This information is current as of May 1, 2017.

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*J Immunol* published online 27 April 2015
http://www.jimmunol.org/content/early/2015/04/25/jimmunol.1402557

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2015/04/25/jimmunol.1402557.DCSupplemental

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MicroRNA-125a Inhibits Autophagy Activation and Antimicrobial Responses during Mycobacterial Infection

Jin Kyung Kim,*† Jae-Min Yuk,*‡ Soo Yeon Kim,*† Tae Sung Kim,*† Hyo Sun Jin,*† Chul-Su Yang,§ and Eun-Kyeong Jo*†‡

MicroRNAs (miRNAs) are small noncoding nucleotides that play critical roles in the regulation of diverse biological functions, including the response of host immune cells. Autophagy plays a key role in activating the antimicrobial host defense against Mycobacterium tuberculosis. Although the pathways associated with autophagy must be tightly regulated at a posttranscriptional level, the contribution of miRNAs and whether they specifically influence the activation of macrophage autophagy during M. tuberculosis infection are largely unknown. In this study, we demonstrate that M. tuberculosis infection of macrophages leads to increased expression of miRNA-125a-3p (miR-125a), which targets UV radiation resistance-associated gene (UVRAG), to inhibit autophagy activation and antimicrobial responses to M. tuberculosis. Forced expression of miR-125a significantly blocked M. tuberculosis–induced activation of autophagy and phagosomal maturation in macrophages, and inhibitors of miR-125a counteracted these effects. Both TLR2 and MyD88 were required for biogenesis of miR-125a during M. tuberculosis infection. Notably, activation of the AMP-activated protein kinase significantly inhibited the expression of miR-125a in M. tuberculosis–infected macrophages. Moreover, either overexpression of miR-125a or silencing of UVRAG significantly attenuated the antimicrobial effects of macrophages against M. tuberculosis. Taken together, these data indicate that miR-125a regulates the innate host defense by inhibiting the activation of autophagy and antimicrobial effects against M. tuberculosis through targeting UVRAG. The Journal of Immunology, 2015, 194: 000–000.

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tophagy is a lysosome-mediated catabolic process that is important for the degradation of unwanted cytoplasmic cargos and the recycling of nutrients for cell survival (1). Accumulating data have revealed the crucial and varied roles of autophagy in human health and disease (2, 3). The autophagy pathway plays diverse roles in the immune response to pathogenic infection (4, 5). A unique function of autophagy is cell-autonomous elimination of intracellular pathogens, including Mycobacterium tuberculosis (6–9). M. tuberculosis is a successful human pathogen that infects and resides in macrophages, where it avoids elimination by arresting phagosomal acidification and transit into a stage of dormancy (10, 11).

The process of macroautophagy is divided primarily into three steps: 1) induction (formation of phagophore), 2) elongation (autophagosome formation), and 3) maturation (fusion of autophagosomes with lysosomes) (12). Numerous autophagy-related genes (Atgs) are involved in each step of the autophagic process, playing key roles not only in the classical activation of macroautophagy but also in the process of phagosomal maturation during microbial invasion (13, 14). Among the Atgs, UV radiation resistance-associated gene (UVRAG), a binding partner of the Beclin 1–class III PI3K complex, is essential for autophagy induction, because of its role in enhancing autophagosome formation (15). Additionally, UVRAG is involved in the late stages of autophagic maturation through interaction with the core class C Vps complex to promote Rab7 GTPase activity (16). It also promotes endocytic vesicle trafficking to accelerate the lysosomal degradation of endocytic cargos (16).

MicroRNAs (miRNAs) are small noncoding RNA molecules (~22 nucleotides in length) that regulate gene expression at the posttranscriptional level. Through binding to the 3′ untranslated regions (UTRs) of target miRNAs, miRNAs typically lead to suppression of protein translation or mRNA degradation (17, 18). Additionally, miRNAs are crucial regulators involved in modulating a variety of biological pathways involved in development, growth, homeostasis, immune regulation, and disease progression (19). Emerging evidence indicates that miRNAs also play critical roles in regulating pathways related to autophagy, especially in cancer (20–22); however, the specific role of miRNAs in relation to the regulation of autophagy during M. tuberculosis infection is largely unknown. The levels of several miRNAs are increased in M. tuberculosis–infected macrophages (23) in serum

[202x80]; UTR, untranslated region; [228x345]Mycobacterium; [263x356](autophagosome formation), and 3) maturation (fusion of autophagosomes with lysosomes) (12). Numerous autophagy-related genes (Atgs) are involved in each step of the autophagic process, playing key roles not only in the classical activation of macroautophagy but also in the process of phagosomal maturation during microbial infection (13, 14). Among the Atgs, UV radiation resistance-associated gene (UVRAG), a binding partner of the Beclin 1–class III PI3K complex, is essential for autophagy induction, because of its role in enhancing autophagosome formation (15). Additionally, UVRAG is involved in the late stages of autophagic maturation through interaction with the core class C Vps complex to promote Rab7 GTPase activity (16). It also promotes endocytic vesicle trafficking to accelerate the lysosomal degradation of endocytic cargos (16).

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samples or PBMCs from tuberculosis patients (24, 25). The expression of hsa-miR-21 was reported to be upregulated in Mycobacterium leprae-infected monocytes (26). Among several miRNAs increased in pulmonary tuberculosis patients (27), we searched for candidate miRNAs that potentially target Atgs and investigated the role of miRNA-125a-3p (miR-125a), which targets UVRAG, in modulation of autophagy and antimicrobial responses during M. tuberculosis infection. We found that infection of macrophages with M. tuberculosis resulted in upregulation of miR-125a through targeting UVRAG, inhibiting autophagosome formation, and promoting intracellular growth of M. tuberculosis. We also investigated the effects of several autophagy-activating drugs or agents on the regulation of miR-125a expression in macrophages infected with M. tuberculosis, and found that a mechanism involving the AMP-activated protein kinase (AMPK) pathway is required for the inhibition of miR-125a expression.

