

Correlation between Parasite Density with TNF- α and IL-10 in Plasmodium Falciparum Infected Patients in East Sumba District, East Nusa Tenggara Province

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Abstract: Introduction: Malaria is caused by female mosquito Anopheles bite transmitting malaria parasite sporozoite stage into the human body. According to World Health Organization (WHO) due to malaria was 429,000 shows case causing malaria death, this need commitment of every country to overcome malaria. Indonesia is still a malaria endemic area and includes a country with a high risk of malaria. Population migration from malaria endemic areas to non-endemic areas of malaria is responsible for malaria transmission, especially in Papua, West Papua, Maluku, North Maluku and East Nusa Tenggara. Malaria cases in East Nusa Tenggara Province 7,05% per 1.000 opulation based on annual parasite incidence (API) and 36,039 positive. East Sumba 12,84% per 1.000 population. Methods: A descriptive cross sectional study was conducted in East Sumba district using the Logical Framework approach. Twenty two people were involved in this study with ranging age 4 to 50 years old. Parasite density was examined by counting parasites on Giemsa-stained thick smears under light microscope. Plasma level of TNF- α and Interleukin-10 were measured by using enzyme linked immunosorbent assay (ELISA) Result: Significant values considered at $p < 0.05$ The result show that parasite density $7.72 \pm 9.51/\mu\text{l}$. TNF- α $27.14 \pm 50.22\text{pg/ml}$ IL-10 $2.20 \pm 3.23\text{pg/ml}$ Conclusion: The result from this study conclude parasitedensity increases of TNF- α and IL-10 cytokine.

1 INTRODUCTION

Malaria one of deadly tropical parasite disease caused by the genus Plasmodium, transmitted by the female Anopheles bites they transmitted parasite into the human (Mota et al, 2017). Data in 2015 people death case cause malaria 429.000, need commitment every country to against malaria (WHO, 2016). Indonesia one of country with a high risk of malaria endemic areas especially in Papua, West Papua, Maluku, North Maluku and East Nusa Tenggara. East Sumba district part of East Nusa Tenggara with high risk of malaria (Kemenkes, 2016).

Glycosylphosphatidylinositol (GPI) is malaria toxin that released along with merozoite and hemozoin when *P. falciparum* schizont-infected erythrocytes rupture causing severe malaria pathology through stimulation of pro-inflammatory responses from NK cells and macrophages as immune cells during innate immunity. Immune

response induced by GPI is mediated by pattern recognition receptors such as TLR2 and TLR4 (Dunst et al, 2017). Intraerythrocytic malaria parasite antigens trigger the early immune response and cytokines production such as TNF- α , IL-1 and IFN- γ from macrophages (Mana et al, 1991). The interleukin-10 (IL-10) is an anti-inflammatory cytokine produced by monocyte/lymphocyte which has been shown to inhibit TNF- α (Burdin et al, 1994) protects against severe malaria anemia (SMA) and cerebral malaria (Terazzaset al 2017).

IL-10 cytokines are found in plasma, produced by monocytes, Th2 cells and B cells, inhibiting cytokine production in Th1 and CD8 + cells. IL-10 anti-inflammatory cytokine protects against severe malaria anemia (SMA) with cerebral malaria (Terazzaset al 2017).

During inflammation production of TNF- α is increased. The dual of cytokines especially TNF- α and IL-10 in the right level play role in protection and healing (Irawati et al, 2008).

In this study, parasite density, plasma level of TNF- α and IL-10 of *P. falciparum*-infected patient and residents in East Sumba District of East Nusa Tenggara Province were measured to find out the correlation between parasite density and both cytokines to determine the immune status of the patients.

2 MATERIAL AND METHODS

2.1 Location and Samples Collection

This research was done in East Sumba district of the East Nusa Tenggara Province. This district that located in tropical region has rainy season during January-April and the rest was dry season, causing this region classified as dry area (BPS Sumba Timur, 2016).

Blood were collected from *P.falciparum*-infected patients who seeked medication in Lindimara Hospital and Public Health Service (Puskemas), and from malaria-suspected residents who were developing fever during samples collection. Only *P.falciparum*-infected blood were used in this study. Three milli liters (mL) of blood were collected from median cubital vein and transferred to the heparin-containing tube, furthermore, plasma were used to measured the cytokines levels.

