ANTIINFLAMMATORY EFFECTS OF LEFLUNOMIDE IN COMBINATION WITH METHOTREXATE ON COCULTURE OF T LYMPHOCYTES AND SYNOVIAL MACROPHAGES FROM RHEUMATOID ARTHRITIS PATIENTS

Extended report

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ABSTRACT

Objectives. To investigate the anti-inflammatory effects of the active leflunomide metabolite A771726 (LEF-M) in combination with methotrexate (MTX) on synovial macrophages from rheumatoid arthritis patients (SM) co-cultured with activated T cell line (Jurkat cell line).

Methods. Pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), adhesion molecule ICAM-1, cyclooxygenase isoenzymes (COX1, COX2) and the NF-κB complex were analysed on SM co-cultured with T cell line, either as intracellular protein expression by immunocytochemistry (ICC) and Western blot (Wb) analysis, as extracellular protein expression by ELISA assay and as mRNA expression by reverse transcriptase-multiplex PCR (RT-MPCR) after treatment with LEF-M (1, 10, 30 µM) alone or in combination with MTX (50 ng/ml).

Results. The most significant intracellular decrease of cytokines was observed by ICC in SM treated with the combination of LEF-M (1, 10, 30 µM) and MTX (50 ng/ml) vs untreated SM (TNF-α 29%, 37%, 49%, IL-1β 56%, 43%, 50%, and IL-6 59%, 62%, 71%, respectively). Furthermore, a significant decrease was confirmed concerning the cytokine levels evaluated by ELISA in the medium of SM treated with the combination LEF-M/MTX (TNF-α 40%, 41%, 44%, IL-1β 10%, 20%, 60%, IL-6 37%, 41%, 49%, respectively). On the other hand, Wb and the RT-PCR analysis confirmed these results. Concordant decreased expression was observed for ICAM-1, COX1, COX2 and NF-κB complex after LEF-M/MTX treatment.

Conclusions. The combination of MTX and LEF-M shows additive inhibitory effects on the production of inflammatory mediators from SM co-cultured with T cell line. These observations might support the positive results obtained in RA clinical studies by combination therapy.

Key words: leflunomide, methotrexate, rheumatoid arthritis, macrophages, TNF-α.

Running title: leflunomide/methotrexate effects on synovial macrophages.

Introduction

Homing of activated lymphocytes and monocytes into the synovial tissue in rheumatoid arthritis (RA) represent the first stage of synovial inflammation that is followed by subsequent degradation of the joints [1, 2] and this is one reason for choosing our experimental model.

Activated lymphocytes and mononuclear cells in rheumatoid arthritis (RA) require roughly an increase de novo synthesis of pyrimidines in order to progress from G1 to the S phase of the cell cycle [3]. Leflunomide (LEF) (N-(4-trifluoro-methylphenyl)-5methylisoxazole-4-carboxamide) mainly through its active metabolite A77 1726 (LEF-M), at low therapeutically applicable doses, reversibly inhibits the enzyme dihydro-orotate dehydrogenase (DHODH), the rate limiting step in de novo synthesis of pyrimidines in different cell lines [4].

However, recent studies suggest that the observed antiinflammatory effects exerted by LEF-M, seem to be strongly related to its ability to inhibit osteoclastogenesis as well as metalloproteinase and inflammatory cytokine production by cultured RA synovial cells and to inhibit the activation following the cell-cell contact between T lymphocyte and monocytes [5-7].

In addition, further studies indicate that LEF-M seems to interfere with the NF-kB complex activation and to down regulate the glycosylation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) [8-10].

Recently, LEF-M was found to influences the trans-endothelial migration of peripheral blood mononuclear cells (PBMC) and to decrease cell adhesion molecules such as monocytic CD44 expression and PBMC-hyaluronan binding by inhibiting DHODH in treated RA patients [11].

All these effects might act together in blocking cell traffic and cell activation into the inflamed RA synovial tissue.

