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Modulation of the phenotype and function of *Mycobacterium tuberculosis*-stimulated dendritic cells by adrenal steroids

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Abstract

Cell-mediated immunity, cytokines induced during the specific immune response and T-cell populations are crucial factors for containing *Mycobacterium tuberculosis* infection. Recent reports suggest a cross-regulation between adrenal steroids (glucocorticoids and dehydroepiandrosterone, DHEA) and the function of antigen-presenting cells (APCs). Therefore, we investigated the role of adrenal hormones on the functional capacity of *M. tuberculosis*-induced dendritic cells (DCs). Cortisol significantly inhibited the functions of *M. tuberculosis*-induced DCs. Interestingly, the presence of DHEA enhanced the *M. tuberculosis*-induced expression of MHC I, MHC II and CD86 and also increased ERK1/2 phosphorylation. Moreover, DHEA improved the production of IL-12 in response to *M. tuberculosis* stimulation, diminished IL-10 secretion and could not modify TNF-α synthesis. Importantly, we observed that DHEA enhanced the antigen-specific T-cell proliferation and IFN-γ production induced by *M. tuberculosis*-stimulated DC. These data show for the first time the relevance of the adrenal axis (especially of DHEA) in the modulation of DC function in the context of tuberculosis, a disease where the induction of a T_h1 environment by APCs is crucial for the development of an effective immune response to the mycobacteria.

Keywords: adrenal steroids; cytokines; dendritic cells; dehydroepiandrosterone; immunity to infection; *Mycobacterium tuberculosis*

Introduction

With over one-third of the world's population infected with *Mycobacterium tuberculosis* and 3 million people killed by the disease per year, tuberculosis (TB) is an emerging global problem. However, in the presence of an effective immune response, only 5–10% of the infected population will develop active TB in their lifetime. The emergence of multidrug-resistant and extensively drug-resistant TB strains coupled with increasing incidence of HIV co-infection threatens to return to an era without any effective way of controlling this disease (1). After inhalation, the bacteria travel to distal regions of the lung and are engulfed by lung dendritic cells (DC) and macrophages. Despite the recognition that T_h1 immunity is critical in preventing progression to active disease, several aspects on how the innate immune response shapes the adaptive T_h1 immune response, how T_h1 immunity participates in the formation of the tuberculous granuloma and why T_h1 immunity is not sufficient to completely eradicate *M. tuberculosis* continue to constitute a conundrum (2). Recent reports suggest a cross-regulation between adrenal steroids (glucocorticoids and dehydroepiandrosterone, DHEA) and the maturation and function of antigen-presenting cells (APCs) (3–5). To date, endocrine regulation of DC function is an issue poorly studied; only one report demonstrated an effect of DHEA-sulphate (DHEA-s) on the maturation of DCs where the presence of DHEA-s during maturation of DCs induced an increased ability of DC to stimulate T_h1 cytokine production by T cells in terms of IFN-γ production (5).

The effects of DHEA are often opposed by the other important adrenal steroid cortisol (6). The DHEA/cortisol ratio is abnormal during various pathological conditions characterized by immune dysfunction, such as AIDS, rheumatoid arthritis and TB (7–9). Specifically in TB, an

enhancer effect of DHEA on *M. tuberculosis*-induced IFN-γ production by PBMC has been demonstrated, together with a reduction in the synthesis of transforming growth factor (TGF)-β, indicating a role for adrenal hormones in the control of TB (10). Moreover, we recently demonstrated an enhancing role for DHEA in the immune response against *M. tuberculosis* during HIV–TB co-infection and immune reconstitution inflammatory syndrome (IRIS) (11).

In this study, we sought to investigate the relevance of the adrenal axis (especially of DHEA) in the modulation of DC function in the context of TB, a disease where the induction of a T_h1 environment by APCs is crucial for the development of an effective immune response to the mycobacteria.

