รายงานวิจัยฉบับสมบูรณ์

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Molecular basis of the dyserythropoiesis by *Plasmodium vivax* infection: An *in vitro* model study in human hematopoietic stem cells

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ABSTRACT

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Project Title : Molecular basis of the dyserythropoiesis by *Plasmodium vivax* infection: An in vitro model study in human hematopoietic stem cells

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The underlying causes of severe malarial anaemias are multifactorial. In previously reports, Plasmodium vivax was found to be able to directly inhibited erythroid cell proliferation and differentiation. However, the molecular mechanisms underlying the suppression of erythropoiesis by *P. vivax* are remarkably complex and remain unclear. In this study, digestive vacuoles (PvDVs) with containing hemozoin prepared from blood stages of *P. vivax* and subjected in study of the effect on erythroid cell (EC) growth. A phosphoproteomic approach was performed to dissect the molecular mechanism of phosphoprotein regulation, which is involved in the inhibitory effect of parasites on erythroid cell development.

Results showed that Intact or lysed PvDV was not able to inhibit gEC growth after exposure for 24 – 72 h compared with gEC growth in media, while lysed PvIE was able to inhibit gEC growth to 73, 66 and 48 % after exposure for 24, 48 and 72 h, respectively. This result was consistent with our previously report. This study describes the first comparative phosphoproteome analysis of growing erythroid cells (gECs), derived from human hematopoietic stem cells, exposed to lysates of infected erythrocytes (IE)/uninfected erythrocytes (UE) for 24, 48 and 72 h. This study utilized IMAC phosphoprotein isolation directly coupled with LC MS/MS analysis. Lysed IE significantly inhibited gEC growth at 48 and 72 h and cell division resulting

in the accumulation of cells in G0 phase. The relative levels of forty four phosphoproteins were determined from gECs exposed to IE/UE for 24-72 h and compared with the media control using the Label-free quantitation technique. Interestingly, the levels of three phosphoproteins: ezrin, alpha actinin-1, and Rho kinase were significantly ($p < 0.05$) altered. These proteins display interactions and are involved in the regulation of the cellular cytoskeleton. Particularly affected was ezrin (phosphorylated at Thr567), which is normally localized to gEC cell extension peripheral processes. Following exposure to IE, for 48-72 h, the ezrin signal intensity was weak or absent. This result suggests that phospho-ezrin is important for actin cytoskeleton regulation during erythroid cell growth and division. These findings suggest that parasite proteins are able to inhibit erythroid cell growth by down-regulation of ezrin phosphorylation, leading to ineffective erythropoiesis ultimately resulting in severe malarial anaemia. A better understanding of the mechanisms of ineffective erythropoiesis may be beneficial in the development of therapeutic strategies to prevent severe malarial anaemia.

Keywords

Plasmodium vivax, ineffective erythropoiesis, anaemia, ezrin, phosphoproteins, haematopoiesis stem cells, erythroid cells

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ชื่อโครงการ: กลไกความผิดปกติการสร้างเม็ดเลือดแดงจากการติดเชื้อ *Plasmodium vivax*: ศึกษาโดยใช้เซลล์ต้นกำเนิดของเม็ดเลือดของคนที่เพาะเลี้ยงในหลอดทดลอง

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ระยะเวลาโครงการ: 2 ปี

สาเหตุการเกิดภาวะโลหิตรุนแรงโรคมาลาเรียเกิดจากหลายปัจจัย จากที่มีการรายงานพบว่าเชื้อมาลาเรียสายพันธุ์ *P. vivax* สามารถยับยั้งการเจริญและการพัฒนาของเซลล์เม็ดเลือดแดง อย่างไรก็ตามกลไกระดับโมเลกุลที่ยับยั้งการพัฒนาของเซลล์เม็ดเลือดแดงตัวอ่อนที่เกิดจากเชื้อสายพันธุ์ *P. vivax* มีความซับซ้อนและไม่ชัดเจน การศึกษาครั้งนี้ ได้เตรียม digestive vacuoles (PvDV) ของเชื้อมาลาเรียสายพันธุ์ *P. vivax* ซึ่งภายในมี hemozoin PvDV ถูกนำมาทดสอบผลกระทบต่อการเจริญของเซลล์เม็ดเลือดแดง ผลการทดลองพบว่า PvDV ไม่สามารถยับยั้งการเจริญของเซลล์เม็ดเลือดแดง ในขณะที่เชื้อที่ถูกทำให้แตก (lysed infected erythrocytes) สามารถยับยั้งการเจริญของเซลล์เม็ดเลือดแดง 73, 66, และ 48 % หลังจากเซลล์เม็ดเลือดแดงตัวอ่อนถูกเลี้ยงในสภาวะที่มี PvDV หรือเชื้อที่แตกเป็นเวลา 24, 48 และ 72 ชั่วโมง ซึ่งผลการทดลองครั้งนี้สอดคล้องกับการศึกษาก่อนหน้านี้ ในการศึกษาครั้งนี้เป็นครั้งแรกที่ศึกษา phosphoproteomic เพื่อศึกษาหากลไกกลที่ควบคุมการเจริญของเซลล์เม็ดเลือดแดงตัวอ่อนลดลงเมื่อถูกเชื้อสายพันธุ์ *P. vivax* โดยใช้วิธี IMAC phosphoprotein และ LC MS/MS จากผลการทดลองพบว่าเชื้อแตกสามารถยับยั้งการเจริญของเซลล์และทำให้เซลล์อยู่ในระยะ G0 ซึ่งเป็นระยะที่เซลล์ไม่มีการแบ่งตัว จากผลการวิเคราะห์ phosphoprotein ของเซลล์เม็ดเลือดแดงตัวอ่อนที่เจริญมาจากเซลล์ต้นกำเนิดของเม็ดเลือดแดงของคนที่ถูกเลี้ยงในสภาวะที่มีเชื้อสายพันธุ์ *P. vivax* ที่แตกเป็นเวลา 24, 48, และ 72 ชั่วโมง โดยเปรียบเทียบ phosphoprotein ของเซลล์เม็ดเลือดแดงที่เลี้ยงในสภาวะไม่มีเชื้อ ผลการทดลองพบ phosphoproteins 44 โปรตีน และที่น่าสนใจมี phosphoproteins ที่ระดับเปลี่ยนแปลงอย่างมีนัยสำคัญที่ $p < 0.05$ ได้แก่ ezrin alpha actinin-1 และ Rho kinase และพบว่าโปรตีนทั้งสามตัวสามารถทำปฏิกิริยากันซึ่งกันและกัน (protein – protein interaction) และมีส่วนเกี่ยวข้องในกลไก

ควบคุมโครงสร้างของเซลล์เม็ดเลือดแดง โดยเฉพาะโปรตีน ezrin ที่ตำแหน่ง Thr567 มีระดับของการ phosphorylation ลดลง และถูกพบที่บริเวณรอบๆ ของเซลล์และส่วนที่ยื่นออกไปของผนังเซลล์ (extension peripheral processes) ซึ่งทำหน้าที่เกี่ยวข้องกับการควบคุม actin ที่เป็นโครงสร้างของเซลล์และเกี่ยวข้องกับการเจริญของเซลล์และการแบ่งเซลล์ จากผลการทดลองแสดงให้เห็นว่า โปรตีนของเชื้อสามารถยับยั้งการเจริญของเซลล์เม็ดเลือดแดงโดยลดระดับการ phosphorylation ของโปรตีน ezrin ซึ่งมีผลกระทบต่อการสร้างเม็ดเลือดแดงให้มีประสิทธิภาพลดลงทำให้ผลิตเม็ดเลือดแดงลดลง ซึ่งนำไปสู่การเป็นสาเหตุหนึ่งของการเกิดโลหิตจางอย่างรุนแรงในโรคมาลาเรียที่เกิดจากสายพันธุ์ *P. vivax* ความรู้และความเข้าใจกลไกการสร้างเม็ดเลือดแดงประสิทธิภาพลดลงจะเป็นประโยชน์ในพัฒนาการรักษาโรคมาลาเรียที่มีภาวะโลหิตจาง

คำหลัก: เชื้อมาลาเรียสายพันธุ์ *P. vivax* ประสิทธิภาพการสร้างเม็ดเลือดแดงลดลง โลหิตจาง เซลล์ต้นกำเนิดเม็ดเลือด เม็ดเลือดแดง ฟอสโฟโปรตีน โปรตีน ezrin

EXECUTIVE SUMMARY

Anemia has frequently been associated with severe malaria and is believed to contribute to the morbidity and mortality of disease. Most published reports on malaria associated anemia focus on *Plamodium falciparum* with *P. vivax* being less well studied (Sina, 2002). However, growing evidence from several geographic regions has demonstrated that *P. vivax* malaria is associated with a higher frequency and more severe anemia (Selvam and Baskaran, 1996; Luxemburger et al., 1997; Mohapatra et al., 2002; Song, et al., 2003; Collins et al., 2003; Echeverri et al., 2003; Kochar et al., 2005; Kochar et al., 2009). The underlying causes of severe malarial anemia are multifactors which are the destruction of parasitized erythrocytes and ineffective erythropoiesis or dyserythropoiesis. Hematologic profiles of pancytopenia and dyserythropoiesis in bone marrow have been reported in vivax malaria patients (Yamakawa et al., 1989; Wickramasinghe et al., 2000). However, the reduction of blood cell production in patients infected with vivax parasites is not completely understood. Our model, in vitro culture of erythrocytes from HSCs previously established has been applied to dissect the complexity of anemia in vivax malaria. Results have revealed for the first time that *P. vivax* can directly inhibited erythroid cell proliferation and differentiation (Panichakul et al., 2012). Interestingly, *P. vivax* is able to perturb the division of erythroid cells (Panichakul, et al., 2012). Moreover inflammatory cytokines, TNF-α and IFN-γ are undetected in erythroid cultures exposed to lysates or intact *P. vivax*, but IL-10 was significantly detected in those cultures (Panichakul et al., 2012). These results suggest that the molecular mechanisms underlying the suppression of erythropoiesis by *P. vivax* are remarkably complex. Several studies report that *P. falciparum* hemozoins directly inhibit erythropoiesis (Skorokhod et al, 2010**;** Casals-Pascual et al., 2006; Lamikanra et al., 2009). In mechanismes of ineffective erythropoiesis by *P. falciparum*, it is found that *falciparum* haemozoin treated erythroid cells enhanced the expression of the transcription factor p53 and cdk-inhibitor p21, and retinoblastoma protein, central regulator of G- to S-phase transition is hypophosphorylated, while GATA-1, master transcription factor in erythropoiesis is reduced (Skorokhod et al., 2010). In another

study, *P. falciparum* hemozoins in vitro inhibite erythroid development independently of inflammatory mediators by inducing apoptotic pathways that not only involve activation of caspase 8 and cleavage of caspase 3 but also loss of mitochondrial potential (Lamikanra et al., 2009). These suggest that parasite hemozoins are associated with the suppression of erythropoiesis. For inhibitory effect of *P. vivax* hemozoins on erythropoiesis, it is still unclear and need to be elucidated. The preparation of *P. vivax* hemozoin is difficult to obtain enough amount of hemozoin for study because *P. vivax* culture is not available in laboratory and vivax hemozoin is formed as scattered granule (Morselt, et al., 1973) which is smaller than *P. falciparum* hemozoin with compress stacking form. In this study, we will design to prepare food vacuoles containing hemozoins from vivax-malaria patient blood for studying inhibition of erythropoiesis. Phosphoproteomic approach will be performed to dissect the molecular mechanism of phosphoprotein expression which involves in the inhibitory effect of parasite food vacuoles on erythroid progenitor cell development. The characterization of phosphorylation is critical to the elucidation of signal transduction pathways, the understanding of the mechanism of ineffective erythropoiesis by *P. vivax*. The basic knowledge of ineffective erythropoiesis from this study will be useful for development of therapeutic strategies to treat severe malarial anemia.

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CHAPTER I
INTRODUCTION

Plasmodium vivax is a risk factor for severe anaemia among patients in *vivax*-endemic areas (Rodriguez-Morales et al., 2006; Genton et al., 2008; Kochar et al., 2010; Ketema & Bacha, 2013; Dhingra et al., 2010; Sharma et al., 2013; Bhattacharjee et al., 2013). Increasing evidence from several reports in endemic areas has reinforced the association between *vivax* malaria, severe anemia and death (Barcus et al., 2007; Tjitra et al., 2008; Kochar et al., 2009; Alexandre et al., 2010; Raposo et al., 2013; Quispe et al., 2014; Douglas et al., 2013; Baird, 2013; McGready et al., 2014). The pathogenesis of anaemia in *vivax*-malaria is unclear. The underlying causes of severe malarial anaemia are multifactors which are the destruction of parasitized erythrocytes, ineffective erythropoiesis or dyserythropoiesis and immunity associated with disease. Evidently, dyserythropoiesis, pancytopenia and degradation of erythroblasts were found in bone marrow from patients infected with *vivax* parasites (Ru et al., 2009; Raina et al., 1998; Yamakawa et al., 1989; Wickramasinghe et al., 2000; Wickramasinghe et al., 1989). Moreover, In vitro culture of erythroid cells from hematopoietic stem cells has been shown that *P. vivax* is able to directly inhibited erythroid cell proliferation and differentiation (Panichakul et al., 2012). The molecular mechanisms underlying the suppression of erythropoiesis by *P. vivax* are remarkably complex.

Phosphoproteome strategy is alternative proteomic method that has been provided the opportunity to investigate the molecular mechanism of signal transduction pathway in each biological processes (Choudhary & Mann, 2010). Regarding to separate the phosphoproteins, the enrichment techniques using metal ion or TiO_2 embedded column are prior steps before the identification and determination of phosphoproteins under LC-based workflow (Carrascal et al., 2008; Ge et al., 2010). Remarkably orchestrated mechanisms in eukaryotic cells are also modulated by various specific signaling proteins under the phosphorylation and dephosphorylation processes of kinase and phosphatase enzymes. This post translational modification in eukaryotic cell machinery is a hallmark regulation pathway which occurs at different

stimuli at particular time. The level of protein phosphorylation vary generally from less than 1% to greater than 90% of specific phosphorylated sites (Macek, et al., 2009). The regulation complex of dynamic signal transduction proteins contributes to the destination of the cells, whereas parasite also influence some inductions through the specific different function of proteins during host-pathogen interaction that especially demonstrate to the roles in pathogenesis (Salinas et al., 2014). In suppression of erythroid development by *P. vivax*, it has not been elucidate which specific mechanisms during parasite exposure to suppress erythroid development then consequentially progress to the pathophysiology of anemia. Here, we described the first comparative phosphoproteome of erythroid cells derived from human hematopoietic stem cells which were exposed to proteins of *P. vivax* using IMAC phosphoprotein isolation directly coupled with LC MS/MS analysis. Interestingly, the phosphoproteins, ezrin involved in the regulation of cytoskeleton pathway in cell development. Ezrin, a member of the ezrin, radixin, and moesin (ERM) subfamily of cytoskeletal proteins is conserved both functionally and structurally (Fievet et al., 2007). By regulation of membrane cytoskeleton, ezrin has roles in several cellular processes such as maintenance of survival, cytokinesis, adhesion, membrane dynamics, motility and integration of membrane transport with signaling pathways (Bretscher et al., 2002). Usually, ezrin exists in an inactive conformation and its function is a crosslinker between the plasma membrane and cellular cytoskeleton (Gary & Bretscher, 1995). Two regions of ezrin, N-terminal and C-terminal regions involved in active conformation are the binding of the N-terminal region to phosphatidylinositol 4,5-biphosphate and the phosphorylation of a threonine at residue 567 (Thr567) in the C-terminal region (Fievet et al., 2007). Rho kinase and protein kinase C (PKC) have been shown to phosphorylate the C-terminal threonine and facilitate conformational activation of ezrin proteins for the F-actin binding site (Matsui et al., 1998; Tran Quang et al., 2000; Ng et al., 2001; Pietromoneco et al., 1998; Simons et al., 1998).

This study demonstrates that the cytoskeleton protein ezrin involves in inhibitory effect of *P. vivax* on erythroid cell growth leading to ineffective erythropoiesis. The basic knowledge of ineffective erythropoiesis from this study will be useful for development of therapeutic strategies for severe malarial anaemia.

OBJECTIVES

1. To investigate the propensity of the food vacuoles of *vivax* parasites on the development of erythroid progenitors.
2. To characterize the phosphorylated proteins in suppressed erythroid cells that are caused by food vacuoles of *vivax* parasites or whole parasites.

CHAPTER II
LITERATURE REVIEW

Plasmodium vivax threatens approximately 2.8 billion people globally, will be difficult to eradicate as well as *Plasmodium falciparum* (Guerra et al., 2010; Carter & Mendis, 2002; Galinski & Barnwell, 2008; Price et al., 2007). The endemic areas of *P. vivax* are the tropic areas in much the same as geographical pattern as *P. falciparum* (Guerra et al., 2010; Guerra et al., 2006). Where *P. falciparum* and *P. vivax* co-exist, the incidence of infection and severity of hematological morbidity and mortality attributable to *P. vivax* tends to peak at a younger age than for *P. falciparum* (Tjitra et al., 2008; Poespoprodjo et al., 2009; Michon et al., 2007; Lin et al., 2010). Increasing evidence from several geographic regions has been reported that *P. vivax* malaria is associated with severe anemia (Selvam and Baskaran, 1996; Luxemburger et al., 1997; Mohapatra et al., 2002; Song, et al., 2003; Collins et al., 2003; Echeverri et al., 2003; Kochar et al., 2005; Kochar et al., 2009; Rizvi et al., 2013; Gehlawat et al., 2013; Sharma et al .,2013; Ketema & Bacha, 2013; Bhattacharjee et al., 2013; Abdallah et al., 2013). In Papua, Indonesia, approximately 25 % of infants hospitalized with *vivax* malaria have severe anaemia (hemoglobin < 5 g/dl) (Tjitra et al., 2008). The pathogenesis of anaemia in *vivax* malaria is still unclear.

Anemia in *vivax* malaria

Direct evidence from vivax malariatherapy patient studies shows that severe anaemia develop much more rapidly than the premature erythrocyte death by *P. vivax* infection (death of infected reticulocytes), thus the other mechanisms of anaemia are likely to be associated (Kitchen, 1938). *Vivax*-infected erythrocytes adhere to uninfected red blood cells or resetting (Chotivanich et al., 1998) but they have limited to adhere toendothelial cells and, thus, sequestration in microvasculature is not a major factor in the pathogenesis of *vivax* malaria (Anstey et al., 2007). *Vivax*-infected cells become more deformable as the parasite matures and to retain the ability to squeeze through splenic slits (Handayani et al., 2009; Suwanarusk et al., 2004). In *vivax* malaria, possibly non-parasitized red cells are hypothesized to be more fragile

than red cells (Handayani et al., 2009; Suwanarusk et al., 2004). Although, removal of uninfected red blood cells in *vivax* patient blood is an important component of *vivax*-associated anemia (Tjitra et al., 2008), the mechanisms of removal are not understood. In addition, activation of innate, cell-mediated and humoral immune systems in response to the presence of *P. vivax* antigens enhances removal of infected and abnormal uninfected red cells (Karunaweera et al., 2003). Cytokine level in patient blood has not been found to correlate with the degree of anemia in *vivax* malaria (Fernandes et al., 2008). Increased phagocytic activity of macrophages in *vivax* malaria results in a highly oxidative environment and this may associate to destruction of non-infected red cells (Griffiths et al., 2001). This is still unknown whether these processes occur in *vivax* anemia.

Patients with *vivax* malaria have bone marrow abnormalities reflecting impaired erythropoiesis. Hematological profiles of dyserythropoiesis and pancytopenia in bone marrow of *vivax* malaria patients have been reported (Yamakawa et al., 1989; Wickramasinghe et al., 2000). In cases of chronic infections with *vivax* parasites, marrow cellularity tends to be ineffective erythropoiesis as indicated presence of morphologically abnormal erythroblasts as a result of cellular injury, and phagocytosis of erythroblasts by marrow macrophages (Wickramasinghe et al., 1989). Using electron microscopy, it was shown that parasitization and degradation of erythroblasts in patients with uncomplicated *vivax* malaria (Ru et al., 2009). In *vivax* malaria, hypoxia of the bone marrow is unlikely to be significant but there is minimal schizont sequestration. Directly toxic effect on erythroblasts or their precursors by *P. vivax* has been proposed (Wickramasinghe et al., 1989; Panichakul et al., 2012). Alternatively *vivax* parasites may exert its effect on bone marrow macrophages leading to increased phagocytic activity or release of cytotoxic molecules damaging hematopoietic cells (Wickramasinghe et al., 1989). The underlying causes of severe malarial anaemia are multifactors which are the destruction of parasitized erythrocytes and ineffective erythropoiesis or dyserythropoiesis. However, the reduction of blood cell production in patients infected with *vivax* parasites is not completely understood. In vitro culture of erythrocytes from HSCs has been applied to dissect the complexity of anaemia in *vivax* malaria and results show that *P. vivax* is able to directly inhibited erythroid cell

proliferation and differentiation (Panichakul et al., 2012). Moreover, *P. vivax* is able to perturb the division of erythroid cells (Panichakul, et al., 2012). This suggests that the molecular mechanisms underlying the suppression of erythropoiesis by *P. vivax* are remarkably complex.