2.2 Microscopy Diagnosis and Determination of Parasite Density

Microcopy examination was done on Giemsa-stained thick and thin blood film using light microscop under 1000x magnification with oil immersion to detect and identify the species of *P.falciparum*. Parasite density were counted per 500 leukocyte based on the following formula:

$$Parasite\ Density = \frac{\sum Parasite \times 8000}{500} \quad (1)$$

Blood drops on the surface glass object, flattened using the other glass object, thin blood smear attach the glass object with a 45 degree in the blood, push blood until tip on ovale, thick and thin blood fixation with metil alcohol dried and then staining used giemsa.

2.3 Procedure of Giemsa Staining

The dried blood preparation fixation with methanol, glass objects are placed on the stain rack, prepared giemsa solution by mixing 3 cc giemsa stock and 97

cc buffer solution, poured 3% giemsa solution to cover the surface of the glass object, leave on for 30 – 45minute, poured clean water slowly on the glass object until clean, dried and than staining used giemsa.

2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma levels of TNF- α and IL-10 were measured by using ELISA according to the manufacturer's protocol (Elabscience, USA), with all samples running in a single assay. The ELISA was performed and analysed by a single operator, and standard curves were derived from cytokine standards. Optical density (OD) value were measured at 450 nm immediately.

Add 100 μ L sample of standard or plasma to each well. Incubate for 90 minutes, remove the liquid. Add 100 μ L of biotinylated detection antibody (Ab). Incubate for one hour at 37 degree, aspirate and wash for three times, add 100 μ L of HRP conjugate. Incubate for 30 minutes at 37 degree, aspirate and wash for 5 times, add 90 μ L of substrate reagent. Incubate for 15 minutes at 37 degree, add 50 μ L of stop solution. Determine the optical density (OD) value at 450 nm immediately.

2.5 Statistical Analysis

All the statistical analyses were done using statistical package for social science (SPSS). The normality data was determine by Kolmogorov Smirnov test with $p > 0,05$. Further analysis was using Pearson correlation test to determine the correlation between parasite density, TNF- α , IL-10 and the ratio of both cytokines.

3 COPYRIGHT FORM

3.1 Microscopy Diagnosis of *P. falciparum* Infection

Microcopy examination was done on Giemsa-stained thick and thin blood film using light microscop under 1000x magnification with oil immersion to detect and identify the species of *P.falciparum*. Parasite density were counted per 500 leucocyte based on the following formula:

$$Parasite\ Density = \frac{\sum Parasite \times 8000}{500} \quad (2)$$

Microscopy diagnosis of Giemsa-stained thin films resulting in 22 out of 46 samples were positive infected with *P. falciparum*, 19 *P. vivax* and 5 mix of both species. Only *P. falciparum* positive samples were used in this study.

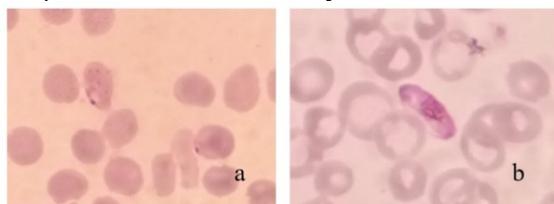


Figure 1: Ringform (a) and gametocyte (b) stages of *P. falciparum* on Giemsa-thin blood film

3.2 Characteristics of Subjects

The results of the characteristics subjects are presented in Table 1. Mean age of malaria positive group *P.falciparum* 16,64 years and negative group 24,10 years. Percentage of gender malaria positive group 50% male, 50% female and malaria negative group 30% male, 70% female.

Table 1: Characteristics of subjects

	Total (%) Positive group <i>P.falciparum</i> (n = 22)	Total (%) Negative group <i>P.falciparum</i> (n = 10)
Number of the subject		
Age (Year), Mean ±SD	16,64 ±10,67	24,10 ± 5,74
Male	11(50,0)	3(30,0)
Female	11(50,0)	7(70,0)

3.3 Parasite Density

The calculation of parasite density *P.falciparum* are presented in Figure 2 below.

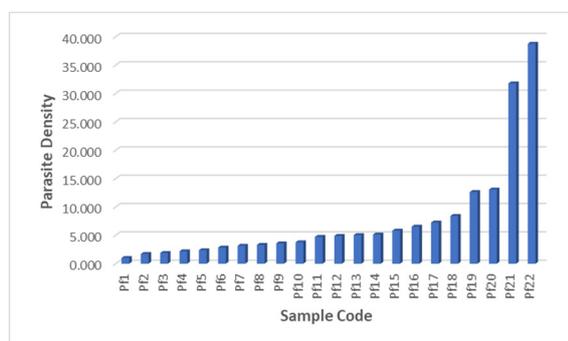


Figure 2: Parasite density in subject infected with *P.falciparum*

The highest parasite density was 38,768 parasites/ μ L while the lowest was 1,008 parasites/ μ L.

