รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษาความสัมพันธ์ของ CD56+ โมโนไซท์ ว่ามีความสัมพันธ์หรือเป็นตัวบ่งชี้ความเสี่ยงหรือมีความไวต่อการเกิดโรคมาลาเรียในประเทศไทยหรือไม่

Are CD56+ blood monocytes associated with, or indicators of malaria risk or susceptibility in endemic areas of Thailand?

โดย ดร.ปฐมาวรรณ ฉิมมา และคณะ

เสร็จสิ้นโครงการมิถุนายน 2556

สัญญาเลขที่ TRG5480007

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สถานส่งเสริมการวิจัย

คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

และมหาวิทยาลัยมหิดล

บทคัดย่อ

รหัสโครงการ: TRG5480007

ชื่อโครงการ: การศึกษาความสัมพันธ์ของ CD56+ โมโนไซท์ว่ามีความสัมพันธ์หรือเป็นตัวบ่งชี้ความเสี่ยงหรือมีความไวต่อการเกิดโรคมาลาเรียในประเทศไทยหรือไม่

ชื่อนักวิจัย: ดร. ปฐมาวรรณ ฉิมมา

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ระยะเวลาโครงการ: 2 ปี

ในผู้ที่มีสุขภาพดีกลุ่มประชากรย่อยโมโนไซท์ที่มีการแสดงออกของโปรตีนบนผิวเซลล์ชนิด $CD56^{low}CD33^{high}$ มีอยู่จำนวนน้อยมาก ลักษณะดังกล่าวบ่งชี้ว่าเซลล์ชนิดนี้เป็นเซลล์ที่เจริญเต็มที่ มีความจำเพาะทั้งลักษณะโปรตีนที่แสดงออกบนผิวเซลล์และการทำหน้าที่ของเซลล์ มีรายงานว่าการเพิ่มจำนวนของเซลล์กลุ่มย่อยนี้พบได้ในผู้ป่วยด้วยโรคที่มีอาการอักเสบเรื้อรัง แต่ในกรณีโรคมาลาเรียซึ่งได้รับผลกระทบจากภาวะอักเสบเช่นกัน ไม่ปรากฏรายงานว่ามีผู้พบหรือทำการทดสอบหน้าที่ของ $CD56^{low}CD33^{high}$ โมโนไซท์ในผู้ป่วยแต่อย่างใด ในการศึกษานี้คณะผู้วิจัยได้ทำการจำแนกลักษณะของ CD56 โมโนไซท์จากตัวอย่างเลือดผู้ป่วยมาลาเรียที่มีสาเหตุจากเชื้อพลาสโมเดียมฟัลซิปารั่มและผู้ที่มีสุขภาพดีที่อาศัยอย่างถาวรในพื้นที่ที่มีการระบาดของโรคมาลาเรีย ในช่วงเวลาก่อนและระหว่างที่มีการระบาดของโรคมาลาเรียในพื้นที่อำเภอแม่สอด จังหวัดตากโดยวิธี immunophenotyping ด้วย marker ของเซลล์ดังนี้คือ CD11, CD14, CD16, CD33, CD56, CD64,CD83, CD123 และ mIFN-γ^{+} นอกจากนี้คณะผู้วิจัยได้มีการทดสอบหน้าที่ในการจับทำลายเม็ดเลือดแดงติดเชื้อโดยวิธีฟาโกไซโตซิสอีกด้วย เมื่อเปรียบเทียบระหว่างผู้ที่มีสุขภาพดีและไม่มีประวัติสัมผัสโรคมาลาเรียกับผู้ที่มีสุขภาพดีที่อาศัยอย่างถาวรในพื้นที่ที่มีการระบาดของโรคมาลาเรียและผู้ป่วย พบว่าในผู้ป่วยมาลาเรียและผู้ที่มีสุขภาพดีที่อาศัยอย่างถาวรในพื้นที่ที่มีการระบาดของโรคมาลาเรีย กลุ่มประชากรย่อย $CD56^{low}$mIFN-γ^{+} โมโนไซท์มีจำนวนมาก และมีระดับการแสดงออกของ HLA-DR and mIFN-γ^{+} อยู่ในปริมาณสูง ซึ่งลักษณะดังกล่าวแสดงให้เห็นว่า CD56 โมโนไซท์ อยู่ในภาวะที่ถูกกระตุ้น นอกจากนี้การตรวจพบ $CD83^{low}$ ซึ่งปกติพบได้บนเดนไดร์ติกเซลล์แสดงให้เห็นว่าเซลล์ดังกล่าวมีแนวโน้มที่จะสามารถพัฒนาไปเป็นเดนไดรติกเซลล์ต่อไปได้อีกด้วย ในการทดสอบหน้าที่ของเซลล์พบว่า CD56 โมโนไซท์ มีความสามารถจับกินเม็ดเลือดแดงติดเชื้อด้วยวิธีฟาโกไซโตซิสต์ได้อย่างดี ในผู้ที่มีสุขภาพดีที่อาศัยอย่างถาวรในพื้นที่ที่มีการระบาดของโรคมาลาเรีย ค่าเฉลี่ยร้อยละของ CD56 โมโนไซท์ ในช่วงที่มีการระบาดของมาลาเรียสูงเป็น 5.14 เท่าของช่วงก่อนการระบาด เป็นที่น่าสนใจว่า HLA-DR^{+}mIFN-$\gamma^{+}CD56^{low}CD14^{high}$ $CD16^{-}$ ฟีโนไทป์ สามารถเหนี่ยวนำให้เกิดขึ้นได้จาก HLA-DR^{low}mIFN-$\gamma^{-}CD56^{-}CD14^{+}$,$CD16^{-}$ โมโนไซท์ของผู้ที่มีสุขภาพดีและไม่มีประวัติสัมผัสโรคมาลาเรีย โดยการช็อคด้วยความร้อนในระยะเวลาจำกัด ผลจากการวิจัยนี้ทำให้พบ กลุ่มประชากรย่อยโมโนไซท์ที่มีลักษณะจำเพาะทางฟีโนไทป์ได้แก่ $CD56^{low}$mIFN-$\gamma^{+}CD83^{low}$ โมโนไซท์ และมีความสามารถสูงในกระบวนการฟาโกไซโตซิส ซึ่งพบได้ทั้งในผู้ป่วยและผู้ที่มีสุขภาพดีที่อาศัยอย่างถาวรในพื้นที่ที่มีการระบาดของโรคมาลาเรีย ผู้ที่มีสุขภาพดีและไม่มีประวัติสัมผัสโรคมาลาเรีย (เมื่อเซลล์ถูกกระตุ้นด้วยความร้อน) กลุ่มประชากรย่อยโมโนไซท์นี้เป็นส่วนหนึ่งในการกำจัดเม็ดเลือดแดงติดเชื้อมาลาเรียและเป็นตัวกำหนดสภาวะเบื้องต้นในการติดเชื้อโดยเฉพาะในผู้ที่ปลอดภูมิคุ้มกันโดยทำหน้าที่ในการจำกัดระดับเชื้อมาลาเรียในร่างกาย

คำหลัก: *Plasmodium falciparum* • Human malaria • Monocyte • Phagocytosis • CD56

Abstract

Project Code: TRG5480007

Project Title: Are CD56+ blood monocytes associated with, or indicators of malaria risk or susceptibility in endemic.

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Project Period: 2 year:

In humans, $CD56^{low}CD33^{high}$ defines a minor subpopulation of mature myeloid cells with unique phenotypic and functional features. This blood monocyte subset is increased in chronic inflammatory diseases but neither the frequency nor function of this monocyte subpopulation has been described in malaria. Here, we characterized the phenotype of CD56 monocytes, by immunophenotyping with CD11, CD14, CD16, CD33, CD56, CD64,CD83, CD123 and mIFN-γ^{+}, from *P.falciparum* infected patients and healthy malaria-exposed individuals living in malaria endemic areas, Mae-Sot, Tak. Blood samples were collected before, and during the transmission season. The phagocytosis activity of these CD56 monocytes was also investigated. Compared to healthy malaria naive controls, high percentages of $CD56^{low}$ monocytes expressing membrane-bound IFN-γ (mIFN-γ^{+}) were detected in malaria patients and healthy malaria-exposed individuals. High expression of HLA-DR and mIFN-γ^{+} indicated that $CD56^{low}$ monocytes were activated and detection of $CD83^{low}$ suggested a phenotype consistent with pre-dendritic cells. $CD56^{low}$ monocytes actively phagocytosed *P. falciparum* infected red blood cells (iRBCs), predominantly comprised a $CD14^{high}CD16^{-}$ phenotype in healthy malaria-exposed individuals, but $CD14^{high}CD16^{+}$ and $CD14^{low}CD16^{+}$ phenotypes in malaria infected patients. In healthy individuals, the mean percentage of $CD56^{low}$ monocytes was 5.41 fold higher during the high than during the low malaria transmission season. Intriguingly, this HLA-DR^{+}mIFN-$\gamma^{+}CD56^{low}CD14^{high}CD16^{-}$ phenotype could be simply produced from HLA-DR^{low}mIFN-γ^{-} $CD56^{-}CD14^{+}$,$CD16^{-}$ blood monocytes from malaria-naive controls following limited exposure to mild heat shock. Therefore, blood monocytes from *P. falciparum* infected patients or healthy subjects living in a malaria endemic area as well as blood monocytes exposed to heat shock in the presence of malaria iRBCs were characterized by the appearance, at very high frequency of a mIFN-$\gamma^{+}CD56^{low}CD83^{low}$ monocytes phagocytic-active phenotype. This MO subset could contribute to the partial clearance of infected cells and might determine the initial course of infection especially in non-immune individuals by limiting the maximum parasite density.

