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Corticosteroids protect infected cells against mycobacterial killing in vitro

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The effect of corticosteroids on human physiology is complex and their use in tuberculosis patients remains controversial. In a high-throughput screening approach designed to discover virulence inhibitors, several corticosteroids were found to prevent cytolysis of fibroblasts infected with mycobacteria. Further experiments with Mycobacterium tuberculosis showed anti-cytolytic activity in the 10 nM range, but no effect on bacterial growth or survival in the absence of host cells at 20 μM. The results from a panel of corticosteroids with various affinities to the glucocorticoid- and mineralocorticoid receptors indicate that the inhibition of cytolysis most likely is mediated through the glucocorticoid receptor. Using live-imaging of M. tuberculosis-infected human monocyte-derived macrophages, we also show that corticosteroids to some extent control intracellular bacteria. In vitro systems with reduced complexity are to further study and understand the interactions between bacterial infection, immune defense and cell signaling.

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1. Introduction

Tuberculosis (TB) is the leading cause of death from a single infectious agent and one of the top 10 causes of death worldwide. WHO estimates that about 10 million people develop TB disease each year [1]. One of the hallmarks of TB is its ability to cause latent infection. In such cases, the infected person has no symptoms, is not contagious, but is at risk for reactivation. The mechanisms of reactivation are poorly understood, but it is widely believed that patients with latent TB treated with immunosuppressive agents such as corticosteroids are at high risk of reactivation and this has been demonstrated in several case-control studies [23]. Agents with immunosuppressive activity are therefore generally contraindicated in TB patients.

Our team recently performed a high-throughput screening campaign to discover substances capable of preventing cytolysis of fibroblast cells upon mycobacterial infection [4]. With this strategy, we expected to discover classic antibacterials with bacteriostatic and bactericidal effects, virulence inhibitors with bacterial targets and substances with impact on the host cell in line with an immunomodulatory mode of action. The screen resulted in 49 substances which met the selection criteria, but among these 38 were excluded as known antibiotics, or for having undesirable properties. Among the substances initially excluded for having undesirable properties were five corticosteroids (data not shown). Although initially rejected for having adverse effects on TB, the corticosteroids had caught our interest and several reports in the literature indicate that corticosteroids may have a beneficial effect for TB outcome. In a joint publication from the American Thoracic Society, Centers for Disease Control and Prevention and Infectious Diseases Society of America, corticosteroid treatment is strongly recommended in the treatment of TB pericarditis and TB of the central nervous system, but not recommended for other...
manifestations [5]. Critchley et al. performed a systematic review and meta-analysis to elucidate if corticosteroid treatment can prevent TB mortality. Steroids reduced mortality by 17% and the authors suggest that they could be reducing mortality for all forms of TB, also pulmonary, although further evidence is needed [6].

Corticosteroids are the major hormones of the hypothalamic-pituitary-adrenal axis. They are best known for their strong anti-inflammatory and immune modulatory activity, but they are also important in fetal development, metabolism, body fluid homeostasis and many other physiological processes. Synthetic glucocorticoids are widely used in treatment of allergy, asthma, autoimmune diseases, sepsis and other inflammatory disorders. The target glucocorticoid receptor (GR) is a versatile transcription factor expressed in nearly all cells in the body. It is expressed in several splice variants and can act on DNA both by transactivation and transcription. Upon activation by cortisol or synthetic corticosteroids, its anti-inflammatory activity is mediated mainly by inhibition of NF-κB and c-Jun-Fos and by induction of proteins such as MAPK phosphatase 1, IκB and annexin I among others, as reviewed in Ref. [7]. The mechanisms of the GR and how it can possess both pro- and anti-inflammatory activity is not fully understood.

Glucocorticoids have vastly different effects in different cell types. While glucocorticoids can induce apoptosis in many cell types throughout the body, other exhibit an anti-apoptotic response to glucocorticoid signaling [8]. In macrophages, glucocorticoids have immunostimulatory effects [9], and act as a potent anti-apoptotic in primary human fibroblasts whereas it is pro-apoptotic in hematopoietic cells. The anti-apoptotic effect by dexamethasone is attributed to a combination of phosphoinositide 3-kinase/Akt signaling and involvement of the BCL2 family protein BCL-XL [10].

The other class of corticosteroids, the mineralocorticoids maintain electrolyte homeostasis in the body. The mineralocorticoid receptor has affinity for both glucocorticoids and mineralocorticoids (e.g. aldosterone) just as the GR also responds to mineralocorticoids [11,12]. Due to the salt-retaining properties of mineralocorticoid receptor signaling, many synthetic glucocorticoids are designed to minimize activation of this receptor [13].

