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miR-1224 Expression Is Increased in Human Macrophages after Infection with Bacillus Calmette-Guérin (BCG)

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ABSTRACT

Tuberculosis (TB) remains a major threat to human health. Understanding the strategies mycobacteria take to overcome immune defense is important in order to control the infection. Micro (mi)RNAs are master regulators of most pathways in the human body. Infection with mycobacterium impacts upon the host metabolic pathways as they are subverted to obtain the nutrition for intracellular TB survival. In this study, we aimed to investigate the effect of Bacillus Calmette-Guérin (BCG) infection on the expression of three miRNAs (miR-1224, -484 and -425), which are important in infection and in the regulation of metabolic pathways.

Peripheral blood monocyte-derived macrophage (MDM) cultures were prepared and infected with BCG at a multiplicity of infection (MOI)=10 or left uninfected as a control. 72h post-infection, RNA was extracted from the cultured cells and cDNA synthesis and real-time PCR performed. Expression levels miRNAs were normalized to the levels of U6 snRNA (*Rnu6*) using the $2^{-\Delta\Delta Ct}$ method.

Infection with BCG resulted in a highly significant increase in miR-1224 expression (24.4±3.8-fold induction) in human MDMs. The induction of miR-484 (1.8±0.3-fold increase) and of miR-425 (1.2±0.2-fold increase) was less increased compared to miR-1224.

Mycobacterium tolerates a hostile microenvironment by escaping from lysosomal degradation and providing a lipid-rich niche by trigger with and re-pattering host metabolism. This study highlighted the potential roles of miRNAs in host responses upon mycobacterium infection.

Keywords: Macrophages; miR-1224; miR-484; miR-425; Monocyte-derived macrophage; Tuberculosis

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INTRODUCTION

Tuberculosis (TB) is one of the most common causes of death from infectious diseases in the world.[1,2] Despite the availability of effective treatments, TB is still a major threat to human health especially in low-income countries.[3,4] It is estimated that one-third of the world's population is infected with *M. tuberculosis* (Mtb). In addition, the number of people who live with latent Mtb infection is estimated at two billion people worldwide.[3,5]

MicroRNAs (miRNAs) are small, non-coding RNAs that have an important role in the regulation of gene expression in most cells and organs in the body.[6] Each miRNA generally acts in concert with others and can potentially target up to several hundred genes in miRNA networks.[7] miRNA dysregulation has been associated with many pathological conditions[8] and has been implicated in the regulation of inflammation during the control of Mtb infection.[9-13] For example, over-expression of miR-144 is seen in T-cells from TB patients[9] and the miR-155/miR-155* ratio was enhanced in peripheral blood mononuclear cells (PBMCs) of TB patients.[14,15]

Macrophages are the first line of immune defense involved in the early clearance of Mtb.[16] Mycobacteria re-program the host's metabolic pathways in order to suite their own nutritional needs enabling their intracellular survival.[17,18] The adaptation of mycobacterium to the new situation follows the host–pathogen interaction and leads to a variety of physiological responses in the host including metabolic re-patterning.[19] During this process, the pathogen triggers metabolic dysregulation via virulence-associated factors leading to modulation of the regulatory networks that control the carbon, nitrogen and lipid metabolism of infected cells.[19,20] This interference may apply at the level of gene expression and energy production including altered expression of transporters, nutrient sensors as well as regulation of metabolic key enzymes in host cells.[19]

Intracellular Mtb uses host triacylglycerol to enable the accumulation of lipid bodies. These serve as a bacterial source of nutrients in the form of cholesterol esters and fatty acids,[21,22] and provide a niche for Mtb to acquire dormancy traits in lipid-loaded macrophages.[17] On the other hand Mtb exploits host-derived fatty acids to expand the acetyl-CoA pool and limiting the metabolic stress from propionyl-CoA.[23]

Previous studies demonstrated that miRNA circuits are used by Mtb to reprogram critical host immune or metabolic pathways to enable its intracellular survival and persistence.[24,25] For example, let-7 regulates the host immune response to Mtb infection by targeting A20 which is an inhibitor of the NF-κB pathway.[26] It was also demonstrated that TB infection up-regulates the miR-33 locus in macrophages and thereby reprograms host lipid metabolism and inhibits autophagy in infected cells.[24]

