Identification of a novel role of ESAT-6-dependent miR-155 induction during infection of macrophages with *Mycobacterium tuberculosis*

Ranjeet Kumar,1 Priyanka Halder,1 Sanjaya K. Sahu,1 Manish Kumar,1 Mandavi Kumar,1 Kuladip Jana,2 Zhumur Ghosh,3 Pawan Sharma,4†
Manikuntala Kundu1 and Joyoti Basu1*

1Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra Road, Kolkata 700 009, India.
2Division of Molecular Medicine, Bose Institute, A.J.C. Bose Centenary Building, P-1/12, CIT Scheme- VII M, Kolkata 700 054, India.
3Bioinformatics Centre, Bose Institute, A.J.C. Bose Centenary Building, P-1/12, CIT Scheme- VII M, Kolkata 700 054, India.
4Immunology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India.

Summary

*Mycobacterium tuberculosis* (M.tb.) replicates in host macrophages to cause tuberculosis. We have investigated the role of miRNAs in M.tb.-infected murine RAW264.7 cells and bone marrow-derived macrophages (BMDMs), focusing on miR-155, the most highly upregulated miRNA. We observed that miR-155 upregulation is directly linked to the attenuation of expression of BTB and CNC homology 1 (Bach1) and SH2-containing inositol 5′-phosphatase (SHIP1). Bach1 is a transcriptional repressor of haem oxygenase-1 (HO-1), whereas SHIP1 inhibits the activation of the serine/threonine kinase AKT. We hypothesize that M.tb.-induced miR-155 induction leads to repression of Bach1, which augments the expression of HO-1, a documented activator of the M.tb. dormancy regulon. SHIP1 repression facilitates AKT activation, which is required for M.tb. survival. In addition, M.tb.-induced miR-155 inhibits expression of cyclooxygenase-2 (Cox-2) and interleukin-6 (Il-6), two modulators of the innate immune response. Importantly, we observed that the virulence-associated secreted protein ESAT-6 plays a key role in miR-155 induction and its subsequent effects on Bach1 and SHIP1 repression. Inhibition of miR-155 hindered survival of M.tb. in RAW264.7 and in murine BMDMs. Thus, our results offer new insights into the role of miRNAs in modulation of the host innate immune response by M.tb. for its own benefit.

Introduction

MicroRNAs (miRNAs) are short 22 bp regulatory RNAs that post-transcriptionally control target mRNA expression or cause translational repression. MiRNAs usually bind to mRNA targets in their 3′-untranslated regions (3′-UTRs). Based on sequence complementarity, this binding leads to translational repression and/or degradation of the mRNA (Baek et al., 2008; Selbach et al., 2008). Among a host of processes, miRNAs play vital roles in development, cancer, neuronal cell fate and immune function in diseased states such as rheumatoid arthritis (Johnston et al., 2005; Rodriguez et al., 2007; Baltimore et al., 2008; Hou et al., 2009; Lee and Dutta, 2009).

Innate immunity constitutes the first line of defence against pathogens. An important branch of innate immune signalling is triggered by ligation of the Toll-like receptors (TLRs) followed by intracellular assembly of signalling scaffolds dependent on adaptor proteins such as MyD88 and TRIF, that activate protein kinases eventually leading to the activation of key transcription factors such as NF-κB (Moresco et al., 2011). Bacterial lipopolysaccharide (LPS)-treated monocytes, macrophages and dendritic cells have been used as models to explore the role of miRNAs in TLR signalling (O’Connell et al., 2007; Tili et al., 2007; Ceppi et al., 2009; Liu et al., 2009; Lu et al., 2009; McCoy et al., 2010; Martinez-Nunez et al., 2011; Zhang et al., 2011). A trio of miRNAs comprising miR-155, miR-146a and miR-21 are now regarded as...
central miRNAs, which regulate inflammatory pathways in myeloid cells (Quinn and O’Neill, 2011). miR-146a plays a role in endotoxin tolerance by virtue of its ability to knockdown IRAK1 and TRAF6 (Taganov et al., 2006). In mature human dendritic cells, miR-155 targets TAB2 to downregulate inflammatory cytokine production (Ceppi et al., 2009). MiR-21 dampens the expression of PDCD4 thereby upregulating IL-10 and negatively regulating the pro-inflammatory response (Sheedy et al., 2010). In addition, miRNAs such as let-7 family members and miR-132 (Androulidaki et al., 2009; Lagos et al., 2010) have also recently been recognized as important regulators of innate immune signalling.

