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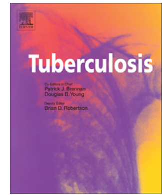

IMMUNOLOGICAL ASPECTS

Effect of cortisol and/or DHEA on THP1-derived macrophages infected with *Mycobacterium tuberculosis*

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ARTICLE INFO

Article history:
Received 26 March 2015
Accepted 19 May 2015

Keywords:
Macrophages
Mycobacterium tuberculosis
Dehydroepiandrosterone
Cortisol
Autophagy

SUMMARY

Tuberculosis (TB) is a major health problem requiring an appropriate cell immune response to be controlled. Macrophages play a central role in the response against *Mycobacterium tuberculosis* (Mtb).

Given our prior studies in which adrenal steroids were found to modify the cellular immune responses from TB patients, it was sensible to analyze the immunomodulatory capability of cortisol and DHEA on macrophages infected with Mtb. The human macrophage-like THP-1 cells were infected with the H37Rv strain of Mtb and treated with Cortisol and DHEA at different doses. We monitored phagocytosis, intracellular-bacterial growth, autophagosoma formation, as well as cytokine gene expression and production.

Cultures exposed to cortisol showed a decreased production of IL-1β, TNF-α, with DHEA being unable to modify the pattern of cytokine production or to reverse the cortisol inhibitory effects. Interestingly the intra-macrophagic bacterial burden was found reduced by DHEA treatment. While this effect was not related to a different cytokine pattern, in terms their production or mRNA expression, DHEA treatment did promote autophagy in Mtb-infected macrophages, irrespective of Cortisol presence.

In essence, the better control of Mtb load by DHEA-treated macrophages seems to be dependent on an autophagic mechanism. The present results are relevant for two reasons as autophagy is not only important for clearance of mycobacteria but also for the prevention of tissue damage.

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1. Introduction

Tuberculosis (TB) constitutes an important health problem today. In 2014, an estimated 8.6 million people developed TB and 1.3 million died from the disease (including 320 000 deaths among HIV-positive people). The number of TB deaths is unacceptably large given that most are preventable [1]. This disease is caused by *Mycobacterium tuberculosis* (Mtb), a facultative intracellular bacterium that is capable of surviving and persisting within host mononuclear cells [2].

In most cases, the immune response against Mtb is adequate and avoids the development of active disease. However, complete clearance of the pathogen is frequently not achieved. Macrophages comprise a niche cell for mycobacterial infection, yet they may also contribute to elimination of bacilli via numerous mechanisms, including the successful acidification and maturation of phagosomes [3–5]. However, Mtb ensures its survival within host macrophages by arresting the maturation pathway that leads to phagosome–lysosome fusion, thus avoiding the phagolysosome that is rich in acid hydrolases capable of microbicidal degradation, and creating a suitable environment for bacillary survival and replication [6–8]. As yet, while Mtb can block phagosome maturation, the induction of autophagy facilitates phagosome-lysosome fusion and the bacilli clearance [9,10].

Among modulators of the immune response, hormones like the steroid hormones of the adrenal and gonadal glands, and neurotransmitters are known to play an influential role in this regard [11,12]. In analyzing the immune-endocrine profile of TB patients, we

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http://dx.doi.org/10.1016/j.tube.2015.05.011
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carried out a series of studies in newly diagnosed, untreated patients with different degrees of lung compromise. Plasma levels of interferon gamma (IFN-γ), interleukin (IL) 10, and IL6 were increased, whereas testosterone and dehydroepiandrosterone (DHEA) levels were profoundly decreased in these patients, more evident in those with an advanced disease. In parallel there were modest increases in the concentrations of cortisol and estradiol [13]. These endocrine changes may partly account for the deficient control of the inflammatory response and the gradual loss of protective responses that TB patients present with disease progression [14].

More recent studies in patients undergoing specific therapy revealed a more balanced cortisol/DHEA and cortisol/DHEAS ratio, implying that etiologic treatment encompasses favorable immune and endocrine changes, which may account for its beneficial effects [15].

Macrophages play a central role in the defense against TB through phagocytosis, intracellular killing of mycobacteria, and antigen presentation to lymphocytes [16,17]. Since adrenal steroids are much likely to modify such responses, it was sensible to analyze the immunoregulatory capability of cortisol and DHEA on macrophages infected with Mtb.

In addressing this issue we have employed a model able to mimic the macrophage-Mtb interaction. Hence, we infected the human macrophage-like THP-1 cells with Mtb strain H37Rv and treated with Cortisol and DHEA at different doses. We monitored phagocytosis, intracellular-bacterial growth, autophagosoma formation, cytokines gene expression and production. This approach provided new insight on the steroid hormones effects on the intracellular fate of Mtb.

2. Materials and methods

2.1. Bacterial cultures

M. tuberculosis strain H37Rv (ATCC 25618) was used in all experiments. For *in vitro* cell infection, frozen H37Rv *M. tuberculosis* was prepared in Middlebrook 7H9 (Difco Laboratories) culture medium supplemented with ADC (Difco Laboratories) during 48 h. Bacilli were then placed in bottles containing 60 ml of the same culture medium and incubated at 70 rpm 35 °C during 7–10 days until reaching a 600 nm OD. To prepare a suspension with single bacteria and disrupt mycobacterial clump formation, bacterial pellets were then resuspended in RPMI with 6% glycerol and vortexed for 5 min in the presence of five sterile 3-ml glass beads. The resulting suspension of mycobacteria was centrifuged at 900 g for 10 min to remove any remaining large clumps. Supernatants with disaggregated mycobacterial stock cultures were then divided into aliquots and stored at −70 °C until use. Mean concentrations of *M. tuberculosis* strain H37Rv stock suspensions were determined by counting colony-forming units (CFU) on 7H10 agar plates in triplicate serial dilutions of declumped stock suspensions between Days 21 and 28. This declumping procedure was performed in each experiment to ensure use of single-bacterial-cell suspensions and to establish the input amounts of bacteria for the infections at 5:1 of multiplicities of infection [MOI], [18].

2.2. Cell preparations

The characteristics of THP-1, a human monocytic leukemia cell line, have been described previously in detail [19]. This cell line was grown in suspension cultures in Tissue Culture Medium RPMI-1640 supplemented with 10% of heat-inactivated fetal bovine serum and kanamycin (60 μg/ml, Gibco) at 37 °C in 5% CO2. THP-1 cells/ml were cultured in complete RPMI-1640 containing 30 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma Chemical Co., St. Louis, MO, USA) and plated for differentiation to macrophages. Twenty four hours later supernatants were removed and complete RPMI-1640 was added for 48 h before the infection.

