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Original Article

Inhibitory Effects of *Leishmania Mexicana infection* on MHC-I Expression in Bone Marrow Derived Dendritic Cells

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Received 09 Jan 2022
Accepted 14 Mar 2022

Keywords:
Bone marrow;
Dendritic cells;
Leishmania Mexicana;
MHC-I Peptide;

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Abstract

Background: *Leishmania* is a parasite causing leishmaniasis with different clinical manifestations depending on the infectious species in many countries worldwide. Although different studies have been taken place to clear the interaction of the parasite with the immune system, many aspects of immunology of leishmaniasis is remained uncertain.

Methods: Bone marrow derived dendritic cells (DCs) were cultured in vitro and divided into different groups (Nottingham Trent University, Nottingham, UK). The groups were separately infected with live or autoclaved *L. mexicana* or loaded with Soluble *Leishmania* Antigen (SLA). The expression of major histocompatibility complex class I (MHC-I) molecule was checked and compared on the cultured DCs using flow cytometry.

Results: Infection of *L. mexicana* caused a significant downregulation in expression of molecules where killed *Leishmania* or SLA could not induce suppression in expression of these molecules.

Conclusion: *L. mexicana infection* results in downregulation of MHC-I expression on bone marrow-derived dendritic cells.

Introduction

Leishmaniasis is an endemic protozoal disease in many areas of the world. Chemotherapy for treatment of leishmaniasis is not fully effective and no protective vaccine has been developed yet. Producing effective *Leishmania* vaccine has long been hindered by the lack of constant stimulation of helper T cells, a pre-requirement for lasting protection that needs to be developed following recovery from natural infection due to the persistence of *Leish-*

mania parasite (1). Dendritic cells are highly specialized APCs that uptake and process antigens. These cells are also essential for effective response against pathogens expanding both CD4 and CD8 effector T cells (2). On the other hand, pathogens develop different mechanisms to interfere with DC's function and down-regulate the affectivity of the immune response. This phenomenon has been observed in HIV, *Plasmodium falciparum* and malaria. The parasite virulence affects the function of DCs increasing intracellular vacuoles and production of some cytokines such as IL-12, TNF-α and IL-10 (3). *L. amasonensis* infection impairs DCs' biological function and differentiation in both human and animal (4). MHC-I molecules are a particularly attractive target for immune evasion by viruses, mycobacterium avium (5) and Chlamedia pneumonia (6) because inhibition of expressing MHC-I can decrease CD8 T cell- mediated recognition of infected cells (7, 8). Previous study on *Leginolla pneumophila*, an intracellular pathogen, have shown a decrease in MHC-I expression on monocytes following infection with this pathogen (9).

In vertebrates, infection of macrophages is a goal for *Leishmania*. The parasite convert to amastigotes and can effectively affect the resistance mechanisms of the macrophage. In immunity to *Leishamnia*, CD8+T cells are shown to play an important role (10). Infection of macrophages with *Leishamnia* induces morphological and functional changes (11). However, no previous studies were conducted to clear the role of intracellular mechanisms of DCs in immune evasion of amastigotes. In this regard the present study was planned to assess the inhibitory effect of *Leishmania* infection on MHC-I expression in these *mysterious* APCs.

Materials and Methods

Animals & antigen

Female BALB/c mice were purchased from Harlan Olac (Oxon, UK) and maintained in accordance with the ethical codes (code Number: IR.BASU.REC.1398.053) of practice for housing and care of animals (Nottingham Trent University, Nottingham, UK).

Leishmania antigen was prepared from *L. mexicana* promastigotes strain (MNYC/BZ/62/M379), according to Dumonteil et al. with some modifications (12). Briefly, late log phase *L. mexicana* parasites were collected by centrifugation, washed with phosphate buffer saline (PBS) four times, and resuspended in 100 mM Tris lysate buffer pH 7.3 containing 1mM EDTA, 0.5 mM PMSF and 2.5 μg/ml leupeptin. Parasites were sonicated, and centrifuged for 20 min at 500 g. The supernatant was centrifuged again for 4 h at 85000 g and then the supernatant was dialyzed against PBS overnight with several changes of the buffer. The SLA was finally filtered (0.22 μm) and the total protein was measured using B.S.A. kit (Sigma).

