Limulus-derived $LALF_{32-51}$ peptide regulates cytokine expression and toll-like receptor in macrophages-derived THP-1 cells exposed to lipopolysaccharide

Ingrid Rodríguez-Alonso[1], Hainer Martínez[2], Manuel Colás[1], Hilda Elisa Garay [3], Osvaldo Reyes[3], ✉ Maribel Guerra[1]

[1]Division of Pharmaceuticals, Center for Genetic Engineering and Biotechnology
Ave. 31 e/ 158 y 190, Cubanacan, Playa, PO Box 6 162, La Habana, Cuba
[2] Quality Control Division
[3]Division of Phyisics & Chemistry
E- mail: maribel.guerra@cigb.edu.cu

RESEARCH

ABSTRACT

The immune system is characterized by the ability to respond to infectious agents without producing a destructive response against itself. We previously showed that a Limulus anti-LPS Factor (LALF)-derived peptide has novel biological properties comprising anti-inflammatory and immunomodulatory capacities. The effectiveness shown by this peptide, in different models of bacterial infection, can be due to a selective up-regulation of elements of innate immunity, which avoid or limit damages caused by inflammation. Here we demonstrate the ability of the $LALF_{32-51}$ peptide to induce the differentiation of the pro-monocytic cell line THP-1 and modulate the innate immune signaling receptors (TLR-2, TLR-4). We also studied peptide-induced gene expression of pro and anti-inflammatory cytokines with a key role in the inflammatory response. Our findings demonstrate that the $LALF_{32-51}$ peptide promotes THP-1 cell differentiation to macrophage linage with a particular phenotype resulting in the abrogation of LPS-induced TNF-α gene expression and the up-regulation of the gene for the TGF-β cytokine. On the other hand, we report for the first time, the up-regulation of the genes of the Toll-like receptors TLR4 and TLR2 and the chemokine IL-8 in THP-1 derived-macrophages by the $LALF_{32-51}$ peptide. Furthermore, a band shift analysis revealed the down-regulation of the transcriptional factor NF-κB in peptide-treated cells challenged with LPS. Our results suggest that the $LALF_{32-51}$ peptide induces an anti-inflammatory phenotype in THP-1 derived-macrophages without impairing their susceptibility to pathogens by up-regulating the expression of several TLRs.

Key Words: *Limulus* anti-LPS factor-derived peptide, THP-1 cells, immunomodulation, cytokines, cellular differentiation, Toll-like receptor

Biotecnología Aplicada 2006;23:99-107

RESUMEN

Modulación de citocinas y TLRs en células THP-1 por el péptido $LALF_{32-51}$. El sistema inmunológico se caracteriza por la habilidad de responder a los agentes infecciosos sin producir daños al organismo. Nosotros informamos previamente que un péptido anti-LPS derivado de Limulus (LALF) ha mostrado propiedades anti-inflamatorias e inmunomoduladoras. La efectividad mostrada por este péptido, en diferentes modelos de infección bacteriana, puede ser debida a una regulación selectiva de elementos de la inmunidad innata los cuales evitan o limitan los daños causados por el proceso inflamatorio. En este trabajo mostramos la habilidad del péptido $LALF_{32-51}$ de inducir la diferenciación de la línea celular pro-monocítica THP-1 y de modular los receptores de señalización de la inmunidad innata TLR-2 y TLR-4. Nosotros también estudiamos la inducción por el péptido de genes de citocinas pro y anti-inflamatorias esenciales en la respuesta inmune. Nuestros resultados demuestran que el péptido $LALF_{32-51}$ promueve la diferenciación de la línea celular THP-1 a un linaje de macrófago con un fenotipo particular que produce la supresión de la expresión del gen de TNF-α inducido por LPS y la sobre-expresión del gen para la citocina TGF-β. En este trabajo se informa por primera vez la regulación de los genes de los receptores TLR4 y TLR2 y de la quimiocina IL-8 en las células THP-1 derivadas a macrófagos por la acción del péptido $LALF_{32-51}$. En células THP-1 tratadas con el péptido $LALF_{32-51}$ y estimuladas posteriormente con LPS se observó la disminución de la expresión del factor transcripcional NF-κB. De conjunto, nuestros resultados sugieren que el péptido $LALF_{32-51}$ induce un fenotipo anti-inflamatorio en las células THP-1 sin dañar su susceptibilidad a los patógenos, dado por la sobre-expresión de varios TLR.

Palabras claves: Péptido LALF, células THP-1, inmunomodulación, citocinas, diferenciación celular, TLR

Introduction

The primary function of the immune system is to identify and eliminate infection, and this is achieved through two major elements; an adaptive component consisting of exquisitely specific T- and B-lymphocytes that undergo clonal selection and expansion in the presence of a foreign antigen, as well as an innate component that is characterized by its rapid mobilization and the activation of an effector mechanism upon microbial challenge [1]. A variety of effector molecules of the innate immune system have been characterized, including cytokines produced by different cellular components and the family of innate immune signaling receptors, known as Toll-like receptors (TLRs). These receptors have proven to be essential in the detection and signaling of infection [2].

Furthermore, cytokines enhance the microbicidal activities of phagocytes, they contribute to the

1. Qureshi ST, Medzhitov R. Toll-like receptors and their role in experimental models of microbial infection. Genes and Immun 2003;4:87-94.

2. Kopp E, Medzhitov R. Recognition of microbial infection by Toll-like receptor. Cur Opin Immunol 2003;15:396-401.

✉ Corresponding author

recruitment of leucocytes towards the site of infection and play a major role in orchestrating the anti-infectious process. While inflammatory cytokines fight infection, their excessive production has severe side effects. Anti-inflammatory cytokines are also produced in large amounts during sepsis and their role is to dampen the inflammatory events, but their excessive production may favor immunodepression, which is observed during severe infections. All these cytokines are part of a complex network of interactions and form part of the infectious disease puzzle [3].

Our understanding of host defense has increased during the past two decades. It is desirable to consider the modulation and control of these mechanisms as therapeutic options. In a setting of severe infections, the aim is usually to block an amplified inflammatory response, which represents the "bad" side of the double-edged sword of host defense. The obvious danger of this treatment strategy is that by impairing the inflammatory reaction we are impairing host defense in patients exposed to infectious agents [4]. Molecules that strengthen or supplement favorable host defense mechanisms have as yet been hardly explored as an alternative approach in the prophylaxis or therapy of infectious diseases. In previous studies, we reported a peptide derived from *Limulus* anti-LPS factor (LALF) that produces considerable therapeutic activity in different models of fulminating sepsis, basically by its immunomodulatory and anti-inflammatory properties [5, 6]. Also, this peptide induces the production of interferon, which has been shown to reduce viral infection. In consequence, this molecule combines the unique properties of an anti-infectious and an anti-inflammatory factor. Here we study the capacity of the $LALF_{32-51}$ peptide to induce THP-1 differentiation to macrophages. THP-1-derived macrophages by the $LALF_{32-51}$ peptide, exhibited an anti-inflammatory profile expressed as the abrogation of TNF gene expression induced by LPS and the up-regulation of the gene for the TGF-β cytokine. Interestingly, $LALF_{32-51}$ up-regulates the expression of TLR4 and TLR2, sensitizing the cell to pathogens. Furthermore, the up-regulation of the IL-8 chemokine might specifically contribute to the recruitment of neutrophiles and may well favor the resolution of infection. The effect displayed by the peptide in THP-1-derived macrophages may play a critical role in the regulation of innate immunity and the subsequent modulation of the immune responses to infection.