Knowledge about the metabolic host cell responses upon mycobacterial infection may provide us a new insight in tuberculosis therapies. We, therefore, selected three miRNAs (miR-1224, -484, -425) which are important in regulating metabolic pathways in mammalian cells and investigated their roles in modulating the metabolic response of macrophages to Mtb infection.

miR-1224 is associated with infectious disease,[27,28] whilst both miR-484 and miR-425 have a major role in the regulation of metabolic pathways.[29,30] This study aimed therefore, to investigate the effect of Bacillus Calmette-Guerin (BCG) infection on the level of miRs -1224, -484 and -425 in human monocyte derived macrophages (MDM) with the ultimate goal to find a biomarker for TB diagnosis.

MATERIALS AND METHODS

Generation of Monocyte-Derived Macrophages (MDM)

MDMs were obtained as previously described.[31] Briefly, peripheral blood was obtained from healthy human donors from the Iranian Blood Transfusion Organization (Tehran-Iran). All human procedures were reviewed and approved by the University of Shahid Beheshti Medical sciences ethics committee (No. sbmu1.REC.1394.127).

PBMCs were isolated by density gradient centrifugation using Ficoll-Plaque (Invitrogen Corp., Carlsbad, CA, USA), and cultured in 75 cm^2 cell culture flasks (Costar, Cambridge, MA) in RPMI 1640 (Gibco; Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS)

(Gibco; Carlsbad, CA, USA), 25mM HEPES (Gibco; Carlsbad, CA, USA), 100 units/mL penicillin (Sigma, Munich, Germany) and 100 ug/mL streptomycin (Sigma, USA) in 5% CO_2. After 4h at 37°C, non-adherent cells, were removed and the adherent monocytes were washed 2-3 times with 1xPBS (Sigma-Aldrich, USA). Monocytes were differentiated into macrophages by cultivation for 7-8 days in RPMI containing 100ng/mL Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Invitrogen) and 10% FBS with one medium change on the 4th day.[31] After day 7-8, macrophages were detached by gentle scrapping in cold PBS and the cells stained with Phycoerythrin (PE)–conjugated anti-human CD86, Fluorescein Isothiocyanate (FITC)–conjugated anti-human CD64 and Allophycocyanin (APC)–conjugated anti-human CD11b (BD Biosciences, San Jose, CA, USA). Cells were incubated with antibodies for 30min, washed twice with fresh media and 10000 cells analyzed by flow cytometry (FACS Calibour, BD, USA). The phagocytosis activity of obtained macrophages was determined by a flowcytometric phagocytosis assay.[32]

BCG Infection of MDMs

Macrophages were infected on days 7 or 8. In brief, bacteria were opsonized by incubation with human AB+ serum (pooled from 30 healthy male) as a source of complement components for 2h at 37°C. Macrophages were infected with opsonized BCG (obtained as a gift from Pasteur Institute of Iran) at Multiplicity of infection (MOI) of 10 (MOI was determined in a separate experiment to result in >70% infection) or were left uninfected as controls. Cells were incubated for 2h at 37°C in a 5% CO_2 incubator and then were washed with 1x PBS containing amikacin (80ug/mL) to eliminate cell-free bacteria. Subsequently, the cells were cultivated for another 72h in medium containing FBS (10% final concentration, Gibco, USA) and 100ng/mL GM-CSF before RNA extraction.

RNA Extraction, cDNA Synthesis and Real-time Quantitative PCR Analysis

Total RNA extraction was performed on MDM 72h post BCG infection using TriZol reagent (Invitrogen, USA). Extracted RNA was reverse transcribed using the miRCURY LNA Universal RT microRNA cDNA Synthesis Kit (Exiqon, Vedbæk, Denmark) according to the manufacturer's instructions.

Real-time PCR assays used the ExiLENT SYBR Green Master Mix kit (Exiqon kit, Sakan Teb PJS, Teheran, Iran) for the miRNAs of interest (miR-1224, miR-484 and miR-425). The real-time PCR program included the following steps: an initial denaturation step at 95°C for 10 min; 45 amplification cycles that consisted of a denaturation step (10s at 95°C) and an annealing step (60s at 60°C). Expression levels of all the miRNAs of interest were normalized to the level of U6 snRNA (*Rnu6*) as a control miRNA using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All experiments were performed at least 3 times and analyzed for significance using analysis of variance (ANOVA). Students t- test was used as a post analysis test and significance was assumed at the 95% confidence level ($p<0.05$). SPSS (v.16) was used for all statistical analysis.