Subtle differences in miRNA expression profiles are likely to have a significant influence on the outcome of an encounter between the host and pathogen. Other than the available literature on the role of TLR ligands in miRNA production in cells of the innate immune system, there is only sketchy knowledge on the role of miRNAs in guiding the outcome of host-pathogen interactions. Tuberculosis (TB) caused by Mycobacterium tuberculosis (M.tb.) remains a global health problem. The outcome of M.tb. infection depends on the balance between innate immune mechanisms designed to eliminate the pathogen, and the pathogen’s ability to skew the innate immune responses in a direction favouring its survival in the host. The mechanisms exploited by virulent M.tb. to tilt the balance of the innate immune response in its favour remain incompletely understood. miRNAs by virtue of their ability to fine tune innate immune signalling, stand out as candidate regulators of M.tb.-induced signalling. In this context, a recent study has investigated the global miRNA response of macrophages infected with M. avium ssp. hominisuis infection (Sharbati et al., 2011). In this study, we have explored the role of miRNAs in influencing macrophage signalling in response to M.tb. infection. Our results demonstrate that miR-155 is the principal miRNA produced by murine macrophages in response to M.tb. infection. MiR-155 induction is dependent on early secreted antigenic target 6 kDa protein (ESAT-6) and is lower in M. bovis BCG (which lacks the esat-6–encoding genomic region) compared with M.tb. H37Rv. We describe the unexpected finding that miR-155 induction benefits the pathogen, and helps in its survival in the early stages of infection in an in vitro model. miR-155 attenuates the SH2-containing inositol 5′-phosphatase (SHIP1) and the transcriptional repressor BTB and CNC homology 1 (Bach1). We hypothesize that SHIP1 attenuation is associated with activation of the serine/threonine kinase AKT, while Bach1 repression upregulates haem oxygenase 1 (HO-1). At the same time, suppression of miR-155 expression with anti-miR-155 demonstrates an attenuating role of miR-155 in the production of cyclooxygenase-2 (Cox-2) and interleukin-6 (IL-6). Most importantly, we demonstrate that inhibition of miR-155 attenuates the ability of M.tb. to survive in macrophages, supporting the contention that miR-155 induction likely offers a survival advantage to M.tb. within its host.

Results
miRNA induction in M.tb.-infected RAW264.7

In order to identify miRNAs that are differentially expressed in M.tb.-infected macrophages, we performed a global analysis using Taqman Low Density Arrays (TLDA; Applied Biosystems, Foster City, CA). MiR-155 was identified as the most highly upregulated miRNA. TLDA analysis (performed in technical replicates) showed a 27-fold induction of miR-155 24 h after infection. In addition, TLDA analysis showed 1.4- to 2-fold induction (24 h post infection) of miR-146a and miR-21, two miRNAs reported to be part of the triumvirate of miRNAs (along with miR-155), which are induced upon challenge of cells of the innate immune system with pathogen-derived effectors. Northern analysis confirmed the induction of these two miRNAs (Fig. S1). miR-155 is the focus of our studies in the present communication. miR-155 is rapidly induced in a dose (moi)- and time-dependent manner, and persists even up to 24 h post infection in RAW264.7 (Fig. 1A).

Recent reports have suggested that lack of miR-155 induction correlates with the ability of a pathogen to suppress the host immune response. MiR-155 induction by the avirulent Francisella novicida but not the virulent F. tularensis is reportedly linked to pro-inflammatory cytokine production (Cremer et al., 2009). The apparent conflict between these findings and our own observations clearly reflecting high induction of miR-155 in cells infected with a pathogenic mycobacterium, prompted us to investigate the consequences of miR-155 expression in M.tb.-infected macrophages in greater detail.

ESAT-6 is linked to miR-155 induction by M.tb.

Previous studies (Rajaram et al., 2011; Schulte et al., 2011) have suggested that sensing of surface-exposed lipoglycans triggers the early miRNA response to bacterial pathogens. We chose to test the involvement of other players in the early miRNA response by examining the effect of the esx1-encoded ESAT-6. Using a mouse 96-well miRNA array (SA Biosciences), we observed that miR-155 was the only miRNA to be induced by ESAT-6. Northern analysis showed that ESAT-6 upregulated miR-155 in a dose- (data not shown) and time-dependent manner (Fig. 1B). qRT-PCR analysis also confirmed that ESAT-6 induces the induction of miR-155 (Fig. S2). Further, ESAT-6 also induced miR-155 in primary bone marrow-derived macrophages (BMDMs) (Fig. 1C). In
order to test the hypothesis that ESAT-6 is a player in miR-155 induction by M.tb., we compared miR-155 induction between H37Rv and the H37Rv:
\texttt{Δ}\textit{esat-6} mutant, or between \textit{M. bovis} BCG and \textit{M. bovis} BCG:2F9 (which harbours the RD1 locus of M.tb.; henceforth referred to as BCG:2F9). Induction of miR-155 was attenuated in RAW26.47 cells (Fig. 1D, Fig. S3) or in BMDMs (Fig. 1E) infected with H37Rv:
\texttt{Δ}\textit{esat-6} compared with H37Rv. In addition, miR-155 induction was higher in RAW264.7 (Figs 1F and S4) or in BMDMs (Fig. 1G) infected with the strain 2F9 compared with \textit{M. bovis} BCG. These results argued in favour of a role of ESAT-6 in miR-155 induction by M.tb. in macrophages.

