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Host induced microRNA hsa-miR-643 promotes Mycobacterium tuberculosis survival inside macrophages by downregulating Gins2

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Abstract

Survival of *Mycobacterium tuberculosis* (Mtb) within macrophages is vital for the tuberculosis pathogenesis. This is facilitated by a battery of Mtb genes whose expressions appeared deregulated after macrophage infection. While Mtb's adaptive response is well-documented, the role of host non-coding RNAs, particularly microRNAs (miRNAs), remains less clear. Building on our previous finding that hsa-miR-643 is significantly upregulated in Mtb-infected THP-1 human macrophages, this study investigates its functional impact. Comparative whole-genome transcriptome analysis revealed that hsa-miR-643 mimics consistently downregulated the expression of *Gins2*, which was validated through qRT-PCR and western blot analysis. Luciferase reporter assays confirmed *Gins2* as a direct target of hsa-miR-643 via its 3'-UTR. Notably, inhibiting hsa-miR-643 promoted apoptosis in THP-1 cells, leading to more efficient clearance of intracellular Mtb. Our results indicate that hsa-miR-643 facilitates Mtb survival by suppressing *Gins2*. Consequently, hsa-miR-643 emerges as a promising therapeutic target to limit mycobacterial progression and combat tuberculosis infection.

Introduction

Despite being curable, tuberculosis (TB) remains a global health crisis, driven by prolonged treatment cycles and the rise of multi-drug resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* (Mtb) strains. These strains are difficult to treat with prevailing regimen of drugs necessitating further research for effective management of the drug resistant TB. As an intracellular pathogen, Mtb primarily targets the lungs, where it is internalized by alveolar macrophages. The ability of pathogenic mycobacterium to adapt to hostile macrophage environment is key to its success as a pathogen. Rather than being cleared, the bacilli subvert host defenses by arresting phagosome maturation and thereby creating a protected niche for replication [1]. While many bacterial genes linked to intracellular survival of the pathogen have been identified [2], the role of host factors, specifically non-coding RNAs, in this interplay is less understood. MicroRNAs (miRNAs) are conserved non-coding RNAs that function as post-transcriptional regulators inhibiting the translation of their target mRNAs [3]. Varying expressions of miRNAs have been found in a broad spectrum of human diseases [4–6]. Recently, miRNAs have been shown to play important roles in successful microbial infections as well as in viral and parasitic infections [7]. During the course of infection bacteria tend to manipulate the host cell by reorganizing cytoskeleton, membrane trafficking, inflammatory response and signal transduction pathways. Despite the stated relevance of miRNAs in the host-pathogen interplay little is known about the role of miRNAs in the survival of *M. tuberculosis* inside host macrophages.

Previously, we identified several miRNAs significantly upregulated in Mtb infected THP-1 macrophages, with hsa-miR-643 showing the highest induction (GSE94007) [8]. This study aims to characterize the targets of hsa-miR-643 and define its role in progression of *M. tuberculosis* inside macrophages. Our findings suggest that hsa-miR-643 promotes the intracellular survival of *M. tuberculosis* by downregulating *Gins2*.

Materials and Methods

Ethics statement.

All experiments were performed as per institutional safety guidelines.

Bacterial strains and culture

M. tuberculosis H37Rv and *M. tuberculosis* H37Ra strains were obtained from departmental stock and cultured in Middlebrook 7H9 liquid culture supplemented with oleic acid albumin dextrose catalase (OADC). Middlebrook 7H11 agar plates supplemented with 10% OADC, 0.05% glycerol were used for solid surface growth. *Escherichia coli* strains were cultured in Luria Bertani (LB) broth and spread on LB agar plate and cultures were incubated at 37°C.

Cell culture and infection

THP-1 cells were cultured in a 6 well culture plate at 37°C with 5% CO2 in RPMI-1640 supplemented with 10% foetal bovine serum (FBS). Cells were treated with 30 ng/ml PMA for 48 hours in culture media to promote adherence and differentiation. For infection the mycobacterial culture (0.6 OD_{600}), dislodged into single cell suspension, were introduced into differentiated THP-1 cell at 10:1 multiplicity of infection (MOI), incubated for 4 hr, washed 5 times 5 min each and incubated in complete RPMI-1640 media with amikacin (200 µg/ml) for 1 hr to kill extracellular mycobacteria. Further, cells were harvested after 24-48 hr.

