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LncRNA SNHG16 Inhibits Intracellular *M. tuberculosis* Growth Involving Cathelicidin Pathway, Autophagy, and Effector Cytokines Production

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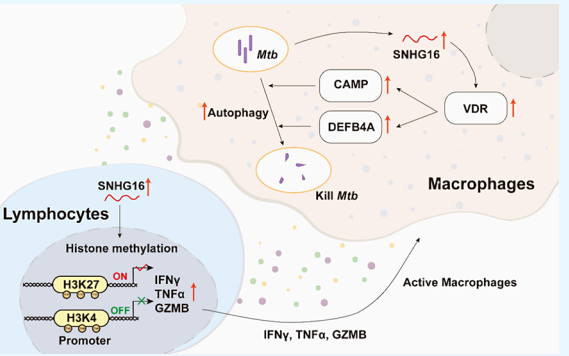
ABSTRACT: Long noncoding small nucleolar RNA (LncRNA) host gene 16 (SNHG16) is associated with certain diseases, including cancers. However, its role and mechanism in *Mycobacterium tuberculosis* (*Mtb*) infection remain unclear. Here, we demonstrated that SNHG16 expression levels were suppressed in peripheral blood mononuclear cells (PBMCs) and $CD14^+$ monocytes of tuberculosis (TB) patients. SNHG16 was up-regulated by acute *Mtb* infection of PBMCs from healthy control (HC) subjects. Such TB suppression of SNHG16 was consistent with an immunosuppressive-like state driven by IL-10 signaling as seen in TB patients. Notably, SNHG16 limited *Mtb* growth in macrophages/monocytes through autophagy and vitamin D receptor (VDR)-dependent cathelicidin (CAMP) antimicrobial pathways. Concurrently, SNHG16 was highly expressed in lymphocytes, including $CD8^+$ and $V\gamma2\ V\delta2$ T-cell subsets in HCs. SNHG16 overexpression in lymphocytes allowed them to control *Mtb* infection in macrophages, and SNHG16 epigenetically increased the expression of anti-*Mtb* effector cytokines in lymphocytes by developing more accessible chromatin states in gene loci encoding IFN-γ, TNF-α, and Granzyme B. Furthermore, the adoptive transfer of SNHG16-overexpressing human PBMCs into *Mtb*-infected SCID mice conferred protective immunity against *Mtb* infection. Thus, SNHG16 drove the induction of pleiotropic effector functions that inhibited intracellular *Mtb* growth in vitro and in vivo, serving as an immunotherapy target in TB.



INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) has been the top killer of single-pathogen infection after the COVID-19 pandemic.[1] Every year, about 10 million people fall ill with TB and 1.5 million people die from TB.[1] In fact, TB is the leading cause of death in HIV-infected persons, and drug-resistant TB accounts for major antimicrobial resistance worldwide.[1] Following aerosol exposure to pathogens, host immune factors are decisive for potential clinical outcomes of active TB, latent TB infection (LTBI), or resisters that exhibit early clearance of the bacillus without an immune signature of infection.[2,3] Thus, clearly characterizing the immune profiles in *Mtb* infection will help identify novel diagnostic markers or develop an effective immunotherapy strategy.

Long noncoding RNAs (LncRNAs) participate in virtually all levels of genome organization, cell structure, and gene expression, regulating the pathogenesis of many diseases.[4,5] Long noncoding small nuclear RNA host genes (Lnc-SNHGs) SNHG16 (small nucleolar RNA host gene 16) is widely associated with human diseases including neuroblastoma and cancers.[6−8] SNHG16 is differently expressed in cancer cells and adjacent normal tissues as well as in the blood of patients and healthy subjects. LncRNA and SNHG16 appear to serve as biomarkers for the diagnosis and prognosis of some cancers including head and neck squamous cell carcinoma,[9] colon adenocarcinoma,[10] and metastatic renal cell carcinoma.[11] Moreover, SNHG16 is also confirmed to be correlated with many autoimmune diseases, such as systemic lupus erythematosus and[12] diabetic retinopathy.[11]

Recently, SNHG16 was identified in exosomes (EV) of body fluids.[13] It was reported that the EV-mediated SNHG16 promotes lymphangiogenesis and lymph node (LN) metastasis in bladder cancers (BCa) in a small ubiquitin-like modifier (SUMO) binding (termed SUMOylation)-dependent manner, implicating SNHG16 as an attractive therapeutic target for LN metastatic BCa.[6] Although SNHG16 has been reported to be involved in a variety of diseases, the role and mechanism of SNHG16 in *Mtb* infection remain largely unexplored.

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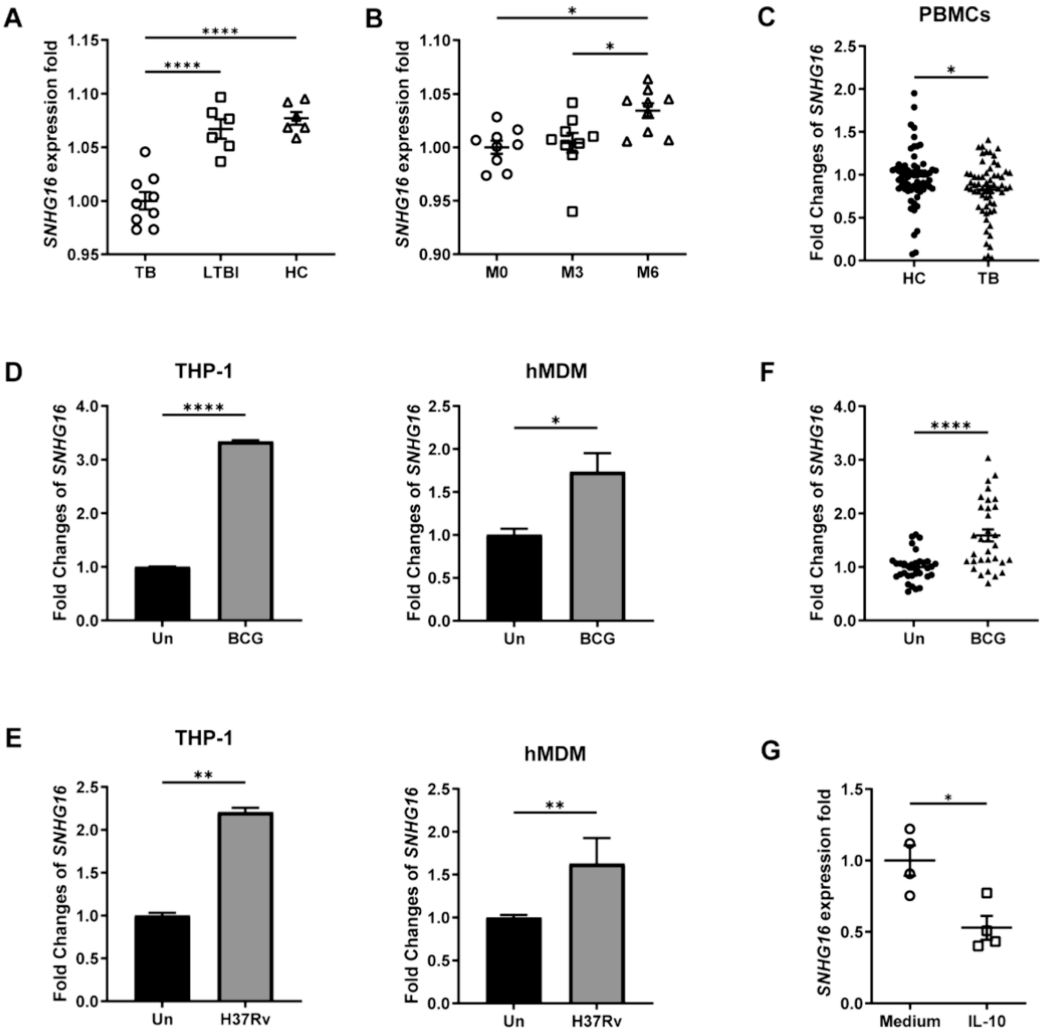

Figure 1. SNHG16 expression was related with *Mtb* infection. (A) Expression levels of SNHG16 in PBMCs were analyzed by the GEO2R platform (GDS4966) in TB patients, LTBIs, and HC subjects. SNHG16 expression levels were normalized to the fold-change of the mean expression level of TB. (B) SNHG16 expression profiles were shown in PBMCs of TB patients at different treatment time courses (platform GPL570) of 0 month (M0), third month (M3), and sixth month (M6). SNHG16 expression levels were normalized to the fold-change of the mean expression level of M0. (C) Expression levels of *SNHG16* in PBMCs isolated from HCs ($n = 60$) and TB patients ($n = 61$) were determined by the q-PCR. (D,E) Expression levels of *SNHG16* were shown in THP-1 and hMDM cells under BCG (D) or H37Rv (E) stimulation (MOI = 10), respectively. (F) Expression levels of *SNHG16* were determined by the q-PCR in PBMCs cells of HCs ($n = 34$) stimulated by BCG (MOI = 1) in vitro. (G) SNHG16 expression levels were analyzed by the GEO2R platform (GDS4551) in human PBMCs cells with IL-10 treatment. SNHG16 expression levels were normalized to the fold-change of the mean expression level of the medium. The results are expressed as the mean ± the SEM $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; data represent 3 independent experiments.

