

Glucocorticoids up-regulate constitutive interleukin-10 production by human monocytes

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Summary

Background IL-10 plays an immunosuppressive role in inflammatory responses. Increased plasma levels of IL-10 have been detected in patients under glucocorticoid (GC) therapy, indicating that steroids may exert their suppressive effect, in part, by increasing IL-10 production.

Objectives The aim was to define possible mechanisms by which steroids up-regulate IL-10 production. To this end, we have analysed *ex vivo* the effect of GCs on the constitutive production of IL-10 by lymphocytes and cells of myeloid origin.

Methods Monocytes and T cells were isolated by a Percoll gradient and B cells were purified by rosetting. Protein and mRNA IL-10 levels were determined by ELISA and by Northern blot, respectively.

Results Monocytes, but not T or B cells, up-regulated the constitutive production of IL-10 following pre-treatment for at least 12 h with physiological doses of dexamethasone (Dex). Up-regulation of IL-10 occurred at both protein and mRNA levels, probably indicating that the effect of Dex was by incrementing gene transcription. Other steroids had similar outcomes, their effects being dose-related, proportional to the steroid potency and totally reversed by the steroid antagonist RU486. Thus, transcript levels of IL-10 were up-regulated by GCs probably through binding of the GC receptor to its specific glucocorticoid response element sequence in the IL-10 promoter. In contrast to monocytes, differentiated immature macrophages and dendritic cells did not vary their constitutive IL-10 production after pre-treatment with Dex.

Conclusion Our results support the fact that steroids up-regulate constitutive IL-10 production by selectively triggering activation signals on monocytes.

Keywords dexamethasone, glucocorticoids, glucocorticoid receptor, GRE, IL-10, monocytes, pre-treatment, RU486

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Introduction

IL-10 is an anti-inflammatory cytokine that is produced by a large variety of cell types including Th0, Th1 and Th2 subsets, B cells, eosinophils, mast cells and monocyte/macrophages, the latter being the most important source in humans [1–3]. Although IL-10 may have immunostimulatory properties, it is best known for its immunosuppressive capacities, mediating the down-regulation of monocyte/macrophage effector functions [4] and the production of pro-inflammatory cytokines such as TNF- α , IFN- γ , IL-12, IL-8 and IL-1 β [5, 6]. In the allergic inflammatory response, the immunosuppressive effects of IL-10 are exerted through its ability to down-regulate IL-4 and IL-5 expression [1], to inhibit IgE production [7], to shorten eosinophil survival [8] and to induce allergen-specific tolerance [9]. Recently, an attenuated IL-10 production was found in severe persistent asthma com-

pared with mild asthma [10], supporting the hypothesis that a deficiency in this cytokine could lead to a more intense inflammatory response. In addition, polymorphic variations in the IL-10 gene promoter associated with low IL-10 production have been shown to be linked with allergies and asthma [11, 12]. A potential therapeutic effect of IL-10 on asthma has also been demonstrated since its administration to sensitized mice inhibited antigen-induced cellular recruitment [13].

Glucocorticoids (GCs) are immunosuppressive drugs widely used in the treatment of autoimmune and allergic diseases. The production of a large number of cytokines with inflammatory properties, such as IL-1, IL-2, TNF- α , IFN- γ , granulocyte macrophage-colony stimulating factor (GM-CSF), IL-6 and IL-12, is down-regulated by GCs. In contrast, data concerning the role of GCs in regulating the production of anti-inflammatory cytokines are contradictory. Most published studies have shown that induced IL-10 expression is either not affected or positively affected by GCs [14–17], whereas a few have reported a down-regulatory effect [18, 19]. *In vivo*, a positive correlation between steroid treatment and increased plasma levels of IL-10 has been found in

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cardiopulmonary by-pass [20], endotoxemia [21], multiple sclerosis [22] and rheumatoid arthritis [23]. Inhaled corticosteroids also increased IL-10 expression in serum [24] and in bronchoalveolar macrophages from asthmatic patients [25, 26]. Moreover, in normal subjects a significant increase in plasma IL-10 was also observed following injection of hydrocortisone, indicating that the positive modulation of GCs was also exerted in normal physiological conditions [27].

