Effect of L-arginine on the growth of *Plasmodium falciparum* and immune modulation of host cells

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**ABSTRACT**

*Background & objectives:* Malaria is a life-threatening disease caused by *Plasmodium* parasites. The life-cycle of *Plasmodium* species involves several stages both in mosquito and the vertebrate host. In the erythrocytic stage, *Plasmodium* resides inside the red blood cells (RBCs), where it meets most of its nutritional requirement by degrading host’s haemoglobin. L-arginine is required for growth and division of cells. The present study was aimed to demonstrate the effect of supplementation of different concentrations of L-arginine and L-citrulline on the growth of parasite, and effect of the culture supernatant on the host’s peripheral blood mononuclear cells (PBMCs).

*Methods:* To examine the effect of supplementation of L-arginine and L-citrulline, *Plasmodium falciparum* (3D7 strain) was cultured in RPMI 1640, L-arginine deficient RPMI 1640, and in different concentrations of L-arginine, and L-citrulline supplemented in arginine deficient RPMI 1640 medium. To have a holistic view of *in vivo* cell activation, the PBMCs isolated from healthy human host were cultured in the supernatant collected from *P. falciparum* culture.

*Results:* Growth of the parasite was greatly enhanced in L-arginine supplemented media and was found to be concentration dependent. However, parasite growth was compromised in L-citrulline supplemented and L-arginine deficient media. The supernatant collected from L-arginine supplemented parasite media (sArg) showed increased FOXP3 and interleukin-10 (IL-10) expression as compared to the supernatant collected from L-citrulline supplemented parasite media (sCit).

*Interpretation & conclusion:* The *in vitro* culture results showed, decreased parasite growth, and decreased expression of programmed cell death-1 (PD-1) (a coinhibitory molecule) and IL-10 in the L-citrulline supplemented media as compared to L-arginine supplemented media. Hence, it was concluded that L-citrulline supplementation would be a better alternative than L-arginine to inhibit the parasite growth.

**Key words** L-arginine; induced nitric oxide synthase (iNOS); interleukin-10; L-citrulline; malaria; *Plasmodium falciparum*; programmed cell death-1
is low, there is downregulation of CD3 zeta chain that hampers the downstream signalling and impairs T-cells functions.23

Role of arginase I and II on immune system has been demonstrated in different diseases.24,26 During malaria infection, the arginase enzyme in blood increases which results in low level of L-arginine in blood.25 Low level of L-arginine is directly related to disease severity and parasitic load. The Plasmodium parasite has arginase enzyme and is capable of metabolizing L-arginine,28,29 but it cannot utilize L-citrulline.8

In malaria, L-arginine have been shown to reverse endothelial dysfunction.30,31 To increase arginine level in blood and to reverse the endothelial dysfunction, the administration of nitric oxide donor like L-arginine, is useful.27,32 The effect of L-arginine on Plasmodium and immune modulation in malaria has not been explored yet.

The activation of immune system is marked by expression of several costimulatory molecules. Programmed cell death-1 (PD-1), an inhibitory co-stimulatory molecule is not expressed on resting T-cells, but an activated T-cell up-regulates its expression.33 In malaria, several studies have reported that PD-1 plays a critical role in immune system modulation and inhibition of T-cell activation.34,35 Cytokines play a decisive role in outcome of any disease. Interleukin-10 (IL-10) secreted by T-regulatory helper cells is a suppressive cytokine that downregulates the immune activation. Interferon gamma (IFNγ) is a pro-inflammatory cytokine, which activates the immune system against different infections.

In the present study, the effect of supplementation of L-arginine and L-citrulline on the growth of P. falciparum culture has been demonstrated. The growth of parasite was rapid in L-arginine supplemented media while its growth was stunted in L-citrulline supplemented media and arginine deficient media. To get an overview of the cell activation (in vivo), the peripheral blood mononuclear cells (PBMCs) were cultured in the supernatant collected from P. falciparum culture, with 1% parasitaemia was used for the experiments. Plasmodium falciparum was cultured in RPMI 1640, L-arginine deficient RPMI 1640 (Himedia Laboratories, India), different concentration of L-arginine, and L-citrulline (1.15 mM; 200 mg/l), (2.30 mM; 400 mg/l), (4.6 mM; 800 mg/l), and (11.5 mM; 1000 mg/l), supplemented in arginine deficient RPMI 1640 media, respectively. The P. falciparum culture supernatant was collected and stored at –80°C until further use.

To determine the parasitaemia, thin smear of the parasitized blood was made and slides were stained with Jaswant Singh Bhattacherji (JSB I and II). The PBMCs were cultured in RPMI 1640 and the culture was supplemented with collected supernatant from the Plasmodium culture (Day 4) in 20:80 (v/v). The cells were harvested and then assayed for viability, surface staining, intracellular staining and quantitative PCRs. All the experiments were performed in triplicates.