2.3. Infection with M. tuberculosis and stimulation of macrophages with cortisol and DHEA

Macrophages were infected with Mtb at Multiplicity of Infection (MOI) of 5:1 (5 bacteria/1 cell) for 3 h, in presence or absence of steroid hormones. In line with our earlier work [20], hormones were employed within the range of physiological concentrations, Cortisol (10^{-6} M, Sigma Chemical Co.) and/or DHEA (10^{-6}or 10^{-7} M, Sigma Chemical Co.) in RPMI with 10% of heat-inactivated fetal bovine serum and kanamycin (60 μg/ml) and cultured at37 °C in 5% CO_2. Cells were then washed 4 times with complete RPMI-1640 to remove extracellular bacilli. According to the study purposes, experimental approaches were as follows:

a) To assess Mtb phagocytosis, the macrophages (1×10^6 cells/wells in chamber slide) were stained by Ziehl Neelsen after 3 h of infection in presence or absence of hormones and 1 h more with RPMI-1640 alone. The rate of infection was measured by quantifying the number of bacteria phagocytosed per cell, 100 cells/field were counted in triplicate.
b) To evaluate the control of mycobacterial intracellular growth, infected macrophages (3×10^5 cells/well in round bottom 96-well polystyrene plates) were incubated for 1 h (Day 0), 24 h (Day 1) and 96 h (Day 4) in presence or absence of hormones. Cells were lysed with 1% SDS followed by 20% BSA, serially diluted in Middlebrook 7H9 medium and plated in triplicate over 7H10 medium to quantify colony forming units (CFU). To take into account the phagocytosis of bacteria, the intracellular growth fold change was calculated as CFU at Day 1 or Day 4 post-infection, and also at Day 0, after 21 days of culture.
c) For cytokine gene expression and production, 5×10^6 cells plated in 24-well dishes under the above described conditions were incubated during 24 h more with hormones. Supernatants were collected to assess cytokine production and cells were preserved for mRNA extraction.
d) For transmission electron microscopy examination, 4×10^6 macrophages cultured in polypropylene tubes under the same conditions were treated during 24 h more with Cortisol and/or DHEA and then fixed and prepared for electron microscopy. In addition, infected cells were treated with muramyldipeptide (MDP, Sigma Chemical Co.) for 24 h post-infection for positive control of autophagy.

2.4. RNA isolation, cDNA synthesis and real-time PCR for cytokines gene expression

Total RNA was isolated by using RNeasy Mini® Kit (Quiagen, Carlsbad, CA, USA) according to the manufacture's recommendations. RNA pellets were dissolved in DEPC sterile water and stored at −80 °C until analysis. RNA quantity was calculating by OD 260 spectrophotometry (NanoVuePlus Spectrophotometer, GE Healthcare, Little Chalfont, UK). The integrity of the purified RNA was determined by 2% agarose gel electrophoresis. cDNA was synthesized from 5 μg of total RNA using 200 U M-MLV reverse transcriptase (USB Corporation, Cleveland, USA) and specific reverse primers (Table 1). Briefly, 5X M-MLV Reaction Buffer, 0.4 mMdNTP (Amersham Biosciences, Piscataway, NJ, USA); 21.5 U RNAasa Inhibitor (RNAguard, Amersham Biosciences), 0.4 μM of each reverse primer; 2 mM $MgCl_2$ (Invitrogen, Camarillo, CA, USA) and DEPC sterile water for 50 μl of final volume. Retrotranscription

Table 1
Real Time nucleotide primer sequence.

Transcripts	Primer forward	Primer reverse	Size (pb)	NCBI reference sequence
TGF-beta1	TACCTGAACCCGTGTTGCTC	GCGAAAGCCCTCAATTTCCC	224	NM_000660.5
IL-23a	CCCAAGGACTCAGGGACAAC	TGGGACTGAGGCTTGGAATC	222	NM_016584.2

programmes consisted of 5 min at 65 °C, 1 h at 40 °C followed by enzyme inactivation at 95 °C for 3 min cDNA was stored at − 80 °C until use it. qPCRwas performed with the ABI PRISM® 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using 10 μl dilution 1/200 of cDNA, 0.4 μM of each primer and 25 μl of SYBR Green PCR Master Mix 2X at a final volume of 50 μl. Thermal cycling conditions were: 2 min at 50 °C, 10 min at 95 °C followed by 45 PCR cycles of denaturing at 95 °C for 15 s, annealing and elongation at 60 °C for 1 min. Fluorescence readings were performed on annealing/elongation steps. Data were expressed as arbitrary units -AU-, where 1 AU equals to 1 μg of standard mRNA [21].

2.5. Cytokine detection

Cytokines IL1-β, IL6, IL10, TNF-α and soluble TNFR type I (sTNFRI) were measured in culture supernatants employing commercially available ELISA kits according to the instructions of the manufacturer (BioLegend Company, CA, USA for IL1-β, IL6, IL10, TNF-α and R&D Systems, Inc. MN, USA for sTNFRI). Detection limits were: 2.0 pg/ml for IL1-β; 4.0 pg/ml for IL6; 2.0 pg/ml for IL10, 2.0 pg/ml for TNF-α and 15.0 pg/ml for sTNFRI.

2.6. Electron microscopy

Cells were prepared for transmission electron microscopy (TEM) by pelleting the various cell preparations by centrifugation for 1 min/6000 rpm. Cells were fixed in 1% glutaraldehyde dissolved in 0.1 M cacodylate buffer (pH 7); postfixed in 2% osmium tetroxide; dehydrated with increasing concentrations of ethanol and gradually infiltrated with Epon resin (Pelco). Thin sections were contrasted with uranyl acetate and lead citrate. In order to confirm autophagosomes, the subcellular localization of its specific marker LC3 protein was investigated by immunoelectronmicroscopy [22]. Briefly, cells were fixed in 4% paraformaldehyde in 0.2 M Sörensen buffer; dehydrated with different concentrations of ethylic alcohol and infiltrated with LR-White hydrosoluble resin (London Resin Co., Hampshire, United Kingdom). Sections of 60 to 80 nm thick were placed on nickel grids. The grids were incubated overnight at 4 °C with specific polyclonal rabbit anti-LC3 antibodies. After rinsing with PBS, the grids were incubated for 2 h at room temperature with goat anti-rabbit IgG (Sigma Chemical Co) conjugated to 5-nm gold particles (Sigma Chemical Co) diluted 1:20 in PBS. The grids were contrasted with uranyl acetate (Electron Microscopy Sciences, Fort Washington, PA) and examined with an M-10 Zeiss electron microscope (Karl Zeiss, Jena Germany). For morphometry, 30 cells from each condition were random selected at day one of incubation with steroids and photographed at 40,000x magnification, then the total number of phagosomes, autophagosomes and lysosomes were counted in each experimental group. From the total number of these vacuolar structures, the number and mean of autophagosomes was quantified and compared among the groups.