Materials and Methods

M. tuberculosis culture

M. tuberculosis H37Rv was provided by R.L. Friedmann (University of Arizona, Tucson, AZ). Mycobacteria were grown in Middlebrook 7H9 (Difco, 271310) medium supplemented with 10% oleic albumin dextrose catalase (OADC), 5% glycerol, and 0.05% Tween 80. The hanced red fluorescent protein (ERFP) was described previously (28). The M. tuberculosis genetically incorporated with the ERFP gene was cultivated in 7H9-OADC plus kanamycin (Sigma-Aldrich, 60615). For all assays, mid-log phase cells (multiplicity of infection 0.4) were used. Bacterial strains were divided into 1 ml aliquots and stored at −70°C. The CFUs were counted on Middlebrook 7H10 agar (Difco, 262710).

Mice and cell culture

Wild-type (WT) C57BL/6 mice were purchased from DBL (Chungbuk, Korea). Trl2, Myd88, and Toll/IL-1R domain-containing adapter inducing IFN-β (Trif)-deficient mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan). The murine macrophage cell lines RAW264.7 (American Type Culture Collection, TIB-71) and J774A.1 (American Type Culture Collection, TIB-67) were maintained in DMEM (Lonza, 12-604F) containing 10% FBS, penicillin (100 IU/ml), and streptomycin (100 µg/ml). Cells were incubated in a humidified atmosphere at 37°C with 5% CO2. For in vivo experiments, C57BL/6 mice and cultured in DMEM for 3–5 d in the presence of M-CSF (R&D Systems, 416-ML). This study was approved by the Institutional Review Board of Chungnam National University. All animal experiments were performed in accordance with the guidelines of the Korean Food and Drug Administration.

M. tuberculosis infection in vitro and in vivo

For in vitro experiments, cells were infected with M. tuberculosis or M. tuberculosis–ERFP at a multiplicity of infection (MOI) of 1:1, 1:5, or 1:10 for 2–4 h. Then, cells were washed with PBS to remove extracellular bacteria, supplied with fresh medium, and incubated at 37°C in the presence of 5% CO2. For in vivo experiments, C57BL/6 mice were i.v. injected with M. tuberculosis (1 × 10^7 CFU/mouse). After 3 wk, mice were sacrificed for harvesting of the lungs, spleens, and livers. Mice were maintained in biosafety level 3 laboratory facilities. The experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Bioleaders Corporation (Daejeon, Korea, protocol no. BLS-ABSL3-13-11).

Reagents, plasmids, and Abs

5-Aminomidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, Apo978), isoniazid (INH, 13377), 3-methyladenine (3-MA, M9281), and DAPI (D9542) were purchased from Sigma-Aldrich. Compound C (171260) and rapamycin (535211) were purchased from Calbiochem. The miR-125a mimic (5′-ACAGUGGGAGUCCUGUGGAGCC-3′) and miR-125a inhibitor (5′-GGCCUCUCGAAUCCUGGCUG-3′) were purchased from Genomed (Solul, Korea). The hemagglutinin (HA)-tagged mouse UVRAG cDNA construct was provided by Dr. J.U. Jung (University of Southern California, Los Angeles, CA). The plasmid encoding mRFP-GFP tandem fluorescent-tagged microtubule-associated protein 1 L chain (3′LC3) (mRFP-GFP-LC3) was provided by Dr. T. Yoshimori (Osaka University, Osaka, Japan). Anti-LC3 (L8918) for Western blotting were purchased from Sigma-Aldrich. Anti-UVRAG (ab70807) was purchased from Abcam. Anti–β-actin (sc-1616) was purchased from Santa Cruz Biotechnology. Alexa Fluor 488–conjugated anti-rabbit IgG (A17041) was purchased from Molecular Probes.

Bioinformatics analysis

Identification of the putative miRNA target was performed using miRanda algorithm (http://www.microrna.org) and TargetScan (http://www.targetscan.org).

RNA preparation, quantitative real-time PCR, and Western blotting

Total RNA was isolated using TRIzol reagent (Qiagen, 79300), and purity of miRNA was performed using the the miRNeasy mini kit (Qiagen, 217004). For the quantification of miR-125a, cDNA was synthesized by reverse transcription using the miScript II RT kit (Qiagen, 218161). Quantitative real-time PCR (qRT-PCR) was carried out using the miScript SYBR Green PCR kit (Qiagen, 218073) with thermal cycling conditions of 95°C for 30 s, followed by 50 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Small nuclear RNA (RNU6) was used for normalization. For qRT-PCR, the primer sequences were as follows: mi-r125a (5′-ACAG-GUGAGGGUCCUGUGGAGCC-3′, Qiagen, MS00011088), miRvug (forward, 5′-GCGAGCCCGAGACTTTGA-3′, reverse, 5′-CATCGTGA-CGTGGCACACAG-3′), and mAmphk (forward, 5′-GATCGGCCGCTACATCTG-3′, reverse, 5′-CAAAGGAGGCGTTCTCGAT-3′), mCdhb (forward, 5′-CTATGAGTGAGCCTTGATGGAG-3′, reverse, 5′-CCT-AGAAGACATTGGCCTGACAGT-3′), and mGp4d (forward, 5′-TGGCAAAAGGAGGATTGTTGCC-3′, reverse, 5′-AGAATGGTGGAT-GGCTTCCCG-3′). For Western blot analysis, proteins were loaded onto 12 or 15% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (Millipore, IPVH0010). Membranes were incubated with anti-UVRAG, anti-LC3, and anti–β-actin. Immunoreactive band analysis was conducted using ECL reagent (Millipore, WBKLI 80 500). ImageJ software was used for densitometric analysis of the blots and film in this study.

