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Modulation of gene expression profile of cytokines and chemokines following infection with avirulent and virulent *Leishmania mexicana*

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Abstract Cutaneous Leishmaniasis, caused by *Leishmania mexicana* complex is associated with unpleasant or disfiguring lesions with no available vaccine. This study aimed to investigate the changes in the gene expression profile of Cytokines (IL-1, IL-6, and TGF-β), and Chemokines (CCL-1, CCL-2, and CCL-5) following infection with avirulent and virulent *L. mexicana* promastigote using tumour human MonoMac-6, U937 monocyte and macrophage cell lines by qPCR analysis, Furthermore, the effect of their Soluble *Leishmania* Antigens was (SLA) also investigated. Results showed that the level of cytokine/chemokine by parasite infection was both cell line- and parasite-virulence dependent. Results of this study illustrated that, the gene expression profile IL-1 and IL-6 expression was significantly up regulated in the three cell lines following the avirulent parasite infection. However, the gene expression of TGF-β was up-regulated following the virulent *L. mexicana* infection in U937 macrophages and MonoMac-6 cell lines. Chemokines CCL-1, CCL-2 and CCL-5 gene expression was down regulated following the infection with *L. mexicana* in U937 monocytes and macrophages. SLA has also an impact on the gene expression profile of ILs and CCLs in stimulated target cells. For example, the gene expression profile of IL-6 was up regulated after stimulation with avirulent SLA in the three cell line. In conclusion virulent but not avirulent *L. mexicana* promastigote significantly up regulated T helper cells type 2 mediators which supported the parasite surviving inside the host, and this *in vitro* model of avirulent *L. mexicana* parasite has potential role of understanding the parasite pathogenicity.
Keywords: avirulent *Leishmania mexicana*, virulent, Chemokines, Cytokines.

دراسة التأثير الناتج عن الإصابة بطفيل اللشمانيا الجلدية Leishmania mexicana علي جينات الـــ Cytokines وجينات الـــ Chemokines في الخلايا الجذعية

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الخلاص الاصابة باللشمانيا الجلدية تحدث نتيجة للاصابة بأنواع من الطفيليات تعود لجنس *Leishmania mexicana complex* بأنواعها المختلفة، والتي تؤدي الى ظهور تقرحات على جلد الشخص في مكان الاصابة. هذا النوع من الاصابات يعد مشكلة كبيرة في جميع المناطق في العالم وخاصة في المناطق الحارة، نظرا لعدم وجود لقاح يمكن استخدامه لتحصين عامة الناس لمنع اصابتهم بهذا المرض. يتميز هذا النوع من الطفيليات الممرضة بالقدرة على تغيير العديد من البروتينات والجزئيات على اسطح الخلايا المناعية مثل الخلايا البلعمية والخلايا احادية النواة و التي لها دور مهم وفعال جدا في عملية الدفاع عن الجسم ومقاومة للكائنات الممرضة. أحدى اهم هذه المكونات مجموعة من البروتينات تدعى *Cytokines* مثل *IL-1, IL-6, IL-10, IL-12β* ومجموعة اخرى تدعى *Chemokines* مثل *CCL-1, CCL-2, CCL-3, CCL-4*. تمت دراسة تأثير الاصابة بطفيل الـــ *Leishmania mexicana* او البروتينات المستخلصة منه على مدى كفاءتة الخلايا الجذعية في مقاومة الاصابة بالمرض. من اهم النتائج المتحصل عليها ان نوع التغيير ومعدله يعتمد على نوع الخلايا الجذعية وكذلك عدائية الطفيل المستخدم في الدراسة. فعلى سبيل المثال وجد ان الجينات المسئولة عن *Cytokine IL-12B* قد ازداد معدلها مقارنة مع الجينات المسئوله عن *IL-10* والتي انخفض معدلها. كما اوضحت نتائج الدراسة الحالية بأن البروتينات المستخلصة من الطفيل الاكثر عدائية لها قدرة عالية على تحوير الاستجابة المناعية للخلايا المصابة لصالح الطفيل، بينما أدى البروتين المستخلص من الطفيل الاقل عدائية إلي تحفيز الاستجابة المناعية للقضاء على الكائن الممرض.

