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The Effect of Leishmania mexicana growth phase on MHC class I down regulation

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The histocompatibility complex (MHC) system is important in immunity to intracellular pathogens such as *Leishmania* parasites. MHC-I expression is regulated after the infection, and it is a pivotal mechanism used by intracellular pathogens to evade the immune response. Very little is known about this mechanism of *Leishmania* infection. In this study, the infection with different growth phases of *Leishmania mexicana* effect on MHC-I expression was evaluated in human cell line U937 and MonoMac-6 by flow cytometry. The results clearly show a wide range of MHC-I downregulation due to the infection of human cells with different phases of *L. mexicana*. MHC-I expression was significantly ($P<0.05$) downregulated when U937 and MonoMac-6 cells were infected with mid log stage at a ratio of 1:10 (cell:parasite), compared to infection with late and stationary phases. The results illustrated that MHC-I expression was down regulated from 88% in control cells to 79.00%, 45.08%, and 1.74% at infection ratios of 1:10, 1:20, and 1:30, respectively. Therefore, the degree of MHC-I downregulation was parasite dosage dependent. The normal MHC-I appearance in parasite infected cells was partially restored after adding fungizone, an anti *Leishmania* agent. In conclusion, the previously documented fluctuations in MHC-I expression responses to Leishmania infection may relate to differences in the parasite's development stages and infection dose.

1. Introduction

It is well established that pathogens use many mechanisms to evade host immune defences. For example, *Leishmania spp* amastigotes coat themselves with host IgG, which favours their ligation to macrophage FcɣR receptors, leading to IL-10 influx and decreasing macrophage responses to IFN-ɣ. Immunosuppression of host responses is another mechanism used by *Leishmania spp* and other microbes. For example, *Mycobacterium tuberculosis* and *M. leprae* (intracellular microbes) have several ways to avoid immune defences that are not fully understood yet, (Zipfel *et al*., 2007). Surprisingly a study by Fernández, *et al*., (2019) has shown that infection of murine dendritic cells with different isolates of *L. mrxicana* can induce different cytokine production profiles.

The importance of MHC-I in activation of the CD8+ T cells pathway in immune recognition and elimination of viruses and other intracellular pathogens, including *Leishmania* parasites have been addressed in several studies (Muller *et al*., 1993; Shrestha & Diamond, 2004; Ali *et al*., 2009).

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Since the MHC-I antigen complex is presented on the surface of target cells, which leads to the activation of CD8 cytotoxic T cells (CTLs). Therefore, pathogens such as *Leishmania* spp have developed sophisticated approaches to eliminate the expression of these molecules to evade immune recognition. Intracellular Pathogens can hide and shelter themselves by being inside infected cells. Interestingly, *Leishmania* spp and the other obligatory intracellular pathogens obtain extra protection by living in those cells, inside small compartments called parasitophorous vacuoles (PV), (Prina *et al*., 1990; Antoine *et al*., 1991; Russell *et al*., 1992). MHC-II molecules have also been identified on infected cell surfaces, but their expression was not altered flowing parasite infection (Lang *et al*., 1994). However, MHC-II expression was down regulated in P388D1 cell line infected with *L. donovani* (Kwan *et al*., 1992).

MHC-I downregulation in antigen presenting cells (APC) was shown to be induced by many viruses, including Varicella zozter virus, Equinherpesvirus-1 (EHV1), and mouse cytomegalovirus (Bertoletti and Gehring 2006; Rehermann 2003). Some parasites can alter the expression of MHC molecules, but only at very late stages of their development as demonstrated by Ma *et al*., (2013). *Toxolasma gondii* was also able to suppress MHC-II in infected melanoma cell lines (Lüder *et al*., 2003). A similar study reported that infection with the same organism has downregulated MHC-II in murine macrophages, and MHC-I expression was not restored in infected cells following treatment with INF-gamma (Luder *et al*., 1998). Although numerous studies have documented the influence of *Leishmania* parasite infection on MHC-I and MHC-II expression, the findings are controversial. For instance, the expression of MHC-II did not change in macrophages isolated from mouse bone marrow after 48 hours of *L. donovani* infection, (Schaible *et al*., 1999). However, other study reported that MHC-II expression was suppressed in human macrophages after infection with *L. amazonensis or L. donovani* (De Souza *et al*., 1995). According to the published data, the parasite's impact on MHC molecule expression was varied. These variations could be due to parasite species differences, or their growth stage during the infection in addition to inconsistencies in other experimental conditions.

