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TGF-β neutralization abrogates the inhibited DHEA production mediated by factors released from *M. tuberculosis*–stimulated PBMC

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Supernatants (SN) from cultures of peripheral blood mononuclear cells (PBMC) of tuberculosis (TB) patients inhibit dehydroepiandrosterone (DHEA) secretion by the adrenal cell line NCI-H295R. To analyze whether TGF-β is involved in this effect, SN of PBMC from healthy controls or patients with severe TB infections, stimulated or not with *Mycobacterium tuberculosis* (Mtb SN), were added to adrenal cells under basal conditions or following stimulation with forskolin. Cortisol and DHEA concentrations were evaluated in supernatants of the adrenal cells cultured with or without the addition of anti-TGF-β. Treatment with Mtb SN from TB inhibited DHEA production, and this effect was reversed when SN were treated with anti-TGF-β. The increase in cortisol production induced by SN from TB patients was not affected by TGF-β neutralization. Mediators released during the anti-TB immune response differentially modulate steroid production by adrenal cells, and TGF-β is a cytokine implicated in the inhibition of DHEA production observed in TB.

Keywords: dehydroepiandrosterone; cortisol; TGF-β; NCI-H295R cell line; tuberculosis

Introduction

Tuberculosis (TB) can be traced back for thousands of years and has probably threatened humanity since its origin. It is estimated that, at present, one-third of the world's population harbors the TB bacillus *Mycobacterium tuberculosis*, and that 10% of the infected individuals will develop active TB during their lifetime. This makes TB the single leading cause of death among all infectious diseases.[1] The infection affects mainly the lungs and begins as a nonspecific inflammatory reaction in the alveoli, followed by a gradual migration of macrophages, T cells, and fibroblasts that form a parenchymal granulomatous reaction directed at limiting mycobacterial spreading.[2] Primary infection is mostly self-resolving, although some bacilli persist in the tissues for months or decades, in a nonreplicative state.[3] Most cases of secondary TB (5% of the infected people) are due to reactivation of old lesions, with pulmonary infiltrates and often parenchymal lung destruction, resulting in cavitary lesions. The affected areas include a few foci in the upper lobes and bilateral lung involvement, characterized by intense inflammation, tissue destruction, and fibrosis.[2,3] The type of active disease that develops is dictated generally by the state of the host's immune system. Infection in immunologically competent subjects induces early and late immune responses that ultimately destroy most tubercle bacilli and usually prevent development of clinical disease.[4,5] The first line of host defense against mycobacteria is conferred by macrophages through nonspecific mechanisms, such as phagocytosis, and production of different cytokines. If the infection cannot be controlled, T cells become involved two to three

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weeks later. T cells contribute to defense against mycobacteria by producing cytokines, including interferon gamma (IFN-γ) and interleukin 2 (IL-2). Due to its substantial macrophage-activating effects, IFN-γ contributes to protective immunity against pathogens by activating macrophages to more effectively eliminate microorganisms, whereas IL-2 supports the proliferation of activated lymphocytes.[6] Although TNF-α is involved in *M. tuberculosis* clearance,[7] it may also account for several common features of TB, such as fever, wasting, and tissue damage.[8–10]

In agreement with the downregulatory role of anti-inflammatory cytokines in infections by intracellular pathogens,[2,6] increased IL-4 and IL-10 production has also been detected in patients with severe TB or anergic disease cases.[11–14] The proposal that cytokines such as IFN-γ play an essential role in the control of the *M. tuberculosis* infection is also reinforced by studies from our laboratory and those of others, indicating that patients with less severe forms of pulmonary TB have a predominant IFN-γ response, whereas the production of IL-4, IL-10, and TGF-β mediators, or potentially toxic compounds, predominate in aggravated disease.[15]

