Glucocorticoid treatment inhibits annexin-1 expression in rheumatoid arthritis CD4\(^+\) T cells

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**Objective.** Annexin-1 (Anx-A1) has been recently shown to play a key role in T-cell activation and to be highly expressed in T cells from RA patients. Here, we investigated the effects of glucocorticoids (GCs) on Anx-A1 expression in T cells in vitro and in vivo.

**Methods.** To evaluate the effects of dexamethasone (Dex) on Anx-A1 expression, human peripheral blood T cells were incubated with Dex and then analysed by real-time PCR and western blotting. Similar experiments were carried out in vivo by measuring Anx-A1 levels in T cells from patients with RA before and after administration of steroids.


**Conclusions.** GCs suppress Anx-A1 expression in T cells in vitro and in vivo. These results provide evidence for a novel pathway by which steroids regulate the adaptive immune response and suggest that Anx-A1 may represent a target for the treatment of autoimmune diseases.

**Key words:** Annexin-1, Glucocorticoids, T cells, RA.

**Introduction**

Glucocorticoids (GCs) are extensively used for the treatment of many inflammatory and immune-mediated diseases [1]. Their wide range of therapeutic effects might be explained by their ability to target cells of both the adaptive and innate immune systems via genomic and non-genomic mechanisms [2, 3]. Studies over the last 20 yrs have increased our knowledge of the molecular mechanisms by which GCs modulate T-cell function and we now know that GCs antagonize both proximal and distal events of the T-cell activation cascade [4, 5]. In all instances, these studies have provided important information about the signalling pathways and the genes modulated by GCs but at same time have raised important and unresolved questions: how can GCs affect several signaling pathways at once? Is there a common molecular target that mediates this wide range of GC effects on T cells?

Annexin-1 (Anx-A1) is an endogenous anti-inflammatory protein, which has been shown to mediate several inhibitory effects of GCs. Recent studies carried out in our laboratory have highlighted a novel role for Anx-A1 in T cells. Anx-A1 plays a homeostatic role in T cells by modulating the strength of TCR signalling. Using both biochemical and genetic approaches, we have shown that high levels of Anx-A1 lower the threshold of T-cell activation [6], whereas low levels of Anx-A1 impair T-cell activation [7]. Despite this novel information on the importance of Anx-A1 as a T-cell modulator and in mediating some of the pharmacological effects of GCs, the role of Anx-A1 in mediating the pharmacological effects of GCs in T cells has so far been largely neglected.

In this study, we investigated the effect of GC treatment on Anx-A1 expression in T cells in man. Our results have shown that GCs decrease Anx-A1 expression in T cells and that this effect correlates with the immunosuppressive effects of these steroids in vitro and in vivo.

**Subjects and methods**

**Reagents**

Anti-human CD3 (clone OKT3) and anti-human CD28 (clone CD28.2) were purchased from eBioscience (San Diego, CA, USA). Endotoxin-free human recombinant Anx-A1 (hrAnx-A1) was prepared as described [8]. The monoclonal antibody for human Anx-A1 has been previously described [9]. Unless otherwise specified, all the other reagents were from Sigma-Aldrich (Saint Louis, MO, USA).