Cell transfection

Oligonucleotides were transfected into BMDMs, RAW264.7 cells, or J774A.1 cells using Lipofectamine 2000 (Invitrogen, 12566014) according to the manufacturer’s instructions.

CFU assay

To assay bacterial viability within macrophages, cells were infected with M. tuberculosis for 4 h and then washed with PBS to remove extra-cellular bacteria. Thereafter, the infected cells were incubated for the indicated time periods. The intracellular bacteria were harvested and inoculated onto Middlebrook 7H10 agar plates containing OADC. After 3 wk, colonies were counted.

Mouse UVRAG/AMPK lentiviral short hairpin RNA production and transduction

Lentivirus production was performed as described previously (7). Briefly, to produce lentiviral short hairpin RNA (shRNA), packing plasmids (pRSV-Rev, pMD2.G, VSVG, and pMID/pRRE purchased from Addgene) and pLKO.1-based target shRNA plasmids (mUVRAG, sc-70884-SH; mAMPK, sc-29674-SH purchased from Santa Cruz Biotechnology) were cotransfected into HEK293T cells using Lipofectamine 2000. Then, 72 h later, the virus-containing supernatant was collected and filtered. For lentivirus infection, BMDMs in DMEM containing 10% FBS were seeded into 24-well plates and infected with lentiviral vectors (MOI of 10) using Polybrene (Sigma-Aldrich, H9268), according to the manufacturer’s protocol. After 2 d, the samples were analyzed for transduction efficiency.

Autoptaphy analysis

LC3 punctate staining and quantification, followed by confocal microscopic analysis, were performed as described previously (7). After the appropriate treatment, BMDMs on coverslips were washed with PBS, fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.25% Triton X-100 in PBS for 10 min, and incubated with anti-LC3 (MBL International, PMD06) for 2 h at room temperature. Cells were washed to remove excess primary Ab and incubated with the appropriate fluorescently labeled secondary Ab for 2 h at room temperature. Nuclei were stained by incubation with DAPI for 5 min. After mounting, fluorescence images were acquired with a confocal laser-scanning microscope (Zeiss, Jena, Germany).
quantitate autophagy, we used fluorescence microscopy to count the number of endogenous LC3 punctate dots using the ImageJ analysis software. Each condition was assayed in triplicate, and at least 200 cells per well were counted. LC3-II protein levels were evaluated by Western blot analysis using an Ab against LC3 (Sigma-Aldrich, L8918). For autophagic flux analysis, RAW264.7 cells were transiently transfected with mRFP-GFP-LC3 and the miRNA control/mimic/inhibitor and then analyzed as previously described (29).

**Plasmid constructs and luciferase assay**

The Uvrag 3'UTR WT, containing the miR-125a–binding elements, were obtained by PCR. A mutant form was cloned into a pmirGLO vector by site-directed mutagenesis using a WT clone. The RAW264.7 cells were seeded into a 12-well plate and cotransfected with the WT or mutant-binding elements together with the control or mimic of miR-125a, respectively, using a Lipofectamine 2000. At 24 h after transfection, a luciferase assay was performed using the Dual-Luciferase reporter system (Promega), according to the manufacturer’s instructions.

**Statistical analysis**

All of the data are presented as mean ± SD of independent experiments. For statistical analyses, paired Student t tests with Bonferroni adjustment were performed. Differences were considered statistically significant at p < 0.05.

**Results**

**M. tuberculosis infection robustly increases miR-125a expression in macrophages in vitro and in mouse lungs in vivo**

We first determined whether miR-125a expression is significantly increased in vivo and in vitro after mycobacterial infection. To examine this, we infected with *M. tuberculosis* in murine BMDMs and RAW264.7 cells and measured the expression of miR-125a using qRT-PCR analysis. As shown in Fig. 1A, *M. tuberculosis*–infected BMDMs showed a gradual increase in expression of miR-125a in an MOI-dependent manner. Additionally, qRT-PCR demonstrates that *M. tuberculosis* infection increased miR-125a expression in both BMDMs and RAW264.7 cells (Fig. 1B, 1C). The peak miR-125a levels were detected 12–24 h after *M. tuberculosis* infection (Fig. 1B, 1C). Moreover, at 3 wk postinfection, the miR-125a levels were highly upregulated in different mouse tissues (Fig. 1D; spleens, lungs, and livers).

The recruitment of adaptor molecules MyD88 or TRIF to TLR after engagement of ligands is crucial for the activation of a variety of inflammatory mediators in macrophages after mycobacterial infection (30). We thus examined the roles of TLR2 and adaptor molecules (MyD88 and TRIF) in the synthesis of miR-125a in BMDMs after *M. tuberculosis* stimulation. Compared with WT BMDMs, the miR-125a levels in *Tlr2*− or *Myd88*−/−-deficient BMDMs were significantly decreased by *M. tuberculosis* infection (Fig. 1E). However, the miR-125a levels in *Trif*-deficient cells were not different from those in WT cells (Fig. 1E). These data show that *M. tuberculosis* infection increases the expression of miR-125a and that this is dependent on TLR2 and MyD88 in macrophages.