In the current study, we showed that SNHG16 drove the induction of pleiotropic effector functions of immune responses, which led to the inhibition of intracellular *Mtb* growth. Mechanistically, SNHG16 regulated VDR-dependent cathelicidin (CAMP) pathways and autophagy to control *Mtb* growth in macrophages/monocytes. Additionally, SNHG16 could enhance the ability of lymphocytes to limit *Mtb* infection in macrophages, and such SNHG16 action was linked to increases in effector cytokines' production in lymphocytes through inducing more accessible chromatin states in gene loci encoding IFN-γ, TNF-α, and Granzyme B. Furthermore, adoptive transferring lymphocytes with SNHG16 overexpression led to a decrease in *Mtb* infection in mice.

RESULTS

SNHG16 Expression was Up-Regulated in Acute *Mtb* Infection but Suppressed in Chronic TB. In an initial effort to assess the role of SNHG16 in TB infection, we compared the expression profiles of SNHG16 in peripheral blood lymphocytes from TB patients, latent TB infections (LTBIs), and healthy controls (HCs) including Chinese cohort microarray data sets using the NCBI GEO2R platform (https://www.ncbi.nlm.nih.gov/geo/).[14] The analysis revealed significantly higher expression levels of SNHG16 in HCs or LTBIs than in TB patients (platform GDS4966, Figure 1A). Moreover, SNHG16 expression levels were significantly higher in TB patients who had been treated for 6 months with anti-TB chemotherapy compared to those treated for 0 or 3 months (platform GPL570, Figure 1B).[14] Here, we also enrolled cohorts of TB patients and HC subjects, collected blood, and measured SNHG16 expression levels in peripheral blood mononuclear cells (PBMCs) using quantitative PCR (q-PCR) methods. Our results showed that chronic TB patients exhibited significantly decreased levels of SNHG16 expression in PBMCs than HC

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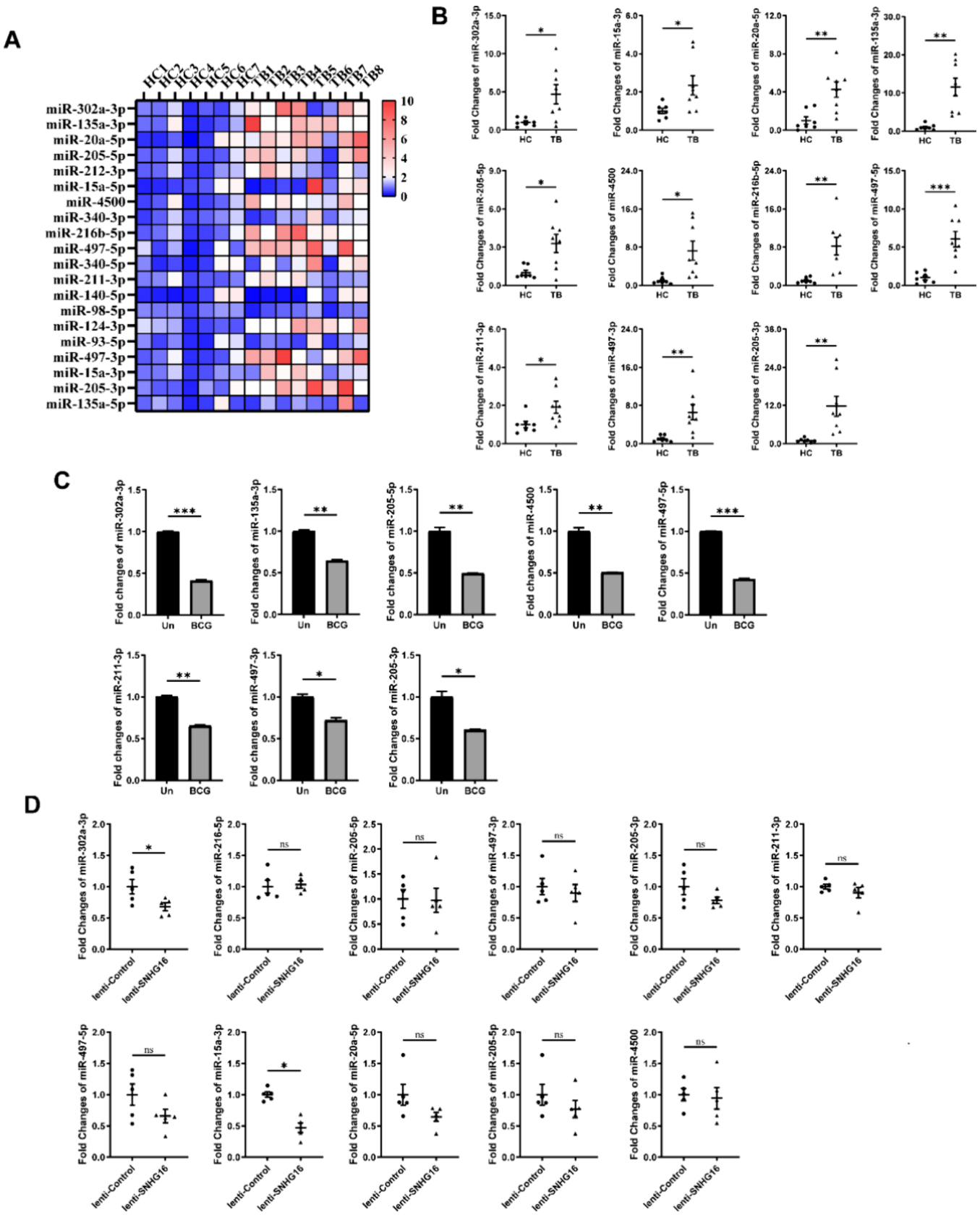

Figure 2. miRNAs interacted with SNHG16. (A) Heatmap of miRNAs expression levels in PBMCs isolated from HCs ($n = 7$) and TB patients ($n = 8$), respectively. (B) Expression levels of miRNAs were detected by q-PCR methods in PBMCs of HCs ($n = 7$) and TB patients ($n = 8$), respectively. (C) Expression levels of miRNA were determined by the q-PCR in THP-1 cells under BCG stimulation (MOI = 10) and unstimulated controls (Un). (D) Expression levels of miRNAs were determined by the q-PCR in PBMCs ($n = 5$) transfected with lenti-SNHG16 and lenti-Control, respectively. The results are expressed as the mean ± SEM *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.; ****$p < 0.0001$; data represent 3 independent experiments.

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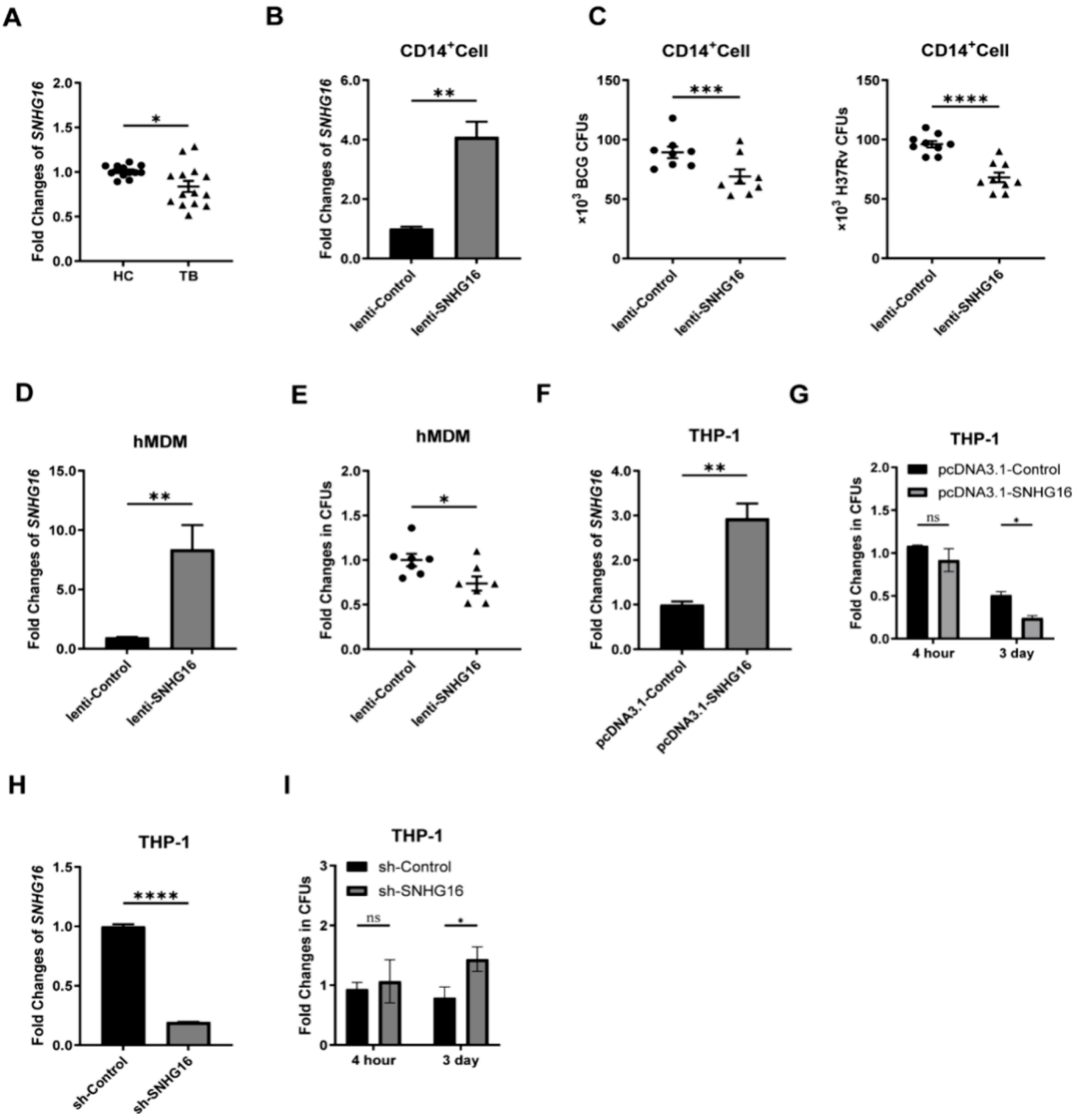

