

CYCLOSPORIN A (CsA) is an immunosuppressor drug that has been used in the treatment of several types of inflammatory diseases. In some of them the inhibition of T-lymphocyte activation does not suitably account for the observed beneficial effect, suggesting that CsA could act on other types of cells. The present study was undertaken to determine the effect of CsA on inflammatory cytokine secretion by U937 monocyte cells. Undifferentiated and dimethylsulfoxide (DMSO) differentiated U937 cells were incubated with different concentrations of CsA (200, 20 and 2 ng/mL) in the presence or absence of phorbol-myristate-acetate (PMA). Interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), IL-6 and IL-8 levels were measured in supernatants using specific enzyme-linked immunosorbent assays. At the highest concentration used (200 ng/mL) CsA decreased the basal and stimulated secretion of all the inflammatory cytokines studied in both undifferentiated and differentiated cells, with the only exception of PMA-stimulated IL-1 secretion by undifferentiated cells. However, only basal secretion of interleukin-8 in both undifferentiated and DMSO-differentiated U937 cells was significantly reduced by CsA at the highest concentration (200 ng/mL). At therapeutic concentrations *in vivo*, CsA exerts a predominant effect on IL-8 secretion by human mononuclear phagocytes.

Key words: Cyclosporin, Cytokine, U937, Macrophage, Monocyte

Effect of cyclosporin A on inflammatory cytokine production by U937 monocyte-like cells

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Introduction

Cyclosporin A (CsA) is an immunomodulatory substance used in the prevention of transplant rejection and in the treatment of autoimmune diseases.¹ Classically, CsA has been reported to exert its immunomodulatory action through its effect on T lymphocytes, mainly helper T lymphocytes, by inhibiting calcineurin dependent interleukin (IL)-2 synthesis.^{2,3} However, it has been suggested that the inhibition of T lymphocyte activation by CsA does not appropriately account for all the effects observed following the *in vivo* administration of the drug.⁴ It is possible that some effects could be due to the action of CsA on other cells.⁵ It should be noted that CsA binds to all human leucocytes. In fact, granulocytes and cells of the mononuclear phagocyte system (MPS) sometimes bind more CsA than lymphocytes, probably because the drug is rapidly internalized.^{2,6}

Cells of the MPS offer an attractive possibility to study the effects of this drug because they participate in a crucial manner in antigen presentation and, in many cases, are the final effectors of the immune system. Many references in the literature report the capacity of CsA to alter the activities of MPS cells. In

general, the capacities, functions and actions of macrophages related to non-specific defense such as chemotaxis, phagocytosis, enzyme release and respiratory burst, are found to be more resistant to CsA.^{7,8} By contrast, those related to immunoregulation or accessory functions, such as monokine production, the expression of histocompatibility antigens and antigen presentation, are more susceptible to the inhibitory action of CsA.^{7,8}

Macrophages synthesize and secrete IL-1 β , tumor necrosis factor (TNF)- α , IL-6 and IL-8.⁹ These monokines are known mediators of the inflammatory response⁹ and are therefore also known as inflammatory cytokines.

The aim of the present study was to evaluate the effect of CsA on the secretion of IL-1 β , TNF- α , IL-6 and IL-8 by U937 monocyte-like cells.

Material and methods

Cell line

U937 cells were kindly supplied by Dr J. Olmos from the Hospital Universitario Marqués de Valdecilla, Santander (Spain). The cell line was kept at 37°C in a

humidified atmosphere with 5% CO₂ in culture with complete medium containing RPMI 1640 (Sigma®), 100 U/mL of penicillin (Sigma®), 100 U/mL of streptomycin (Sigma®), 2 mM L-glutamine (Sigma®) and 10% fetal calf serum (FCS) (Gibco®) in sterile culture flasks (Nunc®) at a concentration between 0.75×10⁵ and 5×10⁵ cells/mL. Cell viability was greater than 90% and the duplication time was between 24 and 48 hours. Cells were regularly screened for lipopolysaccharide (LPS), bacteria, mycoplasma and fungal contamination and found to be negative. Cells were differentiated by incubation over 4 days in complete medium containing 1.3% (0.17 M) dimethylsulfoxide (DMSO).

