

Dynamics of T-cell IFN- γ and miR-29a expression during active pulmonary tuberculosis

Anthony Afum-Adjei Awuah^{1,2}, Bianca Ueberberg³, Ellis Owusu-Dabo^{1,2}, Margaret Frempong² and Marc Jacobsen^{3,4}

¹Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Kumasi, Ghana

²School of Medical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana

³Pediatric Infectious Diseases Group, Department of General Pediatrics, Neonatology, and Pediatric Cardiology, Duesseldorf 40225, Germany

⁴Department of Immunology, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg 20359, Germany

Correspondence to: M. Jacobsen, University Children's Hospital, Moorenstr. 5, 40225 Düsseldorf, Germany; E-mail: marc.jacobsen@med.uni-duesseldorf.de

Received 2 April 2014, accepted 22 June 2014

Abstract

IFN- γ is crucial for protection against *Mycobacterium tuberculosis*. miR-29 was recently shown to non-redundantly inhibit IFN- γ . Here, we investigated IFN- γ and miR-29a expression dynamics of CD4⁺ T cells from patients during active tuberculosis (TB) ($n = 32$) and in household contacts who were latently *M. tuberculosis* infected ($n = 19$) from Ghana. Whereas *M. tuberculosis*-specific IFN- γ expression was similar during TB chemotherapy, superantigen stimulation indicated generally impaired IFN- γ expression in TB patients. No interdependency between miR-29a and IFN- γ expression of T cells was observed. However, miR-29a was differentially expressed in T cells during chemotherapy. We concluded that differential miR-29a expression in active TB was not causative for impaired IFN- γ expression.

Keywords: IFN- γ , miR-29a, TB, T cell

Introduction

Tuberculosis (TB) still remains one of the world's single most infectious diseases estimated to kill ~2 million people annually. About a third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent for human TB. In Ghana, a mean incidence rate of 72 (per 100 000 population) was reported in 2013 (1). It is largely accepted that interactions between T cells—predominant CD4⁺ T helper type 1 cells—and *M. tuberculosis*-infected macrophages play a major role in protecting from TB (2). IFN- γ is a key cytokine in controlling *M. tuberculosis* infection and IFN- γ knockout mice as well as humans defective in the IFN- γ /IFN- γ receptor pathway are highly susceptible to *M. tuberculosis*. Some studies point to impaired IFN- γ response in patients with active TB (3).

miRNAs are small non-coding RNAs that regulate eukaryotic gene expression post-transcriptionally. miRNAs modulate several biological processes including immunity against infection (4). Ma *et al.* (5) first reported non-redundant IFN- γ repression by miR-29a in animal models of infection and others showed that miR-29a exerted its function by inhibiting *Tbet* and *EOMES* (6). Initial results of miR-29a expression in human TB are contrary. One study found decreased miR-29a expression in blood and CD4⁺ T cells of active TB patients (7),

whereas others detected increased miR-29 expression in T cells (8). Notably, Kleinstaub *et al.* (7) showed that miR-29a inhibition *in vitro* by antagonistic miRNAs did not influence IFN- γ expression.

In conclusion, previous studies suggested a role of miR-29a in the immune response against TB, but the exact mechanisms remain elusive. These approaches focused on animal models or were performed in countries with low incidences of TB. The present study concomitantly determined miR-29a and *M. tuberculosis*-specific IFN- γ expression of CD4⁺ T cells from TB patients and healthy but latently *M. tuberculosis* infected (LTBI) contacts in a TB endemic region in Ghana.

Methods

Study design and samples

We conducted a prospective cohort study that was nested with a case-control study. It started from February 2011 and ended in November 2012; 32 clinically confirmed TB patients and 19 *M. tuberculosis*-exposed but healthy household contacts were enrolled from Komfo Anokye Teaching Hospital, Kumasi, South Hospital and Kwame Nkrumah University of

Science and Technology Hospital in Kumasi. Diagnosis of active TB was based on patient history, chest X-ray, sputum smear test and mycobacterial culture. Characteristics of TB patients are summarized in Table 1. Healthy household contacts were termed LTBI since the vast majority had a positive IFN- γ response against purified protein derivative (PPD) tuberculin (Fig. 1). Only one contact had a marginal PPD response. All donors gave written informed consent. This study was approved by the local ethics committee (CHRPE/203/10). We took peripheral blood (30 ml) using heparinized monovettes for immunologic and molecular analysis. The blood samples were quickly transported to the laboratory and laboratory assays began immediately. Chemotherapy for TB patients was initiated immediately after the first blood sample was taken. For the patient study group, blood was taken consecutively (i.e. 1 and 6 months after chemotherapy onset).