Uvrag mRNA is a direct target of miR-125a

To identify specific mRNA targets with miR-125a–binding sites, we performed a bioinformatic analysis using miRanda algorithm (http://www.microRNA.org/) and TargetScan (http://www.targetscan.org/). When we evaluated the scores of specific mRNA targets that potentially have miR-125a–binding sites by bioinformatic analysis, all five mRNA targets involved in autophagy were found in the high-scoring group (Supplemental Table I). RNA accessibility, which is critical for miRNA target recognition, could be calculated by the free energy (ΔG) of the 70 nucleotides flanking the 5' and 3' sides of the predicted miRNA binding sites (31, 32). When the ΔG was calculated using nucleotide sequences surrounding the miR-125a binding sites (http://www.bioinfo.rpi.edu/applications/), the predicted mouse gene Uvrag had the greatest ΔG among five mRNA targets (Supplemental Table I; ΔG = −11.2 kcal/mol). Uvrag mRNA was predicted to be a potential miRNA target of *Mus musculus* mmu-miR-125a (MIMAT0004528) through interaction

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**FIGURE 1.** *M. tuberculosis* infection robustly increases miR-125a expression in macrophages in vitro and in mouse lungs in vivo. (A) BMDMs were infected with *M. tuberculosis* at different MOI for 4 h. (B and C) BMDMs (B) and RAW264.7 cells (C) were infected with *M. tuberculosis* (MOI of 10) for the indicated periods, and miR-125a expression was subsequently examined using qRT-PCR. (D) The expression levels of miR-125a were measured in the spleens, livers, and lungs of normal or *M. tuberculosis*–infected C57BL/6 mice. (E) WT, *Tlr2*−/−, *Myd88*−/−, or *Trif*−/− mice were infected with *M. tuberculosis*, and levels of miR-125a in BMDMs were determined by qRT-PCR analysis. All data above represent mean ± SD from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 two-tailed Student t test. Mtb, *M. tuberculosis*.
with complementary sequences from 503 to 530 at the 3' UTR of mouse Uvrag (Fig. 2A). The 3' UTR of Uvrag was cloned into a luciferase reporter construct (pmirGLO) and reporter assays were performed in RAW264.7 cells. Using 3' UTR luciferase reporter assays, we demonstrated miR-125a–dependent suppression of luciferase activity in RAW264.7 cells expressing the Uvrag 3' UTR reporter (Fig. 2B). Additionally, mutation of the miR-125a–binding site abrogated this inhibition of luciferase activity (Fig. 2B), confirming that UVRAG is a putative target of miR-125a.

To establish the specificity of miR-125a, we further examined whether overexpression of miR-125a decreases UVRAG protein expression in BMDMs. The quantitative densitometric data analysis from the proteins on the Western blot showed that transfection with an miR-125a mimic resulted in a significant decrease in UVRAG protein expression in BMDMs at 6 and 12 h after M. tuberculosis infection (Fig. 2C, 2E; miRNA transfection efficiency is shown in Supplemental Fig. 1A). In contrast, UVRAG levels were significantly increased in uninfected and M. tuberculosis–infected BMDMs, after endogenous miR-125a was blocked by the transfection of an miR-125a inhibitor (Fig. 2D, 2F; quantitative densitometric data analysis; miRNA inhibitor transfection efficiency is shown in Supplemental Fig. 1B). A similar modulation of UVRAG protein expression by overexpression of an miR-125a mimic or inhibitor was observed in RAW264.7 cells before or after M. tuberculosis infection (Supplemental Fig. 2; miRNA transfection efficiency is shown in the left panels). Additionally, we examined whether overexpression of the UVRAG protein rescued the miR-125a–mediated autophagy inhibition in macrophages. As shown in Fig. 2G, transfection of the UVRAG expression plasmid in J774A.1 cells was able to overcome the inhibitory effects of miR-125a in M. tuberculosis–induced autophagy, demonstrating that UVRAG is the target of miR-125a in autophagy inhibition. It was also noted that miR-125a had no effect on the levels of Uvrag mRNA expression in uninfected or M. tuberculosis–infected BMDMs (Fig. 2H). Taken together, these results indicate that miR-125a
posttranscriptionally inhibits UVRAG expression through a direct interaction with its 3’UTR binding site.

miR-125a inhibits induction of autophagy in macrophages

UVRAG plays an essential role in the initiation of autophagy through an association with the Beclin 1/Bcl-2/PI(3)KC3 multi-protein complex (15). Because UVRAG is a target of miR-125a, we examined whether miR-125a regulates the activation of autophagy in macrophages during M. tuberculosis infection or following rapamycin treatment. To examine this, we evaluated the effects of an miR-125a mimic or inhibitor on autophagosome formation by counting LC3 puncta in BMDMs after M. tuberculosis infection. Immunofluorescence analysis revealed that transfection with an miR-125a mimic significantly decreased the number of LC3 puncta in BMDMs at 4 h after M. tuberculosis infection, when compared with the control condition (Fig. 3A). Conversely, the number of LC3+ puncta was greater in M. tuberculosis–infected BMDMs transfected with an miR-125a inhibitor, compared with the control condition (Fig. 3A).

Activation of autophagy requires the Atg3- and Atg7-mediated conjugation of LC3-I to the membrane lipid phosphatidylethanolamine, resulting in the formation of LC3-II (33). Transfection with an miR-125a mimic significantly decreased, whereas transfection with an miR-125a inhibitor significantly increased, the levels of LC3-II/I in BMDMs before and after M. tuberculosis infection (Fig. 3B, 3C: lower, quantitative densitometric data analysis; transfection efficiency of the miRNA mimic and inhibitor is shown in Fig. 3D, 3E). A similar result was observed in RAW264.7 cells (Fig. 3F, 3G).

We further investigated the role of miR-125a on the activation of autophagy in macrophages using rapamycin, a classic autophagy inducer. A similar reduction in the levels of LC3-II/I was detected in BMDMs transfected with miR-125a mimic, whereas increased levels of LC3-II/I resulted from transfection of BMDMs with an miR-125a inhibitor, after rapamycin treatment (Supplemental Fig. 3A, 3B). Moreover, in BMDMs, the miR-125a mimic decreased the number of rapamycin-induced LC3 puncta dots, whereas the miR-125a inhibitor increased the number of puncta (Supplemental Fig. 3C, 3D). These data collectively demonstrate that miR-125a suppresses the activation of autophagy induced by M. tuberculosis infection or by the classical autophagy activator rapamycin in macrophages.

miR-125a inhibits the colocalization of autophagosomes and bacterial phagosomes and enhances intracellular mycobacterial survival in macrophages

Activation of autophagy initiates a bactericidal process that involves the maturation of mycobacterial phagosomes (6). We thus investigated whether miR-125a affected the colocalization of LC3 autophagosomes with mycobacterial phagosomes in BMDMs. As shown in Fig. 4A and 4B, transfection of BMDMs with an miR-125a mimic significantly decreased the colocalization of M. tuberculosis phagosomes and autophagic structures in BMDMs, whereas transfection with an miR-125a inhibitor had the converse effect.