Materials and methods

Reagents and Chemicals

Escherichia coli 0111:B4 LPS, Tri-Reagent, Dulbecco's phosphate buffered saline solution (PBS), 3,3' 5,5' Tetramethylbenzidine tablets (TMB), Bovine serum albumin (BSA) TEMED and amonnium persulfate, were purchased from Sigma Chemical Co.; Vitamin D_3 (Sigma) was kindly donated by Professor Vincent Wilson, Nottingham, U.K. RPMI medium and fetal bovine serum (FBS) were purchased from HyClone and gentamicin was from Gibco. Reverse transcription of RNA was performed using the RNA PCR Core kit (Gene Amp Perkin-Elmer, NJ). [^{32}P] ATP was purchased from CENTIS, Cuba.

Antibodies

The following antibodies were used to perform Flow Cytometry determinations: F5647 (Mouse monoclonal anti-human CD14-FITC conjugate, Sigma); X 0933 (mouse IgG2a-FITC conjugate, isotype control, DAKO).

THP-1 cells

The monocytic leukemia cell line THP-1 (ATCC TIB 202) was grown at 37 °C, in a 5% CO_2 atmosphere, in RPMI 1640 supplemented with 10% FBS and 50 mg/ml of gentamicin. Cell density was maintained at a 5 x 10^5 cells/ml concentration. Flow cytometry analysis was performed to confirm cell differentiation to macrophages. The expression of specific macrophage cell surface antigens was monitored by the CD14 expression.

Peptide synthesis

The $LALF_{32-51}$ peptide (HYRIKPTFRRLKWKYKGKFW) was synthesized manually using Fmoc/tBu solid phase chemistry on Rink Amide MBHA resin (0.54 mmol/g, 0.1 mmol scale) [7]. Side-chain protecting groups were as follows: Arg(Pmc), Lys(Boc), and Trp(Boc). The activation used at least threefold molar excess of Fmoc-amino acids for each coupling cycle. In general, no more than 2h were needed to complete the coupling reaction, which was confirmed by a negative Kaiser test. Cleavage from the resin and de-protection were performed by TFA/TIS/water (95/2.5/2.5) treatment for 2h. Crude peptides were precipitated from tert-buthyl methyl ether and lyophilized.

Purification and characterization of the peptide

The peptide was purified by HPLC on a Vydac C18 column (25 x 250 mm) of up to 99% purity, as demonstrated by analytical HPLC on a Vydac C18 column (4.6 x 100 mm). Peptide molecular mass was verified using mass spectrometry. The low-energy MS/MS spectra was taken using a hybrid quadruple orthogonal acceleration tandem mass spectrometer QTOF from Micromass (Manchester, UK), fitted with a Z-spray nanoflow electrospray ion source.

Cell surface phenotype analysis

Equal amounts of cells (5 x 10^5 cells/samples) were seeded into flat bottom 24-well cell culture plates (Costar). Cells were incubated with 40 mg/ml of $LALF_{32-51}$ or Vitamin D_3 at a final concentration of 10^{-7}M (THP-1 cell differentiation positive control) for 24 h at 37 °C in a 5% CO_2 atmosphere. Finally, cells were processed for immunofluorescence labeling according with the manufacturer´s procedure. In brief, cells were collected in cytometry tubes (Falcon 352054, Becton Dickinson) and washed 3 times with cold PBS. Labeling was performed in a 100 ml reaction volume, using 5 ml of specific mouse monoclonal anti-human CD14-FITC conjugate (F5647) or irrelevant isotype-matched IgG2a (X0933). Samples were incubated in the dark at 4 °C for 30 min. Two additional washes with PBS were needed to eliminate the excess FITC conjugate. Cell fluorescence was analyzed using a flow cytometer FAC Scan Becton Dickinson. Relative fluorescence intensities were recorded as single-

3. Cavaillon JM, Adib-Conquy M, Fitting C, Adrie C, Payen D. Cytokine cascade in sepsis. Scand J Infect Dis 2003;35:535-44.

4. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. The New Engl J of Med 2003;9:138-49.

5. Vallespí MG, Álvarez-Obregón JC, Rodríguez-Alonso I, Montero T, Garay H, Reyes O, *et al.* A *Limulus* anti-LPS factor-derived peptide modulates cytokine gene expression and promotes resolution of bacterial acute infection in mice. Intern Immunopharmacology 2003;3:247-56.

6. Vallespí MG, Colas M, Garay H, Reyes O, Araña MJ. Differential regulation of Th1/Th2 in relevant tissues for sepsis pathogenesis with a *Limulus* anti-LPS factor-derived peptide increases survival in Gram-positive sepsis. Intern Immunopharmacology 2004;4:10-1.

7. Menrrifield RB. Solid phase peptide synthesis. J Am Chem Soc 1963;85:2149-54.

parameter histograms, and the mean fluorescence intensity (MFI) was calculated for each histogram. Results are expressed as MFI indices which correspond to MFI of cells incubated with anti-CD14 Ab/ MFI of cells stained with irrelevant isotype-matched IgG2a. All experiments were repeated three times with similar results.

Preparation of protein extract and Electrophoretic Mobility Shift Assay (EMSA)

The same amount of cells (1 x 10^7 cells/sample) was seeded into 6-well cell culture plates (Costar). The cells were incubated with 40 mg/ml of $LALF_{32-51}$ peptide for 30 min or 24 h and stimulated or not with LPS (0.1 mg/ml) for an additional 30 min. The incubation was at 37 °C in a 5% CO_2 atmosphere. After this period, samples were collected and processed to obtain nuclear extracts as described by Singh and Aggarwal [8]. Hence, cellular pellets were washed twice with cold PBS and suspended in 0.4 ml lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, and 2 mg/mL aprotinin). The cells were allowed to swell on ice for 15 min. Then, 12.5 ml of 10% Nonidet P-40 was added. The tubes were then vigorously vortexed for 10 sec and centrifuged for 1 min at 13 200 g (Sigma Lab. Centrifuges 3K30, Rotor 12 154). The nuclear pellet was resuspended in 25 ml of the extraction buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, and 2 mg/mL aprotinin) and incubated on ice for 30 min with intermittent mixing. After centrifugation for 5 min at 4 °C, the supernatants were used immediately or stored at -70 °C for later use. Protein concentration was determined by the Bradford method [9].

For EMSA we used the Gel Shift Assay System according with the manufacturer's procedure. In short, the NFkB consensus oligonucleotide was labeled with [g^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml) by the action of the T4 polynucleotide kinase in nuclease free water. The labeled oligonucleotide was separated from unincorporated nucleotides by chromatography through a G-25 spin column. Cpm incorporation was measured in a 1214 Rackbeta liquid scintillation counter (LKB-Wallac) and 1 ml of 50 000 -200 000 cpm-labeled NFkB oligonucleotide was incubated with 10 mg of nuclear extract. Positive and negative controls as well as the controls for specific and non-specific binding were included in the experiments. Samples were fractionated on non denaturing 6% polyacrylamide gels at 200V/15cm in Tris Borate Buffer (45 mM Tris borate, 1mM EDTA) at 4 °C. Gel was dried at 80 °C for 30 min and then exposed to X-ray film. All experiments were repeated three times with similar results.