Keywords: *Plasmodium falciparum* • Human malaria • Monocyte • Phagocytosis • CD56

Executive Summary

We have discovered and recently reported that high percentages of cells, unambiguously identified as blood monocytes and expressing $CD56^{low}$, were present not only in patients with acute and uncomplicated malaria attacks, but also in healthy, parasite-free but malaria-exposed, individuals. We also found, in human experimental trials, that this unusually elevated percentage of CD56+ blood monocytes was intimately associated with mosquito bites.

We formulated the hypothesis that seasonal changes in environmental conditions are reflected by drastic alterations in blood monocyte phenotypes, including the increased expression of CD56, HLA-DR and mIFN-γ, at a time when malaria morbidity is on the rise. We have tested this hypothesis in field conditions by determining, in a longitudinal follow-up, the pattern of blood monocyte phenotype modifications in a cohort of individuals living permanently in a seasonal malaria endemic area of Thailand. In Mae-Sot, a region of Thailand where malaria transmission is seasonal, our study has shown that the percentage of CD56+ blood monocytes reached the highest levels at the peak of malaria transmission. We compared the percentages and absolute numbers of $CD56^{low}$ blood monocytes before, and during the transmission season, and, in cases of acute uncomplicated malaria attack, just before treatment.

Due to the flood situation in Mae-Sot during the year 2011 to 2012, unexpected problems were occurred. These are the reasons why we will not be able to acquire a dynamic, accurate and reliable picture of the changes affecting the blood monocyte phenotype. In this point, we will need more data to imply how alterations are associated with, or predictive or malaria morbidity. However, to the best of our knowledge, the results of blood monocyte phenotype obtained during this study, are the first to show that CD56 are found expressed on blood monocytes from all individuals exposed to malaria, but the distribution pattern of CD56 differs markedly between patients and healthy malaria-exposed subjects with no clinically detectable sign of disease. High percentages of $CD14^{high}CD56^{low}$ monocytes are actively involved in the phagocytosis of iRBCs, even in the absence of immune serum, but apparently, in the presence of immune serum, phagocytes could more rapidly process the parasites. Moreover, high *in vitro* phagocytic activity of iRBCs was obtained in experimental conditions, including simultaneously high temperature, immune serum and blood monocytes from malaria-naive individuals. Increased phagocytosis activity was associated with induced expression of $CD56^{low}$ on $CD14^{high}$ MO. Therefore, the subset of mature, activated MO characteristic of healthy malaria-exposed individuals was induced when phagocytosis of iRBC was optimized.

เนื้อหาโครงการวิจัย

1. วัตถุประสงค์ของโครงการ

เพื่อที่จะยืนยันและจำแนกลักษณะจำเพาะของโมเลกุลบนผิวเซลลโมโนไซท์ ที่ค้นพบใหม่ในรายละเอียดมากขึ้น จากผลการวิจัยเบื้องต้นที่ได้จากการศึกษาที่อำเภอแม่สอด จังหวัดตาก โดยมีรายละเอียดดังนี้

(1) เพื่อติดตาม การคงอยู่ และจำนวนโมโนไซท์ที่มีการแสดงออกของโมเลกุลบนผิวเซลล์ที่บ่งบอกถึงภาวะที่มีการกระตุ้นทางภูมิคุ้มกันและตัวรับคีโมไคน์ในประชากรที่มีอายุต่าง ๆ กัน ที่อาศัยอยู่ในพื้นที่ที่มีการระบาดของโรคมาลาเรีย

(2) เพื่อติดตามการคงอยู่ และจำนวน CD56+โมโนไซทที่มีการเปลี่ยนแปลงขึ้นลง ในผู้ที่มีสุขภาพดี อายุต่าง ๆ กันที่อาศัยอยู่ในพื้นที่ที่มีการระบาดของโรคตามฤดูกาล ที่อำเภอแม่สอด จังหวัดตาก

2. วิธีทดลอง : โปรดดูในบทความที่แนบมา

3. ผลการทดลอง : โปรดดูในบทความที่แนบมา

4. สรุปและวิจารณ์ผลการทดลอง : โปรดดูในบทความที่แนบมา

Output ที่ได้จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ:
   - บทความกำลังอยู่ในระหว่างการแก้ไขและปรับเปลี่ยนเนื้อหาบางส่วนเพื่อให้ได้รับการตีพิมพ์จากวารสาร Plos One
2. การเสนอผลงานในที่ประชุมวิชาการ:
   - ได้มีการนำเสนอผลงาน (oral presentation) ในการประชุมวิชาการศูนย์ CENID ในโครงการมหาวิทยาลัยวิจัยแห่งชาติ ครั้งที่ 2 ณ ภูผาผึ้งรีสอร์ท ราชบุรี ระหว่างวันที่ 29-30 กันยายน 2554 เนื่องจากมีข้อมูลที่เชื่อมโยงสอดคล้องกันในการศึกษาการเปลี่ยนแปลงของโมโนไซด์

- ได้มีการนำเสนอผลงาน (Invited speaker) Joint International Tropical Medicine Meeting 2012 ณ โรงแรมเซ็นทราแกรนด์ กรุเทพมหานคร ระหว่างวันที่ 12-14 ธันวาคม 2555
- ได้มีการนำเสนอผลงาน (oral presentation) ในการประชุมวิชาการศูนย์ CENID ในโครงการมหาวิทยาลัยวิจัยแห่งชาติ ครั้งที่ 3 ณ โรงแรมศุโกศล กรุงเทพมหานคร ระหว่างวันที่ 11-12 มีนาคม 2556 เนื่องจากมีข้อมูลที่เชื่อมโยงสอดคล้องกันในการศึกษาการเปลี่ยนแปลงของโมโนไซต์

ภาคผนวก

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1. Manuscript ในหัวข้อ **“ Expansion of $CD14^{++}CD16^{-}CD56^{+}CD83^{+}$ human blood monocytes is associated with high phagocytic activity in healthy malaria-exposed individuals ”**
2. การนำผลงานวิจัยไปใช้ประโยชน์เชิงวิชาการ: มีการต่อยอดงานวิจัยจากผลการศึกษาที่ได้จากโครงการวิจัยนี้ โดยมีความร่วมมือในอนาคตกับสถาบันปาสเตอร์ปารีส ที่จะศึกษาบทบาทของเซลล์โมโนไซต์ในผู้ที่อยู่ในพื้นที่ที่มีการระบาดของโรคมาลาเรีย รวมทั้งโรคติดเชื้ออื่น ๆ

Expansion of $CD14^{++}CD16^{-}CD56^{+}CD83^{+}$ human blood monocytes is associated with high phagocytic activity in healthy malaria-exposed individuals.

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Running title: Human monocytes in malaria

KEYWORDS : *Plasmodium falciparum* • Human malaria • Monocyte • Phagocytosis • CD56

Abstract

In humans, $CD56^{low}CD33^{high}$ defines a minor subpopulation of mature myeloid cells with unique phenotypic and functional features. This blood monocyte

(MO) subset is increased in chronic inflammatory diseases but neither the frequency nor function of this MO subpopulation has been described in malaria. Here, we characterized the phenotype of CD56 MO from *P.falciparum* infected patients and healthy malaria-exposed individuals living in malaria endemic areas. The phagocytosis activity of this CD56 MO was also investigated. Compared to healthy malaria naive controls, high percentages of $CD56^{low}$ MO expressing membrane-bound IFN-γ (mIFN-γ^{+}) were detected in malaria patients and healthy malaria-exposed individuals. High expression of HLA-DR and mIFN-γ^{+} indicated that $CD56^{low}$ MO were activated and detection of $CD83^{low}$ suggested a phenotype consistent with pre-dendritic cells (DCs). $CD56^{low}$ MO actively phagocytosed *P. falciparum* infected red blood cells (iRBCs), predominantly comprised a $CD14^{high}CD16^{-}$ phenotype in healthy malaria-exposed individuals, but $CD14^{high}CD16^{+}$ and $CD14^{low}CD16^{+}$ phenotypes in malaria infected patients. In healthy individuals, the mean percentage of $CD56^{low}$ MO was 5.41 fold higher during the high than during the low malaria transmission season. Intriguingly, this HLA-DR^{+}mIFN-$\gamma^{+}CD56^{low}CD14^{high}CD16^{-}$ phenotype could be simply produced from HLA-DR^{low}mIFN-$\gamma^{-}CD56^{-}CD14^{+}$,$CD16^{-}$ blood MO from malaria-naive controls following limited exposure to mild heat shock. Therefore, blood MO from *P. falciparum* infected patients or healthy subjects living in a malaria endemic area as well as blood MO exposed to heat shock in the presence of malaria iRBCs were characterized by the appearance, at very high frequency of a mIFN-$\gamma^{+}CD56^{low}CD83^{low}$ MO phagocytically active phenotype. This MO subset could contribute to the partial clearance of infected cells and might determine the initial course of infection especially in non-immune individuals by limiting the maximum parasite density.