We further examined whether the colocalization of autophagosomes with M. tuberculosis phagosomes affected intracellular mycobacterial growth. We first established the effects of miR-125a on the intracellular growth of M. tuberculosis in RAW264.7 cells. As shown in Fig. 4C, transfection of RAW264.7 cells with an miR-125a inhibitor significantly inhibited intracellular mycobacterial growth, whereas transfection with an miR-125a mimic increased this growth, as evidenced by the CFU assay. Additionally, transfection with an miR-125a mimic significantly increased the viability of intracellular bacteria in BMDMs, whereas the miR-125a inhibitor decreased the intracellular survival of M. tuberculosis in BMDMs at 3 d postinfection (Fig. 4D). A representative qRT-PCR analysis of the transfection efficiency of the miRNA mimic and inhibitor is shown in Fig. 4E. These data together demonstrate that miR-125a decreases both M. tuberculosis phagosomal maturation and intracellular antimicrobial activity in macrophages.

Autophagy activators suppress miR-125a expression in M. tuberculosis–infected macrophages

We next examined whether known autophagy activators affect miR-125a synthesis in macrophages. AMPK is an important serine-threonine kinase pathway with roles in the regulation of host energy homeostasis and autophagy (34, 35). Our previous studies demonstrated that AICAR, an analog of AMP and activator of AMPK, is a potent activator of antibacterial autophagy during M. tuberculosis infection (7). Additionally, rapamycin is a well-known autophagy activator through inhibition of mammalian target of rapamycin activation and activation of the AMPK pathway (36). We thus used qRT-PCR to investigate the effects of autophagy activators (AICAR or rapamycin) on the expression of miR-125a in primary BMDMs infected with or without M. tuberculosis. Treatment of M. tuberculosis–infected BMDMs with autophagy inhibitors of M. tuberculosis–infected BMDMs resulted in the expression of miR-125a in BMDMs in a dose-dependent manner (Fig. 5A, 5B).

Because our recent studies demonstrated that AMPK activation is involved in AICAR-mediated activation of antibacterial autophagy during M. tuberculosis infection (7), we examined the function of AMPK in the inhibition of miR-125a expression resulting from AICAR treatment. Interestingly, silencing of Ampk by transduction of BMDMs with shRNA specific to Ampk (shAMPK) markedly abrogated AICAR-mediated inhibition of miR-125a expression (Fig. 5C). We also demonstrated that the inhibition of miR-125a levels by the AMPK activator AICAR was dose-dependently reversed by compound C, a pharmacological inhibitor of the AMPK pathway (Fig. 5D). These data suggest that AMPK activation plays an important role in AICAR-mediated inhibition of miR-125a synthesis in M. tuberculosis–infected BMDMs.

We further investigated the inhibitory role of miR-125a on the AICAR-induced autophagic flux in macrophages. Using mRFP-GFP-LC3 plasmid transfection, we measured mRFP-GFP-LC3 delivery to lysosomes in AICAR-treated RAW264.7 cells. As shown in Fig. 5E and 5F, both yellow and red punctae were increased in AICAR-treated RAW264.7 cells, similar to our previous findings (7). However, only yellow punctae increased upon transfection of the cells with the miR-125a mimic, suggesting that autophagosome maturation into autolysosomes was blocked by the overexpression of the miR-125a mimic. In contrast, the red punctae were markedly upregulated upon transfection of the cells with the miR-125a inhibitor, confirming that autophagic flux was increased by the inhibitor (Fig. 5E, 5F). Taken together, these data indicate that miR-125a overexpression blocks the AICAR-induced autophagic flux in macrophages.

UVRAG is required for activation of autophagy and phagosomal maturation in macrophages during M. tuberculosis infection

Numerous studies have demonstrated the role of UVRAG in the activation of autophagy; however, little is known about the specific role of UVRAG in the context of antibacterial autophagy during M. tuberculosis infection. We thus assessed the expression of UVRAG in macrophages before and after M. tuberculosis infection and examined whether UVRAG expression is upregulated in M. tuberculosis–infected BMDMs in response to treatment with activators of autophagy. Fig. 6A showed that the basal level of
UVRAG expression is very low, and that it is induced slightly by *M. tuberculosis* infection. However, treatment of uninfected or *M. tuberculosis*-infected BMDMs by AICAR markedly increased UVRAG protein expression in a dose-dependent manner (Fig. 6A). To examine the role of UVRAG in the activation of autophagy in *M. tuberculosis*-infected BMDMs, we blocked Uvrag mRNA expression by transducing BMDMs with shRNA specific to Uvrag (shUVRAG). Fig. 6B shows the transduction efficiency of shUVRAG.

We next quantified LC3 puncta formation in *M. tuberculosis*-infected BMDMs transduced with shUVRAG or nonspecific shRNA (shNS). We observed that *M. tuberculosis*– or *M. tuberculosis*–AICAR-induced formation of LC3³ autophagosomes was markedly decreased in BMDMs transduced with shUVRAG when compared with those transduced with shNS (Fig. 6C, 6D). Furthermore, as shown in Fig. 6E, silencing of UVRAG resulted in a significant decrease in the colocalization of LC3³ autophagosomes and mycobacterial phagosomes in *M. tuberculosis*-infected...
UVRAG is essential for the antimicrobial response to M. tuberculosis because of its role in the activation of autophagy.

