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Effect of Monocyte Locomotion Inhibitory Factor (MLIF) on the Activation and Production of Intracellular Cytokine and Chemokine Receptors in Human T CD4$^+$ Lymphocytes Measured by Flow Cytometry

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1. Introduction

The supernatant of Axenically cultured Enatamoeba *histolytica* (*E. histolytica)* produces a thermostable factor that was purified and characterized by high resolution chromatography (HPLC) and mass spectrometry (MS-MS), supplemented by the methods of Edman (Edman & Begg, 1967). This revealed a pentapeptide with a molecular weight of 583 Daltons and established the aminoacid sequence (Met - Gln - Cys - Asn - Ser), which was termed Monocyte Locomotion Inhibitory Factor (MLIF). MLIF has powerful and selective anti-inflammatory properties, which were established *in vitro* by Boyden chamber studies. MLIF inhibits locomotion, both random chemokinetic and chemotactic, of mononuclear phagocytes (PM) from normal human peripheral blood but not of neutrophils toward various attractants, such as C5a-Desargues lymphokine and Lymphocyte-derived chemotatic factor (LDCF) (Kretschmer et al., 1985). This factor also depresses the respiratory burst of monocytes and neutrophils activated with zymosan *in vitro*, as measured by chemiluminescence (Rico et al., 1992), and nitric oxide production in mononuclear phagocytes and human polymorphonuclear neutrophils (PMNs) (Rico et al., 2003). Such effects were not accompanied by changes in expression of CD43, a ligand critical in the initial activity of phagocytes, in the membrane of these cells, and did not affect the viability of phagocytes (Kretschmer et al., 1985). In contrast, MLIF does not affect either locomotion or the respiratory burst of zymosan-activated human PMNs (Rico et al., 1998). *In vivo*, MLIF delays the arrival of mononuclear leukocytes in Rebuck chambers applied to the skin of healthy human volunteers (Kretschmer et al., 1985), inhibits cutaneous delayed contact hypersensitivity to 1-chloro-2-4-dinitrobenzene (DNCB) in guinea pigs (Giménez-Scherer et al., 1997) and decreases expression of the adhesion molecules VLA-4 on monocytes and VCAM-1 in the vascular epithelium (Giménez-Scherer et al., 2000). MILF inhibits the expression induced in inflammatory proteins such as MIP-1α and MIP-1β in U-937 cells, which are NF-κB pathway-regulated proteins (Utrera-Barillas et al., 2003). The p65–p50 heterodimer comprises the most abundant form of NF-κB in a PMA-induced system. Temporary studies showed that MLIF induces p50 translocation, which may be explained by the ability of MLIF to induce AMPc synthesis and protein kinase A phosphorylation in NF-κB and IκB followed by NF-κB translocation (Kretschmer et al., 2004). This may also explain the atypical inflammation observed in invasive amoebiasis, in which there is decreased chemotaxis and disequilibrium in cytokine production. This is supported by *in vivo* observations that MLIF notably decreased cellular infiltration and inflammatory cytokine expression.

The selective actions of MLIF upon a variety of cell types suggest that it disrupts an organism's pro- and anti-*inflammatory* network (Giménez-Scherer et al., 1987; Kretschmer et al., 1985, 2001; Rojas-Dotor et al., 2006). A pentapeptide with the same amino acids but in a different sequence, termed a MLIF scramble (Gln-Cys-Met-Ser-Asn), showed no anti-inflammatory properties (Giménez-Scherer et al., 2004). The observed effects of MLIF 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 of the anti-inflammatory properties of the molecule (Soriano-Correa et al., 2006) Figure 1.

It is possible that MLIF is derived from a larger peptide or protein synthesized by the amoeba, which is then degraded by proteases present in the cytoplasm. The lysate of amoebae material, washed and processed according to the method of Aley (Aley et al., 1980), maintains the inhibitory activity, suggesting that the MLIF is produced by the amoeba through de novo synthesis and not due to a complex-degradation process of ingestion and regurgitation of a product present in axenic medium (Rico et al., 1997).

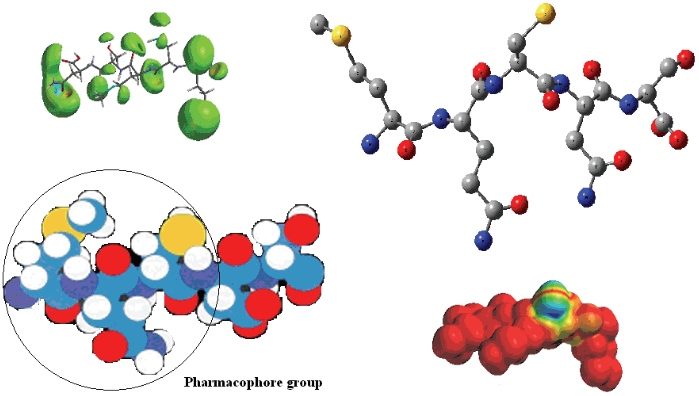

Fig. 1. Molecular Structure of Monocyte Locomotion Inhibitory Factor (Met-Gln-Cys-Asn-Ser). The pharmacophore site, Cys-Asp-Ser, is highlighted (Soriano-Correa et al., 2006)

MLIF seems to be exclusively produced by E *histolytica* and other related amebas, *E. invadens* and *E. moshkovski*, but it is absent in *E. dispar*, as we corroborated through the gene bank in which we only found the MLIF genetic sequence in the E *histolytica*, and not in any other parasites. Infections caused by E *histolytica* induce a transitory cell-mediated immunity-suppressed state in early inflammatory stages in the amebic hepatic abscess (AHA), and a complex cytokine signaling system is activated due to invasion of the parasite (Chadee & Meerovitch, 1984).

