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***Mycobacterium avium* MAV_2941 mimics Phosphoinositol-3-Kinase to interfere with macrophage phagosome maturation**

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Abstract

Mycobacterium avium subsp *hominissuis* (*M. avium*) is a pathogen that infects and survives in macrophages. Previously, we have identified the *M. avium* MAV_2941 gene encoding a 73 amino acid protein exported by the oligopeptide transporter OppA to the macrophage cytoplasm. Mutations in MAV_2941 were associated with significant impairment of *M. avium* growth in THP-1 macrophages. In this study, we investigated the molecular mechanism of MAV_2941 action and demonstrated that MAV_2941 interacts with the vesicle trafficking proteins syntaxin-8 (STX8), adaptor-related protein complex 3 (AP-3) complex subunit beta-1 (AP3B1) and Archain 1 (ARCN1) in mononuclear phagocytic cells. Sequencing analysis revealed that the binding site of MAV_2941 is structurally homologous to the human phosphatidylinositol 3-kinase (PI3K) chiefly in the region recognized by vesicle trafficking proteins. The β 3A subunit of AP-3, encoded by AP3B1, is essential for trafficking cargo proteins, including lysosomal-associated membrane protein 1 (LAMP-1), to the phagosome and lysosome-related organelles. Here, we show that while the heat-killed *M. avium* when ingested by macrophages co-localizes with LAMP-1 protein, transfection of MAV_2941 in macrophages results in significant decrease of LAMP-1 co-localization with the heat-killed *M. avium* phagosomes. Mutated MAV_2941, where the amino acids homologous to the binding region of PI3K were changed, failed to interact with trafficking proteins. Inactivation of the AP3B1 gene led to alteration in the trafficking of LAMP-1. These results suggest that *M. avium* MAV_2941 interferes with the protein trafficking within macrophages altering the maturation of phagosome.

Keywords

M. avium; macrophages; MAV_2941; PIK3C3; AP3B1; phagosome maturation

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1. Introduction

Mycobacterium avium subsp *hominissuis* (hereafter *M. avium*) is commonly associated with opportunistic infections in HIV-1-infected individuals, as well as in immunocompetent patients with underlying chronic lung pathology, and in apparently normal population [21]. Once within the host, *M. avium* infects preferentially mononuclear phagocytes, among other cells. The infection of macrophages is initiated by the binding to complement receptors CR3 and CR1, however, studies have demonstrated that phagocytosis *in vivo* is independent of complement receptors [7, 6, 9]. We have recently identified a new uptake mechanism of *M. avium*, which is dependent on a pathogenicity island [14]. This gene cluster encodes proteins that allow pathogen to enter both environmental amoeba and mammalian macrophages by binding to actin, triggering cytoskeleton rearrangement and phosphorylation of glyceraldehyde 3-phosphate dehydrogenase. Once within phagocytic cells, *M. avium* lives in a vacuole that does not acidify due to the prevention of the association with the H⁺ pump [31]. In addition, the *M. avium* vacuole does not fuse with lysosomes [11], although the mechanisms associated with the ability to interfere with intracellular trafficking have not been fully investigated.

More recently, we described the oligopeptide transporter, OppA, important for bacterial growth in macrophages and in mice. Further investigation revealed an *M. avium* MAV_2941 protein utilizing the OppA transport system for its translocation through the bacterial cell envelop [13]. MAV_2941 is a small protein only present in *M. avium* but not in *Mycobacterium tuberculosis*. In *Mycobacterium avium* subsp *paratuberculosis*, the MAV_2941 open reading frame is truncated and no protein is produced. Due to the observation that MAV_2941 is secreted in the cytoplasm of infected macrophages, and mutations in the functional gene resulted in attenuation of virulence in macrophages, it has been assumed that MAV_2941 has an important function associated with *M. avium* pathogenesis. In the current study, we identified a motif in MAV_2941 similar to PI3K and discovered that MAV_2941 interacts with host partners such as AP3B1, STX8 and ARCN1 trafficking proteins. Studies have demonstrated the role of PI3K in the regulation of endocytic membrane trafficking proteins [30]. The posttranslational modification of the adaptor protein complex AP-3 by inositol pyrophosphates has been documented as well [2]. The AP3B1 plays an essential role in protein sorting at early endosomes and Golgi, and is involved in the sorting of transmembrane proteins targeted to phagosomes and lysosomes [4]. It mediates the recruitment of Clathrin to the membranes, as well as LAMP-1 and LAMP-2 to endosomes [37]. STX8 protein, part of the SNARE complex, also interacts with endosomal membrane and vesicle-associated proteins [8]. The cytosolic vesicle transport protein ARCN1 binds to dilysine trafficking motifs and participates in the transport of proteins from the endosomal reticulum to the trans-Golgi network [36]. In this study, we established the role of MAV_2941 in the inhibition of normal phagosome maturation by demonstrating MAV_2941 binding to vesicle trafficking proteins via competition with PI3K for the target.