In the present report, we wished to know the cellular and molecular mechanisms contributing to the up-regulation of the constitutive production of IL-10 by GCs in the absence of immunological challenges, trying to mimic *in vivo* conditions. By analysing the effect of dexamethasone (Dex) on freshly unstimulated peripheral blood mononuclear cells (PBMCs), we have shown that this steroid increased IL-10 production on monocytes, but not on T and B lymphocytes. The effect was steroid specific and mediated by the activated glucocorticoid receptor (GR).

Methods

Reagents

Water-soluble Dex and hydrocortisone were provided by Sigma Chemical Co. (St Louis, MO, USA). Methylprednisolone (Urbason[®], Hoechst Ibérica, Barcelona, Spain) and deflazacort (Zamene[®], Menarini Laboratories, Barcelona, Spain) were obtained as pharmaceutical suspensions. All GCs were diluted in culture medium. The glucocorticoid antagonist RU486 was provided by Roussel Uclaf (Romainville, France), dissolved in ethanol and diluted in culture medium to obtain a final concentration of ethanol less than 0.01%. Phenol-extracted lipopolysaccharide (LPS) was from Sigma Chemical Co. and TNF- α was from R&D Systems (Abingdon, UK).

Cell purification, differentiation and culture

Cells were obtained from peripheral blood by venipuncture of healthy controls who voluntarily accepted to participate in this study. PBMCs were prepared by Ficoll–Hypaque density centrifugation. Monocytes were isolated by a Percoll gradient [28]. Briefly, a Percoll solution was prepared by mixing 1.65 mL of $10 \times$ Hank's balanced salt solution, without Ca^{2+} and Mg^{2+} (Sigma Chemical Co.), with 10 mL of Percoll (Pharmacia, Uppsala, Sweden). Eight millilitres of the Percoll solution were mixed with 4 mL of PBS with BSA (1 mg/mL) containing 50×10^6 PBMCs in a 15 mL polypropylene tube. The mixture was centrifuged at $345g$ in a fixed angle rotor at room temperature for 25 min without brake. Monocytes were recovered from the cloudy layer in the top 5 mm of the gradient. The monocyte-cell population obtained was typically more than 80% surface CD14⁺ as estimated by flow cytometry. T cells were recovered from the gradient pellet and were more than 95% CD3⁺. B cells were obtained from the tonsils of children with chronic tonsillitis by removing T cells that were rosetted with aminoethylisothiuronium-treated sheep red blood cells. Unrosetted cells in the interphase were collected and were typically more than 98% surface CD19⁺.

Cultures of each cell type were carried out at a concentration of 5×10^6 /mL in RPMI 1640 medium containing

2×10^{-3} M L-glutamine and Hepes (Bio Whitaker, Verviers, Belgium) and supplemented with antibiotics. Cells were incubated at 37 °C and 5% carbon dioxide. After the pre-incubation period with or without steroids, cells were washed twice with pre-warmed RPMI 1640 and cultured in RPMI plus 2.5% of a pool of human AB sera.

Macrophages and dendritic cells (DCs) were differentiated from purified monocytes by culturing at 1×10^6 cells/mL in RPMI supplemented with 10% FCS (ICN Flow, Costa Mesa, CA, USA) for 7 days in the presence of, respectively, GM-CSF (10 ng/mL) or GM-CSF plus IL-4 (10 ng/mL) (R&D). The differentiation stage was assessed by flow cytometry using anti-CD14-FITC (Becton Dickinson, Mountain View, CA, USA), anti-CD1a-PE (Pharmingen, San Diego, CA, USA), anti-DR-FITC (Becton Dickinson) and anti-CD86-PE (Pharmingen) mAb antibodies.

THP-1 human monocytic leukaemia and U-937 cell lines were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and established following the manufacturer's instructions.