Malaria pigment

Postmortem analysis of bone marrow section has shown massive presence of malaria pigment hemozoin in bone marrow macrophages (Wickramasinghe & Abdalla, 2000; Casals-Pascual et al., 2006). Electron microscopic investigations of erythrocytes infected with malaria parasites (Aikawa et al., 1996) have shown that the pigment and also the hemoglobin are only present in food vacuoles of the parasite. In vacuoles, hemoglobin and pigment components are present in variable ratios. The spectrum of the undigested hemoglobin in an infected erythrocyte is identical with the spectrum of hemoglobin from a non-infected erythrocyte, while the progressive development of the parasite the pigment granules or food vacuoles contain an increasing amount of the compound with the absorption maximum at 442 nm that is the pure malaria pigment (Morselt et al., 1973). *P. vivax* hemozoin is formed as scattered granule (Morselt, et al., 1973) which is different from *P. falciparum* hemozoin with compress stacking form. However, the cause of reduction in blood cell production in the bone marrow of patients with vivax malaria is not completely understood. Recently, lysate or intact of both *P. falciparum* and *P. vivax* have shown the inhibition of erythropoiesis, several studies found that *P. falciparum* hemozoins directly inhibit erythropoiesis (Skorokhod et al, 2010**;** Casals-Pascual et al., 2006; Lamikanra et al., 2009), and only one reported that *P. vivax* lysates in vitro suppress erythroid cell development (Panichakul et al., 2012). In mechanismes of ineffective erythropoiesis by *P. falciparum*, it is found that *falciparum* haemozoin treated erythroid cells enhanced the expression of the transcription factor p53 and cdk-inhibitor p21, and retinoblastoma protein, central regulator of G- to S-phase transition was hypophosphorylated, while GATA-1, master transcription factor in erythropoiesis was reduced (Skorokhod et al., 2010). Another study found that *P. falciparum* hemozoins in vitro inhibited erythroid development independently of inflammatory mediators by inducing apoptotic pathways that not only involve activation of caspase

8 and cleavage of caspase 3 but also loss of mitochondrial potential (Lamikanra et al., 2009). Therefore the molecular mechanisms underlying the suppression of erythropoiesis by *P. falciparum* are remarkably complex. For inhibitory effect of *P. vivax* on erythropoiesis, it is still unclear and need to be elucidated. The preparation of *P. vivax* hemozoin is difficult to obtain enough amount of hemozoin for study because *P. vivax* culture is not available in laboratory and vivax hemozoin is formed as scattered granule (Morselt, et al., 1973) which is smaller than *P. falciparum* hemozoin with compress stacking form. The preparation food vacuoles containing hemozoins from *vivax*-malaria patient blood has not been studied.

Phosphoproteomics

Phosphoproteomics is geared the identification and quantification of phosphorylated proteins and the identification of phosphorylation sites (Aebersold & Mann, 2003). Protein phosphorylation is one of the important posttranslational modifications in living cells and this is involved in regulatory functions such as cell cycle control, proliferation, differentiation, transformation, metabolism and receptor-mediated signal transduction (Boris et al., 2008). Two types of enzymes, protein kinases and phosphatases control substrate modification by reversible phosphorylation and dephosphorylation and phosphorylation of proteins occurs almost with adenosine triphosphate (ATP) (Boris et al., 2008). One-third of all cellular proteins are estimated to be phosphorylated, the levels of phosphorylation vary widely and specific sites are phosphorylated from less than 1 % to greater than 90 % (Boris et al., 2008). Protein phosphorylation in signal transduction presents an activating/deactivating switch of protein activity. Phosphorylation plays an important role in the attenuation and termination of the signal progression. Breakdown of phosphorylation balanced control system leads to diseases (Schlessinger, 2000; Pawson & Scott, 2005). In quiescent stage of cells, not all signaling proteins are turned off, but they are sustained at a basal level. The activation of phosphorylation significantly depend on the stimulus applied. In addition, the regulation of the involved kinases and phosphatases lead to changes in the phosphorylation levels of relevant signaling proteins (Boris et al., 2008).

Mass spectrometry (MS), a detector of phosphorylation events, is able to identify each phosphopeptide and localize the phosphorylation molecules in the

peptide sequence. Recently, MS become sufficiently sensitive and robust to be used in large scale in cell signaling research (Boris et al., 2008). The quantitative phosphoproteomics has become a very powerful technological platform which is applied to study in basic cell biology. To enrich phosphopeptides, immobilized metal affinity chromatography (IMAC) with a variety of metal, including Fe3+, Ga3+, Al3+, and Zr3+ are developed for binding phosphopeptides. Titannium dioxide (TiO2) spheres are alternative column for enrichment methods with higher affinity and selectivity (Ikeguchi & Nakamura, 1997). For analysis of phosphopeptide databases, three databases are Phospho.ELM (http://phospho.elm.eu.org), PhosphoSite (www.phosphosite.org), and Phosida (www.phosida.com). UniProt (www.uniprot.org), most of these databases accept all published Data. ScanSite (http://scansite.mit.edu) and NetPhos (www.cbs.dtu.dk/services/NetPhos) are phospho-prediction based on matrix motifs and neural networks, respectively. Phosphopeptide mixtures have to be analyzed by mass spectrometry. The phosphopeptides are separated on a nanoLC column filled with a reversed-phase (C18) material and analyzed in high – resolution mass spectrometers, which the mass-to-charge (m/z) ratio and intensity (MS spectrum) are measured. The mass spectrometer dissociates the peptides and detects the resulting fragment ions in a tandem mass spectrum (Aebersold & Mann, 2003). In this study, phosphoproteomic approach is performed to dissect the molecular mechanism of phosphoprotein regulation which involves in the inhibitory effect of parasites on erythroid cell development. The characterization of phosphorylation is critical to the elucidation of signal transduction pathways, the understanding of the mechanism of ineffective erythropoiesis by *P. vivax*.

Ezrin

Ezrin, radixin, and moesin (ERM) subfamily are cytoskeletal proteins. ERM proteins share highly homologous (-85% identity) N-terminal domains of human erythroid band 4.1 protein (FERM), which interact with plasma membrane proteins (Bonilha 2007). Indirect binding of ERM proteins with multiple transmembrane proteins, such as CFTR (cystic fibrosis transmembrane conductance regulator) and Na+/H+ antiporter, require adaptor proteins, PDZ domain (Bretscher et al., 2002;

Bonilha, 2007). ERM proteins in cytoplasm are maintained in inactive conformation. Interaction between FERM and the C-terminal domains masks active sites in the FERM domain, rendering it for binding to membrane proteins and F-actin. The C-terminal domain is composed of one beta-strand and six helical regions which bind to the area on the FERM domain surface (Bretscher et al., 2002). Function of ERM proteins have been reported as cross-linkers between the plasma membrane and the cortical cytoskeleton. ERM function is found that first, an F-actin-binding site is in the last 34 residues of ezrin (Turunen et al., 1994) and second, the C-terminal residues of ezrin are found to bind tightly to the FERM domain that the F-actin-binding site is masked (Gary & Bretscher, 1995). Ezrin, a member of ERM was first found as a cytoskeletal protein of the intestinal-brush border and the architechture of the cytoskeleton in cellular structures of the intestine (Saotome et al., 2004). Ezrin is conserved both functionally and structurally (Fievet et al., 2007) to regulate membrane cytoskeleton. Ezrin has roles in several cellular processes such as maintenance of survival, cytokinesis, adhesion, membrane dynamics, motility and integration of membrane transport with signaling pathways (Bretscher et al., 2002). Usually, ezrin exists in an inactive conformation, in which the actin-binding and membrane sites are masked by intramolecular interaction of the N-terminal and C-terminal regions (Gary & Bretscher, 1995). Two regions of ezrin involved in active form are the binding of the N-terminal region to phosphatidylinositol 4,5-biphosphate and the phosphorylation of a threonine at residue 567 (T567) in the C-terminal region (Fievet et al., 2007). Rho kinase and protein kinase C (PKC) have been shown to phosphorylate the C-terminal threonine and facilitate conformational activation of ezrin proteins for the F-acin binding site (Matsui et al., 1998; Tran Quang et al., 2000; Ng et al., 2001; Pietromoneco et al., 1998; Simons et al., 1998). Phosphorylation of a conserved threonine residue (Thr 567) are required for ezrin activation, membrane-cytoskeleton linker function (Fievet et al., 2004). Moreover, activation of Rho leads to phosphorylation of ezrin proteins at Thr 567 of C-terminal residue and their accumulation in actin-containing protrusions (Nakamura et al., 1995; Shaw et al., 1998) in Swiss 3T3 cells (Mackay et al., 1997). Ezrin interactions with the PI3K/Akt pathway appeared to mediate cell survival signals in a kidney-derived epithelial cell line (Gautreau A., et al., 1999). Increasing evidence regarding the role of ezrin in cell

proliferation has been reported. Most reports, involving in cell lines and tissues, have reported a direct relationship between cell proliferation and the level of ezrin expression in cells (Chen Z., et al., 2001; Ohtani K., 2002; Crepaldi T., et al., 1997). Anti-ezrin antibodies blocked mouse fibroblasts to entry into S phase, suggesting a proliferative function of ezrin (Kaul et al., 1996). In response to TNF-alpha, ezrin inhibits endothelial cell proliferation via transcriptional repression of cyclin A, a cell cycle regulatory protein (Kishore, 2005). Ezrin, a membrane cytoskeletal cross-linker functions to involve in several growth factor receptors signaling leading to cell survival, differentiation and cell adhesion. However, the role of ezrin during normal erythroid differentiation remains unclear (Monni et al., 2008). Ezrin is expressed in murine erythrocytes and in sp-1 transgenic proerythroblasts. An enforced expression of the N-terminus domain of ezrin in both preleukemic and leukemic cells showed in a reduction in cell number and the appearance of apoptotic cells. This indicated that ezrin play a crucial role in proerythroblasts by the viability and proliferation (Monni et al., 2008). Ezrin protein is a member of band 4.1 protein (FERM). In normal erythrocytes, membrane proteins of cells include protein 4.1, protein 4.2, band 3, spectrin (Cohen & Gascard, 1992). The phosphorylation of erythrocyte protein 4.1 is found to involve in a mechanism which the intracellular malaria parasites alters the mechanical properties of the host plasma membrane and modulates parasite growth in vivo (Chishti et al., 1994). ERM proteins bind directly to the certain cytoplasmic proteins with single transmembrane domains, including ICAM-1, ICAM-2, ICAM-3, CD 43, CD 44, CD 95 and syndecan-2. ICAMs are co-localized with ERM proteins in several cell types (Serrador et al., 1997; 10 Heiska et al., 1998). Interactions of ERM proteins with ICAMs have been unclear.

In this study, we demonstrate that the cytoskeleton protein ezrin involves in inhibitory effect of *P. vivax* on erythroid cell growth. This basic knowledge of ineffective erythropoiesis from this study will be give more understanding development of anaemia in malaria.

CHAPTER III
MATERIALS AND METHODS

Parasite preparation

Vivax parasites from patient blood with 0.05-0.2 % parasitaemia, as determined by examining thick and thin blood smears, were collected. The ethical and methodological aspects of this study for parasite collection from patients attending the malaria clinic in Tha Sae, Chumpon Province, Thailand (MU-IRB 2012/170.2511) have been approved by the Mahidol University Institutional Review Board, Mahidol University, Bangkok, Thailand. Infected erythrocytes (IE) were separated from patient blood using a 60% Percoll solution as previously described (Panichakul et al., 2007). Briefly, whole blood with *vivax* parasites was collected and then filtrated using a Plasmodipur filter (Euro-Diagnostic B.V., Netherlands) to remove white blood cells. To obtain asexual parasites, packed, infected RBCs from 20 ml of patient blood was diluted 1:20 with RPMI1640 (Invitrogen®, CA, USA), layered on 60% Percoll and centrifuged at 1,200g for 20 mins at 20ºC. The purity of IE after isolation was 95% and the pure fraction of isolated IE contained 80 % schizontes and 20 % of other stages. The isolated IEs were used either intact or as lyzed cells prepared by freezing and thawing.

Isolation of digestive vacuoles from *Plasmodium vivax*

The isolation of *P. vivax* digestive vacuoles (PvDV) was performed by modified method from a previous report (Dasari, et al., 2011). The parasites were cultured until mature to late trophozoite or early schizontstages. Mature parasites were adjusted to 20% hematocrit with RPMI (GIBCO, USA) and over layered on 60% Percoll (GE Healthcare Munich, Germany) and centrifuged at 1,200g for 20 min at 4°C. The interphase over Percoll was collected and washed twice with RPMI medium. The number of parasite was counted in hemacytometer. The purity was determined by Giemsa's staining. After isolated schizont stage by using 60% Percoll, these schizonts were cultured in 5 ml of Stemline II medium (Sigma-Aldrich Corporation, Missouri, USA) with 50% AB serum and allowed to release. The

supernatant was centrifuged at 17,000 rpm (Heal Force® Neofuge 18R, China) for 20 min at 4°C to pellet digestive vacuoles (DVs), which were resuspended in 2 ml of RPMI and layered on the top of a 30%, 45% and 60% Percoll step gradient (2 ml of each layers). After centrifugation at 2,500 rpm for 15 min at 4°C, the top 30% and 45% layers were collected and washed twice with RPMI in 2 ml Eppendorf tubes at 17,000 rpm for 10 min at 4°C. Pellets 100 µl were suspended in 1.8 ml of ice cold 42% Percoll containing 250 mM sucrose, 1.5 mM $MgSO_4$ (pH 7.4). The suspension was triturated 10-15 times through a 27-G, 3.5 cm needle and centrifuged at 17,000 rpm for 20 min at 4°C. DVs were recovered as a band of material at the bottom of the tubes. They were washed twice with 2 ml veronal buffered saline (VBS; Virion/Serion, Würzburg, Germany) at 17,000 rpm for 10 min at 4°C. The lysed PvDVs were prepared by freezing and thawing.

The isolation of *P. falciparum* digestive vacuoles (PfDV) was performed by a previous method (Dasari et al., 2011). Briefly, parasite schizonts were cultured in RPMI 1640 medium and allowed to lyse. The parasite culture was centrifuged at 250 g for 5 min and the supernatant was collected and then centrifuged at 5,000 rpm for 10 min. The pellet was resuspended in RPMI and layered on the top of a 30, 45 and 60 % Percoll and centrifuged at 2,500 rpm for 15 min at 4°C. The top 30 and 40 % layers were collected and then washed with RPMI. Pellets were suspended in 1.8 ml of ice cold 42 % Percoll containing with 250 mM sucrose and 1.5 mM $MgSO_4$ (pH 7.4) and then mixed by 27-G, 1-2 cm needle for 8 times. After centrifuged at 13,000 rpm for 10 min at 4°C. The pellet of PfDV was as a black band at the bottom of a tube and washed twice with VBS. The lysed PfDVs were prepared by freezing and thawing.

Isolation of cord blood CD 34$^+$ cells and culture condition

Twenty cases of umbilical cord blood from normal full-term deliveries in Ramathibodi Hospital, Bangkok, Thailand was collected into cord blood bags containing anticoagulant solution (CPDA-1 solution) (Kawasumi Laboratories, Thailand). Cord blood collection (ID 04-52-39) was approved by the Ethical Committee of Research on Human Beings of the Ramathibodi Hospital, Faculty of Medicine, Mahidol University. Haematopoietic stem cells/CD34$^+$ cells were isolated

from cord blood mononuclear cells (MNC) using a CD 34 isolation kit with magnetic microbead selection with Mini-MACS columns (Miltenyi Biotech, Geramany) as described by (Panichakul et al., 2007). The purity of $CD34^+$ cells after isolation was 97% as judged by flow cytometry analysis.

Culture conditions

gECs, 5-day old at a density of 1 x 10^4 cell/well in 24-well tissue culture plates (Corning Incorporated Costar®) were cultured in 0.5 ml of complete medium containing StemlineII medium (Sigma-Aldrich Corporation, Missouri, USA) supplemented with cytokines (Panichakul et al., 2007). Intact or lysed DVs, and lysed infected erythrocytes (IE) or uninfected erythrocytes (UE) were added in ratio 10:1 (DV/IE/UE : gEC) to cell cultures and cultured at 37˚C in 5% CO_2 for 24, 48, 72 h. Viable cells were determined by trypan blue dye exclusion and Propidium iodide was used to determine dead cells.

Cell cycle analysis

After cells were exposed with /without IE for 48 and 72 h, cells were washed with PBS and fixed in 70% ethanol overnight or stored at -20˚C until analysis. Fixed cells were centrifuged at 1,000 g 5 min and washed with PBS. RNase A (Geneaid Biotech Ltd., Taiwan) was added at a final concentration 0.25 mg/ml and cells were then incubated for 10 min at 37˚C. Cells were stained with 0.020 mg/ml propidium iodide (eBioscience) and incubated for 30 min at 37˚C in dark. DNA analysis was performed on a FACS Canto™ flow cytometer (Becton Diskinson). Cell cycle analysis by BD FACSDiva software version 4.1 (Becton Diskinson) was used to determine the percentages of cells in the different cell cycle phases. Experiments were performed three independent times and values are shown as means ± S.D.

Preparation of phosphoprotiens

Cells exposed with/without parasite lysates for 24 – 72 h were prepared phosphoproteins by using Pierce Phosphoprotein enrichment kit (Thermo Scientific, Pierce Biotechnology, IL, USA) and the procedure for enrichment of phosphoproteins was followed in manufacturer's instructions. Four steps of the preparation of

phosphoproteins included cell lysis, phosphoprotein enrichment, phosphoprotein concentration and desalting.

In step of cell lysis, cell pellets, 3-5 x 10^6 cells were added 200 ul of lysis/binding/wash buffer with CHAPS and 1X phosphatase inhibitors (Phosphatase inhibitor cocktail 100X, Roche, UK) and lysed on ice by sonicator (Sonics Vibra cell,sonics & materials INC., USA) with amplitude 70%, break 2 sec, stop 2 sec, 15 cycle in 1 min. After centrifuged (Tomy MX-305 high speed refrigerated microcentrifuge CA, USA) 10,000 g at 4ºC for 10 min, supernatant of cell lysates was collected and proteins were determined by Lowry method (Lowry et al., 1951).

In phosphoprotein enrichment, Proteins of cell lysates were adjusted the concentration to 0.5 mg/ml lysis/binding/wash buffer without CHAPS and applied to each column. Columns were inverted to mix for 30 min at 4ºC and then washed for 3 times with lysis/binding/wash buffer with CHAPS by centrifuge (Allegra X-22R centrifuge, Beckman coulter Inc, USA) at 1000 g for 1 min at 4ºC. One millimeter of elution buffer consisted of 75 mM sodium phosphate, 500 mM sodium chloride, pH 7.5 was added into each column and columns were incubated at room temperature with agitation for 3 min. The eluted proteins were collected by centrifugation at 1000 g for 1 min at 4ºC, and this step was repeated for 4 times. Pool of eluted proteins were frozen at – 80ºC.

Phosphoprotein concentration by using Pierce concentrator (Thermo Scientific), briefly, 4 ml of each sample of eluted proteins were placed into upper chamber of concentrator and then centrifuged 7000 g for 30 min at 4ºC. The concentrated proteins, approximately 100-200 µl were collected from the upper chamber and kept at – 80 ºC.

In the last step, salts and other molecules (< 1000 Da) in samples of the concentrated proteins were removed by Zeba™ spin desalting columns (Thermo Scientific) containing a high-performance resin. Briefly, 100 µl of samples were applied onto columns and then centrifuged 700 g for 30 sec. The desalted samples were collected and proteins of each sample was determined by Lowry method and analysed.

Phosphoprotein analysis

After desalting, 4 ug of phosphoproteins in 10 mM ammonium bicarbonate were reduced with 10 mM DTT (Dithiotheitol) for 30 min at 60 ^{o}C, alkylated with 15 mM IAA (Iodoacetamide) at room temperature for 30 min, and digested with sequencing grade trypsin (Promega, Geramany) for 16 h at 37 ^{o}C. Tryptic phosphopeptides were diluted 0.1 % formic acid to final concentration of 0.25 μg/μl and centrifuged 10,000 rpm at room temperature for 10 min. Phosphopeptide samples were injected into a NanoAcquity system (Waters Corp., Milford, MA) equipped with a Symmetry C_{18} 5 μm, 180-μm x 20-mm Trap column and a BEH130 C_{18} 1.7 μm, 100-μm x 100-mm analytical reversed phase column (Waters Corp., Milford, MA). The samples were initially transferred with an aqueous 0.1% formic acid solution to the trap column with a flow rate of 15 μl/min for 1 min. Mobile phase A was water with 0.1% formic acid, and mobile phase B was 0.1% formic acid in acetonitrile. The peptides were separated with a gradient of 15–50% mobile phase B over 15 min at a flow rate of 600 nl/min followed by a 3-min rinse with 80% of mobile phase B. The column temperature was maintained at 35 °C. The lock mass was delivered from the auxiliary pump of the NanoAcquity pump with a constant flow rate of 500 nl/min at a concentration of 200 fmol/μl of [Glu^{1}]fibrinopeptide B to the reference sprayer of the NanoLockSpray source of the mass spectrometer. All samples were analyzed in once. Analysis of tryptic peptides was performed using a SYNAPT^{TM} HDMS mass spectrometer (Waters Corp., Manchester, UK). For all measurements, the mass spectrometer was operated in the V-mode of analysis with a resolution of at least 10,000 full-width half-maximum. All analyses were performed using positive nanoelectrospray ion mode. The time-of-flight analyzer of the mass spectrometer was externally calibrated with [Glu^{1}]fibrinopeptide B from m/z 50 to 1600 with acquisition lock mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu^{1}]fibrinopeptide B. The reference sprayer was sampled with a frequency of 20 sec. Accurate mass LC-MS data were acquired with data direct acquisition mode. The energy of trap was set at a collision energy of 6 V. In transfer collision energy control, low energy was set at 4 V. The quadrupole mass analyzer was adjusted such that ions from m/z 300 to 1800 were efficiently transmitted. The MS\MS survey is over range 50 to 1990 Da and scan time was 0.5 sec.