Total mRNAs profiling of *M. tuberculosis* infected and hsa-miR-643 mimic transfected THP-1 differentiated macrophages using RNA seq

Total RNA was isolated from Mtb infected and hsa-miR-643 mimics transfected THP-1 differentiated macrophages after 24 hr treatment using RNeasy kit (Qiagene India) as described earlier [9]. RNA was quantified by Nano Drop spectrophotometer and used for comparative transcriptome analysis. Details of this experiment may be obtained from data submitted to NCBI GEO repository (Accession number GSE275580).

Real-time Quantitative polymerase chain reaction (qRT-PCR)

Real-time qRT-PCR was performed using total RNAs from THP-1 differentiated macrophages treated with Mtb, hsa-miR-643 mimics and hsa-miR-643 inhibitors. Total RNA was isolated as described above. 1µg of DNase treated RNA was reverse transcribed to cDNA using manufacturer's protocol. The qRT-PCR was performed using *Gins2* gene specific primers (Forward: 5'-AGCCAAACTCCGAGTGTCT-3' and Reverse: 5'-AGCGCTTGTGTGAGGAAAG-3') using SYBR green PCR master mix (Bio-Rad, India) on StepOnePlus PCR system. Expression levels were normalised by GAPDH primer (Forward 5'-ACCACAGTCCATGCCATCACTG-3', Reverse 5'-TGCTTCACCACCTTCTTGATGT -3') and fold change in

gene expression between treated and untreated sample were calculated using comparative $\Delta\Delta^{Ct}$ method. Experiments were repeated twice and each reaction was set up in triplicate.

Western blot analysis

THP-1 cells were treated for 24 hr with *M. tuberculosis*, hsa-miR-643 mimic and hsa-miR-643 inhibitor and total proteins were extracted as described earlier [10]. Proteins were separated using SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membrane. Membrane was blocked and incubated overnight with primary antibodies, anti *GINS2* (Cat # YI4050884, Invitrogen), anti -*β*-Actin (Cat # GX-5111M, Santa Cruze Biotechnology INC. USA). Next day, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies.The blots were developed using the chemiluminescent substrate (Invitrogen) and the signals were captured on the Chemidoc system (Image Quant LAS 500, GE healthcare). Band intensities were quantified densitometrically using the ImageJ software.

Transient transfection of hsa-miR-643 mimics and inhibitors and growth analysis

hsa-miR-643 mimics and inhibitors were procured from Qiagene, India (Cat # 339173 and Cat # 339121). *Gins2* siRNA (Cat # E-020418-00-0005) and non-specific siRNA (Cat # D-001910-01-05) were procured from Dhermacon, USA. Total 50 pmol mimics and 150 pmol inhibitors per 3×10^6 THP-1 cells were used for transfection of selected miRNA using Lipofectamine 3000 (Invitrogen, USA) as per manufacturer's instructions. THP-1 cells were transfected with pmirGLO vector (Promega, USA), mimics, inhibitors, *Gins2* siRNA and non-specific siRNA as and when needed. *M. tuberculosis* infection was given to pre-transfected THP-1 cells when required. Cells were lysed after 24 hr of infection for RNA and protein analysis. For CFU enumeration, *M. tuberculosis* infected THP-1 cells were lysed, bacilli pelleted, diluted with 7H9 medium and then seeded on 7H11 agar plates for enumeration of bacterial colonies after 3 weeks.

Cloning of *Gins2* 3′-UTR and luciferase assay

Human *Gins2* gene 1.9 kb 3′-UTR region was retrieved from online database (UCSC). Complete 3′-UTR was amplified using *GINS2* 3′-UTR primers (Forward: 5′-AAGCTAGCAGAAAGGCCTGGTGC -3′, Reverse: 5′-ATCTCGAGTCTCCGCAGACGGA-3′). The amplicon was cloned at Nhe1-Xho1 restriction site downstream to the firefly luciferase reporter gene into pmiGLO Dual-Luciferase miRNA Target Expression Vector (Promega USA) to create pmirGLO:*Gins2* 3′-UTR (Fig. 2D). pmirGLO:*Gins2* 3-UTRΔSS was created by deleting the seed sequence region of *Gins2* 3′-UTR by using STY1 restriction digestion. Luciferase assay was performed using Promega's Dual-Glo Luciferase Assay System (Promega, USA) as per kit's protocol. Normalized firefly luciferase activity (Firefly luciferase activity/Renilla luciferase activity) for each construct was compared to that of the pmirGLO vector control without insert. For each transfection, luciferase activity was averaged from three replicates.

Flow cytometry and Annexin V/propidium iodide staining

THP-1 cells were seeded in 6-wells plate (1×10^6cells/well) and transfected with hsa-miR-643 inhibitor and M. tuberculosis. After 24 hr of treatment, adherent cells were washed with ice-cold phosphate buffer saline and trypsinized (0.05% trypsin) to detach cells. Annexin V/propidium iodide staining was performed as per kit's protocol (Cat # 556419, Invitrogen).