Cytokine RNA levels

The same amount of cells (1 x 10^7 cells/sample) was seeded on 6 well cell culture plates (Costar). The cells were incubated with $LALF_{32-51}$ peptide at 40 mg/ml or 10^7 M Vitamin D_3 for 24 h at 37 °C under a 5% CO_2 atmosphere. Afterwards, the samples were stimulated with LPS (0.1 mg/ml) for 10 h for cytokine analyses. In the kinetic studies for IL-8 and TGF-β gene expression, the cells were incubated with the $LALF_{32-51}$ peptide at 40 mg/ml for 3, 6 and 8 h. After this period, samples were collected and the cellular pellet was homogenized in 1 mL of the TRI-Reagent to obtain total RNA according to the manufacturer's instruc-tions. For each sample 1 mg RNA was reverse-transcribed and the cDNA was amplified using a GeneAmp RNA PCR kit following the manufacturer's suggested protocol. PCR assays were conducted in a MiniCycler using the primer pairs and conditions described in table 1. Following PCR, 10 ml of the total amplified product was electrophoresed on ethidium bromide-stained 2% agarose gels and visualized under UV fluorescence. Each gel image was digitalized and analyzed using Kodak Digital Sci.ID (Eastman, Kodak, US). We used 4 h LPS stimulated peripheral blood mononuclear cells (PBMC) as the positive control.

TLRs RNA levels

The same amount of cells (1 x 10^7 cells/sample) was seeded on 6 well cell culture plates (Costar). The cells were incubated with $LALF_{32-51}$ peptide at 40 mg/ml or LPS (0.1 mg/ml) for 3, 8 and 24 h. Also, the cells differentiated with the $LALF_{32-51}$ peptide for 24 h were stimulated with LPS (0.1 mg/ml) for another 3, 8 and 24 h. Samples were then collected and the cellular pellet was homogenized in 1 mL of the TRI-Reagent to obtain total RNA according to the previously described manufacturer's instructions.

8. Singh S, Aggarwal BB. Protein-tyrosine phosphatase inhibitors block tumor necrosis factor-dependent activation of the nuclear transcription factor NFkB. J Biol Chem 1995; 270: 10631-39.

9. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

Table1. PCR primer pairs used for the amplification of human mRNA

Product	Primer sequence	Annealing temp. (°C)	Product Size(bp)
CD14	5′ CCCAAGCTTGGGCAGAGGTTCGGAAGACTTATCG 3′ 5′ GGGGTACCCCTTGACCGTGTCAGCATACTGCC 3′	72	306
TNF-a	5′ CGGGACGTGGAGCTGGCCGAGGAG 3' 5' CACCAGCTGGTTATCTCTCAGCTC 3′	56	450
IL-8	5′ ATGACTTCCAAGCTGGCCGTGGCT 3′ 5′ TCTCAGCCCTCTTCAAAAACTTCTC 3′	60	289
IL-4	5′ AACACAACTGAGAAGGAAACCTTC 3′ 5′ GCTCGAACACTTTGAATATTTCTC 3 '	65	276
IL-10	5′ ATGCCCCAAGCTGAGAACCAAGACCCA 3' 5' TCTCAAGGGGCTGGGTCAGCTATCCCA 3'	65	352
TGF-b	5' GCCCTGGACACCAACTATTGCT 3' 5′ AGGCTCCAAATGTAGGGGCAGG 3'	52	161
IFN-a	5′ GTAGCAGGAGGACCTTGATGC 3 ' 5′ ATGATTTCTGCTCTGACAACC 3 '	57	414
TLR2	5′ TCACCTACATTAGCAACAG 3′ 5′ GATCTGAAGCATCAATCTC 3′	58	368
TLR4	5′ CTTAGACTACTACCTCGATGA 3′ 5′ TAAGCCTTTTGAGAGATTTGA 3′	65	175
b-actin	5' TGACGGGGTCACCCACACTGTGCCCATCTA 3' 5' CTAGAAGCATTGCGGTGGACGATGGAGGG 3'	60	661

TNF-α assay

$5x10^5$ cells/sample were seeded into 24-well cell culture plates (Costar). Cells were incubated with 40 mg/ml of the $LALF_{32-51}$ peptide or 10^{-7} M of Vitamin D_3 for 18 h and then, stimulated or not with LPS (0.1 mg/ml) for an additional 3 h. All incubations were carried out at 37 °C in a 5% CO_2 atmosphere. After this period, samples were collected by centrifugation at 13 200 g (Sigma Lab. Centrifuges 3K30, Rotor 12154) and supernatants were processed for TNF-α determinations. The production of TNF-α was measured using a specific sandwich enzyme-linked immunosorbent assay (ELISA) for human-TNF-α kindly provided by Dr. Wim Buurman (HyCult biotech., Maastricht, The Netherlands) [10].

Statistical methods

The statistical significance of differences in mean fluorescence intensity (MFI) and pro-anti-inflammatory cytokine mRNA expression were determined by 1-factor analysis of variance (ANOVA) followed by Tukey's test for three or more groups. Differences were considered to be significant with $p < 0.05$.

Results

Differentiation of the THP-1 cell line to macrophages by the $LALF_{32-51}$ peptide

Because the monocyte/macrophage system plays a primary role in the initial phases of the innate immune response against invader pathogens, we chose the monocytic cell line THP-1 to study the previously reported immunomodulatory effects of the $LALF_{32-51}$ peptide. We initially focused on the capacity of the peptide to induce differentiation in the human monocytic leukemia THP-1 cell line. The ability of Vitamin D_3 to induce CD14 in THP-1 as indicated by cell differentiation has previously been reported. To define optimal differentiation conditions, we reproduced these findings incubating THP-1 cells with 10^{-7} M to 10^{-9} M of Vitamin D_3 for 24 h and measuring CD14 expression by FACS. Maximum expression of CD14 was observed at 10^{-7} M (data not shown) and this concentration was used in all subsequent experiments. In a first experiment, we treated the cells with different amounts of $LALF_{32-51}$ for 24 h. The effect of the peptide on CD14 expression, as measured by flow cytometry, is shown in figure 1 where cells exposed to Vitamin D_3 are also shown. The treatment with 40 mg/mL of the peptide significantly up-regulated the CD14 expression compared to untreated cells. No statistical significance was observed between 40 mg/mL and higher doses of the peptide. In addition, we evaluated the RNA expression for cytokines TNF-α and IL-8 in THP-1 cells treated with 40 mg/mL of the peptide for 24 h and later challenged them with LPS, since sensitivity to endotoxin is an indicator of cell differentiation in this cell line (figure 2). As in previous studies, TNF-α and IL-8 genes were augmented in the presence of LPS compared to untreated cells. Interestingly, the $LALF_{32-51}$ peptide up-regulated the IL-8 message level, either alone, or in the presence of 0.1 mg/ml LPS, but no expression of the TNF-α gene was observed, even when the CD14 receptor was up-regulated. To correlate TNF-α message level with protein secretion, we measured TNF-α production in additional experiments, (figure 3). A 3- h LPS stimulation following THP-1 differentiation with Vitamin D_3 led to a 10-fold increase in TNF-α levels compared to untreated cells. In contrast, $LALF_{32-51}$ had no effect on LPS-induced TNF-α production in THP-1 cells. Thus, the response to LPS observed with $LALF_{32-51}$ is different to the response observed with Vitamin D_3, at least for the TNF-α cytokine, indicating that Vitamin D_3 and $LALF_{32-51}$ differentiate these cells through different mechanisms.