Preparation of parasite crude lysate

Parasite culture was collected, centrifuged and supernatant was discarded. The pellet was lysed by keeping it in 0.05% of saponin for 30 min at 37°C. The cells were washed thrice with PBS. Protease inhibitor cocktail (Thermo Fisher Scientific, USA) was added and stored at –80°C until use.

Peripheral blood mononuclear cells isolation and culture

A 5 ml blood was drawn from healthy individual. PBMCs were isolated using lymphocyte separation medium, (LSM) (MP Biomedical, CA, USA) as per instructions of the manufacturer. In brief, blood was diluted with equal volume of PBS and layered over 3 ml of LSM, and centrifuged at 400 g for 25 min. PBMCs were collected from the interface and washed twice with PBS. Then they were suspended in PBS, and counted and cultured. The cells were stimulated with Plasmodium crude extract (50 mg) for 8–10 h.36

Surface staining and analysis

The PBMC cells were harvested after eight hour and were labelled with antibody as per manufacturer’s instruction for flow cytometric analyses, and were resuspended in 50 µl of FACS buffer [PBS, 2% foetal calf serum (FCS)] and surface-stained at 4°C with antihuman CD4, PD-1(MIH4), CD69 (BD Biosciences, CA, USA). Stained cells were acquired with a LSR Fortessa (BD Biosciences, CA, USA) cell analyser and analysed by FlowJo LLC software.42
Intracellular staining

The PBMCs (1×10⁶ ml) treated or untreated were suspended in FACS buffer (PBS with 2% FCS). Surface staining was carried out by incubating with the fluorochrome-labelled antibodies, anti-CD4-FITC (fluorescein isothiocyanate) at 4°C for 30 min in the dark. Cells were fixed for 30 min at room temperature in dark with 2% paraformaldehyde, and then treated with Perm/Wash solution at 4°C for 1 h pre-incubation with phycoerythrin (PE)-conjugated anti-FOXP3, anti-IL-4, and anti-IFNγ antibody (e-Bioscience, USA). Thereafter, cells were washed in Perm/Wash solution and in FACS buffer and then suspended in FACS buffer. The cells were acquired using a LSR Fortessa cell analyser.

Quantitative PCR

The PBMCs were stimulated with P. falciparum crude extract and cultured in the supernatant collected from P. falciparum cultured in RPMI 1640, L-arginine or L-citrulline and arginine deficient media. The cultured cells were harvested after overnight incubation at 37 °C. The harvested cells were lysed with TRIzol (Invitrogen, USA) according to the manufacturer’s instruction. RNA was isolated by chloroform-isopropanol method or Qiagen kit and converted to cDNA by reverse transcriptase (Thermo Fisher Scientific, USA). The qPCR was set up with the primer set depicted in Table 1. The relative expression of gene was analysed by methods described by Pfaffl37.

Statistical analysis

All statistical analysis was performed in MS Excel 2003. Student t-test was used as indicated in Figs. 1a and b and considered significant when p ≤ 0.05.

Ethical Approval: The approval of human ethical committee of the National Institute of Malaria Research was obtained (ECR/NIMR/EC/2015/267) for the collection of blood from a healthy individual.

Table 1. Set of the primers used for qPCR.

<table>
<thead>
<tr>
<th>Primer’s name</th>
<th>Primer’s sequence</th>
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<tbody>
<tr>
<td>h-iNOS-F</td>
<td>GCTCTACACCTCCAATGTGACC</td>
</tr>
<tr>
<td>h-iNOS-R</td>
<td>CTGCCGAGATTGAGCCTCATG</td>
</tr>
<tr>
<td>h-arginase 1-F</td>
<td>TCATCTGGGTGATGCTCACAC</td>
</tr>
<tr>
<td>h-arginase 1-R</td>
<td>GAGAATCCTGGGACATCGGGGA</td>
</tr>
<tr>
<td>h-arginase 2-F</td>
<td>CTGGCTTGATGAAAGGGCTTC</td>
</tr>
<tr>
<td>h-arginase 2-R</td>
<td>TGGAGCTGATTGCTATACAGGT</td>
</tr>
<tr>
<td>h-β-actin-F</td>
<td>CACCATTTTGCAATGACCCGTTTC</td>
</tr>
<tr>
<td>h-β-actin-R</td>
<td>AGGTCTTTCGGGATGTCACAGTF</td>
</tr>
<tr>
<td>hIL-10 F</td>
<td>GTTAGCCTCAAGCCTGAGA</td>
</tr>
<tr>
<td>hIL-10 R</td>
<td>CACGCCCTTGCTCTTGGTTT</td>
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</table>