2. Materials and methods

2.1. Bacteria and vectors

M. avium 104 strain, serovar 1, is a clinical isolate obtained from a patient with AIDS. The bacterium has been characterized in several previous studies by our group and other laboratories [12, 34]. The *M. avium* MAV_2941 mutant has been previously described [13]. Bacteria were grown either on Middlebrook 7H10 agar (Hardy Diagnostics) or 7H9 broth (Hardy Diagnostics) depending on the experiment. The medium was supplemented with OADC (oleic acid, albumin, dextrose, and catalase, Hardy Diagnostics), kanamycin (Sigma) 400 µg/ml or ciprofloxacin (Sigma) 0.6 µg/ml according to the assay employed. All plasmid constructs used in this study are listed in Table 1.

2.2. Macrophages

THP-1 cells (ATCC) were used in described experiments. THP-1 monocytes were matured by treating them with 50 ng/ml of phorbol 12-myristate 13-acetate (Sigma) on 24- or 6-well tissue culture plates or 2-chamber slides. Maturation was allowed to occur for 48 h. Then adherent monolayers were washed and replenished with new RPMI-1640 (Life Technology) supplemented with 5% inactivated fetal bovine serum (GEMINI). Viability of cultured cell was determined by the exclusion of trypan blue.

2.3. The Matchmaker Gold Yeast Two-Hybrid vector constructs and screening

To determine the possible interacting partner(s) of MAV_2941 in macrophage cytosol, we initially performed a two-hybrid screening. The MAV_2941 gene was amplified with sense 5'-TTTGAATTCGGGCATGAACCGAAA-3' and antisense 5'-TTTGGATCCTCACCTGCCCCGAACG-3' primers under the following conditions: denaturation at 95°C for 3 min; amplification and quantification program repeated for 35 times: 95°C for 30 sec, 63°C for 30 sec, and extension at 72°C for 3 min. MAV 2941 gene was fused in frame with the GAL4 DNA binding domain by inserting the PCR-generated fragment into the *EcoRI* and *BamHI* sites of pGBKT7. The resultant pGBKT7:MAV_2941 vector was transformed into *Saccharomyces cerevisiae* strain Y2HGold using Yeastmaker Yeast Transformation System 2, according to the manufacturer's instructions (Clontech). The Normalized Mate & Plate™ Universal Human Library, constructed in fusion of GAL4 activation domain of pGADT7 and created in the yeast strain Y187, was purchased from the Clontech. Positive and negative control plasmids pGBKT7-53, pGBKT7-lam, and pGADT7-T were also obtained from Clontech. One ml of the Mate & Plate Library was combined with 4 ml of Bait Yeast Strain and grown in 2xYPDA liquid medium containing 50 µg/ml kanamycin at 30°C for 24 h with slow shaking (50 rpm). To eliminate false positive interactions, the AUR1-C, ADE2, HIS3, and MEL1 integrated reporter activations, controlled by the Gal4-responsive promoter sequences were screened according to the manufacturer's protocols (Clontech). The zygotes were plated on agar plates missing tryptophan (Trp), leucine (Leu), histidine (His), or adenine (Ade), more specifically, on Double Dropout (SD-Leu/-Trp), Triple Dropout (SD- His/-Leu/-Trp), and Quadruple Dropout (SD-Ade/-His/-Leu/-Trp) agar plates. All colonies grown on Quadruple Dropout plates were transferred onto plates containing SD/-Ade/-His/-Leu/-Trp with 20 mg/ml X-a-Galactosidase and SD/-Ade/-His/-Leu/-Trp/X-a-Gal plates with 125 ng/ml Aureobasidin.