Figure 3. SNHG16 regulated CD14$^+$cells/macrophages to control intracellular mycobacteria growth. (A) *SNHG16* expression levels were determined by q-PCR methods in CD14$^+$ cells isolated from PBMCs of HC donors (n = 12) and TB patients (n = 14). (B) After transfection of lentivirus expressing SNHG16 (lenti-SNHG16) and empty lentivirus (lenti-Control), *SNHG16* expression levels were tested by q-PCR methods in CD14$^+$ cells of HC donors. (C) Human CD14$^+$ cells transfected with lenti-SNHG16 and controls (lenti-Control), respectively, were infected with BCG (MOI = 10) and H37Rv (MOI = 2). The values of BCG and H37Rv CFU were counted. (D) *SNHG16* expression levels in hMDM cells of HC donors transfected with lenti-SNHG16 and controls (lenti-Control) were measured by q-PCR methods, respectively. (E) H37Rv CFUs were shown in hMDM cells transfected with lenti-SNHG16 and controls (lenti-Control) at 3 days after H37Rv infection (MOI = 2). (F) *SNHG16* expression levels were determined by q-PCR methods in THP-1 cells after transfection with the plasmid expressing SNHG16 (pcDNA3.1-SNHG16) and empty plasmid controls (pcDNA3.1-Control), respectively. (G) BCG CFU values were shown in THP-1 cells containing plasmids of pcDNA3.1-SNHG16 or pcDNA3.1-Control at 4 h and 3 days after BCG infection (MOI = 10), respectively. (H) *SNHG16* expression levels were tested in THP-1 cells transfected with lentivirus expressing shRNA of SNHG16 (sh-SNHG16) to decrease SNHG16 expression and controls (sh-Control), respectively. (I) BCG CFUs were counted in THP-1 cells transfected with sh-SNHG16 and controls (sh-Control) at 4 h and 3 days after BCG infection (MOI = 10), respectively. The results are expressed as the mean ± SEM *p < 0.05; **p < 0.01; ***p < 0.001.; ****p < 0.0001; data represent 3 independent experiments.

subjects (Figure 1C). The result demonstrated that SNHG16 expression was obviously suppressed in chronic TB.

To examine the effect of acute mycobacterial infection on SNHG16 expression profiles, we infected macrophages with *Mycobacterium bovis* BCG in vitro and measured SNHG16 expression levels using q-PCR methods. Unexpectedly, our results showed that SNHG16 expression levels in BCG-infected THP-1 macrophages were two times greater than those in the control (Figures 1D(left) and S1), and SNHG16 expression levels were also elevated in BCG-infected human monocyte-

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derived macrophage (hMDM) cells (Figures 1D(right) and S1). Furthermore, we infected macrophages with the virulent *Mtb* H37Rv strain and found that SNHG16 expression levels were also significantly increased by about 2 folds in both H37Rv-infected THP-1 and hMDMs compared to controls (Figures 1E and S1). Moreover, we infected PBMCs of HC subjects with the BCG strain and found that SNHG16 expression levels were also significantly increased in BCG-infected PBMCs compared to controls (Figure 1F).

Such TB Suppression of SNHG16 Was Consistent with an Immunosuppressive-like State Driven by IL-10. Since PBMCs or monocytes of TB patients exhibit the antigen-driven overproduction of immunosuppressive-like cytokines such as IL-10,[15,16] we tested the hypothesis that IL-10 signaling contributes to down-regulating SNHG16 in TB. Thus, PBMCs/monocytes from HCs were incubated with IL-10 in culture and assessed for the expression profiles of SNHG16 using the GEO2R platform (GDS4551). Interestingly, IL-10 signaling in PBMCs/monocytes decreased the expression of SNHG16 compared to the control (Figure 1G).[17]

Together, the results above demonstrated that SNHG16 expression was up-regulated in acute *Mtb* infection but suppressed in chronic TB; such TB suppression of SNHG16 was consistent with an immunosuppressive-like state driven by IL-10 signaling as seen in TB patients. The findings implied that SNHG16 might help to up-regulate the immune response against acute *Mtb* infection.

SNHG16 Correlated Inversely with Selected microRNAs in TB and Acute Mycobacterial Infection. It is likely that LncRNAs function as competing RNAs, blocking the binding of miRNAs to mRNAs encoding anti-*Mtb* effector molecules. We therefore assessed the possibility that SNHG16 antagonizes selected miRNA species to regulate immune responses. We sought to identify the expression profiles of miRNAs in comparison with SNHG16 in *Mtb* infection. We found that many miRNA species were differently expressed in PBMCs between HC subjects and TB patients (Figure 2A). Notably, some miRNA species were significantly increased in TB PBMCs (Figure 2B), and their increased expressions coincided inversely with decreases in SNHG16 expression in PBMCs of TB patients (Figure 1C). These results implied that TB-driven down-regulation of SNHG16 contributed to the dominance of some selected miRNAs. Concurrently, we infected THP-1 macrophages with mycobacterial BCG and H37Rv and measured these miRNA expression levels. The results showed that acute mycobacterial BCG infection, while upregulating SNHG16 (Figure 1D,E), indeed inhibited expressions of these selected miRNAs (Figure 2C). Similarly, the expression of some selected miRNAs was also decreased under H37Rv infection (Figure S2). These results implied that SNHG16 can antagonize expressions of selected miRNAs to ensure immune responses to acute mycobacterial infection.

SNHG16 Down-Regulated Expressions of the Autophagy-Antagonizing miR-302a-3p/miR-15a-5p. To directly examine the effects of SNHG16 on miRNA expression, we transfected macrophages with lenti-SNHG16 to increase SNHG16 expression levels and measured the expression of miRNAs. The overexpression of SNHG16 significantly depressed autophagy-antagonizing miR-302a-3p and miR-15a-5p but not others (Figure 2D). These results were consistent with the scenario that SNHG16 can antagonize miR-15a-5p/miR-302p and their antiautophagy actions, as published by other groups.[18−22]

SNHG16 Overexpression Could Significantly Inhibit Mycobacterial Replication/Growth in Macrophages. Macrophages/monocytes function as professional killers for intracellular pathogens.[23] Our results above allowed us to postulate that SNHG16 participates in antimicrobial responses against *Mtb*/BCG infection. To test this, we isolated $CD14^+$ monocytes from TB patients and HC donors and comparatively assessed the expression levels of SNHG16 using q-PCR methods. The results showed that SNHG16 expression was significantly lower in $CD14^+$ cells of TB patients than that of HC subjects (Figure 3A).

To examine whether SNHG16 regulated the effect of macrophages on inhibiting intracellular *Mtb* growth, we constructed a recombinant lentivirus containing noncoding SNHG16 (named lenti-SNHG16) and transfected the lentivirus into $CD14^+$ monocytes to increase SNHG16 expression. We found that the average SNHG16 expression level in primary $CD14^+$ cells transfected with lenti-SNHG16 was about 4 times greater than that in cells with the control lentivirus (lenti-Control) (Figure 3B). Then, those transfected $CD14^+$ cells were infected with BCG and virulent H37Rv strains, respectively, and assessed for mycobacterial CFU counts after 3-day coculture. We found that BCG CFU values in $CD14^+$ cells transfected with lenti-SNHG16 were significantly lower than those with the lenti-Control (Figure 3C, left) while BCG CFU values in $CD14^+$ cells transfected with sh-SNHG16 were obviously higher than those in controls (Figure S3). Consistently, H37Rv CFU values in SNHG16-overexpressing $CD14^+$ cells (Figure 3C, right) were significantly lower than those in the controls. Similarly, when lenti-SNHG16 transfection in hMDMs led to an 8-fold increase in SNHG16 expression (Figure 3D), such SNHG16-overexpressing hMDMs exhibited significantly lower *Mtb* CFU counts after H37Rv infection than the lenti-Control (Figure 3E). These results showed that SNHG16 overexpression increased the ability of monocytes/macrophages to limit intracellular *Mtb* growth.