INTRODUCTION:

The host defense mechanisms against pathogens include a wide spectrum of innate and adaptive responses. In malaria, circulating monocytes (MO) and neutrophils are the first cells to interact with *P. falciparum* infected red blood cells (iRBCs) and, in the spleen and in the liver, the tissue macrophages are crucial for parasite clearance. *In vitro* studies have indicated that MO can remove *P. falciparum* parasites either directly through phagocytosis or indirectly through cytokine release [1] or by Antibody Dependent Cellular Inhibition (ADCI), in association with

immune IgG [2]. It is established that there is a major contribution of myeloid cells to early responses to pathogens [3], but the precise phenotype and the respective functional activities of the various cells involved in innate immune responses following *falciparum* malaria are poorly defined [4].

Blood MO scavenge toxic compounds, are involved in the killing and the disposal of infectious agents and have important roles in homeostasis during the chronic phase of inflammation [5].

MO/macrophages and DCs, which are frequently referred to as the mononuclear phagocyte system, are heterogeneous not only in origin but also in function [6]. A consequence of this heterogeneity is that the contribution of MO, both in the control of pathogens and in the pathophysiology of inflammation, is difficult to dissect as some of their many functions may be attributable to discrete functional MO subsets. For this very reason, the precise identification of the function of the diverse MO phenotypes is necessary to understand the contribution of these cells to the outcome of infection [7].

CD56 is an isoform of the Neural Cell Adhesion Molecule (NCAM) involved in intercellular homophilic adhesion. This molecule is present on the surfaces of endothelial cells and has recently been identified as a new cytoadhesion receptor for *P. falciparum* iRBCs capable of aggregation, leading to macro-aggregate formation involved in the pathogenesis of severe forms of malaria [8]. CD56 is classically considered as a marker of NK cells but the discovery of a small blood MO subset expressing this marker in human blood samples has been recently reported. In healthy individuals, the frequency of $CD56^{low}CD33^{+}$ cells is low, ranging from 0.16 to 3.5% of all mononuclear cells [9]. The pattern of cytokine production by $CD56^{low}CD33^{+}$ MO and other Peripheral blood mononuclear cells (PBMC) overlap but $CD56^{low}CD33^{+}$ MO produce detectable levels of IL-6 and IL-1β and thus define a new MO population with distinct phenotypic and functional features.

A few additional details about $CD56^{low}$ blood MO and MO derived DCs have recently been published [9,10,11,12].

$CD56^{low}$ MO are increased in pathological conditions, in patients with active Crohn's disease [10], and in patients with acute myeloid leukemia, particularly with

monocytic differentiation [13]. In the latter case, $CD56^{low}CD16^{-}$ cells expressing the myeloid markers CD33 and HLA-DR corresponded to adherent MO [9]. Interferon alpha-induced DCs (IFN-DCs) generated *in vitro* from MO also express CD56 on their surface [14]. These observations provide compelling evidence that CD56 is not exclusively a membrane marker limited to NK cells, but that it is also found on certain discrete MO subsets both, in healthy individuals and, in some pathological conditions.

In the present study, we have examined the phenotypic and functional heterogeneity of MO in response to malaria infection or exposure in South East Asia where "knobless" *P. falciparum* isolates are frequently found [15,16,17]. We observed the presence of unusually high percentages of $CD56^{low}$ mature and activated MO in patients with uncomplicated *P. falciparum* malaria and in healthy, malaria-exposed individuals with no blood parasite (i.e. non-infected subjects). In malaria-infected patients these activated cells are present in the $CD14^{high}CD16^{+}$ and the $CD14^{low}CD16^{+}$ subsets, while in healthy malaria-exposed individuals, activated $CD56^{low}$MO are predominant in the $CD14^{high}CD16^{-}$ subpopulation.

We studied the function of these subsets of MO and showed non-opsonic phagocytosis of malaria iRBC is associated with the $CD56^{low}CD14^{high}$ MO sub-population.

RESULTS:

High percentages of $CD56^{+}$ blood MO are found both in patients and in healthy malaria-exposed individuals.

We first identified and gated for MO (R1) using MO marker and granularity measured by CD14 expression and linear side light scatter (SSC), (Figure **1A)**. In order to particular identify MO and demonstrated of their myeloid origin, the expression of CD33 and CD11C were investigated. The expression of CD56 by the populations of blood MO present in the area of interest are also illustrated in Figure **1A**.

As expected, expression of CD56 on blood MO was virtually absent in healthy malaria-naive individuals**.** By comparison, $CD56^{low}$ expression was readily identified

in *P. falciparum* infected patients but also on blood MO from healthy, non-infected malaria-exposed individuals, accounting for a high percentage (45.3%) of the $CD14^+CD33^+CD11C^+CD56^+$ MO in the former subjects **(**Figure **1A)**. Of particular note, no $CD56^{high}$ expression typical of NK cells was found on MO populations.

Further confirmation that the $CD56^{low}$ cells really correspond to blood MO was then obtained by fluorescence microscopy analysis demonstrating the overlap between $CD14^{high}$ (blue) and $CD56^{low}$ (green) fluorescent labels at the surface of the cell membrane (Figure **1B**). Confocal microscopy demonstrated the colocalization of CD14 and CD56 molecules at the surface of a representative blood MO from a healthy, non-infected, malaria exposed individual.

Therefore, it was unambiguously concluded that the $CD14^+CD33^+CD11C^+CD56^{low}$ subset of cells identified by our method obviously corresponds to blood MO and cells delineated in R2 region are referred to as blood MO throughout this study.

In malaria-exposed individuals, high percentages of $CD56^{low}$ blood MO are associated with high levels of mIFN-γ.

In healthy malaria-naive individuals, the mean percentage (±1SD) of total blood MO expressing $CD56^{low}CD14^+$ was 4.9±3.1% whereas the mean percentage detected in patients with uncomplicated acute malaria attacks was 48.0±32.8%. Moreover, the mean percentage of $CD56^{low}CD14^+$ MO found in healthy, non-infected, malaria-exposed individuals was 60.0±27.8%, *i.e.* 12.2 fold higher ($P < 0.0001$) than in healthy malaria-naive controls (Figure **2A)**. Finally, FACS analysis illustrated that MO from healthy non-infected malaria-exposed subjects were activated, demonstrating a higher proportion of $HLA\text{-}DR^+$ cells and a higher intensity of HLA-DR expression compared to blood MO from healthy malaria naive individuals (Table **1**).

The mean percentage of $mIFN\text{-}\gamma^+$ MO was elevated in patients with uncomplicated malaria attacks (32.75±29.35%), and unexpectedly even higher in malaria-exposed but non-infected healthy individuals (54.44±29.21%), as compared to healthy malaria-naive individuals (0.51±0.47%) ($P<0.001$ and $P<0.0001$,

respectively, as illustrated in Figure **2B**). The marked association between the percentages of MO expressing CD56 and the percentages of MO expressing mIFN-γ observed in healthy malaria-exposed individuals (R^2=0.894) as illustrated in Figure **2C** strongly suggested that expression of $CD56^+$ on blood MO was closely associated with MO activation in malaria-exposed healthy individuals.

Blood MO from non-infected malaria-exposed individuals displayed several original and unique characteristics which indicate that MO from individuals living in malaria endemic area are comparatively more mature and activated than blood MO from healthy individuals living in a malaria free area. Therefore, further investigations were carried out to elucidate the main characteristics and function of this original and over-represented MO subset seen in subjects living in malaria endemic areas.

CD56 expression is present on different blood MO subsets in patients and in healthy malaria-exposed individuals.

Three different MO subsets can be identified according to the relative levels of CD14 and CD16 expression [10] (**Figure 3A**). $CD14^{high}CD16^-$ (gate G1), $CD14^{high}CD16^+$ (gate G2) and $CD14^{low}CD16^+$ (gate G3) MO subsets were delineated in R1 region. As expected, in healthy malaria-naive and healthy malaria-exposed, non-infected individuals, most MO belonged to the $CD14^{high}CD16^-$ subset. Healthy malaria-exposed, non-infected individuals displayed remarkably elevated proportions of $CD56^{low}CD14^{high}CD16^-$. In contrast, infected patients' MO contained a low percentage of $CD56^{low}CD14^{high}CD16^-$ MO but high percentages of $CD56^{low}CD14^{high}CD16^+$ and $CD56^{low}CD14^{low}CD16^+$ subsets compared to healthy, non-infected, malaria-exposed individuals (P<0.0001 for comparisons of both subsets as shown in Figures **3A** and **3B**).

In summary, CD56 was expressed on blood MO from all individuals exposed to malaria, but the distribution pattern of CD56 differed markedly between infected patients and healthy malaria-exposed subjects. The presence of significant numbers of $CD56^{low}CD14^{high}CD16^-$ MO in healthy, non-infected but malaria-exposed individuals is unusual and intriguing. Furthermore, a high proportion of mIFN-γ^+ $CD14^{high}CD16^-$

MO in non-pathological conditions is uncommon and led us to evaluate to which extent CD56 expression on blood MO is maintained year round.