However, given the high failure of RA monotherapy and the multifactorial nature of the
pathogenesis of RA, the strategy to combine different therapeutic agents to inhibit the complex processes of the disease is increasing. In particular, LEF has been shown to be useful in combination with MTX in RA patient management [12-14].

MTX is the most common DMARD used in therapy of RA, and it acts by inhibiting dihydrofolate reductase and hence decreasing the supply of reduced folates for purine [15]. A number of antiinflammatory effects exerted by MTX seem related to the induction of extracellular adenosine increase and its interaction with specific cell surface receptors with subsequent inhibitions of IL-8 production by PBMC, IL-6 secretion by human monocytes, leukotriene B4 synthesis in neutrophils and decreased synovial collagenase gene expression [16]. In addition, MTX seems to exert antiinflammatory and antiproliferative activities particularly on activated monocytes [17, 18].

Recently, RA patients treated with combination of MTX and LEF exhibited a significant suppression of several major chemokines including monocyte-derived chemokine (MCP-1) and macrophage-derived chemokine (MDC-1) [19]. Positive correlations among reductions in plasma chemokines and clinical outcome measures were also found [19]. Therefore, we decided to analyze the effects of LEF-M and its combination with MTX in a co-culture of activated T cell line and RA synovial macrophages. The study was focused on the mRNA expression and on the intra- and extra-cellular protein detection for different mediators of the inflammatory reaction, such as cytokines (TNF-α, IL-1β, IL-6), adhesion molecule ICAM-1, cyclooxygenase isoenzymes (COX1, COX2) and the NF-κB pathway, as a complex of transcriptional molecules modulating cellular responses in activated cells [20].

Materials and Methods.

T cell line/synovial macrophages co-culture.

Synovial macrophages were obtained from five RA patients who fulfilled the American College of Rheumatology (ACR) criteria for adult RA [21]. Approval from the ethical committee and informed consent from patients were obtained:

The mean disease duration was 4 ± 2 yrs and disease activity as assessed by the DAS28 score was 4.1. No RA patients have been treated with oral or intra-articular corticosteroids during the 3 months preceding the therapeutic arthroscopic synoviectomy.

The synovial RA tissue samples were gently cut into small pieces (2-5 mm), washed in Dulbecco’s phosphate buffer saline DPBS (Sigma-Aldrich, Sigma Chemical Division, Milan, Italy) and incubated in collagenase (0.75 mg/ml) (Type IV from Clostridium histolyticum) (Sigma-Aldrich, MI, Italy) for 1 hour at 37 °C. The digest was passed through a wire 150 mesh to separate the synovial cells from the debris tissue. Afterwards, the obtained cells were washed 3 times with DPBS, resuspended in RPMI-1640 medium (Sigma-Aldrich, MI, Italy) supplemented with 10% foetal bovine serum (FBS) (containing < 0.5 EU/ml endotoxin), 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin (Sigma-Aldrich, MI, Italy). The synovial cells were seeded into multi-well flat bottom plates (5 x 10^5 cells/well) and cultured in 5% CO2 air humidified atmosphere at 37°C. Viability of the cells (95-97%) was tested by trypan blue exclusion. After 1 hour, non adherent cells were washed out, while adherent cells (synovial macrophages) were cocultured for 24 hours in a trans-well system (Corning-Costar) together with T cell line (Jurkat) previously activated with concanavalin-A (ConA) (1µg/ml) for 20 hours, in absence as well as in presence of different concentrations (1, 10, 30 µM) of A771726 (provided by Aventis Pharma, Deutschland GmbH, Frankfurt am Main Germany). In addition, co-cultures (T cell line/macrophages) were also treated with the three different concentrations of LEF-M (1, 10, 30 µM) in combination with MTX (50 ng/ml).

The utilized concentrations of LEF-M were close to the serum levels obtained with therapeutic doses in RA patents [10]. The MTX dose (50 ng/ml) has already been detected as the concentration achievable in the serum of RA patients treated with low dose MTX [17, 18]. However the capacity of MTX uptake by cultured cells might be underestimated when studies are performed in medium with high concentration of folic acid.
At the end of the co-culture incubation time the Jurkat T cell line were removed with several washes in PBS and synovial macrophages were harvested, washed in PBS, and used for the different test assays. Experiments were performed in triplicate: each experiment, performed with synovial macrophages obtained from an individual patient, was replicated three times.

