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miR-30c is specifically repressed in patients with active pulmonary tuberculosis

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ABSTRACT

Tuberculous pleurisy (PLTB) is a common form of extrapulmonary tuberculosis. It often resolves without chemotherapy being hence considered a rather benign manifestation of the disease. Patients with PLTB mount an effective anti-mycobacterial response, unlike those with active pulmonary TB (pTB) that were shown to present an imbalance in plasma immune and endocrine mediators. In this work, we explored whether expression of the active isoform of the glucocorticoid receptor (hGRα) in the context of the inflammatory-anti-inflammatory responses of TB patients may be associated to microRNA levels. As expected, the inflammatory response triggered in patients coexists with increased circulating cortisol and altered hGRα levels in the peripheral blood mononuclear cells. However, while hGRα expression is significantly downregulated in PLTB, its levels in pTB patients are higher within the control values. These results point out to the existence of an additional mechanism tending to preserve hGRα levels probably to deal with the chronic inflammation observed in pTB. In this regard, we found that miR-30c is strongly downregulated in mononuclear cells of pTB patients compared to PLTB cases, showing an expression profile opposite to that seen with hGRα. Interestingly, low levels of miR-30c are specific for this active form of TB, as its expression is not altered in mononuclear cells from either healthy controls or patients with tuberculous or non-tuberculous pleurisy. Moreover, miR-30c and hGRα also showed an inverse expression pattern in *M. tuberculosis*-stimulated THP-1 macrophage cultures. In sum, our studies identify miR-30c as a specific correlate of pulmonary manifestations of TB, potentially involved in the altered glucocorticoid sensitivity observed in these patients.

1. Introduction

Mycobacterium tuberculosis (Mtb), the causative agent of human tuberculosis, is still a major threat to humankind. About 8 million new cases and more than 1.3 million deaths annually place tuberculosis (TB) among the top three fatal infections [1]. The host response to Mtb is known to play a key role in determining the clinical manifestations and the ultimate disease outcome [2–4]. Such defensive reaction is complex and involves a series of steps dealing with the immune mechanisms put into play and the processes regulating them. In this regard, there is a bidirectional communication between the neuroendocrine and immune systems. In particular, the hypothalamus-pituitary-adrenal (HPA) axis plays a critical role in maintaining immune homeostasis. Endogenous glucocorticoids (GC), acting as key molecules of the HPA axis, take part in coordinating the immune response against infectious agents and other insults and its subsequent modulation to return to homeostasis [5–7]. Previews studies performed by our group showed that TB patients have augmented levels of cortisol -the most important GC in humans- accompanied by increased amounts of compounds with substantial proinflammatory effects [5,8]. These findings suggest an imbalanced immune-endocrine response

Abbreviations: TB, tuberculosis; *Mtb*, *Mycobacterium tuberculosis*; GC, glucocorticoids; GR, glucocorticoid receptor; HPA, hypothalamus-pituitary-adrenal; pTB, pulmonary TB; PLTB, pleural TB or tuberculous pleurisy; noPLTB, non tuberculous pleurisy; HCo, healthy controls; miRNA, microRNA; 3′UTR, 3′ untranslated region; PBMC, peripheral blood mononuclear cells; PE, pleural effusions.

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which may explain the increased inflammation and tissue destruction occurring in these patients.

In immune cells, the GC receptor (hGRα) is located in the cytoplasm in an inactive multiprotein complex form containing chaperones and co-chaperones. Upon GC binding, a molecular rearrangement of the GR heterocomplex occurs, promoting nuclear relocalization, homodimerization and subsequent downregulation of key mediators of the inflammatory response [6,9]. Several distinct molecular mechanisms contributing to modulate glucocorticoid sensitivity have now been identified, so that there is heterogeneity of mechanisms even within a single disease [9]. Among them, genetic susceptibility, post-translational modifications of the hGRα, increased expression of an inactive isoform of the receptor (hGRβ) and defective histone acetylation are the best studied. Recent findings point out that microRNA (miRNA) dependent post-transcriptional regulation might also be involved in controlling GR activity by reducing GR protein levels [10,11]. In line with these findings, bioinformatic analyses performed on the gene encoding the receptor (NR3C1) predicts more than 100 putative miRNAs binding sites in its 3′UTR sequence, a surprisingly high number implying this mechanism as a relevant piece in the regulation of hGRα levels.

miRNAs are endogenous ~23 nt RNAs that play important gene-regulatory roles by pairing to the mRNAs of protein-coding genes to direct their post-transcriptional repression [12]. These small molecules are involved in a wide range of physiological responses, including development, differentiation and homeostasis [12,13], being estimated that they may regulate as much as 60% of mammalian genes (http://www.mirbase.org/). miRNAs are also crucial regulators of the human immune response to infectious agents like mycobacteria. Several studies detected altered expression levels of miRNAs in infected individuals and an increasing amount of data associates specific miRNA expression profiles with different stages and forms of the disease [2,14,15].