\textit{miR-155-dependent downregulation of SHIP1 in M.tb.-infected macrophages}

In an effort to directly analyse the role of miR-155 in the M.tb.-induced immune response, we assessed the expression of selected miR-155 targets. The first target chosen was the Src homology 2-containing inositol 5’-phosphatase 1, SHIP1, a validated, direct target of miR-155. SHIP1 is expressed in haemopoietic cells and hydrolyses the 5’-phosphate of phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3) to negatively regulate the activation of the serine/threonine kinase AKT (Krystal, 2000). It fine tunes lipid phosphate-dependent signalling events and acts in concert with other negative regulators of TLR signalling to promote endotoxin tolerance (Rauh et al., 2004). Deletion of SHIP1 phenocopies many of the effects of miR-155 knockout in mice. Based on reports that AKT is required for the survival of M.tb. in macrophages (Kuijl et al., 2007; Kumar et al., 2010), we contended that miR-155-dependent regulation of SHIP1 could play a role in the survival of M.tb. in macrophages. To begin with, we tested the expression of SHIP1 in M.tb.-treated RAW264.7. A downregulation of SHIP1 was observed in M.tb.-infected macrophages (Fig. 2A) in a dose-dependent manner. Concomitant with a decrease in SHIP1, an increase in AKT phosphorylation was observed in the treated macrophages (Fig. S5). The efficacy of the miR-155 inhibitor was confirmed by analysing attenuation of the luciferase activity in RAW264.7 cells transfected with either control or miR-155 inhibitor along with the SHIP1 3’-UTR construct (Fig. S6A). The status of SHIP1 was then tested in cells transfected with miR-155 inhibitor or control inhibitor followed by infection with M.tb. (Fig. 2B). The expression of SHIP1 was then tested in cells transfected with either control or miR-155 inhibitor along with the SHIP1 3’-UTR construct (Fig. S6A). The status of SHIP1 was then tested in cells transfected with either control or miR-155 inhibitor followed by infection with M.tb. Following M.tb. infection, SHIP1 expression decreased in both cells transfected with miR-155 inhibitor as well as cells transfected with control inhibitor. However, after infection of RAW264.7, an enhanced level of SHIP1 expression was observed in the cells transfected with miR-155 inhibitor compared with control transfected cells (Fig. 2B). The direct involvement of miR-155 in regulating SHIP1 levels in M.tb.-infected macrophages. Enhanced SHIP1 expression was also observed in infected BMDMs, which had been transfected with miR-155 inhibitor compared with
control inhibitor (Fig. S6B). We hypothesize that at least within 24 h post infection, the miR-155-dependent SHIP1 downregulation favours activation of AKT, thereby exerting a pro-survival effect on macrophages, which likely benefits the pathogen.

miR-155-dependent downregulation of Bach1 in M. tb.-infected macrophages

Haem oxygenase-1 (HO-1) encoded by the hmxox1 gene is a stress-responsive gene that responds to oxidative stress by catalysing the degradation of haem to form biliverdin, carbon monoxide (CO) and free iron (Otterbein et al., 2003). The induction of hmxox1 is regulated by two upstream enhancers, E1 and E2 (Alam, 1994; Alam et al., 1995) that contain Maf recognition elements (MAREs) (Kataoka et al., 2001). The MARE can be bound by heterodimeric basic leucine zipper (bZip) factors including Nrf2, BTB and CNC homology 1 (Bach1), Maf and AP-1 families. Bach1 is a basic region leucine zipper transcriptional regulator. It forms heterodimers with the small Maf proteins and the resulting Bach1 heterodimers repress MARE-dependent transcription. With increasing haem levels, the repressor activity of Bach1 is lost, shifting the balance towards activation of hmxox1. It is well documented that there is a rise in hmxox1 expression upon infection of macrophages with M.tb. (Kumar et al., 2008; Shiloh et al., 2008). We reasoned that since Bach1 is a target of miR-155 (Xu et al., 2010), the elevated miR-155 expression could be involved in repression of Bach1 leading to increased hmxox1 expression. In order to test this hypothesis, we determined the levels of Bach1 in M.tb.-infected macrophages. Bach1 levels were decreased in M.tb.-infected macrophages compared with uninfected macrophages (Fig. 3A). This effect was partially reversed in cells treated with miR-155 inhibitor (Fig. 3B). The effect of the miR-155 inhibitor could also be demonstrated in BMDMs infected with M.tb. (Fig. 3C). HO-1 levels were expected to decrease after M.tb. infection in miR-155 inhibitor treated cells. The repression of HO-1 levels in miR-155 inhibitor treated cells infected with M.tb. could be demonstrated in RAW264.7 and BMDMs (Fig. 3 D and E).

The role of ESAT-6 in miR-155-dependent regulation of SHIP1 in RAW264.7

In order to test the role of ESAT-6 in regulation of SHIP1 levels in RAW264.7, we analysed the status of SHIP1 in cells treated with ESAT-6. ESAT-6 downregulated the expression of SHIP1 in a time-dependent manner (Fig. 4A). Concomitantly, there was upregulation of p-AKT (Fig. 4B). In order to further investigate the role of ESAT-6 in the context of the intact bacterium, RAW264.7 was infected with M. bovis BCG or BCG:2F9. The downregulation of SHIP1 was observed in BCG:2F9-infected but not in BCG-infected cells (Fig. 4C), arguing in favour of a role of ESAT-6 in downregulating SHIP1 levels in infected cells.

