosetting is thought to enhance merozoite reinvasion upon rupture of schizont. Based on this hypothesis, the participating uninfected erythrocytes in rosetting complex provide convenience to the released merozoites by offering new uninfected erythrocytes that are ready for invasion. Merozoites released from the ruptured schizont will be able to find targeting host erythrocyte easily without the danger of moving for a long distance, thus reducing the risk of being phagocytosed by the host's phagocytes. As a result, the probability of successful merozoite reinvasion will be much higher compared to condition without rosette formation. This hypothesis is supported by finding that showed positive correlation between rosetting rate and parasitaemia of falciparum malaria isolates (Rowe *et al*., 2002). Nevertheless, there are findings against this hypothesis as well (Clough *et al*., 1998; Deans & Rowe, 2006).

2.6 Roles of rosette formation in causing malaria-related anaemia

Interestingly, rosette formation has been proposed to drive massive destruction of non-parasitized erythrocytes, resulting in severe anaemia. A recent study had demonstrated transfer of haemozoin-generated lipid peroxidation end products from *P. falciparum*-infected erythrocytes to uninfected erythrocytes via rosette formation (Uyoga *et al*., 2012), which results in these modified uninfected erythrocytes being phagocytised and destroyed (Gallo *et al*., 2012; Uyoga *et al*., 2012). These haemozoin-derived products have been shown to reduce an erythrocyte's membrane deformability, which increases its risk of being cleared off circulation (Shorokhod *et al*., 2007).

It is crucial once again to highlight the fact that these postulated roles for rosetting in falciparum malaria remain as hypotheses, and they are neither convincingly validated nor confidently ruled out by research studies thus far. Therefore, there is a possibility that rosetting event does not play any significant role in pathogenesis of malaria. Instead, it is just an intrinsic biological ability of the parasites that poses no significant advantage or disadvantage to their survival. Thus, it is important to conduct in-depth investigations to find out the significance of rosetting event in pathogenesis of malaria.

2.7 A role played by rosetting in vivax malaria?

In general, the roles of rosetting in vivax malaria are assumed by many to be the same as in falciparum malaria. As described previously, the major postulated role of rosetting in falciparum malaria is assisting development of vasculature occlusion. However, *P. vivax* shows rosette formation but does not cause cerebral malaria or other forms of severe malaria as often as *P. falciparum* (Anstey *et al*., 2009). Besides, *P. vivax* does not show significant deep vascular sequestration in the host. Nevertheless, *P.*

vivax has been proven to preferably accumulate in certain organs such as the lungs (Anstey *et al*., 2007; Jayavanth & Bock, 2007; Anstey *et al*., 2009). In addition, cytoadhesion of *P. vivax* happens, albeit less extensively than *P. falciparum* (Carvalho *et al*., 2010). Even so, enhancing vasculature occlusion may not be the major role of vivax malaria rosetting phenomenon.

In vivax malaria, rosette formation may impede phagocytosis, which serves as an immuno-evasion mechanism of the parasite. Meanwhile, severe anaemia, splenic haematoma and splenic rupture are some of the complications reported in vivax malaria (Anstey *et al*., 2009). As *P. vivax* shows lesser degree of sequestration, rosetting alone may lead to erythrocyte pooling in spleen, which ultimately leads to mass destruction of uninfected erythrocytes by spleen filtration, resulting in severe anaemia. It has been demonstrated that both the infected and uninfected erythrocytes of vivax malaria patients show increased fragility, along with the increased deformability experienced by the infected erythrocytes (Handayani *et al*., 2009). The aggregates (rosettes) of such "fragile" erythrocytes under high shear force during splenic filtration may increase the probability of haemolysis. Anaemia will trigger erythropoietic machinery of the host, which results in increased rate of reticulocyte (non-matured erythrocytes) release into the bloodstream to support the haematological function of the host. This may be one of the survival strategies of *P. vivax* since it invades preferably, if not exclusively into the reticulocytes (Hegner, 1938; Kitchen, 1938; Russell *et al*., 2011). Nevertheless, dynamics of splenic function and vivax malaria has yet to be characterized, as with the rosetting properties of *P. vivax*.

Similar to *P. falciparum*, rosetting is believed to assist merozoite reinvasion of *P. vivax*. Upon rupture of schizont, the merozoites have to search for suitable host

erythrocytes to invade and ensure survival. Rosette formation may provide convenience for these merozoites in the search for new host erythrocytes. Nevertheless, for this hypothesis to be applicable in vivax malaria setting, the participating uninfected erythrocytes in rosetting complex should predominantly consist of reticulocytes, the exclusive invasion target of *P. vivax*. Similar to postulated roles of *P. falciparum* rosetting, more in-depth studies are needed to validate these hypothetical roles of rosetting in *P. vivax*.

2.8 Mechanisms of rosette formation in *P. falciparum* model: the receptors

The mechanisms behind the formation of rosettes in malaria are yet to be well-characterized. Nevertheless, many scientists support the hypothesis that *Plasmodium* spp. causes expression of certain proteins on the surface of the infected erythrocytes. These protein ligands (also known as "rosettins") are complementary to the receptor proteins expressed on the uninfected erythrocytes (Sherman, 2005). As a result, the uninfected erythrocytes bind to the infected erythrocyte, forming rosettes. The concept is just like the classical "lock and key" hypothesis.

Rosetting is not observed in all malaria-infected blood samples. Based on this early observation, scientists started looking for trends in rosetting occurrence, and a few interesting correlations have been discovered. There are studies showing that blood group A, AB and B yield higher prevalence of rosetting than blood group O (Carlson & Wahlgren, 1992; Udomsangpetch *et al*., 1993; Rowe *et al.*, 1995; Rowe *et al*., 2007). The A and B trisaccharide antigens have been postulated to be the rosetting receptors on the uninfected erythrocytes (Rowe *et al*., 2007; Vigan-Womas *et al*., 2012; Rowe, 2013). Nevertheless, the relative tendency of rosette formation between blood group A, AB and B is not well characterized and information obtained from previous studies is rather

confusing. Blood group A has been proven by a few studies to show higher frequency of forming rosettes in falciparum malaria (Carlson & Wahlgren, 1992; Udomsangpetch *et al.*, 1993). On the other hand, blood group B has been shown to have the highest frequency of forming rosettes when infected by certain strains of *P. falciparum* (Carlson & Wahlgren, 1992). Meanwhile, blood group AB showed the highest frequency of rosetting when infected with *P. falciparum* of Kenyan strain (Rowe *et al*., 1995). On the other hand, there are studies showing that blood group A forms stronger rosettes than blood group B does (Carlson *et al*., 1994). The prevalence of severe malaria symptoms is also significantly higher in patients with blood group A compared to other blood groups (Lell *et al*., 1999). This confusing situation may be due to the possibility that rosetting phenomenon is not solely affected by the ABO blood group, but other rheological aspects as well. The rosetting receptor requirements of different strains of *P. falciparum* may be different. Based on these accumulated data, it is likely that reliance on human blood group trisaccharides in rosette formation varies among different strains of *P. falciparum*.

Interestingly, all the studies conducted so far have proven that blood group O shows the lowest tendency to form rosettes in falciparum malaria setting, and carries the lowest possibility to develop severe malaria symptoms (Migot-Nabias *et al.*, 2000; Pathirana *et al.*, 2005). This may be responsible for higher percentage of population with blood group O in malaria endemic areas of Africa (O'hUigin *et al.*, 1997). The natural selective pressure exerted by malaria provides survival advantage to those carrying blood group O. Nevertheless, the situation where people with blood groups A, AB or B are wiped off the malaria hyper-endemic regions is not seen. This may be due to the compensatory factors carried by blood group O. For instance, people with blood group O have been shown to carry higher risk of contracting cholera (Harris *et al*.,

2005). Besides the ABO blood group, a few pathological conditions of erythrocytes also confer certain extent of protection against severe malaria to the individuals having the particular genetic mutations. These include alpha-thalassemia, beta-thalassemia, sickle cell trait, G6PD-deficiency, Complement Receptor (CR) 1 deficiency, Southeast Asian Ovalocytosis and other haemoglobinopathies (Carlson *et al*., 1994; Pattanapanyasat *et al*., 1999, Wambua *et al*., 2006; Rowe *et al*., 2007). This may explain the higher prevalence of such genetic defects in malaria endemic regions such as Africa and Southeast Asia, and the presence of such phenotypes may "dilute" the impact exerted by human ABO blood groups on inherited protection against malaria.

Besides the A and B trisaccharide antigens of human blood, a few protein candidates have been postulated as the receptors of rosetting ligands in falciparum malaria, which include the human complement receptor 1 (CR1/CD35), fatty acid translocase (CD36) and heparan sulphate (HS) (Leech *et al*., 1984, Handunnetti *et al*., 1992; Wahlgren *et al*., 1992; Ringwald *et al*., 1993; Chotivanich *et al*., 2004; Vogt *et al*., 2004; Leitgeb *et al*., 2011; Janes *et al*., 2011). Some of these candidates, with CR1 in particular, have been convincingly proven to play important role in rosette formation of *P. falciparum*. Anti-CR1 monoclonal antibody inhibits rosette formation of *P. falciparum* isolates (Rowe *et al*., 1997).

Interestingly, sequestration has been observed to occur slightly earlier than rosetting in the erythrocytic life cycle of *P. falciparum* (Treutiger *et al*., 1998). For sequestration, CD36, HS, intercellular adhesive molecule 1 (ICAM 1), chondroitin sulphate A (CSA), E-selectin and thrombospondins (TSP) are postulated as the required receptors (Ockenhouse *et al*., 1992; Gardner *et al*., 1996; Treutiger *et al*., 1998; Vogt *et al*., 2004; Anstey *et al*., 2007). The sharing of receptors between rosette formation and

sequestration may allow the infected erythrocytes to form rosettes and attach to the endothelial cells of the capillary wall, subsequently resulting in vasculature occlusion as seen in severe falciparum malaria cases.

Right before the initiation of this research project, a research group from Singapore conducted a preliminary study and discovered that human glycophorin C might be involved in rosetting event of *P. falciparum* (Prof. Dr. Peter Preiser, Nanyang Technological University, personal communication). Glycophorin C is a sialoglycoprotein with relatively huge extracellular domain (Smythe *et al*., 1994). The extracellular domain has various potential binding sites for different substances including viruses (Ohyama *et al*., 1993). Various antibodies against different sites of the extracellular domain of glycophorin C have been produced. For example, mouse anti-human glycophorin C IgG clone BRIC 4 antibody acts on amino acid residue 2 to 21 (Smythe *et al*., 1994). On the other hand, mouse anti-human glycophorin C IgG clone BRIC 10 antibody acts on the free amino group at the N-terminus of the domain (Dahr *et al*., 1989; Smythe *et al*., 1994). In view of the nature of glycophorin C's structure (Figure 2.5), there is a need to validate the association of this sialoglycoprotein with rosetting phenomenon of *P. falciparum* and *P. vivax*, and subsequently elucidate the exact region on glycophorin C extracellular domain that is involved in rosetting event.

Fascinatingly, glycophorin C has been proven as a key player in the erythrocytic invasion of *P. falciparum* (Chishti *et al*., 1996; Lobo *et al*., 2002; Mayer *et al*., 2006). Along with human band 3 protein (Castelino *et al*., 1981; Liu *et al*., 1990), glycophorin A (Ridgwell *et al*., 1983) and glycophorin B (Mayer *et al*., 2009; Li *et al*., 2012), glycophorin C is another receptor required by *P. falciparum* merozoites to invade the erythrocyte. Mutations on glycophorin C such as Gerbich negative phenotype and

Leach phenotype confer partial resistance against *P. falciparum* invasion (Chishti *et al*., 1996; Maier *et al*., 2003). If glycophorin C were involved in rosette formation of *P. falciparum*, this sialoglycoprotein may be the link between the parasite's rosetting phenomenon and invasion events. The relative importance of glycophorin C in *P. vivax*'s pathobiology is not well understood. Nevertheless, it would be interesting to find out the relationship between glycophorin C and the rosetting event of *P. vivax*.

In view of the diverse rosetting receptor candidates found so far, the mechanisms and properties of rosettes formation by malaria parasites may be different among various strains of the same species. Such differences may be driven by geographical and host demographic factors as well.

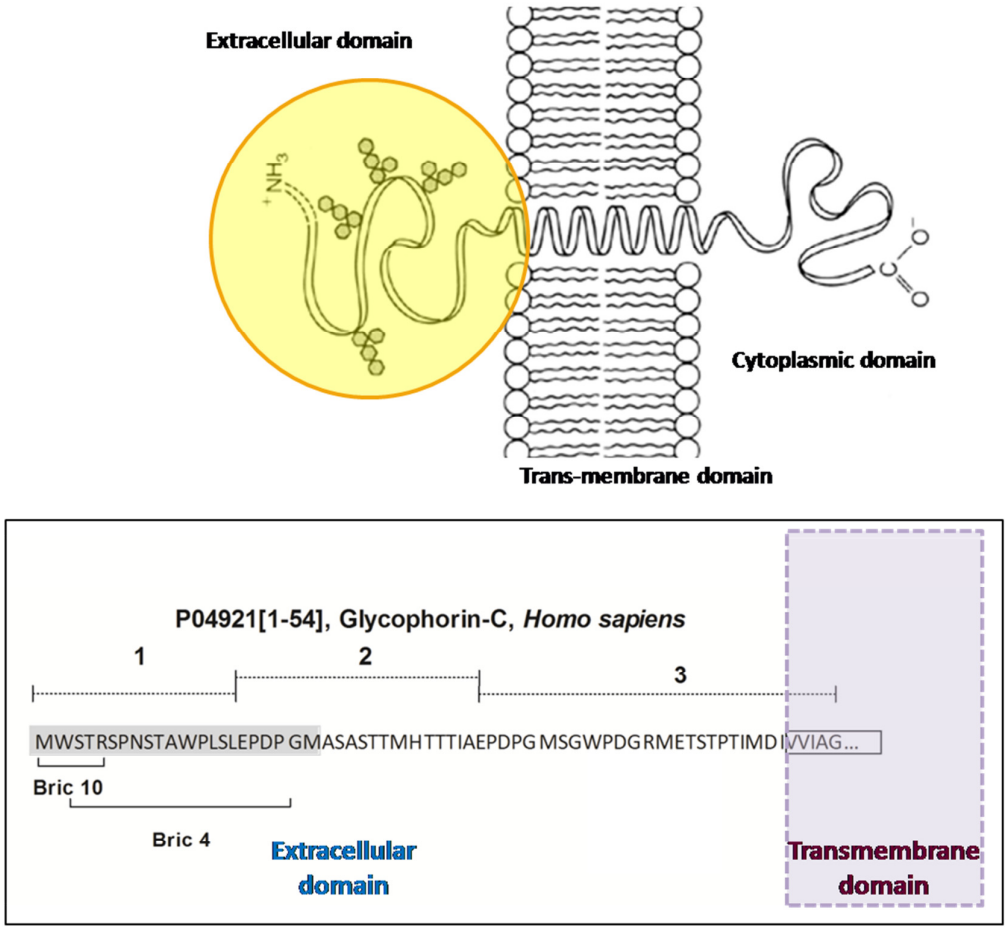

Figure 2.5 Schematic diagram of glycophorin C structure, with the amino acid sequences of the extracellular domain shown in the box underneath the depicted structure. The amino acid residues encoded by exon 1, 2 and 3 of the gene, along with the targeting region of mouse anti-human glycophorin C IgG clone BRIC 4 and clone BRIC 10 antibodies were shown in the box as well.

2.9 Mechanisms of rosette formation in *P. falciparum* model: the ligands

Unlike the receptors involved in rosette formation, the number of candidates suggested and confirmed as rosetting ligands is relatively low (Helmby *et al*., 1993; Rowe *et al*., 1997; Chen *et al*., 1998; Mayor *et al*., 2004; Horata *et al*., 2013). Most of the studies conducted so far have focused on *Pf*EMP1 (Rowe *et al*., 1997; Chen *et al*., 1998; Mayor *et al*., 2004; Horata *et al*., 2013).

*Pf*EMP1 is a group of trypsin-sensitive, variant surface antigens (VSA) encoded by *var* genes (Baruch *et al*., 1995). The haploid genome of *P. falciparum* erythrocytic stage contains around 60 *var* genes (Gardner *et al*., 2002). For any individual infected erythrocyte, only one variant of *var* protein is expressed. Through this antigenic variation strategy, the parasites can evade host's immune responses. Surely, host's immune responses will be elicited following recognition of these parasitic antigens expressed on the surface of infected erythrocytes. However, the antibodies raised against the *Pf*EMP1 are mostly variant specific (Marsh & Howard, 1986; Newbold *et al*., 1992). Therefore, the partial immunity arises from previous exposure to a particular strain of *P. falciparum* may confer protection against severe malaria during the second infection by only against the very same strain of parasite. Without doubt, such partial immunity will not protect the host from contracting falciparum malaria (Chen *et al*., 2000). In addition, this partial immunity may be specific to only a particular parasite strain, resulting in very limited protection against the infection. Such strategy enables establishment of chronic *P. falciparum* infection (Kyes *et al*., 2007).

The Duffy-binding-like-alpha (DBLα) domain of *Pf*EMP1 (also known as *Pf*EMP1- DBLα) is supported by many studies to be the rosetting ligand (Rowe *et al*., 1997; Chen *et al*., 1998; Mayor *et al*., 2004; Mayor *et al., 2009*; Horata *et al*., 2009).

However, the *Pf*EMP1- DBLα of different strains of *P. falciparum* shows varied binding affinity to different rosetting receptors, such as CR1 (Rowe *et al*., 1997; Mayor *et al., 2009*), heparan sulphate, and blood group A trisaccharide (Chen *et al*., 1998; Chen *et al*., 2000). This reflects the diverse nature of receptor-ligand interaction in rosetting event of *P. falciparum*. Today, *Pf*EMP1 is still the focus of rosetting research in *P. falciparum* model, with particular attention on elucidating the specific region on *Pf*EMP1 that serves as the rosetting ligand (Horata *et al*., 2009; Angeletti *et al*., 2012; Angeletti *et al*., 2013; Adams & Rowe, 2013).

The "surface receptor-ligand" theory for rosette formation mechanism sounds simple and straight-forward. However, this surface receptor-ligand theory cannot explain the phenomenon of giant rosette formation where the uninfected erythrocytes are seen adhering to a rim of uninfected erythrocytes that are adhered to the infected erythrocyte (Udomsangpetch *et al*., 1995). Since the outer rims of erythrocytes do not get into contact with the surface of the infected erythrocyte, the ligands involved in rosette formation are not located exclusively on the surface of the infected erythrocytes. The proteins responsible for rosetting may be secreted out of the infected erythrocytes to the surrounding before adhering to the nearest surface of any erythrocytes.

2.10 Current knowledge about rosetting event of *P. vivax*

In contrast to the comprehensive studies on rosetting studies of *P. falciparum*, the rosetting event of *P. vivax* has not been deeply investigated. Hence, knowledge on the basic properties of rosettes formed by *P. vivax*-infected erythrocytes is limited.

Based on the limited amount of investigations conducted (Udomsangpetch *et al*., 1995; Chotivanich *et al*., 1998), it has been shown that rosettes formed by *P. vivax* are

sensitive to a variety of reagents such as heparin and enzyme trypsin. Besides, the rosetting event of *P. vivax* is dependent of divalent ions such as calcium (Ca^{2+}) and magnesium (Mg^{2+}). In addition, a few studies showed that the rosetting event of *P. vivax* is independent of the human ABO blood groups. The relationship between rosetting event and vivax malaria severity is not intensively studied. Recently, *P. vivax* rosetting has been correlated to anaemia in vivax malaria patients from Manaus, Brazil (Marín-Menéndez *et al.*, 2013). Besides, receptors involved in rosetting event of *P. vivax* are yet to be explored. Similarly, ligands involved in *P. vivax* rosetting event remain as a mystery. Obviously *P. vivax* has been neglected for a long time in malaria research.

2.11 Difficulties in implying rosetting knowledge of *P. falciparum* onto *P. vivax*

As mentioned earlier, different species of *Plasmodium* may behave differently in cellular biology and pathobiology. It would be difficult, if not impossible to completely derive knowledge gained from studies on a particular species to other species of the same genus. Certain differences are so distinct that hypotheses on biological properties of a species based on extrapolation of findings on another species are hardly possible. For instance, receptor-ligand interaction of rosetting event in *P. falciparum* is well studied, with the light of focus shone towards the *Pf*EMP1 protein. However, *Pf*EMP1 has no orthologs in *P. vivax*, raising concern that the rosetting receptors deduced from studies on *P. falciparum* may not be relevant to *P. vivax*'s settings. In view of the lack of understandings on the rosetting properties of *P. vivax*, coupled with the fact that *P. vivax* are different from *P. falciparum* in many aspects, there is a need to specifically investigate the biological properties of rosette formed by *P. vivax* isolates, and subsequently decipher the roles played by this phenomenon in pathogenesis.

2.12 Methods available for conducting rosetting-related experiments

To investigate rosetting phenomenon of malaria parasites, a good technique of visualizing and characterizing the rosettes is required. This technique must be accurate, convenient to prepare, simple and workable under various conditions, including field settings. There are a few methods available from previous studies conducted on this topic. Different study groups have applied different techniques for their studies. Each method poses distinctive strength and weaknesses, which are elaborated as follow.

Giemsa-stained thin blood smear was used to visualize and characterize rosettes of *P. falciparum*-infected blood samples (Le Scanf *et al*., 2008). This technique is simple, easy to prepare and cheap. However, it is almost impossible to differentiate between a real rosettes and a mere clump of erythrocytes under thin smear examination. Application of this technique on rosetting characterization raises doubts on its relative accuracy and practicality.

The second method used for rosetting study is fluorescent microscopy using wet mount preparation. This is the most frequently used method for rosetting studies (Udomsangpetch *et al*., 1989; Carlson *et al*., 1990b; Chen *et al*., 1998; Chotivanich *et al*., 1998; Clough *et al*., 1998; Treutiger *et al*., 1998; Deans & Rowe, 2006). This technique applies fluorescent dyes such as acridine orange or ethidium bromide to stain the parasite's nucleic acids. Thus, rosettes are visualized. This method is accurate and produces good visual evidence for rosetting phenomenon. However, fluorescence microscope may not be available in every laboratory setting, especially laboratories in rural areas, where malaria is more likely to be endemic. Besides, fluorescent dyes must be stored and handled properly to ensure long shelf life and optimal performance.

The third method involves unstained wet mount examination using light microscope (Udomsangpetch *et al*., 1992). This is also a convenient and cheap technique. However, a good phase contrast microscope, coupled with an experienced microscopist is required to optimally differentiate between malaria parasites and artifacts under unstained condition.

The fourth technique involves application of wet mount preparation from malaria culture suspension of 5% haematocrit concentration, stained supravitally with 10% Giemsa (Chotivanich *et al*., 1998). This method visualizes the parasites as seen from thin blood smear under microscopic examination, enabling rosetting investigation to be conducted with simple equipment. Nevertheless, when this method was tried prior to the initiation of this research project, it was found that the formula stated conferred a crowded cell population under the wet mount, which hindered optimal visualization and characterization of the rosettes. Besides, crenation happened rapidly after the preparation (less than 5 minutes post-preparation), causing characterization of rosettes difficult to be implemented with this method. As the wet mount is placed on the microscope stage and shone by the light for examination, heat generated from the light source causes water evaporation of the wet mount medium, making the originally isotonic extracellular milieu hypertonic to the cells, and hence the cellular crenation. Therefore, the ratio of cell density to water content of the wet mount suspension must be adjusted to resist fast development of crenation. Obviously, an improved method is needed for optimal visualization and characterization of the rosettes.

2.13 Additional haematological aspect that deserves attention

Since research study on rosetting phenomenon is considered as a form of haematological investigation, it is crucial and beneficial for the project to simultaneously look into other haematological aspects of the recruited samples. One particular haematological aspect that deserves attention in this project is the reticulocyte profile of the sample. As mentioned earlier, reticulocyte is the immature erythrocyte that serves as the exclusive invasion target for *P. vivax*. Reticulocytes are differentiated from the matured erythrocytes (normocytes) by having reticulated matters (remnants of ribosomes and RNA) within the cytoplasm (Brecher, 1949; Rowan, 1991). Besides, the size of a reticulocyte is slightly larger than that of a normocyte, and the shape of a reticulocyte tends to be slightly irregular instead of the biconcave morphology seen in normocytes (Brecher, 1949; Rowan, 1991; Riley *et al*., 2001) (Figure 2.6). In order to validate the hypothesis that rosette formation enhances merozoites reinvasion, reticulocyte characterization is undoubtedly an essential part of this study.

There are a few methods available for reticulocyte characterization (NCCLS, 2004). The gold standard technique for reticulocyte profiling is microscopic examination on thin smear prepared from blood suspension stained supravitally with reticulocyte stains (Rowan, 1991; Riley *et al*., 2001; NCCLS, 2004). The commonly used reticulocyte stains include new methylene blue (NMB) and brilliant cresyl blue (Brecher, 1949; Koepke & Koepke, 1986). This technique works by visualizing the reticulated matters within the cytoplasm of the reticulocytes, thus enabling differentiation of reticulocytes from other erythrocytes. This old, gold standard technique is cheap and easy to prepare. However, this method is time consuming with inconsistent performance as significant variations were observed in reticulocyte staining and visualization by the reticulocyte stains produced by different manufacturers, as well

as inter-batch variation by the very same company (Rowan, 1991). Furthermore, this technique is inaccurate when applied on samples infected with haemoprotozoa such as *Plasmodium* spp. and *Babesia* spp. This is due to the fact that reticulocyte stains cannot optimally stain the parasites, causing difficult differentiation between the haemoprotozoa and reticulocyte's reticulated matters.

Other methods are automated techniques, which include automated blood cell counter and flow cytometry (Rowan, 1991; NCCLS, 2004). Nevertheless, the principles behind this technique are the same as the gold standard technique, which involve staining and visualization of the reticulated matters within the reticulocytes. The automated technique is fast but expensive. Besides, it requires stable power supply in the laboratory setting (Ali *et al*., 2010). This particular requirement poses problems to many laboratories in rural areas and developing countries whose power supply is usually unstable. More importantly, this sophisticated technique is also inaccurate when applied on blood samples infected with haemoprotozoa (Rowan, 1991; Riley *et al*., 2001). Obviously, a much better technique is needed for optimal characterization of reticulocytes on malaria-infected blood samples.

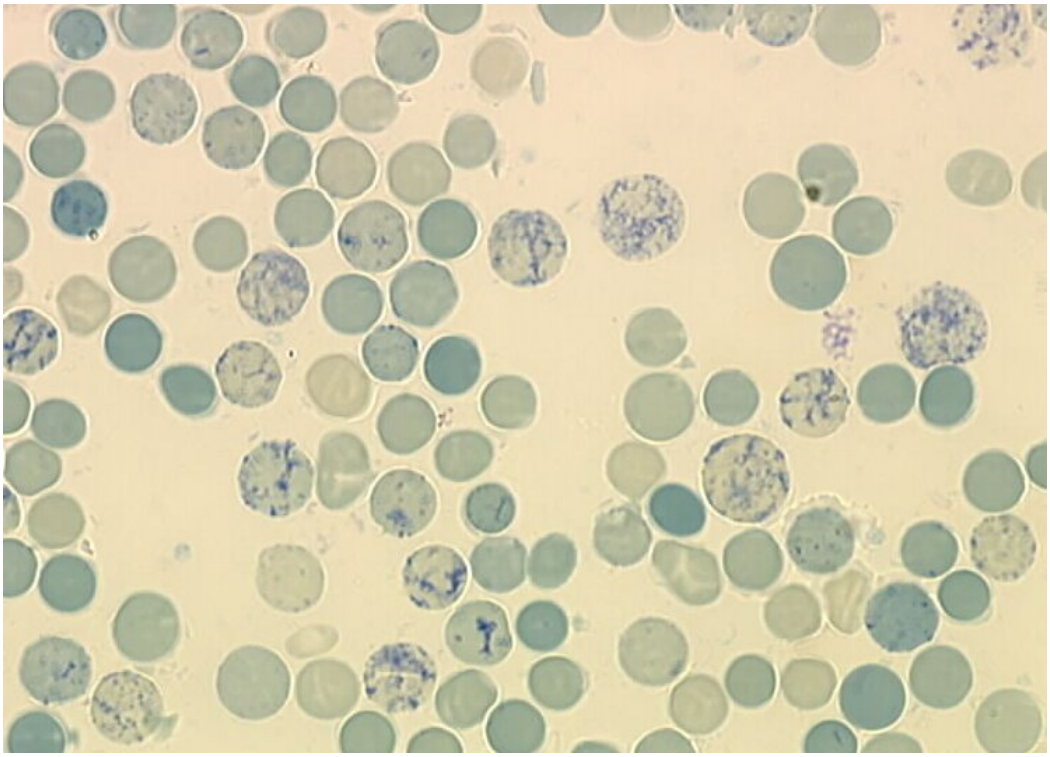

Figure 2.6 Smear prepared from blood stained supravitally with new methylene blue (NMB). The reticulocytes are characterized by the presence of reticulated matters within the cytoplasm, slightly larger size, and slightly irregular shape. (Diagram source: Website of Faculté de médecine, Université de Rennes 1: https://facmed.univ-rennes1.fr/resped/hemato/CC/CD/cd/an/dossiers/retie/sheetse.htm).

