Effect of the monocyte locomotion inhibitory factor (MLIF) produced by *E. histolytica* on cytokines and chemokine receptors in T CD4+ lymphocytes

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**ABSTRACT**

*Entamoeba histolytica* produces Monocyte Locomotion Inhibitory Factor (MLIF), which may contribute to the delayed inflammation observed in amoebic hepatic abscesses. Leukocytes are affected through the modulation of cytokine expression and/or production. We evaluated the effects of MLIF on the activation and production of intracellular cytokines in human CD4+ T lymphocytes by flow cytometry. Cells were stimulated for 24 h with PMA, MLIF, or PMA+MLIF. Cellular activation was measured using anti-CD69. Th1/Th2 production was studied by the expression of intracellular cytokines and cytokine/chemokine receptors. MLIF increased CD69 and induced the over-expression of the IL-1β, IFN-γ, IL-2, IL-4, and IL-10 intracellular cytokines; PMA+MLIF inhibited Th1 cytokine (IFN-γ) and increased Th2 cytokines (IL-4 and IL-10). The co-expression of the cytokine and chemokine receptors IFN-γ/CCR5 and IL-1β/CCR5 was inhibited by PMA+MLIF and Th2 co-expression was increased.

MLIF effects varied depending on the conditions. MLIF alone activated the Th1 and Th2 cytokines and cytokine/receptor expression; however, PMA+MLIF increased the expression of Th2 but inhibited it in Th1.

**Key terms:** Amebic hepatic abscess (AHA); CC chemokine receptors (CCR4, CCR5); monocyte locomotion inhibitory factor (MLIF); *Entamoeba histolytica* (*E. histolytica*); 1-phorbol-12 myristate-13 acetate (PMA), interleukin (IL), T helper 1 lymphocyte (Th1); T helper 2 lymphocyte (Th2)

**INTRODUCTION**

Activation of lymphocytes is a complex, yet finely regulated cascade of events that results in the expression of cytokine receptors, the production and secretion of cytokines, and the expression of several cell surface molecules, eventually leading to divergent immune responses. Parasite-specific immune responses are regulated by cytokines and chemokines. They modulate and direct the immune expression but may also contribute to an infection induced by the pathogenesis and parasite persistence (Talvani et al., 2004). Parasitic infections frequently result in highly polarized CD4+ T cell responses, characterized by dominant Th1 or Th2 cytokine production profiles. Although it was previously thought that these infections were strictly dependent on signaling by differentiated cytokines, as IFN-γ, IL-12 and IL-4, recent data indicate that this polarization may be primarily decided by a series of different factors, intrinsic to the pathogen-antigen-presenting-cell interaction that direct T cell priming and all this is influenced by the local environment (Katzman et al., 2008). The infection caused by *E. histolytica* parasite is associated with an acute inflammatory response (Chadee and Meerovitch, 1984). However, it is not
completely clear how *E. histolytica* triggers the host inflammatory response and how host-parasite interaction starts, modulates, and eventually turns off these events.

Axenically cultured Enatamoeba histolytica (*E. histolytica*) produces a pentapeptide (Met-Gln-Cys-Asn-Ser) called Monocyte Locomotion Inhibitory Factor (MLIF) with a molecular weight of 583 Daltons. *In vitro*, it inhibits the locomotion of human monocytes and does not affect the neutrophil polymorfonucleares and the respiratory burst in either human neutrophils or monocytes, nor does it affect their viability (Rico et al., 1992). *In vivo*, mononuclear leukocyte arrival is delayed in Rebuck windows of human skin chambers (Kretschmer et al., 1985), while in guinea pigs and gerbils, MLIF inhibits cutaneous dinitrochlorobenzene (DNCB) delayed hypersensitivity (Giménez-Scherer et al., 1997). In addition, MLIF decreases VLA-4 adhesion molecule expression in monocytes and VCAM-1 adhesion molecule expression in vascular epithelium (Giménez-Scherer et al., 2000). The selective actions of MLIF upon a variety of cell types suggested that it disrupts an organism’s pro- and anti-inflammatory network (Kretschmer et al., 1985, Giménez-Scherer et al., 1987, Kretschmer R et al., 2001). A pentapeptide with the same amino acids but in a different sequence (MLIF scramble, Gln-Cys-Met-Ser-Asn) showed no anti-inflammatory properties (Giménez-Scherer et al., 2004). Such effect could be attributed to the chemical activity of the peptide. Ongoing studies in quantum chemistry have revealed that a pharmacophore group in the MLIF sequence (Cys-Asn-Ser) could be responsible for most anti-inflammatory properties of the molecule (Soriano-Correa et al., 2006).