Cell cultures

5×10⁵ cells together with different stimuli and/or CsA at different concentrations were added to each well of the culture dishes (Costar® 3513, 12 dishes 22.6 mm ø) with complete medium: Lipopolysaccharide from *E. coli* (Sigma®) at 100 µg/mL, 10 µg/mL and 1 µg/mL; phorbol-12-myristate-13-acetate (PMA) (Sigma®) at 10⁻⁴M, 10⁻⁵M and 10⁻⁶M; human gamma-interferon (Sigma®) at 1000, 100 and 10 U/mL and CsA (Sandoz®) at three non-toxic *in vivo* concentrations: 200, 20 and 2 ng/mL.

Cells were kept at 37°C in a humidified atmosphere with 5% CO₂ for 18 h, after which supernatants were centrifuged at 500 g for 10 min to sediment cells in suspension and then stored at -70°C until assayed.

Cytokine determination

The inflammatory cytokines were determined by direct double-sandwich enzyme-linked immunosorbent assay (ELISA) in the supernatants. BIOTRAK® (Amersham, UK) commercial kits specific for human IL-1β, TNF-α, IL-6 and IL-8 were used; these showed no cross-reactivity with one another, or with other cytokines. They had a coefficient of variation less than 10%, and the following respective sensitivity limits: 0.3 pg/mL, 4.8 pg/mL, 0.35 pg/mL and 4.7 pg/mL. All samples were measured in duplicate. Results were expressed in picograms/10⁶ cells. None of the components of the culture medium (RPMI 1640, antibiotics, glutamine or fetal calf serum) showed immunoreactivity with the four types of cytokines studied.

Statistical analysis

All data are expressed as means ± SEM. For each cytokine *t*-test for paired data was used to compare the differences in cytokine secretion between CsA treated and untreated cells; *p* values < 0.05 were considered significant.

Results

Differentiation of the U937 line

The data (not shown) confirming that suitable differentiation of the monocyte cell line toward macrophage cells had been achieved were as follows:

- A decrease in the cellular proliferation rate without changing cell viability.
- An increase in adherence to the culture flasks and among cells.
- An increased cytoplasm, the disappearance of nuclear polylobulation and a decrease in nuclear atypias and the number of nucleoli.
- An increase in the content of macrophage enzymes (non-specific esterases).
- An increase in cytoplasmic RNA content.

Determination of type of stimulus and most effective concentration on inflammatory cytokine secretion

In order to study the most suitable type of stimulus for inducing the secretion of inflammatory cytokines and the most effective concentration, U937 cells were used under both differentiated and undifferentiated conditions. Three classic stimuli were studied at three different concentrations: lipopolysaccharide (LPS), phorbol myristate acetate (PMA) and gamma-interferon (γ-IFN). The mean values of the results of the study carried out in duplicate are shown for each cytokine in Fig. 1. Overall, the data obtained indicate that the most potent stimulus of cytokine secretion was PMA at a concentration of 10⁻⁵M.

Secretion of inflammatory cytokines by U937 cells

Once the required experimental conditions had been established, inflammatory cytokine secretion by U937 cells was studied (Table 1). In basal conditions U937 cells secrete large amounts of IL-8, moderate amounts of IL-6 and TNF-α and low amounts of IL-1β. The stimulation with PMA induces a large increase in the secretion of all cytokines. Only IL-6 secretion was stronger in differentiated than in undifferentiated cells.

Effect of CsA on inflammatory cytokine secretion

The results on the effect of CsA on cytokine secretion under both basal and stimulated conditions are shown in Table 1. These data show the mean values obtained in four different assays. At therapeutic *in vivo* concentrations (200 µg/mL) CsA decreased basal and stimulated secretion of all the inflammatory cytokines studied in both undifferentiated and differentiated