Protein identification

The differential quantitation of proteins and peptides based on MS signal intensities of individual LC – MS was analysed. For proteins quantitation, DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare (Johansson et al., 2006; Thorsell et al., 2007) was used. Acquired LC-MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ions signal intensities in MS mode. The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software (Matrix Science, London, UK, (Perkins et al., 1999)). For protein identification, the data was searched against the NCBI *Homo sapiens* database with following parameters: enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (±0.6 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages.

Bioinformatic analysis

All MS/MS data was search against human protein database using Swissprot protein database. The heatmap visualization was constructed using free web-based analysis MEV program (Saeed et al., 2003) and protein cluster was analyzed by distance metric selection with Person correlation parameters calculate K-Means. Different levels of phosphoproteins were analyzed by significant T test at p value = 0.5. The gene ontology analysis was performed using UniprotKB http://www.uniprot.org (Magrane, 2011) and PANTHER http://www.pantherdb.org databases for biological processes and molecular function classification (Mi et al., 2013). The protein-protein interactions mapping was conducted under the data visualization with statistical analysis at low confident score in STRING database http://www.string-db.com (Franceschini et al., 2013). Gene biological categorization was performed and selected at p value < 0.01. Analysis of the protein-protein interaction network was performed using the KEGG (Kyoto Encycloprdid of Gens and Genomes) PATHWAY database (Kanehisa et al., 2014; Kanehisa et al., 2000).

Immunofluorescence assay

The distribution of threonine-phosphorylated ERM and ERM was evaluated by immunofluorescence. Cells were fixed in 4 % formaldehyde in PBS (phosphate buffer saline) at 4ºC for 20 min, washed with PBS, permeabilized with 0.1 % Triton X-100 in PBS for 10 min and blocked in 2 % human serum in PBS for 20 min at room temperature. Mouse anti-ezrin (abcam®, Cambridge, UK), and rabbit anti- phospho-ezrin (Thr567)/radixin (Thr564)/mosin (Thr41A3) ERM (Cell Signaling Technology, Danvers, MA, USA) antibodies diluted in 1 % human serum/PBS in ratios 1:100, were added and incubated at room temperature for 1.5 h. Goat anti-rabbit and anti-mouse antibodies conjugated with Alexa Fluor green 488 and Alexa Fluor Red 594 (Molecular probes) diluted in 2 % human serum/PBS was added for 2 h. After washed with PBS, stained cells were mounted with anti-fade medium containing DAPI (Molecular probes). Cells were examined using a laser scanning confocal microscope (LSM 510 Meta, Zeiss, Jena, Germany) with a 63x objective at zoom 2. Immunofluoresence intensity of 1,000 cells from each culture condition was determined using ImageJ software (Rasband, 2014). The mean of intensity from 1,000 cells were presented as intensity ratios, calculated from the intensity of IE/UE-exposed gECs divided by the intensity of gECs in control cultures.

Statistical evaluation

Data of cell growth were analysed using the SPSS program version 17. The unpaired Mann – Whitney – Wilcoxon test was to compare means between independent groups as appropriate and results are reported as statistically significant if the *P* – value was less than 0.01.

CHAPTER IV
RESULTS

Effect of parasite digestive vacuoles on erythroid cell growth

Digestive vacuoles (DVs) were prepared and isolated from blood stages of *P. vivax* and used to study of the effect on erythroid cell (EC) growth. DVs observed under microscope were found the vesicles contained hemozoin crystals and DV size was 1 to 2 μm as shown in Figure 1. Cultures of gECs 5 day-old were exposed to intact or lysed PvDVs/PfDV and lysates of IE/UE at a ratio of 1:10 (gEC:DV/IE/UE) and incubated for 24, 48 and 72 h. Effect of PvDV or PfDV on gEC growth for 24 – 72 h was showed in figure 2. Inhibitory effect of intact or lysed PvDV on gEC growth compared with gEC growth in media was not significantly different with $p < 0.01$, while lysed PvIE was able to inhibit gEC growth to 73, 66 and 48 % after exposure for 24, 48 and 72 h, respectively, and this inhibitory effect of PvIE compared with gEC growth in media was siginificant at $p < 0,01$ as shown in figure 2A. Intact or lysed PfDV and lysed PfIE were not able to inhibit gEC growth after exposure for 24 – 72 h, compared with gEC growth in media as shown in figure 2B.

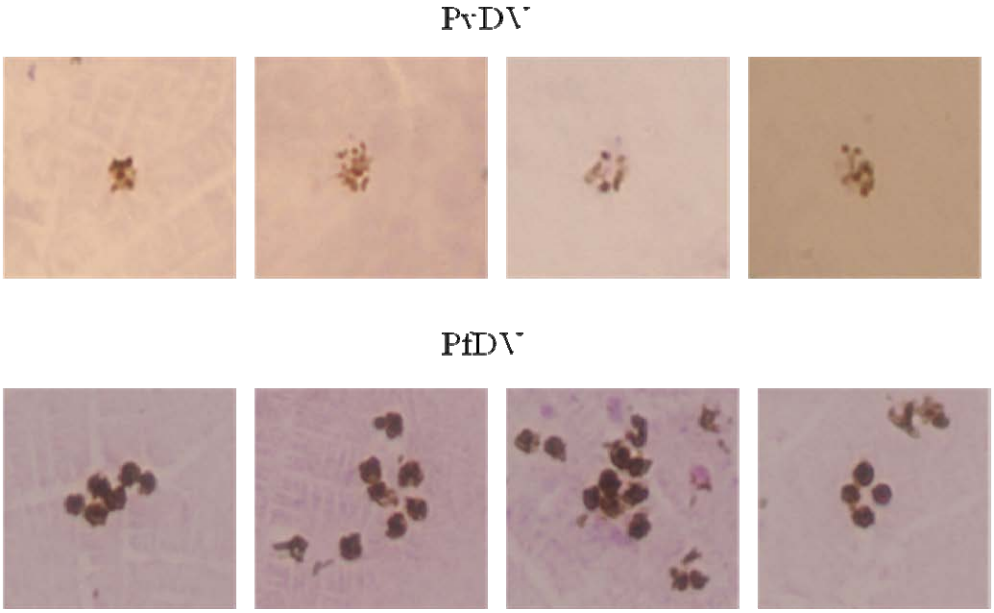
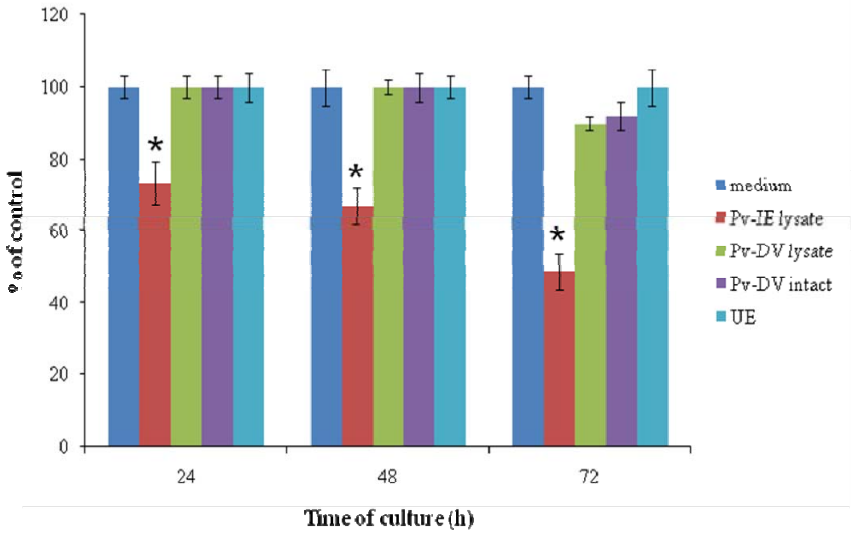


Figure 1 Digestive vacuoles of *P. vivax* and *P. falciparum*. *P. vivax* or *P. falciparum* digestive vacuoles (PvDV or PfDV), isolated from late stage schizonts and stained with Giemsa.

a



b

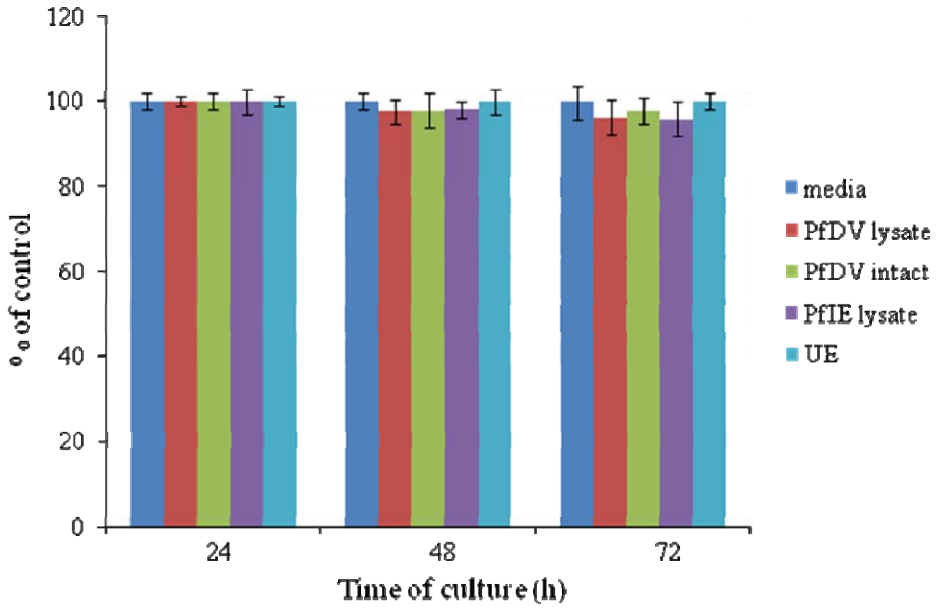


Figure 2 Effect of parasite digestive vacuoles on erythroid cell growth. gECs exposed to intact or lysed PvDV (*P. vivax* digestive vacuoles) in graph A and PfDV (*P. falciparum* digestive vacuoles) in graph B for 24, 48 and 72 h, and controls, including lysates of PvIE (*P. vivax* infected erythrocytes) or PfIE (*P. falciparum* infected erythrocytes), and UE (uninfected erythrocytes from normal donor blood)**.** *Statistically significant at $P < 0.01$.

Inhibition of erythroid cell growth in *in vitro* cultures by *P. vivax*

It was previously reported that *P. vivax* parasites inhibited erythroid cell growth and perturbed erythroid cell division in 3 day *in vitro* cultures (Panichakul, et al., 2012). However, the molecular mechanisms underlying the inhibition of erythroid development by *P. vivax* were unclear. In this study, the underlining mechanism of the inhibitory effect on gEC growth by *P.vivax* was examined using growing erythroid cells (gECs), derived from human cord blood HSCs/$CD34^+$, and infected erythrocytes (IE), isolated from patient blood. gECs from 5-day old cultures were exposed for 24, 48 and 72 h to lysed IE or UE, at a ratio of IE/UE: gEC 10:1. Cell aggregation was found only in cultures of gEC with lysed IE, compared culture control without lysed IE as shown in figure 3. Lysed IE significantly inhibited gEC growth at 48 and 72 h (*P*-value < 0.01), compared with lysed UE and media controls (Figure 4a). No difference in cell death at 24, 48 and 72 h was observed in cultures with and without IE/UE, as shown in Figure 4b. Interestingly, lysed IE dramatically increased the number of cells in the G0 phase and decreased the number of cells in the mitotic fraction (G2/M and > 4n) at 48 and 72 h (Figure 4c). This indicates that parasite proteins were able to inhibit erythroid cell growth and division resulting in the accumulation of cells in a resting phase but did not induce cell death.

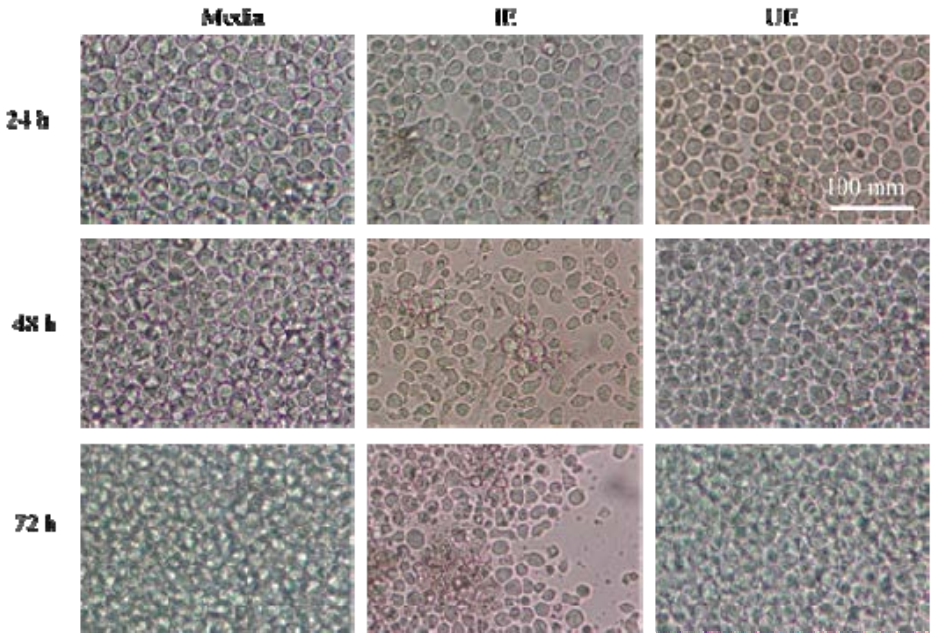
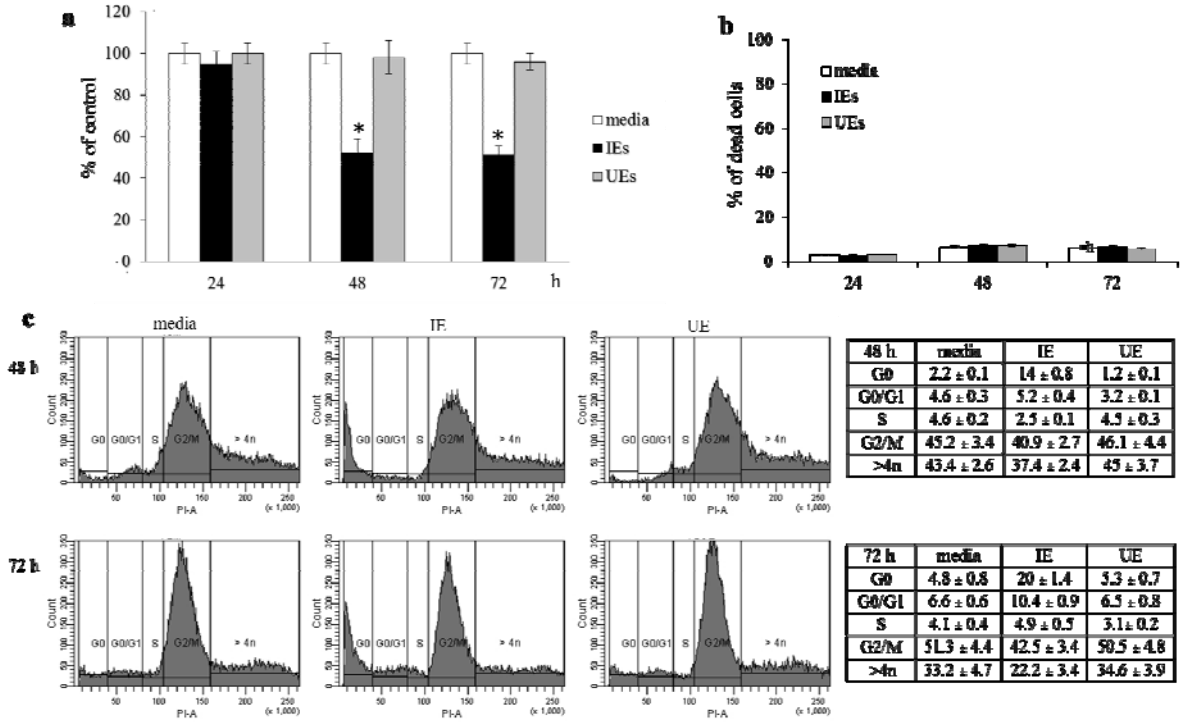


Figure 3. Erythroid cell aggregation in culture of gECs exposed to *P. vivax*. Erythroid cells, 5-day old, were cultured with IE/UE lysates at a ratio of 1:10 (gEC:IE/UE) for 24, 48 and 72 h. Cell aggregation was observed under inverted microscope with 200x magnification.



48 h	media	IE	UE
G0	2.2 ± 0.1	1.4 ± 0.8	1.2 ± 0.1
G0/G1	4.6 ± 0.3	5.2 ± 0.4	3.2 ± 0.1
S	4.6 ± 0.2	2.5 ± 0.1	4.5 ± 0.3
G2/M	45.2 ± 3.4	40.9 ± 2.7	46.1 ± 4.4
>4n	43.4 ± 2.6	37.4 ± 2.4	45 ± 3.7

72 h	media	IE	UE
G0	4.8 ± 0.8	20 ± 1.4	5.3 ± 0.7
G0/G1	6.6 ± 0.6	10.4 ± 0.9	6.5 ± 0.8
S	4.1 ± 0.4	4.9 ± 0.5	3.1± 0.2
G2/M	51.3 ± 4.4	42.5 ± 3.4	50.5 ± 4.8
>4n	33.2 ± 4.7	22.2 ± 3.4	34.6 ± 3.9

Figure 4. Inhibitory effect of *P. vivax* on erythroid cell growth and cell cycle. gECs, 5-day old, were cultured with IE/UE lysate at a ratio of 1:10 (gEC:IE/UE) for 24, 48 and 72 h. **(a)** Cell growth is presented as means ± SD of percentage of control, compared with a control containing medium. **(b)** Cell death is illustrated as means ± SD of percentage of dead cells, and **(c)** cell cycle is illustrated as means ± SD of percentage of DNA content in each phase of G0, G0/G1, S, G2/M and > 4n, using propidium iodide staining. Means ± SD were calculated from five independent experiments. **P*-value < 0.01 compared with media control.

Characterization of phosphoproteins from erythroid cells after exposure to *P. vivax*

To analyze the mechanism of *P. vivax* inhibition on gEC growth, following exposure to IE / UE for 24, 48 and 72 h, phosphoproteins were enriched and analyzed by LC-MS/MS. The Decyder program module was used for quantitation of the MS/MS intensity in each culture condition and results were compared to the human protein database. Phosphoproteins were classified based on biological function, molecular function, and protein class. Forty four phosphoproteins were identified from gECs exposed to IE/UE and media control at 24, 48 and 72 h (Figure 2). The molecular functions of the 44 phosphoproteins were classified according to the gene ontology analysis (Figure 5a) and include those involved in catalysis (40 %), binding (28.6%), structural molecules (17%), enzyme regulators (5.7%), translation regulators (2.9 %), transcription regulators (2.9 %) and transporters (2.9 %). The biological processes of these phosphoproteins (Figure 5b) were found to be predominantly in metabolic processes (23.3 %), cellular processes (15.6 %), transport (8.9 %), and cell cycle (4.4%). In addition, protein class categories were analyzed for these phosphoproteins indicating functions in cytoskeleton (10.6 %), proteases (4.3 %), kinases (4.3 %) and structural proteins (2.1 %) (Figure 5c).

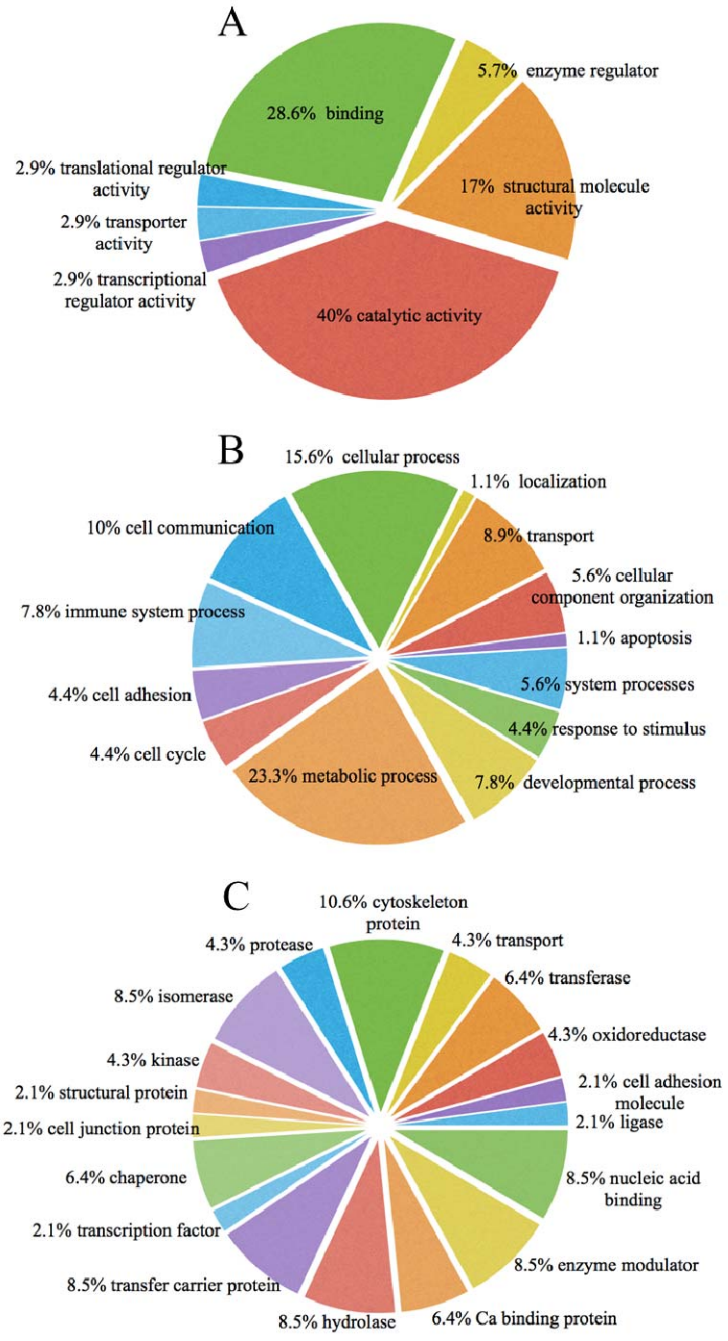


Figure 5. Classification of phosphoproteins identified in erythroid cells. Phosphoproteins found following exposure to infected erythrocytes (IE)/uninfected erythrocytes (UE) and in media control were categorized according to **(a)** molecular functions, **(b)** biological processes, and **(c)** protein classes.