RESULTS

Isolated human monocytes were cultured with Granulocyte-macrophage colony-stimulating factor (GM-CSF) for 7-8 days by which time they assumed the characteristic fried egg morphology of mature macrophages (Figure 1A and B). Flow cytometry analysis showed enhanced expression of the macrophage-specific markers CD86 (86.0±2.7%), CD64 (75.0±2.8%) and of CD11b (68.0±2.0%) (Figure 1C).

The phagocytosis activity of MDMs was assessed by flow cytometry and cells could successfully engulf FITC-labeled BCG (88.24%±3.72 uptake) at an MOI=10 (Figure 2).

72h after infection, RNA was extracted and the quantity and quality of the extracted RNA was confirmed by Agilent Bioanalyzer and Nanodrop, respectively. BCG infection increased the level of miR-1224 (24.4±3.8-fold induction). In contrast, there was no significant effect of BCG infection on the expression of miR-425 (1.2±0.2-fold induction) and miR-484 (1.8±0.3-fold induction) (Figure 3).

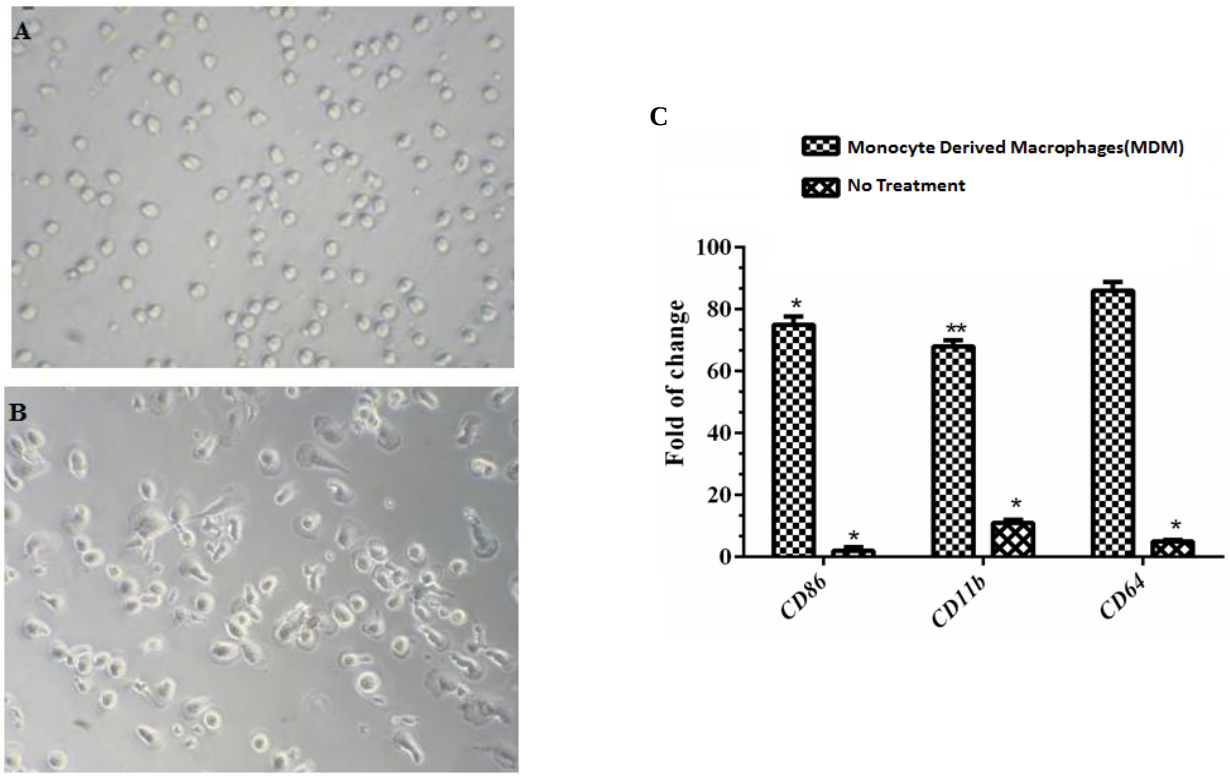

Figure 1. In Vitro Generation of monocyte-derived macrophages. Primary human monocytes were isolated from buffy coats and cultured in Roswell Park Memorial Institute media(RPMI) (A) Isolated human monocytes were maintained