Generation of DC from murine bone marrow

DCs were generated using a method adapted from Inaba et al. (13). Briefly, hind limbs (femurs and tibias) were harvested aseptically from female BALB/c mice and placed in sterile PBS supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, and 0.25 μg/ml fungizone. The marrow was collected and gently resuspended to make a single cell suspension. The cells were washed twice in serum-free RPMI medium. The pellet was resuspended in DC medium (RPMI 1640 medium supplemented with 2 mM glutamine,

Five percent FBS, 10 mM HEPES, 20 mg/ml gentamicin sulfate, 50 μM 2-ME, 50 IU/ml penicillin, 50 μg/ml streptomycin, 0.25 μg/ml fungizone, and 20 ng/ml mGM-CSF) at a concentration of 1×10^6 leukocytes/ml and seeded in 24-well plates at 1 ml/well and incubated at 37°C and 5% CO_2 in air-humidified atmosphere. The nonadherent cells (T cells, B cells, granulocytes) were removed on days 2 and 4, and the remaining cells were cultured in fresh DC medium. Clusters of loosely adherent DCs cultured for 7–9 days were dislodged by gently washing medium over each well using a Pasteur pipette. The cells were collected, washed twice,

and resuspended in serum-free RPMI at 1×10^7 cells/ml and stored on ice for Characterization by flow cytometry using specific antibodies.

Characterization of DC

Flow cytometry analysis was performed on freshly isolated (day 0) and 7- and 9-day cultured bone marrow cells. Antibodies against CD11c (N418 hamster hybridoma; American Type Culture Collection, Manassas, VA), CD80, CD40, CD45R, CD4, CD8, MHC class II, and macrophage/monocyte Ags (Serotec, Oxford, U.K.) were used for staining, and the cells were analyzed by a flow cytometer (Beckman Coulter).

BM-DC phenotyping

5×10^5 per tube DCs were harvested for FACS analysis. Cells were washed twice in PBS + 0.1%BSA + 0.02%NaN_3. Rat anti-mouse CD80, Macrophage/Monocyte marker (F4/80), DEC205, I-A (murine class II) and CD45, and hamster anti-mouse CD11c monoclonal antibodies were added. Appropriate isotype controls were used in each experiment. The cells were incubated on ice for 30 minutes with primary antibodies. Cells were then washed twice in PBS + 0.1% BSA + 0.02% NaN_3 and incubated for 30 minutes on ice with FITC coupled goat anti-rat IgG or goat anti-hamster IgG as secondary antibodies as appropriate. Finally, the cells were washed in PBS + 0.1% BSA + 0.02% NaN_3 and resuspended in 500 µl of sheath fluid, and analysed by FACS.

Expression of MHC-I

To assess the effect of *L. mexicana* infection on expression of MHC-I, 1×10^6/ml DCs were cultured in 24-well plate and divided in three groups. 10×10^6 of live *L. mexicana*, 10×10^6 of autoclaved *L. mexicana* and 10mg/ml SLA were then added to the wells accordingly in duplicate. lg/ml LPS was added to all of the wells and they were incubated at 37°C with 5% CO_2 for 24h. The cultured DCs were stained with FITC conjugated gout anti mouse MHC-I antibody and the expression of MHC-I on the DCs was analyzed using a flow cytometer (Beckman Coulter-US).

Expression of MHC-I on DCs infected with live L. mexicana

Bone-marrow cells were cultured in presence of GM-CSF for 6 days with wash every 2 days. DCs were harvested and investigated in two groups. The first group was infected with 10 times number of live *L. mexicana* to DCs for 24 hours. No parasite was added to the second group. On day 7 both groups were checked for the expression of MHC class I by FACS analysis using FITC conjugated examined MHC class I antibody. The first group was loaded with 1µg/ml LPS and PBS was added to the second group. On day 7 both groups were checked for the expression of MHC-I. The expression of the MHC-I was compared between DCs loaded with SLA and infected with live *L. mexicana*.