**Patients**

All patients gave written informed consent and the study was approved by the ethics committee of the host institution. Five RA patients were studied before and 48 h after intramuscular steroid therapy [120 mg Depo-Medrone (Pfizer, Sandwich, UK); methylprednisolone acetate], which was administered to control their synovitis. All patients fulfilled 1987 ARA criteria for RA and had established disease [10]. Four of the five patients were female, median age 55 yrs, median disease duration 10 yrs. Details are shown in the Table 1 and Fig. 2; none of the patients were taking oral steroids. Peripheral blood mononuclear cells were prepared from peripheral blood and CD4\(^+\) cells were selected from peripheral blood using positive selection as previously described [6]. Briefly, peripheral blood was subjected to Ficoll density centrifugation (Ficoll-Paque Plus, Amersham Biosciences, Little Chalfont, UK). Adherent cells were removed from the mononuclear cells by adherence to serum-coated plastic. Non-adherent cells were incubated with mouse anti-human CD4 antibody (RFT4), washed in buffer (phosphate buffered saline, 0.5% BSA, 2 mM EDTA pH 7.2) and incubated with goat anti-mouse antibody conjugated to a magnetic bead (Miltenyi Biotech GmbH (Bergisch Gladbach, Germany)). Cells were run through a MACS column (Miltenyi Biotech) and CD4\(^+\) cells collected. The purity of the cells was assessed by flow cytometry. The percentage of CD3\(^+\) CD4\(^+\) cells following the 10 depletions was 98% (range 97–99.3%).
Cytokine ELISA
To measure IL-2 production, purified peripheral blood T cells (10⁶ cells/ml) were stimulated by plate-bound anti-CD3 and anti-CD28 for 24 h in 24 well plates. IL-2 levels were measured in culture supernatant by using an antibody-pair set (eBioscience) following the manufacturer’s protocol.

RT–PCR analysis
Total RNA was extracted from cells with Quiaquick mini spin columns [Quiagen GmBH (Hilden, Germany)] according to the manufacturer’s protocol. Total RNA from 2 to 5 x 10⁶ cells was used for first-strand cDNA synthesis with random primers [Invitrogen (Carlsbad, CA, USA)] and AMV reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. Real-time PCR was carried out by using TaqMan Universal PCR Master Mix and fluorescent primers obtained from the Applied Biosystems web site (Assay-on-demand Gene Expression products, available at http://www.appliedbiosystems.com/) as previously described [6].

Western blotting analysis
T cells were incubated with dexamethasone (Dex) (0.1 μM) at 37°C for various time-periods, and then analysed by immunoblotting as previously described [6].

Immunohistochemistry
Cryostat tissue sections of synovial tissue from patients with RA or tonsils from healthy control volunteers were analysed using mouse monoclonal anti-Anx-A1 [9] or anti-CD3 (UCHT-1). Immunohistochemical analyses and subsequent image visualization were carried out as previously described [11].

Results
GCs inhibit Anx-A1 expression in CD4⁺ T cells
To investigate the effect of GCs on Anx-A1 expression in CD4⁺ T cells, we incubated peripheral blood human CD4⁺ cells from healthy control volunteers with Dex for varying lengths of time and then collected the cells for protein and mRNA expression analysis. As shown in Fig. 1A and B, Dex inhibited Anx-A1 protein and mRNA in a time-dependent manner with the greatest reduction occurring at 12 h. This effect was concentration-dependent (Fig. 1C) and not due to cell necrosis or apoptosis as demonstrated by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay or propidium iodide/annexinV staining and was also observed in murine T cells (data not shown). We hypothesized that the reduction of Anx-A1 expression by Dex might be responsible for the inhibitory effect of this steroid on IL-2 production by stimulated CD4⁺ cells. To test this hypothesis, we performed a rescue experiment by adding hrAnx-A1 to CD4⁺ cells that had been incubated with Dex for 12 h (the time after which Anx-A1 expression is almost abolished). As shown in

<table>
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<th>Number</th>
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<td>82</td>
<td>2</td>
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Fig. 1D, incubation of CD4<sup>+</sup> cells with Dex for 12 h followed by stimulation with anti-CD3/CD28 markedly inhibited IL-2 production. Addition of hrAnx-A1 to Dex-treated cells 2 h before anti-CD3/CD28 stimulation produced an almost complete reversal of the inhibitory effect of the GC.

**GCs suppress Anx-A1 expression in CD4<sup>+</sup> T cells from RA patients**

To confirm that these findings were clinically relevant and not simply an in vitro phenomenon, we also measured Anx-A1 expression in CD4<sup>+</sup> cells after in vivo treatment with steroids. We have previously shown that CD4<sup>+</sup> cells from patients with RA express high levels of Anx-A1. We confirmed these results by analysing Anx-A1 expression in T cells from the synovial tissue of patients with RA by immunohistochemistry. Co-staining of synovial tissue with monoclonal anti-Anx-A1 and anti-CD3 (Fig. 2A, second and third panels, respectively) showed a high degree of expression and co-localization. Finally, we measured Anx-A1 expression in CD4<sup>+</sup> cells from RA patients before and after a treatment regimen with 120 mg intramuscular Depo-Medrone (methylprednisolone acetate). Consistent with the in vitro results, administration of the steroid in RA subjects induced a decrease in CD4<sup>+</sup> cell Anx-A1 expression ranging from 50% to 80% (Fig. 2B).