In this work we sought to find a link between the expression of hGRα and miRNAs putatively capable of regulating its levels in different manifestations of TB. We demonstrated that miR-30c is specifically downregulated in mononuclear cells from patients with active pulmonary TB (pTB) but not in patients with tuberculous pleurisy, a highly favorable form of the disease [16,17]. Interestingly, expression of miR-30c showed an inverse relationship with hGRα levels both in pTB patient samples compared to PLTB; and in an *in vitro* model using the THP-1 cell line, reinforcing the association between the levels of miR-30c and hGRα expression.

2. Materials and methods

2.1. miRNA selection criteria

A bioinformatic approach was employed to select miRNAs associated to TB that may putatively target the GR transcript. The miRWalk2.0 web-interface was employed to identify putative miRNA binding sites in the 3 UTR of NR3C1 (ID:2908) by combining information from 4 existing miRNA-target prediction softwares (miRWalk2.0, miRanda, RNA22, and Targetscan) [18]. Conserved miRNA binding sites predicted by all of them (118 out of the 1842 documented by at least one software) were further analyzed (Suppl. Table 1). Then, candidates were selected based on phylogenetic data and expression levels: 34 out of the 118 candidates belong to miRNAs families broadly conserved among vertebrates [19] and only 19 correspond to highly abundant miRNA guide strands (miRbase). miARNs studied in this work, miR-29a, miR-30c, miR-124, and miR-181a/b, were finally selected based on previously reported association to Mtb infection (Suppl. Table 2).

2.2. Study groups

33 subjects were included in this study from people attending at Carrasco Hospital from Rosario city between 2010 and 2014. Participants were enrolled upon obtaining their written consent. This work was approved by the Ethical Committee of the School of Medical Sciences, Rosario National University.

Blood samples from 9 newly diagnosed pulmonary TB patients (pTB) of moderate to severe disease were obtained before initiation of treatment. The control population was composed of 9 healthy volunteers (HCo), BCG vaccinated in no contact with TB patients.

Fifteen patients with pleural effusions (PE) were also included in the study, 9 of them of tuberculous origin (TBPL) and the 6 remaining ones due to a nontuberculous but an infection-associated etiology (n = 3) or cancer (n = 3), regarded as non-tuberculous pleurisy (NoTBPL). In the latter 3 cases, malignant cells were observed during histopathologic examination of pleural biopsies. TBPL effusions were defined as exudates with a positive Ziehl Nielsen stain or positive Lowenstein Jensen culture of pleural exudates or pleural biopsy specimens. All pleural effusions were classified as exudates according to established criteria [20]. PE samples from all patients were obtained during diagnostic thoracocentesis before the initiation of chemotherapy. Samples were subjected to routine biochemical analysis, including tests for total protein, glucose and lactate dehydrogenase. Blood samples from these patients were obtained before the procedure.

Patients and HCo revealed no statistical differences in age and sex distribution (Suppl. Table 3) and none of them had other respiratory disease, immunocompromising diseases or concomitant therapies.

2.3. Mononuclear cell isolation

Mononuclear cells from both peripheral blood (PBMC) and pleural effusions of patients were isolated by Fycoll-Paque plus density gradient centrifugation (Amersham Biosciences) at 1800 rpm for 30 min. Collected cells were washed three times (15 min at 1200 rpm) and finally resuspended in 1 ml of PBS. Cells ($5–8 \times 10^6$ cells/ml) were stored with TRIzol® (Invitrogen) at −80 °C until use.

