ภูมิตอบสนองต่อเชื้อมาลาเรียชนิดพลาสโมเดียมไวแวกซ์

Native Immune Response against Plasmodium vivax Infection

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ภูมิตอบสนองต่อเชื้อมาลาเรียชนิดพลาสโมเดียมไวแวกซ์: กรณีศึกษาเปรียบเทียบระหว่างคนไข้ในประเทศไทยและประเทศจีน

องค์ความรู้ในด้านการศึกษากลไกภูมิคุ้มกันทางด้าน Cell-mediated immunity ต่อเชื้อมาลาเรียชนิด *P. vivax* นั้นมียังมีจำกัด ในอดีตได้มีผู้ศึกษาพบว่า คนไข้ที่ติดเชื้อมาลาเรียทั้งสองชนิด (mixed infection ระหว่าง *P. falciparum* และ *P. vivax*) จะเกิดความรุนแรงของโรคได้น้อยกว่าคนไข้ที่ติดเชื้อ *P. falciparum* เพียงชนิดเดียว ดังนั้นแสดงให้เห็นว่า เชื้อ *P. vivax* น่าจะมีบทบาทที่สำคัญในการกระตุ้นระบบภูมิคุ้มกันทางด้านเซลล์ให้มีประสิทธิภาพในการกำจัดและทำลายเชื้อทั้งสองชนิด ส่งผลให้เกิดการลดลงของระดับความรุนแรงของโรค งานวิจัยในโครงการ เราได้ทำการศึกษากลไกการกระตุ้นเซลล์ในระบบภูมิคุ้มกันชนิดต่างๆ ต่อเชื้อมาลาเรียชนิด *P. vivax* ผลการทดลองแรกเราพบว่าเซลล์ชนิด regulatory T cells เพิ่มขึ้นมากอย่างเห็นได้ชัดในขณะเกิดไข้มาลาเรียในระยะเฉียบพลันซึ่งเราเชื่อว่า ความไม่สมดุลกันระหว่างการกระตุ้นเซลล์ชนิดนี้ให้มีระดับที่สูงขึ้นในขณะติดเชื้ออาจเป็นกลไกที่สำคัญของเชื้อชนิด *P. falciparum* และ *P. vivax* ให้สามารถใช้หลบหลีกภูมิตอบสนองต่อร่างกายได้ เรายังพบว่าเซลล์ชนิด $\gamma\delta$ T cells มีระดับเพิ่มสูงขึ้นอย่างชัดเจนในคนไข้ *P. vivax* ซึ่งมีมากกว่าคนไข้มาลาเรียชนิด *P. falciparum* และจากการทดลองในหลอดทดลองยังสนับสนุนให้เราเชื่อว่า เซลล์ชนิด $\gamma\delta$ T cells เป็นเซลล์ที่ออกมาทำลายเชื้อมาลาเรียในช่วงต้นของการติดเชื้อ ผลการทดลองต่อมาเราได้ทำการเปรียบเทียบระดับ cell phenotypes ในกลุ่มคนไข้จีนตอนกลางที่ติดเชื้อมาลาเรียชนิด *P. vivax* เนื่องจากประเทศจีนตอนกลางเป็นบริเวณที่มีการระบาดของโรคมาลาเรียชนิด *P. vivax* เพียงชนิดเดียวเท่านั้น ไม่พบการติดเชื้อมาลาเรียชนิดอื่นๆ โดยการศึกษาเราพบว่าเมื่อคนไข้ได้รับเชื้อในระยะเฉียบพลัน เซลล์บางชนิดมีระดับต่างกัน เช่น regulatory T cell มีระดับต่ำลงในกลุ่มคนไข้จีน ซึ่งแสดงให้เห็นว่าระดับการกดภูมิคุ้มกันโดย *P. vivax* มีระดับที่ต่ำกว่ากลุ่มคนไทย แต่ทั้งสองเชื้อชาติมีระดับ $\gamma\delta$ T cells สูงขึ้น ซึ่งจากผลการทดลองนี้ทำให้เราสามารถสรุปเบื้องต้นได้ว่า $\gamma\delta$ T cells เป็นเซลล์ที่ได้รับการกระตุ้นโดยเชื้อมาลาเรีย *P. vivax* ส่วนภูมิคุ้มกันทางด้าน antibody ที่จำเพาะต่อเชื้อ *P. vivax* ในคนจีนมีระดับที่สูงและมีความจำเพาะมากกว่าคนไทย และสิ่งที่น่าสนใจก็คือ ในคนไทยเราพบความเป็น cross-reactivity ของ antibody ที่มีต่อเชื้อ ผลการทดลองดังกล่าวสามารถชี้ชัดได้ว่า คนไทยที่อาศัยอยู่ในบริเวณที่มีการระบาดของโรคมาลาเรียเคยมีประวัติการติดเชื้อ *P. falciparum* มาก่อนแทบทุกคน จึงสามารถตรวจพบantibodyต่อเชื้อ *P. falciparum* ดังนั้นการศึกษานี้ก่อให้เกิดองค์ความรู้ใหม่ทางด้านภูมิคุ้มกันวิทยาต่อเชื้อมาลาเรียชนิด *P. vivax* ที่จำเพาะเพื่อนำไปสู่การวิจัยและพัฒนารูปแบบใหม่ของวัคซีนต่อโรคมาลาเรียในอนาคต

Native immune response against *Plasmodium vivax* infection: a comparative study between Thailand and China

Cell-mediated immunity induced by *P. vivax* has not been investigated widely. A study has shown the severity reduction induced by *P. falciparum* among mixed *P. falciparum* and *P. vivax* infection, indicated the role of host immunity against *P. vivax* may efficiently destroy malaria parasite during infection. In this project, we performed the study on functional mechanisms of cell mediated immune responses by showing various cell phenotypes during *P. vivax* infection. Our first finding has shown that regulatory T cell is increased during acute *P. vivax* infection. This indicates that the parasites can evade the immune responses via induction of regulatory T cells. The next finding, we have found a significant elevation of $\gamma\delta$ T cells in acute *P. vivax* infection higher than that of *P. falciparum* infection. Moreover, the *in vitro* study supports the early activation of $\gamma\delta$ T cells in response to *P. vivax* parasites. We characterize lymphocyte phenotypes in the Central China, where *P. vivax* is the only cause of malaria infection. Our study has shown that regulatory T cell in the Chinese patients is reduced in contrast to the level of $\gamma\delta$ T cell which is increased during acute *P. vivax* infection. This can be primarily summarized that $\gamma\delta$ T cells play role against *P. vivax* infection. The antibody mediated immunity against *P. vivax* infection among Chinese patients is higher and more *P. vivax*-specific than those of the Thai patients. This suggests the cross-reactivity of anti-malaria antibodies among Thai patients. These findings provide information to understand cell-mediated immune responses against malaria infection, leading to various innovative designs of malaria vaccine in the future.

Executive Summary

Basic Research

1. Research title

Native immune response against *Plasmodium vivax* infection: a comparative study between Thailand and China

2. Principal investigators

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3. Field of research

Immunology in *Plasmodium vivax* malaria

4. Problems and importance of the problems

Malaria infection is a major cause of morbidity and mortality in many countries. Two species of the pathogenic parasite, *Plasmodium falciparum* and *Plasmodium vivax* are commonly causing infections. Nevertheless, the pathogenicity and pathogensis of these two organisms are different. Investigations are extensive in falciparum infection due to more severity comparing to that of vivax malaria. Only a few researches have focused on the pathogenesis of vivax infection although the organism is considered one of the major problems in Ethiopian, Latin American and Southeast Asian countries, particularly Thailand. *Plasmodium vivax* has a typical character in which this organism can remain inside the host for several months or years. Relasping of the infection is common and can cause severe morbidity. During the parasitaemic vivax infection, although host immunity both antibody and cell-mediated responses should play roles against the organisms, however, the mechanism remains unclear. To understand the modulation of host immune system during acute *P. vivax* infection, it is important to investigate the role of *P. vivax*-specific T cells during the disease activation process in comparison with the convalescence period. We hypothesized that *P. vivax* parasites activate lymphocytes to enter a slow expansion of the *P. vivax*-specific lymphocytes rendering the rise of natural protective immunity. Nonetheless, this specific activation by *P. vivax*-derived products eventually triggers the activated lymphocytes into apoptosis. These events are conceivably redundant during the onset of infection, particularly in the

case of relapsing *P. vivax* infection. Therefore, the more often the infection is relapsed the lower expansion of the *P. vivax*-specific T cells occur. Ultimately, the immunity to *P. vivax* is compromised by the parasites, i.e. low antibody response.

Objectives

The objective of this study is to establish the understanding of the mechanisms of host immune regulation during *P. vivax* infection by determining *P. vivax*-specific T cell activation. In addition, this study also investigates the role of natural and inducible regulatory T cells activation during *P. vivax* infection. Immunity is controlled by and involved with various genetic codes, therefore, a comparison among the populations having different genetic background, living in similar malaria endemicity awaited the investigation.

Methodology:

1. Sample collection

Acute *P. vivax*-infected volunteers registered at Malaria Clinic in Mae Sot and Mae Kasa districts, Tak province will be informed of the details of the project prior to recruitment. Healthy volunteers living outside the endemic area and without previous history of malaria infection are recruited by individual consent and blood sample collected as for baseline controls.

2. Preparation of peripheral blood mononuclear cells (PBMC)

Heparinized blood will be carefully overlayered on Lymphoprep™ (AXIS-Shied PoC AS, Norway) and PBMC will be separated by gradient centrifugation. The PBMC pellet will be finally resuspended in RPMI-1640 containing 10% fetal calf serum (FCS). The viability will be determined by tryphan blue exclusion and the number will be counted in hematocytometer.

3. Antigen preparations

Infected blood of *P. vivax* at schizont stage from volunteers will be filtered through the Plasmodipur filter® in order to remove leukocytes and used as source of antigen. Parasites in the samples will be separated by centrifugation on the 60% Percoll®. The cells in the interface layer between medium and Percoll® will be collected, washed 2 times and the pellets will be stored at -70°C and used as malaria antigens.

4. Activation of T cells *in vitro*

PBMC at 2×10^5 /ml in HEPES-buffer RPMI-1640 supplemented with 10% inactivated AB serum will be activated with optimal concentrations of malaria antigens and cultured under *in vitro* conditions for a week. The cells will be harvested on day 5 and 7 for further analysis.

Control wells will be cultured with RPMI-1640 medium alone as negative control, with RBC antigens as base line stimulation, and with Phytohemagglutinine-A (PHA) (Sigma, UK) as mitogenic activation.

5. Flow cytometric analysis

PBMC from all individuals will be stained as well as activated lymphocytes *in vitro* will be harvested and stained for three-color flow cytometry with various mAb to CD3, CD4, CD8, CD25, CD45RO, and CD19. The stained cells will be analysed on FACScan using the CELLQUEST software (Becton Dickinson, USA).

6. Data analysis

Data will be analysed with SPSS programme (SPSS Inc, USA). Differences in percentage of T and B cell phenotypes of individuals between and within the groups will be compared using parametric analysis *t* test.

Expected outcomes:

The overall goals of this work are

1. To produce two PhD graduate students of Mahidol University

   Ms. Patchanee Choothong

   Faculty of Medical Technology, Mahidol University

   Ms. Sriwipa Chuangchaiya

   Faculty of Tropical Medicine, Mahidol Univerisy

2. To train a young researcher with focus in Malaria Immunology

   Dr. Kulachart Jangpatarapongsa

   Thesis dissertation on 24 February 2006.

Expected publications: 4-5 international papers

[1] **Jangpatarapongsa K, Chootong P**, Sirichaisinthop J, Sattabongkot J, Tangpradubkul S, Hisaeda H, Troye-Blomberg M, Cui L, **Udomsangpetch R**. *Plasmodium vivax* alter the balance of myeloid and plasmacytoid dendritic cells and induction of regulatory T cells. *Eur. J. Immunol* .

[2] **Chuangchaiya S, Jangpatarapongsa K, Chootong P,** Sirichaisinthop J, Sattabongkot J, Pattanapanyasat K, Chotivanich K, Troye-Blomberg M, Cui L, **Udomsangpetch R.** Immune response to *Plasmodium vivax* has a potential to reduce malaria severity. *Clinical and Experimental Immunology.*

[3] **Chootong P, Jangpatarapongsa K**, Sirichaisinthop J, Sattabongkot J, **Udomsangpetch R.** Characteristic of immunity to malaria infection: Comparative T cell responses in two major human malaria. *Microbes and Infection*.

[4] Xia H, **Jangpatarapongsa K,** Fang Q, Hu K, Yuan Y, Peng M, Sattabongkot J, Gao Q, Cui L, Li B, **Udomsangpetch R.** Immune responses against *Plasmodium vivax* infection: The study in the Central of China. *Malaria Journal*.

[5] **Jangpatarapongsa K,** Xia H, Hu K, Zhiyong T, Miao J, Fan Q, Cui L, Sattabongkot J, Gao Q, Cui L, Li B, **Udomsangpetch R**. Natural immune response against *Plasmodium vivax* c-terminal merozoite surface protein-1 (MSP-1) and apical membrane antigen-1 (AMA-1) and cross-reactivity between *P. vivax* and *P. falciparum* infection: A study in Thailand and China. Parasite International.

(เอกสารลับ)

<u>รายงานผลการดำเนินการวิจัยฉบับสมบูรณ์</u>

ชื่อทุนที่ได้รับ ทุนองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา (BRG4980009)

ชื่อโครงการ ภูมิตอบสนองต่อเชื้อมาลาเรียชนิดพลาสโมเดียมไวแวกซ์: กรณีศึกษาเปรียบเทียบระหว่างคนไข้ในประเทศไทยและประเทศจีน

Native Immune Response against *Plasmodium vivax* Infection: a comparative study between Thailand and China

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ความสำคัญและที่มาของปัญหาที่ทำการวิจัยโดยย่อ

ในปัจจุบันโรคมาลาเรียยังคงเป็นปัญหาทางสาธารณสุขอย่างมาก โดยเฉพาะอย่างยิ่งในเขตพื้นที่ชายแดนระหว่างไทยกับพม่า เชื้อมาลาเรียที่ก่อให้เกิดโรคในมนุษย์และพบได้เป็นส่วนใหญ่ในประเทศไทย ได้แก่ *Plasmodium falciparum* (PF) และ *Plasmodium vivax* (PV) ลักษณะเด่นของเชื้อ PV ก็คือ เมื่อเชื้อเข้าสู่ร่างกายคน แล้วจะเข้าไปแฝงตัวอยู่ในตับและมีชีวิตได้นานหลายเดือนถึงหลายปี ทำให้ผู้ป่วยเกิดโรคมาลาเรียชนิดเดิมได้อีก โดยไม่มีการถูกยุงกัด และอาจเป็นอยู่ในเวลาเวลาหลายเดือนหรือหลายปี ในขณะที่เชื้ออยู่ในตับ (liver stage) และกระแสเลือด (blood stage) ร่างกายก็มีกลไกทางภูมิคุ้มกันทั้งในแบบ Cell-mediated immune response ที่จะช่วยกำจัดเชื้อให้หมดไปจากร่างกาย ในปัจจุบันการศึกษากลไกภูมิคุ้มกันทางด้าน Cell-mediated immunity ต่อเชื้อมาลาเรียชนิด PV ในคนยังไม่มีใครทำการศึกษา ซึ่ง

การศึกษาที่มีมาแล้วเป็นงานวิจัยในกลุ่มผู้ป่วยที่ติดเชื้อ PF หรือเป็นมาลาเรียของหนูเท่านั้น เนื่องจากเชื้อ PV ไม่มีการระบาดในประเทศกลุ่มแอฟริกาและปัจจุบันยังไม่สามารถทำการเลี้ยงเชื้อในห้องปฏิบัติการได้

เนื่องจากความรู้พื้นฐานทางด้านภูมิคุ้มกันวิทยาทางด้าน Cell-mediated immunity และ Humoral immunity ต่อเชื้อมาลาเรียชนิด PF มีมากมาย แต่กลไกทั้งสองต่อเชื้อ PV นั้นมีอย่างจำกัด ในอดีตได้มีผู้ศึกษาพบว่า คนไข้ที่ติดเชื้อมาลาเรียทั้งสองชนิด (mixed infection ระหว่าง PF และ PV) จะเกิดความรุนแรงของโรคได้น้อยกว่าคนไข้ที่ติดเชื้อมาลาเรีย PF เพียงชนิดเดียว (Luxemburger et. al 1997) ดังนั้นแสดงให้เห็นว่า เชื้อ PV น่าจะมีบทบาทที่สำคัญในการกระตุ้นระบบภูมิคุ้มกันทางด้านเซลล์ให้มีประสิทธิภาพในการกำจัดและทำลายเชื้อทั้งสองชนิด ส่งผลให้เกิดการลดลงของระดับความรุนแรงของโรค

เนื่องจากประเทศจีนตอนกลางได้พบการระบาดของโรคมาลาเรียที่พบเพียง *Plasmodium vivax* ชนิดเดียวเท่านั้น จึงมีความน่าสนใจที่จะต้องทำการศึกษาภูมิคุ้มกันที่จำเพาะต่อเชื้อชนิดนี้เท่านั้น เพื่อเปรียบเทียบกับคนไทยซึ่งมีการระบาดของโรคมาลาเรียทั้ง 4 ชนิดซึ่งได้แก่ *P. falciparum, P. vivax, P. malariae, P. ovale* ทำให้เราไม่สามารถศึกษาภูมิคุ้มกันที่จำเพาะได้อย่างชัดเจนเพียงในประเทศไทย ดังนั้นการศึกษาของเรามีวัตถุประสงค์หลักเพื่อหาภูมิตอบสนองต่อเชื้อมาลาเรียชนิดพลาสโมเดียมไวแวกซ์ที่แตกต่างกันระหว่างประเทศไทยและประเทศจีน ในบริเวณที่มีการระบาดของโรคมาลาเรียนั้นซึ่งเป็นบริเวณที่มี genetic factor ต่างกัน (Rolf et al. 1998; Yao et al. 2002)

ในด้านการศึกษาวิจัยในโครงการ เราได้ทำการศึกษากลไกการกระตุ้นเซลล์ในระบบภูมิคุ้มกันชนิดต่างๆ โดยเฉพาะอย่างยิ่งเชื้อมาลาเรียชนิด PV ซึ่งเมื่อเร็วๆ นี้ Jangpatarapongsa et al, 2006 พบว่า Cell-mediated immunity มีบทบาทที่สำคัญเป็นอย่างมากต่อการควบคุมเชื้อมาลาเรียในขณะเกิดไข้ โดยเฉพาะอย่างยิ่งเซลล์เม็ดเลือดขาวชนิดทีเซลล์จดจำ (memory T cell) และ แกมม่าเดลต้าทีเซลล์ (gamma delta T cells) ซึ่งมีบทบาทที่สำคัญทั้งทางด้าน innate และ adaptive immunity การศึกษาข้างต้นยังได้พบว่าเซลล์ชนิดนี้เพิ่มขึ้นมากอย่างเห็นได้ชัดในขณะเกิดไข้มาลาเรียในระยะเฉียบพลัน ดังนั้นจึงทำให้เกิดโจทย์วิจัยว่า gamma delta T cells มีบทบาทต่อการควบคุมเชื้อมาลาเรียได้อย่างไรทั้งใน liver stage และ blood stage และใช้กลไกใดมากำจัดเชื้อไม่ให้แพร่กระจายได้อย่างรวดเร็ว

นอกจากเซลล์ชนิด gamma delta T cells แล้วเซลล์ที่มีบทบาทต่อการดำรงชีวิตของเชื้อ PF ให้อยู่ในกระแสเลือดได้ คือ regulatory T cells ซึ่งมีบทบาททางด้านการกดภูมิคุ้มกัน เมื่อเร็วๆ นี้ Walther, et al. 2005 พบว่า เชื้อ PF สามารถกระตุ้นเซลล์ชนิดนี้ให้เพิ่มจำนวนได้ ซึ่งกลุ่มวิจัยจึงเชื่อว่า ความไม่สมดุลกันระหว่างการกระตุ้นเซลล์ชนิดนี้ให้มีระดับที่สูงขึ้นในขณะติดเชื้อ เมื่อเปรียบเทียบกับระดับเซลล์ชนิดอื่นๆ เช่น memory T cell และ gamma delta T cell อาจเป็นกลไกที่สำคัญของเชื้อชนิด PF และ PV ให้สามารถใช้หลบหลีกภูมิตอบสนองต่อร่างกายได้ ดังนั้นจึงสนใจที่จะศึกษากลไกการตอบสนองของเซลล์ชนิดต่างๆ เหล่านี้ว่ามีความเกี่ยวข้องกันอย่างไร งานวิจัยชิ้นนี้จึงตั้งขึ้นมาเพื่อสร้างความรู้ใหม่ที่จะนำมาประยุกต์ใช้พัฒนาวัคซีนป้องกันเชื้อโรคมาลาเรียชนิด PV และ PF โดยระบบภูมิคุ้มกันทางด้านเซลล์ และนำความรู้ที่ได้มาพัฒนาทางด้านการรักษา และการพัฒนาวัคซีนป้องกันมาลาเรียได้ต่อไปในอนาคต

วัตถุประสงค์ของโครงการ

1. เพื่อศึกษาหาระดับ Cell-mediated immunity ต่อเชื้อ PV ที่มีอยู่ในร่างกายของกลุ่มคนที่ติดเชื้อมาลาเรียชนิด PV ในกลุ่มคนไข้ที่ติดเชื้อในประเทศไทยและประเทศจีน

2. เพื่อศึกษากลไกที่แตกต่างกันการกระตุ้นระบบ Cell-mediated immunity ต่อเชื้อ PV, PF และ mixed infection ในกลุ่มคนไข้ไทยและจีน โดยทำการศึกษาการกระตุ้นเซลล์ชนิดต่างๆ เช่น gamma delta T cell, regulatory T cell และ dendritic cell เป็นต้น

3. เพื่อศึกษากลไกการทำลายเชื้อมาลาเรีย PV โดยเซลล์เม็ดเลือดขาวชนิด gamma delta T cells, regulatory T cells เป็นต้น

ผลการทดลอง

1. การตรวจวัดระดับ cell phenotypes ในคนไข้ที่ติดเชื้อมาลาเรียชนิดไวแวกซ์ ในประเทศไทย *(ผลการทดลองโดยละเอียดอยู่ในเอกสารแนบท้ายฉบับที่ 1)*

เราได้ทำการหา cell phenotypes ชนิดต่างๆ เช่น T cells, B cells, memory T cells, regulatory T cells และ gamma delta T cells ใน Peripheral blood mononuclear cell (PBMC) ที่แยกจากเลือดคนปกติและคนไข้ที่ติดเชื้อมาลาเรียชนิดพลาสโมเดียมไวแวกซ์ รวมทั้งเซลล์เม็ดเลือดขาวที่เกี่ยวข้องอื่นๆ เช่น dendritic cells โดยนำค่าที่ได้มาทำการหาความสัมพันธ์กับการก่อโรคมาลาเรียชนิดนี้ โดยผลการทดลองเบื้องต้นพบว่า ขณะที่คนไข้มีการติดเชื้อและกำลังมีไข้ในระยะเฉียบพลัน เซลล์เม็ดเลือดขาวชนิด memory T cells, gamma delta T cells และ regulatory T cells มีจำนวนเพิ่มสูงขึ้นอย่างเห็นได้ชัด นอกจากนี้เรายังพบกลไกการกดภูมิคุ้มกันของ regulatory T cells ที่พบว่ามีความสัมพันธ์กับการลดลงอย่างมีนัยสำคัญของกลุ่ม dendritic cells ในคนไข้ที่ติดเชื้อพลาสโมเดียมไวแวกซ์

(ตีพิมพ์ในวารสาร European Journal of Immunology และเป็นงานวิจัยที่ถูกยกย่องว่าเป็น The Best Immunological Research ของวารสารในเครือ Wiley-Blackwell ดังเอกสารแนบหมายเลข 1 และ 2)

2. การเปรียบเทียบระดับ cell phenotypes ในคนไข้ที่ติดเชื้อมาลาเรียชนิดไวแวกซ์และชนิดฟัลซิพารั่ม *(ผลการทดลองโดยละเอียดอยู่ในเอกสารแนบท้ายฉบับที่ 3)*

การศึกษาหา cell phenotype ดังกล่าวเราได้ทำการเปรียบเทียบทั้งในกลุ่มคนไข้ที่ติดเชื้อมาลาเรียชนิดพลาสโมเดียมฟัลซิพารั่ม ซึ่งพบว่าเชื้อพลาสโมเดียมชนิดไวแวกซ์สามารถกระตุ้นระดับ cell phenotype ชนิดต่างๆ โดยเฉพาะอย่างยิ่งกลุ่มเซลล์ชนิด $\gamma\delta$ T cells ที่มีระดับเพิ่มสูงขึ้นอย่างชัดเจนในคนไข้มาลาเรีย PV เมื่อเปรียบเทียบกับคนไข้มาลาเรียชนิด PF

*(ได้รับการตอบรับให้ตีพิมพ์ในวารสาร **Clinical and Experimental Immunology** ดังเอกสารแนบหมายเลข 3)* นอกจากนี้ยังได้นำผลงานนี้ไปเสนอที่ 2nd European Congress of Immunology ณ กรุงเบอร์ลิน ประเทศเยอรมนี ซึ่งที่ประชุมได้มอบรางวัล Travel award ให้ นส. ศรีวิภา ช่วงไชยา (นศ. ทุนปริญญาเอกกาญจนาภิเษก โดย สกว.) เพื่อเดินทางไปเสนอผลงานดังกล่าวที่กำลังจะจัดขึ้นในวันที่ 13-16 กันยายน 2552 นี้ *(ดังเอกสารแนบหมายเลข 8)*

เรายังได้ทำการศึกษา ผลของการกระตุ้น lymphocytes เพื่อศึกษาหาผลของ lymphocyte stimulation ทั้งการศึกษาหา cell proliferation และ cell apoptosis หลังจากการกระตุ้นด้วยเชื้อมาลาเรียจากการทดสอบในหลอดทดลอง ผลการทดลองข้างต้นเราได้ manuscript ฉบับที่ 2 และรอการตีพิมพ์ในวารสาร Infection and Immunity *(**ผลการทดลองโดยละเอียดอยู่ในเอกสารแนบท้ายฉบับที่ 4 ซึ่งเป็น manuscript รอส่งตีพิมพ์ในวารสารวิชาการนานาชาติ**)* *นอกจากนี้*ผลงานดังกล่าวยังได้นำเสนอในที่ประชุม American Society of Tropical Medicine and Hygiene Meeting *(**ดังเอกสารแนบท้ายหมายเลข 9**)*

3. การศึกษาระดับ cell phenotypes และระดับแอนติบอดีในคนไข้ที่ติดเชื้อมาลาเรียชนิดไวแวกซ์ในกลุ่มคนไข้จากประเทศจีน *(ผลการทดลองโดยละเอียดอยู่ในเอกสารแนบท้ายฉบับที่ 5)*

เราได้ทำการเปรียบเทียบระดับ cell phenotypes ในกลุ่มคนไข้จีนตอนกลางที่ติดเชื้อมาลาเรียชนิด PV เนื่องจากประเทศจีนตอนกลางเป็นบริเวณที่มีการระบาดของโรคมาลาเรียชนิด PV เพียงชนิดเดียวเท่านั้น ไม่พบการติดเชื้อมาลาเรียชนิดอื่นๆ โดยการศึกษาเราพบว่า เมื่อคนไข้ได้รับเชื้อในระยะเฉียบพลันเซลล์บางชนิดมีระดับต่างกัน เช่น regulatory T cell มีระดับต่ำลงในกลุ่มคนไข้จีน ซึ่งแสดงให้เห็นว่าระดับการกดภูมิคุ้มกันโดยไวแวกซ์มาลาเรียมีระดับที่ต่ำกว่ากลุ่มคนไทย แต่ทั้งสองเชื้อชาติมีระดับ $\gamma\delta$ T cells สูงขึ้น ซึ่งจากผลการทดลองนี้ทำให้เราสามารถสรุปเบื้องต้นได้ว่า $\gamma\delta$ T cells เป็นเซลล์ที่ได้รับการกระตุ้นโดยเชื้อมาลาเรียชนิดพลาสโมเดียมไวแวกซ์ ทำให้เราสนใจศึกษากลไกการกระตุ้นและทำลายเชื้อมาลาเรียโดยเซลล์ชนิดนี้ต่อไป

*(จากผลการศึกษาเปรียบเทียบดังกล่าวทำให้เราได้ **manuscript ที่รอการแก้ไขเพื่อส่งตีพิมพ์ในวารสาร Malaria Journal ดังเอกสารแนบหมายเลข 6)***

นอกจากผลการศึกษาทางด้าน cell-mediated immunity ที่ต่างกันแล้ว เรายังพบความแตกต่างของระบบ Humoral immunity ซึ่งเราพบว่า ภูมิคุ้มกันทางด้าน antibody ที่จำเพาะต่อเชื้อ *P. vivax* ในคนจีนระดับที่สูงและมีความจำเพาะมากกว่าคนไทย และสิ่งที่น่าสนใจก็คือ ในคนไทยเราพบความเป็น cross-reactivity ของ antibody ที่มีต่อเชื้อ ในขณะที่เราจะไม่สามารถพบผลดังกล่าวได้ในคนจีน *(**manuscript รอการแก้ไขเพื่อส่งตีพิมพ์ในวารสารวิชาการนานาชาติ ดังเอกสารแนบท้ายหมายเลข 6)*** ผลการทดลอง

ดังกล่าวสามารถชี้ชัดได้ว่า คนไทยที่อาศัยอยู่ในบริเวณที่มีการระบาดของโรคมาลาเรียเคยมีประวัติการติดเชื้อ *P. falciparum* มาก่อนแทบจะทุกคน จึงสามารถตรวจพบ antibody ต่อเชื้อ *P. falciparum* ตรงกันข้ามกับคนจีนซึ่งไม่เคยได้รับเชื้อ *P. falciparum* มาก่อนจึงไม่สามารถตรวจพบระดับ antibody ต่อเชื้อชนิดนี้ได้ **(ผลการทดลองดังกล่าวได้ส่งไปนำเสนอในที่ประชุมระดับนานาชาติ 14th International Congress of Immunology ณ ประเทศญี่ปุ่น เดือนสิงหาคม 2553)**

4. การศึกษากลไกการกระตุ้นและทำลายเชื้อมาลาเรียชนิดพลาสโมเดียมไวแวกซ์โดยเซลล์ชนิด γδ T cells *(ผลการทดลองโดยละเอียดอยู่ในเอกสารแนบท้ายฉบับที่ 7)*

งานวิจัยที่ได้ทำควบคู่กันไปคือการศึกษาคุณสมบัติการกระตุ้นเซลล์ชนิด γδ T cells โดยได้นำเซลล์ของคนปกติมาทำการกระตุ้นให้เซลล์มีคุณสมบัติเป็น γδ T cells โดยใช้ Isopentenylpyrophosphate (IPP) ให้ได้จำนวนมากพอในการนำไปทำการทดลองเลี้ยงร่วมกับเชื้อพลาสโมเดียมไวแวกซ์ที่เก็บได้จากคนไข้ที่เข้ามารับการรักษา ณ มาลาเรียคลินิก อำเภอแม่สอด จังหวัดตาก โดยคนไข้ได้รับการตรวจวินิจฉัยโรคเบื้องต้นด้วยกล้องจุลทรรศน์และวิธีทาง PCR จากการศึกษาการกระตุ้น PBMC จากคนปกติจำนวน 7 ราย ให้มีคุณสมบัติเป็น γδ T cells ด้วยสาร IPP พบว่า เปอร์เซ็นต์ γδ T cells มีจำนวนสูงขึ้นมากกว่า 60% เมื่อเปรียบเทียบกับที่ไม่ได้กระตุ้น หลังจากนั้นจะนำ γδ T cells ที่ได้ไปทำการเลี้ยงร่วมกับเชื้อพลาสโมเดียมไวแวกซ์ เพื่อการศึกษากลไกการฆ่าเชื้อพลาสโมเดียมไวแวกซ์โดยเซลล์ชนิด gamma delta T cells โดยวิธีการวัดทาง Flow cytometry และการตรวจนับจำนวนเชื้อที่ตายไป เซลล์อีกส่วนหนึ่งจะถูกนำไปกระตุ้นด้วย recombinant proteins ที่ผลิตขึ้นเอง ซึ่งได้แก่ PvMSP1, PvAMA1 เพื่อศึกษากลไลการกระตุ้น gamma delta T cells โดยมาลาเรียแอนติเจนชนิดต่างๆ ผลการทดลองเบื้องต้นเราพบว่า จำนวนของ gamma delta T cells จะเริ่มแบ่งตัวในวันที่ 2 หลังจากที่กระตุ้นด้วยตัวเชื้อมาลเรีย โดยจะมี marker ที่บ่งบอกถึง early (CD69+) และ late (CD25+) activation ในวันที่ 1 และ 3 หลังจากการกระตุ้น ส่วนผลของการดูกลไกการฆ่าเชื้อมาลาเรียโดย gamma delta T cells พบการเพิ่มขึ้นของ $CD107a^+$ และการหลั่งสาร IFN-gamma ได้ทำการทดลองแล้วและกำลังอยู่ในระหว่างการวิเคราะห์ผลทางสถิติ

นอกจากนี้เรายังได้ทำการศึกษากลไกอย่างเดียวกันที่มีต่อเชื้อพลาสโมเดียมฟัลซิพารั่ม โดยเราพบว่า expression of CD107a+ gamma delta T cells มีการเพิ่มขึ้นอย่างรวดเร็วที่ day 0 และลดลงอย่างรวดเร็วในวันต่อมา ซึ่งแสดงให้เห็นชัดเจนว่า กลไกการฆ่าและทำลายเชื้อของ gamma delta T cells นั้นจะเป็นแบบ early response

ผลการทดลองข้างต้นได้ถูกส่งไปเสนอที่ 2nd European Congress of Immunology ณ กรุงเบอร์ลิน ประเทศเยอรมนี ซึ่งที่ประชุมได้มอบรางวัล Travel award ให้ ดร.กุลชาติ จังภัทรพงศา เพื่อเดินทางไปเสนอผลงานดังกล่าวที่กำลังจะจัดขึ้นในวันที่ 13-16 กันยายน 2552 นี้ด้วยเช่นกัน ***(ดังเอกสารแนบหมายเลข 10)*** นอกจากนี้ ดร.กุลชาติ จังภัทรพงศา ยังถูกรับเชิญให้ไปบรรยายผลงานวิจัยดังกล่าวในงานประชุมวิชาการของสำนักงานคณะกรรมการการอุดมศึกษาครั้งที่ ๒ ของ สกอ. ที่จัดขึ้นในวันที่ 27-29 สิงหาคม 2552 ***(ดังเอกสารแนบหมายเลข 11)***

เนื่องจากการศึกษาหาภูมิคุ้มกันเพื่อการพัฒนาวัคซีนต่อโรคมาลาเรียในอนาคต เรายังต้องการข้อมูลทางภูมิคุ้มกันวิทยาต่อโรคมาลาเรียชนิด *P. vivax* ที่จำเพาะ ซึ่งประเทศจีนเป็นแหล่งตัวอย่าง *P. vivax* ที่ดีและมีคุณค่าในการศึกษามาก เพราะทางสถาบันของจีนมักไม่อนุญาติให้คนภายนอกประเทศมาใช้ตัวอย่างผู้ป่วยได้ง่ายๆ ทำให้วิชาการด้านนี้ขาดข้อมูล แต่ทางโครงการของเรานั้นได้ร่วมมือกับนักวิจัยที่มหาวิทยาลัย Bengbu และที่ Wuxi Institute of Parasitic Diseases มานานแล้ว ซึ่งผลการทดลองที่ได้นี้ทำให้เราสนใจที่จะมีความร่วมมือกับกลุ่มที่ทำงานวิจัยทางด้านมาลาเรียในประเทศจีนต่อไป

ผลงานที่เกิดขึ้นจากโครงการวิจัย

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ และ manuscript ที่รอการตอบรับ (submitted) หรือรอส่งตีพิมพ์ (In preparation)

[1] **Jangpatarapongsa K**, **Chootong P**, Sirichaisinthop J, Sattabongkot J, Tangpradubkul S, Hisaeda H, Troye-Blomberg M, Cui L, **Udomsangpetch R**. *Plasmodium vivax* alter the balance of myeloid and plasmacytoid dendritic cells and induction of regulatory T cells. *Eur. J. Immunol* (2008) Oct;38(10):2697-705 (Impact factor=4.865)

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[2] **Jangpatarapongsa K,** Promwan S, Dabkam K, Hongeng S, Chotivanich K, Polpanich D, Sattabongkot J, Sirichaisinthop J, Cui L, **Udomsangpetch R.** Killing mechanism of *Plasmodium vivax* parasites by gd T cells. Commission of Higher Education Congress I. Sep 2008 at Chonburi. Thailand (Poster Presentation)

[3] **Jangpatarapongsa K,** Promwan S, Dabkam K, Hongeng S, Chotivanich K, Polpanich D, Sattabongkot J, Sirichaisinthop J, Cui L, **Udomsangpetch R.** Killing mechanism of

Plasmodium vivax parasites by gd T cells. Commission of Higher Education Congress I. Sep 2008 at Chonburi. Thailand (Oral Presentation)

3. ผลงานที่ถูกนำเสนอในที่ประชุมระดับนานาชาติ

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[3] **Chuangchaiya S, Jangpatarapongsa K,** Chootong P, Sirichaisinthop J, Sattabongkot J, Pattanapanyasat K, Chotivanich K, Troye-Blomberg M, Cui L, **Udomsangpetch R.** Plasmodium vivax controls Plasmodium falciparum survival by modulating host immune response. 2nd European Congress of Immunology Sep 2009 at Berlin, Germany

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[5] **Jangpatarapongsa K,** Loharungsikul S, Treeratanapaiboon L, Promwan S, Dabkam K, Hongeng S, Chotivanich K, Polpanich D, Sattabongkot J, Sirichaisinthop J, Cui L, **Udomsangpetch R.** Killing mechanism of *Plasmodium vivax* parasites by gd T cells.