IL-10 protein quantification

The protein levels of IL-10 were measured by an in-house sandwich ELISA. Microtitre wells were coated overnight with 6 μ g/mL of affinity-purified anti-human IL-10 mAb (R&D) and blocked with 2% casein in TBS (50×10^{-3} M Tris, 150×10^{-3} M NaCl, pH 7.4) for 3 h at 37 °C. Samples and IL-10 standards (R&D) were diluted in TBS containing 1% casein and incubated for 18 h at 4 °C. After washing with TBS/Tween-20 (0.05%), wells were incubated for 2 h with biotinylated anti-human IL-10 Ab (R&D), washed, incubated for 1 h with streptavidin–alkaline phosphatase conjugate (Caltag, Burlingame, CA, USA) and revealed using *p*-nitrophenyl phosphate as substrate. Absorbance was determined at a wavelength of 405 nm. The ELISA detection range was 7.5–4000 pg/mL.

Northern blot analysis

Total cellular RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction. Ten micrograms of RNA were denatured at 65 °C for 15 min in electrophoresis buffer (20×10^{-3} M MOPS, 5×10^{-3} M sodium acetate, 10^{-3} M EDTA; pH 7.0) containing 5% formaldehyde, 50% formamide and ethidium bromide at 30 μ g/mL. RNA was then size-fractionated by electrophoresis through 1.2% agarose gels containing 2% formaldehyde. RNA integrity and equivalent amounts of total RNA/gel were assessed by monitoring 28S and 18S ribosomal RNA (rRNA) under ultraviolet irradiation. RNA was capillary transferred to nylon membranes (Zetaprobe; BioRad, Hercules, CA, USA) and fixed by baking for 2 h at 80 °C.

The probes used were a 0.7-kb pair *Bgl*II–*Hind*III insert of a human IL-10 cDNA (ATCC, Rockville, MD, USA) and a 0.7-kb pair *Eco*RI insert of a human 28S rRNA cDNA (ATCC). The DNA was labelled with [α -³²P]-dCTP (3000 Ci/mmol; Institute of Isotopes, Budapest, Hungary) by the random hexamer priming method (Stratagene, La Jolla, CA, USA). Hybridization was performed at 42 °C for 42 h in 50% deionized formamide, 5 $\times$ Denhardt's solution,

5 × sodium saline phosphate EDTA buffer (SSPE), 1% SDS, 5% dextran sulphate and 100 µg/mL salmon sperm DNA. The membranes were rinsed and washed 3 times with 0.2 × sodium saline citrate buffer (SSC) and 0.5% SDS for 30 min at 65 °C and exposed to radiograph film at -70 °C. Bands on the autoradiograms were integrated by scanning densitometry with the Bio Image Intelligent Quantifier program (Ann Arbor, MI, USA), and values were normalized as a percentage of the control 28S rRNA signal.

Statistical analysis

The Kolmogorov–Smirnov test was used to assess the normal distribution of the data. The Student's *t*-test and the Wilcoxon signed rank test were used for paired comparisons of normally and not normally distributed data, respectively. A *P*-value of less than 0.05 was considered to be a significant difference between groups. The SPSS 9.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for all calculations.

Results

GCs increase IL-10 production by unstimulated human monocytes

PBMCs and isolated T cells, B cells and monocytes were directly cultured *ex vivo* for 24 h with Dex. Levels of IL-10 production by all cell populations analysed did not vary by adding different doses of the GC (data not shown). However, when the cells were pre-incubated for 12 h with Dex (10^{-6} M), washed and cultured in medium alone for 24 h, an up-regulation of the constitutive IL-10 production was observed in PBMCs and monocytes, but not in T and B cells, as compared with pre-incubation in medium alone (Fig. 1). This effect was not observed unless the steroid was removed after the pre-incubation period. Peak IL-10 responses were reached when monocytes were pre-incubated with Dex for at least 12–18 h, declining at longer pre-incubation periods (Fig. 2). No selective cell death by apoptosis was detected during the pre-incubation period with Dex as assessed by cytofluorometric analysis (data not shown). Dex also increased mRNA IL-10 levels in steroid-pre-incubated monocytes as shown by Northern blot analysis (Fig. 3), indicating that one mechanism by which Dex exerts its positive effect is by modulating IL-10 transcript levels.