2.14 Problem statement

Rosette formation is definitely not a new topic in malaria research. Many studies have been conducted to understand this biological phenomenon. Nevertheless, there is an obvious imbalance in attention allocation to medically important malaria parasites when it comes to malaria rosetting research. Although *P. vivax* is the second most prevalent cause of global malaria burden, attention given to this species is far lesser that that received by *P. falciparum*. Therefore, more studies are required to fill the rosetting knowledge gap between *P. falciparum* and *P. vivax*.

Throughout the course of this research project, the following questions were raised: does *P. vivax* form rosettes as frequently as *P. falciparum*? Is there any correlation between rosetting event and clinical settings of the patient, such as parasitaemia and reticulocyte reading of the patient? How similar, and of course, how different are rosetting events of *P. vivax* and *P. falciparum*? Which type of blood samples should be used for this experiment? What are the requirements, such as the receptors needed by *P. vivax* to form rosettes? What are the roles (if any) played by rosetting event in *P. vivax* infection? And to investigate these questions, which is the most suitable technique for this research project?

METHODOLOGY

Due to the nature and requirements of this research project, fieldworks were conducted and based in Shoklo Malaria Research Unit (SMRU), Thailand, using malaria samples from the Thai-Myanmar border. Part of the work for technique development was carried out in the Singapore Immunology Network (SIgN) of Agency for Science, Technology and Research (A*STAR), Singapore. Data analysis and interpretation was conducted in the University of Malaya, Malaysia.

3.1 Technique development for reticulocyte visualization

This work was intended to develop a field technique that is suitable for haematological investigations, as stated in objective 1.5.1.

3.1.1 Blood sample collection and processing

Blood samples were collected from infected and uninfected donors after obtaining written informed consent following ethical guidelines in the approved protocols (OXTREC 027-025, University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, UK and MUTM 2008-215 from Medical Ethic Committee of Faculty of Tropical Medicine, Mahidol University). Blood samples were collected into BD Vacutainer® with lithium heparin anticoagulant. All samples were processed freshly upon collection or stored at 4°C for not more than 24 hours prior to processing.

3.1.2 Preliminary study: NMB smear versus NMB wet mount

A preliminary study using only uninfected blood samples were conducted to compare reticulocyte counting between the traditional NMB smearing technique and the NMB wet mount technique. Good correlation between the two methods does not ultimately indicate the good agreement between the methods. Therefore the Bland-

Altman plot was used to analyze correlation and the degree of agreement (pattern of bias) between two methods.

From this preliminary study, the reticulocyte counts of both methods were comparable to each other (Figure 3.1). As a result, subsequent comparison of NMB wet mount technique and the Giemsa wet mount technique was feasible.

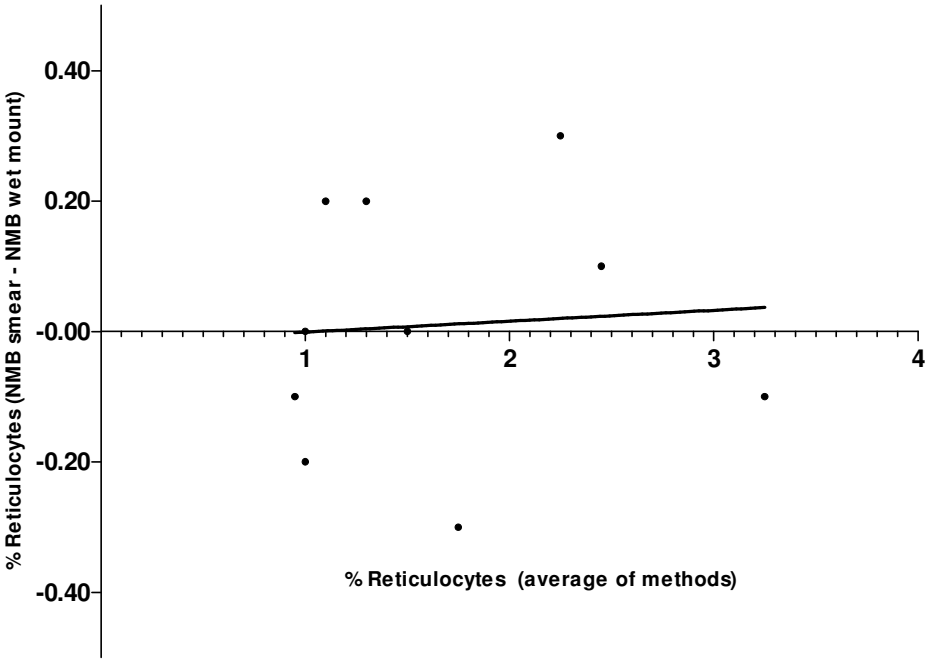

Figure 3.1 Bland-Altman plot showing comparison of reticulocyte counting between the gold standard NMB smearing technique and NMB wet mount technique. Slope = -0.1850 to 0.2184 (95% CI); slope not significantly deviated from zero (P = 0.8531). Both methods are comparable to each other.

3.1.3 NMB smear (gold standard method)

Blood samples were supravitally stained with NMB (Sigma-Aldrich®) stock solution in a 1:1 volume ratio. After incubation over a period of 10 minutes at room temperature, a thin blood smear was prepared from the stained blood suspension. The blood film was air-dried prior to examination under light microscope (Olympus IX53®).

3.1.4 NMB wet mount method

For this technique, 50 µl of each blood sample was stained supravitally with 50 µl NMB stock solution (1:1volume ratio) for 10 minutes at room temperature. The stained blood cell suspension was centrifuged and the pellet was resuspended at 3% haematocrit with plain McCoy's 5A medium (Gibco®) [could be substituted with RPMI 1640 medium (Gibco®)]. Subsequently, 7.5 µl of the stained suspension was dropped onto a glass slide and then covered with a 22 x 32 mm (0.17 mm thickness) glass cover slip.

3.1.5 Giemsa wet mount method

Another 50 µl of each unstained blood sample was stained supravitally with 2.5 µl of a filtered solution of Giemsa (Sigma-Aldrich®) (final staining concentration = 5%) for 15 minutes at room temperature, followed by gentle mixing. Then, a wet mount was prepared as described above for NMB wet mount technique.

3.1.6 Reticulocyte counting

All specimens, especially the wet mounts were examined immediately with oil immersion light microscopy. Reticulocyte percentage number was determined by counting the number of reticulocytes per 1000 erythrocytes (ICSH, 1998).

3.2 Rosetting visualization and characterization

This work was conducted to characterize the rosetting properties of *P. vivax* (objective 1.5.2), as well as to compare the rosetting properties between *P. vivax* and *P. falciparum* (objective 1.5.3).

3.2.1 Experiments on fresh malaria samples

For the collected malaria-positive blood samples, ABO blood group of each sample was determined via standard haemagglutination with TransClone® Anti-A and Anti-B antibodies (Bio-Rad, California, USA). Thick and thin blood smears were prepared from each blood sample to determine species of malaria parasites involved, parasitaemia and the predominant erythrocytic stage of the parasite.

Malaria-infected blood samples with at least 70% of the parasite population being "ring" stages were matured *in vitro* in Mc Coy's 5A medium enriched with 20% homologous serum at 5% haematocrit concentration, as described previously (Russell *et al*., 2012). These samples were examined at different time intervals that represented ring, early trophozoite, late trophozoite and schizont stages. Visualization and characterization of rosettes were conducted using the Giemsa wet mount method described above. The wet mounts were examined immediately using light microscope under oil immersion. Rosetting rate and rosetting prevalence were determined.

3.2.2 Experiments on cryopreserved malaria samples

Vivax malaria blood samples with at least 70% of parasite population in ring form were cryopreserved using the glycerolyte method as described previously (Christofinis & Miller, 1983; Borlon *et al*., 2012). Prior to cryopreservation, vivax malaria samples with parasitaemia lower than 0.1% were subjected to 75% Percoll

density gradient centrifugation to concentrate the parasitized erythrocytes. The cryopreserved samples were then thawed using the sodium chloride method (Blomqvist, 2008). Subsequently, the thawed samples were matured *in vitro* and rosetting assay was conducted as described above. Rosetting rate of each cryopreserved isolate and the rosetting prevalence of cryopreserved isolates were determined.

3.3 Rosetting erythrocyte preference study

This study was conducted to investigate the potential roles of rosetting in vivax malaria, as stated in objective 1.5.4. Blood samples infected with either *P. vivax* or *P. falciparum* were matured *in vitro* until at least 60% of the parasite population reached schizogony. Subsequently, 100 µl of each culture suspension was used as a control where its rosetting rate was determined. Erythrocytes infected with mature-stage parasites were concentrated using magnetic activated cell sorting column (MACS^{TM}) (Miltenyi Biotec). The concentrated infected erythrocytes (volume = x) were subsequently mixed with 0.5x volume of uninfected packed normocytes and 0.5x volume of uninfected packed reticulocytes. The red cell mixture was then suspended in enriched McCoy's 5A medium as described above.

Preformed rosettes were dissociated mechanically via two minutes of vigorous vortexing. In a preliminary study, it was observed that the formed rosettes dissociated when they were subjected to vigorous vortexing (around 1500 rpm) for as brief as one minute. After that, the freed infected erythrocytes resumed rosetting within seconds, and the rosetting rate recovered slowly back to the original value within five minutes (Table 3.1).

The vortexed cellular suspension was then incubated at 37°C for 30 minutes prior to rosetting assay. Subsequently, the types of erythrocyte subsets involved in rosette formation of *P. vivax* and *P. falciparum* were determined.

Table 3.1 Recovery of rosetting rate of *P. falciparum* and *P. vivax* isolates after vortexing (preliminary study). Samples were collected from various Thai-Myanmar border refugee camps in Tak Province, Thailand.

Malaria Isolates	Original Roseting rate (%)	Rosetting rate 1s post-vortex (%)	Rosetting rate 1min post-vortex (%)	Rosetting rate 2 min post-vortex (%)	Rosetting rate 3 min post-vortex (%)	Rosetting rate 4 min post-vortex (%)	Rosetting rate 5 min post-vortex (%)	Rosetting rate 6 min post-vortex (%)	Rosetting rate 7 min post-vortex (%)
P. vivax									
BPD 093	27.5	1.0	5.0	14.5	23.5	27.0	27.5	27.5	27.5
PID 403733	26.0	2.0	6.0	11.5	19.5	26.0	26.0	26.0	26.0
PID 403897	27.5	2.5	7.0	15.0	21.0	27.0	27.0	27.5	27.5
PID 403891	29.5	0.5	8.0	13.0	24.0	29.0	30.0	29.0	29.5
PID 403926	23.0	1.5	4.5	9.5	17.5	23.0	23.0	23.0	23.0
PID 401198	15.0	0.5	5.0	9.0	13.0	14.0	15.0	15.0	15.0
BPD 097	26.0	1.0	15.0	20.0	24.0	24.0	26.0	26.0	26.0
VHX 521	51.0	0.5	19.0	35.0	44.0	50.5	51.0	51.0	51.0
BPD 095	0	0	0	0	0	0	0	0	0
P. falciparum									
MMA 2163	7.5	0	1.5	5.0	5.5	7.0	7.5	7.5	7.5
MRC 0153	0	0	0	0	0	0	0	0	0
TH 004069	10.0	1.0	2.0	4.5	7.5	10.0	10.0	10.0	10.0
PID 101059	20.5	1.0	2.5	5.5	12.5	18.0	20.5	20.5	20.5
PID 104150	12.5	0.5	3.0	6.5	9.0	12.5	12.5	12.5	12.5
NHP 1437	6.0	0.5	2.0	4.0	5.0	5.5	6.5	6.0	6.0
MMA 3052	10.0	1.0	2.0	3.0	7.0	10.0	10.0	10.0	10.0
NHP 1440	9.0	1.5	2.5	5.0	7.0	8.0	9.0	9.0	9.0
NHP 1439	12.0	2.0	4.5	6.0	9.5	12.0	12.0	12.0	12.0

3.4 Elucidating rosetting receptors of *P. vivax* and *P. falciparum*

These experiments were conducted to characterize the rosetting properties of *P. vivax* (objective 1.5.2), as well as to compare the rosetting properties between *P. vivax* and *P. falciparum* (objective 1.5.3).

3.4.1 Anti-human glycophorin antibodies

For this experiment, three types of antibodies were chosen, namely, mouse anti-human glycophorin A (CD235a) IgG (Abcam, Cambridge, UK), mouse anti-human glycophorin C (CD236R) IgG clone BRIC 4 (Thermo Scientific Pierce, USA), and mouse anti-human CD236R IgG clone BRIC 10 (Abcam, Cambridge, UK). Preliminary trials clearly showed that the intact anti-glycophorin C and A antibodies caused considerable haemagglutination. Therefore it was necessary to use Fab fragments of anti-CD236R and anti-CD235a antibodies. Antibody Fab fragments of these antibodies were prepared using the Pierce® Fab Micro Preparation Kit-Resin Kit (Thermo Scientific Pierce, USA).

The antibody Fab fragment preparation was to ensure specific binding of the antibodies to target sites due to the proximity of the selected targeting sites to each other, as well as to other proteins, which may be potential binding sites for any biological event. By preparing this step, the possibility of rosetting inhibition due to steric hindrance caused by the large complete immunoglobulin structure could be ruled out. Besides, this preparation can prevent haemagglutination, which was seen in the preliminary studies when these antibodies were applied directly as complete immunoglobulin.

When the *in vitro* matured culture suspension showed at least 70% of the parasite population being late erythrocytic stages, four aliquots of the culture suspension were prepared. The aliquots were vortexed vigorously for two minutes to mechanically dissociate any pre-formed rosettes.

After that, each aliquot was mixed with one type of the antibody Fab fragments prepared earlier (final concentration = 1000 µg/ml), and one remaining antibody-free aliquot served as the negative control. The aliquots were mixed well by vortexing, and incubated for 30 minutes at 37°C. Subsequently, rosetting assay was conducted on each aliquot as described above.

3.4.2 Anti-human complement receptor 1 (CR1/ CD35) antibody

Steps similar to those used in experiment with mouse anti-human glycophorin antibody Fab fragments were used in this experiment. As this antibody did not cause haemagglutination in preliminary trials, antibody fab fragment preparation was exempted. Mouse anti-human CD35 IgG (BD Pharmigen^{TM}) with final antibody concentration of 20 µg/ml (within the concentration range of effective *P. falciparum* rosetting inhibition described in previous study) was used (Rowe *et al*., 1997). Untreated aliquot of culture suspension served as the negative control. Indeed, this experiment itself also served as a positive control to the experiment with mouse anti-human glycophorin antibody Fab fragments.

3.4.3 Haematopoietic cell culture and CD236R knockdown; Rosetting inhibition

Cryopreserved human umbilical cord blood mononuclear cells (HUMC) were expanded using a method described previously (Giarratana *et al.*, 2011) with few modifications. Briefly, the CD34+ haematopoietic progenitors were isolated by

immuno-magnetic selection using CD34 MicroBead kit (MiltenyiBiotec). The CD34+ cells were harvested during 3 weeks in IMDM Glutamax (Gibco®) supplemented with 330 g/ml of human holotransferin (Sigma®), 10 g/ml recombinant human insulin (SAFC Biosciences®), 2 IU/ml heparin (Sigma®) and 5% AB serum. Between day 0 and 7, the following additional supplementation were incorporated: 10^{-6} M of hydrocortisone (StemCell Technologies™), 100 ng/ml of SCF (Peprotech®), 5ng/mL of IL-3 (Peprotech®) and 3 UI/ml of Epo (StemCell Technologies™), between day 7 and day 11, 100 ng/mL of SCF and 3 UI/mL of Epo, and after day 11 only Epo was added.

One day after the immuno-magnetic sorting, the CD34+ cells were transduced (MOI of 5) with shRNA lentitvector MISSION™, shRNA Target Set NM_002101 (Glycophorin C [CD236R]) (Sigma®) in the presence of 8 g/ml of Polybrene (Sigma®). Fifteen days after the transduction, GFP positive and negative cells were sorted using Influx BD flow cytometer (BD biosciences). The day after, GFP positive and negative fraction were incubated with *P. vivax* late stages isolated by magnetic sorting as described previously (Malleret *et al*., 2011). The level of CD236R expression in each subset was measured by flow cytometry using anti-human CD236R clone BRIC4 (Thermo scientific) antibody as primary staining and anti-mouse e660 (eBioscience) as secondary staining. The cells were acquired on LSRII flow cytometer (BD Biosciences) and analysed with FlowJo software (Tree Star).

3.5 Anti-malarial drug challenge assay

This study was conducted to investigate the potential roles of rosetting in vivax malaria, as stated in objective 1.5.4. Seven anti-malarial compounds were used in the study, namely artesunate, chloroquine, mefloquine, amodiaquine, lumefantrine, quinine and methylene blue. Drug well plate with different compound concentrations of each

drug were prepared using serial dilution technique. For serial dilution, as the drugs show different hydrophilicity, different solvents were used to dissolve and dilute the drug compounds. Double distilled water was used for serial dilution of chloroquine, amodiaquine and methylene blue, 70% ethanol was used for serial dilution of artesunate, mefloquine, and quinine, whereas a combination of Triton X-100, Linoleic acid and absolute ethanol in 1: 1: 1 ratio was used for serial dilution of lumefantrine.

When the *in vitro* matured culture suspension showed at least 70% of the parasite population being late erythrocytic stages, the culture suspension was transferred to the prepared drug plate and incubated at 37°C for 2 hours prior to rosetting assay. Non-treated culture suspension of each recruited malaria isolate was used as the control.

3.6 Human white blood cell (WBC) challenge assay

This study was conducted to investigate the potential roles of rosetting in vivax malaria, as stated in objective 1.5.4. Malaria-infected blood samples collected in heparin collecting tubes were centrifuged at 1800 rpm for 5 minutes. After that, buffy coat (WBC rich region) of the blood sample was carefully removed and collected, subsequently filtered through a CF11 cellulose powder column. The filtered packed erythrocytes were then divided into two; one part used for *in vitro* cultivation at 3% haematocrit suspension (control group), whereas the other part was mixed with the collected buffy coat prior to *in vitro* maturation with similar condition as mentioned previously. Rosetting assay was conducted when at least 70% of the parasite population reached late stages.

3.7 Statistical analysis and interpretation

Rosetting rate is defined as the percentage of infected erythrocytes that form rosettes in 200 recruited infected erythrocytes. Giant rosette is defined as a rosetting

complex with the participation of at least 10 uninfected erythrocytes (Udomsangpetch *et al*., 1989; Udomsangpetch *et al*., 1995). Percentage of giant rosette formed was calculated. The percentage of recruited isolates that showed rosetting phenomenon was expressed as rosetting prevalence.

As most of the data were not normally distributed, non-parametric analysis such as Mann-Whitney U, Kruskal-Wallis (more than two groups) and Friedman test (repeated measures) were utilised. In the latter two tests Dunn's post hoc analysis was used. All statistical analysis used Prism 5 for Windows (version 5.01), Software Mackiev™. Correlation analysis was conducted to study relationship between rosetting rate and parameters of interest. Bland-Altman analysis was applied to study degree of agreement between two techniques under comparison (Bland & Altman, 1995). The experiments covered in this research project, with the number of samples used are summarized in Figure 3.2.

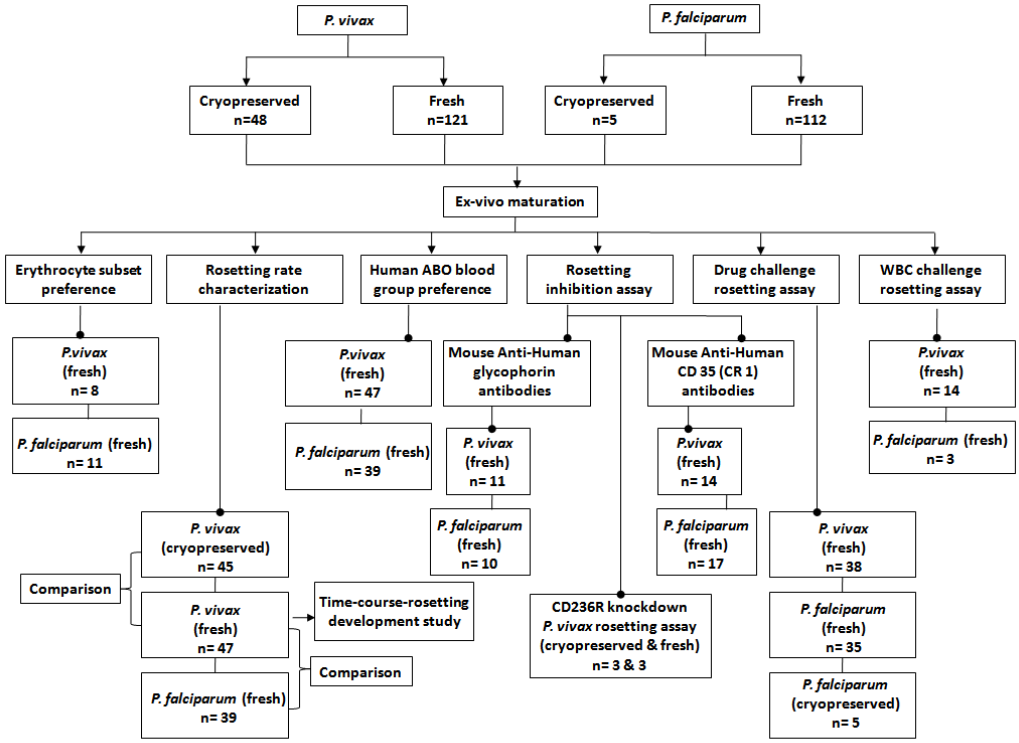

Figure 3.2 Flow chart showing the experiments conducted in this research project with the number of samples used.

RESULTS

4.1 Technique development for reticulocyte visualization

For comparison between NMB wet mount and Giemsa wet mount techniques, a total of 103 malaria-infected blood samples from the Thai-Myanmar border were used. The samples consisted of 69 isolates of *P. vivax*, 32 isolates of *P. falciparum*, 1 isolate of *P. malariae*, and 1 isolate of mixed infection of *P. vivax* and *P. falciparum*. The highest reticulocyte count based on the average value of the two studied methods was 6.45% and the lowest being 0.07%.

The precipitated reticulated matters within the cytoplasm of the reticulocytes were clearly visualized by either the NMB (Figure 4.1A) or the Giemsa (Figure 4.1B) wet mount method. Giemsa produced a lighter staining pattern by specifically staining only the reticulated matters of the reticulocytes. On the other hand, NMB produced a non-specific purplish coloration (background staining) in all erythrocytes regardless of their infection status or haematological stage (Figures 4.1A & 4.2A). Nevertheless, both wet mount methods enabled clear differentiation between reticulated matters of reticulocytes and parasitic matters (Figure 4.2A & B). Besides, the irregular shape and larger size of reticulocyte relative to normocyte were clearly shown using thes techniques.

The mean reticulocyte count in *P. vivax* isolates (1.3% ± 2SD 1.05) was not significantly different from that in *P. falciparum* isolates (1.57% ± 2SD 0.88) ($p = 0.19$). Both the NMB wet mount and Giemsa wet mount methods were significantly correlated (Pearson R = 0.97; 95% CI 0.8799 to 0.9934; $r^2 = 0.96$; $p < 0.0001$) (Figure 4.3). Bland-Altman analysis indicated a good agreement between these two methods (95% limit of

agreement 20.30% and 0.54%) without significant pattern of bias (Bias = 0.05 SD of Bias 0.2) (Figure 4.4).

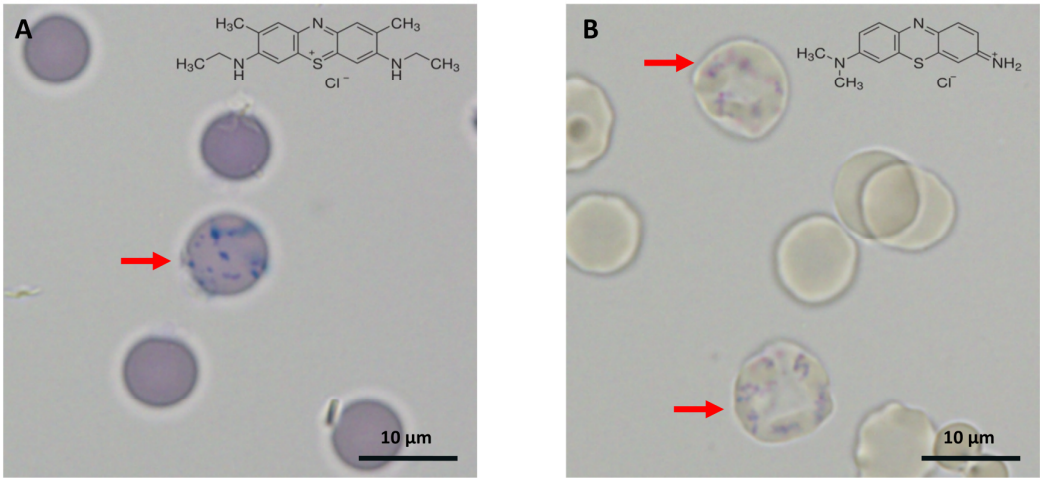

Figure 4.1 Erythrocytes under wet mount prepared from suspension stained supravitally with NMB (A) and Giemsa (B) respectively. The reticulocytes (indicated by red arrows) are erythrocytes with darkly stained reticulated matters within the cytoplasm. Chemical structure of respective staining molecules is indicated at the upper right corner of each picture.

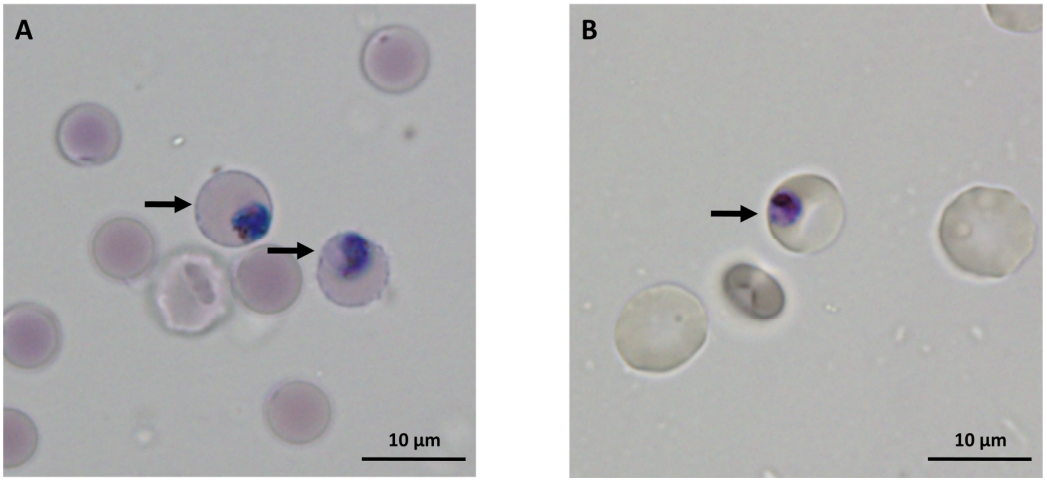

Figure 4.2 ***P. vivax*-infected erythrocytes under wet mount prepared from suspension stained supravitally with NMB (A) and Giemsa (B) respectively.**

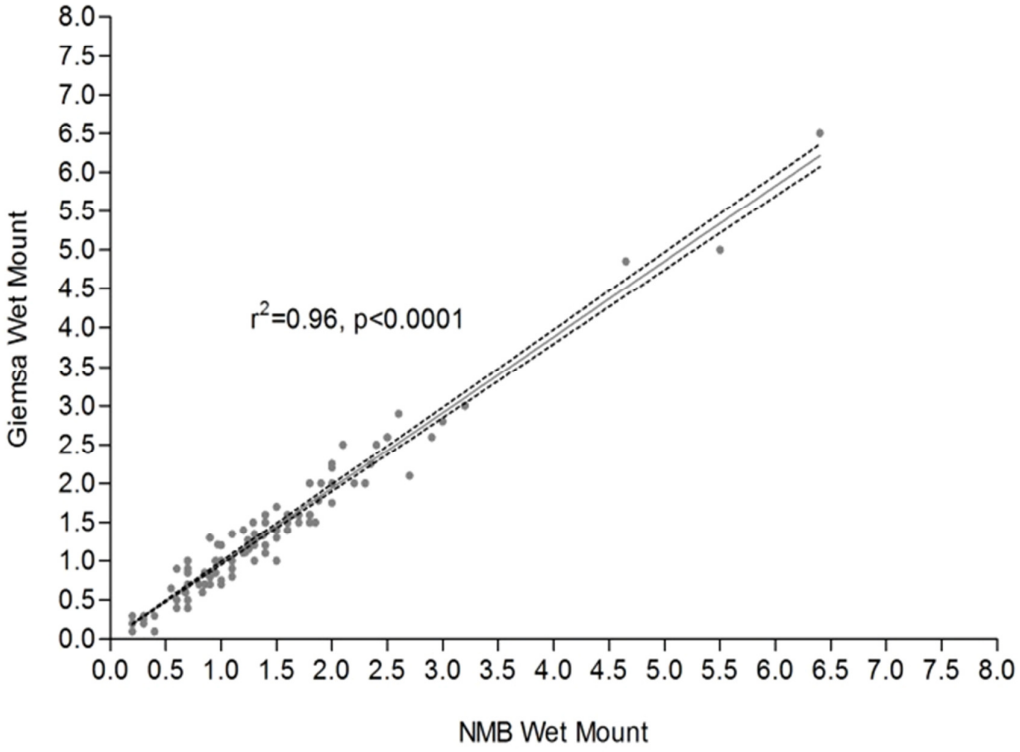

Figure 4.3 Linear regression of reticulocyte readings between NMB and Giemsa wet mount methods.

