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Altered microRNA expression levels in mononuclear cells of patients with pulmonary and pleural tuberculosis and their relation with components of the immune response

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ABSTRACT

Different lines of evidence demonstrate that microRNAs (miRNAs) play an important role in host–pathogen interactions. In this study we investigated the expression patterns of several miRNAs, most of them involved in regulating inflammatory responses, in patients with tuberculosis (TB). In order to understand the events occurring at the site of infection, we employed mononuclear cells obtained from both peripheral blood (PBMC) and pleural fluids (PFMC) of patients. Interestingly, we found that the miRNA signature of each compartment is different, with a strong down-regulation in PFMCs of miR-223, miR-144* and miR-421. In addition, we observed that miR-146a expression is also down-regulated in tuberculosis patients, both in PBMCs and PFMCs while miR-424 levels are elevated only in the peripheral compartments. We also showed that systemic expression of these miRNAs changes upon specific treatment and is associated with IL-6 levels, a cytokine playing a substantial role in TB immunopathology. Present results contribute to a better knowledge of the host responses in TB pathogenesis, pointing out the role of miRNAs in this disease.

1. Introduction

Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis, is responsible for 8 million new cases and nearly 1.5 million deaths per year in the world (WHO Reports, 2010). Different T-lymphocyte subsets are involved in the immune response against the bacillus, but production of interferon-gamma by T cells seems to be fundamental for disease control (Dorhoi et al., 2011). Although mainly affecting the lung tissue, tuberculous pleuritis may also occur (Barnes et al., 1989). In addition of being a highly favorable form of the disease (Roper and Waring, 1955), TB pleuritis offers the possibility of obtaining immunocompetent cells present in the pleural fluid (Sahn, 1998; Ferrer, 1997; Toossi et al., 2011) and hence identifying the events occurring in situ; allowing a better understanding of the response against infection.

The host response to Mtb is known to play a major role in determining the clinical manifestations and the ultimate disease outcome (Dorhoi et al., 2011). Such defensive reaction is complex and involves a series of steps dealing with the immune mechanisms put into play and the processes regulating them. 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 (Baltimore et al., 2008; Lindsay, 2008).

Recently, two independent groups identified a series of miRNA differentially expressed in PBMCs of Chinese patients with pulmonary TB by using miRNA expression profiling (Liu et al., 2011; Wang et al., 2011). Liu and coworkers showed that the expression of several miRNAs was significantly altered during active TB with miR-144* being mainly expressed in T cells. Meanwhile, Wang et al. identified another set of miRNAs differentially expressed in PBMCs of active TB patients, further showing that miR-424 and miR-365 levels were significantly raised in patients with active TB compared to healthy controls.

The dissimilar results seen in both studies suggest that systemic miRNA expression may depend on the particular features of the group under analysis in which the individual background cannot be disregarded, particularly when considering the racial variations in the susceptibility to TB (Stead et al., 1990; Stead, 1992).

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In this study we investigated the expression patterns of several miRNAs, most of them involved in the regulation of inflammatory responses, in PBMCs from Caucasian patients with pulmonary TB. We also analyzed whether their expression pattern varied depending on cells were obtained from the peripheral or pleural compartments and the eventual changes that may occur following specific anti-mycobacterial therapy. In addition, the potential relationship of microRNA expression patterns with components of the inflammatory response was also evaluated. It was found that the miRNA signature of each compartment is different and that its expression in PBMCs changes upon specific treatment showing some association with the levels of cytokines involved in the host reaction against infection.

2. Materials and methods

2.1. Study groups

Patients (3 females and 21 males) with no HIV co-infection and newly diagnosed pulmonary TB of moderate to severe disease were included. Age of patients ranged from 18 to 71 years – one case (37 ± 16 years, mean $\pm$ standard deviation). The control population was composed of 20 healthy volunteers (HCo) BCG vaccinated in no contact with TB patients. Patients and HCo revealed no statistical differences in age and sex distribution and none of them had other respiratory disease, immunocompromising diseases or concomitant therapies. Anti-tuberculosis therapy consisted in six months of rifampicin and isoniazid, initially supplemented by two months of pyrazinamide and ethambutol. Blood samples were obtained from all participants at 8 a.m.; in TB patients before initiation of treatment (t0) as well as at 2, 4, and 6 months (t2, t4, t6) after starting the specific therapy.

Samples from patients with pleural tuberculosis ($n = 7$) 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. All pleural effusions were classified as exudates according to the established criteria (Light et al., 1972). Blood sample from these patients was obtained before the procedure.