2. Inflammation

Inflammation is the body's reaction against invasion by an infectious agent, an antigenic stimulus or even just physical injury. This response induces the infiltration of leukocytes and plasma molecules into regions of infection or injury. Its main effects include increased blood flow to the region, increased vascular permeability allowing the passage of large serum molecules such as immunoglobulin and leukocyte migration through the vascular endothelium toward the inflamed area. Inflammation is controlled by cytokines, factors produced by mast cells, platelets and leukocytes, chemokines and plasma enzyme systems such complement, coagulation and fibrinolysis. Cytokines stimulate the expression of adhesion molecules by endothelial cells, and these adhesion molecules bind to leukocytes and initiate their attraction to areas of infection. Microbial products, such as peptides with N-formilmetionil, chemokines, and peptides derived from complement such as C5a, and leukotrienes (B4), act on leukocytes to stimulate their migration and their microbicidal abilities. The composition of cells involved in inflammatory processes changes with time and goes from neutrophil rich to mononuclear cell rich, reflecting a change in the leukocytes attracted (Roitt, 1998; Abbas & Lichtman, 2004). Macrophages attracted to the site of infection are activated by microbial products and interferon-gamma (IFN-γ) which cause them to phagocytose and kill microorganisms (Figure 2).

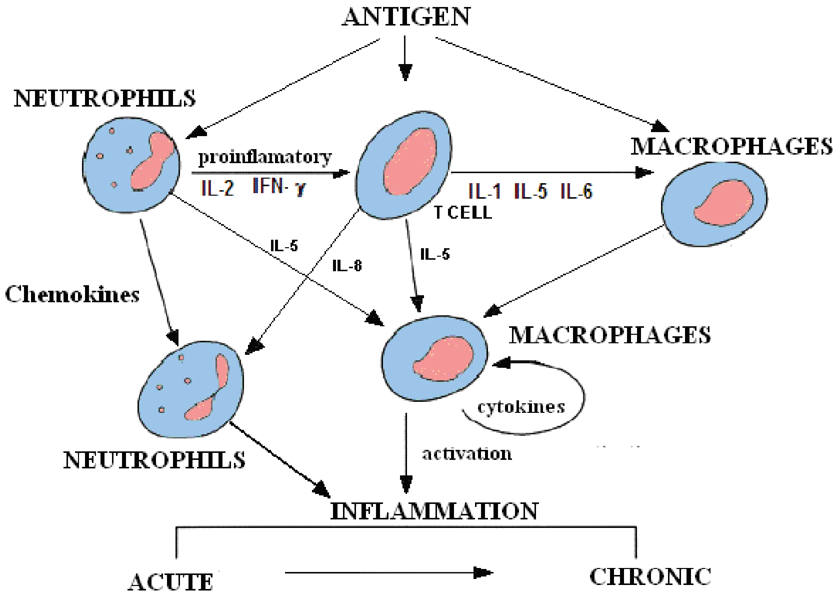

Fig. 2. Cytokines play an important role in the development of acute or chronic inflammatory responses. Interleukin 1 (IL-1), IL-6, tumor necrosis factor alpha (TNF-α) and IL-12, in addition to cytokines and chemokines, have redundant and pleiotropic effects, which together contribute to the inflammatory response. If the antigen is eliminated, inflammatory cells become apoptotic or return to the circulation. If the antigen persists for several days, it will induce chronic inflammation, recruit mast cells, eosinophils, lymphocytes and macrophages, and induce the production of antibodies and cytokines. These cells are often found in damaged tissue. (Luscinskas & Gimbrone, 1996)

Chemokines are small polypeptides that activate and direct the migration of monocytes, neutrophils, eosinophils and activated T lymphocytes from the bloodstream to sites of infection. They also regulate pro-inflammatory signals by binding to specific receptors belonging to the superfamily of seven trans-membrane domain alpha protein-coupled G (such as trimeric guanosine triphosphate (GTP)), and these can also be used as markers to differentiate chemokines and their receptors can also be used as markers of differentiation of helper T cell populations, pro-inflammatory (Th1) or anti-inflammatory (Th2) (Mosmann & Fong, 1989). Th1 cells express on their cell surface CCR5 chemokine receptor but not CCR3, whereas Th2 cells express the chemokine receptor CCR3 but not CCR5 (Sallusto et al., 1998). It has been shown that several inflammatory chemokine receptors, such as CCR1, CCR2, CCR3, CCR5 and CXCR3, are expressed shortly after signaling through the T cell receptor (TCR) in Th1 and Th2 cells. In contrast to CCR7, CCR4 and CCR8, which are over-expressed after activation through the TCR, these changes in chemokine receptor expression can be used to modify the migratory behavior of activated Th cells, and to establish the hierarchy of action between the different chemokine receptors (Loetscher et al., 1998; Zingoni et al., 1998).