The data above led us to examine the role of UVRAG in the antimicrobial response to M. tuberculosis infection. To examine the role of UVRAG in the antimicrobial response to M. tuberculosis infection, we transduced BMDMs with shUVRAG or shNS and then infected cells with M. tuberculosis in the presence or absence of 3-MA, an inhibitor of autophagy induction. BMDMs were then treated with AICAR or INH for 3 d and subjected to an intracellular CFU assay. Because we found that INH treatment led to the induction of UVRAG expression in M. tuberculosis–infected BMDMs (data not shown), we treated BMDMs with INH to examine the role of UVRAG in INH-induced antimicrobial responses.

As shown in Fig. 7, either AICAR or INH treatment resulted in an inhibition of intracellular mycobacterial survival in BMDMs after 3 d of infection, which is consistent with previous findings (7, 28). Additionally, 3-MA treatment led to an inhibition of the antimicrobial responses induced by AICAR or INH. Importantly, silencing of UvrAg in macrophages significantly abrogated the antimicrobial responses induced by AICAR or INH treatment (Fig. 7, for shUVRAG). It was also noted that intracellular mycobacterial growth in untreated or 3-MA–treated BMDMs was markedly increased in cells transduced with shUVRAG, when compared with those transduced with shNS (Fig. 7). These data collectively demonstrate that UVRAG is critically involved in the AICAR- or INH-induced M. tuberculosis killing effects in M. tuberculosis–infected BMDMs via a mechanism involving activation of autophagy.

**Discussion**

Accumulating evidence suggests that miRNAs are important translational regulators of genes involved in multiple facets of immune system function, including immune cell differentiation, effector function, and modulation of the host defense against pathogenic infection (37–39). Autophagy is fundamentally important for the maintenance of intracellular homeostasis and is required for the degradation of intracellular components via a lysosome-dependent pathway (40, 41). UVRAG is an essential molecule in the initiation and maturation steps of the autophagy pathway (15, 16). It is now evident that autophagy can target numerous intracellular bacteria, including the vacuolar pathogen M. tuberculosis (4–6). However, the biological roles of miRNAs in the specific context of autophagy during M. tuberculosis infection remain largely unknown. The current study demonstrates that miR-125a (miR-125a-3p; a mature form of miR-125a) can control the expression of UVRAG, thereby modulating activation of the autophagic pathway. We found that miR-125a modulated the induction of autophagy and the maturation of autophagosomes during M. tuberculosis infection by inhibiting UVRAG protein expression and this effect is mediated through the miR-125a complementary sequences contained in the 3’UTR of UvrAg.

We demonstrated that the expression of miR-125a is increased by M. tuberculosis H37Rv (a virulent strain of M. tuberculosis) infection in macrophages. In THP-1 macrophages infected with virulent (H37Rv) and avirulent (H37Ra) strains of M. tuberculosis, nine miRNA genes (miR-30a, miR-30e, miR-155, miR-1275, miR-3665, miR-3178, miR-4484, miR-4668-5p, and miR-4497) were differentially expressed (23). Both ESAT-6 and CFP-10 Ags are required for various pathogenic and immunogenic aspects of mycobacterial infection (42, 43). We also found that miR-125a expression in BMDMs was increased by stimulation with ESAT-6 or an avirulent (H37Ra) M. tuberculosis strain (data not shown). Recent study showed that miR-155 is highly upregulated in

**FIGURE 4.** miR-125a inhibits the colocalization of autophagosomes and bacterial phagosomes and enhances intracellular mycobacterial survival in macrophages. (A) BMDMs transfected with control, miR-125a mimic, or inhibitor were infected with M. tuberculosis–ERFP for 4 h, then fixed, and stained with an anti-LC3 Ab, followed by Alexa Fluor 488–conjugated goat anti-rabbit IgG to detect autophagosomes and DAPI. Scale bar, 5 μm. (B) The percentage of colocalization of LC3 and M. tuberculosis. (C and D) RAW264.7 cells (C) and BMDMs (D) were transfected with control, miR-125a mimic, or inhibitor and then infected with M. tuberculosis (for 24 h, RAW264.7 cells; for 3 d, BMDMs). Intracellular survival of M. tuberculosis was assessed by enumerating CFU. (E) The incubation time and culture conditions were the same as in (D). The expression levels of miR-125a in BMDMs by qRT-PCR analysis are shown. Data represent mean ± SD from at least three experiments. *p < 0.05, **p < 0.01, ***p < 0.001 two-tailed Student t test. Mtb, M. tuberculosis.
FIGURE 5. AMPK activation leads to inhibition of miR-125a synthesis and the overexpression of miR-125a blocks the AICAR-induced autophagic flux in macrophages. (A–D) The expression levels of miR-125a were measured by qRT-PCR analysis. BMDMs were infected with M. tuberculosis and then treated with AICAR (0.1, 0.5, or 1 mM) for 16 h (A) or rapamycin (10, 20, or 30 μg/ml) for 8 h (B). (C) BMDMs were transduced with shNS or shAMPK for 3 d. After transduction, cells were infected with M. tuberculosis and treated with AICAR (0 or 0.5 mM) for 16 h. Semi-quantitative RT-PCR analysis was performed to assess transduction efficiency (inset). (D) BMDMs were pretreated with the AMPK inhibitor compound C (5, 10, or 25 μM for 1 h) and then incubated with AICAR (0.5 mM) for 24 h. (E) RAW264.7 cells were cotransfected with control, miR-125a mimic, or inhibitor and an mRFP-GFP-LC3 and then treated with AICAR (0.5 mM) for 16 h. Cells were fixed and then analyzed for LC3 by confocal microscopy. Scale bar, 5 μm. (F) Quantification of yellow puncta/total red puncta (%) per cell. Data represent mean ± SD from at least three independent experiments (A–D and F). *p < 0.05, **p < 0.01, ***p < 0.001 two-tailed Student t-test, relative to the solvent control (A and B) and shNS (C). A, AICAR; CC, compound C; M, Mtb, M. tuberculosis; Rapa, rapamycin; SC, solvent control; shAMPK, short hairpin RNA specific to Ampkα; U, untreated.