**Discussion**

GCs still represent the most important and frequently used class of immunosuppressive and anti-inflammatory drugs for the therapy of many immune-mediated inflammatory diseases. A large number of studies have shown that some of the pharmacological effects of GCs are mediated by Anx-A1. Historically, the effects of Anx-A1 have largely been studied in cells of the innate immune system where this protein has anti-inflammatory effects. Demonstrated initially using passive immunization strategies [12, 13] it has been more recently substantiated in Anx-A1-deficient mice, which have an exaggerated inflammatory response and partial or complete resistance to the anti-inflammatory effects of GCs [14, 15]. The clinical relevance of these findings has also been confirmed by several studies in humans in whom it has been shown that the amount of cell-associated Anx-A1 in neutrophils and monocytes is increased after systemic or local administration of GCs [16, 17].

Given that Anx-A1 expression is positively up-regulated by GCs in cells of the innate immune system [13, 18], we had predicted, at the beginning of this programme of work, that this protein might inhibit naïve T-cell activation, thereby accounting for the immunosuppressive effects of GCs. However, our recent studies have shown that Anx-A1 plays a positive modulatory role in the adaptive immune response by modulating the strength of TCR signalling. Stimulation of T cells in the presence of high levels of hrAnx-A1 increases their activation and proliferation, whereas Anx-A1 deficient T cells showed an impaired response to either TCR or phorbol 12-myristate 13-acetate/ionomycin stimulation.

In the present study, we show that GC treatment of CD4<sup>+</sup> cells suppresses Anx-A1 expression in a time- and concentration-dependent fashion. This reinforces the hypothesis that Anx-A1 might represent an important target for immunosuppressive therapy. If Anx-A1 is a positive modulator of T-cell responses, then the reduced expression of this protein would induce a state of reduced responsiveness to TCR stimulation: it is noteworthy that such a phenotype has been described in Anx-A1 deficient T cells [7].

Another interesting observation made in this study is the lag period of 6–12 h required to bring about GC-mediated down-regulation of Anx-A1 expression. This time lag has long been recognized as an intriguing aspect of the immunoregulatory effect of GCs on T-cell activation. Studies in the last 10 yrs have indicated distinct mechanisms to account for this phenomenon, including up-regulation of I<sub>κ</sub>B, NFAT, or cells subjected to prolonged exposure to GCs [19–21]. However, the latter mechanism would not explain the delayed effect of steroids on the activation of transcription factors such as Nuclear Factor of Activated T cells (NFAT) and AP-1. The results presented here indicate that GC regulation of Anx-A1 expression could be one of the mechanisms responsible for this delayed phenomenon. This conclusion is reinforced by our recent data with hrAnx-A1, which produced an increase in NF-κB, NFAT and AP-1 activation in anti-CD3/CD28 stimulated T cells.

Previous studies on the effects of Anx-A1 on cells of the innate immune system have led to the model whereby GCs lead to Anx-A1 up-regulation in these cells with consequent anti-inflammatory effects. We now report that the immunosuppressive effects of GCs on T cells are associated with, and at least partially mediated through, down-regulation of Anx-A1. It is therefore tempting to propose a unified model by which Anx-A1 can mediate both the anti-inflammatory and immunosuppressive effects of GCs on cells of the innate and adaptive immune system, an effect attained through reciprocal regulation of Anx-A1 expression. Further analysis of this model could provide a better understanding of the activation and involvement of the Anx-A1 system in relation to the mechanisms of GC therapeutic efficacy, and may have an impact on the future development of more selective anti-inflammatory and immunosuppressive drugs.

**Rheumatology key message**

- Glucocorticoids inhibit Anx-A1 expression in T cells.
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References