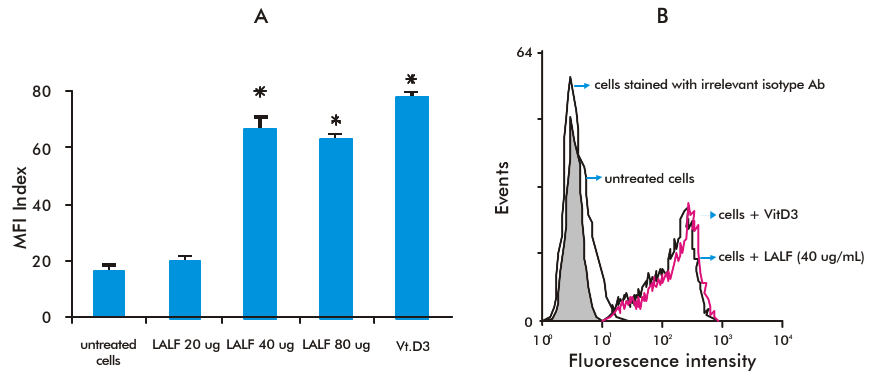

Figure 1. THP-1 cell differentiation by FACScan flow cytometry. A, The THP-1 cells were treated with 10^{-7} M Vitamin D_3 or 20, 40 and 80 mg/ml of the $LALF_{32-51}$ peptide for 24 h. Then, the cells were processed for labeling with anti-human CD14 monoclonal antibody F5647. The results are mean ±SD from three independent experiments of MFI indices which correspond to the following ratio: MFI of cells incubated with specific Ab CD14/ MFI of cells stained with irrelevant isotype-matched IgG Ab. (*) The symbol indicates a significant difference compared to untreated THP-1 cells at $P < 0.05$. B, Results are expressed as a histogram of fluorescence intensity. Data presented correspond to one of the three independent experiments.

$LALF_{32-51}$ reprogramming LPS-response of THP-1-derived macrophages

In previous studies we have reported the ability of the $LALF_{32-51}$ peptide to modulate the pattern of pro and anti-inflammatory cytokines and to promote the

10. Dentener MA, Bazil V, Von Asmuth EJ, Ceska M. Involvement of CD14 in lipopolysaccharide in-induced tumor necrosis factor, IL-6 and IL-8 release by human monocytes and alveolar macrophages. J Immunol 1993;150:2885-91.

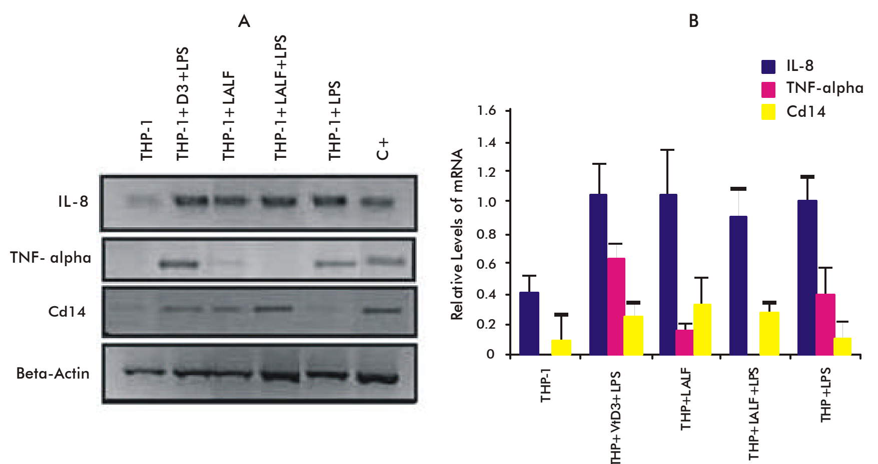

Figure 2. Effect of the $LALF_{32-51}$ peptide on TNF-a, IL-8 and CD14 expression. Cells ($1x10^7$) were stimulated with 40 mg/ml of $LALF_{32-51}$ or Vitamin D_3 10^{-7} M for 24 h. Following a 10 h induction with LPS (0.1 mg/ml) total RNA was isolated and gene expression was determined by RT-PCR, as described in Materials and Methods. We used 4 h LPS stimulated PBMC as the positive control. (A) One significant experiment of three independent assays is represented. (B) Transcript levels were expressed as quantities relative to the housekeeping gene Beta-Actin; bars, ±SD.

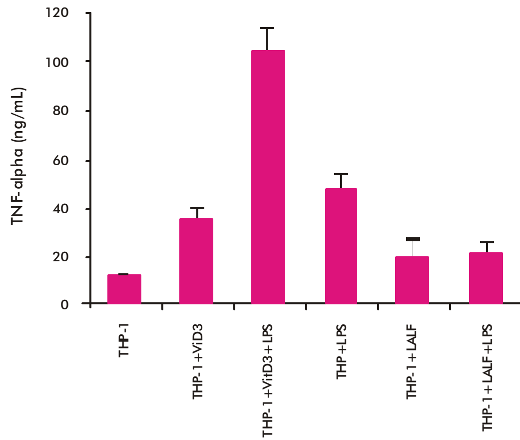

Figure 3. Effect of the $LALF_{32-51}$ peptide on TNF-a release. $5x10^5$ THP-1 cells were incubated with 40 mg/ml of $LALF_{32-51}$ or Vitamin D_3 (10^{-7} M). Then LPS was added for an additional 3 h and supernatants were collected and assayed for the presence of soluble TNF-a by specific ELISA. LPS stimulation following THP-1 differentiation with Vitamin D_3 led to a 10-fold increase in TNF-a levels in relation to untreated cells. In contrast, $LALF_{32-51}$ had no effect on LPS-induced production of TNF-a in THP-1 cells. These results are representative of three independent experiments; bars; ±SD.