Expression of MHC-I molecules on bone marrow derived DCs following espouser to autoclaved L. mexicana

DCs were in vitro cultured from BALB/c mouse bone marrow cells and infected with autoclaved *L. mexicana*.

Expression of MHC-I on DCs treated with SLA

Expression of MHC-I was evaluated in mouse DCs treated with SLA. Balb/c mouse DCs were cultured in vitro and divided into two groups. The first group was treated with 10mg/ml *L. mexicana* SLA and the second group was used as control. Both groups were cultured in duplicate. The expression of MHC-I molecules on the DCs was evaluated using anti-mouse MHC-I antibody. To induce the DCs to be matured and express MHC-I, two different groups of DCs were first pulsed with 1µg/ml LPS and one of the groups was treated with SLA and the other groups was used as control. The expression of MHC-I was evaluated on the DCs using the same antibody.

Time course in expression of MHC-I on DCs infected with live L. mexicana

Mouse bone marrow derived DCs were cultured in-vitro. DCs were harvested and examined at four times for two groups. Expression of MHC-I on *L. mexicana* infected DCs was checked after 1, 3, 5 and 18 hours of infection. The first group infected with *L. mexicana* and the second group was used as control.

Statistics

The data were statistically analyzed using SPSS version 20 (IBM Corp., Armonk, NY, USA). Due to the normal distribution of the data, one-way ANOVA followed by post hoc Tukey's test were performed to analyze the results in all groups. *P* values ≤0.05 were considered significant in the assays.

Results

Characterization of bone-marrow derived DCs

The cell's phenotype was determined with a number of Abs and FACS analysis. The ratio of DC/macrophage was 75% (Fig. 1).