In addition to their immunological effects, many cytokines produced during the immune response also influence several neuroendocrine mechanisms, for example, activation of the hypothalamus–pituitary–adrenal (HPA) axis, as part of a well-regulated defense reaction.[16–18] Inflammatory cytokines can stimulate the synthesis of corticotrophin-releasing hormone in the hypothalamus, leading to the release of adrenocorticotropin hormone and the subsequent production of adrenal steroids, glucocorticoids (GCs), and dehydroepiandrosterone (DHEA),[16–18] or they can directly influence the activity of the adrenal gland.[19,20] At physiologic concentrations, GCs are able to shift the immune response from a proinflammatory to an anti-inflammatory cytokine pattern, and can also facilitate humoral immune responses, partly by inhibiting IFN-γ–producing cells.[16–18] Conversely, DHEA stimulates T helper functions by enhancing the capacity of activated T cells to produce IL-2, thus counteracting the inhibitory effect of GC on IL-2 synthesis. At the same time, DHEA seems to synergize with GC anti-inflammatory effects, as this adrenal androgen, like GC, is has potent antiphlogistic activity.[21]

Studies in *M. tuberculosis*–infected mice have shown that stimulation of the HPA axis contributes to disease reactivation.[22] Increased GC levels in *M. tuberculosis*–infected A/J mice were also found to counteract the development of protective Th1 responses,[23] whereas administration of DHEA and androstenediol, a steroid derivative, improves the course of experimental TB infection.[24] Work from the same group also documented important adrenal changes during experimental TB.[25] *In vitro* studies demonstrated that treatment of peripheral blood mononuclear cells (PBMC) from TB patients with physiological concentrations of cortisol inhibits mycobacterial antigen–driven lymphoproliferation and IFN-γ production, whereas DHEA suppresses TGF-β production.[26]

To better approach the clinical situation, we evaluated several immune and endocrine parameters in untreated TB patients with different degrees of lung involvement. Patients were HIV-negative, newly diagnosed, and presented with mild, moderate, or advanced disease. As our laboratory and those of others have shown,[14,27] IFN-γ, IL-10, IL-6, and growth hormone plasma levels were increased in TB patients, compared with healthy controls, in parallel with modest increases in concentrations of cortisol, estradiol, prolactin, and thyroid hormones and a profound decrease in testosterone and DHEA levels.[28] Because of the well-known effects of cytokines on endocrine functions,[16–18] the presence of a partly skewed profile of hormonal alterations may be related to cytokines released during the TB-specific immune response; consistent with this, supernatants of *M. tuberculosis*–stimulated PBMCs from TB patients significantly inhibited DHEA secretion by a human adrenal cell line.[28] TGF-β may be among the cytokines potentially involved in this effect because this cytokine can inhibit DHEA production,[29] and large amounts of TGF-β are detected in supernatants of cultures of *M. tuberculosis*–stimulated PBMCs from TB patients.[30] The study presented here provides evidence that treatment of these supernatants with anti-TGF-β–specific antibodies reverses the inhibition of DHEA production by adrenal cells.

Materials and methods

Study groups

Patients (one female and five males) with no HIV coinfection and newly diagnosed lung TB were

recruited for these studies. Diagnosis of *M. tuberculosis* infection was based on clinical and radiological data together with the identification of TB bacilli in sputum. The age of the patients ranged from 26 to 62 years (42.6 ± 20.1, mean ± SD, years). Disease severity was determined by radiological pattern and was classified as advanced TB. The control population was composed of seven healthy volunteers (healthy controls (HCo), three females, four males) of comparable age without any known prior contact with TB patients. None of the HCo had clinical or radiological evidence of active pulmonary TB, of any other respiratory disease, or of acute, chronic, or immunocompromising diseases or therapies. Additional exclusion criteria included diseases that affect the adrenal glands, the HPA or hypothalamus–pituitary–gonadal axes, corticosteroid treatment, pregnancy, and age below 18 years. Blood samples were obtained from all donors at entry into the study, and in the case of TB patients, before initiation of anti-TB treatment. This work was approved by the Ethical Committee of the Facultad de Ciencias Medicas, Universidad Nacional de Rosario. Participants were enrolled upon obtaining written consent.