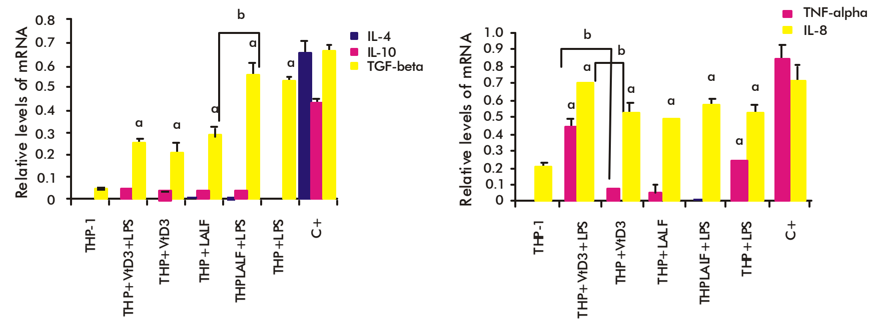

Fig 4. Differential regulation of pro-anti-inflammatory cytokine mRNA expression. Cells ($1x10^7$) were stimulated with 40 mg/mL of $LALF_{32-51}$ or Vitamin D3 10^{-7} M for 24 h. Following a 10 h induction with LPS (0.1 mg/mL) total RNA was isolated and gene expression of TNF-α, IL-8, TGF-β, IL-4 and IL-10 was determined by RT-PCR, as described in Materials and Methods. Transcript levels were expressed as quantities relative to the housekeeping gene Beta-Actin. The Control group (C+) refers to peripherical blood mononuclear cells stimulated with LPS. Data are mean ±SD from three independent experiments. (a) The symbol indicates a significant difference compared to the THP-1 cells at $P < 0.05$. (b) The symbol indicates a significant difference between THP-1 cells differentiated with $LALF_{32-51}$ or Vitamin D3 and later stimulated with LPS at $P < 0.05$.

resolution of the invader pathogen in animal models of infectious diseases. We then questioned whether $LALF_{32-51}$ might have the ability to modulate the response to LPS in human monocytes. The THP-1 cells were treated with the peptide for 24 h and later challenged with LPS. The mRNA expression for TNF-α, IL8, IL-10, IL-4 and TGF-β were analysed by RT-PCR. As shown in figure 4, the analyses of pro and anti-inflammatory cytokines showed that the $LALF_{32-51}$ peptide induces the stimulation of IL-8 and TGF-β mRNA in THP-1-derived macrophages, similarly to that observed with Vitamin-D_3 differentiated cells or LPS- stimulated THP-1. On the other hand, the peptide showed no expression of cytokines TNF-α, IL-10 and IL-4. We also analysed the kinetic expression for IL-8 and TGF- genes. Figure 5 shows similar kinetics for both genes, suggesting that, under our experimental conditions, TGF-β is not enough to down-regulate IL-8. In addition, the pattern of cytokines after LPS challenge showed the up-regulation of the TGF-β gene, either THP-1 stimulated with $LALF_{32-51}$ or with Vitamin D_3. However, the TGF-β expression increased significantly when the cells were pre-treated with the peptide in contrast to cells differentiated with Vitamin D_3. These results suggest that the $LALF_{32-51}$ peptide has a direct impact on monocyte differentiation leading to a type of macrophage with an anti-inflammatory phenotype.

Toll-like receptors are up-regulated by the peptide $LALF_{32-51}$ in THP-1-derived macrophages

Because THP-1 cells express TLRs and are thought to play primary roles in the initial phases of the innate immune response, we analysed whether $LALF_{32-51}$ is able to modulate TLR2 and TLR4 in response to endotoxin in these cells. The LALF peptide up-regulated TLR2 mRNA expression in THP-1 cells and both receptors were enhanced by a 3 h LPS treatment (figure 6A). We had previously reported the induction of IFN-α in human peripherical blood mononuclear cells (PBMC) stimulated with $LALF_{32-51}$. Hence, several cytokines including IFN-α may alter TLRs expression. We then studied the ability of the peptide to induce IFN-α gene expression in THP-1 cells. Interestingly, THP-1 stimulated with the LALF peptide increased the expression of the gene for IFN-α, either alone or in the presence of LPS, (figure 6B).

The $LALF_{32-51}$ peptide modulates the transcriptional factor NFkB in THP-1-derived macrophages

It is well documented that several microbial products induce NFkB activation in monocytes and regulate cytokine expression. We therefore tested the involvement of NFkB in the response to LPS in $LALF_{32-51}$-derived macrophages. Two groups of THP-1 cells were incubated with 40 mg/mL of the peptide for two time periods of 30 min and 24 h. The THP-1

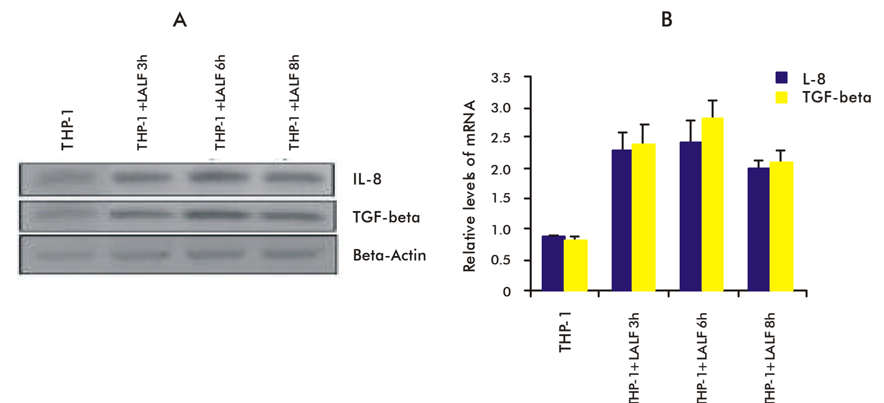

Figure 5. Effect of the $LALF_{32-51}$ peptide on IL-8 and TGF-β gene expression. Cells ($1x10^7$) were stimulated with 40 mg/mL of $LALF_{32-51}$ for 3, 6 and 8 h. Then total RNA was isolated and gene expression of IL-8 and TGF-β was determined by RT-PCR, as described in Materials and Methods. (A) One significant experiment of two independent assays is represented. (B) Transcript levels were expressed as quantities relative to the housekeeping gene Beta-Actin; bars, ±SD.

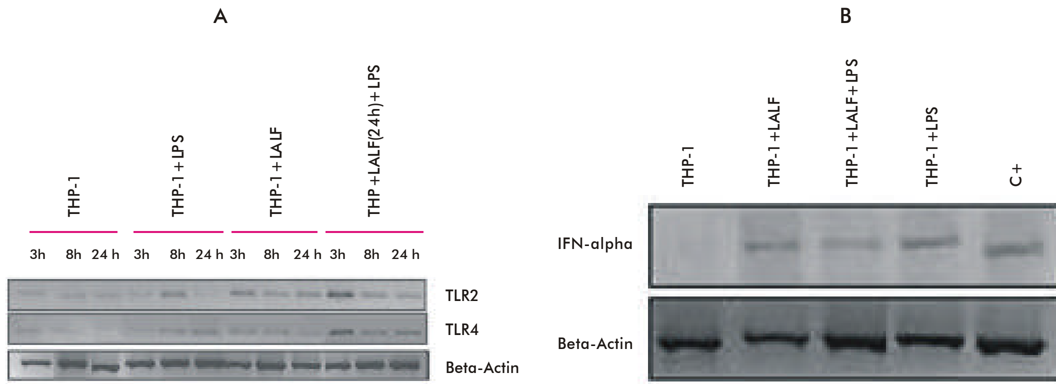

Figure 6. TLR mRNA expression in LALF$_{32-51}$-differentiated THP-1 cells. A, Cells (1x10^{7}) were stimulated with 40 mg/mL of LALF$_{32-51}$ or 0.1mg/mL LPS for 3, 8 and 24 h. Also, the cells were pre-treated with the peptide (40 mg/mL) for 24 h and later stimulated with LPS for an additional 3, 8 and 24 h. Total RNA was isolated and gene expression of TLR2 and TLR4 was determined by RT-PCR, as described in Materials and Methods. B, THP-1 cells were treated with LALF$_{32-51}$ or LPS as indicated above, and then total RNA was extracted and IFN-a gene expression analysed by RT-PCR. The results shown are significant and are drawn from three separate experiments.

cells treated for 30 min with the LALF peptide increased NFκB binding activity slightly compound to untreated cells, but the binding activity was strongly increased after 24 h of incubation with the peptide, (figure 7A). However, the NF-κB binding activity was completely abolished when the THP-1 cells were pre-treated with the peptide for 24 h and then stimulated with LPS for 30 min, (figure 7A). The transcriptional factor NFkB was also activated in LPS-stimulated cells, but its activity decreased following a 2 h treatment with the endotoxin (figure 7B).