The colonies that grew of blue color in presence of X-a-Gal and Aureobasidin were processed for identification of inserts using Matchmaker Insert Check PCR Mix 2 (Clontech). The positive-interacting plasmids were isolated using Easy Yeast Plasmid isolation kit (Clontech) and sequenced at the Center for Gene Research and Biocomputing (CGRB) facility, Oregon State University. Results were analyzed by comparison to the GenBank human database and three host proteins AP3B1, STX8 and ARCN1 were identified. To confirm the results obtained in initial screening, the interactions between MAV_2941 and host proteins were further performed with the following co-transfections of pGADT7:MAV_2941 with pGBKT7: AP3B1, pGBKT7: STX8 or pGBKT7: ARCN1 constructs in the corresponding yeast strains, using method described above.

2.4. MAV_2941 mutant construct

At first, we constructed the PCR-based mutations in the MAV_2941 gene by simply placing the desired nucleotide change in the primer sequence [13]. Next, we generated deletion of PRPP amino acids between 16–21aa position using the QuikChange® mutagenesis kit (Agilent Technologies) according to the manufacturers protocol. The sense 5'-CGAGCACCGTCCAGCCTCCCGACACATC-3' and antisense 5'-GATGTGTCGGGAGGCTGGACGGTGCTCG-3' primers were designed using the QuikChange® Primer Design Program. The mutations and deletion in MAV_2941 were confirmed by sequencing before cloning. Modified MAV_2941 gene was then cloned into pLenti6.3/V5-TOPO vector to express mutated protein in THP-1 cells as described below.

Lentiviral strategy for overexpression of proteins of interest in THP-1 cells—

Using the ViralPower™ HiPerform™ Lentiviral Expression and Delivery Systems (Life Technologies), we examined interactions between *M. avium* MAV_2941 protein and vesicle-trafficking AP3B1, STX8 and ARCN1 proteins as well as interactions between *Homo sapiens* phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3) and AP3B1, STX8 and ARCN1 proteins. The HIS-tagged *M. avium* MAV_2941 gene fused to Red Fluorescent protein was cloned and expressed in the virion vector pLenti6.3/V5-TOPO (Table 1). Two constructs of phosphatidylinositol 3-kinase containing an accessory domain (homologous with MAV_2941) and C2 catalytic domain (with no homology to MAV_2941) were HIS-tagged, cloned into the virion vector and named pLenti/HIS: PIK3C3 and pLenti/HIS: PIK3C3(–), respectively. The AP3B1, STX8 and ARCN1 gene sequences were fused with C-terminal V5-tag of pLenti6.3/V5-TOPO vector and resulting vectors were called pLenti/V5:AP3B1, pLenti/V5:SXT8, and pLenti/V5:ARCN1. The lentiviral expression constructs of pLenti/HIS:RFP, pLenti/HIS:RFP:MAV_2941 mutated and pLenti/HIS: PIK3C3(–) served as controls for protein-protein interaction studies. The lentiviral stocks of created constructs were produced in 293FT cell line, according to the manufacturer's protocol (Life Technology). Briefly, confluent monolayers of 293FT cells (80–90%) were transfected with the packaging mix (Life Technology) prepared as follow: 9 µg of Virapower^{MT} reagent and 3 µg of lentivirus expression plasmids were diluted in 1.5 ml Opti-MEM I medium without serum and then combined with 1.5 ml Opti-MEM medium containing 36 ml Lipofectamine 2000. The transfected cells were incubated at 37°C in 5% CO₂ and virus-containing supernatants were collected after 72h. Immunofluorescence microscopy was performed to observe protein co-localization in THP-1 cells infected with

lentiviral particles. The expression of AP3B1, STX8 and ARCN1 proteins was visualized using V5 primary (1:200 dilution) and FITC-conjugated secondary (1:1000 dilution) antibodies; Whereas, MAV_2941 and PIK3C3 proteins were observed using anti-HIS primary (1:200 dilution) and Texas-Red secondary antibodies (1:1000 dilution). All antibodies were purchased from Santa Cruz Biotechnologies (Dallas, Texas).