The interaction between chemokines and chemokine receptors is an important step in controlling leukocyte migration into inflammation sites. Chemokines also mediate a variety of effects independent of chemotaxis, including the induction and enhancement of Th1 and Th2-associated cytokine responses (Campbell et al., 2000). Th1 responses preferentially induce CCR5 and CXCR3 while Th2 responses induce CCR4 and CCR8 (Sebastiani et al., 2001, Loetscher et al., 1998, Andrew et al., 2001). These differential patterns of chemokine receptor expression suggest a mechanism for the selective induction of migration and activation of Th1 and Th2 cells during inflammation and, perhaps, normal immune homeostasis (D Ambrosio et al., 1998, Sallusto et al., 1998). Cellular immunity-inflammation-related cytokines (represented by interleukin [IL]-1β, IL-2, and interferon gamma [IFN]-γ) are characteristic of a Th1 response, while anti-inflammatory cytokines (typically IL-4 and IL-10) are associated with a Th2 response (Mosmann and Fong 1989). During inflammation, leukocytes are orchestrated and regulated by the mononuclear leukocyte Th1/Th2 derived cytokine network. Thus, it was interesting for us to evaluate the effects of MLIF on lymphocyte activation and Th1/Th2 cytokine production. Additionally, it has been suggested that *E. histolytica* invasion occurs within a territory where the Th1 response can be inhibited, this is, in an unbalanced environment where Th1 < Th2. In this work, we evaluated the *in vitro* effect of MLIF on the activation and production of Th1/Th2 intracellular cytokines (IL-1β, IL-2, INF-γ, IL-4, and IL-10) and the relation with the chemokine receptors CCR4 and CCR5 in human CD4+ T cells.

**MATERIALS AND METHODS**

**Cell purification**

Thirty ml of venous heparinized blood were obtained from healthy, nonsmoking adult volunteer donors (n= 30) of both sexes. Blood was diluted 1:2 with phosphate buffered saline (PBS; 0.15 M phosphate buffer), then 10 ml of sample was layered over 4 ml of Ficol-Hypeaque (Sigma Chemical Co., St. Louis, MO) gradient (δ=1.077) and centrifuged at 700g for 30 min at 21°C (Böyum, 1968). The cellular interface peripheral blood mononuclear cells (PBMC) were removed and washed two times with PBS. CD4+ T cells were purified using the CD4+ T cell isolation kit II, as an indirect magnetic labeling system
for the isolation of untouched CD4+ T cells from human PBMCs (Miltenyi Biotec, Germany), and LS column and MidiMACS separator. Briefly, 1 x 10^7 PBMC cells were placed in propylene tubes with 80 μl PBS-albumin-EDTA and 20 μl cocktail of biotin-conjugated antibodies against CD8, CD11b, CD16, CD19, CD36, CD56, CD123, TCRγδ, and CD235a (Glycophorin A) and incubated for 10 min at 4°C. These cells were subsequently magnetically labeled with Anti-Biotin MicroBeads for depletion. The CD4+ lymphocytes obtained were 95% pure.

Monocyte Locomotion Inhibitory Factor (MLIF)

MLIF (Met-Gln-Cys-Asn-Ser) 96% pure was commercially obtained (American Peptide Co., Sunnyvale, CA, USA). All working solutions were tested for endotoxin (LPS<0.3 pg) using Limulus assay (Amoebocyte Lysate Endosafe KTA Charles River Endosafe INC, Charleston, SC, USA) and kept at -70 °C until use.

