Induction of specific immune responses against the *Plasmodium vivax* liver-stage via in vitro activation by dendritic cells

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Abstract

Due to chronic morbidity, the risk of increasing drug resistance and the existence of the hypnozoite stage in *Plasmodium vivax* malaria, there is a need to find out how hosts develop immunity to compromise the malaria parasites. Here we focused on an in vitro model for immunotherapy and vaccine development. Immunosuppressive mechanisms in malaria include inhibition of T cell response and suppression of dendritic cell function. Using in vitro activation of lymphocytes by malaria antigen-pulsed dendritic cells could overcome the limitation of antigen presentation during acute infections. Here we showed that the sporozoite-pulsed dendritic cell could elicit cytotoxicity against liver stage of *P. vivax*. Analysis using immunophenotypic markers showed maturation of the dendritic cells and stimulation of cytotoxic T cells. Functional assay of the in vitro-activated cytotoxic T cells showed enhancement of specific killing of the *P. vivax* exoerythrocytic stages within infected hepatocytes. This model may be useful for vaccine development against human malaria.

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1. Introduction

Malaria is one of the most important infectious diseases worldwide, as approximately 40% of the world’s population are at risk. The major parasites that cause human malaria include *Plasmodium vivax* and *Plasmodium falciparum*. It has been estimated that outside Africa most malaria cases are caused by *P. vivax* [1]. Vivax malaria not only causes debilitating illness, but also clinical relapses even years after first successful treatment. This is due to the failure in destroying the dormant liver-stage parasites, hypnozoites [2], and the increasing incidences of drug resistance in *P. vivax* [3].

Several malaria vaccine trials have focused on blood and sexual stages [4,5], but rarely investigated the pre-erythrocytic stage. The *P. vivax*-infected hepatocytes are of interest in the development of vaccine-induced immunity because of their relapsing property in humans. Hypnozoite is one of the targets in vaccine development against *P. vivax*. The pre-erythrocytic stage parasite completes its cycle for approximately 5.5 days in the hepatocyte, which allows the detection by immune effector cells [6]. However, because of the lack of knowledge in the parasite–host response relationship, there have been limited studies in the vaccine development against *P. vivax* liver stage [6,7].

It is widely accepted that primary effector cells against liver stage malaria are CD8+ T cells having specificity to parasite-derived epitopes in association with MHC class I molecules of the infected hepatocytes [6]. However, very weak natural CD8+
T cell responses against liver stage antigens have been observed in both vivax and falciparum malaria-exposed persons [8,9]. Although researchers have been interested in a vaccine against liver stage malaria because it can prevent clinical symptoms and transmission, the success has been limited largely due to the immune tolerance mechanisms in the liver that cause poor and short-lived CD8+ T cell responses [10]. Since the conventional subunit malaria vaccines have many limitations including incorrect native structural conformation, narrow and limited range of both MHC class I and II binding motifs, inability for long-term persistence, and limited numbers of effective exogenous adjuvants [11,12], they are not efficient in the field study in endemic areas [13]. The whole organism approaches such as radiation-attenuated sporozoites (RAS) have been used because of their outstanding efficacy [14]. Yet, RAS immunization was less effective in P. vivax infections compared to a P. falciparum model [14]. Therefore, these novel approaches for malaria vaccine development require further studies [15–17]. One promising venue is dendritic cell-based vaccine, since it could overcome the problem of boosting immunity from a RAS vaccine, especially in endemic areas [18,19].

Dendritic cells, the most potent antigen presenting cells, prime-specific T cell responses and play important roles in responses against various pathogens [20]. In malaria, dendritic cells induce cellular immune responses against both liver and blood stages [21,22]. At the onset of sporozoite infection, dendritic cells are recruited to the liver, suggesting a role in liver-stage immunity [23]. In previous studies, antigen-pulsed dendritic cells have been used for the induction of immune responses against liver-stage parasites, but these studies are limited only in murine malaria [16–18,24,25]. In contrast, the role of dendritic cells in human malaria has rarely been explored [26].

Here this is the first report describing the induction of in vitro specific cytotoxic responses against the pre-erythrocytic stage of P. vivax by using monocyte-derived dendritic cells pulsed with crude extract of P. vivax sporozoites. We show that sporozoite-pulsed dendritic cells were able to activate cytotoxic lymphocytes, leading to specific killing of the infected hepatocytes. This may be a useful model for longitudinal studies of cell-mediated responses specific to the liver-stage parasites in the future.