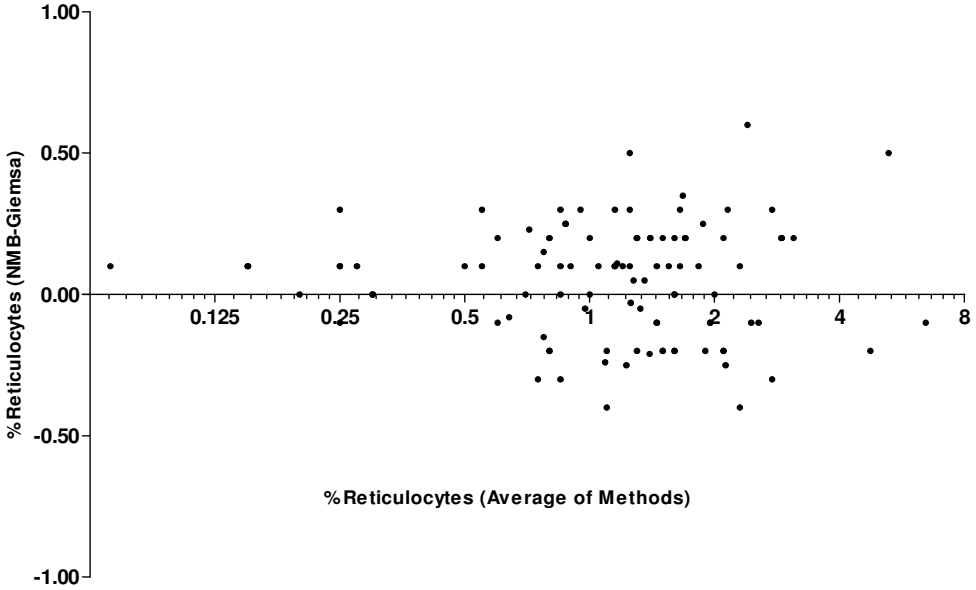

Figure 4.4 Bland-Altman comparison of reticulocyte readings between NMB wet mount technique and Giemsa wet mount technique.

4.2 Technique development for rosetting visualization

The Giemsa wet mount technique, which was modified from a previous study (Chotivanich *et al.*, 1998) and developed for the reticulocyte characterization, was successfully applied for rosette visualisation as well. The malaria parasites were stained clearly, and the rosettes were visualised (Figure 4.5) by using only simple equipment and materials such as Giemsa stain, glass slides, glass cover slips and compound light microscope. By using the Giemsa wet mount, true rosettes can be differentiated easily from a mere overlap or clump of erythrocytes due to the fluidic surrounding of wet mount setting.

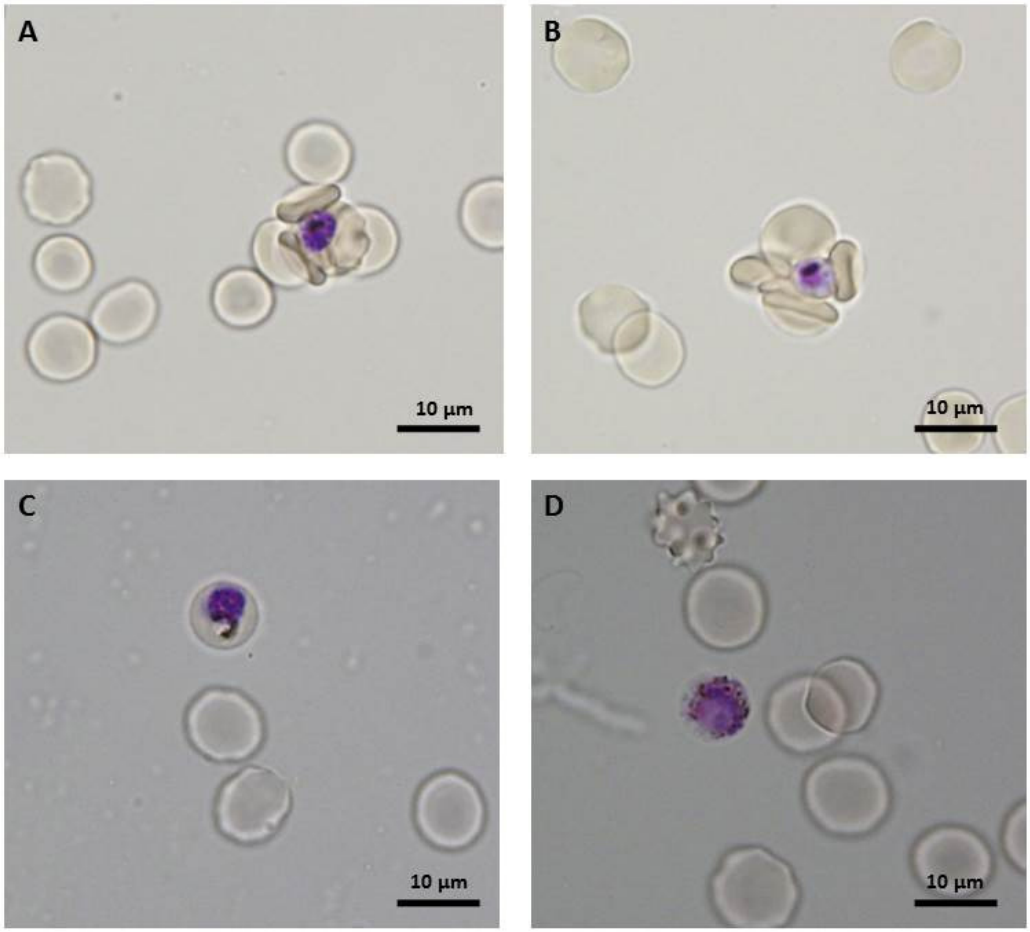

Figure 4.5 ***P. vivax*-infected erythrocytes that form rosettes (A & B) and that do not form rosettes (C & D) from Giemsa wet mount preparation.**

4.3 Rosetting characterization

4.3.1 Rosetting development of *P. vivax*

Rosette formation of *P. vivax* increased with the parasite erythrocytic maturation (Figure 4.6). In this study, no early stages (ring forms) were found to be involved in rosette formation. Rosette formation of *P. vivax* was noted 24 hours post cultivation, represented by early trophozoite stages. The rosetting rate increased markedly between early the trophozoite stage and the late trophozoite stage. After 30 hours the rosetting development continued, albeit with much lower rate, until the parasite reached schizogony at hour 44 (Figure 4.6). Such rosetting development trend is similar to that reported previously for *P. falciparum* (Figure 4.6) (Udomsangpetch *et al*., 1989). Beside the asexual stages, gametocytes of *P. vivax* were also found to be involved in rosette formation (Figure 4.6). Involvement of gametocyte-infected erythrocytes in rosette formation was found in *P. falciparum* isolates as well (Figure 4.6).

4.3.2 Giant rosette characterization

Giant rosettes were found occasionally. Around 10% of the rosettes (223 out of 2233 rosettes) in *P. vivax* isolates were giant rosettes (Figure 4.7A). On the other hand, only around 0.5% of the rosettes (4 out of 759 rosettes) found in *P. falciparum* isolates were giant rosettes (Figure 4.7B).

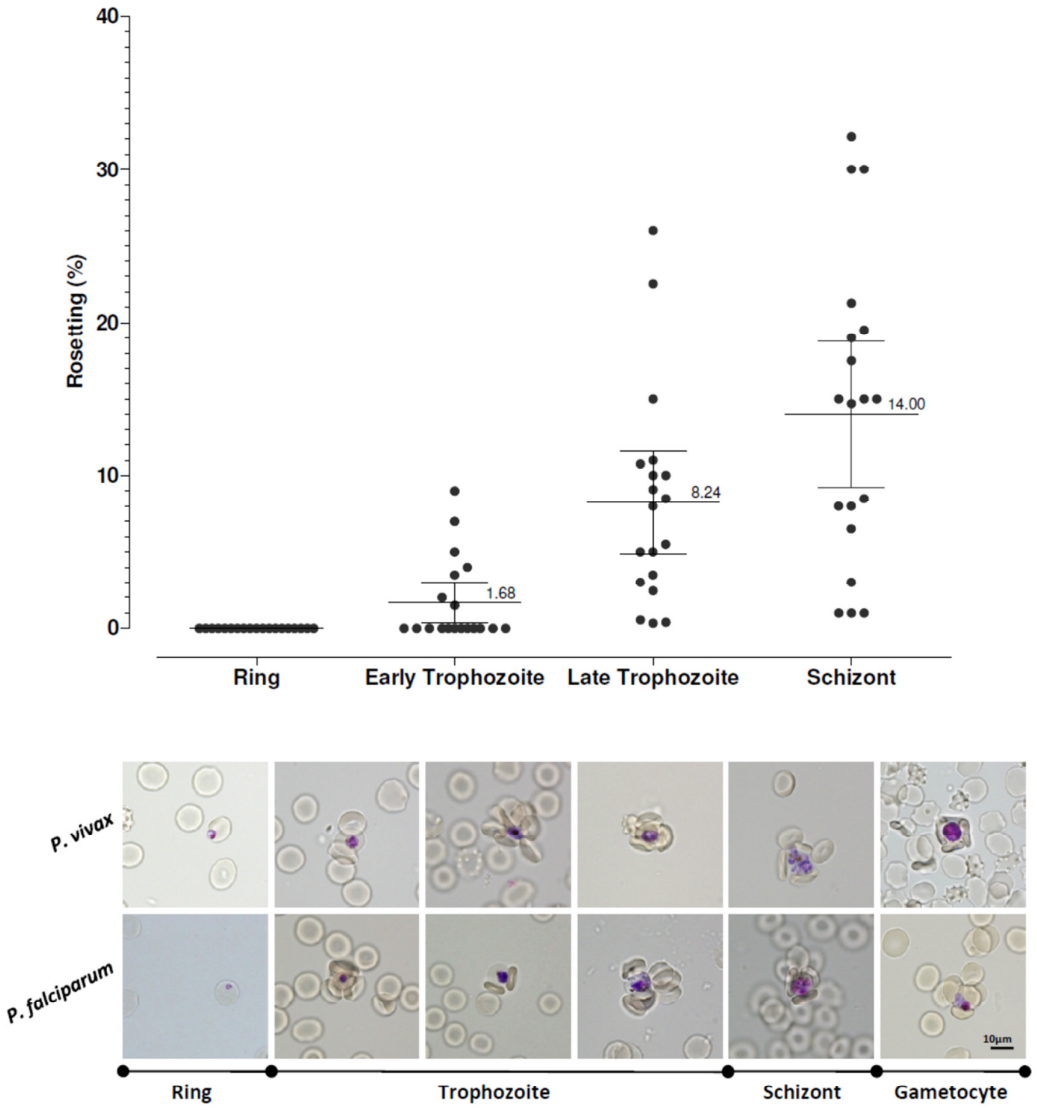

Figure 4.6 Plot showing rosetting development of rosette-forming *P. vivax* isolates along the erythrocytic maturation. Rosettes formed by different stages of *P. vivax* and *P. falciparum* are shown beneath the plot.

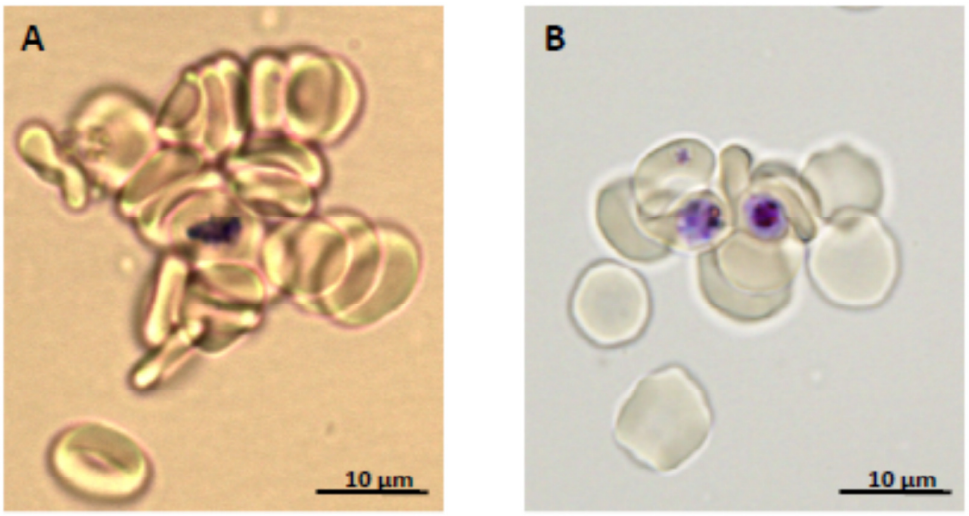

Figure 4.7 Giant rosettes formed by erythrocytes infected with *P. vivax* (A) and *P. falciparum* (B).

4.3.3 Rosetting prevalence of *P. vivax*

At the beginning of this research project, considering the high cost of conducting overseas fieldwork research, only cryopreserved *P. vivax* isolates from the Thai-Myanmar border were used. However, after conducting several experiments and data interpretation, it was found that the cryopreserved vivax malaria isolates differed from the fresh vivax malaria isolates in rosetting capability. For the cryopreserved vivax malaria isolates, 19 out the 45 isolates examined showed rosetting phenomenon, with rosetting prevalence of 42.22%. The highest rosetting rate recorded from this batch of cryopreserved samples was 30.00% with the lowest being 0.20% (only 1 rosette found after examining 500 infected erythrocytes). The rosetting prevalence obtained from these cryopreserved samples was much lower than that reported in a previous study using fresh vivax malaria isolates from Thailand (rosetting prevalence recorded = 71.00%) (Udomsangpetch *et al*., 1995). Therefore, a decision was made to repeat the experiment with the fresh malaria isolates from the same area.

When the experiment was repeated with fresh vivax malaria isolates, the rosetting prevalence recorded was as high as 91.49% (43 out of 47), with the highest rosetting rate recorded as 61.00% and the lowest being 2.00%. When the rosetting data between the fresh vivax malaria isolates and the cryopreserved vivax malaria isolates were compared using the Mann-Whitney U test, significant difference between the two groups was noted; P (two-tailed) < 0.0001 (Figure 4.8). The rosetting capability of fresh *P. vivax* isolates was significantly higher than that of the cryopreserved isolates. These results indicate that cryopreservation affects rosetting capability of the vivax malaria isolates. This finding is different from that of fresh and cryopreserved *P. falciparum* isolates reported earlier (Kyriacou *et al*., 2007).

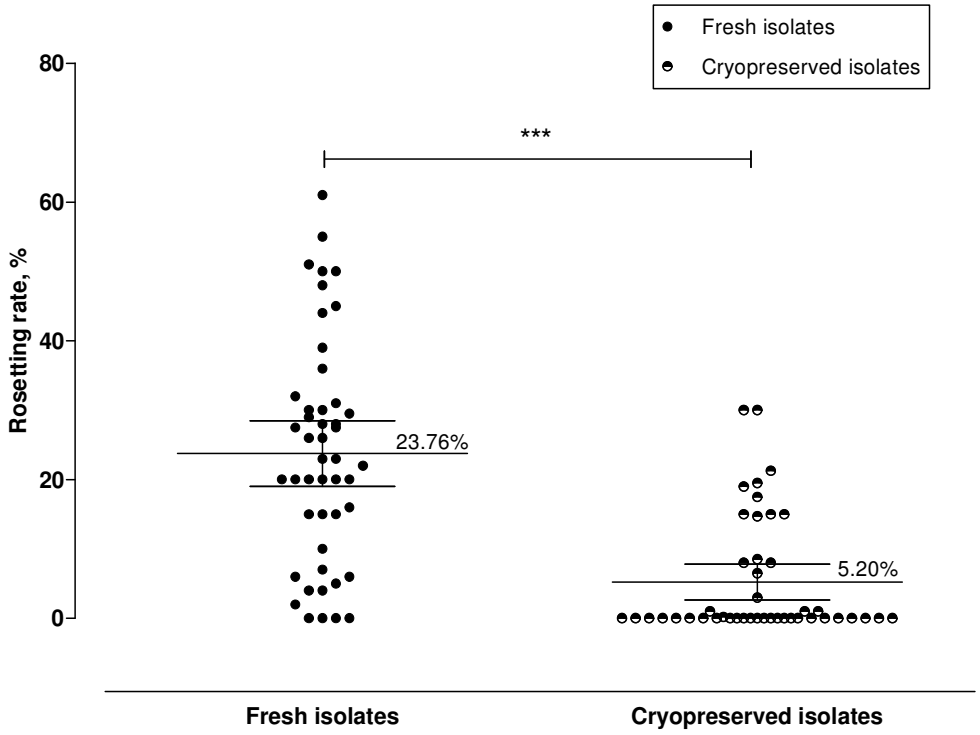

Figure 4.8 Plot showing significant difference in rosetting rates between fresh and cryopreserved *P. vivax* isolates, with the mean values of respective groups. Mann-Whitney U test was performed, P (two-tailed) < 0.0001.

4.3.4 Rosetting prevalence of *P. falciparum*

The rosetting prevalence of fresh *P. falciparum* isolates of the Thai-Myanmar border was studied as well. The rosetting prevalence recorded was 79.49%; with the highest rosetting rate being 28.00% and the lowest being 3.00%. Interestingly, when the median rosetting rates between fresh isolates of *P. vivax* and *P. falciparum* were compared using Mann-Whitney U test, the value of the fresh *P. vivax* isolates (24.5%) was significantly higher than that of *P. falciparum* (9.0%), P (two-tailed) < 0.05 (Figure 4.9).

4.3.5 Correlation study of rosetting phenomenon and different clinical parameters

For both *P. vivax* and *P. falciparum* isolates, no significant correlation was found between rosetting rate and parasitaemia of the isolates (Figure 4.10). The parasitaemia range for *P. vivax* and *P. falciparum* was 0.01 – 1.70% and 0.10 – 11.00%, respectively. Similarly, no significant relationship was found between rosetting rate and peripheral reticulocyte counting of the isolates in both *P. vivax* and *P. falciparum* isolates (Figure 4.11). Besides, no significant correlation was found between rosetting rate and ABO blood groups of vivax and falciparum malaria patients from this region (Figure 4.12).

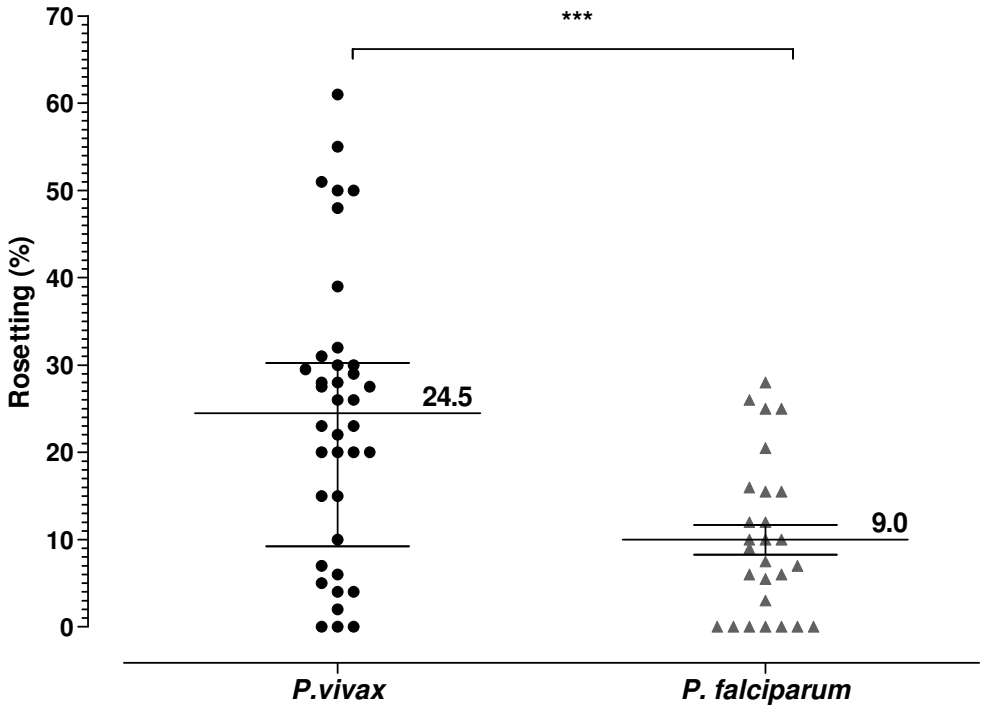

Figure 4.9 Comparison of rosetting rate between *P. vivax* and *P. falciparum* isolates from the Thai-Myanmar border. For *P. vivax* isolates, 95% CI (18.68 to 29.45) and for *P. falciparum* isolates, 95% CI (6.46 to 13.50); Mann-Whitney U test was conducted, there is significant difference between the medians of rosetting rate between isolates of *P. vivax* and *P. falciparum*; P (two tailed) < 0.05.

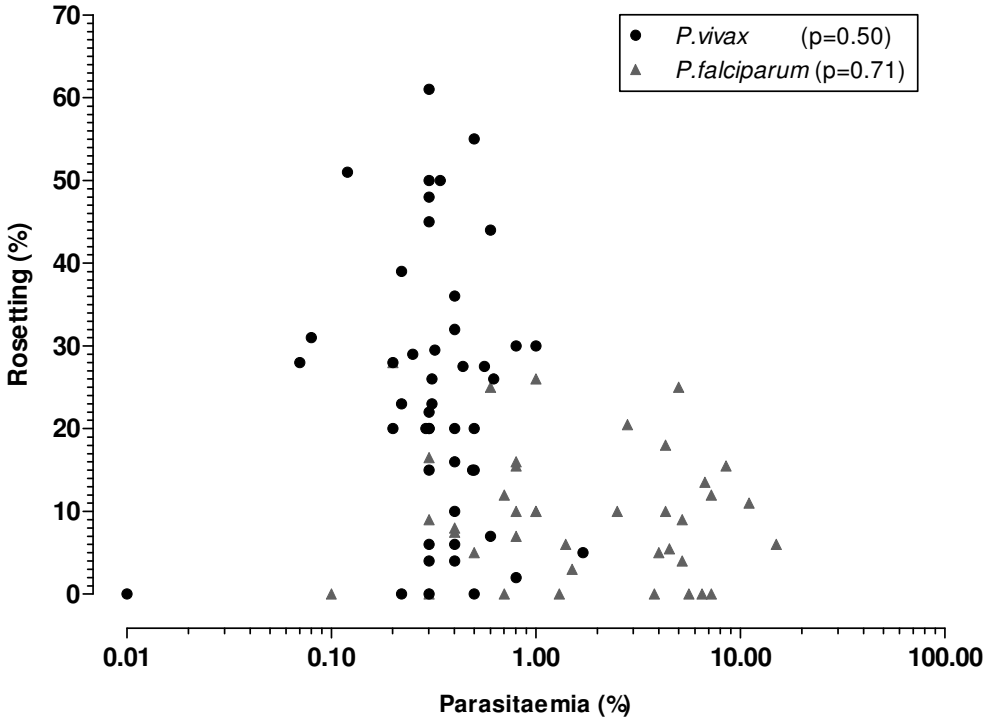

Figure 4.10 Correlation of rosetting rate and parasitaemia in vivax malaria and falciparum malaria isolates from the Thai-Myanmar border. Spearman correlation test was performed. For *P. vivax* isolates, Spearman r = -0.1013; 95% CI (-0.39 to 0.20); P (two-tailed) = 0.4983 i.e. no significant correlation found. For *P. falciparum* isolates, Spearman r = -0.0626; 95% CI (-0.38 to 0.27; P (two tailed) = 0.7051. In both species, no significant correlation was found.

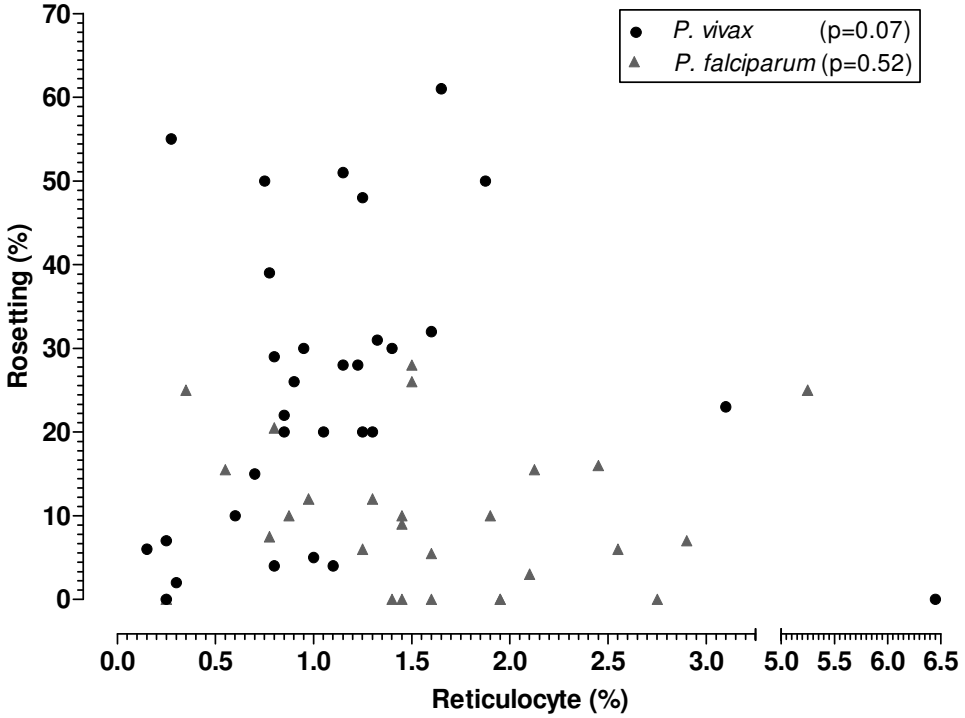

Figure 4.11 Correlation of rosetting rate and peripheral reticulocyte count in vivax malaria and falciparum malaria cases from the Thai-Myanmar border. Spearman correlation test was performed. For *P. vivax* isolates, Spearman r = -0.1013; 95% CI (-0.39 to 0.20); P (two-tailed) = 0.4983. For *P. falciparum* isolates, Spearman r = -0.0626; 95% CI (-0.38 to 0.27; P (two tailed) = 0.7051. In both species, no significant correlation was found.

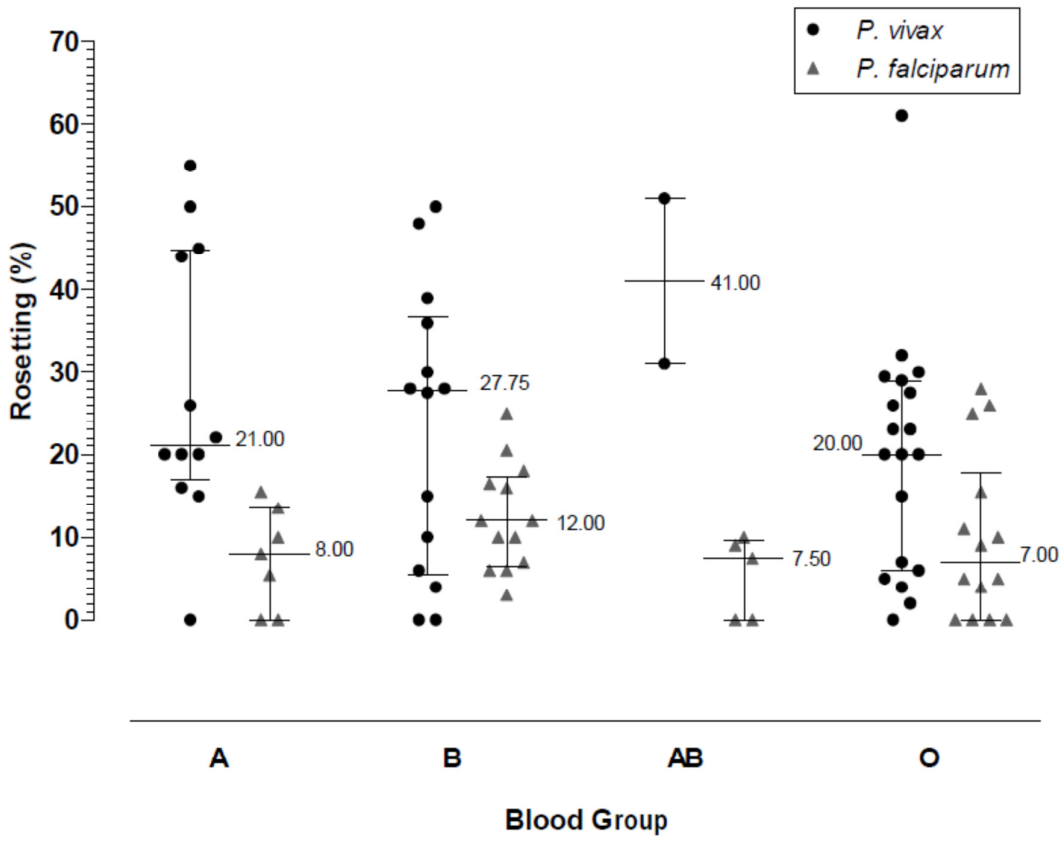

Figure 4.12 Plot showing rosetting rate of *P. vivax* and *P. falciparum* isolates in different human ABO blood groups. Kruskal-Wallis with Dunn's multiple comparison test was conducted. For *P. vivax* isolates (P = 0.2672) and for *P. falciparum* (P = 0.2033), there was no significant difference in rosetting rate between different human ABO blood groups.