2.1 Cytokines, soluble mediators

Cytokines are small peptide proteins with hormone-like activity that play a central role in communication between cells of the immune system. They are soluble mediators and regulators of innate and specific immunity. Additionally, cytokines promote growth and differentiation of leukocytes and blood cell precursors. Cytokines are key mediators of inflammation in many diseases, such as rheumatoid arthritis, lupus erythematosus, asthma and allergies (Ruschpler & Stiehl, 2002; Ivashkiv, 2003, D'Ambrosio et al., 2002, 2003). The host defenses against infectious pathogens are highly cytokine-dependent mechanisms mediated by humoral or cellular immunity. Each mechanism preferentially acts against intra or extracellular pathogens, viruses or worms. These host defense responses are strictly regulated by cytokines secreted by T helper populations, Th1 and Th2 (Kawakami, 2002). Cytokines have autocrine activity, increasing the proliferation, differentiation and effector functions of their own cell subset, and may additionally have far ranging effects on other cell types. T helper lymphocytes, the main orchestrators of the immune response, are subdivided into T_{helper} 1 (Th1) and T_{helper} 2 (Th2) subsets by the range of cytokines they secrete. Th1 cells mainly secrete the cytokines that promote cellular immunity and the inflammatory process, such as Interleukin-2 (IL-2) and Interferon-gamma (IFN-γ). (Mosmann, 1997). In contrast, Th2 cells secrete IL-4, IL-5 and IL-10, which direct the immune response toward a more humoral (antibody-mediated) response and impair differentiation toward the Th1 phenotype (Figure 3).

In the case of several infectious diseases, like-Leishmaniasis and HIV, the development of Th1-dependent immunity protects against the infectious agent. The development of Th2 dependent immunity, in contrast, was determined to protect the parasite or virus. Down-regulation of the immune response is a frequent parasitic strategy. Monitoring the immune response polarization toward a Th1- or Th2-type response is important for the development of effective vaccines. Because of the interplay between cytokines and the cells that respond to them, looking at changes in levels of soluble cytokines, changes in cell surface cytokine receptor expression and expression of intracellular cytokines by individual cell subpopulations is crucial to the understanding of cytokine biology. (Clark at al., 2011; Campanelli et al., 2010).

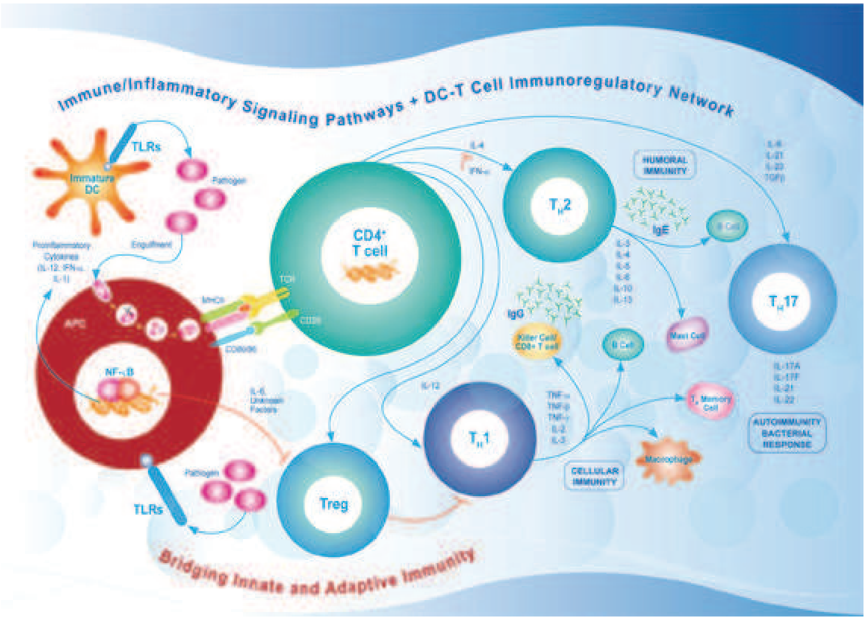

Fig. 3. Antigen-presenting cells (APCs) communicate with two types of helper T cells, Th1 and Th2. They first produce cytokines, such as IFN-γ, TNF-α and IL-2, which are responsible for inflammation, and Th2 cells produce cytokines involved in the production of antibodies. The balance of activation of between Th1 and Th2, maintained by IFN-γ and IL-10, determines the nature of an immune response. Th17 cells are another recently identified subset of CD4⁺ T helper cells. They are found at the interfaces between the external environment and the internal environment, such as the in the skin and the lining of the gastrointestinal tract. Regulatory T cells respond to the presence of IL-2 by rapid proliferation. Because IL-2 is secreted by effector T cells, this provides a negative-feedback mechanism, in which inflammatory T-cell activity (e.g., by Th1 cells) is restrained by the resulting expansion of regulatory T cells (Image taken from www.imgenex.com)