CD56 expression levels change as a function of malaria transmission seasons in non-infected, malaria-exposed, healthy individuals.

When the mean percentage of $CD56^{low}$ MO present in the blood of 15 malaria-exposed, healthy individuals was evaluated during the peak of malaria transmission, a mean of 68.5±24.7% was found. By comparison, the mean percentage of $CD56^{low}$ MO detected in 19 donors during the non-transmission season was 6.7±3.4%, i.e. 10.2 fold lower (P<0.0001 by Median test, as illustrated in Table **2a**). In addition, 5 healthy individuals living permanently in Mae Sot, Thailand, agreed to be blood sampled twice; once during the non-transmission season, and a second time, 2.5 months later, during the peak of malaria transmission. In both occasions, blood smears from the 5 donors were found free of blood parasite forms and none of the donors reported to have been sick during the weeks before the samplings. A 5.4 fold increase in the mean percentages of $CD56^{low}$ MO was observed between the two periods of investigations, confirming, at the individual level, the drastic changes in $CD56^{low}$ MO percentages (mean % and 95% confidence intervals were 6.8% [1.4%-12.2%] and 36.9% [29.3%-44.5%], during the non-transmission and the transmission period respectively, P=0.001 by paired test). The percentages of $CD56^{low}$ MO found during the two

Further characterization of blood MO was then obtained by using additional markers and investigating with LSRII flow cytometer. For these analyses, we were following the gating of the $CD56^{low}CD14^{+}$ population then the selection of $CD11C^{+}CD33^{+}$ cells and thereafter the analysis of staining by additional markers including mIFN-γ, HLA-DR, CD16, CD64, CD83, and CD123 as indicated in Figure **4B**.

$CD56^{low}CD14^{+}CD33^{+}CD11C^{+}$ cells were found positive for CD123 (an antigen known as the IL-3 receptor alpha chain expressed at high levels only on plasmacytoid DCs and basophils and at lower levels on MO), for CD64 (the Fc-γ receptor I), and for CD83 (an immunoglobulin superfamily member frequently used as a marker for mature DCs).

Altogether, the levels of expression of these different markers confirmed that during the peak of malaria transmission, circulating $CD56^{low}$MO are activated, become more mature and display a phenotype approaching that of DCs.

We formulated the hypothesis that various environmental stimuli, including mosquito bites, might account for the drastic changes observed in the phenotype and percentages of blood MO expressing $CD56^{low}$ and we looked for the functional activity of this singular subpopulation of blood MO.

MO of healthy malaria-exposed individuals are actively involved in the phagocytosis of iRBCs.

Whereas the phagocytosis of non-infected RBC was only marginal, $CD56^{low}$ MO were found able to rapidly engulf iRBC, in the absence of parasite-specific antibodies, and the results of non-opsonic phagocytosis assays were checked in kinetic studies. CD56 expression was rapidly lost after phagocytosis and for this reason, the engulfment of iRBCs by blood MO was best followed-up by analysis of $CD14^{high}$ (which account for virtually all $CD56^{low}$ MO) and by that of $CD14^{low}$ MO (which correspond to the CD56 negative subset of blood MO). The parasite DNA content present in $CD14^{high}$ and $CD14^{low}$ blood MO from non-infected, malaria-exposed, representative healthy individuals was compared by FACS analysis between non-malaria and the malaria transmission seasons.

On the one hand (Figures **5A** and **5B**), after 10 min of assay, the non-opsonic phagocytosis activity of $CD14^{high}$ MO did not markedly differ between seasons. As illustrated by data from a representative donor (out of 8 individuals tested during the non-transmission season), 47.2% of $CD14^{high}$ MO phagocytosed iRBCs versus 39.6% of $CD14^{high}$ MO obtained from another representative donor (out of 10 individuals tested during the transmission season). After 30 min of phagocytosis assay, 28.2% of the $CD14^{high}$ MO tested during the non-transmission season had engulfed iRBCs compared to 49.4% of $CD14^{high}$ MO (i.e. 1.7 fold more) tested during the transmission season (Figures **5A** and **5B**).

On the other hand, after 10 min of non-opsonic phagocytosis assay, only 9.9% of $CD14^{low}$ MO obtained during the non-transmission season had phagocytosed

iRBCs, whereas 35.6% (3.6 fold more) of the same MO subset were involved in phagocytosis during the transmission season (Figures **5A** and **5B**). After 30 min of assay, 21.2% of $CD56^{low}$ $CD14^{low}$ MO had engulfed iRBCs during the low transmission period and up to 40.3% (i.e. 1.9 fold more) of these MO were involved in iRBCs phagocytosis during the high transmission season (Figures **5A** and **5B**).

Overall, the above results of non-opsonic phagocytosis assays indicated that, in addition to $CD14^{low}$ MO, which are classically considered as the MO subset responsible to perform phagocytosis, $CD14^{high}$ MO also showed a marked capacity to engulf iRBCs. These data also suggest that, during the non-transmission season, $CD14^{high}$ MO (i.e. most $CD56^{low}$ MO) were possibly involved more rapidly and at higher percentages in the phagocytosis of iRBCs than $CD14^{low}$ MO. After 30 min of assay, both subsets were more consistently involved in iRBC phagocytosis activity during the transmission period than during the non-transmission season.

In addition, the percentages of all blood MO involved in the non-opsono-phagocytosis of iRBCs were closely correlated with the percentages of $CD56^{low}$ MO able to perform non-opsonic phagocytosis activity, as illustrated by Figure **5C**.

Demonstration by confocal microscopy studies that $CD56^{low}CD14^{high}$ blood MO are the cells involved in iRBCs ingestion.

Overall, cells identified as $CD56^{low}$ blood MO from healthy individuals living in the malaria-endemic area of Mae Sot were found extremely efficient at ingesting non-opsonized iRBCs, particularly during the high transmission season. In order to assess that phagocytosis of parasites was directly attributable to the subset of $CD56^{low}$ MO, additional confocal studies were carried out as shown in Figure **6**.

A cell identified as a MO by direct observation (bright field) was stained by anti-CD56 (in green) and anti-CD14 (in blue) antibodies. This cell had obviously engulfed a parasite as shown by the bright HE staining of the parasite DNA/RNA, a result demonstrating the active involvement of $CD56^{low}$ blood MO in the phagocytosis of iRBCs (as illustrated by merged image in Figure **6**).

Increased levels of $CD56^{low}$ blood MO are induced in short term cultures.

When blood MO from malaria-naive European individuals were maintained at a temperature of 41±1°C, for no more than 30 minutes, the percentage of blood MO found involved in the phagocytosis of iRBCs was elevated (Table **3)**.

Time course experiments confirmed that expression of HLA-DR was initially high at the start of our culture conditions but gradually decreased during phagocytosis assays (data not shown). However, increases in the percentage of $CD56^{low}CD14^{high}$ MO during exposure to heat-shock (i.e. in experimental conditions aimed at "mimicking" the temperature-induced stress of a clinical episode of fever) were associated with an increase in iRBCs phagocytosis by the blood MO, reinforcing the indications of an association between the active and inducible phagocytosis activity and $CD56^{low}CD14^{high}$ MO (Table **3)**.

DISCUSSION:

This study represents a first attempt to describe the phenotypic and functional heterogeneity of MO in response to *falciparum* malaria infection and/or exposure in a malaria endemic area of Thailand. Phenotyping by flow cytometry was used to highlight the relative contribution of different MO subsets in human malaria to functional activity, using methods previously found helpful to delineate characteristics of immune cells, and simultaneously correlate phenotypes with corresponding functional activities [18]. In order to unambiguously identify MO among peripheral blood leukocytes, criteria recognized as characteristic of mononuclear phagocytes were used throughout the present study, including mononuclearity, myeloid nature, and phagocytic potential.

Firstly, our results indicate major changes in the respective proportions of the different blood MO subsets of infected patients and healthy malaria-exposed individuals compared to malaria-naive subjects. They also demonstrate dramatically high levels of pro-inflammatory MO expressing the $CD14^{low}CD16^{+}HLA\text{-}DR^{++}$ phenotype in infected patients [18]. Secondly, a very high percentage of $CD56^{low}$ MO was observed in the $CD16^{+}$ and $CD16^{-}$ MO of malaria-infected patients and healthy malaria-exposed individuals respectively, compared to malaria-naive individuals. NK cell contamination could not explain these results because the gating area used in the present study was based on a reliable methodology and on the use of well-established MO markers, including CD33 and HLA-DR, which are not found on NK cells.

Moreover, NK cells are identified as $CD56^{high}CD16^{++}$ cells whereas we found a $CD56^{low}$ staining on blood MO.

The very high level of $CD56^{low}$ MO in healthy malaria-exposed individuals was a remarkable new finding given that, in the reports published so far, elevated percentages of $CD56^{low}$ MO were detected in patients with inflammatory diseases [13]. To our knowledge, high percentages of this MO subset have never been reported to similar levels in healthy individuals. In addition to CD56 expression, an increase in CD83, mIFN-γ and HLA-DR expression indicated that these MO correspond to myeloid cells with a distinct and stimulated phenotype. Of note, elevated percentages of $CD56^{low}$ expression on $CD16^{-}$ MO were not associated with clinically detectable signs of disease.