Methods

In vitro differentiation and culture of DC

DC were differentiated from PBMC as described earlier (12). Briefly, PBMC were separated from 15 healthy blood donors using Ficoll Hypaque density gradient and monocytes were purified by Percoll gradients. Monocytes were cultured for 5 days in RPMI 1640 medium supplemented with 1% HEPES 2 mM L-glutamine, 1% streptomycin and penicillin (Sigma-Aldrich), 10% fetal bovine serum (Natocor), recombinant cytokines IL-4 (15 ng ml^{-1}; BD Biosciences) and granulocyte macrophage colony-stimulating factor (30 ng ml^{-1}) to obtain immature DCs. Immature DC were stimulated with heat-killed *M. tuberculosis* (H37Rv, 10 μg ml^{-1}, BEI Resources, American Type Culture Collection, Manassas, VA, USA) in the presence or absence of DHEA or cortisol at the indicated concentrations for 16–24 h. Cell-free culture supernatants were collected and stored at −80°C for further ELISA assessments. This work was approved by the Ethics Committee from the School of Medicine, University of Buenos Aires and conforms to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). Written informed consent was obtained, according to the local Ethics Committee.

Evaluation of DC phenotype

For the analysis of cell surface antigens, the following mAbs were used: HLA-ABC, HLA-DR, CD1a, CD80, CD86 and CD83 (all from BD Biosciences). Cells were analyzed in a flow cytometer. Cells were acquired in a FACSCanto flow cytometer and analyzed with FACSDiva software (BD Biosciences).

Western blotting

DC were stimulated for 20 min with *M. tuberculosis* in the presence or absence of DHEA at the indicated concentrations and thereafter cells were washed and solubilized in lysis buffer to prepare whole cell extracts as described (13). Equivalent amounts of protein were analyzed by 10% SDS–PAGE, transferred to nitrocellulose (Hybond ECL Nitrocellulose Membrane, Amersham Biosciences) and incubated with anti-phospho-ERK, anti-ERK1/2, anti-Iκ-Bα (Santa Cruz Biotechnology) or anti-β-actin (Sigma-Aldrich) antibodies. Bound antibodies were revealed with HRP-conjugated affinity purified anti-mouse antibody (GE Healthcare) or HRP-conjugated affinity purified anti-rabbit antibody (Santa Cruz Biotechnology), using enhanced chemiluminescence (ECL, Amersham Biosciences) and Kodak BioMax films.

Assessment of APC function of DCs

The ability of adrenal hormones to modulate *M. tuberculosis*-presenting DCs to stimulate T cells was assessed by using an autologous DC–T-cell co-culture. Briefly, CFSE-stained lymphocytes were plated in 96-well round-bottomed tissue culture plates (2×10^5 per well), and DC were added to give different DC:T-cell ratios. Supernatants of DC–T-cell co-cultures were collected on day 4, and IFN-γ levels determined by ELISA. T-cell proliferation was assessed by flow cytometry by CFSE dilution assays at day 6. Also, IFN-γ-producing T lymphocytes were determined by intracellular staining by flow cytometry. As a positive control, cells were stimulated with PHA.

Statistical analysis

Comparisons between groups were done by using non-parametric Kruskal–Wallis and Mann–Whitney tests. Analysis of related samples was performed by the Wilcoxon signed-rank test. Values of $P < 0.05$ were considered significant.

Results

We investigated the modulation of the functional capacity of *M. tuberculosis*-activated DCs by adrenal hormones. To achieve this, we initially assessed the cytokine profile (IL-12, IL-10, TNF-α) produced by DCs after inducing their maturation overnight with *M. tuberculosis* in the presence or absence of cortisol (10^{-6} M) or DHEA (10^{-7} M) alone or in combination. We observed, as expected, a significant increase on the production of IL-12 ($P < 0.05$), TNF-α ($P < 0.05$) and IL-10 ($P < 0.0001$, Fig. 1), after *M. tuberculosis* stimulation. Addition of cortisol to the cultures significantly inhibited the *M. tuberculosis*-induced secretion of the three cytokines studied (Fig. 1A–C). Remarkably, DHEA improved the production of IL-12 in response to *M. tuberculosis* stimulation ($P < 0.05$, Fig. 1A), could not modify TNF-α synthesis (Fig. 1B) and diminished the IL-10 secretion ($P < 0.05$, Fig. 1C). We could not observe a reversion of the inhibitory effects induced by cortisol by DHEA treatment (data not shown).