3.4 Plasma level of TNF- α

The results of measurements of TNF- α levels are shown in Figure 3 below.

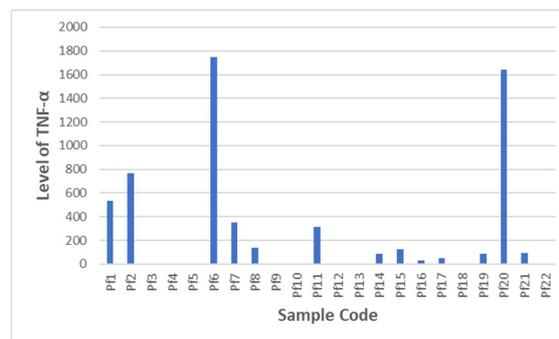


Figure 3: Plasma level of TNF- α in *P. falciparum*-infected subjects

The highest levels of TNF- α were found in Pf6 1750.94 pg / mL and Pf20 1641.59 pg / mL samples. The lowest TNF- α levels Pf3, Pf4, Pf5, Pf9, Pf10, Pf12, Pf13, Pf18, Pf22 were below the standard absorbance values of the ELISA measurement.

3.5 Plasma Level of IL-10

The plasma level of IL-10 by ELISA is shown in Figure 4.

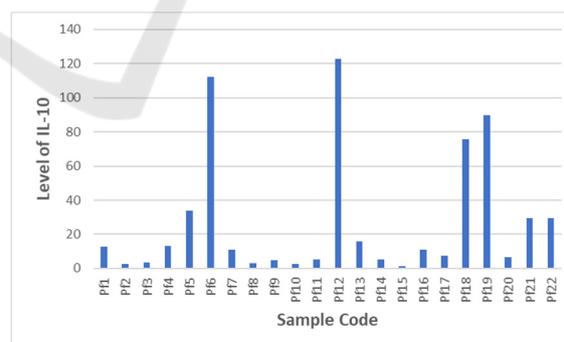


Figure 4: Plasma level of IL-10 levels in *P. falciparum*-infected subjects

The highest levels of IL-10 were found in samples of Pf6 with 112.04 pg / mL and Pf12 with 122.76 pg / mL. The lowest level of IL-10 were Pf2 with 2.52 pg / mL, Pf10 with 2.40 pg / mL and Pf15 with 1.05 pg / mL.

The levels of parasite density, TNF- α and IL-10 vary widely, therefore, to explain the correlation between parasite density and the levels of TNF- α and IL-10, the discussion is grouped into 3 groups and picked up several samples as follows:

1. Samples with high parasite density. 2. Samples with high TNF- α level or high ratio of TNF- α : IL-10, and 3. Samples with high IL-10 levels or low TNF- α : IL-10 ratio.

Table 2: Grouping of representative samples with high parasite densities, high TNF- α and high IL-10 levels

Samples with high parasite density				
Code	Parasite density	TNF- α	IL-10	Ratio
Pf22	38.768	0	29,292	0
Pf21	31.776	96,251	29,292	3,2
Pf20	13.072	1.641,5	6,531	251
Pf19	12.624	85,0	89,7	0,9
Samples with high level of TNF- α				
Code	Parasite density	TNF- α	IL-10	Ratio
Pf1	1.008	531.227	12.822	41.43
Pf2	1.712	767.29	2.526	303.7
Pf6	2.848	1750.948	112.041	15.62
Samples with high level of IL-10				
Code	Parasite density	TNF- α	IL-10	Ratio
Pf5	2.368	0	33.739	0
Pf12	4.896	0	122.764	0
Pf18	8.408	0	75.506	0

	Low
	Moderate
	High

4 DISCUSSION

The higher levels of parasite density were observed in uncomplicated P.falciparum infection compared to the severe malaria infection. In the present study high parasite density was followed by high TNF- α and IL-10 levels. Activation of Th1 and Th2 balance for parasite killing (Irawati, 2008). Several studies have shown the involvement of pro-inflammatory cytokine in patogenesis of severe falciparum malaria where high plasma TNF- α and IL-10 show cerebral malaria (CM) and severe malaria anemia (SMA) (Parera, et al 2013)

Several studies indicated TNF- α is a critical mediators of malarial fever P.falciparum. TNF- α released in intermittent burst with the schizont

rupture. Not all proinflammatory cytokines are equally relevant for the development of cerebral malaria (Angulo, 2002). Cytokines play an important role in human immune responses to malarial disease. Balance between pro and anti inflammatory cytokine are differ in each stage of Plasmodium infection (Andrade, 2010).