**Immunocytochemistry assay**

Macrophages were harvested mechanically and then incubated on glass slides for 45 minutes at 4°C. Therefore, the cellular spots were fixed in acetone for 30 seconds, air dried and stored at −20°C until use. After rehydration in PBS, to prevent non-specific antibody binding, the spots were incubated with rabbit normal serum for 20 minutes. The following step was the incubation with anti TNF-α ( lot n° C161), IL-1β ( lot n° J100), IL-6( lot n° G170) and ICAM-1( lot n° C221) antibodies 1:100 diluted (Santa Cruz Biotechnology, CA, USA) at room temperature for 45 minutes. Linked antibodies were detected by biotynilated universal (pan specific) antibody and subsequently horseradish peroxidase streptavidin (Vector Laboratory Inc, Burlingame, CA, USA). Each step was followed by two washes in PBS. Staining reaction was developed by diaminobenzidine (DAB) system (Ylem S.r.l., Rome, Italy). Finally, slides were counterstained with haematoxylin, fixed with ethanol and cover slipped with Eukitt. Controls were treated identically, however the primary antibodies were omitted.

**Image Analysis**

Image analysis was performed with the Leica Q-Win image analysis system (Leica, Cambridge, UK). For each sample about 100 cells were analysed, and pixels/µm² (positive area) were quantified by the Leica Q-Win software. The single cells were randomly selected by the operators, using the cursor and then automatically measured as positive area.

**Immune-enzymatic assay**

After 24 hours of treatment the culture medium was harvested and stored at −20°C until analysis. The enzyme immunoassay for quantitative determination of TNFα, IL-1β and IL-6 was carried out with a microplate kit system (Diaclone, France) (CV intra : 2.15; CV extra: 3.85). The results were obtained with a multiwell plate automatic processor (Thecno Genetics, Milan, Italy).

**Western Blot analysis**

After the different treatments, macrophage pellets were lysed in a buffer containing 20mM Tris-HCL pH 8, 150 mM NaCl, 1 mM phenylmethylsulphonylfluoride (PMSF), 5mg/ml aprotinin and 0.5% Nonidet P-40 (Promega, Milano, Italy) for one hour at 4°C. The lysates were then centrifuged for 10 minutes at 13,000 rpm. Protein samples (20 µg) were diluted with sample buffer and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteins were transferred to a Hibond-C-nitrocellulose membrane (Amersham Pharmacia Biotech Europe, Freiburg, Germany). After transfer, the membrane was blocked for one hour at room temperature in PBS containing 5% non-fat powdered milk.

For immunoblot analysis, the membrane was incubated with anti TNF-α, IL-1β and IL-6 polyclonal antibodies 1:500 diluted (Santa Cruz, Biotechnology, CA, USA) overnight at 4°C, washed in 0.05%PBS/Tween 20 pH 7.4 (PBS/T) and finally, incubated for one hour at room temperature with secondary horseradish peroxidase labelled goat antibody (Santa Cruz, Biotechnology). After three further washes with PBS/Tween, bound secondary antibody was detected by emitting chemiluminescent reaction (Amersham Pharmacia Biotech Europe, Freiburg, Germany).