Statistical analysis

Data were collected from three independent experiments. Experiments were set up in duplicates at each point. The mean values and standard deviations were plotted for each data set using GraphPad Prism Version 8.0.2.

Results

Transcriptome analysis of Mtb infected and hsa-miR-643 treated THP-1 cells

PMA differentiated THP-1 cells were separately treated with Mtb and hsa-miR-643 mimics and harvested after 24 hrs for mRNA profiling as described in the methods. Transcriptome data revealed deregulation of several genes in comparison to the untreated control. Expression analysis of genes was done by using iDEP.96 online software [21]. Data were analysed at FDR (false discovery rate) cutoff of 0.05 and at minimum log fold change (FC) cutoff of 2. Typically, FDR of 0.05 means that the expected proportion of false positives among differentially expressed genes is 5%. A comparison of data from three groups identified 800 differentially expressed genes using a threshold of log2 fold-change > 1.8 (upregulated) or < -1.8 (downregulated) for inclusion in this study. Genes that were found to be down regulated could be the potential targets of the hsa-miR-643. A large number of genes were found to be downregulated in the mimics-treated sample compared to the infected sample. Comparisons of differentially regulated genes were drawn as follow: **a**- differentially regulated THP-1 genes after infection with Mtb, **b**- differentially regulated THP-1 genes after transfection with hsa-miR-643 mimics, **c**- commonly differentially regulated THP-1 genes after infection with Mtb and transfection with hsa-miR-643 mimics. Commonly downregulated genes in both samples were included in this study. A total of 113 target genes were found to be downregulated and 362 genes to be upregulated in the infected and transfected samples. Selected few commonly downregulated genes are enlisted in the table (Table 1) and among these, *Gins2* was selected for further study, as it was previously identified as a target of immune response modulators in macrophages [7]. Further, several selected genes were independently validated to reaffirm RNA sequencing results (Fig. 1). Details of transcriptome data are available online (GEO accession number GSE275580).

hsa-miR-643 downregulates Gins2 expression

Gins2 was examined as hsa-miR-643 target and it was hypothesised that the enhanced expression of hsa-miR-643 likely downregulate the expression of *Gins2* in THP-1 cells after Mtb infection. Quantitative

RT-PCR and western blot analysis confirmed that *Gins2* expression significantly decreased in THP-1 cells treated with Mtb or hsa-miR-643 mimics (Fig. 2). Conversely, treating cells with an hsa-miR-643 inhibitor led to a significant increase in *Gins2* mRNA and protein levels (Fig. 2). Notably, no reduction in *Gins2* was observed when cells were infected with the non-pathogenic H37Ra strain, indicating that *Gins2* downregulation is specifically linked to hsa-miR-643 induction during pathogenic Mtb infection.

hsa-miR-643 binds to the Gins2 3'-UTR region

Using STarMir software (http://sfold.wadsworth.org/starmir.html), a specific binding site for hsa-miR-643 was predicted within the 3'-UTR of *Gins2* (Fig. 3A) [12]. To validate this, a 1.9kb fragment of the *Gins2* 3'-UTR was cloned into a pmirGLO dual-luciferase vector to create pmirGLO:*Gins2* 3'-UTR (Fig. 3B). The THP-1 cells were transfected with the pmirGLO:*Gins2* 3'-UTR vector and subsequently challenged with hsa-miR-643 mimics to record the ensuing luciferase activity. Transfection with hsa-miR-643 mimics significantly reduced luciferase activity, whereas a mutant construct lacking the miRNA seed sequence (pmirGLO:*Gins2* 3'-UTRΔSS) restored activity to basal levels (Fig. 3D). These results confirm that hsa-miR-643 directly regulates *Gins2* by binding to its 3'-UTR.

hsa-miR-643 inhibition promotes THP-1 apoptosis and reduces intracellular Mtb survival

Because apoptosis is a key host mechanism for killing intracellular mycobacteria, and hsa-miR-643 is a known regulator of the cell cycle and programmed death [13, 14], we evaluated the effect of an hsa-miR-643 inhibitor on infected THP-1 cells. Flow cytometric analysis revealed that hsa-miR-643 inhibition markedly increased the apoptotic population to 15%, compared to 3.09% in uninfected controls and 5.52% in Mtb-infected cells. When hsa-miR-643-inhibited cells were subsequently infected with Mtb, apoptosis further increased to 20% (Fig. 4A). This suggests that the inhibitor antagonizes Mtb-induced hsa-miR-643 expression, thereby augmenting *Gins2* levels, a result corroborated by western blot analysis (Fig. 2C). We further assessed the impact of this pathway on bacterial viability using Colony Forming Unit (CFU) assays. At 24 hours post-infection, THP-1 cells treated with the hsa-miR-643 inhibitor exhibited a significant decline in bacterial load compared to untreated cells (Fig. 4C). Conversely, silencing *Gins2* with siRNA resulted in an increased bacterial burden (Fig. 4C). Collectively, these data demonstrate that hsa-miR-643 facilitates Mtb survival by suppressing *Gins2* and inhibiting host cell apoptosis.