A similar positive effect on IL-10 production was observed with other GCs, such as the synthetic steroids methylprednisolone (MP) and deflazacort (DFZ) and the natural GC cortisol (hydrocortisone, HC). As can be observed in Fig. 4a, the up-regulation of IL-10 protein levels on human monocytes by different GCs was dose-dependent and correlated with the steroid potency. Dex, which has a high GC potency, was very efficient up-regulator of IL-10 even at the low 10^{-8} M concentration, whereas the other two synthetic steroids, MP and DFZ, with a lower steroid potency, required their use at a high concentration of 10^{-5} M to achieve similar effects. The levels of IL-10 were only slightly increased by a high dose of the natural corticosteroid HC according to its lowest steroid potency. Addition of the GR antagonist RU486 significantly abolished the positive effect of all drugs

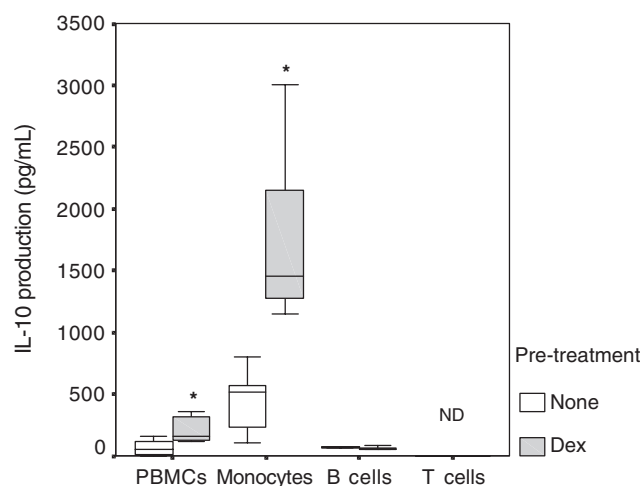


Fig. 1. Modulation of IL-10 production by Dex pre-treatment on different peripheral blood cell populations. PBMCs, monocytes, T and B cells (5×10^6 cells/mL) were pre-incubated with or without Dex (10^{-6} M) for 12 h. After washing, cells were cultured for an additional period of 24 h in the absence of the steroid. Culture supernatants were collected and assayed for IL-10 production by ELISA. Statistical comparisons were performed with the Wilcoxon signed rank test for monocytes and with the Student's paired *t*-test for PBMCs and B cells. Data are represented as the median and range of seven different experiments for PBMCs and monocytes and of five different experiments for B and T cells. **P* < 0.05. ND, not detected.

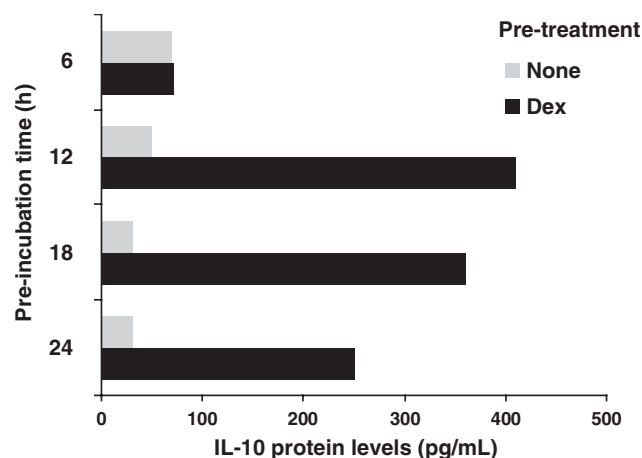


Fig. 2. Effect of the pre-incubation time on Dex-mediated up-regulation of IL-10 production by monocytes. Monocytes (5×10^6 cells/mL) were pre-treated for different periods of time with or without Dex (10^{-6} M). After washing, cells were cultured for an additional period of 24 h in the absence of the steroid. Culture supernatants were collected and assayed for IL-10 production. Data are from a single experiment representative of three independent experiments with similar results.

analysed, indicating that GC-mediated IL-10 up-regulation on human monocytes is steroid-specific and is mediated by the GR (Fig. 4b). In contrast, female sex steroids, such as oestradiol and progesterone, were unable to up-regulate IL-10 production by pre-cultured monocytes (data not shown).