4.4 Rosetting erythrocyte preference study

A total of 11 falciparum and 8 vivax malaria isolates were used in this study. For both *P. falciparum* and *P. vivax* incubated in a reticulocyte-enriched environment, only few rosettes were noted involve reticulocyte (Figure 4.13 A-D). A total of 562 and 391 rosettes were examined in *P. vivax* and *P. falciparum* isolates, respectively. Only around 10% of the rosettes examined for both species of malaria parasites had reticulocyte involvement even under a reticulocyte-rich milieu (Figure 4.14). In contrast, all rosettes found from *P. vivax* isolates had normocyte involvement, whereas 388 out of the 391 rosettes (99.23%) in *P. falciparum* isolates showed normocyte involvement.

When the rosetting rates of each isolate under reticulocyte-enriched environment and non-reticulocyte-enriched setting (control) were compared using Wilcoxon signed-rank test, no significant difference was found for the *P. vivax* isolates. On the other hand, rosetting rates of *P. falciparum* isolates showed significant reduction when they were subjected to reticulocyte-enriched setting (P (one-tailed) < 0.01) (Figure 4.15). Although reticulocytes were seen occasionally in few *P. vivax* rosetting complexes from the reticulocyte-enriched settings, the formation of rosettes almost exclusively involved normocytes despite the even chance to encounter reticulocytes. As there may be concerns regarding the difference between cord blood reticulocytes and peripheral reticulocytes, the reticulocyte enrichment experiment was repeated by using three fresh isolates of *P. vivax* and reticulocytes isolated from two adult donors. No significant difference was found between the cord blood and peripheral-derived reticulocytes in their lack of involvement in *P. vivax* rosetting. (Figure 4.16). Instead, rosettes of *P. falciparum* and *P. vivax* consist predominantly of normocytes, as shown in the experiments when both reticulocytes and normocytes were provided equally for the infected erythrocytes to form rosettes.

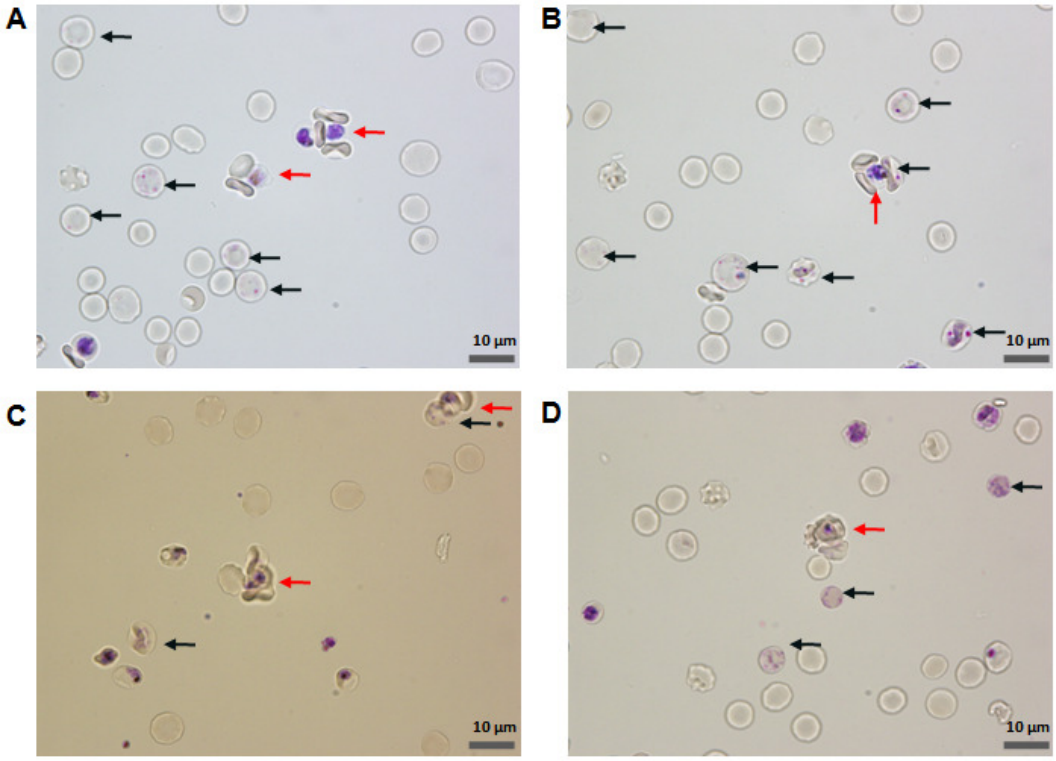

Figure 4.13 Rosettes captured during the rosetting erythrocyte subset preference study in *P. vivax* isolates (A & B) and *P. falciparum* isolates (C & D). Rosettes are indicated by red arrows whereas reticulocytes are indicated by the black arrows.

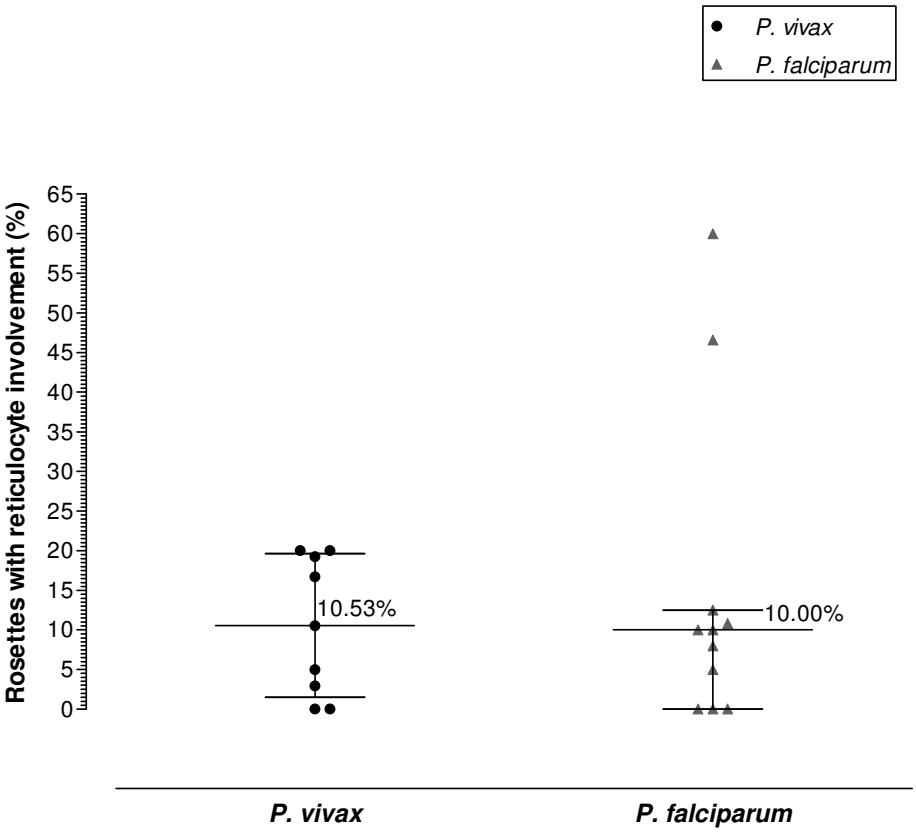

Figure 4.14 Plot showing the percentage of rosettes with reticulocyte involvement in each recruited isolate under reticulocyte-enriched setting. Median value of each group is shown in the plot.

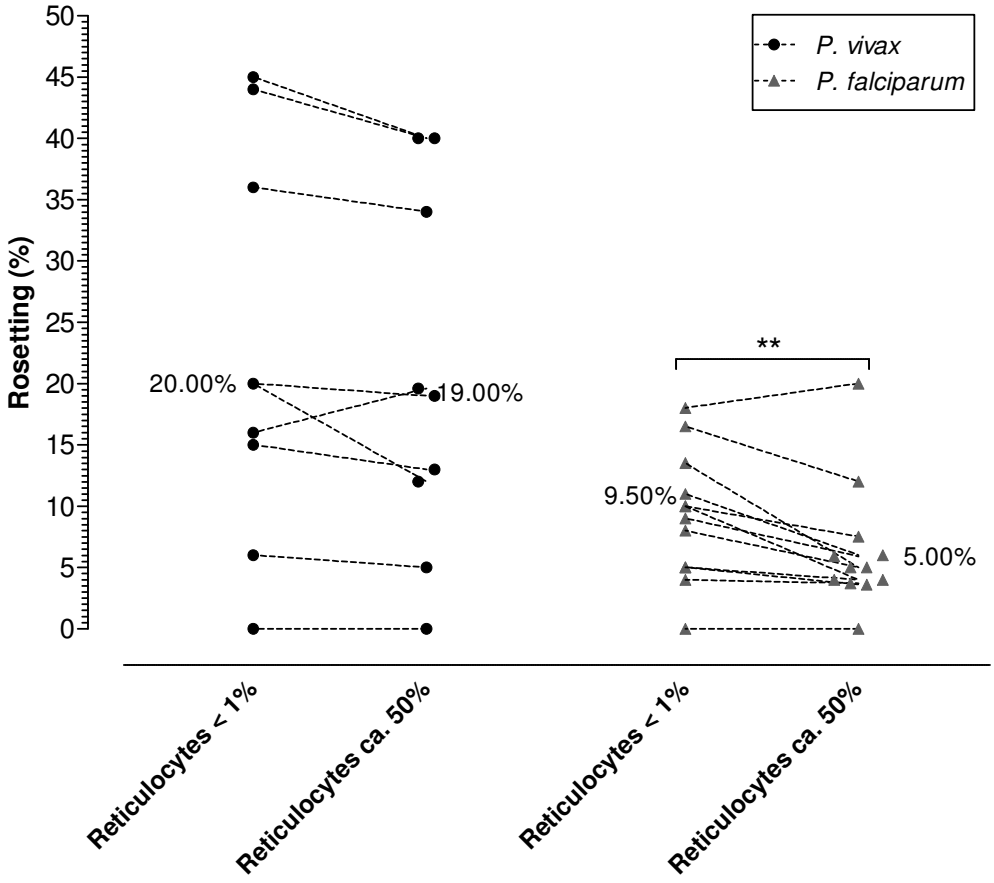

Figure 4.15 Plot showing differences in rosetting rate of *P. vivax* and *P. falciparum* isolates under environment with less than 1% reticulocytes and environment with approximately 50% reticulocytes. Differences in rosetting rate of *P. vivax* and *P. falciparum* isolates in environment with less than 1% reticulocytes and environment with approximately 50% reticulocytes (achieved by concentrating host reticulocytes on a 75% percoll gradient). Lines connect paired observations. Altering the reticulocyte concentration had little effect on *P.vivax* rosette formation; however the number of normocytes attached to the *P.vivax* IRBC was notably reduced in the treatment of enriched reticulocytes. Interestingly an increase in the available reticulocytes reduced the ability of *P. falciparum* to rosette ($P < 0.01$).

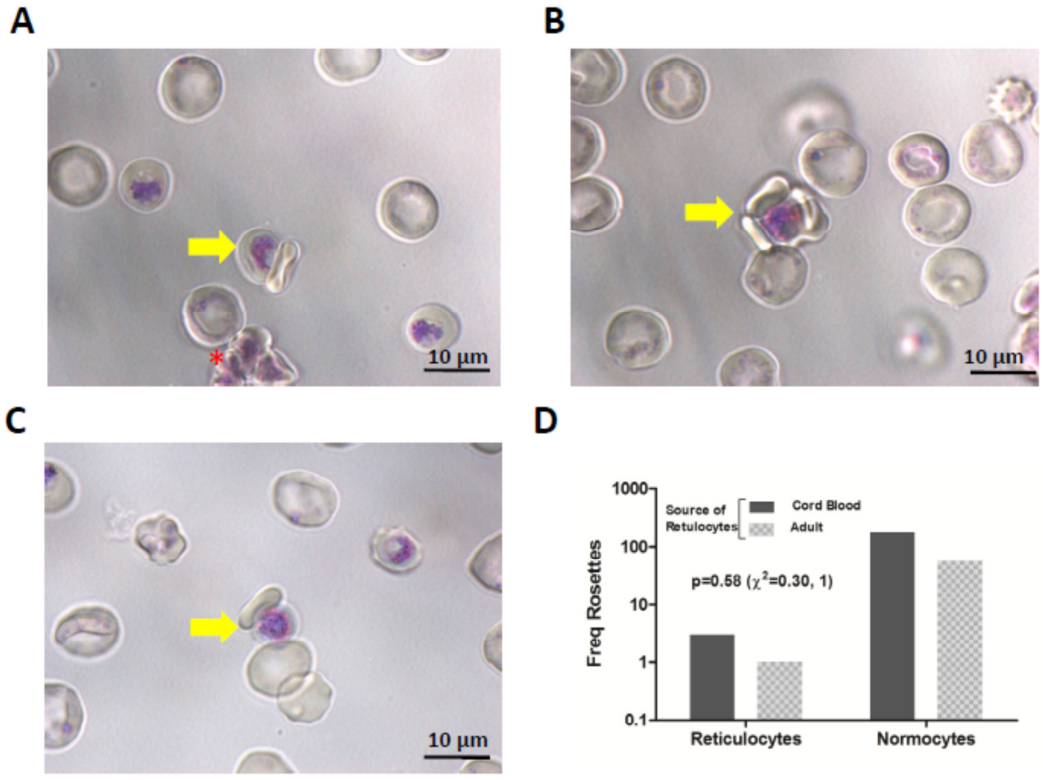

Figure 4.16 Type of erythrocytes involved in rosetting using different sources of blood (peripheral adult blood or cord blood). Concentrated late stage *P. vivax* were exposed to >50% reticulocytes collected from adult peripheral blood (A-C). In rare cases type II and III reticulocytes were found to clump with each other (red asterisk) and not with the parasites (similar to that of cord blood reticulocytes). Note also that only biconcave normocytes (no reticular Giemsa staining pattern) rosette with the parasites (yellow arrow). 58 and 183 rosettes were observed from the adult and cord blood respectively. The frequency of these rosettes being associated with at least one reticulocyte was scored (D). Reticulocytes were rarely involved with rosetting. Additionally, there was no significant association (chi-square with Yates's correction) between the types of erythrocyte involvement and the sources of reticulocyte used.

4.5 Elucidating rosetting receptors of *P. vivax* and *P. falciparum*

4.5.1 Mouse anti-human glycophorin antibodies (Fab fragments)

The rosetting phenomenon of *P. vivax* and *P. falciparum* isolates were significantly inhibited by Fab fragments of mouse anti-human glycophorin C (CD236R) IgG clone BRIC 4 (Figure 4.17B). Based on the Friedman analysis with Dunn's multiple comparison test, rosetting of *P. vivax* was significantly reduced by 27%; $P < 0.05$. Meanwhile, rosetting of *P. falciparum* was inhibited by at least 50%; $P < 0.05$. Interestingly, rosetting events of both *P. vivax* and *P. falciparum* were not affected by the Fab fragments of mouse anti-human CD236R IgG clone BRIC 10, which acts specifically on the amino terminus of glycophorin C (Figure 4.17A). Besides, rosetting of both species of malaria parasites was not affected by the Fab fragments of mouse anti-human glycophorin A (CD235a) IgG (Figure 4.18).

4.5.2 Mouse anti-human complement receptor 1 (CR1/ CD35) antibody

Based on paired t-test, significant rosetting inhibition was seen in *P. falciparum* isolates; P (two-tailed) < 0.0001 in the presence of anti-human CD35 antibody. This result is parallel to findings from a previous study (Rowe *et al*., 1997). However, rosetting of *P. vivax* isolates was not affected by the mouse anti-human CD35 antibody; P (two-tailed) = 0.2722. The mouse anti-human CD35 antibody significantly reduced rosetting of *P. falciparum*, but not in the case of *P. vivax* (Figure 4.19).

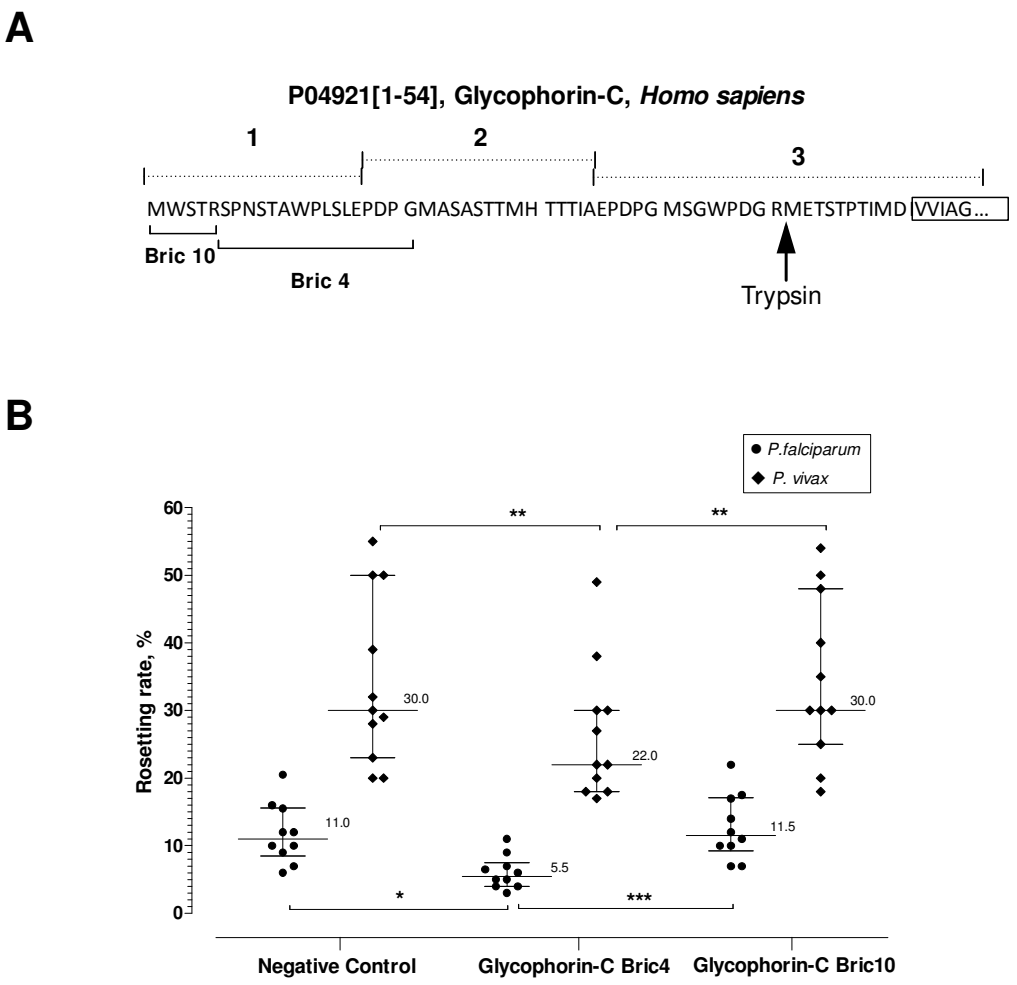

Figure 4.17 Schematic diagram of the target sites of anti-glycophorin C antibody clone BRIC 10 and anti-glycophorin C antibody clone BRIC 4 on human glycophorin C. The target site of enzyme trypsin on this sialoglycoprotein is shown as well (A). Plot showing the extent of rosetting inhibition exerted by the Fab fragments of the antibodies used on *P. vivax* and *P. falciparum* isolates studied (B). One way ANOVA with Dunn's multiple comparison test was conducted to study the difference between the groups. The Fab fragment of mouse anti-human glycophorin C antibody clone BRIC 4 produced significant reduction in rosetting of *P. falciparum* isolates ($P < 0.0001$) and *P. vivax* isolates ($P < 0.0001$).

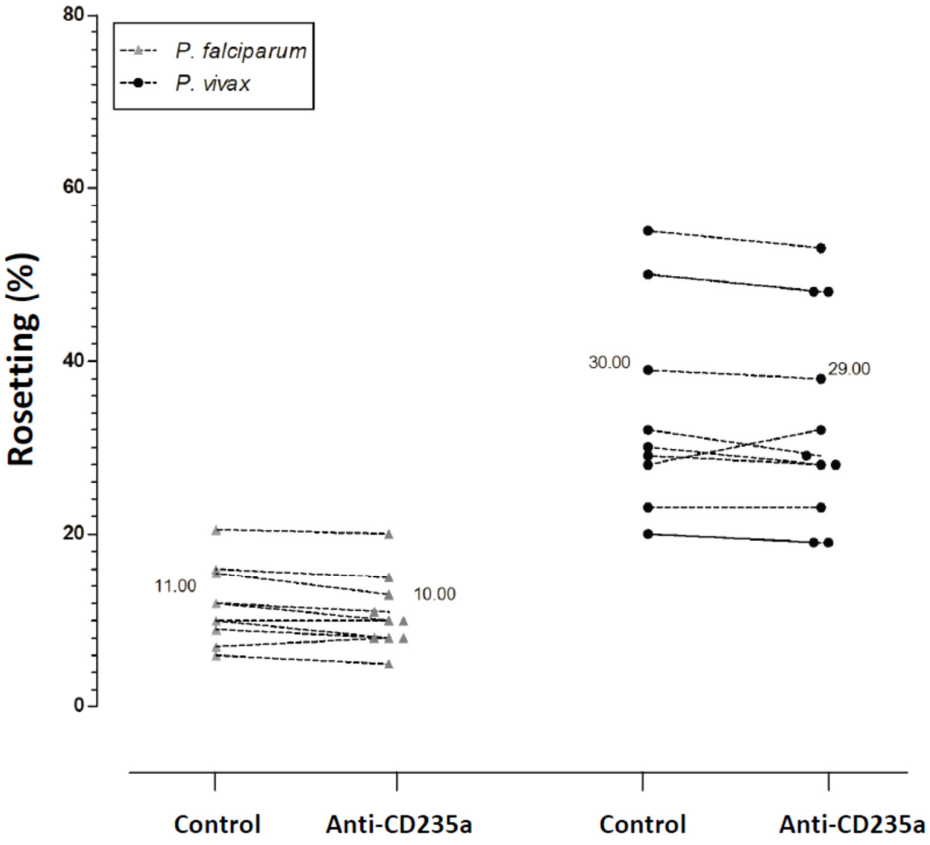

Figure 4.18 Comparison of rosetting rates between the control and cells incubated with Fab fragments of mouse anti-human glycophorin A antibody from the recruited *P. falciparum* and *P. vivax* isolates. Mann-Whitney U test was conducted. There is no significant difference between the control group and the "anti-glycophorin A" group in *P. vivax* (P = 0.6687) and *P. falciparum* (P = 0.5692) isolates studied.

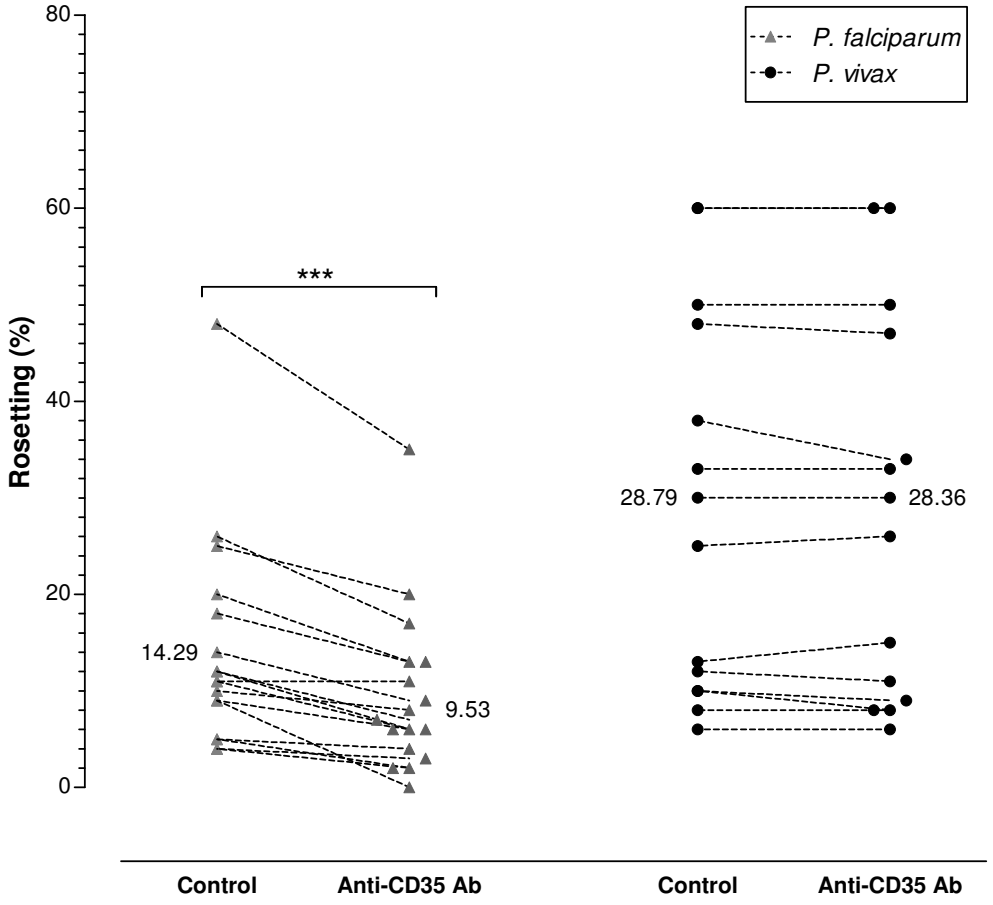

Figure 4.19 Plot showing the extent of rosetting inhibition by mouse anti human CD35 antibody. Paired t test was conducted. For *P. falciparum* isolates, P (two tailed) < 0.0001; t = 5.808; df = 16; R = 0.8236. For *P. vivax* isolates, P (two tailed) = 0.2722; t = 1.147; df = 13; R = 0.3031. The antibody significantly reduced rosetting rate of *P. falciparum* isolates.

4.5.3 Haematopoietic cell culture and CD236R knock down; Rosetting inhibition.

Although the above antibody Fab-mediated inhibition assays provide useful clues to the identity of the host rosetting receptor, such an approach is limited by potential off-target effects (such as steric hindrance or changes to membrane rigidity) (Bei & Duraisingh, 2012). To limit these unwanted off-target effects, a transgenic method to knockdown the expression of CD236R was employed to produce normocytes that are mostly deficient in this receptor (Figure 4.20A). Phenotyping of GFP positive and GFP negative cells (according to BRIC4 region recognition (Figure 4.17A)) showed a knockdown of 81.5% ± 2.5% in CD236R expression (Figure 4.20B). It should be noted that the method employed in this experiment was different from the one used recently in a previous study to knock down the expression of Glycophorin A (where piromycin and neomycin rather than GFP was used for the selection) (Bei *et al*., 2010; Bei & Duraisingh, 2012). Nevertheless, both methods have the same knock down efficiency. It is important to note that the erythrocytes generated from the CD236R knockdown had a normal phenotype in terms of seven other characteristic erythrocytic receptors (Figure 4.21).

Initially this work needed to be conducted in Singapore, thus the experiment had to rely on cryopreserved isolates of *P.vivax*. Of the three *P.vivax* isolates used only one showed very high levels of rosetting (>50%). Despite this limitation, differences in rosetting between the control normocytes (CD236R positive) and CD236R knockdown normocytes were immediately evident. After examination of 200 fields (1000x magnification) and counting 50 *P. vivax*-infected erythrocytes per treatment, only 4 of 50 were rosetting with CD236R knockdown erythrocytes compared to 27 out of 50 in the control (chi-square = 22.6, d.f = 1, $P < 0.0001$ [chi-square with Yates's correction]). Since usage of cryopreserved samples may raise confounding effects to the rosetting

results (Figure 4.8), the experiment was repeated in the Thai-Myanmar border by using three fresh isolates. It was found that the CD236R knockdown erythrocytes significantly reduced the formation of rosettes ($P < 0.001$) (Figure 4.20D). It is important to understand that although the cells were sorted on GFP, this protein has about 26 hours half-life in mammalian cells. Therefore, the signal gradually diminishes as the erythrocyte matures (Corish & Tyler-Smith, 1999). Consequently the normoblasts (Heilmeyer stage 0) and early reticulocytes (Heilmeyer stage I, II and III) have bright signals. Mature reticulocytes (Heilmeyer stage IV) and normocytes have little or no discernable signal (Figure 4.20C). An unintended benefit of this age related GFP signal output was that in the four rosettes observed in the CD236R knockdown, no rosette was associated with GFP positive cells (reticulocytes), thus further supporting earlier data on *P.vivax* rosette normocytic preference.