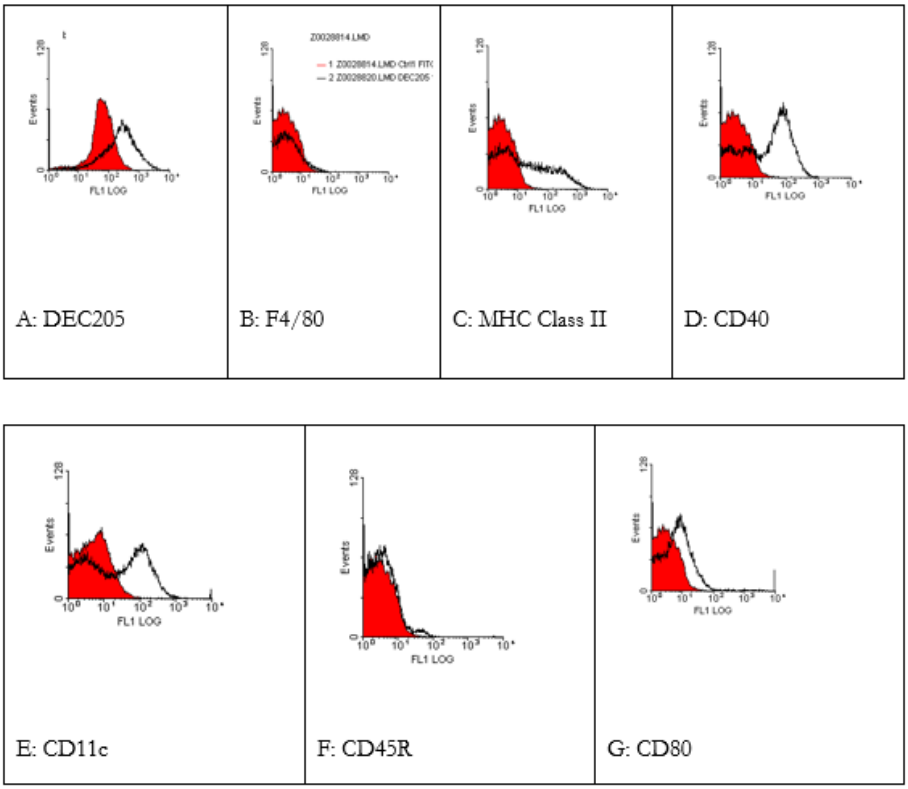

Fig. 1: DC phenotypic analysis

On day 6 the DCs were split into a number of groups stained with Abs and phenotyped by FACS analysis. Red line: control; Black line: Ab stained

Infection of DCs with L. mexicana

DCs were generated from mouse bone marrow cells and infected with *L. mexicana*. The infection of the cells and conversion of *Leishmania* promastigotes to amastigotes were checked under inverted microscopy (Fig. 2).

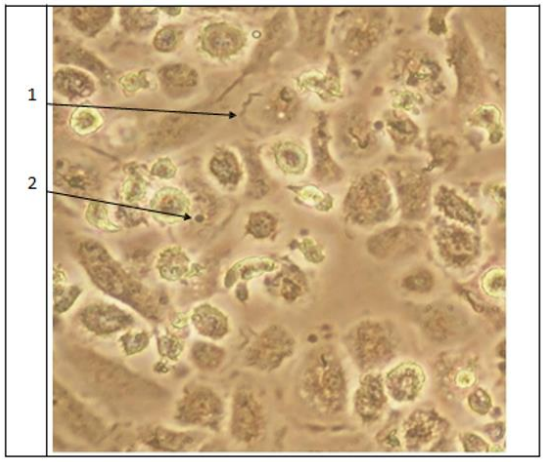

Fig. 2: Infection of DCs with *L. mexicana*. 1- *Leishmania promastigotes* outside of DCs 2- *Leishmania* amastigotes inside of DCs

Expression of MHC-I molecules on in-vitro cultured DCs

Expression of MHC-I molecules was evaluated on DCs derived from mouse bone marrow. MHC-I molecules were successfully expressed on the DCs (Fig. e 3a). To more stimulate the DCs for maturation and expression of MHC-I, the bone marrow derived DCs were also pulsed separately with l g⁄ml LPS and checked for expression of MHC-I (Fig. 3b). In both groups, MHC-1 were significantly expressed on the cells and no significant upregulation was observed in the group treated with LPS ($P \leq 0.05$).