Lymphocyte activation, as measured early on by mitogenic assay, was used as an indicator of immune function. Mitogenic assays measure the proliferative response of isolated mononuclear cells to *in vitro* stimulation with mitogenic lectins (Phytohaemagglutinin, Concanalin A, and Pokeweed Mitogen) or certain specific antigens (Streptokinase, PPD). The proliferative index of activation is a proportion determined by the relative uptake of radiolabel nucleotides (^{3}H-thimidine) by the mitogen-stimulated culture compared to a basal nonstimulated culture. Actively proliferating cells incorporate more radionucleotides than weakly proliferating cells. Non-proliferating cells should have little or no incorporation of radionucleotides. These assays are often 48-72 hours in length and require licensing, storage and disposal of radioactive waste. A similar flow cytometry-based assay utilizes the uptake of the non-radioactve nucleotide bromo-deoxyuridine (BrdU) and detection with a fluorescent anti-BrdU antibody. These assays are somewhat non-specific and provide little information regarding cytokine production or cell communication. These tests have recently been supplanted with flow cytometry-basad assays for measuring changes in cells surface markers and assays for measuring the expression of intracellular cytokine. Flow cytometers are laser-based cell counters that are capable of distinguishing 3, 4, 5 or more (depending of flow cytometer), different fluorescence emissions, each associated with a particle identified by its light scatter proprieties. Fluorescence dyes with distinct fluorescence emissions are attached to monoclonal antibody that recognizes distinct cell surface antigens.

Traditionally, cytokines have been measured by radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). Unfortunately, these techniques are limited by their detection range and an inability to simultaneously measure multiple analytes (García, 1999).

Using extremely sensitive multiparameter flow cytometers, Multiplexed Cytokine Immunoassay Kits overcome both of these limitations. Multiplexing is the simultaneous assay of many analytes in a single sample. Applications for flow cytometry are diverse, ranging from simple cell counting and viability to more complex studies of immune function, apoptosis and cancer, stem cells, separation of cells populations such as monocytes and T and B lymphocytes, measuring changes in cell surface markers, cell cycle analysis, cellular activation, and measuring the expression of intracellular cytokines (Collins et al., 1998; McHugh, 1994; Spagnoli,et al., 1993; Trask et al., 1982).

3. Cell activation

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 response, 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 Th1 or Th2 cytokine dominated production profiles. Although it was previously thought that these infections were strictly dependent on signaling by cytokines, such as IFN-γ, IL-12 and IL-4, recent data indicate that this polarization may be primarily directed by a series of different factors intrinsic to the pathogen–antigen-presenting-cell interaction that directs T cell priming, and that all of this is influenced by the local environment (Katzman et al., 2008). The infection caused by the *E. histolytica* parasite is associated with an acute inflammatory response (Chadee & Meerovitch, 1984). However, it is not completely clear how *E. histolytica* triggers the host inflammatory response or how host-parasite interactions start, modulate, and eventually turn off the inflammatory response.

During inflammation, leukocytes are orchestrated and regulated by the mononuclear leukocyte Thl/Th2 derived cytokine network. Thus, it was interesting for us to evaluate the effects of MLIF on lymphocyte activation and Thl/Th2 cytokine production. Additionally, it has been suggested that *E. histolytica* invasion occurs within a territory where the Thl response can be inhibited, this is, in an unbalanced environment where Thl < Th2. In this experiment, we evaluated the *in vitro* effect of MLIF on the activation and production of Thl/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. Peripheral blood samples were obtained from healthy, nonsmoking adult volunteer donors of both sexes. The peripheral blood mononuclear cells were obtained by Ficoll-Hypaque (Sigma Chemical Co., Louis, MO) and CD4+ T lymphocytes were obtained by negative selection technique (MACS® Reagents, Kit isolation, Human cell T CD4+). The purity of lymphocytes was analyzed by flow cytometry. The flow cytometry measures and analyzes the optical properties of individual cells pass through a laser beam. Depending on how cells interact with the laser beam, the cytometer measures five parameters for each cell: size (forward scatter, FSC), complexity (side scatter, SSC) and three fluorescence emissions (FL-1, FL-2 and FL -3). An electro-optical system converts the voltage signals, which is translated into a digital value which is stored in a computer, the data are then retrieved and analyzed with the software that combines information from different cells in statistical charts, which measure individual parameters (histograms) or two parameters at a time (dot plot, density or contour). For our study, purified lymphocytes were analyzed in a dot plot of SSC vs. FSC marking the region corresponding to lymphocytes, excluding debris and dead cells. In a dot plot is compensated for fluorescence with anti CD3-FITC and anti-CD4-PE (cluster of differentiation (CD) and marker for T lymphocytes CD4+).

Test samples of at least 10.000 events were acquired under these conditions. With this procedure, we obtained a population of CD4+ lymphocytes with 96% purity. (Figure 4)