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4. การสร้างนักวิจัยรุ่นใหม่

[1] **ดร. กุลชาติ จังภัทรพงศา** นักวิจัยระดับหลังปริญญาเอก

ปัจจุบันได้รับการบรรจุในตำแหน่ง อาจารย์ ภาควิชาจุลชีววิทยาคลินิก คณะเทคนิคการแพทย์ มหาวิทยาลัยมหิดล

[2] **ดร. พัชนี ชูทอง** นักศึกษาปริญญาเอกจากโครงการวิจัยนี้ จบการศึกษาปี พ.ศ. 2552

ปัจจุบันได้รับการบรรจุในตำแหน่ง อาจารย์ ภาควิชาจุลชีววิทยาคลินิก คณะเทคนิคการแพทย์ มหาวิทยาลัยมหิดล

[3] **น.ส. ศรีวิภา ช่วงไชยยะ** นักศึกษาปริญญาเอก โครงการปริญญาเอกกาญจนาภิเษก จากโครงการวิจัยนี้ กำลังจะจบการศึกษาภายในปี พ.ศ. 2553 นี้

ศาสตราจารย์ ดร. รัชนีย์ อุดมแสงเพ็ชร

หัวหน้าโครงการ

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Plasmodium vivax parasites alter the balance of myeloid and plasmacytoid dendritic cells and the induction of regulatory T cells

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Immunity induced by *Plasmodium vivax* infections leads to memory T-cell recruitment and activation during subsequent infections. Here, we investigated the role of regulatory T cells (Treg) in coordination with the host immune response during *P. vivax* infection. Our results showed a significant increase in the percentage of FOXP3$^+$ Treg, IL-10-secreting Type I Treg (Tr1) and IL-10 levels in patients with acute *P. vivax* infection as compared with those found in either naïve or immune controls. The concurrent increase in the Treg population could also be reproduced *in vitro* using peripheral blood mononuclear cells from naïve controls stimulated with crude antigens extracted from *P. vivax*-infected red blood cells. Acute *P. vivax* infections were associated with a significant decrease in the numbers of DC, indicating a general immunosuppression during *P. vivax* infections. However, unlike *P. falciparum* infections, we found that the ratio of myeloid DC (MDC) to plasmacytoid DC (PDC) was significantly lower in acute *P. vivax* patients than that of naïve and immune controls. Moreover, the reduction in PDC may be partly responsible for the poor antibody responses during *P. vivax* infections. Taken together, these results suggest that *P. vivax* parasites interact with DC, which alters the MDC/PDC ratio that potentially leads to Treg activation and IL-10 release.

Key words: Dendritic cell · IL-10 · Malaria · *Plasmodium vivax* · Regulatory T cell

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Introduction

Malaria is a common tropical disease causing deaths among *Plasmodium falciparum*-infected children mainly in Sub-Saharan Africa [1]. *P. falciparum* causes malignant tertian malaria that accounts for most malaria-associated deaths, whereas *P. vivax* causes relapsing fever and the infection rarely becomes fatal. Although a better understanding of immunity is needed for the design of effective vaccines, immune regulation in the host during malaria infection is not fully understood, and few studies have been conducted in patients with *P. vivax* infections. Our recent study has shown that anti-*P. vivax* antibody levels were very low in immune individuals living in endemic area and in patients with acute *P. vivax* malaria. For the cell-mediated arm, an acute *P. vivax* infection was associated with the activation of memory T cells belonging to either a cytotoxic or helper phenotype [2]. Additionally, previous evidence [3, 4] shows that immunization with pre-erythrocytic antigens can induce IFN-γ release. This suggests that *P. vivax* can activate the immune system via the Th1 pathway. However, a possible suppressing mechanism arises from the activation of regulatory T cells (Treg) as has been shown in a murine malaria study [5].

Treg constitutively express CD25, which is the IL-2/α chain receptor [6]. Co-presentation of CD25 with forkhead box protein P3 (FOXP3) dictates the immune-suppressive role of Treg *via* the release of IL-10 and TGF-β [7]. Treg have been shown to alter the balance between myeloid dendritic cells (MDC) and plasmacytoid dendritic cells (PDC) in blood, which eventually determines the outcome of T-cell responses [8]. The increase in MDC/PDC ratio is associated with the activation of the Th1 pathway, whereas a decreased ratio is associated with the activation of the Th2 pathway [8–10]. In a murine malaria model, the parasites have been shown to evade the immune response *via* activation of Treg [11]. A recent study confirms this trend during *P. falciparum* infections where Treg activation was correlated with higher rates of parasite growth [12]. However, the role of Treg during the course of *P. vivax* infection has not been investigated. Here, we compared Treg population and IL-10 levels among acute *P. vivax* patients, immune and naïve controls. Results revealed significant association between vivax infections with increased levels of Treg and IL-10. In contrast, *P. vivax* infections led to a general reduction in DC and lowered the MDC/PDC ratio, suggesting a possible immunosuppressive role of Treg during acute *P. vivax* infections.

Results

Activation of Treg during *P. vivax* infection

The percentage of $CD4^+CD25^+$ T cells was quantified and the results are shown in Fig. 1A. Immune individuals living in endemic areas had a similar level of Treg as observed in naïve controls. However, the level was significantly elevated during acute *P. vivax* infections (mean, quartile percentages = 13.8%, 11.3–16.2) when compared with that of naïve controls (7.5%, 6.3–8.8, $P = 0.001$) and immune controls (4.3, 4.7–9.9, $P = 0.001$).

The expression of $FOXP3^+$ on $CD4^+CD25^+$ T cells wa analyzed to differentiate the $FOXP3^+$ Treg [13]. The result showed that $FOXP3^+$ Treg in the acute *P. vivax* malaria patient (20.8%, 12.9–68.3) were approximately 7- and 3-fold higher tha that of immune controls (2.7%, 1.9–4.6, $P<0.001$) and naïv controls (6.3%, 2.4–10.2, $P<0.001$), respectively. Interestingly the level of $FOXP3^+$ Treg in the malaria immune controls wa significantly lower than that in naïve controls ($P = 0.4$) (Fig. 1B)

IL-10 and the activation of Treg

It is known that Treg produce the anti-inflammatory cytokin IL-10. Results indicated that IL-10 was 4-fold higher during acut *P. vivax* infections than those measured in immune control ($P<0.001$) and 2-fold higher than that of naïve control ($P = 0.005$) (Fig. 2A). The levels of IL-10 in plasma collecte from immune controls were significantly lower than that seen i naïve controls ($P = 0.008$). As expected, the level of IL-1 producing Treg ($CD4^+CD25^+IL\text{-}10^+$ or type 1 Treg or "Tr1 cells) of *P. vivax* infected patients was also significantly highe than that of immune controls ($P = 0.001$) and naïve control ($P = 0.004$) (Fig. 2B). Moreover, there was a significant associa tion in the numbers of $FOXP3^+$ Treg and Tr1 cells in acut *P. vivax* patients ($R^2 = 0.3$, $P = 0.006$) and immune control ($R^2 = 0.4$, $P = 0.01$). This association was not found in naïv controls (Fig. 2C).

Treg stimulation by *P. vivax* antigens

Infection with *P. vivax* resulted in an increase levels of Treg an plasma IL-10. To reproduce these phenomena *in vitro*, periphera blood mononuclear cells from naïve controls were stimulate with *P. vivax* antigens and the number of $FOXP3^+$ Treg wa determined on day 5 (Fig. 3A). The results indicated that th number of $FOXP3^+$ Treg was significantly increased when PBM was stimulated with 50 μg/mL of *P. vivax* antigen as compare with that of normal red blood cells (NRBC) ($P = 0.01$) and that o phytohemagglutinine ($P = 0.01$). Consistent with enzyme-linke immunosorbent assay (ELISA) results, the levels of Tr1 cell measured from flow cytometric (FCM) analysis was found t increase after stimulation with 50 μg/mL of *P. vivax* antigen a compared with that of NRBC ($P = 0.048$) (Fig. 3B). Moreover, i reverse transcriptase-polymerase chain reaction (PCR) we als detected the expression of IL-10 in naïve PBMC after 5 days o stimulation with *P. vivax* antigens (data not shown).

Blood DC during acute *P. vivax* infection

Circulating DC defined as $HLA\text{-}DR^+$MDC and $HLA\text{-}DR^+$PD were determined in acute *P. vivax* patients and naïve control

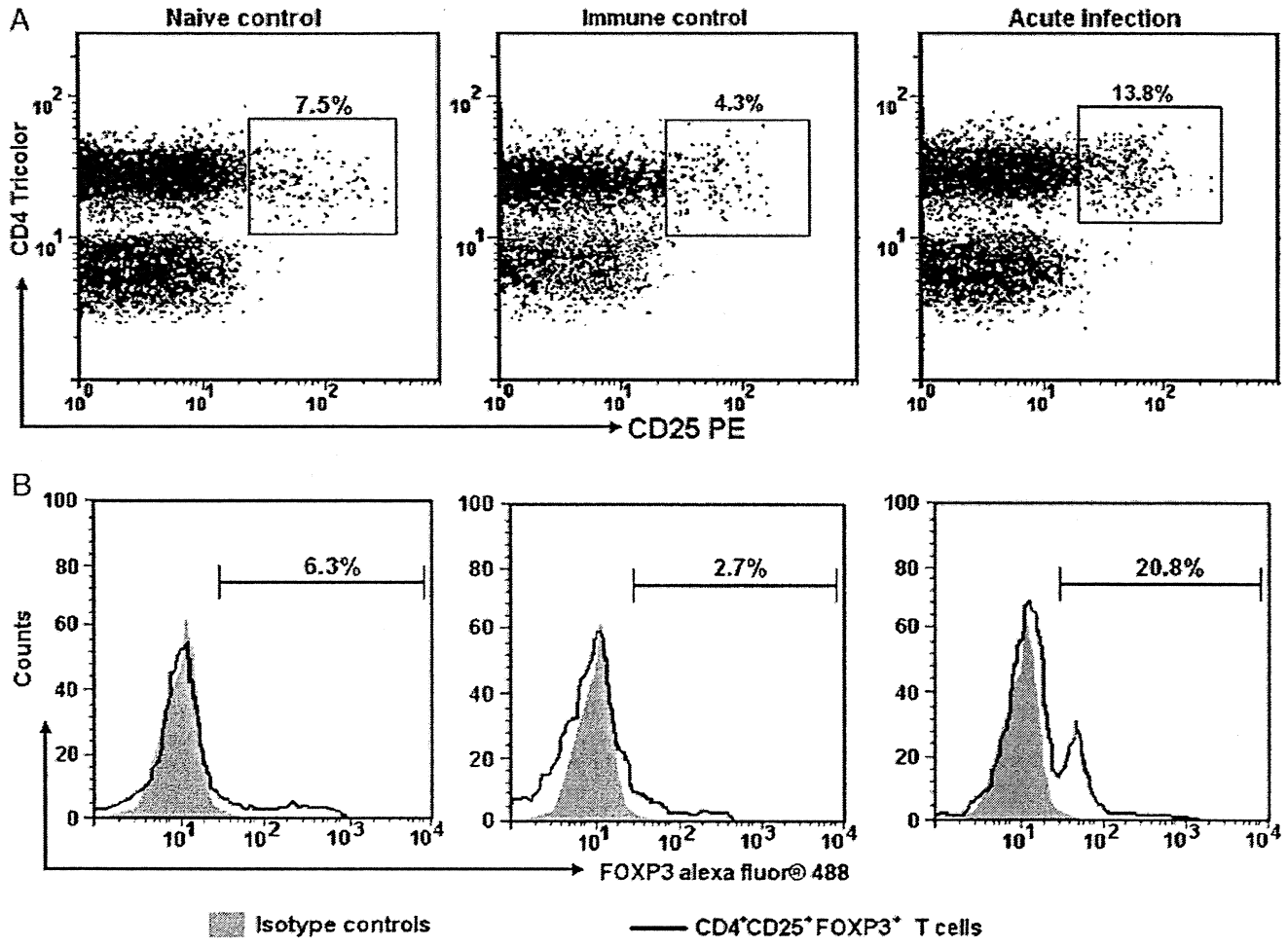

Figure 1. Increased numbers of FOXP3$^+$ Treg in patients with acute *P. vivax* infections. FCM analyses of cryopreserved PBMC stained with anti-CD4 Tricolor, anti-CD25 PE, and anti-FOXP3 Alexa Fluor® 488 from the indicated groups and lymphocyte populations were gated. (A) CD4$^+$CD25$^+$ T cells in the lymphocyte population are indicated by the boxed areas. (B) Histograms of FOXP3 expression in the CD4$^+$CD25$^+$ T cells. The percentages of CD4$^+$CD25$^+$ (A) and CD4$^+$CD25$^+$FOXP3$^+$ (B) cells are shown. All figures show one representative sample out of seventeen.

(Fig. 4A–C). The results showed that the absolute numbers of both MDC and PDC were significantly reduced in *P. vivax* patients than those of naïve controls ($P = 0.09$ and 0.03, respectively) (Fig. 4D). Consequently, the MDC/PDC ratio was significantly different between naïve controls and acute *P. vivax* patients ($P = 0.01$) (Fig. 4E).

During infection, *P. vivax* parasites may activate Treg via *P. vivax*-primed MDC or PDC of the host. Therefore, the association between MDC or PDC with Treg was analyzed. However, there was no association between PDC or MDC with FOXP3$^+$ Treg or Tr1 cells (data not shown).

Discussion

Infections by pathogens can lead to either the Th1 or Th2 response. Th1 response often leads to the resolution of malaria infection and therefore is favored in vaccine design [14]. IFN-γ produced by CD8 T cells and/or NK cells inhibits parasite development, thereby contributing to the protection against pre-erythrocytic stages of both *P. falciparum* and *P. vivax* [15]. However, there are studies showing the induction of IL-10 in malaria patients, suggesting that Th2 pathway may also be involved in malaria immunity [16]. Quite different from the findings in *P. falciparum*, we found that *P. vivax* infections rarely resulted in significant production of parasite-specific antibodies, but that acute *P. vivax* infections induced Treg activation. This result is consistent with two recent findings in both *P. yoelii* and *P. falciparum*, suggesting that parasites may use a similar mechanism of immune evasion [11, 12]. Although the interactions among malaria-specific T cells, B cells and Treg remain poorly characterized, our result suggested that the increased frequency of Treg during acute *P. vivax* infection may lead to suppression of both cell- and antibody-mediated immunities, which may benefit parasite survival in hosts. There are evidence indicating that Treg can suppress T-cell responses through the production of IL-10 and TGF-β [17, 18], whereas they may suppress B-cell maturation and differentiation directly [19, 20] or indirectly through the down-regulation of IL-2 or IL-4 production in responder lymphocytes. This may account for low parasite-specific antibody levels seen in *P. vivax* patients [2].

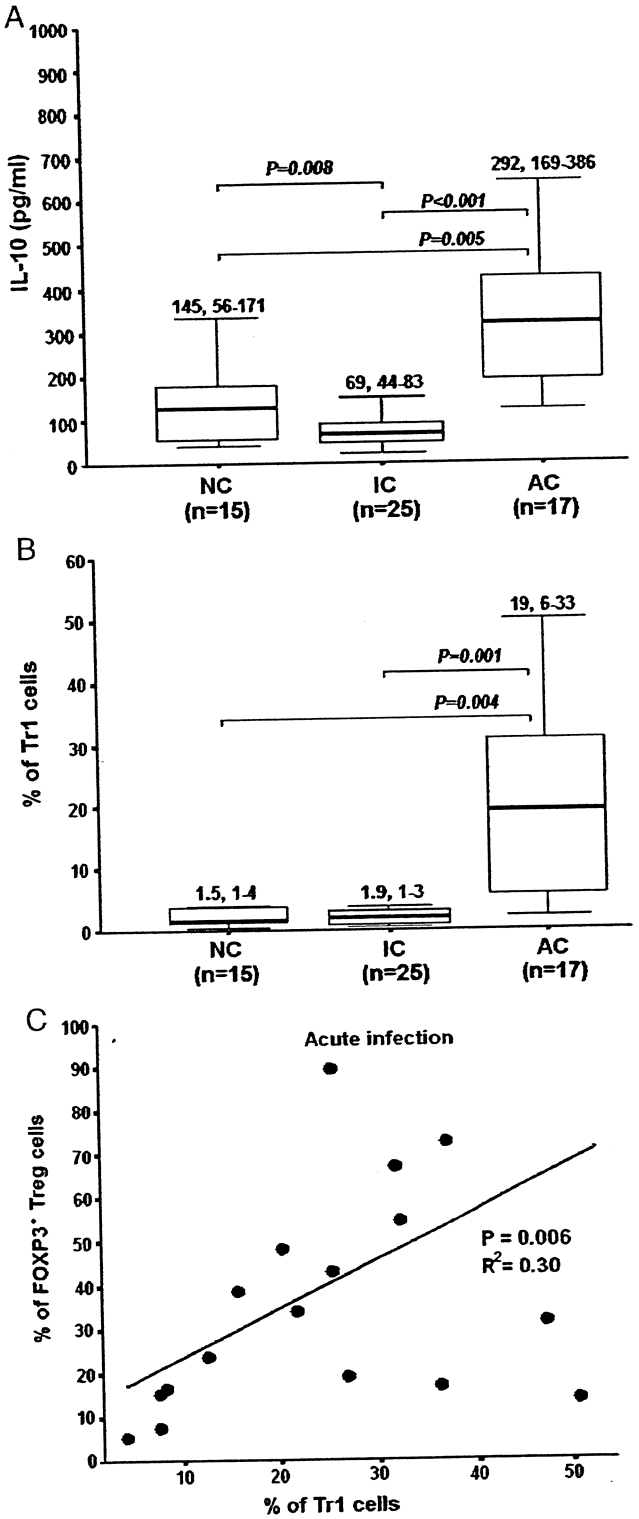

Figure 2. Comparison of the levels of plasma IL-10 (A) and Tr1 cells (B) between naïve controls (NC), immune controls (IC), and patients acutely infected with *P. vivax* (AC). The correlation between Tr1 cells and FOXP3$^+$ Treg in lymphocytes from patients acutely infected with *P. vivax* (C). Data are shown as mean (thick horizontal line), inter-quartile range (box plot), maximum and minimum (upper–lower lines) (A and B). The numbers represent mean, inter-quartile range. (Data were log transformed and independent sample t test was used to calculate P value).

In this study, patients with acute *P. vivax* infection wer observed to exhibit higher plasma levels of IL-10 than those o naïve and immune controls. Similar findings have been reporte in both *P. vivax* and *P. falciparum* infections [21–24]. Eve though there is evidence that CD8 Treg produce IL-10 to suppres T-cell responses [25], our result showed a significant correlatio between elevated levels of Tr1 and FOXP3$^+$ Treg, a result furthe corroborating our earlier finding of increased levels of tota CD4$^+$ T cells during acute *P. vivax* infections [2]. In parallel, w also found a concurrent increased levels of Treg and expression o IL-10 when PBMC from naive controls were stimulated wit *P. vivax* antigens *in vitro*. This result is also consistent wit findings that the number of Treg is increased in cord bloo mononuclear cells of placental *P. falciparum* infections and IL-1 elevation after stimulation with *P. falciparum* antigens [26] Altogether, these results suggest that Treg may be a direct sourc of IL-10 production and/or enhance the IL-10 production by D [27]. Recent report showing that the stimulated FOXP3$^+$ Tre produce many cytokines including IL-10 resulting in activation o monocytes/macrophages can support the former hypothesis [28] Our finding showed significant association between differen Treg populations during acute *P. vivax* infection, despite the fac that some acutely infected cases (23%) appeared to have elevate proportion of Tr1 cells with comparatively low levels of FOXP3 Treg. Previous studies have demonstrated that Tr1 cells gene ated *in vitro* do not express FOXP3$^+$, but it was found to maintai the suppressor function [29, 30]. This suggests that FOXP expression is not a prerequisite for the suppressive function of Tr cells. On the other hand, Tr1 cells specific for desmoglein3, th dominant autoantigen in pemphigus vulgaris, are shown t constitutively express FOXP3 [31]. Therefore, the association c Tr1 cells with FOXP3$^+$ Treg remains controversial and need further investigation [32].

Among the three groups of volunteers, the lowest levels c IL-10 and Tr1 cells were observed in the immune controls. Thi suggests that the activation of Treg is transient during *P. viva* infections and that subsequent decline in Treg after parasit clearance could be due to higher levels of memory T cells tha were maintained after the infection [2]. Our study did not find correlation of CD4$^+$CD25$^+$ and FOXP3 levels. This result is in lin with a recent study showing no correlation between CD25$^+$ cell and FOXP3 level in human CD4$^+$ cells [33].

The immunoregulatory effect of malaria infections is relate with the down-regulation of antigen presenting DC. Consisten with earlier studies documenting the immunosuppressive effec of malaria parasites on the maturation and differentiation of D [34, 35], we found significantly lower levels of DC (both PDC an MDC) in acute *P. vivax* patients. This finding coincides with th recent study showing the decreasing numbers of MDC and PD among children with severe *P. falciparum* infection [36]. Anothe reason could be the reallocation of cells away from the peripher blood, *e.g.* to the spleen. The evidence of lymphopenia in bot *P. falciparum* and *P. vivax* infection supports such an explanatio [37–39]. The levels of MDC and PDC in the peripheral blood ar recovered during post-treatment [36].

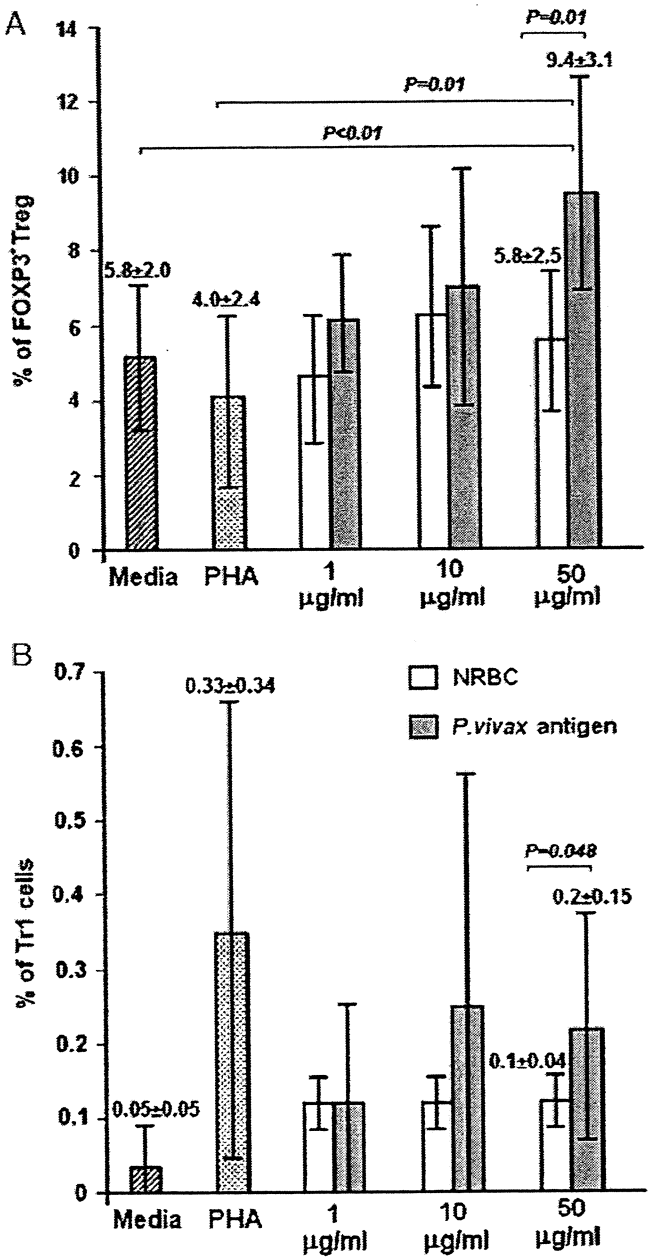

Figure 3. Increased numbers of FOXP3$^+$ Treg (A) and Tr1 cells (B) in lymphocytes after stimulation with *P. vivax* antigens. PBMC from naïve controls cultured with NRBC or PV schizont lysate at the indicated concentrations were analyzed for FCM analysis. Data represent mean percentage ± SD of five experiments. Data were log transformed and independent sample t test was used to calculate P value.

It has been shown that the depletion of PDC abrogated the secretion of specific polyclonal IgG in response to influenza virus [40]. Therefore, the reduction in PDC level during *P. vivax* infection may account for the low level of *P. vivax*-specific antibodies in vivax patients [2]. This suggests that PDC are critical for the generation of plasma cells and antibody responses during parasite/pathogen infections. However, unlike that of *P. falciparum*-infected patients [36], the percentage of PDC was higher than that of MDC during acute *P. vivax* infections, resulting in a lower MDC/PDC ratio. This may suggest that *P. vivax* parasites suppress host immune response through elevated levels of PDC and Treg. Two pieces of evidence show that the stimulation of PDC induces naïve CD4 and CD8 T-cell differentiation into Th2 cells and IL-10 producing CD8 T-cells [8, 41].

Taken together, we found that acute *P. vivax* infections were associated with higher levels of Treg and IL-10, but lower levels of DC in the blood. This suggests that Treg are activated by parasites during acute infections, which then play an immunosuppressive role of host cell- and antibody-mediated immunities, resulting in retarded parasite clearance. While the overall levels of DC were reduced, the balance between the two cell types (MDC and PDC) was altered. In addition, our previous study also revealed an increase in the levels of γδ T cells during acute *P. vivax* infection. Therefore, the interactions among different DC, T cell phenotypes and *P. vivax* antigens await further investigations.

Materials and methods

Study population

Blood samples were collected from 17 patients with acute *P. vivax* infections at two malaria clinics in Mae Sot and Mae Kasa, Tak province, Thailand. Diagnosis of *P. vivax* malaria infection was based on the examination of Giemsa-stained thick blood films. PCR with four human malaria (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) species-specific primers was performed on DNA isolated from blood samples to verify malaria infections. Only single *P. vivax* infection was recruited in the experiments. Blood samples were collected from 25 additional people residing in the same *P. vivax*-endemic area. The subjects defined as "immune controls" have had recent malaria infections and had anti-*P. vivax* antibodies determined by ELISA. These immune controls did not have *P. vivax* infections at the time of blood collection as determined by both microscopic and PCR analyses. Fifteen healthy adults living in Bangkok without previous malaria exposure or antibodies to malaria parasites were recruited to serve as "naïve controls". The clinical characteristics of the subjects are listed in Table 1. This study was approved by the Committee on Human Rights Related to Human Experimentation, Mahidol University, and the Ministry of Health, Thailand. Informed consent was obtained from each individual before the blood sample was taken.

Preparation and cryopreservation of PBMC

Venous blood from *P. vivax* patients, immune controls, and naïve controls was collected in heparinized tubes and PBMC were separated by gradient centrifugation using Lymphoprep™ (AXIS-Shied PoC AS, Oslo, Norway) according to the manufacturer's

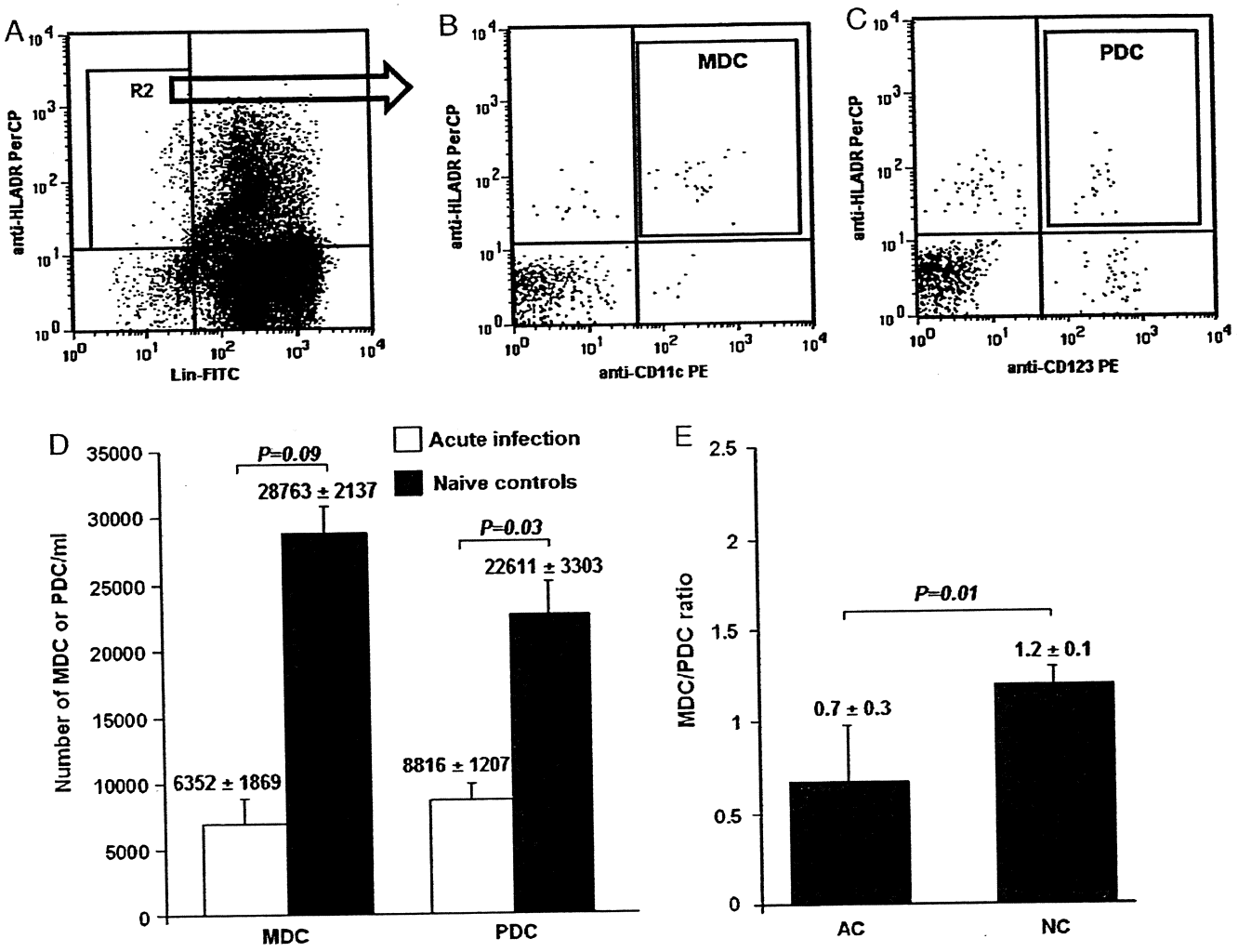

Figure 4. (A–C) FCM analyses of whole blood stained with anti-HLA-DR PerCP and Lin FITC, anti-CD11c RPE (for MDC), or anti-CD123 RPE (for PDC R2 shows the population of HLA-DR$^+$Lin$^-$. (D) Number of MDC and PDC. (E) MDC/PDC ratio in the peripheral blood of patients acutely infected with *P. vivax* (AC) and naïve controls (NC). Numbers represent mean ± SD. (number of samples: acute infections = 17 and naïve controls = 15) Sample test was used to calculate *P* value.

Table 1. Information and clinical data of *P. vivax* patients, immune and naïve controls[a)]

	No. of cases	Age (years)	Sex		Hematocrit (%)	Temperature (°C)	Parasitemia (%)
			M	F			
AC	17	38±11 (15–52)	7	10	43±7 (35–63)	37.5±1.3 (35–40)	0.4±0.3 (0.03–1.2
IC	25	38±13 (18–77)	16	9	46±3 (43–48)	36.8±0.4 (36–37)	0
NC	15	29±7 (22–43)	8	7	42±4 (37–45)	37.0±0.5	0

[a)] Mean ± standard deviation (range). AC, acute *P. vivax* infection; IC, immune controls; NC, naïve controls.

recommendations. The PBMC pellet was resuspended at a concentration of 10^6 cells/mL in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, USA). The viability of PBMC, normally above 98%, was determined by tryphan blue exclusion. PBMC in 10% dimethyl sulfoxide were cryopreserved in liquid nitrogen until analysis.

Parasite cultures and antigen preparations

Crude *P. vivax* antigens were obtained from *P. vivax*-infecte red blood cells. Briefly, *P. vivax*-infected blood was depleted c white blood cells by filtering through a sterile column of CF1 cellulose (Whatman, Maidstone, UK) and the RBC were washe

with RPMI-1640 by centrifugation at 1190*g* for 5 min. The parasites were cultured in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 24–30 h at 5% hematocrit in McCoy's 5A medium (Invitrogen) supplemented with 25% human AB serum until they matured to the schizont stage (≥six nuclei). These mature parasites were enriched by centrifugation on 60% Percoll (Pharmacia, Uppsala, Sweden) at 1190*g* for 10 min. The enriched infected red blood cell pellets were sonicated for 40 s at 150 watts and the protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, USA). The vials were then aliquoted and stored at −70°C until use. Uninfected RBC were processed similarly and stored at −70°C to be used as a negative control.

In vitro stimulation of PBMC

PBMC from five healthy donors were used for *in vitro* stimulation. PBMC (2×10^5 cells/well) in RPMI-1640 supplemented with 25 mM HEPES, 1.8 mg/mL D-glucose, 2 mM glutamine, 40 mg/mL of gentamicin, and 10% heat-inactivated fetal bovine serum were cultured for 5 days at 37°C in a humidified chamber with 5% CO_2, 5% O_2, and 90% N_2 in the presence of *P. vivax* antigens at a concentration of 1, 10, or 50 μg/mL. Medium alone, equivalent concentration of RBC extracts or 2 μg/mL of phytohemagglutinine were used as negative and positive controls, respectively. All the experiments were performed in duplicates. After 5 days of activation, the cells were harvested and stained for Treg marker, CD25 and FOXP3 or IL-10.

Intracellular staining and FCM analysis

For the three-color FCM analysis, PBMC (10^5 cells/vial) were stained with a combination of fluorochrome-conjugated monoclonal antibodies (mAbs): RPE-Cy5-labeled anti-CD4 (Caltag, Burlingame, USA) and RPE-labeled anti-CD25 (Immunotech, Marseille, France) for 30 min at 4°C and washed with phosphate-buffered saline (PBS). After staining, the cell pellets were fixed with a fixative solution and washed with a permeabilizing solution according to the manufacturer's recommendations (BioLegend, San Diego, USA). After incubation in the permeabilizing buffer for 20 min at room temperature, the cells were incubated with Alexa fluor® 488-labeled anti-FOXP3 (BioLegend) or FITC-labeled anti-IL-10 for 30 min and then washed with PBS. Cells were fixed with the fixative solution for data acquisition and analysis on FACSCalibur using the CELLQUEST software (Becton Dickinson, San Jose, USA).

Phenotyping of circulating blood DC

Two hundred microliters of whole blood from *P. vivax*-infected patients were stained with an antibody mixture containing lineage-specific mAbs to CD3, CD14, CD19, CD20, CD56, and CD66b conjugated with FITC (lin-FITC) (Caltag), antibodies to CD11c (Serotec, Oxford, UK) and CD123 conjugated with RPE (BioLegend), and antibodies to HLA-DR conjugated with PerCP (Becton Dickinson). Stained cells were treated with RBC lysis solution. At least 2×10^5 cells were analyzed in a FACSCalibur flow cytometer (Becton Dickinson). HLA-DR$^+$CD123$^+$lin$^-$ cells were defined as PDC and HLA-DR$^+$CD11c$^+$lin$^-$ as MDC. The absolute number of circulating MDC and PDC was obtained by multiplying the percentage of MDC or PDC by the number of leukocytes per milliliter of blood.

Determination of IL-10 by ELISA

Polystyrene immunoplates (Corning, Corning, USA) were coated with 25 μL of 1 g/mL of anti-human IL-10 mAb (Mabtech, Nacka, Sweden) diluted in PBS (pH 7.4) and incubated overnight at 4°C. Each well was blocked with 50 μL of 0.1% bovine serum albumin for 90 min at room temperature. After five washings with PBS, 25 μL of plasma (1:2 dilution) or PBS control were added into duplicate wells and incubated overnight at 4°C. The plates were then washed for five times with PBS, after which 25 μL of anti-human IL-10 conjugated with biotin (dilution 1:2000) were added and incubated for 90 min at 37°C, followed by the addition of 25 μL of streptavidin-alp-PQ (dilution 1:2000) (Mabtech) at 37°C for 90 min. After five final washings, 25 μL of *p*-nitro phenylphosphate (Sigma, Saint Louis, USA) were added and incubated for 30–60 min in the dark at room temperature. Enzyme activity was measured by an automated microplate reader, V-Max (Molecular Device, Sunnyvale, USA) at 405 nm.

Determination of IL-10 gene expression by reverse transcriptase-PCR

RNA was extracted from six samples after *in vitro* stimulation using Trizol® solution (Invitrogen) according to the manufacturer's recommendation. Total RNA was reverse transcribed to cDNA; primers of the IL-10 gene and amplification conditions were as described previously [42]. The expression of *IL-10* gene (138 bp) was determined in 1.5% agarose gels containing ethidium bromide. Primers of β-*actin* were designed as described previously [43] and used as a control (790 bp).