Mononuclear cell isolation and in vitro *stimulation*

PBMC were isolated from freshly obtained EDTA-treated blood. In brief, blood was diluted 1:1 with culture medium (CM): RPMI 1640 (PAA Laboratories GmbH, Austria) containing standard concentrations of L-glutamine, penicillin, and streptomycin. The cell suspension was layered over a Ficoll-paque plus gradient (density 1.077, Amersham Biosciences, Piscataway, NJ) and centrifuged at 400 *g* for 30 min at room temperature (19–22 °C). PBMC recovered from the interface were washed three times with CM and resuspended in CM containing 10% of heat-inactivated pooled normal AB human serum (RPMI, PAA Laboratories GmbH). Cells were cultured in quadruplicate in flat-bottomed microtiter plates (5×10^6 cells/well in 1 mL) with or without the addition of whole sonicated, heat-killed H37Rv *M. tuberculosis* (Mtb; 8μg/mL, kindly provided by J.L. Stanford, London). PBMC cultures were incubated for 36 h at 37 °C in a 5% CO_2 humidified atmosphere.

Human adrenal cell line NCI-H295R cultures

The human adrenal cell line NCI-H295R (kindly provided by M. Ehrhart-Bornstein, Dresden, Germany)[31] was cultured in DMEM/F12 medium supplemented with L-glutamine and HEPES (Gibco), $NaHCO_3$ (1.2 g/L), insulin (379.47 ng/mL), hydrocortisone (3.625 ng/mL), estradiol (2.724 ng/mL), transferrin (10 μg/mL), selenite (5 ng/mL) (all from Sigma-Aldrich), penicillin (100 U/mL), streptomycin (100 μg/mL; Biochrom, Germany), and 2% heat-inactivated FCS (Gibco). Cells (70,000 cells/cm^2) were cultured until 60–70% confluence (1.5×10^5 cells/well/400μL CM) in flat-bottom 24-well plates (Corning Costar, Cambridge, MA). Five days later, when cells were at the exponential growth phase, the medium was removed and 200 μL from pools of supernatants obtained from Mtb-stimulated or unstimulated PBMC and 200 μL of fresh medium were added to the cells. In a series of cultures, forskolin (FK; 2×10^{-5} M; Tocris, Biotrend, Germany) was used to stimulate the adrenals cells. Each treatment was assayed in quadruplicate. Supernatants from these cultures were obtained after 48 h and frozen at −20 °C until used for hormone determinations.

In summary, adrenal cells received either (1) CM alone (RPMI, as described in the previous paragraph); (2) supernatants from PBMC obtained without further stimulation (basal); or (3) supernatants from mycobacterial antigen-stimulated PBMC (Mtb SN).

TGF-β neutralization

In a first step, adrenal cell cultures at the exponential growth phase were treated with recombinant TGF-β (Sigma Aldrich, St. Louis, MO) at a range of concentrations corresponding to the amounts detected in TB patients and HCo (4,338 to 9,916 pg/mL). This set of experiments demonstrated that 6,100 pg/mL of TGF-β, the average concentration in supernatants of antigen-stimulated PBMC from patients with a severe disease, caused a 64% inhibition of DHEA production compared with untreated cultures (data not shown). Thus, this concentration was used as reference to determine the dilution of the anti-TGF-β antibody (Santa Cruz Biotechnology, CA) necessary to neutralize TGF-β in the supernatants. On the basis of the results of pilot neutralization studies, the relations used in the final experiments were 25:1, 10:1, and 1:1 (a 1:1 ratio corresponds to 6,100 pg/mL anti-TGF-β: 6,100 pg/mL TGF-β). Parallel cultures in which adrenal cells were treated only with the various anti-TGF-β

concentrations were also performed for comparison purposes.

Hormone determinations

Cortisol and DHEA concentrations in culture supernatants of adrenal cells were determined using commercially available ELISA kits, according to the instructions of the manufacturer (DRG Systems, Marburg, Germany). The detection limits were 2.5 ng/mL for cortisol and 0.1 ng/mL for DHEA.

Statistical analysis

Data are shown as mean ± SEM of four independent determinations (corresponding to four individual cultures per group). Statistical comparisons were performed by the Kruskall–Wallis and Mann–Whitney U tests. $P < 0.05$ was considered statistically significant.