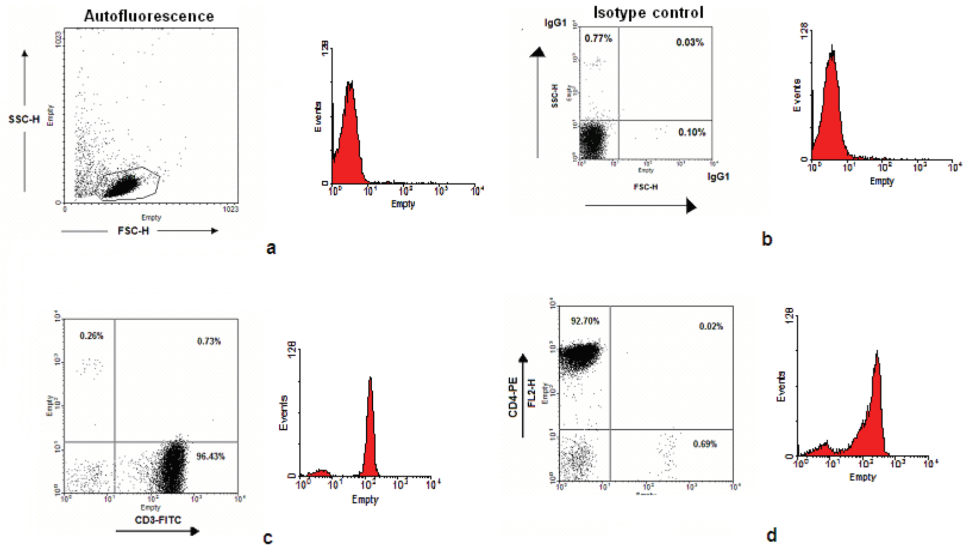

Fig. 4. Simple analysis of CD4+ T cells obtained from healthy individuals by flow cytometry. The X-axis shows staining with fluorescein isothiocyanate (FITC), and the Y-axis shows staining for phycoerythrin (PE). a) Autofluorescence; b) Isotype control, staining with mouse IgG1-FITC; c) Staining for subpopulations of CD3 coupled to FITC; d) Staining for subpopulations of CD4 coupled to PE. All stains show simple representation of the histogram and are an example of 6 experiments ± SE

The presence or absence of chemokine receptors on cell surfaces also provides information regarding the cell's state of activation. Chemokine receptors can by analyzed by flow cytometry using fluorescently labeled anti-receptor antibodies or fluorescently-labeled chemokines. Combining these reagents with antibodies against the activation marker CD69 enables analysis of cell activation within specific cell population. Figure 5 shows that the best activation was obtained with 50 ng of phorbol 12-myristate 13-acetate (PMA) and 50 µg of MLIF.

CD69 is a cell surface activation marker expressed on T cells, B cells, and activated NK cells. MLIF is able to induce expression of this marker, suggesting that it activates CD4$^+$ T lymphocytes. T-lymphocyte activation is also associated with an up-regulation of cell surface chemokine receptors. (Figure 5)

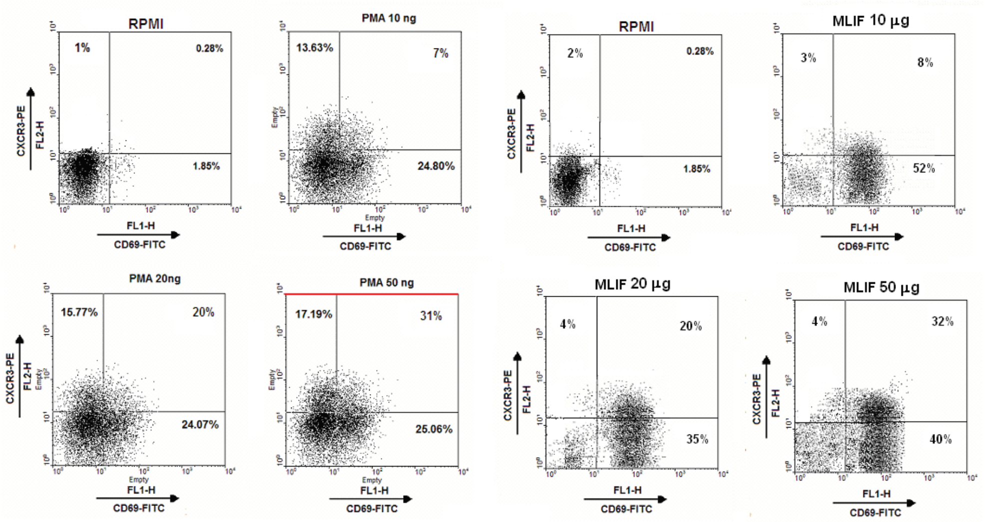

Fig. 5. Expression of the chemokine receptor CXCR3 and the activation marker CD69

Cell surface expression of the chemokine receptor CXCR3 and the activation marker CD69 on CD4$^+$ T cells after 24 hours of treatment with RPMI medium alone or activation with PMA and MLIF at different concentrations. The cell population positive for both CXCR3 and CD69 were identified using a FITC-labeled anti-CD69.

4. Cell surface molecules

Cellular activation may modify the expression of chemokines and chemokine receptors, which are essential for leukocyte recruitment during inflammation. Once activated, T lymphocytes acquire different migratory capacities and are necessary for efficient immune response regulation (Mackay, 1993; Katakai et al., 2002). CCR5 is a receptor that regulates normal activation, and it was expressed along with the tested Th1 cytokines. However, MLIF exposure inhibited these cells and induced significant decreases in production of IFN-γ and IL-1β. IFN-γ exerted a strong influence on Th1/Th2 polarization, and also affected chemokine receptor expression. MLIF induced an increase in CCR5 and CCR4 expression; however, this increase was only significant for the first. The observed CCR5 increase was greater in CCR4+ cells than in CCR4- cells (31% vs. 7%). The increases in CCR5 expression cannot be considered as a pro-Th1 response. The chemokine receptors, which are key factors in immune regulation, are influenced by MLIF. Th2 cells exhibited high CCR4 expression levels in response to MLIF and, when co-expressed, the increase was even greater, demonstrating that MLIF possessed an additive effect on these markers (Figure 6) (Rojas-Dotor et al., 2009).