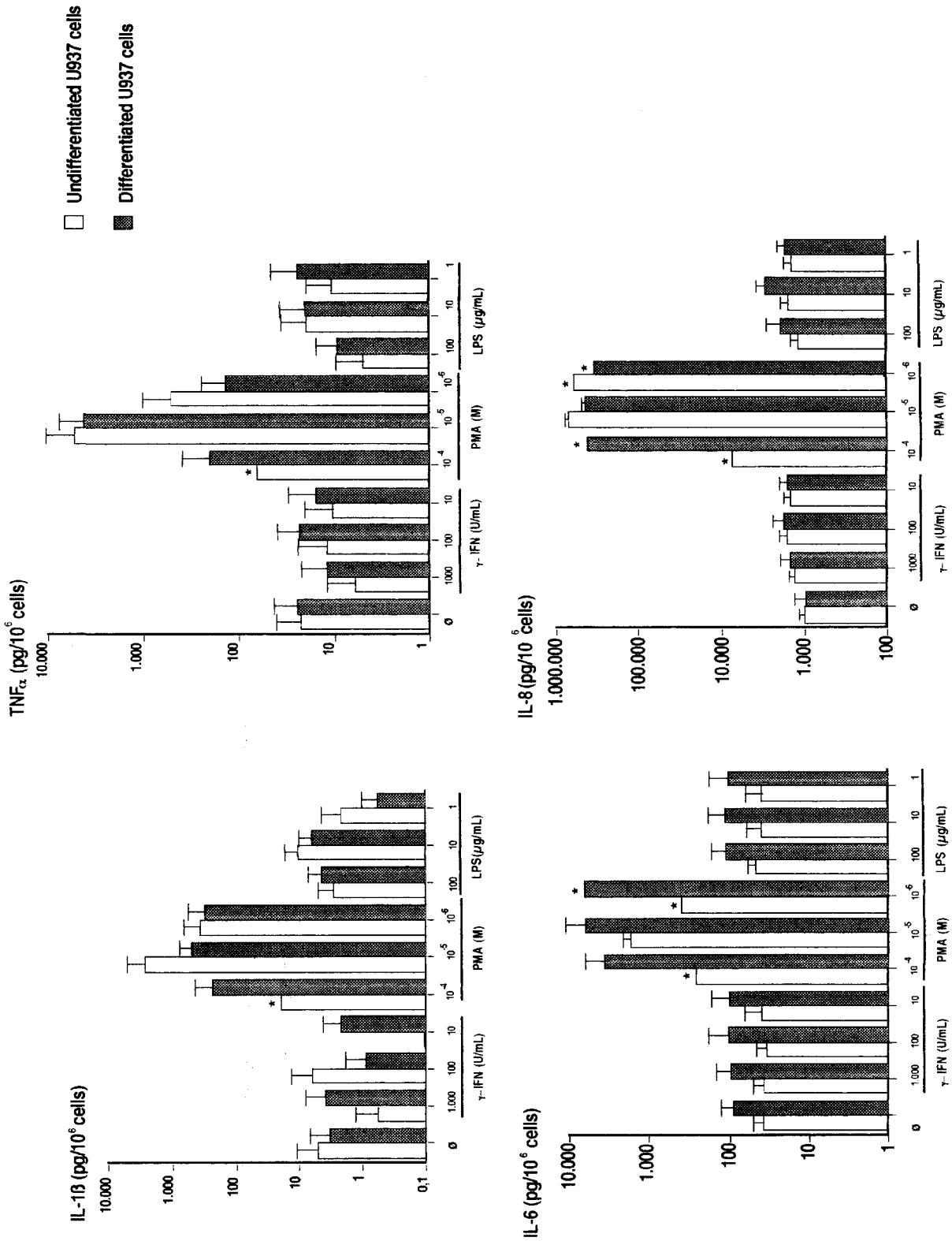


FIG. 1. Effect of different stimuli on IL-1 β , TNF- α , IL-6 and IL-8 secretion by U937 cells. Overall the most potent stimulus for cytokine secretion is PMA at a concentration of 10⁻⁵M. Note the logarithmic scale. (*) Only one valid assay. Definition of abbreviations: IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol myristate acetate, TNF, tumor necrosis factor.

Table 1. Effect of CsA on basal and stimulated IL-1 β , TNF- α , IL-6 and IL-8 secretion by U937 cells (pg/10⁶ cells). The CsA reduction of inflammatory cytokine secretion has statistical significance only in the case of basal IL-8 secretion in both undifferentiated and differentiated cells. Data are expressed as mean \pm SEM