2.5. Pull-down assay

To confirm the protein interactions, THP-1 macrophages were seeded in 75 cm² flasks at 80 – 90% confluency and co-infected with lentiviruses expressing recombinant fusion proteins with the following combinations: RFP:MAV_2941 and AP3B1, STX8 or ARCN1 proteins; PIK3C3 and AP3B1, STX8 or ARCN1 proteins. The HIS:RFP and HIS:PIK3C3(–) were used as controls for the protein binding assay. Lentiviral co-infections with the mutated HIS:RFP:MAV_2941 and AP3B1, STX8 or ARCN1 were used as controls. Macrophages were lysed with CelLytic™ M reagent (Sigma), and HIS-tagged proteins were isolated directly from a crude cell lysate using 30 µl of paramagnetic pre-charged HIS-nickel particles, according to the manufacturer's protocol (Promega, Fitchburg WI). Briefly, macrophage lysates were incubated with the pre-charged HIS-nickel particles overnight at 4°C, and next day particles were pulled down with a magnetic holder. The captured complexes were washed three times with phosphate-buffered saline (PBS), resuspended and boiled in Laemmli sample buffer containing mercaptoethanol for 5 min. Proteins were resolved by electrophoresis in 12.5 % Tris-HCl gels and processed for western blot analysis. The HIS-tagged-interacting proteins were detected with V5 antibody (1:200, Santa Cruz Biotechnology).

2.6. Fluorescent imaging of LAMP1

Phagocytic cells (10⁵) were seeded in 2-chamber slides, and duplicate wells were infected with fluorescein-labeled live or heat-killed (100°C) *M. avium* 104 with multiplicity of infection [27] of 10 for 1 h at 37°C. Some monolayers were inactivated for AP3B1 with a gene specific siRNA or transfected with MAV_2941 recombinant protein before infection. The MAV_2941 recombinant protein was made as follow: the MAV_2941 gene was fused in frame with the TAT peptide/Protein Transducing Domain (PTD) and HIS-tag by inserting the PCR-generated fragment into the *EcoRI* and *HindIII* sites of pET28b TAT v1 vector kindly provided by Dr. Steven F. Dowdy at University of California, San Diego [35]. The overexpressed protein was purified with HIS-columns (Clontech). Forty eight hours after infection, cells were fixed in 4% formaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 10 min and blocked in 2% bovine serum albumin. Slides were then incubated with either rabbit anti-LAMP-1 or Rab5 antibody (1:200) (Santa Cruz) for 2h, washed three times and incubated with Texas-Red labeled goat anti-rabbit IgG (1:1000, Molecular Probe, Eugene OR) for 1h. The slides were mounted and observed under a Leica DM4000B fluorescent microscope (Leica).

2.7. Inactivation of AP3B1 by siRNA

LAMP-1 trafficking on the phagosome membrane has been demonstrated to be dependent on the presence of AP3B1[37]. To examine the role of AP3B1 in LAMP-1 localization

during *M. avium* infection, THP-1 macrophages were transfected with AP3B1 siRNA consisting of pool of five target-specific 19–25 nt siRNAs to knockdown gene expression (Santa Cruz Biotechnology). Transfection was performed using Santa Cruz Biotechnology reagents according to the manufacturer's recommendations. Briefly, macrophages (10^5 /ml) were seeded in 2-chamber or 6-well plate as described above, washed once with siRNA transfection medium and replaced with transfection medium containing AP3B1 siRNA transfection reagent. Control siRNA (scrabbled sequences)-transfection was used as negative control. AP3B1 and β -actin expressions were determined by western blot analysis. The silenced monolayers were then infected with heat-killed *M. avium* and visualized for Lamp1 marker.

2.8. Statistical Analysis

Each experiment, with two technical replicates, was repeated three times unless otherwise indicated. Comparisons between control and experimental groups were submitted to statistical analysis to determine the significance. The results express as means $\pm$ standard deviations analyzed by Student's t test. P values of <0.05 were considered significant.