with granulocyte-macrophage colony-stimulating factor (GM-CSF) and the classical morphology (fried egg shaped) associated with mature macrophages was observed at day 7-8 (B). Flow cytometric analysis showed 86.0±2.7%, 75.0±2.8% and 68.0%±2.0% expression level of CD86, CD64 and CD11b in mature macrophages (C). Results are presented as mean±S.E.M of three independent experiments each repeated twice.

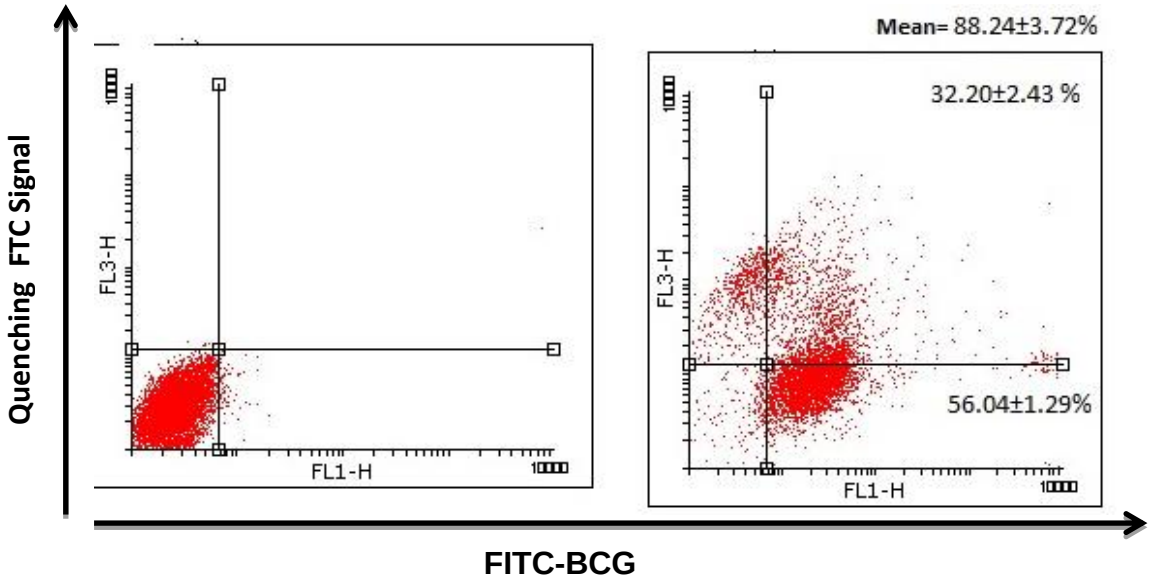

Figure 2. Uptake of FITC-Bacillus Calmette-Guérin (BCG) by mature differentiated macrophages. Representative result showing BCG uptake by differentiated macrophages. The mean±S.E.M. of three independent experiments (88.24±3.72%) is indicated above the right-hand figure.

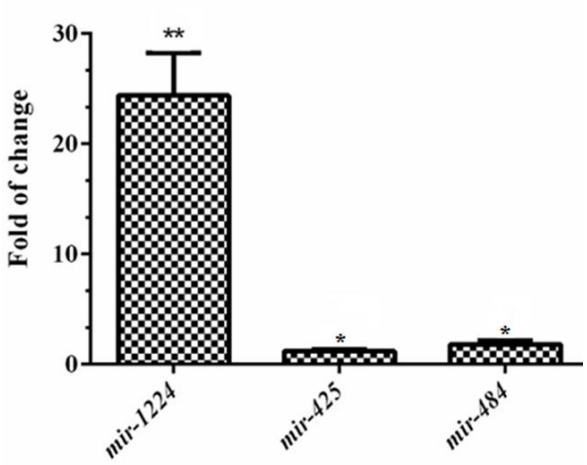

Figure 3. Real-time RT–PCR analysis of miR-1224; miR-484; miR-425 expression in macrophage cell cultures 72 h post infection with BCG. Infection resulted to increased expression of miR-1224 (24.4± 3.8-fold increase), miR-484 (1.81±0.3-fold increase) and miR-425 (1.18±0.2-fold increase) compared to the control uninfected group. Data represent mean±S.E.M of three independent experiments each repeated in triplicate (*p< 0.05; **p< 0.01 versus control).