Discussion

This study highlights a novel mechanism by which *Mycobacterium tuberculosis* manipulates host cell signaling to ensure its survival within macrophages. While miRNAs are known to influence microbial infections [15–17], their specific roles in the Mtb intracellular persistence remain largely undefined [18]. Our findings establish a critical regulatory axis involving hsa-miR-643 and its target, *Gins2*, in the suppression of host cell apoptosis. Previous research identified hsa-miR-643 as one of the most

significantly upregulated miRNAs during Mtb infection [8]. Through a comparative transcriptome analysis (GEO: GSE275580) of Mtb-infected and hsa-miR-643-transfected THP-1 cells, we identified *Gins2* as a potential target. Using dual-luciferase assays and western blotting, we confirmed that hsa-miR-643 directly binds to the 3'-UTR of *Gins2*, negatively regulating its expression (Figs. 2 & 3). A hallmark of virulent Mtb (H37Rv) is its ability to evade macrophage apoptosis, a process that would otherwise lead to bacterial clearance [11, 20]. In contrast, avirulent strains like H37Ra induce significantly higher levels of apoptosis. Our data suggest that *Gins2* levels positively correlate with this apoptotic response. Notably, inhibition of hsa-miR-643 restored *Gins2* levels and increased the apoptotic population of infected THP-1 cells to 20% (from 5.52% in Mtb only samples). CFU analysis demonstrated that inhibiting hsa-miR-643 significantly limits Mtb survival, whereas *Gins2* knockdown via siRNA facilitated bacterial survival (Fig. 4). While *Gins2* is traditionally recognized for its role in the eukaryotic GINS complex and DNA replication [19], and is often implicated in tumor progression [22], this study is the first to report its involvement in the host-pathogen interface of tuberculosis. By downregulating *Gins2*, hsa-miR-643 effectively attenuates host apoptosis, providing a conducive niche for Mtb replication. Although hsa-miR-643 likely targets other genes (such as CYP11B1 and APOL6 [12, 13]) that contribute to mycobacterial progression, the hsa-miR-643/ *Gins2* pathway represents a significant survival strategy. Consequently, targeting hsa-miR-643 may offer a promising therapeutic avenue to enhance host-mediated clearance of Mtb.

Beyond DNA replication, *Gins2* specifically influences cell fate [22]. In many contexts, high *Gins2* levels promote proliferation and inhibit apoptosis, whereas its downregulation, as seen when targeted by hsa-miR-643, leads to cell cycle arrest and triggers programmed cell death. The discovery that *M. tuberculosis* exploits the hsa-miR-643/*Gins2* axis to avoid host-mediated killing opens several clinical avenues. Unlike traditional antibiotics that target the bacteria, using an hsa-miR-643 inhibitor could serve as a host-directed therapy (HDT). This makes it a highly promising strategy to clear intracellular pathogens, such as, *M. tuberculosis*, without causing the bacteria to develop antibiotic resistance. By blocking this miRNA, *Gins2* levels remain high, restoring the macrophage's ability to undergo apoptosis and naturally clear the infection. hsa-miR-643 levels could be developed as a biomarker to distinguish active TB from latent infections or to monitor a patient's response to treatment. Restoring host cell defense mechanisms via miRNA modulation could work synergistically with standard anti-TB drugs, potentially shortening treatment duration or helping to combat multidrug-resistant (MDR) strains.

In conclusion, we have shown that hsa-miR-643 downregulates *Gins2*, which in turn attenuates the host cell apoptosis and facilitates the *M. tuberculosis* survival inside macrophages. However, since hsa-miR-643 targets many genes the role of other gene(s) facilitating mycobacterial progression inside macrophages cannot be ruled out and is subject for larger study plan. Since *Gins2* is also an established oncogene in various cancers (e.g., lung, breast, leukemia), drugs or inhibitors developed for oncology targeting this axis might be repurposed or adapted for infectious disease management.

Declarations

Conflict of interest:

Authors declare no conflict of interest.