Regulation of LPS- and TNF- α -induced IL-10 expression by Dex

We also considered it of interest to analyse the effect of steroids on LPS- and TNF- α -induced IL-10 production by

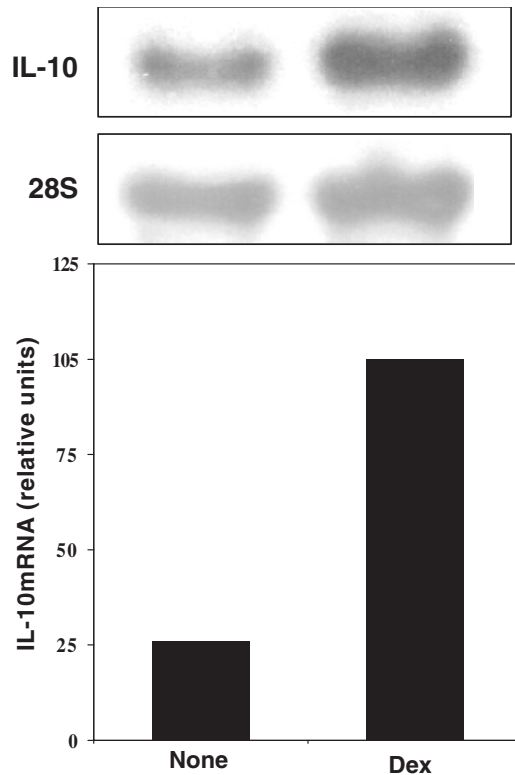
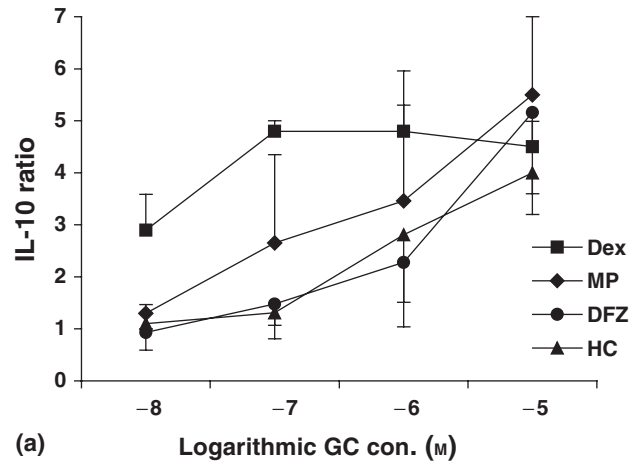


Fig. 3. Effect of Dex on IL-10 mRNA expression on monocytes. Monocytes (5×10^6 cells/mL) were pre-treated for 12 h with or without Dex (10^{-6} M). After washing, cells were cultured for an additional period of 4 h in the absence of the steroid. Total RNA was extracted and Northern blot analysis was performed by hybridization with an IL-10 probe and, subsequently, with a ribosomal 28S probe as a constitutive standard (upper panel). The IL-10 hybridization signal was integrated by scanning densitometry and expressed as relative units normalized to the 28S signal (lower panel). Results shown are representative of two separate experiments.

human monocytes. After a pre-incubation period of 12 h with or without Dex (10^{-6} M), monocytes were cultured for 24 h with LPS ($1 \mu\text{g/mL}$) or TNF- α (10 ng/mL). As shown in Fig. 5a, steroid pre-treatment did not affect IL-10 production induced by TNF- α stimulation. In contrast, pre-incubation with Dex significantly diminished IL-10 protein levels by LPS stimulation compared with Dex-untreated cells. The presence of the steroid during the whole culture period considerably down-regulated IL-10 levels produced by TNF- α and LPS-stimulated monocytes (Fig. 5b). Therefore, Dex exerts different regulatory functions on the constitutive and induced IL-10 production by monocytes.