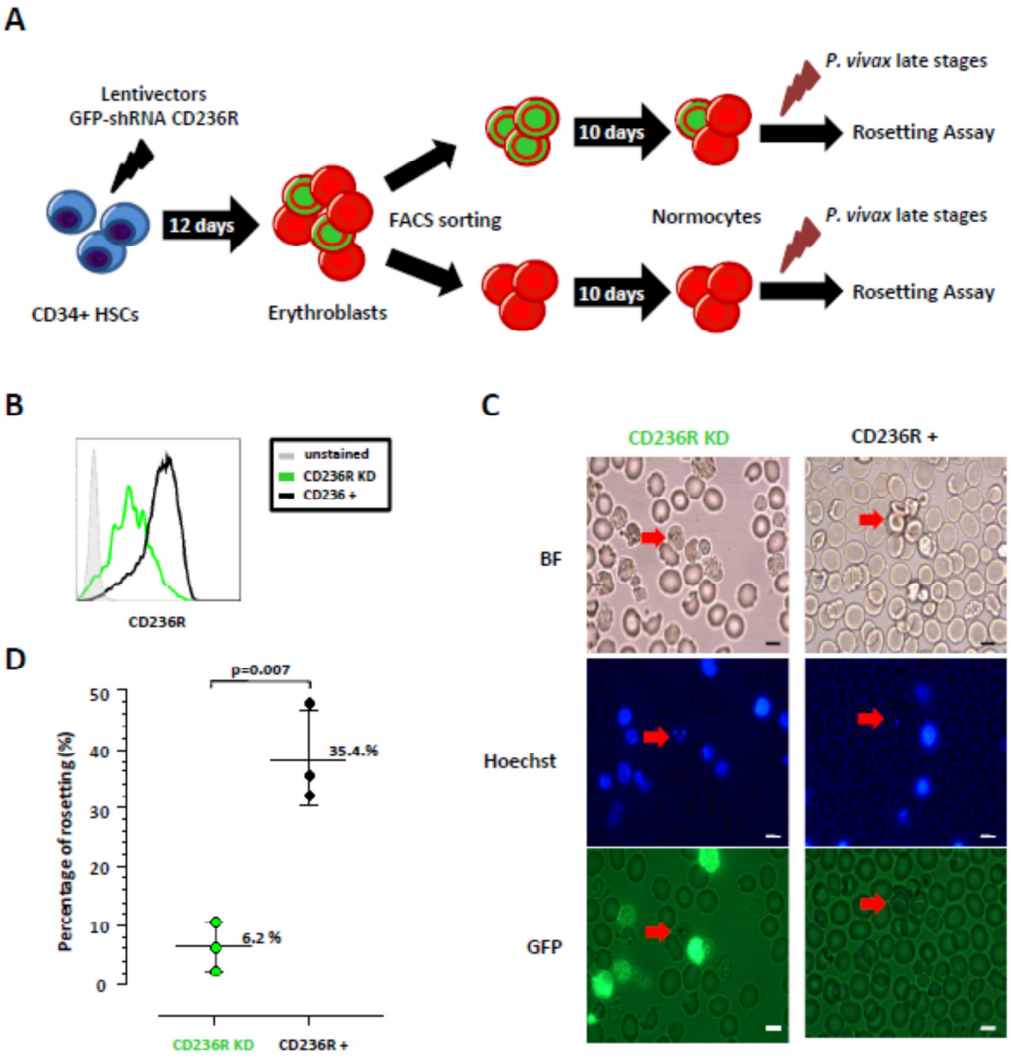

Figure 4.20 Transgenic approach to investigate the role of CD236R in *P. vivax* rosetting. Experimental design of *P. vivax* rosetting assay with cultured erythrocytes (cRBCs) generated from CD34+ haematopoietic stem cells (HSCs). The stable knockdown of CD236R (Glycophorin C) is obtained using Sigma® lentivector with GFP and short hairpin RNA (shRNA) against CD236R expression. The 2 subsets of cells: CD236R knockdown and CD236R+ cells were separated by flow cytometry using GFP expression one day before performing the rosetting assay with *P. vivax* schizont-infected erythrocytes that were isolated by magnetic sorting (A). Flow cytometry histograms showing CD236R expression in GFP positive cells (CD236R knockdown cells in green line) and GFP negative (CD236R+ cells in black line) and unstained cells

in grey line (B). Erythrocyte infected with *P. vivax* schizont forms rosette with CD236R knockdown (CD236R KD) and non-CD236R knockdown (CD236+) erythrocytes using bright field (BF), Hoechst and GFP detection (C). Rosetting rates of erythrocytes infected with *P. vivax* schizonts in the presence of CD236R knockdown cultured RBCs (cRBCs) and CD236+ cRBCs respectively (D). A significant difference in rosetting rates between the two types of cRBCs was found.

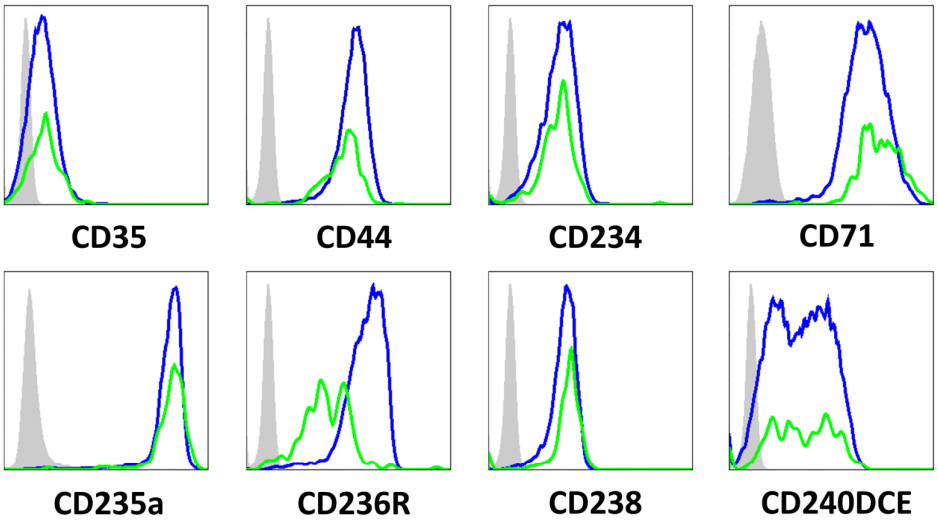

Figure 4.21 Flow cytometry histograms showing phenotypes of erythroblasts generated from cord blood CD34+ haematopoietic stem cells. Plots showing surface expression of erythrocytic markers in GFP positive cells (CD236R knock down cells in green line), GFP negative (CD236R+ cells in blue line) and unstained cells in grey line. This set of plots highlights that the CD236R knockdown cells have a normal phenotype in terms of seven other characteristic erythrocytic receptors (CD35, CD44, CD234, CD71, CD235a, CD238 and CD240DCE).

4.6 Anti-malarial drug challenge assay

Within the concentration range tested, after two hours of incubation, rosetting rates of both *P. falciparum* and *P. vivax* increased with the elevation of drug concentrations. For *P. falciparum* isolates, there were significant differences between the rosetting rate of control and that of chloroquine (from 192.97 ng/ml onwards) (Figure 4.22A), mefloquine (from17.53 ng/ml onwards) (Figure 4.22C), amodiaquine (from 9.41 ng/ml onwards) (Figure 4.22E), artesunate (from 2.38 ng/ml onwards) (Figure 4.22G), quinine (from 1262.64 ng/ml onwards) (Figure 4.22I), lumefantrine (from 28.75 ng/ml onwards) (Figure 4.22K), and methylene blue (from 1.25 ng/ml onwards) groups (Figure 4.22M); $P < 0.05$. For *P. vivax* isolates tested, there were significant differences between the rosetting rate of control and chloroquine (from 385.94 ng/ml onwards) (Figure 4.22B), mefloquine (from 70.13 ng/ml onwards) (Figure 4.22D), amodiaquine (from 9.41 ng/ml onwards) (Figure 4.22F), artesunate (from 4.75 ng/ml onwards) (Figure 4.18H), quinine (from 157.83 ng/ml onwards) (Figure 4.22J), lumefantrine (from 3.59 ng/ml onwards) (Figure 4.22L), and methylene blue (from 0.625 ng/ml onwards) groups (Figure 4.22N); $P< 0.05$. Prior to this experiment, a preliminary test with the anti-malarial drugs was performed on uninfected erythrocytes from three uninfected donors (with blood group A, B and O respectively). Clumping and crenation of the erythrocytes were not detected in any of the drug concentrations used. Similarly, during the study, crenated cells and clumping of uninfected erythrocytes were not found. This observation implies that the range of drug concentrations used in this study did not affect the osmolarity of the suspension and induce formation of artifacts that may affect the results of the study.

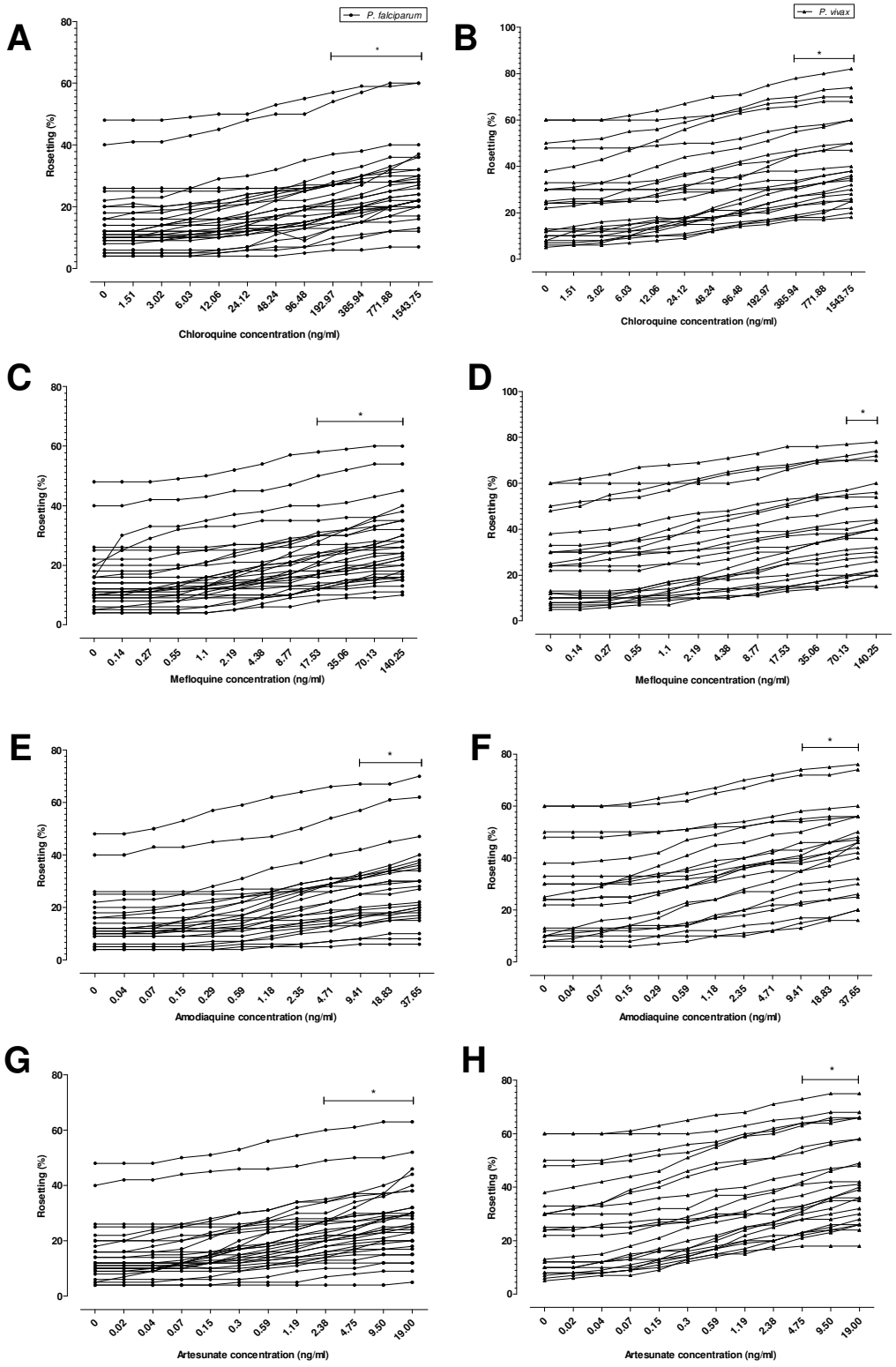

Figure 4.22 Plots showing changes of rosetting rate upon brief exposure to different drug concentrations.

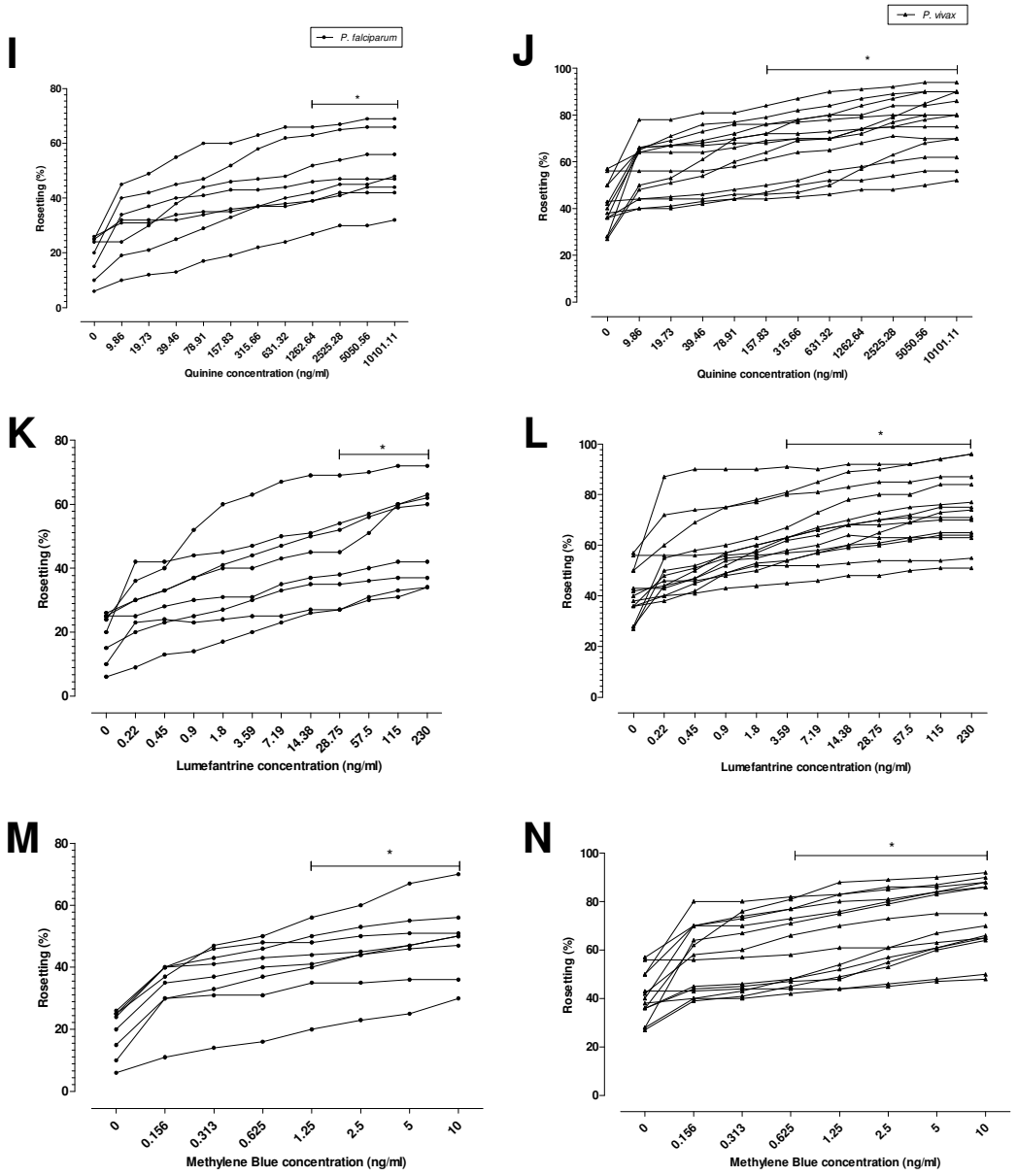

Figure 4.22, continued Plots showing changes of rosetting rate upon exposure to different drug concentrations. One way ANOVA with Dunn's multiple comparison test was performed to compare the rosetting rates after exposure to various drug concentrations with those of the control. Areas under * are the ranges of drug concentrations yielding rosetting rates that were significantly higher than those of the drug-free control ($P<0.05$).

4.7 Human white blood cell (WBC) challenge assay

For all the recruited *P. vivax* and *P. falciparum* isolates, parallel comparison of rosetting rate between the WBC-supplied and WBC-free cultivation systems was done. For each isolate, rosettes found in both cultivation settings showed similar size range (participating uninfected erythrocytes ranged from 2 to 25 cells) (Figure 4.23 A & B). No significant difference was found in comparison of giant rosette formation between the batch matured with WBCs and the batch matured without WBCs (Figure 4.24). Therefore, the size of rosettes was not influenced by the presence of WBCs. Besides, autoagglutination of infected erythrocytes was not found in all isolates tested. In addition, infected erythrocytes were rarely found to be attached to the WBCs.

From the recruited *P. vivax* isolates, the rosetting rates obtained from culture suspension with WBCs were significantly higher than those from WBC-free culture suspension; P (two tailed) <0.0001 (Figure 4.25). A similar trend was found on *P. falciparum* isolates as well; P (two tailed) <0.05 (P = 0.0212) (Figure 4.25).

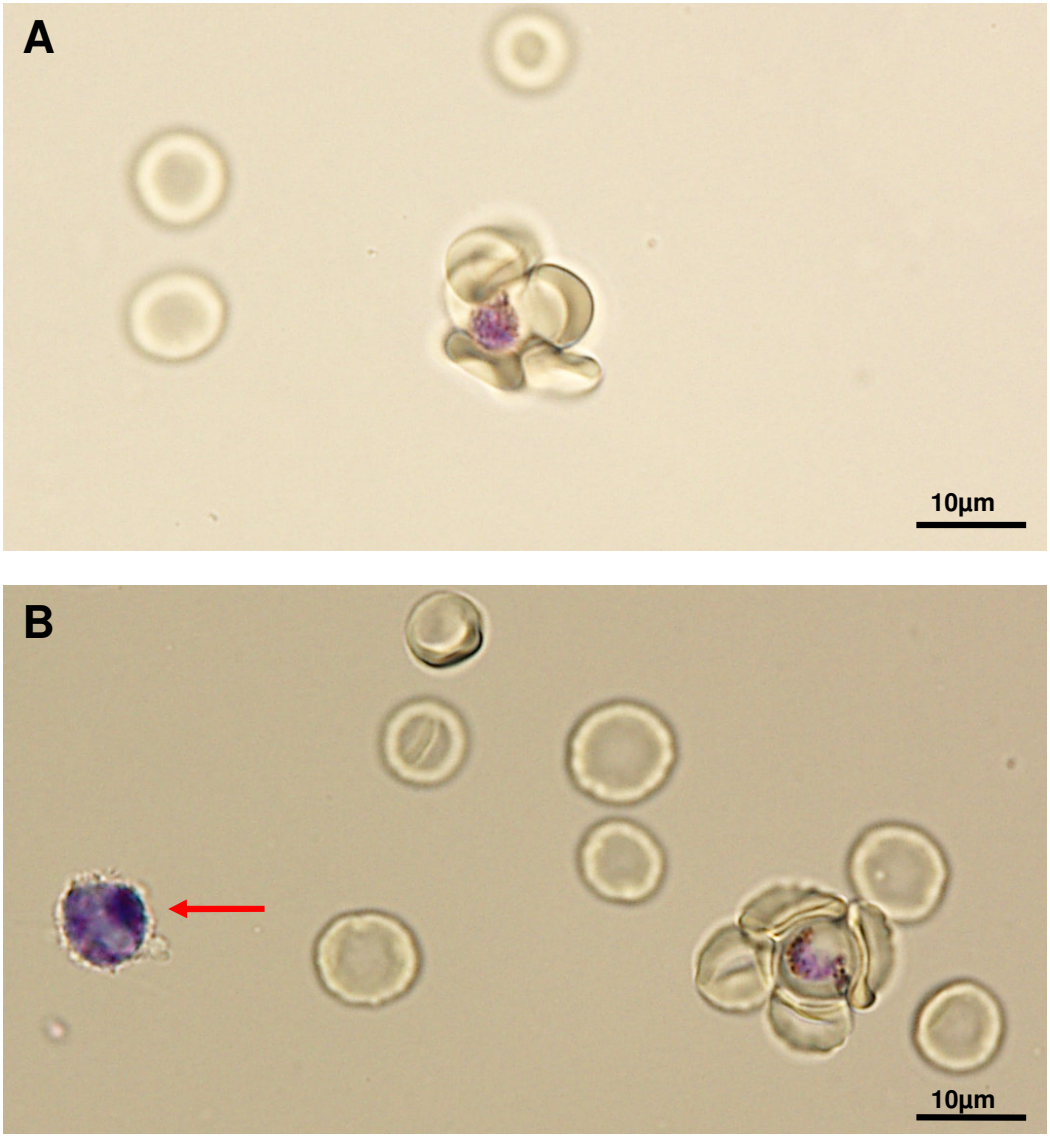

Figure 4.23 Rosettes formed by a *P. vivax* isolate matured *ex vivo* without WBCs (A), and in the presence of WBCs (red arrow) (B) respectively.

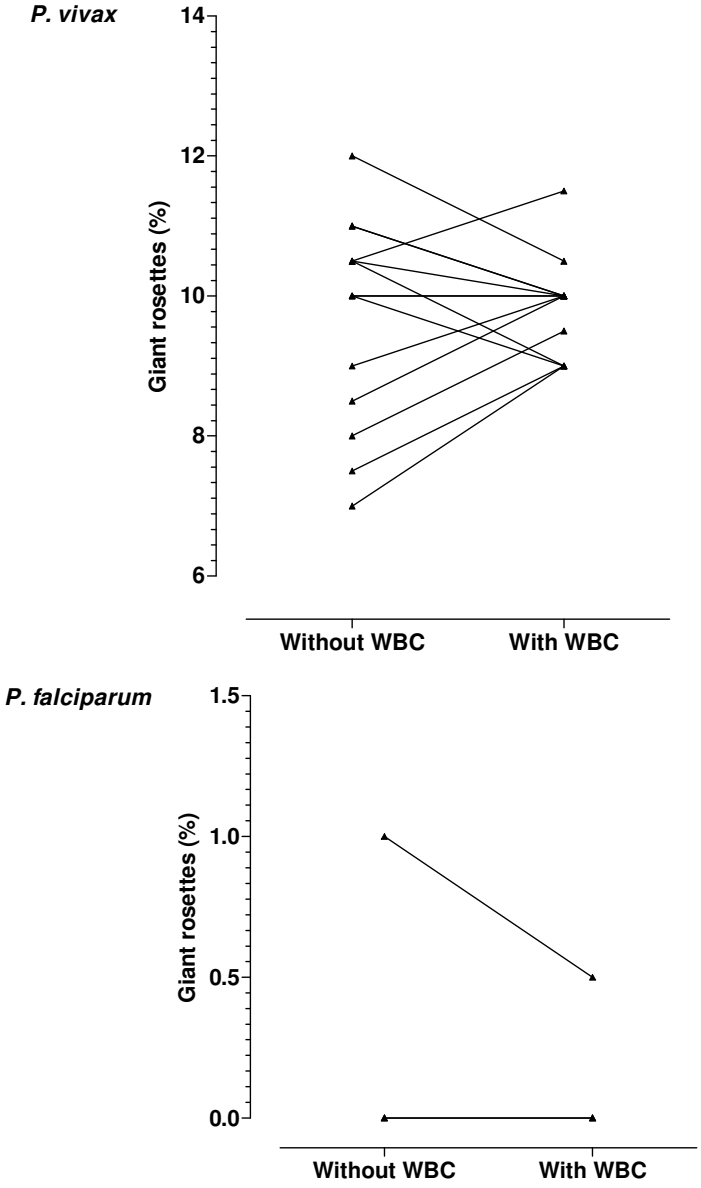

Figure 4.24 Plots showing giant rosettes found in recruited malaria isolates under WBC-free condition and condition with WBCs. Paired t-test was conducted. For *P. vivax* isolates tested, P (two tailed) = 0.6752; t = 0.4287; df = 13; R = 0.1181. For *P. falciparum* isolates recruited, P (two tailed) = 0.4226; t = 1.000; df = 2; R = 0.5773. No significant difference in the relative abundance of giant rosette formation between WBC-supplied setting and WBC-free setting for both *P. vivax* and *P. falciparum* isolates recruited.

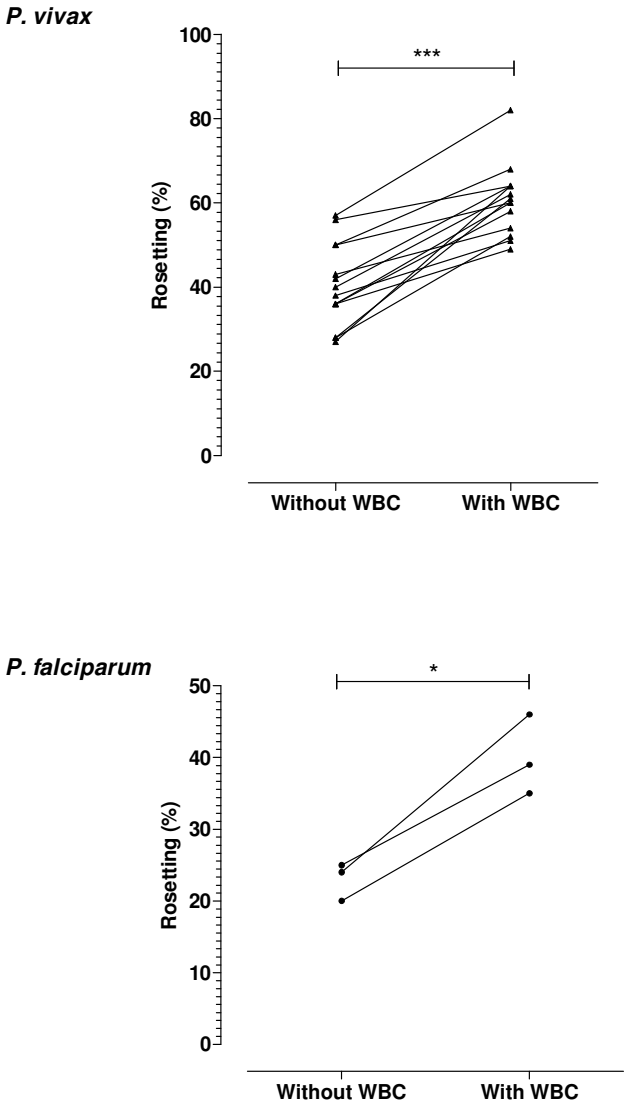

Figure 4.25 Plots showing rosetting rate comparison of malaria isolates that were subjected to *in vitro* maturation with WBCs and without WBCs. Paired t-test was conducted to study the comparison. For *P. vivax* isolates, P (two tailed) < 0.0001; t= 8.799; df = 13; R= 0.925. For *P. falciparum* isolates, P (two tailed) = 0.0212 (P < 0.05); t= 6.755; df= 2; R= 0.978. Rosetting rate was significantly increased with the presence of WBCs in both *P. vivax* and *P. falciparum* isolates.

DISCUSSION

5.1 Technique development for reticulocyte and rosette visualization

Approximately half the world's population reside in malaria endemic regions (Breman *et al*., 2004), and most of these areas suffer from severe limitations of health infrastructure. Therefore, clinical haematology in these regions is largely dependent upon differential stains and microscopy rather than flow cytometry or other automated methods used in developed countries. The most widely practiced method for reticulocyte counting involves smearing supravitally stained blood on slides prior to microscopic examination, rather than using the wet mounting method developed in this study. Unfortunately, reticulocyte assessment of NMB-stained smears is complicated by the presence of artifactual ribosomal matter resulting from current or past (due to splenic pitting) malaria parasite infection. Furthermore, erythrocytes containing the early 'ring' forms of *Plasmodium* spp. and degraded dead malaria parasites can easily be mistaken for reticulocytes on NMB smears.

These issues are not entirely solved with the use of flow cytometry methods. Although DNA-specific stains enable exclusion of parasitized erythrocytes from the analysis (Nunez, 2001), reticulocytes infected with malaria parasites (especially *P. vivax* merozoites post invasion) are also 'gated out' from the total reticulocyte population. Consequently, reticulocyte quantification of both methods may not reflect the actual haematological condition of malaria patients. Due to this problem, even though a field-based flow cytometer (BD Accuri^{TM} C6) was available, the flow cytometry comparison with this newly developed Giemsa-wet mount method was not done, since the presence of malaria parasites would significantly confound the results.

Application of wet mounting technique (NMB and Giemsa) kept malaria parasites alive during the examination period. The active movement of live parasites was easily differentiated from the static reticular matter of reticulocytes. Dead parasites were distinguished from reticulocytes based on the darker staining pattern of the latter. In most cases, parasitic and host reticular matters possess discernible differences in thickness, opacity, and refractive indices; light travelling through these entities is subjected to different degrees of optical interference. Such optical effects are further magnified by the liquid medium of wet mount, which has refractive index higher than that of air. However, such optical phenomena are not available on the dry smear. Hence, the optical differentiation of live parasites, dead parasites, and reticulocytes is best contrasted in wet mounting method. The preference for wet mount staining concurs with earlier work that used wet mounts of supravitally stained blood from vivax malaria patients to confirm the preference of *P. vivax* for reticulocyte invasion (Vryonis, 1939). While the results from the present research project show that wet mount preparation from both staining methods (NMB and Giemsa) provided almost identical reticulocyte counts, the Giemsa method is marginally preferable to NMB due to its low cost and longer shelf life. However, Giemsa requires longer staining time (15 minutes) than NMB (10 minutes). Based on this study, a 5% Giemsa staining formulation is recommended for the staining procedure.