الكلمات المفتاحية: اللشمانيا الجلدية، Cytokines, Chemokines.

Introduction

Leishmaniasis is a disease occurred following *Leishmania spp* infection. This disease is endemic in tropical and subtropical areas, where the environmental conditions are supporting the presence of the disease transmission vector. *Leishmania* parasite species, have developed unique mechanisms that favour their survival inside the host immune system. Cytokines have been considered as the key factor in the immune response to *Leishmania* infection, [1-2]. Cytokines such as IL-1β and TNF-α and IL-12 can be produced by macrophages which harbour the parasites. In addition to their direct contribution to *Leishmania* infection control or disease exacerbation, cytokines can also up regulate the expression of some chemokines, which in turn induce leukocyte recruitment to the site of the infection [3]. For example, IL-1 and TNF-α secreted from infected macrophages or neutrophils induce the expression of chemokines in fibroblasts, endothelial and epithelial cells [4]. The cytokines and chemokines can also work collectively to control the *Leishmania* infection. A study by [5] reported that IL-12 was an important for the induction of XCL-1, CXCL-10 and CCL-2 expression in lymph nodes which required for *L. major* infection control. Chemokines can be divided into two types. Firstly, Th1 related chemokines such as CXCL-9 and CXCL-10, which are induced by IFN-γ and secondly, Th2 related chemokines such as CCL-22 and CCL-6, which are induced by IL-4 and IL-13 [6].

Chemokines are involved in both innate and adaptive immune responses to *Leishmania* infection. The immune response to *Leishmania* infection starts immediately following parasite delivery by an infected sand fly (or by deliberate injection in the case of experimental models), which involves parasite interaction with DCs and macrophages *via* their TLRs [7]. In humans, as soon as PMNs cells arrive at the site of *L. major* entry, they start to secrete IL-8 (also known as CXCL-8 chemokine), and CXCL-1 in infected mice. These chemokines in turn attract more PMNs to the site of infection [8-9]. Chemokines also have an indirect role in the adaptive immune response to *Leishmania* infection via the activation of DCs. DCs act as APCs and are considered as a bridge between innate and adaptive immune response. They can become activated by chemokine secretion, which causes up-regulation of the expression of some co-stimulatory molecules (CD54, CD40, CD80 and CD86) and IL-12 production [10]. Unlike the mouse model, little is known on the effect of *Leishmania* parasite infection on the network of cytokines and chemokines function in humans. Therefore, the modulation of interleukin and chemokine expression was investigated in this study, using U937 monocytes, U937 macrophages and MonoMac-6 monocyte cell lines infected or stimulated with pathogenic (virulent) and non-pathogenic (avirulent *L. mexicana* promastigote, or their antigens using qPCR analysis.

Materials and Methods

Target cells and *L. mexicana* parasite

MonoMac-6 cell line (DSMZ no. ACC124) were cultured in a complete RPMI medium, supplemented with 10% FCS, 2mM L-Glutamine, 1mM Na pyruvate, 1% non-essential amino acids, 9μg/ml bovine insulin and Penicillin-Streptomycin 200μg/ml., under 95% humidity at 37°C in a 5% CO2 incubator. U937 monocyte cell line were cultured in RPMI 1640 supplemented with 10% FCS, 2mM L-Glutamine and 200μg/ml of Penicillin-Streptomycin. Cells were grown under 95% v/v humidity at 37°C in a 5% CO2 incubator. U937 monocytes were induced to differentiate into human macrophage-like cells using Phorbol 12-myristate 13-acetate (PMA, Sigma, UK) as described by [11]. The *L. mexicana* (MNYC/BZ/62/M379) strain was obtained from Dr V. Yardley, London School of Hygiene and Tropical Medicine (LSHTM). The virulency of the parasites promastigote was maintained by continuously passaging them in susceptible Balb/c mice. The maintenance and care of mice were according to the guidelines of the Home Office. Mice were killed when they developed the required lesion. After two passages of amastigotes in Drosophila Schneider media (Lonza, UK), supplemented with 10% HIFCS at 25°C, obtained promastigote were further cultured and frozen in liquid nitrogen for future work. Avirulent *L. mexicana* promastigote were obtained as published in [11]. All experimental work has been done at Interdisciplinary Biomedical Research Centre labs at Nottingham Trent University.