Culture cells

Five x 10^5 CD4+ T cells were placed in 24-well plates in: 1) RPMI-1640 medium alone (supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml streptomycin, 5 μg/ml gentamicin, and 1mM sodium pyruvate,) (Gibco Laboratories, Grand Island, NY, USA), or 2) in medium supplemented with 1-phorbol-12 myristate-13 acetate (PMA) (50 ng/ml) (Sigma, Chemical Co, St. Louis MO), or 3) in medium with MLIF (50 μg/ml), or 4) PMA+MLIF for 24 h at 37 °C, 5% CO₂ (Freshney et al., 1987). Cell viability was ≥ 90% by Trypan blue dye (Sigma) exclusion. The optimal concentrations of MLIF and PMA were determined by dose-response curves.

Expression of CD-69, chemokine receptors, and intracellular cytokines

The activation of lymphocytes, both in vivo and in vitro, induced the expression of CD69. This molecule was involved in lymphocyte proliferation, expression of different surface molecules and functions as a signal-transmitting receptor in lymphocytes (Cambiaaggi et al., 1992, Reddy et al., 2004). The cells were first stained to detect their surface markers using monoclonal antibodies (mAb) labeled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): anti-CD69, anti-CD3, anti-CD4, anti-human CCR4, and anti-human CCR5, mouse IgG isotype control (PharMingen San Diego, CA, USA), followed by a permeabilization step and staining with the following anti-human mAbs: anti- IL-1β, anti-IL-2, anti-IFN-γ, anti-IL-4, and anti-IL-10 (PharMingen San Diego, CA, USA). The cells were also stained to detect chemokine receptors and cytokine with the following combinations of mAb: anti-IL-1βPE/anti-CCR5FITC, anti-IL2FITC/anti-CCR5PE, anti-IFNγ PE/ anti-CCR5FITC, anti-IL-4PE/anti- CCR4FITC and anti-IL-10FITC/anti- CCR4PE (PharMingen). Briefly, 5 X10^5 CD4+ T cells from each group were incubated in 24-well plates for 24 h; 10 μg/ml brefeldin A were added and incubated for the last 6 hours. After incubation, cells were centrifuged for 5 min at 400g and supernatants were aspirated without disturbing pellets. Cells were washed with PBS/0.5% albumin/2mM EDTA. They were then marked with mAb, and incubated for 20 min at 4°C in the dark, and fixed with 1% p-formaldehyde (Sigma Chemical Co., St. Louis, MO) according to the manufacturer’s instructions (PharMingen). Acquisition of 10,000 events was conducted in flow cytometry FACScalibur (BD Biosciences, Palo Alto, CA). For analysis, Win MDI 2.8 software was used (Miltenyi, et al., 1990).

STATISTICAL ANALYSIS

All data are presented as mean ± standard error of the mean (SEM). n=3 for figure 1 and 2, n= 6 for the other figures. Statistical comparison among groups was performed using the Mann-Whitney U tests. Differences were considered as statistically significant when p<0.05.
RESULTS

Determination of CD69 expression by CD4+ T cells

To determine the activation cell by MLIF, CD4+ lymphocytes were cultured in presence of RPMI, MLIF, PMA, or PMA+MLIF. The results showed that 2% of CD4+ T lymphocytes expressed CD69 constitutively (control). When the cells were stimulated with MLIF, 38% of CD4+ T lymphocytes expressed CD69 with a significant difference (p < 0.002). When stimulated with PMA, 46% expressed CD69, and with PMA+MLIF the expression marker was present in 28% (p<0.05%). PMA+MLIF significantly decreased the expression of CD69 (figure 1).

CCR4, CCR5, and CCR4/CCR5 expression

After testing the ability of MLIF to activate CD4+T cells, we demonstrated the different expressions of CCR4, CCR5, and CCR4/CCR5 chemokine receptors.

In unstimulated cells (RPMI), 16% IL-1\(\beta\) of CD4+ T cells expressed CCR4, 18% expressed CCR5 and 12% expressed CCR4/CCR5. In stimulated with MLIF cells (MLIF) 19% of CD4+ T cells expressed CCR4, 25% expressed CCR5, and 43% expressed CCR4/CCR5. In summary, MLIF up-regulated the chemokine receptors, being statistically significant for CCR5 (p<0.05) and CCR4/CCR5 (p<0.05), but not for CCR4 (figure 2).