Results

TGF-β concentration was determined in supernatants of 36-h cultured PBMC from TB patients and controls. PBMC were either nonstimulated or stimulated with Mtb. Basal and *M. tuberculosis*–driven TGF-β production by PBMC from TB patients (5785.5 ± 797.6 pg/mL and 6757.7 ± 790.5 pg/mL, respectively) was higher than in cultures from HCo (4404.2 ± 1096.5 and 5692.1 ± 994.2 pg/mL, respectively), but the trend did not reach statistical significance.

After confirming that the human adrenal cell line NCI-H295R (with or without FK stimulation) efficiently grows in the medium used to obtain the PBMC supernatants, pools of supernatants obtained from Mtb-stimulated or unstimulated PBMC of HCo and TB patients were added to the adrenal cells. Forty-eight hours later, supernatants from the human adrenal cell line were collected to determine the concentration of the adrenal steroids DHEA and cortisol. As seen in Figure 1, SN from unstimulated PBMC from HCo enhanced unstimulated and FK-stimulated DHEA secretion when compared with corresponding controls, but the secretion of the hormone decreased when adrenal cells were incubated with supernatants derived from Mtb-stimulated PBMC (Fig. 1A). Supernatants from Mtb-stimulated PBMC from TB patients also decreased DHEA production (Fig. 1B). However, and opposite to the effect of supernatants obtained from healthy donors, the supernatants from PBMC of TB patients that had not been further stimulated with the mycobacterial antigen *in vitro* exerted an inhibitory effect when adrenal cells were stimulated by FK.

Furthermore, it should be noted that SN from nonstimulated PBMC from HCo induced a four-fold increase in DHEA production by FK-stimulated adrenal cultures relative to the effect on spontaneous hormone release, whereas treatment with comparable SN from TB patients increased DHEA production by two-fold only.

Nonstimulated and FK-stimulated cortisol secretion by adrenal cells was augmented by SN from

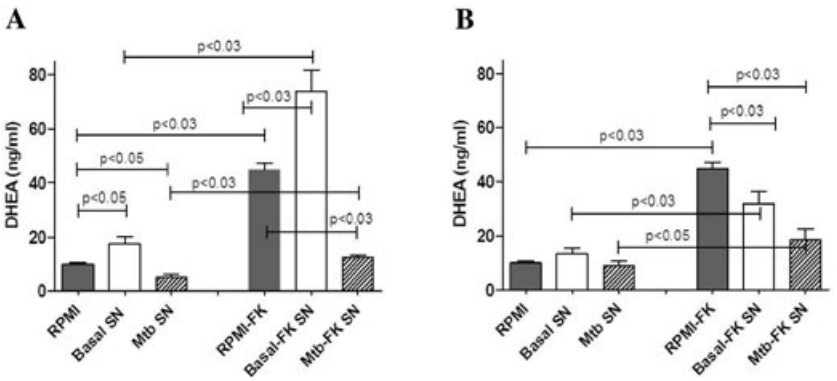

Figure 1. **Effect of supernatants of PBMC on DHEA production by the human adrenal cell line NCI-H295R. Supernatants from cultures of PBMC stimulated with *M. tuberculosis* antigen (Mtb SN) or not (basal SN) from healthy controls (panel A) and TB patients (panel B) were collected. The supernatants were added to NCI-H295R adrenal cells. Another series of adrenal cell cultures received the supernatants together with forskolin (FK) to stimulate basal hormone production. The concentration of DHEA in the medium in which adrenal cells were cultured was determined 48 h later in duplicate. Bars and lines represent means ± SEM of four individual culture/group. Horizontal lines indicate comparisons between groups and statistically significant differences.**

PBMC from both HCo and TB patients that were not further stimulated *in vitro* with the mycobacteria sonicate (Fig. 2). SN from Mtb-stimulated PBMC of HCo and TB patients induced a significant increase in cortisol secretion by adrenal cells that were not exposed to FK. Interestingly, the supernatants derived from Mtb-stimulated PBMC of TB patients were effective in significantly increasing cortisol secretion even when adrenal cells were already stimulated by FK (Fig. 2).