3. Results

3.1. MAV_2941 is homologous to PI3K binding site

Since MAV_2941 is secreted in the cytosol of infected macrophages, and mutations in the functional gene result in deficiency of *M. avium* to grow as the wild-type bacterium inside the phagocytes, we hypothesized that MAV_2941 protein would interfere with the phagocyte function by mimicking an eukaryotic protein(s). To address this possibility, a genome-wide comparison was performed against human as well as amoeba, which is a host/reservoir for *M. avium* in the environment. We included non-annotated contigs of water- and soil-borne amoeba species in our search from the Sanger Institute (<http://www.sanger.ac.uk>) database. The search, using the *Dictyostelium discoideum* OmniBlast Server, identified high-scoring segment match between MAV_2941 protein and contig JC3V2, annotated as phosphatidylinositol kinase-like kinase (PIKK; Gene ID: 8623319 tra1) (Fig. 1A). The further structural homology search, using ExPASy Proteomics (<http://ca.expasy.org>) ClustalW alignment, identified *Homo sapiens* phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3) (Fig. 1B), a homolog to *D. discoideum* PIKK and structurally similar to *M. avium* MAV_2941 protein (Fig. 1C). Predicted 3D structure of MAV_2941 (Fig. 1D–a) was generated using SWISS_MODEL (<http://swissmodel.expasy.org>), BioMolecular Modeling (<http://bmm.cancerresearchuk.org/>) and POLIVIEW-3G (<http://polyview.cchmc.org>) software. The crystal structure of PIK3C3 protein (Fig. 1D–c) is available in the Protein Data Bank (PDB; <http://www.rcsb.org/pdb/home/home.do>). PyMOL molecular visualization software (<http://www.pymol.org>) was used to label homologous amino acids on both proteins in order to visualize the similar positioning of matching structures (Fig. 1D–b and 1D–d). As observed, there is a significant homology and similarity between MAV_2941, amoeba PIKK and its *Homo sapiens* homolog PIK3C3.

3.2. MAV_2941 interacts with vesicle trafficking proteins

To identify the interacting partner of secreted MAV_2941 protein [13], the gene was synthesized with a translational fusion of the DNA-binding domain and used as the bait in the Yeast-Two-Hybrid screening against the Mate & Plate™ Universal Human cDNA Library. Out of 5×10^6 transformants screened, 37 clones grew in the absence of tryptophan (Trp), leucine (Leu), histidine (His), adenine (Ade) and expressed X-a-Gal. These clones were further screened on 125 ng/ml Aureobasidin plates in the presence of X-a-Gal and absence of Ade, His, Leu, and Ttp. After elimination of 32 clones, the five positive clones were sequenced. The selected host proteins were additionally retested for specificity by constructing the host cDNAs in the bait pGBKT7 vector and MAV_2941 gene in the prey pGADT7 vector (Table 1). Three true positive clones, AP-3 complex subunit beta-1 (AP3B1), syntaxin-8 (STX8) and archain 1 (ARCN1) were identified to have specific interaction with MAV_2941 (Fig. 2A).

3.3. MAV_2941 binds to the vesicle trafficking proteins in macrophages

To further analyze the interaction of MAV_2941 with the vesicle trafficking proteins AP3B1, STX8 and ARCN1 in THP-1 phagocytes, we used the lentiviral expression system for co-localization studies (Fig. 2B), followed by the pulled-down assay (Fig. 3D). We also evaluated the interaction of the *Homo sapiens* PIK3C3 accessory domain of PI3K with the vesicle trafficking proteins AP3B1, STX8 and ARCN1 in phagocytic cells (Fig. 2C) and confirmed the interaction by pull-down assay (Fig. 2E). THP-1 cells expressing lentiviral particles of MAV_2941 as well as PIK3C3 (in red) showed granular fluorescence in the cytosol, and mainly co-localized with AP3B1, STX8 and ARCN1 (in green) as observed in the merged images of Figure 3B and C (in yellow). The Western blot analysis of HIS:RFP-fusion MAV_2941 and HIS-fusion PIK3C3, and bound AP3B1, STX8 and ARCN1 proteins (visualized with V5-tag) showed specific binding (Fig. 2D and E). Conversely, the pull-down assay from macrophages transfected with the control HIS:RFP protein did not result in specific binding to vesicle-trafficking proteins (Fig. 2D or F). The PIK3C3(−) binding assay was also performed as a control experiment to determine if the sequence that is not homologous to the MAV_2941 protein would interact with the vesicle-trafficking proteins. As shown in Figure 2E, PIK3C3(−) did not pull V5-tagged overexpressed AP3B1, STX8 or ARCN1 proteins. In addition, MAV_2941 with modifications in the amino acids homologous to the binding region of PIK3C3 were unable to bind to AP3B1, STX8 and ARCN1 (Fig. 2F).