2.7. Statistical analysis

There were 5 independent rounds of experiments. Data are shown as means ± SEM. Statistical comparisons were performed by the Kruskall-Wallis and Mann-Whitney U tests. A *p* value <0.05 was considered statistically significant except the morphometric study, where the Student t test was used. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of steroid hormones on phagocytosis in cells infected with M. tuberculosis

Adherent cells were infected with Mtb H37Rv at an infection ratio of 5 bacteria/1 macrophage for 3 h and treated with Cortisol (Gc) and/or DHEA. As depicted in Figure 1, Cortisol decreased the phagocytosis of Mtb while the phagocytic capacity of macrophages was increased by DHEA but it was not significant. The combination of Cortisol 10^{-6} M and DHEA at 10^{-6} M significantly reverted the Cortisol inhibitory effect.

3.2. Effects of steroid hormones on the intracellular-bacterial growth of cells infected with M. tuberculosis

After infection, cultures treated with Cortisol or DHEA showed significant differences at Day 0, where treatment with DHEA at 10^{-7} M resulted in increased CFU numbers respect cultures exposed to Mtb alone (Figure 2, panel A). The same was true when comparing cultures treated with Gc + DHEA vs. those only infected with Mtb (Figure 2, panel A).

Considering culture timing, macrophages exposed to Mtb alone showed an increase in the CFU numbers from day 1 to day 4 By opposite, in cultures exposed to DHEA at 10^{-7} a trend to a decreased bacterial low as time elapsed was found, but differences remained statistically insignificant (Figure 3 panel A). Assessment at day 4 revealed that cultures treated with Gc + DHEA had significantly reduced CFU numbers with reference to their untreated counterparts (Figure 2 panel C). Moreover, at day 4 there was a significant CFU decrease ($p < 0.01$) respect to day 1 in the cultures infected and treated with Gc + DHEA (Figure 3 panel B).

3.3. Effects of steroids hormones on cytokines and sTNFR release in cells infected with M. tuberculosis H37Rv

Cells were incubated for 24 h in the presence or absence of Cortisol (10^{-6} M) and/or DHEA (10^{-6} M or 10^{-7} M) before *M. tuberculosis* infection (MOI 5:1). In cultures left uninfected and treated with hormones (Gc and/or DHEA), cytokines and sTNFR were undetectable (data not shown). As shown in Figure 5, Mtb-infected cultures had increased amounts of TNF-α, sTNFR, IL1-β and IL10. Except for sTNFR levels, which were significantly higher in Cortisol-treated cultures, this hormone decreased TNF-α, IL1-β and IL-10 levels respect to infected cultures left without hormones (Figure 4). In general terms, values in DHEA-treated cultures remained within the range seen in cells exposed to Mtb (Figure 4). Cultures treated with Gc + DHEA continued to show lesser amounts of TNF-α, IL1-β and IL10 together with higher quantities of sTNFR (Figure 4). IL6 levels were undetectable at this infection time point.

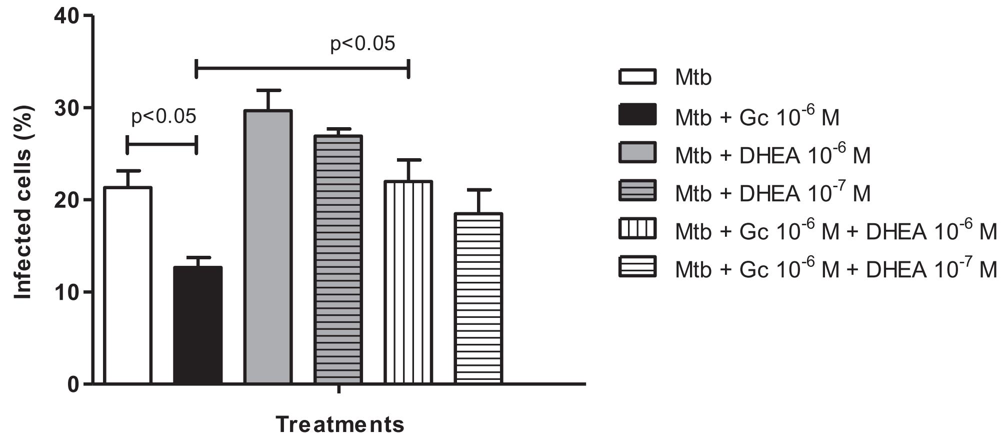

Figure 1. Effects of steroid hormones on phagocytosis by cells infected with *M. tuberculosis*. Cells were infected with *M. tuberculosis* H37Rv at an infection ratio of 5 bacteria/1 macrophage for 3 h and treated with Cortisol and/or DHEA. Non-phagocytosed bacteria were washed away and macrophages were fixed and stained. The values are means ± SEM (n = 5); $p < 0.05$. Mtb: *M. tuberculosis*; Gc: Cortisol 10^{-6} M; DHEA: DHEA 10^{-6} M or 10^{-7} M.