RNA extraction, cDNA synthesis and Quantitative Real-Time PCR analysis

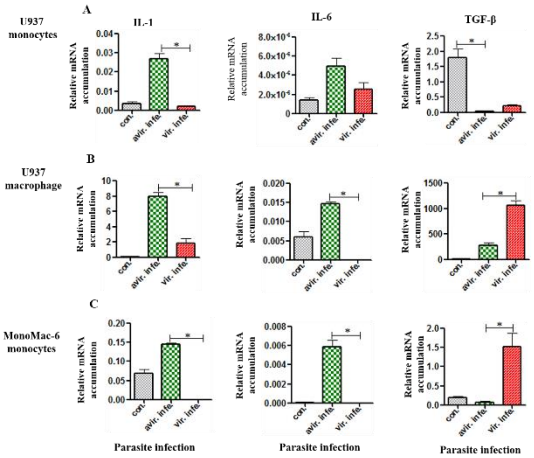
Total mRNA was extracted from target cells, U937 macrophages, U937 monocytes, and MonoMac-6, after infected with a ratio of 1:30 (cell: promastigote) late log phase of virulent and avirulent *L. mexicana* promastigote, or stimulated with 10μg per ml virulent or avirulent *L. mexicana* antigens (SLA), prepared as described [11] for 24 hours using a Qiagen kit, according to the manufacturer's protocol. Before RNA extraction, infected cells were harvested and free parasites were removed by centrifugation, and U937 macrophages were harvested using trypsin solution. After infection or stimulation with virulent or avirulent *L. mexicana* or their antigens, a fixed number of target cells was subjected to RNA extraction. The synthesis of single-stranded cDNA was performed using the M-MLV reverse transcriptase method (Promega, UK), following the manufacturer's protocol. The gene expression profile of IL-1, NM-000576, F.P.AA GCTGAGGAAGATGCTGGT.R.P.CGTTATCCCA T GTGTCGAAG; IL-6, NM-000600, F.P.TCAGCC CTGAGAAAGGAGAC. R.P.CCATCTTTGGAAGG TTCAGG and TGF-β, NM-00066, F.P. ACAATT CCTGGCGATACCTC.R.P. ACAACTCCGGTGAC ATCAAA cytokines. And CCL1, NM-002981, F.P.ATGCAGGTACCCTTCTCCAG. R.P.AACACA GGATTGCCTCAG; CCL2, NM-002982, F.P.GCAGAAGTGGGTTCAGGATT .R.P.TGGGTTGT GGAGTGAGTGTT and CCL5, NM-000579, F.P.CGGTGTCGAAATGAGAAGAA. R.P.TAGGG AGCCCAGAAGAGAAA Chemokines in control, infected, or stimulated target cells were

investigated using quantitative polymerase chain reaction (qPCR). The comparative threshold method (Ct) was used in order to quantify the results obtained by qPCR. In this method, Ct values of genes of interest were compared with the Ct values of endogenous housekeeping gene (ACTB, NM-001101, F.P.GGCATGGGTCAGAAGGATT. R.P.AGAAG GTGCCAGATT). For each gene of interest the relative expression was calculated as $2^{-\Delta\Delta CT}$. The Mann-Whitney U-test was used for statistical analysis and a value of $P < 0.05$ was taken as significant.