3.4. MAV_2941 binding to AP3B1 prevents the association of LAMP-1 with phagosomes

In agreement with previous studies [23], only 17.5% of the wild type *M. avium* vacuoles in THP-1 phagocytes were associated with LAMP-1 (Fig. 3a and Table 2). In contrast, the co-localization of LAMP-1 with the heat-killed *M. avium* (Fig. 3b) was significantly greater (91%). The co-localization rate of LAMP-1 significantly decreased with heat-killed *M. avium* vacuoles in macrophages expressing the transfected MAV_2941 protein when compared with vacuoles containing heat-killed *M. avium* alone (Fig. 3c). In addition, because AP3B1 is essential for trafficking of cargo proteins (including LAMP-1) to the phagosome and lysosome-related organelles, we decided to examine LAMP-1 localization in

AP3B1-inactivated macrophages that was infected with the heat-killed *M. avium* (Fig. 3d). Using the siRNA interference system, we inhibited the expression of the AP3B1 gene. As shown in Figure 4, the protein level of AP3B1 was significantly reduced in macrophages transfected with siRNA when compared with macrophages transfected with the scrambled siRNA control. It became clear that in absence of AP3B1, LAMP-1 docking to the heat-killed fluorescein-labeled *M. avium* vacuoles was significantly lower (39.5% of the vacuoles, Fig. 3d and Table 2) than in wild-type macrophages infected with the heat-killed *M. avium* (91%, Fig. 3b and Table 2). As a control, we also visualized the distribution of mycobacteria and the early endosome trafficking protein Rab5 and determined percentage of acquired Rab5 in phagosomes containing fluorescein-labeled *M. avium*. As shown in Table 2, almost all mycobacteria-containing phagosomes were positive for Rab5 in all experimental groups, suggesting that MAV_2941 interference with AP3B1 protein results in defective trafficking of LAMP-1 in macrophages.

4. Discussion

Previous work has identified MAV_2941 as an *M. avium* secreted protein that translocate into the macrophage cytoplasm [13]. *M. avium* MAV_2941 is a small protein with sequence identity to the phosphatidylinositol kinase-like kinase (PIKK) of the environmental host amoeba *Dictyostelium*. The amoeba PIKK is similar to the accessory domain of *Homo sapiens* PIK3C3. Since *M. avium* infects amoeba in the environment, it is likely that the pathogen evolved strategies to survive in the environmental protozoan. In fact, similar phenomenon has been evidenced previously in a work describing *M. avium*-acquired genes that allow the bacterium to infect amoeba [19]. It is clear that the lifestyle in the amoeba environment has been a significant pressure for *M. avium* evolution. It has been shown that *M. avium* grows within *Acanthamoeba castellanii* and inhibits the fusion of lysosomes with vacuoles [10]. Both *Acanthamoeba* and *Dictyostelium* have endocytic pathways that resemble the ones in macrophages [8]. The function of phosphatidylinositol 3-kinase (PI3K) in amoeba has been shown to be quite similar to mammalian cells, including a role in actin polymerization, bacterial phagocytosis and endocytic compartment trafficking [19, 33]. *Homo sapiens* PIK3C3 is also known as hVPS34 (vacuolar protein sorting 34) and it is the only member of the class III PI3K. The function of PI3K in the phagosome maturation has been described [24], and many pathogens alter macrophage signaling at this level to divert phagolysosomal trafficking and normal phagosome maturation [25]. Upon infection with *M. tuberculosis*, PIK3C3 activity has a critical role in the recruitment of cytosol proteins to the early phagosomal membranes, controlling the phagosome maturation process [17]. *M. tuberculosis* lipoarabinomannan and PI3P phosphatase inhibit PI3P generation affecting the membrane trafficking and sorting events within the endosomal system, and consequently normal phagosome biogenesis [15]. *M. tuberculosis* infection promotes targeting of small GTPases to the phagosome membranes via lipid-mediated interaction with PI3K and, thus, altering the phagolysosome biogenesis [32].

Based on the motif similarity between MAV_2941 and the accessory domain in PIK3C3 [28], we hypothesized that MAV_2941 might have a motif that mimics the binding site of PIK3C3 and is potentially recognized by trafficking-associated host protein(s) partners. The observation that MAV_2941 enable the pathogen to survive in macrophages and the