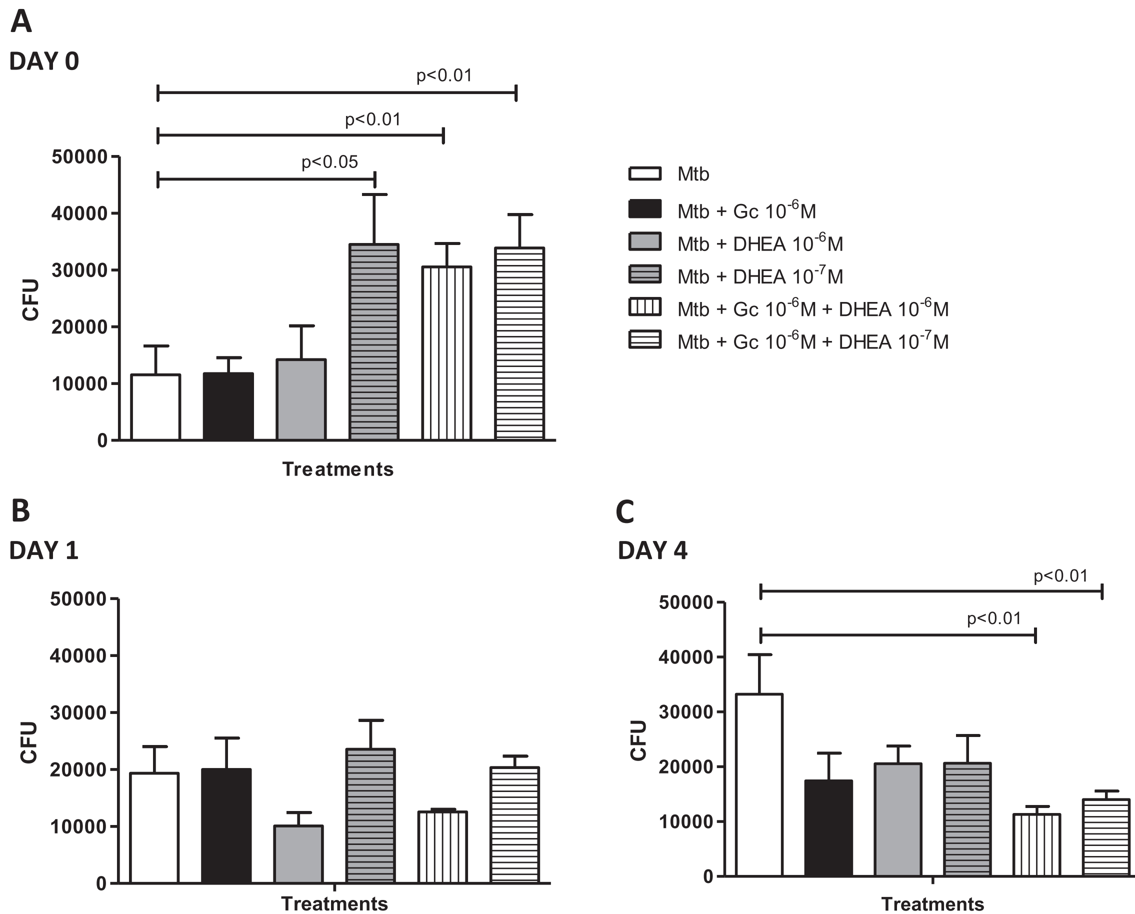

Figure 2. Effects of steroid hormones on CFU in cells infected with *M. tuberculosis*. Cells were infected with *M. tuberculosis* H37Rv at an infection ratio of 5 bacteria/1 macrophage for 3 h and treated with Cortisol and/or DHEA. Non-phagocytosed bacteria were washed away and macrophages were treated again with steroid hormones or medium. The values are means ± SEM (n = 5); Mtb: *M. tuberculosis*; Gc: Cortisol 10^{-6} M; DHEA 10^{-6} M or 10^{-7} M.

3.4. Effects of steroids hormones on cytokine gene expression in cells infected with M. tuberculosis

We next proceeded to analyze gene expression levels for a couple of cytokines also relevant in the macrophage response against pathogens. Data from Figure 6 revealed that Mtb induced the expression of TGF-β and IL23, with cortisol inhibiting the expression of the latter cytokine. DHEA failed to reverse such inhibitory effect. The pattern of IL23 expression in DHEA-treated cultures was similar to the one seen in *M. tuberculosis*-infected cultures (Figure 5 panel A). Infection with Mtb also led to a significant expression of TGF-β, although in this case treatment with Cortisol and/or DHEA resulted in no gross changes when compared with infected cultures left without hormones (Figure 5 panel B).

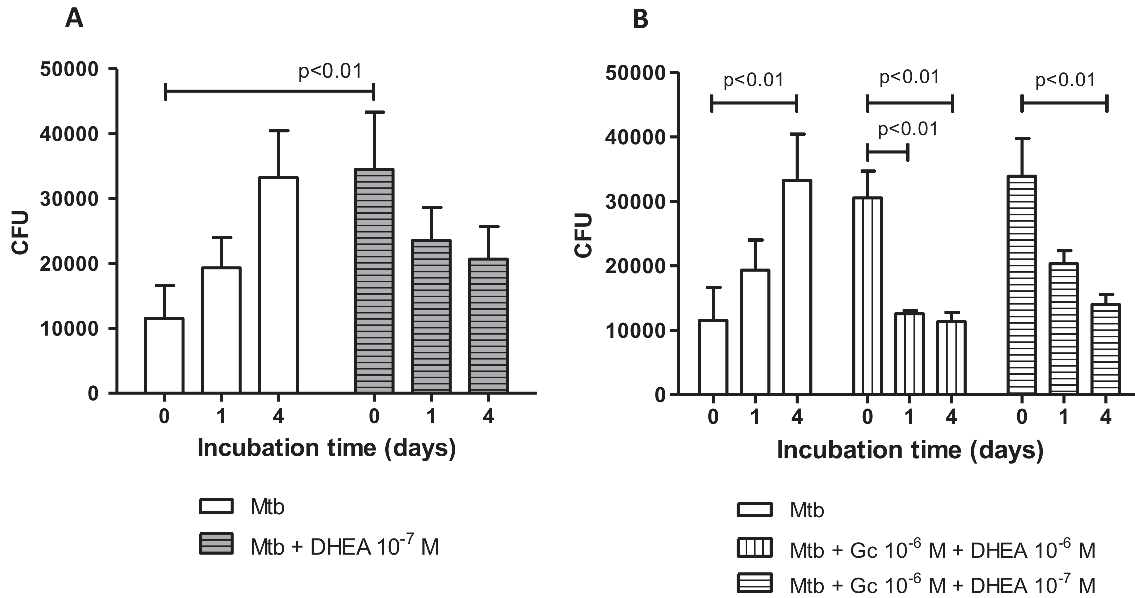

Figure 3. Effects of DHEA with or without Cortisol on CFU in cells infected with *M. tuberculosis*. A) Cells were infected with *M. tuberculosis* H37Rv for 3 h and treated with DHEA 10^{-7} M. Non-phagocytosed bacteria were washed away and macrophages were treated again with steroid hormones or medium. **B)** Cells were infected with *M. tuberculosis* H37Rv for 3 h and treated with Cortisol and DHEA. Non-phagocytosed bacteria were washed away and macrophages were treated again with the steroid hormone or medium. The values are means ± SEM (n = 5); Mtb: *M. tuberculosis*; Gc: Cortisol 10^{-6} M; DHEA 10^{-6} M or 10^{-7} M.