2.4. RNA isolation, cDNA synthesis and qPCR

Total RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacture's recommendations. Mature miRNA levels were determined by stem-loop RT-qPCR [21]. cDNA was synthesized from 1 μg of total RNA using Superscript III reverse transcriptase (Invitrogen), oligodT and specific primers (Suppl. Table 4). PCR reactions were performed in a StepOne Real-Time PCR System (Thermo Fisher Scientific) using SYBRGreen I (Roche) to monitor dsDNA synthesis. Transcript levels were calculated by the ΔΔCt method relative to THP1 expression. CycA (GeneID: 5478) was also measured and used as endogenous control for mRNA determinations while U6 snRNA was employed for miRNAs quantifications.

2.5. Cytokine and hormone assessments

Plasma was obtained from EDTA-treated blood. IFNγ- (IFNγ-OptEIA BD Biosciences), IL-6 (Amersham Biosciences) and cortisol (hormones-DRG Instruments GmbH) plasma concentrations were assessed according to the manufacturer instructions. C reactive protein (CRP) levels were determined by CRP Ultrasensitive Turbitest (Wiener Lab). All samples were processed individually and assayed in duplicate. Detection limits were: 0.1 pg/ml for IL-6;

4.7 pg/ml for IFNγ; 3.3 mg/ml for CRP and 2.5 ng/ml for Cortisol.

2.6. Cell culture

The THP-1 cell line was grown in suspension cultures in Tissue Culture Medium RPMI 1640 supplemented with 10% of heat-inactivated fetal bovine serum and penicillin/streptomycin (20 Ul/ml, 20 μg/ml, Sigma Chemical Co) at 37 °C in 5% CO_2. For differentiation to macrophages, cells were plated in 12-well dishes (10^6 cells per well, n = 4) on RPMI containing 20 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma Chemical Co). Twenty four hours later, supernatants were removed and complete RPMI was added for 48 h before treatment. Macrophages were then cultured in presence or absence of gamma irradiated Mtb-H37Rv (*iMtb*, 8 μg total protein/ml per well) and/or Cortisol (10^{-6} M, Sigma Chemical Co.) for either 3 or 24 hs at 37 °C in 5% CO_2. Cells were then washed 3 times with PBS before collecting with TRizol for RNA extraction.

2.7. Statistical analysis

An initial Kruskall Wallis analysis of variance was carried out to assess between-group differences followed by the Dumm post-hoc comparison approach when applicable. Spearman correlation coefficients were also calculated. Data were considered statistically significant when $p < 0.05$.

3. Results

3.1. pTB patients present distinct features in elements of the GC response as compared to PLTB cases

To dissect the GC response in TB patients, we first measured cortisol plasma levels and the expression of the active isoform of the GR (hGRα) in PBMCs of patients. As expected, we observed a subtle but significant raise in cortisol levels of samples from both pTB and PLTB compared to HCo (Fig. 1A). Interestingly, a statistically significant decrease in hGRα levels was found in PBMCs of PLTB patients respect to HCo while expression in pTB samples was barely diminished and did not differ from HCo (Fig. 1B).

Downregulation of hGRα expression upon infection has already been described in the literature [22,23], and may be viewed as a physiological mechanism to favor the response needed to eradicate the infectious insult. Indeed, TB patients showed high levels of circulating pro-inflammatory mediators such as CRP and IL-6 (Suppl. Fig. 1), as seen in earlier studies [8,24,25]. In this context, differences in hGRα expression between pulmonary and pleural TB patients may be a reflection of a mechanism present in pTB attempting to potentiate the GC response.

3.2. mir30c is specifically downregulated in PBMC of patients with pTB

miRNA regulatory circuits tend to be involved in the fine tuning of fundamental cellular processes. In this work we studied whether these molecules may bear some relation with GR expression in tuberculosis. As mentioned above, many putative binding sites for miRNAs have been predicted in the 3′UTR of the gene encoding the GR. We selected 5 miRNAs (miR-29a, miR-30c, miR-124, miR-181a and miR-181b) based on phylogenetic data, expression levels and documented associations with TB, using selection criteria described in Materials and Methods (section 2.1). miRNAs levels were measured in PBMC of both pulmonary and pleural TB patients and their expression pattern was compared with those found in HCo. We also analyzed miRNA expression in patients with non-tuberculous pleurisies to evaluate the specificity of the response.