Data analysis

All data were analyzed using the SPSS program (Version 10.0, Chicago, USA). The percentages of CD4$^+$CD25$^+$, FOXP3$^+$ Treg, Tr1 phenotypes, plasma IL-10 levels, and number of MDC and PDC both from volunteers and *in vitro* stimulation were log transformed to produce normal distributions. Parametric analysis was performed using transformed data as follows: the mean percentage differences for Treg phenotypes, IL-10 levels, and number of MDC and PDC between the groups (*i.e.* naïve controls

vs. immune controls, naïve controls *vs.* acute infection, and immune controls *vs.* acute infection) were analyzed using the independent samples *t* test. The Spearman approach was used to evaluate the correlation of Tr1 with FOXP3$^+$ Treg and the correlation of MDC or PDC levels with Treg. The results were considered statistically significant ($P<0.05$) at the 95% confidence interval.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **FCM:** flow cytometric · **FOXP3:** forkhead box protein P3 · **MDC:** myeloid DC · **NRBC:** normal red blood cell · **PDC:** plasmacytoid DC · **Tr1:** IL-10-secreting Type I Treg

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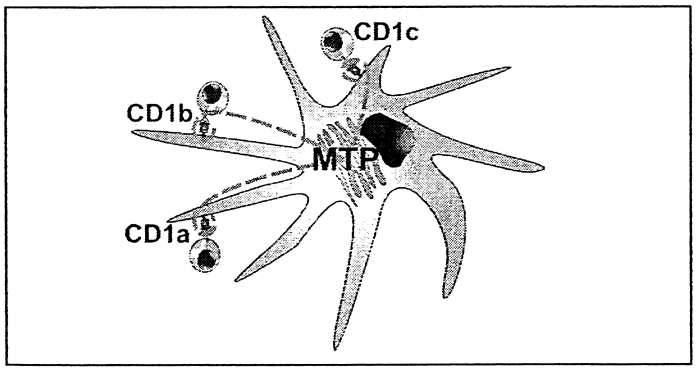
ImmunoDigest

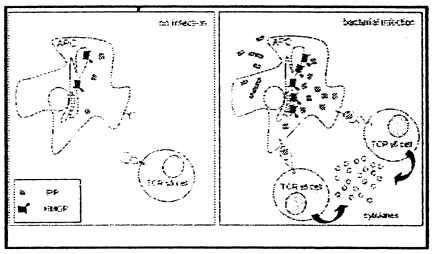
MTP regulates lipid antigen presentation beyond CD1d molecules

While the majority of CD1 family members present lipid antigen to T cells with diverse TCR-α and -β chains, CD1d lipid antigen presentation is restricted to the natural killer T (NKT) cells. It is well established that lipid antigen presentation by CD1d is regulated by the microsomal triglyceride transfer protein (MTP). What is not known, however, is whether MTP is also involved in regulating lipid antigen presentation by other CD1 molecules, namely CD1a, CD1b and CD1c. Kaser et al. reported that MTP regulates both endogenous and – surprisingly – exogenous (microbial) lipid antigen presentation by CD1a, CD1b, and CD1c. As MTP is resident in the endoplasmic reticulum, the authors conclude that MTP can ultimately function distally, regulating the presentation of exogenous, endosomally-loaded, microbial lipid antigens.



Kaser, A. et al., Eur. J. Immunol. 2008. 38: 2351–2359
http://www3.interscience.wiley.com/journal/120775914/abstract
DOI: 10.1002/eji.200738102

What activates TCR Vc9-Vd2 cells during bacterial infection?



Human TCR Vγ9-Vδ2 cells recognize non-peptidic ligands in the absence of MHC and CD1 restriction. Kistowska et al. investigated the mechanisms of TCR Vγ9-Vδ2 cell activation during bacterial infections. By infecting DC and monocytes with different bacteria, the authors show that TCR Vγ9- Vδ2 cells are activated by mevalonate metabolites of host-origin. Bacterial infection induces rapid activation of the mevalonate pathway by modifying a key enzyme and promoting the synthesis of a stimulatory endogenous metabolite. Thus TCR Vγ9-Vδ2 cells, by sensing the host mevalonate pathway altered during infection, immediately provide effector functions, when bacteria-specific T cells are not yet available.

Kistowska, M. et al., Eur. J. Immunol. 2008. 38: 2200–2209
http://www3.interscience.wiley.com/journal/120775995/abstract
DOI: 10.1002/eji.200838366

Wiley Authors Once Again Receive Nobel Prize in Chemistry

Hoboken, N.J., October 8, 2008—John Wiley & Sons, Inc. (NYSE: JWa & JWb) is pleased to announce that all three 2008 Chemistry Nobel laureates are part of our publishing community. We congratulate Dr. Osamu Shimomura, Dr. Martin Chalfie, and Dr. Roger Y. Tsien on their award for having discovered green fluorescent protein and related marine photoproteins, and for having developed them into highly useful tools for chemical, biological, and medical analysis. The original discovery of green fluorescent protein was reported by Osamu Shimomura in 1962 in Wiley's Journal of Cellular and Comparative Physiology.

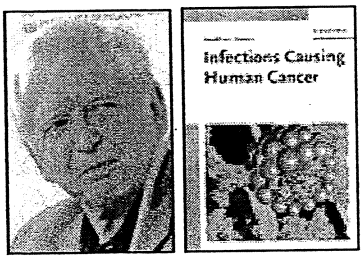
Wiley Authors Win Nobel Prize in Physiology or Medicine for 2008

Hoboken, N.J., October 7, 2008 — John Wiley & Sons, Inc, is pleased to announce that the Nobel Assembly at Karolinska Institutet has awarded the Nobel Prize in Physiology or Medicine for 2008 to Prof. Harald zur Hausen and jointly to Prof. Françoise Barré-Sinoussi and Prof. Luc Montagnier.

"We are honored that all three Nobel laureates are part of our publishing community. We congratulate Prof. Harald zur Hausen, Prof. Françoise Barré-Sinoussi and Prof. Luc Montagnier for this recognition of their lifetime achievements which have changed the course of science and medicine, as well as the lives of human beings," said Mike Davis, Vice President and Managing Director, Life Sciences.

Prof. Harald zur Hausen, of Germany, receives the Nobel Prize in Physiology or Medicine for his studies of human papilloma viruses causing cervical cancer. He is Editor-in-Chief of the International Journal of Cancer, has contributed several articles to Wiley–Blackwell journals, and is the book author of Infections Causing Human Cancer, published by Wiley-VCH in 2006.

Prof. Françoise Barré-Sinoussi and Prof. Luc Montagnier, of France, were awarded the Nobel Prize in Physiology or Medicine for discovering the human immunodeficiency virus. Both contributed various articles to Wiley–Blackwell journals and were involved in numerous book projects.



Stem Cells: How Do They Behave When Cultured Under Physiological Conditions?

Embryonic stem (ES) cells hold promise for providing unlimited quantities of specialized cells to treat a wide range of diseases. Little is known about their proliferation and differentiation at low partial pressures of oxygen (pO_2), even though the early embryo is normally exposed to such conditions. Powers and co-workers investigate the effects of oxygen on undifferentiated mouse ES cell growth, phenotype retention, and cellular energetics. Growth rate is maximal at intermediate pO_2 and declines modestly at the extremes over the range of 285–0 mmHg. When cultured at low pO_2 under conditions that normally maintain the stem cell state, expression of self-renewal genes decreases, but pluripotency is maintained. Following a decrease to low pO2, aerobic metabolism decreases and anaerobic metabolism increases so that the total ATP generation rate remains constant. This work helps us understand the behavior of ES cells at physiological oxygen levels.

Powers, D. E. et al.,Biotechnol and Bioeng. 2008. 101: 241-254.
http://www3.interscience.wiley.com/journal/119815678/abstract
DOI.1002/bit.21986

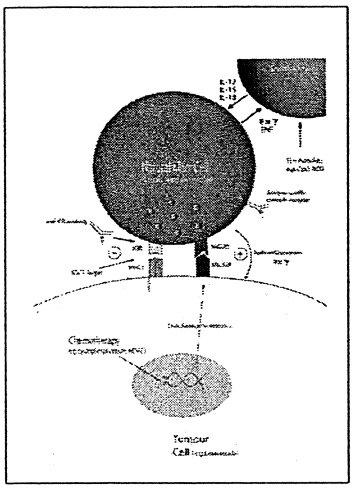
Is it Possible to Completely Camouflage the Surface of Red Blood Cells?

The ever increasing shortage of donated human blood has prompted the development of hemoglobin-based oxygen carriers (HBOCs) for use in transfusion medicine. HBOCs in development range from polymerized hemoglobins to particle encapsulated hemoglobins. However, by taking hemoglobin outside of its native environment—the red blood cell (RBC)—HBOCs exhibit increased NO scavenging compared to RBCs. Previous work addressed this problem by PEGylating bovine RBCs (bRBCs) in order to camouflage potential antigens while maintaining hemoglobin its native environment (Gundersen and Palmer, 2007. Biotech Bioeng 96:1199–1210). PEGylated bRBCS should be physical capable of oxygen transport w the human vasculature but ca we really fool the immune sys by camouflaging xenogenic ce with methoxypolyethylene gly In this B&B issue, Gundersen Kennedy and Palmer demons that we unfortunately cannot nature yet. The primary xenoa tigen, Gala(1,3)Gal, still rema exposed on the surface of the and is still immunoreactive at tested levels of PEGylation. Th authors recommend a compl fresh approach using siRNA t knockout Gala(1,3)Gal synthe creating a immunologically si natural, hemoglobin carrier.
Gundersen, S. I. et al., Biotec and Bioeng. 2008. 101: 337-3
http://www3.interscience.wiley.cor journal/117946382/abstract
DOI: 10.1002/bit.21908

5-aminolaevulinic acid a photodynamic therapy reduce HSV-1 replicatio in HaCat cells through a apoptosis-independent mechanism

In this study, the photo-inact on of herpes simplex virus ty (HSV-1) in human keratinocy using 5-aminolaevulinic acid (5-ala) was investigated. We demonstrated that ALA-PDT ment acts on HSV-1 replicati only in post-absorption cond reducing HSV-1 replication b about 70%. We did not detec evidence of apoptosis in the reduction observed after ALA treatment in keratinocytes. S data suggest that the target c photo-inactivation appears t viral replication and not a ce response.
http://www3.interscience.wiley.co journal/121667791/abstract
Ayala et al. Photoderm. Photoimm Photomed. 2008 24: 237-243.

Viewpoints on NK cells



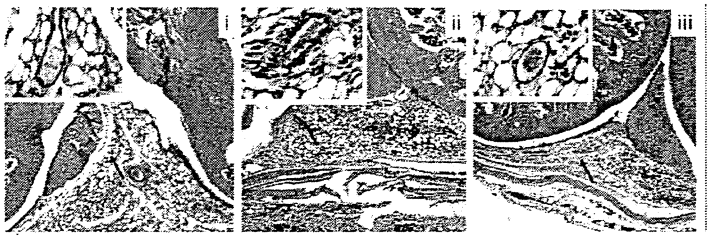
Following on from EJI's successful first Viewpoints on Treg cells (http://www3.interscience.wiley.com/journal/117954013/issue), the latest discoveries and advances in the field of NK cells are presented in the November issue of EJI. Experts such as *James Di Santo*, *Hans-Gustaf Ljunggren* and *Mark Smyth* discuss aspects ranging from NK cell education and adaptive immunity crosstalk through to immunoevasion and potential therapeutic targets. Find out more about the latest Viewpoint series at www.eji-journal.eu
In addition, published articles on NK cells in recent years in EJI include:

Allelic expression patterns of KIR3DS1 and 3DL1 using the Z27 and DX9 antibodies
John Trowsdale and colleagues
http://www3.interscience.wiley.com/journal/114122280/abstract
DOI 10.1002/ 10.1002/eji.200636773

Germ-line and rearranged Tcrd transcription distinguish bona fide NK cells and NK-like T cells
Immo Prinz and colleagues
http://www3.interscience.wiley.com/journal/114231942/abstract
DOI 10.1002/ 10.1002/ eji.200737354

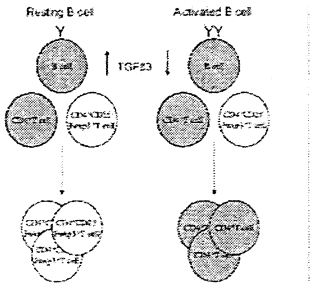
Blocking Chemokine Receptors May Help Arthritis

Coelho et al. have shown, in murine models of monarticular antigen-induced arthritis, that blockage of the chemokine receptors known as CXCR1/CXCR2 prevents tissue inflammation, local cytokine production, joint damage, and inflammatory pain. The mechanism by which the drugs (reparixin or DF2162, allosteric inhibitors of CXCR1/CXCR2) act in the system is by preventing the ability of neutrophils to adhere to the vascular endothelium in the joint, thus preventing their extravasation and consequent joint damage. In addition to preventing neutrophil influx and consequent joint damage, the compounds prevented the local production of tumor necrosis factor (TNF), suggesting that neutrophils and their CXCR1/CXCR2 receptors contribute to the local production of TNF . The authors believe their research suggests that compounds, such as antagonists of CXCR1/CXCR2 which prevent the interaction between endothelial cells and neutrophils, may be useful for the treatment of human arthritis. As well as preventing tissue inflammation and joint damage, these compounds appear to have the added benefit of ameliorating the pain associated with arthritis, as measured by diminished hypernociception.
Coelho, F. M. et al, Arthritis Rheum. 2008. 58: 2329-2337.
http://www3.interscience.wiley.com/journal/121358012/abstract
DOI 10.1002/art.23622



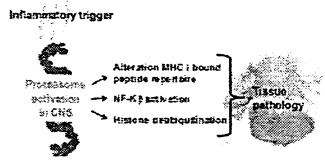
Balancing tolerance and immunity by B cells

B cells are not only precursors for antibodyproducing plasma cells but are also APC. Shah and Qiao demonstrate another role for B cells in the maintenance of a population of Treg. Resting B cells are shown to support the survival and expansion of CD41CD251Foxp31 Treg via the production of TGF-b3. Production of TGFb3 by B cells is in turn down-regulated by TLR signaling or via BCR cross-linking. Reduced TGF-b3 secretion by activated B cells results in decreased survival and expansion of Treg but increased expansion of effector CD41 T cells. Thus, in addition to humoral immune responses, B cells play an important role in balancing T-cell tolerance and immunity, and in preventing autoimmune diseases.



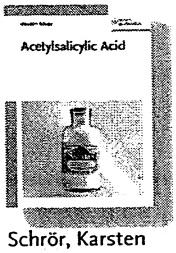
Shah, S. et al., Eur. J. Immunol. 2008. 38: 2488–2498
http://www3.interscience.wiley.com/journal/121408230/abstract
DOI: 10.1002/eji.200838201

Proteasomal activation in the CNS: A link between inflammation and neurodegeneration

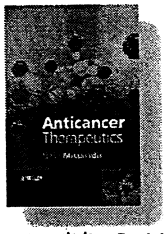


Multiple sclerosis (MS) is an inflammatory and autoimmune disease of the CNS leading to axonal and neuronal loss. Using the standard experimental model of MS, namely EAE, Fissolo et al. demonstrated increased and altered proteasomal activity in macro- and microglia in the CNS, as well as in lymphoid tissues, in comparison with activity in non-EAE controls. Inhibition of the proteasome with or without inhibition of lysosomal proteases led to amelioration of EAE.
This protective effect is most likely due to a reversal of the effects of proteasomal activation, such as increased availability of antigenic peptides, up-regulation of proinflammatory molecules and changes in histone de-ubiquitination. These novel findings indicate that understanding proteasomal activity in the CNS may be a key to a better understanding of neurodegenerative diseases.
Fissolo, N. et al., Eur. J. Immunol. 2008. 38: 2401–2411
http://www3.interscience.wiley.com/journal/121407419/abstract
DOI: 10.1002/eji.200838413

Books of immunological interest



Schrör, Karsten
Acetylsalicylic Acid
ISBN-10: 3-527-32109-8
ISBN-13: 978-3-527-32109-4
November 2008



Missailidis, Sotiris
Anticancer Therapeutics
ISBN-10: 0-470-72303-3
ISBN-13: 978-0-470-72303-6
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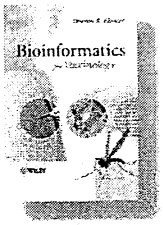
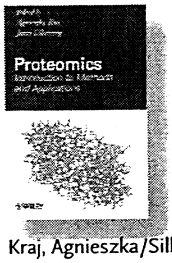
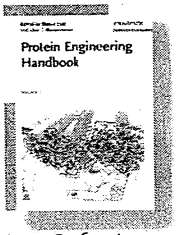


Flower, Darren R.
Bioinformatics for Vaccinology
ISBN-10: 0-470-02711-8
ISBN-13: 978-0-470-02711-0
November 2008



Kraj, Agnieszka/Silberring, Jerzy (Hrsg.)
Introduction to Proteomics
ISBN-10: 0-470-05535-9
ISBN-13: 978-0-470-05535-9
November 2008



Lutz, Stefan /
Bornscheuer, Uwe Theo (Hrsg.)
Protein Engineering Handbook
ISBN-10: 3-527-31850-X
ISBN-13: 978-3-527-31850-6
November 2008

What's new and updated in Current Protocols in Immunology

Performing aseptic survival surgery in rodents can be challenging. This unit describes some basic principles to assist clinicians, researchers, and technicians in becoming proficient in performing aseptic rodent surgery.

Curr. Protoc. Immunol. 82: 1.12.1-1.12.14.

Unit 2.4 Production of Polyclonal Antisera

Much of modern biology and biochemistry relies on the availability of highly specific antibodies for use such ubiquitous techniques as immunohistochemistry, ELISAs, immunoprecipitation, and immunoblo Thus, the generation of large quantities of specific antibodies directed to proteins or peptides of intere is essential to the success of both basic and applied research programs. In addition, with the advent of antibody-based proteomic strategies for profiling protein expression and post-translational modificatio a requirement for timely production of specific antibodies has emerged. Polyclonal antibodies derived f animals immunized with purified proteins or peptides are particularly valuable for use in the laboratory This unit provides protocols for the production of polyclonal antisera specific for protein antigens in rabbits, rats, mice, and hamsters.
Curr. Protoc. Immunol. 82: 2.4.1-2.4.10.

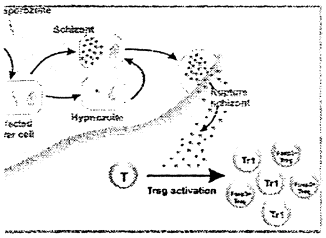
Unit 7.10 Measurement of Proliferative Responses of Cultured Lymphocytes

Measurement of proliferative responses of human lymphocytes is a fundamental technique for the assessment of their biological responses to various stimuli. Most simply, this involves measurement o the number of cells present in a culture before and after the addition of a stimulating agent. This unit contains several different prototype protocols to measure the proliferative response of lymphocytes following exposure to mitogens, antigens, allogeneic or autologous cells, or soluble factors. Each of th protocols can be used in conjunction with an accompanying support protocol which contains methods pulsing cultures with [3H]thymidine and determining incorporation of [3H]thymidine into DNA or asse cell proliferation by nonradioactive methods, e.g., reduction of tetrazolium salts (MTT). The protocols described here provide an estimate of DNA synthesis and cell proliferation in an entire cell population, but do not provide information on the proliferation of individual cells. A protocol for CFSE labeling allc specific subpopulations of cells to be separated viably for further analysis.
Curr. Protoc. Immunol. 82:7.10.1-7.10.24.

Unit 20.9 ErbB2 Transgenic Mice: A Tool for Investigation of the Immune Prevention and Treatment of Mammary Carcinomas

The epidermal growth factor receptor belongs to a superfamily of receptor tyrosine kinases (RTK) that includes ErbB2. ErbB2 is involved in normal physiological processes, such as embryogenesis, cell proliferation, differentiation, adhesion motility, and apoptosis, while its malfunction or overexpression responsible for development defects, diabetes, and cancer. The human ortholog of ErbB2 is referred a Her-2 (human ErbB2) while the rat ortholog is referred as neu (rat ErbB2). As ErbB2 is directly involve carcinogenesis, mice transgenic for the rat neu oncogene allow straightforward assessment of the abil of drugs and vaccines to inhibit the progression of neu-driven cancer. Information from this model ma provide indications on the efficacy of similar treatments in patients. This commentary provides key information regarding the use of these transgenic mouse models for evaluation of the efficacy of anti-t strategies.
Curr. Protoc. Immunol. 82:20.9.1-20.9.10.

alaria parasites impair the st immune system

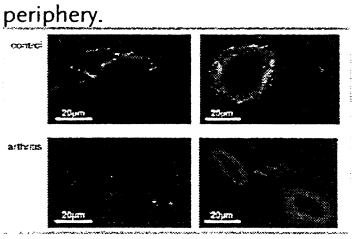


laria is famous amongst munologists for its chameleon- camouflage ability and sion of the immune system. gpatarapongsa et al. explored various mechanisms exploited malaria parasites to elude immune system. Acute smodium vivax infection ds to activation of two Treg populations, FOXP31 Treg l Tr1 cells. The authors report association between the level Treg, IL-10, plasmacytoid and myeloid DC during ite P. vivax malaria. While overall levels of DC were uced, the balance between the DC types was found to be ered. These findings indicate munosuppression of both cell- l antibody-mediated immunity ulting in hindered parasite arance.

gpatarapongsa, K. et al., Eur. J. munol. 2008. 38: 2697–2705
://www3.interscience.wiley.com/ nal/121426028/abstract
I: 10.1002/eji.200838186

Disrupted Joint–Immune System–Brain Communication in Arthritis

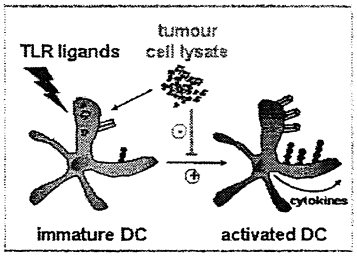
Investigating experimental arthritis in rats, del Rey et al (p. 3090) have provided the first evidence that changes in cytokine expression in the hypothalamus are observed during a specific peripheral immune response. In their study, this response resulted later in the development of type II collagen (CII)–induced arthritis, a model that has many features in common with human arthritis. Additionally, the researchers found that the cytokine-mediated brain–joint communication is disrupted during the development of the disease. This disruption results in an interruption of two main anti-inflammatory pathways controlled at the central nervous system (CNS) level—the hypothalamus–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS)—which may contribute to aggravated disease. The authors noted that although defects in these pathways have been previously described in patients with rheumatoid arthritis (RA), it was not known that central mechanisms might be involved in these alterations. While it was already known that proinflammatory cytokines are expressed in parallel in the CNS and in the periphery following acute immune challenge, the researchers observed that immunization with CII antigens induces increased hypothalamic expression of interleukin-1 (IL-1) and IL-6 prior to the enhanced production of these cytokines in the periphery. Paradoxically, such increase in the brain disappears when arthritis is manifested and there is maximal production of proinflammatory cytokines in the periphery.



del Rey, A. et al., Arthritis Rheum. 2008. 58: 3090-3099.

DOI. 10.1002/art.23869

You are what you eat: Disrupted cells interfere with TLR-mediated DC activation



Effective anti-tumour adaptive immunity requires efficient delivery of tumor-associated antigens in the context of appropriateinnate immune activation. Both freeze-and-thaw-disrupted tumour cells and DC activation by TLR ligands have been studied extensively for antigen delivery and innate immune activation, respectively. Nevertheless, little is known about DC activation in response to TLR ligands in the presence of freeze-and-thaw-disrupted tumour cells. Tirapu et al. reported that disruption of membrane integrity does not affect the uptake of tumourcell material by DC, but suppresses the responsiveness of DC to TLR-mediated activation. The inhibitory activity associated with disrupted cells is neither cell type- nor species-specific, is instantly accessible upon freeze-and-thaw-disruption, and appears to be independent of phosphatidylserinemediated inhibition of DC activation.

Tirapu, I. et al., Eur. J. Immunol. 2008. 38: 2740–2750
http://www3.interscience.wiley.com/journal/121416311/abstract
DOI: 10.1002/eji.200838284

25 years of HIV research on virology, virus restriction, immunopathogenesis, genes and vaccines

Summarising the findings of the important recent 25th Anniversary HIV meeting held at the Institut Pasteur in Paris, this review by Scherer, Douek & McMichael provides an important overview of current research endeavouring to find the elusive cure for this devastating condition. We are grateful to the authors for their special attention to this important commission that complements the excellent historical perspective provided earlier this year by Robin Weiss. Both papers are of especial interest in the light of the recent Nobel announcements regarding Francoise Barré-Sinoussi & Luc Montagnier.

Scherer, E., et al., Clin. Exp. Immunol. 154: 6-14.
http://www3.interscience.wiley.com/journal/121392066/abstract
DOI: 10.1111/j.1365-2249.2008.03750.x

The T-cell receptor repertoire of regulatory T cells

The CD4+ CD25+ regulatory population of T cells (Treg cells) is the key component of the peripheral tolerance mechanism that protects us from a variety of autoimmune diseases. Experimental evidence shows that Treg cells recognize a wide range of antigenic specificities with increased reactivity to self antigens, although the affinity of these interactions remains to be further defined. In this review, Pacholczyk & Kern discuss how different features of the Treg repertoire influence our understanding of Treg specificities and the role of self reactivity in the generation of this population.

Rafal Pacholczyk and Joanna Kern Immunology 125: 450-458
http://www3.interscience.wiley.com/journal/121511255/abstract
doi: 10.1111/j.1365-2567.2008.02992.x

Insights into Langerhans cell function from Langerhans cell ablation models

Kaplan, D. H. et al., Eur. J. Immmunol.2008. 38: 2369-2376.
http://www3.interscience.wiley.com/journal/121407401/abstract
DOI: 10.1002/eji.200838397

Induction, function and regulation of IL-17-producing T cells

Mills, K. H. G. Eur. J. Immmunol.2008. 38: 2636-2649.
http://www3.interscience.wiley.com/journal/121489807/abstract
DOI: 10.1002/eji.200838535

Regulatory B cells as inhibitors of immune responses and inflammation

Bouaziz, J.-D., et al., Immunol. Rev. 224: 201-214.
http://www3.interscience.wiley.com/journal/121370576/abstract
DOI: 10.1111/j.1600-065-X.2008.00661.x

Early T-cell responses in tuberculosis immunity

Winslow, G. M., et al., Immunol. Rev. 225: 284-299.
http://www3.interscience.wiley.com/journal/121414293/abstract
DOI: 10.1111/j.1600-065-X.2008.00693.x

Cytokine Gene Polymorphisms in Recurrent Spontaneous Abortions: A Comprehensive Review

Choi, Y. K. and Kwak-Kim, J. Am. J. Reprod. Immunol. 60: 91-110.
http://www3.interscience.wiley.com/journal/120751213/issue
DOI: 10.1111/j.1600-0897.2008.00602.x

Lessons learnt from many years of experience using anti-D in humans for prevention of RhD immunization and haemolytic disease of the fetus and newborn

Translation of novel immunotherapies into the human setting represents an ongoing challenge for researchers. Pooling of knowledge from relevant arenas – such as trials involving monoclonal/recombinant antibodies – may be key to developing the means to both predict desired responses, and anticipate deleterious ones. Anti-D therapy for haemolytic disease of the newborn represents an established therapy where alternatives to plasma-derived polyclonal IgG, in the form of monoclonal/recombinant antibodies, have been sought. As it involves a well-established intervention, this transition may represent an ideal means by which to observe the particular response characteristics engendered by antibodies produced by, for instance, different expression systems. This review by Kumpels et al. summarises trial data hitherto and speculates on what might be learned – with particular reference to the TGN1412 trail in which unintended consequences played such a drastic role.

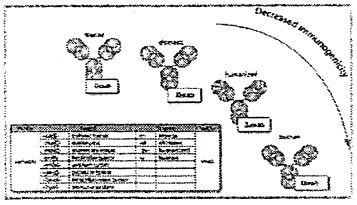
B. M. Kumpel Clin Exp Immunol 154: 1-5
http://www3.interscience.wiley.com/journal/121385811/abstract
doi: 10.1111/j.1365-2249.2008.03735.x

A chromatic explosion: the development and fu of multiparameter flow cytometry

Multiparameter flow cytomet has matured tremendously since the 1990s, giving rise tc a technology that allows us to study the immune system in unprecedented detail. In this article, Chattopadhyay, Hogerkorp & Roederer reviev the development of hardware reagents, and data analysis tc for multiparameter flow cytor and discuss future advances the field. Finally, we highlight new applications that use thi technology to reveal previous unappreciated aspects of cell biology and immunity.

Pratip K. Chattopadhyay, Car Magnus Hogerkorp and Mar Roederer Immunology 125: 441-449
http://www3.interscience.wiley.co journal/121493771/abstract
doi: 10.1111/j.1365-2567.2008.0

Antibody humanization



Monoclonal antibodies (mAbs) are increasingly used to treat human diseases, but as they are predominantly generated from mouse hybridomas their application has been hampered by their immunogenecity. To minimize antigenic reactions, researchers from Genmab in The Netherlands established a transgenic mouse platform for the routine production of fully humanized mAbs. In an overview of recent clinical research, the authors describe efficacy and mechanism of action of three novel humanized mAbs derived from this platform. Zanolimumab, Ofatumumab and Zalutumumab are designed to target CD4, CD20 and the epidermal growth factor receptor overexpressed on cancer cells and to attract cytotoxic reactions against them. All three humanized mAbs show improved efficacy in treatment trials of T-cell lymphomas, B-cell leukemias and head and neck cancer.

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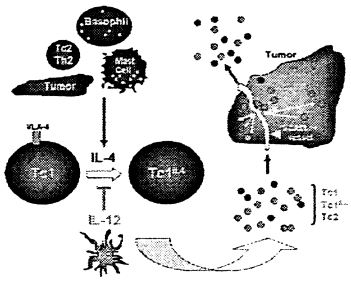
http://www3.interscience.wiley.com/journal/121377802/abstract
DOI. 10.1002/biot.200800110

A new angle in IL-4 and IL-12/IFN-g antagonism

The ability of IL-4 to antagonize antitumour immunity is well recognized. What is less known, however, is the role of IL-4 in effector CD8 T-cell trafficking. Sasaki et al. showed that IL-4 suppresses the migration of protective CD8 T cells into tumor lesions. IL-4 is expressed by various immune cells as well as cancer cells. In tumor-bearing animals, IL-4 suppresses expression of the integrin VLA-4. Suppression of VLA-4 renders antitumor CD8 effector T cells incapable of binding to their molecular counterpart, vascular cell adhesion molecule-1 (VCAM-1) which is expressed on the tumor-associated vasculature. As a consequence, these antitumor effector T cells fail to infiltrate tumor and prevent disease progression. Interestingly, the inhibitory effect of IL-4 on VLA-4 expression is reversed by IL-12 but not IFN-g. The current results suggest that there may be yet another level to the intricacies of IL-4, IL-12 and IFN-g interactions.



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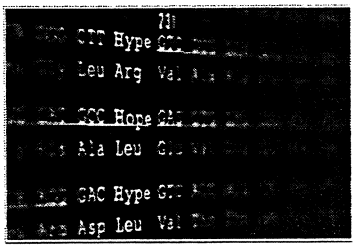
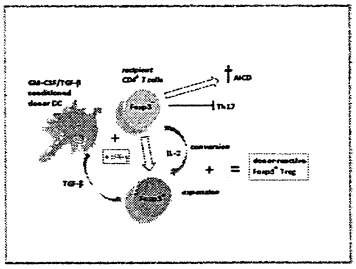
Hype or Hope After Genome-wide SNP Studies in Rheumatology?



An editorial by van der Helm-van Mil et al. takes a critical look at new genome-wide association studies of single-nucleotide polymorphisms in rheumatology. The authors ask whether these studies are raising hopes for future understanding of diseases and the development of personalized medicine, or if they are mainly hype, built on our fascination with impressive technologies. Using specific examples, the authors noted that single genes, in particular those with small odds ratios, play a limited role in predicting risk of disease onset. Additionally, they stated that very few studies have been performed on the effect of genetics on the progression of disease or response to therapy. When several genetic variations with moderate risk are combined, the risk for disease may increase, but the portion of the population affected will diminish, they continued. Their examples brought the authors to the conclusion that combinations of several genetic variants can provide important pathogenetic insights, but they are uncertain if this is a basis for meaningful clinical predictions. Regarding the combination of genetic context with environmental factors, they said the impact of a gene variant increases considerably with this combination. A direct correlate of findings from genomewide association studies is that criterion-based syndromes can now be subdivided, or sometimes merged into new categories that are more meaningful from pathogenetic perspectives. The most important drawback of testing many variants without any prior hypothesis is the chance of obtaining false-positive findings, they noted, recommending replication in independent case–control studies. Noting that the ultimate goal of a genetic association study is to identify biologic pathways that may help to create new therapies, the researchers offered a 3-step process. However, they noted that, "thus far, the findings have not resulted in novel diagnostic tools that significantly refine the currently available measures for risk stratification and fit easily into daily clinical practice."

van der Helm-van Mil, A. H. M. et al., Arthritis Rheum. 2008. 58: 2591-2597.
http://www3.interscience.wiley.com/journal/121391305/abstract
DOI .1002/art.23751

Making regulatory T cells ex vivo: IFN-g is a good cytc after all



Transplantation saves lives ar improves the quality of life foı thousands of organfailure pat Transplantation is, however, not without cost to the patien non-specific immunosuppres results in complications such increased risk of infection anc cancer. The induction of imm logical tolerance for long-term graft survival in the absence of lonı term non-specific immunosuppression remains elusive. A promis new development lies in immunotherapy with ex vivo generated o panded regulatory T cells (Treg). Feng et al. described a novel pro for selection of donor-reactive Foxp31 Treg ex vivo by activating C T cells with GM-CSF/TGF-b-conditioned DC in the presence of IFI Interestingly, this results in the preferential death of effector cells, suppression of Th17 responses, expansion of naturally occurring and direct conversion of non-Treg precursors. More importantly, cells prevent transplant rejection without additional manipulation supporting their further development for the clinical transplantati setting and treatment of autoimmune diseases.

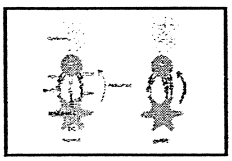
Feng, G. et al., Eur. J. Immunol. 2008. 38: 2512–2527
http://www3.interscience.wiley.com/journal/121408240/abstract
DOI: 10.1002/eji.200838411

Automated Analysis of Flow Cytometry Data

Advances in the optics and electronics of flow cytometers have lec their widespread introduction in many areas of biology and clinica research. However, tools used in the analysis of the data generate have been slower to advance. The automated processing tool dev ped by Jeffries and co-workers uses a number of different algorith and processing techniques to define and describe subpopulation cells. They demonstrate the usefulness of this software when appl to the immunophenotyping of T-cells in a typical clinical research study. Their effort highlights the different features of the APT whic used to objectively define populations of FOXP3+ cells in HIV-infe individuals as well as different CD8T cell populations in CMV infe children in The Gambia.

Jeffries, D. et al., Cytometry A. 2008, 73A: 857-867.
http://www3.interscience.wiley.com/journal/120735832/abstract
DOI. 10.1002/cyto.a.20611

A novel regulator at the DC–T cell interface: gp49B



DC express several cell-surface, ITIM harbouring immune inhibitory receptors that maintain adequate DC developr and/or function; however, little is known about these receptors' immunoregulatory functions in the context of T-cel tivation. In this issue, Kasai et al. demonstrated that gp49B, an ITIM-harboring receptor for avb3 integrin, is expres on DC, and that gp49B-deficient DC induce enhanced proliferation of, and IL-2 secretion by, antigen-specific CD4+ CD8+ T cells in a cell–cell contact manner. The inhibitory role of gp49B at the DC–T cell interface is further demon ted by the accelerated, lethal acute GVH disease in gp49B-deficient recipients, indicating that gp49B negatively reg tes DC function in vitro and in vivo. Previous studies have revealed that gp49B attenuates cytokine and chemokine production by mast cells macrophages. The present findings highlight the versatility of the inhibitory role of gp49B at the DC–T cell interface in a physiological settin

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Immune response to *Plasmodium vivax* has a potential to reduce malaria severity

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Summary

Plasmodium falciparum infection causes transient immunosuppression during the parasitaemic stage. However, the immune response during simultaneous infections with both *P. vivax* and *P. falciparum* has been investigated rarely. In particular, it is not clear whether the host's immune response to malaria will be different when infected with a single or mixed malaria species. Phenotypes of T cells from mixed *P. vivax–P. falciparum* (PV-PF) infection were characterized by flow cytometry, and anti-malarial antibodies in the plasma were determined by an enzyme-linked immunosorbent assay. We found the percentage of $CD3^+\delta2^+$-T cell receptor (TCR) T cells in the acute-mixed PV-PF infection and single *P. vivax* infection three times higher than in the single *P. falciparum* infection. This implied that *P. vivax* might lead to the host immune response to the production of effector T killer cells. During the parasitaemic stage, the mixed PV-PF infection had the highest number of plasma antibodies against both *P. vivax* and *P. falciparum*. Interestingly, plasma from the group of single *P. vivax* or *P. falciparum* malaria infections had both anti-*P. vivax* and anti-*P. falciparum* antibodies. In addition, antigenic cross-reactivity of *P. vivax* or *P. falciparum* resulting in antibodies against both malaria species was shown in the supernatant of lymphocyte cultures cross-stimulated with either antigen of *P. vivax* or *P. falciparum*. The role of $\delta2 \pm$ TCR T cells and the antibodies against both species during acute mixed malaria infection could have an impact on the immunity to malaria infection.

Keywords: antibody response, $CD4^+$ T cells, $CD8^+$ T cells, gamma delta T cells, infections

Introduction

Mixed malaria infection is common and has been reported in many parts of the world where malaria is endemic [1–5]. In regions with low malaria endemicity regions, especially in Thailand, mixed infection with *Plasmodium falciparum* (PF) and *P. vivax* (PV) is common. These co-infections can be either simultaneous or sequential. Previous studies have shown that mixed PV-PF malaria infection is less severe than the single *P. falciparum* infection in terms of lower frequency of anaemia, treatment failure and clinical outcomes for the patients [6]. Moreover, mixed PV-PF malaria infection is approximately a quarter as severe as single *P. falciparum* infection [7]. It is conceivable that interaction between the host's immunity and the two malaria species may take place during acute infection.

The immune mechanism plays an important role in resisting malaria and other infectious diseases [8]. Immunity to malaria induced by different plasmodia species may give various outcomes. Immunity to *P. falciparum* is still controversial. Previous study has shown immunosuppression in acute *P. falciparum* leading to a lower absolute number of $CD3^+$ T cells [9], although the overall percentages of $CD4^+$ and $CD8^+$ T cells are not changed [9–11]. On the other hand, during acute *P. vivax* infection, the percentage of $CD4^+$ but not $CD8^+$ T cells is elevated, whereas the number of antibodies against this parasite is low [12].