	IL-1	TNF- α	IL-6	IL-8
Undifferentiated				
Basal	5.3 \pm 5.3	22.0 \pm 11.7	38.4 \pm 7.9	979.5 \pm 281.4
+ CsA (2 ng/mL)	10.8 \pm 10.8	0.0 \pm 0.0	37.6 \pm 13.1	1596.7 \pm 323.8
+ CsA (20 ng/mL)	3.6 \pm 3.6	8.4 \pm 8.4	34.0 \pm 12.7	1337.8 \pm 343.9
+ CsA (200 ng/mL)	0.5 \pm 0.5	6.7 \pm 6.7	25.2 \pm 4.2	477.0 \pm 185.6*
+PMA 10 ⁻⁵ M	2717.1 \pm 1733.1	5409.7 \pm 4967.3	1705.1 \pm 313.5	712,123.4 \pm 48,360.1
+ CsA (200 ng/mL)	2857.6 \pm 1258.8	4727.8 \pm 1427.8	731.4 \pm 66.0	469,104.9 \pm 109,861.8
DMSO differentiated				
Basal	3.3 \pm 3.3	17.8 \pm 6.3	90.4 \pm 27.6	948.8 \pm 447.6
+CsA (2 ng/mL)	3.1 \pm 3.1	0.0 \pm 0.0	99.7 \pm 33.3	1732.8 \pm 747.9
+CsA (20 ng/mL)	1.1 \pm 1.1	0.0 \pm 0.0	91.4 \pm 34.2	1452.6 \pm 643.2
+CsA (200 ng/mL)	2.6 \pm 2.6	6.9 \pm 6.1	67.1 \pm 20.6	526.4 \pm 342.6*
+ PMA 10 ⁻⁵ M	496.0 \pm 136.2	4389.3 \pm 3456.0	6343.7 \pm 3135.6	446,567.3 \pm 40,043.9
+CsA (200 ng/mL)	144.0 \pm 144.0	4372.5 \pm 1717.6	2035.1 \pm 1072.6	299,909.3 \pm 43,399.6

**p*<0.05 determined by *t*-test for paired data to compare cytokine secretion between CsA treated and untreated cells. Definition of abbreviations: CsA, cyclosporin A; DMSO, dimethylsulfoxide; IL interleukin; PMA, phorbol myristate acetate; TNF- α , tumor necrosis factor alpha.

cells, with the only exception being PMA-stimulated IL-1 secretion by undifferentiated cells. However, only basal secretion of interleukin-8 in both undifferentiated and DMSO-differentiated U937 cells was significantly reduced by CsA at the highest concentration used (200 ng/mL).

Effect of CsA on cell viability and cell proliferation capacity

The inhibitory effect of CsA might be due to an effect on cell viability and/or proliferation. Subsequently, a duplicate study was performed in which the proliferation capacity and cell viability of U937 cells subjected to the same stimuli as to those used in the study were evaluated. As shown in Table 2, CsA did not affect either viability or proliferation capacity, either in undifferentiated U937 cells or in cells differentiated with DMSO. Therefore the results on cytokine secretion cannot be interpreted in these terms. In this study, the effects of several stimulatory substances

were also assessed. Both PMA and LPS, but not gamma-interferon, were shown to decrease cell viability.

Discussion

The use of the U937 cell line in the evaluation of different macrophage functions is well documented.¹⁰ This human cell line grows continuously in suspension and was initially obtained from a pleural effusion in a patient with histiocytic lymphoma.¹¹ Its phenotype corresponds to immature monocyte cells arrested in a differentiation state close to the myelomonocytic stem cell.¹¹ It should be recalled that macrophages are highly differentiated cells and therefore have low proliferation capacity while cell lines show a strong duplication rate. Thus, the first step in our work was to differentiate the human U937 monocyte cell line. In the presence of several substances U937 cells undergo a process of differentiation and therefore acquire morphological and

Table 2. Effect of CsA and of different stimuli on cell proliferation and viability. In this study we started out with a viable cell concentration of 250,000 mL, obtaining the data after 18h of culture. CsA did not affect either the viability or proliferation capacity of either differentiated or undifferentiated cells. Additionally, both LPS and PMA, but not interferon, decreased cell viability

	Undifferentiated U937 cell line		Differentiated U937 cell line	
	Viable cells/mL	Viability (%)	Viable cells/mL	Viability (%)
Basal conditions	370,000	91	317,000	95
PMA 10 ⁻⁵ M	240,000	84	128,000	76
LPS (100 μ g/mL)	335,000	88	280,000	88
γ -interferon (1000 U/mL)	400,000	94	285,000	92
CsA (200 ng/mL)	385,000	94	300,000	93
CsA (200 ng/mL) + PMA 10 ⁻⁵ M	240,000	77	115,000	81

Definition of abbreviations: CsA, cyclosporin A; LPS, lipopolysaccharide; PMA, phorbol myristate acetate.

functional characteristics similar to those of macrophages.¹² Since one of the interests of the present work was to assess the effect of PMA on cytokine secretion, DMSO was chosen as a differentiating agent because it displays differentiating capacity on the U937 cell line.¹³ Differentiation was evaluated by kinetic, morphological, cytochemical and molecular techniques. The results obtained, which were similar to those reported by other authors,^{11,13} allowed us to conclude that the protocol used is indeed able to promote the differentiation of this cell line.