Fig. 2 shows the RT-qPCR analysis of miR-29a, miR-30c, and miR-181a and b in PBMCs of patients and HCo. miR-124 levels were undetectable in our samples using the standard miRNA quantification technique described by Chen and coworkers [21]. Interestingly, the expression of miR-30c is significantly diminished only in PBMC of patients with pTB (Fig. 2B), suggesting that this miRNA may be a specific correlate for this form of the disease. Furthermore, we observed that miR-30c expression negatively correlates with IFNγ levels, a key player in TB pathogenesis ($r = -0.482$, $p < 0.05$; Suppl. Fig. 2). Interestingly, even though the hGRα 3′-UTR contains many predicted binding sites for miR-30, recent findings strongly suggest that this miRNA regulates hGRα expression by an indirect mechanism [26].

When analyzing the other miRNA families, we observed that the expression of miR-181 and miR-29 was strongly decreased in samples from patients with different types of lung diseases (Fig. 2A, C and D). The bulk of results suggest that these miRNAs may be associated with non TB-specific regulatory responses.

Strikingly, a different miR-30 and -181 expression pattern was observed in cells obtained from pleural fluids. On average, miRNA expression in these samples was lower compared to those obtained in the peripheral compartment (Suppl. Fig. 3, to provide statistical significance). This phenomenon may be due to environmental signals present in the pleural compartment, as patients with pleurisies from different etiology showed very similar miRNA expression profiles.

3.3. miR-30c and hGRα show an inverse expression pattern in iMtb-stimulated macrophage cultures treated with cortisol

To expand the study on the miR30c-hGRα network we next employed an *in vitro* model using the human macrophage-like THP-

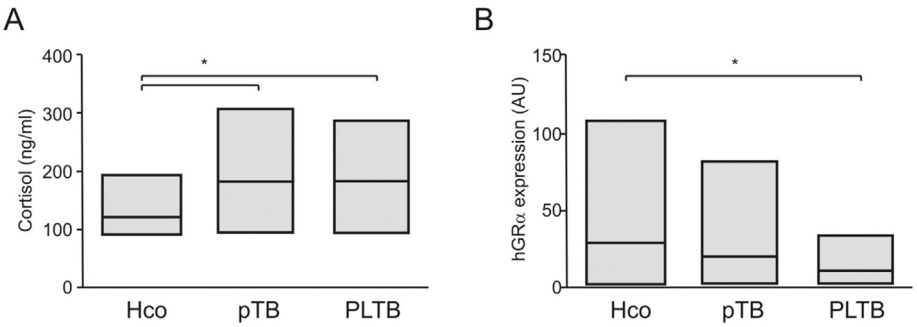

Fig. 1. Cortisol levels and hGRα expression in active pulmonary tuberculosis and tuberculous pleurisy patients.(A) Plasma levels of cortisol in samples from healthy controls (HCo) and patients with active pulmonary TB (pTB) and pleural TB (PLTB); **(B)** hGRα expression levels in PBMC of HCo and patients. Box plots show maximum and minimum values in each group and the line represents the median values. Significant differences: *$p < 0.05$.