Maturation of DC induced by mycobacteria often involves activation of mitogen-activated protein kinase (MAPK) such as ERK1/2 and nuclear factor (NF)-κB pathways (14, 15). Within this setting, we observed that *M. tuberculosis* induced the phosphorylation of ERK1/2 and the degradation of IκBα (Fig. 1D). The addition of DHEA enhanced the activation of ERK1/2 induced by the antigen and notably, DHEA inhibited the degradation of IκBα, therefore indicating a diminished translocation of NF-κB to the nucleus (Fig. 1D). Culture of DCs with DHEA alone resulted in no changes in the above-mentioned signaling molecules.

Since the expression of antigen-presenting proteins and co-stimulatory molecules are strongly related to DC function (16), we assessed the phenotype of DCs after inducing their maturation overnight with *M. tuberculosis* in the presence or absence of cortisol or DHEA alone or in combination,

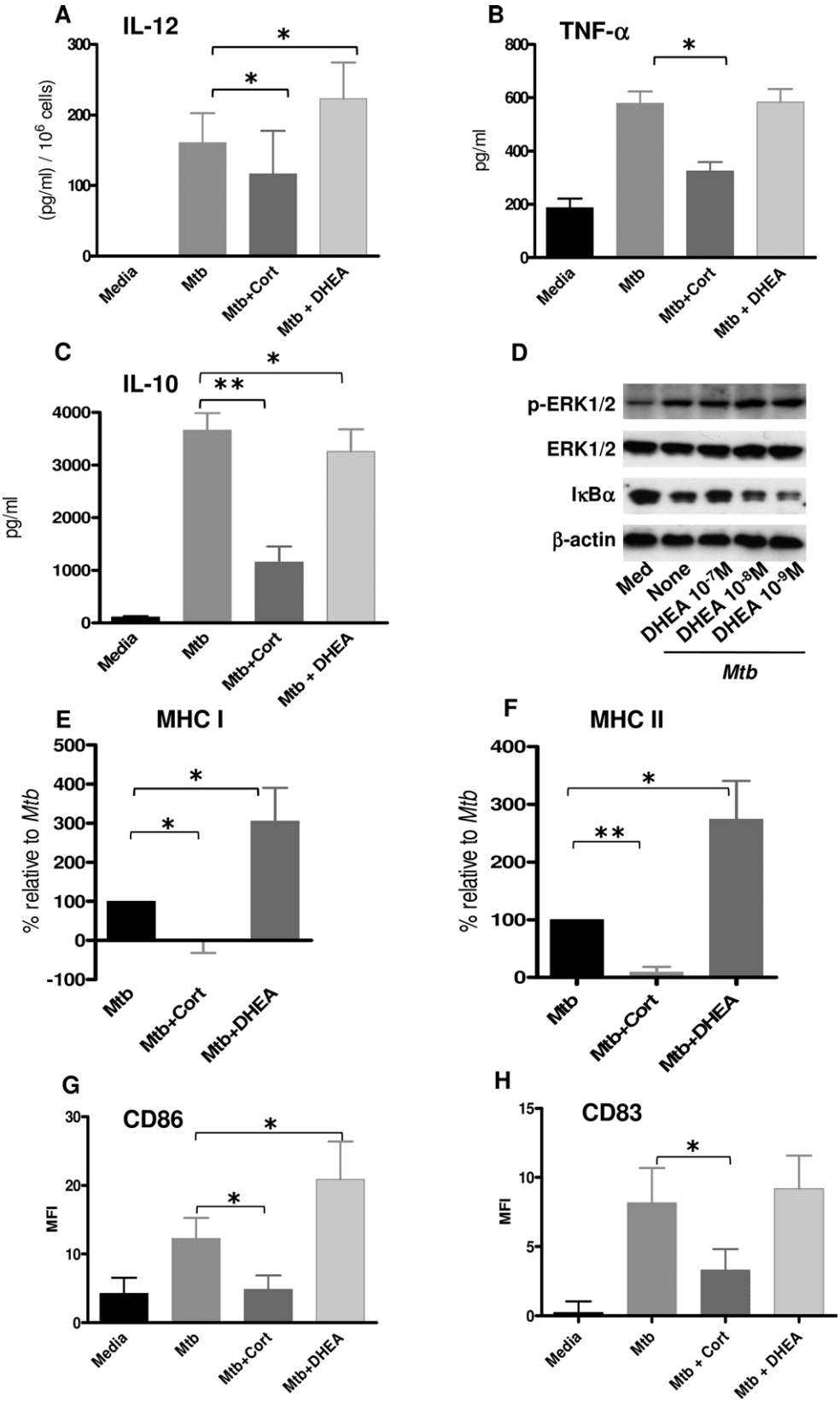

Fig. 1. Adrenal hormones modulate the *Mtb*-induced DC maturation. (A–C). DC were cultivated with *Mtb* antigen (10 μg ml^{-1}) in the presence or absence of cortisol (1×10^{-6} M), DHEA (1×10^{-8} M) or both and cell-free supernatants were collected after 24 h. The levels of the cytokines IL-12 (A), TNF-α (B) and IL-10 (C) were determined by ELISA. Data are representative of eight independent experiments performed with DC from eight different donors. (D) DC were stimulated with *Mtb* as indicated and then lysed after 20 min of stimulation. Samples were separated

by flow cytometry. Stimulation by *M. tuberculosis* induced a significant increase on the expression of MHC I, MHC II, and CD86 and CD83 ($P < 0.05$, Fig. 1E–H, respectively). Interestingly, the presence of DHEA during the culture period enhanced the *M. tuberculosis*-induced expression of MHC I ($P < 0.05$, Fig. 1E), MHC II ($P < 0.05$, Fig. 1F) and CD86 ($P < 0.05$, Fig. 1G), without affecting CD83 expression (Fig. 1H). Cortisol treatment significantly inhibited the above-mentioned functions exerted by *M. tuberculosis*-induced DCs (Fig. 1E–G) and this effect could not be reverted by DHEA addition (data not shown).