To authenticate the SNHG16 regulation of *Mtb* infection, we utilized plasmids containing noncoding SNHG16 (pcDNA3.1-SNHG16) and lentivirus containing SNHG16 shRNA (Sh-SNHG16) to increase and knock-down SNHG16 expression levels in THP-1 macrophages, respectively, during mycobacterial infection. We first determined whether changes in SNHG16 expression levels altered mycobacterial entrance. At 24 h after the transfection of pcDNA3.1-SNHG16 into THP-1 cells, SNHG16 expression levels increased by >3 times compared to the control (Figure 3F). These transfected THP-1 cells with high-level SNHG16 were then cocultured for 4 h with BCG at MOI = 10, then washed intensely to remove free unbound BCG, and then lysed for measurements of BCG CFU counts. There were no significant differences in BCG CFU counts between SNHG16-overexpressed THP-1 cells and controls (Figure 3G). Concurrently, while the transfection of Sh-SNHG16 led to >70% knock-downs of SNHG16 at 24 h (Figure 3H), there were also no significant differences in BCG CFU counts between the Sh-SNHG16-transfected THP-1 cells and the controls at 4 h after BCG infection (Figure 3I). These results suggested that SNHG16 overexpression did not alter BCG entry into target cells—macrophages.

We then determined whether SNHG16 overexpression could regulate BCG replication/growth at 3 days after BCG coculturing with THP-1 cells. Thus, prior to BCG infection, THP-1 cells were transfected 24 h with pcDNA3.1-SNHG16 or Sh-SNHG16 and their controls. At 3 days after BCG infection,

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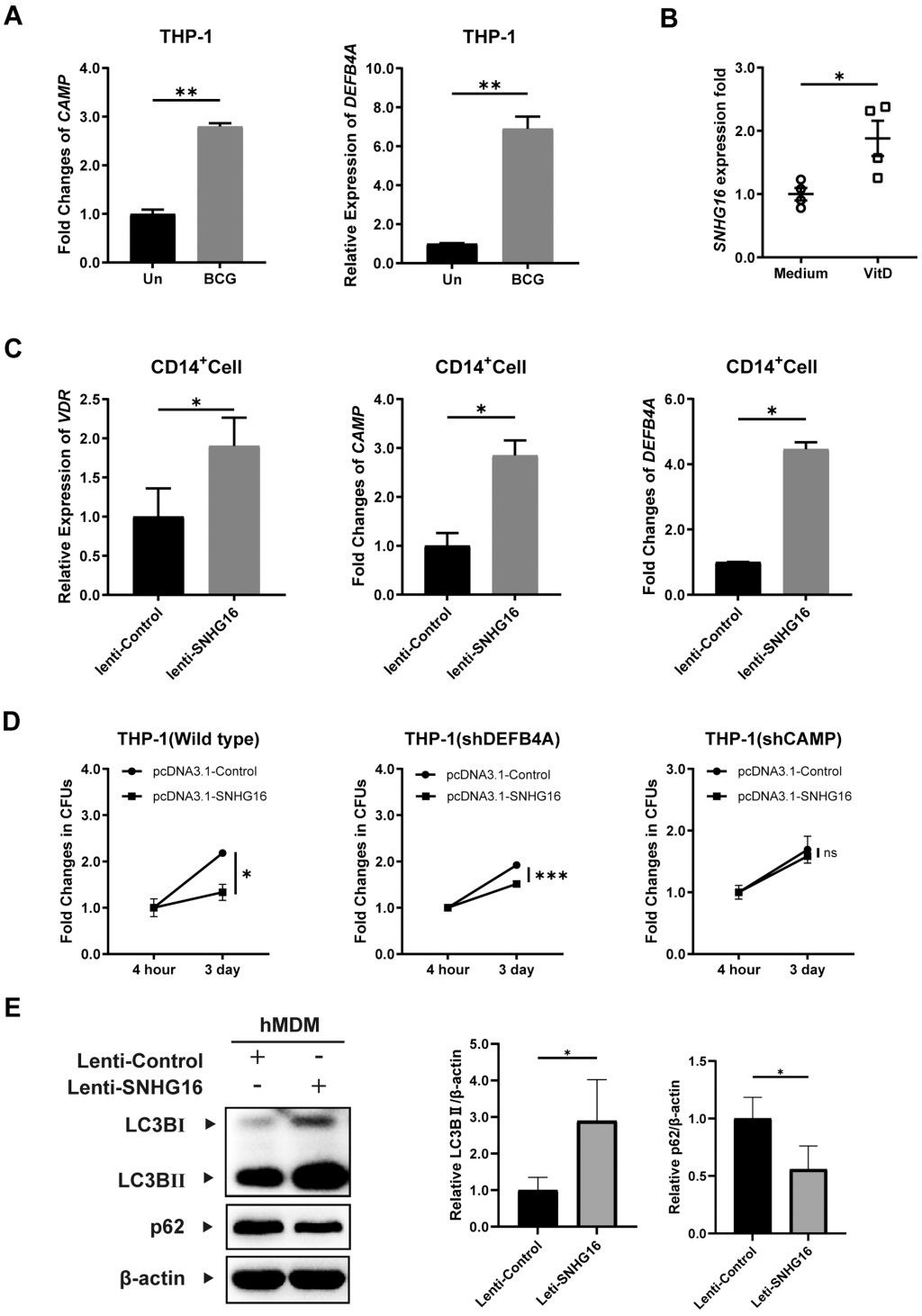

Figure 4. SNHG16 activated VDR-related signal pathways and inhibited intracellular mycobacteria growth dependent on the CAMP pathway. (A) Expression levels of *CAMP* and *DEFB4A* in THP-1 cells were detected by q-PCR methods after BCG infection (MOI = 10), respectively. (B) SNHG16 expression levels in PBMCs were analyzed using the GEO2R platform (GDS4860) under VitD stimulation and medium (M) control. SNHG16 expression levels were normalized to the fold-change of the mean expression level of the M. (C) Expression levels of *VDR*, *CAMP*, and *DEFB4A* were determined by q-PCR methods in human $CD14^+$ cells with lentivirus transfection of lenti-SNHG16 and lenti-Control. (D) BCG CFUs were counted in SNHG16-overexpressed cells of wild-type THP-1 cells, *DEFB4A* knock-down cells (shDEFBFA), and *CAMP* knock-down cells (shCAMP) at 3 days after BCG infection (MOI = 10), respectively. (E) Representive western blot (WB) results of LC3BI/II and P62 proteins in hMDM cells transfected with lenti-SNHG16 or lenti-Control, respectively. The results are expressed as the mean ± SEM $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.; $****p < 0.0001$; data represent 3 independent experiments.

transfected cells were lysed to measure the BCG CFU counts. BCG CUF counts in SNHG16-overexpressing THP-1 cells were significantly lower than those in the controls (Figure 3G). Consistently, SNHG16 knock-down after BCG infection of the Sh-SNHG16-transfected cells led to significantly higher CFU counts than the controls (Figure 3I). These results therefore

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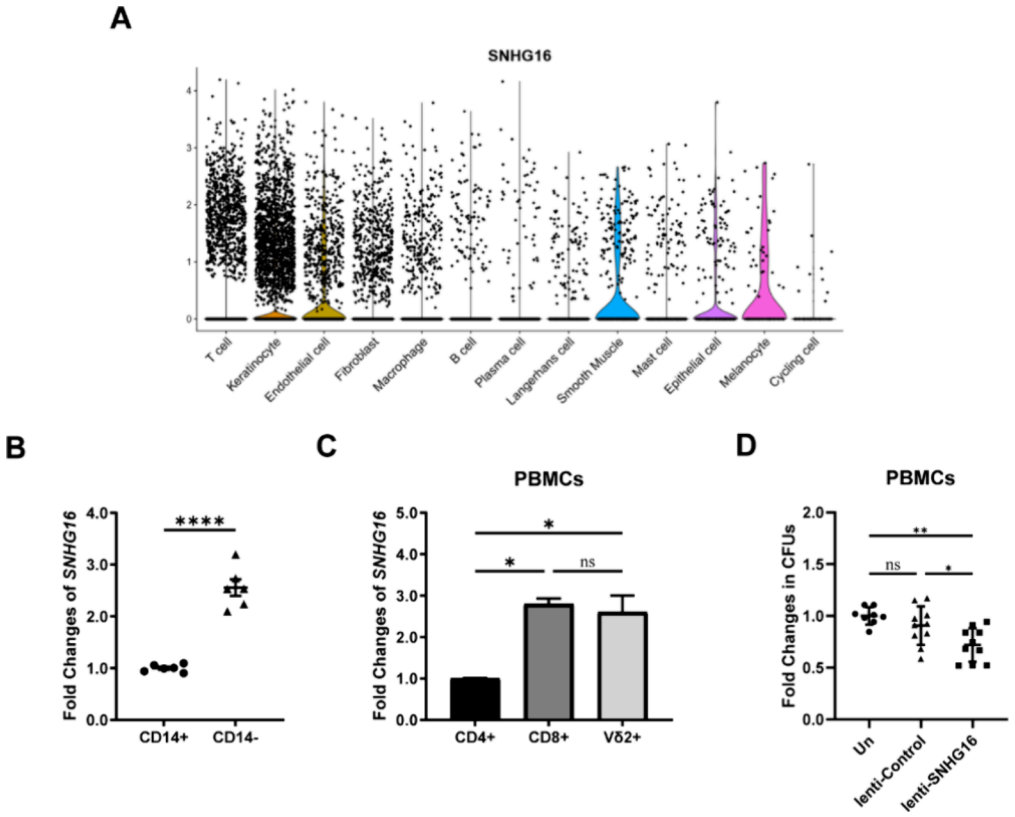

Figure 5. SNHG16 differently expressed in CD14$^-$cells. (A) Violin plots showing the expression of SNHG16 by cell subtypes (data from GEO under Accession Number GSE173706). Each dot represents the gene's expression in a single cell. (B) Expression levels of *SNHG16* were determined by q-PCR methods in CD14$^+$ and CD14$^-$ cells isolated from PBMCs of HC subjects ($n = 6$). (C) *SNHG16* expression levels were tested by q-PCR methods in CD4$^+$, CD8$^+$, and Vγ2$^+$ T cells isolated from PBMCs of healthy donors, respectively. (D) Fold changes of BCG CFU values were compared in PBMCs of healthy subjects ($n = 10$) transfected with lentivirus of lenti-SNHG16 and controls at 3 days after BCG infection (MOI = 1), respectively. the results are expressed as the mean ± SEM *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; data represent 3 independent experiments.

demonstrated that SNHG16 could inhibit BCG replication and growth in THP-1 macrophages.