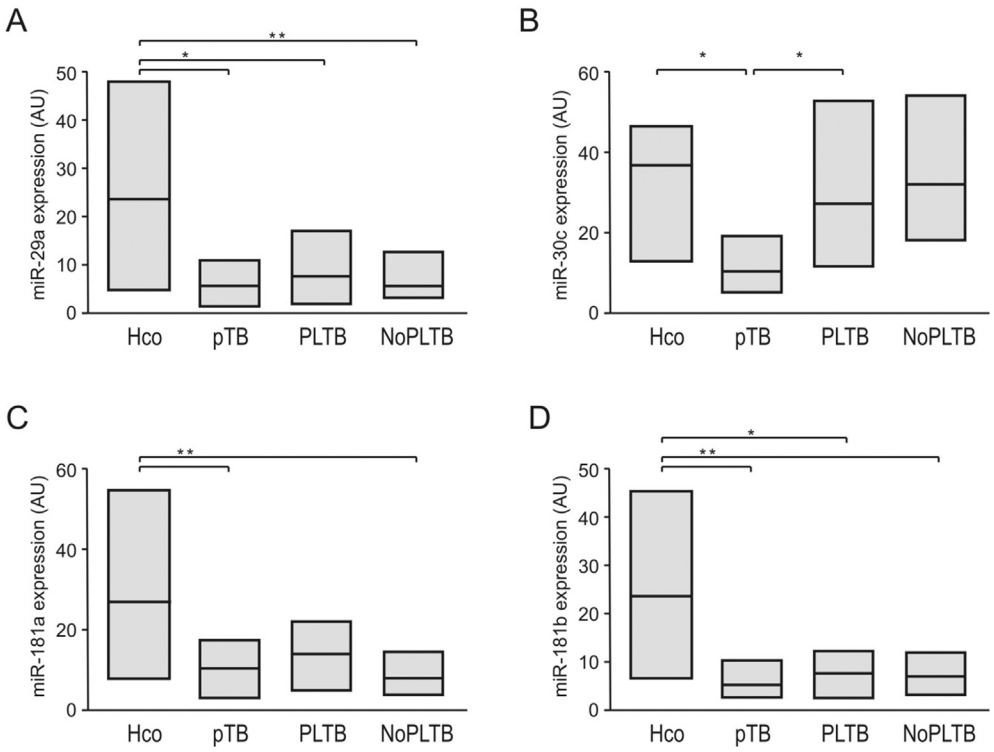

Fig. 2. Systemic miRNA expression in patients with different types of lung diseases. miR-29a **(A)**, miR-30c **(B)**, miR-181a **(C)** and miR-181b **(D)** expression levels in PBMC of healthy controls (HCo) and patients with pulmonary TB (pTB), pleural TB (PLTB) and non-tuberculous pleurisy (NoPLTB). Box plots show maximum and minimum values in each group and the line represents the median values. Significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$.

1 cell line and *iMtb* to stimulate the cells. We first analyzed gene expression levels of cytokines relevant to the macrophage response against pathogens. *iMtb* induced the expression of IL-1β, IL-6 and IL-12 in 24 h cultures (Suppl. Fig. 4A–C) while IL-23 levels showed a more substantial raise, being significantly increased even 3 h after treatment (Suppl. Fig. 4D). It follows that cultures exposed to *iMtb* expressed markers of classical activation as well as key mediators of inflammation.

iMtb also led to a significant raise in the expression of miR-155, miR-146a and miR30c (Fig. 3A–C). Actually, levels of both miR-30c and miR-30a, as well as the joint quantification of all members of the miR-30 family show the same expression profile in our cell line model (Suppl. Fig. 5). In accordance with our results, these miRNAs have already been described as induced by *Mtb* in macrophages [27]. Their levels are elevated by many different types of infectious stimuli (i.e, LPS, Suppl. Fig. 6A–C), as part of the inflammatory immune responses mounted by the host [28–31]. Interestingly, hGRα transcript levels in the cell line showed a significant increase 3 h following stimulation with *iMtb* with the trend loosing statistical significance at the 24 h time point evaluation (Fig. 3D). As above mentioned, GC response is regulated by a plethora of molecular mechanisms. In this model, we speculate that early induction of hGRα expression is controlled at the transcriptional level by the key mediators of the inflammatory response. However, the fact that the raising tendency is reverted 24 h after treatment, when miR-30c is significantly upregulated, suggests a link between these two molecules. Similar results were obtained in LPS-stimulated THP1 cells, in which the hGRα expression levels tend to decrease after LPS treatment (Suppl. Fig. 6D).

Finally, we analyzed the effect of cortisol addition to stimulated cell cultures. This condition shares some resemblance to what happen in pTB patients, where infection proceeds with increased circulating levels of GC. Cortisol has a powerful effect on this type of phagocytic cells, significantly decreasing the levels of IL-23 (Fig. 4A) and other key mediators of the immune response such as IL1β, IL6 and TNFα (data not shown). Interestingly, in this condition, expression of miR-30c is significantly downregulated while hGRα levels increase (Fig. 4B and C), in agreement with the results obtained in pTB patients. The inverse expression pattern between miR-30c and hGRα observed in our experiments provides additional evidence in favor of a putative role of miR-30c in modulating hGRα expression.

4. Discussion

Pulmonary TB is the most frequent clinical manifestation of tuberculosis but extrapulmonary forms of the disease may also occur, mainly involving lymph nodes and pleura [32]. The fact that PLTB is a rather benign form of the disease [17] provides a useful model to analyze the immune-endocrine profile involved in a situation dealing with a better control of the disease.

Upon Mtb infection, the host mounts a complex response essential for maintaining the physiological homeostasis and pathogen clearance. Activation of the HPA axis contribute not only to optimize this response but also to protect the individual against its potentially damaging effects. Our work demonstrates that pTB patients present distinct features in the GC response as compared to patients with PLTB or other lung pathologies.