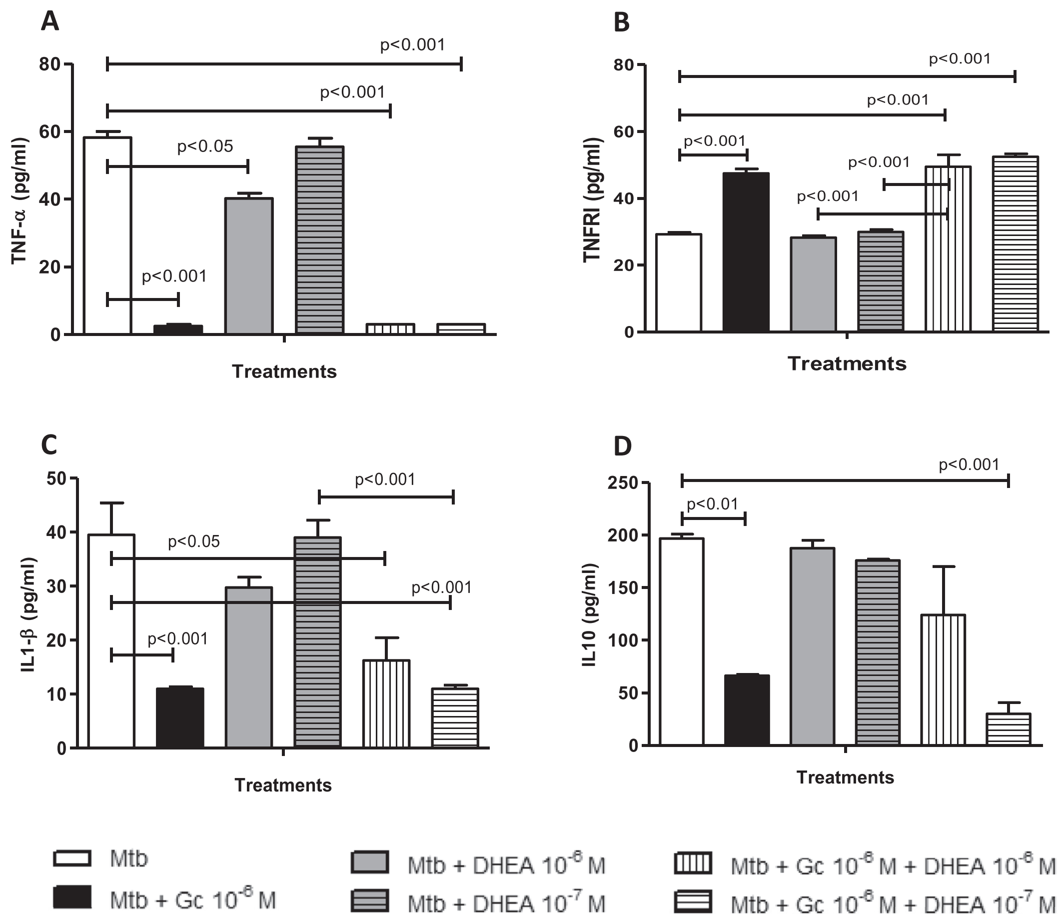

Figure 4. Effects of steroid hormones on cytokine release and sTNFRI levels in cells infected with *M. tuberculosis* H37Rv. Cells were incubated for 24 h in the presence or absence of Cortisol (10^{-6} M) and/or DHEA (10^{-6} M or 10^{-7} M) before Mtb infection (MOI 5:1). Production of TNFα, sTNFRI, IL1-β and IL10 (pg/ml) was measured in culture supernatants using ELISAs kits. The values are means ± SEM (n = 5). Mtb: *M. tuberculosis*; Gc: Cortisol 10^{-6} M; DHEA: DHEA 10^{-6} M or 10^{-7} M.

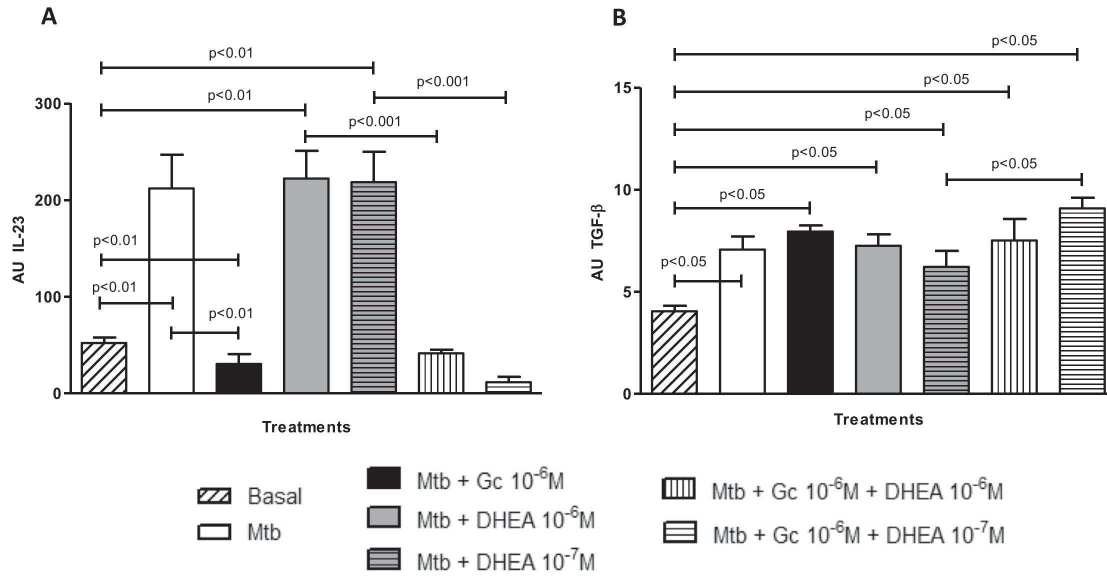

Figure 5. Effects of steroid hormones on cytokine gene expression in cells infected with *M. tuberculosis* H37Rv. Cells were incubated for 24 h in the presence or absence of Cortisol (10^{-6} M) and/or DHEA (10^{-6} M or 10^{-7} M) before Mtb infection (MOI 5:1). Gene expression of IL23 and TGF-β was measured in cells using real time RT-PCR. The values are means ± SEM (n = 5), of arbitrary units (**AU**). Mtb: *M. tuberculosis*; Gc: Cortisol 10^{-6} M; DHEA: DHEA 10^{-6} M or 10^{-7} M.