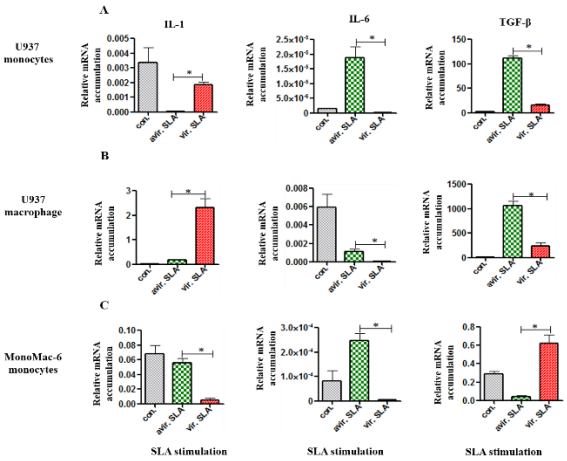
Results

The effect of *L. mexicana* infection and SLA stimulation on gene expression profile of interleukins (ILs) in susceptible human cell lines

Figure 1 shows the effect of *L. mexicana* promastigote infection on the gene expression profile of cytokines (ILs) measured by qPCR it was varied depending on the cell line tested. Results clearly show that the gene expression profile of IL-1 and IL-6 was significantly up-regulated in U937 monocytes infected with the avirulent parasite, while TGF-β expression was down regulated after the parasite infection. Infection of U937 macrophages with *L. mexicana* also caused a significant up-regulation of IL-1, IL-6 flowing the infection with avirulent parasite compared to virulent parasite. In contrast, TGF-β was significantly up-regulated following the virulent parasite infection. The gene expression of IL-1, IL-6 and TGF-β in MonoMac-6 cell line was similar to their expression in infected U937 macrophages. Stimulation of targeted cells with 10µg SLA per ml affect ILs expression (Figure 2). For example, IL-6 expression was up-regulated after stimulation with avirulent SLA, and down regulated after stimulation with virulent SLA in the tested cell lines. IL-1 expression was up-regulated following the stimulation with virulent SLA in U937 monocyte and macrophages. TGF-β expression was up-regulated after stimulation with avirulent SLA. The results also show that the gene expression profile of IL-1 and IL-6 cytokines in MonoMac-6 cell line was up-regulated following the stimulation with avirulent SLA, in contract, TGF-β was up-regulated after stimulation with virulent SLA.



Finger 1: The effect of *L. mexicana* infection on gene expression profile of the cytokines (ILs). Target cells were infected with virulent or avirulent *L. mexicana* promastigote 24 hours. RNA was extracted and converted to cDNA, which was used as templet for qPCR analysis. Bars represent 3 independent experiments. * P<0.05 was calculated according to Mann-Whitney test.

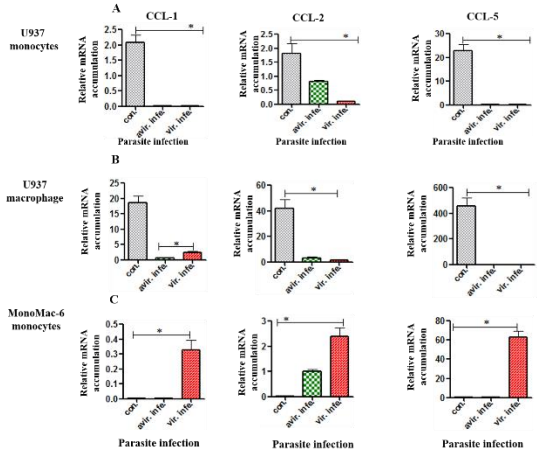


Finger 2: Finger 1: The effect of *L. mexicana* SLA stimulation on gene expression profile of the cytokines (ILs). Target cells were stimulated with virulent or avirulent *L. mexicana* promastigote 24 hours. RNA was extracted and converted to cDNA, which was used as templet for qPCR analysis. Bars represent 3 independent experiments. * P<0.05 was calculated according to Mann-Whitney test.