2. Materials and methods

2.1. Sporozoite production

Production of P. vivax sporozoites has been described previously [27]. In brief, blood was drawn from positive cases following consent of the patients and kept in heparin. The blood was fed to 5- to 7-day-old female Anopheles dirus (Bangkok Colony) mosquitoes. Sporozoite-containing salivary glands were dissected 3 weeks later and pooled salivary glands were grounded in an aseptic solution containing 200 U/mL penicillin, and 200 μg/mL streptomycin. The sporozoite suspension was subsequently centrifuged and the number of sporozoites counted.

2.2. Sporozoite antigen preparations

The isolated sporozoites were washed and resuspended in a serum-free minimal essential medium (MEM) at 10^7 sporozoites/mL. Sporozoite extract was prepared by freezing and thawing for four times and the solution was centrifuged at 4 °C at 300×g for 10 min. The supernatant was collected and sterilized with a 0.45 μm filter. Protein concentration was determined by using the Bradford assay (Bio-Rad, Hercules, CA, USA).

2.3. Infection of hepatocytes in vitro

Human hepatocyte cell line, HC-04, susceptible to both sporozoite of P. falciparum and P. vivax, was used to produce the exoerythrocytic stages of malaria parasites [28]. Briefly, 5×10^4 hepatocytes per well were plated in a 96-well tissue culture plate (Nunc, Norway) and cultured at 37 °C with a complete medium (MEM: Ham’s F12 Gibco BRL, 1:1 v/v) supplemented with 10% fetal bovine serum (Gibco BRL), 100 U/mL penicillin, and 100 μg/mL streptomycin. At 48 h, the medium in each well was aspirated and replaced with 2×10^-4 sporozoites in 25 μL MEM+Ham’s F12 complete medium containing 10% heat-inactivated group B human serum, 200 U/mL penicillin and 200 μg/mL streptomycin. After incubation for 3 h, the hepatocytes were washed three times with the complete medium and the number of aspirated sporozoites in the washed medium from each well was counted. About 100 μL of fresh complete medium was added to the infected hepatocytes. The culture was maintained for 2 days to allow growth of the liver-stage parasites.

2.4. Generation of human dendritic cells in vitro

Blood was obtained from healthy donors after receiving the informed consent (Mahidol University IRB number 76/2003). Peripheral blood mononuclear cells (PBMCs) were used for the generation of dendritic cells as described previously [29] with modifications. Briefly, PBMCs were isolated from non-immune donors by gradient centrifugation using the Ficoll-paque solution (Nycomed, Oslo, Norway). The PBMCs were then panned in 24-well tissue culture plates and incubated under culture conditions for 3 h. Non-adherent cells were removed by washing and the adherent PBMCs were further cultured in serum free X-VIVO medium (BioWhitaker, Walkersville, MD) supplemented with 100 ng of granulocyte–macrophage colony stimulating factor (GM-CSF) and 100 ng of interleukin-4 (IL-4) (R&D systems, Minneapolis, MN, USA). The cells were cultured for 7 days, and the medium was changed every other day.

2.5. Antigen pulsing and maturation of dendritic cells

Dendritic cells cultured for 7 days were washed by serum-free X-VIVO medium, and 75 μg/mL sporozoite extracts were added and incubated under culture conditions for an additional 18 h. Then, supernatant was aspirated and the sporozoite-pulsed
dendritic cells were incubated with 50 ng/mL tumor necrosis factor-alpha (TNF-α) (R&D systems) and 1 μg/mL *Salmonella* lipopolysaccharide (LPS) (Sigma) for 24 h. The immunophenotypes of the sporozoite-pulsed dendritic cells from day 9 cultures were characterized by flow cytometry. An aliquot of the pulsed dendritic cells was cryopreserved in a cell freezing solution (Cell Banker, Nippon Zenyaku, Kohriyama, Japan) for restimulation of T cells. The phenotypes and function of cryopreserved dendritic cells have been shown to be well preserved after thawing [30].

2.6. Flow cytometry

The sporozoite-pulsed dendritic cells were stained with fluorochrome-conjugated antibodies to CD83, CD80, CD86, CD40, CD1a, and HLA-DR (BD Biosciences, San Jose, CA, USA). The stained cells were then analyzed by flow cytometry (FACScan) using the Cellquest software (Becton Dickinson, Franklin Lakes, NJ, USA).