SNHG16 Overexpression Activated Vitamin D Receptor-Dependent Antimicrobial Genes and Inhibited Intracellular Mycobacteria Growth via Autophagy and VDR-Dependent Cathelicidin (CAMP) Pathways. As widely reported, vitamin D receptor (VDR)-dependent activation pathways promote the ability of macrophages to produce cathelicidin (CAMP) and β-defensin 2 (DEFB4A), which contribute to the lysosomal killing of intracellular bacteria.[24] Here, we found that mycobacterial BCG infection-driven increases in SNHG16 expression (Figure 1D) were associated with significantly increased antimicrobial peptide production of CAMP and DEFB4A (Figure 4A).

Given that SNHG16 overexpression could inhibit BCG replication/growth in macrophages, we wanted to know whether the SNHG16-driven antimycobacterial response involved the activation of VDR-related antimicrobial genes. We, therefore, examined whether VitD treatment of human PBMCs coincided with changes in SNHG16 using the GEO2R platform (GDS4860). We found that VitD treatment led to significant increases in SNHG16 expression in human PBMCs (Figure 4B).[25] Then, we sought to examine whether SNHG16 regulated the expression of VDR-related antimicrobial genes. We increased SNHG16 expression in macrophages by transfection of lentivirus lenti-SNHG16 and measured VDR pathway gene expression using the q-PCR. The results showed that the expression levels of *VDR*, *CAMP*, and *DEFB4A* genes in SNHG16-overexpressing cells were about 2-, 3-, and 4.2-fold greater than those in cells with lentivirus of the lenti-Control, respectively (Figure 4C). Conversely, in SNHG16 expression decreased cells transfected with Sh-SNHG16, the expression levels of *CAMP* and *DEFB4A* genes were significantly reduced compared to controls (Figure S4).

To determine whether SNHG16 depended on CAMP or DEFB4A to inhibit intracellular mycobacteria growth, we transfected lentivirus lenti-SNHG16 to increase SNHG16 expression in wild-type THP-1 cells, DEFB4A-deficient THP-1 (shDEFB4A),[26] or CAMP-deficient THP-1 (shCAMP),[26] and then infected each of these cell types with mycobacterial BCG for 3 days before measuring BCG CFU counts. In SNHG16-overexpressing macrophages, BCG CFU counts were only half of those in cells transfected with the lenti-Control (Figure 4D). Similarly, BCG CFU counts in the DEFB4A-deficient THP-1 cells with SNHG16 overexpression were also significantly decreased compared to controls (Figure 4D). However, in the CAMP-deficient THP-1 cells with SNHG16 overexpression, BCG CFU counts were similar to those in CAMP-deficient cells transfected with the lenti-Control (Figure 4D), indicating a loss of SNHG16-driven reduction of BCG infection. These results implied that SNHG16 appeared to depend on the CAMP pathway to inhibit intracellular mycobacterial growth.

As recently reported, CAMP could activate autophagy in macrophages.[27] This raised the possibility that SNHG16 could affect the autophagy process. To address this, we measured the expression levels of autophagy-related LC3BI/II and P62

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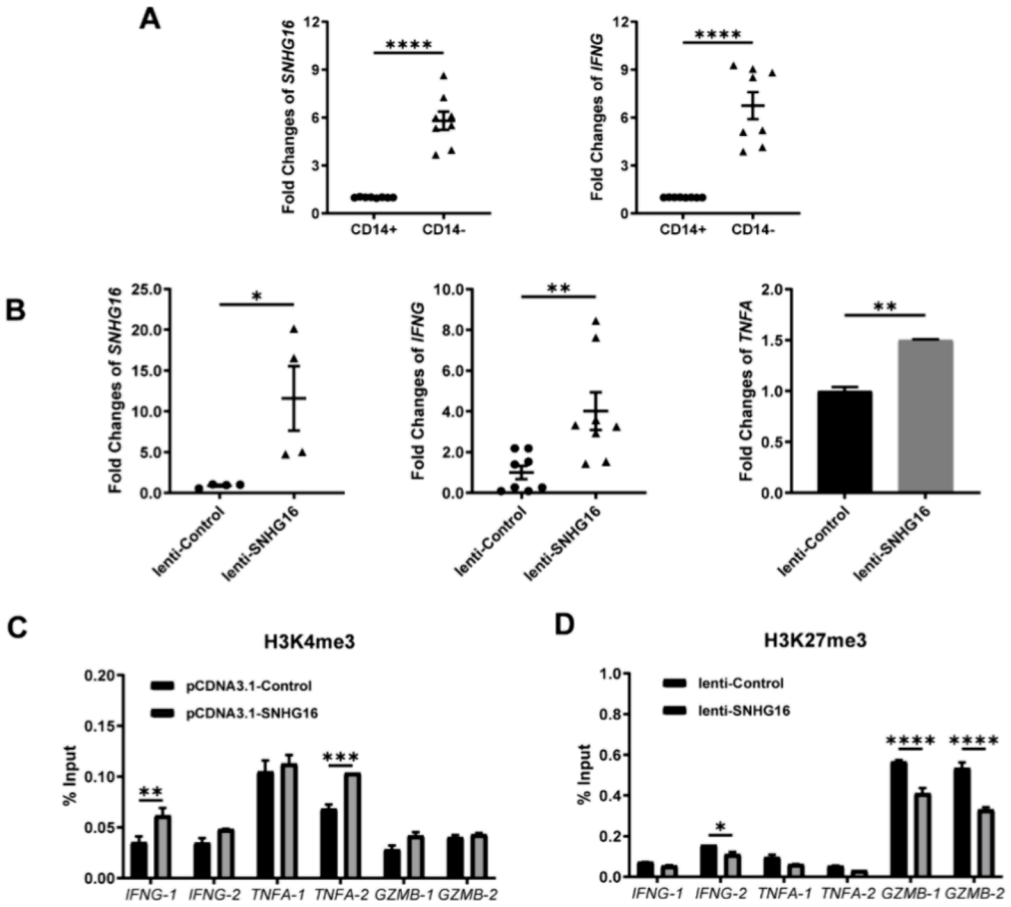

Figure 6. Overexpression of SNHG16 induced a more accessible chromatin state in IFN-γ, TNF-α, and Granzyme B coding gene locus. (A) Expression of *SNHG16* and *IFNG* were determined by the q-PCR in $CD14^+$ and $CD14^-$ cells isolated from PBMCs of healthy subjects ($n = 6$) with *Mtb* antigen PPD stimulation or medium, respectively. (B) Expression levels of *SNHG16*, *IFNG*, and *TNFA* encoding genes were measured by the q-PCR in human PBMCs transfected with lenti-SNHG16 and controls, respectively. (C,D) H3K4Me3 (C) and H3K27Me3 (D) histone modifications in the promoter of IFN-γ, TNF-α, and Granzyme B coding gene locus were analyzed in ChIP-qPCR in 293T cells, which were transfected with the plasmid containing SNHG16-coding gene or empty plasmid as a control. Values derived from three independent experiments were normalized by background signals and input chromatin. The results are expressed as the mean ± SEM *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.; ****$p < 0.0001$; data represent 3 independent experiments.

proteins in SNHG16-overexpressing hMDMs using western blot (WB) methods. The results showed that the protein expression levels of LC3BI/II and the ratios of LC3BII/β-actin in SNHG16-overexpressing hMDMs were significantly higher than those in cells transfected with the lenti-Control (Figure 4E). The expression level of P62 was lower in macrophages with lenti-SNHG16 compared to controls (Figure 4E). These results demonstrated that SNHG16 overexpression could inhibit intracellular mycobacterial growth via autophagy and VDR-dependent cathelicidin CAMP pathways.