Table 1. Information and clinical data* of mixed *Plasmodium vivax–P. falciparum* (PV-PF), single *P. vivax*, single *P. falciparum* infections and naive controls

	Naive controls	Mixed species	*P. vivax*	*P. falciparum*
No. of patients	50	17	63	63
Age (year)*	39 ± 5·1	27 ± 9	31 ± 13	28 ± 8·2
Male	45	14	51	54
Female	5	3	12	9
Temperature (°C)*	36·5 ± 0·5	38·5 ± 0·8	37·3 ± 0·8	38 ± 1·3
Parasitaemia (%)*	0	1·8 ± 2†	0·2 ± 0·2	1·9 ± 2·1
		0·07 ± 0·1‡		
Haematocrit (%)*	42 ± 5·3	37·6 ± 7·3	40 ± 4·2	41 ± 8·5

*Mean ± standard deviation. †*P. falciparum*; ‡*P. vivax*.

γδT cells play a role in linking the innate and adaptive immunities against the broad range of parasites [13–15]. These γδT cells recognize non-peptide phosphoantigens of the microbes leading to the release of cytokines such as tumour necrosis factor (TNF)-α and interferon (IFN)-γ, and therefore exert the effector function, i.e. cytotoxicity and natural killing [16–18]. Generally, $CD3^+\delta2^+$T cells predominate in the peripheral blood in response to many infectious agents such as *Mycobacterium* spp. [19], *Pseudomonas aeroginosa* and *Escherichia coli* [14]. In cases of *P. vivax* malaria, the elevation of $CD3^+\delta2^+$ T cells is observed in peripheral blood but not in *P. falciparum* malaria infection [12]. The natural immune response against malaria in hosts during acute mixed PV-PF malaria infection has been investigated rarely. So far, only one study from Ethiopia has shown that γδT cells are increased in mixed PV-PF malaria infection and single *P. falciparum* infection, but not in *P. vivax* infection [10]. However, successful immunity to malaria required both cell-mediated and humoral immune responses. Therefore, in this study, we characterized the natural immune response during acute mixed PV-PF malaria infection in patients who live in areas of Thailand where malaria is endemic. Understanding both cell-mediated and humoral responses may disclose the roles of the host's immunity to the two malaria species.

Materials and methods

Sample collection

Blood samples were collected in 20 μl of heparin from 17 acutely mixed PV-PF malaria-infected individuals, 63 *P. vivax*- and 63 *P. falciparum*-infected individuals at malaria clinics in Mae Sot, Tak province, Thailand; 50 malaria naive volunteers from non-malaria endemic areas were recruited as naive controls. Diagnosis of malaria infection was based on the examination of Giemsa-stained thick and thin blood films. Recruitment criteria were: age ≥ 15 years; body temperature ≤ 40°C; systolic blood pressure ≥ 90 mm; haematocrit ≥ 25%. Clinical data are shown in Table 1. This study was approved by the Committee on Human Rights Related to Human Experimentation, Mahidol University, Bangkok. Informed consent was obtained from each individual before blood samples were taken.

Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were separated from the collected blood by gradient centrifugation at 800 *g* for 20 min using Lymphoprep™ (AXIS-Shield PoC AS, Oslo, Norway). PBMCs were washed twice with RPMI-1640 by centrifugation at 800 *g* for 10 min and resuspended in RPMI-1640 containing 10% fetal calf serum (FCS). The viability of the PBMCs was determined by trypan blue exclusion dye. PBMCs (10^7 cells/ml) diluted in Cell banker® (Nihon Zenuaku Kohgyo, Japan) were stored in liquid nitrogen until further analysis.

Antigen preparation

White blood cells were depleted from *P. vivax*-infected blood by filtering through a Plasmodiper® (Whatman, Maidstone, UK). The red blood cells were washed twice with RPMI-1640 by centrifugation at 1190 *g* for 5 min. The parasites were cultured at 5% haematocrit in McCoy's medium (Gibco, Carlsbad, CA. USA) supplemented with 25% human antibody serum for 24–30 h in 5% CO_2 until a mature schizont of *P. vivax* appeared [20]. *P. falciparum* culture was performed as described previously [21] in RPMI-1640 medium supplemented with 10% human serum until a mature schizont of *P. falciparum* appeared. *P. vivax* and *P. falciparum* parasites were separated by centrifugation on 60% Percoll®. The infected red blood cell (iRBCs) pellets were pulsed for 40 s on ice at 150 watts and stored at −70°C to be used in a lymphocyte stimulation assay and enzyme-linked immunosorbent assay (ELISA). The protein concentration of the *P. vivax* schizont extract (PvSE), and *P. falciparum* schizont extract (PfSE) was determined by a Bradford assay (Bio-Rad, Hercules, CA, USA). Uninfected red blood cells (uRBC) were processed similarly and used as control protein.

Antigenic cross-stimulation of lymphocytes

To investigate the antigenic cross-reactivity between *P. vivax* and *P. falciparum* parasites. PBMCs from a single *P. vivax* or *P. falciparum* infection were stimulated with PfSE and PvSE. The PBMCs were cultured at (2×10^5 cells/100 µl/well) in a HEPES-buffer RPMI-1640 supplemented with 10% FCS. The PvSE, PfSE and uRBC at 10 µg/100 µl and 100 µg/100 µl were added to each well. The cell quality control of the PBMCs was tested with a medium containing 10 µg/100 µl of phytohaemagglutinin (PHA) and incubated at 37°C in 5% CO_2 for 5 days. The culture supernatant was stored at –20°C for further investigation of cross-reactivity.

Flow cytometric analysis

Phenotyping of T cells was performed by three-colour flow cytometry (FACScan; Becton Dickinson, Oxford, UK). PBMCs (10^5 cells) were stained with the following three-colour combinations of fluorescent dye conjugated with antibodies that were specific to T cell surface markers: fluorescein isothiocyanate (FITC)-conjugated antibody to CD4, R-phycoerythrin (R-PE)-conjugated antibody to CD3 and R-PE-cyanine 5 (R-PE-Cy5) conjugated antibody to CD8; and FITC-conjugated antibody to $\delta 2^+$ TCR T cells and R-PE antibody to CD3 (Caltag, Burlingame, CA, USA) for 30 min at 4°C. After staining and washing, the cells were fixed with 1% paraformaldehyde in PBS pH 7·4 and analysed using CellQuest software (Becton Dickinson, San Jose, CA, USA).

Determination of antibodies against parasite antigen of *P. vivax* and *P. falciparum* antigen

Two methods were used to determine anti-malarial antibodies.

Enzyme-linked immunosorbent assay. Fifty ml of 10 µg/ml (PBS, pH 7·4) PvSE and PfSE were incubated overnight at 4°C in a 96-well polystyrene immunoplate (Corning, NY, USA). The immunoplate was blocked with 100 µl/well of blocking buffer (0·5% boiled casein in PBS and 0·05% Tween 20) for 2 h at room temperature. After three washings, 50 µl of serum (1 : 100 dilution in PBS pH 7·4) were added into duplicate wells and incubated overnight at 4°C. After washing with 0·05% Tween 20 in PBS, pH 7·4, 50 µl of horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (Caltag) and 50 µl of 2,2′-azino-di-(3-ethylbenzthiaoline sulphonic acid) containing 50% hydrogen peroxide (Kirkepaard & Perry Laboratories, Gaithersburg, MD, USA) were added sequentially to each well and incubated for 1 h at room temperature. Enzyme activity was measured by microplate reader, Wallac Victor (Perkin Elmer, Jügesheim, Germany) at 405 nm. The levels of anti-*P. falciparum* and anti-*P. vivax* antibodies were expressed as a ratio increase in a median optical density (OD) compared to naive controls.

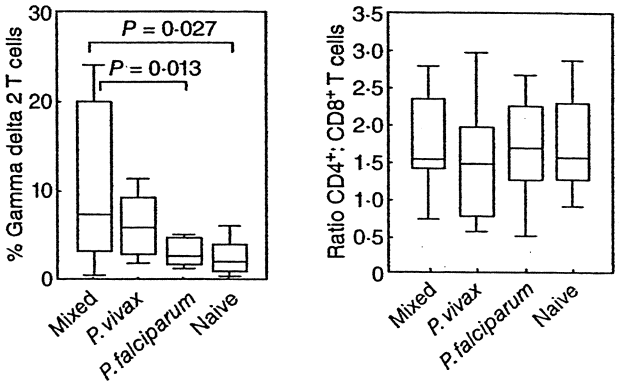

Fig. 1. Phenotyping of T cells in mixed *Plasmodium vivax–P. falciparum* (PV-PF), *P. vivax*, *P. falciparum* malaria infection and malaria naive controls determined by flow cytometry. Data are shown as interquartile ranges (box plots), maximum and minimum (upper–lower line) and bars indicate median. (a) $CD3^+\delta 2^+$ T cells; (b) $CD4^+$: $CD8^+$ T cells.

Immunofluorescence assay (IFA). The pellets of infected red blood cells by *P. vivax* and *P. falciparum* parasites were spotted, dried and fixed on the multi-well slides. The cultured supernatant from PvSE-stimulated lymphocytes and PfSE-stimulated lymphocytes was added to the multi-well slides and incubated in a humidified box at room temperature for 1·5 h. The slides were then washed three times for 5 min. The multi-well slides were then incubated with goat anti-human IgG conjugated to FITC (Serotec, Oxford, UK). The slides were washed three times and mounted with a coverslip using 50% glycerol in PBS and then analysed with a fluorescence microscope.

Statistical analysis

The phenotypes of lymphocyte were analysed using the SPSS program (version 11·5; SPSS Inc., Chicago, IL, USA). To compare the phenotype of T cells in different groups, these data were log-transformed in order to obtain a normal distribution. Statistical significance was determined by one-way analysis of variance (ANOVA). Non-parametric analysis (two independent samples) and Mann–Whitney *U*-test were used to determine the significance levels of anti-*P. falciparum* and anti-*P. vivax* antibodies. The results were considered significant at $P < 0{\cdot}05$.

Results

Phenotypes of T cells

Flow cytometry was used to identify subsets of T cells, including $CD4^+$, $CD8^+$ and $CD3^+\delta 2^+$ (Fig. 1). The median percentage of $CD3^+\delta 2^+$ T cells was significantly higher in acute mixed PV-PF malaria infection (7·4%) compared with

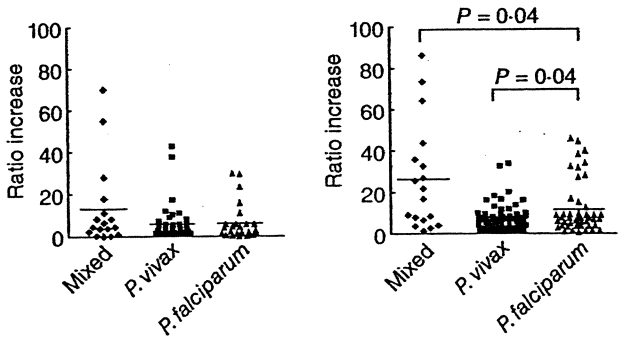

Fig. 2. Antibodies to *Plasmodium vivax* (PV) and *P. falciparum* (PF) in mixed PV-PF, *P. vivax*, *P. falciparum* malaria infection, and malaria naive controls determined by enzyme-linked immunosorbant assay. Data are shown in median ratio and bars indicate median of the ratios. (a) Anti-*P. vivax* antibodies; (b) anti-*P. falciparum* antibodies.

acute *P. falciparum* infection (2·3%, $P < 0·01$) and naive controls (1·8%, $P < 0·03$). There was no difference in $CD3^+\delta2^+T$ cells between acute *P. vivax* and the mixed malaria infections (7·4% *versus* 5·9%, $P > 0·05$). The median percentage of $CD4^+$ and $CD8^+$ T cells in the mixed malaria infection (36·7% and 24·6%, respectively) was similar to that of the single *P. vivax* (29·2% and 25·5%, $P > 0·05$) and *P. falciparum* infection (44·8% and 23·5%, $P > 0·05$) and these levels were not different from the naive controls (44·7% and 24·0%, $P > 0·05$).

Antibodies to *P. falciparum* and *P. vivax* protein extracts

Antibodies to the parasite protein extracts in the plasma were determined and are shown in Fig. 2. Both mixed malaria and single malaria infections had antibodies against *P. vivax* and *P. falciparum* antigens. Interestingly, the mixed infection had the highest plasma levels of antibodies to both *P. vivax* and *P. falciparum* antigens [13-fold ($P > 0·05$) and 24-fold ($P < 0·04$), respectively] compared with those of the naive controls. The single *P. falciparum* group had an increased ratio of antibodies to the *P. vivax* (sixfold, $P > 0·05$) and *P. falciparum* infection (10-fold, $P > 0·05$). The single *P. vivax* group had a sixfold increase of anti-*P. vivax* and anti-*P. falciparum* antibodies compared with the naive control. Of the three malaria infection groups, anti-*P. falciparum* antibodies were significantly higher in the mixed PV-PF malaria infection ($P < 0·04$) compared with each single malaria infection group, as shown in Fig. 2b. Antibodies specific to *P. vivax* protein extracts were low in all groups, as shown in Fig. 2a.

Cross-reactivity of antibodies to *P. vivax* and *P. falciparum* parasites

The plasma antibodies of the single malaria infections, either *P. vivax* or *P. falciparum*, showed reactivity with the other malaria species regardless of the species that had currently caused the infection. Interestingly, the supernatant collected from the PvSE- or PfSE-stimulated lymphocytes of the single *P. falciparum* infection produced both anti-*P. vivax* and anti-*P. falciparum* antibodies. Similarly, the supernatant collected from the PfSE- or PvSE-stimulated lymphocytes of the single *P. vivax* infection also produced both anti-*P. vivax* and anti-*P. falciparum* antibodies. The antibodies showed strongly positive with mature stages of both parasites as determined by the IFA (data not shown). The supernatant collected from the malaria PvSE- or PfSE-stimulated lymphocytes of the malaria naives showed no reactivity with any parasite.

Discussion

The objective of this study was to characterize the profiles of T cells and the response of antibodies to blood stage antigens of both *P. vivax* and *P. falciparum* during acute mixed-malaria infection. Our study gave novel evidence of the natural immune response of γδT cells against mixed malaria infection, were modulated by *P. vivax* and their role in the reduction of malaria severity caused by *P. falciparum*. The percentage of $CD3^+\delta2^+T$ cells were increased significantly in acute mixed and single *P. vivax* infections compared with these of the naive controls, whereas only a low level of these cells was found in acute *P. falciparum* patients. The CD4 : CD8 ratio did not show any difference between malaria patient groups, which was in line with previous studies [9–11]. These results suggested the role of $CD3^+\delta2^+T$ cells in the development of the cell-mediated immunity during mixed malaria infection.

The γδT cells inhibit development of the pre-erythrocytic stage of *P. yoelii* in a mouse model lacking αβ T cells [22], and the α-galactosyl-ceramide (a-GalCer)-activated natural killer (NK) T cells protect mice from *P. yoelii* infection [23]. Moreover, γδT cells, NK, NK T cells and macrophages harboured in the liver where the pre-erythrocytic stage of malaria are presented may boost the immunity to malaria infection [24] more effectively than those malaria having only the erythrocytic stages.

The γδT cells control the expansion of *P. chabaudi* in a mouse model [25] and inhibit growth of *P. falciparum* parasites [26] through the release of granulysin [8]. The elevation of $CD3^+\delta2^+$ T cells in acute *P. vivax* infection conceivably plays a similar role to that in *P. falciparum* infection. Interestingly, a greater elevation of the $CD3^+\delta2^+$ T cells was shown in the mixed malaria infection and the single *P. vivax* infection compared with that of the *P. falciparum* infection. By contrast, a previous study in Ethiopia showed that there was no difference in $CD3^+\delta2^+$ T cells in mixed PV-PF malaria infection compared with single *P. vivax* or *P. falciparum* infection. However, the $CD3^+\delta1^+$ T cells, having a killer effector function, were more numerous in the single *P. falciparum* and mixed PV-PF malaria infections [10].

Our data showed no correlation between the percentage of $CD3^+\delta2^+$ T cells and the number of *P. vivax* parasites or the number of the *P. falciparum* parasites. Nevertheless, the lower number of *P. falciparum* parasites in the mixed malaria infection, as opposed to that in the single *P. falciparum* infection, suggested that *P. vivax* might activate the effector killer function of the $CD3^+\delta2^+$ T cells resulting in the inhibition of *P. falciparum* growth.

When *P. vivax* was co-infected with *P. falciparum* in the malaria infection, patients had a higher fever than those with single *P. falciparum* or single *P. vivax* infections [27]. High temperature is shown to kill *P. falciparum* parasites in *in vitro* studies [28,29]. In addition, serum from a *P. vivax*-infected donor during paroxysm inhibits maturation of *P. falciparum* schizonts [30]. Together, these data support the notion that a mechanism by which *P. vivax* controls the expansion of *P. falciparum* in mixed malaria infection could be via the induction and persistency of high fever in patients, particularly the high systemic temperature in the organs where *P. falciparum* sequestered. Therefore, if this can be established, the vaccine development imperative against *P. falciparum* infection and severity may derive from the combination of *P. falciparum* antigen and the antigen candidate from *P. vivax*.

Antibodies to malaria, although short-lived, are the primary mechanisms of defence against parasitic infection [31]. Induction and maintenance of anti-malarial antibodies requires repetitive infections [32,33]. Evidence such as the existence of the asymptomatic parasitaemic individual confirms development of immunity against malaria [34,35]. Recently, a study has shown that the incidence of severe malaria in patients with mixed PV-PF malaria infection is 4·2 times less than that in *P. falciparum* infection alone [7].

In this study we have shown that the anti-*P. falciparum* and anti-*P. vivax* antibody levels in the mixed malaria infection were higher than those of the single *P. vivax* or *P. falciparum* infections. Our data provide a new basis to support previous findings that, on one hand, development of a cross-immune reactivity between *P. vivax* and *P. falciparum* during acute mixed infection could be due to the activation of a pool of memory T cells having specificities to both *P. vivax* and *P. falciparum* antigens. These cells co-existed in the residents of the endemic areas where there is regular exposure to malaria parasites.

On the other hand, the antigenic cross-stimulation by *P. vivax* antigens sharing common epitopes with *P. falciparum* [36] results in the cell-mediated and antibody responses at high levels against *P. falciparum* during the acute phase of infection. Further investigations in the different geographical endemic areas are needed to verify the two categories. To stratify this finding further, we performed an *in vitro* T cell stimulation assay using PvSE and PfSE antigens derived from *P. vivax* and *P. falciparum*, respectively, to stimulate the PBMCs from acute single *P. vivax* or single *P. falciparum* infections with the two antigens. Both anti-*P. vivax* and anti-*P. falciparum* antibodies, tested by the IFA, were found in the supernatant collected from these assays, regardless of the parasite species which caused the infection. This suggested that the patients had developed antigen-specific memory T cells against both *P. vivax* and *P. falciparum* parasites which were activated upon re-exposure to either *P. vivax* or *P. falciparum* antigens. Supporting evidence from a study in Thailand shows the antibody cross-reactivity from a single *P. vivax*-infected patient against both the schizont extract of *P. falciparum* parasite and the $PfMSP1_{19}$ parasite protein [30]. In addition, the cross-reactivity between anti-PvMSP5 and anti-PfMSP5 antibodies was observed in single *P. vivax* or single *P. falciparum* infections [37]. Our findings were also supported by the evidence from an epidemiological observation and cross-sectional study [2], during a wet season, which showed that the dominant parasite is *P. falciparum* whereas *P. vivax* dominates during the dry season. Overall immunity, effector T cells and anti-malaria antibodies to malaria among the residents of endemic areas would be strengthened by the existence of *P. vivax*.

In summary, our results indicate the possibility of *P. vivax* suppressing *P. falciparum* parasites, because *P. vivax* induces $CD3^+\delta2^+$T cells which are effector T killer cells. *P. vivax* infection also elevates anti-*P. falciparum* antibodies during the acute phase, and induces a very high fever. These findings suggest that the interaction between the host and *P. vivax* parasites could offer protection as demonstrated in the mixed PV-PF malaria infection. However, further clinical and experimental research is needed in order to verify these assumptions. Furthermore, in single *P. vivax* or *P. falciparum* infection, similar levels of T helper type 1 (Th1)/Th2 cytokine responses are shown [38]. Clarification of such responses to mixed malaria infection in man, i.e. conversion between Th1- and Th2-type responses, are of interest and require further investigation.

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Disclosure

None.

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Characteristic of Immunity to Malaria: Comparative Lymphocyte Responses in *P. falciparum* and *P. vivax* Infection

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Infection with *P. vivax* induces memory and regulatory T cell activation during infection. Here, we investigated the characterization of lymphocyte response during *P. vivax* infection compared with *P. falciparum* by using *in vitro* stimulation assays. Our result showed the elevation of $CD4^+$, $CD8^+$ and $\gamma\delta^+$T cells in acute *P. vivax* patients and after stimulation with crude *P. vivax* antigens by significantly showing in $CD4^+$ T cells. The acute *P. vivax* patients showed antibody responses, approximately sixty percent of *P. vivax* patients had a two-fold increase of antibody level compared with naive controls. Further, antibody levels increased in corresponding to antibody level in each patient after stimulation with *P. vivax* antigens. In comparison, the level of $CD8^+$ and $\gamma\delta$ T cells increased in acute *P. falciparum* patients whereas $CD4^+$ T cells were significantly reduced. The stimulation of PBMCs from acute patients showed no significantly response of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells. Importantly, crude *P. falciparum* antigens significantly induce B-cell expansion and antibody production *in vitro*, indicating a major role of humoral immunity in *P. falciparum* infection. T-cell apoptosis is one mechanism to impair cell-mediated immunity in malaria. $CD4^+$ and $CD8^+$ T cell apoptosis were increased in both acute *P. falciparum* and *P. vivax* patients and they were significantly heightened upon antigen stimulation. In this study, we compared the natural response of lymphocyte between *P. vivax* and *P. falciparum* infection. Crude *P. vivax* antigens stimulated lymphocyte expansion by predominantly increase of $CD4^+$ cells and induced antibody response. However, T cells of *P. falciparum* patients lowly responded to crude antigens. The expansion of B-cell and antibody levels upon stimulation suggests the major role of humoral immunity in development of naturally acquired immunity in *P. falciparum*. The induction of $CD4^+$ T cell apoptosis is common strategy in two human malaria for impairment of natural immune response.

Malaria is one of the most serious infectious diseases widely distributed in tropical and subtropical countries. Global burden is estimated more than 500 million cases per year. Most of these cases are caused by *P. falciparum* and *P. vivax* [21]. *P. falciparum* is a significant cause of mortality; severe anemia, cerebral malaria, and acute renal failure. In contrast with *P. falciparum*, *P. vivax* infection cause relapsing

56 fever but is rarely fatal. (22). The severity of malaria infected patients is mostly
57 associated with immune mediated diseases.

58 Both antibodies and T cells play an important role in protective immunity to malaria
59 parasites [19, 25,31,32]. Stimulation of autologous T/B cell mixture with low
60 concentration of *P. falciparum* antigen gave rise to IgG secretion *in vitro* cultures
61 [15]. Lymphocytes from malaria exposed and non-exposed donors responded to *P.*
62 *falciparum* parasite [5]. However, it is difficult to acquire long lasting immunity
63 despite repeated exposure of parasites to people living in malaria endemic areas [9,
64 16]. Suppression of malaria-specific T cells proliferation and induction of T cells
65 apoptosis were present during acute *P. falciparum* infection [1, 4]. Additionally, host
66 immune responses to the parasites were affected on the pathogenesis. High level of
67 pro-inflammatory cytokines such as tumor necrosis factor–α (TNF-α), interleukin
68 (IL)-1 and IL-6 are responsible to severe complicated *P. falciparum* patients [18].

69 Very few studies in immunity to *P. vivax* infection have been conducted. Most
70 studies in immunity to malaria were reported in *P. falciparum*. One reason for this
71 could be the difficulty of maintaining *P. vivax* cultures as a source for the preparation
72 of parasites antigens. Since, a characteristic having hypnozoite stage for *P. vivax*, we
73 hypothesized that the natural immune response to *P. vivax* infection differs from that
74 of *P. falciparum*. There have been some studies of human immune response to *P.*
75 *vivax* circumsporozoite protein (CSP) [7, 8]. The most antibodies response in
76 naturally infected *P. vivax* was targeted to epitopes in non-repeat region of CSP [28].
77 It is believed that CSP-specific $CD4^+$ and $CD8^+$ T cell responses, along with CSP-
78 specific antibodies, will be important in immunity to *P. vivax* sporozoite/liver stages.

In immunity to blood stage of *P. vivax,* recently, our study has been shown that memory T cells were maintained in immune villagers and elevated during acute *P. vivax* infection. However, low level of specific antibody to blood stage of *P. vivax* was detected in acute and convalescent period of infection [13]. This indicated the suppressive immunity in blood stage of *P. vivax* infection that needed for further study.

Therefore, this study aims to demonstrate the natural response of lymphocytes and characteristic of lymphocytes responses to *P. vivax* and *P. falciparum* parasites. We compared the status of lymphocyte proliferation in both human malaria and identified the phenotypes of T cells responding to schizont protein extract, including the apoptosis induction by parasites.

MATERIALS AND METHODS

Sample collection. The acutely *P. vivax* and *P. falciparum* infected volunteers who registered at Malarial Clinics in Mae-Sod and Mae-Kasa districts, Tak province have been informed the details of the project prior blood samples were taken. Selecting criteria of the projects were as followings: (1) systolic blood pressure was not less than 90 mm, (2) body temperature was not higher than 40°C, (3) Hematocrit was not less than 25% and (4) all patients have to be the age of 15 or above. Those who were not fitting the criteria and pregnant patients were excluded. After the patients were selected, 20 ml of peripheral blood were taken. The healthy volunteers who live outside the endemic area and without previous history of malarial infection were included as naive controls.

101 This study was approved by the Committee on Human Rights Related to Human
102 Experimentation, Mahidol University, and the Ministry of Health, Thailand. Informed
103 consent was obtained from each individual before the blood sample was taken.

104 **Parasite cultures and antigen preparations.** *P. vivax* and *P. falciparum* infected
105 blood collected from the same endemic area was used as PvSE and PfSE in *in vitro*
106 stimulation assays and ELISA, respectively. Briefly, *P. vivax* infected blood cells was
107 depleted of white blood cell by filtering through Plasmodiper (Whatman, Maidstone,
108 UK) and the red blood cells were washed with RPMI-1640 by centrifugation at 1190×
109 g for 5 min. The parasites were cultured for 24-30 hrs at 5% haematocrit in McCoy's
110 medium (GIBCO, Carlsbad, USA) supplemented with 25% human AB serum at. *P.*
111 *falciparum* cultures were performed as described previously [35] in RPMI-1640
112 medium supplemented with 10% human serum. Both *P. vivax* and *P. falciparum*
113 cultures were kept at 5% O_2 and 90% N_2. After the parasites had matured to schizont
114 stage, antigens were separated by centrifuged in 60% Percoll®. The cells in the
115 interface layer between medium and Percoll® were collected, washed twice and the
116 pellets were stored at -70°C to bed used for *in vitro* stimulation assays and for
117 antibody detection.

118 **Lymphocyte purification and *in vitro* stimulation assay.** Heparinized blood was
119 mixed with equal volume of HEPES-buffer in RPMI-1640 medium (GIBCO,
120 Carlsbad,USA). The diluted blood was carefully overlayered on Lymphoprep™
121 (AXIS-Shied PoC AS, City, Norway) and PBMCs were separated by gradient
122 centrifugation at 800 x g for 20 mins. After centrifugation, the PBMCs were collected
123 from the interface between plasma/medium and Lymphoprep™ following washed

twice with RPMI-1640 at 800 x g for 10 mins. The PBMC pellet was finally resuspended in RPMI-1640 containing 10% heated-fetal calf serum (FCS). The viability was determined by Tryphan Blue exclusion and the number was counted in hematocytometer. PBMCs were cultured at $2x10^6$ cells/ well in HEPES-buffer RPMI-1640 supplemented with 10% FCS. The lysate of *P. vivax* schizont extract (PvSE), *P. falciparum* schizont extract (PfSE) and lysate of uninfected red blood cells (uRBC) at 10 μg or 100 μg were added in each well. Control cells were cultured with Media and 10 μg of Phytohemagglutinine-A (PHA) and incubated at 37 in 5% CO_2 for 5 days.

Lymphocyte proliferation assays. 1×10^6 peripheral blood mononuclear cells (PBMCs) were resuspended in HEPES-buffer RPMI-1640 supplemented with 10% heat-inactivated FCS and incubated with 2.5 μM of 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) at 37°c for 10 mins. 100 μl of CFSE-labeled cells were plated into each well of 96-well round-bottom tissue culture plates. PvSE or PfSE were added into PBMCs and cultured under *in vitro* conditions for 5 days. Control wells were cultured with RPMI-1640 medium alone as negative control, with uRBC antigens as base line stimulation, and with 10 μg of PHA as mitogenic activation of PBMCs. The percentage of lymphocyte proliferation in parasite culture was obtained from the total of divided cells in PvSE or PfSE cultures subtracted with uRBC stimulation.

Flow cytometry analysis. B and T cells phenotyping was performed by three-color flow cytometry (FACscan Becton Dickinson, Oxford, UK). $CD4^+$, $CD8^+$ T cells were defined with a combination of fluorochorme-conjugated Mabs, Tricolor labeled anti-CD3, PE labeled anti-CD4 and FITC labeled anti-CD8 (Caltag, Burlingame, USA).

147 For $\gamma\delta^+$ T cells, PBMCs were stained with mouse IgG1 anti-human TCR Vγ9 (A13) or
148 TCR Vδ2 (BB3), (kindly provided by Dr. L. Moretta, Centro Di Biotechnologie
149 Avanzate, Genova, Italy) for 1 h at 4 °C and washed twice with phosphate buffered
150 saline (PBS). Goat $F(ab)^2$ anti-mouse IgG antibodies conjugated with FITC were used
151 to detect the anti-γ9 and δ2 TCR antibodies. B cells were defined by staining with
152 FITC labeled anti-CD19 (Serotec, Oxford, UK).

153 **Determination of antibody response by enzyme linked-immunosorbent assays**
154 **(ELISA).** The purified *P. falciparum* and *P. vivax* parasite were diluted in PBS and
155 coated in 96 well plates. Blocking buffer, 2% BSA in PBST were added in each well
156 and incubated for 2 hrs for protecting of non-specific binding. Sera samples or the
157 stimulated *P. vivax* or *P. falciparum* cultures, 100 µl of 1:100 diluted was added and
158 incubated at room temperature for 1.5 hr. The plates were then washed three times for
159 5 min for each wash. The secondary antibodies, goat anti-human IgG (FC) conjugated
160 peroxidase (Serotec, Oxford, UK), were added and incubated for 1.5 hr, washed three
161 times as before and then added substrate ABTS, incubated for 1 hr and measure
162 Optical Density (OD) values at 405 wavelength by ELISA plate reader.

163 **Data analysis.** All data analyzed using the SPSS program (Version 11.5 Chicago,
164 USA). Non-Parametric analysis (two independent samples); Mann-Whitney test was
165 used for comparison of lymphocytes response between PvSE or PfSE stimulation and
166 media control. The results were considered statistically significant values at $P< 0.05$.

167 **RESULTS**

168 **The proliferative response of human lymphocytes in malaria.** The function of
169 lymphocytes during *P. vivax* and *P. falciparum* infection was assessed by

identification of divided cell number. CFSE-labeled lymphocytes were determined after 5 days of PBMC stimulation with crude schizont protein extract at varied concentration from 1 μg to 100 μg. The stimulation of naive PBMCs with *P. vivax* antigens showed small increase of lymphocyte proliferation compared with medium control (PvSE1 = 4.45±2.5%, PvSE10 = 7.49±5.4 %, PvSE100 = 7.61±5.6±%, figure 1). Unlike *P. vivax* stimulation, the significant increase of divided cells was observed after stimulation naive PBMCs with *P. falciparum* antigens (PfSE1 = 9.36±4.18%, PfSE10 = 10.15±3.5%, PfSE100 = 15.28±3.11%, Figure 1). These results indicate the higher mitogenic index in *P. falciparum* than those in *P. vivax* parasites.

To evaluate the natural response of lymphocytes during *P. vivax* or *P. falciparum* infection, PBMCs of acute malaria patients were stimulated with crude shizont antigen for 5 days and measured the number of divided cells. The results showed that lymphocyte of *P. vivax* patient expanded upon stimulation with PvSE compared with un-stimulation (PvSE1 = 3.89±2.87%, PvSE10 = 7.49±4.17%, PvSE100 = 7.18±5.23%, figure 1). Moreover, the proliferative response of lymphocyte was also showed in *P. falciparum* stimulation, approximately 5-8% of lymphocytes were stimulated by *P. falciparum* antigens (PfSE1 = 5.67±3.5%, PfSE10 = 8.19±3.11%, PfSE100 = 7.23±2.87%, figure 1). These results suggest a boosting of lymphocyte expansion in repeated infection with plasmodium.

Phenotyping of T-cells response to *P. vivax* and *P. falciparum*. To identify which subset of T cells response to parasites, the percentage of responded T cells ($CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells) were phenotyped in acute *P. falciparum* and *P. vivax* patients and after stimulation with 100 μg of schizont protein extract. PBMCs in acute *P.*

193 *vivax* patients were reduced compared to naive controls (*P. vivax* = 23.34×10^6cell/ml,
194 naive controls = 25.56×10^6cell/ml, n=8). The percentage of $CD4^+$, $CD8^+$ T cells and
195 $\gamma\delta^+$ T cells in acutely infected *P. vivax* patients showed small increase ($CD4^+$ T cells =
196 47.2±3.79%, $CD8^+$ T cells = 26.77±3.48% and $\gamma\delta^+$ T cells = 2.13 ±3.45%, n=8)
197 compared with naive controls ($CD4^+$ T cells = 44.04±2.32%, $CD8^+$ T cells =
198 26.4±2.02% and $\gamma\delta^+$ T cells = 1.19±1.21 %, n=8).

199 PBMCs in acutely infected *P. falciparum* patients was significantly reduced
200 compared with naive controls (acute patients = 14.1×10^6 cell/ml, naive = 25.56×10^6
201 cell/ml, p=0.009, n=8). Phenotyping of T cell population showed significant
202 reduction of $CD4^+$ T cells ($CD4^+$ T cells = 34.93±4.9%, p=0.008, figure 2) but small
203 increase of $CD8^+$ T cells and $\gamma\delta^+$ T cells ($CD8^+$ T cells = 35.84±9.9%, $\gamma\delta^+$ T cells =
204 3.01±1.45 %), compared with naive controls ($CD4^+$ T cells = 44.04±2.32%, $CD8^+$ T
205 cells = 26.4±2.02%, $\gamma\delta^+$ T cells = 1.19±1.21 %, figure 2).

206 To demonstrate the first exposure to human parasites, naive PBMCs were
207 stimulated with crude schizont protein extract *in vitro* cultures for 5 days and
208 assessed the number of responded cells. The results showed small expansion of $CD4^+$
209 T cells (stimulation = 47.69±4.89%, un-stimulation = 44.04±2.32%, figure 2A) $CD8^+$
210 T cells (stimulation = 28.76±3.4%, un-stimulation = 26.4±2.02%, figure 2B) and $\gamma\delta^+$
211 T cells (stimulation = 3.6±2.7%, un-stimulation =1.19±1.21%, figure 2C) in *P. vivax*
212 cultures. The strong expansion of $CD4^+$ T cells was significantly shown in *P.*
213 *falciparum* cultures compared with un-stimulation (stimulation = 63.44±2.9%, un-
214 stimulation = 44.04±3.79%, p=0.005). However, only small number of naive $CD8^+$ T
215 cells (stimulation = 29.01±3.91%, un-stimulation = 26.4±2.2%, figure 2B) and naive

$\gamma\delta^+$ T cells responded to *P. falciparum* antigens (stimulation= 2.09±2.08, un-stimulation = 1.91±1.21, figure 2C).

The natural response of T cells to malaria antigens were demonstrated by stimulation of PBMCs from malaria patients with crude schizont antigens *in vitro*. The results showed highly response of $CD4^+$ T cells, (stimulation = 57.40±8.05%, un-stimulation = 47.69±4.89%, p=0.003, figure 2A) small increase of $CD8^+$ T cells (stimulation= 32.54±4.49%, un-stimulation = 28.76±3.4%, figure 2B) and $\gamma\delta^+$ T cells upon stimulation with *P. vivax* antigens comparing to un-stimulation (stimulation = 4.21±3.45%, un-stimulation = 3.06±2.7%, figure 2C). Stimulation of PBMCs from *P. falciparum* patients with crude schizont antigens showed small increase of $CD4^+$ T cells (stimulation = 42.01±6.1%, un-stimulation = 34.93±4.9%, figure 2A) $CD8^+$ T cells (stimulation = 41.50±4.22%, un-stimulation = 35.84±9.9%, figure 2B) and $\gamma\delta^+$ T cells (stimulation = 7.18±1.95%, un-stimulation = 2.09±2.08%, figure 2C).

The response of B-cells during *P. falciparum* and *P. vivax* infection. Our previous study showed that anti-*P. vivax* antibody levels were very low in acutely infected *P. vivax* patients [13]. To demonstrate the function of B-cells during *P. vivax* infection, B cell number in acute *P. vivax* patients and the response of B-cells to *P. vivax* antigens after stimulation *in vitro* were demonstrated compared with naive controls. The population of B cells was slightly decreased in acutely infected *P. vivax* and *P. falciparum* patients compared to naive controls (*P. vivax* = 16.15±9.65%, *P. falciparum* = 18.89±6.28%, naive = 20.13±1.41%, figure 3). The stimulation of naive B-cells with *P. vivax* antigens in cultures showed small increase of B-cells compared with un-stimulation (stimulation = 23.18±6.38, un-stimulation = 20.13±1.41%, figure

3). Contrastly, naive B-cells strongly respond to *P. falciparum* antigens, indicating the mitogenic effect of *P. falciparum* parasites on B-cell function (stimulation = 32.24±6.28%, un-stimulation = 20.13±1.41%, p=0.003, figure 3).