Reticulocyte quantification using the Giemsa wet mount method has some limitations. A bright halo effect called spherical aberration may arise (Figure 5.1). This effect is especially pronounced during the usage of cover slips with thickness that is incompatible to the objectives of light microscope (Gibson & Lanni, 1992; Wan *et al*., 2000). Use of immersion oil with incompatible refractive index can also induce this optical effect (Gibson & Lanni, 1992; Wan *et al*., 2000). Nevertheless, this problem can

be alleviated by using an appropriate glass cover slip (0.17 mm thickness). Nikon immersion oil type A with refractive index of 1.515 at 23°C allowed optimal visualization with minimal spherical aberration. Another potential problem of the Giemsa-wet mount method is the cell movement that can impede cell counting. However, this problem can be solved by using the appropriate volume of suspension with glass cover slips of appropriate size in preparing the wet mounts. In this research project, it was found that 7.5 µl cell suspension of 5% haematocrit with cover slip of 22 x 32 mm (0.17 mm thickness) produced an even cell distribution with minimal cell movement. In addition, the wet mounts should not be examined on a slanted bench top. The wet mounts should be checked immediately after preparation, and counting must be performed as soon as possible. As the heat of the microscope light source accelerates water loss, cell crenation will be noted if the wet mount is examined for longer than 20 minutes. Riming the outside of the coverslip with Vaseline and storage in a cool dark place will allow for examination at a later time (6 hours after preparation).

The Giemsa-wet mount technique developed here was indeed a technique modified from a method described in a previous study on rosetting characterization (Chotivanich *et al*., 1998). The modified protocol applied in this research project enables optimal visualization of malaria parasites with minimal usage of laboratory resources (Lee *et al*., 2013b). Besides, the viable parasites can be kept alive while the cellular morphology of host erythrocytes and parasites are visualized and characterized. In addition, this improved formula enables longer investigation duration of the wet mount on microscope. Using 5% instead of 10% Giemsa staining formulation applied in previous study (Chotivanich *et al*., 1998), cellular crenation rate was much lower when the wet mount was observed under microscope.

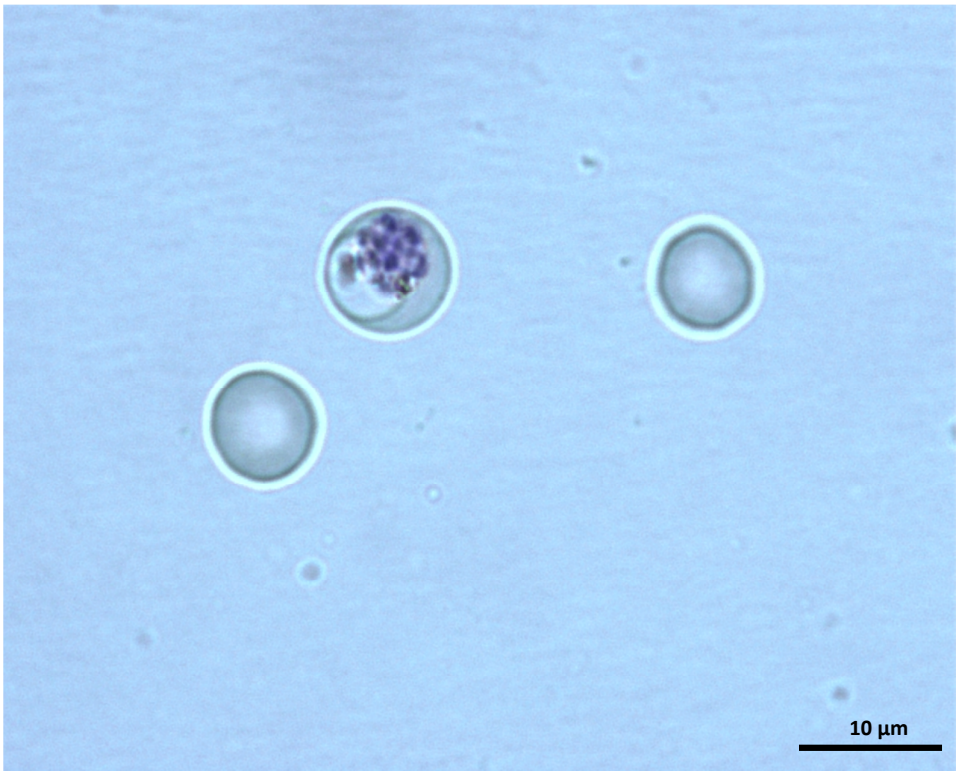

Figure 5.1 ***P. vivax*-infected and uninfected erythrocytes under wet mount prepared from suspension stained supravitally with Giemsa.** Spherical aberration, which is a "halo" rim effect around each erythrocyte, is clearly demonstrated in this picture.

5.2 Rosetting characterization of *P. vivax*

Similar to *P. falciparum*, rosetting phenomenon is found only after *P. vivax* matures into the early trophozoite stage. As the parasites require time to synthesize and transport various proteins onto the surface of the infected erythrocytes, it is not surprising to see such rosetting trend. Indeed, it has been shown that the rosetting ligand of *P. falciparum*, *Pf*EMP1, starts appearing on the surface of the infected erythrocytes when the parasite matures into late ring form, which concurs with the emergence of cytoadherence properties of *P. falciparum* (Gardner *et al*., 1996; Kyes *et al*., 2000; Kriek *at al*., 2003). Nevertheless, as mentioned earlier, the rosetting ligand of *P. vivax* has yet to be discovered.

Prior to this research project, gametocytes were believed to be unable to form rosettes (Lowe *et al*., 1998). Rosette formation of gametocytes was deemed illogical in the transmission cycle of malaria since gametocytes should be available freely in the peripheral circulation instead of posessing cytoadherence properties. Such belief was considered as a supporting point for the hypothetical role of rosetting in enhancing the asexual merozoite reinvasion (Lowe *et al*., 1998). In this research project, it has been shown that gametocytes of *P. vivax* and *P. falciparum* can be involved in rosette formation. Before going deeper into the discussion on this topic, it should be emphasized here that rosette formation and deep vein sequestration are two separate biological phenomena of malaria parasites, as highlighted in previous study (Adams *et al*., 2014). Therefore, they should not be regarded as mutually dependant events. Developing gametocytes of *P. falciparum* (stage I to IV), but not matured gametocytes (stage V), have been found to sequester in bone marrows (Smalley *et al*., 1981; Rogers *et al*., 2000). Since mature gametocyte-infected erythrocytes must be available in peripheral circulation in order to sustain the transmission cycle via *Anopheles* mosquito

bites, rosette formation may serve as a physical shield, protecting the infected erythrocytes from host's immuno-recognition and phagocytic destruction by the phagocytes. Nevertheless, this remains as a postulation and should be further verified with more experiments.

Giant rosettes were found more frequently in *P. vivax* than *P. falciparum* isolates. Such finding may arise from the difference in relative stability of parasite-derived rosetting ligands in anchoring onto the host erythrocyte membrane. The rosetting ligand of *P. falciparum* is securely anchored on the surface of uninfected erythrocyte membrane. Only uninfected erythrocytes that come into direct contact with the infected erythrocyte will involve in rosette formation. On the other hand, the rosetting ligands of *P. vivax* may be more loosely anchored on the surface of infected erythrocytes. As uninfected erythrocytes come into contact with the infected erythrocyte, the loosely bound ligands may be transferred onto the surface of uninfected erythrocytes, enabling another rim of uninfected erythrocytes to bind with the uninfected erythrocytes that are already involved in rosetting complex. The principle is analogically similar to the transfer of pollens from flower to bees. As the rosetting ligands are not tightly anchored on the erythrocyte membrane, they will not stay anchored on the surface of these uninfected erythrocytes for a very long time within circulation. Hence, the scenario where uninfected erythrocytes also form "rosette-like clumping" in *P. vivax* isolates was not seen.

Previously, a few studies reported that cryopreservation does not affect the rosetting capability of *P. falciparum* isolates (Kinyanjui *et al*., 2004; Kyriacou *et al*., 2007; Ribacke *et al*., 2013), and rosetting-related experiments were conducted with cryopreserved isolates (Vigan-Womas *et al*., 2008). These previous studies indicate that

the structural integrity of *P. falciparum* rosetting ligands is well preserved during the cryopreservation and thawing processes. On the other hand, the effect of cryopreservation on *P. vivax* rosetting was not studied. From this research project, it was found that the rosetting capability of cryopreserved *P. vivax* isolates was much lower than that of fresh *P. vivax* isolates of the same geographical origin. The integrity of certain surface proteins may not be well preserved during the cryopreservation and thawing processes. The ligands responsible for rosette formation of *P. vivax* may be one of such proteins. Consequently, the rosetting capability of cryopreserved *P. vivax* isolates is lowered. This discovery highlights another biological difference between *P. vivax* and *P. falciparum*.

Based on this study, rosetting prevalence of *P. vivax* is higher than that of *P. falciparum*, indicating that rosetting phenomenon is common among *P. vivax*. Such finding is contradictory to previous belief that *P. vivax* was one of the species with low rosetting capability (Sherman, 2005). However, the rosetting ability of *P. vivax* may vary from one geographical strain to another. Another point worth mentioning is the relatively higher rosetting rates shown by individual *P. vivax* isolates (2.00% to 61.00%; median = 24.5%) as compared to *P. falciparum* isolates (3.00% to 28.00%; median = 9.0%). Rosetting and endothelial cytoadhesion are the contributing forces towards deep vascular sequestration, which is believed to assist the matured parasites in immune-evasion (Berendt *et al*., 1994; Amante *et al*., 2010). Although *P. vivax* has been shown to preferably accumulate in certain organs such as lungs (Anstey *et al*., 2007; Jayavanth & Bock, 2007; Anstey *et al*., 2009), the extent of its cytoadhesion is much lower than that of *P. falciparum* (Carvalho *et al*., 2010). As a result, deep vascular sequestration in vivax malaria may not happen as frequently or extensively as that seen in falciparum malaria. Thus, the matured trophozoites of *P. vivax* need alternative strategies to escape

host's immune responses. Rosette formation may act as one of those immune-evasion strategies for the matured stages. This may be a reason that the rosetting rate median of *P. vivax* isolates is higher than that of *P. falciparum*.

Unlike a few previous studies from African countries and one recent report from Brazil that had found significant correlation between rosetting rate and clinical parameters such as patient's parasitaemia as well as different forms of severe malaria (Rowe *et al*., 1995; Rowe *et al*., 2002; Le Scanf *et al*., 2008; Doumbo *et al*., 2009; Marín-Menéndez *et al*., 2013), no significant correlation was found between rosetting rate and parasitaemia, or similarly, and peripheral reticulocyte count from this research project. Such discrepancy may reflect the virulence differences among parasites of different strains or geographical origins. More importantly, it is difficult to conduct a meaningful correlation study between rosetting rate and the clinical parameters prevalent at sample collection. As these clinical data are obtained in "snap-shot" mode, the actual, meaningful correlation between rosetting and clinical presentations may not be reflected from the collected data. Besides, haematological data such as peripheral reticulocyte count, haematocrit and platelet reading can be influenced by various confounding factors such as underlying disorders and nutritional problems, especially in places like Thai-Myanmar border (Stoltzfus, 2001; Kemmer *et al*., 2003; Boel *et al*., 2010), where sampling for this research project was conducted. In addition, disease severity on a patient depends on how prompt the patient attend to doctor for treatment. With various confounding factors, such correlation study is difficult to be implemented. Indeed, a previous study using clinical samples from Papua New Guinea reported no significant correlation between falciparum malaria severity and rosetting phenomenon (al-Yaman *et al*., 1995).

5.3 Elucidating rosetting receptors of *P. vivax* and *P. falciparum*

An important finding from this project is the lack of significant correlation between rosetting and human ABO blood groups among both *P. vivax* and *P. falciparum* isolates. The lack of significant correlation between rosetting rate and ABO blood groups in *P. vivax* isolates was similar to that reported previously (Chotivanich *et al*., 1998). This finding further confirms that human ABO blood group antigens do not play significant roles in rosetting of *P. vivax*. The lack of association between human ABO blood groups and CR1 with *P. vivax* rosetting should not be surprising as the ligand responsible for this interaction in *P. falciparum* (*Pf*EMP1) is absent from *P. vivax*. On the other hand, human ABO blood groups have been incriminated as one of the coreceptors for *P. falciparum* rosetting (Udomsangpetch *et al*., 1993; Rowe *et al*., 1995; Barragen *et al*., 2000; Rowe *et al*., 2007; Tekeste & Petros, 2010; Vigan-Womas *et al*., 2012). The contradictory finding from this research project may be due to several reasons.

Firstly, there are a few rosetting coreceptors for *P. falciparum*. The *P. falciparum* strains of the region under study may have evolved to prefer other antigen candidates over the human A or B blood group antigens as the rosetting coreceptors. This is analogically similar to the rosetting phenomenon of *P. falciparum* VarO strain, which is CR1-independent but ABO-dependent (Vigan-Womas *et al*., 2012). The ABO blood group polymorphisms of the human population may be another contributing factor to the different finding yielded from this project. For instance, there are different subgroups for blood group A alone (Poskitt & Fortwengler, 1974), and some of these subgroups have been demonstrated to show varied affinity to the DBL1a1-CIDR1c double domain of *Pf*EMP1, the rosetting ligand of *P. falciparum* (Vigan-Womas *et al*.,

2012). Nevertheless, genetic characterization of the human population is beyond the scope covered by this research project.

In this research project, rosetting phenomenon of *P. vivax* was found to be independent of CR1. Once again, this finding shows that the biological properties of *P. vivax* are not entirely the same as *P. falciparum*. The efforts to shortlist novel receptors mediating *P. vivax* rosetting were aided by findings from preliminary studies, in which *P. falciparum* rosetting is mediated by CD236R (Glycophorin C) in a *Pf*EMP1-independent manner (Prof. Dr. Peter Preiser, personal communication). In this research project, by specifically blocking the BRIC 4 region (amino acid residues 2 – 21) of CD236R, rosette formation of *P. falciparum* and *P. vivax* was significantly inhibited. As most of the BRIC 4 region is located in segment one of CD236R (Figure 4.15A), it is unlikely that the Gerbich and Yus phenotypes (which cause major deletions at exon 2 and 3) will have any effect on rosetting capability. It is interesting to note that enzyme treatment of erythrocytes with trypsin completely removes two of the extracellular domains of CD236R (segment 1 and 2) (Figure 4.15A), resulting in complete abrogation of rosetting in *P. vivax* (Russell *et al*., 2011).

To further confirm the importance of CD236R as a key player in *P. vivax* rosetting, expression of CD236R on normocytes was blocked using knock down experiment with haematopoietic stem cells. Rosetting assays using these CD236R-knock down erythrocytes clearly showed a significant reduction of rosetting phenomena in *P. vivax* as compared to normocytes (from the same culture) that expressed CD236R. One problem with the idea that CD236R is important to rosetting, is that presentation of this receptor is decreasing during reticulocyte maturation to normocyte, albeit still highly expressed in both erythrocyte subsets (Malleret *et al*., 2013). As *P. vivax* does

not rosette well with reticulocytes, one has to question what prevents the interaction between reticulocyte CD236R with the yet to be discovered *P. vivax* rosetting ligands. Based on previous study on characteristics of reticulocytes (Malleret *et al*., 2013), it is possible that the rigid biochemical properties of reticulocytes (reticulocytes are stiffer than normocytes) inhibit close bond formation with the *P. vivax*-infected erythrocytes. Close examination of *P. vivax* rosetting images (especially in Figure 4.5B and 4.12A) reveals the tight association between normocytes and infected erythrocytes (indicated by the flat face of the biconcave disk to the infected erythrocyte). The globular and stiff reticulocytes prevent intimate contact with the infected erythrocytes. Furthermore, attempt to promote rosetting by manipulating reticulocytes into direct contact with the *P. vivax*-infected erythrocytes using micropipette technique failed to induce rosetting (Lee *et al*., 2014).

Another point worth mentioning is the significant reduction, instead of complete inhibition of rosetting by the anti-CD236R clone BRIC4 Fab and CD236R-knock down erythrocytes. The results from both experiments indicate that the BRIC4 region of CD236R plays significant role as one of the coreceptors in rosette formation of *P. falciparum* and *P. vivax*. Nevertheless, it is definitely not an exclusive rosetting receptor in any of the two species studied.

5.4 The biological role of *P. vivax* and *P. falciparum* rosetting

To date, the roles of rosetting in pathogenesis of malaria remain to be validated. The question that remains to be answered for this biological phenomenon is whether it confers and advantage to the intra-erythrocytic parasites. The results obtained in this research project are inadequate to provide a definite answer to this question. Nonetheless, the collected data have shown that rosetting does not directly facilitate

reinvasion of *P. vivax* merozoites upon rupture of schizonts, backed by two lines of evidence.

Firstly, *P. vivax* infected erythrocytes preferentially forms rosettes with normocytes and not reticulocytes. If rosetting was to significantly facilitate merozoite reinvasion, the erythrocytes infected with parasites of mature stages should preferentially adhere to uninfected reticulocytes, which would subsequently improve the invasion success rate by granting proximate and immediate access to reticulocytes, which are relatively scarce in the peripheral circulation. However, observations of naturally occurring rosettes from fresh isolates and reticulocyte enrichment study in this research project clearly show that rosettes favour normocytes instead of reticulocytes. Since *P. vivax* merozoites exclusively invade reticulocytes, there would be little, if no advantage for *P. vivax*-infected erythrocytes to adhere to normocytes, which are not receptive to reinvasion. On the other hand, *P. falciparum* merozoites can invade both reticulocytes and normocytes. Therefore, its preferential rosette formation with normocytes does not rule out the possibility that rosetting may enhance reinvasion success of *P. falciparum* merozoites. Nevertheless, previous studies have shown that the rosette-forming strains of *P. falciparum* have no invasion advantage over the non-rosette forming strains (Clough *et al*., 1998; Deans & Rowe, 2006). Furthermore, unpublished data (Asst. Prof. Dr. Bruce Russell, personal communication) from an earlier study on *P. vivax* invasion showed that the rosetting rate within an isolate bears no correlation with invasion success.

The second line of evidence against the "rosette-assisted invasion" hypothesis is that *P. vivax* and *P. falciparum* start rosetting at early trophozoite stage, which is at least 24 hours prior to the release of merozoites from the ruptured schizont. While some may

suggest that the developing parasite is getting an "early warm up" on the enrolment of receptive erythrocytes, such an effort would be futile as the shear forces within host's circulation and physical barriers such as the splenic red pulp would disrupt and dissociate rosettes numerous times within the 24 hours-time interval between the initiation of rosetting and schizont rupture. Perhaps even more convincingly, the gametocytes of *P. vivax* and *P. falciparum* have been observed to be involved in rosette formation as well. This particular finding is contrary to previous report (Lowe *et al*., 1998), which backed the "rosette-assisted invasion" hypothesis with the finding that the sexual stages were excluded from rosette formation.

Clearly, the data from this research project, coupled with data from previous studies suggest that rosetting does not directly assist in targeting or invasion of malaria parasites into the uninfected host erythrocytes. Therefore, another postulated role of rosetting as a "shield" for immune response evasion and protection against harmful substances may be the more likely role of rosetting in malaria pathogenesis (Wahlgren *et al*., 1989). Indeed, results from two experiments of this research project (the anti-malarial drug and human WBCs challenge studies) seem to support this "rosetting immune-evasion hypothesis". From the experiments conducted, rosetting rates of *P. falciparum* and *P. vivax* isolates were elevated with the presence of anti-malarial drugs and human WBCs.

In the presence of "threats" such as chemicals (e.g. anti-malaria drugs) or host's WBCs, the *Plasmodium*-infected erythrocytes may be stimulated to form rosettes. The rosette may act as a primitive protective shield. Rosette formation will hinder phagocytosis of the infected erythrocyte by the host's phagocytes. Besides, rosette may slow down the harmful substances from reaching the infected erythrocytes. In view of

such “rosetting-reflex” by the presence of WBCs, rosetting may contribute more significantly towards mechanical vascular occlusion *in vivo* for falciparum malaria cases, along with deep vein sequestration.

Based on the results, it is postulated that rosette formation may be an intrinsic, reflex-like response by the malaria parasite upon encounter of life-threatening entities. Such mechanism may be intended as a “shield-like” protective measure for the parasite, albeit not a completely effective one. The findings from this research project serve as preliminary evidence for this rosetting role. For future studies, other non-anti-malarial drug compounds can be tested. Besides, parasite strains that are not forming rosettes under normal physiological condition such as the *P. falciparum* HB3-HBEC and FCR3-CSA strains (Adams *et al*., 2014) can be used for further investigation of this aspect.

Prior to this research project, there were only a few studies regarding effects of anti-malaria drugs or other chemical compounds on rosetting and none of these used *P. vivax* (Udomsangptech *et al*., 1996; Goldring *et al*., 1999; Kyriacou *et al*., 2007). It is interesting to point out that the results obtained from the anti-malaria drug exposure assays of this research project were different from those of previous studies conducted on *P. falciparum* (Udomsangptech *et al*., 1996; Goldring *et al*., 1999). Such discrepancy may arise from the difference in research design. In this study, the infected erythrocytes were matured until at least 70% of the parasite population reached late trophozoite stage. After that, the culture suspension was transferred into the drug plates and incubated for two hours prior to rosetting assay. Since rosetting is noted to develop optimally at the mature erythrocytic stages, incubation of the parasites with drugs after maturing them allows assessment of the effect of drugs on rosetting in a relatively “parasite growth-

independent" manner. In other words, any observed effect of drugs on rosetting was not due to arrested parasite's growth by the drugs.

In one of the previous studies (Udomsangpetch *et al*., 1996), infected erythrocytes were incubated with the drugs for a range of 2-24 hours prior to drug-free *in vitro* maturation for another 24-30 hours. Subsequently rosetting development was evaluated along the parasite's erythrocytic maturation. Such design may inhibit synthesis machinery of many important proteins within the parasites during the early erythrocytic stages, hence causing inhibition of rosette formation. As mentioned in previous section, *Pf*EMP1, the rosetting ligand for *P. falciparum*, is noted to appear on the surface of infected erythrocytes from the late ring stage onwards (Gardner *et al*., 1996; Kyes *et al*., 2000; kriek *et al*., 2003). Therefore, if the protein synthesis of the parasite is arrested at its early developmental stage, various biological activities of this parasite will be subsequently inhibited. Another study (Goldring *et al*., 1999) applied method similar to that used in this study, albeit with longer incubation period (4 hours). Nevertheless, the drug concentrations applied in previous studies were much higher than those used in this study. For example, the lowest mefloquine concentration used by Goldring *et al*. was 200 ng/ml (Goldring *et al*., 1999), whereas the highest mefloquine concentration used in this study was 140.25 ng/ml. The mefloquine concentration used by Udomsangpetch *et al* in their *in vitro* study was 250 ng/ml (Udomsangpetch *et al*., 1996). The electrical charges carried by the drug molecules may affect the rosetting efficiency of the infected erythrocyte when the drug concentration is higher than a certain threshold. Nevertheless, this effect of electrical charges of a compound on *Plasmodium* rosetting remains to be validated.

CONCLUSION

From this research project, a novel technique for visualization and characterization of reticulocytes and malaria-infected erythrocyte rosettes was successfully developed. This convenient and economical technique provides a simple and accurate way of conducting reticulocyte characterization on haemoprotozoa-infected blood samples and malaria rosette related experiments.

Rosetting phenomenon of *P. vivax* has been studied and characterized. Rosette formation is commonly seen in *P. vivax*, where its development along the parasite erythrocytic maturation resembles that of *P. falciparum*. Nevertheless, *P. vivax* rosetting poses several differences from *P. falciparum* rosetting. Giant rosettes are more commonly found in *P. vivax* compared to *P. falciparum*. In addition, gametocytes of *P. vivax* and *P. falciparum* are found to be involved in rosette formation as well. Unlike *P. falciparum*, cryopreservation affects the rosetting capability of *P. vivax* isolates.

Human ABO blood group-independent rosetting in *P. falciparum* and *P. vivax* isolates from the Thai-Myanmar border shows that rosetting phenomenon is a multi-factorial biological event that involves multiple receptors and varies from one geographical strain to another. Unlike *P. falciparum*, *P. vivax* rosetting is independent of CR1. The BRIC 4 region of CD236R is the newly found rosetting coreceptor for *P. vivax* and *P. falciparum*. As with *P. falciparum*, there is likely to be more than one host receptor involved in *P. vivax* rosetting. This finding contributes to the search of *P. vivax* rosetting ligand.

The almost exclusive preference for normocytes in rosetting indicates that rosetting does not directly assist merozoite reinvasion of *P. vivax*. Instead, rosetting is

more likely to play a role as protection for the parasitized erythrocytes against external harms such as the host's WBCs and anti-malarial drugs. The high frequency of rosetting in patient isolates suggests that it confers *P. vivax* with a survival advantage.

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APPENDICES

Appendix 1 5% Giemsa- wet mounting technique

- Blood cell suspension of 5% heamatocrit (cell density) is prepared using PBS (incomplete McCoy's and RPMI medium can be used as alternatives to PBS).
- For 95 µl of cell suspension, 5 µl of filtered Giemsa stock is added, and stained for 15 minutes.
- 7.5 µl of the stained suspension is dropped onto a pre-cleaned glass slide, and covered with glass coverslip (22 x 32 mm).
- The wet mount is examined with light microscope using oil immersion magnification.

Appendix 2 NMB reticulocyte staining protocol

- 1 volume of blood is pipette into a microcentrifuge tube.
- 1 volume of NMB stain stock is pipette into the blood, and mixed well.
- The blood suspension is stained for 15 minutes.
- For traditional smearing method, a thin smear is prepared from the stained blood.
- For wet mounting method, a 7.5 µl of this stained suspension is used for wet mount preparation.

Appendix 3 Malaria parasite thawing

- Cryopreserved blood vial is thawed in 37°C water bath.
- The volume of cryopreserved sample is measured (volume = X).
- 0.2 X volume of 12 % NaCl solution is mixed with the blood slowly, on drop by drop basis.
- The well- mixed suspension is left for 5 minutes.
- 10 X volume of 1.6 % NaCl solution is mixed with the suspension slowly, on drop by drop basis.
- The suspension is centrifuged at 1800 rpm for 5 minutes.
- Supernatant is removed.
- 10 X volume of 0.9 % NaCl solution is mixed with the suspension, on drop by drop basis.
- The suspension is centrifuged again at 1800 rpm for 5 minutes.
- Supernatant is removed.
- The volume of packed red blood cells is measured for further usage.

Appendix 4 Malaria parasite cryopreservation

- The volume of packed parasite- infected blood cells is measure (volume = X).
- 0.33 X volume of glycerolyte is mixed with the blood cells slowly, on drop by drop basis.
- The mixture is left for 5 minutes.
- 1.3 X volume of glycerolyte is mixed with the suspension slowly, on drop by drop basis.
- The mixture is then transferred into cryopreservation vials (around 0.8 – 1.0 ml each vial).
- These vials are placed into a polystyrene box and sealed properly.
- The box of vials is then left in -80°C freezer overnight.
- The frozen vials are transferred into liquid nitrogen tank for long time storage.

Appendix 5 Concentration technique of erythrocytes infected with *Plasmodium* ring forms

- Infected blood is filtered using CF 11 column.
- Blood smear examination is performed.
- If the parasite population consists of more than 50 % ring forms, this technique can be applied.
- The blood sample is centrifuged at 1800 rpm for 5 minutes.
- Excessive supernatant is removed and only 5 ml of supernatant is kept.
- The blood is then mixed well with the left supernatant.
- The 5 ml suspension is then added slowly onto the 75 % Percoll as gently as possible.
- The sample is centrifuged at 2800 rpm for 15 minutes.
- The ring band is collected.
- The efficiency of concentration is then confirmed with a blood smear examination.
- If the yield is successful, the packed red blood cells is then cryopreserved.

Appendix 6 Concentration technique of reticulocytes from cord blood

- Cord blood obtained from donors can be kept for as long as l month prior to this technique application.
- Blood is filtered with CF 11 column twice.
- The filtered blood can be kept in pure Mc Coy's medium at 4°C before further processing.
- 5 ml blood of 50 % hematocrit value is prepared.
- This 5 ml blood is mixed with 6 ml of 65% Percoll in a Falcon tube as gently as possible.
- The mixture is centrifuged at 2800 rpm for 15 minutes.
- The ring band is collected and resuspended in Mc Coy's medium.
- The suspension is centrifuged again at 1800 rpm for 5 minutes.
- Supernatant is removed, and blood cell pellet is collected.
- A blood smear from this pellet is examined.
- The reticulocyte harvest is considered good if the reticulocytes accommodate at least 30 % of the whole population.
- The pellet can then be resuspended in 5 ml of plain McCoy's medium.
- If the reticulocyte harvest is not as good, the concentration technique can be repeated by combining several low grade harvests prior to the concentration technique.

Appendix 7 AB serum preparation

- Blood is drawn from donors with AB blood group, and collected in sterile 50 ml Falcon tubes.
- The blood is left at room temperature overnight to ensure complete coagulation of blood.
- Coagulated blood is centrifuged for 10 minutes at 1800 rpm.
- Serum is extracted with Pastuer pipettes and collected in new sterile Falcon tubes.
- Transferred serum is heat inactivated at 56 °C for 1 hour.
- Heat inactivated serum is then aliquoted accordingly into collecting tubes (10 ml each).
- The tubes are sealed.
- The serum is stored in -20 °C freezer.