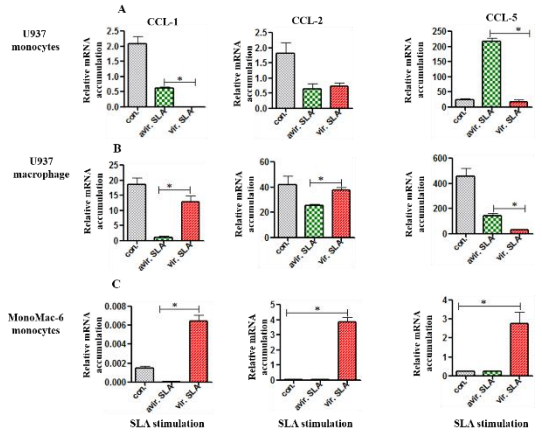
The effect of *L. mexicana* infection and SLA stimulation on gene expression profile of chemokines (CCLs) in susceptible human cell lines.

The infection with the *Leishmania* parasite has significantly affected the gene expression profile of CCL-1, CCL-2 and CCL-5 chemokines (figure 3) in U937 monocytes and macrophages. Interestingly, avirulent *L. mexicana* infection of U937 monocytes caused a remarkable down regulation of CCL-1, CCL-2 and CCL-5 that were highly expressed in control (non-infected) cells. Results also show that the tested chemokines expression was cell line depends. For example, infection of MonoMac-6 cells was significantly up-regulate the gene

expression of CCL-1, CCL-2 and CCL-5, and the level of the up-regulation induced by virulent *L. mexicana* was significantly more compared to avirulent parasites infection. Stimulation of target cells with *Leishmania* parasite SLA has a significant effects on the gene expression profile of tested chemokines depending on the virulency of parasites used for SLA preparation, (Figure 4). The level of expression of CCL-1 and CCL-2 chemokines was significantly higher after stimulation of U937 macrophages with virulent SLA compared to stimulation with avirulent SLA. Unlike U937 monocytes, stimulation of MonoMac-6 with virulent *L. mexicana* SLA caused a significant up-regulation of CCL-1, CCL-2 and CCL-5 compared to avirulent SLA.



Finger 3: The effect of *L. mexicana* infection on gene expression profile of the chemokines (CLLs). Target cells were infected with virulent or avirulent *L. mexicana* promastigote 24 hours. RNA was extracted and converted to cDNA, which was used as templet for qPCR analysis. Bars represent 3 independent experiments. * P<0.05 was calculated according to Mann-Whitney test.



Finger 4: Finger 1: The effect of *L. mexicana* SLA stimulation on gene expression profile the chemokines (CCLs). Target cells were stimulated with virulent or avirulent *L. mexicana* promastigote 24 hours. RNA was extracted and converted to cDNA, which was used as templet for qPCR analysis. Bars represent 3 independent experiments. * P<0.05 was calculated according to Mann-Whitney test.

Discussion

The immune response to foreign antigens is modulated by complex interactions between immune host cells and invading pathogens. The model of virulent and avirulent parasites produced in the current study [11] was further used to investigate and compare the ability of both sets of parasites to modulate the gene expression profile of target cells. The novelty of this investigation is the use of avirulent *L. mexicana* in parallel with its virulent counterpart which may be the best control.

To investigate the *in vitro* modulation of ILs, and CCLs, target cells were infected with the late log phase of virulent and avirulent *L. mexicana* for 24 hours. The expression of the target genes in infected cells was evaluated using qPCR technique. Although the final host for *Leishmania* spp in the mammalian host is tissue macrophages, the entry of *Leishmania* promastigote into the host blood exposes them to other types of immune cells including monocytes [12]. Therefore, in this study three types of cell lines: MonoMac-6, U937 monocytes and U937 macrophages were used to compare their responses to infection with virulent and avirulent of *L. mexicana* promastigote and their SLA.

The vast majority of published studies have agreed that resistance to *Leishmania* parasite infection is partly dependent on the suppression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12B [13]. On the other hand, susceptibility to *Leishmania* parasite infection is associated with the up regulation of anti-inflammatory responses such as IL-4, IL-13 and TGF-β [14-15]. IL-10 is one of the anti-inflammatory cytokines produced by monocytes, macrophages, DCs and B cells [16]. According to [17], the biological function of IL-10 is to inhibit the production of pro-inflammatory cytokines by infected macrophages, which enhances parasite infection.

