Leishmaniasis is a neglected tropical disease caused by a spectrum of protozoan parasites that belong to the genus *Leishmania* and exists in two morphological forms. While promastigotes are found in the vector and cultures, amastigotes are present in the mammalian host's mononuclear phagocytic cells. Among *Leishmania* spp., *L. donovani* invades the reticuloendothelial system causing visceral leishmaniasis (VL), known also as Kala azar, black fever, and Dum Dum fever. Notably, VL is manifested by fever, lymphadenopathy, hepatosplenomegaly, anemia, and skin pigmentation; the disease progresses to a fatal illness if left untreated[1]. According to recent WHO reports, the incidence of VL is estimated as 50,000 to 90,000 new cases per year worldwide, with only 25-45% of cases being reported[2].

The *Leishmania* amastigotes subvert the host immune response to promote their persistence and reproduction. One of the most important immune targets of *Leishmania* are the macrophages that develop from circulating monocytes recruited to the infected or traumatized tissues[3]. They are recognized in almost all tissues of the body and they can change to several phenotypes according to the tissue and the infectious agent[4]. They play crucial roles in the innate and adaptive immune responses, and represent the first line of defense against many invaders. They kill pathogens through the nitric oxide (NO) and reactive oxygen intermediates (ROI)[5], present antigens and prime the effector mechanisms, regulate the immune response, and promote tissue repair and homeostasis after infection or trauma[6]. All these capabilities are the product of macrophages polarization to different phenotypes depending on the stimulus, the cellular

Original Article

*Leishmania donovani* promotes macrophages polarization towards M2 phenotype *in vitro*: A new approach to identify a new therapeutic target

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**ABSTRACT**

**Background:** The immune response against *L. donovani* depends significantly on infected macrophages. Since *Leishmania* amastigotes deploy several immune suppressive mechanisms to escape host immune responses, macrophages polarize towards the classically activated macrophages (M1) or the alternatively activated macrophages (M2). The balance between both types is crucial in shaping the infection outcome.

**Objective:** The aim of this study is to explore the macrophage polarization behavior in response to *L. donovani* infection, and to examine the differential expression of IL-10 and TNF-α by each phenotype.

**Material and Methods:** *Leishmania*-infected phorbol 12-myristate 13-acetate (PMA)-treated human leukemia monocytic cell line (THP-1) was used as an *in vitro* model of *Leishmania* infection. *Leishmania* stationary phase promastigotes were used to infect the macrophages at different multiplicities of infections (MOIs) i.e., ratio of macrophages to stationary phase promastigotes at 1:1, 1:10, and 1:20; and time points of 24 and 48 h post infection (PI). While CD68, CD40, HLA-DR were used as markers for M1; CD68 and CD163 were used to characterize M2. Both M1 and M2 phenotypes were analyzed using flow cytometry. To evaluate the behavior of polarization, IL-10 and TNF-α were tested in both phenotypes, in addition to the assessment of percentage of infected macrophages.

**Results:** The percentage of M1 exhibited significant decrease followed by non-significant increase, while M2 showed significant increase correlating with the MOIs. Both phenotypes expressed MOI-dependent increase in IL-10, but only M1 significantly expressed TNF-α. Besides, M2 phenotype predominated M1, in a time and MOI dependent manner.

**Conclusion:** *Leishmania* infection induces macrophages polarization towards M2, with significant production of IL-10. These results extend knowledge regarding the immunomodulation exerted by *Leishmania* amastigotes to defeat the immune system.

**Keywords:** drug target; immunomodulation; *in vitro*; M1; M2; macrophages polarization; visceral leishmaniasis.

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Macrophage polarization was reviewed by Mosser and Edwards[8] who described M1 and M2 as the two edges of a spectrum. Stimulation of M1 is achieved by microbial products, pro-inflammatory cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granulocyte macrophage colony-stimulating factor (GM-CSF)[9]. In contrast, M2 are detected in repair circumstances and are triggered by interleukin (IL)-4, IL-10, IL-13, and colony-stimulating factor (CSF)[10]. Notably, M1 are distinguished with high antigen presentation and more production of pro-inflammatory mediators which promote T helper type 1 (Th1) response[10,11]. On the other hand, M2 produce several regulatory and chemotactic substances via arginase pathway[12,13]. Besides, they promote a Th2 response that plays important roles during parasitic infections, tissue repair, immune tolerance, and tumor development[12,13]. Interestingly, several M2-like macrophage subsets have been documented; M2a, M2b, and M2c, all of which share high IL-10 production and insignificant pro-inflammatory cytokine expression[7].