DMSO-differentiated U937 cells retain their capacity to be stimulated later on with different agents,¹³ among them PMA.¹⁴ It should be noted here that differentiation and activation are not incompatible concepts and that it is in fact possible to employ several substances¹² to activate cells differentiated with other agents. Here, we evaluated the effect of three known macrophage stimulating agents and observed that for this purpose the most suitable agent was PMA at a concentration of 10^{-5} M. Although according to the literature lower concentrations of this substance are usually used,^{11,13} in each experimental system it is necessary to obtain evidence of the most effective concentration in the induction of a given effect. In the present study the above concentration was used. Although at this concentration PMA directly decreases cellular viability, from a quantitative point of view the increase that it induces in cytokine secretion cannot be attributed to the elimination of cytokines by dead cells.

The cytokine secretion pattern observed under both basal and PMA-stimulated conditions in this cell line was similar to that of tissue macrophages such as human alveolar macrophages.^{9,15} Different studies have documented the production of inflammatory cytokines by U937 cells using bioassays,¹⁶ immunoassays¹⁷⁻²⁰ or molecular biology techniques,^{16,19} although none of them used a protocol identical to the one used here. From the quantitative point of view, the results obtained here are similar to those reported by Hass *et al.* regarding IL1, TNF and IL-6¹⁷ and by Bosco *et al.*¹⁹ regarding IL-8, although the former authors¹⁷ did not detect IL-6 secretion, possibly due to the stimulation conditions. By contrast, the values described by Jiang *et al.*¹⁸ for IL-1, TNF and IL-6 and by Nishimura *et al.*²¹ for TNF are lower than our own, although both authors also used different protocols. A different protocol was also used by Srivastava *et al.*,²⁰ who reported much higher amounts of spontaneously secreted IL-8 in comparison with the present values. However, none of these works evaluated the release of the four cytokines studied here jointly.

Only basal secretion of interleukin-8 in both undifferentiated and DMSO-differentiated U937 cells was significantly reduced by CsA at the highest concentra-

tion used (200 ng/mL). Our group has previously demonstrated that CsA decreases IL-8 secretion in human alveolar macrophages,²² however Mrowietz *et al.* have found that human monocyte interleukin-8 secretion is not inhibited by CsA²³ but they only studied stimulated secretion and used higher and *in vivo* toxic concentrations of CsA. The effect of CsA on the U937 cell line is not well documented; in fact, only two works have evaluated the action of this immunomodulator on cytokine secretion.^{10,24} In this sense, Tadmori *et al.*²⁴ have shown that CsA decreases TNF- α secretion in U937 cells, although these authors used extremely high concentrations, and Keicho *et al.*¹⁰ report the inhibitory effect of this immunomodulator on IL-1 α secretion. In these two works incubations were higher than 24 h.^{10,24}

The CsA reduction of IL-8 secretion by human macrophages is an interesting finding that may help to explain the efficacy of CsA treatment in diseases like psoriasis or pulmonary fibrosis in which neutrophilic infiltration is a key pathogenetic event.^{7,25,26} It has been demonstrated that CsA inhibits lymphocyte chemotaxis²⁷ and pulmonary recruitment of inflammatory leukocytes⁵ and that interleukin-8—a chemokine that recruits and activates neutrophils—is a cyclosporin A binding protein.²⁸ Furthermore the cyclosporin binding proteins or cyclophilins are chemotactic for eosinophils and neutrophils²⁹ and their chemotactic activity is inhibited by CsA.²⁹ Therefore it seems that CsA modulates chemotaxis.²⁹ In the opinion of some authors, blocking of chemotaxis may be the principal reason for the therapeutic effect of cyclosporin in patients with diseases like psoriasis.³⁰

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