4.1 Samples with High Parasite Density

There are 4 samples categorized as having high parasite densities (> 10,000/ μ l blood) with different levels of TNF- α and IL-10 as listed in the Table 2.

■ Subject Pf21

This subject with high parasite density has low TNF- α and IL-10 levels, make the ratio of TNF- α : IL-10 also low (0). The low anti-inflammatory response managed the host immunity to suppress the levels of proinflammatory cytokine (TNF- α) to the level below standard (0). Possibility in defecting or mutations in TNF- α encoding gene has occured that can result in polymorphisms of the gene (Qidwai and Khan, 2011).

■ Subject Pf20

This subject has high parasite density with high TNF- α levels but moderate IL-10 level, thus the ratio of TNF- α : IL-10 is high. The blood sample was collected at the Baing Health Center when the subject was seeking medication due to suffering from fever. Microscopy diagnosis has confirmed the positive infection of P. falciparum. The immune response of patient is indicated by high levels of TNF- α . Fever is a clinical manifestation of the TNF- α response. Fever and TNF- α maintain parasitic density within safe limits. Such immunity explains a host defense mechanism that depends on parasite density (Kwiatkowski 1989; 1991; 1994). TNF- α has several other biological effects, such as the deployment of neutrophils and monocytes to the site of infection and activating these cells to exclude pathogens, stimulating the expression of vascular endothelial cell adhesion molecules for leukocytes, stimulating macrophages to secrete chemokines and inducing chemotactic and leukocyte deployment, stimulating the hypothalamus inducing fever (Bratawidjaja, 2014). High parasitic density induces TNF- α production very strongly. The excessive level of

TNF- α indicate that parasites escape the immune mechanism. The host fights infection by producing anti-inflammatory cytokines, IL-10, but is unable to kill parasites mimicking the immunotolerance against pathogen. These conditions can lead to splenomegaly and hepatomegaly and maybe specific in East Sumba Regency.

- Subject Pf19

The host responds to infection by producing high level of IL-10 to suppress TNF- α production, however, IL-10 produced by Th2 cells is unable to help B cells to produce anti-parasitic antibodies that are strong enough to eliminate parasites, so parasites continue to increase. A research conducted by Shabani et al (2017) described that density of *P. falciparum* which >10,000 parasites μ l of blood can cause Cerebral Malaria (CM) and Severe Malaria Anemia (SMA). In anemia, the parasite density increases (Maina et al, 2010). *P. falciparum* infects old erythrocytes. When erythrocytes infected with schizont stage rupture release merozoites to invade other erythrocytes, thus the more erythrocytes infected by the schizont result in reducing the number of erythrocytes. On the other hand, the production of new erythrocytes is not as fast as the invasion of parasites. Parasitic density is related to age, and clinical malaria such as fever, chills, headache and splenomegaly are associated with parasite density (Pryblyski et al, 1999). Peripheral parasitic density is also associated with plasma TNF- α level in pregnant women (Ifeanyichukwu et al, 2017).

4.2 Samples with High TNF- α Level

- Subject Pf code 1

This subject has low parasite density, high TNF- α levels and moderate IL-10 levels, thus the ratio of TNF- α :IL-10 is moderate. High TNF- α level plays a role in parasite killing mechanism, because TNF- α levels can inhibit parasite growth (Kwiatkowski, 1991), causing low parasitic density. Moderate levels of IL-10 indicate the host responds against infection to balance the TNF- α . Subject Pf2 has similar immune status to Pf 1.

- Subject Pf6

This subject has low parasite density, but high level of TNF- α and IL-10 and moderate

ratio of both cytokines. This situation shows that the host is fighting infection by producing IL-10 to compensate for TNF- α production. Parasite killing mechanism has also occurred. The presence of an anti-inflammatory response indicates a tendency for patients to recover from malaria infection.

4.3 Sample Group with High IL-10 Level

Subjects Pf5, Pf12 and Pf18 had defective TNF- α coding genes (Qidwai and Khan, 2011), causing very low TNF- α levels and unreadable by the system on measurements with the ELISA method. High respond of IL-10 indicates a strong fight against infection followed by a decrease in parasite density, thus indicates healing is very likely.

5 CONCLUSIONS

In summary, proinflammatory cytokine and anti-inflammatory cytokine are both required for adequate protection, Th-1 cytokine are important in controlling early parasite malaria, although they need to be counterbalanced later in the infection by a Th-2 response which leads to antibody production.

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