Funding:

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Author Contribution

Garima: performed most experiments, data analysis, writing first draft. Ruchi, Rajendra and Gunjan: assisted in cloning and expression analysis. Mustkim: data analysis. Bhupendra: Conceptualization of the project, funding, writing, review and overall Project management and supervision.

Acknowledgment:

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Data Availability

Ttranscriptome data were generated from this study and is available online (GEO accession number GSE275580).

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Table

Table No.1 Commonly downregulated genes in THP-1 cells after infection with Mtb and transfection with hsa-miR-643 mimics.

S. No	Commonly downregulated genes	Fold changes
1.	FCGBP	-1.93
2.	GINS2	-1.82
3.	CX3CR1	-1.73
4.	SLC29A1	-1.66
5.	CCL21	-1.22
6.	GPR34	-1.22
7.	GSTA4	-0.92
8.	PAK7	-0.85
9.	SYT-7	-0.65
10.	MRVI1-IP-3	-0.65

Figures

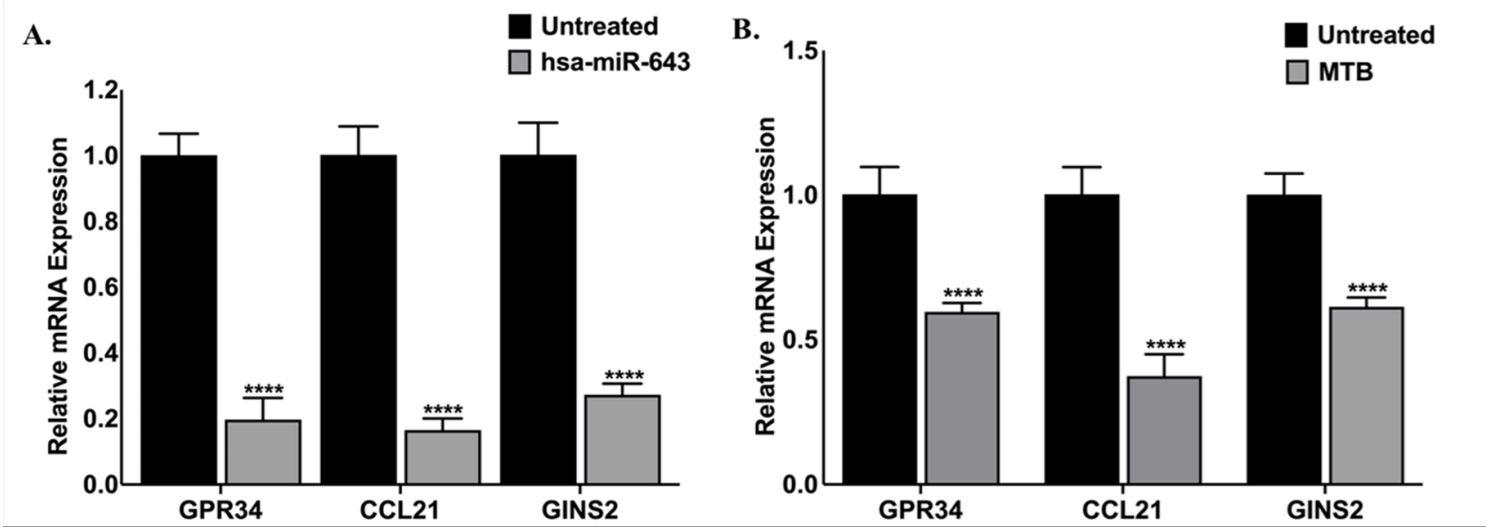


Figure 1

qPCR validation of putative target gene downregulation. Relative expression levels of target genes were quantified in THP-1 macrophages. **(A)** Untreated cells compared to cells transfected with hsa-miR-643 mimic. **(B)** Untreated cells compared to *Mycobacterium tuberculosis* (MTB)-infected cells. Data represent mean ± SD from two independent experiments performed in triplicate. Statistical significance was determined using Sidak's multiple comparisons test (∗∗∗∗P< 0.0001).