In spite of all new techniques in biomedical science, infection with *Leishmania spp* is stile demanding to be diagnosed and treated (Mann *et al*., 2021). Therefore, the current study was designed to determine the impact of infection dose, and *L. mexicana* growth phase on MHC-I appearance in target cells.

1 Materials and Methods

Characterisation of Parasite Growth. The *L. mexicana* parasite that was used belongs to the (MNYC/BZ/62/M379) strain. The parasite was cultured in fresh Drosophila Schneider medium, as described by Ali *et al*., (2013). Three cultures were initiated for every experiment and the parasite density was daily examined. Three stages were identified in the *L. mexicana* growth curve according to the number of parasites per milliliter: mid-log ($1*10^7$), late log ($2*10^7$) and stationary ($3*10^7$) phases. The effect of every growth stage on MHC-I expression was assessed.

Host cells: RPMI 1640 complemented with 10% HIFCS, 2 mM L-glutamine, and 200 µg/ml Penicillin-Streptomycin were used to cultivate target human cell lines MonoMac-6 and U937. During the experiment, cells were kept in an incubator at 37 ºC and 5% CO2.

Infection of target Cells and Flow Cytometry Analysis. Using the flow cytometry approach, the expression of MHC-I in *L. mexicana* infected target cells was examined. MonoMac-6 and U937 cells were at plated $2x10^5$ per well, and *L. mexicana* from the mid log, late log, and stationary stages were used to infect target cells for 24 hours. The cells were then fixed using 2% paraformaldehyde. Blocking reagent (Miltenyi Biotec, UK) was added to target cells. Cells were stained with a primary antibody extracted from the W6/32HK Hybridoma cell line for 15 minutes after that, the stained cells washed using FACS buffer. Target cells were subjected to a secondary goat anti mouse IgG2a FITC antibody or isotype control antibody (Serotec, UK) for 15 minutes. After washing, 300 µl of Isoton was added to the cells just before flow cytometer (Beckman Coulter) analysis.

Effect of Fungizone on MHC-I Expression

To assess if *L. mexicana* infected cells can revert MHC-I appearance after fungizone treatment, host cells were grown in 24 well plates, and parasites 1:30 (cell: parasite), were added to the target cells.

Four groups of cells were created: one of them was considered a control, two hours after infection, the second group was fixed with 2% paraformaldehyde, the third received 4 µg of fungizone two hours after infection, and the fourth group was infected for a whole day. The MHC class I labeling technique was applied, as previously mentioned, to the evaluated MHC-I restoration in both infected and non-infected cells.The results were analysed using the Kaluza software provided by the company.

Statistical Analysis: The Student's t-test was used for all statistical analyses in the current study.

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2 Results

Parasite Impact on MHC-I. Cells were infected with various growth stages of *L. mexicana*, and MHC-I appearance was examined. The findings demonstrated that U937 monocyte infection at a concentration of 1:10 with *L. mexicana* from the mid log growth stage decreased MHC-I expression to 29.44%, compared to 85.92% in non-infected cells, however, MHC-I downregulation was not significant using late and stationary phases (84.2% and 78.50%), respectively (Figure 1,A). Median fluorescence intensity (MFI) analysis showed a significant MHC-I downregulation in U937 cells due to infection with parasites at different growth phases, (Figure 1, B).

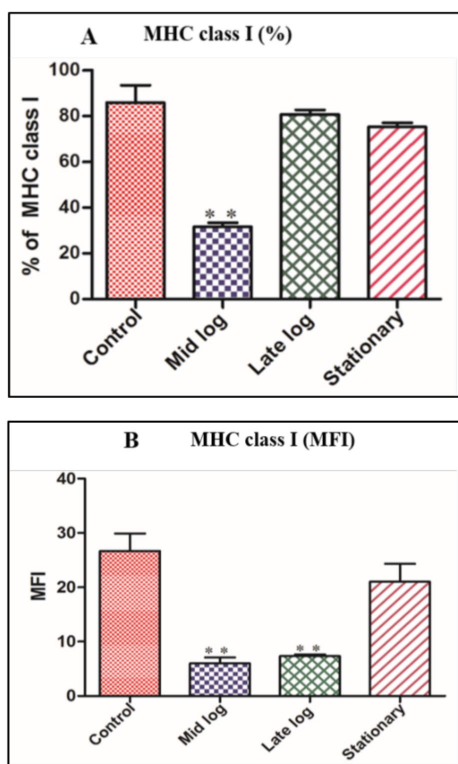

Figure 1: shows MHC-I expression in the infected U937 cell line. (A): a dot plot of MHC-I expression in control and infected U937 cells obtained using a flow cytometer. (B): median fluorescence intensity in target cells. **P≤0.01, P≤0.05, +SEM.