RAW264.7 cells and BMDMs in response to M. tuberculosis infection and ESAT-6 stimulation, and it plays a role in the down-regulation of BTB and CNC homology 1 and SHIP1 to modulate host innate immune responses (44). Additionally, another study reported that miR-99b is highly upregulated in M. tuberculosis H37Rv–infected dendritic cells and macrophages, and it contributes to the enhancement of intracellular bacterial growth and downregulation of proinflammatory cytokines (45). Together with the present data, these studies suggest that the expression of multiple miRNAs is increased by M. tuberculosis infection, which may contribute to host evasion mechanisms.

Our data provide evidence for an important role for miR-125a in regulating UVRAG expression. We found that the enforced expression of miR-125a suppressed UVRAG protein expression, whereas transfection of cells with an inhibitor of miR-125a resulted in an increase in UVRAG expression in M. tuberculosis–infected macrophages. Additionally, a role for miR-125a in UVRAG suppression was shown in the inhibition of the activity of a reporter construct containing the consensus sequences for miR-125a. The inhibitory effect of miR-125a upon reporter activity was abolished in the absence of the miR-125a consensus sequences. An earlier report showed that UVRAG was a tumor suppressor candidate that played a role in Beclin 1–dependent autophagy through its interaction with Beclin 1 (15). Additionally, UVRAG is essential for the coordination of autophagic maturation and endocytic trafficking, promoting the transport of the endocytic cargo to the late endosomal/lysosomal compartments (16). Our data suggest that miR-125a is involved in targeting UVRAG to modulate its unique function in autophagy induction during mycobacterial infection.

The observed effects on UVRAG expression prompted us to examine whether miR-125a influences the activation of autophagy induced by M. tuberculosis, rapamycin, and the AMPK activator AICAR. We demonstrated that the autophagy induction and autophagic flux were inhibited by overexpression of miR-125a, whereas these were enhanced by inhibition of miR-125a by transfection with miR-125a inhibitors. Emerging evidence emphasizes the importance of the link between autophagy and miRNAs during pathogenic infections. Previous studies demonstrated that miR-155 promotes the
The autophagic response and maturation of bacillus Calmette–Guérin phagosomes through binding to the 3′-UTR of Ras homolog enriched in brain, a negative regulator of autophagy (46). Recent studies also showed that several pathogens, including mycobacteria, inhibit IFN-induced autophagy in macrophages through induction of miR-155 and miR-31 to sustain WNT and sonic hedgehog signaling (47). These mammalian target of rapamycin–mediated epigenetic modifications result in diminished cellular levels of PP2A, a phosphatase, followed by prolonged activation of WNT and sonic hedgehog signaling, leading to inhibition of IFN-induced JAK-STAT signaling, thus contributing to evasion of autophagy during infection (47). These results together establish a role for miRNA-mediated modulatory mechanisms employed by pathogens to fine-tune or escape from autophagy activation. Our data demonstrate that overexpression of the UVRAG protein in J774A.1 cells was sufficient to overcome the inhibitory effects of miR-125a on M. tuberculosis–induced autophagy, suggesting that UVRAG is the rate-limiting target of miR-125a for autophagy inhibition during M. tuberculosis infection.

**FIGURE 6.** UVRAG is required for activation of autophagy and phagosomal maturation in macrophages during M. tuberculosis infection. (A and B) Western blot analysis of UVRAG expression. (A) BMDMs were infected with M. tuberculosis and then treated with AICAR (0.1, 0.5, or 1 mM) for 16 h. (B) BMDMs were transduced with shNS or shUVRAG and then infected with M. tuberculosis. (C–E) BMDMs were transduced with shNS or shUVRAG for 3 d and then infected with M. tuberculosis–ERFP prior to AICAR treatment (0.5 mM for 16 h). (C) BMDMs were fixed and stained with an anti-LC3 Ab, followed by Alexa Fluor 488–conjugated goat anti-rabbit IgG and DAPI. The colocalization of M. tuberculosis with LC3 was detected by confocal microscopy. Scale bar, 5 μm. Quantitative analyses of LC3 punctae (D) and the percentage of colocalization of LC3 and M. tuberculosis (E) are shown. Data represent mean ± SD from at least three independent experiments (D and E). *p < 0.05, **p < 0.01 two-tailed Student t test, relative to shNS (D and E). A, AICAR; M, Mtb, M. tuberculosis; SC, solvent control; U, untreated.