In healthy malaria-exposed individuals, CD56 and CD83 expression were largely confined to the $CD14^{high}CD16^{-}$ MO subset, whereas in patients with uncomplicated acute malaria attacks, $CD56^{low}CD83^{low}$ mature and activated MO were mostly detected in the $CD16^{+}$ MO sub-population. $CD56^{low}CD83^{low}$ MO were found at high levels both in the $CD14^{high}CD16^{+}$ and the $CD14^{low}CD16^{+}$ MO subsets, suggesting that $CD56^{low}CD83^{low}CD16^{+}$ MO could reflect, or be associated with, malaria pathology.

In malaria endemic areas it is well established that the blood infection rate is always markedly lower than the mosquito inoculation rate [19]. A large number of mosquito bites do not result in new blood infections and so, healthy malaria-exposed individuals receive frequent sporozoite inoculations without becoming ill. This exposure to pre-erythrocytic parasite stages may be one reason for the important changes found in MO phenotypes, as compared to naive subjects. Other possible reasons are the numerous bites of other mosquito species, such as *Culex,* and *Aedes*, as well as the exposure to a wide range of bacterial and viral infections. In addition, in a recent clinical study, we found that after experimental mosquito bites of Caucasian volunteers, compared to baseline values, $CD56^{low}$ MO were present at high levels and persisted for weeks (unpublished data).

$CD14^{low}$ MO from the same healthy malaria-exposed individuals were apparently not as actively involved in the phagocytosis of iRBCs as $CD14^{high}$ MO, but

addition of pooled immune African serum induced a massive increase in the percentage of MO involved in phagocytosis and disposal of engulfed parasites.

The phagocytosis assays demonstrate that, in the absence of immune serum, blood MO from malaria-exposed but healthy individuals with no detectable blood parasite have a remarkable, and so far not mentioned, capacity to engulf iRBCs.

Finally, these observations might be of relevance with regard to our previous parasite growth inhibition assays [18] because, blood MO from malaria-exposed individuals differ phenotypically and therefore might also display substantially different functional activities compared with MO obtained from malaria-naive individuals. Of note, in the present work, $CD56^{low}CD83^{low}$ MO from healthy malaria exposed individuals were involved in the non-opsonophagocytosis of iRBCs. In contrast, $CCR2^{+}CX3CR1^{+}$ MO from patients enrolled in the same area displayed sustained ADCI activity (and low parasitemia), as demonstrated in our previous investigations. [20]

Phagocytosis of malaria iRBCs is intimately associated with the $CD56^{low}CD14^{high}$ MO sub-population and intriguingly this phenotype can be induced *in vitro* by exposure of $CD56^{-}CD14^{+}$ MO to heat shock and hyperimmune sera.

A report from Thailand demonstrated that the plasma levels of IFN-□ were increased in adults sufferring from mild or severe *P. falciparum* malaria [21]. Remarkably, following *in vitro* exposure to IFN-□, CD14+ MO steadily increased both their CD56 and CD83 expression profile [22], [23] supporting the notion that the MO phenotype is modulated during *falciparum* infection and revealing a critical role for linking innate and adaptive immunity.

MO, macrophages and immature DCs contain preformed intracellular CD83 [24] and our observation of a rapid *in vitro* induction of CD56 after a brief heat shock of blood MO suggests that this might also be the case for this latter marker.

On the one hand, cells expressing CD56 mutually upregulate CD56 through CD56 homophilic binding [25]. On the other hand, CD83 is an adhesion receptor with a counter-receptor expressed on MO and the interaction between the two structures plays a critical role in the induction and regulation of immune responses [26]. Therefore, the presence at high levels of preactivated $CD56^{low}CD83^{low}$ blood MO may

be advantageous to ensure the rapid induction of effective immune responses against invading malaria blood stages and the early control of parasite replication.

MO are potentially relevant sources of DC during many inflammatory responses [27] and in most infections, they might serve as precursors of "emergency" DCs [28] In Mae Sot inhabitants, the phenotypic expression of blood Mo suggests a possible maturation toward DC and this might be relevant for the resolution of malaria infections because human myeloid DC can modulate innate immunity by enhancing NK cell activity [29].

The outcome of protozoan infections is crucially dependent on the order, timing and relative strength of appropriate immune responses [30]. In addition, early signals delivered by cells of the innate immune system as a consequence of pathogen encounter, can shape the subsequent adaptive immunity [31]. Therefore, understanding pathways of induction of different MO subsets *in vivo* and their significance with regard to the outcome of infection will lead to define how the innate immune response contributes to host defense in malaria and hopefully, how interventions to modulate these immune responses may reduce morbidity and/or mortality from malaria.

MATERIALS AND METHODS:

Healthy Individuals and Patients

Three groups of healthy individuals, corresponding either to malaria-naive or to *P. falciparum*-exposed individuals recruited either in non-transmission or during transmission season in Thailand, were studied. Eight healthy malaria-naive individuals (*i.e.* people with no record of previous exposure to malaria infection) were recruited among permanent residents of Bangkok, a malaria free area. Non-infected, but *Plasmodium*-exposed healthy individuals (referred to as healthy malaria-exposed subjects) were recruited among permanent residents of the Tak Province, a malaria endemic area at the border between Thailand and Myanmar. Fifteen of these healthy malaria-exposed individuals were recruited during the malaria transmission season and 19 were recruited during the non-transmission season. Heparin blood samples from malaria-naive or malaria-exposed individuals were obtained and tested for absence of *Plasmodium* infection at the blood bank of Siriraj Hospital, Bangkok and

at Tak Hospital respectively. Fifty four patients from the same Tak endemic area with acute uncomplicated *P. falciparum* malaria attacks at the time of blood sampling, during the peak of malaria transmission period, were included in this work. The study protocol was approved by the Research Ethics Committee, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Written informed consent was obtained from all participants after clear and detailed explanations of the present immunological studies had been given in local language.

Parasite Cultures

TM267, a local *P. falciparum* laboratory strain, was used throughout the study. Parasite cultures were maintained essentially as described by Trager and Jensen[20] in fresh group O^+ human RBCs supplemented with RPMI medium 1640 (Sigma®, Germany) containing 10% heat-inactivated human AB^+ serum at 37°C with 5% CO_2. Group AB sera used in parasite cultures were obtained from malaria-naive donors whose sera did not inhibit *in vitro* parasite growth.

Monocyte Phenotyping

Indirect immunofluorescence and FACS analysis were performed with 200 µl of whole blood. We used mice monoclonal antibodies (mAb) against human CD14 conjugated to peridinin chlorophyll protein (PerCP), or allophycocyanin (APC) (all from BD PharMingen®, USA unless otherwise indicated), human CD16 conjugated to phycoerythrin (PE), and human CD33, human CD56, human CD64, human CD83, human CD123, human HLA-DR, human mIFN-γ conjugated to fluorescein isothiocyanate (FITC). Moreover, Recommended concentrations of mAbs were added to the samples and incubated for 15 minutes, at room temperature. Red cells were lysed using FACS lysing solution (BD Bioscience®, USA) and cells were washed twice with 0.01% sodium azide in 2% fetal calf serum (staining media). The cell pellet was resuspended in 500 µl of staining medium containing 1% fresh paraformaldehyde. Samples were stored overnight at 4°C in the dark and immunofluorescence measured with a FACsCalibur flow cytometer (Becton Dickinson®, USA) and data analyzed using the Cell Quest® programme. Instrument

settings and compensations were adjusted as indicated by Fagnoni and colleagues [32]. In brief, we first established the instrument setting by acquiring the unstained blood cells then the whole blood stained with isotype controls were used to define the limited area of control negative. Compensation was performed on whole blood monocytes stained with each fluorochrome-conjugated mAb and the mixing of two by two and three different single stain positive controls. The MO gated area was initially created according to linear forward light scatter (FSC) versus linear side light scatter (SSC) parameters then data from 2×10^4 viable nucleated cells was acquired to define the CD14 and CD33 gated areas. The acquisition limit was set to include $5\text{-}10 \times 10^3$ cells to be analyzed within the MO gate. The populations of interest were obtained and analyzed by sequential gating.

To investigate expression of more markers, we also performed MO surface analyses by using a LSRII® (Becton Dickinson, USA) flow cytometer, measuring expression of 6 different markers simultaneously. The protocols for the staining procedures are similar to the protocols described above. The additional mAbs used were anti-human CD14 conjugated to APC-Cy 7, anti-CD11C conjugated to APC, anti-mIFN-γ conjugated PerP-Cy 5.5, anti-CD33 conjugated to PE Cy5.5 and anti-CD56 PE-Cy 7 (Bio-legend, USA). Acquisitions were carried out with the FACS Diva software and data were analyzed by Flowjo software.