Finally, we sought to investigate whether DHEA could affect the ability of DCs to activate antigen-specific T cells. To achieve this, monocyte-derived DC were loaded with *M. tuberculosis* in the presence or absence of DHEA at different concentrations, and afterward co-cultured in the presence of syngeneic lymphocytes with the aim of assessing *M. tuberculosis*-specific proliferation and IFN-γ production by T cells. We observed that *M. tuberculosis*-activated DCs promoted T-cell proliferation, which was enhanced by DHEA (Fig. 2A and B). Furthermore, DHEA also increased the production of IFN-γ by activated T cells in response to *M. tuberculosis* stimulation (Fig. 2C), and also the frequency of IFN-γ-producing T lymphocytes (depicted in Fig. 2D).

Discussion

So far, there are no reports describing the effect of adrenal hormones on DC function in the context of TB infection. Several authors demonstrated an effect of cortisol and DHEA on DC maturation and function, showing a decrease in cytokine production, antigen presentation, T-cell co-stimulation and activation among other functions induced by corticosteroids (3, 4, 17–20) and an enhancement by DHEA of DCs' capacity to induce T-cell allostimulation (3) but not in the framework of an intracellular infection such as TB. Our data indicate that DHEA immune intervention may positively affect DC function since we observed an induction of a pro-inflammatory cytokine milieu, increment on the expression of molecules involved in antigen presentation and T-cell co-stimulation, along with an enhancement on the *M. tuberculosis*-specific T-cell stimulation by DCs.