identification of MAV_2941-interacting host molecules as the trafficking associated AP3B1, syntaxin-8 and archain 1 proteins, suggested that MAV_2941 most likely was competing for a common binding site of PI3K on the three identified trafficking proteins. The AP3B1 protein belongs to the family of cytosolic adaptor complexes involved in intracellular protein sorting and vesicle formation. AP3B1 facilitates vesicle trafficking from both the Golgi network and endosomal compartments [16]. It has been experimentally demonstrated that AP3B1 is involved in initiation of macroautophagy and biogenesis of lysosomes [20]. In addition, deficiency of AP3B1 has been shown to alter the distribution of SNARE proteins [26]. The SNARE proteins have been implicated in different intracellular trafficking steps and in mediating membrane fusions. Syntaxin 8 is one of the main components of the SNARE complex, with a role in the fusion of late endosomes and suppression of its function [1, 29]. *M. tuberculosis* has been shown to interact with SNARE proteins [17] and to alter phagosome maturation by blocking the syntaxin 6-dependent delivery of cargo [18]. ARCN1, a cytosolic protein associated with Golgi non-clathrin-coated vesicles, mediates protein transport through a trans-Golgi network and is involved in vesicular organelles, such as endosome and lysosome, trafficking[36]. The impairment in ARCN1 function results in defective intra-Golgi transport of resident proteins [36]. These three human proteins are highly conserved in *Dictyostelium* amoeba, and the role of PI3K in protein sorting and trafficking from endosomes to lysosomes has been well characterized in *D. discoideum* [5, 8].

It has been reported, that homozygous genomic deletion in AP3B1 is associated with defective organelle membrane protein trafficking [22], and with the misplacement of LAMP-1 and LAMP-2 on the cell surface instead of the vacuole membrane [37]. Our studies clearly demonstrated that MAV_2941 interferes with the host-cell trafficking proteins and consequently with the binding of LAMP-1 to the phagosome membrane. *M. avium* appears to use an additional mechanism when compared to *M. tuberculosis* to alter the phagosome maturation and fusion with lysosome, and the strategy was probably acquired in environmental amoeba and adapted to mammalian macrophages. The roles of STX8 and ARCN1 in *M. avium* infection are still unknown, and further investigation may reveal additional interactions.

In conclusion, we identified host trafficking proteins targeted by the secreted MAV_2941 effector protein and characterized a new mechanism by which MAV_2941 competes with similar domain in the binding of phosphatidylinositol 3-kinase to trafficking proteins subverting normal phagosome maturation process.

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Fig. 1. MAV_2941 protein structural homology to phosphatidylinositol 3-kinase
(A) Homology of *M. avium* MAV_2941 protein (A0QGT9) to the *Dictyostelium* amoeba PIKK (Q54T85) identified with WASH-U BLAST (gapped alignments) at ExPASy Proteomics Server. Alignment identified 24% identical and 24 similar positions. *, Exact match of amino acids; : Structurally highly similar amino acids; . Structurally similar.
(B) *Dictyostelium* PIKK alignment with the protein sequence of phosphoinositide-3-kinase from *Homo sapiens* (Q8NEB9) identified 14% identical and 54 similar positions.

(C) MAV_2941 homology to *Homo sapiens* PIK3C3 accessory domain with 14% identical and 27 similar positions.

(D) The predicted MAV_2941 (a) and annotated PIK3C3 (3LS8) (c) PDB files were imported into PyMOL molecular visualization software and homologous amino acids were labeled in pink. These labeled homolog sequences were then extracted from the protein structures to visualize similar positioning of the matching amino acids in MAV_2941 (b) and PIK3C3 (d) proteins.

Fig. 2. Protein-protein interaction studies for *M. avium* MAV_2941

(A) Interaction between *M. avium* and host proteins was identified using a yeast two-hybrid system. The Matchmaker Gold yeast two-hybrid reporter strain containing the bait pGBKT7:MAV_2941 construct was screened against the target universal human library and identified true positive AP3B1, STX8 and ARCNI clones on quadruple dropout medium SD/- Ade/-His/-Leu/-Trp supplemented with X-a-Gal and Aureobasidin A. To confirm the specificity of binding, reverse interactions were tested by constructing pGBKT7:AP3B1, pGBKT7:STX8 and pGBKT7:ARCNI bait and pGADT7:MAV_2941 prey vectors. The known interactions between murine p53 (53) and T-antigen (T), and lamin (Lam) and T-antigen serve as positive and negative controls, respectively.