Fig. 1: Growth of Plasmodium falciparum (3D7) in RPMI 1640, L-arginine deficient RPMI 1640, and in different concentrations of L-arginine, and L-citrulline supplemented in arginine deficient RPMI 1640 medium on different days: (a) Parasitaemia level of P. falciparum cultured in different concentration of L-arginine; and (b) L-citrulline; (c) Growth trend in the parasitaemia of P. falciparum cultured in different media vs days; and (d) Plasmodium falciparum stages in different media (images taken by NIKON Model: TiU at 100x). Culture experiments were repeated thrice and statistical significance was calculated using unpaired t-test (**p<0.001 and *p<0.005).
RESULTS

Parasite growth and L-arginine supplementation

L-arginine and L-citrulline are nitric oxide donors. L-arginine is required for growth and proliferation of cells. In order to observe the effect of these nitric oxide donors on parasite’s growth *P. falciparum* (3D7 strain) was cultured in RPMI 1640 (200 mg/l of arginine), arginine deficient RPMI 1640 and different concentrations of L-arginine and L-citrulline supplemented in L-arginine deficient RPMI 1640. Parasite cultured in RPMI 1640 served as control. It was found that parasite growth was more vigorous in media supplemented with L-arginine and the parasitaemia increased with increase in L-arginine concentration. The parasitaemia in 800 and 1000 mg/l did not vary significantly (*p* > 0.06). To ascertain if further increase in the concentration of L-arginine increases the parasitaemia, the L-arginine concentration was increased to 1200, 1500 and 2000 mg/l. The growth of parasite was lower in higher concentrations as compared to 1000 mg/l (*p* > 0.07). The growth of the parasite was greatly inhibited in L-citrulline supplemented media (Figs. 1a and b). At a concentration of 1000 mg/l of L-citrulline, slight increase in parasitaemia was observed, but it was not significant. Even supplementation of L-citrulline at higher concentration (1200, 1500, 2000 mg/l) did not assist parasite growth. The different stages of parasite in thin smear were counted in RPMI 1640, L-arginine deficient media L-arginine and L-citrulline supplemented media. It was observed that parasite cultured in RPMI 1640 (control media) were mainly in trophozoite stage whereas in L-arginine, deficient and L-citrulline supplemented media parasite was mainly confined to ring stage. On the other hand, supplementation of L-arginine further enhances the growth of the parasite to schizont stage as summarized in Table 2.

Table 2. Different stages of parasite (after 72 h) cultured in different media, counted in 10 different settings

<table>
<thead>
<tr>
<th>Media (mg/l)</th>
<th>Rings</th>
<th>Schizonts</th>
<th>Trophozoites</th>
<th>Gametocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg (200)</td>
<td>11</td>
<td>2</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Arg (400)</td>
<td>16</td>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Arg (800)</td>
<td>17</td>
<td>16</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Arg (1000)</td>
<td>26</td>
<td>14</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>RPMI</td>
<td>6</td>
<td>1</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Arg-</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cit (200)</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
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<td>Cit (400)</td>
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<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cit (800)</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Cit (1000)</td>
<td>14</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Effects of parasite culture supernatant on PBMCs

As the culture supernatant contained several soluble factors secreted by parasite, it was important to investigate the effect of these supernatant on PBMCs. The PBMCs cultured with the supernatant of different media were analysed for the expression of PD-1, an inhibitory co-stimulatory molecule (Fig. 2). The expression of PD-1 was found to be increased in all the different media. However, its expression was significantly higher in the PBMC cultured in supernatant collected from L-arginine supplemented parasite media (sArg) followed by supernatant collected from L-citrulline supplemented parasite media (sCit) while its expression was similar in supernatant collected from parasite cultured in RPMI (sRPMI) and supernatant collected from arginine deficient parasite media Arg- media (Fig. 2a). The PBMCs were further checked for the intracellular cytokines cultured in presence of different supernatant. The PBMC cultured in sArg had marginally increased expression of FOXP3 as compared to sCit, sArg and sRPMI media (Fig. 2b). The analysis of the expression of IFNγ showed that cells cultured in sArg had the highest induction of IFNγ expressing CD4+ T-cell, while cells cultured in sCit, sArg and sRPMI had similar induction of IFNγ.

Effect on nitric oxide synthase and arginase in PBMC

Further to know the effects of supernatant, in the induction of induced nitric oxide synthase (iNOS), arginase I, arginase II and IL-10, qPCR was carried out (Fig. 3). About 6-folds and 4-folds increase was observed in induc-
ened. Also, the number of schizonts were more in arginine supplemented media as compared to RPMI which had more trophozoites. The parasite in arginine deficient and L-citrulline supplemented media was mostly in ring stage. However, formation of gametocytes was not observed in any of the cases. Presence of gametocytes in culture is a sign of parasite in stress. The inability of parasite to utilize L-citrulline inhibits the growth of parasite. L-citrulline can be utilized by macrophages to produce nitric oxide without hampering its phagocytic activities.