In accordance with previous studies showing that plasma GCs are elevated during tuberculosis [8,24], our results show an increase in serum cortisol levels in both pTB and PLTB patients (Fig. 1A). Different lines of evidence demonstrate that increased GC levels associates with a downregulation in the expression and ligand binding activity of GR [22,23,33]. Our observations that

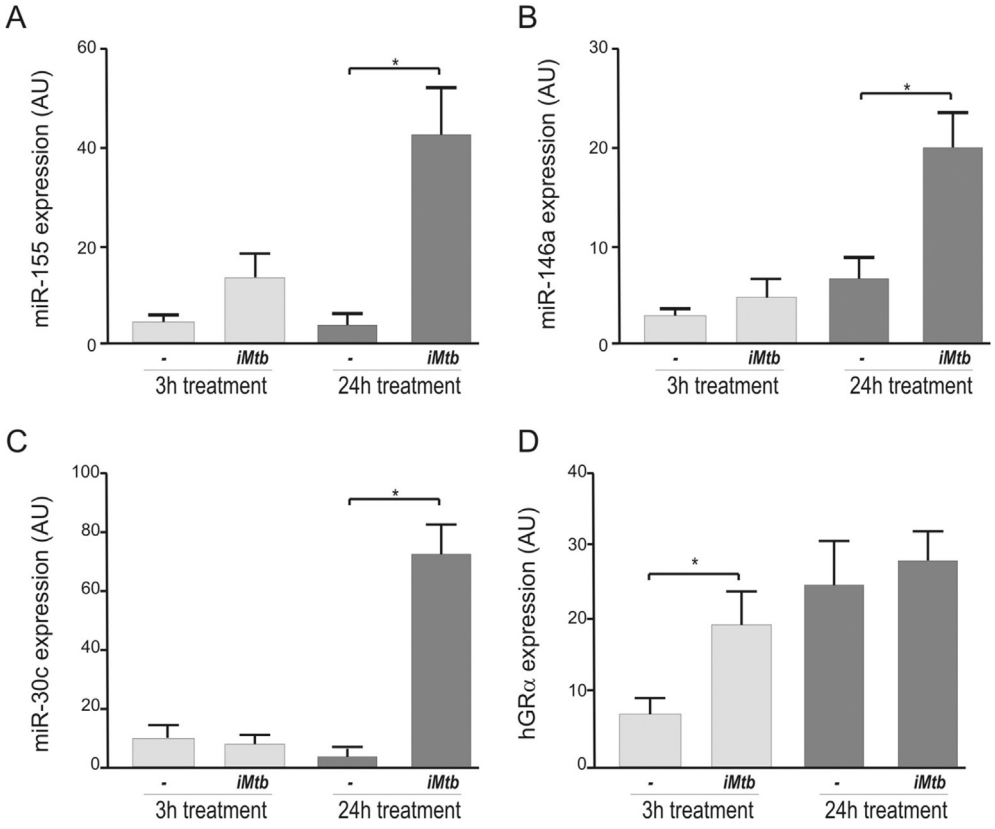

Fig. 3. Expression profile of miRNAs and hGRα in iMtb-stimulated cultures. Levels of miR-155 **(A)**, miR-146a **(B)**, miR-30c **(C)** and hGRα **(D)** in the THP-1 cell line assessed in the presence or absence gamma irradiated *M. tuberculosis*-H37Rv (*iMtb*), 3 h and 24 h after treatment. Data present the mean of 4 biological replicates ± s.e.m. Significant differences: *p < 0.05.