The results of figure (2) represented MHC-I expression in MonoMac-6 cells following infection with various growth phases of *L. mexicana*, which gave a similar pattern of results. MHC-I expression was 83.12% in control cells after infection with *L. mexicana* in the mid, late and stationary phases was downregulated to 38.64%, 73.48%, and 42.48% after infection respectively (Figure 2, A). The MFI analysis was comparable to the ratio of MHC class expression (Figure 2, B). In summary, the results presented in figures 1 and 2 demonstrated that expression of MHC-I in infected cells was growth phase dependent.

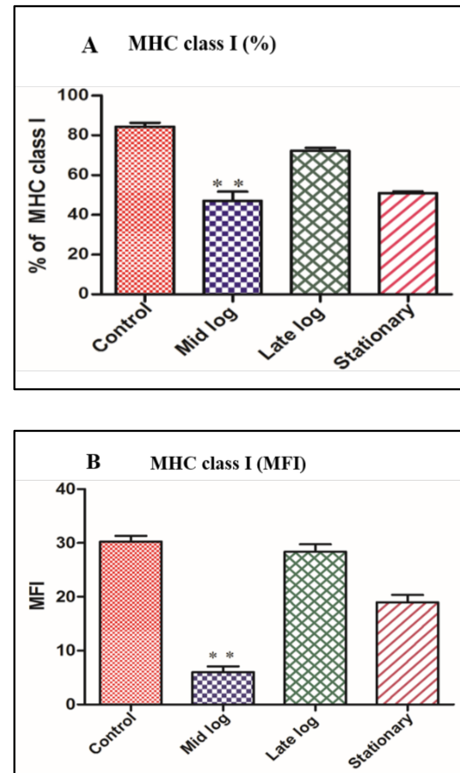

Figure 2: shows MHC-I expression in the infected MonoMac-6 cell line. (A): a dot plot of MHC-I expression in control and infected MonoMac-6 cells obtained using a flow cytometer. (B): median fluorescence intensity in target cells. **P≤0.01, P≤0.05, +SEM.

Restoration of MHC-I expression. To demonstrate if MHC-I downregulation after parasite infection can be restored again, monocyte U937cells were infected with stationary phase *L. mexicana* at a concentration of 1:30 for two hours. After that, infected cells were treated with a 4μg fungizone agent. The results of Figure (3),showed that MHC- I expression significantly downregulated in infected U937 cells with *L. mexicana* for either two or twenty-four hours. However, following two hours of exposure to the parasite. However, treatment of infected U937 cells with fungizone significantly boosted MHC-I expression.

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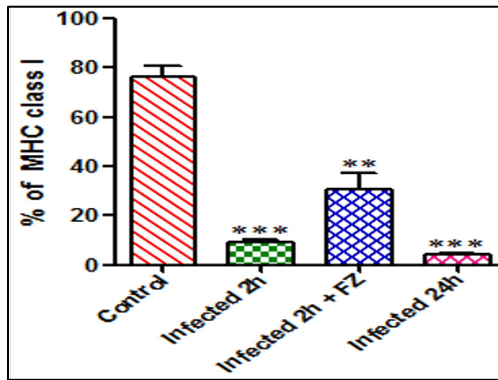

Figure 3: MHC-I restoration in infected cells. Target cells were infected using 24 well plates, infected, and treated as described in the method section. Results showed restoration of MHC-I after fungizone treatment. **P≤0.01 and ***P≤0.000 according to the student's t-test. + SEM.