During leishmaniasis, macrophages perform a dual role of acting as the primary habitat for amastigotes, and at the same time they represent the ultimate killer. Macrophage polarization towards M1 or M2 was proved to shape the infection outcome. Both M1 and M2 phenotypes are triggered during infection[14], where they promote Th1 and Th2 responses respectively, which in turn strengthen M1 or M2 in a positive feedback loop to stabilize the immune phenotype[15]. Response of Th1 leads to production of pro-inflammatory cytokines such as IL-12, TNF-α, and IFN-γ that promote M1 with NO release, hence supporting protective immunity through parasite elimination[16]. In contrast, Th2 response leads to release of anti-inflammatory cytokines like IL-4, IL-10, and IL-13, that trigger M2 with arginase release; thus, parasite survival is supported through counteracting the killing mechanisms of macrophages[17,18].

Since IL-10 is the chief cytokine, it regulates the protective immune response during VL through direct action on CD4+ T cells and inhibition of IL-12, IFN-γ, TNF-α, IL-4, and IL-5. In addition, IL-10 inhibits upstream activities originated by antigen presenting cells (APCs)[19,20]. It was proved to limit the antigen presenting strategies in macrophages through inhibiting the expression of class II major histocompatibility complex (MHC II) leading to decrease in T cell stimulation[19,20]. As reviewed by Bunn et al[21], IL-10 is produced by Th2, Th1, Th17, T regulatory cells (Tregs), T regulatory type 1 (Tr1), dendritic cells (DCs), macrophages, B cells, neutrophils, and natural killer cells (NKs). Although the production of IL-10 by Tr1 promotes parasite survival, it was proved to protect tissues from TNF-α-mediated tissue damage[22].

One of the very important cytokines produced by M1 macrophages is TNF-α which in association with other mediators, can guide Th1 response and drive type I inflammation[23-25]. It is secreted by Th1 cells, NK cells, and mast cells and it is a central player in the immune response[26]. The anti-parasitic importance of TNF-α emerges from its role in activation of macrophages to exert a leishmanicidal effect on the intracellular amastigotes[27]. It is one of the most important elements in hepatic granuloma formation, and consequently the limitation of infection; however, its role in the spleen is linked to architectural damage[28]. Additionally, it has been linked to IL-10 increase, which in turn helps to establish the infection through catalyzing the arginase activity over iNOS, thus preventing the production of NO[29].

The purpose of the present study is to explore the polarization behavior of macrophages. First, the in vitro settings for L. donovani infection were established using THP-1 cells differentiated into macrophages. Then, this model was used to explore the influence of macrophages alone in response to Leishmania infection without the effect of other immune cells. Infection with L. donovani was done at different MOIs, and macrophages were harvested at 24 and 48 h PI; then both phenotypes were analyzed using flow cytometry. Evaluation of IL-10 and TNF-α phenotype-specific expression was also assessed.

MATERIAL AND METHODS

This descriptive analytical in vitro study was conducted at Medical College of Georgia, Augusta University, Augusta, GA, USA, during the period from January 2017 to January 2018.

Study plan: Macrophages were infected with Leishmania stationary phase promastigotes, with MOIs of 1:1, 1:10, and 1:20, while uninfected macrophages were used as controls. Cells were collected at 24 and 48 h PI to explore the polarization towards M1 and M2 using flow cytometry and to estimate the expression of IL-10 and TNF-α in each phenotype. Additionally, Giemsa-stained preparations were used to detect percentage of infected macrophages at MOIs (1:10 and 1:20) at 24 and 48 h PI.

Material and chemicals: Table (1) presents all chemicals used in this study.

Leishmania culture: L. donovani promastigotes were grown in M199 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 1 ml hemin solution was added to each 500 ml bottle. Hemin...
solution was prepared using 2.5 ml triethanolamine, 5 mg hemin, in 2.5 ml distilled water (DW). The PH of the media was adjusted to 7.0, and the parasites were grown in a standard incubator at 26°C.[30]

The THP-1 cells culture and differentiation to macrophages: The cell line was grown in a 37°C, 5% CO₂ incubator, in Petri dishes using RPMI-1640 media supplemented with 10% heat inactivated FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol. Differentiation of THP-1 cells into macrophages was induced by overnight addition of 100 ng/ml PMA to the media. Cells were washed 3 times with PBS prior to infection.[31]