SNHG16 was Highly Expressed in $CD14^-$ Lymphocytes. Given that SNHG16 could regulate anti-*Mtb* innate immunity in monocytes and macrophages, we extended our studies to examine SNHG16 effector functions in other cell populations. We first analyzed SNHG16 expression patterns in the single-cell sequencing database[28] of many different human cell types. The results showed that SNHG16 is widely expressed in many cell populations, including T cells (Figure 5A). Then, we isolated $CD14^+$ monocytes and $CD14^-$ lymphocytes from human PBMCs, and we measured SNHG16 expression levels, respectively. We found that SNHG16 was highly expressed in $CD14^-$ lymphocytes, and their expression levels were even significantly higher than those in $CD14^+$ monocytes (Figure 5B).

To examine SNHG16 expression profiles in T-cell populations, we isolated $CD4^+$, $CD8^+$, and $V\delta2^+$ T cells and measured SNHG16 expression levels in these cells. We found that SNHG16 expression levels in $CD8^+$ and $V\delta2^+$ T cells were about 3 folds of those in $CD4^+$ cells (Figure 5C). Since $CD8^+$ and $V\delta2^+$ T cells can differentiate into cytotoxic T lymphocytes (CTL), the high-level SNHG16 expression in these cells may regulate the CTL effector function.

SNHG16 Overexpression Promoted the Ability of $CD14^-$ Lymphocytes To Mount the Effector Function of Controlling Mycobacterial Infection in Macrophages. We then sought to evaluate whether SNHG16 could affect the effector function of $CD14^-$ lymphocytes including $CD8^+$ and $V\delta2^+$ T cells. We transfected lenti-SNHG16 into human PBMCs for the overexpression of SNHG16, then isolated $CD14^-$ lymphocytes, and then cocultured them with BCG-infected THP-1 macrophages for 3 days before measuring BCG CFUs. BCG CFU counts in SNHG16-overexpressing lymphocyte groups were significantly lower than those in control groups (Figure 5D). Thus, the above results demonstrated that SNHG16 was highly expressed in $CD14^-$ lymphocytes and

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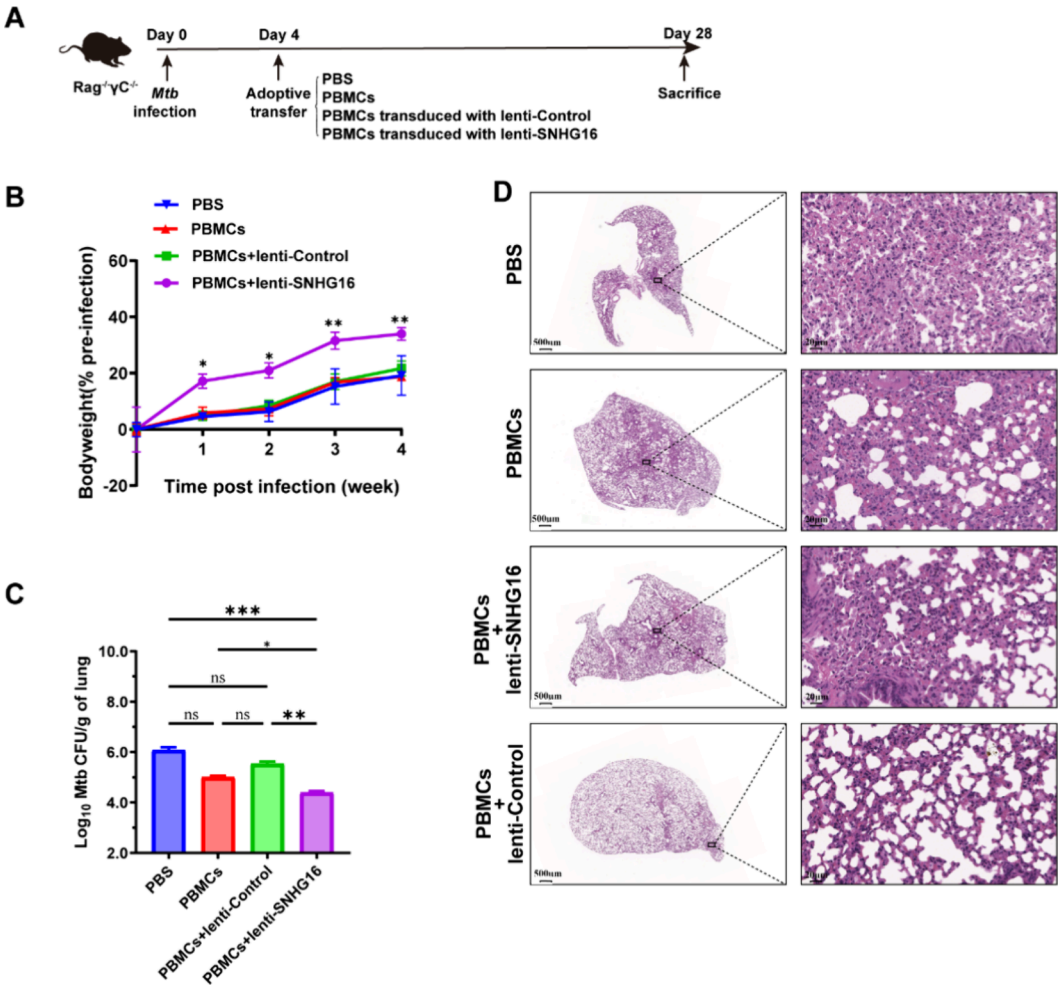

Figure 7. SNHG16 inhibited *Mtb* H37Rv growth in SCID mice. (A) Schematic with the human PBMCs adoptive transfer strategy. PBMCs cells (1×10^7 cells) treated with lentivirus of lenti-SNHG16, lenti-Control, or medium, and the same volume of PBS was adoptively transferred intravenously (i.v.) into *Mtb* H37Rv-infected SCID mice. (B) Data shown were the mice weights at different time points after *Mtb* H37Rv infection. (C) Data shown were *Mtb* H37Rv CFUs in the lung of SCID mice treated with PBMCs transfected by lenti-SNHG16 and other controls, respectively. (D) Representive histological results of lung tissues from *Mtb* H37Rv-infected mice were presented, which were collected on 28th day postinfection. The results are expressed as the mean ± SEM $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.; $****p < 0.0001$; data represent 3 independent experiments.

that SNHG16 overexpression promoted the ability of $CD14^-$ lymphocytes to mount the effector function of limiting mycobacterial growth in macrophages.

SNHG16 Overexpression Induced More Accessible Chromatin States in the Genetic Loci of IFN-γ, TNF-α, and Granzyme B, Consistent with High Expressions of These Cytokines. We then sought to dissect mechanisms whereby SNHG16 regulated antimycobacterial effector functions of $CD14^-$ lymphocytes. Notably, in the setting of the mycobacterial antigen PPD, the expression levels of IFN-γ and SNHG16 were both higher in $CD14^-$ lymphocytes than those in $CD14^+$ monocytes (Figure 6A). These results suggested that the SNHG16 expression might be consistent with effector cytokine IFN-γ production.

To examine whether SNHG16 regulated effector cytokines' production, we overexpressed SNHG16 in human PBMCs by transfection of lenti-SNHG16 and then measured expression levels of effector cytokines. The SNHG16 and IFN-γ expressions in the SNHG16-overexpressing PBMCs were about 12-folds and 4-folds greater than those in the PBMCs control, respectively (Figure 6B). Similarly, the expression of TNF-α in the SNHG16-overexpressing PBMCs was also significantly higher than that in the control (Figure 6B). Moreover, the expression of IFN-γ in SNHG16 decreased PBMCs and was obviously lower than that in the control (Figure S5). These results suggested that SNHG16 regulated effector cytokine expression in lymphocytes.

In eukaryotes, chromatin packages and organizes the genome to protect it from environmental insults and orchestrate all DNA-based processes, including DNA repair, transcription, chromosome segregation, and suppression of transposable elements.[29] To examine how SNHG16 influenced effector cytokine expression, we measured chromatin states of effector cytokine-coding gene loci via ChIP-qPCR in 293T cells, which were transfected with either lenti-SNHG16 or empty lentivirus as a control.

Trimethylation of histone H3 lysine 4 (H3K4me3) is associated with transcriptional start sites and has been proposed to regulate transcription initiation.[30] H3K4me3 usually maintains repression. Trimethylations of lysine 4 (K4) and 27 (K27) on histone H3 have been shown to associate with active and repressed gene expression, respectively. We found that SNHG16 overexpression, but not control, increased H3K4me3 at the promoter-loci of IFN-γ and TNF-α (Figure 6C).

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Accordingly, trimethylation of lysine 27 on histone H3 (H3K27me3), a transcription-suppressor histone modification,[31] was decreased at the promoter-loci of the genes encoding IFN-γ and Granzyme B in SNHG16-overexpressing cells compared to controls (Figure 6D).

The data suggest a hypothetical model in which SNHG16 may induce H3K4me3 and repress H3K27Me3 at the promoter-loci of the genes encoding effector cytokines IFN-γ, TNF-α, and Granzyme B, and therefore allow chromatin to program accessible states and enhance transcription of IFN-γ, TNF-α, and Granzyme B in T cells.