Discussion

Monocyte / macrophage play a major role in the pathogenesis of inflammatory diseases since they are the main target for Gram-negative and Gram-positive bacteria. In previous papers, we have described the effect of the LALF$_{32-51}$ peptide modulating the host's defenses and promoting a type of immune response that is effective in fighting acute bacterial infection and sepsis. The present study provides experimental evidence of the ability of LALF$_{32-51}$ to differentiate THP-1 towards macrophages, reprogramming their response to LPS and switching the

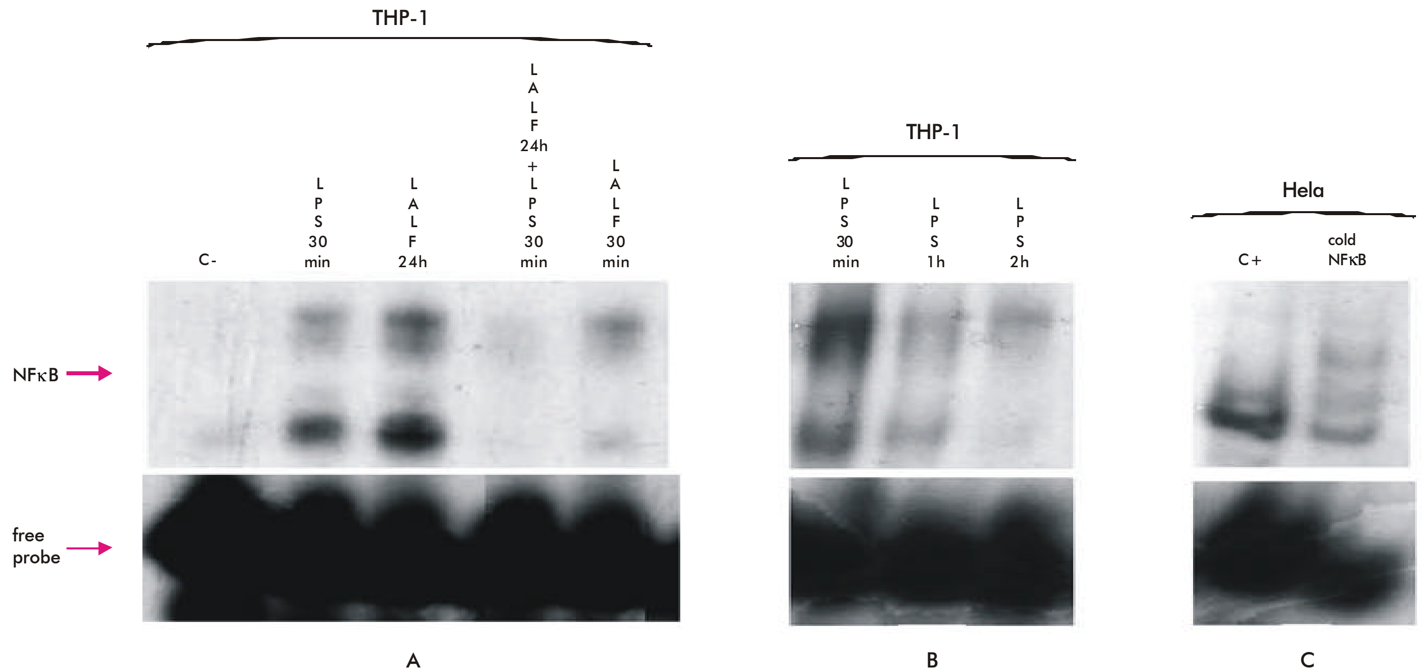

Figure 7. Effect of the LALF$_{32-51}$ peptide on the LPS-mediated activation of NFkB. 1x10^{7} THP-1 cells were incubated with 40 mg/mL of LALF$_{32-51}$ for 24 h. Then, LPS (0.1 mg/mL) was added for an additional 30 min. Following stimulation, nuclear extracts were obtained and assayed for NFkB DNA-binding activity by EMSA as described in materials and methods. A, Pre-incubation of THP-1 cells with LALF$_{32-51}$ completely abolishes LPS-induced NFkB activation. Higher NFkB binding activity is reached 24 h after LALF$_{32-51}$ stimulation. The control group (C-) refers to non-stimulated THP-1 cells. B, higher NFkB binding activity is reached 30 min after LPS stimulation. C, HeLa nuclear extract is used as the EMSA positive control (C+) and cold NFkB indicates the specific binding of NFkB oligonucleotides. The data of a representative experiment (n=3) are shown.

differentiation towards a particular type of macrophages, down-regulating TNF-α and increasing TGF-β gene expressions. This effect might play an important role in balancing an appropriate immune response against invader pathogens and preventing an excessive inflammatory reaction. The pro-monocytic cell line THP-1 is largely known to differentiate into macrophage lineage with Vitamin D_3 and this process leads to the up-regulation of the CD14 receptor, which mediates the endotoxin responsiveness of those cells, as evidenced by the production of different cytokines, e.g. TNF-α [11]. Accordingly, our results demonstrated up-regulation for CD14 and LPS-induced TNF production in Vitamin D_3-treated cells. In this study we report, for the first time, the capacity of a LALF-derived peptide to differentiate the pro-monocytic cell line THP-1 to macrophages, confirmed by the up-regulation of the cell surface receptor CD14. Interestingly, the increase in CD14 expression was not positively correlated with LPS-induced TNF-α production in peptide-treated THP-1 cells, but an up-regulation of the expression for IL-8 and TGF-β was observed. Vitamin D receptor (VDR) belongs to a nuclear receptor super-family that mediates the genomic actions of Vitamin D_3 and regulates gene expression by binding with vitamin D response elements in the promoter region of the cognate gene [12]. It has also been demonstrated that the combined stimulation of THP-1 cells with Vitamin D_3 and LPS up-regulates the mCD14, TLR4, MD-2 and MyD88 expression by these cells. This mechanism might be strongly involved in increased IL-8 and TNF-α production by cells leading to a pro-inflammatory phenotype [13]. Nevertheless, $LALF_{32-51}$ completely abolishes the LPS -induced TNF-α production in THP-1 cells and significantly increases the TGF-β gene expression, which leads to macrophage differentiation with an anti-inflammatory phenotype. In all, these findings indicate the regulatory effects of the LALF peptide on LPS-induced macrophage response in controlling the modulation of pro and anti-inflammatory cytokines, and strengthens our hypothesis that different pathways are used by the LALF peptide and Vitamin D_3 to exert their biological functions.