Isolation of Peripheral Blood Mononuclear Cells

PBMC from healthy individuals were isolated by centrifugation of whole blood (2400 rpm, 30 min, room temperature) over a Histopaque-1077 gradient (Sigma®, Germany). PBMCs were collected from the interface and washed two times with PBS. These PBMC were used in phagocytosis of iRBCs assay. To avoid the contamination of RBCs that can interfere in phagocytosis experiments, red cells were lysed with BD FACS lysing solution (BD Bioscience®, USA) at room temperature. Then cold PBS was used to wash cells and cell pellets were resuspended in RPMI 1640.

Phagocytosis of *P. falciparum* iRBCs.

Phagocytosis assays were performed using PBMC from 8 healthy malaria-naive and 18 healthy *Plasmodium* exposed**,** but non-infected**,** individuals. Aliquots of 250 µl of PBMC isolated after histopaque centrifugation and containing between 1 and 1.5 x 10^5 MO were used. They were placed in polystyrene round bottom tubes with RPMI 1640, at 37°C in a stirred water bath for 30'. Normal RBCs or synchronized parasitized RBCs (with >90 % trophozoite and schizont forms) were stained with dihydroethidiumbromide (HE, nucleic acid staining dye) used at a final concentration of 5 µg/ml for 20 minutes at 37°C. Then the cells were washed 2 times with cold PBS and resuspended in RPMI 1640 at 10 % final hematocrit. HE-labeled RBCs or iRBCs were added at a ratio of target cells to MO of 100:1. Cells were incubated for 30' in a water bath set at 37°C. After phagocytosis, cells were processed as described by Tippett [1]. In our study, instead of using CD14-PerCP to identify the MO, mononuclear phagocytic cells were gated using CD14-APC as a counterstaining dye. Both $CD14^{high}$ and $CD14^{low}$ percentages of phagocytic cells were determined by gating the areas of $CD14^{+}$ and then analyzed for HE bright staining expression on the FL2 channel. In preliminary experiments, the fluorescence intensity of HE expression was found proportional to the amount of parasite RNA/DNA engulfed by MO and this measure of phagocytosis was used throughout this study.

Confocal analysis

Image analysis by confocal microscopy was included in this study. For MO surface staining, PBMC from either naive or malaria-exposed individual were suspended in RPMI with 10% albumax and plated on Lab-Tech chambers (Nunc®, France) for 30' then washed 3 times with PBS containing 1% Triton X-100. In a previous study, we showed that washing after adherence of cells for 30' substantially reduced the numbers of B and T cells which can adhere to the surface of Lab-Tech chambers after longer incubation times[18]. After washing, CD14-PerCP, CD56-FITC and/or CD33-APC and respective isotype controls were added at dilution of 1:50. Lab-Tech chambers were incubated for 30' at room temperature, followed by 3 washing steps with PBS-1% Triton X-100. Each slide was mounted with cold PBS

containing 40% glycerol before analysis by confocal microscopy. For kinetic studies of non-opsono-phagocytosis by confocal microscopy, we prepared samples as for cytometry analyses with the exception that the experiments were performed in the Lab-Tech chambers. After phagocytosis, slides were washed 3 times with PBS-1% Triton X-100 and air dried and stained as described above.

The samples were analyzed by confocal laser scanning microscopy using a Zeiss scanning head mounted on a LSM 510 Meta, Zeiss confocal microscope (Zeiss, Jena, Germany). Z-stack images were collected at 0.4 μm steps with sequential laser excitation to eliminate bleed-through and with confocal parameters selected to minimize the thickness of the calculated optical section. Images are presented as maximum-intensity projections of the Z-stacks.

Statistical analyses:

Univariate and multivariate analyses were carried out using SPSS16® (IBM® Corporation, NY, USA) or JMP® (SAS Institute Inc. Cary, USA). Parametric or non-parametric tests were used to compare the different groups for normally or non-normally distributed data respectively and a *P* value of less than 0.05 was considered as statistically significant.

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Authorship :

Conceived and designed the experiments: PC, CR, JLP. Performed the experiments: PC, PuS, PaS. Analyzed the data: PC, CR. Provided blood samples from healthy individuals and patients: RR, SC. Wrote the paper PC, CR, DJR, KP, PLD. Contributed to the overall design and concept of the study KP and PLD.

Conflict-of-interest disclosure: All the authors concurred to this submission and disclose any commercial affiliations, and none of them is aware of any conflict of interest.

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FIGURES AND FIGURE LEGENDS:

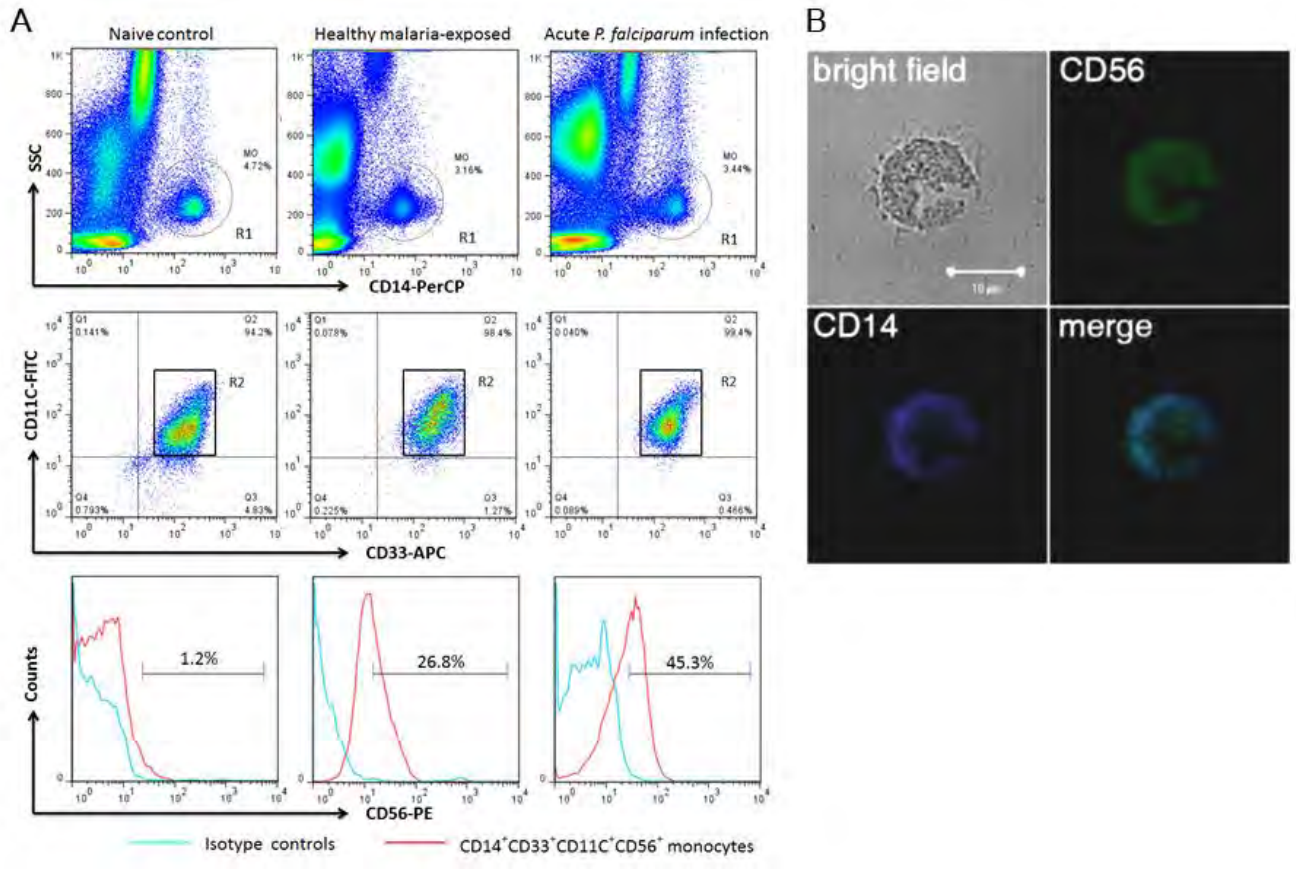

Figure 1: CD56^{low} MO identifications

(A) Representative results of FACS analysis carried out on healthy, malaria naive or healthy, malaria-exposed individuals and patients. Fresh whole blood stained with anti-CD14 PerCP, anti-CD11C FITC, anti-CD33 APC and anti-CD56 PE were gated. Region "R1" represents the selected MO gating, based on monocyte marker (CD14) and the granularity (SSC). Region "R2" represents the myeloid origin of these cells, demonstrated by CD11C and CD33 expressions. Histogram of CD56 expression on $CD14^+CD33^+CD11C^+$ MO from each group of donors are shown. The percentages of $CD14^+CD33^+CD11C^+CD56^+$ MO (red line) over isotype (blue line) are indicated. No $CD56^{high}$ expression was found in the gated area studied, hence NK cell contaminants were excluded. **(B)** Result of confocal study showing co-expression of CD14 and CD56 markers on a representative blood MO from a healthy non-infected malaria-exposed subject. A blood MO is shown in bright field, after CD56 and CD14 staining and the resulting merged study confirmed by confocal microscopy that $CD14^+$ cells are $CD56^+$