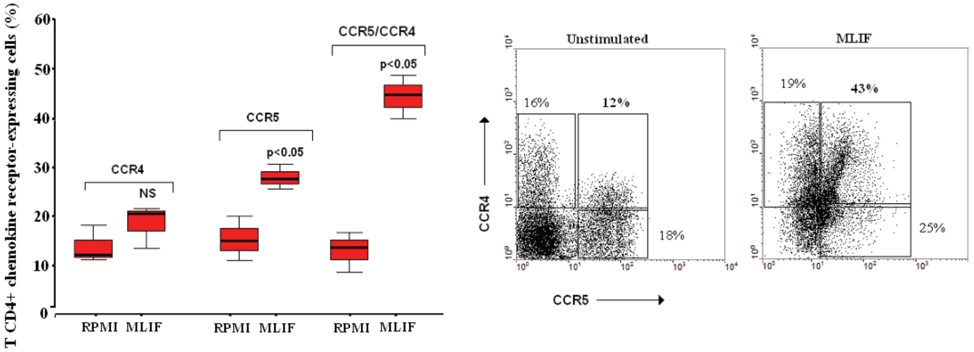

Fig. 6. Expression profiles of CCR4, CCR5, and CCR4/CCR5 on isolated CD4^{+} T cells. 5 × 10^5 CD4^{+} T lymphocytes were cultured for 24 h with RPMI or MLIF (50 µg/mL). Cells were stained with PE or FITC anti- human CCR4, anti-human CCR5, or anti-human CCR4/CCR5 mAbs. Box plots represent range, 25th and 75th percentiles, and 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 a Mann-Whitney Test. Dot plots show the co-expression of CCR4/CCR5, and bold numbers are the mean of three independent experiments

5. Intracellular cytokines

The effect of MLIF upon the production of intracellular cytokines was evaluated using a quantitative method of flow cytometry. This was used to assess the production of IL-lβ, IL-2, IFN-γ, IL-4, and IL-10. CD4^{+} T cells were cultured in 24-well plates in RPMI-1640 medium (supplemented with fetal calf serum (FCS), L-glutamine, streptomycin, gentamicin, and sodium pyruvate) with PMA alone or in conjunction with MLIF for 24 h at 37 ºC with 5% CO_2. Cell viability was ≥ 90% determined by trypan blue dye (Sigma) exclusion. Once CD4^{+} T lymphocytes were activated, we determined if the effect of MLIF on cytokine production was related to a Th1 or Th2 cytokine pattern. To stain for intracellular cytokine expression, lymphocytes are labeled with anti-CD antibodies to identify cells by their subset, such as helper lymphocytes, B lymphocytes and cytotoxic lymphocytes. The cells are then stabilized by fixation with formaldehyde. Holes are punched in the cell membrane by detergent to enable the passage of anti-cytokine antibodies to the interior of the cells. By three-color flow cytometry analysis, activated T- lymphocytes can be subdivided into several different populations according to their staining characteristics. CD4 and CD3 positive and negative cells populations are identified using a FITC or PE-labeled anti-CD4 or CD3 antibody, which labels the cell surface. Following the permeabilization step, intracellular cytokines are stained with anti-human mAbs directed against IL-1β, IL-2, IFN-γ, IL-4, and IL-10, and Th1 and Th2-associated cytokine-producing lymphocytes can be counted on a flow cytometer. This procedure helps to differentiate between Th1 (IFN-γ producing) and Th2 (IL-4 producing) cells in specific cell populations. MLIF increased the expression of IL-lβ, IL-2, IFN-γ, IL-4, and IL-10. Following PMA+MLIF treatment, the production of IFN-γ and IL-1β was inhibited compared to treatment with PMA alone. MLIF possessed the ability to nonspecifically activate CD4+ T cells, and it induced an increase in pro- and anti-inflammatory cytokine production (IL-1β, IL-2, IFN-γ, IL-4, and IL-10) (Rojas-Dotor et al., 2006). In contrast, in PMA +MLIF-incubated cells, we found that IFN-γ and IL-1β production was inhibited and production of IL-10, the prototypical anti-inflammatory cytokine, was increased (Figure 7) (Rojas-Dotor et al., 2009). It is probable that MILF induces a signaling cascade, which results in the activation of transcription factors, such as nuclear factor kB (NF-kB) (Kretschmer et al., 2004). After its translocation into the nucleus, NF-kB binds to genomic sites that regulate a large number of genes implicated in cytokine production. In this way, *E. histolytica* could potentially first establish an acute transitory reaction involving pro-inflammatory cytokines, followed by an increase and dominant pattern of anti-inflammatory signals mainly through increased IL-10. IL-10 could cause the decreased inflammatory reaction observed in the advanced states of invasive amoebiasis (Kretschmer et al., 1985).