To demonstrate this phenomenon during malaria infection, B-cells of acutely infected *P. vivax* and *P. falciparum* patients were stimulated with crude schizont antigens *in vitro* and assessed the total number of $CD19^{+}$B-cells. The result showed that stimulation of PBMCs with *P. falciparum* induced a significant expansion of B-cells (stimulation = 26.2±7.61%, un-stimulation = 18.89±6.28%, p=0.007, figure 3) whereas *P. vivax* accelerated small population of B-cell expansion (stimulation = 20.99±3.01%, un-stimulation = 16.15±4.65%, figure 3).

The effect of *P. falciparum* and *P. vivax* parasites on T-cell apoptosis. The significant reduction of PBMCs in acutely infected *P. falciparum* and *P. vivax* patients indicate some mechanisms to withdraw lymphocyte during malaria infection. One strategy of evasive immunity by malaria parasite was the induction of lymphocyte apoptosis. To demonstrate this phenomenon *in vitro*, the level of Anexin $V^{+}CD4^{+}$ and Anexin $V^{+}CD8^{+}$ T cells were detected in acute *P. vivax* and *P. falciparum* infection and after stimulation of PBMCs with crude schizont antigen *in vitro*. The result showed that the level of T cell apoptosis both $CD4^{+}$ T cells and $CD8^{+}$ T cells was higher in *P. falciparum* and *P. vivax* patients than naive controls ($CD4^{+}$ T cells, *P. falciparum* = 4.95±3.81%, *P. vivax* = 5.67±4.33%, naive = 1.11±0.84%; $CD8^{+}$ T cells, *P. falciparum* = 6.64±3.21%, *P. vivax* = 4.20±2.11%, Naive = 1.6±1.43%).

To re-produce apoptosis phenomenon *in vitro*, PBMCs from naive were stimulated with *P. falciparum* and *P. vivax* antigens and the number of apoptosis T cells were determined on day 5. The result showed that apoptotic T cells both $CD4^+$T and $CD8^+$ T cells were higher in *P. falciparum* ($CD4^+$ T cells, stimulation = 4.57±2.13%, un-stimulation = 1.11±0.84%, figure 4A; $CD8^+$ T cells, stimulation = 2.26±2.27%, un-stimulation = 1.6±1.43%, figure 4B) and *P. vivax* stimulation than in control ($CD4^+$ T cells, stimulation = 2.23±2.14%, un-stimulation = 1.11±0.84%, figure 4A; $CD8^+$ T cells, stimulation = 2.09±1.98%, un-stimulation = 1.6±1.43%, figure 4B). Stimulation of PBMCs from acute patients with *P. falciparum* antigens showed significantly elevation of $CD4^+$ T cells apoptosis (stimulation = 11.57±4.64%, un-stimulation = 2.58±1.11%, P = 0.009, figure 4A) and small increase of $CD8^+$ T cells apoptosis (stimulation = 4.67±4.64%, unstimulation = 2.43±1.7%, figure 4B). Similarly, $CD4^+$ T cells apoptosis (stimulation = 9.23±5.49%, un-stimulation = 2.24±1.81%, p=0.008, figure 4A) and $CD8^+$ T cells apoptosis (stimulation = 3.83±2.33%, un-stimulation = 2.19±1.43%, figure 4B) was also highly obtained in *P. vivax* patient cultures.

The antibody response in *P. falciparum* and *P. vivax* infection. Immunity induced by *P. falciparum* and *P. vivax* stimulated the population of B-cell expansion during infection (figure 3). To evaluate the function of responded B-cell for their efficiency to produced malaria specific antibodies, the anti-*P. falciparum* antibodies and anti- *P. vivax* antibodies in both human malaria were determined in individual sera by conventional ELISA method. B-cell of all acute *P. falciparum* patients produced specific anti-*P. falciparum* antibodies with a mean OD, and standard deviation of 2.1±0.27, approximately 4-fold increase in antibody levels compared with naive controls (0.52±0.15, figure 5C). Anti-*P. vivax* antibody levels in acutely

285 infected *P. vivax* patients was classified into two groups based on the difference of
286 antibody levels from naive controls. The high responder (H) showed antibody levels
287 higher than mean±2SD of naive controls, 5 in 8 of patients showed two fold increase
288 of antibody titer (1.2±0.32, figure 5A). Three of 8 patients were classified into low
289 responder (L), anti-*P.vivax* antibody levels ≤mean ± SD (0.22±0.11, figure 5A).

290 The function of B-cell during malaria infection was demonstrated *in vitro*. PBMCs
291 of naive controls were stimulated with *P. falciparum* and *P. vivax* antigens and were
292 assessed antibody levels. The result showed that crude *P. vivax* (0.28±0.13, figure 5B)
293 and *P. falciparum* antigen (0.97±0.16, figure 5D) did not significant induce antibody
294 secretion in cultures compared with medium control. The stimulation of PBMCs from
295 acute *P. falciparum* patients with crude antigens induced anti-*P. falciparum*
296 antibodies (1.4±0.22, figure 5A) over what was found in medium control (0.57±0.12,
297 figure 5A) and in naive control cultures (0.97±0.16, figure 5B). In comparison with *P.*
298 *vivax*, the stimulation of PBMCs with *P. vivax* antigens showed that high responder
299 had anti-*P. vivax* antibodies higher in corresponding to antibody level in each patient
300 (1.4±0.29, figure 5B). This result indicates a boosting of antibody levels during *P.*
301 *vivax* infection. However, the low responder did not respond to antigens after
302 stimulation, keeping anti- *P. vivax* antibody in low levels (0.19±0.13, figure 5B).

303 **DISCUSSIONS**

304 The short-lived of naturally-acquired immunity in malaria lead one to assess the
305 natural response of lymphocyte during *P. falciparum* and *P. vivax* infection by
306 stimulation of patient lymphocytes with crude antigens from *P. falciparum* or *P. vivax*
307 infected red blood cells for reflection to secondary response *in vitro* of primed cell *in*

308 *vivo*. We showed the different characterization of T cell response between two human
309 malaria; *P. falciparum* and *P. vivax* infection. The stimulation of PBMCs from
310 acutely infected *P. vivax* patients with crude antigen accelerated the expansion of
311 lymphocytes. $CD4^+$ T cells were the predominant population response to *P. vivax*
312 antigens. Quite different from the finding in *P. vivax*, we found that T cells ($CD4^+$,
313 $CD8^+$, $\gamma\delta^+$ T cells) of acute *P. falciparum* patients did not significant response to
314 crude schizont antigens. $CD4^+$ T cell were significant reduced in acute *P. falciparum*
315 patients, indicating the impairment in the role of T cells during infection. The
316 assessment of B-cells and antibody response in malaria patients showed that B-cells
317 play a major function during *P. falciparum* and *P. vivax* infection. The small
318 expansion of B-cells upon crude antigen stimulation showed anti-*P. vivax* antibody
319 levels significantly higher than in medium control. Similarly, the significant
320 expansion of B-cells upon *P. falciparum* stimulation resulted in induces high anti- *P.*
321 *falciparum* antibody levels. Some strategies occur beyond the expansion of
322 lymphocyte as we showed the elevation of $CD4^+$ T-cell apoptosis in acutely infected
323 *P. falciparum* and *P. vivax* patients and after stimulation with crude schizont antigens.

324 There has been reported the mitogenic property of Plasmodium. The stimulation of
325 PBMCs from naive controls with crude *P. falciparum* and *P. vivax* antigens showed
326 that high percentage of divided cells in cultures with crude *P. falciparum* antigens
327 which support in line with previous studies [5]. Contrastly, small expansion of
328 lymphocyte was obtained in *P. vivax* cultures. B-cells were the population responded
329 to *P. falciparum* antigen, approximately 12 % of B-cell expanded in stimulation. This
330 suggests the presence of mitogenic component in infected erythrocyte of *P.*

331 *falciparum* whereas it lowly contain in *P. vivax* parasite. Therefore, our study support
332 the phenomenon; high level of immunoglobulin in Plasmodium infection [2, 6, 10].

333 The natural response of lymphocytes during *P. vivax* infection was produced by
334 stimulation of *P. vivax*-exposed PBMCs with crude antigens. Lymphocyte of *P. vivax*
335 patients expanded after stimulation of patient PBMCs with crude *P. vivax* antigens.
336 This result was associated with the elevation of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells.
337 Interestingly, $CD4^+$ T cells were the predominantly responded to *P. vivax* antigens. In
338 association with the previous study, we have shown that memory $CD4^+$ and $CD8^+$ T
339 cells pool in malaria immune person were maintained and activated during acute and
340 convalescent *P. vivax* infection patients [13]. Therefore, the elevation of $CD4^+$, $CD8^+$
341 and $\gamma\delta^+$T cells after stimulation of PBMCs with malaria antigen can be explained as a
342 model for the expansion of memory or effector cells in re-infection or relapsing with
343 *P. vivax* parasites. In the other hands, immunity induced by *P. vivax* lead to Treg and
344 IL-10 secreting T cells activation during acute infection The activation of Treg can
345 suppress Th1 or Th2 function and support parasites growth in human malaria
346 infection [12, 33]. Interestingly, the number of Treg in individual immune control
347 living in *P. vivax* area was lower than naive controls [12]. This phenomenon indicated
348 that the repeated infection does not boost Treg response. Therefore, the activation of
349 Treg during acute infection is one strategy of parasites for immune suppression and
350 limiting the generation of memory T cells. Taken together, our study clearly support
351 that the repeated exposure to *P. vivax* increased population of memory T cells as well
352 as regulatory T cells. The particular *P. vivax* schizont antigen or specific epitopes
353 induce memory T cells or Treg should be identified for the future development of

354 successful vaccine. Moreover, it is not only the phenotype and the level of T cell
355 response but also the function of responded T cells for parasite killing as well as the
356 longevity of responded T cell after stimulation with *P. vivax* antigens need to further
357 study.

358 Immunity induced by *P. falciparum* significantly suppressed lymphocyte
359 proliferative response during infection [4, 29]. Here, we identified lymphocyte levels
360 in acutely infected *P. falciparum* patients. The result showed significantly reduction
361 of $CD4^+$ T cells in patients compared with naive controls. Stimulation of *P.*
362 *falciparum* exposed PBMCs with antigen showed no significant expansion of $CD4^+$,
363 $CD8^+$ and $\gamma\delta^+$ T cells, indicating the impairment of boosting activated T cells in
364 repeated infection. This phenomenon can be explained the limitation of memory T
365 cell pool in residence *P. falciparum* areas. People naturally exposed to malaria
366 infection frequently have rather weak cellular and humoral responses to vaccine
367 candidates; circumsporozoite surface protein (CSP) and merozoite surface protein-1
368 (MSP-1) [16, 28]. One explanation could be the polymorphic of immunodominant T-
369 or B-cell epitopes. Therefore, the induction or maintenance of T cell response to
370 malaria antigen in general or to specific malaria antigen is difficult. Further, malaria
371 antigens induce T cell apoptosis during infection [1, 30]. All strategies indicate the
372 suppression of T cell response during *P. falciparum* infection and explain the taking
373 long time to develop *P. falciparum* immunity in individual in endemic areas. The
374 response of $\gamma\delta^+$ T cells in both naive and patients may reflect the non-specific
375 stimulation of malaria component, phosphoantigen IPP which is support by previous
376 study [22].

377 Antibody response to blood stage antigen is short-lived in children and adult in
378 malaria endemic area [19]. Here, we assessed anti-*P. falciparum* antibodies in acute
379 patients and in cultures after stimulation of PBMCs from acute patient with crude
380 schizont antigens. Anti-*P. falciparum* antibody levels in patients were higher than in
381 naive controls. Further, crude *P. falciparum* antigens induced antibody levels upon
382 stimulation, indicating the presence of memory B cells and they were boosted in
383 repeated infection. Therefore, humoral immunity may play a greater protective
384 function than cell-mediated immunity in *P. falciparum* infection. The finding of B-
385 cell epitopes in crude *P. falciparum* antigens will provide a strategy for vaccine
386 development in the future. In comparison with *P. vivax*, antibody response in acute
387 patients was classified in two groups (high and low responders) based on the antibody
388 levels difference from medium control. High responder increased in antibody
389 response corresponding to antibody level in each patient after *P. vivax* stimulation
390 whereas anti-*P. vivax* antibody in low responder still keep in low levels after
391 stimulation which is associated with our previous finding, anti-P. vivax antibody
392 levels were very low in the acutely P. vivax infected patients [13]. Interestingly, no
393 correlation between the response of $CD19^{+}$B-cells to crude antigens and antibody
394 levels in *P. vivax* simulation has been shown. Patients did not have significant
395 expansion of B-cell after *P. vivax* stimulation developed high antibody levels. This
396 result indicates that the present of antibody may be provided by long-lived plasma
397 cells which rarely circulate in peripheral blood. The re-exposure with *P. vivax* induced
398 plasma cell to produce antibodies. Very low level of anti-*P. vivax* antibodies may be
399 resulted from the impairment in the generation of memory B-cells. One possibility
400 could be the activation of B-cell apoptosis as showed in animal model, the majority of

401 apoptosis cells in the spleen of infected *P. chabaudi chabaudi* AS are B-cells.
402 Moreover, the effect of other immune cells on B-cell function in individual patients
403 should be explained. The significant reduction of PDCs in acute *P. vivax* patients may
404 impair antibody response as showed evidence in influenza virus [14, 24]. The deletion
405 of PDCs abrogated the secretion of specific polyclonal IgG. Further, the significant
406 increase in the percentage of $FOXP3^+$ Treg and IL-10 secretion type I T reg in acute
407 *P. vivax* infection may also result in suppress B-cell response. It has been showed that
408 Treg can directly suppress B-cells or whether they must suppress Th cells to suppress
409 B-cell responses [17]. However, the specific epitopes of *P. vivax* antigens suppresses
410 humoral immunity or induce memory responses could be more explanation during
411 infection.

412 Apoptosis was increased in patient suffering from acute malaria and asymptomatic
413 individuals living in endemic area which represent of chronic infection. High and
414 permanent exposure to the immune system of the *P. falciparum* antigens might induce
415 a chronic state of T cell activation as described in mitogens or superantigens [20]. The
416 relationship between *P. falciparum* exposure and the level of apoptotic cells showed
417 induction or amplification of apoptosis phenomenon. On the other hand, during the
418 course of immune response, T cells clones must be down regulated by inducing
419 apoptosis after antigens are eliminated. Our study carried out the stimulation *in vitro*
420 assay to demonstrate the induction of T cell apoptosis by parasites. We showed that
421 addition of *P. falciparum* to naive and *P. falciparum* patient's culture consistently
422 increased the percentage of $CD4^+$ T cells which was associated with the significant
423 reduction of peripheral $CD4^+$ T cells in acute patients and low $CD4^+$ T cell expansion
424 in stimulation cultures whereas apoptosis cell was low in medium and PHA control.

425 Therefore, the elevation of apoptotic cells should be the affect from parasite induction
426 as was shown in previous studies; the addition of *P. falciparum* schizont-rich extract
427 induced lymphocyte apoptosis *in vitro* [30]. The frequency of apoptotic cells in the
428 spleen cells infected with *P. chabaudi chabaudi* increased > 50 % during parasite
429 growth was seen to be under control [27]. Taken together, these data suggest that the
430 induction of T cell apoptosis is one mechanism of immunosuppression by *P.*
431 *falciparum* parasites. Unlike acutely infected *P. vivax* patients, $CD4^+$ cells apoptosis
432 increased in naive and *P. vivax* patient cultures in association with the expansion of
433 $CD4^+$T cells clones as well as boosting of humoral immunity. Therefore, the induction
434 of T cell apoptosis in *P. vivax* infection could be a regulating mechanism of immune
435 response; turning off immune system. The induction of T cell apoptosis when specific
436 T cells clones were activated and parasite were eliminated.

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570 **Figure legend**

571 **FIG. 1.** Comparison of lymphocyte response in *P. falciparum* and *P. vivax* infection.
572 PBMCs from acutely infected *P. vivax* and *P. falciparum* patients were stimulated
573 with PvSE and PfSE at 1, 10 and 100 μg, respectively. Media and PHA are control of
574 stimulation *in vitro* cultures. The percent of divided cells in PvSE or PfSE culture was
575 obtained from the total of divided cells in PvSE or PfSE cultures subtracted with
576 divided cell in uRBC stimulation. Each point represents the percent of divided cell in
577 each individual. Horizontal bars represent mean value for each group. ● = *P.*
578 *falciparum* patients, ○ = *P. vivax* patients, ▲= naive controls. Numbers along the
579 bottom represent the amount of crude *P. falciparum* or *P. vivax* antigen: 1, 10, 100
580 showed 1 μg, 10 μg and 100 μg of PvSE or PfSE, respectively. M= media controls
581 Asterisk indicate statistical significant at $P < 0.05$ with the non-parametric two
582 independent test.

583

584 **FIG. 2.** Phenotype of T cells upon *P. falciparum* or *P. vivax* stimulation. PBMCs
585 from naive control acutely infected *P. vivax* and *P. falciparum* patients were
586 stimulated with 100 μg PvSE and 100 μg PfSE, respectively. Media and PHA act as
587 stimulation control. The percentage of $CD4^+$ T cells (a), $CD8^+$ T cells (b) and $\gamma\delta^+$ T
588 cells (c) was obtained from the percent of T cell stimulated by malaria protein
589 subtracted with uRBC stimulation. The data is shown as mean values of eight
590 experiments from naive controls or malaria infected patients. Asterisk indicate
591 statistical significant at $P < 0.05$ with the non-parametric two independent test. □ =
592 un-stimulation; ■ = stimulation.

593

594 **FIG. 3.** The response B-cells in *P. falciparum* and *P.vivax* infections. PBMCs from
595 naive controls and acutely *P. vivax* or *P. falciparum* infected patients were stimulated
596 with 100 μg PvSE and PfSE, respectively. Media and PHA act as stimulation control.
597 The percent of B cells upon antigen stimulation was obtained from the percent of B
598 cells stimulated by malaria antigen subtracted with uRBC stimulation. The data is
599 shown as mean values of eight experiments from naive control or malaria infected
600 patients. □ = un-stimulation, ■ = stimulation. Asterisk indicate statistical significant at
601 $P < 0.05$ with the non-parametric two independent test.

602

603 **FIG.4.** Influence of malaria antigen on T cell apoptosis. The percentage of apoptotic
604 cells from *P. vivax* and *P. falciparum* infected patients upon culture with 100 μg of
605 PvSE and 100 μg of PfSE, respectively. Media and PHA were used as a stimulation
606 control. The percentage of apoptotic cells were obtained from the median percentage
607 of apoptotic T cells stimulated by PvSE or PfSE subtracted with uRBC. The data were
608 shown as mean values of eight experiments from naïve controls or acutely infected
609 patients. □ = un-stimulation, ■ = stimulation. (a) $CD4^+$ T cell apoptosis; (b) $CD8^+$ T
610 cell apoptosis. Asterisk indicate statistical significant at $P < 0.05$ with the non-
611 parametric two independent test.

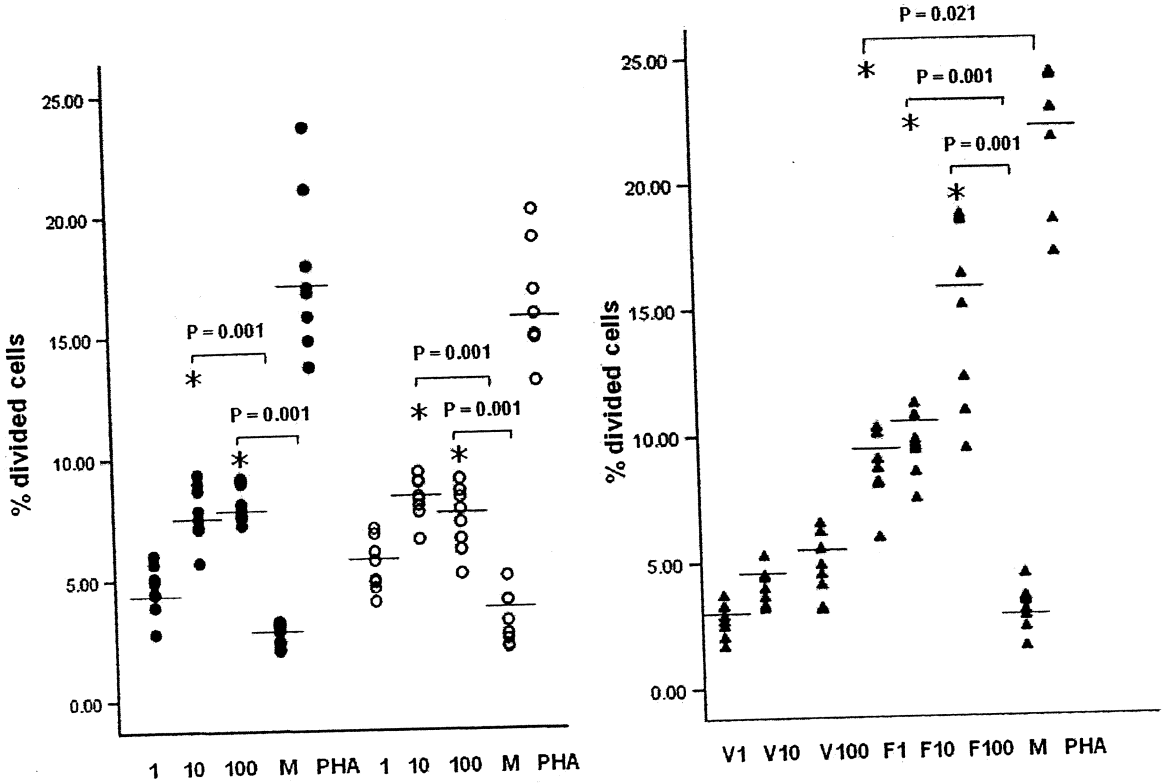
612

613 **FIG.5.** Malaria antigen induced immunoglobulin *in vitro*. Scatter diagram of
614 antibodies in culture supernatant of PBMCs exposed to PvSE or PfSE antigens and

615 patient sera. Dots represent the mean optical density value in triplicate wells for each
616 patients or supernatant. The optical density value was obtained from the optical
617 density value in each patients or PvSE or PfSE stimulation substracted with optical
618 density value in uRBC. Each point represents the optical density (OD) value in
619 individual. Horizontal bars represent mean OD value for each group. (A) Acute *P.*
620 *falciparum* patients, (B) Acute *P. vivax* patients. Symbols; ○ = *P. vivax* or *P.*
621 *falciparum* patients ● = naive controls; and = medium controls. Asterisk indicate
622 statistical significant at $P < 0.05$ with the non-parametric two independent test.

623 (A)

(B)

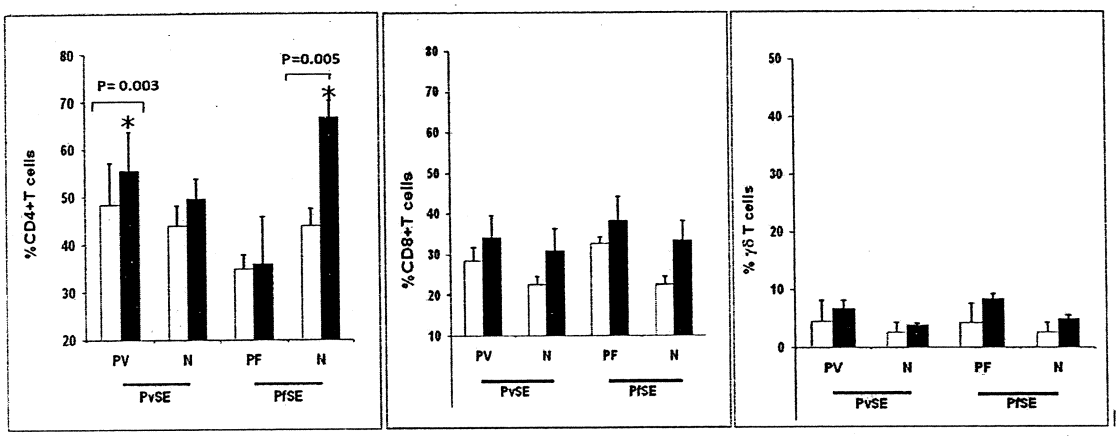


624

625

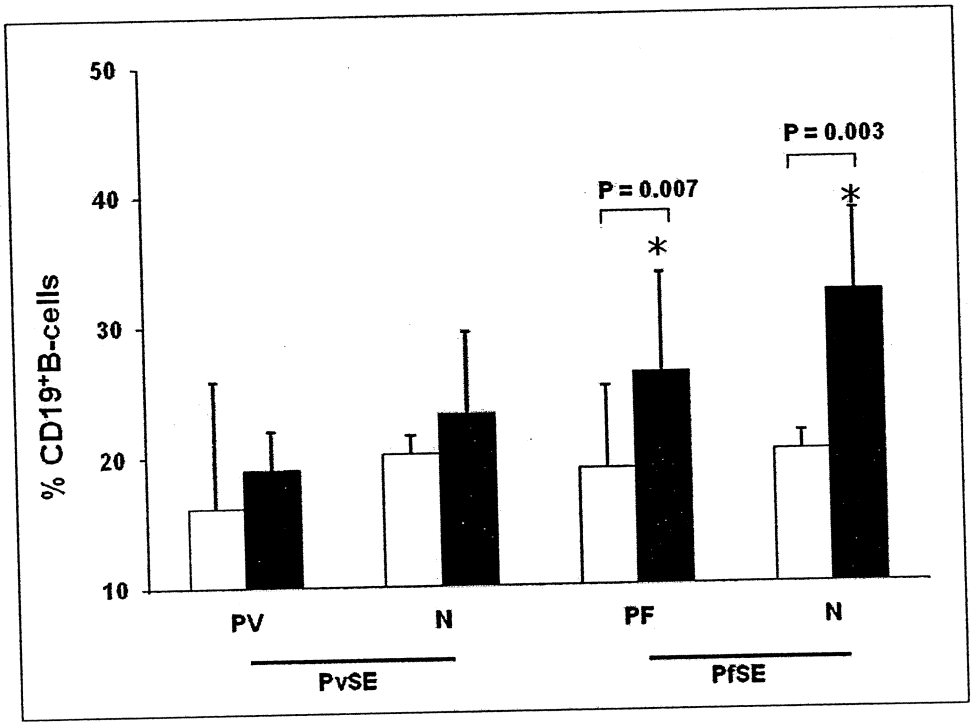
FIG 1

626 (A) (B) (C)



627

628 FIG 2

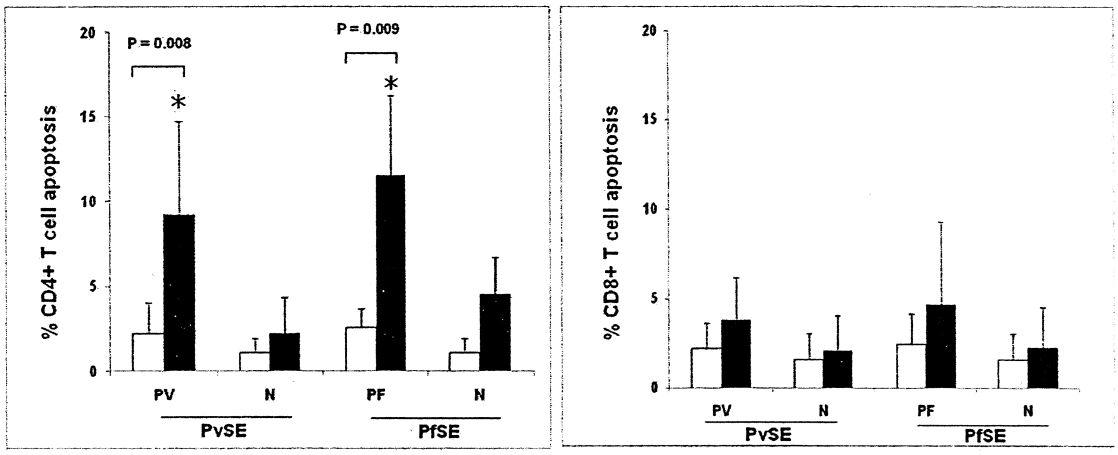


629

630

FIG 3

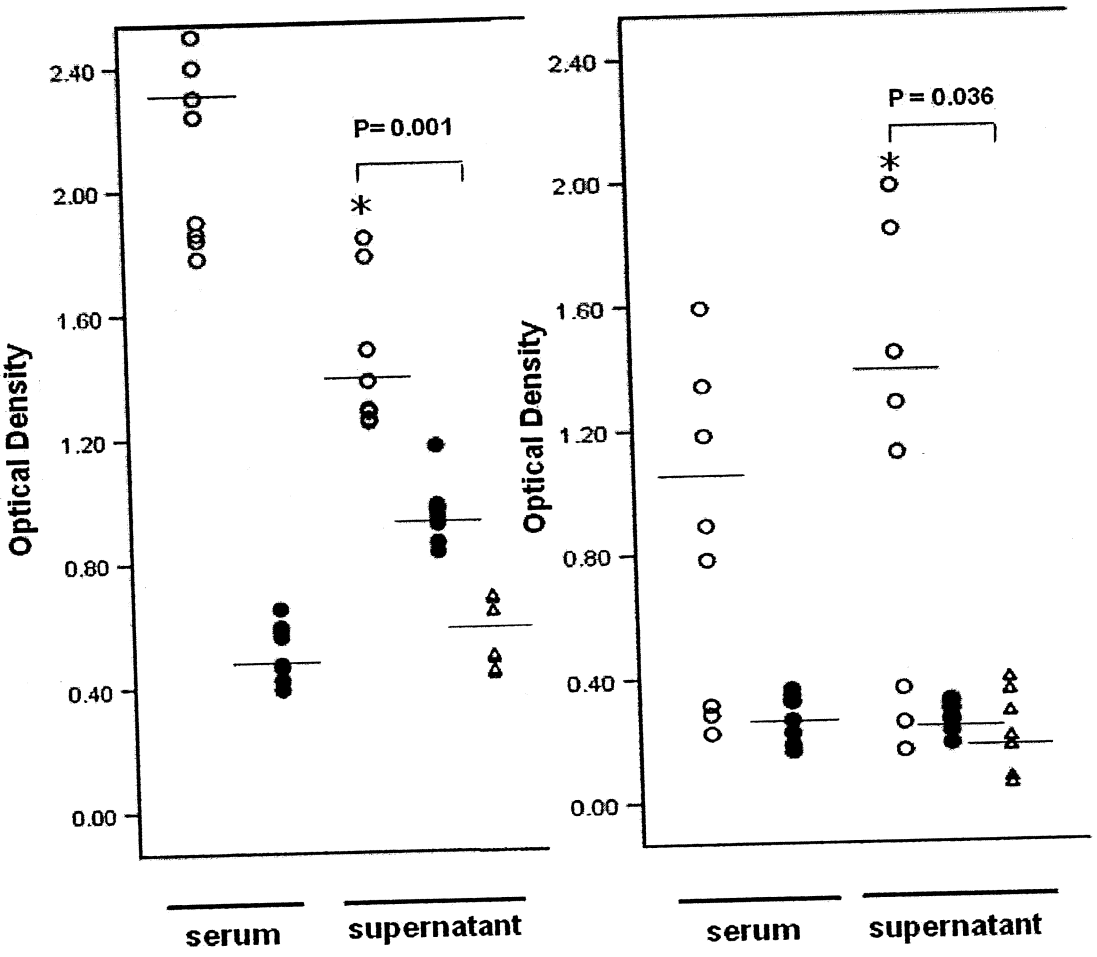
631 **(A)** **(B)**



632

633 FIG 4

634 (A) (B)



635

636 FIG 5

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Immune response to *Plasmodium vivax* infection: A study in the Central of China

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Summary

Immunity induced by *P. vivax* infection may leads to activation of T cells during "relapse" or "re-infection". This study aims to characterize memory T cells, γδ T cells, regulatory T cells, B cells, NK cells and NK T cells in patients with acute *P. vivax* infection living in completely *P. vivax* infection cohort in Central of China. Lymphocytes were collected from patients infected by *P. vivax*, immune controls and naive controls. Four-color flow cytometry were used for determination of cell phenotypes. We found the level of both memory $CD4^+$ and $CD8^+$ T cells were elevated in blood circulation during acute infection. The level of γδ T cells increased twice times comparing with normal controls. This suggests that memory cells and γδ T cells play role in protection against *P. vivax* infection. Our finding showed that B, NK and NK T cells were decreased during acute infection. However, there was the activation of regulatory T cells during acute infection, suggests one immune suppression mechanism by *P. vivax* parasites. However, the suppression mechanisms or the interaction between B, NK and NK T cells during malaria infection has not been elucidated.

Our results provide a further insight into the interaction between *P. vivax* parasites and host cell-mediated immunity to *P. vivax* infection that could be important for the future development of a successful vaccine and anti-malarial drug designation.

Key words: malaria immunity, *Plasmodium vivax*, T cells, B cells, gamma delta T cells, NK cells, NKT cells,

Introduction

Malaria is a common tropical disease associated with mortality among children infected with *Plasmodium falciparum*, mainly in African countries (1). In contrast with *P.falciparum* malaria, benign *P.vivax* infection causes relapsing fever but is rarely fatal (2). This may be because a pattern of unique host-parasite relationship exists during the infection. Until now very few studies have investigated host immunity during *P.vivax* infection. One reason for this could be the difficulty maintaining *P.vivax* cultures as a source for the preparation of parasite materials. Therefore, in the past, progress in *P.vivax* research has been limited. The current incidence of *P.vivax* infection has increased gradually in tropical countries, but whether this is due to a slow development of drug resistance among the *P.vivax* parasites remains to be verified. The collection of more information from areas where *P.vivax* is endemic should be encouraged.

Several studies have been shown the status of natural immune responses against malaria infection in both of cell-mediated and humoral-mediated immune responses against malaria infection in malaria endemic areas around the world. However, this study was performed in a vivax malaria-endemic area of central of China which has a completely *P. vivax* infection cohort unlike other areas having mixed species, for example, in Thailand, Ethiopia (3-5). Recent study has shown increasing of memory T cells among P. vivax infected patients in endemic areas of Thailand and this can be maintained during 2 months follow up (6). Another study has found that the infection by *P. vivax* infection caused of increasing of TH2 cells (7). This was in contrast with the recent study performing in Ethiopia, which has found that malaria parasites both *P. falciparum* and *P. vivax* has no affected to the percentage of CD4, CD8, CD3 and B cells. However, these finding have shown the status of cell mediated immune response in *P. vivax* infection among mixed species endemic areas.

Our study aimed to characterize the acquired cell-mediated immunity following *P.vivax* infection who living in completely *P. vivax* infection cohort without other *Plasmodium. spp* infection, and aim of establishing the nature of host responses in relation to the severity of the *P.vivax* infection.

Materials and Methods

2.1 Study population

Blood samples were collected from 37 patients with acute *P. vivax* infections (AC) at Wuhe County Hospital, Guzhen County Hospital, and The First Hospital of Bengbu in Anhui province, China. The diagnosis of *P. vivax* malaria infection was based on the examination of Giemsa-stained thick blood films. Polymerase chain reaction (PCR) with species-specific primers was performed on DNA isolated from the blood samples to verify *P. vivax* infections. Only *P. vivax* infected patients were recruited in the experiments.

Blood samples were collected from additional 21 peoples residing in the same *P. vivax*-endemic area. These subjects serving as "immune controls" (IC) did not have acute *P. vivax* infections at the time of blood collection as determined by both microscopy and PCR assay. A further 17 healthy adults living in Bengbu city area without previous malaria exposure or antibodies to malaria parasites were recruited to serve as "naïve controls" (NC). The clinical characteristics of the subjects are listed in Table 1. This study was approved by Ethical Approval Committee of Biomedical Institute of Anhui Medical University. Informed consents were obtained from each individual before a blood sample was taken.

2.2 Preparation of peripheral blood mononuclear cells (PBMC)

Venous blood from *P. vivax* patients, immune controls, and naïve controls were collected in heparinized tubes and PBMC were separated by gradient centrifugation using Ficoll-Hypaque lymphocyte isolation (TIAN JI HAO YAO BIOLOGICAL MANUFACTURE, Tianjing, China) according to the manufacturer's recommendation. The PBMC pellet was resuspended at a concentration of 10^6 cells/ml in RPMI-1640 (GIBCO, Carlsbad, USA) supplemented with 10% heat-

inactivated fetal calf serum (FCS) (HANGZHOU SIJIQING ORGANISM ENGINEERING MATERIALS, Hangzhou, China) and number of PBMC was counted.

2.3 Parasite cultures and antigen preparations

P. vivax-infected red blood cells (iRBC) purified from infected blood were used as antigens for in vitro stimulation. Briefly, *P. vivax* infected blood was depleted of white blood cells by filtering through a sterile column of CF11 cellulose (Whatman®, Maidstone, UK) and the red blood cells were washed with RPMI-1640 by centrifugation at 1190 g for 5 minutes. The parasites were cultured for 24 - 30 hours at 5% hematocrit in McCoy's 5A medium (GIBCO) supplemented with 25% human AB serum. For *P. falciparum* infected blood, a buffy-coat separation was used to get rid of the white blood cells and the parasites were cultured at 5% hematocrit in RPMI1640 medium supplemented with 10% human serum. Both *P. vivax* and *P. falciparum* parasites were maintained in an incubator containing 5% CO_2, 5% O_2 and 90% N_2 until matured to schizont stage (≥6 nuclei). The late stage iRBC were enriched by centrifugation using 60% Percoll (GE Healthcare, Uppsala, Sweden) at 1190 g for 10 minutes.

The enriched iRBC pellets were sonicated for 40 seconds at 150 watts and the protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, USA). The vials were then aliquoted and stored at -70°C until use. Uninfected RBCs were processed similarly as above and the protein concentration equal to the malaria antigens was stored at -70°C to be used as a negative control.