We then proceeded to analyze the production of DHEA and cortisol by adrenal cells when the PBMC supernatants had been treated with anti-TGF-β antibodies. Treatment of the adrenal cells with the mycobacterial antigen or anti-TGF-β antibodies alone did not affect DHEA production, independent of whether FK was also added. For a better appreciation of the effect caused by TGF-β neutralization, only results obtained in FK-stimulated adrenal cells are shown in Figure 3.

Treatment with anti-TGF-β reversed the inhibitory effect of SN from Mtb-stimulated PBMC from HCo and TB patients on DHEA production. In the case of SN from HCo, such an effect was already achieved when anti-TGF-β antibody was added at a 10:1 relation. However, the amount of antibody required to abrogate the inhibitory effect mediated by SN from TB patients had to be increased to 25:1. A trend to normalize DHEA production was also seen in adrenal cells exposed to SN from unstimulated PBMC of TB patients treated with anti-TGF-β (25:1 ratio).

When analyzing cortisol production, cultures exposed to SN from Mtb-stimulated PBMC from HCo treated with anti-TGF-β released significantly more cortisol than their untreated counterparts only when the antibody was used at a relation 10:1 (Fig. 4). No major differences in cortisol production were detected in cultures treated with SN from TB patients, independent of whether anti-TGF-β was added (Fig. 4).

Discussion

The host response to an infectious challenge involves a generalized defense reaction, characterized by changes in immune, metabolic, endocrine, and neural functions aimed at inhibiting pathogen growth and the accompanying inflammation.[32,33] From a teleological standpoint, these neuroimmunoendocrine changes may have an important adaptive value, but when the immune response fails to eradicate the pathogen, a chronic infection is established, leading to misdirected responses with harmful consequences.

Alterations in the concentration of adrenal steroids in patients with pulmonary TB, such as increased cortisol and decreased DHEA levels, are likely to influence the immune response, possibly by contributing to the gradual loss of the effect of Th1 cytokines.[15] In fact, increased levels of GCs

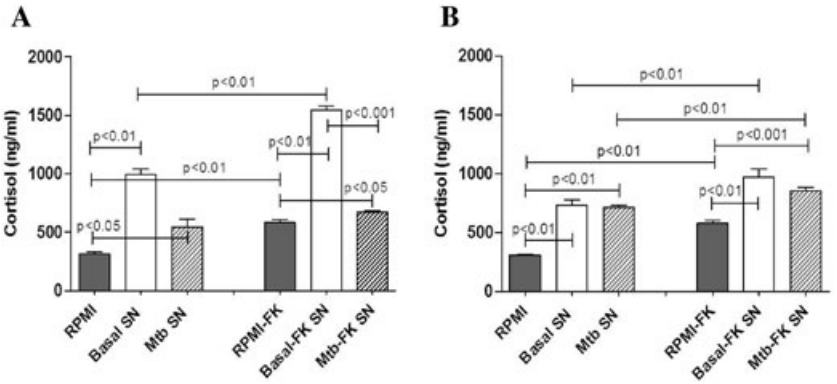

Figure 2. **Effect of supernatants of PBMC on cortisol production by the human adrenal cell line NCI-H295R. Supernatants from cultures of PBMC stimulated with *M. tuberculosis* antigen (Mtb SN) or not (basal SN) from healthy controls (panel A) and TB patients (panel B) were collected. The supernatants were added to NCI-H295R adrenal cells. Another series of adrenal cell cultures received the supernatants together with forskolin (FK) to stimulate basal hormone production. The concentration of cortisol in the conditioned medium of the adrenal cells was determined 48 h later by duplicate. Bars and lines represent means ± SEM of four individual culture/group. Horizontal lines indicate comparisons between groups and statistically significant differences.**