**mRNA analysis**

After treatments at the different culture conditions, a part of samples were harvested, washed in DPBS twice and treated with Trizol mRNA lysis buffer for total RNA extraction. All samples were
analysed by reverse transcriptase PCR (RT-MPCR) with multiplex system (MBL, Maxim’s kits, 780 Dubuque Av. So. S. Francisco CA 94080, U.S.A) for a temporal and spatial distribution of mRNA expression of different cytokines (TNF-α, IL-1β, IL-6, IL-8,GM-CSF), COX-1, COX-2, NF-kB sub unity p50/p52 and NF-kB inhibitors. This method is an accurate and valid system to detect multiple gene expression by amplifying all the genes under the same conditions. The PCR primers designed by Maxim have similar Tm and no obvious 3’-end overlap to enhance multiple amplification. The 680 bp (TNF-α), 555 bp (IL-1β), 424 bp (GM-CSF), 360 bp (IL-6), 300 bp (IL-8), 161 bp (TGF-β), 278 bp (COX-2), 218 bp (COX-1), 183 bp (NF-kB inhibitors), 143 bp (NF-kB p50/p52) PCR products can be generated from human RNA or the positive control, which included in the kits.

Statistical Analysis

The results were analysed by the ANOVA non-parametric test (Bonferroni test) and represent mean values ± standard deviations (SD) of five different experiments. Each experiment, performed with synovial macrophages obtained from an individual patient, was repeated three times (triplicate).

Results.

Effects of LEF-M alone and with MTX on cytokine production in co-cultured synovial macrophages evaluated by ICC, ELISA and Wb analysis.

Synovial macrophages co-cultured with activated T cell line showed a significant reduction in cytokine content, evaluated as intra-cytoplasmatic protein expression by immunocytochemistry (ICC) following the treatment with LEF-M at different concentrations (1, 10, 30 µM) (Fig.1). The observed changes included a dose-dependent decrease for TNF-α of 13% (p<0.05), 24% and 40% (p<0.01) and for IL-1β of 16% (p<0.05), 39% (p<0.01) and 58% (p<0.001) following the treatment with 1, 10, 30 µM of LEF-M, respectively. For IL-6 we observed a not dose-dependent decrease of 46%, 43%, 44% (p<0.001) when compared with controls (untreated co-culture) (Fig. 2A).

The combined treatment with LEF–M and MTX induced a further reduction of the cytokine intra-cytoplasmatic expression at the level of macrophages (TNF-α of 29% (p<0.05), 37% (p<0.01), 49% (p<0.001); IL-1β 56%, 43%, 50% (p<0.001); IL-6 59%, 62%, 71% (p<0.001)). In particular, a dose-dependent decrease of TNF-α expression was observed, whereas the decrease of IL-1β and IL-6 was not found dose-dependent (Fig. 2B).

A decrease of cytokine concentrations was confirmed in the media of the co-culture after the treatment with LEF-M (different concentrations), when compared with untreated cells (TNF-α 37% (p<0.01), 28%, 27% (p<0.05); IL-1β 6%, 8% (ns), 26% (p<0.05); IL-6 21% (p<0.05), 29% (p<0.01), 14% (ns)). The reduction of IL-1β levels was dose-dependent, in particular in relation to the doses of 30 µM, while the decrease of TNF-α and IL-6 was quite similar for all LEF-M concentrations (Fig. 2C). Interestingly, the treatment of the co-culture with LEF-M in combination with unidoose of MTX, induced a further significant cytokine reduction, particularly for TNF-α 40%, 41% (p<0.01), 44% (p<0.001) and IL-1β 10%, 20% (p<0.05), 60% (p<0.001) and for IL-6 37%, 41% (p<0.01), 49% (p<0.001) (Fig. 2D).

The data obtained with Western blot analysis for both LEF-M alone and LEF-M in combination with MTX were performed with densitometry analyses and confirmed a down-regulation of cytokine intracytoplasmatic expression versus untreated cells; a stronger decrease of signal was evident in the cells treated with LEF-M/MTX. The data were expressed as area and normalized versus control (Fig.3A, 3B).

The comparative analysis of data obtained from immunocytochemistry, Western blot and ELISA assay confirm a reduction of the cytokine production in the SM co-cultured treated with LEF-M in combination with MTX.
Effects of LEF-M alone and with MTX on cytokine mRNA expression in co-cultured synovial macrophages evaluated by multiplex RT-PCR.
The effects of LEF-M, alone or in combination with MTX, on mRNA expression in co-cultured SM were assessed by multiplex RT-PCR analysis for different gene expression. A decrease of the mRNA for all cytokines when compared with the untreated co-cultured SM was detected. The mRNA expression decrease in a not dose-dependent manner (Fig. 4A). Interestingly, the combination of LEF-M and MTX induced a further evident and additive down regulation of the mRNA expression for all the tested cytokines (Fig. 4B).