The results from our high-throughput screening prompted us to further investigate the effect of corticosteroids on cells infected by mycobacteria. We show that corticosteroids not only prevent cytosis of mycobacteria-infected lung fibroblast cells, but also reduce intracellular bacterial growth in human monocyte-derived macrophages even though they have no effect on bacterial growth or survival in the absence of host cells. The results indicate that the effect most likely is mediated by the GR. Although corticosteroids have a systemic and complex impact on human physiology, the in vitro experiments presented can help us understand the host response to mycobacterial infection on a cellular level.

2. Materials and methods

2.1. Cell culture

Human lung fibroblast cell line MRC-5 (ATCC® CCL-171™) was maintained and expanded in Advanced Dulbecco’s Modified Eagle Medium (A-DMEM) (Life technologies) supplemented with 5% new-born calf serum and 0.3% (w/v) I-glutamine at 37 °C with 5% CO2 until 80% confluent.

Wild-type Mycobacterium marinum M (ATCC BAA-535™) and a RD1 knockout with M. marinum M background (a kind gift from Fredric Carlsson, Lund university) were grown in 7H9 medium (BD Difco) supplemented with 0.05% Tween80 and 10% ADS (0.5% albumin, 0.2% dextrose and 0.085% saline) as standing cultures at 30 °C. M. tuberculosis H37Rv, H37Rv harboring the pCherry3 plasmid carrying mCherry gene under a constitutive promoter (Psymc) [14] and H37Ra were also grown in 7H9 medium supplemented with 0.05–0.10% Tween80 and 10% ADS as standing cultures at 37 °C.

2.2. Compounds

A chemical library of 28,000 compounds provided as stock solutions in dimethyl sulfoxide (DMSO) from the Laboratories for Chemical Biology Umeå and Chemical Biology Consortium Sweden library was screened at 20 μM to identify hits that would prevent M. marinum-induced cytosis of MRC-5 fibroblasts as described previously [4]. Budesonide (Cayman chemical), Fluorocortisone acetate, deoxycorticosterone acetate, dexamethasone (MP Biomedicals), flumethasone, flunisolide (Santa Cruz), hydrocortisone, aldosterone, betamethasone, triamcinolone acetate (Acros Organics), fluocinolone acetonide (abcam) and desonide (Sigma-Aldrich) were dissolved in DMSO to 10 mM and stored at 4 °C until used in experiments. Working solutions of isoniazid (Fluka) and rifampicin (G Biosciences) were freshly prepared in DMSO for each experiment.

2.3. Activity of corticosteroids on M. tuberculosis

Twelve corticosteroids were assayed at concentrations ranging from 0.001 μM to 0.5 μM on MRC-5 fibroblast cells infected with M. tuberculosis H37Rv in 96-well plates as described previously [4]. Corticosteroid impact on MRC-5 cell viability was plotted as bar graphs based on average and standard deviation of triplicates. In order to determine if corticosteroids have bactericidal or bacteriostatic activity, bacterial growth and survival was assayed with corticosteroid concentrations up to 20 μM in 7H9 media as described in Ref. [4].

2.4. Preparation of human monocyte-derived macrophages (hMDMs)

Peripheral blood mononuclear cells (PBMCs) were isolated fromuffy coats obtained from healthy volunteers who gave their written informed consent for the use of their blood for scientific purposes (Linköping University Hospital blood bank, Linköping, Sweden) as described in Ref. [15]. PBMC isolation was performed using LymphoPrep (Axis-Shield), according to the instructions from the manufacturer. The mononuclear cells were seeded in culture flasks and later differentiated into hMDMs as previously described [16].

2.5. Infection of hMDMs monitored by IncuCyte live cell microscopy

The hMDMs obtained from two different donors were washed, trypsinated and resuspended in DMEM (Gibco), supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM I-glutamine (Gibco) and 10% non-heated inactivated human serum pooled from 5 donors (Linköping University Hospital). The hMDMs were seeded in a 384-well plate (8000 cells/well) and infected with M. tuberculosis H37Rv:pCherry3 reporter strain at a multiplicity of infection (MOI) of 1:1 in the presence of 0.5 μM of corticosteroid. To control wells, 0.4% DMSO or 0.1 μg/ml isoniazid or 0.1 μg/ml rifampicin was added. The plate was incubated at 37 °C with 5% CO2. At different time points (day 1, 2, 3, 4 and 5) images from each well were acquired with an IncuCyte live-cell microscope (Essen Biosciences). Custom analysis scripts within the IncuCyte Software were used to measure fluorescence signal from M. tuberculosis H37Rv:pCherry3 reporter strain. In this custom
script, intensity and particle size cut-offs were used to discriminate background noise and identical parameters were applied for each image acquisition. For each well and time point, mean integrated fluorescence intensities were calculated with the IncuCyte software. Average values obtained from day 1 replicate wells were set to 100% and the average values from the corresponding wells at other time points were normalized accordingly. The bacterial growth from day 1 to day 5 was plotted as line graphs based on average and standard deviation of triplicates. In addition, difference in bacterial growth at day 5 was plotted as bar graphs based on average and standard deviation of triplicates.