Adoptive Transfer of SNHG16-Overexpressing PBMCs to *Mtb*-Infected SCID Mice Conferred Protective Immunity against *Mtb* Infection. Based on the strong data above, we hypothesized that SNHG16 would mediate better control of *Mtb* infection in vivo. To address this, we developed an adoptive cellular transfer strategy using *Mtb*-infected SCID mice. First, we transfected lenti-SNHG16 into human PBMCs to enhance SNHG16 expression. Next, we adoptively transferred these SNHG16-overexpressing PBMCs to *Mtb*-infected SCID mice at day 4 after *Mtb* infection (Figure 7A). As controls, 3 groups of mice received empty lentivirus-transfected PBMCs, PBMCs only, and PBS.

Since weight loss is a common symptom of human TB, we evaluated body-weight changes after the *Mtb* challenge of different groups of mice. We found that the mouse test group receiving transfer of SNHG16-overexpressed PBMCs showed significantly more gains in mean body weight than control groups of mice starting from the first week after *Mtb* infection (Figure 7B). Moreover, the mouse test group showed significantly lower bacterial burdens in the lungs, compared with the control groups in the fourth week after *Mtb* infection (Figure 7C).

Consistently, the mouse test group displayed fewer pathological lesions and fewer inflammatory cell infiltrations in the lungs compared to control groups (Figures 7D and S6). Thus, these results demonstrated that SNHG16-overexpressing human PBMCs could confer immune control of *Mtb* infection in SCID mice. The findings suggested that SNHG16 played an active role in the protective immunity against *Mtb* infection.

DISCUSSION

SNHG16 is mostly overexpressed in cancer patients and associated with the development and progression of various malignancies. However, SNHG16 expression patterns in lymphocytes of TB patients remain unclear. Here, we demonstrated that SNHG16 expression was suppressed in PBMCs of TB patients compared to HC subjects, largely different from what has been seen in cancer patients or diabetic patients.[32,33] Our results consistently showed that SNHG16 expression levels in PBMCs of healthy and LTBI subjects were significantly higher than those in TB patients.[14] Moreover, SNHG16 expression levels in PBMCs of TB patients increased after 6-month treatment compared to the levels before treatment.[14] We also discovered that mycobacteria BCG infection/stimulation in vitro increased SNHG16 expression levels in PBMCs of healthy subjects. Our results suggest that immunosuppressive-like response such as IL-10 elevation in chronic TB,[15,16] contributes to the TB-driven down-regulation of SNHG16 as the cytokine IL-10 treatment of cultured PBMCs reduced SNHG16 expression. This speculation was also supported by the finding that acute *Mtb* infection in culture without IL-10 or other immunosuppressive-like cytokines led to a high-level expression of SNHG16 and a favorable anti-*Mtb* effector function. Consistently, TB-driven down-regulation of SNHG16 coincided with up-regulation of immune-antagonizing microRNAs including miR-302a-3p/miR-15a-5p, whereas acute BCG infection inhibited the expression of these microRNAs, with SNHG16 being directly down-regulating miR-302a-3p/miR-15a-5p. Therefore, SNHG16 expression patterns were closely correlated with *Mtb* infection, TB outcome, and progression, with the potential of serving as a biomarker for *Mtb* infection and TB development.

The current study demonstrated that SNHG16 regulatory functions are mainly linked to macrophages' innate immunity and T-cell effector response against *Mtb* infection, although it can widely be expressed in diverse cell types or wrapped in EVs and even in serum.[13,34] In the setting of innate immunity, SNHG16 overexpression could significantly inhibit mycobacterial replication/growth in macrophages despite a lack of effects on mycobacterial entry. Mechanistically, SNHG16 induced autophagy to control intracellular *Mtb* and BCG growth, as characterized by an increase of LC3BII expression and a decrease of P62 expression. While SNHG16 overexpression significantly enhanced the expression of VitD-dependent antimicrobial molecules of cathelicidin (CAMP) and β-defensin 2 (DEFB4A), the knock-down/deficiency of CAMP but not DEFB4A led to a loss of SNHG16-induced immune control of mycobacterial infection in macrophages. Thus, the antimicrobial effect of SNHG16 might depend more upon the CAMP pathway, which plays an important role in the induction of autophagy. Concurrently, SNHG16 inhibited the expression of miR-302a-3p and miR-15a-5p. Because miR-302a-3p was reported to suppress autophagy,[20−22] SNHG16 may counteract depressing miRNAs to ensure innate immunity. Here, we first characterized the effector function of SNHG16 linked to innate immunity in hMDM and macrophage cell lines, and it would be better for further studies in primary macrophages, especially lung-resident macrophages.

On the other hand, the current study showed that SNHG16 also regulated T-cell/cytokine effector functions against *Mtb* infection. Here, SNHG16 was highly expressed in $CD14^-$ lymphocytes, particularly in CD8 T, and Vγ2 Vδ2 T-cell subsets in HC. Thus, SNHG16 may help to drive these T-cell subsets differentiating into anti-*Mtb* effector phenotypes including CTL in anti-TB immune responses. In fact, we demonstrated that SNHG16 overexpression promoted the ability of $CD14^-$ lymphocytes to mount effector functions of controlling mycobacterial infection in macrophages. In addition, SNHG16 overexpression increased the expression of effector cytokines IFN-γ, TNF-α, and granzyme B. Such SNHG16-induced cytokine expression appeared to take place by means of epigenetic regulation inducing a more accessible chromatin state in their genetic loci. Thus, SNHG16-expressing lymphocytes may help produce more effective cytokines to activate macrophages/monocytes controlling intracellular *Mtb* growth. Furthermore, adoptive transfer of SNHG16-overexpressing human PBMCs (mainly lymphocytes) into SCID mice conferred protective immunity, characterized by lower *Mtb* CFU in the lung, less pathological damage, and more body-weight gains than control mice. Of course, more experiments are needed to fully characterize the effect mechanisms of SNHG16 when SNHG16 is taken as a potential immunotherapy target in TB treatment.

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Table 1. Oligonucleotide Sequence of PCR Primers

	forward primer	reverse primer
primers for qRT-PCR		
SNHG16	5′-GCAGAATGCCATGGTTTCCC-3′	5′-GGACAGCTGGCAAGAGACTT-3′
CAMP	5′-AGGATTGTGACTTCAAGAAGGACG-3′	5′-GTTTATTTCTCAGAGCCCAGAAGC-3′
DAFB4A	5′-GGT GTT TTTGGTGGTATAGGCG-3′	5′-AGGGCAAAAGACTGGATGACA-3′
IFNG	5′-CATCACGTCATACCAGCCATTT-3′	5′-CATCACGTCATACCAGCCATTT-3′
TNFA	5′-CCTCTCTCTAATCAGCCCTCTG-3′	5′-GAGGACCTGGGAGTAGATGAG-3′
GZMB	5′-CCACTCTCGACCCTACATGG-3′	5′-GGCCCCCAAAGTGACATTTATT-3′
β-ACTIN	5′-GCCCTGAGGCACTCTTCCA-3′	5′-TGTTGGCGTACAGGTCTTTGC-3′
primers for ChIP		
IFNG-1	5′-AGAGTCAACATTTTACCAGGGCGAA GTGGG-3′	5′-GTTTCCTTTAGACTCCTTGGGTCCTT TGACG-3′
IFNG-2	5′-TGGGATTCTTTGAAGGCACT-3′	5′-TGCCCCTTTGTAAAGGTTTG-3′
TNFA-1	5′-GGGAGTGTGAGGGGTATCCT-3′	5′-AACCAGCGGAAAACTTCCTT-3′
TNFA-2	5′-CCCTCCAGTTCTAGTTCTATC-3′	5′-GGGGAAAGAATCATTCAACCAG-3′
GZMB-1	5′-CCAAACGTGCTTCCTTTCGG-3′	5′-CCTGGACTCAGCTCTAGGGA-3′
GZMB-2	5′-CACTTCATAGGCTTGGGTTCCTG-3′	5′-CTCTGGGTGCTTGTGTGAGAATC-3′

CONCLUSIONS

Taken together, while SNHG16 appears to serve as a potential marker predicting TB development, SNHG16 can drive the induction of pleiotropic anti-*Mtb* effector functions of macrophages and T-cell subsets. SNHG16 augments the ability of macrophages to limit *Mtb* growth via autophagy and VDR/CAMP-dependent antimicrobial pathway; SNHG16 overexpression in lymphocytes/T cells can epigenetically up-regulate the expression of anti-TB cytokines and control *Mtb* infection in macrophages. As far as we know, the current study provided the first evidence that SNHG16 overexpression could control *Mtb* growth both in vitro and in vivo.

MATERIALS AND METHODS

Human Subjects. This study was approved by both the institutional review boards for human subject research and the institutional biosafety committees of Shanghai Pulmonary Hospital (SPH). All subjects are adults (≥18y) and signed written informed consent. Active TB patients were enrolled at the SPH (Shanghai, China). Age- and sex-matched uninfected volunteers without clinical and immunological evidence of TB were recruited as HCs. All donors were tested for hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV). Individuals with HBV, HCV, and HIV infections and other infectious diseases, cancers, and diabetes were excluded.[3,35] This study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki. The studies involving humans were approved by the Ethics Committee of SPH (No. K22-143Z). The studies were conducted in accordance with local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. In these experiments, we did not use any statistical methods to predetermine sample size, since the experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessments.