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Giemsa-Stained Wet Mount Based Method for Reticulocyte Quantification: A Viable Alternative in Resource Limited or Malaria Endemic Settings

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Abstract

The quantity of circulating reticulocytes is an important indicator of erythropoietic activity in response to a wide range of haematological pathologies. While most modern laboratories use flow cytometry to quantify reticulocytes, most field laboratories still rely on 'subvital' staining. The specialist 'subvital' stains, New Methylene Blue (NMB) and Brilliant Crésyl Blue are often difficult to procure, toxic, and show inconsistencies between batches. Here we demonstrate the utility of Giemsa's stain (commonly used microbiology and parasitology) in a 'subvital' manner to provide an accurate method to visualize and count reticulocytes in blood samples from normal and malaria-infected individuals.

Citation: Lee W-C, Russell B, Lau Y-L, Fong M-Y, Chu C, et al. (2013) Giemsa-Stained Wet Mount Based Method for Reticulocyte Quantification: A Viable Alternative in Resource Limited or Malaria Endemic Settings. PLoS ONE 8(3): e60303. doi:10.1371/journal.pone.0060303

Editor: Fabio T. M. Costa, State University of Campinas, Brazil

Received January 11, 2013; **Accepted** February 25, 2013; **Published** April 2, 2013

Funding: This study received financial support from the following funds: WCL, YLL, and MYF were supported by University of Malaya, High Impact Research Fund (E000051-20001). BR, RS, and LR were supported by funding from SIgN and from the Horizontal Programme on Infectious Diseases under the Agency for Science, Technology and Research (A*STAR, Singapore). KS, CC, and FN were sponsored by The Wellcome Trust of Great Britain, as part of the Oxford Tropical Medicine Research Programme of Wellcome Trust-Mahidol University. LR is an adjunct professor in the Department of Parasitology, University of Malaya, Kuala Lumpur, Malaysia. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Bruce Russell, Laurent Renia, and Francois Nosten are all Academic editors of PLOS ONE. The authors agree to abide by the rules, ethical standards, and practices of PLOS; furthermore this does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Patient reticulocyte profiles provide important data on erythropoietic activity, reticulocyte release into peripheral circulation and erythrocyte maturation rate. Rapid and objective reticulocyte counts by way of flow cytometry (using florescent stains such as Thiazole Orange) [1–2] has largely replaced microscopic examination using 'subvital' ——reticulocyte stains such as New Methylene Blue (NMB) and Brilliant Crésyl Blue [2–4]. However, the use of flow cytometry in developing countries and field laboratories is problematic due to the high cost of the equipment, limited access to servicing and unreliable power supply [5]. Perhaps the most significant confounder facing flow cytometry performance in the developing world is the possibility that a patient sample contains intraerythrocytic malaria parasites [6]. Therefore reticulocyte counts conducted in laboratories of malaria endemic areas are still reliant on the traditional microscopic method of subvital staining with a commercial NMB solution. Unfortunately, NMB is a specialist stain with limited shelf life and inconsistent resupply. Moreover, NMB stains produced by different manufacturers as well as inter-batch variations can yield significant inconsistency in reticulocyte identification [7]. Therefore, there is a need to develop an alternative staining methodology for microscopic reticulocyte quantification that is inexpensive and accurate. One of the most commonly encountered stains in the field is Giemsa's stain (Giemsa). This inexpensive purple stain is used for a range of histological and microbiological applications (i.e. identification of *Chlamydia* spp., Spirochetes and Trypanosomes) as well as its important use in malaria diagnosis. Here we compare a wet mounting method for subvitally stained Giemsa and NMB for the detection and quantification of reticulocytes in a malaria endemic field setting.

Materials and Methods

Blood sample collection, processing and ethics

Blood samples from infected and uninfected donors were collected after obtaining written informed consent following ethical guidelines in the approved protocols; OXTREC 027-025 (University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, UK) and MUTM 2008-215 from Ethic committee of Faculty of Tropical Medicine, Mahidol University; with specific provision for the samples used in this study. Blood samples were collected into the BD Vacutainer® with Lithium Heparin Anticoagulant. All samples were processed freshly upon collection or stored at 4°C for not more than 24 hours prior to processing.

In earlier studies we determined that reticulocyte counts were not affected by wet mounting of NMB subvital stained blood samples relative to the standard smearing of the same sample, thus

allowing the direct comparison of the NMB-wet mount method with the Giemsa-wet mount method, as detailed below.

NMB wet mount method

A 50 µl blood samples were subvitally stained with 50 µl NMB (Cat#R4132; Sigma-Aldrich®)After incubation over a period of 10 minutes at room temperature, the stained blood cells were centrifuged and the pellet was resuspended at 5% hematocrit with plain McCoy's 5A medium (RPMI medium can be substituted). Subsequently 7.5 µl of the stained suspension was dropped onto a glass slide and then covered by a 22×32 mm (0.17 mm thickness) glass cover slip.

Giemsa wet mount method

Another 50 µl of each unstained blood sample was stained subvitally with 2.5 µl of filtered Giemsa (Cat#G4507; Sigma-Aldrich®.) for 15 minutes at room temperature, followed by gentle mixing. The wet mount was prepared as described above for the NMB.

Reticulocyte counting

All blood smears and wet mounts were examined immediately with light microscope under oil immersion magnification. Reticulocyte percentage number was determined by counting the number of reticulocytes per 1000 erythrocytes [8].

Statistical Analysis

Comparison of reticulocyte counts (percentage of reticulocytes in 1000 erythrocytes counted) between the NMB-wet mount method and Giemsa-wet mount method was performed using Pearson correlation and Bland-Altman analysis [9]. The relationship between the mean percentage reticulocyte count and parasite species (*P. vivax* and *P. falciparum*) were compared with an unpaired Student's t-test. Welch's correction was not necessary for this t-test as the F- test showed that the variances were not significantly different ($F = 1.4$, $p = 0.27$)

Results

A total of 103 malaria patient blood samples were tested. Of these, 69 samples were from *Plasmodium vivax* infections, 32 cases of *Plasmodium falciparum*, 1 mixed infection of *P. vivax* and *P. falciparum*, and 1 case of *Plasmodium malariae* infection. The highest reticulocyte count based on the average of the two methods was 6.45%, the lowest being 0.07%.

The stained precipitated reticular matter of the reticulocytes was clearly evident using either NMB (Fig 1A) or Giemsa (Fig 1B) wet mounting methods, however the latter method produced a lighter staining pattern. Importantly both methods enabled the clear differentiation between reticular and parasitic matter (Fig 1C and D). It is notable that the NMB produced a non-specific colouration (background stain) in the all the erythrocytes irrespective of their infection status or age (Fig 1A and C). The mean reticulocyte count in the *P. vivax* samples (1.3%+/−SD 1.05) was not significantly different to the *P. falciparum* isolates (1.57%+/−SD 0.88)($p = 0.19$).

Both the NMB and Giemsa methods were significantly correlated (Pearson $R = 0.97$, 95% CI 0.8799 to 0.9934, $r^2 = 0.96$, $p<0.0001$) (Fig 1E). Bland-Altman analysis indicated a good agreement between the two methods (95% limit of agreement −0.30% and 0.54%) with no significant pattern of bias (Bias = 0.05 SD of Bias 0.2) (Fig 1F),

Discussion

Approximately half the world's population resides in malaria endemic regions [10], and most of these areas suffer from severe limitations to health infrastructure. Therefore, clinical haematology in these regions is largely dependent on differential stains and microscopy rather than flow cytometry methods used in developed countries. The most widely practiced method for reticulocyte counts involves smearing subvitally stained blood on slides prior to microscopic examination, rather than using the wet mounting methods developed in this study. Unfortunately, reticulocyte assessment of NMB stained smears is complicated by the presence of artifactual ribosomal matter resulting from current or past (due to splenic pitting) malaria parasite infection. Furthermore, erythrocytes containing the early 'ring' forms of *Plasmodium* spp. and the degraded dead malaria parasites can be easily mistaken for reticulocytes on NMB smears. These issues are not entirely solved with the use of flow cytometry methods. Although DNA-specific stains enable exclusion of parasitized erythrocytes from the analysis [11], reticulocytes infected with malaria parasites (especially *P. vivax* merozoites post invasion) are also 'gated out' from the total reticulocyte population. Consequently, reticulocyte quantification from both methods may not reflect the actual haematological condition of malaria patients. So, although we had access to a field based flow cytometer (BD Accuri™ C6) we decided against a thiazole orange comparison with our Giemsa method; as we were concerned that malaria parasites would significantly confound the results.

Our use of the wet mounting method (NMB and Giemsa), kept viable malaria parasites alive during the examination period. The active movement of living parasites were easily differentiated from the static reticulate matter of reticulocytes. Dead parasites were differentiated from reticulocytes based on the darker staining pattern of the latter. In most cases, parasitic and host reticular matter possess a discernible difference in thickness, opacity, and refractive indices; light that has travelled through these entities is subjected to different degrees of optical interference. Such optical effects are further magnified by the liquid medium of wet mount, which has refractive index higher than that of air. However, such optical phenomena are not available on the dry smear. Hence, the optical differentiation of living parasites, dead parasites, and reticulocytes is best contrasted in wet mounting method. Certainly, our preference for wet mount staining concurs with earlier work that used wet mounts of subvitally stained blood from vivax malaria patients to confirm the preference of *Plasmodium vivax* for reticulocyte invasion [12].

While our results show that wet mount preparation from both staining methods (NMB and Giemsa) provided almost identical reticulocyte counts, the Giemsa method is marginally preferable to NMB due to its low cost and longer shelf life. However, Giemsa requires longer staining time (15 minutes) than NMB. Based on this study, a 5% Giemsa solution is recommended for the staining procedure.

Reticulocyte quantification with the Giemsa wet mount method has some limitations. A bright halo effect called spherical aberration may arise using this method. This optical effect is especially pronounced during the usage of cover slips with thickness that is incompatible to the objectives of light microscope [13–14]. Usage of immersion oil with incompatible refractive index can also induce this optical effect [13–14]. Nevertheless, this problem can be alleviated by using an appropriate glass cover slip (0.17 mm thickness). Nikon immersion oil type A with refractive index of 1.515 at 23°C allowed an optimal visualization with minimal spherical aberration. Another potential problem of the

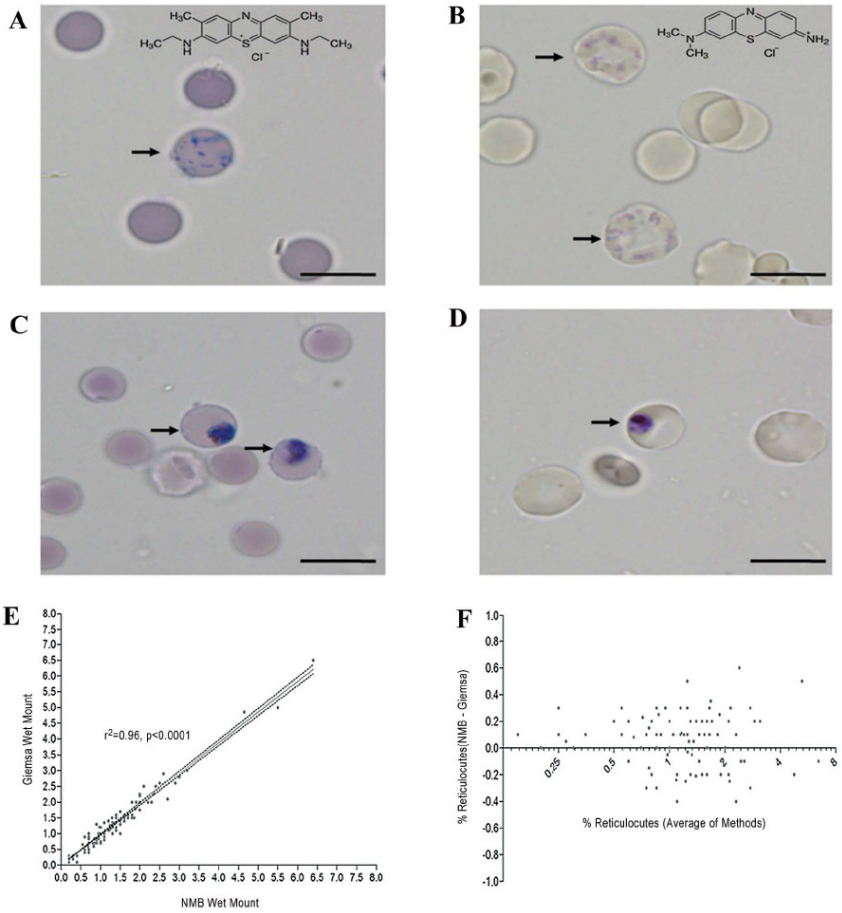

Figure 1. Wet mounted erythrocytes subvitally stained with New Methylene Blue (NMB) (A). A reticulocyte containing the dark reticular matter is indicated by an arrow. The chemical structure of NMB is inserted at the top right hand corner. Wet mounted erythrocytes subvitally stained with Giemsa (B). Two reticulocytes are indicated by arrows. The chemical structure of Giemsa is inserted at the top right hand corner. Wet mounts of *Plasmodium vivax* (trophozoite stage) infected erythrocytes subvitally stained with NMB (C) and Giemsa (D). The parasitized red cells are indicated by the arrows. The horizontal scale bar at the bottom right of each photomicrograph represents 10 µm. Linear Regression (E) and Bland-Altman (F) comparison of the percentage of reticulocytes (number of reticulocytes per 1000 erythrocytes) detected by Geimsa and NMB wet mount methodologies.
doi:10.1371/journal.pone.0060303.g001

Giemsa-wet mount method is the cell movement that can impede cell counting. However, this problem can be solved by using the appropriate volume of suspension with glass cover slips of appropriate size in preparing wet mounts. Here, we show that 7.5 µl of cell suspension of 5% hematocrit value with cover slip of 22×32 mm (0.17 mm thickness) allows an even cell distribution with minimal cell movement. In addition, the wet mounts should not be examined on a slanted bench top. The wet mounts should be checked immediately after preparation, and counting must be performed as soon as possible. As the heat of the microscope light source accelerates water loss, cell crenation will be noted if the wet mount is examined for longer than 20 minutes. Riming the outside of the coverslip with Vaseline and storage in a cool dark place will allow for examination at a later time (<6 hours after preparation).

Conclusion

Subvitally, Giemsa is a convenient and cost effective alternative to NMB for the characterisation of reticulocytes in resource limited or malaria endemic settings.

Acknowledgments

We wish to thank the staff and patients at the Shoklo Malaria Research Unit.

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Author Contributions

Involved in ethics submission and clinical sampling collection: FN CC. Conceived and designed the experiments: WCL BR YLL LR. Performed the experiments: WCL BR CC KS RS. Analyzed the data: WCL BR YLL CC KS RS FN LR. Contributed reagents/materials/analysis tools: YLL MYF LR FN KS. Wrote the paper: WCL BR YLL MYF CC RS FN LR.

blood 2014 123: e100-e109
doi:10.1182/blood-2013-12-541698 originally published online March 20, 2014

Glycophorin C (CD236R) mediates vivax malaria parasite rosetting to normocytes

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

e-Blood

RED CELLS, IRON, AND ERYTHROPOIESIS

Glycophorin C (CD236R) mediates vivax malaria parasite rosetting to normocytes

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Key Points

- *P vivax* infected cells rosette exclusively to normocytes. Thus, rosetting does not directly facilitate *P vivax* merozoite invasion.
- Glycophorin C (CD236R) mediates vivax malaria parasite rosetting. This finding will help in the search for the *P vivax* rosette ligand.

Rosetting phenomenon has been linked to malaria pathogenesis. Although rosetting occurs in all causes of human malaria, most data on this subject has been derived from *Plasmodium falciparum*. Here, we investigate the function and factors affecting rosette formation in *Plasmodium vivax*. To achieve this, we used a range of novel ex vivo protocols to study fresh and cryopreserved *P vivax* (n = 135) and *P falciparum* (n = 77) isolates from Thailand. Rosetting is more common in vivax than falciparum malaria, both in terms of incidence in patient samples and percentage of infected erythrocytes forming rosettes. Rosetting to *P vivax* asexual and sexual stages was evident 20 hours postreticulocyte invasion, reaching a plateau after 30 hours. Host ABO blood group, reticulocyte count, and parasitemia were not correlated with *P vivax* rosetting. Importantly, mature erythrocytes (normocytes), rather than reticulocytes, preferentially form rosetting complexes, indicating that this process is unlikely to directly facilitate merozoite invasion. Although antibodies against host erythrocyte receptors CD235a and CD35 had no effect, Ag-binding fragment against the BRIC 4 region of CD236R significantly inhibited rosette formation. Rosetting assays using CD236R knockdown normocytes derived from hematopoietic stem cells further supports the role of glycophorin C as a receptor in *P vivax* rosette formation. (*Blood*. 2014;123(18):e100-e109)

Introduction

In malariology, rosetting is defined by the adherence of uninfected erythrocytes to a *Plasmodium* spp.-infected erythrocyte. Although the role of rosetting phenomenon remains unknown, 2 major hypotheses have been proposed to explain its importance to the survival and fitness of the malaria parasite. First, the "rosette-assisted invasion" hypothesis, which supposes that rosetting facilitates the encounter of newly emergent merozoites with receptive uninfected red cells bound to the schizont.[1-3] Second, that uninfected cells rosetting shield the infected red blood cell (RBC) from the host immune system.[2,4]

Since its discovery in the 1980s,[5,6] rosetting phenomenon has been observed in the 4 major causes of human malaria.[1,7-10] However, almost all rosetting studies have focused on *P falciparum* and its possible role in the pathogenesis of severe disease.[11-17] A renewed interest in vivax malaria and a better appreciation of its importance to public health has led to an increased number of studies examining particular aspects of *P vivax* pathogenesis.[18-25] Certainly in the case of *P vivax*, apart from some initial descriptions on *P vivax* rosetting[8,10] and its association with anemia,[26] little has been done to investigate the importance of rosetting to the survival of *P vivax* within the human host and the molecular mechanisms associated with the formation of rosettes in this species.

Due to the technical challenges associated with *P vivax* research, the properties, as well as the postulated roles of rosetting in vivax malaria have been extrapolated from experiments conducted on *P falciparum*.[3,4] However, comparisons between these 2 species are problematic, especially when considering the receptor ligand interactions involved in the formation of rosettes. This is because one of the ligands associated with rosette formation in *P falciparum* (*P falciparum* erythrocyte membrane protein 1 [*Pf*EMP1]) has no orthologs in *P vivax*. Thus, all of the receptors on the host red cell corresponding to *Pf*EMP1, such as complement receptor 1 (CD35),[27] blood group antigens (A and B),[28-30] heparan sulfate,[31] and thrombospondin (CD36),[32,33] may not be relevant to *P vivax* rosette formation.

Submitted December 2, 2013; accepted February 21, 2014. Prepublished online as *Blood* First Edition paper, March 20, 2014; DOI 10.1182/blood-2013-12-541698.

Presented in part at the International Malaria Symposium, Sabah, Malaysia, April 16-17, 2013, and the Gordon Research Conference (Malaria), Lucca, Italy, August 4-9, 2013.

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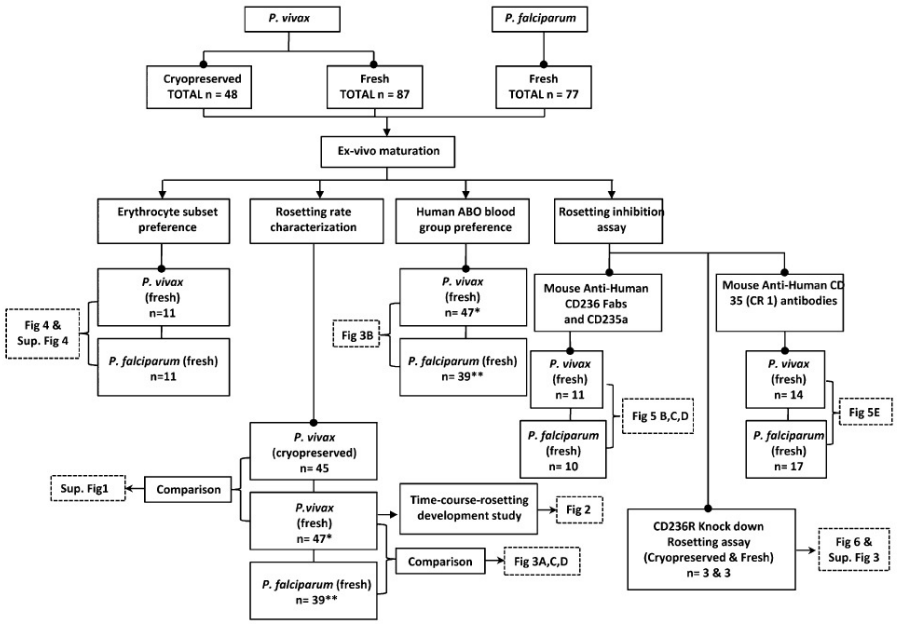

Figure 1. Experimental overview. Flowchart showing the summary of methodology applied in this study, and the respective results (figures) are shown in boxes with dotted lines. Note that $^{*}P < .05$ and $^{**}P < .001$ indicate these isolates are the same, and used for more than 1 experiment.

Recent advances in our ability to manipulate ex vivo isolates of *P vivax*[25] have enabled us to conduct detailed investigations on the rosetting phenomenon in this species, allowing us to conduct side-by-side comparisons with rosetting in *P falciparum*. Specifically, this study has 2 major objectives. First, to evaluate the postulated roles of rosette formation in *P vivax*, and second, to elucidate erythrocytic receptors that are involved in *P vivax* rosette formation.

Methods

A summary of the methodology applied, number of isolates used, and corresponding figure index is shown in a flowchart (Figure 1).

Blood sample collection

A total of 87 fresh isolates of *P vivax* and 77 fresh isolates of *P falciparum* were used in this study. Another 48 cryopreserved *P vivax* isolates were also used. All isolates were obtained from malaria patients presenting at the clinics of the Shoklo Malaria Research Unit (SMRU) in northwestern Thailand. The clinical samples were collected and tested in accordance with protocols approved by The Center for Clinical Vaccinology and Tropical Medicine at University of Oxford (OXTREC 58-09 and OXTREC 04-10), in consultation with the Ethics Committee of the Faculty of Tropical Medicine at Mahidol University. The study was conducted in accordance with the Declaration of Helsinki. Blood samples were collected using BD Vacutainer with lithium heparin anticoagulant. ABO blood group of each sample was determined via standard hemagglutination with TransClone anti-A and anti-B antibodies (Bio-Rad, Hercules, CA). A thick and thin blood smear was prepared from each blood sample to determine the species of malaria parasites involved, parasitemia, and the predominant erythrocytic stage of the parasite. Reticulocyte concentrations were prepared from human cord blood using the method outlined by Russell et al.[24]

Rosetting assay on fresh samples

Plasmodium sp. infected blood samples with at least 70% of parasite population in "ring" forms were cultivated at 3% hematocrit using McCoy's 5A medium enriched with 20% homologous serum, using the method described by Russell et al.[24] Samples were checked frequently, and sampled at ring, early trophozoite, late trophozoite, and schizont stages. The presence of rosettes and living parasites were detected and quantified using a novel Giemsa subvital staining methodology,[34] modified from techniques applied in a previous study.[8] Briefly, the sampled culture suspension was stained with Giemsa (the final stain concentration was 5%) for 15 minutes. A small volume of this suspension (7.5 μl) was used to make a wet mount with 22 × 32 mm (0.17 mm thickness) glass cover slip. The wet mount was examined immediately with light microscope under oil immersion magnification. Rosetting rate was then determined by examining 200 infected erythrocytes (in McCoy's 5A medium enriched with 20% homologous serum).

Rosetting assay on cryopreserved samples

Vivax malaria blood samples with at least 70% of parasite population in "ring" forms were cryopreserved using the glycerolyte method as described previously.[22,35] Prior to cryopreservation, vivax malaria samples with parasitemia lower than 0.1% were subjected to 75% Percoll density gradient centrifugation to concentrate the parasitized erythrocytes. The cryopreserved samples were then thawed using the sodium chloride method.[36] The thawed

samples were then matured, and rosetting assay was conducted as described above. Rosetting prevalence of each cryopreserved isolate was determined.

Erythrocyte subset preference study

Blood samples infected with either *P vivax* or *P falciparum* were cultivated and matured until at least 60% of the parasite population reached schizogony. One hundred μL of each culture suspension was used as a control where its rosetting rate was determined. Matured parasites from the remaining culture suspension were then concentrated with magnetic activated cell sorting column (Miltenyi Biotec). The concentrated infected packed erythrocytes (volume = ×) were then mixed with 0.5 × volume of uninfected packed normocytes, and 0.5 × volume of uninfected reticulocytes. The cell mixtures were suspended in enriched McCoy's 5A medium as described above and mixed well by vortexing to disrupt rosettes. Importantly, in a preliminary study (data not shown), we found that rosettes were disrupted even after 1 minute of vortexing (we used 2 minutes to be absolutely sure of total disruption). The free infected RBCs (IRBCs) started reforming rosettes within seconds, and the vortexed isolate returned to the original rosetting frequency within 5 minutes. To ensure maximal rosette formation, we incubated the vortexed blood mixtures at 37°C for 30 minutes. Thereafter, rosetting assay was conducted as described above. Types of erythrocytes involved in each rosette were studied, and the erythrocyte subset preference for rosetting in *P vivax* and *P falciparum* were then determined.

Erythrocyte receptor-blocking study using Ag-binding fragments and antibodies

Preliminary trials clearly showed that the intact anti-glycophorin C and A antibodies caused considerable hemagglutination (not observed with CD35 antibodies). Therefore, it was necessary to use Ag-binding (FAB) fragments fragments of anti-CD236R and CD235a monoclonal antibodies.

Mouse anti-human CD236R clone BRIC 4 IgG (Thermo Scientific Pierce), mouse anti-human CD236R clone BRIC 10 IgG (Abcam, Cambridge, United Kingdom), and mouse anti-human glycophorin A IgG (Abcam) were used for this experiment. Fab fragments of the respective antibodies were prepared using the Pierce Fab Micro Preparation Kit and Resin Kit (Thermo Scientific Pierce). *Plasmodium* sp. infected blood samples were cultured and matured as described above. Then, the culture suspensions were vortexed vigorously for 2 minutes to mechanically dissociate any preformed rosettes. Each culture suspension was divided into four 1.5 mL microcentrifuge tubes, where each tube of culture suspension was added with one type of the prepared antibody or Fab fragments (1 mg/mL final concentration), and one treatment-free tube was the negative control for the experiment. The mixtures were incubated for 30 minutes at 37°C. Finally, rosetting assay was conducted on each tube of suspension as described above. In addition to the Fab treatments, we also used mouse anti-human CD35 antibody (BD Pharmigen) as a positive control.

Hematopoietic cell culture and CD236R knockdown experiments

Cryopreserved human umbilical cord blood mononuclear cells were expanded using a method described by Giarratana et al[37] with few modifications. Briefly, the $CD34^+$ hematopoietic progenitors were isolated by immunomagnetic selection using CD34 MicroBead Kit (Miltenyi Biotec, Singapore). The $CD34^+$ cells were harvested during 3 weeks in IMDM GlutaMAX (Gibco), supplemented with 330 μg/mL of human holotransferin (Sigma-Aldrich, Singapore), 10 μg/mL recombinant human insulin (SAFC Biosciences), 2 IU/mL heparin (Sigma-Aldrich), and 5% AB blood group serum. Between days 0 and 7, the following additional supplementation were incorporated: 10^{-6} M of hydrocortisone (Stemcell Technologies), 100 ng/mL of stem cell factor (PeproTech), 5 ng/mL of IL-3 (PeproTech), and 3 UI/mL of erythropoietin (Epo) (Stemcell Technologies); between days 7 and 11, 100 ng/mL of stem cell factor and 3 UI/mL of Epo; and after day 11 only Epo was added.

One day after the immunomagnetic sorting, the $CD34^+$ cells were transduced (MOI of 5) with shRNA (short hairpin RNA) lentivector MISSION, shRNA Target Set NM_002101 (Glycophorin C [CD236R]) (Sigma-Aldrich) in the presence of 8 μg/mL of Polybrene (Sigma-Aldrich). Fifteen days after the transduction, green fluorescent protein (GFP) positive and negative cells were sorted using BD Influx Flow Cytometer (BD Biosciences). The day after, GFP positive and negative fractions were incubated with *P vivax* late stages isolated by magnetic sorting as described previously.[38] The level of CD236R expression in each subset was measured by flow cytometry using anti-human CD236R, clone BRIC 4 (Thermo Scientific Pierce) antibody as primary staining, and anti-mouse e660 (eBioscience) as secondary staining. The cells were acquired on LSRII Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Statistical analyses

In most cases, the rosetting rate was defined as the percentage of 200 infected erythrocytes that formed stable adhesion with at least 1 uninfected erythrocyte after 15 minutes of postvortex incubation (as observed under 100× oil immersion). A giant rosette is defined as a rosetting complex with the participation of at least 10 uninfected erythrocytes.[9-11] As most of the data were not normally distributed, nonparametric analysis such as Mann-Whitney *U*, Kruskal-Wallis (more than 2 groups), and Friedman tests (repeated measures) were used. In the latter 2 tests, Dunn post hoc analysis was used. All statistical analysis used Prism 5 for Windows (version 5.01), Software MacKiev.

Results

Cryopreservation and rosetting

At the beginning of this study, for purposes of practicality, we used cryopreserved isolates of *P vivax*. However, it soon became evident that the rates of rosetting in these cryopreserved-then-thawed samples were considerably lower than those previously reported from fresh isolates.[10] Subsequently, we decided to focus our work on fresh isolates rather than cryopreserved (with the exception of the knockdown experiment). Certainly, when we compared our data on rosetting prevalence from fresh isolates with cryopreserved isolates, we showed that cryopreservation was associated with a lower incidence of rosette formation; whereas the median rosetting rate for fresh isolates was 23.80% (interquartile range [10.00% to 31.00%]), the median rosetting rate for cryopreserved isolates was 0.00% (interquartile range [0.00% to 14.85%]) on P (2-tailed) $< .0001$, Mann-Whitney U test (see supplemental Figure 1 on the *Blood* Web site).