Infection of PMA-THP-1 cells with Leishmania: Stationary phase promastigotes were centrifuged and washed once with PBS, then counted in a hemocytometer and added to PMA-treated THP-1 cells according to the planned MOIs, i.e., the different ratios of macrophages to promastigotes (1:1, 1:10, and 1:20). Cells were incubated with *Leishmania* at 37°C for 24 h after which the cells planned for 24 h time point were washed and fixed. Cells intended for the 48 h time point were washed and fresh media was added till the desired time point (48 h) was reached. Uninfected macrophages were used as control groups for both time points.[32]

**Giemsia stain:** Cells were cultured and differentiated in 6-well plates with coverslips placed at the bottom of each well. Infection was performed with MOIs of 1:10 and 1:20, for 24 and 48 h PI. After the desired time points were reached, the coverslips were gently removed and fixed by 100% methanol for 2 min, followed by Giemsa stain for 3 min, then washed in DW and left to dry.[33] The slides were mounted using Fluoroshield mounting media and visualized using EVOS light microscopy (40x). The infection was evaluated by dividing the number of infected macrophages by the total number of macrophages as a percentage, in each of several high-power fields.

**Flow cytometry:** At the desired time points, cells were harvested, counted, fixed, and permeabilized with the BD Cytofix/Cytoperm kit. Fc receptors (FcRs) were blocked with a monoclonal antibody against mouse cell surface antigens CD16 and CD32 for 15 min and then stained according to standard protocols.[34] The following antibodies were used at the concentrations recommended by the manufacturer: anti-CD68 APC, anti-CD40 FITC, anti-IL-10 PE/cy7, anti-TNF-α APC/cy7, anti-CD163 Percp/cy5.5, and anti-HLA-DR PE. Macrophages were gated on the basis of forward and side scatter parameters, and 100,000 events were collected with a flow cytometer (BD Systems, San Jose, CA, USA). Data were analyzed with FlowJo.
**RESULTS**

**Leishmania infection of PMA-treated THP-1 cells:** The cultured THP-1 cells proved to be a good candidate for *L. donovani* habitat. The effect of *L. donovani* dose and incubation time on the macrophages’ response was visualized. At 48 h PI, MOI 1:20 exhibited significant increase in the percentage of infected macrophages compared to 1:10 condition at 24 h PI (*P* ≤ 0.02) (Fig. 1a, b).

**Effect of Leishmania on M1 cells:** The M1 macrophage phenotype was explored based on specific M1 markers (CD68, HLA-DR, and CD40), by using flow cytometry. Cells were gated first using CD68 and HLA-DR, then the double positive populations were further gated by CD40, at 24 and 48 h PI (Fig. 2a). Results were calculated as fold change of the control. The M1 cells (CD68+ HLA-DR+ CD40+) were found to exhibit significant, MOI-dependent decrease in their percentage at 24 h PI (*P* ≤ 0.02).

Fig. 1. Survival and replication of *Leishmania* inside PMA-treated THP-1 cells was estimated by quantification of *Leishmania*-infected macrophages in Giemsa-stained preparations. Images demonstrating macrophages (yellow arrow heads) with amastigotes (red arrow heads) located intracellularly (a). Means±SD from 3 independent experiments showed significant increase in the percentage of infected macrophages in the 1:20 MOI at 48 h PI than the 1:10 MOI at 24 h PI (b). *: Significant (*P* = 0.02).

Fig. 2. Leishmaniasis promotes initial significant decrease, followed by non-significant increase in M1 phenotype. CD68+ HLA-DR+ CD40+ were used for characterization of M1. Cells were gated first using CD68 and HLA-DR, then the double positive populations were further gated by CD40 (a). The M1 cells showed MOI dependent decrease relative to the control at 24 h PI, *: Significant (*P*<0.0001) (b). Non-significant increase at 48 h PI (c). Data are presented as means±SEM (b, c).

0.7 (FlowJo Software LLC, Ashland, OR, USA). The cytokine expression was calculated by multiplying the percentage of its expression by the percentage of that phenotype, then presented as fold change of the control.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Comparisons of multiple experimental groups were conducted using one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test as a post hoc test. Data were presented as the mean ± standard error of the mean (SEM). Statistical significance was considered when *P*≤0.05.

Ethical consideration: The experiments in this study were approved by the Institutional Review Board of Augusta University according to the joint supervision mission (JS-3069).
< 0.0001) (Fig. 2b), then they showed non-significant MOI-dependent increase at 48 h PI (Fig. 2c).