The role of ESAT-6 in miR-155-dependent regulation of Bach1 in RAW264.7

Bach1 was downregulated in ESAT-treated RAW264.7 (Fig. 5A) and increased upon inhibition of miR-155 (Fig. 5B). These results supported a role of ESAT-6 in miR-155-dependent Bach1 regulation in RAW264.7. At the same time, HO-1 levels were upregulated in ESAT-6 treated cells (Fig. 5C). Bach1 levels decreased in BCG-infected RAW264.7 compared with uninfected cells. However, there was a more pronounced decrease in Bach1 in RAW264.7 cells infected with BCG:2F9 expressing ESAT-6, compared with BCG-infected cells (Fig. 5D). In harmony with the decrease in Bach1, HO-1 levels increased in M.tb.-infected RAW264.7 (Fig. S7). The induction of HO-1 was lower in BCG compared with M.tb., whereas it was higher in BCG:2F9 compared with BCG (Fig. S7). Further, in miR-155 inhibitor-treated cells, ESAT-6 treatment showed higher Bach1 levels compared with cells treated with control inhibitor (Fig. S8A). Concomitantly, there was a decrease in hmxox1 (Fig. S8B) and...
HO-1 protein (Fig. S8C). Taken together, these observations support the contention that ESAT-6 plays a role in augmenting HO-1 expression by inhibiting *Bach1* expression in a miR-155-dependent manner.

**miR-155 regulates the production of cyclooxygenase-2 in *M. tb*-infected macrophages**

The balance between the production of the eicosanoids prostaglandin E2 (PGE2) and lipoxin A4 has been reported to determine the cellular fate of macrophages infected with *M. tb*. *M. tb* infection elicits the expression of prostaglandin E synthase (PGES) [or cyclooxygenase-2 (COX-2)] in macrophages (Rand *et al.*, 2009). COX-2 activity leading to generation of PGE2 limits the production of anti-inflammatory lipoxin A4 (LXA4) and prevents necrosis of the infected macrophage (Chen *et al.*, 2008). Infection of prostaglandin PGES−/− macrophages *in vitro* with *M. tb*. H37Rv results in higher bacterial burden compared with the wild-type macrophages (Chen *et al.*, 2008). Considering the important role of COX-2 in mycobacterial infection, we asked the question whether miR-155 influences the expression of *Cox-2* after infection. Transfection of RAW264.7 macrophages with miR-155 inhibitor augmented the expression of *Cox-2* after infection (Fig. 6A). This result suggests that miR-155-mediated inhibition of *Cox-2* could favour the survival of *M. tb*. in macrophages.

**miR-155 regulates the production of IL-6 in *M. tb*-infected macrophages**

We next wanted to test the global role of miR-155 production in *M. tb*-infected macrophages through direct or
indirect effects by analysing the regulation of selected cytokines or chemokines in the absence of presence of miR-155 inhibitor. We tested the expression of Tnf-α, Tgf-β, Il-12 p40, Il-6, Ccl5 (RANTES) and Ccl2 (MCP-1) by qRT-PCR. Out of these, Il-6 was found to be differentially regulated in the absence or presence of miR-155 inhibitor. Transfection of RAW264.7 macrophages with miR-155 inhibitor augmented the expression of Il-6 (Fig. 6B). This was evident at the level of the IL-6 protein released into the supernatant as well (Fig. S9). IL-6 plays a key role in the granuloma maintenance response to mycobacterial trehalose dimycolate (Welsh et al., 2008).

In addition, there is evidence that virulent M.tb. strains elicit reduced levels of pro-inflammatory cytokines (including TNF-α and IL-6) and RNI from macrophages compared with less virulent ones. This phenomenon has been described for the hypervirulent HN878 strain of M. tuberculosis and the hypervirulent mce1 mutant of M.tb. (Shimono et al., 2003; Manca et al., 2004; Reed et al., 2004). The dampening of Il-6 induction could therefore be a response favouring the pathogen.

miR-155 expression is required for growth of M.tb. in macrophages

Considering the fact that miR-155 modulated important immune function regulatory molecules of macrophages infected with M.tb., we asked the question whether miR-155 expression affects the growth of M.tb. in macrophages. In order to test the role of miR-155, RAW264.7 or BMDMs were transfected with either control inhibitor or miR-155 inhibitor, followed by infection with M.tb., cells were lysed after infection and viable counts (cfu) were determined by plating out the bacteria. Significant differences were not observed at 0 h post infection (Fig. 7A