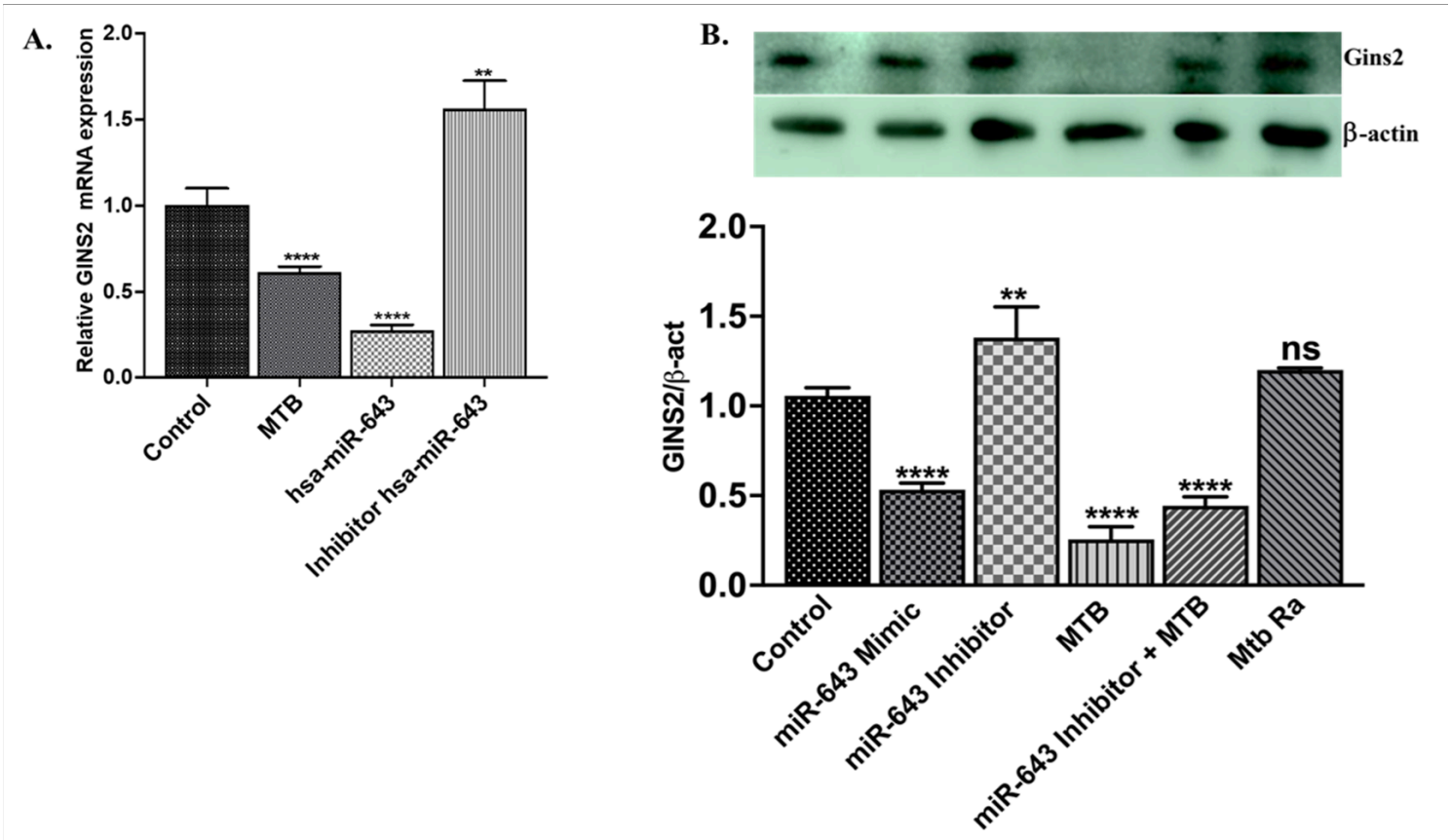


Figure 2

Regulation of *Gins2* expression by MTB and has-miR-643 in THP-1 cells. (A) Relative *Gins2* mRNA expression following treatment with MTB and hsa-miR-643 mimics. Significance was calculated by Sidak's multiple comparisons test (∗∗P < 0.01 and ∗∗∗∗ P < 0.0001). **(B)** Western blot analysis of GINS2 protein levels after treatment with Mtb H37Ra, Mtb, miR-643 mimics, or miR-643 inhibitor. Data show mean ± SD from two independent experiments, with normalization to β-Actin. Significance was calculated by Tukey's multiple comparisons test (∗∗P < 0.01 and ∗∗∗∗ P < 0.0001).