PBMC stimulation and IFN- γ quantification

PBMCs were isolated from 30 ml heparinized whole blood by density centrifugation (Biochrom, Berlin, Germany) according to the manufacturer's suggestions. PBMCs (2×10^5) were stimulated with PPD of *M. tuberculosis* H37Rv (Statens Serum Institute, Copenhagen, Denmark) ($10 \mu\text{g ml}^{-1}$) and the staphylococcal enterotoxin B (SEB) superantigen ($100 \mu\text{g ml}^{-1}$; kindly provided by C. Steeg, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany) in 96-well titer plates containing X-Vivo 15 medium (supplemented with 1%

penicillin/streptomycin; Lonza, Cologne, Germany), respectively. After incubation (5 days at 37°C , 5% CO_2), culture supernatants were harvested and stored at -20°C before IFN- γ quantification by ELISA. The IFN- γ concentration in culture supernatants was quantified using the ELISA for human IFN- γ kit (MABTECH, Nacka Strand, Sweden) following the manufacturer's recommended protocol.

Enrichment of CD4⁺ T cells

CD4⁺ T cells were enriched from PBMCs (1.5×10^7) using the magnetic cell sorting system (IMag, BD Biosciences, Heidelberg, Germany) following the manufacturer's instructions. The purity of enriched T-cell populations was generally higher than 95% as determined by flow cytometry.

Isolation, reverse transcription and miRNA expression analysis

miRNAs were isolated from T cells (minimum of 5×10^6) using the mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. For reverse transcription and quantitative PCR, the TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Assays (Applied Biosystems/Life Tech Technologies, Carlsbad, CA, USA) specific for miR-29a and the housekeeping control gene RNU48 were used following the manufacturer's instructions. All samples were tested in triplicates. Mean fold change in miR-29a expression relative to RNU48 was calculated using $2^{-\Delta\text{Ct}}$ (Ct = threshold cycle).

Statistical analysis

All data were analyzed using Graph Pad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The confidence interval was set at 95%. In all statistical tests, a *P* value (two tailed) of <0.05 was considered as statistically significant. The D'Agostino and Pearson omnibus normality test was used to test for normality of all groups. The Mann-Whitney *U*-test was used to compare median difference between IFN- γ expression of LTBI and TB patients at all time points. It was also used to compare median differences between IFN- γ expression for TB patients at time point 0 and time point 1. The unpaired *t*-test was used to compare mean PPD-induced IFN- γ expression

Table 1. Patient characteristics

	Active pulmonary TB	LTBI
Number of participants	32	19
Mean age (SD)	36.3 ± 10.58	35.7 ± 13.58
Male (<i>n</i>)	24	6
BCG vaccination (<i>n</i>)	14	6
Concomitant disease (<i>n</i>)	5	0
Infectious (HIV)	1	0
Non-infectious	4	0

Out of the total number of patients recruited, five had concomitant diseases (one HIV positive patient, two with hypertension, one scrotal hernia and one case of dysmenorrhea).