3 Discussion

The approaches of MHC-I downregulation in APCs following infection with *Leishmania spp* are not fully understood compared to virus approaches, (Petersen *et al*., 2003). Mechanisms of MHC-I downregulation differ according to the type virus, for instance, Abendroth *et al*., (2000) have shown varicella-zoster virus (VZV) infection has induced MHC class II downregulation which was reversed as results of treatment with IFN-gamma. Other research suggested that the viral products could be the cause of MHC-I downregulation. For instance, HIV-1 Nef protein has been reported to increase the pathogenicity of HIV by changing MHC-I appearance in APCs (Le Gall *et al*., 1988; Schwartz *et al*., 1996).

Based on previous and relevant studies, this is the only study that considered the effect of *L. mexicana* growth stages, and dose of infection, on MHC-I expression, in order to understand some of the Contadiatctory findings in the published literature. MHC-I are presented on the plasma membranes of immune cells and they play a significant part in immune recognition of intracellular infections, (Ellner and Daniel 1979; Rodrigues *et al*., 2003).

L. mexicana different growth stages have been identified in two ways: First, according to the parasite number per ml. Second, by determining the virulency-associated gene expression, such as the GP63 and LPG2 genes, which are crucial for parasite attachment to host cells and suppress the innate immunity by obstructive complement activation (Isnard et. al., 2012). Above genes were highly expressed at the stationary stage compared to the mid log phase (Wozencraft *et al*., 1987; Ali *et al*., 2013). In addition, Silva's group (2012) have a similar pattern of results since some other genes such as CPC, CPB2, and CPB2.8, which have a significant role in parasite presence inside infected cells, were highly expressed in the stationary stage.

Although the target cells in the current study were both monocytes, small differences were observed in the expression of MHC-I. This could be due to their variation in responding to antigens. In addition, the mid log *L. mexicana* effect was more obvious in U937 compared to MonoMac-6 cells. These differences between the cells were also noted by Rappocciolo *et al*., (2003), who stated that there were variations in MHC-I appearance on the surface of NBL-6 and EEL cells after infection with EHV1. It is worth mentioning that, the infectivity of *Leishmania* promastigotes was conferred by the ability of the stationary phase to produce the amastigote, these results were similar to Isnard *et al*., (2012) findings. Our study has also confirmed that, MHC-I downregulation as a results of parasite infection was dose dependent (date not shown), since *L. mexicana* infection of target cells with 1:10 (cell: parasite) has a limited effect on MHC-I expression compared to infection ratios of 1:20 and 1:30.

Reiner *et al*., 's study (1987), showed that MHC I and II were down regulated in macrophages infected with *L. donovani*. However, macrophages isolated from susceptible Balb/c and resistant C57BL/6 mice infected with *L. amazonensis* for 12 and 48 hours did not affect MHC I expression, while MHC II was highly expressed on the PVs (Lang *et al*., 1994). Another study by Rezvan *et al*., (2022) stated that MHC-I appearance was downregulated in dendritic cells after *L. mexicana* infection. Moreover, Luder *et al*., (1998) have stated MHC-I and MHC-II were down regulated in Balb/c macrophages as results of *T. gondii* infection.

Interestingly, expression of MHC I was restored as a result of adding the fungizone at (4 µg per ml) for 24 hours to infected cells. Our finding confirmed *L. mexicana*'s ability to manipulate histocompatibility complex presence in infected cells, which cause an alteration of CTL activity. These results are in line with Ali *et al*., (2009) and Asteal (2011), who reported similar findings using DCs infected with the same parasite then treated with 7.5 µg of fungizone. Our findings have confirmed the complexity manipulation pathways that led to MHC class I downregulation after *L. mexicana* infection. Since several studies have stated that intercellular pathogens with large DNA have developed different tools and mechanisms to escape from CTL activity, these mechanisms include variations in the assembly, transportation, localization and biosyntheses of MHC-I molecules (Peterlin and Trono 2003). Meanwhile, *Leishmania* species have a big DNA and intracellular pathogens,

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consequently, they are expected to use similar strategies that used by viruses to avoid immune system resistance, including MHC class I downregulation. These mechanisms needed more investigation in order to eliminate the differences in the published data.

4 Conclusions

This study's findings established that infection with *L. mexicana* has downregulated MHC-I expression in the human cell lines MonoMac-6 and U937. This was further confirmed by using the anti-*Leishmania* agent Fungizone**.** In addition, MHC-I expression was *L. mexicana* growth phase and dose dependent.

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Contrast of Interest: The authors declare that there are no conflicts of interest.

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