Forty four phosphoproteins, identified from gECs exposed to IE/UE for 24 – 72 h, are presented using a heat map visualization (Figure 6). The relative levels of these phosphoproteins was evaluated as high and low compared with level of phosphoprotein present in gECs from media control. The patterns of phosphoproteins from gECs exposed to IE/UE or in media for 24, 48 and 72 h were analyzed in 5 clusters and categorized using the Kmean clustering method (Figure 6). Interestingly, the relative level pattern of phosphoproteins in cluster 1 was low from gECs exposed to IE for 24 – 72 h. To identify potential phosphoproteins from 5 clusters involved in inhibition of gEC growth and division by IE, the relative levels of phosphoproteins in each condition and time point were determined using statistical analysis with paired t-test at *P*-value $\leq$ 0.05. The lists of selected phosphoproteins with significant differences in relative level (*P*-value $\leq$ 0.05) were evaluated for biological processes and molecular functions using the KEGG PATHWAY database, as shown in Table 1. Interestingly, three of the phosphoproteins identified, ezrin, alpha actinin-1 and Rho kinase were reported to function in the regulation of the cellular cytoskeleton (Table 1). We observed that ezrin, alpha actinin-1 and Rho kinase from gECs are all included in cluster 1 and that the relative levels of these three phosphoproteins were significantly low from gECs exposed to IE for 72 h, compared with gECs in media only. These findings suggests that ezrin, alpha actinin-1 and rho kinase in the phosphoproteome of gECs may have roles relevant to the cellular pathology caused by *P. vivax* during erythroid cell growth.

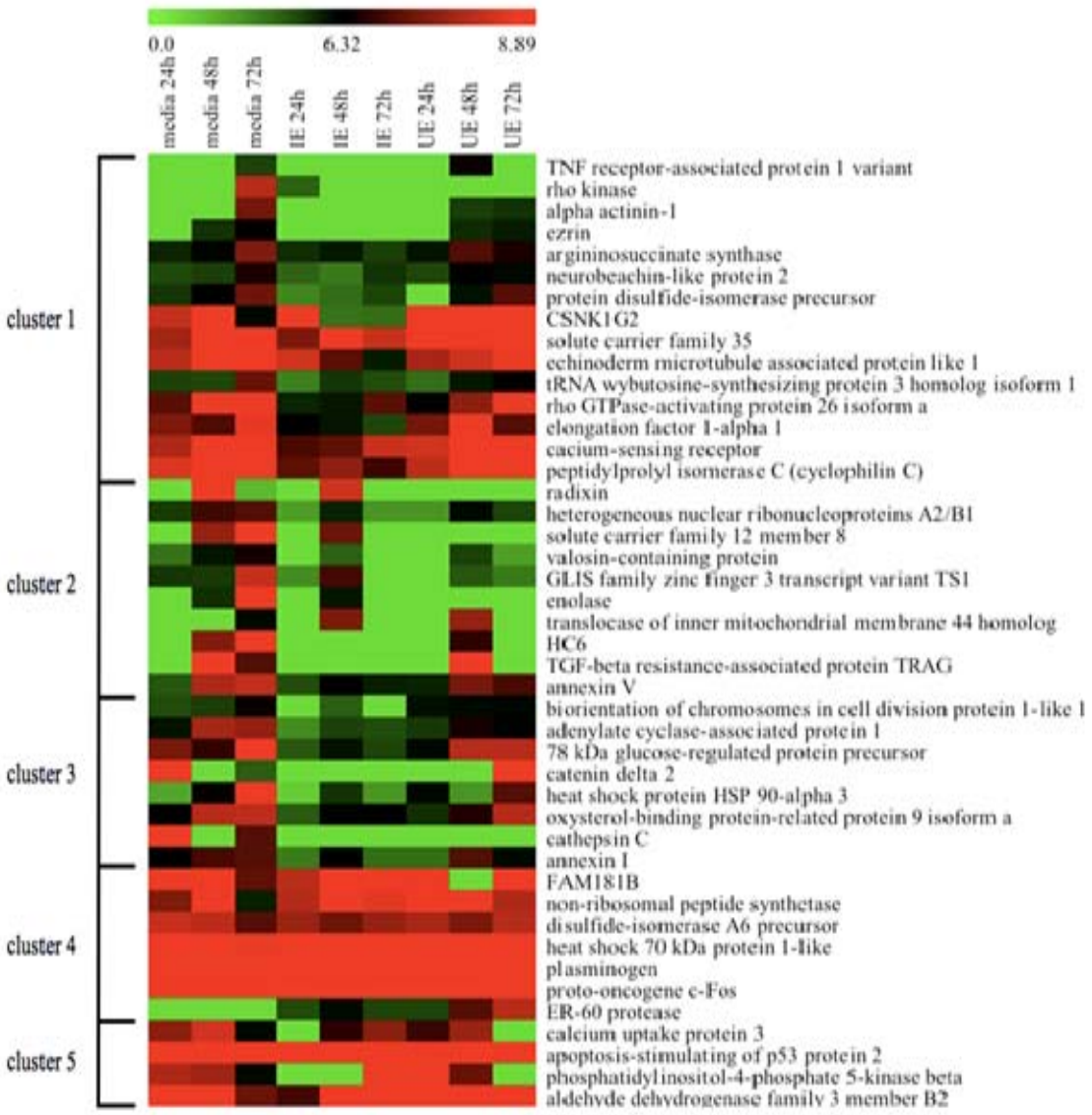


Figure 6. Comparison of phosphoproteins levels in erythroid cells untreated and exposed to *P. vivax*. Heatmap of relatively high (red) and low (green) phosphoproteins from gECs exposed to lysates of infected erythrocytes (IE) and uninfected erythrocytes (UE) for 24 -72 h and gECs in media control. The heatmap and clustering were generated using the web-based analysis program MEV.

Table 1 Evaluation of biological processes and molecular function of selected phosphoproteins using the KEGG PATHWAY database.

cluster	KEGG pathway
	Protein processing in endoplasmic reticulum[a]
2	Valosin-containing protein
4	ER-60 protease[b]
4	Heat shock 70 kDa protein 1-like
4	Disulfide-isomerase A6 precursor
	Regulation of actin cytoskeleton[a]
1	Rho kinase
5	Phosphatidylinositol-4-phosphate 5-kinase type II beta
1	Ezrin[b]
1	alpha actinin-1[b]
2	Radixin
	Leukocyte transendothelial migration[a]
1	Rho kinase
1	Ezrin[b]
1	alpha actinin-1[b]
	Focal adhesion
1	Rho kinase
1	alpha actinin-1[b]
	MAPK signaling pathway
4	Heat shock 70 kDa protein 1-like

[a] KEGG pathway analysis under STRING database at p value ≤ 0.01

[b] The differential relative level of phosphoproteins under significant t-test at p value ≤ 0.05

Exposure to a pathogen, like malaria, has the ability to disturb the cellular processes in particular cell types. The alteration of protein expression and function inside the cells are the result of host-pathogen interactions. The exploring protein-protein interactions of gECs exposed to IE lysate were analyzed using web based free database, STRING and three significant relative levels of phosphoproteins including ezrin, alpha actinin-1and Rho kinase were specifically found in the networks (Figure 7a). Moreover, the finding of phosphatidylinositol-4-phosphate 5-kinase type II beta in cluster 5 (Figure 6) also links the association of three proteins (Figure 7a). Interestingly, the protein-protein interaction of ezrin, alpha actinin-1, Rho kinase and phosphatidylinositol-4-phosphate 5-kinase type II beta was found in a specific relevant interaction pathway of IE-exposed gEC. Likewise, the alteration of defined phosphoprotein levels in gECs during exposure to IE for 24 to 72 h was performed as the dynamic relative level of phosphoproteins as shown in Figure 7b. Levels of the phosphoproteins ezrin, alpha actinin-1 and rho kinase are reduced following 48 to 72 h of IE exposure to gECs, compared with media controls. In contrast, abundance of phosphatidylinositol-4-phosphate 5-kinase type II beta was elevated under the same conditions. This showed that the altered relative level of these 4 phosphoproteins was a result of the interactions between IE and gEC proteins. To investigate whether reduced abundance of ezrin is involved in the mechanism of ineffective erythropoiesis in malaria the ezrin protein-protein interaction network was analyzed, using the KEGG PATHWAY database. The ezrin interactome and its involvement in various cellular functions is shown in Figure 4c. This analysis revealed that the ezrin protein has important roles in various pathways though its function in the regulation of actin cytoskeleton may be the most relevant to malaria. While ezrin is associated with several pathways, only regulation of the actin cytoskeleton is shared among alpha actinin-1, Rho kinase and phosphatidylinositol-4-phosphate 5-kinase type II beta (Figure 7c).

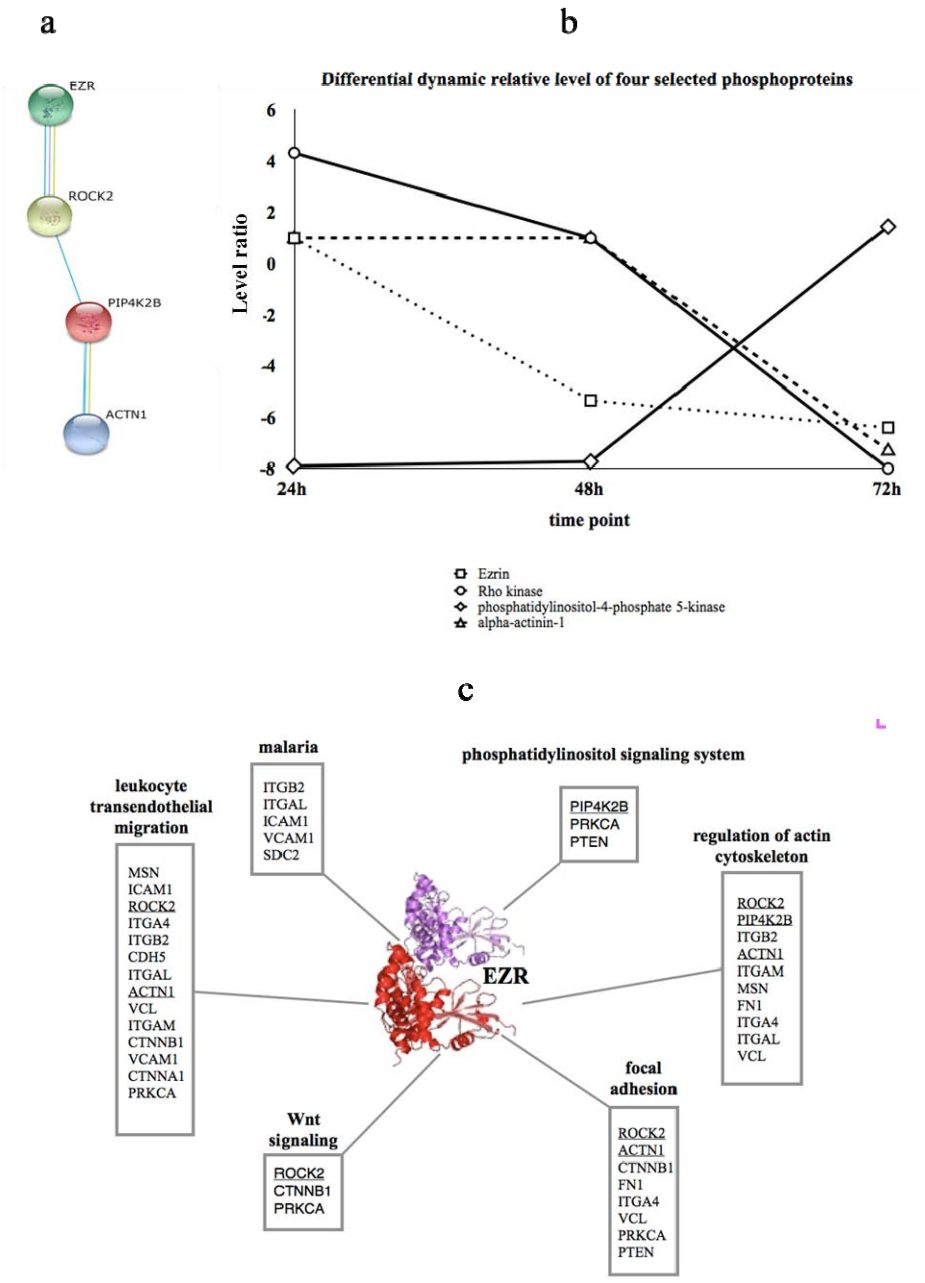


Figure 7. Relative levels and interactions of selected phosphoproteins. (a) Interactions of ezrin, Rho kinase, phosphatidylinositol-4-phosphate 5-kinase type II beta and alpha actinin-1 analyzed using the STRING database **(b)** relative levels of selected phosphoproteins, ezrin, Rho kinase, phosphatidylinositol-4-phosphate 5-kinase type II beta and alpha actinin-1, and **(c)** interaction of ezrin with regulatory pathways and cellular cytoskeleton generated using the KEGG PATHWAY database.

Inhibitory effect on erythroid cell growth by *P. vivax* is regulated by ezrin phosphorylation

Protein phosphorylation is involved in several regulatory functions in living cells. Using phosphoproteomic and bioinformatics analysis, ezrin was identified and determined to have to function in regulation of actin cytoskeleton. One ezrin phosphopeptide from gECs contained the amino acid sequences DKYKTLRQIR, corresponding to residues 563-572. The phosphorylation site of this peptide analyzed from MS/MS data was predicted to be tyrosine 565 (Y565). Ezrin, and phospho-ezrin were evaluated by immunofluorescence using specific antibodies against ezrin and phospho-ezrin Thr567 (Matsui et al., 1998), as shown in Figure 8. Unfortunately, the antibody against phospho-ezrin Y565 is not available and data for phosphorylation at this ezrin residue was not reported. Results of immunofluorescence showed that ezrin and phospho-ezrin Thr567 localized to cell extensions peripheral processes of gECs. gECs in culture with UE or in media displayed strong signals for phospho-ezrin Thr567. In contrast, the signal strength for phospho-ezrin Thr567 was markedly reduced following exposure to IE for 48 and 72 h (Figure 8a and 8b). Quantitation of signals using ImageJ software for ezrin and phospho-ezrin Thr567 from 1,000 gECs from each culture, confirm the decreased level of ezrin proteins in cells exposed to IE (Figure 9). The intensity ratios of phospho-ezrin Thr567 in IE-exposed gECs compared to media controls were less than 1 (0.17 and 0.26, at exposure times 48 and 72 h, respectively). In contrast, the same analysis using UE-exposed gECs gave intensity ratios for phospho-ezrin Thr567 of approximately 1 (0.99 and 0.93, at exposure time 48 and 72 h, respectively). Ezrin signals in IE/UE-exposed gECs compared to gECs in media at 48 and 72 h were nearly 1 (0.86 and 0.95 for IE-exposed gECs, and 0.9 and 0.98 for UE-exposed gECs, respectively), as shown in figure 9. These results indicate that ezrin phosphorylation at the carboxy-terminal threonine residue 567 was decreased in gECs exposed to IE. This suggests that parasite proteins were able to inhibit erythroid cell growth by preventing the phosphorylation of the C-terminal region of ezrin.

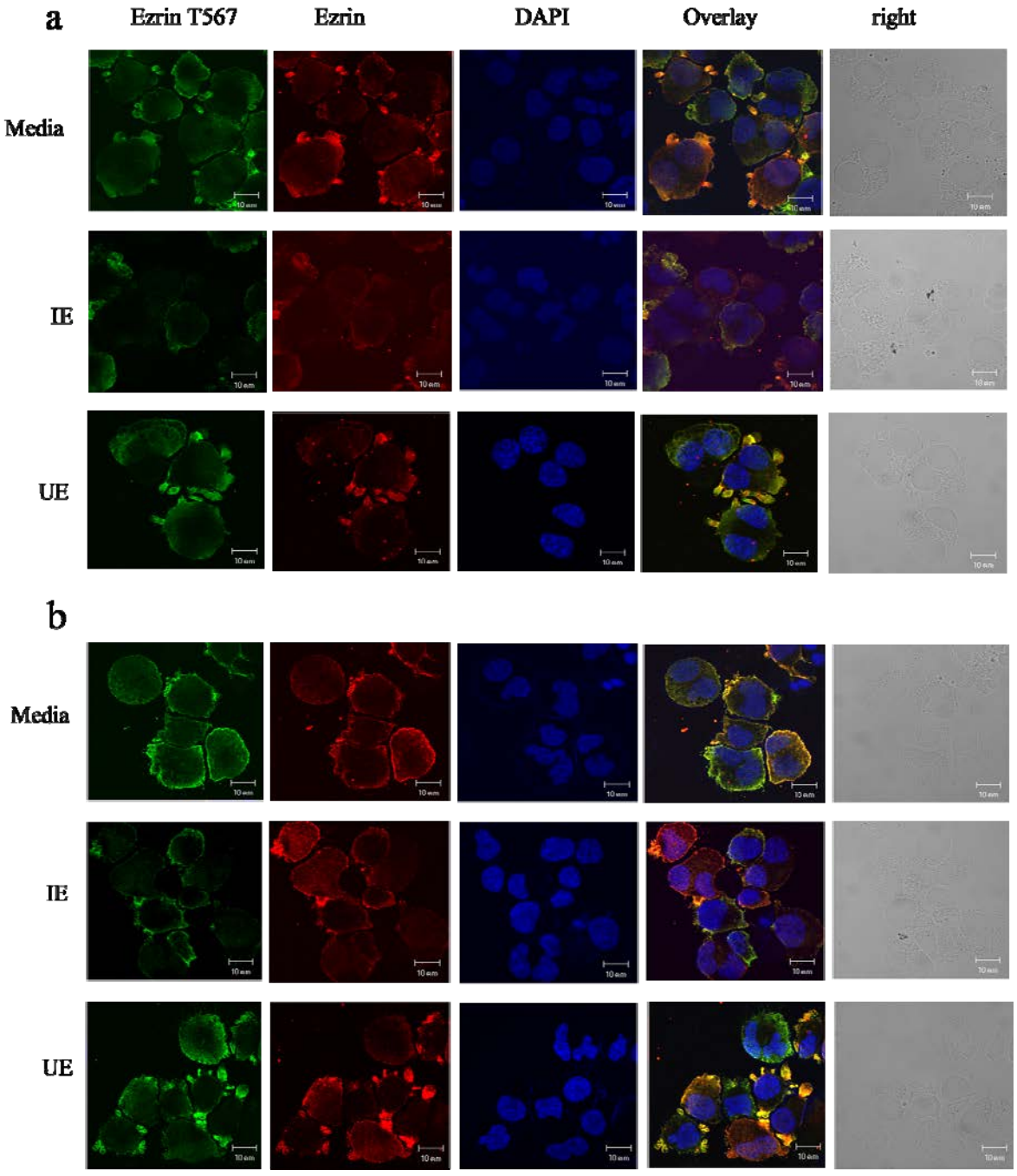


Figure 8. Localization of ezrin and phospho-ezrin Thr 567 in untreated erythroid cells and those exposed to *P. vivax*. Immunofluorescence, ezrin phospho-Thr567 (green), ezrin (red), and nucleus (blue) from gECs exposed to lysates of IE/UE and cells in media for 48 h **(a)** and 72 h **(b)** detected with using specific antibodies against ezrin and phospho-ezrin Thr567. Images were captured using a confocal microscope with 63x magnification and zoom 2.

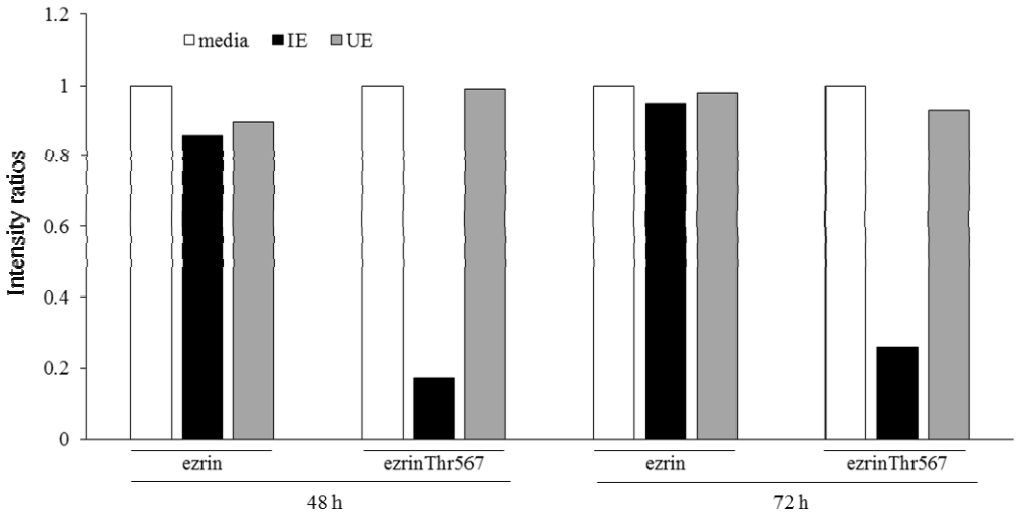


Figure 9. Intensity ratios of ezrin and phospho-ezrin Thr567 from gECs exposed to IE/UE relative to signals from gECs in medium control. The intensity of ezrin and phospho-ezrin Thr567, from 1,000 gECs cells in each condition, exposed to IE/UE or in media were determined using ImageJ software (http://imagej.nih.gov). Intensity ratios were calculated using the mean intensity of IE/UE-exposed gECs normalized to the mean intensity from gECs in media.