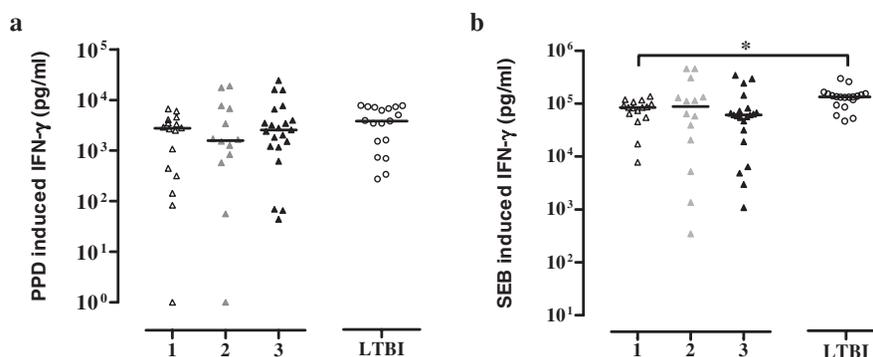


Fig. 1. IFN- γ responses of TB patients and LTBI. (a) PPD- and (b) SEB-induced IFN- γ response of TB patients at different time points [1 = at diagnosis ($n = 16$, open triangles); 2 = 1 month ($n = 14$, grey triangles); and 3 = 6 months ($n = 21$, black triangles) after chemotherapy onset] and LTBI ($n = 19$, open circles). Significant differences are indicated with asterisks ($*P < 0.05$).

in TB patients and LTBI. The Mann–Whitney U -test was used to compare median difference between SEB-induced IFN- γ expression in TB patients and LTBI. The Kruskal–Wallis test was used to compare median PPD- and SEB-induced IFN- γ expression in TB patients at all time points. The difference in the mean of miR-29a differential expression between TB patients and LTBI was tested using the unpaired t -test. The Kruskal–Wallis test was used to compare median differential expression of miR-29a in TB patients at all time points. Correlation between PPD- and SEB-induced IFN- γ and miR-29a was tested using the Spearman correlation test for TB patients and LTBI.

Results and discussion

We compared *M. tuberculosis* PPD-specific IFN- γ expression in pulmonary TB patients over a period of 6 months as well

as of healthy LTBI contacts. Median IFN- γ expression after *in vitro* re-stimulation with PPD was similar between different treatment time points (Fig. 1a) and a tendency of decreased PPD-specific IFN- γ expression in TB patients compared with LTBI were detected ($P = 0.094$) (Fig. 1a). Since antigen-specific IFN- γ expression may be affected by generally impaired T-cell responses (e.g. T-cell anergy described for active TB) (3), we concomitantly determined superantigen (SEB)-induced IFN- γ expression. SEB-induced IFN- γ was comparable in TB patients during chemotherapy (Fig. 1b), whereas significantly less IFN- γ was detected in TB patients (prior to chemotherapy) compared with LTBI contacts ($P = 0.002$) (Fig. 1b). We concluded that T cells from TB patients (prior to chemotherapy) showed generally decreased IFN- γ expression compared with LTBI contacts not restricted to *M. tuberculosis*-specific T cells. CD4⁺ T cells are the main