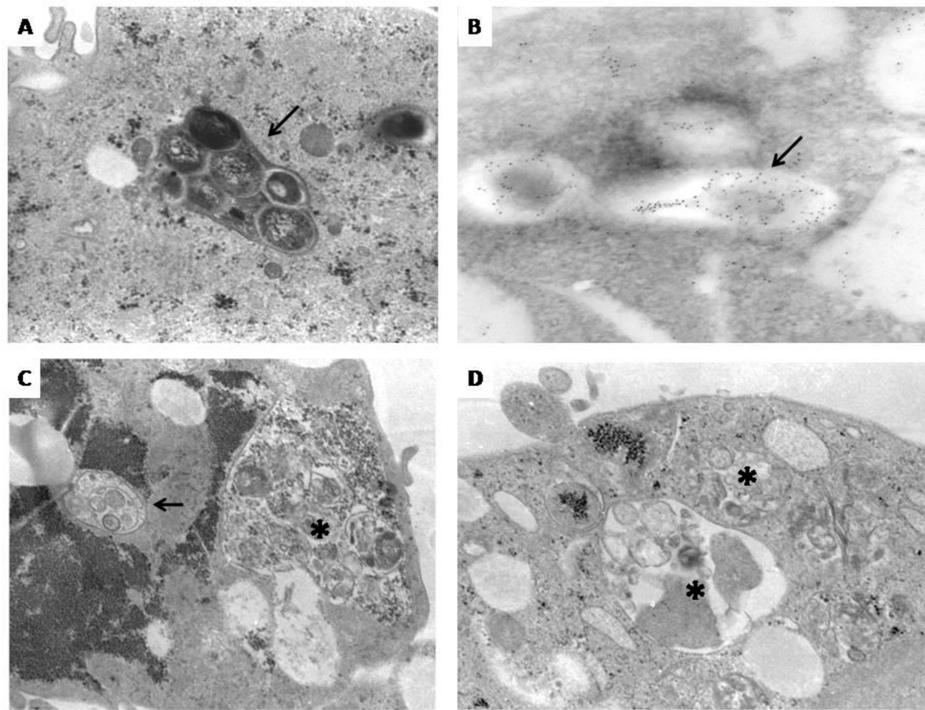

Figure 6. Representative electronmicroscopy and immunoelectronmicroscopy micrographs from infected macrophages incubated during 24h with adrenal steroids. A) The cytoplasm from an infected macrophage shows multi-vesicular structures corresponding to an autophagosome (arrow) (31,000x). B) These vacuoles show small black dots that correspond to immunogold labeling to the specific autophagosome specific marker LC3 (arrow) (63,000x). C) The cytoplasm from an infected macrophage incubated with DHEA (10^{-7} M) shows isolated (arrow) or conglomerate of autophagosomes (asterisk) (20,000x). D) Large autophagosomes (asterisks) are seen in the cytoplasm of macrophage incubated with Gc 10^{-6} M + DHEA 10^{-7} M (25,000x).

3.5. Autophagosome formation

Infected macrophages showed numerous cytoplasmic vacuoles, like primary lysosomes, phagosomes and autophagosomes. These latter organelles corresponded to multivescicular structures in which it was possible to recognize phagosomes associated with mitochondria, primary lysosomes or endoplasmic reticulum vacuoles. Autophagosomes were confirmed by the detection of the protein LC3 by immunoelectron microscopy an specific marker of these structures (Figure 6).

The total number of lysosomes, phagosomes and autophagosomes were assessed in 30 cells by experimental condition and the

number of autophagosomes was compared with the total number of cytoplasmic vacuoles. Control infected macrophages showed a mean of 13 ± 2 autophagosomes. A similar number was found when analyzing infected macrophages incubated with cortisol, whereas DHEA-treated macrophages displayed increased autophagosome numbers, in 10^{-6} M and 10^{-7} M concentrations, respectively, $p < 0.01$ (Figure 7). Some of these cells show bigger phagosomes forming large conglomerates (Figure 6).

Respect to cells infected and treated with both hormones (Gc + DHEA), the Gc 10^{-6} M + DHEA 10^{-6} M combination displayed values similar to infected cultures without hormones. Unlike this, Gc 10^{-6} M + DHEA 10^{-7} M had a higher amount of autophagosomes, some of them larger than those exhibited by infected macrophages without steroid hormones in the culture medium (Figure 6). Uninfected cells revealed no autophagosomes (data not shown).

4. Discusion

Macrophages engulf invading bacteria and kill them by the intracellular degradation system. However, some species of microorganisms, including *M. tuberculosis*, have strategies for evading intracellular degradation. These microorganisms can thrive in host phagocytes, favoring the development of infectious disease [23,24].

As with all complex organisms, single biological systems rarely work in isolation. There is extensive cross-talk between the immune and endocrine axis, together with the neural system, to form the major communication network in the body [25]. Cortisol is a glucocorticoid hormone secreted by the adrenal gland and plays a role in the stress response being also immunosuppressive [26]. DHEA and its sulphated precursor (DHEAS) have opposing actions to cortisol and may protect individuals from negative effects of aging and inflammation. They are secreted from the adrenal cortex and, in smaller amounts from testes and ovaries [27]. Confirming and extending former observations we here observed that cortisol has an immunosuppressive/anti-inflammatory effect given the decreased production of IL-1β, TNF-α in cultures exposed to this steroid. While, DHEA was unable to modify the pattern of cytokine production or to reverse the cortisol inhibitory effects; interestingly the intra-macrophagic bacterial burden was found reduced by DHEA treatment. This result adds further support to the beneficial effect of DHEA seen in previous studies from our group and other laboratories [13,14,28,29]. Studies during experimental tuberculosis also proved a protective effect of androstenediol and DHEA when administrated for 3 weeks, which is coincident with the phase of adrenal hyperplasia. Collectively, it follows that adrenal steroids are implicated in the pathogenesis of tuberculosis [30].

As regards the hormonal effects on phagocytosis, while cortisol decreased such function DHEA seemed to increase it in a dose-dependent manner. It is known that Mtb surface proteins interact with different receptors from host cell surface to mediate invasion beyond phagocytosis; i.e.,Toll-like receptors, mannose receptors, scavenger receptors, surfactant protein A and D receptors and complement receptors 1, 3 and 4 [31–33]. Whether DHEA is likely to modify receptor expressions and hence favoring Mtb entry without phagocytosis, remains to be established.

In relation to the reduced intra-macrophagic bacterial burden seen in single DHEA-treated cultures such findings could not be ascribed to a different cytokine pattern, in terms of their production or mRNA expression, suggesting that other mechanisms are accounting for the diminished bacterial load.