2.7. Stimulation of T cells by the sporozoite-pulsed dendritic cells

The pulsed dendritic cells were co-cultured with freshly isolated lymphocytes from the autologous donor at a ratio of 1:10. 20 IU/mL interleukin-2 (IL-2) (R&D systems) was added to the culture on days 3 and 5. After 7 days of IL-2 stimulation, pulsed dendritic cells were added into the culture again at the same number for restimulation of the T lymphocytes. The same amount of IL-2 was added on days 3 and 5 during the stimulation. On day 7 of co-culture, the stimulated T lymphocytes were tested for cytotoxic activity.

2.8. IFN-γ detection

To determine whether T lymphocyte activation had occurred following stimulation by the pulsed dendritic cells. Supernatants from day 3 dendritic cell-lymphocyte co-culture were tested for IFN-γ production by ELISA using the standard IFN-γ detection kit (BD Biosciences).

2.9. Characterization of the activated T cells

Phenotypes of the activated cytotoxic T cells were characterized by flow cytometry using monoclonal antibodies to CD3, CD25, CD56, TCR-α/β, TCR-γ/δ (Immunotech, Marseille, France), CD4, CD8 (BD Biosciences), and monoclonal antibody (MAb) against IFN-γ for intracellular cytokine staining (Caltag Laboratories, Burlingame, CA, USA). Phase-contrast microscopy was used for gross examination of the T cell proliferation during in vitro activation and compared with that of the unstimulated culture.

2.10. Cytotoxicity assay

The antigen-specific dendritic cell-stimulated lymphocytes at $2 \times 10^7$, $1 \times 10^7$ and $5 \times 10^4$ cells/50 μL/well were co-cultured for 4 h with the day 2 sporozoite-infected HC-04 in 96-well tissue culture plate. At the end of the incubation, the effector cells were removed by vigorously washing three times with 200 μL MEM+F12 medium. The viability of the remaining cells in each well after washing was subsequently analyzed by adding 100 μL of MEM+F12 medium and 10 μL of Alamar Blue™ (10% FBS; Serotec, Kidlington, Oxon, UK). The Alamar Blue™ was used to determine the quantity of the live cells in the culture by incubating with the culture for 3 h and the fluorescence intensity was determined by using SoftmaxPro program. The reduction of fluorescence intensity indicates the degree of cytotoxicity of the target cells in the culture [31]. To evaluate specific killing of the infected hepatocytes by activated T cells, the fluorescence intensity ($L$) from co-culturing with uninfected hepatocytes was subtracted by that from co-culturing with infected hepatocytes with the same number of effector cells ($L$ uninfected well – $L$ sporozoite infected well). When comparing the difference in cytotoxicity in each experiment, the $L$ of specific killing from lymphocytes generated by culturing with the sporozoite-pulsed dendritic cells ($L_{PDC}$) in vitro was subtracted by that from lymphocytes with non-pulsed dendritic cells ($L_{NDC}$) and calculated as follows:

$$L_{PDC} = (L_{PDC} \text{ uninfected well} – L_{PDC} \text{ sporozoite–infected well}) – (L_{NDC} \text{ uninfected well} – L_{NDC} \text{ sporozoite–infected well}),$$

where $L_{PDC}$ is the level of $L$ from the non-infected HC-04 (uninfected well) or from the $P. vivax$-infected HC-04 (sporozoite-infected well) co-cultured with the lymphocytes stimulated by sporozoite-pulsed dendritic cells. $L_{NDC}$ is the level of $L$ from the non-infected HC-04 (uninfected well) or from the $P. vivax$-infected HC-04 (sporozoite-infected well) co-cultured with the lymphocytes stimulated by non-pulsed dendritic cells as control.

2.11. Statistical analysis

Differences of the fluorescence unit of specific killing after subtracted from uninfected hepatocyte control among effector cells were compared by using the Kruskal–Wallis $H$ test. The differences in specific killing between the lymphocytes activated by pulsed and non-pulsed dendritic cells were compared using the Mann–Whitney $U$ test. The differences in IFN-γ production between lymphocytes activated by sporozoite-pulsed and non-pulsed dendritic cells were compared by a two-tailed, unpaired Student’s $t$ test. A $p$ value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS for Windows program.