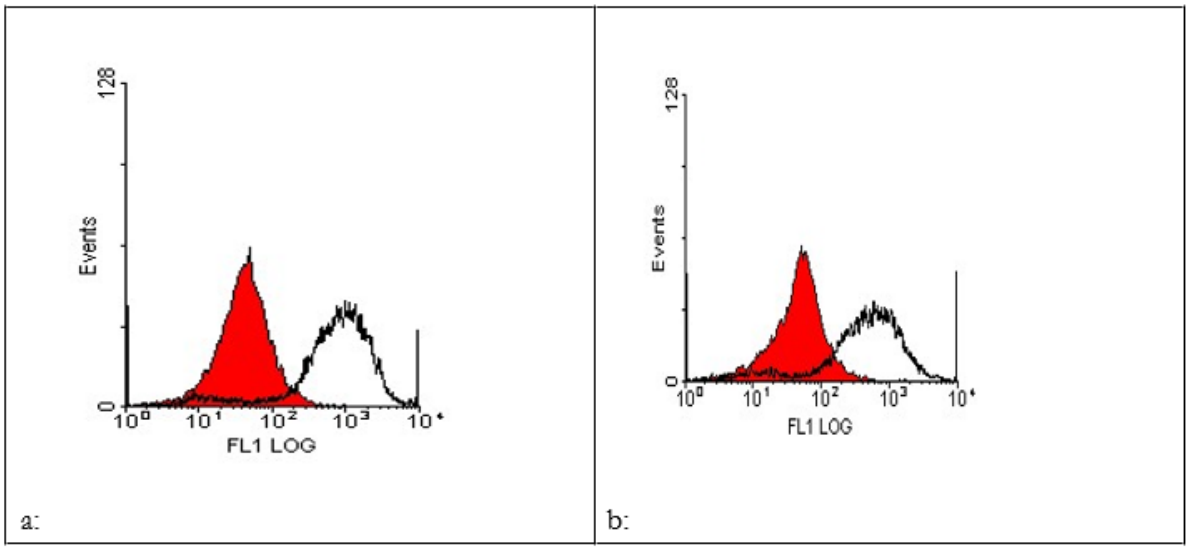

Fig. 3: Expression of MHC-I molecules on DCs
A: Both groups had no pulse with LPS. DCs stained with isotype control antibody (red) and DCs stained with FITC conjugated anti-MHC-I antibody **B**: LPS was added to the DCs culture. LPS pulsed DCs stained with isotype control antibody (red) and LPS pulsed DCs stained with FITC conjugated anti-MHC-I antibody.

Expression of MHC-I molecules on bone marrow derived DCs after espouser to autoclaved L. mexicana

Mouse bone marrow derived DCs were in vitro co-cultured with autoclaved *L. mexicana*. The autoclaved parasites were engulfed by the DCs but no down regulation on MHC-I was observed (Fig. 4).

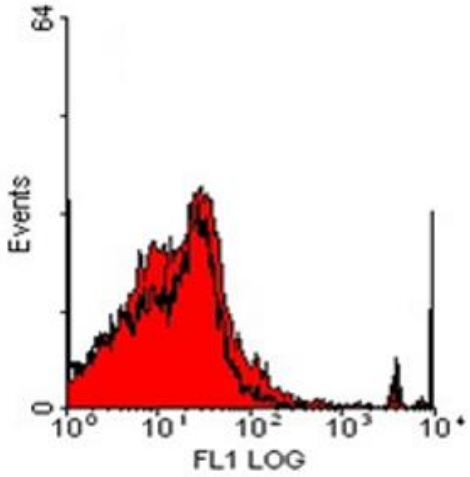

Fig. 4: Impact of autoclaved *L. mexicana* on expression of MHC-I on in vitro cultured DCs
The graph compares the level of MHC-I expression on normal DCs stained with anti-mouse MHC-I antibody (red graph) and DCs co-cultured with autoclaved *L. mexicana* and stained with anti-mouse MHC-I antibody (black graph)

Expression of MHC-I on DCs treated with SLA

The first group was treated with 10mg/ml *L. mexicana* SLA and the second group was used as control. No significant difference in expression of MHC-I molecules was observed between the two groups ($P \leq 0.05$)(Fig. 5).

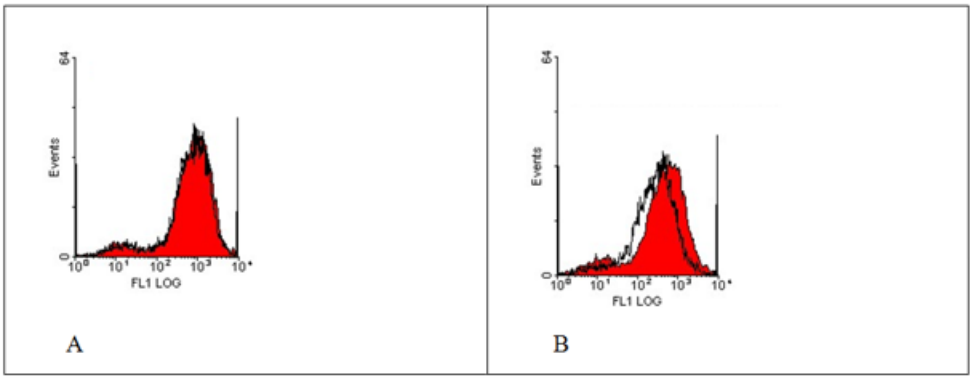

Fig. 5: Expression of MHC class I molecules on DCs loaded with SLA
A: The first group was treated with 10mg/ml SLA and the second group was used as control. No changes in expression of MHC-I molecules was observed after SLA treatment. **B:** Another two groups of DCs were first pulsed with 1µg/ml LPS and then one group treated with SLA and the other group was used as control. No significant difference in expression of MHC-I molecules was also observed between the two groups