CHAPTER V
DISCUSSION AND CONCLUSION

Anaemia has been frequently observed to be a consequence of *vivax* infection (Selvam & Baskaran, 1996; Luxemburger et al., 1997; Mohapatra et al., 2002; Song, et al., 2003; Collins et al., 2003; Echeverri et al., 2003; Kochar et al., 2005; Kochar et al., 2009; Rizvi et al., 2013; Gehlawat et al., 2013; Sharma et al .,2013; Ketema & Bacha, 2013; Bhattacharjee et al., 2013; Abdallah et al., 2013). Recently, transmission electron microscope analysis of two *vivax* malarial cases with symptom of anemia demonstrated the presence of *vivax*-infected erythroblasts and subsequent degradation of erythroblasts. Interestingly, *vivax* parasites were found in bone marrow but could not be detected in peripheral blood (Ru et al., 2009). Consistent with this observation, an in vitro study has revealed that *P. vivax* can directly inhibited erythroid cell proliferation and differentiation through altered division of erythroid cells (Panichakul et al., 2012). Digestive vacuoles (DVs) prepared from blood stages of *P. vivax* and observed under microscope were found the vesicles contained hemozoin crystals and DV size was 1 to 2 μm as shown in Figure 7. The intact/lysed DV was used to study inhibitory effect on gEC growth. Our study found that intact or lysed PvDV was unable to inhibit gEC growth, while lysed PvIE was able to inhibit after exposure for 24 - 72 h. This result is consistent with our previously report (Panichakul et al., 2012). However, the mechanisms underlying the suppression of erythroid development by *P. vivax* appear to be complex and poorly characterized. In this study, phosphoprotein analysis was used to investigate the underlying the suppression of erythroid development by *P. vivax*. Using LC-MS/MS analysis in combination with gene ontology information from the human protein database, 44 phosphoproteins from gECs exposed and non-exposed to IE/UE were identified and categorized according to molecular function, biological process and protein class. Interestingly, the relative level of phosphoproteins was significantly lower following exposure of gECs to parasite proteins, compared to non-exposed cells. Phosphoproteins that displayed significant decreases include ezrin, alpha actinin-1 and Rho kinase. These phosphoproteins were determined to be in the same cluster 1 with significantly low

level pattern of phosphoproteins and exhibited a similar pattern of abundance in response to IE exposure. These results suggest that the phosphoproteins ezrin, alpha actinin-1 and Rho kinase may have roles in the inhibitory effect on erythroid cell growth and division following *P. vivax* exposure. The protein-protein interaction networks determined using the KEGG human protein database revealed that ezrin functions in several cellular pathways, including regulation of actin cytoskeleton.

Ezrin, a member of ERM (ezrin, radixin and moesin) proteins, is localized to cell extension peripheral processes and able to interact with transmembrane proteins and the cytoskeleton. Ezrin functions to regulate cytoskeletal assembly, membrane-protein function and membrane transport (Vaheri et al., 1997; Bretscher et al., 1997; Bretscher et al., 2002). Recent studies, have implicated ezrin as a signal transducer involved in a wide variety of cellular functions, including cell survival, adhesion, morphogenesis, motility, cytokinesis and cellular proliferation (Louvet-Vallee, 2000; Gautreau A., et al., 2002). Increasing evidence indicates a direct relationship between cell proliferation and the level of ezrin expression in cells (Chen Z., et al., 2001; Ohtani K., 2002; Crepaldi et al., 1997). Microinjection of anti-ezrin antibodies into the cytoplasm blocked the entry of mouse fibroblasts into S phase, confirming the function of ezrin in proliferation (Kaul et al., 1996). In response to TNF-alpha, ezrin inhibits endothelial cell proliferation through transcriptional repression of cyclin A, a cell cycle regulatory protein (Kishore et al., 2013). Our analysis also found that ezrin in erythroid cells appears to function in the regulation of cell growth and division. Erythroid cells with growth inhibited by *P. vivax* display low levels of ezrin phosphoprotein.

The phosphorylation of a threonine at residue 567 (T567) is necessary for the active conformation of ezrin and enhances binding with F-actin (Fievet et al., 2007). The results of this study also find that phosphorylation of ezrin, possibly at Thr567, is involved in the regulation of erythroid cell growth. Although our analysis identified the position of the phosphorylation site in an ezrin-phosphopeptide as tyrosine 565, the phosphorylation of ezrin threonine 567 was also detected using a specific antibody. A source of error in phosphorylation site localization on peptides is the presence and intensity of ions for these species in the MS/MS spectra (Beausoleil et al., 2006). However, both residues 565 and 567 are contained in the phosphopeptide

563-572 from ezrin, which was detected in this study. It is also possible that the anti-ezrin Thr567 monoclonal antibody may recognize the phosphorylation of Tyr565 as well as Thr567. This antibody was produced using phosphopeptide of human ezrin containing residues 564-568 (KYKTL) (Matsui et al., 1998) and contains both Tyr565 and Thr567. Previous reports have demonstrated the specificity of this antibody for phospho-ezrin Thr567 (Zheng et al., 2011; Zhu et al., 2007; Chuan et al., 2006) but it is not known if it can recognize phosphorylation at other sites.

Three phosphoproteins in this study, ezrin, Rho kinase and alpha actinin-1 are present at significantly low levels in parasite-exposed cells. Investigation of the KEGG PATHWAY database demonstrates that ezrin has important roles in various pathways, particularly, regulation of actin cytoskeleton, which has implications to malaria, as shown in Figure 7c. Ezrin exerts its biological functions through protein-protein interactions and its active form is regulated by Rho kinase/ROCK2, which directly interacts with membrane proteins (Matsui et al., 1998; Ren et al., 2009). Ezrin binds to adhesion-related proteins with single transmembrane domains such as ICAM-1, CD44 and CD43 through their cytoplasmic tails to modulate cell morphology (Legg & Isacke, 1998; Louvet-Vallee, 2000). Cytoadhesion molecules such as ICAM1 contribute to cytoadhesive phenotype/rosetting and high intensity of rosetting is found in anaemia cases with *P. vivax* infection (Marín-Menéndez et al., 2013). Both ICAM-1 and VCAM-1, soluble adhesion molecules, are detected at high levels in serum from *falciparum* patients with severe malaria (Jakobsen et al., 1994). This study also found abnormal erythroid cell aggregation in culture of gECs exposed to IE lysates (Figure 3). Taken together, this suggests that during interaction of vivax parasite proteins with adhesion molecules on erythroid cells, ezrin regulates cell adhesion by connecting membrane adhesion receptors to the actin-based cytoskeleton. This erythroid cell adhesion may contribute to down regulation of erythroid cell production leading to the development of anaemia. For other adhesion molecules, integrin alpha L chain (ITGAL) and integrin α2β1 has been found a higher probability to involve in severe thrombocytopenia in *vivax* malaria (Campos et al., 2013). The expression of molecules sharing an epitope with human ITGB2/LFA-1 integrin, or CD18 leukocyte integrin on the *falciparum*-parasitized erythrocyte surface could be involved in the pathogenesis of severe disease (Tacchini-Cottier et al., 1995).

However, further studies are need to explore the association of these intercellular adhesion molecules with ezrin in *vivax* malaria.

In addition, ERM proteins are members of the band 4.1 superfamily, FERM (four-point one, ezrin, redixin, moesin) (Gould et al., 1989; Lankes et al., 1991; Funayama et al., 1991). Phosphorylation of erythrocyte protein 4.1 is involved with the modification of the host erythrocyte membrane by *falciparum* parasites (Chishti et al., 1994)]. Moreover, tyrosine phosphorylation of band 3, band 4.2, catalase and actin in *P. falciparum* infected erythrocytes are predicted to be part of the regulatory mechanism to modify the erythrocyte membrane (Pantaleo et al., 2010). This study found low levels of alpha actinin-1 in gECs in the presence of IE. The subsequent reassembly of actin structure in response to parasite suppression is currently being investigated. This poorly understood aspect of ezrin function suggests that parasites inhibit the ezrin protein allowing the assembly of complex cellular structures in erythroid cells leading to dyserythropoiesis, inhibition of erythroid cell growth and division resulting in the accumulation of cells in a resting G0 phase. This study demonstrates that *vivax* parasites suppress development of erythroid progenitor cells through a mechanism that includes decreased ezrin phosphorylation. *P. vivax* is able to enter bone marrow, as previously reported, (Sharma & Varma, 2013; Ru et al., 2009; Imirzalioglu et al., 2006; Wickramasinghe & Abdalla, 2000) and parasites or its products bind to erythroid progenitor cells, resulting in decreased ezrin phosphorylation, leading to suppression of erythroid development, and ultimately anaemia. This is the first analysis suggesting that ezrin contributes to the suppression of erythroid cell growth by *vivax* parasites. Further investigation of this mechanism should help to better understand pathogenesis of anaemia in acute or chronic *P. vivax* infection.

Conclusions

This analysis demonstrates that the pathogenesis of anaemia in *vivax* malaria is mediated by parasite suppression of human erythroid cell growth and division. Inactivation of the ezrin protein, leading to ineffective erythropoiesis and dyserythropoiesis, appears to be a key event resulting in the development of severe

anaemia. The understanding of the pathogenesis of anaemia in *vivax* malaria should help in the development of therapeutic strategies to treat severe anaemia malaria.

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OUTPUT

1. This study has been published with the title of ***Plasmodium vivax*** **inhibits erythroid cell growth through altered phosphorylation of the cytoskeletal protein ezrin.** This manuscript was submitted in Malaria J., 2015, 14:138. Impact factor 3.49.,

2. Oral presentation with title of กระบวนการ **Phosphorylation ที่ Plasmodium vivax ใช้สำหรับการติดเชื้อในเซลล์เม็ดเลือดแดง** in meeting " Functional Proteomics กับงานวิจัยทางการแพทย์ " at Ramathibodi hospital, Mahidol University, Bangkok, Thailand. 24 March 2015.

APPENDIX

1. Ethics
2. One published article in
3. Curriculum vitae

Ethic

COA. No. MU-IRB 2012/170.2511

Certificate of Approval
Mahidol University Institutional Review Board (MU-IRB)

Title of Project: Enhancing Vivax Malaria Research in Thailand

Principal Investigator: Professor Dr. Rachanee Udomsangpetch

Name of Institution: Faculty of Science

Approval includes: Annual Report version received date 22 October 2012

Mahidol University Institutional Review Board is in full compliance with International Guidelines for Human Research Protection such as Declaration of Helsinki, The Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Date of Renewal (4th): 25 November 2012

Date of Expiration: 24 November 2013

Signature of Chair: .....

(Professor Dr. Rutja Phuphaibul)
Vice Chair for Chair

Signature of Head of the Institute: .....

(Professor Prasit Palittapongarnpim)
Vice President for Research

Office of the President, Mahidol University, 999 Phuttamonthon 4 Rd., Salaya, Phuttamonthon District, Nakhon Pathom 73170. Tel. (662) 8496223-5 Fax. (662) 8496223

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เอกสารแสดงความยินยอม (ฉบับบภาษาไทย)

เอกสารแสดงความยินยอมฉบับนี้จัดทำขึ้นสำหรับผู้เข้าร่วมโครงการวิจัยมาลาเรียชนิด พลาสโมเดียมไวแวกซ์ ณ ศูนย์ควบคุมโรคติดต่อนำโดยแมลง อำเภอท่าแซะ จังหวัดชุมพร

ผู้วิจัยหลัก: ศาสตราจารย์ ดร. รัชนีย์ อุดมแสงเพ็ชร
หน่วยงาน: ภาควิชาพยาธิชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล
ผู้สนับสนุนโครงการ: ทุนองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา(BRG)
ชื่อโครงการวิจัย: การเพิ่มศักยภาพการวิจัยโรคมาลาเรียชนิด พลาสโมเดียม ไวแวกซ์ ในประเทศไทย

เอกสารแสดงความยินยอมฉบับนี้ประกอบด้วย 2 ส่วน

- <u>เอกสารคำอธิบายโครงการวิจัยแก่ผู้เข้าร่วมโครงการ (เด็กอายุ 15 ปีขึ้นไป)</u>
- คำยินยอม (สำหรับลงชื่อเพื่อเข้าร่วมโครงการ)

ส่วนที่1: คำแนะนำโครงการ

บทนำ

ผู้รับผิดชอบโครงการ ดร. รัชนีย์ อุดมแสงเพ็ชร ซึ่งขณะนี้ดำรงตำแหน่งเป็นศาสตราจารย์ ประจำอยู่ที่ภาควิชาพยาธิชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดลถนนพระราม 6 กรุงเทพฯ เรากำลังทำวิจัยเกี่ยวกับมาลาเรียซึ่งเป็นโรคที่พบได้ทั่วไปในประเทศไทย เราจะแนะนำให้ท่านทราบเกี่ยวกับวัตถุประสงค์ของโครงการ และท่านจะได้รับอะไรบ้างเมื่อท่านเข้าร่วมโครงการ ถ้าท่านไม่สามารถอ่านเอกสารฉบับนี้ได้ไม่ว่ากรณีใดก็ตาม นักวิจัยจะทำหน้าที่เป็นผู้อ่านและอธิบายให้ท่านฟัง ท่านสามารถถามคำถามได้ตลอดเวลา ถ้าท่านพบว่าไม่

เข้าใจความหมายของคำ และถ้าท่านมีปัญหาในเวลาต่อมา ท่านก็สามารถติดต่อผู้รับผิดชอบโครงการคือ ดร. รัชนีย์ อุดมแสงเพ็ชร หรือ แพทย์หรือเจ้าหน้าที่ได้

วัตถุประสงค์

มาลาเรียเป็นโรคหนึ่งที่พบได้บ่อยครั้งในประเทศไทย โรคนี้นำโดยยุงก้นปล่องซึ่งกินเลือดคนในเวลากลางคืน ยุงก้นปล่องนี้ จะติดเชื้อมาลาเรียได้เฉพาะเมื่อกินเลือด คนที่มีแกมมีโตไซต์ในเลือดเท่านั้น และในปัจจุบันก็พบอุบัติการณ์ของเชื้อมาลาเรียที่ดื้อต่อยาต้านมาลาเรียเพิ่มมากขึ้น และเพื่อศึกษาการดื้อยาของเชื้อมาลาเรียและพัฒนาการรักษาโรคนี้นั้น เราต้องพัฒนาการเลี้ยงเชื้อในห้องปฏิบัติการ เราหวังว่าจะมีอาสาสมัครถึงประมาณ 10 คน ต่อสัปดาห์ที่จะร่วมโครงการวิจัยนี้ เฉพาะท่านที่ป่วยและมีอายุ 15 ปีขึ้นไปเท่านั้น ที่จะอาสาเข้าร่วมโครงการ เนื่องจากทางมาลาเรียคลินิกได้ตรวจพบเชื้อมาลาเรียในเลือดของท่านหรือบุตรหลานของท่าน โดยขออนุญาตเจาะเลือดท่าน 20 ซีซี (ประมาณ 4 ช้อนโต๊ะ) จากเส้นเลือดดำเส้นหนึ่งที่แขนของท่านหรือบุตรหลานของท่าน และนำเลือดติดเชื้อมาลาเรียไปเลี้ยงในห้องปฏิบัติการเพื่อทำการศึกษาโปรตีนของเชื้อมาลาเรียเพื่อพัฒนาวิธีการควบคุมโรคมาลาเรียต่อไป

วิธีการคัดเลือกอาสาสมัคร

ผู้ที่ติดเชื้อมาลาเรียชนิดพลาสโมเดียมไวแวกซ์ ที่มีอายุ 15 ปีขึ้นไป และสนใจเข้าร่วมโครงการ ก่อนที่เราจะอธิบายรายละเอียดเกี่ยวกับการศึกษานี้ ขอย้ำให้ท่านเข้าใจว่า ท่านหรือบุตรหลานของท่าน (อายุ 15-19ปี) สามารถเข้าร่วมโครงการวิจัยนี้ได้เมื่อท่านยินยอมเท่านั้น แม้เราจะต้องการให้พวกท่านเข้าร่วมโครงการ แต่การตัดสินใจนี้ขึ้นอยู่กับตัวท่านเอง ถึงแม้ท่านจะตัดสินใจไม่เข้าร่วมโครงการ เจ้าหน้าที่มาลาเรียคลินิกจะปฏิบัติต่อท่านเหมือนเดิม หากท่านไม่เข้าร่วมโครงการ ท่านหรือบุตรหลานของท่านจะได้รับการรักษาที่พึงได้รับตามปกติ และถ้าท่านตัดสินใจเข้าร่วมโครงการวิจัยแล้ว ท่านอาจเปลี่ยนใจในการที่จะเข้าร่วมต่อ หรือไม่อนุญาตให้บุตรหลานเข้าร่วมโครงการต่อได้ หลังจากการศึกษาวิจัยได้เริ่มไปแล้ว

วิธีการ

เมื่อท่านและบุตรหลานของท่านที่อายุ 15 ขึ้นไป สนใจและยินดีเข้าร่วมโครงการ และเจ้าหน้าที่มาลาเรียคลินิกตรวจพบเชื้อมาลาเรียในเลือดของท่าน หรือบุตรหลานของท่านในขณะที่ป่วย เราจะตรวจอาการแสดงชีพของท่าน (เช่นความดันโลหิต การหายใจ และอุณหภูมิร่างกายที่วัดทางปาก) และปริมาณเม็ดเลือดแดงในเลือดของท่าน (โดยการเจาะเลือดจากปลายนิ้ว) เพื่อให้แน่ใจว่าท่านมีสุขภาพดีพอที่จะเข้าร่วมการศึกษานี้ เราจะขออนุญาตเจาะเลือด 20 ซีซี (ประมาณ 4 ช้อนโต๊ะ) จากเส้นเลือดดำเส้นหนึ่งที่แขนของท่าน หรือบุตรหลาน หลังจากนั้นเรา

จะใส่เลือด 5 ซีซีลงในถ้วยเพื่อให้ยุงกิน ซึ่งเป็นวิธีการที่จะทำให้ยุงติดเชื้อ ส่วนเลือดที่เหลือจะทำการเลี้ยงในห้องปฏิบัติการเพื่อศึกษาโปรตีนของเชื้อมาลาเรีย หลังจากเจาะเลือดแล้ว ท่านจะได้รับยารักษาโรคมาลาเรียตามนโยบายที่ทางคลินิกกำหนดไว้

ความเสี่ยง

ความเสี่ยงที่ท่านหรือบุตรหลานจะได้รับบาดเจ็บจากการเก็บเลือดมีโอกาสน้อย แต่อาจเกิดรอยช้ำเล็กน้อยที่บริเวณเข็มเจาะขณะเก็บตัวอย่างเลือด เราจะใช้เข็มอันใหม่เจาะเลือดทุกครั้ง ถ้าท่านหรือบุตรหลานของท่านได้รับการบาดเจ็บซึ่งเป็นผลโดยตรงจากการเข้าร่วมโครงการวิจัยนี้ เราจะให้การรักษาท่านหรือบุตรหลานของท่าน โดยท่านไม่ต้องเสียค่าใช้จ่ายใดๆ ท่านจะไม่ได้รับเงินชดเชยนอกเหนือจากการรักษาทางการแพทย์ และโปรดเข้าใจด้วยว่าสิ่งนี้มิใช่การละเว้นหรือสละสิทธิ์ตามกฎหมายของท่านหรือบุตรหลานของท่านแต่อย่างใด ถ้าท่านมีข้อสงสัยเกี่ยวกับเรื่องนี้ โปรดสอบถามผู้วิจัยหลักก่อนการตัดสินใจเข้าร่วมโครงการ

การสิ้นสุดการเข้าร่วมการศึกษา

ท่านหรือบุตรหลานของท่านอาจจะไม่สามารถเข้าร่วมการศึกษานี้ ถ้าท่านหรือบุตรหลานตั้งครรภ์ หรืออายุต่ำกว่า 15 ปี เมื่อท่านและบุตรหลานเข้าร่วมการศึกษานี้แล้ว ท่านอาจถอนตัวได้ตลอดเวลา ถ้าท่านหรือบุตรหลานมีเลือดจาง (มีเม็ดเลือดไม่สูงพอ) หรือเรามีปัญหาในการเจาะเลือดจากเส้นโลหิตดำของท่าน เราจะหยุดการเจาะเลือดของท่านทันที

ประโยชน์ที่อาจเกิดขึ้นต่อตัวผู้เข้าร่วมวิจัยและต่อผู้อื่น

<u>การเข้าร่วมโครงการของท่าน</u> ช่วยให้เราได้รับข้อมูลใหม่ๆ เกี่ยวกับเชื้อมาลาเรียที่จะใช้เป็นตัวนำให้เกิดความสามารถในการต่อต้านโรคได้ <u>ตัวของท่านเองอาจมีภาวะสุขภาพดีขึ้นแต่ไม่สามารถรับรองได้</u> และการศึกษานี้จะมีส่วนช่วยในการผลิตนักวิจัยรุ่นใหม่ให้กับประเทศไทย ความรู้ที่ได้จะเป็นประโยชน์อย่างยิ่งในการพัฒนาการรักษาให้มีประสิทธิภาพมากยิ่งขึ้นซึ่งจะเกิดประโยชน์แก่ผู้ป่วยในอนาคต ดังนั้น โครงการวิจัยนี้จึงเป็นประโยชน์อย่างยิ่งต่อการควบคุมมาลาเรีย