THP-1 cells pre-treated with the peptide and stimulated with LPS slightly increased IL-8 mRNA expression. This could be favorable for the rapid elimination of pathogenic microorganisms that invade the body, because this cytokine mainly functions as a neutrophil chemo-attractant and activating factor, initiating microbicidal functions that serve to contain infection [14]. The TGF-β cytokine, one of the most recognized monocyte-deactivating cytokines [15], was significantly up-regulated. A surprising and counter intuitive finding was that TGF-β did not inhibit LPS-stimulated IL-8 mRNA expression. To address this question we performed kinetic studies in peptide-treated THP-1 cells. We showed similar kinetic expressions for both cytokines. This finding contrasts with studies reporting that the addition of TGF-β to cell cultures before LPS stimulation inhibited the secretion of IL-8 [16,17]. However, it has also been reported that TGF-β did not significantly inhibit *Mycobacterium bovis*- stimulated IL-8 production in human monocytes [18]. One possible explanation is that the effect of TGF-β on IL-8 stimulation may depend on the stimulus and other cellular factors present in the environment. Recently, other studies indicate that the IL-8 response observed with LPS or *Salmonella Typhimurium* in THP-1 cells requires the independent activation of multiple pathways. Therefore, the prevention of IL-8 release appears to require the blockage of more than one intracellular mediator [19]. It is important to understand the mechanism of how IL-8, TGF-β and TNF-α are differentially regulated by $LALF_{32-51}$ at molecular levels. This suggests that $LALF_{32-51}$-induced gene expression in human monocytes/macrophages should be done by DNA microarray analysis.

Although the production of pro-inflammatory cytokines is important for mediating the initial host defense against the invading pathogens, an inability to regulate the nature or duration of the host's inflammatory response can be detrimental, as in endotoxin shock [20]. An emerging concept suggests that the clinical expressions of septic shock can be due to an imbalance in the production of pro-and anti-inflammatory cytokines, and merely blocking one cytokine might be ineffective [21]. Thus, the balance of pro-anti-inflammatory cytokines observed in response to LPS in $LALF_{32-51}$-differentiated macrophages may be appropriate in promoting a type of immune response that is effective against acute bacterial infection and sepsis.

To extend that finding, we addressed the modulation of the transcriptional factor NF-κB, an essential component involved in TLR2 -and TLR4-mediated intracellular signaling pathways. The $LALF_{32-51}$ peptide activated early NF-κB DNA binding in THP-1 cells, correlating with macrophage differentiation and TLR up-regulation. Surprisingly, we did not observe either TNF-α message levels or protein production in peptide-treated cells. At present it is not clear how this occurs, but we might hypothesize that the LALF peptide may modulate the expression of several transcriptional factors required for the optimum induction of TNF-α. Several authors have shown that different nuclear transcriptional factors, including the nuclear factor of activated T cells (NF-AT), Egr-1, and Ets are involved in the effective transcriptional activation of TNF-α [22-25]. Further studies are needed to identify cellular and molecular signaling events involved in the regulation of TNF-α by the $LALF_{32-51}$ peptide. These investigations may highlight the potential therapeutic use of this molecule.

In contrast, we demonstrated that in the presence of an infectious stimulus, such as LPS, the $LALF_{32-51}$ peptide inhibits NF-κB activation corresponding to the abrogation of LPS-induced TNF-α production. Modulation of macrophage activation occurs through the release of anti-inflammatory mediators such as IL-10 and TGF-β [26,27]. These immune modulators attenuate the inflammatory response through the down-regulation of the transcriptional factor NF-κB [28]. The cytokine pattern analysed suggests that this effect may be explained, at least in part, by the ability of TGF-β to inhibit pro-inflammatory cytokines at the transcriptional levels, particularly through the inhibition of transcriptional factor NF-κB, since cytokines IL-10 and IL-4 did not modulate during the treatment with the peptide. Cytokine IL-8 has been well described to be regulated by NF-κB. Nevertheless,

11.Tobias PS, Soldau K, Kline L, Lee JD, Kato K, Martin TP, *et al.* Cross-linking of LPS to CD14 on THP-1 cells mediated by LPS-binding protein. J Immunol 1993;150: 3011-21.

12.Pramanik R, Asplin JR, Linderman C, Favus MJ, Bai S, Coe FL. Lipopolysaccharide negatively modulates vitamin D action by down-regulating expression of vitamin D-induced VDR in human monocytic THP-1 cells. Cellular Immunology 2004;232:137-43.

13.Tamai R, Sugawara S, Takeuchi O, Akira S, Takada H. Synergistic effects of lipopolysaccharide and interferon-gamma in inducing IL-8 production in human monocytic THP-1 cells accompanied by up-regulation of CD14, Toll-like receptor 4, MD-2 and MyD88 expression. J Endotoxin Res 2003;9:145-53.

14.Baggionili M, Dewald B, Moser B. Interleukin-8 and related chemotactic cytokines-CXC and CC chemokines. Adv Immunol 1994;55:179-97

15.Wahl SM, Swisher J, McCartney-Francis N, Chen W. TGF-β: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. J of Leukocyte Biology 2004;76:1-10.

16.Ehrlich LC, Hu S, Sheng WS, Sutton RL, Rockswold GL, Peterson PK, *et al.* Cytokine regulation of human microglial cell IL-8 production. J Immunol 1998;160:1944-8.

17.Smith WB, Noack L, Khew-Goodall Y, Isenmann S, Vadas MA, Gamble JR. TGF-β inhibits the production of IL-8 and the transmigration of neutrophils through activated endothelium. J. Immunol 1996; 157: 360-8. Menrrifield RB. Solid phase peptide synthesis. J Am Chem Soc 1963; 85:2149-54.

18.Méndez-Samperio P, García E, Vázquez A, Palma J. Regulation of Interleukin-8 by Interleukin-10 and transforming growth factor b in human monocytes infected with *Mycobacterium bovis*. CDLI 2002;9:802-7.

19.Vitiello M, D' Isanto M, Galdiero M, Raieta K, Tortora A, Rotondo P, *et al*. Interleukin-8 production by THP-1 cells stimulated by *Salmonella enterica serovar Typhimurium* porins is mediated by AP-1, NF-kB and MAPK pathways. Cytokine 2002;27:15-24.

20.O'Neill LA, Dinarello CA. The IL-1 receptor/Toll-like receptor superfamily: crucial receptors for inflammation and host defense. Immunol Today 2000;21: 206-9.

21.Bone RC. Immunologic dissonance: a continuing evolution in our understanding of the systemic inflammatory response syndrome and multiple organ dysfunction syndrome. Ann Intern Med 1996;125:680-7.

22.Kramer B, Meichle A, Hensel G, Charnay P, Kronke M. Characterization of a Krox-24/Erg-1-responsive element in the human tumor necrosis factor promoter. Biochim Biophys Acta 1994;1219:413-21.

23.Kramer B, Wiegmann K, Kronke M. Regulation of the human TNF promoter by the transcription factor Ets, J Biol Chem 1995;270:6577-83.