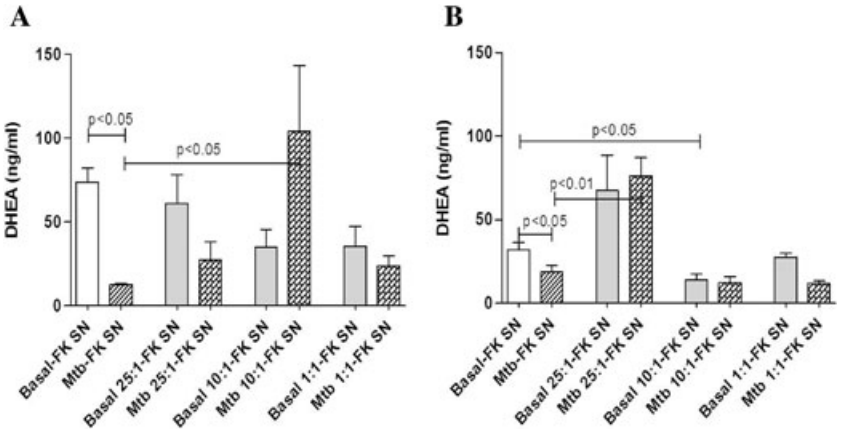

Figure 3. Effect of neutralizing TGF-β in supernatants of PBMC on DHEA production by the human adrenal cell line NCI-H295R. Supernatants from cultures of PBMC stimulated or not with *M. tuberculosis* antigen (Mtb SN and basal SN, respectively) from healthy controls (panel A) and TB patients (panel B) were collected. The supernatants were treated with anti-TGF-β at a ratio of 25:1, 10:1, or 1:1 (a ratio 1:1 corresponds to 6,100 pg/mL anti-TGF-β: 6,100 pg/mL TGF-β) and added to NCI-H295R adrenal cells cultured with forskolin (FK). The concentration of DHEA in the conditioned medium was determined in duplicate 48 h later. Bars and lines represent the mean ± SEM of four individual cultures/group. Horizontal lines indicate comparisons between groups and statistically significant differences.

exert anti-inflammatory effects, favoring an immune response that would be inefficient against intracellular pathogens.[34,35] DHEA counteracts the inhibitory effects of GCs, but also exerts potent anti-inflammatory actions.[21] Thus, the altered cortisol/DHEA relation, at the expense of the markedly reduced levels of DHEA, will favor an inhibition of cellular-mediated immune responses. The potential repercussion of the cortisol/DHEA balance on immune perturbations during TB was recently investigated by analyzing the relation between cortisol and DHEA levels and the *in vitro* immune response to mycobacterial antigens of PBMCs from patients with active TB. We have found that plasma DHEA levels are positively correlated with IFN-γ values, whereas an inverse correlation between the cortisol/DHEA ratio and IFN-γ levels was detected.[36]

The findings reported here extend previous observations that mediators released at different phases of the anti-TB immune response differentially modulate steroid production by adrenal cells.[28] We found that the effects vary depending on the source of the supernatants and the steroid hormone under analysis. Although SN of nonstimulated PBMC from healthy controls enhanced DHEA secretion, SN from Mtb-stimulated mononuclear cells exerted the opposite effect. Cells from TB patients also produced factors that inhibit DHEA production by FK-stimulated adrenal cells and, interestingly, without further *in vitro* stimulation of the PBMC with mycobacterial antigens. Thus, mycobacterial stimulation induces the production of mediators capable of reducing DHEA secretion by adrenal cells. In the case of TB patients, *in vivo* infection with the bacilli is enough to stimulate PBMC to release such products *in vitro*. As opposed to the effects on DHEA production, PBMC supernatants from HCo and TB patients showed a general trend of increased cortisol production, though this increase was less evident in cultures treated with SN of nonstimulated PBMC from TB patients (Fig. 2).