Expression of various co-stimulatory molecules on T-cells and cytokine secreted by these cells, defines the outcome of a disease. Studies have shown that the expression of inhibitory molecules like PD-1 weakens the immune activation leading to severity of the disease and generation of regulatory T-helper cells also suppresses the immune system by secretion of IL-10. PD1 expression, secretion of IFNγ and induction of IL-10, iNOS, Arg I and II was investigated in PBMCs cultured in different media. As evident from the results, stimulation of PBMCs and expression of PD-1 was highest in sArg. The high expression of PD-1 in sArg suggests the presence of more parasite antigens or higher amount of soluble factors, which might be a result of increased parasite growth. Similarly, the expression of PD-1 was higher in sCit as compared to sRPMI and sArg, which might be the result of the stress conditions when parasite was cultured in L-citrulline supplemented media. The expression of PD-1 was similar in sRPMI and sArg. The results suggested that when arginine is supplemented during Plasmodium infection it aids parasite growth and drives the immune system towards suppression.

PBMC culture results indicated that CD4+FOXP3+ cells were greatly induced when cultured in sArg suggesting that the increased parasite antigen in the supernatant leads to the induction of T-regulatory cells. The induction of CD4+FOXP3+ cells was similar in sRPMI, sCit and sArg media. The evaluation results of the secretion of IFN by T-cells, suggested that the IFNγ secreting CD4 cells was greatly induced when cultured in sArg but in other media, the percentage of these cells remained almost similar. The induction results of arginase I and II gene clearly indicated that the parasite was able to induce both Arg I and II by >4-folds, when cultured in sArg. Induction of arginase enzyme has been linked to immune suppression. The induction of these genes were significantly reduced in sCit and lowest in sArg. Interestingly, the induction of iNOS was only 4-folds in sRPMI, while, it was 6-folds in sArg. This result further supports that the P. falciparum antigen drives arginase induction suppressing iNOS induction. In various studies, iNOS induction has

DISCUSSION

L-arginine is a semi-essential amino acid which is generated in urea cycle. L-citrulline is not required by our body and it is not a part of any protein. It plays an important role in urea cycle. It can help in replenishment of L-arginine by action of ASS an ALT within cell. The effect of administration of arginine to malaria patient has been shown to reverse the endothelial dysfunction and increase in nitric oxide level. Arginine supplementation has been tried in cerebral malaria (CM) cases. A study by Alkaitis et al has demonstrated that the hypo-argininemia in children with CM is linked with decreased rate of plasma arginase appearance. They also observed decrease in citrulline, ornithine and other components of urea cycle. However, its effect on Plasmodium parasite has not been explored yet. Besides the effect of L-citrulline, which is also a nitric oxide donor, has not been studied in malaria.

In this study, the effect of supplementation of these amino acids in parasite culture media was shown. Growth of parasite was enhanced in presence of L-arginine and the parasitaemia increases with increase in concentration of arginine in media. However, at concentration beyond 1000 mg/l the growth of the parasite was not significant. The increase in parasitaemia on L-arginine supplementation suggests that parasite uses it for its growth and proliferation. It was observed that in media supplemented with L-arginine the erythrocytic cycle of parasite was short-
been linked with immune activation while induction of arginase has been linked to immune suppression\(^2^1\). Higher induction of IL-10 in sArg suggests that the parasite antigen suppress the immune system. Induction of IL-10 was low in sCit and sArg\(^+\). In this study, it was shown for the first time that L-citrulline, a nitric oxide donor, might be used as a supplement in chemotherapy as it retards the parasite growth and revives the immune system from immune-suppression by decreasing PD-1 and IL-10 expression.

**CONCLUSION**

The exogenous supply of arginine favoured the growth of the parasite in concentration dependent manner; but, beyond 1000 mg/l, no increase in parasitaemia was observed. However, the growth of the parasite was low in L-citrulline supplemented media and the supernatant collected from L-citrulline did not induce higher amount of IL-10 and arginase I and II. Increased parasitaemia causes higher secretion or accumulation of parasite antigens, resulting in induction of inhibitory molecules like PD-1, IL-10 and induction of arginase I and II. These factors collectively contribute to suppression of immune system. Hence, it was concluded that L-citrulline supplementation would be a better alternative than L-arginine to inhibit the parasite growth and prevent immune suppression. Further, studies are underway to confirm the results in animal models.

**Conflict of interest:** None.

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**REFERENCES**

24. Ghosh S, Navarathna DH, Roberts DD, Cooper JT, Atkin AL, Petro TM, et al. Arginine-induced germ tube formation in Cam-
*dida albicans* is essential for escape from murine macrophage line RAW 264.7. *Infect Immun* 2009; 77: 1596–605.


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