24.McCaffrey PG, Goldfeld AE, Rao A. The role of NFATp in cyclosporin A-sensitive tumor necrosis factor-α gene transcription. J Biol Chem 1994;269:30445-50.

our results show that NF-κB is down-regulated by the LALF peptide while IL-8 mRNA is up-regulated. It is well established that the stimulation of monocytes results in the activation and assembly of multiple transcription factors to adjacent sites in the promoters of inducible genes. In addition to NF-κB, other transcription factors participate in the regulation of the IL-8 gene expression by binding to *cis*-acting DNA elements in the same promoter. There is evidence of a functional association of NF-κB with the activator protein-1 (AP-1) and the cAMP response element (CRE)-binding protein (CREB) [29]. In a particular situation, a deficient NF-κB activity might be replaced by a cooperative effect with other transcription factors; we might speculate that possibly LALF activates some of these factors, ensuring the activation of the IL-8 genes. Furthermore, an increase in the TLR2 and TLR4 cell surface expression by the LALF treatment in LPS-activated THP-1 cells was observed, which supports the notion that the induction of TLR2 and TLR4 could contribute to the stimulation of IL-8 production in response to LALF in THP-1 cells. Cellular activation by Gram-negative and Gram-positive bacteria through TLRs initiates a signaling cascade also resulting in mitogen-activated protein (MAP) kinase activation, mediating AP-1 and CREB activation essential for IL-8 expression [30]. Recently, a similar effect for the Dialyzable Leukocyte Extract on the modulation of NF-κB and IL-8 has been also documented by other authors [31].

Here we provide evidence that the LALF peptide in the normal host stimulates a mild inflammatory and non-specific immunostimulatory event. In contrast, in the presence of a septic event the LALF peptide inhibits early transcriptional factor activity and cytokine transcription associated with septic challenge, thereby blunting the host's inflammatory response to the injury. These events might lead to an enhanced response to bacterial infections, giving a putative molecular explanation for the protective effect of the $LALF_{32-51}$ peptide observed during prophylactic treatment in animal models of infectious diseases [5, 6, 31, 32].

Toll-like receptors (TLR) are pattern recognition receptors used by cells of the innate immune system to detect the presence of a wide variety of pathogens. These receptors specifically recognize pathogen-associated molecular patterns (PAMPs). Monocytic-like THP-1 cells regulate TLR mRNA levels in response to a variety of stimuli including LPS, bacterial lipoproteins, live bacteria and cytokines [32]. Human polymorphisms in TLR2 and TLR4 are associated, respectively, with an increased susceptibility to *S. aureus* infections [33] and LPS hyporesponsiveness [34], underscoring the importance of the intact PAMP recognition system in human health. To identify the role of $LALF_{32-51}$ on TLR regulation, we studied the expression of TLR2 and TLR4 in THP-1 cells. Interestingly, $LALF_{32-51}$-differentiated THP-1 expressed higher levels of TLR2 compared to undifferentiated cells, and the expression for both receptors was enhanced early after post-challenging with endotoxin. This finding shows the ability of the LALF peptide to modulate receptors involved in the innate immune response sensitizing the cells to microbial stimulation. In our previous observations the LALF peptide induced IFN-α in human peripherical blood mononuclear cells PBMCs [35]. Here we demonstrated the capacity of $LALF_{32-51}$ to induce gene expression for IFN-α in THP-1 cells, both alone and in the presence of LPS. The effect of $LALF_{32-51}$ on the regulation of TLR message level may be due either to direct effects or to autocrine stimulation by secreted elements such as IFN-α, which regulate TLR2 and TLR4 gene expression in human macrophages [36]. More recently, it has been reported that IFN-α up-regulates TLR3 expression and enhances TLR3-mediated antiviral cytokine expression in immune, epithelial and endothelial cells [37]. Therefore, we do not discard that other Toll-like receptors such as TLR2 and TLR4 could also be up-regulated by the $LALF_{32-51}$ peptide. Collectively, the up-regulation of TLR2, TLR4 and IFN-α in THP-1 cells after peptide treatment may lead to an enhanced sensitivity of macrophages, especially towards Gram-negative and Gram-positive bacteria and viral infections, due to the crucial role of those elements in enhancing innate immune sensing.

Considering the modulating effects of $LALF_{32-51}$ on the production of the pro-inflammatory cytokines TNF-α and IL-8, associated with the up-regulation of anti-inflammatory cytokine TGF-, together with the increase in both TLR2 and TLR4 expression and the suppression of NF-κB activity, we conclude that these results provide new insights into the biological activities of $LALF_{32-51}$.

Acknowledgements

We would thank like to Dr. Vincent G. Wilson from the School of Biomedical Science, Queens Medical Centre, Nottingham for providing the Vitamin D_3 reagent. We would also like to thank Dr. Eduardo Penton and Amelia Rodríguez for their critical reading of the manuscript.

25.Ye J, Aeidler P, Young S, Martinez A, Robinson VA, Jones W, *et al.* Activation of mitogen-activated protein kinase p38 and extracellular signal-regulated kinase is involved in glass fiber-induced tumor necrosis factor- production in macro-phages. J Biol Chem 2001;276:5360-7.

26.Malefyt De W, Abrams R, Bennett B, Fidgor C, Vries JE. IL-10 inhibits cytokines synthesis. by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991;174:1209-20.

27.Letterio JJ, Roberts AB. Regulation of immune responses by TGF-β. Annu Rev Immunol 1998; 16:137-61.

28.Trepicchio W, Wang L, Bozza M, Dorner A. IL-11 regulates macrophage effectors function through the inhibition of nuclear factor-kB. J Immunol 1997;159:5661-70.

29. Hershko DD, Robb BW, Luo G, Hasselgren PO. Multiple transcription factors regulating the IL-6 gene are activated by cAMP in cultured Caco-2 cells. Am J Physiol Regul Integr Comp Physiol 2002;283:R1140-8.

30.Rehli M. Of mice and men: species variations of Toll-like receptor expression. Trends in Immunology 2002;23:375-8.

31.Ojeda M, van' t Veer C, Fernandez CB, Araña MJ, Buurman WA. Dialyzable Leukocyte Extract differentially regulates the production of TNF-a, IL-6 and IL-8 in bacterial component-activated leukocytes and endothelial cells. Inflamm Res 2005;54:74-81.

32.Zarember KA, Godowski PJ. Tissue expression of human toll-like receptors and differential regulation of toll-like receptor mRNA in leukocytes in response to microbes, their products and cytokines. J Immunol 2002; 168:554-61.

33.Lorenz E, Mira JP, Comish KL, Arbour NC, Schwartz DA. A novel polymorphism in the toll-like receptor 2 gene and its potential association with staphylococcal infection. Infect Immun 2000; 68: 6398-406.

34. Arbour NC, Lorenz E, Schurre BC, Zabner J, Kline JN, Jones M, *et al*. TLR4 mutations are associated with endotoxin hyporesponsiveness in human. Nat Genet 2000; 25:187-93.

35. Vallespi MG, Glaria LA, Reyes O, Garay H, Ferrero J, Araña MJ. A Limulus antilipopolysaccharide factor-derived peptide exhibits a new immunological activity with potential applicability in infectious diseases. CDLI 2000;7:669-75.

36. Sirén J, Pirhonen J, Julkunen I, Matikainen S. IFN-a regulates TLR-dependent gene expresión of IFN-a, IFN-b, IL-28 and IL-29. J Immunol 2005;174:1932-7.

37. Tissari J, Sirén J, Meri S, Julkunen I, Matikainen S. IFN-a enhances TLR3-mediated antiviral cytokine expresión in human endotelial and epithelial cells by up-regulating TLR3 expression. J Immunol 2005; 174:4289-94.

Received in march, 2006. Accepted for publication in june, 2006.