Th1/Th2 intracellular cytokine production

We wondered if the effect of MLIF on the expression of intracellular cytokine was related to Th1 or Th2 cytokines pattern. Therefore, the production of IL-1\(\beta\), IL-2, IFN-\(\gamma\), IL-4, and IL-10 by flow cytometry was first analyzed. Constitutive expression (PBS) of intracellular cytokines was 3%, 5%, 0.7%, 4%, and 1%, respectively; MLIF increased the expression of all of them to 24%, 31%, 10%, 28%, and 16%, respectively. Cytokines production after PMA treatment (69%, 52%, 22%, 46%, and

Fig 1: CD69-activation marker expression in T CD4+ cells: 5 \times 10^5 CD4+ T lymphocytes were obtained from human peripheral blood by Ficoll-Hypaque gradient. They were purified with the negative selection technique and incubated for 24 h with RPMI, MLIF (50 \mu g/mL), PMA (50 ng/mL), or PMA+MLIF. Cells were stained with FITC anti-CD-69 mAbs (10 mL X 10^5 cells). Quadrant markers were set based on a fluorescent IgG isotype control. Histograms show mean values ± SEM. The numbers in each quadrant of the dot plots correspond to the percentage of CD69 activation marker expression in T CD4+ cells; bold numbers show the mean of three independent experiments. Asterisk indicates the statistically significant differences between the percentages of CD69-producing cells among the groups when *p < 0.05 (Mann-Whitney Test).
32%, respectively) was significantly different (p<0.05) when compared to the constitutive expression. With the treatment of PMA+MLIF the production of cytokines was 57%, 41%, 4%, 50% and 38%, respectively. MLIF inhibited the production of IFN-γ induced for PMA (p<0.002) (figure 3).

**Cytokine and chemokine receptor co-expression**

The presence and up-regulation of cytokines and chemokines receptors were studied with MLIF (Th1/CCR5 and Th2/CCR4). Briefly, in control CD4+ T cells, 2% co-expressed IL-1β/CCR5, IL-2/CCR5, and IFN-γ/CCR5, while 3% co-expressed IL-4/CCR4, and 1% co-expressed IL-10/CCR4. After stimulating CD4+ T cells with MLIF, 15% cells co-expressed IL-1β/CCR5, 21% IL-2/CCR5, and 16% IFN-γ/CCR5, while 18% co-expressed IL-4/CCR4 and 16% IL-10/CCR4. PMA increased the expression of all of them (24%, 28%, 23%, 32%, and 31%, respectively) and the combination PMA+MLIF showed that MLIF inhibited significant IL-1β/CCR5 (p<0.05) and IFN-γ/CCR5 (p<0.002) induced by PMA (figure 4).

**DISCUSSION**

Different diseases (rheumatoid arthritis, HIV infection, liver infection, parasitism etc.) have been associated with the Th1/Th2 balance but the mechanisms have not been completely clarified (Leng 2008, Power 2000, Katzman 2008, Shields 1999, Kaur 2008). Down-regulation of the immune response is a common parasite strategy.

*E. histolytica* produces the Monocyte Inhibitory Locomotion Factor (MLIF), a pentapeptide with powerful anti-inflammatory, selective properties tested *in vitro* and *in vivo* (Kretschmer et al., 1985). MLIF seems to be exclusive of the *Entamoeba histolytica* and other related amebas (*E. invadens, E. moshkovsi* (Kreschmer et al, 1985)), but is absent in *E. dispar* (Silva et al., 2000), as we corroborated through the gene bank, where we only found the MLIF genetic sequence in the *Entamoeba histolytica*, and to date not in other parasites.