Up-regulation of IL-10 expression by Dex is dependent on the stage of myeloid differentiation

As Dex-mediated up-regulation of constitutive IL-10 expression in PBMCs was restricted to monocytes, we thought it of interest to assess the effect of the steroid on other cell types of myeloid origin, such as macrophages and DCs as well as on the cell lines THP-1 and U937. Immature macrophages and DCs were obtained following a 7-day incubation period of monocytes in the presence of GM-CSF or GM-CSF plus IL-4, respectively. Monocyte-derived macrophages expressed high



(a) IL-10 ratio vs. Logarithmic GC con. (M)

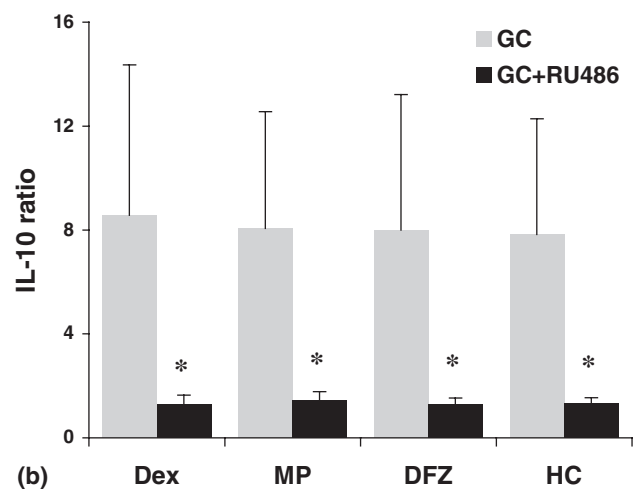


Fig. 4. Up-regulation of IL-10 production by different GCs is dose-dependent, steroid-specific and mediated by the GR. Monocytes (5×10^6 cells/mL) were pre-incubated for 12 h with the synthetic steroids Dex, methylprednisolone (MP) and deflazacort (DFZ) and of the natural corticosteroid cortisol (hydrocortisone, HC). (a) Different concentrations (10^{-8} – 10^{-5} M) of GCs were tested. (b) Pre-cultures were carried out with the GCs at a concentration of 10^{-5} mol/L in the presence or absence of RU486 (10^{-6} M). After washing, cells were cultured for an additional period of 24 h in the absence of the steroid. Culture supernatants were collected and assayed for IL-10 production by ELISA. Statistical comparisons were performed with the Student's paired *t*-test. Results are represented as the mean $\pm$ SEM of the ratio of supernatant IL-10 secretion obtained in the presence/absence of GC pre-incubation. Data were obtained from three independent experiments in (a) and from five independent experiments in (b). **P* < 0.05.

levels of surface CD14 and low levels of CD1a, whereas DCs were typically negative for CD14 and highly positive for CD1a. Both populations had a higher expression of DR-surface molecules and co-stimulatory molecules, as CD86, than monocytes. As shown in Fig. 6, immature macrophages and DCs produced significant quantifiable constitutive amounts of IL-10, which were not incremented by Dex pre-treatment. The cell line THP-1 expressed low levels of IL-10 whereas this cytokine could not be detected in the supernatants of U937 cells. Both cell lines did not up-regulate their constitutive production of IL-10 by Dex pre-treatment (Fig. 6). These results suggest that *in vitro* IL-10 up-regulation by Dex depends on the stage of myeloid differentiation, this being restricted to monocytes.