สิ่งตอบแทนที่ผู้ป่วยจะได้รับ

ในการที่ท่านเสียเวลาการทำงานเพื่อเดินทางมาที่หน่วยมาลาเรีย ให้เราเจาะเลือดสำหรับโครงการนี้ ท่านจะได้รับค่าพาหนะจำนวน 150 บาท

การรักษาความลับ

ข้อมูลทั้งหลายที่เกี่ยวข้องกับโครงการศึกษานี้ จะถือเป็นความลับตลอดไป <u>จะไม่เปิดเผยชื่อ สกุล ที่อยู่และข้อมูลส่วนตัวของท่าน</u> การที่ท่านหรือบุตรหลานของท่านมีส่วนร่วมในการศึกษาวิจัยครั้งนี้ ก็จะเก็บไว้เป็น

ความลับ ท่านและนักวิทยาศาสตร์ที่ดำเนินการวิจัยนี้เท่านั้นที่จะทราบผลการศึกษา ข้อมูลต่างๆอาจจะได้รับการพิจารณาทบทวน โดยผู้แทนจากกระทรวงสาธารณสุข คณะกรรมการการพิจารณาการวิจัยในคน แห่งมหาวิทยาลัยมหิดล ข้อมูลทางวิจัยและข้อมูลทางคลินิกที่เกี่ยวข้องกับท่าน อาจจะถูกนำไปใช้ร่วมกันกับนักวิจัยและกลุ่มนักวิทยาศาสตร์ท่านอื่น ในลักษณะของการปาฐกถาและการตีพิมพ์ โดยที่จะไม่มีการระบุชื่อท่านหรือบุตรหลานของท่าน

การแลกเปลี่ยนข้อมูลการวิจัย

ความรู้ที่ได้รับจากโครงการนี้จะได้รับการบอกกล่าวจากท่านก่อนที่จะเผยแพร่สู่สาธารณชน เฉพาะข้อมูลที่เป็นความลับเกี่ยวกับท่านจะไม่ถูกเผยแพร่ โดยทางเราจะติดประกาศให้ท่านทราบถึงผลการวิจัยซึ่งท่านสามารถติดตามได้ ณ มาลาเรียคลินิก โดยจะประกาศให้ทราบทุกครึ่งปีจนกระทั่งโครงการวิจัยสิ้นสุดลง ถ้าท่านอยากทราบเกี่ยวกับผลการวิจัย ท่านสามารถสอบถามได้กับ ดร. รัชนีย์ อุดมแสงเพ็ชร โดยตรงหรือเจ้าหน้าที่ในมาลาเรียคลินิค

สิทธิในการปฏิเสธหรือถอนตัว

ท่านอาจปฏิเสธที่จะเข้าร่วมโครงการหรือไม่เข้าร่วมโครงการ ถ้าท่านตัดสินใจเข้าร่วมโครงการวิจัยแล้วท่านอาจเปลี่ยนใจในการที่จะเข้าร่วมต่อ หรือไม่อนุญาตให้บุตรหลานเข้าร่วมโครงการต่อได้ หลังจากการศึกษาวิจัยได้เริ่มไปแล้ว ถ้าท่านต้องการถอนตัวจากการศึกษานี้โปรดแจ้งต่อผู้วิจัยอาวุโส ณ คลินิคมาลาเรีย

การยินยอม

<u>ท่านสามารถถอนตัวจากโครงการได้ทุกเวลาโดยไม่มีผลกระทบต่อตัวท่าน</u> ข้อมูลที่เป็นความลับประกอบไปด้วย ชื่อสกุล ที่อยู่ และวันที่เข้าร่วมโครงการของท่าน และข้อมูลทั้งหลายจะถูกเก็บ ณ มหาวิทยาลัยมหิดล 20ปีเป็นอย่างน้อย

ข้อสงสัย

ถ้าท่านมีข้อสงสัยเกี่ยวกับการวิจัยนี้ โปรดสอบถาม ศาสตราจารย์ ดร. รัชนีย์ อุดมแสงเพ็ชร หรือ ถ้าท่านมีข้อสงสัยในภายหลัง ศาสตราจารย์ ดร. รัชนีย์ อุดมแสงเพ็ชร (ภาควิชาพยาธิชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล โทร 02 2015576) จะตอบคำถามท่านได้

ส่วนที่ 2

ใบยินยอมให้ทำการวิจัยโดยได้รับการบอกกล่าวและเต็มใจ

การวิจัยเรื่อง การเลี้ยงเชื้อมาลาเรียชนิดพลาสโมเดียมไวแวกซ์ เพื่อพัฒนางานวิจัยมาลาเรียในประเทศไทย

วันให้คำยินยอม วันที่..... เดือน..... พ.ศ.....

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ วิธีการวิจัย อันตรายที่อาจเกิดขึ้นจากการวิจัย รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียด และมีความเข้าใจดีแล้ว

ผู้วิจัยรับรองว่าจะตอบคำถามต่างๆที่ข้าพเจ้าสงสัยด้วยความเต็มใจ ไม่ปิดบัง ซ่อนเร้น จนข้าพเจ้าพอใจ

ข้าพเจ้ามีสิทธิที่จะบอกเลิกการเข้าร่วมโครงการวิจัยนี้เมื่อใดก็ได้ และเข้าร่วมโครงการวิจัยนี้โดยสมัครใจและการบอกเลิกการเข้าร่วมการวิจัยนี้จะไม่มีผลต่อการรักษาโรคที่ข้าพเจ้าพึงได้รับตามปกติ

ผู้วิจัยรับรองว่าข้อมูลส่วนตัวของข้าพเจ้าจะถูกปกปิดไว้และจะเปิดเผยได้เฉพาะในรูปที่เป็นสรุปผลการวิจัย การเปิดเผยข้อมูลส่วนตัวของข้าพเจ้าต่อหน่วยงานต่างๆที่เกี่ยวข้อง กระทำได้เฉพาะกรณีจำเป็นด้วยเหตุผลทางวิชาการเท่านั้น

ผู้วิจัยรับรองว่าหากเกิดอันตรายใดๆอันเนื่องจากการวิจัยดังกล่าวข้าพเจ้าจะได้รับการรักษาพยาบาลโดยไม่คิดมูลค่าตามมาตรฐานวิชาชีพและจะได้รับการชดเชยรายได้ตามที่สูญเสียไประหว่างการรักษาพยาบาลดังกล่าว ตลอดจนเงินทดแทนความพิการที่อาจเกิดขึ้น

ผู้วิจัยรับรองว่าหากมีข้อมูลเพิ่มเติมที่ส่งผลกระทบต่อการวิจัย ข้าพเจ้าจะได้รับแจ้งให้ทราบโดยไม่ปิดบังซ่อนเร้น

ข้าพเจ้าเข้าใจดีว่ามีโอกาสที่เลือดที่ข้าพเจ้าบริจาคในการศึกษานี้ อาจถูกนำไปใช้ในการวิจัยโรคมาลาเรียด้านอื่นๆได้ (ถ้าท่านประสงค์จะอนุญาตให้เราใช้เลือดของท่านหรือบุตรหลานของท่าน ในการศึกษามาลาเรียด้านอื่นๆ เราจะขอให้ท่านเซ็นชื่อในเอกสารบริจาคเลือดและองค์ประกอบ และเราขอให้ท่านมั่นใจได้ว่าเลือดของท่านจะไม่ถูกซื้อขายในเชิงพาณิชย์ ท่านไม่ต้องเซ็นเอกสารดังกล่าวนี้ถ้าท่านไม่ต้องการ ถ้าท่านไม่เซ็นเอกสารนี้ เราจะไม่นำเลือดของท่านไปใช้ในการศึกษาด้านอื่นนอกเหนือจากที่ได้อธิบายไว้ในเอกสารแสดงความยินยอมฉบับนี้)

ข้าพเจ้าได้อ่านข้อความข้างต้นแล้ว และมีความเข้าใจดีทุกประการ และได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม _____ผู้ยินยอม

ลงนาม _____พยาน

ลงนาม _____พยาน

ในกรณีที่ผู้ยินยอมตนให้ทำการวิจัยไม่สามารถอ่านและเขียนหนังสือได้ จะต้องได้รับการยินยอมในขณะที่ยังมีสติสัมปชัญญะ และระบุข้อความไว้ตามนี้ ข้าพเจ้าไม่สามารถอ่านหนังสือได้ แต่ผู้วิจัยได้จัดหาล่ามอ่านข้อความในใบยินยอมนี้ให้แก่ข้าพเจ้าฟังจนเข้าใจดีแล้ว ข้าพเจ้าจึงลงนามหรือประทับลายนิ้วหัวแม่มือของข้าพเจ้าในใบยินยอมนี้ด้วยความเต็มใจ

ลายนิ้วหัวแม่มือของผู้ยินยอมตน

ลงนาม _____พยาน

ลงนาม _____พยาน

ข้อมูลของอาสาสมัคร (ปกปิดเป็นความลับ)

ชื่ออาสาสมัคร_____

วัน เดือน ปี ที่เกิด_____

อายุของอาสาสมัคร_____ เพศ_____ สัญชาติ _____

ที่อยู่ของอาสาสมัคร_____

_____

โทรศัพท์_____

แบบสอบถาม

เคยเป็นมาลาเรียมาแล้วในเดือนที่ผ่านมา _____ ครั้ง

(สำหรับผู้ป่วยหญิง) อยู่ในระหว่างตั้งครรภ์หรือไม่ _____

การตรวจร่างกาย

ความดันเลือด _____มม. ของปรอท

การหายใจ _____ครั้งต่อนาที

อุณหภูมิร่างกาย _____องศาเซลเซียส

ความเข้มข้นของเลือด _____%

หมู่เลือด _____

ลายเซ็นอาสาสมัคร_____วันเดือนปี _____

ชื่อตัวบรรจง_____

(เอกสารหนึ่งชุดได้มอบให้กับอาสาสมัคร)

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Plasmodium vivax inhibits erythroid cell growth through altered phosphorylation of the cytoskeletal protein ezrin

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Abstract

Background: The underlying causes of severe malarial anaemia are multifactorial. In previously reports, *Plasmodium vivax* was found to be able to directly inhibited erythroid cell proliferation and differentiation. The molecular mechanisms underlying the suppression of erythropoiesis by *P. vivax* are remarkably complex and remain unclear. In this study, a phosphoproteomic approach was performed to dissect the molecular mechanism of phosphoprotein regulation, which is involved in the inhibitory effect of parasites on erythroid cell development.

Methods: This study describes the first comparative phosphoproteome analysis of growing erythroid cells (gECs), derived from human haematopoietic stem cells, exposed to lysates of infected erythrocytes (IE)/uninfected erythrocytes (UE) for 24, 48 and 72 h. This study utilized IMAC phosphoprotein isolation directly coupled with LC MS/MS analysis.

Results: Lysed IE significantly inhibited gEC growth at 48 and 72 h and cell division resulting in the accumulation of cells in G0 phase. The relative levels of forty four phosphoproteins were determined from gECs exposed to IE/UE for 24-72 h and compared with the media control using the label-free quantitation technique. Interestingly, the levels of three phosphoproteins: ezrin, alpha actinin-1, and Rho kinase were significantly ($p < 0.05$) altered. These proteins display interactions and are involved in the regulation of the cellular cytoskeleton. Particularly affected was ezrin (phosphorylated at Thr567), which is normally localized to gEC cell extension peripheral processes. Following exposure to IE, for 48-72 h, the ezrin signal intensity was weak or absent. This result suggests that phospho-ezrin is important for actin cytoskeleton regulation during erythroid cell growth and division.

Conclusions: These findings suggest that parasite proteins are able to inhibit erythroid cell growth by down-regulation of ezrin phosphorylation, leading to ineffective erythropoiesis ultimately resulting in severe malarial anaemia. A better understanding of the mechanisms of ineffective erythropoiesis may be beneficial in the development of therapeutic strategies to prevent severe malarial anaemia.

Keywords: *Plasmodium vivax*, Ineffective erythropoiesis, Anaemia, Ezrin, Phosphoproteins, Haematopoiesis stem cells, Erythroid cells

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Background

Plasmodium vivax is a risk factor for severe anaemia, among patients in vivax-endemic areas [1-7]. Increasing evidence has established an association between vivax malaria, severe anemia, and death [8-16]. The pathogenesis of severe anaemia in vivax-malaria remains unclear and is likely caused by multiple underlying factors. These include the destruction of parasitized erythrocytes, ineffective erythropoiesis or dyserythropoiesis, and immunity associated with disease. Evidence for dyserythropoiesis, pancytopenia and degradation of erythroblasts was found in bone marrow from patients infected with *P. vivax* parasites [17-21]. Moreover, *in vitro* cultures of erythroid cells derived from haematopoietic stem cells has demonstrated that *P. vivax* is able to directly inhibit erythroid cell proliferation and differentiation [22]. The molecular mechanisms underlying the suppression of erythropoiesis by *P. vivax* are remarkably complex and poorly understood.

The phosphoproteome strategy is alternative proteomic method that allows investigation into the molecular mechanisms of signal transduction pathways [23]. The separation and enrichment of phosphoproteins utilizes metal ion or TiO_2 embedded columns prior to the identification and determination of phosphoproteins under liquid chromatography–mass spectrometry (LC-MS) based techniques [24,25]. Many molecular pathways in eukaryotic cells are modulated by specific signaling proteins that are controlled, by phosphorylation and dephosphorylation, through the activity of kinase and phosphatase enzymes. This post-translational control of eukaryotic cellular machinery is a hallmark of pathways that respond to different stimuli. The level of protein phosphorylation at specific sites varies from less than 1% to greater than 90%, depending on conditions [26]. The regulation of complex and dynamic signal transduction proteins contributes to the destination of targeting proteins and the signal transduction of cell growth, and exposure to parasites can also influence signaling pathways. This occurs through specific modulation of regulatory proteins during the host-pathogen interaction, especially proteins with roles in pathogenesis [27]. The specific mechanism involved in the suppression of erythroid development by *P. vivax* has not been elucidated. However, it is known that during parasite exposure, suppressed erythroid development is a key aspect in the pathophysiology of anaemia. Here, this study describes the first comparative phosphoproteome of erythroid cells, derived from human haematopoietic stem cells, exposed to proteins of *P. vivax*. This analysis utilized IMAC phosphoprotein isolation, directly coupled with LC MS/MS analysis. Interestingly, the phosphoprotein ezrin, involved in the regulation of cytoskeleton during cell development, was identified using this procedure. Ezrin, a member of the ezrin, radixin, and moesin (ERM) subfamily of cytoskeletal proteins and is conserved both functionally and structurally in mammalian cells [28]. Through the regulation of the cytoskeleton ezrin has a role in several cellular processes, such as maintenance of survival, cytokinesis, adhesion, membrane dynamics, motility, and integration of membrane transport with signaling pathways [29]. Usually, ezrin is present in an inactive conformation and functions as a crosslinker between the plasma membrane and cytoskeleton [30]. Two regions of ezrin, the N-terminal and C-terminal domains are involved in the active conformation. The N-terminal domain binds to phosphatidylinositol 4,5-biphosphate, which facilitates phosphorylations of threonine 567 (Thr567) in the C-terminal domain [28]. Rho kinase and protein kinase C (PKC) are capable of phosphorylating the C-terminal threonine in ezrin, promoting the formation of the F-actin binding site in active conformation [31-35]. This study has identified the involvement of the cytoskeleton protein ezrin in the inhibitory effect of *P. vivax* on erythroid cell growth, leading to ineffective erythropoiesis. The molecular mechanism characterized in this study, relevant to ineffective erythropoiesis, should have utility in the development of therapeutic strategies for severe malarial anaemia.

Methods

Parasite preparation

Plasmodium vivax parasites from patient blood with 0.05-0.2% parasitaemia, as determined by examining thick and thin blood smears, were collected. The ethical and methodological aspects of this study for parasite collection from patients attending the malaria clinic in Tha Sae, Chumpon Province, Thailand (MU-IRB 2012/170.2511) have been approved by the Mahidol University Institutional Review Board, Mahidol University, Bangkok, Thailand. Infected erythrocytes (IE) were separated from patient blood using a 60% Percoll solution as previously described [36]. Briefly, 20 ml of whole blood from patients were collected and passed through a Plasmodipur filter (Euro-Diagnostic B.V., Netherlands) to remove white blood cells. To separate IE, patient blood, after filtration, was diluted 1:20 with RPMI1640 (Invitrogen®, CA, USA), layered on 60% Percoll, and centrifuged at 1,200 g for 20 min at 20°C. The purity of IE after isolation was 95%, containing 80% schizonts and 20% other stages. The isolated IEs were used as lysed cells prepared by freezing and thawing as previously described [22].

Isolation of cord blood CD 34^+ cells and culture conditions

Haematopoietic stem cells (HSCs)/CD 34^+ cells were isolated from normal human umbilical cord blood as previously described [22]. Cord blood collected from normal full-term deliveries at Ramathibodi Hospital (ID 04-52-39) was approved by the Ethical Committee of

Research on Human Beings of the Ramathibodi Hospital, Faculty of Medicine, Mahidol University. Briefly, HSCs/$CD34^+$ cells from cord blood were isolated using a CD 34 isolation kit with magnetic microbead Mini-MACS columns (Miltenyi Biotech, Geramany). The purity of $CD34^+$ cells after isolation was 97% as determined by flow cytometry analysis.

Growing erythroid cells (gECs), derived from HSCs/$CD34^+$ cells, at a density of 5 x 10^5 cell/well in 24-well tissue culture plates (Corning Incorporated Costar®, NY, USA) were cultured in 1 ml of complete medium containing Stemline II medium (Sigma-Aldrich Corporation, Missouri, USA) supplemented with cytokines as previously described [36]. Lysates of IE or uninfected erythrocytes (UE) from normal donor blood were added to gEC cultures, at a ratio of 1:10 (gEC:IE/UE), on day 5 and cultured at 37°C in 5% CO_2 for 24, 48, 72 h. Viable cells were determined by trypan blue dye exclusion and dead cells were stained with 2 μg/ml propidium iodide (eBioscience) and analysed by flow cytometry.

Cell cycle analysis

gECs exposed to IE/UE for 48 and 72 h were washed with PBS and fixed with 70% cold ethanol overnight at -20°C. After fixed cells were washed with PBS, cells were added with 5 μg/ml RNase A (Geneaid Biotech Ltd., Taiwan), stained with 20 μg/ml propidium iodide (eBioscience) and incubated for 30 min at 37°C in dark. DNA analysis was performed on a FACS Canto™ flow cytometer (Becton Dickinson). Cell cycle analysed by BD FACSDiva software version 4.1 (Becton Dickinson) was used to determine the percentages of cells in the different cell cycle phases. Experiments were performed five independent times and values are shown as means ± S.D.

Preparation of phosphoprotiens

Phosphoproteins were prepared from gECs, after exposure to IE/UE lysates, using Pierce Phosphoprotein enrichment kit (Thermo Scientific, Pierce Biotechnology, IL, USA) followed the manufacturer's instructions. Briefly, 5 x 10^6 cells were added to 200 μl of lysis/binding/wash buffer with CHAPS and 1X phosphatase inhibitors (phosphatase inhibitor cocktail 100X, Roche, UK) and lysed on ice by sonication (Sonics Vibra cell,sonics & materials INC., USA) with amplitude 70% utilizing 15 cycles of 2 sec disruption followed by 2 sec on ice. Following centrifugation (Tomy MX-305 high speed refrigerated microcentrifuge, CA, USA), 10,000 g at 4°C for 10 min, supernatants from cell lysates were collected and proteins concentrations were determined by Lowry method [37].

For phosphoprotein enrichment, cell lysates were adjusted to a concentration to 0.5 mg/ml in lysis/binding/wash buffer without CHAPS and applied to the column. Columns were inverted to mix for 30 min at 4°C and then washed three times with lysis/binding/wash buffer with CHAPS using centrifugation (Allegra X-22R centrifuge, Beckman Coulter, Inc, USA) at 1,000 g for 1 min at 4°C. One millimetre of elution buffer, consisting of 75 mM sodium phosphate, 500 mM sodium chloride, pH 7.5, was added to each column and then incubated at room temperature, with agitation for 3 min. The eluted proteins were collected by centrifugation at 1,000 g for 1 min at 4°C, and this step was repeated four times. The pool of eluted proteins was concentrated using a Pierce concentrator (Thermo Scientific). Briefly, 4 ml of each sample of eluted proteins was placed into the upper chamber of the concentrator followed by centrifugation at 7,000 g for 30 min at 4°C. The concentrated proteins, approximately 100-200 μl, were collected from the upper chamber and stored at -80°C. In the final step, salts and other molecules (<1,000 Da) were removed using Zeba™ spin desalting columns (Thermo Scientific). One hundred microlitres of each sample was applied to columns and then centrifuged at 700 g for 30 sec. The desalted samples were collected and the proteins content of each sample was determined by the Lowry method and used for phosphoprotein analysis.