As regards to the mechanisms dealing with mycobacterial clearance, the induction of both apoptotic and necrotic cell death has been reported during the onset of tuberculosis in Mtb–infected macrophages. Necrotic cell death may cause dissemination of intracellular pathogens, from the site of infection. In contrast, apoptotic cell death is accompanied by complete packaging of the pathogens within apoptotic bodies, which are immediately engulfed by neighboring phagocytes [34–38]. Added to it, autophagy has also been implicated in several physiological processes, with perturbations in the autophagy phenomenon being associated to different diseases including the infectious ones [39–41]. Vesicle expansion and formation of autophagosomes is controlled by two ubiquitin-like conjugation processes: the Atg12-Atg5-Atg16L1 complex formation, and the conjugation of Atg8/LC3 to phosphatidylethanolamine. Finally, the newly formed autophagosomes fuse with lysosomes in a process that requires recruitment of the small GTPase Rab7 [42–44]. Studies have demonstrated that stimulation of autophagy suppressed the intracellular survival of Mtb *in vitro*. Upon infection of macrophages, Mtb block phagosome maturation in order to survive but induction of autophagy facilitates phagosome-lysosome fusion and degradation of pathogen [9]. In our hands, a lower dose of DHEA promoted autophagy in Mtb-infected macrophages, regardless of cortisol, suggesting that the CFU decrease from 1-4 days may be achieved through this mechanism.

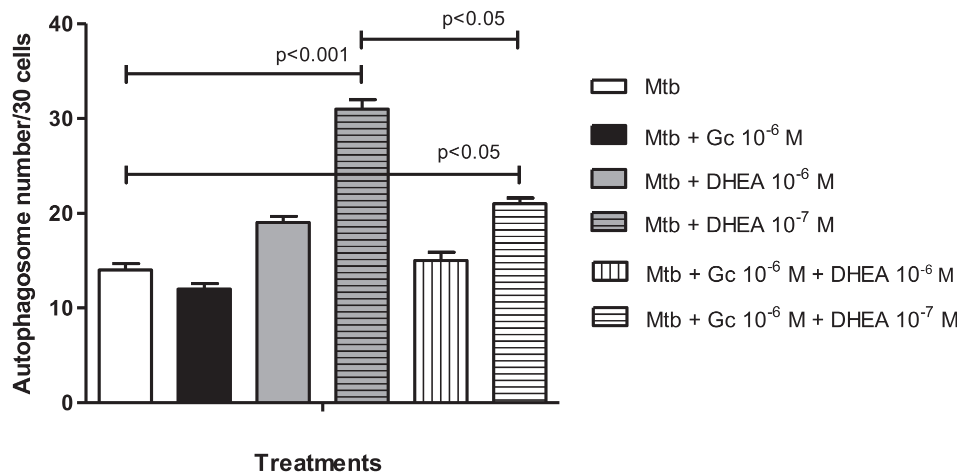

Figure 7. Effects of steroid hormones on autophagosome formation in cells infected with *M. tuberculosis* H37Rv. Cells were incubated for 24 h in the presence or absence of Cortisol (10^{-6} M) and/or DHEA (10^{-6} M or 10^{-7} M) before Mtb infection (MOI 5:1). Quantification of autophagosome numbers is represented in the graph. Values correspond to means ± SEM (n = 30 cells). Mtb: *M. tuberculosis*; Gc: Cortisol 10^{-6} M; DHEA: DHEA 10^{-6} M or 10^{-7} M.

In conclusion, DHEA in physiological conditions (that is in presence of cortisol) leads to autophagy induction and hence a better Mtb control. These results sound interesting since autophagy is not only important for bacterial clearance but also for the prevention of tissue damage [45]. Last but not least, present results add support to the use of DHEA as an adjuvant therapy for TB.

Funding: None.

Competing interests: None declared.

Ethical approval: Not required.

Acknowledgments

This study was supported by grants from the Consejo Nacional de Ciencia y Tecnología (CONACYT 253053) of México, Agencia de Promoción Científica y Tecnológica Argentina (PID160, ANPCyT) and Secretaria de Estado de Ciencia, Tecnología e Innovación de la Provincia de Santa Fe, Argentina [2010-140-11]. We thank Esmeralda Juarez PhD for her advice in Microbiology topics.