![Fig. 5. ESAT-6 modulates Bach1 in a miR-155-dependent manner in RAW264.7.
A. RAW264.7 cells were treated with recombinant ESAT-6 (5 µg ml⁻¹) for different periods of time and cell lysates were immunoblotted with Bach1 antibody.
B. Cells were transfected with control inhibitor (ctrl) or miR-155 inhibitor (miR-155). Transfected cells were treated with ESAT-6 for 6 h followed by immunoblotting with Bach1 antibody. Blots were reprobed with tubulin antibody. The fold change in Bach1 expression in miR-155-transfected cells over cells transfected with control inhibitor (ctrl) is shown.
C. Cells were transfected with ESAT-6 as described above and cell lysates were immunoblotted with HO-1 antibody. The blot was reprobed with tubulin antibody. The fold change in HO-1 over uninfected cells is shown.
D. Cells were left uninfected or infected with BCG or BCG:2F9 at a moi of 5. Cell lysates were immunoblotted with Bach1 antibody. Blots were reprobed with tubulin antibody. The fold change in Bach1 over uninfected cells is shown. Data are representative of the results obtained in at least three (A) or two (B–D) independent experiments.](image)

![Fig. 6. Inhibition of miR-155 enhances the expression of Cox-2 and Il-6 in M.tb.-infected RAW264.7 and hinders the survival of M.tb. in macrophages. RAW264.7 cells were transfected with either control inhibitor (ctrl) or miR-155 inhibitor (miR-155). After 20 h, transfected cells were either left uninfected or infected with M.tb. H37Rv at a moi of 5. RNA was isolated and expression of Cox-2 (A) or Il-6 (B) was assessed by qRT-PCR using Gapdh expression for normalization. Relative expression is shown with respect to the expression in uninfected cells. Data are shown as mean ± SD of three separate determinations. Similar results were obtained in at least three independent experiments. * P < 0.05, ** P < 0.01.](image)
and B). Relative to control inhibitor-transfected cells, there was significant reduction in bacterial colony-forming units (cfu) in miR-155 inhibitor-transfected RAW264.7 cells 24 h post infection (Fig. 7B). In similar experiments performed with BMDMs, colony-forming units were significantly reduced in cells transfected with miR-155 inhibitor compared with cells transfected with control RNA at 24 and 48 h post infection (Fig. 7D and E).

**Discussion**

The mechanisms exploited by virulent M.tb. to tilt the balance of the innate immune response in its favour remain incompletely understood. The role of miRNAs in controlling the outcome of an encounter between host and pathogen has been investigated in a limited manner in the context of intracellular pathogens. With this in view, we undertook an analysis of the miRNAs regulated when M.tb. infects murine RAW264.7 cells. Our results show that a core set of miRNAs (miR-155, miR-146a and miR-21) previously identified to act as first-line innate immune effectors is upregulated following challenge of macrophages with M.tb. Considering the fact that miR-155 was the most highly regulated, we focused on this particular miRNA in the present study. Bacterial components such as surface lipoglycans likely have a role in miR-155 induction as described by Rajaram *et al.* (2011). However, we show for the first time that initial sensing of an intracellular pathogen is not confined to sensing of surface-exposed lipoglycans. The induction of miR-155 depends at least in part on the presence of the secreted antigen, ESAT-6. Using exogenous ESAT-6, M.tb. or an *esat*-6 knockout strain, *M. bovis* BCG or BCG expressing ESAT-6, we provide evidence that miR-155 induction requires ESAT-6. Mir-155 transcription is dependent on NF-κB, while exogenous ESAT-6 inhibits TLR/MyD88-dependent NF-κB activation (Pathak *et al.*, 2007). This would appear contradictory. However, it is pertinent to point out that miR-155 induction is dependent on several other transcription factors such as AP-1 and SMAD4 (Kong *et al.*, 2008; Yin *et al.*, 2008). In addition, regulation could also be at the post-transcriptional level (Ruggiero *et al.*, 2009). Therefore, the overall effect of ESAT-6 could be dependent on its ability to influence these pathways as well.

Rajaram *et al.* (2011) have suggested that miR-155 induction is low in M.tb.-infected human macrophages, 6 h post infection. Our results show markedly increased levels of miR-155 24 h post infection. More work is needed to understand the role of miR-155 in the host response to M.tb.
required to understand the similarities and differences in the response of human and murine macrophages with regard to miRNA production. It is also pertinent to note that stimulation of PBMCs from active tuberculosis patients with purified protein derivative (PPD) leads to activation of miR-155 (Wu et al., 2012), suggesting a global role of miR-155 in the immune response to tuberculosis that deserves further investigation.

A link between miR-155 and the innate immune response is well established through experiments, showing increased expression following stimulation of macrophages or monocytes with LPS or lipoprotein, or in the splenocytes of mice infected with Salmonella enteritidis-derived LPS. (Taganov et al., 2006; O’Connell et al., 2007; Tili et al., 2007). We demonstrate that the induction of miR-155 correlates directly with suppression of its targets SHIP1 and Bach1. Based on the knowledge that SHIP1 attenuates AKT activation, we hypothesize that miR-155-dependent inhibition of SHIP1 favours AKT activation, which is required for survival of M.tb. in macrophages. Shiloh et al. (2008) have hypothesized HO-1-induced CO production supports long-lasting activation of the dormancy regulon. We rationalize that a link exists between miR-155 induction, HO-1 expression and activation of the dormancy regulon, based on our observation that miR-155 downregulates the hmx1 transcriptional repressor, Bach1.