Our results show that DHEA increased IL-12 production from *M. tuberculosis*-stimulated DCs, which may favor the development of T_h1 responses, crucial for the protective immune response to the mycobacteria. In contrast, DHEA treatment was not able to influence TNF-α production, vital to shape microbicidal granulomas and to control *M. tuberculosis* infection. This result becomes relevant when analyzing the importance of DHEA in the control of latent TB, a condition where the granuloma is a fundamental structure to restrict mycobacterial growth in the lung (21). In line with these observations, we previously demonstrated an enhancing role for DHEA in the immune response against *M. tuberculosis* during HIV–TB co-infection and IRIS since DHEA improved T_h1 *M. tuberculosis*-specific responses in patients dually infected with TB and HIV (11).

One immune-limiting mechanism during TB is the inhibitory and anti-inflammatory cytokine IL-10. IL-10 is produced by many hematopoietic cells and a major role is to suppress macrophage and DC functions, which are required for the capture, control and initiation of immune responses to pathogens such as *M. tuberculosis* (22). Infection by M. *tuberculosis* induces high levels of IL-10 by DCs (23). We observed that both DHEA and cortisol diminished IL-10 secretion induced by *M. tuberculosis* stimulation without altering DC viability (data not shown). These results are in contrast to those published by Toebak *et al.* (19), who measured IL-10 production by DCs upon ligation of CD40 observing no changes after hydrocortisone or dexamethasone treatment of monocyte-derived DCs. On the other hand, it has been shown that DHEA negatively regulates the production of IL-10 during aging (24) and in several murine models (revised in ref. 9). We propose a scenario where the cell reaches its maximum after *M. tuberculosis* stimulation, and the addition of cortisol may weaken the capacity of DCs to secrete IL-10.

Several authors have demonstrated the involvement of the MAPK and NF-κB signaling pathways in *M. tuberculosis*-induced DC maturation (14, 25, 26). Our data show that DHEA signaling induces MAPK activation in *M. tuberculosis*-stimulated DCs, therefore contributing to the T_h1 immune responses necessary to eliminate the bacteria. Of note, our demonstration that DHEA at supra-physiological concentrations inhibited IκBα degradation suggests a diminished NF-κB translocation to the nucleus, therefore negatively affecting DC activation. However, previous studies have shown that DHEA-s can inhibit the ability of reactive oxygen species to stimulate degradation of the Iκ-Bα protein (27). Since different NF-κB subunits exert distinct roles in regulating inflammatory and T-cell stimulatory functions in DCs, where the p50 and cRel NF-κB subunits would regulate genes important for DC-induced T-cell responses (e.g. CD40, IL-12 and IL-18) but not for genes encoding inflammatory cytokines (e.g. TNF-α, IL-1α and IL-6) and the RelA subunit would modulate the expression of inflammatory cytokine genes but not T-cell stimulatory genes (28), the observed responses modulated by DHEA after *M. tuberculosis* stimulation may be due to the integration of different signaling pathways rather than the NF-κB signaling pathway alone.

Regarding the surface DC molecules involved in antigen presentation and co-stimulation, we observed that CD86, a well-known activation marker for DCs, was augmented by DHEA, supporting the enhancing role of this adrenal hormone on the functional capacity of DCs in TB. Also, we observed

on SDS–PAGE, transferred to a polyvinylidene difluoride membrane and then analyzed by western blotting. The p-ERK and ERK proteins were detected by their specific antibodies. The activation of NF-κB was determined by detecting the degradation of IκBα using a specific antibody. Data are representative of three independent experiments performed with DC from three different donors. (E–H) DC were cultured with *Mtb* in the presence of DHEA (1×10^{-8} M) and/or cortisol (1×10^{-6} M) for 16 h and analyzed for the expression of surface markers. The change of the mean fluorescence intensity for MHC I (E), MHC II (F), CD86 (G) and CD83 (H) were determined by flow cytometry. (E and F) The percentage of MHC I and MHC II increment relative to *Mtb* was calculated as follows: % of MFI relative to *Mtb* = {[(*Mtb* hormone – Media) – (*Mtb* – Media)]/(*Mtb* – Media)} × 100. Each bar represents the mean ± SEM of 6–8 independent experiments performed with cells from different donors. Asterisks indicate comparisons between conditions. *Mtb*, *Mycobacterium tuberculosis*. *$P < 0.05$; **$P < 0.01$.