Our studies also indicate that TGF-β is implied in the inhibition of DHEA production by adrenal cells. In fact, treatment with anti-TGF-β abrogated the inhibitory effects of SN from stimulated PBMC from HCo and TB patients on DHEA release. However, higher concentrations of the neutralizing antibody were necessary to reverse the inhibition caused by products from cells obtained from patients with active TB. These results agree with the finding that culture supernatants from PBMC of TB patients contained larger amounts of TGF-β than those from HCo counterparts. We cannot explain at present why a higher concentration of the antibody did not abrogate the inhibitory effect of supernatants from HCo. One possibility is that the relation of TGF-β

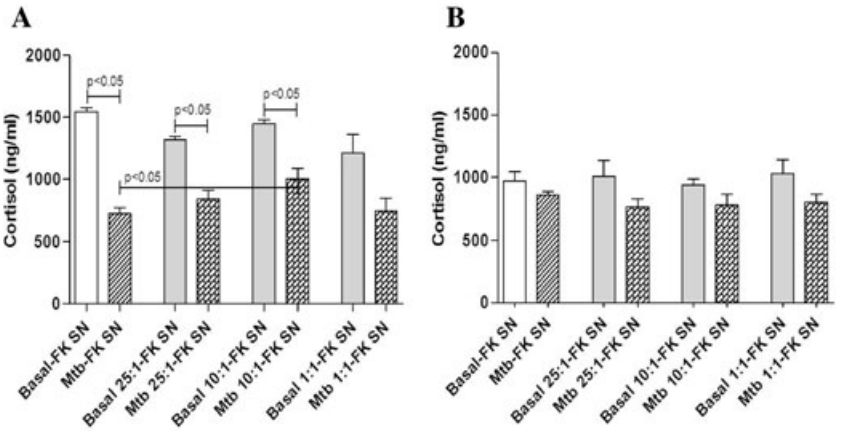

Figure 4. Effect of neutralizing TGF-β in supernatants of PBMC on cortisol production by the human adrenal cell line NCI-H295R. Supernatants from cultures of PBMC stimulated or not with *M. tuberculosis* antigen (Mtb SN and basal SN, respectively) from healthy controls (panel A) and TB patients (panel B) were collected. These supernatants were treated with anti-TGF-β at a ratio of 25:1, 10:1, or 1:1 (a ratio 1:1 corresponds to 6,100 pg/mL anti-TGF-β: 6,100 pg/mL TGF-β) and added to NCI-H295R adrenal cells cultured with forskolin (FK). The concentration of cortisol in the conditioned medium was determined in duplicate 48 h later. Bars and lines represent means ± SEM of four individual culture/group. Horizontal lines indicate comparisons between groups and statistically significant differences.

antibody to TGF-β might be critical, and that higher concentrations result in prozone-like effects.

TGF-β is a key cytokine in TB immunopathology because it inhibits macrophages and downregulates IFN-γ production.[37] Our previous studies showed that *M. tuberculosis*–stimulated PBMCs of TB patients with a severe form of the disease produce more TGF-β than those from healthy controls or from patients with a mild or moderate disease.[30] Extending these findings, we now add evidence involving TGF-β in the immunoendocrine communication during TB, particularly in the impaired DHEA secretion that these patients present.

A parallel, interesting finding was that neutralization of TGF-β in supernatants of Mtb-stimulated PBMC from HCo resulted in a relatively small but significant increased cortisol production by adrenal cells. These results indicate that Mtb-stimulation of PBMC from healthy donors triggers the production of soluble factors capable of increasing cortisol production, an activity that is manifested when TGF-β is neutralized. The fact that no such effect on cortisol production is observed when the supernatants are derived from TB patients might indicate that these supernatants are qualitatively different, and that other products, which are different from TGF-β, are responsible for the increase in cortisol production by adrenal cells. This observation adds an extra level of complexity to the intricate network of immunoneuroendocrine interactions. Furthermore, the studies reported here show direct effects of immune products on adrenal cells. However, the actions of cytokines exerted upstream from this gland on the other components of the HPA axis have to be considered.

Conclusions

In chronic infectious diseases such as TB, excessive and/or prolonged cytokine production may affect immune–endocrine communication, favoring the establishment of an adverse state, characterized by important alterations in essential biological functions, together with perpetuated tissue damage. We have shown that mediators released during different phases of the anti-TB immune response differentially modulate steroid production by adrenal cells. Furthermore, to the well-known immunopathological role of TGF-β during TB, the results reported here suggest that this cytokine may be an important link in the communication between the immune and the endocrine systems, in particular, as a mediator of the disturbed cortisol/DHEA balance.

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Conflicts of interest

The authors declare no conflicts of interest.

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