Effects of LEF-M alone and with MTX on ICAM-1 protein expression evaluated by ICC.
The ICC analysis showed that the treatment with LEF-M induced a decreased expression of the ICAM-1 protein at all the tested concentrations when compared with untreated controls. The treatment with LEF-M (different doses) in combination with MTX (unidose) confirmed a more evident additive decrease of the ICAM-1 expression (Fig. 5A, B, C). These data were confirmed by image analysis (data not shown).

Effects of LEF-M alone and with MTX on COX-1, COX-2 and NF-kB complex mRNA expression
In the present study, the analysis with RT-MPCR for different gene expression showed an evident dose-dependent decrease of the COXs mRNA expression in co-cultured SM after treatment with LEF-M (Fig. 6).
In addition, the mRNA expression for the NF-kB subunit p50/p52 disappeared after 24 hours in treated co-cultured SM at all LEF-M concentrations and the mRNA expression of NF-kB inhibitors decreased in a dose-dependent manner (Fig 6).
Interestingly, in the combination treatment with LEF-M and MTX, a total loss of the mRNA signal was observed for all gene expression here analyzed (data not shown).

Discussion.
The present study shows that the pro-inflammatory activity of cultured RA synovial macrophages is enhanced in presence of activated T-cell line and that the addition of the active metabolite of LEF (A771726), exerts a significant anti-inflammatory effect by decreasing the macrophage production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), the adhesion molecule ICAM-1, COXs, and the NF-kB expression, as a complex of transcriptional molecules modulating cellular responses in activated cells.
The treatment of these co-cultures with the combination of LEF-M and MTX, induced a further significant decrease of the inflammatory mediators above mentioned, supporting a possible additive action of MTX, as already postulated few years ago [22]. The effects of the combination (LEF-M/MTX) versus the presence of the LEF-M alone, was found particularly impressive and significant on the production of the pro-inflammatory cytokines (TNFα, IL-1β and IL-6) by the activated RA macrophages.
Since in the combination experiments the MTX was unidose (50 ng/ml), the observed inhibitory influences here reported were mainly driven by LEF-M, as its dose-related effects (1,10 and 30 µM) still evident after the MTX addition. Furthermore, in our previous studies, no significant antiinflammatory effects by MTX (50 ng/ml) alone were found on cultured RA macrophages, when compared to activated monocytic cells, suggesting a less potent action of MTX on differentiated cells (i.e. macrophages) [17, 18].
The results of the study strongly support a recent clinical investigation showing that RA patients treated with combination of MTX and LEF exhibited a significant suppression of several major chemokines including monocyte-derived chemokine (MCP-1) and macrophage-derived chemokine (MDC-1) [19].
These RA patients have been on treatment with MTX 15 mg/week for not less than 3 months before entry to the study and a loading dose of 100 mg/day of LEF was given for 3 days, followed by 10...
mg/day for the rest of the study period. Moreover, positive correlations among reductions in plasma chemokines and the clinical outcome measures were also found [19]. The study suggests that the combination therapy with LEF and MTX exhibited anti-inflammatory activity in the suppression of chemokine expression and subsequent recruitment of inflammatory cells into the inflammatory sites in RA [19]. In the next studies it would be of interest to utilize an experimental model that involves the RA synovial macrophages in co-cultures with PBL of the same RA patients in order to investigate the effects of LEF-M and MTX combination therapy.

Several clinical studies have recently confirmed that the combination therapy with LEF and MTX improves the clinical responses in active RA patients and that is safe [13, 14, 23]. Therefore, the results of the present in vitro study, seem to confirm the recent observation of a significantly reduced number of macrophages, together with decreased expression of TNF-α, IL1-β, and ICAM-1, in synovial tissue samples obtained from patients with RA after four months of treatment with LEF or MTX [24].