Phosphoprotein analysis

After desalting, 4 μg of phosphoproteins in 10 mM ammonium bicarbonate were reduced with 10 mM DTT (Dithiotheitol) for 30 min at 60°C, alkylated with 15 mM IAA (iodoacetamide) at room temperature for 30 min, and digested with sequencing grade trypsin (Promega, Geramany) for 16 h at 37°C. Tryptic phosphopeptides were diluted with 0.1% formic acid to final concentration of 0.25 μg/μl and centrifuged 10,000 rpm at room temperature for 10 min. Phosphopeptide samples were injected into a NanoAcquity system (Waters Corp., Milford, MA) equipped with a Symmetry C_{18} 5 μm, 180-μm x 20-mm Trap column and a BEH130 C_{18} 1.7 μm, 100-μm x 100-mm analytical reversed phase column (Waters Corp., Milford, MA). The samples were initially transferred with an aqueous 0.1% formic acid solution (mobile phase A) to the trap column with a flow rate of 15 μl/min for 1 min. The peptides were separated with a gradient of 15-50% mobile phase B in acetonitrile with 0.1% formic acid over 15 min at a flow rate of 600 nl/min followed by a 3 min rinse with 80% of mobile phase B. The column temperature was maintained at 35°C. The lock mass was delivered from the auxiliary pump of the NanoAcquity system with a constant flow rate of 500 nl/min with 200 fmol/μl of [Glu^1] fibrinopeptide B delivered to the reference sprayer of the NanoLockSpray source of the mass spectrometer. All samples of tryptic peptides were analysed by using a SYNAPT™ HDMS mass spectrometer (Waters Corp., Manchester, UK), which was operated in the V-mode of

analysis with a resolution of at least 10,000 full-width half-maximum, using positive nanoelectrospray ion mode. The time-of-flight analyzer of the mass spectrometer was externally calibrated with [Glu^1] fibrinopeptide B from m/z 50 to 1600 with acquisition lock mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu^1] fibrinopeptide B. The reference sprayer was sampled with every 20 sec. Accurate mass LC-MS data were acquired with data direct acquisition mode. The energy trap was set at the collision energy of 6 V. In the transfer collision energy control, low energy was set at 4 V. The quadrupole mass analyzer was adjusted such that ions from m/z 300 to 1800 were efficiently transmitted. The MS\MS survey was over range from 50 to 1990 Da and scan time was 0.5 sec.

Protein identification

The differential quantitation of proteins and peptides based on the MS signal intensities of individual LC-MS samples was analysed using DeCyder MS differential analysis software (DeCyderMS, GE Healthcare) [38,39]. Acquired LC-MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ions signal intensities in MS mode. The analysed MS/MS data from DeCyderMS were submitted for database search using the Mascot software (Matrix Science, London, UK) [40]. For protein identification, data was compared against the NCBI *Homo sapiens* database with the following parameters: enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (±0.6 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages.

Bioinformatic analysis

All MS/MS data were compared against the human protein set using Swissprot protein database. The heatmap visualization was constructed using the web-based analysis MEV program [41] and the protein cluster was analysed by distance metric selection with Pearson correlation parameters calculate K-Means. Different levels of phosphoproteins were analysed using the significant *t*-test at P-value ≤ 0.05. The gene ontology analysis was performed with UniprotKB (protein knowledgebase) [42] and PANTHER (classification system) [43] databases for biological processes, molecular function and classification. The mapping of protein-protein interactions was conducted using data visualization with statistical analysis at a low confidence score in the STRING database (known and predicted protein-protein interactions) [44]. Gene biological categorization was performed and selected at P-value ≤ 0.01. Analysis of the protein-protein interaction network was performed using the KEGG (Kyoto Encyclopedia of Genes and Genomes) PATHWAY database [45,46].

Immunofluorescence assays

The distribution of threonine-phosphorylated ezrin and ezrin in gECs after exposure to IE/UE and in control media was verified by immunofluorescence. Cells were fixed in 4% formaldehyde in PBS (phosphate buffer saline) at 4°C for 20 min, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked in 2% human serum in PBS for 20 min at room temperature. Mouse anti-ezrin (Abcam®, Cambridge, UK) and rabbit anti-phospho-ezrin (Thr567)/radixin (Thr564)/mosin (Thr41A3) ERM (Cell Signaling Technology, Danvers, MA, USA) antibodies, diluted in 1% human serum/PBS at a ratio 1:100, were added and incubated at room temperature for 1.5 h. Cells were incubated with goat anti-rabbit and anti-mouse antibodies, conjugated with Alexa Fluor green 488 and Alexa Fluor Red 594 (Molecular probes), respectively, diluted in 2% human serum/PBS were for 2 h. After washing with PBS, stained cells were mounted with anti-fade medium containing DAPI (Molecular probes). Cells were examined using a laser scanning confocal microscope (LSM 510 Meta, Zeiss, Jena, Germany) with a 63x objective at zoom 2. Immunofluoresence intensity of 1,000 cells from each culture condition was determined using ImageJ 1.48v/Java software [47]. The mean of intensity from 1,000 cells were presented as intensity ratios, calculated from the intensity of IE/UE-exposed gECs divided by the intensity of gECs in control cultures.

Statistical evaluation

Data for cell growth was analysed using the SPSS program (version 17). The unpaired Mann-Whitney-Wilcoxon test was used to compare means between independent groups for significantly statistic evaluation of cell growth, P-value < 0.01.

Results

Inhibition of erythroid cell growth in *in vitro* cultures by *P. vivax*

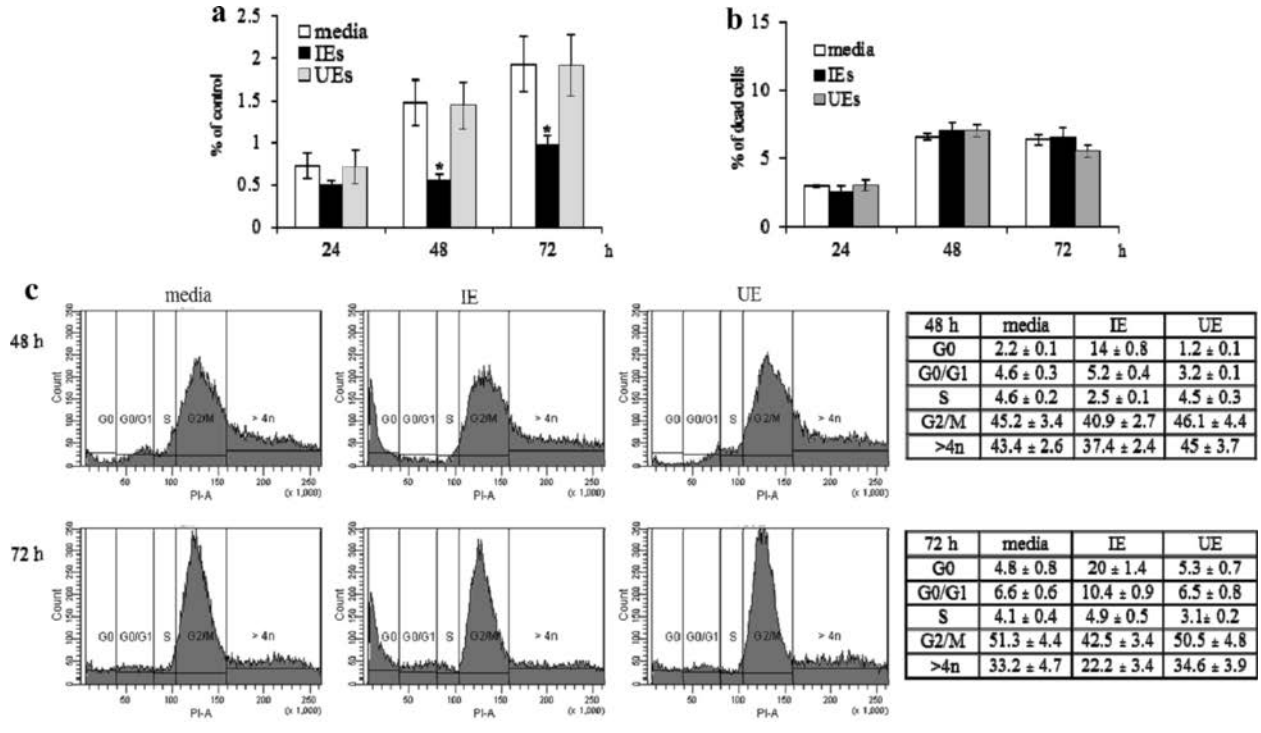
It was previously reported that *P. vivax* parasites inhibited erythroid cell growth and perturbed erythroid cell division in three-day *in vitro* cultures [22]. However, the molecular mechanisms underlying the inhibition of erythroid development by *P. vivax* were unclear. In this study, the underlining mechanism of the inhibitory effect on gEC growth by *P.vivax* was examined using growing erythroid cells (gECs), derived from human cord blood HSCs/CD34$^+$, and infected erythrocytes (IE), isolated from patient blood. gECs from 5-day old cultures were exposed for 24, 48 and 72 h to lysed IE or UE, at a ratio of IE/UE: gEC 10:1. Lysed IE significantly inhibited gEC

growth at 48 and 72 h (P-value < 0.01), compared with lysed UE and media controls (Figure 1a). No difference in cell death at 24, 48 and 72 h was observed in cultures with and without IE/UE, as shown in Figure 1b. Interestingly, lysed IE dramatically increased the number of cells in the G0 phase and decreased the number of cells in the mitotic fraction (G2/M and > 4n) at 48 and 72 h (Figure 1c). This indicates that parasite proteins were able to inhibit erythroid cell growth and division resulting in the accumulation of cells in a resting phase but did not induce cell death.

Characterization of phosphoproteins from erythroid cells after exposure to *P. vivax*

To analyse the mechanism of *P. vivax* inhibition on gEC growth, following exposure to IE/UE for 24, 48 and 72 h, phosphoproteins were enriched and analysed by LC-MS/MS. The DecyderMS program module was used for quantitation of the MS/MS intensity in each culture condition and results were compared to the human protein database. Phosphoproteins were classified based on biological function, molecular function, and protein class. Forty four phosphoproteins were identified from gECs exposed to IE/UE and media control at 24, 48 and 72 h (Figure 2). The molecular functions of the 44 phosphoproteins were classified according to the gene ontology analysis (Figure 2a) and include those involved in catalysis (40%), binding (28.6%), structural molecules (17%), enzyme regulators (5.7%), translation regulators (2.9%), transcription regulators (2.9%) and transporters (2.9%). The biological processes of these phosphoproteins (Figure 2b) were found to be predominantly in metabolic processes (23.3%), cellular processes (15.6%), transport (8.9%), and cell cycle (4.4%). In addition, protein class categories were analysed for these phosphoproteins indicating functions in cytoskeleton (10.6%), proteases (4.3%), kinases (4.3%) and structural proteins (2.1%) (Figure 2c).

Forty four phosphoproteins, identified from gECs exposed to IE/UE for 24 – 72 h, are presented using a heatmap visualization (Figure 3). The relative levels of these phosphoproteins was evaluated as high and low compared with level of phosphoprotein present in gECs from media control. The patterns of phosphoproteins from gECs exposed to IE/



48 h	media	IE	UE
G0	2.2 ± 0.1	14 ± 0.8	1.2 ± 0.1
G0/G1	4.6 ± 0.3	5.2 ± 0.4	3.2 ± 0.1
S	4.6 ± 0.2	2.5 ± 0.1	4.5 ± 0.3
G2/M	45.2 ± 3.4	40.9 ± 2.7	46.1 ± 4.4
>4n	43.4 ± 2.6	37.4 ± 2.4	45 ± 3.7

72 h	media	IE	UE
G0	4.8 ± 0.8	20 ± 1.4	5.3 ± 0.7
G0/G1	6.6 ± 0.6	10.4 ± 0.9	6.5 ± 0.8
S	4.1 ± 0.4	4.9 ± 0.5	3.1± 0.2
G2/M	51.3 ± 4.4	42.5 ± 3.4	50.5 ± 4.8
>4n	33.2 ± 4.7	22.2 ± 3.4	34.6 ± 3.9

Figure 1 Inhibitory effect of *P. vivax* on erythroid cell growth and cell cycle. gECs, 5-day old, were cultured with IE/UE lysate at a ratio of 1:10 (gEC:IE/UE) for 24, 48 and 72 h. **(a)** Cell growth is presented as means ± SD of percentage of control, compared with a control containing medium. **(b)** Cell death is illustrated as means ± SD of percentage of dead cells, and **(c)** cell cycle is illustrated as means ± SD of percentage of DNA content in each phase of G0, G0/G1, S, G2/M and > 4n, using propidium iodide staining. Means ± SD were calculated from five independent experiments. *P-value < 0.01 compared with media control.

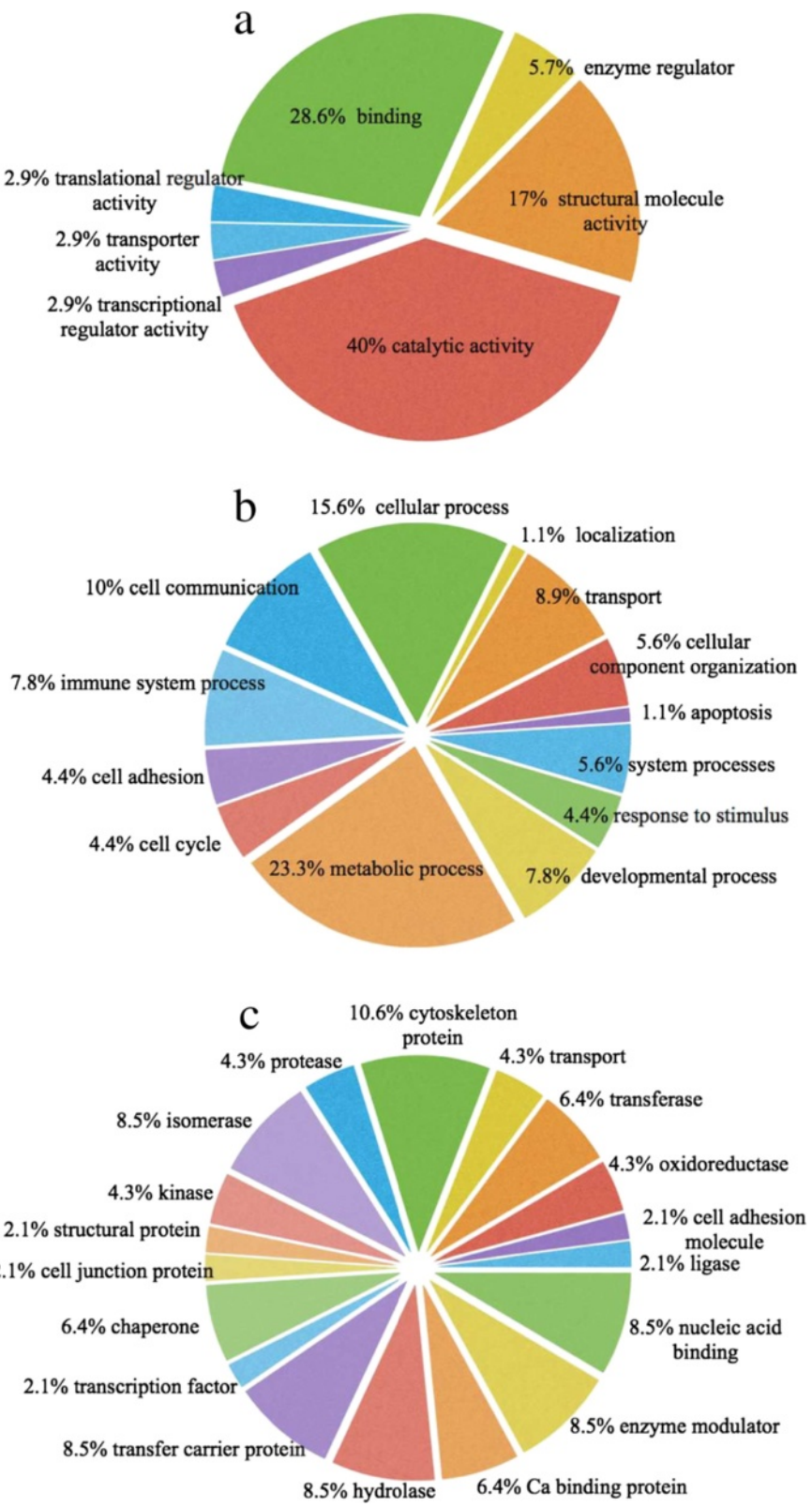


Figure 2 Classification of phosphoproteins identified in erythroid cells. Phosphoproteins found following exposure to infected erythrocytes (IE)/uninfected erythrocytes (UE) and in media control were categorized according to **(a)** molecular functions, **(b)** biological processes, and **(c)** protein classes.

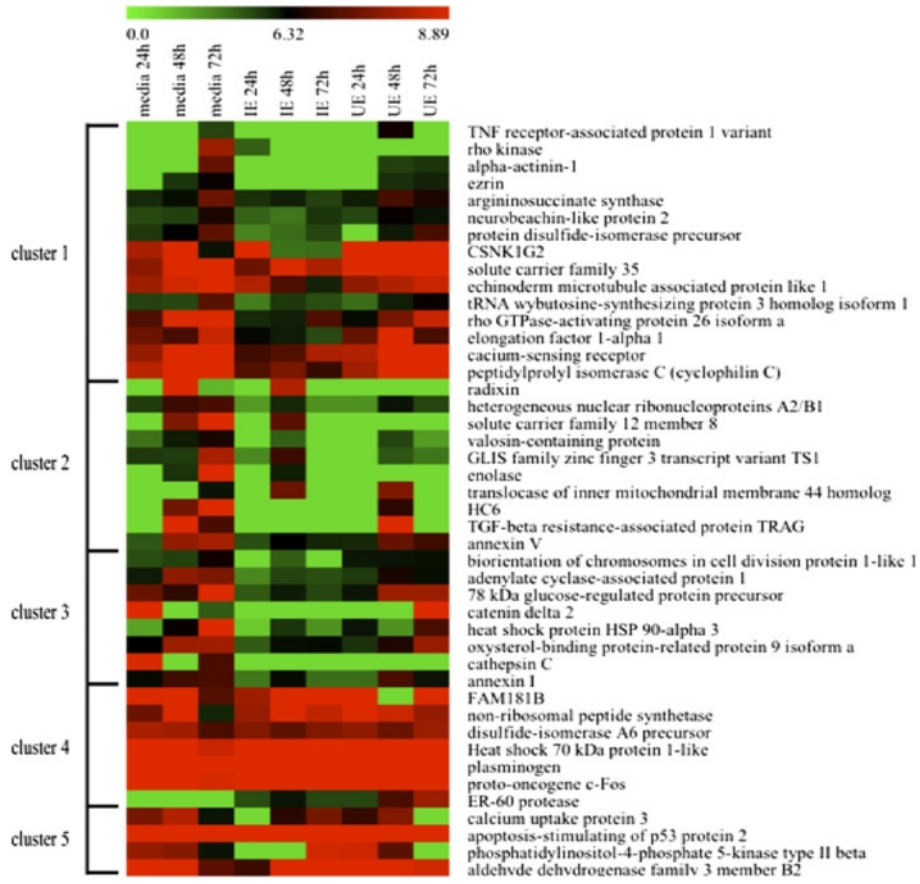


Figure 3 Comparison of phosphoprotein levels in erythroid cells untreated and exposed to *P. vivax*. Heatmap of relatively high (red) and low (green) phosphoproteins from gECs exposed to lysates of infected erythrocytes (IE) and uninfected erythrocytes (UE) for 24-72 h and gECs in media control. The heatmap and clustering were generated using the web-based analysis program MEV.

UE or in media for 24, 48 and 72 h were analysed in five clusters and categorized using the K-mean clustering method (Figure 3). Interestingly, the relative level pattern of phosphoproteins in cluster 1 was low from gECs exposed to IE for 24 – 72 h. To identify potential phosphoproteins from five clusters involved in inhibition of gEC growth and division by IE, the relative levels of phosphoproteins in each condition and time point were determined using statistical analysis with paired *t*-test at *P*-value ≤ 0.05. The lists of selected phosphoproteins with significant differences in relative level (*P*-value ≤ 0.05) were evaluated for biological processes and molecular functions using the KEGG PATHWAY database, as shown in Table 1. Interestingly, three of the phosphoproteins identified, ezrin, alpha actinin-1 and Rho kinase were reported to function in the regulation of the cellular cytoskeleton (Table 1). Ezrin, alpha actinin-1 and Rho kinase from gECs were observed to be all included in cluster 1 and the relative levels of these three phosphoproteins were significantly low from gECs exposed to IE for 72 h, compared with gECs in media only. These findings suggests that ezrin, alpha actinin-1 and rho kinase in the phosphoproteome of gECs may have roles relevant to the cellular pathology caused by *P. vivax* during erythroid cell growth.

Exposure to a pathogen, like parasites, has the ability to disturb the cellular processes in particular cell types. The alteration of protein expression and function inside the cells are the result of host-pathogen interactions. The exploring protein-protein interactions of gECs exposed to IE lysate were analysed using web based free database, STRING and three significant relative levels of phosphoproteins including ezrin, alpha actinin-1and Rho kinase were specifically found in the networks (Figure 4a). Moreover, the finding of phosphatidylinositol-4-phosphate 5-kinase type II beta in cluster 5 (Figure 3) also links the association of three proteins (Figure 4a). Interestingly, the protein-protein interaction of ezrin, alpha actinin-1, Rho kinase and phosphatidylinositol-4-phosphate 5-kinase type II beta was found in a specific relevant interaction pathway of IE-exposed gEC. Likewise, the alteration of defined phosphoprotein levels in gECs during exposure to IE for

Table 1 Evaluation of biological processes and molecular function of selected phosphoproteins using the KEGG PATHWAY database

Cluster	KEGG pathway
	Protein processing in endoplasmic reticulum[a]
2	Valosin-containing protein
4	ER-60 protease[b]
4	Heat shock 70 kDa protein 1-like
4	Disulfide-isomerase A6 precursor
	Regulation of actin cytoskeleton[a]
1	Rho kinase
5	Phosphatidylinositol-4-phosphate 5-kinase type II beta
1	Ezrin[b]
1	alpha actinin-1[b]
2	Radixin
	Leukocyte transendothelial migration[a]
1	Rho kinase
1	Ezrin[b]
1	alpha actinin-1[b]
	Focal adhesion
1	Rho kinase
1	alpha actinin-1[b]
	MAPK signaling pathway
4	Heat shock 70 kDa protein 1-like

[a]KEGG pathway analysis under STRING database at p value ≤ 0.01.
[b]The differential relative level of phosphoproteins under significant *t*-test at p value ≤ 0.05.