3. Results

3.1. Infection of hepatocytes

Fig. 1 showed an HC-04-containing intrahepatic $P. vivax$ after maintaining under the culture condition for 2 days post-sporozoite infection. The parasite reacted with the MAb to $P. vivax$ circumsporozoite protein (PvCSP) VK210 type. The
day 2-infected HC-04 cells were subsequently used as target cells for cytotoxicity assay.

3.2. The generation of dendritic cells

Sporozoite-pulsed dendritic cells were induced to maturation by adding 50 ng/mL TNF-α and 1 μg/mL LPS. The mature dendritic cells were confirmed by morphology and subsequently by flow cytometric analysis using fluorochrome-conjugated antibodies against surface antigens. The results showed that 31.54% (21.75–60.71%) of the antigen-pulsed dendritic cells showed CD80+ (Fig. 2A) and 67.46% (55.31–92.89%) were CD86+ (Fig. 2B). Varied numbers (average 50.15%, 7.58–89.57%) of the antigen-pulsed dendritic cells were strongly CD83+ (Fig. 2A, B), confirming maturation of the cells during in vitro activation. Co-stimulatory molecule CD40 was also expressed in 29.36% (18.87–57.67%) of dendritic cells (Fig. 2C), indicating the capacity for T cell stimulation. We also found that 49.15% (17.03–88.28%) of these dendritic cells were also CD1a+ and 60.66% (43.59–77.73%) were strongly HLA-DR+ (Fig. 2C, D). These antigen-pulsed dendritic cells were then used as antigen-specific stimulators for T cell activation.

When compared with monocytes prior to culturing, the monocytes were found to express very low amount of dendritic cell maturation markers and costimulatory molecules (data not shown). Additionally, after the addition of maturation factors, the expression of surface markers of non-pulsed dendritic cells was not found to be significantly different from that of antigen-pulsed dendritic cells. The only difference between these two cell populations was found after antigen addition, indicating similar capacity of pulsed and non-pulsed dendritic cells in T cell stimulation (data not shown).

3.3. Induction of T cell response

IFN-γ production was determined in the culture supernatant collected from lymphocytes co-cultured with the sporozoite-pulsed dendritic cells. The IFN-γ production was observed in the co-cultivation of lymphocytes with antigen-

![Fig. 1. Infection of hepatocytes in vitro. HC-04 human hepatocytes were infected with sporozoites of *P. vivax* and stained with anti-PvCSP antibody 2 days after the infection. The early EE-stage parasite expressed CSP as visualized by immunofluorescence staining (green). Hepatocytes were counterstained with Evans blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image1)

![Fig. 2. Flow cytometric analysis of dendritic cells. Dendritic cells pulsed with sporozoite extracts and stimulated with TNF-α and LPS, were immunophenotyped by using fluorochrome-conjugated antibodies to various surface markers, CD83, CD86 and CD80 (A and B), CD1a and CD40 (C), and HLA-DR (D). % of each marker expression is shown in the upper right corner.](image2)
pulsed or non-pulsed dendritic cells. The results showed that the pulsed dendritic cells (428.33 ± 140.59 pg/mL) induced significantly more IFN-\(\gamma\) production than that from lymphocyte controls (168.75 ± 68.84 pg/mL) (Fig. 3A; \(P = 0.026\)). In addition, the high IFN-\(\gamma\) level was found to be associated with the appearance of multifocal proliferation of lymphocytes observed microscopically (data not shown). We also observed that the \(L_{\text{PDC}}\), lymphocytes co-cultured with sporozoite-pulsed dendritic cells, were 1.26±0.12 times that of \(L_{\text{NDC}}\), lymphocytes co-cultured with non-pulsed dendritic cells. The activated lymphocytes, after co-culturing with sporozoite-pulsed dendritic cells, showed expanded CD8+IFN-\(\gamma\)+cell populations (26.81%) when compared with the unstimulated lymphocyte control (19.36%) (Fig. 3B). In addition, the level of CD3+TCR\(\alpha/\beta^+\) population was also increased accompanied by a drastic reduction of the CD56\(^+\) cell population (data not shown). However, the level of IFN-\(\gamma\) production from \(L_{\text{PDC}}\) was positively correlated, albeit not significantly, with the specific fluorescence unit in the killing of infected hepatocytes after being subtracted by that of control (\(R = 0.50, P > 0.05, n = 3\)).