Concerning ICAM-1 expression, LEF-M by depleting the pyrimidine pool seems to down regulate the glycosylation of adhesion molecules, further reducing cell-cell contact activation and homing of inflammatory cells during the inflammatory reaction [10, 25]. LEF-M was found to influence the trans-endothelial migration of peripheral blood mononuclear cells (PBMC) by inhibiting DHODH in treated RA patients (mainly monocytes) [11]. Fluorocytometric analysis of PBMC subsets within the migrated population showed a decrease of monocytes, but not of B or T cells, after LEF-M treatment. Furthermore, incubation with LEF-M of the PBMC from the RA patients also decreased other cell adhesion molecules such as monocytic CD44 expression and PBMC-hyaluronan binding [11].

The present study shows that the combination of LEF-M and MTX induces an additive decreased expression of ICAM-1 on macrophages co-cultured with activated T cell line. Therefore, a further anti-inflammatory mechanism exerted by both drugs in RA, seems related to the decrease of possible cell-cell contact activation [26].

Recently, dynamic gadolinium enhanced magnetic resonance imaging (DRMRI) analysis of synovial tissue in patients with RA treated with LEF as well as with MTX showed for both drugs a significant reduction of the inflammatory reaction [27].

Because the DRMRI correlates with the infiltrating leucocytes in the RA synovial tissue, it was confirmed that LEF as well as MTX exert anti-inflammatory activity mainly on monocytes/macrophages. Actually, very similar effects on basic mechanisms of inflammation have been observed for both LEF-M and MTX in combination or in single treatment [10,15,17,18,28].

The results of the present study support an additive effect of MTX when combined with LEF-M concerning the anti-inflammatory activity with effects on co-cultured macrophages with T cell line. However, the additive or synergistic effect of the combination should be confirmed in a study comparing each drug alone at different doses as well as their combination.

The significant decrease of inducible COX2 but also of COX1 found for the first time in the present study on the mRNA expression, confirms earlier observations that suggest a wide anti-inflammatory effect for both LEF-M, as here shown, and for MTX (data not shown) [10,29,30].

Since LEF-M has also been shown to suppress the activation of nuclear factor κB (NF-κB), a potent mediator of inflammation when stimulated by inflammatory agents, the present study suggests that the inhibition is realized at the level of the mRNA expression, supporting another important target of the LEF mechanisms of action [31-33].

The more detailed effects of LEF-M and MTX (alone or in combination) on the NF-kB complex activation, IκB-α phosphorylation (ser 32) and NF-κB DNA-binding are further matter of an ongoing study.

In conclusion, the combination of MTX and LEF-M shows additive inhibitory effects on the production of inflammatory mediators by SM co-cultured with T cell line. These observations might support the positive results obtained in RA combination clinical studies and might allow a reduced daily dosage of both drugs when combined [34,35].
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Figure legends

Fig. 1
Example of intracytoplasmatic detection of TNF-α, IL-1 and IL-6 by immunocytochemistry assay in co-cultured synovial macrophages (SM) untreated (A, B, C respectively) and treated with LEF-M (30 μM) (D, E, F respectively). (magnification 100x).

Fig. 2
A. Evaluation of TNF-α, IL-1β and IL-6 intracytoplasmatic expression in co-cultured SM treated with LEF-M 1, 10, 30 μM and untreated (control) by immunocytochemistry and image analysis. Results are expressed as mean values ± SD of different synovial macrophage populations obtained from five different RA patients. Each experiment, obtained from an individual patient, was performed 3 times. *= p<0.05 vs control (cnt); **= p<0.01 vs cnt; ***=p<0.001 vs cnt.