In addition to its effects on SHIP1 and Bach1, miR-155 negatively regulates two important immune regulators Cox-2 and Il-6 probably through indirect effects involving pathways, which are yet to be understood. Considering that PGES $^\nu$: mice harbour higher burdens of M.tb., suppression of Cox-2 by miR-155 appears to be yet another miR-155-dependent mechanism favouring the survival of the pathogen. In addition, miR-155 inhibits the expression of the pro-inflammatory mediator Il-6. Again, this is unlike the case of the non-pathogenic F. novicida, which induces higher levels of IL-6 in miR-155 expressing cells compared with miR-155-negative cells, supporting a pro-inflammatory role of miR-155 in Francisella infection. Our results also differ from the report of Kurowska-Stolarska et al. (2011) who show that miR-155 enhances IL-6 production from M-CSF-differentiated human macrophages. Our findings suggest that in the context of M.tb. infection in murine macrophages, miR-155 exerts the opposite effect.

Mir-155 has been reported to oscillate after TNF-$\alpha$ stimulation. These oscillations could result in positive or negative effects on target proteins which are important mediators of NF-$\kappa$B signalling, such as IKKe, or cellular death pathways, such as FADD. Mir-155 expression is regulated by multiple transcription factors. It is therefore important to elucidate the mechanism and the temporal pattern of miR-155 induction by M.tb. in order to have comprehensive knowledge about its role in infection. In addition, the effects of miR-155 cannot be viewed in isolation. A host of miRNAs most likely engage in orchestrated feedback loops to guide the fates of the pathogen and its host. These are subjects of ongoing investigations.

The central finding of the present study is that expression of miR-155, occurring early following infection of murine macrophages with M.tb., sets off a chain of events that favours establishment of infection. Work with other pathogens has shown that miR-155 expression is an early event in the innate immune response, and is set off by cell surface-exposed lipoglycans. Our results uncover hitherto unappreciated roles of miR-155 in dampening the innate immune response and describe for the first time that a secreted protein, ESAT-6, functions as a direct effector of miR-155 production.

### Experimental procedures

#### Antibodies and reagents

Beta-actin antibody, EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] and 1-methyl imidazole were obtained from Sigma Chemical Co., St. Louis, MO. Ultrahyb, miRNA isolation kit and RNA molecular weight markers were from Ambion. Antibodies specific for SHIP1 (Cat. No. 2728) and phosphorylated AKT (Cat. No. 9271) were from Cell Signaling Technology, Beverly, MA. Antibodies specific for AKT (sc-8312), Bach1 (sc-27121) and tubulin (sc-23948) were from Santa Cruz Biotechnology; anti-HO1 antibody was from Enzo Life Sciences, Ann Arbor, MI (ADI-OSA-150-D). DIG-labelled LNA-DNA mixed oligonucleotide probes against mature murine miRNA-155 and U6 RNA were obtained from Exiqon. M-CSF was a product of Prospec Protein Specialists, East Brunswick, NJ.

#### Cell culture and transfection

The murine macrophage cell line RAW264.7 was obtained from Dr Dipshikha Chakravortty, Indian Institute of Science, Bangalore and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM glutamine and penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO$_2$. BMDMs were prepared from C57BL/6 mice and cultured for 7–8 days in the presence of M-CSF to allow them to mature (Boone et al., 2004; Gómez-Muñoz et al., 2004).

Transfections were carried out using Lipofectamine 2000 (Invitrogen). Briefly, cells were seeded 24 h before transfection in 12-well plates at a density of 3 x 10$^5$ cells per well and the mmu-miR-155 inhibitor (Ambion, AM17010 assay ID 13058) or miRNA inhibitor negative control (Ambion, AM 17000) was transfected at a final concentration of 150 nM. For confirmation of the downregulation of miR-155, co-transfections of SHIP1 3′-UTR construct (gift from Prof. David Baltimore, California Institute of Technology, USA), β-galactosidase expressing construct and miRNA-155 inhibitor (or miRNA inhibitor negative control) were carried out. Cells were lysed after 48 h of transfection and luciferase activity was measured using a luciferase assay kit (Promega) according to the manufacturer’s protocol.
activity was normalized by measuring beta-galactosidase activity using beta-galactosidase assay kit (Promega).

**Bacterial strains**

*Mycobacterium tuberculosis* strain H37Rv, and the Δesat-6 mutant (H37Rv::Δesat-6) (both from the laboratory of Professor David Sherman, SBRI, Seattle) were grown in Middlebrook 7H9 broth (Difco) supplemented with 10% ADC (Becton Dickinson). Recombinant cosmid vector RD1-2F9 harbouring the RD1 locus of *M. tuberculosis* (Brodin et al., 2005) was a gift from Professor Stewart Cole of the École Polytechnique Fédérale de Lausanne (EPFL), Switzerland. *M. bovis* BCG (Copenhagen) was transformed with cosmid 2F9 as described by Chatterjee et al. (2011). *M. bovis* BCG Copenhagen and BCG:2F9 were grown in Middlebrook 7H9 (Difco) supplemented with 10% OADC (Becton Dickinson) and 40 mM sodium pyruvate. Briefly, 50 μg ml⁻¹ hygromycin was used for culturing H37Rv::Δesat-6, while 50 μg ml⁻¹ hygromycin and 100 μg ml⁻¹ ampicillin were used for culturing BCG-2F9.