2.4 *In vitro* stimulation

PBMC from other 5 healthy donors were used for *in vitro* stimulation. PBMC in RPMI-1640 supplemented with 25 mM HEPES, 2 mM glutamine, 40 μg/ml gentamicin and 10% heat treated FCS were cultured at $2x10^5$ cells/well in round bottom 96 well plate (Costar, Corning, USA) for 5 days in humidified incubator at 37°C in 5% CO_2, in the presence of *P. vivax* antigen or *P. falciparum* antigen at a concentration of 1, 10, 50 μg/ml. Medium alone, equivalent concentration of RBC and 2 μg/ml of anti-CD3 [OKT] were used as negative and positive controls, respectively. All samples were performed in triplicates. After 5 days of activation the cells were harvested and added H^3-Thymidine at the concetntration of for 16-18 hrs. The proliferation of PBMC was determined by β-counter FJ-353 (262 Factory, Xian, China).

2.5 Surface and intracellular staining and flow cytometric (FCM) analysis

Fifty microliter of whole blood were stained with combination of fluorochrome-conjugated mAbs as showed in Table 2 and the stained cells were incubated for 30 minutes at 4 °C followed by treated with RBC lysing solution by incubating at room temperature for 15 min and washed with PBS (8g NaCl, 0.2g KCl, 1.4g Na_2HPO_4, 0.24g KH_2PO_4, in 1L distilled H_2O). After staining, the cell pellets were analysed by FACSCalibur using the CELLQuest software (Becton Dickinson, San Jose, USA) .

For intracellular staining, cells were washed with a permeabilizing solution according to the manufacturer's recommendation (BioLegend, San Diego, USA). The cells were incubated in the permeabilizing buffer for 20 minutes at room temperature. Then Alexa fluor® 488-labeled anti-Foxp3 (BioLegend, San Diego, USA) was incubated for 30 minutes at room temperature after which the cells were washed with PBS. Cells were fixed with 2% of Paraformaldehyde in PBS for acquisition and analysis on FACSCalibur.

2.6. Data analysis

All data were analyzed by the SPSS programme (Version 11.5, Chicago, USA). The percentages of cell phenotypes were log transformed. Parametric analysis independent *t* test was then performed on the transformed data as follows; The difference of mean percentages of cell phenotypes among each following groups i.e. naïve controls vs. immune controls, naïve controls vs. acute infection, immune controls vs. acute infection. Data in the results and tables showed median and interquartile ranges (25^{th}-75^{th} percentile). Data in in vitro study showed mean ± 2 standard errer (SE). The results also showed mean difference (MD), 95% Confident Interval (CI) and P value. The results were considered significant at $P<0.05$.

Results

T cells phenotyping

The CD4+ memory T cells were quantified and the results showed that the median for the percentage of CD4+CD45RO+ T cells in the acute infection (27%) was significantly higher than in the naive controls (20%) (MD=8.8, 95%CI=0.5-17.1, P=0.02), however, this was not significant difference when compared with immune control (22%) (P=0.1).

The proportion of CD8+ memory T cells was not significantly higher among immune controls (18%) comparing with native control (13%) (P=0.2). Similar raised proportion of memory CD8+ T cells was also seen in patients with acute infection (17%) (P>0.05).

The result of percentage of γδ T cells showed that the median of CD3+δ2+ was statistically significant higher in the acute infection (4%) comparing with naïve controls (2%) (MD=2.1, 95%CI=-0.1-4.2, P=0.03) and immune controls (2%) (MD=3.2, 95%CI=1.0-5.4, P=0.005).

The level of CD3−CD19+ B cells in blood circulation was detected. The median of levels was significantly lower in immune controls (6%) comparing with naïve controls (9%) (MD=3.5, 95%CI=0.9-6.0, P<0.001). However, after infection this level was higher (9%) to the same level as showed in naïve controls (9%).

Among CD3+ T cells, the percentage of regulatory-T cells expressing $CD4^{+}CD25^{hi}$ was quantified and showed that the significant higher among immune controls (3%) than healthy controls (2%) MD=0.9, 95%CI=0.1-1.7, P=0.02). Moreover, the level was significantly elevated in acute *P. vivax* infection (4%) comparing with those of the healthy controls (2%) (MD=2.0, 95%CI=0.7-3.0, P=0.002), and the immune controls (3%) (MD=1.3, 95%CI=0.9-2.7, P=0.03).

And among CD4+ T cells, the expression of $FOXP3^{+}$ on $CD4^{+}CD25^{hi}$ T cells was examined and analyzed to verify the regulatory type-T cells (8). However, we found in contrast result with

$CD4^+CD25^{hi}$, The median level of $CD4^+CD25^{hi}FOXP3^+$ T-cells in the acutely infected patients were not significant lower in acute infection (7%) (P=0.2) and the immune controls (10%) (P=0.1) comparing with the naïve controls (14%). We also found a few cases (7%) of *P. vivax* infected patients and 6% of immune villagers has very high level of $CD4^+CD25^{hi}FOXP3^+$ T-cells (>30%) which we will not use these data to calculate.

The percentages of NK and NKT cells were identified. The percentages of NK cells were not significant decreased after infection (6%) comparing with immune controls (15%) and naïve control (16%) (P>0.05), this could be the high level of NK cells in some immune controls and acute patients. However, in contrast to NK cells, the percentages of level of NKT cells showed the significantly low during acute infection (3%) comparing with immune controls (2%) (MD=1.4, 95%CI=-0.5-3.0, P=0.05) and naïve controls (5%) (MD=1.4, 95%CI=-0.2-3.0, P=0.03).

In vitro activation

The PBMC from *P. vivax* infected patients at acute infection and followed up on day 7 and day 14 was co-cultured with difference concentration of *P. vivax* or *P. falciparum* antigens. We used *P. falciparum* in parallel with *P. vivax* crude antigen to determine the cross activation and reaction between species. We found the obviously difference no. of count per minute by using H3-thymidine between co-cultured PBMC with nRBC, *P. vivax* and *P. falciparum* crude antigen at 10 ug/ml. Hence, we used only this concentration for further experiments (data not shown). The results showed that PBMC from *P. vivax* infected patients at day 0 after infection had highest proliferated (5781), this levels was similar to positive controls (5396) (Figure 3).

All patients's PBMC at day 7 folowed up had no response with any stimulator even anti-CD3 OKT. After day 14 followed up, PBMC were proliferated by co-culturing with both *P. vivax* and *P. falciparum* antigen. There was no responding to *P. falciparum* crud antigen in any PBMC activation.

Discussion

The objective of this work was to characterize T cell phenotypes in patients during acute *P. vivax* infection among Chinese population living in Central of China, north areas of Anhui Province. Our major finding showed that upon acute infection, both CD4+ and CD8+ memory T cells were elevated in blood circulation. However, there was only CD8+ memory T cells maintained at elevated levels in immune individual of P. vivax infection, During infection there also were γδ T cells increased. Our result also showed the activation of regulatory T cells during acute infection, this also one immune suppression mechanism by *P. vivax* parasites.

Our finding showed that $CD4^+$ memory T cells were elevated in acutely *P. vivax* infected patients. This result in line with recent study performed in Western border of Thailand which has shown $CD4^+$ memory T cells were elevated during *P. vivax* infected patients and convalescent period among Thai population (6). However, those levels of Thai patients (38%) were higher than Chinese patients (27%) for memory $CD4^+$ T cells and the level of memory $CD8^+$ T cells was also higher among Thai patients (26%) than Chinese patients (17%). In human, $CD4^+$ T cells that express CD45RO have been detected in the peripheral blood circulation of persons who treated by attenuated *P. falciparum* sporozoites. The study has shown that the $CD4^+$CD45RO T cells produced IL-4 and play role in the protective immunity against *P. falciparum* infection (9). This study has been confirmed by recent study, showed that memory $CD4^+$ T cell ($CD45RO^+CD27^-$) T cell produce IL-4, and shown in long-term protection against *P. falciparum* infection (10). The finding that there were high levels memory T cells during acute infection, suggests that memory T cells were not suppressed by the parasites. The reasons why the acutely infected patients had such high levels of memory cells might be due to previous infection by *P. vivax* parasites.

Our finding showed that the expression of memory $CD8^+$ T cells was increased during the acute infection, suggests that cytotoxic memory T cells are activated by the parasite. These memory

$CD8^+$ T cell responses could have been influenced by an earlier exposure to liver stage antigens or by cross-presentation of blood stage antigens, since the similar elevated levels of memory CD8+ T cells in immune controls. However, present study has shown only that cytolytic $CD8^+$ T cells play a role in protection against the malaria liver stage (11) .

A recent study has shown that $CD4^+$ T cells are essential for the differentiation of $CD8^+$ memory T cells (12). This is supported by the finding (13) that T-helper cells play a role in the differentiation and expansion of memory $CD8^+$ T cells. These findings are consistent with our data showing induction of both memory $CD4^+$ and $CD8^+$ T cells during acute *P. vivax* infection. The function of these memory T cells during acute infection could either be through cytotoxicity or they could act as T-helper cells regulating antibody response against the *P. vivax* parasite. We hypothesize that the increased levels of memory T cells may control parasitaemia during acute infection through an influence on P. *vivax* specific antibody responses rather than cytotoxicity. However, the mechanisms whereby the memory cells control *P. vivax* parasites at the liver stage and blood stage await further investigation.

In this study, memory $CD8^+$ T cells were readily detectable in the immune controls. This is most likely due to frequent exposure to *P. vivax* parasites at liver stage, hypnozoite.

γδ T cells have been reported to be elevated in peripheral blood and spleen (14-17) of individuals with acute or convalescent *P. falciparum* infection and also in spleen of mice with malaria (18). An increase of γδ T cells has been observed in non immune *P. vivax* patients during clinical paroxysms (19,20). Moreover, γδ T cells are increased in the spleen of humans who died from cerebral malaria (17). Moreover, recent study has also confirmed that γδ T cells are elevated during acute *P. vivax* infection in Thailand (6). In similar phenomenon was found in our study, we found the level of γδ T cells was elevated during acute infection. So, this indicates that γδ T cells play role against *P. vivax* infection. Recent finding found that γδ T cells kill *P. falciparum* parasites

by cytolytic pathway and our group is studying in *P. vivax* infection (21). *In vitro* stimulation with *P. falciparum* extracts of blood mononuclear cells not previously exposed to malaria also results in γδ T cell activation, with a majority of Vγ9/Vδ2 cells responding (22,23). Activation of γδ T cells from malaria naïve donors inhibit parasite replication in erythrocytes *in vitro* and also induced the production of pro-inflammatory cytokines (24,25), suggesting that these cells have protective function and their mechanism involves both cytotoxic and regulatory functions against malaria infection.

Our finding showed an increase of Treg during acute *P. vivax* infection. In support to a recent study which has shown the increase number of Treg upon *P. vivax* infection among Thai infected patients [submitted], moreover, Treg also increased during *P. falciparum* infection and correlated with higher rates of parasite growth *in vivo* (26). The increased frequency of Treg during an acute *P. vivax* infection may lead to the suppression of protective immune response both of cell- and antibody-mediated immunity, which may result in improved parasite survival. One possible mechanism is Treg cells may directly suppress B cells (27) or the proliferation of Th cells, resulting in down-regulation of IL-2 or IL-4 production by responder lymphocytes and suppression of B cell differentiation to plasma cells. This could be the reason why this study found the decreasing of B cells and the level of anti-*P. vivax* antibody among *P. vivax* infected patients was low during acute *P. vivax* infection (6). However, the interaction among malaria specific T cells, B cells and Treg cells remains poorly characterized.

Recent study has shown that the NK cells from both healthy and *P. falciparum* infected individuals directly lyse parasitised erythrocytes *in vitro* (20,28), and this killing mechanism requires contact between NK cells and *P. falciparum* infected erythrocytes (20,29). Our study showed no significant decreased level of NK cells, our result support the study among Thai population (7), and in Ethiopian population (3). Therefore, the expression of NK cells during acute infection may not

depend on geographical location, genetic factors difference or level of malaria endemicity. Recent studies indicated that NK cells are the first cells in peripheral blood that produce IFN-γ within 18 hours *in vitro* after exposure to infected erythrocytes followed and this activation was dependent on IL-12 (20). That may be the reason that why we found that NK cells level were not changed in peripheral blood during acute infection which was in contrast to gamma delta T cells. Our result suggests that NK cells protect earlier against *P. vivax* infection than gamma delta T cells and NKT cells.

In vitro stimulation of PBMC from *P. vivax* infected patients at day 0 and showed higher level of proliferation after 5 days co-cultured with *P. vivax* then developed higher again at day 14. One explanation for this result could be the patients did not well develop immune response against malaria antigens during first week infection. We did not find the cell proliferation by *P. falciparum* antigen, this could be there was no cross-reactivity between *P. vivax* and *P. falciparum* antigens. Moreover, our study also confirmed the specificity to *P. vivax* antigen, since this area of central of China is *P. vivax* endemic area and only *P. vivax* prevalence.

In conclusion, our findings showed *P. vivax* infection induced the increasing memory T cells, particularly the CD8 phenotypes and γδ T cells, could play significant roles in the development of cell-mediated immunity against *P. vivax* infection. The decreasing of NK and NK T cells suggests that the early protection by NK and NKT cells caused of the transients activation after infection. Another reason may be explained by the increasing of regulatory T cells during acute infection, caused of the suppression of NK and NKT cells. Moreover, the suppression by regulatory T cells could be affected to the reduction of B cells levels during acute infection. However, the interaction between regulatory T cells and NK, NKT and B cells need to be elucidated.

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Table 1.

Information and Clinical data of *P.vivax* patients, immune and naïve controls *

	N	Sex		Age	Parasitemia	Temperature
		M	F	(years)	(%)	(°C)
P. vivax infection	37	10	17	29±10	0.05±0.08	38.5±0.7
Immune controls	21	11	6	33±11	0	36.5±0.5
Naïve controls	17	10	11	29±5	0	36.5±0.5

*mean ± standard deviation (SD)

Table 2.

List of the combination of fluorochrome-conjugated with anti-human mAbs were used for FCM analysis

	Combination of fluorochrome-conjugated mAbs			
Tube no.	FITC	RPE	RPE-Cy5	APC
1	anti-CD45RO[a]	anti-CD4[a]	anti-CD8[a]	anti-CD3[a]
2		anti-CD19[a]	anti-CD3[a]	
3	anti-gamma9[b]		anti-CD3[a]	
4	anti-CD56[a]		anti-CD3[a]	
5	anti-Foxp3[c]	anti-CD25[b]	anti-CD3[a]	

[a]Caltag, Burlingame, USA; [b]Immunotech, Marseille, France,

[c] This mAb was conjugated with Alexa fluor® 488 (BioLegend, San Diego, USA)

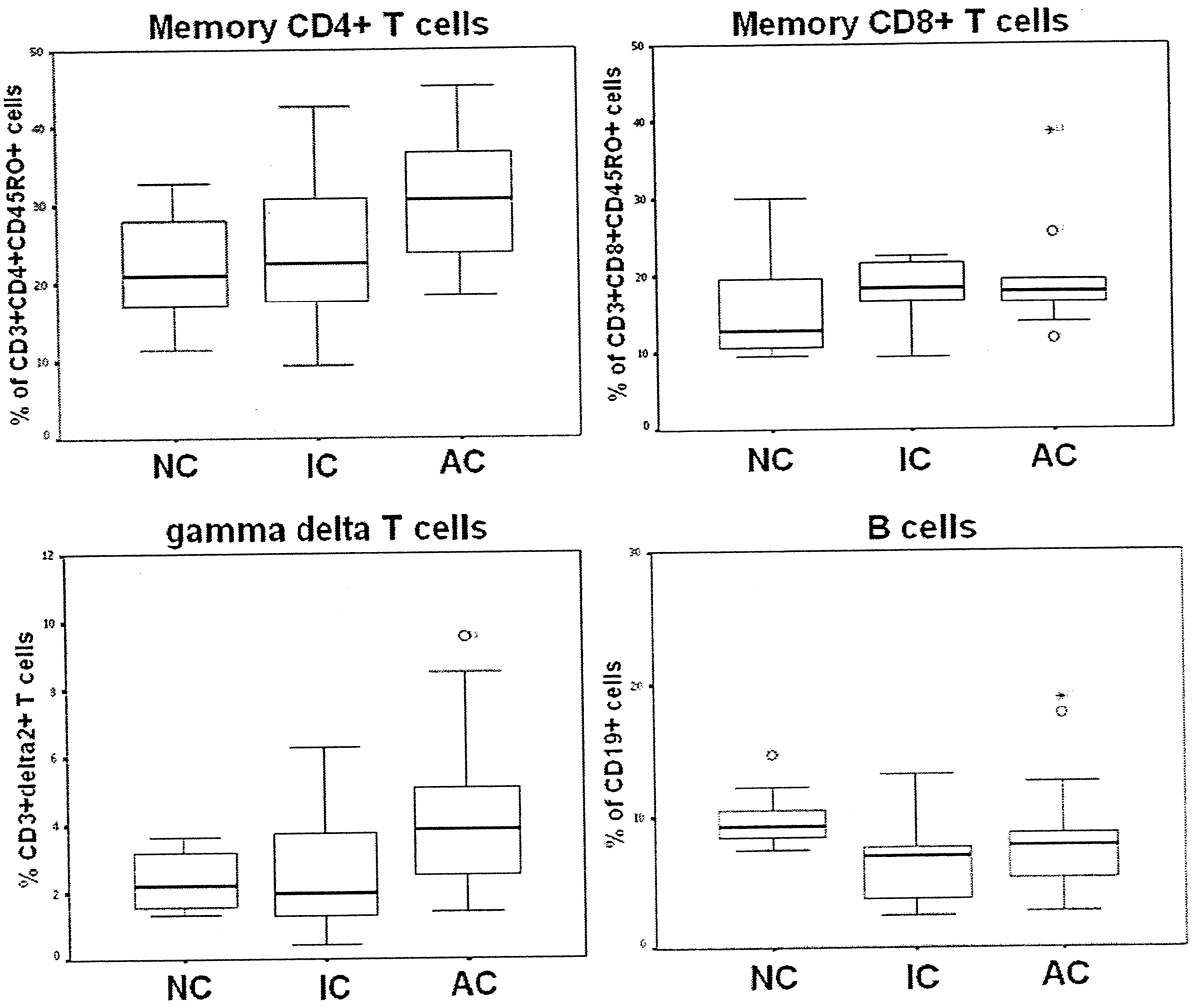
Memory CD4+ T cells
% of CD3+CD4+CD45RO+ cells
NC
IC
AC
Memory CD8+ T cells
% of CD3+CD8+CD45RO+ cells
NC
IC
AC
gamma delta T cells
% CD3+delta2+ T cells
NC
IC
AC
B cells
% of CD19+ cells
NC
IC
AC

Figure 1

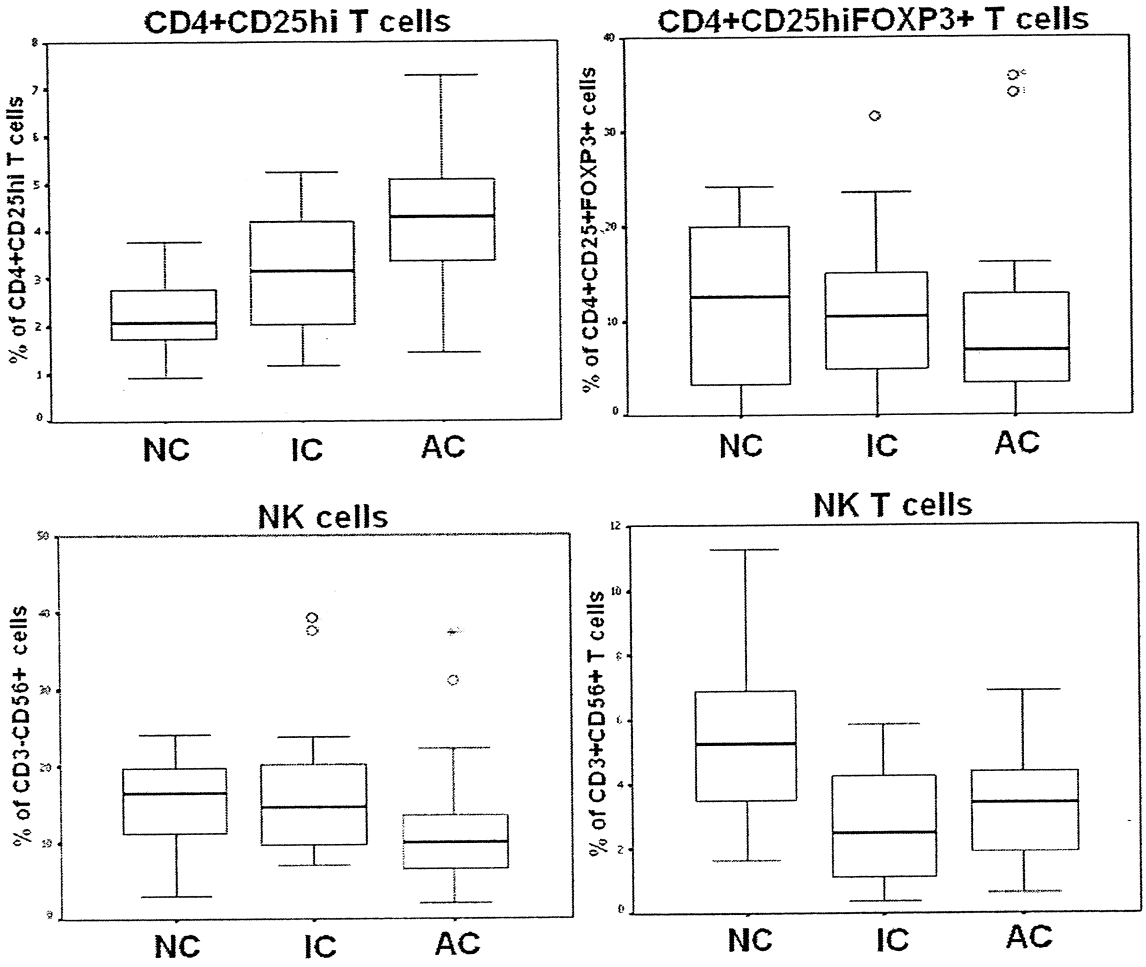
CD4+CD25hi T cells
% of CD4+CD25hi T cells
NC
IC
AC
CD4+CD25hiFOXP3+ T cells
% of CD4+CD25+FOXP3+ cells
NC
IC
AC
NK cells
% of CD3-CD56+ cells
NC
IC
AC
NK T cells
% of CD3+CD56+ T cells
NC
IC
AC

Figure 2

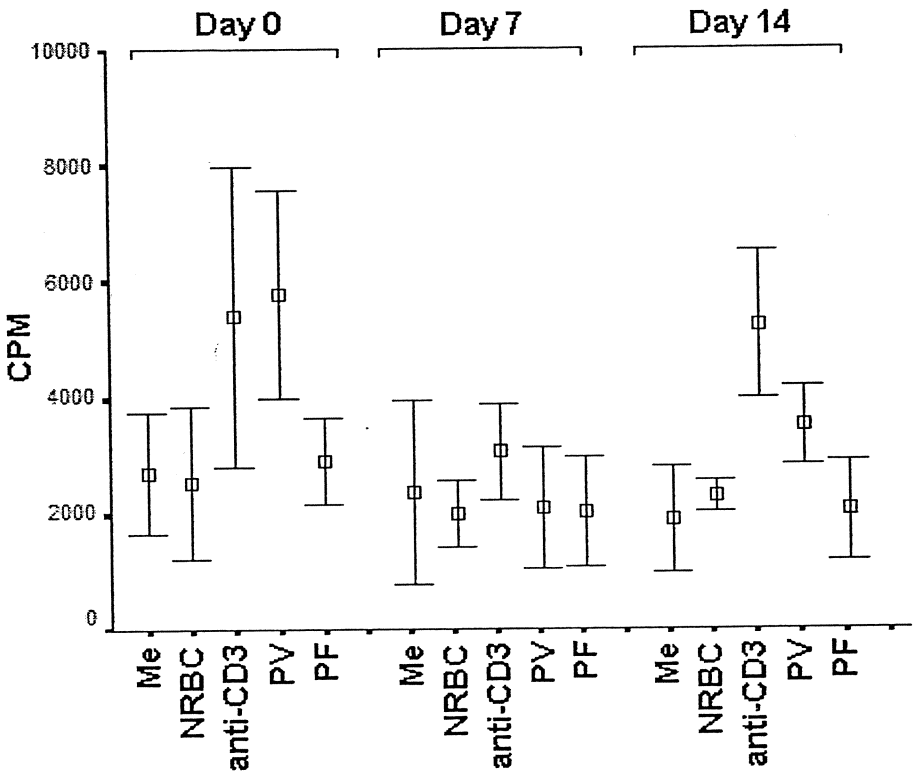
Day 0
Day 7
Day 14
10000
8000
6000
4000
2000
0
CPM
Me
NRBC
anti-CD3
PV
PF
Me
NRBC
anti-CD3
PV
PF
Me
NRBC
anti-CD3
PV
PF

Figure 3

Natural immune response against *Plasmodium vivax* c-terminal merozoite surface protein-1 (MSP-1) and apical membrane antigen-1 (AMA-1) and cross-reactivity between *P. vivax* and *P. falciparum* infection: A study in Thailand and China

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Introduction

Malaria is a common tropical disease associated with mortality among children infected with *Plasmodium falciparum*, mainly in African countries [1]. In contrast with *P.falciparum* malaria, benign P.vivax infection causes relapsing fever but is rarely fatal [2]. In spite of the current incidence of P. vivax infection, the mechanism of cellular and humoral immune responses against P. vivax parasite remained poorly understood. Most study of human malaria immunity is in P. falciparum infection. The reasons for this could be the difficulty to access P. vivax-infected cases and to maintain the parasite in vitro for studies. Therefore, progress in P. vivax research has been limited. Immunity to *P. vivax* has been studied in various endemic areas. Antibody response to different parasite proteins is reported [3-5]. However, only few recombinant proteins can be used due to lack of the parasite materials [6]. The collection of more information from areas where *P.vivax* is endemic should be encouraged.

Malaria infection induces strong humoral immune responses in residents of endemic area. The level of total antimalarial antibodies increases with age and depends on the length and intensity of exposure to malaria. Antibody-mediated inhibition of parasites is more efficient during blood stage than in liver stage infections [7]. Antibodies also mediate antibody-dependent cellular cytotoxicity and phagocytosis involving polymorphonuclear cells, neutrophils or platelets [8].

This study was performed in 1) a vivax malaria-endemic area of central of China which has a completely *P. vivax* infection cohort 2) malaria endemic area of western border of Thailand having mixed *Plasmodium spp.* infection incidence (50.6% was caused by *P. vivax*, 48.9% by *P. falciparum*, 0.1% by *P. malariae*, and 0.4% by mixed infection) [9].

To understand the natural immune response during *P. vivax* infection, we determine antibodies in the sera to proteins extracted from P. vivax parasites, and to P. vivax-derived proteins produced by E.coli system consisting of PvMSP1(19) and PvAMA-1 [10-13]. Our study aimed to characterize the level of IgG antibody following *P.vivax* infection comparing between two malaria endemic areas having difference geography and incidence of *Plasmodium spp.* Infection.

Materials and Methods

Study population

Plasma samples were collected from 76 patients with acute P. vivax infections (AC) at Wuhe County Hospital, Guzhen County Hospital, The Frist City Hospital, Bengbu city, Anhui province, China. The diagnosis of P. vivax malaria infection was based on the examination of Giemsa-stained thick blood films. Polymerase chain reaction (PCR) with species-specific primers was performed on DNA isolated from the blood samples to further verify P. vivax infections.

Blood samples were collected from additional 32 peoples residing in the same *P. vivax*-endemic area. These subjects serving as "immune controls" (IC) did not have acute *P. vivax* infections at the time of blood collection as determined by both microscopy. A further 20 healthy adults living in Bengbu city area without previous malaria exposure or antibodies to malaria parasites were recruited to serve as "naïve controls" (NC). The clinical characteristics of the subjects are listed in Table 1. This study was approved by Ethical Approval Committee of Biomedical Institute of Anhui Medical University. Informed consents were obtained from each individual before a blood sample was taken.

Parasite cultures and antigen preparations

P. vivax-infected red blood cells (iRBC) purified from infected blood was used as crude antigens for coating. Briefly, *P. vivax* infected blood was depleted of white blood cells by filtering through a sterile column of CF11 cellulose (Whatman®, Maidstone, UK) and the red blood cells were washed with RPMI-1640 by centrifugation at 1190 g for 5 minutes. The parasites were cultured for 24 – 30 hours at 5% hematocrit in McCoy's 5A medium (GIBCO,

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Carlsbad, USA) supplemented with 25% human AB serum. *P. vivax* parasites were maintained in an incubator containing 5% CO_2, 5% O_2 and 90% N_2 until matured to schizont stage (≥6 nuclei). The late stage iRBC were enriched by centrifugation using 60% Percoll (GE Healthcare, Uppsala, Sweden) at 1190 g for 10 minutes. The enriched iRBC pellets were sonicated for 40 seconds at 150 watts and the protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, USA). The vials were then aliquoted and stored at -70°C until use. Uninfected RBC were processed similarly as above and the protein concentration equal to the malaria antigens was stored at -70°C to be used as a negative control.

Protein expression and purification

PVMSP1 (19)

Briefly, blood filtered paper from isolated P. vivax infected patients was used to get genomic DNA then PCR was used to get MSP1(19) gene by using designed primers in Table. Sequences were cut by using BamHI and XhoI and verified. Verified sequences were cloned into pET28a vector. Protein was expressed in E. coli BL21 by culturing in LB medium. IPTG was used for protein induction and cell pellet was collected and sonicated. Protein was purified by using native condition as described by manufacturer's protocol [Qiagen, USA]. Supernatant was collected and protein was purified by Ni-NTA beat (bind to 6His-tag). Finally, protein was eluted and checked expression by SDS-PAGE.

PVAMA1

PCR was used to get PVAMA-1 gene by using designed primers in Table. Sequences were cut by using NdeI and XhoI enzymes and verified. Verified sequences were cloned into pET22b vector. Protein was expressed in E. coli BL21 by culturing in LB medium. IPTG was

used for protein induction and cell pellet was collected and sonicated. Protein was purified by using denaturing condition as described by manufacturer's protocol [Qiagen, USA]. Supernatant was collected and protein was purified by Ni-NTA beat (bind to 6His-tag). Finally, protein was eluted and checked expression by SDS-PAGE.

Results

Naturally anti-*P. vivax* IgG antibody responses after infected with *P. vivax* comparing between Thai and Chinese *P. vivax* infected patients

Anti-Crude PV

There was significantly difference of base line level of IgG among naïve controls against *P. vivax* crude antigen between Chinese (0.07) and and Thai (0.03) (MD=0.07, 95%CI=0.04-0.1, P<0.001). After infection by *P. vivax*, the significant elevation of IgG was found both Chinese (0.85) and Thai (0.14) infected patients. However, *P. vivax* infected patients from Chinese was found the increasing in the level of IgG higher than Thai infected patients. In similar to naïve control, the immune controls among Chinese patients (0.8) had higher level of IgG against *P. vivax* crude antigen than Thai patients (0.07).

Anti-*P. vivax* MSP-1(19)

Base lines of mean level of total IgG to PvMSP1(19) among healthy controls of Thai and Chinese population living outside (naïve controls) and inside (immune controls) endemic area was compared. We found that there was higher levels mean of total IgG among Thai (0.03) than Chinese naïve controls (0.3) (P<0.001). Moreover, the level of antibodies in peoples living in *P. vivax* endemic area was significant different between Thai (0.31) and China population (0.06) (P<0.001). We found two samples of Thai healthy control had IgG to PvMSP1(19) as high as *P. vivax* infected patients as shown in above figure, so we excluded this samples to calculate the value. Among both population, the mean level of total IgG to PvMSP-1(19) was significantly

increased during acute *P. vivax* infection in both of Thai (0.8) (P<0.001) and Chinese (0.5) (P<0.001). Interestingly, we found the significantly higher of these levels among Thai than China P. vivax infected patients (P=0.02).

Anti-*P. vivax* AMA-1

The mean level of total IgG against PvAMA-1 among healthy controls living outside the P. vivax endemic area was very low both of Thai (0.1) and Chinese population (0.03). However, there was the significant higher among Thai than Chinese (P<0.000). There was higher level among Thai (0.2) than Chinese immune comtrols (0.1) (P<0.001). We also found non significant difference between healthy donors among Thai peoples living inside and outside endemic areas (P>0.05), which was contrasted with Chinese (P<0.001). During acute P. vivax infection, there was significant higher comparing with naïve controls ((Thai P=<0.001, Chinese P<0.0.001). However, among *P. vivax* patients population, we found no significant different between Thai (0.3) and China (0.2) population (P=-0.15). We also found a sample of P. vivax infected patient had obviously high level of IgG to PvAMA-1, so we did not use this data to calculate.

Cross-reactivity between *P. vivax* and *P. falciparum* antigens; comparative study between Chinese and Thai *P. vivax* infected patients

To examine the cross-reactivity between *P. vivax* and *P. falciparum* antigens, the *P. vivax* and *P. falciparum* crude antigens were used as coated antigen on the plate. The median percentage of base line level of IgG in naive controls among Chinese was very low (0.003) and significantly lower than that of Thai (0.04) donors (MD=0.07, 95%CI=0.04-0.1, P<0.001). Moreover, we also found the significant higher level of IgG against *P. falciaprum* antigens in

immune controls among Thai (0.18) than Chinese (0.01) (MD=0.6, 95%CI=0.45-0.75). After infection with *P. vivax*, patients have had higher level of IgG against *P. falciparum* (Chinese=0.07, Thai=0.36).

Discussion

In this study, we have evidence that Thai villagers having had *P. vivax* infection and living in the endemic area do not produce high level of crude *P. vivax*-specific IgG [14]. However, in an assay with proteins extracted from the parasites. Since this has been tested with the complex proteins in the crude extract from *P. vivax*-infected erythrocytes, we have confirmed the presence of antibodies to *P. vivax* antigens by testing with recombinat *P. vivax* proteins, i.e. $PvMSP1_{19}$ and PvAMA1, and compared the level of natural antibodies between two *P. vivax* endemic areas, Chinese and Thai. The species specific antibodies could tell us the cross-reactivity between *P. falciparum* and *P. vivax* which lead to the examination of epidemiological status in those areas.

Various immunoglubulins are produced and IgG is the most important [15], Previously, a passive transfer of immune IgG to Gambian children provides protection [16]. The immunity against blood stage of *P. falciparum* infection is associated with class and subclass of IgG antibody [17]. Similarly, IgG1 and IgG3 are predominant among *P. vivax*-infected patients with history of malaria [18]. Recent study has shown anti-*P. vivax* merozoite surface protein 1 (MSP-1) IgG among subjects with distinct degrees of malaria exposure in endemic area. The IgG1 and IgG3 against *P. vivax* are higher among the subjects with a 1 year-exposure period than the group with >19 years of exposure [19]. PvMSP119 is shown to induce immunity in non-human primates [6].

Our study showed the significant lower of base line level of anti-crude *P. vivax* IgG among Chinese than Thai. We found the higher level of anti-crude *P. vivax* IgG in Chinese than Thai immune controls. Moreover, this level was very high in similar to that showed in the acute *P. vivax* infection. This could be the *P. vivax* endemicity in China was more than Thai, even these two area are hypo-endemic area.

However, in contrast to what we found in anti-crude *P. vivax* IgG, the base line level of anti-MSP-1 and anti-AMA-1 IgG was significantly lower in naïve controls of China than Thai. This could be hypothesized that the IgG among Thai donors can recognize a part of peptide of MSP-1 or AMA-1, therefore they can develop IgG against these peptides. Another possible reason could be all of these malaria naïve donors have had malaria experiences, but asymptomatic conditions among Thai healthy donors, therefore, they can develop and maintain the low level of antibodies in their circulation.

Crude *P. falciparum* antigens was used in this study to determine the species specific antibody and the cross-reactivity. We found the very low level of base line IgG antibody among both of healhy controls and immune controls of Chinese donors. After infected with *P. vivax*, Chinese patients also develop a bit of *P. falciparum* antibodies. These results were in contrast to those found in Thai. The higher level of base line of anti-*P. falciparum* IgG was found among healhy and immune controls. Moreover, development of anti-P. falciparum IgG in Thai *P. vivax* infected patients were much more higher than Chinese. This could be suggested the accuracy of species specific of total IgG. Moreover, his could be told us the higher risk or incidence of *P. falciparum* infection in Thailand was more than China. This also could be told us the *P. falciparum* infection experience in their life during staying in malaria endemic area. Therefore,

Thai patients living in malaria endemic area could develop *P. falciparum* antibody and maintain this level in their body.