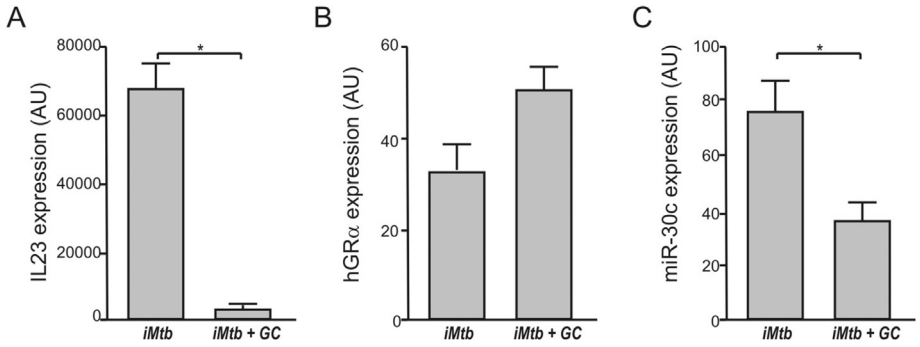

Fig. 4. Inverse expression of miR-30c and hGRα in iMtb-stimulated cultures treated with cortisol. Levels of IL23 **(A)**,hGRα **(B)** and miR-30c **(C)** in THP-1 cell cultures stimulated with *iMtb* for 24 h in the presence or absence of 10^{-6} M Cortisol. Data present the mean of 4 biological replicates ± s.e.m. Significant differences: *p < 0.05.

hGRα expression is significantly decreased in PBMC of PLTB patients are in close agreement with the results obtained by other investigators (Fig. 1B). The fact that hGRα levels in pTB patients are higher suggests that the less efficient defensive mechanisms seen in pTB, are partly due the GC-mediated signaling through hGRα acting to inhibit the immune protective response.

We then evaluated whether miRNAs could be involved in modulating GR expression in the context of TB pathogenesis. During the last few years there has been an increased interest in studying the role of miRNAs in the immune system. Studies conducted by many groups have demonstrated that miRNAs are important in both adaptive and innate immunity, by influencing the differentiation of various immune cell subsets as well as their immunological functions [28,29].

Several studies characterized miRNAs of different surrogate tissues from Mtb-infected individuals and controls but common biomarker candidates have not been identified so far, mainly because of the heterogeneity of the experimental designs and population biases [15,34].

miRNA dependent regulation might play an important role in controlling GR activity, as suggested by the high number of miRNAs binding sites predicted to target its 3′UTR sequence. In our study we focused on miR-29a, miR-30c, and miR-181a/b as different lines of evidence link these miRNAs with TB pathogenesis. Ma and coworkers showed that infection of mice with *Listeria monocytogenes* or *Mycobacterium bovis* bacillus Calmette-Guérin downregulates

miR-29 expression in T cells and demonstrated that this miRNA controls innate and adaptive immune responses to intracellular bacterial infection by targeting IFN-γ [35]. Studies conducted by many groups have also identified miR-29, as well as miR-181, as differentially expressed in TB by using miRNA expression profiling [34,36–38].

Consistent with the literature, miR-181a, miR-181b and miR-29a were all downregulated in TB patients, regardless of the clinical form under study (Fig. 2A, C and D). These miRNAs were also repressed in NoPLTB patients, suggesting that they may be playing a role in the immune-endocrine regulation of many lung disorders.

miRNAs belonging the miR-30 family were also found significantly altered in TB patients, as shown by diverse high throughput screenings [36,39]. Our results demonstrate that miR-30c levels are significantly downregulated in PBMC of pTB patients compared to HCo, PLTB or NoPLTB patients (Fig. 2B). The fact that low levels of miR-30c coexist with augmented hGRα expression in pTB patients compared to PLTB cases is consistent with an association between miR-30c and hGRα expression in this pathological condition.

Results by studying THP-1 cell cultures were also informative in demonstrating that miR-30c expression in relation with mycobacteria exposure may be different depending on the cell population under analysis. Whatever the case, results shown in Fig. 4, using this *in vitro* model are also in agreement with the inverse relation between miR-30c and hGRα expression levels. Further experiments should be performed to ascertain whether the association observed could be explained by a direct or indirect mechanism. In this regard, a recent report indicates that miR-30a negatively regulates hGRα in normal and injured induced podocytes by an indirect effect on the transcriptional activity of GR. In this work, Xie and colleagues showed that expression of hGRα is downregulated by miR-30a interference, however, miR-30a mimics failed to repress luciferase activity from a reporter harboring the 3′UTR of the GR transcript [26]. In our hands, miR-30a and miR-30c are both upregulated after *iMtb* stimulation (Suppl. Fig. 5), suggesting that all members of this miRNA family contribute to modulate hGRα expression after *Mtb* infection.

Our findings raise the possibility that miR-30c down-regulation may favor hGRα expression specifically in pTB patients, as a process attempting to improve GC sensitivity. In essence, the fine tuning of GC function will help to ultimately keep a proper balance between anti-inflammation without affecting immune protection. In sum, present results contribute to shed light on host responses in TB pathogenesis, pointing out to the role of miRNAs in this regard.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2017.04.004.

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