**Leishmaniasis and increased expression of TNF-α and IL-10 by M1:** In this model, M1 cells (CD68+ HLA-DR+ CD40+) were further gated by TNF-α. The percentage of TNF-α+ M1 cells was multiplied by the percentage of M1 cells to detect the percentage of TNF-α+ M1 relative to the whole population, then fold change of the control was calculated. Interestingly, at 24 h PI, MOI of 1:20 showed significant increase of TNF-α relative to all other conditions (P<0.0007) (Fig. 3a). Moreover, at 48 h PI, the cells with MOI 1:20 exhibited significant increase in TNF-α expression relative to all other conditions (P<0.0001) (Fig. 3b). Regarding IL-10 expression, M1 cells were further gated using IL-10. The results were presented as fold change of the control. MOI dependent increase in IL-10+ M1 cells was noticed at both time points. At 24 h PI, MOI 1:20, the percentage of IL-10+ M1 cells to the whole population showed significant increase compared to all other conditions (P=0.0007) (Fig. 3c). Furthermore, at 48 h PI, MOI 1:20 exhibited significant increase compared to the other groups (P=0.0001) (Fig. 3d).

**Leishmaniasis and macrophage promotion towards M2 phenotype:** To characterize M2, CD163 and CD68 were used for gating (Fig. 4a). At 24 h PI, CD68+, and CD163+ cells (M2) were noticed to exhibit MOI dependent insignificant increase in their percentages (Fig. 4b). Then, at 48 h PI, MOI 1:20, M2 experienced significant increase in their percentage when compared to the control and MOI 1:1 (P=0.02), (Fig. 4c).
Leishmaniasis induces IL-10, but not TNF-α, expression by M2: Expression of IL-10 by M2 macrophages was explored by flow cytometry, using the same method of calculation. At 24 h PI, IL-10 expression by M2 showed significant increase in both 1:10 and 1:20 MOIs ($P=0.001$) (Fig 5a). Similarly, at 48 h PI, both 1:10 and 1:20 MOIs expressed significant increase in IL-10+ M2, relative to the control and 1:1 MOI ($P=0.001$) (Fig 5b). In contrast, Non-significant MOI dependent increase was noticed in TNF-α+ M2 at both time points (Fig. 5c, 5d).

Predominance of M2 cells over M1 cells during leishmaniasis: To explore the switch of macrophages towards M2 in our model, M1 and M2 percentages were compared at MOI 1:20 at both time points, both as fold change of the control. Significantly, M2 percentage showed increase compared to M1 percentage ($P<0.0001$) (Fig. 6a), and ($P=0.01$) (Fig. 6b) at 24 and at 48 h PI, respectively.

DISCUSSION

Macrophages are a double-edged sword during Leishmania infection. Although they represent a critical element of the immune system to control leishmaniasis, they provide a safe habitat for this parasite. Thus, the infection outcome depends greatly on the interaction between the virulence of the parasite and the immune response driven by the macrophages[35]. Macrophages are exploited in several ways by Leishmania to establish the infection, one of these mechanisms is the promotion of macrophages polarization towards M2 phenotype[36,37]. Although animal models were used to reach such conclusions, the clear M1/M2 dichotomy is not obvious enough in animal models as in humans[4].

THP-1 cells are an immortalized monocyte-like cell line derived from a case of acute monocytic leukemia[38] which when stimulated by PMA develop into macrophage-like cells with increased adherence and lack of division[39]. In this study, PMA-treated THP-1 cells were used as a successful in vitro model and L. donovani was able to replicate inside them. They helped to highlight the polarization machinery, taking in consideration the study conducted by Baek et al[30], who reported that PMA-treated THP-1 cells infected with L. donovani showed higher infection ratios compared to bone marrow-derived macrophages (BMDM). Also, another study done by Shiratori et al.[40] confirmed that PMA-treated THP-1 cells are distinctive in the morphology and expression
of polarization marker genes. In addition, they proved that M1 genes are up-regulated 6 h after stimulation while M2 genes are enhanced 48 h after the stimulus to limit the inflammatory response and protect tissues. This was obvious in our results when M2 percentages showed significant increase at 48 h PI, which was lacking significance at 24 h PI. Therefore, to overcome Leishmania, macrophages shift first to M1 which has potent leishmanicidal activity through NO release, after which, the parasite exerts its immune evasion strategies and promotes switch of polarization towards M2 which inhibits inflammation, promotes parasite persistence and infection development\cite{16,17}.

Both phenotypes were found to be induced at different percentages during infection and both phenotypes can produce IL-10 and TNF-α. IL-10 production is the hallmark of M2 polarization, promoting anti-inflammatory responses and helping parasite persistence, while TNF-α, although activating the anti-parasitic mechanisms, it works on promotion of IL-10 with M2 polarization dominance\cite{16,17}.