Infection caused by *Entamoeba histolytica* induces a transitory cell-mediated immunity-suppressed state in early inflammatory stages in the amebic hepatic abscess (AHA), and a complex

![Fig 2: Expression profiles of CCR4, CCR5 and CCR4/CCR5 on isolated CD4+ T cells: 5 × 10⁵ CD4+ T lymphocytes were cultured for 24 h with RPMI or MLIF (50 mg/mL). Cells were stained with PE or FITC anti- human CCR4, anti human -CCR5, or anti-human CCR4/CCR5 mAbs. Box plots represent range, 25th and 75th percentiles, vertical lines represent the 10th and 90th percentiles of data. Horizontal bars show significant statistical differences among the different groups. NS = no significant difference. Values (p) were calculated using Mann-Whitney Test. Dot plots show the co-expression of CCR4/CCR5 and bold numbers are the mean of three independent experiments.](image-url)
cytokine signaling system is activated due to invasion of the parasite (Eckmann et al., 1993).

The effect of MLIF upon the production of cytokines was previously evaluated in a short study using the ELISA method (Rojas et al., 2006). Consistent with the present research, the previous study showed that MLIF was able to modify the production of Th1/Th2 cytokines in the supernatants of cultures of T CD4+ lymphocytes stimulated with PMA or MLIF. We found that MLIF+PMA inhibited the IL-1β, IL-5, and IL-6 production without affecting the IL-10 production.

In the present study, we demonstrated that MLIF per se possessed the ability to unspecifically activate T CD4+ cells and induce an increase in pro- and anti-inflammatory cytokine production (IL-1β, IL-2, IFN-γ, IL-4, and IL-10). In contrast, in PMA +MLIF-incubated cells, we found that IFN-γ and IL-1β production was inhibited; whereas it increased the

**Fig 3: Intracellular cytokine production**: $5 \times 10^5$ CD4+ T lymphocytes were cultured for 24 h in the presence of RPMI, MLIF, PMA, or PMA+MLIF. Brefeldin A (cellular transport inhibitor (10 μg/mL) was added during the last 6 h of culture. Cells were permeabilized and stained with anti-human cytokine mAbs (IL-1β, IL-2, IFNγ, IL-4, and IL-10) and mouse anti-IgG as isotype control. FACSscan dot plots are representative of control and of the cells treated; the numbers in each quadrant indicate the mean of the 6 independent experiments. In A, B, C, D, and E the histograms represent control (white), MLIF (diagonals), PMA (dotted) PMAM+ MLIF (black) and represent mean values ± SEM. Asterisk shows comparison among groups, *$p < 0.05$ (Mann-Whitney Test). Bold numbers (dot plots) represent the mean.
production of IL-10 (the prototype of an anti-inflammatory cytokine).

It is probable that MILF generates a signaling cascade, which finally activates transcription factors such as nuclear factor kB (NF-kB) (Kretschmer et al., 2004); after its translocation into the nucleus, it binds to sites regulating a large number of cytokine production-implicated genes. In this way, *E. histolytica* may establish, first an acute transitory reaction involving pro-inflammatory cytokines, followed by an increase and dominant pattern of anti-inflammatory signals mainly through the increase in interleukin-10. IL-10 could cause a decrease of the inflammatory reactions observed in the advanced states of invasive amebiasis (Kretschmer et al., 1985). Additionally, cell activation may modify the expression of chemokines and chemokine receptors, which, in alternate fashion, are essential for leukocyte recruitment during inflammation. Once activated, T lymphocytes acquire different migratory capacities and are, in fact, key for an efficient immune-response regulation (Mackay, 1993, Katakai et al., 2002). CCR5 is a receptor that regulates normal T-cell activation, and it was expressed in the tested Th1 cytokines. Nonetheless, when these were exposed to MLIF (PMA+MLIF) they were inhibited and the decrease was significant, at least for IFN-γ and IL-1β. IFN-γ exerted a strong influence on Th1/Th2 polarization and also affected chemokine receptor expression; MLIF per
Fig 4: Cytokine, and chemokine receptor co-expression: Cells were cultured with RPMI, MLIF, PMA, and PMA+MLIF for 24 h at the previously mentioned concentrations. Brefeldin A was added during the last 6 h of culture. Cells were first stained to detect the surface cell molecules with anti-human CCR5 or anti-CCR4 mAbs, then they were permeabilized and stained with anti-cytokine mAbs (IL-1β/CCR5, IL-2/CCR5, IFNγ/CCR5, IL-4/CCR4, and IL-10/CCR4) and were analyzed on a flow cytometer. A,B,C,D, and E. FACScan dot plots are representative staining of the control and the treated cells, bold numbers represent the mean of the 6 additional experiments. The histograms represent control (white), MLIF (diagonals) PMA (dotted) PMAM+ MLIF (black) and represent mean values ± SEM. Asterisks show the comparison among of groups, *p <0.05, **p<0.002 (Mann-Whitney Test).