In this study (Figure 1) shows infection of target cells with avirulent parasites caused significant up-regulation of IL-1 and IL-6 compared to virulent infection, a finding that highlights the role of the pro-inflammatory response in controlling *L. mexicana* infection. Infection with virulent parasites induced less down regulation compared to avirulent parasite infection. This agrees with [18], who reported that chronic infection with *L. mexicana*, requires IL-10, which in turn suppressed IL-12 production from macrophages, decreased IFN-γ in T cells and nitric oxide production in infected cells. Other studies have reported that in human and experimental models of Cutaneous Leishmaniasis (CL), high levels of IL-10 production are strongly associated with non-healing forms of the disease [16]. The biological role of IL-12 in controlling *Leishmania* infection has been reported through activation of Th1 type (CD4 T cells) immune response and secretion of IFN-γ [19]. In experimental models, treatment of resistant mice infected with *L. major* with anti-IL-12 antibodies caused failure of these mice to

overcome their infection. Meanwhile treatment of susceptible Balb/c mice with recombinant IL-12 cytokines enabled them to control the infection [20]. Results from the current study (Figure 1) show that highly expressed IL-1 and IL-6 following infection with avirulent *L. mexicana* in comparison to virulent infection in the three cell line. These findings reflect the multiple roles that each cytokine can play during the course of the parasite infection, using different host cells under the same experimental conditions.

It is well established that the expression or production of IFN-γ is highly associated with resistance to *Leishmania* infection [21]. The biological role of these cytokines is related to activation of the innate immune response through enhancement of microbicidal activity such as iNOS (inducible nitric oxide synthase) expression and NO secretion by macrophages [22]. In the present study, infection of U937 monocytes and MonoMac-6 cell line with virulent promastigote down regulated TNF-α when compared to infection with avirulent parasites (data not shown). This may inhibit NO secretion in the early stages after infection with the virulent parasite [23] thus enhancing their survival inside infected cells. The other interesting finding from this study is the expression of TGF-β, well known as an important immune regulatory cytokine. Infection of MonoMac-6 monocytes with virulent L. mexicana induces high expression of this cytokine, but infection with avirulent parasites induced its down regulation. Results also showed that although TGF-β was up regulated following infection with both sets of parasites, its expression was several hundred fold more in U937 macrophages infected with virulent compared to avirulent parasites, This may explain survival of virulent L. mexicana inside the host cells. As mentioned above, no investigations have been reported on the effect of SLA on ILs gene expression profile *in vitro*. Chemokines are group of polypeptides, and group CC Chemokines (which have their first two cysteines adjacent to each other) were chosen to be investigated in this study because they act on a large group of cells, such as monocytes and macrophages, [24].

Since the biological role of chemokines is to recruit immune cells to the site of infection, understanding their gene regulation by modulation following *Leishmania* infection is an important tool in infection control [25-26]. Results presented in Table 2 show that all tested CCLs were up regulated in U937 macrophages infected with parasites. The level of up regulation was variable depending on virulence of the parasite and the type of CCLs. For example pro-inflammatory CCL-1 chemokine was significantly up regulated following infection of U937 macrophages with virulent *L. mexicana* compared to infection with avirulent parasites.

Generally, stimulation with virulent SLA caused more up regulation of CCLs compared to avirulent SLA in U937 macrophages and MonoMac-6 cells. This could be due to the presence of large amounts of *Leishmania* surface proteins such as lipophosphoglycan (LPG) and glycoprotein gp63 (GP63) in virulent SLA which could modulate CCLs expression. This needs to be confirmed by further analysis.

Conclusion

Collectively, the results of this study have addressed some aspects of the complexity of the immune response to *Leishmania* spp, which is characterised by potent modulation of gene expression profile of ILs and CCLs in susceptible human cell lines infected with virulent or avirulent *L. mexicana* promastigote. Most importantly, the results illustrate differences between infection with virulent and avirulent *L. mexicana* promastigote using the same cell line. Moreover, the results have clearly shown that differences were not only induced by infecting cell lines with avirulent and virulent *L. mexicana*, but also with their antigens (SLA), which may have a potential application as vaccine candidates.

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