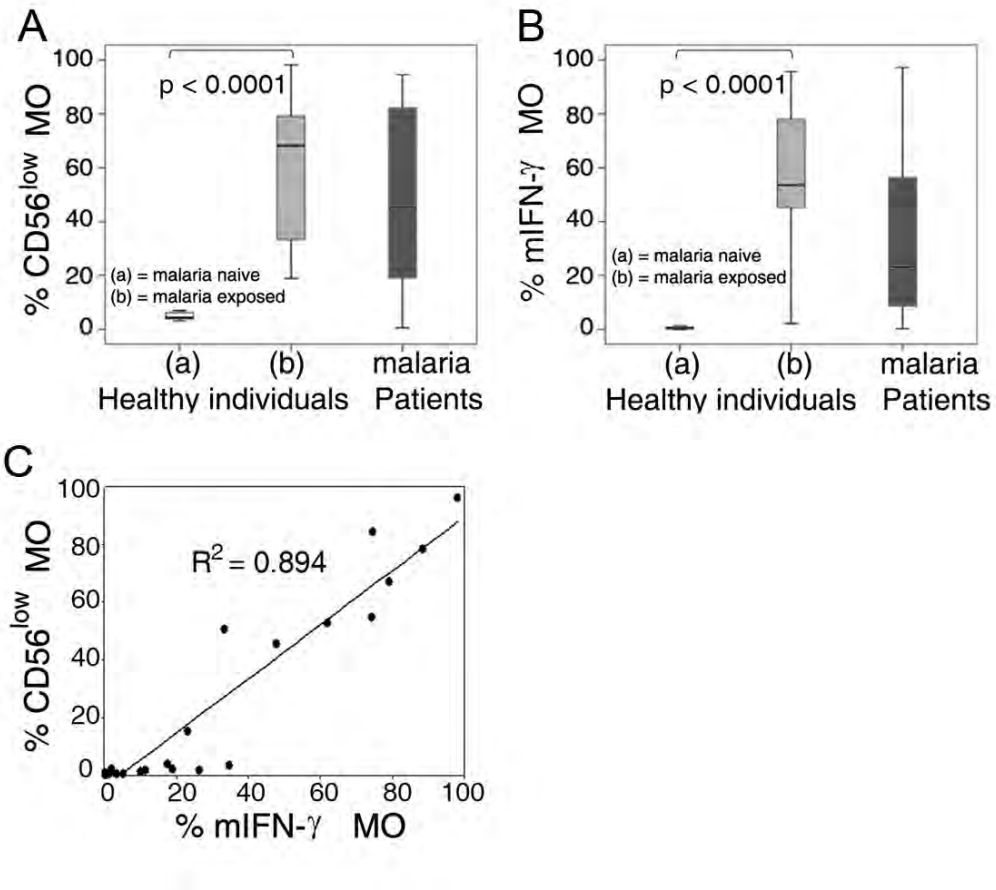

Figure 2: High percentages of CD56$^+$ MO are associated with high level of mIFN-γ$^+$.

Box plots show the percentages of CD56$^+$ blood MO **(A)** and the percentages of mIFN-γ$^+$ blood MO **(B)**. (a) corresponds to malaria naive individuals. The light grey box plot (b) corresponds to MO from healthy non-infected malaria exposed individuals and the dark grey box plot corresponds to MO from patients with uncomplicated malaria attack. The horizontal line corresponds to the median value and the box plots show the 25 and the 75% percentiles. **(C)** The apparent association between CD56$^+$ and mIFN-γ$^+$ markers identified on blood MO from healthy malaria naive and from healthy non-infected malaria exposed individuals strongly suggested the co-expression of the two markers.

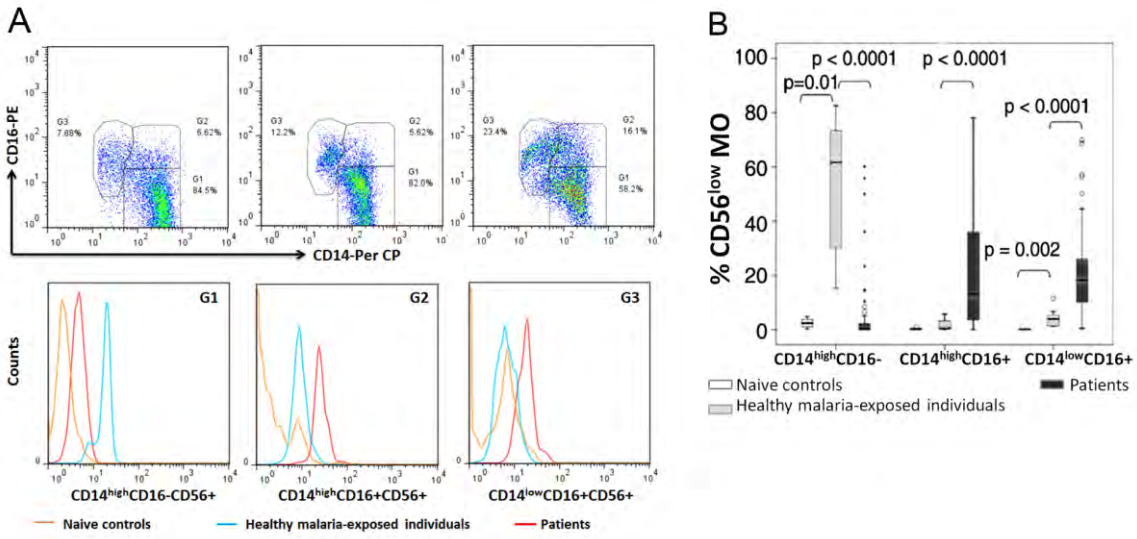

Figure 3: **Relative levels of CD56 expression found on the 3 main subsets of blood MO.**

(A) Representative results of FACS analysis carried out on healthy, malaria naive or healthy, malaria-exposed individuals and patients. Dot plot analyses show the distributions of MO subpopulations; gate G1 represents the $CD14^{high}CD16^{-}$ MO, gate G2 represents the $CD14^{high}CD16^{+}$ MO and gate G3 represents $CD14^{low}CD16^{+}$ MO. Histogram analyses illustrate the distribution of CD56 on each subset of MO. The pattern of staining is shown for 3 representative individuals: a healthy malaria naive individual is shown in orange line, a healthy non-infected malaria-exposed individual is shown in blue line and a patient with uncomplicated malaria is shown in red line. **(B)** CD56 percentages found in classical ($CD16^{-}$) and inflammatory ($CD16^{+}$) blood MO. 8 healthy malaria-naive subjects (white box), 15 non-infected malaria exposed individuals (grey box) and 54 patients with acute uncomplicated malaria (black box) were tested. In each of the three main blood MO subset ($CD14^{high}CD16^{-}$ MO; $CD14^{high}CD16^{+}$ MO and $CD14^{low}CD16^{+}$ MO respectively), the percentages of CD56 found in malaria naive, malaria-exposed and patients are indicated. The horizontal line corresponds to the median value and the box plots show the 25 and the 75% percentiles.

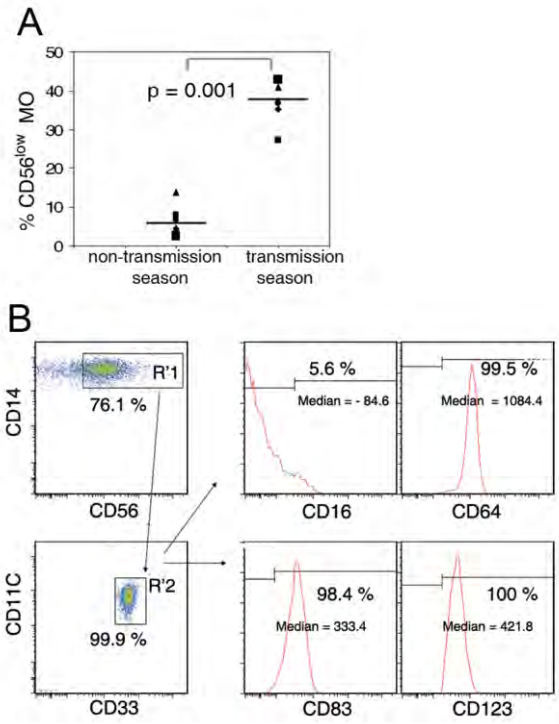

Figure 4: Drastic differences in the percentages of CD56^{low} blood MO were found during the non-malaria transmission season and during the transmission season.

(A) The same 5 healthy non-infected malaria-exposed individuals, living permanently in Mae Sot and with no recent history of malaria infection, were tested twice, at a time when no malaria infection was detectable (horizontal bars indicate mean values. N=5; *P* = 0.001 by paired t-Test).

(B) Further characterization of CD56^{low} blood MO from malaria exposed individuals (during transmission season). Cells found positive for CD14 and CD56 (in R'1) were also positive for CD33 and CD11C (in R'2) and most of them were also CD123, CD64 and CD83 positive, whereas a minority of the CD56^{low} MO were stained by anti-CD16 antibodies.