se induced an increase in the CCR5 and CCR4 receptors, only significant in the first. CCR5 increase was stronger in CCR4+ than in CCR4- cells (31% vs. 7%). This does not allow considering the increase as a pro-Th1 response. We demonstrated that these molecules, which are key factors in immune regulation, are affected by MLIF. Th2 exhibited high CCR4 expression levels in response to MLIF and when co-expressed, this expression increased to an even higher level, demonstrating that MLIF possessed an additive effect on these markers, at 24 h in these experimental conditions.

The precise mechanisms that MLIF uses to cause these biological effects are unknown; however, it is known that MLIF interacts with human leukocytes by means of a mannose-containing receptor (Kretschmer et al., 1991), and that it causes an increase in the number of pericentriolar microtubules, as well as in cytoplasmic AMPc concentration, without concomitant GMPc diminution (Rico et al., 1995). MILF inhibits the expression induced in inflammatory proteins such as MIP-1α and MIP-1β in U-937 cells, which are NF-kB pathway-regulated proteins, as described in literature (Utreras-Barillas et al., 2003). The p65-p50 heterodimer comprises the most abundant form of NF-kB in a PMA-induced system. Temporary studies showed that MLIF induces p50 translocation; this
can also be explained by the fact that MLIF induces AMPc synthesis and protein kinase A phosphorylation in NF-kB, IkB, followed by NF-kB translocation (Kretschmer R et al., 2004).

A still unanswered question is whether MLIF is a real inhibitor or a competitive antagonist - or both - depending on cells and conditions (e.g. values of MLIF alone are higher than in resting RPMI conditions, but MLIF+PMA values are lower than those for active PMA cells). This is the tendency for pro-Th1 factors. On the contrary, MLIF+PMA rendered the highest values for Th2 representatives as IL-10. Despite details in the molecular and cellular microenvironment, the overall in vivo effect was inhibiting. MLIF effects have proven to be somehow sui-generis in many studies, as for example: MLIF inhibits locomotion and phagocytosis in mononuclear cells, but only phagocytosis in neutrophil PMN, and not in eosinophils; it inhibits cytoskeleton function over-polymerization the microtubule, opposite to what happens with colchicines. In spite the amount of changes in the cells by MLIF, there is an excellent cellular viability, therefore it apparently does not lead to apoptosis or necrosis.

Given the level of activity of the studied cytokines, we observed that MLIF acted the promotion of the cell populations that express IL-2/IL-10 or IFN-γ/IL-10 and CCR4/CCR5. This type of behavior has been
reported before and it is associated with pro-
and anti-inflammatory functions (Katsikis et al., 1995). In previous works, it was 
observed that MLIF inhibited the expression 
induced in the CC, MIP-1α, MIP-1β, and 
3-09 chemokines, the CCR1 receptor (Utrera-
Barrillas et al., 2003), and the IL-1β, IL-5, 
and IL-6 cytokines (Rojas-Dotor et al., 2006). This behavior can be related with 
the atypical inflammation observed in invasive 
amebiasis, in which there is a decrease in 
chemotaxis and/or disequilibrium in 
cytokine production. This is supported by 
observations in vivo in which MLIF notably 
decreased cellular infiltration and 
inflammatory cytokine expression. 

The effect of MLIF observed in this 
study could be explained by Th1 inhibition, 
as was observed with IL-1β/CCR5, IFN-γ/ 
CCR5 and an increase of Th2, as was found 
in IL10/CCR4 expression, resulting in the 
predominantly anti-inflammatory Th1<Th2 
pattern.

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