**Infection**

Bacteria were grown up to mid-log phase and washed thoroughly prior to infection. Bacterial clumps were removed by passing the washed suspension through a 27-gauge syringe. Infections were carried out for 4 h at the indicated multiplicities of infection (moi). Cells were washed and treated with gentamicin for 2 h in order to remove adhered bacteria. Incubations in DMEM containing 10% FBS were carried out for 20 h post infection (unless otherwise stated) or as indicated.

**Bacterial cfu determinations**

For the quantification of mycobacterial growth within macrophages cells were lysed after infections and colony-forming units were calculated after plating serial dilutions on Middlebrook 7H11 agar supplemented with OADC, using standard procedures.

**Western blotting**

Cells were washed with cold phosphate-buffered saline and lysed in lysis buffer (Cell Signaling Technology). Proteins were separated by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) containing 1% Tween 20 (TBST) for 1 h at room temperature, washed and subsequently incubated overnight at 4°C with primary antibodies in TBST supplemented with 5% (w/v) bovine serum albumin or non-fat dry milk. Following three washes of 5 min each with TBST, the blots were incubated with horse radish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology or Cell Signaling Technology, Beverly, MA) followed by development with chemiluminescence detection reagent according to the manufacturer’s protocol (Cell Signalling Technology).

**RNA extraction**

RAW264.7 cells (6 × 10⁶ to 8 × 10⁶) were lysed in Qiazol lysis reagent (Qiagen). RNA was isolated using the mirVana miRNA isolation kit (Ambion). In some instances, the RT-qPCR-Grade miRNA isolation kit (SA Biosciences, Part No. 1033A) was used for isolating miRNA.

**Northern blotting**

The non-radioactive Northern blotting of miRNA was carried out as described by Kim et al. (2010). Briefly, total (or small RNA) was separated on 8 M urea-15% polyacrylamide gels and transferred to positively charged nylon membranes (Roche Applied Science). RNA was cross-linked by incubating the membrane in EDC cross-linking solution for 90 min at 60°C. For DIG-labelled LNA probes, pre-hybridization was carried out at 37°C in ULTRAhyb hybridization buffer (Ambion) for 1 h, followed by hybridization at 37°C for 16 h with slow rotation using 0.25 nM DIG-labelled LNA probe for miR-155 (Exiqon, Product No. 39471-15) (Pall and Hamilton, 2008). After washing the membranes, anti-DIG-AP Fab fragment (Roche Applied Science) was used for detection. After stripping the membranes, reprobing was carried out using DIG-labelled U6 probe (Exiqon, Product No. 99002-15).

Radioactive probes were prepared by phosphorylating 10 pmol of each DNA probe (Table S1) with 20 pmol of [γ-³²P]-ATP and T4 polynucleotide kinase (Fermentas) followed by purification using Quick Spin Columns (Roche Applied Bioscience). Pre-hybridization was performed in 6× SSC, 10× Denhardt’s solution and 0.2% SDS for 1 h at 65°C, followed by hybridization in 6× SSC, 5× Denhardt’s solution, 0.2% SDS containing 4 × 10⁵ cpm 5′-end labelled probe for 16 h at 37°C with slow rotation.

**Real-time quantification of RNA**

Global analysis of miRNA expression was performed using Taqman Low Density Array (TLDA) in technical replicates at Life Technologies, Gurgaon, India. Two independent experiments were carried out for this purpose. For the quantification of miR-155, cDNA was synthesized using the Taqman MicroRNA reverse transcription kit (Applied Biosystems) and specific reverse transcription primers for mmu-miR 155 and housekeeping small RNA snoRNA142 (Applied Biosystems). Real-time PCR was carried out using the Taqman universal PCR master mix (Applied Biosystems) in a 7500 Real-time PCR system (Applied Biosystems). The relative expression of the target gene normalized to the endogenous reference gene was quantified using the comparative Ct method. Alternatively, cDNA was synthesized from miRNAs using the RT-miRNA First Strand Kit (SABiosciences, Cat. No. MA-03) and miRNAs were assayed in 96-well plate format (SABiosciences, Cat. No. MAM-001-C2) according to the manufacturer’s instructions.

For quantification of gene expression, cDNA was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas) and SYBR Green-based real-time PCR was carried out using the primers provided in Table S2.

**Expression and purification of His-ESAT6**

*Escherichia coli* BL21 (DE3) transformed with pET 23b- carrying the ESAT6 gene was grown in LB medium containing 100 μg ml⁻¹ ampicillin. IPTG was added at a concentration of 0.25 mM when
the $A_{600}$ reached 0.6–0.7, and the cells were harvested after 4 h by centrifugation at 4°C. The cell pellet from 50 ml bacterial culture was resuspended in 3 ml Bugbluster HT (Novagen) supplemented with 0.5 mM EDTA and 100 $\mu$M Pefabloc (Roche Applied Science) and incubated on a shaking platform for 20 min at room temperature, followed by centrifugation at 16 000 g for 20 min at 4°C. The soluble fraction containing ESAT6 was loaded on a Ni²⁺-NTA-agarose column equilibrated in 50 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl (buffer A). After washing the column with 50 mM imidazole in buffer A, ESAT6 was eluted with 250 mM imidazole in buffer A. The purified protein was stored at –70°C.