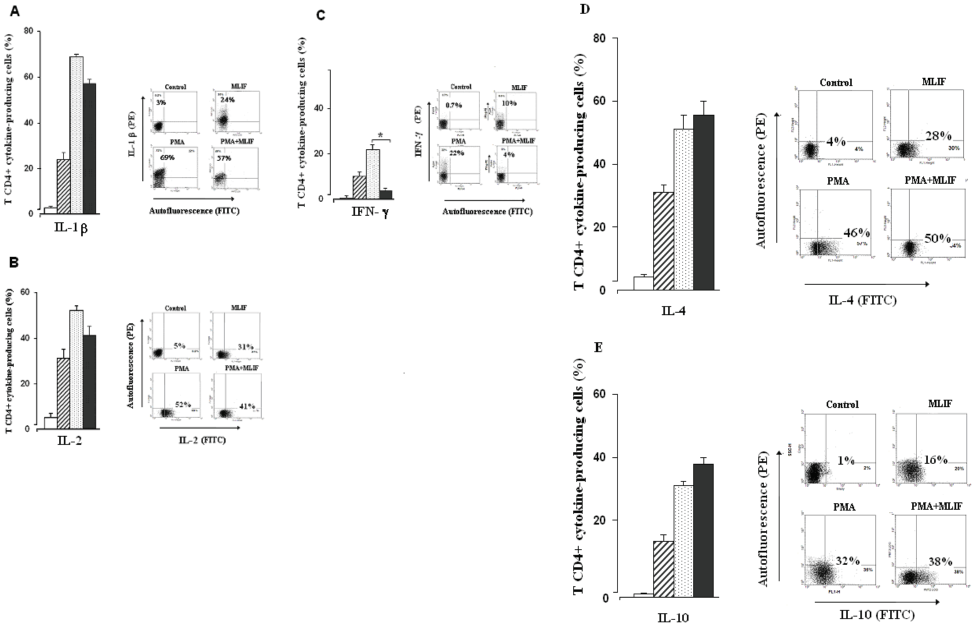

Fig. 7. Intracellular cytokine production

5 × 10^5 CD4+ T lymphocytes were cultured for 24 h in the presence of RPMI, MLIF, PMA, or PMA+MLIF. Brefeldin A, a cellular transport inhibitor 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) or mouse anti-IgG as an isotype control. FACScan dot plots are representative of control and treated cells. 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), and PMAM+ MLIF (black) treated cells and untreated cells represent mean values ± SEM. Asterisk shows comparison among groups, $*p < 0.05$ (Mann-Whitney Test). Bold numbers (dot plots) represent the mean.

6. Cytokines and chemokine receptors

The presence and regulation of cytokines and chemokines receptors were studied with MLIF. 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). 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 then they were marked with mAb, and incubated for 20 min at 4°C in the dark, and fixed with 1% p-formaldehyde according to the manufacturer's instructions (PharMingen). Acquisition of 10,000 events was conducted in flow cytometry FACScan (BD Biosciences, Sa Jose, USA). For analysis, Facs Diva and Win MDI 2.8 software were used. The results showed that CD4$^+$ T cells control 2% co-expressed IL-lβ/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 8).

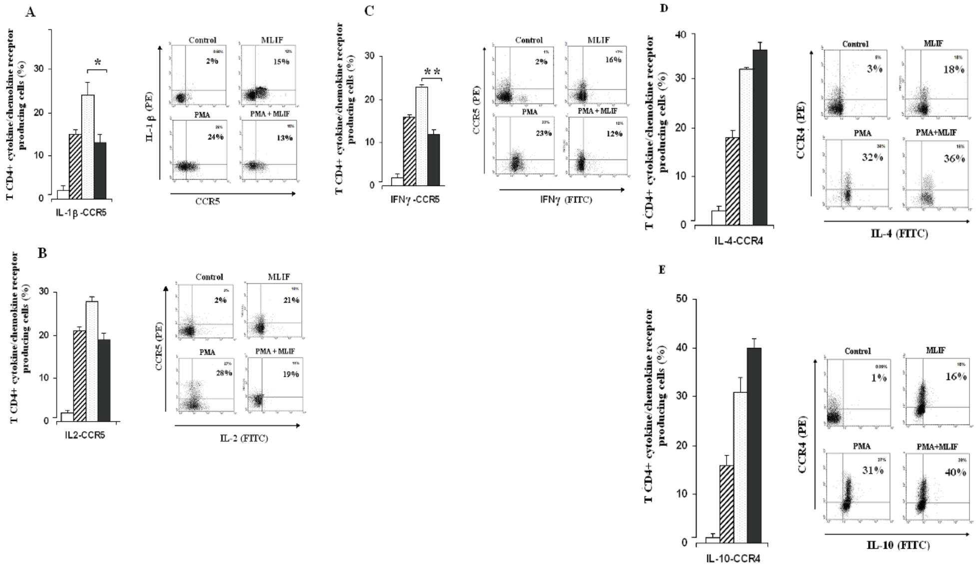

Fig. 8. Cytokine and chemokine receptor co-expression

Cells were cultured with RPMI, MLIF, PMA, or PMA+MLIF for 24 h at the previously mentioned concentrations. Brefeldin A was added during the last 6 h of culture. The cells were first stained to detect the surface cell molecules with anti- human CCR5 or CCR4 mAbs. They were then permeabilized and stained with mAbs directed against IL-1β and CCR5, IL-2 and CCR5, IFNγ and CCR5, IL-4 and CCR4, or IL-10 and 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), and PMAM+ MLIF (black) treated cells and untreated cells represent mean values ± SEM. Asterisks indicate significant differences between the groups, $^*p < 0.05$, $^{**}p < 0.002$ (Mann-Whitney Test).