Cell Isolation and Culture. As previously described, human PBMCs were isolated by density gradient centrifugation using the Ficoll-Paque PLUS medium (Cytiva, USA) from buffy coats prepared from the peripheral blood of healthy donors or active TB patients and cultured in RPMI 1640 media supplemented with 2 mM glutamine, 50 U/ml of penicillin, and 50 mg/mL of streptomycin and containing 10% fetal bovine serum.[36]

Cell isolation kits were used to isolate and enrich $CD14^+$, $CD4^+$, $CD8^+$, and $V\delta2^+$ cells (Miltenyi Biotec, Germany, 130-050-201, 130-097-048, 130-045-201, and 130-092-892) from fresh PBMCs of healthy donors by positive selection according to the protocols. The purity of enriched cells was more than 95% (Figures S7).

hMDMs were differentiated from freshly isolated $CD14^+$ cells in media containing RPMI-1640 media, supplemented with 10% heat-inactivated fetal bovine serum and 50 ng/mL of human GM-CSF (Novoprotein, China, CCR7) for 7 days.[26]

Generation of Lentivirus Expressing SNHG16 and Infection. The plasmid containing the SNHG16 encoding gene fragment (pcDNA3.1-lncRNA SNHG16) was provided by Prof. Qifeng Yang lab at Shandong University.[8] To construct lentivirus overexpressing SNHG16, the DNA fragment of the SNHG16 encoding gene was digested from the plasmid of pcDNA3.1-lncRNA SNHG16 and was cloned into pCDH-CMV-MCS-EF1-puro lentiviral vectors. Lentiviral shRNA for knocking down SNHG16 was cloned into pLKO.1-puro. For the control group, the empty lentiviral vector of pCDH-CMV-MCS-EF1-puro and pLKO.1-puro were used in the assay. 293T cells were transfected with the lentivirus constructs using the transfection reagent (Yeasen, China) according to the manufacturer's protocol. After 24 h, media were discarded, and fresh media were added. On days 4 and 5, the cell suspension was collected, pooled, and filtered through a 0.45 mm filtration unit (Merck/Millipore, Germany). Then, the virus particles were harvested and stored for use as previously described.[26]

Mycobacteria Culture and Infection of Host Cells. The *M. bovis* bacillus Calmette−Guerin (BCG) Danish strain (ATCC 35733) and *M. tuberculosis* H37Rv were grown at 37 °C in Difco Middlebrook 7H9 broth or on Middlebrook 7H10 agar (BD, USA) supplemented with 10% oleic acid−albumin−dextrose−catalase-enriched Middlebrook (OADC) (BD, USA), 0.2% glycerol, and 0.05% Tween-80 for 3−4 weeks. *Mycobacteria* were cultured in the ABSL-II level laboratory of the Shanghai Pulmonary Hospital.

The phorbol 12-myristate 13-acetate (PMA)-treated human macrophage cell line THP-1, $CD14^+$, and hMDM cells were infected with BCG at a multiplicity of infection (MOI) of 10 for 4 h. Cells of THP-1, $CD14^+$, and hMDM cells were infected with

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H37Rv at a MOI of 2 for 4 h. After infection, extracellular bacilli were removed by washing four times with PBS buffer.[26]

In the intracellular *Mycobacterial* growth inhibition assay, host cells were first transduced with lentivirus to increase SNHG16 expression (named lenti-SNHG16) or empty lenti-Control before being infected with BCG or H37Rv. The infected cells were cultured for 3 days or given time and then were lysed in sterile PBS with sodium dodecyl sulfate (SDS; Sigma-Aldrich) in a working concentration of 0.3 g/L. Serial dilutions were performed for quantitative culturing. *Mycobacteria* viability was quantified via counting CFUs.[37]

Real-Time Quantitative Polymerase Chain Reaction (q-PCR) Analysis for Gene Expression. Total ribonucleic acid (RNA) was extracted from PBMCs or freshly isolated cell subsets using RNA column enrichment procedures (Zymo Research, USA). RNA was reverse-transcribed to complementary DNA (cDNA) (TaKaRa, Japan). The cDNA was used to amplify the target gene fragment in triplicate reactions for each gene (TaKaRa, Japan). Sequences of q-PCR primers are listed in Table 1. β-actin was used as an internal control gene for normalization.

Total miRNA was extracted from freshly isolated PBMCs or THP-1 cells using a miRcute miRNA isolation kit (Tiangen, China). Relevant cDNA was synthesized with a miRcute miRNA first-strand cDNA synthesis kit (Tiangen, China). q-PCR primers were purchased from a company (Tiangen, China). The q-PCR was performed using the miRcute Plus miRNA q-PCR kit (SYBR Green) (Tiangen, China). The relative expression level of miRNA was normalized by U6.

Western Blotting. hMDM cells were transduced by lenti-SNHG16 or empty lentivirus and infected with BCG at MOI = 10 for 4 h. After being cultured for 3 days, cells were lysed by incubation in the RIPA lysis buffer on ice for 5 min. Next, lysates were separated by SDS−PAGE and transferred to a polyvinylidene difluoride membrane (Merck/Millipore, Germany). After blocking with 5% BSA, the membrane was incubated with Abs against LC3 (Abcam, U.K.), P62 (Abcam, U.K.), or β-actin (Sangon Biotech, China) overnight at 4 °C, followed by incubation with the respective secondary Abs.[26]

ChIP Assay. ChIP was performed using the SimpleChIP Enzymatic Chromatin IP Kit (CST, USA) according to the manufacturer's protocol. 293T cells were incubated (10 min at room temperature) in 1% formaldehyde and added to the tissue culture medium, which was then quenched with glycine. Nuclei were then incubated with micrococcal nuclease (Boehringer) (25 units/ml) to generate chromatin fragments with average lengths of 100−500 bp. Sheared chromatin was immunoprecipitated by using specific antibodies, H3K4me3 (Active Motif, USA, 39915), H3K27me3 (Active Motif, USA, 39155), or Negative control IgG mAb (CST, USA, 3900) at 4 °C overnight followed by immobilized protein A presaturated with sheared salmon sperm DNA (2 h at 4 °C). These protein A agarose bead-bound complexes were pelleted and washed extensively by using low-salt buffer, high-salt buffer, LiCl buffer, and twice with 1 × 0.1 M Tris-Cl, pH 7.5/10 mM EDTA buffer. DNA−protein complexes were eluted from the immunoprecipitating antibody with 0.1 M $NaHCO_3$ containing 1% SDS, followed by incubation at 65 °C overnight in 200 mM NaCl to reverse cross-links. DNAs purified by proteinase K digestion, phenol−chloroform extraction, and ethanol precipitation were assayed by the PCR amplification target at IFN-γ, TNF-α, and Granzyme B/GZMB encoding gene promoters of *IFNG*, *TNFA*, and *GZMB*. The primers of target gene promoters are listed in Table 1.

Adoptive Cellular Transfer in H37Rv-Infected SCID Mice. Four-week-old female SCID mice (Gempharmatech Co., Ltd.) were infected via i.n. with 5×10^4 CFU of H37Rv in 20 μL of PBS. After 4 days, 1×10^7 PBMCs transduced with lenti-SNHG16, empty lentivirus, or treated with the transduction medium for 3 days were transferred into each H37Rv-infected recipient mouse via i.p. injections in 0.2 mL of PBS. At day 28 postinfection, the lungs of all mice were harvested, homogenized in PBS, and plated on 7H10 agar at 10-fold series dilution to enumerate H37Rv bacilli. All animal experiments were approved by the Ethics Committee of SPH and performed according to the guidelines of the National Animal Protection and Ethics Institute.

Statistical Analysis. Statistical analysis was performed with GraphPad Prism 9.0. Differences between groups were assessed by *t* test, nonparametric *t* test, or one-way ANOVA, followed by Dunnett's test or Tukey's multiple comparison test indicated in each figure.

■ ASSOCIATED CONTENT

Data Availability Statement

NCBI GEO2R platform is open sources at https://www.ncbi.nlm.nih.gov/geo/. All data supporting the findings can be requested from the corresponding author.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c07053.

SNHG16 expression enhanced with mycobacteria infection; expression of miRNAs in H37Rv-infected macrophages; BCG growth in $CD14^+$ cells after the down-regulation of SNHG16; expression of CAMP and DEFB4A in $CD14^+$ cells after the down-regulation of SNHG16; expression of IFNG in human PBMCs after the down-regulation of SNHG16; cell numbers in mice lung sections; and frequencies of $CD14^+$, $CD4^+$, and $V\delta2^+$ cells after sorting by magnetic beads (PDF)

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Author Contributions

G.H., X.W., and X.J. have contributed equally to this work. G.H., X.W., and H.S.: conceptualization. G.H., X.J., and Y.P.: formal analysis. Q.S., W.S., H.S., and F.W.: funding acquisition. G.H., X.J., and Y.P.: investigation. G.H., X.J., and E.Y.: methodology. H.S.: project administration. X.W. and X.C.: resources. W.S., H.S.: supervision. X.J., J.W., L.Z., and Y.W.: validation. L.S. and H.S.: writing—original draft. H.S. and F.W.: writing—review and editing.

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Notes

The authors declare no competing financial interest.

This study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki. The studies involving human samples were approved by Ethics Committee of Shanghai Pulmonary Hospital (No. K22-143Z). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

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