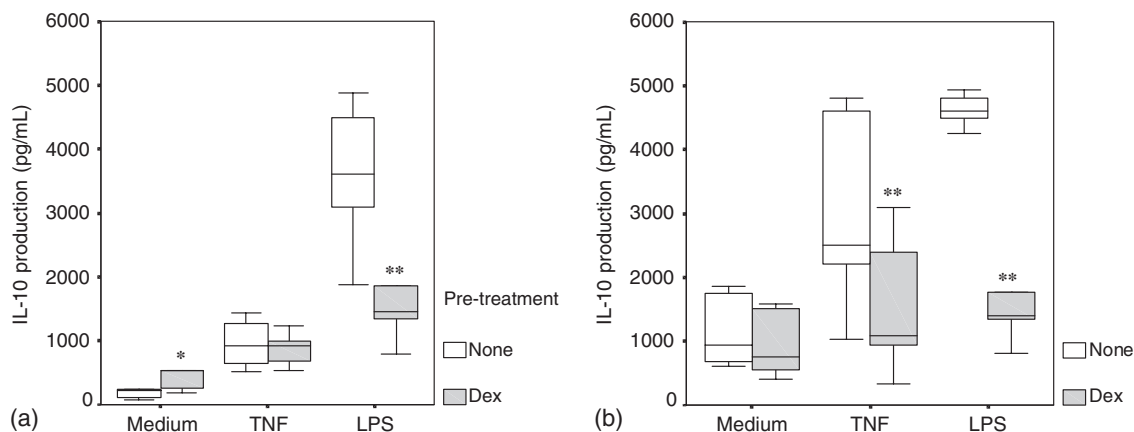


Fig. 5. Dex-mediated regulation of LPS- and TNF- α -induced IL-10 protein expression. (a) Monocytes (5×10^6 cells/mL) were pre-incubated for 12 h with or without Dex (10^{-6} M), washed and cultured for 24 h more in the absence of the steroid with or without LPS (1 μ g/mL) or TNF- α (10 ng/mL). (b) Monocytes were cultured for 24 h in medium alone or with LPS (1 μ g/mL) or TNF- α (10 ng/mL) in the presence or absence of Dex during the whole culture period. IL-10 protein levels in culture supernatants were measured by ELISA. Statistical analyses were performed with the Wilcoxon signed rank test for the IL-10 constitutive production by monocytes and with the Student's paired *t*-test for the LPS- and TNF- α -induced production. Results are presented as the median and range of five different experiments. **P* < 0.05 ***P* < 0.01.

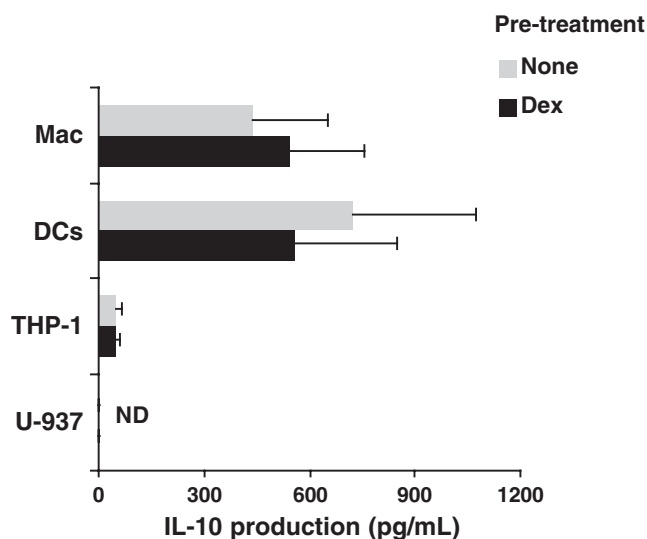


Fig. 6. Modulation of IL-10 production by Dex pre-treatment on different cell types of myeloid origin. Macrophages (Mac) and dendritic cells (DCs), differentiated from peripheral monocytes, and the cell lines THP-1 and U937 were pre-incubated for 12 h with or without Dex (10^{-6} M). After washing, cells were cultured for an additional period of 24 h in the absence of the steroid. Culture supernatants were collected and assayed for IL-10 production by ELISA. Statistical comparisons were performed with the Student's paired *t*-test. Data are represented as mean $\pm$ SEM of seven different experiments for Mac and DCs and of five different experiments for THP-1 and U-937 cell lines. ND, not detected.

Discussion

The purpose of this study was to know the mechanism by which steroids incremented *in vivo* the concentration of circulating IL-10 levels as this has been well established in previous reports [20–23]. To this end, we have analysed, for the first time, the effect of GCs on the constitutive production of this cytokine by *ex vivo* unstimulated cells. Other previous works had analysed the effect of these hormones on IL-10 secretion induced by polyclonal activators [14–19]. Our data show that the synthetic steroid Dex, up-regulates the

spontaneous production of IL-10 by unstimulated human monocytes, whereas this treatment has no effect on the basal production of IL-10 by peripheral T or B lymphocytes. This finding indicates that the *in vivo* steroid-induced up-regulation of IL-10 is mediated by selectively triggering activation signals on monocytes.