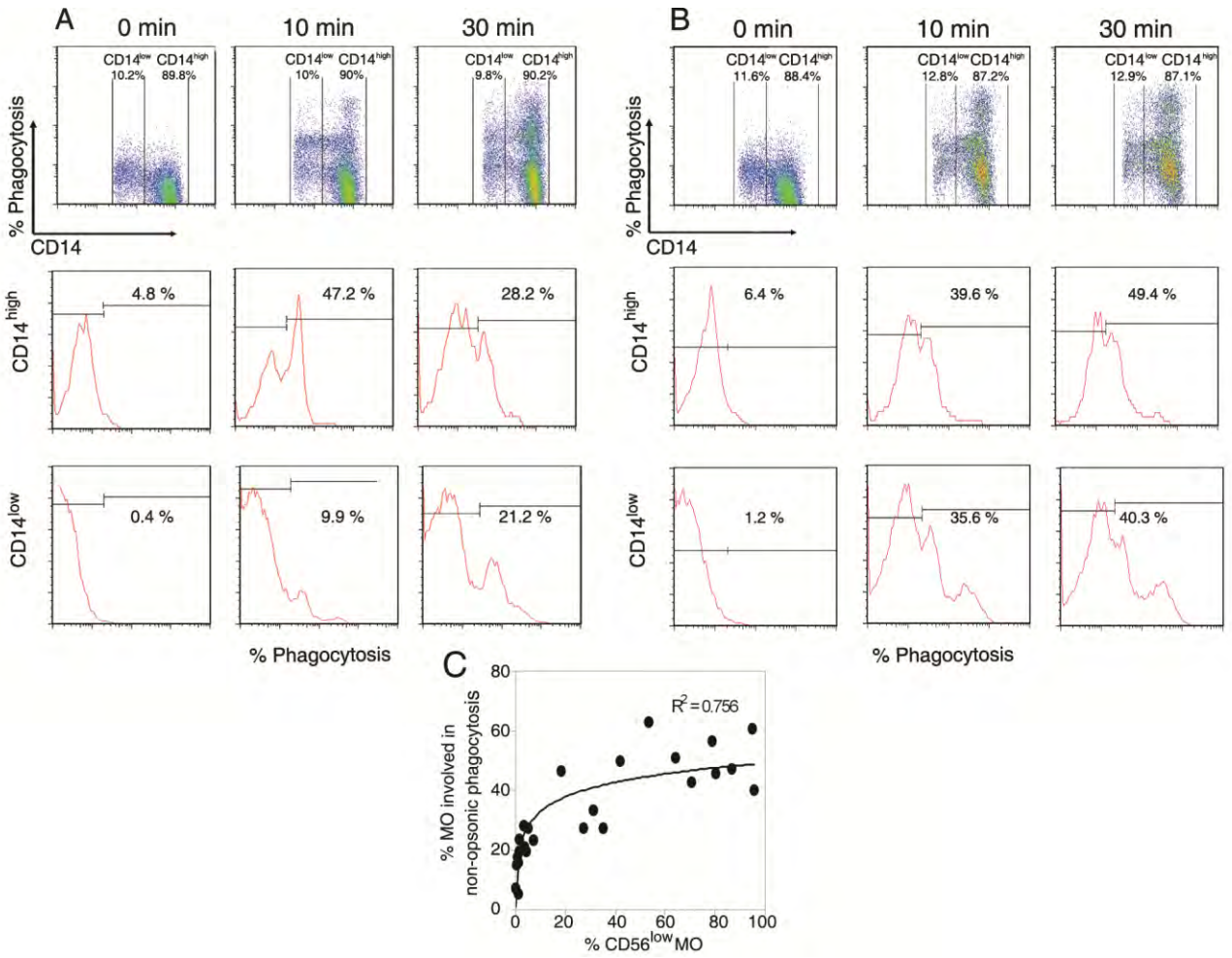

Figure 5: Kinetics of non-opsonic phagocytosis of iRBC by blood MO from representative, healthy and non-infected malaria-exposed individuals.

Phagocytosis tests were done with blood MO obtained either during the non-transmission season **(A)** or during the transmission season **(B)**. FACS analysis was carried out 3 times, at the start of the assay (0 min), after 10 min and after 30 minutes. Figures show the pattern of CD14 staining and the percentage of phagocytosis of iRBCs. In addition, counts of $CD14^{low}$ and of $CD14^{high}$ are shown for MO having or having not engulfed parasites and results of non-opsonophagocytosis are illustrated in each MO subset. **(C)** This picture shows the simultaneous increase in the percentages of $CD56^{low}$ MO and the percentages of the entire population of blood MO involved in the non-opsonic phagocytosis of iRBC in a 30 minutes assay with cells obtained from healthy non-infected malaria-exposed individuals.

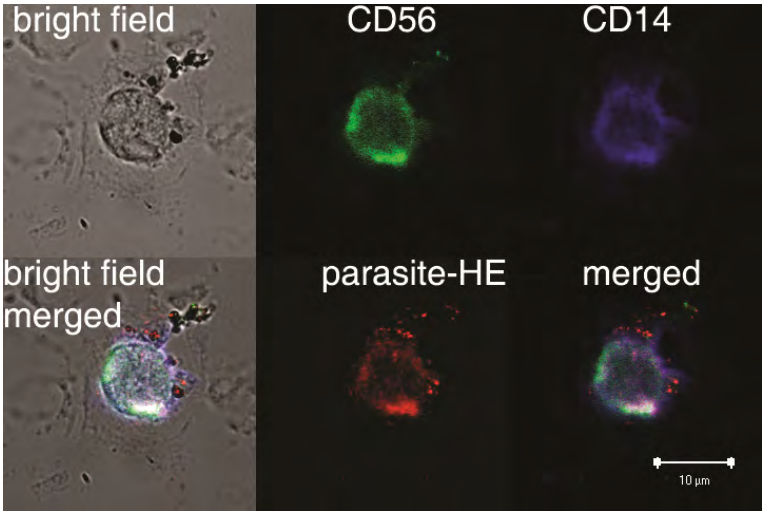

Figure 6: CD56^{low} blood MO having ingested malaria parasites.

After a phagocytosis assay, pictures of a blood MO are shown in bright field (upper left corner of Figure **6**), and following staining with either anti-CD56 (green) or anti-CD14 (violet) monoclonal antibodies. The DNA/RNA of malaria parasites was stained by HE ("parasite-HE"). A merged picture is shown in bright field (lower left corner) and again, as a final confocal merged picture (lower right corner).

TABLES:

Table 1. FACS analysis of myeloid and activation marker expression on blood MO of healthy malaria naive and healthy, non-infected but *P. falciparum* exposed individuals.

Subjects tested	Monocytes %		Fluorescence intensity (FI)	
	CD33	HLA-DR	CD33	HLA-DR
8 Healthy malaria naive individuals	99.8±0.2	4.6±4.4	46.3±17.4	51.1±28.9
10 Healthy, non-infected malaria-exposed subjects	99.5±1.3	99.6±0.4**	30.4±5.8*	154.6±25.6**

Significance levels were determined by independent t-tests between healthy malaria naive and healthy malaria-exposed individuals, *$P = 0.037$, and **$P < 0.0001$.

Table 2. Levels of $CD56^{low}CD33^{+}$ blood MO found in healthy malaria naive and healthy malaria-exposed individuals during the low and high malaria transmission seasons in Mae Sot.

Healthy individuals tested:	Malaria transmission seasons	Mean % ± SD in blood samples		Fluorescence Intensity (geometric mean values)		
		MO	$CD56^{low}$ MO ($CD14^{+}CD33^{+}$)	$CD14^{low}$	$CD14^{high}$	$CD56^{low}$
Malaria naive	No transmission (N= 8)	5.6±2.0	1.8±1.4	92.1±44.6	199.5±38.6	46.5±16.3
Malaria-exposed	No transmission (N= 19)	5.4±1.5	6.7±3.4	93.7±1.7	183.8±49.6	46.5±8.1
Malaria-exposed	Peak transmission (N= 15)	5.7±2.1	68.5±24.7**	36.5±2.9	132.7±22.9	19.2±3.6

The individuals tested were either healthy malaria naive (N= 8) or healthy, non-infected but malaria-exposed volunteers tested either when there was no malaria transmission (N= 19) or during the peak of malaria transmission (N= 15). $**P < 0.0001$.

Table 3: Experimental induction of increased percentages of $CD56^{low}$ MO and non-opsonophagocytosis of iRBC.

Test conditions	Phagocytosis %		$CD56^{low}$ $CD14^{high}$		$HLA\text{-}DR^{+}$ $CD14^{high}$	
	blood MO	$CD14^{high}$	%	FI	%	FI
MO+ sera* + 37 °C	nd	nd	2.4±0.3	30.3±2.5	25.7±4.1	18.9±5.1
MO+ sera* + 41°C	5.3±1.6	3.6±0.8	4.9±1.7	25.9±2.5	69.5±25.6	100.3±100.8
MO+ sera*+ iRBC +41°C	27.8±11.2	20.7±4.3	11.2±3.1	31.0±9.7	22.3±8.8	26.7±12.8

*10% hyper immune sera were added into the culture media.

The mean±SD of representative results obtained from 3 independent experiments performed with 3 different malaria naive European individuals are indicated.

Each phagocytosis assay was carried out in duplicate.