This work was approved by the Ethical Committee of the School of Medical Sciences, Rosario National University. Participants were enrolled upon obtaining their written consent.

2.2. Mononuclear cell isolation

PBMCs and PFMCs 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 $-80\,^{\circ}C$ until use.

2.3. 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 (Chen et al., 2005). cDNA was synthesized from 1 μg of total RNA using Superscript III reverse transcriptase (Invitrogen) and specific primers (Supplementary Table). PCRs were performed in a Mastercycler® ep realplex thermal cycler (Eppendorf) using SYBRGreen I (Roche) to monitor dsDNA synthesis. The relative transcript level for each sample was calculated with respect to THP1 expression and was normalized using the U6 snRNA cDNA quantification.

2.4. Cytokine assays

Plasma was obtained from EDTA-treated blood. IL-1β (Invitrogen), and IL-6 (Amersham Biosciences) plasma concentrations were determined according to the manufacturer instructions. All samples were processed individually and assayed in duplicate.

2.5. Statistical analysis

An initial Kruskall Wallis analysis of variance was carried out to determine difference between group distributions with the Dumm method being for post hoc comparisons. Spearman correlation coefficients were also calculated. Paired comparisons at different times of anti-tuberculosis treatment were done by Friedman analysis of variance and Wilcoxon test. Data were considered statistically significant when $p < 0.05$.

3. Results

3.1. Expression of miRNAs in mononuclear cells from TB patients differs between the site of infection (PFMC) and the peripheral compartment (PBMC)

Our study was focused on miR-424, miR-365, miR-144*, miR-421, miR-223 and miR-146a given their potential involvement either in TB or diseases accompanied by an important inflammatory response (Liu et al., 2011; Wang et al., 2010, 2011; Sharbati et al., 2011). We also tested the expression patterns of several other miRNAs previously associated with bacterial infection, i.e., miR-29a, miR-142 and miR-155 (Baltimore et al., 2008; Wang et al., 2011; Ma et al., 2011; Fu et al., 2011) but their levels did not change in TB patients (data not shown). Fig. 1 shows the real-time RT-qPCR analysis of these six miRNAs in PBMCs from TB patients and healthy controls (HCo). The results obtained in PFMCs from 7 patients with pleural TB were also included. As can be seen, a statistically significant raise in the levels of miR-424 was found in PBMCs of pulmonary TB patients with respect to HCo. By contrast, the miR-146a expression appeared to be decreased in PBMCs of pulmonary and extrapulmonary TB patients. No statistical differences between TB patients and HCo were observed when analyzing the remaining four miRNAs. However, in all cases patients showed higher miRNA expression levels, suggesting a tendency of these miRNAs to be up-regulated in their PBMCs.

Strikingly, a different miRNA expression pattern was observed when analyzing cells obtained from pleural fluids. On average, miRNA expression in these samples was lower compared to those obtained in the peripheral compartments (Fig. 1). Expression of miR-144*, miR-421 and miR-223 was significantly lower in PFMCs of pleurisy patients when compared to values seen in PBMCs (Fig. 1) while expression of miR-146a did not differ between the two compartments.

3.2. miR-424 and miR-146a expression changes upon anti-tuberculosis treatment and shows association with IL-6 levels

We next analyzed the expression profile throughout the course of anti-tuberculosis treatment in samples of patients completing a successful therapy ($n = 11$), in clinical, radiological and bacteriological terms. As depicted in Fig. 2, miR-424 in treated patients lowered to levels within the normal range with values at 2 and 6 months after treatment (t2, t6) being significantly different from those recorded at diagnosis. By opposite, miR-146a was moderately increased during treatment reflecting in a significant difference