B. Evaluation of TNF-α, IL-1β and IL-6 intracytoplasmatic expression in co-cultured SM treated with LEF-M 1, 10, 30 μM in combination with MTX (50 ng/ml) and untreated (control) by immunocytochemistry and image analysis. Results are expressed as mean values ± SD of different synovial macrophage populations obtained from five patients. Each experiment, obtained from an individual patient, was performed 3 times. *=p<0.05 vs control (cnt); **= p<0.01 vs cnt; ***=p<0.001 vs cnt.

C. Evaluation of TNF-α, IL-1β and IL-6 levels in supernatants of co-cultured SM treated with LEF-M 1,10, 30 μM and untreated (control) by ELISA assay. *=p<0.05 vs control (cnt); **= p<0.01 vs cnt.

D. Evaluation of TNF-α, IL-1β and IL-6 levels in supernatants of co-cultured SM treated with LEF-M 1,10, 30 μM in combination with MTX (50 ng/ml) and untreated (control) by ELISA assay. *=p<0.05 vs control (cnt); **= p<0.01 vs cnt; ***=p<0.001 vs cnt.

Fig. 3
A. Western blot analysis of TNF-α, IL-1β and IL-6 protein expression in co-cultured SM untreated (control) and treated with LEF-M 1,10 and 30 μM. right: densitometry analyses.

B. Western blot analysis of TNF-α, IL-1β and IL-6 protein expression in co-cultured SM untreated (control) and treated with LEF-M (1,10 and 30 μM) in combination with MTX (50 ng/ml). right: densitometry analyses.

Fig. 4
A. Expression of cytokine mRNAs evaluated by multiplex RT-PCR.
Lane 1= positive control; lane 2= negative control; lane 3= co-cultured SM untreated; lane 4= co-cultured SM treated with LEF-M (1μM); lane 5= LEF-M (10 μM); lane 6= LEF-M (30 μM); lane 7= markers of molecular weight. Expression of the housekeeping gene β-actin (bottom)

B. Expression of cytokine mRNAs evaluated by multiplex RT-PCR.
Lane 1= co-cultured SM untreated; lane 2= co-cultured SM treated with LEF-M (1μM) in
combination with MTX (50 ng/ml); lane 3 = LEF-M (10µM) and MTX (50 ng/ml); lane 4 LEF-M (30 µM) and MTX (50 ng/ml); lane 5 = positive control; lane 6 negative control; lane 7 = markers of molecular weight. Expression of the housekeeping gene β-actin (bottom).

Fig. 5
Example of intracytoplasmatic detection of ICAM-1 evaluated by immunocytochemistry assay. 
A: ICAM-1 detection in co-cultured SM untreated; B: ICAM-1 detection in co-cultured SM treated with LEF-M (30 µM) alone; C: ICAM-1 detection in co-cultured SM treated with LEF-M (30 µM) in combination with MTX (50 ng/ml) (magnification 50x).

Fig. 6.
Expression of COXs, NF-kB inhibitors and NF-kB complex mRNAs evaluated by multiplex RT-PCR.
Lane 1 = positive control; lane 2 = untreated co-cultured SM (control); lane 3 = co-cultured SM treated with LEF-M (1µM); lane 4 = LEF-M (10 µM); lane 5 = LEF-M (30 µM); lane 6 = markers of molecular weight.
Intracytoplastmatic detection with ICC
Fig. 2B

Intracytoplasmatic detection with ICC
Evaluation in supernatants with ELISA test
Fig. 2D

Evaluation in supernatants with ELISA test
Fig. 3A

TNF-α

IL-1β

IL-6

cnt  1 μM  10 μM  30 μM

[Bar chart showing densitometry analyses for TNF, IL1, and IL6 under different conditions: cnt, 1 μM, 10 μM, 30 μM]
Fig. 3B

Graph showing the densitometry analyses of TNF-α, IL-1β, and IL-6 with methotrexate at different concentrations (cnt, 1 uM, 10 uM, 30 uM).
Fig. 4A

TNF-α
IL-1β
GM-CSF
IL-8
TGF-β

β-act
Fig. 4B

TNF-α →
IL-1β →
IL-6 →
IL-8 →

β-act →

1  2  3  4

1  2  3  4