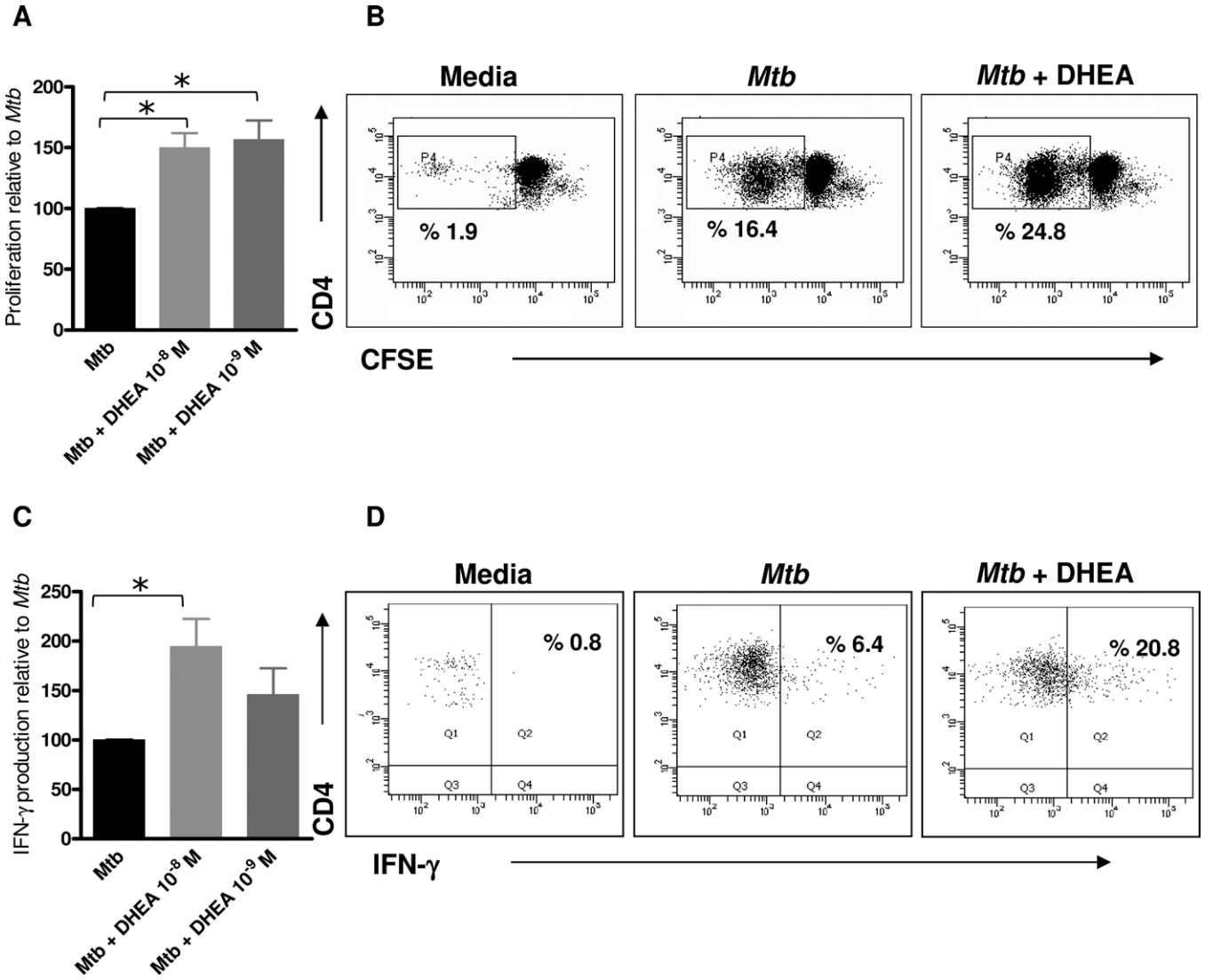

Fig. 2. DHEA enhances the antigen-specific T-cell activation induced by *Mtb*-stimulated DCs. (A and B) Autologous lymphocytes were co-cultured with medium, DHEA 10^{-8} M- or DHEA 10^{-9} M-treated DCs pulsed with *Mtb* (10 μg ml^{-1}) at a 1:125 ratio. T-cell proliferation was determined by CFSE dilution of $CD4^+$ T cells by flow cytometry after 3 days of culture. (A) Each bar illustrates the mean ± SEM of the percentage for $CFSE^{dim}$ $CD4^+$ T cells relative to *M. tuberculosis* for each condition, calculated as follows: Proliferation relative to *Mtb* = {[(*Mtb* hormone – Media) – (*Mtb* – Media)]/(*Mtb* – Media)} × 100. Asterisks indicate comparisons between each condition against *Mtb*-specific response. (B) Representative flow cytometry graphs showing the $CFSE^{dim}$-proliferating $CD4^+$ T cells after culturing lymphocytes with DCs alone (left), *Mtb*-pulsed DCs (middle) or DHEA-treated *Mtb*-pulsed DCs (right). (C and D) IFN-γ responses by $CD4^+$ T lymphocytes after co-culture with DCs as described above. (C) The levels of IFN-γ relative to *M. tuberculosis* after culturing autologous lymphocytes with DCs for 3 days were determined by ELISA. Bars indicate the mean ± SEM of four independent experiments. (D) Representative intracellular staining and flow cytometry analysis of IFN-γ-producer T cells after co-culturing autologous lymphocytes with DCs as in (A). Data are representative of four independent experiments performed with cells from four different donors. *Mtb, Mycobacterium tuberculosis*. $^*P < 0.05$.

that MHC class I and II molecules were up-regulated by DHEA treatment after *M. tuberculosis* stimulation of DCs, suggesting an impact not only on T_h responses but also on $CD8^+$ lymphocytes, possibly influencing both their cytotoxic and cytokine-producing capacities. These observations are in agreement with our results showing an increment on the functional capacities of specific T cells after DHEA treatment of DCs and also with our previous data showing an enhancement of *M. tuberculosis*-specific immune responses in HIV–TB co-infected individuals (11). Finally, the expression of CD83, a marker for DC activation, was not modified by DHEA addition, suggesting that this hormone fails to modify the maturation status of DCs in the context of TB infection.