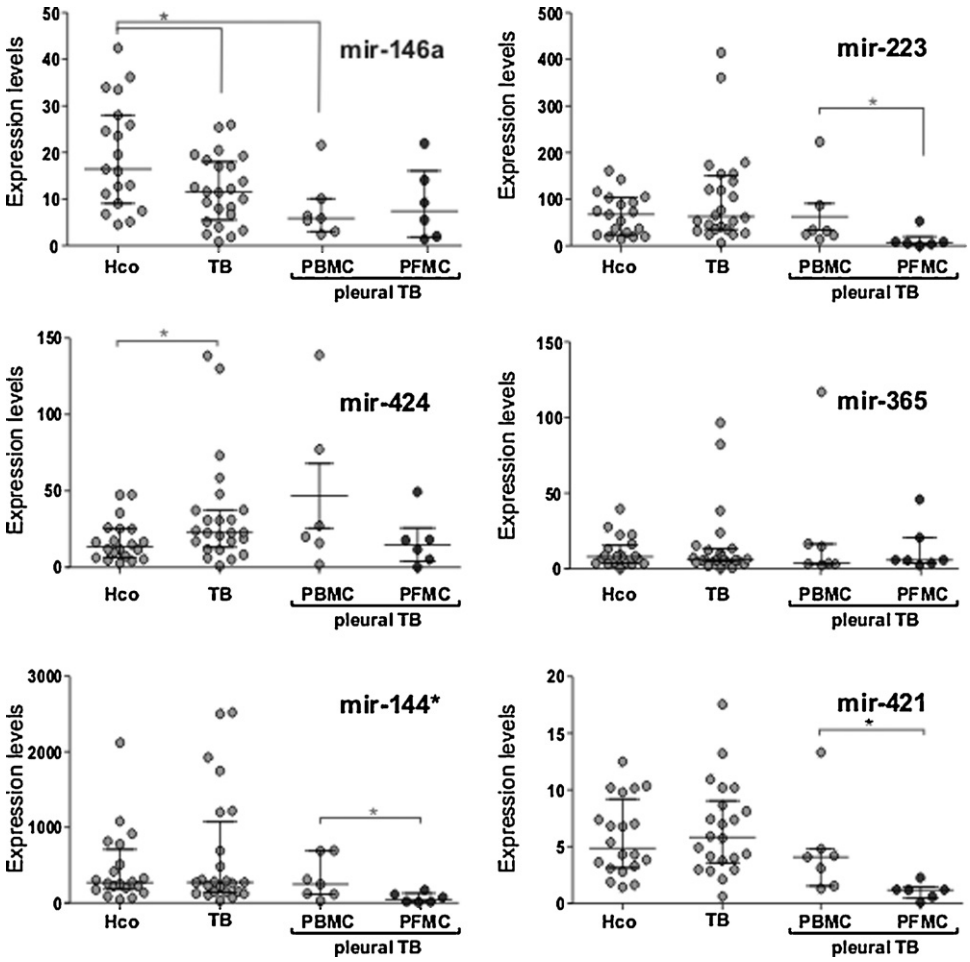

Fig. 1. Local and systemic expression of miR-146a, miR-223, miR-424, miR-365, miR-144* and miR-421 in mononuclear cells of TB patients. miRNA expression levels were determined by RT-qPCR in PBMC of patients and HCo and in PFMC of cases with pleural TB. The lines represent the median with 25–75 percentiles values. Significant differences: $^{*}p < 0.05$.

when comparing t2 values with those seen at the time of inclusion (Fig. 2). Assessment of the remaining miRNAs revealed no differences among the different time point evaluations (data not shown).

Blood samples were also analyzed for the levels of cytokines involved in the host reaction against infection showing that active TB patients had increased amounts of circulating IL-1β and IL-6 which decreased upon specific treatment (Table 1), as recorded in former studies (Santucci et al., 2011; Bongiovanni et al., 2012). Pairwise correlations between these circulating mediators and miRNAs revealed that IL-6 positively correlated with miR-424 both at diagnosis (t0) and two months after treatment (t2), whereas a negative correlation between this cytokine and miR-146a was found at the end of anti-tuberculosis treatment (t6) (Table 2).

4. Discussion

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 (Bartel, 2009). These small molecules are involved in a wide range of physiological responses, including development, differentiation and homeostasis (Bartel, 2009; Zamore and Haley, 2005) being estimated

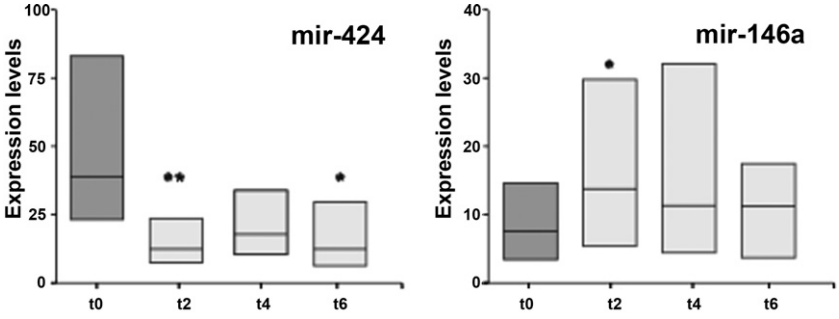

Fig. 2. miR-146a and miR-424 levels during anti-mycobacterial therapy. miRNAs expression levels determined by RT-qPCR in PBMC of patients at diagnosis (t0) and 2, 4 and 6 months after treatment (t2, t4, t6). Box plots show 25–75 percentiles of data values in each group and the line represents the median values. Intragroup comparisons showed significant differences with respect t0: $^{*}p < 0.05$ and $^{**}p < 0.01$ respectively.