3. Results and discussion

TB in general and antibiotic-resistant TB in particular, is difficult to treat and new drugs and treatment strategies are much needed. In a previous study, we therefore screened 28,000 compounds to identify substances with anti-cytolytic activity in MRC-5 fibroblast cells infected with mycobacteria. This investigation resulted in 8 novel compounds that prevent cytolysis of fibroblasts infected with M. marinum as well as M. tuberculosis H37Rv without having any bactericidal or bacteriostatic effect [4]. However, after the initial screening, 38 hits had been discarded as already known antibacterials or assumed not to be suitable for TB treatment. Amongst these initially discarded hits, one group of substances caught our attention — the corticosteroids. Budesonide, fluocortisone acetate, flumethasone, flunisolide and hydrocortisone base were all preventing cytolysis of MRC-5 cells infected with M. marinum (data not shown). These hits were discarded in our earlier publication since corticosteroids generally are regarded as a risk factor for reactivating latent TB [2,3,17,18]. However, recent clinical studies suggest that usage of corticosteroids during active TB infection might be beneficial [5,6].

We decided to further investigate the effects of corticosteroids in our fibroblast infection assay by evaluating the five initial corticosteroid screening hits as well as additional seven corticosteroid compounds in M. tuberculosis H37Rv infected MRC-5 fibroblasts and comparing the MRC-5 viability in the end of a two-day infection. All corticosteroids except deoxycorticosterone acetate and aldosterone prevented cytolysis of the MRC-5 cells at concentration range from 0.001 μM to 0.1 μM (Fig. 1). Interestingly, deoxycorticosterone acetate and aldosterone are potent activators of the mineralocorticoid receptor but have little or no glucocorticoid activity [13,19]. In contrast, all corticosteroids with anti-cytolytic activity have glucocorticoid activity as well as some also have additional mineralocorticoid activity [13,19]. These results suggest that the glucocorticoid anti-cytolytic activity observed is mediated by the GR rather than the mineralocorticoid receptor. Nieuwenhuis and co-workers described the molecular mechanism of how dexamethasone protects fibroblasts from apoptosis involving both phosphoinositide 3-kinase/Akt signaling and the BCL2 family protein BCL-Xl [10], but further experiments are needed to elucidate if similar mechanisms are involved in the anti-cytolytic activity we observe.

Since fibroblasts are not natural host cells of M. tuberculosis, we decided to test the effect of corticosteroids in a biologically more relevant intracellular infection model. We infected hMDMs obtained from two different donors with 1 MOI of M. tuberculosis H37Rv:pCherry3 and followed bacterial growth from day 1 to day 5 using an IncuCyte live-cell microscope (Essen Bioscience). Despite the fact that none of these corticosteroids were bacteriostatic or bactericidal up to 20 μM in 7H9 medium (Supplementary Fig. 1), they significantly reduced the intracellular bacterial growth in hMDMs cells at 0.5 μM (Fig. 2). Corticosteroids with glucocorticoid activity significantly reduced bacterial growth in hMDMs obtained from both donors (Fig. 2), whereas also corticosteroids with...
mineralocorticoid activity but low glucocorticoid activity (deoxycorticosterone acetate and aldosterone) seemed to have some activity in cells from donor 1 (Fig. 2). This is not very surprising since numerous single nucleotide polymorphisms in a number of genes are well known to determine the individual response to inhaled corticosteroids. For instance, asthma treatment with corticosteroids has a large inter-individual variability with numerous patients not responding to the treatment at all [20,21]. Moreover, 11β-hydroxylation of deoxycorticosterone produces a corticosteroid with glucocorticoid activity [12], and aldosterone can to some degree bind the GR resulting in GR-mediated cellular response [11].

The corticosteroid system itself is very complex and the level of complexity increases drastically with TB infection. A massive immune response, inflammation, pharmacokinetic interactions between corticosteroids and TB drugs as reviewed in Ref. [22], as well as rifampicin itself activating the GR [23], forms an overwhelming complexity.

We cannot form an opinion whether corticosteroid use in TB is beneficial or not based on the experiments presented and this was never the aim of the study. Instead, we attempted to simplify the

![Image of Figure 2](image-url)
complexity, and by studying the effect of corticosteroids on infected cells in vitro we show that glucocorticoids protect against mycobacterial killing. We believe simplified model experiments will be key to decipher the immense complexity of corticosteroid effects on TB infection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.02.044.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.02.044.

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