Acknowledgements

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List of Tables

Table 1 Information and Clinical data of *P.vivax* patients, immune and naïve controls [a]

	No.	Sex		Age	Hct	Temperature	Parasitemia
		M	F		(%)	(°C)	(%)
Chinese Naïve controls							
Immune controls							
Acute *P. vivax*							
Thai Naïve controls	27	15	12	32 ± 12 (15 - 70)	41 ± 3 (37 - 48)	37.5 ± 0.5	0
Immune controls	53	40	13	40 ± 15 (16 - 77)	47 ± 7 (34 - 64)	36.8 ± 0.4 (36 - 37)	0
Acute *P. vivax*	52	32	20	32 ± 12 (15 - 70)	41 ± 6 (30 - 63)	37.8 ± 1.3 (34 - 40)	0.4 ± 0.4 (0.02 - 1.2)

Table 2 the PCR primers

Name	Sequences 5'→ 3' (enzymes)(vectors)
PvMSP1(19) Forward	CG GGATCC aat gtg caa act cag tta tta ac (BamHI) (PET28a)
PvMSP1(19) Reverse	CCG CTC GAG gct gga gga gct aca gaa aac (Xho I)
PvAMA Forward	GGAATTC CAT ATG acc gtt gag aga agc aca cg (Nde I) (PET22b)
PvAMA Reverse	CCG CTC GAG tag tag cat ctg ctt gtt cg (XhoI)

Figure legends

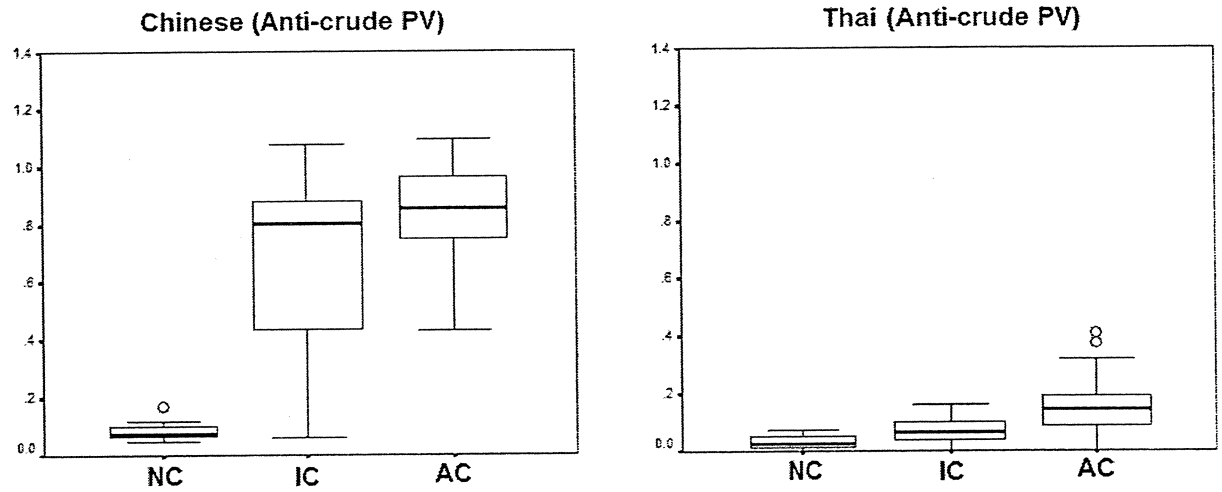

Fig. 1 Absorbance (405 nm) value of IgG antibody reacting with crude *P. vivax* antigens in the naïve controls (NC), immune controls (IC), acute *P. vivax* infection (AC) comparing between Chinese and Thai. Data are shown in median, interquartile ranges (box plots), maximum and minimum (upper-lower lines).

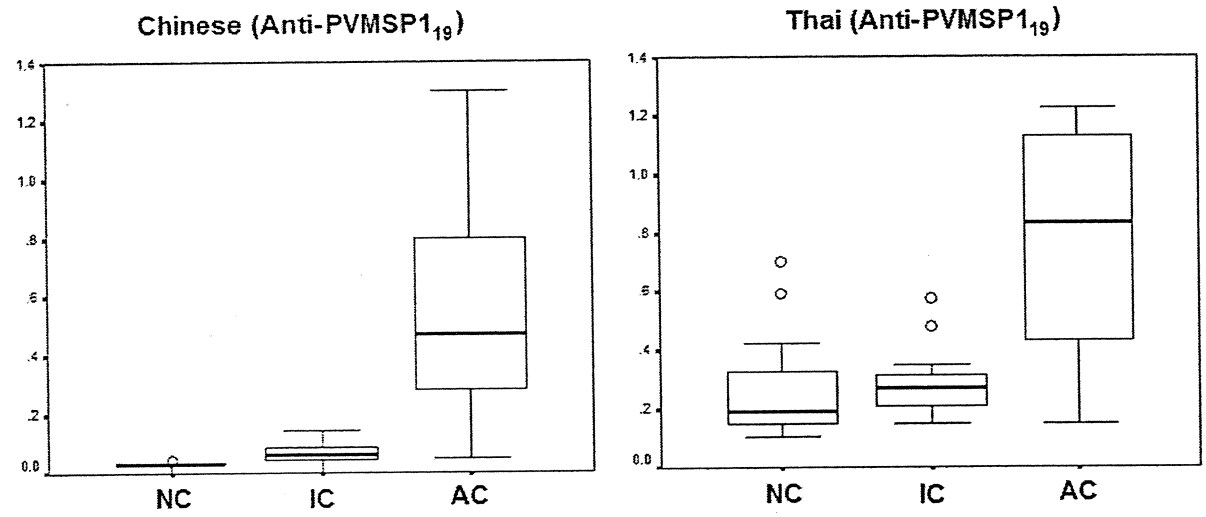

Fig. 2 Absorbance (405 nm) value of IgG antibody reacting with recombinant *P. vivax* MSP-1_{19} protein in the naïve controls (NC), immune controls (IC), acute *P. vivax* infection (AC) comparing between Chinese and Thai. Data are shown in median, interquartile ranges (box plots), maximum and minimum (upper-lower lines).

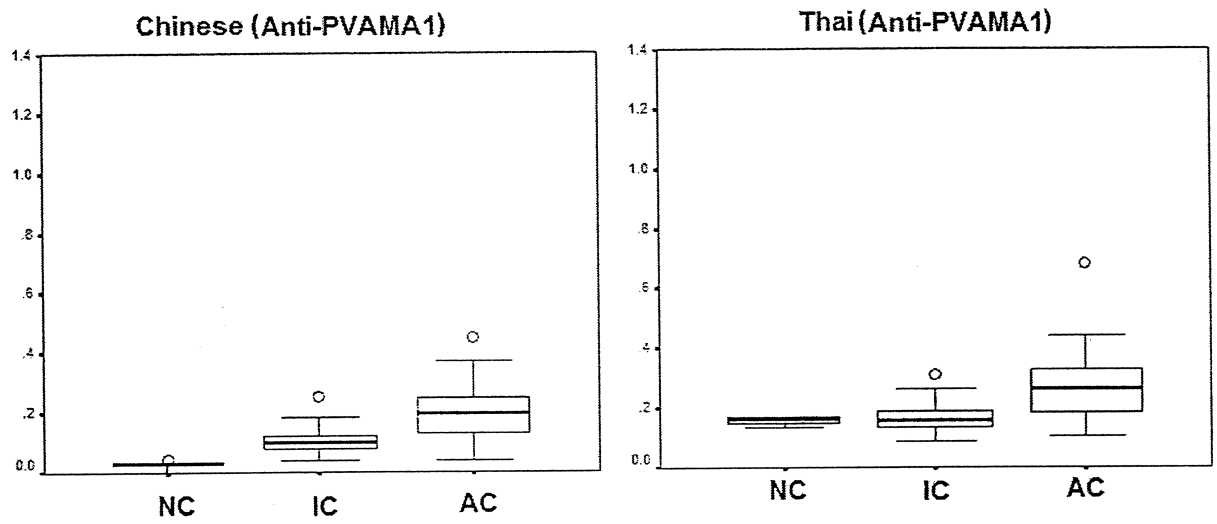

Fig. 3 Absorbance (405 nm) value of IgG antibody reacting with recombinant *P. vivax* AMA-1 protein in the naïve controls (NC), immune controls (IC), acute *P. vivax* infection (AC) comparing between Chinese and Thai. Data are shown in median, interquartile ranges (box plots), maximum and minimum (upper-lower lines).

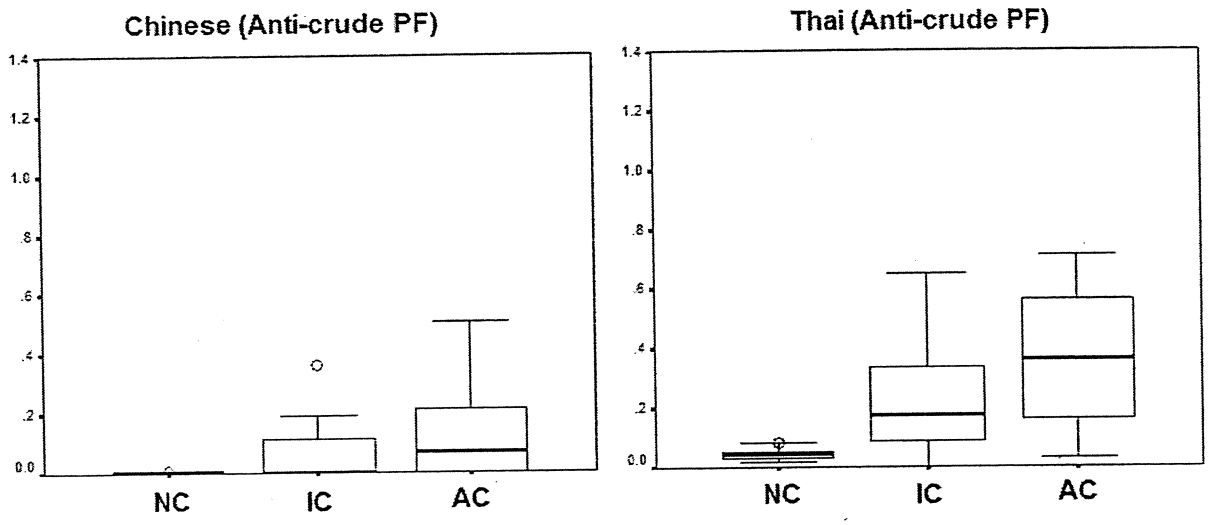

Fig. 4 Absorbance (405 nm) value of IgG antibody reacting with crude *P. falciparum* antigen in the naïve controls (NC), immune controls (IC), acute *P. vivax* infection (AC) comparing between Chinese and Thai. Data are shown in median, interquartile ranges (box plots), maximum and minimum (upper-lower lines).

Killing mechanism of *Plasmodium vivax* parasites by γδ T cells

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Introduction

In Thailand, major malaria endemic areas are located along the Thai-Myanmar and Thai-Cambodian borders. Malaria prevalence in these areas is increased indicating the potential risk of drug resistance. In certain area of Srakaew, *P. vivax* infection is >90% of the infected cases. In most cases, villagers have immunity to the parasites, which may help reducing disease severity. However, this does not prevent subsequent infections. One of the major interests is that how host responds in order to eliminate the parasites, and how possible can the immune response protect host from the infection and severe manifestations. Measuring level of the antibody to *P. vivax* parasite among indigenous villagers of endemic area will be a good parameter for evaluation of the baseline immunity. Cell-mediated immune response, having specificity to a particular antigen of *P. vivax* parasite, elicited during the repeated episodes of infection will determine how efficient the patients can protect themselves from the disease.

T cell activation by malaria parasites plays an important role in elicitation of protective immunity against the disease. Various T cell populations categorized by a number of phenotypic markers are responsible for different functions. Function of various mononuclear leukocytes determines effectiveness of the cell-mediated response to malaria. Depending on the antigen presentation and stimulation of the specific parasite proteins, particular T cell phenotypes will expand. As shown earlier that expansion of gamma/delta T cells play roles in innate immunity to malaria infection (Stevenson and Riley 2004). Our previous study has shown a similar increase of gamma/delta T cells in acute and convalescent *P. vivax* infection (Jangpatarapongsa et al. 2006). However, function of these T cells is not verified, whether they are for or against the *P. vivax* parasite. Antigen-specific T cells recruited during *P. vivax* infection can either eliminate or promote the parasites. There is an immune suppression caused by the presence of *P. falciparum*

parasite during acute infection (Hisaeda et al. 2005). However, there is limited information about how patients primarily respond to acute *P.vivax* infections, and much less understanding about how immune surveillance would be in a carrier having hypnozoites in the liver.

Antigen recognition by human T cells is mediated through two types of T cell receptor (TCR) consisting of alpha/beta and gamma/delta chain which are associated with CD3 surface molecules. The vast majority of lymphocytes in the peripheral blood (>90%) express the alpha/beta TCR whereas about 6-8% bear the gamma/delta TCR. gamma/delta T produce various cytokines and act as MHC restricted and non-restricted cytotoxic effector cells (Haas et al. 1993). gamma/delta T cells are elevated in acute or convalescent *P. falciparum* infection (Bordessoule et al. 1990; Ho et al. 1990; Roussilhon et al. 1990; Ho et al. 1994). An increase of gamma/delta T cells is in non-immune *P. vivax* patients during paroxysms (Perera et al. 1994), and gamma/delta-memory T cells in acute and convalescent period (Jangpatarapongsa et al. 2006). Activation of gamma/delta T cells but not alpha/beta T cells from malaria naïve donors inhibits blood stage parasite replication (Elloso et al. 1994; Troye-Blomberg et al. 1999) and induces production of pro-inflammatory cytokines (Troye-Blomberg et al. 1999). Recent study has shown that gd T cells can inhibit the growth of *P. falciparum* in vitro by cytolytic pathways suggesting that these cells have protective function involving both cytotoxic and regulatory mechanisms against malaria infection. However, the direct killing mechanism of these cells during *P. vivax* infection has not yet been investigated. This studies aims to study and compare the killing mechanism of γδ T cells against two major malaria species: *P. falciparum* and *P. vivax.*

Materials and methods

Isolation and activation peripheral blood mononuclear cells (PBMC)

Venous blood from five healthy donors was collected in heparinized tubes and PBMC were separated by gradient centrifugation using Lymphoprep™ (AXIS-Shied PoC AS, Oslo, Norway) according to the manufacturer's recommendations. The PBMC pellet were resuspended in RPMI 1640 supplemented with 10%fetal calf serum (FCS), and then plated in 24-well plates at a concentration of 1×10^6/ml together with 30 mg/ml Isopentenylpyrophosphate (IPP) for 2 weeks with addition of 20 U/ml rhIL-2 every 3 days exclusion. At day 14, cells were washed twice in TCM, counted, phenotyped by flow cytometry and used as effector cells in the parasite reinvasion/growth inhibition assays. PBMC at the same amount of gamma delta were used to compared. % growth inhibition of the parasite was calculated according to the following equation (Farouk et al. 2004): %parasite growth inhibition = (% parasitemia in control – % parasitemia in test) / (% parasitemia in control) ×100.

Parasite cultures

P. falciparum (AMB47) and *P. vivax* infected blood collected from endemic area wa used for intact parasites in the studies. *P. vivax* infected blood was depleted of white blood cells by filtering through a sterile column of CF11 cellulose (Whatman®, Maidstone, UK) and the red blood cells were washed with RPMI-1640 by centrifugation at 1190 g for 5 minutes. Both parasites were adjusted to be 2% parasitemia, 5% Hematocrit (Hct) before co-culturing with PBMC or γδ T cells. Co-cultured parasites were maintained in an incubator containing 5% CO2, 5% O_2 and 90% N_2. Uninfected RBC was processed similarly as above was used as a base line.

***In vitro* stimulation of PBMC**

PBMC ($1x10^5$, $1x10^6$ and 2 x 10^6 cells/well) in RPMI-1640 supplemented with 25 mM HEPES, 1.8 mg/ml D-glucose, 2 mM glutamine, 40 mg/ml of gentamicin and 10% heat-inactivated FCS were cultured for 3 days at 37°C in a humidified chamber with 5% CO_2, 5% O_2 and 90% N_2 in the presence of Intact *P. falciparum* or *P. vivax* parasites at a 2% parasitemia and 5% Hct. Medium alone, $1x10^6$ cells/well of NRBC extracts or were used as negative control and base line level, respectively. All experiments were performed in duplicates. After activation, the cells were harvested and stained for the gamma deta T cells and interested markers.

Intracellular staining and flow cytometric (FCM) analysis

For surface marker by the three-color FCM analysis, harvested cells were stained with a combination of fluorochrome-conjugated monoclonal antibodies (mAbs): RPE-Cy5-labeled anti-CD3 (Caltag, Burlingame, USA) and RPE-anti-gamma9 (Immunotech, Marseille, France) and FITC-labeled anti-CD69 or FITC-labeled anti-CD25 for 30 min at 4°C and washed with phosphate-buffered saline (PBS). Cells were fixed with 1% paraformaldehyde and data acquisition and analysis on FACSCalibur using the CELLQUEST software (Becton Dickinson, San Jose, USA).

For intracellular, after staining with RPE-Cy5-labeled anti-CD3 (Caltag, Burlingame, USA) and RPE-anti-gamma9 (Immunotech, Marseille, France), the cells were fixed with 1% paraformaldehyde and washed with a permeabilizing solution. After incubation in the permeabilizing buffer for 20 min at room temperature, the cells were incubated with FITC-labeled anti-CD107a and then washed with PBS.

Results

Growth inhibition of *P. falciparum* parasites by γδ T cells

The median percentage of parasites growth inhibition was increased everyday when intact *P. falciparum* parasites were co-cultured with PBMC (Fig. 1A) and gamma delta T cells (Fig. 1B). the % growth inhibition was dose dependence manner. At day 2, we found the higher of growth inhibition in $1x10^6$ cells/well of gamma delta T cells than PBMC. However, the growth inhibition was not difference if we put more amounts of cells co-culturing with parasites.

Activation of γδ T cells by *P. falciparum* parasites

The median level of $CD3^+gamma9^+$ T cells in PBMC was decreased increased at day 2 stimulation (Fig 2A). However, gamma delta T cells were maintained and increased after day 2 stimulation with intact *P. falciparum* parasites (Fig. 2B). Moreover, the highest level of $gamma9^+$ T cells was intact parasites co-culturing with 10^5 cells/well. The high amount of cells resulted in decreasing of gamma9 T cells activation. The similarly results were shown in both PBMC and enriched gamma9 T cells. Therefore, the activation of gamma9 T cells was dose dependent manner.

Early activation of γδ T cells by *P. falciparum* parasites

Normally, $CD69^+$ molecule was expressed at the early stage of activation. The gamma9 T cells activation level from Fig. 1 was also confirmed the cells activation status. In concordance with gamma9 T cells activation, we found the early activation marker ($gamma9^+CD69^+$ T cells)

were expressed at day 2 in PBMC (Fig 3A), and day 1 in gamma delta T cells after activation with intact *P. falciparum* parasites (Fig. 3B). Moreover, the early activated marker was increased when amount of gamma delta T cells was increased (Fig 3B).

Activation of lysosomal-associated membrane protein-1 (LAMP-1) intracellularly expression in γδ T cells by *P. falciparum* parasites

Lysosome associated membrane protein-1 (LAMP-1 or CD107a) are major protein components of the lysosomal membrane. Protein components of the lysosomal membrane also mediate a number of essential functions of this compartment, including the acidification of the lysosomal lumen, transport of amino acids, fatty acids, and carbohydrates resulting from the hydrolytic degradation (Eskelinen 2006). In addition, lysosomal membrane proteins may be involved in the interaction and fusion of the lysosomes with themselves as well as with other cell components, including endosomes, phagosomes, and the plasma membrane (Fukuda 1991).

In this study, we determined the cytolytic activities by quantification of the levels of gamma9^{+}CD107a^{+} T cells expressed intracellular of gamma delta T cells (Fig. 4). In concordance with the activated marker (CD69^{+}), the median percentage of gamma9^{+}CD107a^{+} T cells was increased in gamma delta T cells co-culturing with intact *P. falciparum* parasites which was higher than was found in PBMC. Interestingly, the expression of CD107a+ was highest in lower amount of cells (10^{5} cells/well).

Gamma/delta T cells in acute *P. vivax* infected patients

Gamma/delta T cells are activated during acute *P. vivax* infection and the high level is maintained after treatment (Fig.5). We hypothesize that the gamma/delta T cells can eliminate the parasites and therefore control parasitemia to subside the disease severity.

Activation of γδ T cells by *P. vivax* parasites

At the first donors, the percentage of $CD3^+gamma9^+$ T cells expressing on lymphocytes was increased after PBMC co-culturing with intact *P. vivax* parasites (Fig. 6). However, the level of $CD3^+gamma9^+$ T cells was increased after enriched gamma delta T cells was co-cultured with intact P. vivax parasites at day 2 and maintained at day 3. We also found the increasing of $CD3^+gamma9^+$ T cells was increased at high amount of cells, suggesting that this level was dose dependence.

Early and late activation of γδ T cells by *P. vivax* parasites

The gamma9 T cells activation was also confirmed the cells activation status. In similar to what we found in *P. falciparum*, $CD3^+gamma9^+$ T cells of the PBMC co-culturing with intact *P. vivax* parasites also expressed $CD69^+$ at early stage (day 1) (Fig. 7A). Moreover, we found the early activation marker ($gamma9^+CD69^+$ T cells) was expressed at day 1 in gamma delta T cells after activation with intact *P. vivax* parasites (Fig. 7B). Moreover, the early activated marker was increased when amount of gamma delta T cells was increased (Fig 7A,B).

In contrast to early stage activation, the $CD25^+$ expressing on $CD3^+gamma9^+$ T cells was expressed at day 3 activation in PBMC co-culturing with intact *P. vivax* parasites (Fig. 7C).

However, the level of $CD3^{+}gamma9^{+}CD25^{+}$ T cells in gamma delta T cells co-culturing with *P. vivax* parasites was expressed at the first day stimulation (Fig. 7D). The activation was also dose dependence manner.

Activation of lysosomal-associated membrane protein-1 (LAMP-1) intracellularly expression in γδ T cells by *P. vivax* parasites

In comparison with what we found in *P. falciparum*, we also determined the cytolytic activities expressing of $CD107a^{+}$ intracellularly of gamma delta T cells in PBMC or gamma delta T cells co-culturing with intact P. vivax parasites (Fig. 8). The level of $CD3^{+}gamma9^{+}CD107a^{+}$ T cells was increased at day 0 and rapidly decreased at day later (Fig. 8A). In contrast to what we found in PBMC, the level of $gamma9^{+}CD107a^{+}$ T cells was increased in gamma delta T cells co-culturing with intact *P. vivax* parasites at day 2 and rapidly decreased at day later.

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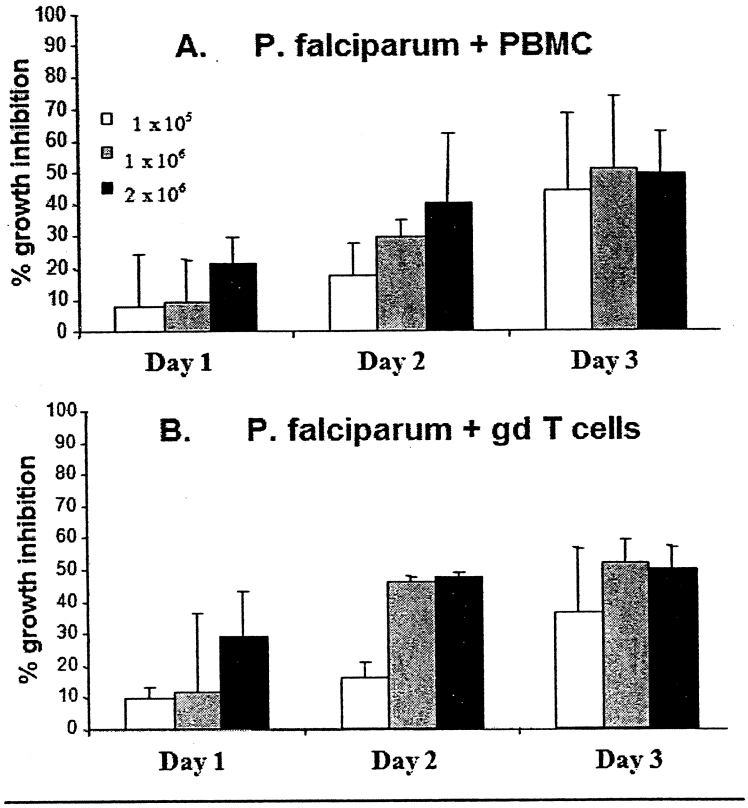

Fig. 1. % *P. falciparum* parasites growth inhibition after co-culturing with PBMC (A) or γδ T cells (B) at different cell number from $1x10^5$, $1x10^6$ and $2x10^6$ cells/well. parasitemia was counted everyday from day 0 until day 3. Data represent median percentage (blocks) ± SD (vertical bars) from five experiments.

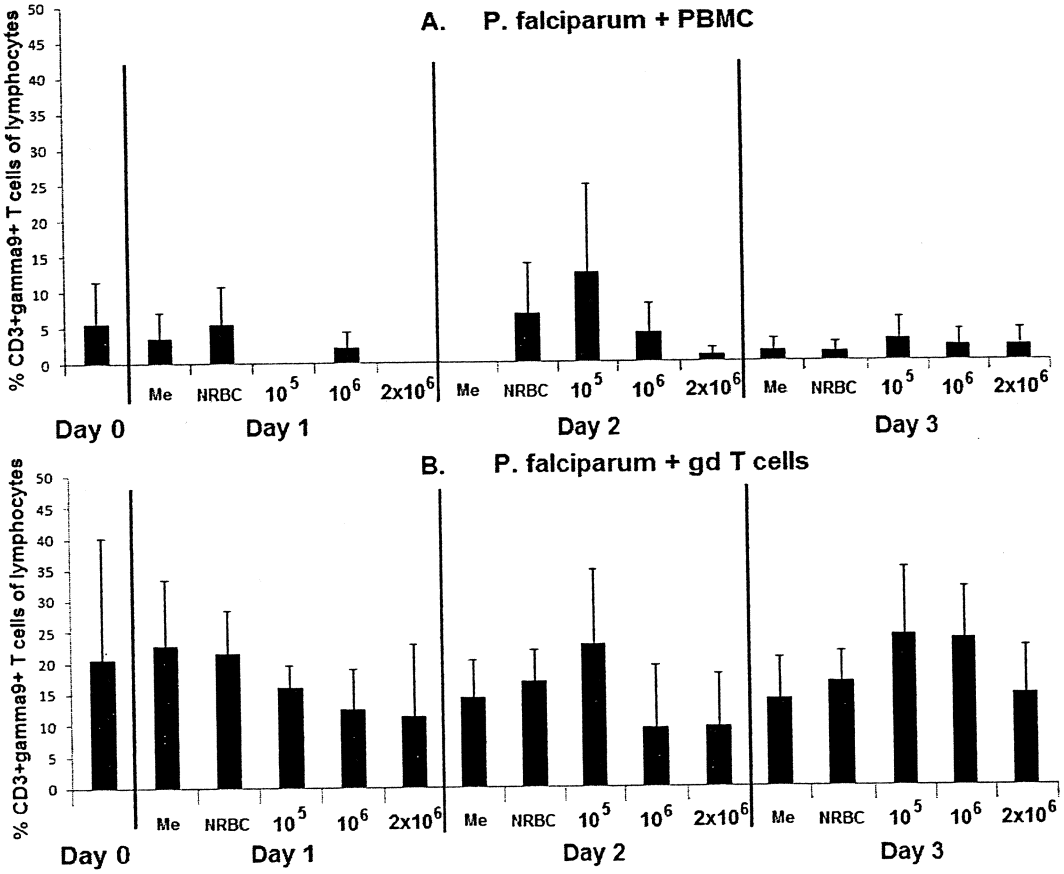

Fig. 2 % $CD3^{+}gamma9^{+}$ T cells of lymphocytes shown by flow cytometry. *P. falciparum* at 2% parasitemia, 5% Hct was co-culturing with **PBMC (A)** or **γδ T cells (B)** at different cell number 10^5, 10^6 and $2x10^6$ cells per well. Medium alone (Me) was used as negative controls and normal red blood cells (NRBC) was used as base lines levels. Data represent median percentage (blocks) ± SD (vertical bars) from five experiments.

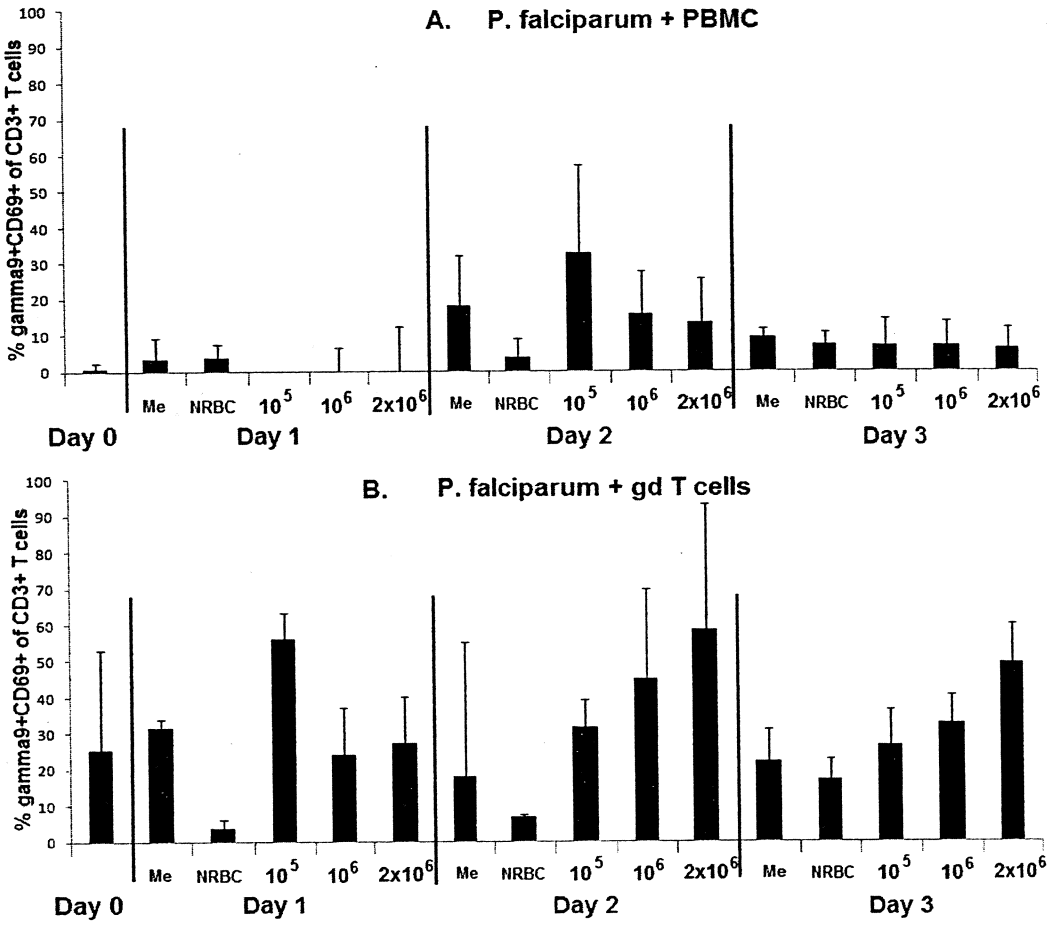

Fig. 3 % gamma9$^+$CD69$^+$ T cells of CD3$^+$ shown by flow cytometry. *P. falciparum* at 2% parasitemia, 5% Hct was co-culturing with **PBMC (A)** or **γδ T cells (B)** at different cell number 10^5, 10^6 and $2x10^6$ cells per well. Medium alone (Me) was used as negative controls and normal red blood cells (NRBC) was used as base lines levels. Data represent median percentage (blocks) ± SD (vertical bars) from five experiments.

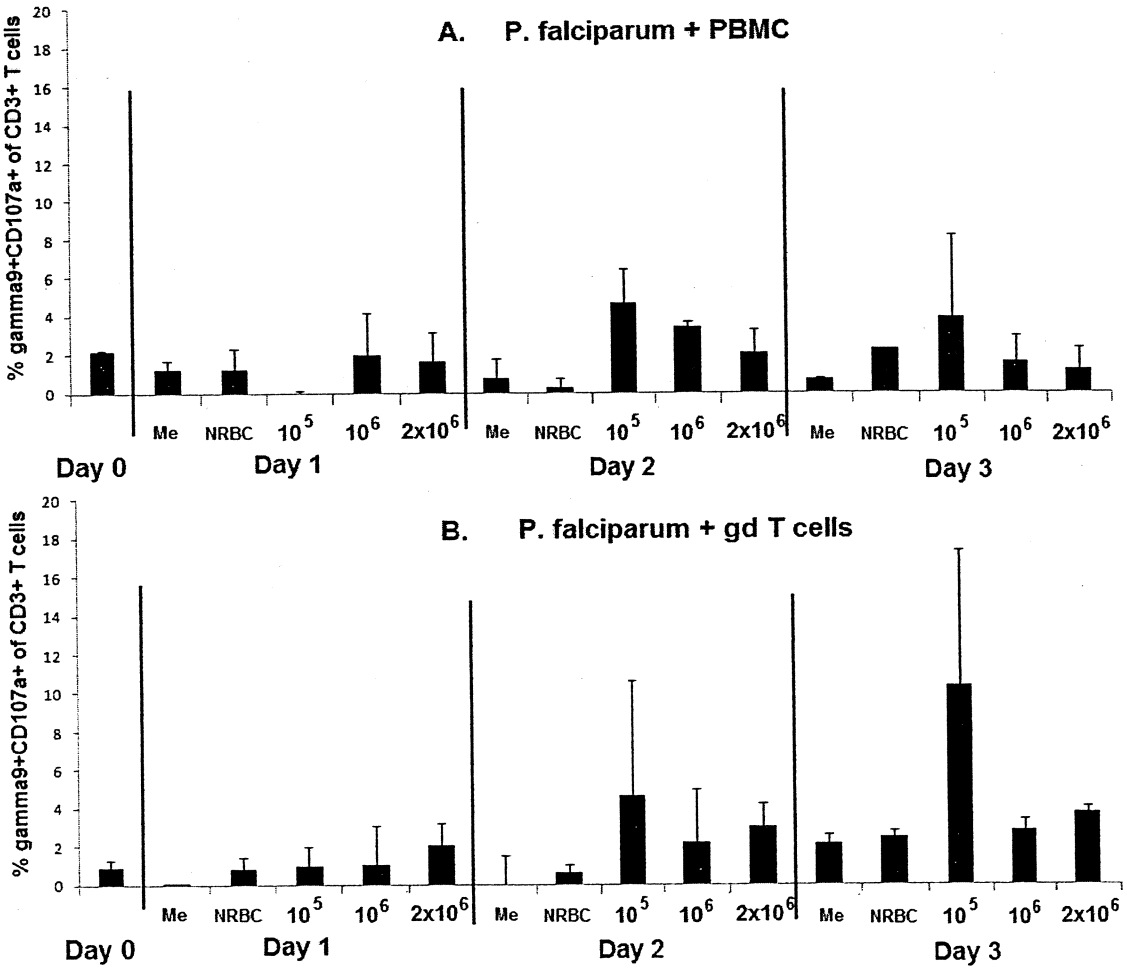

Fig. 4 % gamma9$^+$CD107a$^+$ T cells of CD3$^+$ shown by flow cytometry. *P. falciparum* at 2% parasitemia, 5% Hct was co-culturing with **PBMC (A)** or □□ **T cells (B)** at different cell number 10^5, 10^6 and $2x10^6$ cells per well. Medium alone (Me) was used as negative controls and normal red blood cells (NRBC) was used as base lines levels. Data represent median percentage (blocks) ± SD (vertical bars) from five experiments.

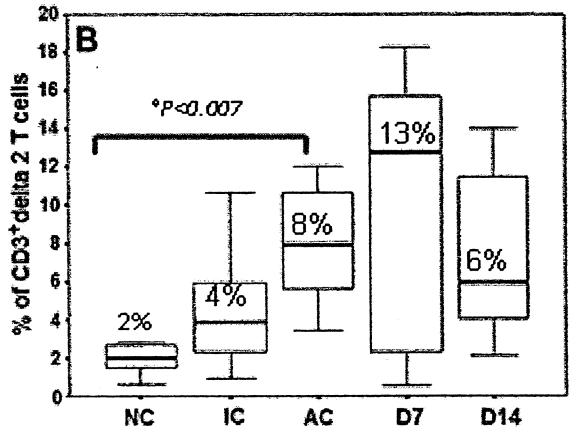

Fig. 5 Gamma/delta T cells shown by flow cytometry. Naïve controls (NC)(N=27), malaria-exposed donors (IC)(N=25), acute *P. vivax* infection (AC)(N=17), the day 7 (D7), and day 14 (D14) after treatment. Median, interquartile ranges (box plots), maximum and minimum (upper-lower lines) are shown.

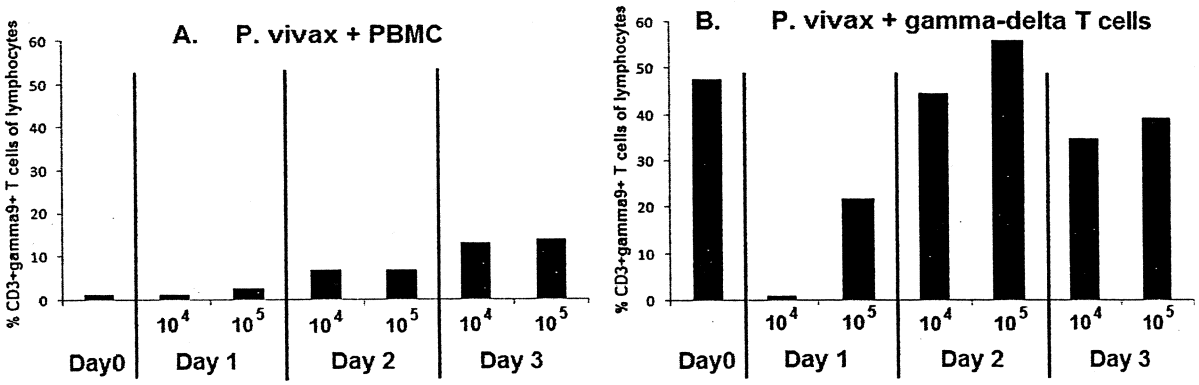

Fig. 6 % $CD3^+$ $gamma9^+$ T cells of lymphocytes shown by flow cytometry. Intact *P. vivax* parasites at 2% parasitemia, 5% Hct were co-culturing with **PBMC (A)** or **γδ T cells (B)** at different cell number 10^5, 10^6 and $2x10^6$ cells per well. Medium alone (Me) was used as negative controls and normal red blood cells (NRBC) was used as base lines levels. Data represent median percentage (blocks) ± SD (vertical bars) from five experiments.