Expression of MHC-I on DCs infected with live L. mexicana

Expression of MHC-I molecules were evaluated on bone marrow derived DCs infected with live *L. mexicana*. The results clearly showed a significant downregulation in MHC-I expression on DCs infected with *L. mexicana* ($P \leq 0.05$) (Fig. 6a). Similar results were obtained by addition of SLA (Fig. 6c). Comparison of MHC-I expression on DCs pulsed with SLA (Fig. 5) and those infected with live *L. mexicana* (Fig. 6) confirmed that downregulation of MHC-I expression only occurred in DCs infected with live *L. mexicana* and *Leishmania* proteins cannot induce downregulation in expression of these molecules.

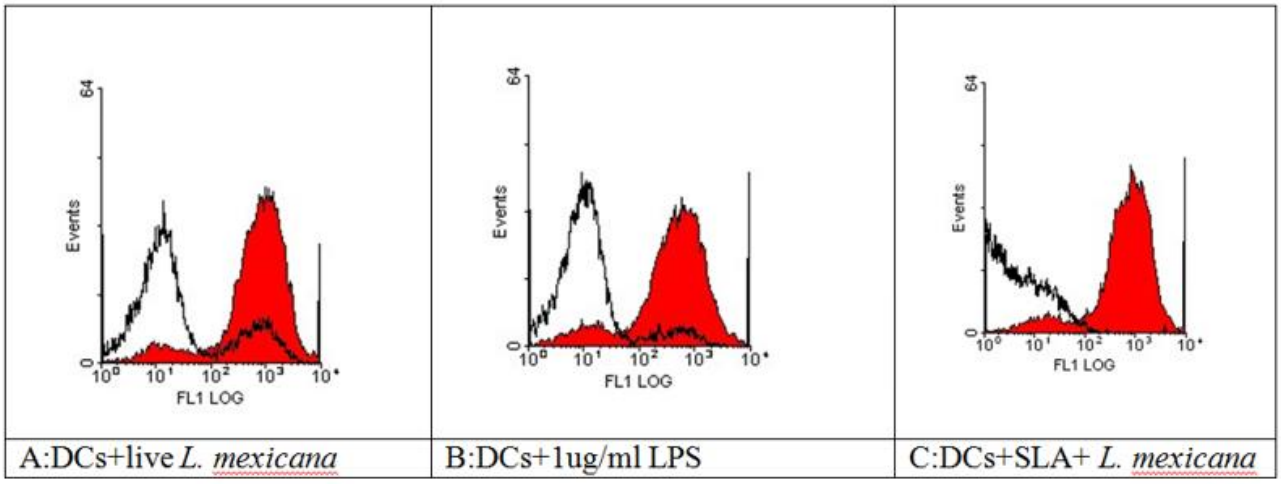

Fig. 6: Expression of MHC class I molecules in live *Leishmania* infected DCs
A: non-infected DCs (red graph) show high expression of MHC class I where the expression of these molecules in *Leishmania* infected DCs (black graph) is highly down regulated. **B:** The first group was loaded with 1µg/ml LPS and PBS was added to the second group. On day 7 both groups were checked for the expression of MHC-I. There was no difference between normal DC (red graph) and DCs loaded with LPS (black graph) **C:** the expression of the MHC-I was compared between DCs loaded with SLA and infected with live *L. mexicana*. There was a significant decrease in expression of MHC-I in *Leishmania* infected DCs (black graph) compared to those treated with SLA (red graph)

Time course of downregulation in expression of MHC-I on DCs infected with live L. mexicana

The results showed that downregulation of MHC-I molecules started about 3hrs of postinfection (Fig. 7b), completed in 5hrs (Fig. 7c) and continued as after 18 h of infection, expression of MHC-I molecules was completely downregulated (Fig. 7d).