3.4. Cytotoxicity of the activated T cells

Cytotoxic activity of effector T cells co-cultured with the sporozoite-infected HC-04 was determined as described in the methods. The reduction of fluorescence intensity was correlated with the degree of target cell cytotoxicity in the culture. For the validation of specific killing, fluorescence intensity of the non-infected HC-04 control was subtracted from that of the remaining infected HC-04 after co-culturing with the effector T cells. The specific killing activity by each effector cells calculated from fluorescence intensity showed significant difference (Fig. 4A; \(P = 0.038\)). However, one of the six cases showed spontaneous killing of the uninfected hepatocytes, and was therefore excluded from the study. To confirm the difference in cytotoxic activity in each experiment, the specific killing from \(L_{\text{PDC}}\) was subtracted by that from \(L_{\text{NDC}}\). The \(L_{\text{PDC}}\) showed significantly greater cytotoxic activity than the \(L_{\text{NDC}}\) (Fig. 4B; \(P = 0.014\)).
4. Discussion

We investigated the in vitro induction of cytotoxic lymphocytes by sporozoite-pulsed dendritic cells using human malaria, *P. vivax*. Previous studies in mouse malaria models have shown that dendritic cells can modulate protective immunity against the pre-erythrocytic stages [16,17,25]. Yet, several studies have shown defects in dendritic cell function during infection or after being exposed to blood-stage human malaria [26,32]. For the elimination of liver stage parasite, the major aim is to induce specific T cell responses against *P. vivax*-infected hepatocytes [33,34]. In this study, we generated dendritic cells in vitro from peripheral blood monocytes of malaria naïve healthy persons. Immunophenotypes of the dendritic cells analyzed by flow cytometry showed the expression of costimulatory molecules essential for stimulation of specific T cells including CD86, CD80, and CD40. The successful differentiation of dendritic cells provided a possibility for further studies of in vitro induction of T cell immunity against malaria parasites.

Co-culturing of sporozoite extract-pulsed dendritic cells and T lymphocytes successfully induced clonal proliferation in vitro. The level of cell proliferation was positively correlated with the level of IFN-γ release during the activation as determined by quantitative ELISA assays. The role of IFN-γ in the elimination of infected hepatocytes has been suggested previously [35,36]. Our study using in vitro activated lymphocytes by sporozoite-pulsed dendritic cells, which were able to eliminate *P. vivax*-infected hepatocytes as quantified by the Alamar Blue hydrolysis assay, has provided further supporting evidence for the correlation between IFN-γ production and cytolysis of infected cells. Moreover, at the same effector/target ratio, the sporozoite-pulsed dendritic cells could potentiate higher cytotoxic activity of the T-lymphocytes to specifically lyse vivax-infected hepatocytes. Variation of results among experiments may be due to factors such as differences in MHC-dependent cytotoxicity and epitope specificity [37–39]. Although soluble proteins are less effective antigen sources than cell-associated antigens for dendritic cell pulsing [40], we have shown that sporozoite-pulsed dendritic cells could successfully induce cytotoxicity against *P. vivax* pre-erythrocytic stage when primed with a higher concentration of soluble antigens, consistent with findings in another model [41]. CSP is a shared antigen between sporozoite and EE stage (Fig. 1) although the epitopes for antibody and T cell recognition are different. The liver cells are them first target of sporozoite invasion, and the outcome of malaria infection is therefore dependent on how good the sporozoites have developed to the EE stage. The immunotherapy against malaria should therefore stop the parasite at the earlier stage of development. The results shown in this study introduced an alternative direction for vaccine development. Further, sporozoite-pulsed dendritic cells might not boost complete immune responses against antigens that are exclusively expressed only by the liver-stage parasites [6,42], but at least our results indicated that between the sporozoite and liver-stage parasites do share certain antigens.

Dendritic cells have been suggested for vaccination in various infections [43]. For the protozoa infection, particularly *Leishmania* infection, dendritic cell-based vaccination has been investigated extensively [44,45]. In murine malaria, previous studies have demonstrated the importance of dendritic cell immunization against both blood stage [17,46] and liver-stage parasite [16,18,24,25,47]. Here we show for the first time that sporozoite-pulsed dendritic cells in vitro could elicit antigen-specific T lymphocyte immunity against liver stage of vivax malaria. This technique of antigen-specific priming of dendritic cells in vitro may be useful in future studies towards the development of individual ex vivo vaccine-primed dendritic cells.

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