Table 1
Plasma levels of IL-1β and IL-6 in HCo and TB patients at different stages of anti-TB treatment (mean ± s.e.m.).

	IL-1β (pg/ml)	IL-6 (pg/ml)
Healthy controls	nd	3.11 ± 1.23
TB, diagnosis (t0)	0.99 ± 0.41	31.03 ± 9.50[†]
TB, 2 months after treatment (t2)	nd	6.30 ± 1.65[**]
TB, 4 months after treatment (t4)	nd	3.06 ± 0.59[**]
TB, 6 months after treatment (t6)	nd	3.51 ± 0.82[**]

nd: not detectable.
[†] Between-group comparisons: $p < 0.001$ with respect HCo.
[**] Intragroup comparisons with respect t0: $p < 0.01$.

that they may regulate as much as 60% of mammalian genes (http://www.mirbase.org/). The clinical significance of miRNA dependent regulation has already been demonstrated in various types of cancers (Lu et al., 2005). However, the knowledge of their role in infectious diseases is still incomplete.

In our study we investigated the expression patterns of several miRNAs in PBMCs and PFMCs of TB patients. Interestingly, we found that the miRNA signature of each compartment was quite different, pointing out to a compartmentally distinct type of regulatory processes. Three out of six miRNAs tested (miR-223, miR-421 and miR-144*) were expressed in low levels in cells obtained from pleural fluids (Fig. 1), while miRNA expression in PBMCs from the same patients did not differ from HCo. Interestingly, miR-146a was down-regulated in both PBMCs and PFMCs of patients, even though it was not identified in any of the high throughput experiments performed by other groups (Liu et al., 2011; Wang et al., 2011). miR-146a has been previously described as a negative regulator of the immune response (Baltimore et al., 2008; Lindsay, 2008) and its systemic down-regulation may be associated with the exacerbated inflammatory response observed in TB patients (Bottasso et al., 2007). Additionally, we found that miR-424 was significantly up-regulated in PBMCs of pulmonary TB patients, in line with previous studies (Wang et al., 2011).

When evaluating miRNA levels throughout anti-mycobacterial therapy, miR-424 and miR-146a expression situated within normal values after 2 months treatment (Fig. 2). In line with the important degree of tissue inflammation that coexists with TB (Bottasso et al., 2007), patients had increased levels of IL-6 and IL-1β in circulation, which decreased throughout specific treatment (Table 1) (Santucci et al., 2011; Bongiovanni et al., 2012). The positive association of miR-424 with IL-6 may be explained when considering that this miRNA promotes monocyte-macrophage differentiation (Rahimian et al., 2011). Additionally, the negative relation between miR-146a and IL-6 is consistent with the known role on this miRNA in NF-κB down-regulation (Taganov et al., 2006).

The fact that expression in PBMCs of most miRNAs analyzed showed no strong differences between patients and HCo may imply that miRNA levels in periphery are tightly controlled by a complex network of interactions precluding them from large variations. Slight changes may be rather dependent on the population sample under analysis, like the immunological status and genetic background of patients. As regards changes seen at the pleural space, when considering the preserved immune response of patients with pleural TB (Roper and Waring, 1955; Toossi et al., 2011), the local down-regulation of miRNAs putatively associated with repression of the inflammatory response such as miR-146a, miR-223, and miR-144* (Baltimore et al., 2008; Lindsay, 2008; Liu et al., 2011), may contribute to the favorable prognosis of this clinical form.

Present results provide a stimulating background for a deeper study on the role of miRNA in the complex mechanisms of TB pathogenesis and its potential implications in strategies for disease control.

Table 2
Correlation analysis of cytokines levels with miRNA expression values in TB patients.

Significant correlations	*r*	*p*
miR-424, TB at diagnosis (t0) vs IL-6	0.77	0.0092
miR-424, TB after treatment (t2) vs IL-6	0.79	0.0061
miR-146a, TB after treatment (t6) vs IL-6	−0.89	0.0188

r: Spearman's coefficient of correlation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2012.08.008.

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