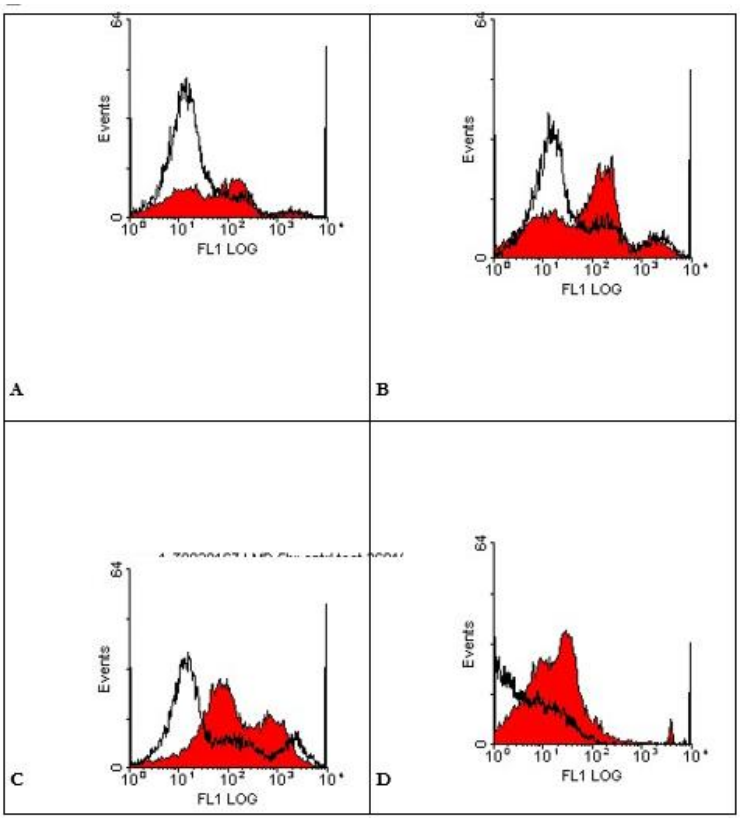

Fig. 7: Time course of MHC-I expression on DCs infected with live *L. mexicana*
The first group infected with *L. mexicana* (black graph) and the second group was used as control (red graph). **A:** Expression of MHC-I on DCs after 1h postinfection. **B:** Expression of MHC-I on DCs after 3hrs postinfection. **C:** Expression of MHC-I on DCs after 5hrs postinfection. **D:** Expression of MHC-I on DCs after 18hrs postinfection

Discussion

In the present study, the effect of *L. mexicana* infection on the expression of MHC-I molecules in bone marrow-derived DCs was evaluated. A significant downregulation occurred in expression of MHC-I in DCs after infection of *L. mexicana.*

Prevention of MHC expression in virus-infected cells is a known mechanism, which helps the virus to escape from the immune responses (15). Although this mechanism is not so clear for intracellular parasites, there are some evidences for downregulation of MHC-II in intracellular parasite infected cells. In this regards, suppression effects of *Leishmania* infection on MHC-II molecules is reported in DCs infected with *L. donovani* (16). Inhibition of MHC expression for class II molecules are also reported for other parasites such as *Toxoplasma gondii* in macrophages (17). Instead, *L. mexicana* amastigotes have no effect on expression of MHC molecules in their host cells (14) where *L. major* amastigotes are capable of upregulating the expression of MHC class II (18) indicating a species-specific behavior on the expression of MHC molecules in *Leishmania* infected cells. The potency of *Leishmania* infected macrophages to stimulate CD4+T cells is also succumbed by the infection (19). The effect of parasitic infection on expression on MHC-I molecules was not clearly known.