**IL-6 analysis**

IL-6 was measured in the supernatants of infected cells using the IL-6 ELISA kit from Ray Biotech. All measurements were done at least in duplicate.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5 software. Data have been expressed as means ± SD. Student’s $t$-test was used when comparing two groups. Differences in values were considered significant for $P < 0.05$.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Expression of miR-21 and miR-146a in RAW264.7 infected with M.tb. H37Rv. RAW264.7 cells were left uninfected or infected with M.tb. H37Rv at a moi of 5 for 24 h. Bacteria were washed as described under ‘Methods’ and RNA was isolated. 32P-labelled probe specific for miR-21 or miR-146a was used for Northern blotting. As a loading control, the blots were reprobed with a probe specific for U6 RNA. Similar results were obtained in two independent experiments.

**Fig. S2.** Expression of miR-155 in RAW264.7 treated with ESAT-6. RAW264.7 cells were left untreated (−) or treated (+) with ESAT-6 (5 μg ml−1) for 6 h. Expression of miR-155 was measured by qRT-PCR. Data were normalized with respect to snoRNA142. Data represent mean ± SD of triplicates. *P < 0.05. Similar results were obtained in two independent experiments.

**Fig. S3.** Expression of miR-155 in RAW264.7 infected with *M. tuberculosis* H37Rv or Δesat-6. RAW264.7 cells were left uninfected or infected with the indicated strains at a moi of 5. Expression of miR-155 was measured by qRT-PCR performed in triplicate. Data were normalized with respect to snoRNA142. Data are expressed relative to the expression of miR-155 in H37Rv set at 100. *P < 0.05. 3 determinations.

**Fig. S4.** Expression of miR-155 in RAW264.7 infected with *M. bovis* BCG or BCG:2F9. RAW264.7 cells were left uninfected or infected with *M. bovis* BCG or BCG:2F9 at a moi of 5. Expression of miR-155 was measured by qRT-PCR performed in triplicate. Data were normalized with respect to snoRNA142. Relative miR-155 expression is shown with respect to the expression in uninfected cells (considered to be 1). Data represent mean ± SD of triplicates. *P < 0.05. Similar results were obtained in two independent experiments.

**Fig. S5.** M.tb.-induced phosphorylation of AKT. RAW264.7 cells were infected with M.tb. at different moi. Cells were lysed and immunoblotted with phospho-AKT antibody. The blot was reprobed with AKT antibody. The blot is representative of two independent experiments.

**Fig. S6.** 3′-SHIP1-UTR luciferase reporter assay and SHIP1 regulation in infected BMDMs. (A) Shown is an analysis of expression of luciferase reporter in the presence of control (filled bar) or miR-155 inhibitor (open bar). Luciferase expression in the presence of the control inhibitor is set at 100. (B) Cells were transfected with either control inhibitor (ctrl) or miR-155 inhibitor (miR-155) and then infected with M.tb. Cell lysates were immunoblotted with SHIP1 antibody, and blots were reprobed with beta actin antibody. The fold change in SHIP1 was calculated with respect to infected cells transfected with control inhibitor. Data are representative of the results obtained in at least two independent experiments.

**Fig. S7.** HO-1 expression in infected RAW264.7. Cells were left uninfected or infected with the indicated strains at a moi of 5 for 20 h. Expression of HO-1 was analysed in the lysates by Western blotting. Data represent the results obtained in two independent experiments.

**Fig. S8.** Bach1, hmox1 and HO-1 expression in RAW264.7 cells transfected with miR-155 inhibitor and treated with ESAT-6. RAW264.7 cells were transfected with either control inhibitor (ctrl) or miR-155 inhibitor (miR-155). After 20 h, transfected cells were treated with ESAT-6 (5 μg ml−1) for 6 h. RNA was isolated and expression of Bach1 (A) or hmox1 (B) was assessed by qRT-PCR using Gapdh expression for normalization. Data are shown as mean ± SD of three separate determinations. *P < 0.01. (C) Cells were transfected with control inhibitor (ctrl) or miR-155 inhibitor (miR-155). Transfected cells were treated with ESAT-6 for 6 h followed by immunoblotting with HO-1 antibody. Blots were reprobed with actin antibody. The fold change in HO-1 expression is shown with respect to cells transfected with control inhibitor (ctrl).

**Fig. S9.** Role of miR-155 in IL-6 release by M.tb.-infected RAW264.7. RAW264.7 cells were transfected with either control inhibitor (ctrl) or miR-155 inhibitor (miR-155). Transfected cells were infected with M.tb. H37Rv and IL-6 was measured in the supernatant 20 h post infection. *P < 0.001.

**Table S1.** Probes for Northern blotting.

**Table S2.** Primers for qRT-PCR.

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