**FIGURE 7.** UVRAG is essential for activation of antimicrobial responses against M. tuberculosis through a mechanism involving autophagy activation. (A and B) BMDMs were transduced with shNS or shUVRAG for 3 d and then infected with M. tuberculosis, followed by treatment with 3-MA (2 h, 10 μM). Cells were then washed and treated with AICAR (A) or INH (0.5 μg/ml) (B) for 24 h. Intracellular survival of M. tuberculosis was determined by CFU counts. Semiquantitative RT-PCR analysis was performed to assess transduction efficiency (upper). All data represent mean ± SD from three independent experiments. ***p < 0.001 two-tailed Student t test, relative to the shNS (A and B). SC, solvent control.
To date, multiple agents, cytokines, or pattern recognition receptor signals have been shown to potentiate antibacterial autophagy in response to *M. tuberculosis* infection, leading to intracellular killing of *M. tuberculosis* in macrophages (8, 48, 49). Our recent studies have shown that AMPK activation by AICAR leads to activation of autophagy and phagosomal maturation in *M. tuberculosis* infection (7). We found that either AICAR or rapamycin suppressed the expression of miR-125a in *M. tuberculosis*-infected macrophages. Additionally, the inhibition of miR-125a levels is mediated through AMPK activation, because knockdown of AMPK counteracted the inhibition of miR-125a expression. Moreover, UVRAG expression was upregulated in *M. tuberculosis*-infected BMDMs after AICAR treatment. These data suggest that the AMPK-induced autophagy activation is, at least partially, mediated through an inhibition of miR-125a and an enhancement of UVRAG expression during mycobacterial infection. Additionally, AMPK is known to play an essential role in metabolism and inflammation in immune cells and other cell types (34, 50). Thus, it would be of interest in future studies to examine whether AMPK-induced downregulation of miR-125a is associated with modulation of other biological functions during infection and inflammation.

So far, the exact function of UVRAG in relationship to the host defense against *M. tuberculosis* has been largely unknown. We found that UVRAG is essentially required for *M. tuberculosis* and AMPK-induced activation of autophagy as well as phagosomal maturation in macrophages. Additionally, knockdown of UVRAG enhanced intracellular mycobacterial growth and also attenuated the *M. tuberculosis* killing effects induced by autophagy-activating and antimicrobial agents. Previous studies showed that the interaction between UVRAG and the class C Vps complex is important for stimulation of Rab7 GTPase activity (16), which is essential for maturation of endosomes and autophagosomes, as well as in the fusion with lysosomes (51). It is known that virulent mycobacteria are able to arrest phagosomal-lysosomal fusion and interfere with the recruitment of Rab7 in mycobacterial phagosomes (52). Together with the current data, UVRAG plays a crucial role in the autophagy activation and antimicrobial responses during mycobacterial infection, presumably through promotion of Rab7 activity and the phagolysosomal fusion of *M. tuberculosis* in infected macrophages.

Previous studies demonstrated that miR-125a is associated with some human cancers, with roles in the regulation of apoptosis and tumor suppression (53–56). However, the role of miR-125a in the regulation of autophagy and host defense has not been reported. In this study, we demonstrated the previously unrecognized role of miR-125a in inhibition of autophagy activation and phagosomal maturation of *M. tuberculosis* in the host innate immune cells. Activation of autophagy by the AMPK pathway leads to an inhibition of miR-125a, but an upregulation of the expression of UVRAG, which is essential for *M. tuberculosis*-induced autophagy induction, phagosomal maturation, and antimicrobial responses.

Acknowledgments

We thank Dr. Joyoti Basu (Bose Institute, West Bengal, India) for critical reading of manuscript, Dr. H.-M. Lee (Chungnam National University, Daejeon, Korea) for helpful discussion and technical assistance, Dr. C.-H. Lee and Dr. D.-H. Choi (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea) for excellent technical assistance, Dr. R.L. Friedmann (University of Arizona, Tucson, AZ) for provision of mycobacterial strains, Dr. J.U. Jung (University of Southern California, Los Angeles, CA) for provision of HA-tagged UVRAG cDNA clone, Dr. S.-J. Shin (Yonsei University, Seoul, Korea) for provision of *M. tuberculosis*-ERFP, Dr. S. Akira (Osaka University, Osaka, Japan) for provision of TLR2, MyD88, and TRIF-deficient mice, and Dr. T. Yashimori (Osaka University) for provision of mRFP-GFP-LC3 plasmid.
Figure S1. MiR-125a mimic induces, but inhibitor reduces, expression levels in a dose-dependent manner in BMDMs. (A and B) BMDMs were transfected with miR-125a mimic (A) or inhibitor (B) at indicated concentration for 24 h. The expression levels of miR-125a were measured by real-time PCR. All data above represent the means ± SD from 3 experiments.
Figure S2. Effects of expression levels of miR-125a and UVRAG by mimic and inhibitor in RAW264.7 cells. (A and B) RAW264.7 cells were transfected with miR-125a mimic (A) or inhibitor (B) for 24 h. The expression levels of miR-125a were measured by real-time PCR (left). After transfection, the cells were infected with, or without Mtb and the cell lysates were analyzed by Western blot analysis using an anti-UVRAG antibody (right). Data are shown as mean ± SD of three independent experiments.
Figure S3. MiR-125a suppresses rapamycin-induced autophagy. (A and B) BMDMs were transfected with miR-125a mimic (A) or inhibitor (B) for 24 h. After transfection, the cells were treated with or without 20 μg/ml rapamycin. Cell lysates were analyzed by western blot analysis using LC3 antibody. (C) BMDMs were transfected with control, miR-125a mimic or inhibitor and then infected with Mtb for 4 h. The cells were fixed and stained with LC3 antibody followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG, and the colocalization of Mtb with LC3 was detected by confocal microscopy. (D) Quantitative data of LC3 puncta analysis in rapamycin-treated BMDMs. All data above represent the means ± SD from 3 experiments. **P < 0.01 (two-tailed Student’s t test), relative to control (D). Un, untreated; Rapa, rapamycin.
Supplemental Table 1. Predicted ΔG (-kcal/mol) of the 3' flanking regions, 70 nucleotides, of the potential mir-125a-3p targeting mRNA sites

<table>
<thead>
<tr>
<th>Predicted mouse gene</th>
<th>3' flanking region (70bp) ΔG (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVRAG</td>
<td>-11.16</td>
</tr>
<tr>
<td>ATG10</td>
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</tr>
<tr>
<td>ULK2</td>
<td>-10.78</td>
</tr>
<tr>
<td>ATG9a</td>
<td>-9.31</td>
</tr>
<tr>
<td>PTEN</td>
<td>-8.62</td>
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