References

[1] World Health Organization. Global tuberculosis report. WHO report 2014. Available at: http://apps.who.int/iris/bitstream/10665/137094/1/9789241564809_eng.pdf?ua=1.
[2] Comstock GW. Epidemiology of tuberculosis. Am Rev Respir Dis 1982;125:8–15.
[3] Hestvik AL, Hmama Z, Av-Gay Y. Mycobacterial manipulation of the host cell. FEMS Microbiol Rev 2005;29:1041–50.
[4] Russell DG. Mycobacterium tuberculosis: here today, and here tomorrow. Nat Rev Mol Cell Biol 2001;2:569–77.
[5] Jordao L, Bleck CK, Mayorga L, Griffiths G, Anes E. On the killing of mycobacteria by macrophages. Cell Microbiol 2008;10:529–48.
[6] Clemens DL, Horwitz MA. Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. J Exp Med 1995;181:257–70.
[7] Rohde K, Yates RM, Purdy GE, Russell DG. *Mycobacterium tuberculosis* and the environment within the phagosome. Immunol Rev 2007;219:37–54.
[8] de Chastellier C. The many niches and strategies used by pathogenic mycobacteria for survival within host macrophages. Immunobiology 2009;214:526–42.
[9] Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. Cell 2004;119(6):753–66.
[10] Castillo EF, Dekonenko A, Arko-Mensah J, Mandell MA, Dupont N, Jiang S, Delgado-Vargas M, Timmins GS, Bhattacharya D, Yang H, Hutt J, Lyons CR, Dobos KM, Deretic V. Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. Proc Natl Acad Sci USA 2012;109(46):E3168–76.
[11] Cutolo M, Straub RH, Bijlsma JW. Neuroendocrine-immune interactions in synovitis. Nat Clin Pract Rheumatol 2007;3:627–34.
[12] Loria RM. Beta-androstenes and resistance to viral and bacterial infections. Neuroimmunomodulation 2009;16(2):88–95.
[13] del Rey A, Mahuad CV, Bozza VV, Bogue C, Farroni MA, Bay ML, Bottasso OA, Besedovsky HO. Endocrine and cytokine responses in humans with pulmonary tuberculosis. Brain Behav Immun 2007;21:171–9.
[14] Bottasso O, Bay ML, Besedovsky H, Del Rey A. Adverse neuro-immune-endocrine interactions in patients with active tuberculosis. Mol Cell Neurosci 2013;53:77–85.
[15] Bongiovanni B, Díaz A, D'Attilio L, Santucci N, Dídoli G, Nannini JL, Gardeñez W, Bogue C, Besedovsky H, del Rey A, Bottasso O, Bay ML. Changes in the immune-endocrine response in patients with pulmonary tuberculosis undergoing specific treatment. Ann N Y AcadSci 2012;1262(1):10–5.
[16] Pieters J. *Mycobacterium tuberculosis* and the macrophage: maintaining a balance. Cell Host Microbe 2008;3:399–407.
[17] Fenton MJ, Vermeulen MW. Immunopathology of tuberculosis: roles of macrophages and monocytes. Infect Immun 1996;64:683–90.
[18] Carranza C, Juárez E, Torres M, Ellner J, Sada E. Mycobacterium tuberculosis growth control by lung macrophages and CD8 cells from patient contacts. Am J Respir Crit Care Med 2006;173:238–45.
[19] Tuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer 1980;26:171–6.
[20] Mahuad C, Bay ML, Farroni MA, Bozza V, Del Rey A, Besedovsky H, Bottasso OA. Cortisol and dehydroepiandrosterone affect the response of peripheral blood mononuclear cells to mycobacterial antigens during tuberculosis. Scand J Immunol 2004;60:639–46.
[21] D'Attilio L, Trini E, Bongiovanni B, Dídoli G, Gardeñez W, Nannini L, Giri A, Bottasso A, Bay ML. mRNA expression of alpha and beta isoforms of glucocorticoid receptor in peripheral blood mononuclear cells of patients with TB and its relation with components of the immunoendocrine response. Brain Behav Immun 2011;25:461–7.
[22] Rivas-Santiago B, Schwander SK, Sarabia C, Diamond G, Klein-Patel ME, Hernandez-Pando R, Ellner JJ, Sada E. Human {beta}-defensin 2 is expressed and associated with *Mycobacterium* tuberculosis during infection of human alveolar epithelial cells. Infect Immun 2005;73:4505–11.
[23] Vergne I, Chua J, Lee HH, Lucas M, Belisle J, Deretic V. Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 2005;102:4033–8.
[24] Divangahi M, Chen M, Gan H, Desjardins D, Hickman TT, Lee DM, Fortune S, Behar SM, Remold HG. Mycobacterium evades macrophage defenses by inhibiting plasma membrane repair. Nat Immunol 2009;10:899–906.
[25] Straub RH, Cutolo M, Zietz B, Scholmerich J. The process of aging changesthe interplay of the immune, endocrine and nervous systems. Mech Ageing Dev 2001;122(14):1591–611.
[26] Phillips AC, Carroll D, Gale CR, Lord JM, Arlt W, Batty GD. Cortisol, DHEAsulphate, their ratio, and all-cause and cause-specific mortality in theVietnam experience Study. Eur J Endocrinol 2010;163(2):285–92.
[27] Baylis D, Bartlett D, Patel H, Roberts H. Understanding how we age: insights intoinflammaging. Longev &Healthspan 2013;2:1–8.
[28] Mahuad C, Bozza V, Pezzotto SM, Bay ML, Besedovsky H, del Rey A, Bottasso O. Impaired immune responses in tuberculosis patients are related to weight loss that coexists with an immunoendocrineimbalance. Neuroimmunomodulation 2007;14(3–4):193–9.
[29] D'Attilio L, Bozza VV, Santucci N, Bongiovanni B, Dídoli G, Radcliffe S, Besedovsky H, del Rey A, Bottasso O, Bay ML. TGF-β neutralization abrogates the inhibited DHEA production mediated by factors released from M. tuberculosis-stimulated PBMC. Ann N Y Acad Sci 2012;1262:1–9.
[30] Hernandez-Pando R, De La Luz Streber M, Orozco H, Arriaga K, Pavon L, Al-Nakhli SA, Rook GA. The effects of androstenediol and dehydroepiandrosterone on the course and cytokine profile of tuberculosis in BALB/c mice. Immunology 1998;95(2):234–41.
[31] Ernst JD. Macrophage receptors for *Mycobacterium tuberculosis*. Infec Immun 1998;66:1277–81.
[32] Rooyakkers AW, Stokes RW. Absence of complement receptor 3 results in reduced binding and ingestion of *Mycobacterium tuberculosis* but has no significant effect on the induction of reactive oxygen and nitrogen intermediates or on the survival of the bacteria in resident and interferon-gamma activated macrophages. Microb Pathog 2005;39:57–67.
[33] Ocampo M, Aristizábal-Ramírez D, Rodríguez DM, Muñoz M, Curtidor H, Vanegas M, Patarroyo MA, Patarroyo ME. The role of Mycobacterium tuberculosis Rv3166cprotein-derived high-activity binding peptidesin inhibiting invasion of human cell lines. Protein Eng Des Sel 2012;25(5):235–42.
[34] Fratazzi C, Arbeit RD, Carini C, Remold HG. Programmed celldeath of *Mycobacterium avium*serovar 4-infected human macrophages preventsmycobacteria from spreading and induces mycobacterial growth inhibition byfreshly added, uninfected macrophages. J Immunol 1997;158:4320–7.
[35] Danelishvili L, McGarvey J, Li YJ, Bermudez LE. *Mycobacterium tuberculosis* infection causes different levels of apoptosis and necrosis in human macrophages and alveolar epithelial cells. Cell Microbiol 2003;5:649–60.
[36] Gil DP, León LG, Correa LI, Maya JR, París SC, García LF, Rojas M. Differential induction of apoptosis and necrosis in monocytes from patients with tuberculosis and healthy control subjects. J Infect Dis 2004;189:2120–8.
[37] Zhang J, Jiang R, Takayama H, Tanaka Y. Survival of virulent *Mycobacterium tuberculosis* involves preventing apoptosis induced by Bcl-2 upregulation and release resulting from necrosis in J774 macrophages. Microbiol Immunol 2005;2005(49):845–52.
[38] Iyoda T, Takada M, Fukatsu Y, Kumokoshi S, Fujisawa T, Shimada T, Shimokawa N, Matsunaga T, Makino K, Doi N, Terada H, Fukai F. A novel mechanism underlying the basic defensive response of macrophages against *Mycobacterium* infection. J Immunol 2014;192(9):4254–62.
[39] Pengo N, Scolari M, Oliva L, Milan E, Mainoldi F, Raimondi A, Fagioli C, Merlini A, Mariani E, Pasqualetto E, Orfanelli U, Ponzoni M, Sitia R, Casola S, Cenci S. Plasma cells require autophagy for sustainable immunoglobulin production. Nat Immunol 2013;14(3):298–305.
[40] Deretic V. Autophagy in immunity and cell-autonomous defense against intracellular microbes. Immunol Rev 2011;240(1):92–104.
[41] Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature 2011;469(7330):323–35.
[42] Deretic V, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. Nat Rev Immunol 2013;13:722–37.
[43] Perot BP, Ingersoll MA, Albert ML. The impact of macroautophagy on CD8(+) T-cell-mediated antiviral immunity. Immunol Rev 2013;255:40–56.
[44] Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol Rev 2012;249:158–75.
[45] Deretic V. Autophagy in tuberculosis. Cold Spring Harb Perspect Med 2014;4(11). http://dx.doi.org/10.1101/cshperspect.a018481.