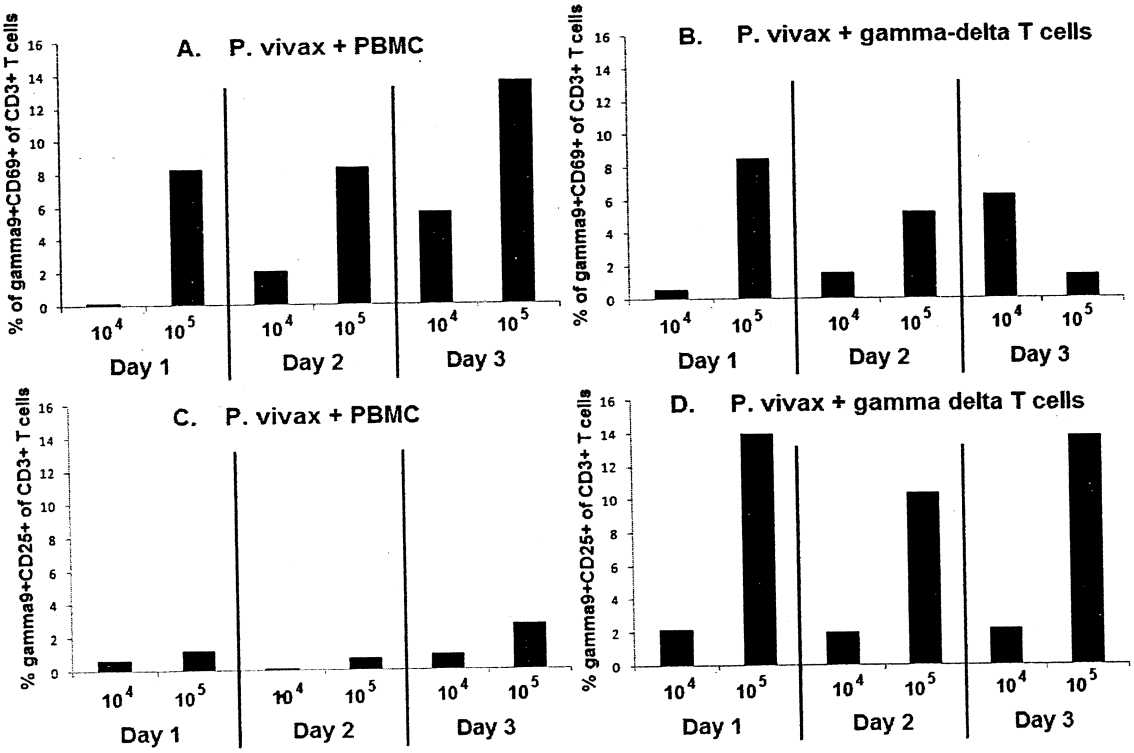

Fig. 7 % gamma9$^+$CD69+ (A,B) and **CD25$^+$ T cells (C,D)** of CD3$^+$ shown by flow cytometry. Intact *P. vivax* parasites at 2% parasitemia, 5% Hct were co-culturing with **PBMC (A,C)** or **γδ T cells (B,D)** at different cell number 10^5, 10^6 and $2x10^6$ cells per well. Medium alone (Me) was used as negative controls and normal red blood cells (NRBC) was used as base lines levels. Data represent median percentage (blocks)

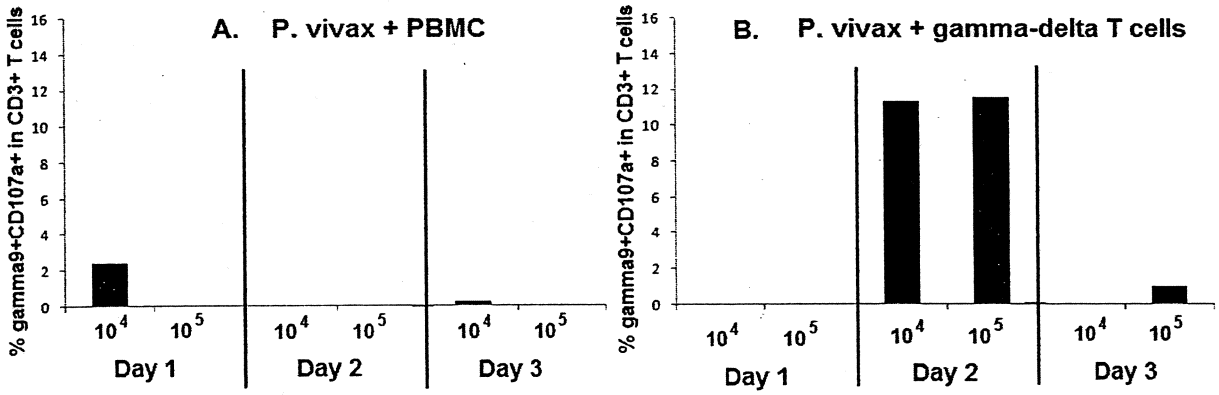

Fig. 8 % gamma9$^+$CD107a$^+$ T cells of CD3$^+$ shown by flow cytometry. Intact *P. vivax* parasites at 2% parasitemia, 5% Hct were co-culturing with **PBMC (A)** or **γδ T cells (B)** at different cell number 10^5, 10^6 and $2x10^6$ cells per well. Medium alone (Me) was used as negative controls and normal red blood cells (NRBC) was used as base lines levels. Data represent median percentage (blocks)

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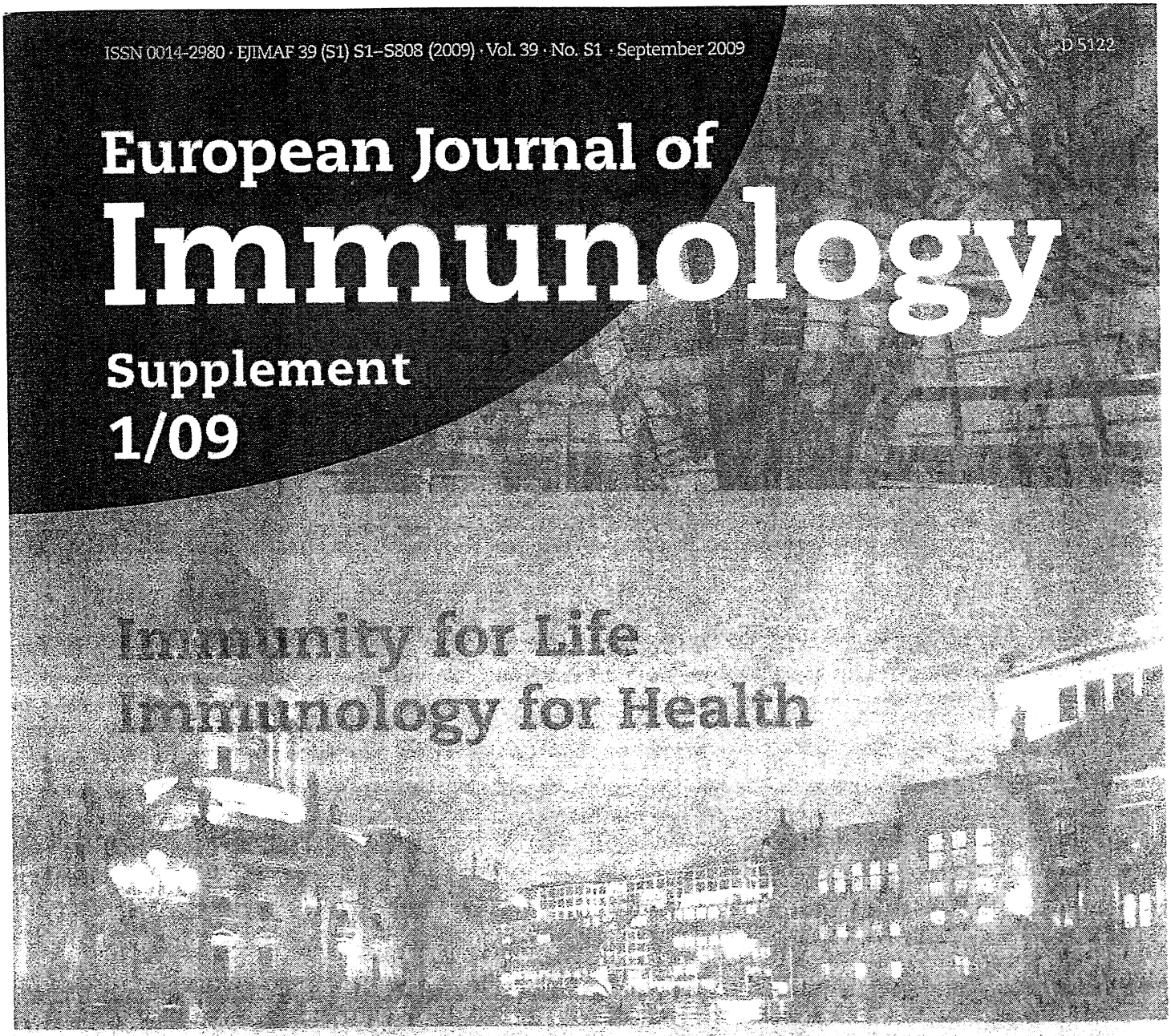

Abstracts

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PA13/60 KILLING MECHANISM OF PLASMODIUM VIVAX PARASITES BY GAMMA-DELTA T CELLS

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Objectives & methods: An increasing of gamma-delta T cells during acute *P. vivax* infection and convalescent period has been reported. Moreover, the activ of gamma-delta T cells leads to the inhibition of blood stage *P. falciparum* parasites *in vitro*. To determine the killing mechanisms of *P. vivax* parasites by ga delta T cells comparing with what has been found in *P. falciparum*, the gamma-delta T cells were enriched by Isopentenylpyrophosphate (IPP) from naïve P Different number of gamma-delta T cells and normal PBMC were incubated with intact of *P. vivax* parasites and protein extract of *P. vivax* parasites, recomb $PvMSP1_{19}$ and PvAMA1 proteins. Gamma-delta T cells was daily determined the cytokine and granzyme intracellular releasing by Flow cytometry until day turing.
Results: Among the enriched gamma-delta T cells, the percentage of cells expressing $CD69^+$ and $CD25^+$ was elevated after co-culturing with intact and th teins of *P. vivax* parasites. The overall gamma-delta T cells showed proliferation at day 3 after the co-cultivation. Moreover, the gamma-delta T cells expressing gamma$^+$ and $CD107a^+$ (lysosomal associated membrane proteins: LAMP-1) elevated from the first day of PBMC collection after co-culturing with the intac *P. vivax* antigens. This level was correlated with the significantly decreasing number of parasites and the increasing percentage of parasite growth inhibition
Conclusion: Our results showed the activation of gamma-delta T cells during *P. vivax* infection *in vitro*. This suggests that gamma-delta T cells could be stimul by *P. vivax* parasites and these actively activated gamma-delta T cells could kill the parasites via mechanism of granzyme and cytokines at the early stage of cell vation. This study provides more understanding in activation of the innate immunity during acute malaria infection which may lead to the selection of appro malaria proteins as vaccine candidates in the future.

PA13/61 ANALYSIS OF INVARIANT NKT CELLS OF PATIENTS WITH ATOPIC DERMATITIS BY FLOW CYTOMETRY

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Objectives: Several evidence suggest that invariant NKT cells (iNKT) connect innate and acquired immune system. They are able to produce both Th1 and cytokines after stimulation. Atopic dermatitis (AD) is a chronic inflammatory skin disease. Th1-like and Th2-like cytokines have been implicated in the pathoj sis of AD, but there are controversial data on their role in AD.
Methods: The frequency and absolute number of iNKT cells in mononuclear cells (PBMCs) of peripheral blood of patients with atopic dermatitis (AD) (n=43) healthy controls (n=13) were determined by flow cytometry using anti-CD3 and monoclonal antibody specific for the CDR3 loop of the invariant TCR α cha iNKT cells (clone:6B11). Furthermore, after PMA/ionomycin stimulation for 4 hours, intracellular IFNγ and IL-4 cytokines were detected in CD4+CD8- , CD4- (DN), CD4-CD8+ and CD4+CD8+ subsets of iNKT cells by five colour flow cytometry in patients with AD (n=10) and healthy controls (n=10).
Results: Both frequency and absolute number of iNKT cells were significantly lower in patients with AD ($P< 0.01$) compared to healthy controls. The frequ of DN subpopulation was significantly lower in AD patient ($P< 0.01$). There was a positive correlation between the frequency of DN cells and iNKT cells bo AD patients ($R=0.726$ and $P< 0.001$) and healthy controls ($R=0.693$ and $P< 0.001$). In the intracellular IFNγ level there were no significant difference in a the iNKT subsets of AD patients, however the intracellular IL-4 level was significantly higher in DN subpopulation of iNKT cells of AD patients compared to he controls ($P< 0.05$).
Conclusion: The frequency, the number of iNKT cells and the cytokine producing capacity of the CD4/CD8 iNKT subsets are different in peripheral blood obta from AD patients compared to healthy controls.
Our result suggest that the DN iNKT cell subset can serve as a source of IL-4 that promotes the Th2 differentiation in AD patients and might play a role in the pa genesis of this disease.

PA13/62 ISOLATION OF MURINE INTRAHEPATIC IMMUNE CELLS EMPLOYING A MODIFIED PROCEDURE FOR MECHANICAL DISRUPTION AND FUNCTIONAL CHARACTERIZATION OF THE B, T AND NATURAL KILLER T CELLS OBTAINED

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Introduction: Intrahepatic immune cells (IHIC) are known to play central roles in immunological responses mediated by the liver, and isolation and phenot characterization of these cells is therefore of considerable importance.
Aims: In the present investigation, we developed a simple procedure for the mechanical disruption of mouse liver that allows efficient isolation and phenot characterization of IHIC. These cells are compared with the corresponding cells purified from the liver after enzymatic digestion with different concentratio collagenase and DNase.
Results: The mechanical disruption yielded viable IHIC in considerably greater numbers than those obtained following enzymatic digestion. The IHIC isol employing the mechanical disruption were heterogeneous in composition, consisting of both innate and adaptive immune cells, of which B, T, natural killer (I NK T cells, granulocytes and macrophages were the major populations (constituting 37.5%, 16.5%, 12.1%, 7.9%, 7.9% and 7.5% of the total number of cells re ered respectively). The IHIC obtained following enzymatic digestion contained markedly lower numbers of NK T cells (1.8%). The B, T and NK T cells among I isolated employing mechanical disruption were found to be immunocompetent, i.e. they proliferated in vitro in response to their specific stimuli (lipopolysac ride, concanavalin A and alpha-galactosylceramide respectively) and produced immunoglobulin M and interferon-gamma.
Conclusions: Thus, the simple procedure for the mechanical disruption of mouse liver described here results in more efficient isolation of functionally compe IHIC for various types of investigation.

PA13/63 THE FUNCTION AND CHARACTERISTICS OF SUPERANTIGEN SEB-ACTIVATING $CD8^+$NKT CELLS

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Nature Killer T cells (NKT) are a special T cell population with co-expresses NK and T cell surface markers. Murine NKT cells include $CD4^+$ NKT and $CD4^-CD8^-$ cells. $NK1.1^+$ NKT cells may release large amounts of IL-2, IL-4, IFN-γ and IL-10 after they are activated. It has been reported that α-Galactorsykeramide (α-Gal a glycolipid, may induce proliferation of NKT cells with the role of immune regulation by stimulating mouse spleen cells. This study demonstrated that supera gen staphylococcal enterotoxin B (SEB) , a kind of peptide, can activate the NKT cells with the function of immune tolerance. The response ability of SEB-activa effect cells to ConA, LPS and IL-2 had significantly decreased compared with that of normal lymphocytes. The effect cells exerted an inhibitory effect for response of normal lymphocytes to ConA and IL-2. There was a significantly increase in the percent of $CD8^-NK1.1^+$ and $TcRV\beta8^+NK1.1^+$ NKT cells identified fi the SEB-activated cells. Based on the cell distribution detected in the upper part of the FACS picture, expression of CD69 molecule existed in 68.95% of the c from large-scale selection. The percent of $CD8^+NK1.1^+$ and $TcRV\beta8^-NK1.1^+$ NKT cell subsets in the giant lymphocytes were enhanced to 84.0 and 38.9 fo respectively. Under a light microscope at x400 magnification, the SEB-activating lymphocytes in size were larger than not only the ConA-activated cells but also adherent macrophages with an increase of 5 fold observed under a microscope. There were a few granules seen in cytoplasm. The value of cytoplasm vs nuclei less than 1.0 and they are non-adherent cells. The differentiation pathway of the SEB-activating $CD8^+$ and $TcRV\beta8^-$NKT cells was not relative to a NK source. T were produced directly from T cell population and were considered as a subsets of T lymphocytes. Our results suggest that the superantigen SEB can act on $CD8^+$NKT cell and $TcRV\beta8^+$NKT cells. And the two NKT cell subsets may play a critical role in SEB mediated tolerance.

PA13/64 INTESTINAL INTRAEPITHELIAL γδ T CELL SHAPE THEIR CELLULAR ENVIRONMENT THROUGH TCR-DEPENDENT PRODUCTION OF CHEMOKINES AND CYTOKINES

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γδ T cells in the intestinal intraepithelial compartment (γδ iIEL) show an intrinsic activated phenotype. We hypothesised that their T cell receptor γδ (TCRγδ implicated in the activation of γδ iIEL. Because the TCR γδ ligands in mice are not well described, monoclonal antibodies (mAb) directed against the γδ TCR the clone GL3 which binds the δ subunit of TCR γδ, are important tools to specifically activate γδ T cells. Using cytometric Indo-1AM measurement, we could de calcium flux of intestinal and peripheral γδ T cells from TCRd-H2BeGFP reporter mice. Stimulation with anti-γδ clone GL3 or anti-CD3 clone 2C11 elicited ac tion of γδ T cells suggesting that TCR γδ and CD3 molecules in γδ T cells are functional and signalling competent.

sion was found in the B-cell zone after low-dose injection only. A much stronger T-cell proliferation was induced after high-dose injection of SRBC. This newly formed T cells did not respond to the second encounter with SRBC in the footpad. This unresponsiveness remained even after transferring the T cells into a naïve host and challenging them again with SRBC.
Conclusion: We conclude that the absent DTH reaction after high-dose injection of SRBC is not due to lower numbers of T cells. We hypothesise that the newly formed T cells are either unable to migrate into the skin or are regulatory T cells which suppress the DTH reaction in the skin.

V55 LIVER SINUSOIDAL ENDOTHELIAL CELLS INDUCE ANTI-INFLAMMATORY CD4$^+$ T CELLS SUPPRESSING MURINE AUTOIMMUNE HEPATITIS

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Objectives: Cellular mechanisms that maintain the intrahepatic immune balance are crucial in viral or autoimmune liver diseases and for allograft acceptance. For naive CD8$^+$ T cells, liver sinusoidal endothelial cells (LSEC) have been shown to act as non-professional antigen presenting cells and thus induce tolerance by inhibition of cytotoxicity. In this study we investigated consequences of CD4$^+$ T cell priming by LSEC.
Methods: Priming by LSEC was investigated in bone marrow chimeric mice expressing MHC class II exclusively on non-hematopoietic cells. We studied the cytokine expression of LSEC primed CD4$^+$T cells (T_{LSEC}) and determined the stability of their phenotype *in vivo* by adoptive transfer into congenic mice and immunogenic antigen application. Investigating suppressive capacities of T_{LSEC} we performed an *in vitro* suppression assay. The ability of T_{LSEC} to influence proinflammatory reactions *in vivo* was analyzed in a model of T cell-mediated autoimmune hepatitis. Hepatic inflammation was monitored by ALT levels and histologic analyses. The migration pattern of T_{LSEC} was investigated by an *in vivo* homing assay.
Results: We demonstrated that LSEC induce proliferation of naive CD4$^+$ T cells *in vitro*. Although the expression of CD45RB was downregulated in T_{LSEC}, these cells did not produce effector cytokines. This phenotype of T_{LSEC} remained stable *in vivo*. The *in vivo* migration pattern of T_{LSEC} was different from cells activated by professional antigen presenting cells isolated from the spleen, since they showed enhanced homing into lymph nodes and the intestine while they were also present in the liver. Interestingly, T_{LSEC} negative for CD25 and Foxp3, suppressed the proliferation of naive CD4$^+$ T cells *in vitro*. They did neither support a DTH reaction nor a hepatic inflammation and were even able to suppress hepatitis.
Conclusion: Priming of naive CD4$^+$ T cells by LSEC leads to an anti-inflammatory phenotype here referred to as T_{LSEC}. Thus liver sinusoidal endothelia may directly contribute to limiting hepatic inflammation and supporting local tolerance towards exogenous antigens or endogenous self-antigens.

V56 SPECIFIC T-CELL RESPONSES IN LESIONS AND PERIPHERAL BLOOD FROM PATIENTS WITH BURULI ULCER

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Buruli ulcer disease (BUD), caused by *Mycobacterium (M.) ulcerans*, is a neglected bacterial infection of the poor in remote rural areas. BUD is a mutilating disease leading to severe disability; it is the third most common mycobacterial infection in immunocompetent people after tuberculosis and leprosy most endemic in West Africa.
There is some evidence that a T helper type 1-mediated immune response is protective against *M. ulcerans* but the role and distribution of antigen-specific T cells in BUD lesions is hardly defined. In addition, analysis and diagnosis of specific T-cell immunity against *M. ulcerans* is hampered by concomitant infection with other atypical mycobacteria, *M. tuberculosis*, and/or *M. bovis BCG* vaccination.
Here we determine the T-cell distribution of two ulcerative lesions and peripheral blood from a BUD patient using a quantitative PCR method for analyses of T-cell receptor Vβ (TCR-BV) chains and compare the antigen-specific T-cell response in peripheral blood of children infected with *M. ulcerans* or other mycobacteria using *in vitro* restimulation with mycobacterial lysates and intracellular cytokine analyses.
TCR-BV chain analyses in two lesions from a BUD patient reveal differential expression of certain chains depending on the distance from the lesion center. Skewed TCR-BV chains are different between two distinct lesions from the same BU patients. This suggests that T cells, which infiltrate the BUD lesions, are oligoclonally expanded but a predominantly infiltrating subtype could not be identified.
Antigen-specific T-cell cytokine analyses of peripheral blood mononuclear cells from patients with BUD (n = 26) and other mycobacterial infections (n = 9) reveal IFN-γ, IL-2, and TNF-α secretion after stimulation with lysates and purified protein derivates from a non-toxic *M. ulcerans* strain, *M. tuberculosis*, and *M. avium*. Despite crossreactivity against different mycobacterial lysates, ratios of specific T cells (percentage of *M. ulcerans*-specific T cells/*M. tuberculosis*- specific T cells in individual donors) were significantly different between the BUD patients and donors with other mycobacterial infections. Therefore concomitant measurement of T-cell cytokine expression after restimulation with different mycobacterial lysates can help to distinguish early infection with *M. ulcerans* from infection with other mycobacteria.

V57 *PLASMODIUM VIVAX* ALTERS IMMUNOSUPPRESSION CAUSED BY *PLASMODIUM FALCIPARUM* INFECTIONS

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Objectives: *Plasmodium falciparum* infection causes transient immunosuppression during parasitemic stage. However, immune response during simultaneous infection with both *P. vivax* and *P. falciparum* has not been investigated. In particular, it is not clear whether host immune response to malaria will be different when compare an infection with single malaria species versus mixed malaria species.
Methods: Human blood mononuclear cells from mixed *P. vivax-P. falciparum* infection were characterized by flow cytometry for the immunomodulatory role of T cells. In addition, antibodies to parasite-derived proteins and to PfMSP-1_{19} and PvMSP-1_{19} recombinant proteins were determined.
Results: We found that CD3$^+$delta 2$^+$-TCR T cells, T-killer cell phenotype, were significantly higher in the acute-mixed *P. vivax-P. falciparum* infection compared with either single *P. vivax* or *P. falciparum* infection. Interestingly, mixed malaria-infection had the highest antibodies against both *P. vivax* and *P. falciparum* compared with those antibodies obtained from the single malaria infection.
Conclusion: This suggests that co-infection with *P. vivax* could induce effector T-killer cells. In addition, antimalarial antibodies found in the mixed infection could have protective role against disease severity in *P. falciparum* infection as shown by the lower parasitemia in the mixed infection group. These findings imply that *P. vivax* may help resolving the severity of *P. falciparum* infection.

V58 PHENOTYPIC CHARACTERIZATION OF PERIPHERAL BLOOD MEMORY CD8+ T CELL SUBSETS, WITHIN DIFFERENT GROUPS OF PATIENTS SUFFERING AMERICAN TEGUMENTARY LEISHMANIASIS (ATL) IN THE NORTHWEST OF ARGENTINA. FIRST REGIONAL REPORT

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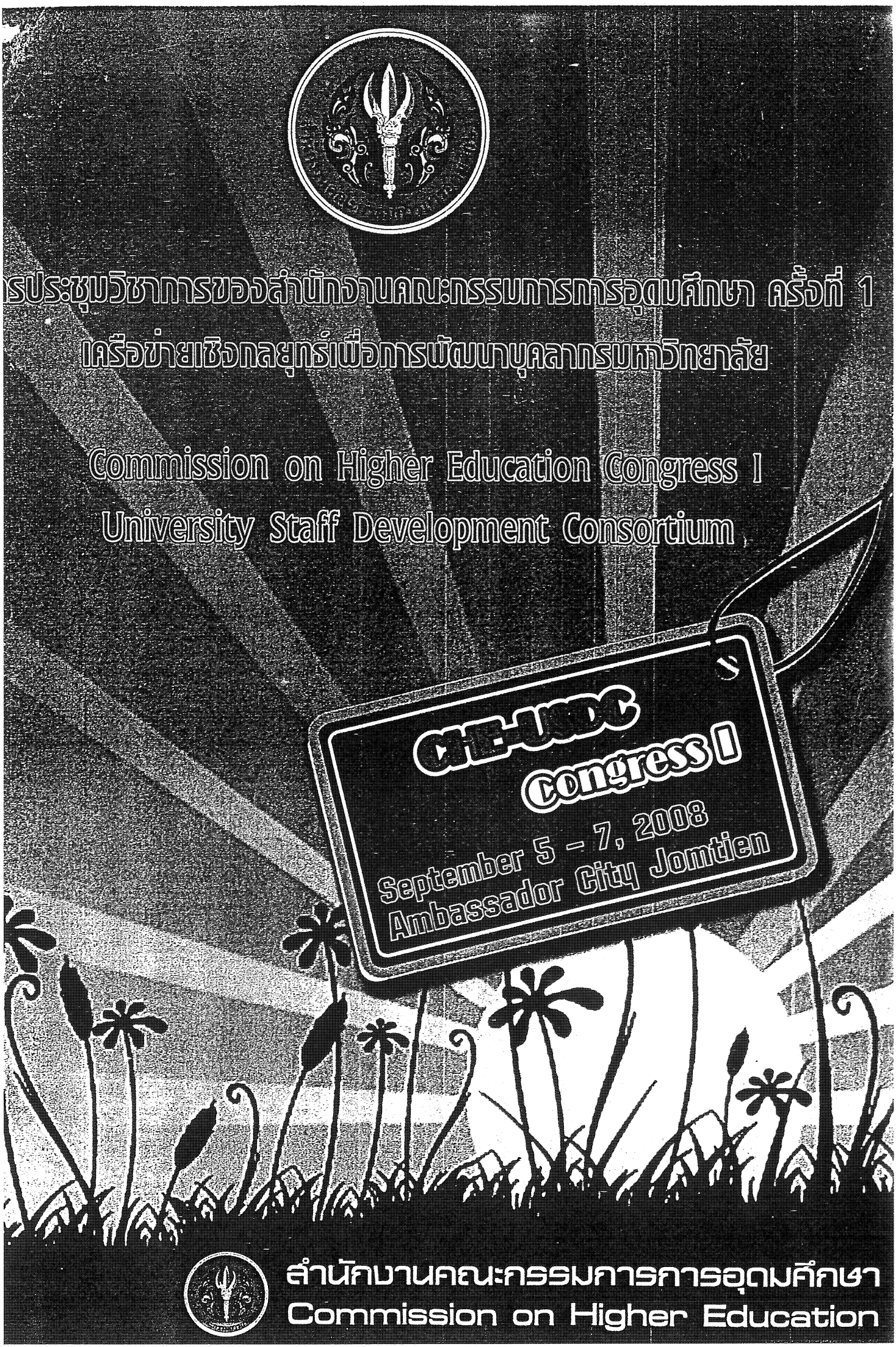
Aim: Phenotypic characterization of CD8+ T peripheral cells from patients suffering ATL is the purpose of this work in order to better understand their role within the pathology outcome.
Methods: Study groups: 1) 9 patients with diagnosis of ATL (infection duration: 5-20 years); 2) 5 ATL patients that received 2 or more complete therapy regimens but suffered frequent relapses (infection duration: 5-20); 3) 2 acute ATL patients (infection duration: < 1 month); 4) 6 healthy subjects. Isolated, cryopreserved and thawed peripheral blood mononuclear cells were stained with: Anti-CD3,-CD8,-CD57,-CD45RO,-CD27,-CD127,-CD45RA,-CD28 -FITC,-PE or -PerCP labelled monoclonal antibodies (BD, Pharmingen). Results were evaluated by flow cytometry (FACScan cytometer, CellQuest software).
Results: Lower percentages of CD127, CD27, CD28 and "early" CD8+ T cells (CD27+, CD28+) were observed in the first two groups of patients compared to the control group. Likewise, increase of "late" (CD27-, CD28-) and CD57+ T cells was observed, indicating the presence of highly differentiated cells. As shown in the table, these differences were more accentuated in group 1. On the other hand, no differences were found between acute ATL patients and the control group.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Commission on Higher Education

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Killing mechanism of *Plasmodium vivax* parasites by γδ T cells

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[5]National Nanotechnology Center, National Science and Technology Development Agency, Pathumthani, Thailand. [6] Department of Entomology, AFRIMS, Bangkok, Thailand. [7]Center of Malaria Research and Training, Ministry of Public Health, Saraburi, Thailand. [8]Department of Entomology, The Pennsylvania State University, PA 16802, USA

Objectives and Method

An increasing of γδ T cells during acute *P. vivax* infection and convalescent period has been reported (Jangpatarapongsa K, *et al.* 2006). Moreover, the activation of γδ T cells leads to the inhibition of blood stage *P. falciparum* parasites *in vitro* (Troye-Blomberg, *et al.* 1999). To determine the killing mechanisms of *P. vivax* parasites by γδ T cells comparing what has been found in *P. falciparum*, the γδ T cells were enriched by Isopentenylpyrophosphate (IPP) from naïve PBMC. The different number of γδ T cells of normal PBMC was incubated with intact *P. vivax* parasites, recombinant $PvMSP1_{19}$ and PvAMA1 proteins. After 5 days co-culturing, γδ T cells was determine the cytokine and granzyme intracellular releasing by Flow cytometry.

Results

The percentage of γδ T cells in lymphocytes was elevated after PBMC co-culturing with intact *P. vivax* parasites. Moreover, the enriched γδ T cells were found the proliferation at day 2 after co-culturing. At day 1 and 3 of γδ T cells stimulation, we found the high level of $CD69^+$ and $CD25^+$ expressing on γδ T cells respectively. This could tell us the early stage of γδ T cells activation among lymphocyte populations by *P. vivax*.

As expected, by comparing with *P. falciparum*, we also determine the cytolytic activities of γδ T cells by the intracellular expression of $CD107a^+$ during co-culturing with intact *P. vivax* parasites. The level of enriched γδ T cells expressing $CD107a^+$ was elevated at day 0 and rapidly decreased on further days. In contrast to what we found in normal PBMC stimulation, the increasing of γδ T cells expressing $CD107a^+$ was increased at day 2 and rapidly decreased on further days.

Conclusion and Discussion

Our results showed the activation of γδ T cells during *P. vivax* infection *in vitro*. We also showed the killing mechanism of γδ T cells against *P. vivax* infection by releasing of granzyme at early stage activation. These suggest that γδ T cells may play role against *P. vivax* parasites at early stage of infection in blood stage.

Publications Output

Jangpatarapongsa K, Chootong P, Sattabongkot J, *et al. Plasmodium vivax* parasites alter the balance of myeloid and plasmacytoid dendritic cells and induction of regulatory T cells. Eur. J. Immunol (accepted).

Xia H, Jangpatarapongsa K, Fang Q. et al. Immune response to *Plasmodium vivax* infection: A study in Thailand and the Central of China (manuscript in preparation).

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Program & Abstracts

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KILLING MECHANISMS OF *PLASMODIUM VIVAX* PARASITES BY GAMMA-DELTA T CELLS

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ORAL PRESENTATION

Objectives & Methods: An increasing of gamma-delta T cells during acute *P. vivax* infection and convalescent period has been reported. Moreover, the activation of gamma-delta T cells leads to the inhibition of blood stage *P. falciparum* parasites *in vitro*. To determine the killing mechanisms of *P. vivax* parasites by gamma-delta T cells comparing with what has been found in *P. falciparum*, the gamma-delta T cells were enriched by Isopentenylpyrophosphate (IPP) from naïve PBMC. Different number of gamma-delta T cells and normal PBMC were incubated with intact of *P. vivax* parasites and protein extract of *P. vivax* parasites, recombinant $PvMSP1_{19}$ and PvAMA1 proteins. Gamma-delta T cells was daily determined the cytokine and granzyme intracellular releasing by Flow cytometry until day 5 culturing.

Results: Among the enriched gamma-delta T cells, the percentage of cells expressing $CD69^+$ and $CD25^+$ was elevated after co-culturing with intact and the proteins of P. vivax parasites. The overall gamma-delta T cells showed proliferation at day 3 after the co-cultivation. Moreover, the gamma-delta T cells expressing IFN-gamma$^+$ and CD107a+ (lysosomal associated membrane proteins: LAMP-1) elevated from the first day of PBMC collection after co-culturing with the intact and P. vivax antigens. This level was correlated with the significantly decreasing number of parasites and the increasing percentage of parasite growth inhibition.

Conclusion: Our results showed the activation of gamma-delta T cells during P. vivax infection in vitro. This suggests that gamma-delta T cells could be stimulated by P. vivax parasites and these actively activated gamma-delta T cells could kill the parasites via mechanism of granzyme and cytokines at the early stage of cell activation. This study provides more understanding in activation of the innate immunity during acute malaria infection which may lead to the selection of appropriate malaria proteins as vaccine candidates in the future.

Publication Outputs: **Jangpatarapongsa K**, Chootong P, Sirichaisinthop J, Sattabongkot J, Tangpradubkul S, Hisaeda H, Troye-Blomberg M, Cui L, Udomsangpetch R. *Plasmodium vivax* alter the balance of myeloid and plasmacytoid dendritic cells and induction of regulatory T cells. **(2008) *Eur. J. Immunol* Oct;38(10):2697-705**

Xia H, **Jangpatarapongsa K**, Qiang F, Kaiming H, Sattabongkot J, Qi G, Cui L, Li B, Udomsangpetch R. Immune response to *Plasmodium vivax* infection: A study in the Central of China. (In preparation)

Jangpatarapongsa K, Loharungsikul S, Treeratanapiboon L, Promwan S, Dabkam K, Hongeng S, Chotivanich K, Polpanich D, Sattabongkot S, Sirichaisinthop J, Cui L, Udomsangpetch R. Killing mechanism of *Plasmodium vivax* parasites by gamma-delta T cells. (in preparation)

Vivax Malaria Research III+ Conference
Bangkok, Thailand
30 September 2009 – 1 October 2009

AGENDA

30 September 2009 — **Pullman Bangkok King Power Hotel**

0800 – 0930:	Registration, Coffee and Pastry Break	Infinity Room 1 Foyer, Ground Floor
0930 – 1030:	Welcome & review of draft report Dr. John Adams et al. University of South Florida Department of Global Health	Infinity Room 1, Ground Floor
1030 – 1100:	Mid Morning Break	Infinity Room 1 Foyer, Ground Floor
1130 – 1230:	Discussion of draft report	
1230 – 1330:	Lunch	Cuisine Unplugged, Ground Floor
1330 – 1515:	Afternoon Presentations	Infinity Room 1, Ground Floor

Diagnosis, clinical management, pathogenesis

Plasmodium vivax infected red cells cytoadherence to glycosaminoglycan
Dr. Kesinee Chotivanich, Mahidol University

Comparison of _P. vivax_ with _P. falciparum_ symptoms and development of exposure related immunity in the Low Transmission region of the Peruvian Amazon
Oralee Branch, New York University

Cytoadhesion of _Plasmodium vivax_ infected erythrocytes
Laurent Renia, Singapore Immunology Network

Epidemiology, vectors, environmental control

Impact of global malaria programme on _P.vivax_ prevalence in Cambodia 2001-2002
Frederic Ariey, Institut Pasteur

A role for G6PD Mahidol Mutation in Protection Against Malaria in South-East Asia
Rick Paul and Anuvaj Sakuntubhai, Institut Pasteur

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Active surveillance of malaria in military areas of operation (AO) along Northern Thai-Myanmar and Thai-Cambodia
COL Jariyanart Gaywee, Ph.D

Vivax malaria in China
Cao Jun, Ph.D

Situation of vivax malaria in Thailand
Jeeraphat Sirichaisinthop, MD, MPH, Vector Borne Disease Training Center, Bangkok

Geographic and Genetic of Thai *Plasmodium vivax* isolates
Usa Lek-Uthai, Mahidol University

Immunity, preclinical discovery, vaccine development

Mapping epitopes of the *Plasmodium vivax* Duffy binding protein with naturally acquired inhibitory antibodies.
Dr. Patchanee Chootong, Mahidol University

Natural immunity against *P. vivax* infection
Dr. Kulachart Jangpatarapongsa, Mahidol University

Drugs, Resistance, Targets and Development

Potential use of 8 aminoquinolines for malaria elimination on islands
Dennis Shanks, Director, Australian Army Malaria Institute

Drugs for Liver Stages
Dennis Kyle, Professor, University of South Florida, Department of Global Health

Ex vivo studies on fresh and cryopreserved clinical isolates of *Plasmodium vivax*: Practical considerations and its relevance to antimalarial sensitivity studies
Bruce Russell, Singapore Immunology Network

1515 – 1600: Break

1600 – 1745: Afternoon Discussion — Infinity Room 1, Ground Floor

1800 – 1930: Dinner — Infinity Room 2, Ground Floor

VMR3+ is supported by a grant from the Bill and Melinda Gates Foundation

1 October 2009 **Armed Forces Research Institute of Medical Sciences**
Vet Med conference room, Building 5

0800 – 0900: Coffee and Pastry

0900 – 11:30: Discussion

11:30: Meeting Adjournment

VMR3+ is supported by a grant from the Bill and Melinda Gates Foundation