The precise mechanisms through which MLIF causes these biological effects are unknown, but 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 and cytoplasmic AMPc concentration without concomitant GMPc diminution (Rico et al., 1995). Recent studies show that the MLIF does not interfere with programmed cell death or necrosis (Rojas-Dotor et al., 2011).

Given the level of activity of the studied cytokines, we observed that MLIF acted to promote cell populations that express IL-2/IL-10 or IFN-γ/IL-10 and CCR4/CCR5 chemokine receptor. These effects have been previously reported and are associated with pro- and anti-inflammatory functions (Katsikis et al., 1995). In previous work, MLIF was found to inhibit the induction of CC, MIP-1α, MIP-1β, and I-309 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 may be associated with the atypical inflammation observed in invasive amoebiasis in which there is a decrease in chemotaxis and disequilibrium in cytokine production. This conclusion is supported by observations *in vivo* in which MLIF notably decreased cellular infiltration and inflammatory cytokine expression.

7. Conclusion

Entamoeba *histolytica* produces Monocyte Locomotion Inhibitory Factor (MLIF), a pentapeptide with proven anti-inflammatory properties both *in vitro* and *in vivo*. MLIF may contribute to the exiguous inflammation observed in late amebic liver abscess, through effects exerted directly on monocytes, such as decreased locomotion and respiratory burst, or indirectly by modulating the production and expression of cytokines involved in mononuclear cell recruitment to the inflammatory focus. We evaluated the effect of MLIF on the expression of pro and anti-inflammatory $CD4^+$ T cells after 24 h of incubation with RPMI, MLIF, PMA or PMA + MLIF. MLIF treatment increased expression of CD69 by these cells, from which we can infer that MLIF acts as an inducer or activator of $CD4^+$ cells under these experimental conditions. The expression of the cytokines IL-1β, IFN-γ, IL-2, IL-4 and IL-10 and co-localization with the chemokine receptors IL-1 β/CCR5, IFN-γ/CCR5, IL-2/CCR5, IL-4/CCR4 and IL-10/CCR4 are induced by MLIF. While PMA-induced production of IL-1 β and IFN- γ was inhibited by MLIF, IL-2 production was not affected, in contrast to the expression of IL-10, which was increased by MLIF. The inhibitory effect of MLIF could be explained by two different and independent mechanisms: inhibition of pro-inflammatory cytokines such as IL-1 β and IFN-γ or increased expression of IL-10, with the concomitant increase in the suppressive effects attributed to IL-10.

MLIF acts at the beginning of the inflammatory process as a nonspecific activator, inducing the production of both pro and anti-inflammatory cytokines. As inflammation progresses, Th2 cytokine production prevails, which may inhibit Th1 cytokines. The observed effect of MLIF in this study could be explained by Th1 inhibition, as decreases in IFN-γ, IL-1β, cytokine and IL-1β/CCR5, IFN-γ/CCR5 cytokine and chemokine receptor co-expression were observed along with increases in the Th2 factors IL-4/CCR4 and IL-10/CCR4, resulting in a predominantly anti-inflammatory Th1<Th2 pattern.

The effects of MLIF on the expression of cell surface molecules and intracellular cytokine expression was made possible by the availability of a range of monoclonal anti- antibodies coupled to fluorochromes, such as FITC or PE, and analysis by flow cytometry.

8. Acknowledgements

The research was supported by the Consejo Nacional de Ciencia y Tecnología (CONACYT), México (No. 38104-M). We also wish to acknowledge American Journal Experts (AJE) for the critical review of the manuscript in English (EE.UU).

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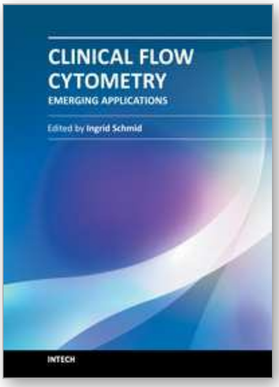

Clinical Flow Cytometry - Emerging Applications
Edited by M.Sc. Ingrid Schmid

ISBN 978-953-51-0575-6
Hard cover, 204 pages
Publisher InTech

Published in print edition May, 2012

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Sara Rojas-Dotor (2012). Effect of Monocyte Locomotion Inhibitory Factor (MLIF) on the Activation and Production of Intracellular Cytokine and Chemokine Receptors in Human T CD4+ Lymphocytes Measured by Flow Cytometry, Clinical Flow Cytometry - Emerging Applications, M.Sc. Ingrid Schmid (Ed.), ISBN: 978-953-51-0575-6, InTech, Available from: http://www.intechopen.com/books/clinical-flow-cytometry-emerging-applications/effect-of-monocyte-inhibitory-locomotion-factor-mlif-on-the-activation-and-production-of-intracellul

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