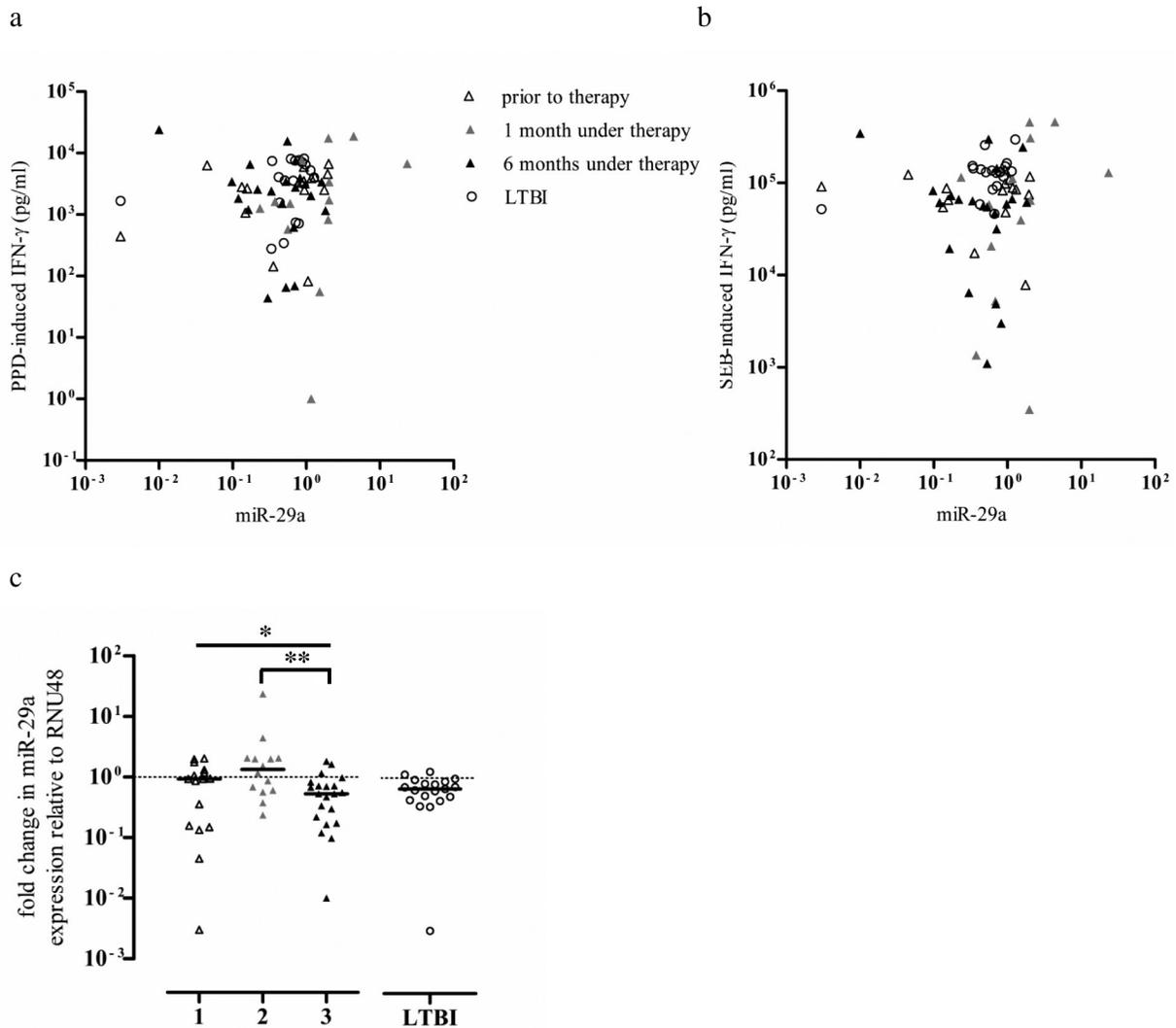


Fig. 2. Differential expression of miR-29a and comparison with T-cell produced IFN- γ in TB patients and LTBI contacts. Correlation of PPD-induced (a) and SEB-induced (b) IFN- γ secretion with miR-29a expression in TB patients at different time points [prior to therapy ($n = 16$), 1 month ($n = 14$) and 6 months under therapy ($n = 21$)] and LTBI ($n = 19$). (c) Differential expression of miR-29a between TB patients at different time points [1 = diagnosis ($n = 16$), 2 = 1 month ($n = 14$) and 3 = 6 months ($n = 21$) after chemotherapy onset] and LTBI ($n = 19$). Significant differences are indicated with asterisks (* $P < 0.05$, ** $P < 0.01$).

source of IFN- γ after PPD-specific *in vitro* re-stimulation (9) and miR-29 has been described to inhibit IFN- γ expression of CD4⁺ T cells (5). Marginal levels of IFN- γ expressed by NK cells or CD8⁺ T cells may also add to total concentrations in PPD or SEB re-stimulated PBMC supernatants, but this IFN- γ expression was also described to be inhibited by miR-29 (5).