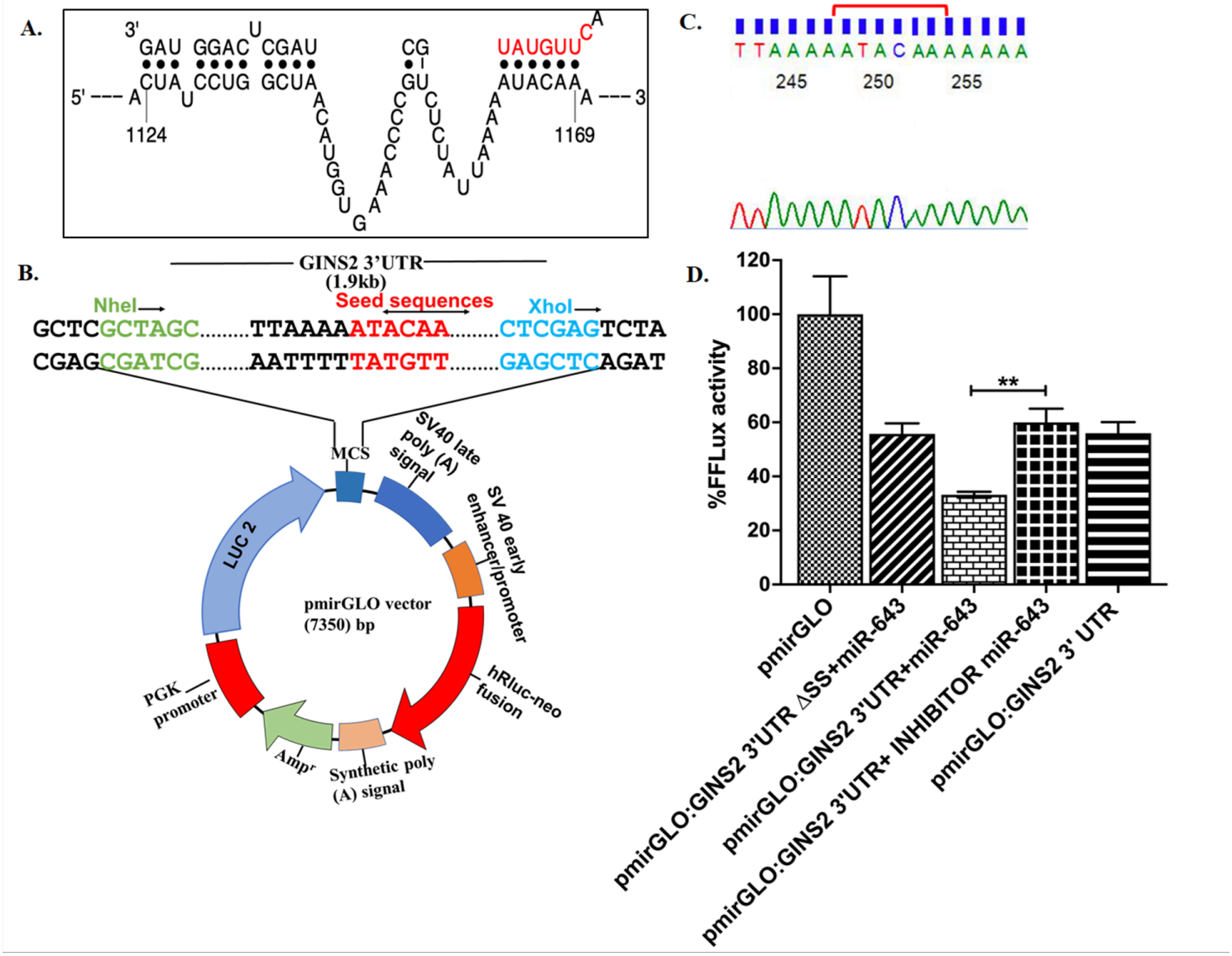


Figure 3

Validation of *Gins2* as a direct target of hsa-miR-643. (A) In silico prediction of the hsa-miR-643 binding site (red) within the Gins2 3'-UTR. **(B)** Schematic diagram of the pmirGLO:Gins2 3'-UTR reporter vector with the critical seed sequence highlighted in red. **(C)** DNA sequencing chromatogram confirming successful cloning of the wild-type Gins2 3'-UTR seed sequence into the pmirGLO vector. **(D)** Dual-luciferase reporter assay in THP-1 cells. Cells were co-transfected with indicated pmirGLO constructs (wild-type or seed-sequence deleted; ΔSS) alongside hsa-miR-643 mimics or inhibitors. Relative firefly

luciferase activity was normalized to Renilla luciferase activity, with the empty pmirGLO vector control set at 100%. Data represent mean ± SD from two independent experiments performed in triplicate. ∗∗P < 0.01 by Tukey's multiple comparisons test.