Isolated monocytes pre-cultured with Dex significantly produced more IL-10 than monocytes pre-incubated without the steroid. Pre-incubation with Dex was a mandatory requisite since the presence of Dex during the whole culture period did not significantly change the constitutive production of IL-10. Furthermore, the pre-treatment time period appeared to be critical since peak responses were obtained when these cells were pre-incubated with the steroid for 12 h. It is known that steroids are plurifunctional drugs which, in general, may exert positive and negative effects, the former being anticipatory and produced at basal steroid levels. These functional characteristics of steroids may explain the necessity of removing the drug from the monocyte cultures in order to observe a positive action on IL-10 production, since a prolonged exposure of cells to the steroids could allow these hormones to achieve a putative suppressive effect. In fact, it is known that GC-mediated regulation depends on the intracellular levels of the GR and its co-activators, some of which, such as SRC-1, are hormone modulated [29–31]. This may also explain the different effects of GCs in monocytes and in T and B cells, because a tissue-specific expression of GR and co-regulators has also been described [31, 32]. Similarly, to '*in vitro*' findings, an '*in vivo*' pre-exposure of cells to GCs may be required for IL-10 up-regulation since serum peak levels of this cytokine were observed 4 h following steroid administration [20, 27]. The steroid specificity of our observation is supported because the effect correlated with the steroid potency and was dose-dependent. Furthermore, it was reverted by the GR antagonist RU486.

Steroids up-regulate IL-10 production in monocytes by incrementing IL-10 mRNA concentration as we have shown by Northern blot analysis, indicating that Dex is probably acting at a transcriptional level. Enhancement of transcription by GCs is mediated by binding of the activated GR to a

glucocorticoid response element (GRE) in the promoter of the target genes. Therefore, Dex-induced GR activation and binding to the GRE motif described in the IL-10 promoter [33, 34] may be involved in the GC-dependent IL-10 up-regulation on monocytes, although activation of other transcription factors may contribute to this outcome. In fact, the transcription factor Sp1, which is known to interact with the GR–GRE motif [35] and which is an important regulator of the basal transcription of IL-10 [36], may certainly play a role in Dex-mediated IL-10 transcription. Nevertheless, we cannot rule out that Dex also operates at a post-transcriptional level modifying IL-10 mRNA half-life, protein translation or secretion.

Pre-treatment with GCs also influences mitogen and TNF- α -induced IL-10 production by monocytes. TNF- α -dependent production of IL-10 was not affected by steroid pre-treatment whereas these drugs significantly down-regulated cytokine production induced by LPS. This differential regulation is certainly due to diverse effects of steroids on the specific pathways triggered by each activator. Previous published data are contradictory, since both positive [15, 17] and negative effects [18, 25] have been described on the LPS-induced IL-10 levels produced by human monocyte/macrophages. Different experimental conditions, such as dose and time of GC addition, may account for these variable results.

In addition, we tested the effect of Dex on constitutive IL-10 production by immature macrophages and DCs differentiated from peripheral monocytes. These cells spontaneously produce considerable amounts of IL-10 but, in contrast to monocytes, production of this cytokine was not affected by Dex pre-treatment. We cannot discard that the negative effect of Dex in macrophages and DCs is due to a recent exposure to cytokines. A recent article described an increased production of IL-10 by immature DCs following continuous exposure to Dex during the differentiation process [37]. Variable results have been published on the effect of steroids on IL-10 secretion by mature DCs activated with different stimuli [38–41]. Further studies are necessary to clarify the effect of steroids on IL-10 secretion by these cells.

In view of our results, it is feasible that steroids may exert anti-inflammatory effects indirectly by increasing the constitutive production of IL-10 by monocytes, which in turn could diminish a broad spectrum of immunoresponses.

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