Consequently, we determined miR-29a expression of CD4⁺ T cells from TB patients and LTBI. Both *M. tuberculosis*-specific and SEB-induced IFN- γ concentrations were compared with miR-29a expression from individual donors. We detected no significant correlation either for PPD-induced IFN- γ [TB patients (R (correlation coefficient) = 0.20; *P* = 0.157); LTBI (R = 0.37; *P* = 0.118)] (Fig. 2a) or for SEB-induced IFN- γ [TB patients (R = 0.20; *P* = 0.167); LTBI (R = 0.22; *P* = 0.367)] (Fig. 2b). These results were consistent with own previous studies that detected no correlation between miR-29a expression and IFN- γ levels of IFN- γ release assays of children with TB (7). However, the study by Kleinstueber *et al.* quantified miR-29a of whole blood, which rendered confounding effects due to differential miR-29a expression of distinct blood cell populations possible (7). Furthermore, the present study was performed in a country with high incidence rates of TB. Although our results did not exclude a role of miR-29a in IFN- γ regulation, a major inhibitory effect of miR-29a on CD4⁺ T cells during acute TB and latent *M. tuberculosis* infection was not likely. It is tempting to speculate that besides miR-29, additional factors contribute to IFN- γ regulation.

Marked interindividual variability of miR-29a expression (especially in the group of TB patients) was detected and although median miR-29a expression was slightly higher in TB patients, there was no significant difference compared with LTBI (Fig. 2c). These results contradict previous studies that either detected decreased (7) or increased miR-29 expression (8) in TB patients compared with LTBI. Notably, analyses of miR-29a expression during antituberculous chemotherapy showed significant differential miR-29a expression (*P* = 0.015) (Fig. 2c). Comparisons between single time points revealed a significant decrease of miR-29a expression between the second time point (1 month after onset of chemotherapy) and the third time point (6 months after therapy onset) (*P* = 0.004). We concluded that miR-29 expression in CD4⁺ T cells is differentially

expressed between TB disease stages. Although an influence on IFN- γ expression was not detected, other functional effects, e.g. on T-cell polarization (7, 10), cannot be excluded. Further analyses are needed to clarify the exact role of miR-29a in CD4⁺ T cells during chronic infections like TB.

Funding

The German Leprosy and TB relief association (DAHW) funded the MCCR/NATCELRES project to M. Jacobsen.

Acknowledgements

We also want to acknowledge the Kumasi Centre for Scientific Research in tropical medicine (KCCR), Kumasi, Ghana, and the University Children's Hospital, Dusseldorf, Germany, for their support for this study.

References

- 1 WHO. 2013. *WHO Tuberculosis Report*. WHO, Geneva, Switzerland.
- 2 Flynn, J. L. and Chan, J. 2001. Immunology of tuberculosis. *Annu. Rev. Immunol.* 19:93.
- 3 Boussiotis, V. A., Tsai, E. Y., Yunis, E. J. *et al.* 2000. IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. *J. Clin. Invest.* 105:1317.
- 4 Baltimore, D., Boldin, M. P., O'Connell, R. M., Rao, D. S. and Taganov, K. D. 2008. MicroRNAs: new regulators of immune cell development and function. *Nat. Immunol.* 9:839.
- 5 Ma, F., Xu, S., Liu, X. *et al.* 2011. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon- γ . *Nat. Immunol.* 12:861.
- 6 Steiner, D. F., Thomas, M. F., Hu, J. K. *et al.* 2011. MicroRNA-29 regulates T-box transcription factors and interferon- γ production in helper T cells. *Immunity* 35:169.
- 7 Kleinstueber, K., Heesch, K., Schattling, S. *et al.* 2013. Decreased expression of miR-21, miR-26a, miR-29a, and miR-142-3p in CD4⁺ T cells and peripheral blood from tuberculosis patients. *PLoS One* 8:e61609.
- 8 Fu, Y., Yi, Z., Li, J. and Li, R. 2014. Deregulated microRNAs in CD4⁺ T cells from individuals with latent tuberculosis versus active tuberculosis. *J. Cell. Mol. Med.* 18: 503.
- 9 Mueller, H., Detjen, A. K., Schuck, S. D. *et al.* 2008. Mycobacterium tuberculosis-specific CD4⁺, IFN γ +, and TNF α multi-functional memory T cells coexpress GM-CSF. *Cytokine* 43:143.
- 10 Liston, A., Papadopoulou, A. S., Danso-Abeam, D. and Dooley, J. 2012. MicroRNA-29 in the adaptive immune system: setting the threshold. *Cell. Mol. Life Sci.* 69:3533.