24 to 72 h was performed as the dynamic relative level of phosphoproteins as shown in Figure 4b. Levels of the phosphoproteins ezrin, alpha actinin-1 and rho kinase are reduced following 48 to 72 h of IE exposure to gECs, compared with media controls. In contrast, abundance of phosphatidylinositol-4-phosphate 5-kinase type II beta was elevated under the same conditions. This showed that the altered relative level of these 4 phosphoproteins was a result of the interactions between IE and gEC proteins. To investigate whether reduced abundance of ezrin is involved in the mechanism of ineffective erythropoiesis in malaria, the ezrin protein-protein interaction network was analysed using the KEGG PATHWAY database. The ezrin interactome and its involvement in various cellular functions is shown in Figure 4c. This analysis revealed that the ezrin protein has important roles in various pathways though its function in the regulation of actin cytoskeleton may be the most relevant to malaria. While ezrin is associated with several pathways, only regulation of the actin cytoskeleton is shared among alpha actinin-1, Rho kinase and phosphatidylinositol-4-phosphate 5-kinase type II beta (Figure 4c).

Inhibitory effect on erythroid cell growth by *P. vivax* is regulated by ezrin phosphorylation

Protein phosphorylation is involved in several regulatory functions in living cells. Using phosphoproteomic and bioinformatics analysis, ezrin was identified and determined to have to function in regulation of actin cytoskeleton. One ezrin phosphopeptide from gECs contained the amino acid sequences DK**Y**K**T**LRQIR, corresponding to residues 563-572. The phosphorylation site of this peptide analysed from MS/MS data was predicted to be tyrosine 565 (Y565). Ezrin, and phospho-ezrin were evaluated by immunofluorescence using specific antibodies against ezrin and phospho-ezrin Thr567 [31], as shown in Figure 5. Unfortunately, the antibody against phospho-ezrin Y565 is not available and data for phosphorylation at this ezrin residue was not reported. Results of immunofluorescence showed that ezrin and phospho-ezrin Thr567 localized to cell extensions peripheral processes of gECs. gECs in culture with UE or in media displayed strong signals for phospho-ezrin Thr567. In contrast, the signal strength for phospho-ezrin Thr567 was markedly reduced following exposure to IE for 48 and 72 h (Figure 5a and b). Quantitation of signals using ImageJ software for ezrin and phospho-ezrin Thr567 from 1,000 gECs from each culture, confirm the decreased level of ezrin proteins in cells exposed to IE (Additional file 1). The intensity ratios of phospho-ezrin Thr567 in IE-exposed gECs compared to media controls were less than 1 (0.17 and 0.26, at exposure times 48 and 72 h, respectively). In contrast, the same analysis using UE-exposed gECs gave intensity ratios for phospho-ezrin Thr567 of approximately 1 (0.99 and 0.93, at exposure time 48 and 72 h, respectively). Ezrin signals in IE/UE-exposed gECs compared to gECs in media at 48 and 72 h were nearly 1 (0.86 and 0.95 for IE-exposed gECs, and 0.9 and 0.98 for UE-exposed gECs, respectively), as shown in Additional file 1. These results indicate that ezrin phosphorylation at the carboxy-terminal threonine residue 567 was decreased in gECs exposed to IE. This suggests that parasite proteins were able to inhibit erythroid cell growth by preventing the phosphorylation of the C-terminal region of ezrin.

Discussion

Anaemia has been frequently observed to be a consequence of vivax infection [4,7,10,48-58]. Recently, transmission electron microscope analysis of two vivax malarial cases with symptom of anaemia demonstrated the presence of *P. vivax*-infected erythroblasts and subsequent degradation of erythroblasts. Interestingly, *P. vivax* parasites were found in bone marrow, but could not be detected in peripheral blood [17]. Consistent with this observation, an *in vitro* study has revealed that *P. vivax* can directly inhibit erythroid cell proliferation and differentiation through

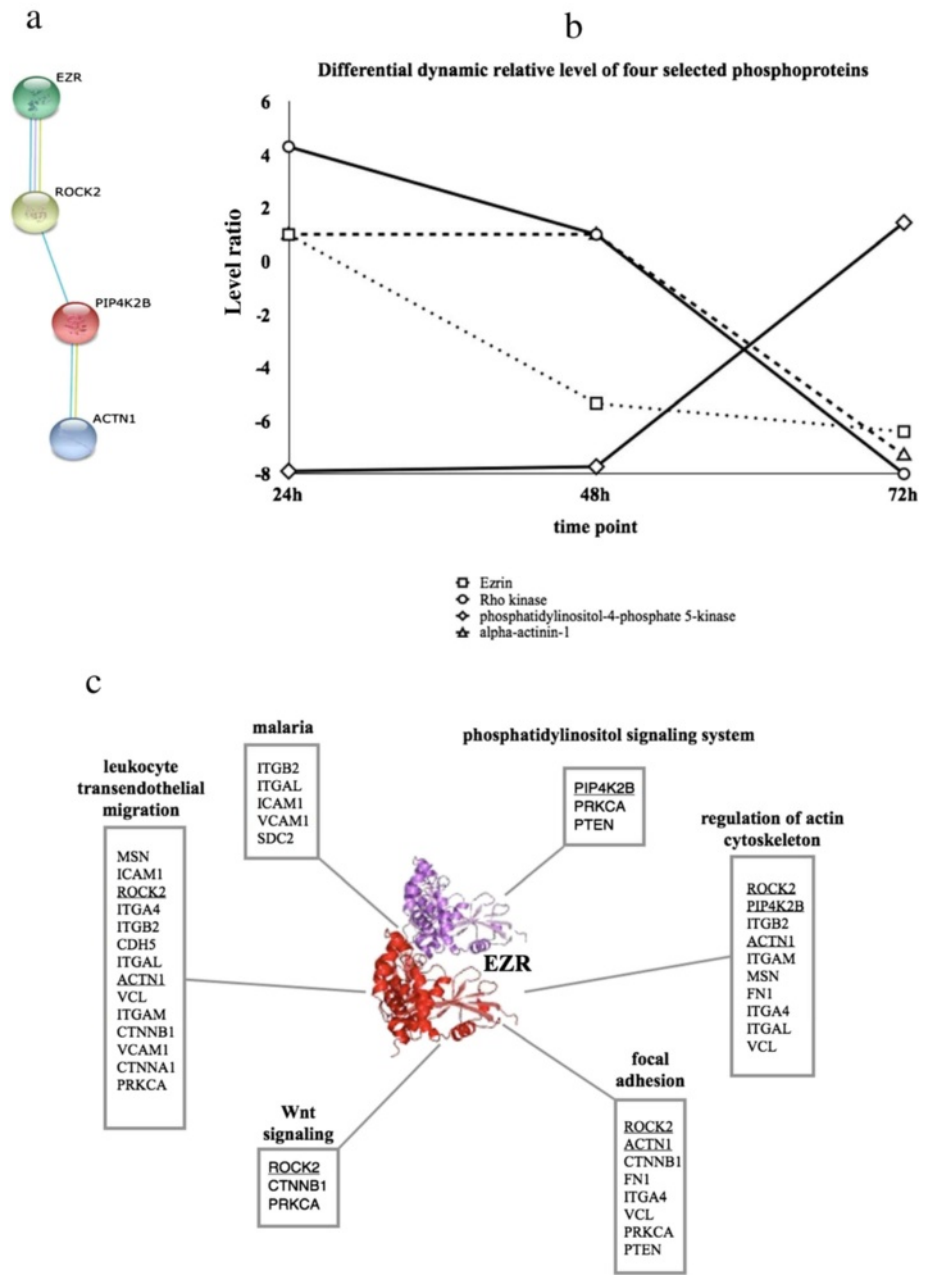


Figure 4 Relative levels and interactions of selected phosphoproteins. (a) Interactions of ezrin, Rho kinase, phosphatidylinositol-4-phosphate 5-kinase type II beta and alpha actinin-1 analysed using the STRING database **(b)** relative levels of selected phosphoproteins, ezrin, Rho kinase, phosphatidylinositol-4-phosphate 5-kinase type II beta and alpha actinin-1, and **(c)** interaction of ezrin with regulatory pathways and cellular cytoskeleton generated using the KEGG PATHWAY database.

altered division of erythroid cells [22]. However, the mechanisms underlying the suppression of erythroid development by *P. vivax* appear to be complex and poorly characterized. In this study, phosphoprotein analysis was used to investigate the underlying the suppression of erythroid development by *P. vivax*. Using LC-MS/MS analysis in combination with gene ontology information from the human protein database, 44 phosphoproteins from gECs exposed and non-exposed to IE/UE were identified and categorized according to molecular function, biological process and protein class. Interestingly, the relative level of phosphoproteins was significantly lower following exposure of gECs to parasite proteins, compared to non-exposed cells. Phosphoproteins that displayed significant decreases include ezrin, alpha actinin-1 and Rho kinase. These phosphoproteins were determined

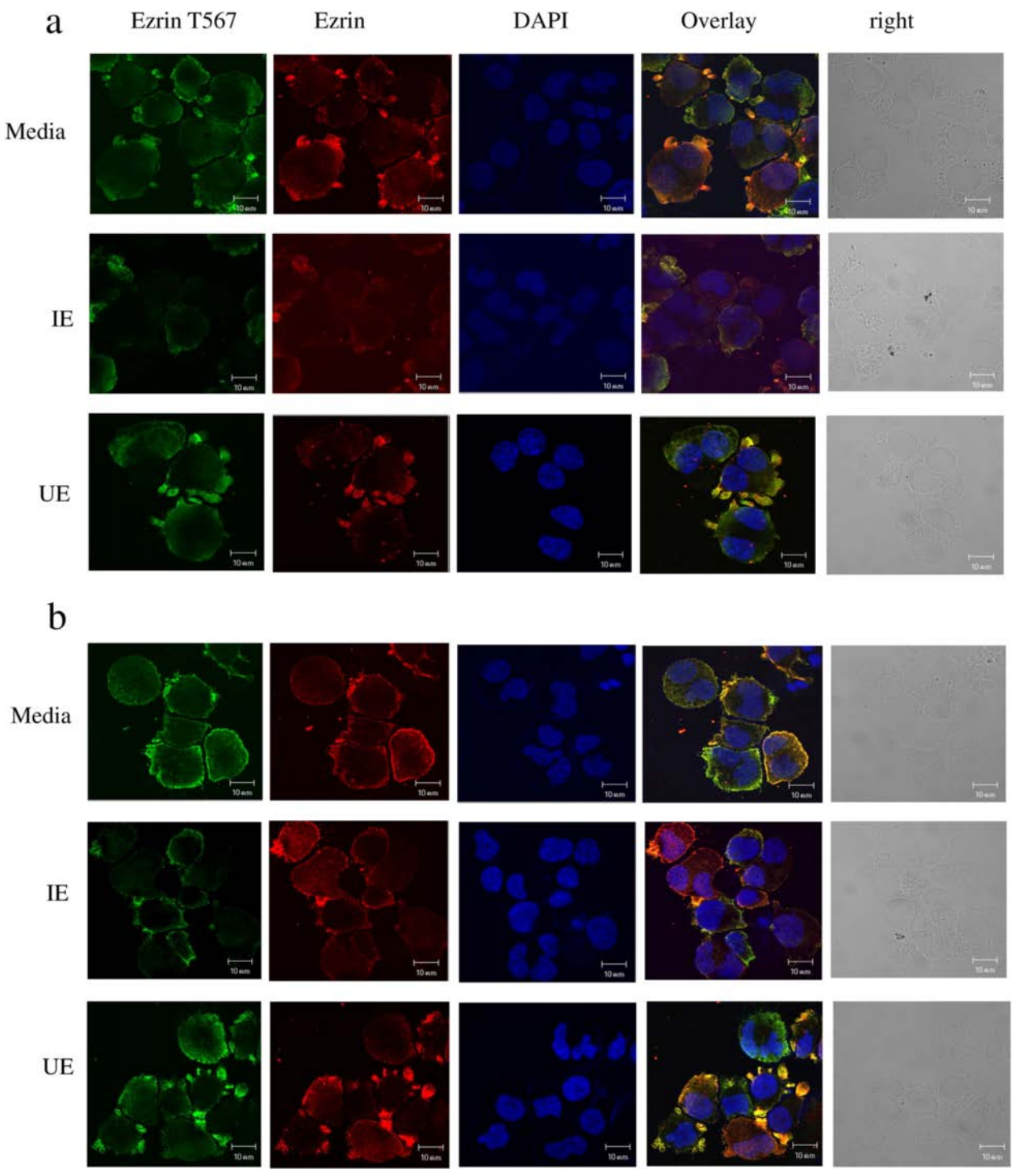


Figure 5 Localization of ezrin and phospho-ezrin Thr 567 in untreated erythroid cells and those exposed to *P. vivax*. Immunofluorescence, ezrin phospho-Thr567 (green), ezrin (red), and nucleus (blue) from gECs exposed to lysates of IE/UE and cells in media for 48 h **(a)** and 72 h **(b)** detected with using specific antibodies against ezrin and phospho-ezrin Thr567. Images were captured using a confocal microscope with 63x magnification and zoom 2.

to be in the same cluster 1 with significantly low level pattern of phosphoproteins and exhibited a similar pattern of abundance in response to IE exposure. These results suggest that the phosphoproteins ezrin, alpha actinin-1 and Rho kinase may have roles in the inhibitory effect on erythroid cell growth and division following *P.*

vivax exposure. The protein-protein interaction networks determined using the KEGG human protein database revealed that ezrin functions in several cellular pathways, including regulation of actin cytoskeleton.

Ezrin, a member of ERM (ezrin, radixin and moesin) proteins, is localized to cell extension peripheral processes and able to interact with transmembrane proteins and the cytoskeleton. Ezrin functions to regulate cytoskeletal assembly, membrane-protein function and membrane transport [29,59,60]. Recent studies, have implicated ezrin as a signal transducer involved in a wide variety of cellular functions, including cell survival, adhesion, morphogenesis, motility, cytokinesis and cellular proliferation [61,62]. Increasing evidence indicates a direct relationship between cell proliferation and the level of ezrin expression in cells [63-65]. Microinjection of anti-ezrin antibodies into the cytoplasm blocked the entry of mouse fibroblasts into S phase, confirming the function of ezrin in proliferation [66]. In response to TNF, ezrin inhibits endothelial cell proliferation through transcriptional repression of cyclin A, a cell cycle regulatory protein [67]. This study also found that ezrin in erythroid cells appears to function in the regulation of cell growth and division. Erythroid cells with growth inhibited by *P. vivax* display low levels of ezrin phosphoprotein.

The phosphorylation of a threonine at residue 567 (T567) is necessary for the active conformation of ezrin and enhances binding with F-actin [28]. The results of this study also find that phosphorylation of ezrin, possibly at Thr567, is involved in the regulation of erythroid cell growth. Although our analysis identified the position of the phosphorylation site in an ezrin-phosphopeptide as tyrosine 565, the phosphorylation of ezrin threonine 567 was also detected using a specific antibody. A source of error in phosphorylation site localization on peptides is the presence and intensity of ions for these species in the MS/MS spectra [68]. However, both residues 565 and 567 are contained in the phosphopeptide 563-572 from ezrin, which was detected in this study. It is also possible that the anti-ezrin Thr567 monoclonal antibody may recognize the phosphorylation of Tyr565 as well as Thr567. This antibody was produced using phosphopeptide of human ezrin containing residues 564-568 (K**Y**K**T**L) [31] and contains both Tyr565 and Thr567. Previous reports have demonstrated the specificity of this antibody for phospho-ezrin Thr567 [69-71] but it is not known if it can recognize phosphorylation at other sites.

Three phosphoproteins in this study, ezrin, Rho kinase and alpha actinin-1 are present at significantly low levels in parasite-exposed cells. Investigation of the KEGG PATHWAY database demonstrates that ezrin has important roles in various pathways, particularly, regulation of actin cytoskeleton, which has implications to malaria, as shown in Figure 4c. Ezrin exerts its biological functions through protein-protein interactions and its active form is regulated by Rho kinase/ROCK2, which directly interacts with membrane proteins [31,72]. Ezrin binds to adhesion-related proteins with single transmembrane domains such as ICAM-1, CD44 and CD43 through their cytoplasmic tails to modulate cell morphology [61,73]. Cytoadhesion molecules such as ICAM1 contribute to cytoadhesive phenotype/rosetting and high intensity of rosetting is found in anaemia cases with *P. vivax* infection [74]. Both ICAM-1 and VCAM-1, soluble adhesion molecules, are detected at high levels in serum from falciparum patients with severe malaria [75]. This study also found abnormal erythroid cell aggregation in culture of gECs exposed to IE lysates (Additional file 2). Taken together, this suggests that during interaction of *P. vivax* proteins with adhesion molecules on erythroid cells, ezrin regulates cell adhesion by connecting membrane adhesion receptors to the actin-based cytoskeleton. This erythroid cell adhesion may contribute to down regulation of erythroid cell production leading to the development of anaemia. For other adhesion molecules, integrin alpha L chain (ITGAL) and integrin α2β1 has been found a higher probability to involve in severe thrombocytopenia in vivax malaria [76]. The expression of molecules sharing an epitope with human ITGB2/LFA-1 integrin, or CD18 leukocyte integrin on the *Plasmodium falciparum*-parasitized erythrocyte surface could be involved in the pathogenesis of severe disease [77]. However, further studies are need to explore the association of these intercellular adhesion molecules with ezrin in vivax malaria.

In addition, ERM proteins are members of the band 4.1 superfamily, FERM (four-point one, ezrin, redixin, moesin) [78-80]. Phosphorylation of erythrocyte protein 4.1 is involved with the modification of the host erythrocyte membrane by *P. falciparum* [81]. Moreover, tyrosine phosphorylation of band 3, band 4.2, catalase and actin in *P. falciparum*-infected erythrocytes are predicted to be part of the regulatory mechanism to modify the erythrocyte membrane [82]. This study found low levels of alpha actinin-1 in gECs in the presence of IE. The subsequent reassembly of actin structure in response to parasite suppression is currently being investigated. This poorly understood aspect of ezrin function suggests that parasites inhibit the ezrin protein allowing the assembly of complex cellular structures in erythroid cells leading to dyserythropoiesis, inhibition of erythroid cell growth and division resulting in the accumulation of cells in a resting G0 phase. This study demonstrates that *vivax* parasites suppress development of erythroid progenitor cells through a mechanism that includes decreased ezrin phosphorylation. *Plasmodium vivax* is able to enter bone marrow, as previously reported [17,20,57,83], and parasites or its products bind to erythroid progenitor cells, resulting in decreased ezrin phosphorylation, leading to suppression of erythroid

development, and ultimately anaemia. This is the first analysis suggesting that ezrin contributes to the suppression of erythroid cell growth by *P. vivax*. Further investigation of this mechanism should help to better understand pathogenesis of anaemia in acute or chronic *P. vivax* infection.

Conclusions

This analysis demonstrates that the pathogenesis of anaemia in vivax malaria is mediated by parasite suppression of human erythroid cell growth and division. Inactivation of the ezrin protein, leading to ineffective erythropoiesis and dyserythropoiesis, appears to be a key event resulting in the development of severe anaemia. The understanding of the pathogenesis of anaemia in vivax malaria should help in the development of therapeutic strategies to treat severe anaemia malaria.

Additional files

Additional file 1: Intensity ratios of ezrin and phospho-ezrin Thr567 from gECs exposed to IE/UE relative to signals from gECs in medium control. The intensity of ezrin and phospho-ezrin Thr567, from 1,000 gECs cells in each condition, exposed to IE/UE or in media were determined using ImageJ software (http://imagej.nih.gov). Intensity ratios were calculated using the mean intensity of IE/UE-exposed gECs normalized to the mean intensity from gECs in media.

Additional file 2: Erythroid cell aggregation in culture of gECs exposed to *P. vivax*. Erythroid cells, 5-day old, were cultured with IE/UE lysates at a ratio of 1:10 (gEC:IE/UE) for 24, 48 and 72 h. Cell aggregation was observed under inverted microscope with 200x magnification.

Abbreviations

(gECs): Growing erythroid cells; (IE): Infected erythrocyte; (UE): Uninfected erythrocyte; (ERM): Ezrin ezrin radixin and moesin; (HSCs): Haematopoietic stem cells; (Thr567): Threonine 567; (LC-MS): Liquid chromatography mass spectrometry.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TP designed the study, collected parasites from patient blood, performed cell culture and other experiments, performed statistical analysis, and wrote the manuscript. SP performed bioinformatic analysis. SR supported laboratory facilities for proteomic analysis. AP and SK performed LC-MS/MS. SH and RU contributed substantially to design of experiments and gave advice for writing of the manuscript. All authors read and approved the final manuscript.

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Books Panichakul T. & Sukasem C. (2011). Emerging and Re-emerging Diseases by Viral Infection.

Symposia

1. Tengchaisri T. and Sirisinha S. Establishment and characterization of a new human cholangiocarcinoma cell line from Thai patient with intrahepatic bile duct tumor.

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