Rosetting properties of *P vivax*

Rosette formation of *P vivax* increased with the parasite erythrocytic maturation (Figure 2A). In this study, no early stage (ring form) was found to be involved in rosette formation. Rosette formation of *P vivax* was noted 24 hours postcultivation, represented by early trophozoite stage. The rosetting rate increased markedly between early trophozoite stage and the late trophozoite stage. After 30 hours, the rosetting development continued, albeit at a much lower rate, until the parasite reached schizogony at hour 44.

Giant rosettes were found occasionally in *P vivax* isolates; 10% of the rosettes found in *P vivax* isolates were giant rosettes. On the other hand, only 0.5% of the rosettes found in *P falciparum* isolates were giant rosettes. Besides the asexual stages, gametocytes of *P vivax* were found to be involved in rosette formation (Figure 2B). Involvement of gametocyte-infected erythrocytes in rosette formation was found in *P falciparum* isolates as well (Figure 2B).

Rosetting rate and rosetting prevalence

Rosetting occurs as frequently in isolates of *P vivax* (91.5%; 43 of 47) as it does in *P falciparum* (79.5%; 31 of 39) confirming earlier

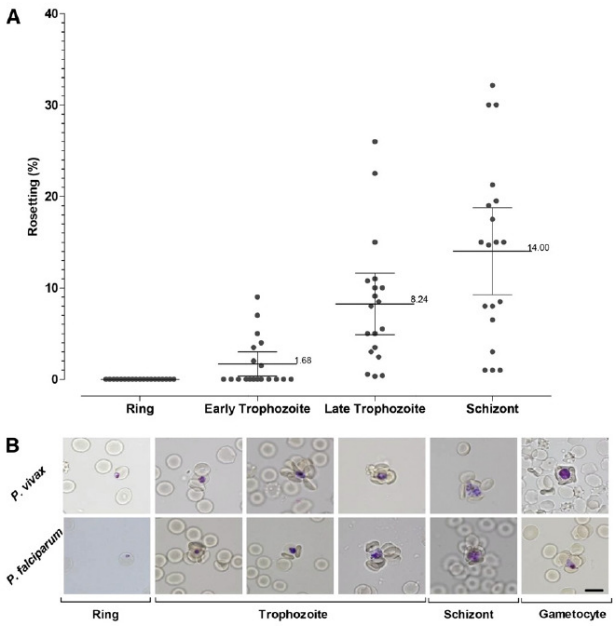

Figure 2. Rosetting kinetics. (A) Plot showing the kinetics of rosetting development in 47 *P vivax* isolates matured ex vivo. (B) Representative images of rosettes formed by different stages of *P vivax* and *P falciparum* are shown after Giemsa subvital staining process.[34]

studies by Udomsanpetch et al.[10] Interestingly, the median rate of rosetting was significantly higher in *P vivax* (24.5%) when compared with *P falciparum* (9.0%) isolates from the same area in Thailand ($P < .001$) (Figure 3A). No significant correlation was found between the rosetting rate and parasitemia or ABO blood groups in both vivax and falciparum malaria patients (Figure 3B-C). The parasitemia range for *P vivax* and *P falciparum* was 0.01% to 1.70% and 0.10% to 11.00%, respectively.

Erythrocyte subset preference study

The availability of reticulocytes in the peripheral blood of vivax or falciparum malaria patients had no bearing on the incidence of rosettes (Figure 3D). Close inspection of rosettes from 9 vivax malaria (562 rosettes) and 11 falciparum (391 rosettes) malaria isolates revealed that only ~10% of rosettes involved attachment of reticulocytes (and in these cases only 1 reticulocyte per rosette could be observed); the majority of rosettes were exclusively composed of mature erythrocytes (Figure 4A).

As reticulocytes only make up a small portion of the peripheral blood (<2%), we wanted to ensure that the lack of reticulocyte involvement in rosette formation was not only a function of the lower probability of parasitized cells encountering the reticulocyte (rare) versus the commonly found normocyte. To test this, we added magnetic activated cell sorting column-concentrated *P vivax* and *P falciparum* IRBCs to blood samples containing enriched reticulocytes (50% $^{+/-}$10). When the reticulocyte-enriched experiment group was compared with the paired rosetting data from the control group (the nonenriched blood) using Wilcoxon Signed-Rank test, rosetting rates for *P vivax* was unaffected, whereas *P falciparum* rosetting rates actually decreased in the enriched reticulocyte treatments $P < .01$ (1-tailed, Mann-Whitney *U* test) (Figure 4B-C). Although we did observe the occasional *P vivax* rosette with reticulocytes attached in the enriched treatment, the formation of rosettes almost exclusively involved normocytes, despite the even chance to encounter reticulocytes. Since there may be concerns regarding the difference between cord blood reticulocytes and adult reticulocytes, we repeated the reticulocyte enrichment experiment using 3 more fresh isolates of *P vivax* and reticulocytes isolated from 2 adult donors. We found no significant difference between the adult and cord-blood–derived reticulocytes in their lack of involvement in *P vivax* rosette formation (supplemental Figure 2).

Rosetting inhibition assay

Fab and antibody blocking experiment. In vivax malaria isolates tested, Fab of mouse anti-human CD236R IgG (clone BRIC 4) (Figure 5A shows where this Fab clone binds) significantly reduced rosetting rate by 27% ($P < .05$; Friedman analysis with the Dunn multiple comparison test) (Figure 5B). Interestingly, BRIC 4 Fabs reduced rosette formation in falciparum malaria isolates by at least half (Figure 5C). Importantly, the Fab fragment of another anti-CD236R antibody had no effect on rosette formation in the same paired experiments demonstrating the specificity of the inhibition of BRIC 4 antibodies (Figure 5B). Anti-glycophorin A Fab fragments had no effect on rosette formation for both species (Figure 5D).

CD35 blocking experiment

Complement receptor 1 or CD35 is 1 of the erythrocytic receptors involved in rosetting of *P falciparum*.[27] Certainly in the

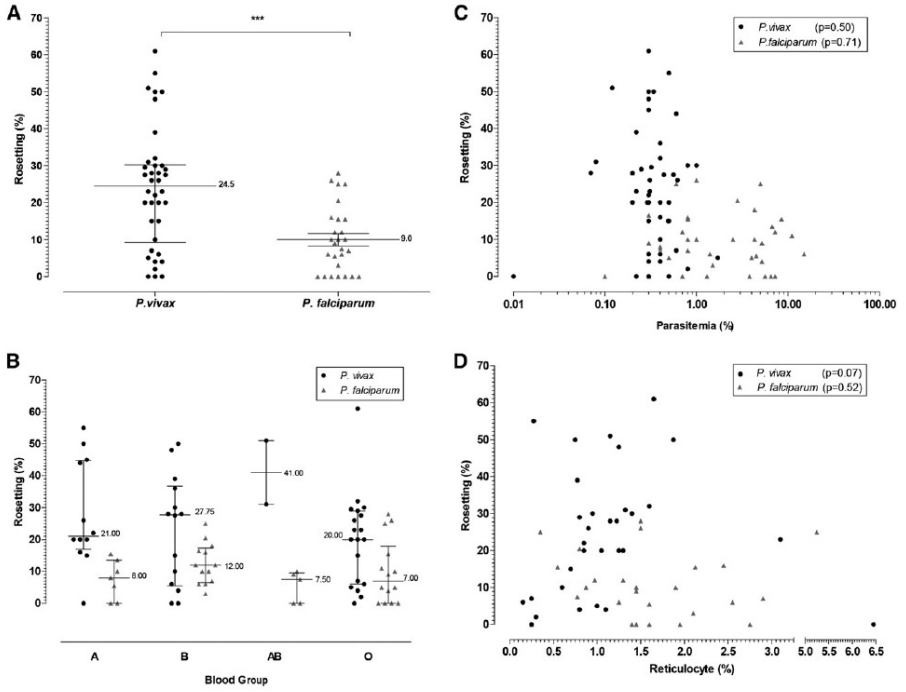

Figure 3. Factors affecting rosetting formation in *P vivax* and *P falciparum*. (A) Comparison of rosetting rate between *P vivax* and *P falciparum* isolates from the Thailand-Myanmar border. The median percentage of *P vivax* IRBCs is significantly higher than *P falciparum*; *P* (2-tailed) < .001. (B) Rosetting rate of *P vivax* and *P. falciparum* isolates found in different human ABO blood groups. No significant association was found between ABO phenotype of the malaria and occurrence of *P vivax* (*P* = .28) or *P falciparum* (*P* = .20). (C) Plot of rosetting rate of *P vivax* and *P falciparum* isolates against the original parasitemia of malaria patients presenting for treatment. No significant correlation was observed between patient parasitemia and rosetting for *P vivax* (Spearman *r* = −0.10; 95% CI [−0.39 to 0.20]; *P* = .50) or *P falciparum* (Spearman *r* = −0.06; 95% CI [−0.38 to 0.27]; *P* = .71). (D) Rosetting rate of *P vivax* and *P falciparum* isolates against peripheral reticulocyte counts in malaria patients. No significant correlation was observed between rosetting and patient reticulocyte counts for either *P vivax* (Spearman *r* = 0.33; 95% CI [−0.04 to 0.62]; *P* [2-tailed] = .07) or *P falciparum* (Spearman *r* = −0.13; 95% CI [−0.49 to 0.28]; *P* = .52).

P falciparum isolates we tested, the rosetting rate was significantly inhibited (33.3%) by the mouse anti-human CD35 antibody ($P < .0001$, $R = 0.8236$; 95% CI [3.026 to 6.504]). However, CD35 had no effect on rosette formation in the *P vivax* isolates tested (Figure 5E).

Hematopoietic cell culture and CD236R knockdown

Although the above Fab-mediated inhibition assays provide useful clues to the identity of the host rosetting receptor, such an approach is limited by potential off-target effects (such as steric hindrance or changes to membrane rigidity).[39] To limit these unwanted "off-target effects," we employed a transgenic method to knockdown the expression of CD236R to produce normocytes mostly deficient in this receptor (Figure 6A). Phenotyping of GF- positive and GFP-negative cells (according to BRIC 4 region recognition [Figure 5A]) showed a knockdown of 81.5% ± 2.5% in CD236R expression (Figure 6B). It should be noted that the method we used to knockdown the expression of glycophorin A was different to the one recently used by Bei et al[40] (where puromycin and neomycin rather than GFP was used for selection) but with the same knockdown efficiency. It is important to note that the erythrocytes generated from the CD236R knockdown had a normal phenotype in terms of 7 other characteristic erythrocytic receptors (supplemental Figure 3).

Initially, this work needed to be conducted in Singapore rather than in Mae Sod; therefore, we had to rely on cryopreserved isolates of *P vivax* to conduct this experiment. Of the 3 *P vivax* cryopreserved isolates used, only one showed very high levels of rosetting (>50%). Despite this limitation, differences in rosetting between the control normocytes (CD236R positive) and CD236R knockdown normocytes was immediately evident. After examination of 200 fields (×1000) and counting 50 *P vivax* IRBCs per treatment, only 4 of 50 were rosetting with CD236R knockdown erythrocytes compared with 27 of 50 in the control (χ-square = 22.6, d.f. = 1, $P < .0001$ [χ-square with Yates correction]). Because we were concerned about possible confounders associated with the use of cryopreserved isolates (supplemental Figure 1), we repeated this experiment, this time using 3 fresh isolates. As per the original experiment, we found that the CD236R knockdown erythrocytes significantly reduced the formation of rosettes ($P = .007$) (Figure 6C-D). It is important to understand that although the cells were sorted on GFP, this protein has about 26 hours

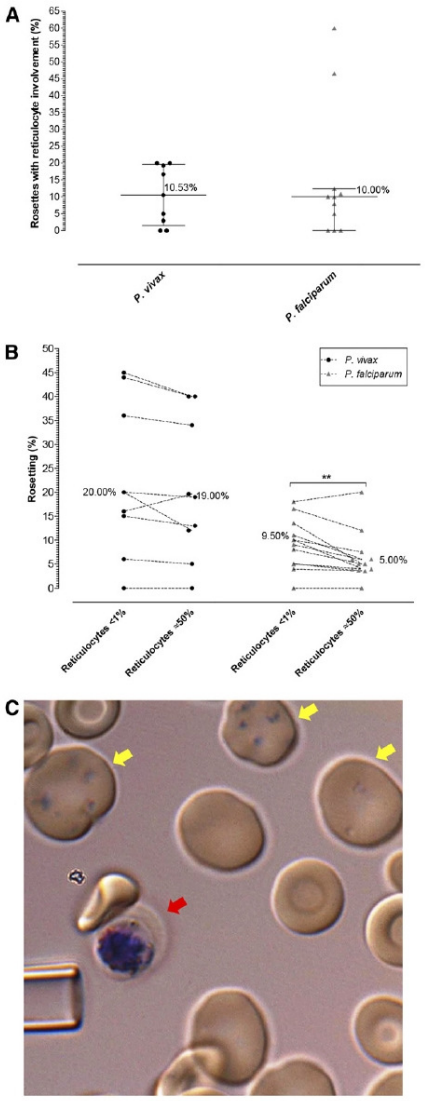

Figure 4. *P vivax* rosetting preference. (A) Percentage of rosettes associated with at least 1 reticulocyte in *P vivax* and *P falciparum* isolates in patient isolates already containing late-stage parasites (no ex vivo maturation). (B) Differences in rosetting rate of *P vivax* and *P falciparum* isolates in environment with <1% reticulocytes and approximately 50% reticulocytes (achieved by concentrating host reticulocytes on a 75% Percoll gradient). Lines connect paired observations. Altering the reticulocyte concentration had little effect on *P vivax* rosette formation; however, the number of normocytes attached to the *P vivax* IRBC was notably reduced in the treatment of enriched reticulocytes. Interestingly, an increase in the available reticulocytes

half-life.[41] Therefore, in mammalian cells, the signal gradually diminishes as the erythrocyte matures. Consequently, the normoblasts (Heilmeyer stage 0) and early reticulocytes (Heilmeyer stages I, II, and III) have bright signals. Mature reticulocytes (Heilmeyer stage IV) and normocytes have little or no discernable signal (Figure 6C). An unintended benefit of this age-related GFP signal output was that in the 4 rosettes observed in the CD236R knockdown, no rosette was associated with GFP-positive cells (reticulocytes), thus further supporting our data on *P vivax* rosette normocytic preference.

Discussion

The biological role of *P vivax* and *P falciparum* rosetting

Although the role of rosetting in the pathogenesis of malaria remains controversial, the real mystery surrounding this phenomenon is what advantage it brings to the intra-erythrocytic parasite. While not providing a definitive answer to this question, our data provides compelling evidence that rosetting does not facilitate the invasion of merozoites. We have 2 lines of evidence disputing the "rosette-assisted invasion" hypothesis.

Foremost and most convincingly, our data shows that *P vivax* preferentially forms rosettes with mature red cells and not reticulocytes. If *P vivax* rosettes were to truly assist in merozoite invasion, one would hypothesize that its schizonts would preferentially adhere to uninfected reticulocytes; thereby improving invasion success by the provision of immediate access to proximal reticulocytes (which are generally scarce in the peripheral circulation). However, the observations of naturally occurring rosettes from fresh isolates and our reticulocyte enrichment experiment clearly show that rosettes favor normocytes rather than reticulocyte attachment. As *P vivax* merozoites exclusively invade reticulocytes, there would be little advantage for the parasite species to have its schizonts binding with normocytes, which are not receptive to invasion. As *P falciparum* merozoites can invade both reticulocytes and normocytes, its preferential rosette formation with normocytes does not discount the possibility that rosetting may improve the invasion success of merozoites in this species. However, convincing data from Clough et al shows that rosette-forming strains of *P falciparum* have no invasion advantage over nonrosetting strains.[42] Unpublished data (supplemental Figure 4) derived from an earlier study on *P vivax* invasion,[24] clearly shows that the rate of rosetting within an isolate bears no correlation with invasion success.

A second line of evidence against the "rosette-assisted invasion" hypothesis is that *P vivax* and *P falciparum* start rosetting at early trophozoite stage, at least 24 hours prior to the expulsion of merozoites from the segmenting schizont. Whereas some may suggest that the developing parasite is getting an "early start" on the collection of receptive cells, such an effort would be wasted as the shear forces present in the host circulation, and physical barriers such as the splenic red pulp would undoubtedly disrupt the rosette numerous times within the 24-hour time interval between the initiation of

Figure 4 (continued) reduced the ability of *P falciparum* to rosette ($P < .01$). (C) Reticulocytes were rarely associated with rosettes irrespective of the group or species observed. Here, a single normocyte is rosetting on a *P vivax* schizont (red arrow). The yellow arrows point to Heilmeyer stage IV reticulocytes subvitally stained with Giemsa. The tip of a glass micropipette with an internal diameter of 6 um is shown for scale.

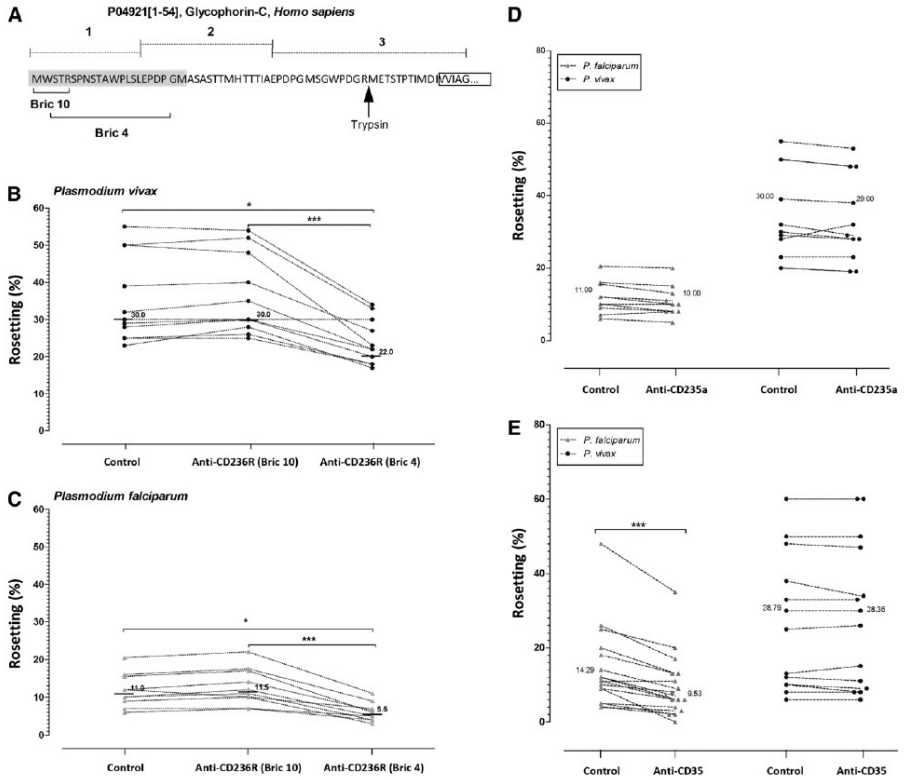

Figure 5. Antibody blocking rosette formation. (A) Schematic diagram of the target sites of anti-CD236R antibody clone BRIC 10 and anti-CD236R antibody clone BRIC 4 on human CD236R structure. The target site of trypsin on this sialoglycoprotein is shown by the black arrow. (B-C) Rosetting inhibition in *P vivax* and *P falciparum* caused by Fab fragments specifically targeting the BRIC 10 and BRIC 4 locations on CD236R. BRIC 4 showed a significant reduction in rosetting of *P falciparum* isolates ($P < .0001$) and *P vivax* isolates ($P < .0001$) studied. A paired Student *t* test was conducted. (D) Rosetting inhibition by mouse anti-human CD35 antibody. Unlike in *P vivax*, this antibody significantly reduced rosetting rate of *P falciparum* isolates tested ($P < .0001$). (E) Comparison of rosetting rates between the control and cells incubated with Fab fragments of mouse anti-human glycophorin A antibody from *P falciparum* and *P vivax* isolates recruited. There was no significant difference between the control group and the "anti-glycophorin A" group in *P vivax* and *P falciparum* isolates studied.

rosette formation and schizont rupture. Perhaps even more convincing is our observation that *P vivax* and *P falciparum* gametocytes also form rosettes, a new finding contrary to the earlier report from Lowe et al.[1]

Clearly, data from this and earlier studies[24,42] suggest that rosetting does not directly assist in the targeting or invasion of malaria parasites into uninfected host erythrocytes. Although we do not have data to support the alternative hypothesis, which proposes that rosetting shields the iRBC from the host immune system (first postulated by Wahlgren et al),[3] we agree that this is the most likely reason for such an adaptation in *Plasmodium* spp. It certainly seems intuitive that iRBC containing mature stages (and the associated plethora of parasite antigens expressed on the red cell membrane) would greatly benefit from a cloak of host RBCs. Again, this hypothesis is relatively speculative, and we hope that future studies will focus on the role of rosetting in the protection of *Plasmodium* spp. from the host immune system.

The observation that rosetting occurs as frequently in *P vivax* as it does in *P falciparum*, raises a number of important questions regarding the clinical significance of rosetting in vivax malaria. Although our data are from de-identified samples (thus not allowing direct clinical correlates with the rosetting rate), we do know that all our samples were collected from uncomplicated cases of vivax malaria. As such, it is difficult to speculate on the direct role of rosetting on *P vivax* pathogenesis. We certainly hope that future clinical investigations into severe vivax malaria includes rosetting as an important parasitologic parameter.

Erythrocyte receptors mediating rosetting in fresh isolates of *P vivax*

Whereas numerous studies have shown that the human ABO blood group and CR1 (CD35) are key determinants on *P falciparum* rosetting,[7,27-30] this does not seem to be the case with *P vivax*. The

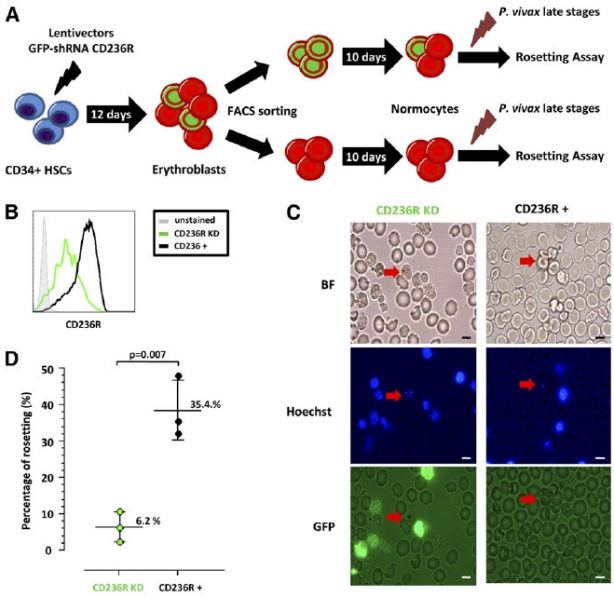

Figure 6. Transgenic approach to investigate the role of CD236R in *P vivax* rosetting. (A) Experimental design of schizont *P vivax* rosetting assay with cultured RBCs (cRBCs) generated from $CD34^+$ hematopoietic stem cells. The stable knockdown of CD236R (glycophorin C) is obtained using Sigma lentivector with GFP and shRNA against CD236R cassette expression. The 2 subsets of cells: CD236R knockdown and $CD236R^+$ cells were separated by flow cytometry using GFP expression, 1 day before performing the rosetting assay with *P vivax* schizonts isolated by magnetic sorting. (B) Flow cytometry histograms showing CD236R expression in GFP-positive cells (CD236R knockdown cells [green line]), GFP-negative ($CD236R^+$ cells [black line]), and unstained cells (gray line). (C) Plots showing schizont *P vivax* rosetting with cRBC with CD236R knockdown (CD236R KD) and without knockdown ($CD236^+$) with bright field (BF), Hoechst, and GFP detection. (D) Frequency of schizonts with or without rosetting in the presence of CD236R knockdown cRBCs or of $CD236^+$ cRBCs showing a significant difference in proportion of schizonts able to form rosettes between the 2 different types of cRBCs (CD236R KD and $CD236^+$).

absence of association between *P vivax* rosetting and the ABO blood group agrees with the findings from an earlier study by Chotivanich et al.[8] The lack of association between the ABO blood group and CR1 in *P vivax* should not be surprising as the ligand responsible for this interaction in *P falciparum* (PfEMP1) is absent from *P vivax*. Our efforts to shortlist novel receptors mediating rosetting in *P vivax* were aided by unpublished data from one of the authors of this study, which indicated that CD236R modulates *P falciparum* rosetting in a PfEMP1-independent manner. Certainly, our results show that specifically blocking the BRIC 4 region (amino acid residues 2-21) of CD236R significantly inhibits rosette formation in *P vivax* and *P falciparum*. As most of the BRIC 4 region is located in segment 1 of CD236R, it is unlikely that the Gerbich and Yus mutations (which cause major deletions to regions 2 and 3) will have any effect on its ability to form rosettes (Figure 5A). Although the Leach mutation would indeed remove the residues of interest, we did not attempt to source erythrocytes from this rare phenotype, as the mutant cells are elliptical (not biconcave) in shape, and have a profoundly modified biomechanical profile that would confound any conclusions regarding their ability to rosette (irrespective of the complete absence of CD236R in the Leach-type cells). It is interesting to note that trypsin treatment of erythrocytes completely removes 2 extracellular domains of CD236R (1 and 2) (Figure 5A), resulting in the complete abrogation of rosetting in *P vivax*.[24]

To better understand the importance of CD236R as a key host receptor for *P vivax* rosetting, we knocked down its expression in normocytes derived from hematopoietic stem cells. Rosetting assays using these CD236R knockdown erythrocytes clearly show the significant reduction of rosetting phenomena in *P vivax* compared with normocytes (from the same culture) expressing this receptor.

One problem with the idea that "CD236R is important to rosetting," is that the presentation of this receptor decreases during reticulocyte maturation to normocyte; however, it is still highly expressed in both subsets.[43] As *P vivax* does not rosette well with reticulocytes, one has to question as to what prevents the interaction between reticulocyte CD236R and the yet to be discovered *P vivax* rosetting ligands. We postulate that the rigid biomechanical properties of reticulocytes (reticulocytes are significantly stiffer than normocytes),[43] inhibit the formation of close bonds with the *P vivax* IRBC. Close examination of images of *P vivax* rosettes, especially in Figure 4C, show the tight association of the normocyte and infected cell (ie, the flat face of the biconcave disk to the IRBC). The globular and stiff reticulocyte inhibits intimate contact with the *P vivax* IRBC. Certainly, our efforts to manipulate reticulocytes into direct contact with *P vivax* cells (using a micropipette) failed to promote rosetting.

Conclusion

Rosetting is a common property of mature intra-erythrocytic malaria parasites, occurring as frequently in *P vivax* as it does in *P falciparum*. The high frequency of rosetting in patient isolates suggests that it provides *P vivax* with a survival advantage. Our ex vivo experiments indicate that in the case of *P vivax*, this advantage is most likely to be associated with immune invasion rather than facilitating the invasion of reticulocytes. Although ligands and receptors primarily involved in the formation of rosettes in *P vivax* still remain a mystery, we provide strong evidence that CD236R is involved, whereas receptors traditionally involved in *P falciparum* rosettes, such as ABO antigens and CD35, are not. We also wish to

stress that as with *P falciparum*, there is likely to be more than 1 host receptor involved in *P vivax* rosetting. Future work should focus on the discovery of *P vivax* ligands that modulate the attachment of normocytic CD236R and other possible receptors involved in the rosetting process.

Acknowledgments

The authors thank the staff of SMRU who assisted in the management of this research study. B.R. and L.R are adjunct Professors at the Department of Parasitology, University of Malaya, Kuala Lumpur, Malaysia. We would like to thank the SIgN flow cytometry core lead by Dr Anis Larbi and his team members, Ivy Low Nurhidaya Shadan, Seri Mustafahi, and Nurhidaya Shadan.

This work was supported by the University of Malaya Research Grant (PV044-2012-A) (W.C.L.), University of Malaya, High Impact Research Fund UM-MOHE (UM.C/625/1/HIR/MOHE/CHAN/14/3), the Ministry of Higher Education Malaysia (Y.L.L. and M.Y.F.), the SIgN and the Horizontal Programme on Infectious Diseases (L.R.), and a Young Investigator Grant (BMRC YIG Grant No:13/1/16/YA/009) under the Agency for Science, Technology, and Research (Singapore) (B.M.). SMRU is part of the Mahidol Oxford University Research Unit, supported by The Wellcome Trust of Great Britain. And additional funding was provided by the National University of Singapore Faculty Start-Up Grant and the Singapore National Medical Research Council (NMRC/CBRG/0047/2013) (B.R. and B.M.).

Authorship

Contribution: W.C.L., B.R., B.M., M.M., R.Z., R.S., J.S.C., L.A., and F.T.M.C. carried out laboratory work, collected, and analyzed the data; R.M., F.N., Y.L.L., and M.Y.F. performed the clinical management of patients, ethical clearance, and collection and processing of the blood samples; W.C.L., B.R., P.P., Y.L.L., M.Y.F., B.M., and L.R. participated in data interpretation and helped to draft the manuscript; and all authors read and approved the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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