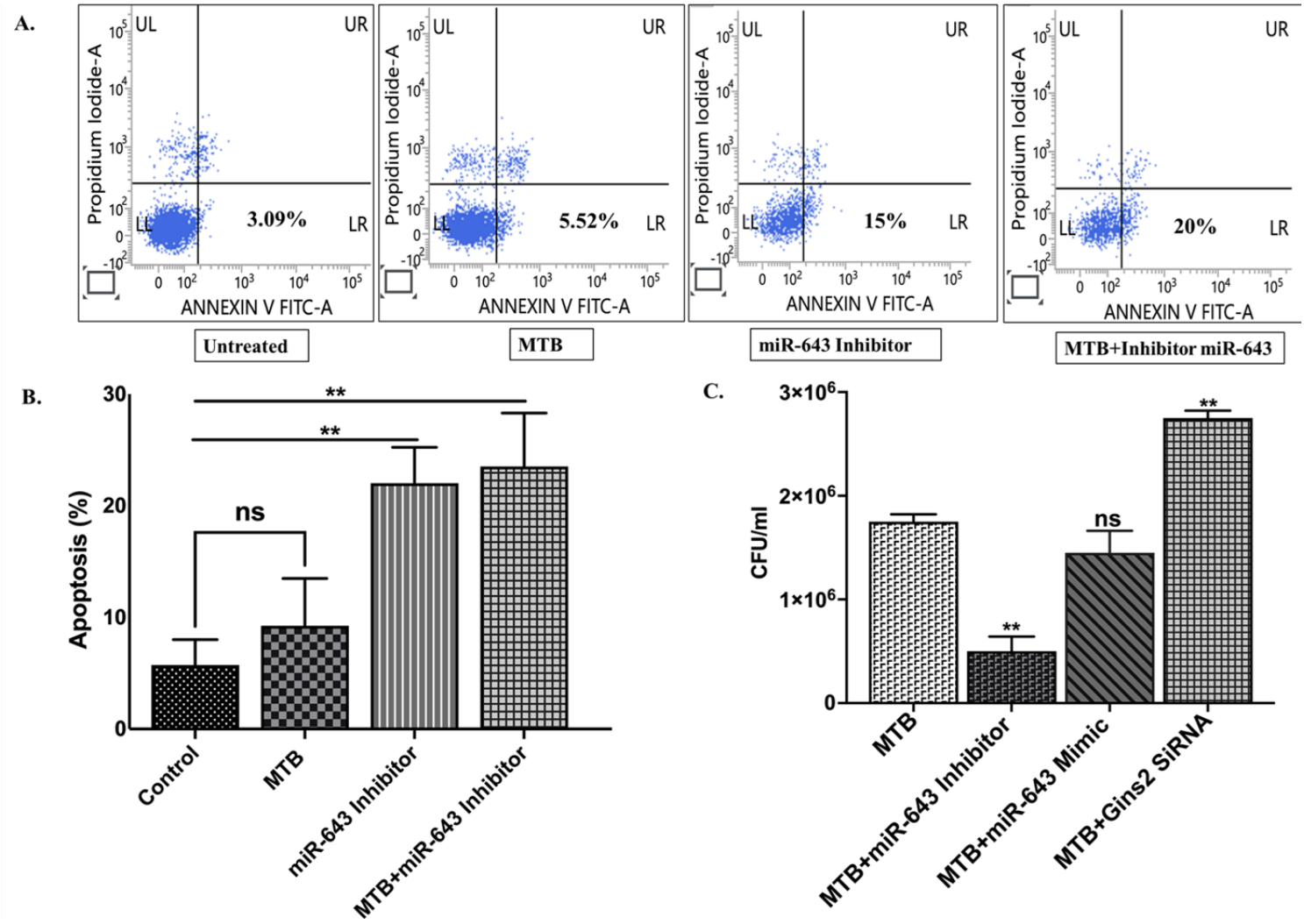


Figure 4

Modulation of THP-1 cell apoptosis and intracellular *M. tuberculosis* (Mtb) survival by has-miR-643 and *Gins2* inhibition. (A) Flow cytometry (FACS) analysis of THP-1 cell apoptosis using Annexin V-FITC/PI staining. Quadrants represent viable cells (lower left; Annexin V^-/PI^-), early apoptotic cells (lower right; Annexin V^+/PI^-), and dead/late apoptotic cells (upper right; Annexin V^+/PI^+). Representative plots are shown from two independent experiments. **(B)** Quantification of THP-1 cell apoptosis from three independent replicates. **(C)** Intracellular survival of Mtb in THP-1 cells. Macrophages were transfected with hsa-miR-643 inhibitor or Gins2 siRNA, infected with Mtb for 24 h, and bacterial load was quantified by Colony Forming Unit (CFU) enumeration. Data were obtained from two separate cultures performed in triplicates. Statistical significance in **(B)** and **(C)** was determined using Dunnett's multiple comparisons test. Data are presented as mean ± SD; ** $P < 0.01$ compared to the untreated control.