In the present study, the results showed a downregulation of MHC-I in DCs infected with *L. mexicana* exactly similar to that hap-

pens in virus-infected cells. Therefore, suppression of MHC-I expression could be accounted as an important strategy for *Leishmania* to evade the immune response. In this study infection of DCs with killed *L. mexicana* had no effect on the expression of MHC-I. Similarly, stimulation of DCs with SLA did not alter the expression of these molecules in the host cells. Viability of the parasites had no effect on their recognition by DCs as the killed parasites were perfectly recognized and phagocytosed by DCs but infection with the killed parasites had no effect on the expression of MHC-I. Therefore, the live parasite can actively manipulate the expression mechanisms of MHC-I molecules and when the parasites are killed this potency of the parasite disappears.

These results indicated that only live *L. mexicana* can affect the expression of MHC-I molecules as none of either SLA or killed parasite were able to downregulate MHC-I molecules in the infected cells.

Different strategies are used by *Leishmania* to escape from the immune system including manipulation of host's immune cells and prevention of MHC-peptide complexes from expressing on the cell surface by which the parasite impairs antigen presentation. The disposition of MHC-II molecules caused by *Leishmania* infection rafts the fluidity in macrophages and results in impairment of antigen presentation and T cell priming (20, 21). Sequestration of antigens from the MHC-II pathway defecting antigen processing of *Leishmania*-infected macrophages is also reported after infection with *L. pifanoi* and *L. amazonensis* amastigotes (22, 23). In bone marrow macrophages infected with *L. donovani,* the capacity of surface MHC class II-peptide complexes to engage with the T cell receptor is modulated defecting the potency of the macrophages to activate the antigen-specific T cells (24). However, other mechanisms underlying the inhibitory effects of the parasite for expression of MHC molecules is still not known, and these mechanisms and the underlying cellular signaling employed for evasion of the immune repose by *Leishmania* is thought to be species dependent.

We measured the down regulation rate of MHC-I after 1, 3, 5 and 18 hours post infection. After 1 hour of infection, expression of MHC-I molecules started to become down-regulated. As the time went on, the expression of MHC-I molecule was more down-regulated where after 18 hours, the expression of MHC-I molecules on the infected DCs was very low. These results confirmed that the live parasite along with converting to the amastigote form, actively interfere with the process of MHC-I expression. There are a few studies indicating that DCs receptors play different roles in phagocytosis of the two forms of amastigote and promastigote of *L. mexicana* after 3, 6 or 24 hours of incubation; after 3h incubation, DCs receptors (DCs-SIGN) participate in phagocytosis of promastigote but amastigotes (25).

Effects of *L. mexicana* infection on function and signaling of DCs showed that the parasite inactivates signaling cascades responsible for cytokine expression inducing the immune response against the parasite via reducing MHC expression on the cell surface resulting in downregulation of antigen presentation capacity. In this regard, primary DCs, after infection with *L. mexicana* promastigotes, were not able to mature and did not express the B7 co-stimulatory molecules (B7.1 & B7.2). These cells express low levels of IL-12p70, increased levels of IL-10 and were not able to promote Th1 cells (26). Although the parasite affects the cell's signaling, other functions of the DCs, such as modulation in the chemokines and cytokines and phagosome maturation expression are effective during infection.

Conclusion

The results address an effective interfere of *Leishmania* with MHC-I expression in *L. mexicana* infected DCs. Infection of DCs with live

parasite significantly downregulated the expression of MHC-I molecules where neither killed parasite nor SLA rendered downregulation effects on expression of these molecules. Downregulation of MHC-I expression in infected DCs increased as the time of infection went on and the parasite converted to the amastigote. So, this phenomenon can be postulated as a mechanism for *Leishmania* to evade the immune system. However, the mechanism by which *Leishmania* downregulates the expression of these molecules on infected DCs is an important question and still needs to be answered.

Acknowledgements

We would like to thank BU-Ali Sina University and the Nottingham Trent University for their financial support and supplying the materials.

Conflict of Interest

We declare that there is no conflict of interest.

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