DISCUSSION

In this study, we report that infection of peripheral blood-derived human MDM with BCG markedly enhanced the expression of miR-1224 with a small but significant effect on miR-484 after 72 hrs. In contrast, BCG infection had no effect on miR-425 expression.

miRNAs act as master regulators of gene expression, having a critical role in the modulation of most biological processes and are crucial for maintaining cellular homeostasis.[6] Alteration of miRNA profiles upon infection with Mtb have been reported previously[33-35] in a number of different cell lines[34,36] and in primary monocyte cell cultures.[33,37]

Initial array-based profiling of miRNAs in response to Mtb infections in human macrophages was performed by Furci and colleagues in 2013 who reported a set of miRNAs including mir-145, mir-222, mir-27a and mir-27b which were significantly different in infected cells compared to controls.[33] Elizabeth and colleagues in 2016, quantified the expression level of a group of miRNAs, including let-7e, mir-21, mir-29a, mir-155, mir-146a, mir-146b, mir-210, mir-223 and mir-1224 in the J774a.1 mouse macrophage cell line during infection with the most relevant mycobacterial strains. They reported that mir-146b and mir-1224 expression increased in response to different strains of mycobacterium infection.[36]

In another study by Rajaram in 2011, it was shown that the expression of mir-125b up regulated and, in contrast, that of mir-155 down regulated in response to Mtb infection in human monocyte derived macrophages.[37]

Previous studies reporting miRNA expression profiles in response to Mtb infection have reported inconsistent results. However, we selected three crucial miRNA that regulate the host's metabolic and energy production machinery and their immunological pathways. The expression of these miRNAs was quantified following infection with Mtb.

Dysregulation of miRNA expression has been associated with many pathological conditions[38] and miR-1224 has been involved in regulating inflammatory responses[1] particularly in negatively regulating tumor necrosis factor α (TNF-α) expression.[40] TNF-α plays an important role in host defense during *M. tuberculosis* infection[41,42] by promoting phagosome-lysosome maturation and thereby increasing T cell-dependent anti-mycobacterial host defense mechanisms.[43] On the other hand, TNF stimulates apoptosis of mycobacterium-infected macrophages,[44,45] which subsequently leads to cross-priming of CD8+ T cells.[46] Our result confirms data from another study that reported overexpression of miR-1224 in the mouse macrophage cell line (J744A.1) after infection with a different Mtb species.[47]

This result raises the possibility that intracellular Mtb may down-regulate host cell TNF production in order to evade the host antibacterial immune responses. In fact, Mtb contains genes that encode components

that down-regulate macrophage TNF-α expression such as A20.[48-50] miR-1224 is also involved in the regulation of many lipid-related genes and subsequent changes in lipid metabolism.[40]

A modest overexpression of miR-484 and miR-425 was observed following infection with BCG in macrophages. These two miRNAs are implicated in the regulation of various metabolic pathways and are associated with metabolic syndrome.[29,30] miR-484 targets the mitochondrial fission protein 1 (Fis1)[29] whilst loss of miR-425 is linked to insulin resistance.[30] Changes in the expression of these miRNAs associated with metabolic re-patterning may indicate that the mycobacterium is subsuming the host's metabolism to enable nutrient uptake and survival.[18] Changes in the level of mir-425 expression was previously reported by Furci upon infection of human macrophages with virulent *Mtb* H37Rv and *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG).[33]

The results indicate a small induction of both miR-484 and of miR-425 but neither of these changes were large. This may reflect a true failure of BCG infection to modulate the expression of these miRNAs or that the optimal time-point for their induction was missed by only using a single time point for analysis. Furthermore, we did not measure the expression of downstream metabolic targets of these miRNAs. Further experiments are required to establish the functional role, if any, of these miRNAs. In addition, the expression of these miRNAs in lung samples and serum in large cohorts of TB patients may help establish whether these could act as surrogate markers of TB infection and host metabolic reprogramming. Our data show that Mtb induce miRNAs that target the host metabolic and energy production machinery to post-transcriptionally limit host immune responses

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