It has been suggested that the development of TB is due to failure of immune regulation or inappropriate immune regulation (29). The disturbances in immune regulation may speculatively involve the subversion of a protective T_h1 response, including the generation of $CD8^+$ CTL, by several mechanisms including T_h2-like cytokines, TGF-β, Treg or other regulatory cells, and hitherto undescribed mechanisms afflicting the protective T_h1 pathways (30), including DC maturation and differentiation. Thus, a more effective and shorter

treatment may require modulation of the immune system and a switch away from an immunopathologic phenotype to a protective one. In this regard, our previous findings suggesting an enhancement of T_h1 responses concomitant with a down-modulation of Treg function by DHEA treatment (11) could set a scenario where treatment of TB could be enhanced by using DHEA as a treatment adjuvant for mycobacterial diseases, especially in the context of HIV infection.

The data presented here show for the first time the relevance of the adrenal axis (especially of DHEA) in the modulation of DC function in the context of TB, a disease where the induction of a T_h1 environment by APCs is crucial for the development of an effective immune response to the mycobacteria. Because of its immune modulatory effects, DHEA supplementation or treatment with DHEA derivatives may be a novel and inexpensive approach to treat conditions where a skewing of the immune response toward an unfavorable cytokine profile may contribute to pathology, as is the case for mycobacterial infections (9). DHEA has also been proposed as a vaccine adjuvant, since this approach has proved successful, with DHEA administration augmenting the immune response of aged mice to a range of vaccines (9). While further studies are necessary to get a deeper understanding of the mechanisms involved in DHEA enhancement of anti-TB immune responses, the possibility of using DHEA or DHEA derivatives as potential adjuvant therapy in TB should be considered.

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