Regarding TNF-α, it has a central role in resistance to Leishmania infection. Its protective role in L. major was credited to macrophages activation to produce NO, leading to parasite clearance and suppression of visceralization\cite{41}. During L. braziliensis infection, TNF-α was found to control the parasite number in the skin, lymph nodes, and spleen\cite{42}. TNF-α knockout mice cannot tolerate infection with Leishmania spp.\cite{43}, and patients receiving TNF-α antagonists also have higher susceptibility to leishmaniasis\cite{44}. Interestingly, endogenous TNF-α was found to present initial resistance to L. major in IFN-γ knockout (GKO)-1 mice\cite{45}, but increased levels of TNF-α promote T cells to express IL-10 as a homeostatic response\cite{46}, which was noticed in this study when the increase in TNF-α expression by M1 phenotypes was accompanied by IL-10 increased expression from both phenotypes. Noteworthy, TNF-α expression by M2 did not show significant increase in relation to the MOI or time points, although a significant increase was noticed in M1. The production of TNF-α is attributed to the pro-inflammatory nature of M1 and its products which are concerned with killing of pathogens, and this is not the case in M2. Although M1 percentage showed significant MOI-dependent decrease at 24 h and non-significant change at 48 h, M1 continued to express higher TNF-α at both time points, because the decrease in M1 does not necessarily preclude their ability to produce pro-inflammatory cytokines such as TNF-α. In contrast, non-significant change in TNF-α production was reported in a study done on L. infantum infected THP-1 cells\cite{46}. The variability in TNF-α results may be attributed to the fact that in the present study, the differential expression of TNF-α by M1 phenotype was measured, which accounts for the microbicidal functions and consequently produces higher levels of TNF-α, while in the referred study, they measured the cytokine in the culture supernatants, produced by the whole macrophages population.

Although IL-10 inhibits the potentially damaging inflammatory responses, it inhibits antigen presentation by macrophages, thus affecting T cells effector functions during chronic infections, favoring parasite survival\cite{47}. In human VL, the suppressive role of IL-10 was found to result in the severe decrease in accumulation of macrophages derived from monocytes through the migration inhibitory factor (MIF)\cite{48}. Intriguingly, Babiker et al.\cite{49} demonstrated no difference between IL-10 production in THP-1 cells and whole blood samples from VL patients, both infected with live L. donovani promastigotes. Bunn et al.\cite{50} demonstrated conventional CD4+ T cells and DCs as major sources of IL-10 and absence of IL-10 led to control of the parasitic burden but the damage in the splenic architecture was accelerated. They also proved that absence of IL-10 receptor (IL-10R) was linked to increase in the parasite-specific CD4+ T cells and better control of the parasite growth, but also with increased damage of the splenic architecture. In this study, although M1 showed initial increase, M2 predominated M1 and IL-10 production was enhanced. This finding is reinforced by other studies in which monocytes and macrophages from VL patients showed decreased oxidative burst and antigen presentation, and M2 phenotype was enhanced with higher expression of CD163 and IL-10\cite{51}. Additionally, the significant increase in M2 cells in this study goes in line with the study done by Dasgupta et al.\cite{52}, who found that L. donovani infected THP-1 cells express more macrophage inhibitory protein (MIP)-1, which is linked to M2\cite{53}. Chronic inflammatory environment in the spleen of VL patients is thought to condition macrophages towards M2, and IFN-γ in such settings promotes STAT3-induced response supporting parasite replication and disease progression\cite{50}. Additionally, CD163, as M2 marker, was recommended by Silva and colleagues to be used as a biomarker of the severity of human VL\cite{51}.

Macrophages polarization is an active process and polarized macrophages can reverse from one phenotype to another under certain conditions\cite{52}. M2 repolarization was found to occur more quickly than M1 when exposed to Toll like receptor (TLR) ligands such as LPS or IFN-γ or by expression of certain microRNAs (miRNAs) such as miR-155\cite{53,54}. In contrast, treatment of M1 with IL-4 couldn’t reverse the polarization to M2\cite{55}. Several miRNAs such as miR-9, miR-124 could suppress M1 phenotype and trigger M2\cite{56}. Thus, extensive studies are encouraged to understand the polarization process and to accurately identify the ultimate balance between both phenotypes, which can help immune-based therapeutic development.

In conclusion, PMA-treated THP-1 cells can successfully host survival, and replication of L. donovani. Differential polarization of macrophages
during *Leishmania* infection confirms plasticity of these cells. M2 phenotype takes the upper hand over M1 in response to *L. donovani* infection with significant production of IL-10 by both phenotypes. Switch of macrophages from one to another phenotype still needs extensive research to help determining the perfect balance. Strategies of shaping macrophages polarization could be promising therapeutic modalities that require more attention.

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