(B) The interactions of MAV_2941 with vesicle-trafficking AP3B1, STX8 and ARCNI proteins, and (C) the interactions of *Homo sapiens* PIK3C3 with AP3B1, STX8 and ARCNI proteins were investigated by using the ViralPower™ HiPerform™ Lentiviral Expression and Delivery Systems (Life Technology), where *M. avium* and human genes construct vectors were expressed in pLenti6.3/V5-TOPO, transferred and delivered into the THP-1 cells. The AP3B1, STX8 and ARCNI proteins were visualized using V5 primary and FITC conjugated secondary antibodies; whereas, MAV_2941 and PIK3C3 proteins were developed with HIS primary and Texas-Red secondary antibodies. The resulting yellow dots

from the red- and green-labeled protein overlap on the merged micrographs indicate co-localization of the studied proteins. Bar, 10 μ m. **(D)** THP-1 cells co-expressing lentiviral constructs of HIS:RFP: MAV_2941 and V5:AP3B1, V5:STX8 or V5:ARCN1 proteins were lysed and pulled-down using the paramagnetic pre-charged HIS-nickel particles. Samples were then washed and processed for western blot analysis using HIS antibody. The HIS:RFP: MAV_2941 bound complexes visualized with V5 antibody confirmed binding of AP3B1, STX8 and ARCN1 proteins to *M. avium* protein. The pull-down with HIS-RFP was used as a control for this assay; **(E)** THP-1 cells co-expressing lentiviral constructs of HIS:PIK3C3 and V5:AP3B1, V5:STX8 or V5:ARCN1 proteins were lysed, pulled-down with HIS-nickel particles and visualized with HIS antibody. The HIS: PIK3C3 bound complexes visualized with V5 antibody on the western blot confirmed binding of AP3B1, STX8 and ARCN1 proteins. The pull-down assay with the non-accessory domain of PI3K was used as a control; **(F)** The HIS:RFP:MAV_2941 mutated protein fails to pull-down V5:AP3B1, V5:STX8 or V5:ARCN1 proteins from THP-1 cells co-expressing bacterial and host lentiviral constructs.

Fig. 3. Fluorescence microscopy for the late endosomal marker LAMP-1 in THP-1 cells
Macrophages were infected with fluorescein-labeled live *M. avium* 104 (a) or heat-killed *M. avium* 104 (b, c, d). Before the heat-killed *M. avium* infection, selected wells were transfected with MAV_2941 protein containing TAT transduction domain (c), or the AP3B1 gene was knocked down (d). Arrows indicate the co-localization of fluorescein-labeled bacteria and LAMP-1 protein, visualized by Texas-Red. Bar, 10 μ m.

Fig. 4. The western blot demonstrating AP3B1 knockdown using the interference siRNA system THP-1 cells ($10^5/\text{ml}$) were seeded in 6-well plate, washed with siRNA transfection medium and replaced with AP3B1 siRNA transfection reagent before *M. avium* infection. Transfection with the control siRNA (scrabbled sequences) was used as a negative control. Briefly, cells were lysed in CellLytic™ M lysis buffer supplemented with protease inhibitor cocktail (Sigma) and pre-cleared samples were separated on 12% Tris-HCl gels. Membranes were blocked with 2% BSA for 1h and incubated with AP3B1 primary antibody at a 1:250 dilution for 1h. After, membranes were probed with the corresponding IRDye® secondary antibody (Li-Cor Biosciences, Inc) at a dilution of 1:5,000 for 30min, and proteins were quantified via semi-quantitative western blot on the Odyssey Imager (Li-Cor). The photon emission means were recorded for each band to quantify the signal intensity. Data shown is representative of two independent experiments. (1) AP3B1 siRNA transfection, (2) Scrambled siRNA (control) transfection. The β -actin serves as a loading control.

Table 1**Plasmids**

Name	Description	Source
pGBKT7	Yeast two-hybrid bait vector for expressing proteins fused to the GAL4 DNA-binding domain	Clontech
pGADT7	Yeast two-hybrid pray vector for expressing proteins fused to the GAL4 activation domain	Clontech
pGADT7-library	Normalized Mate and Plate™ Universal Human Library	Clontech
pGBKT7-53	Control bait vector for positive interaction. Murine p53 fused with the Gal4 DNA-binding domain	Clontech
pGBKT7-lam	Control bait vector for negative interaction. Lamin fused with the Gal4 DNA-binding domain	Clontech
pGADT7-T	Control pray vector for positive/negative interaction. The Gal4 activation domain fused with SV40 large T-antigen	Clontech
pGBKT7:MAV 2941	The MAV_2941 gene fused to the GAL4 DNA- binding domain	This study
pGADT7:MAV 2941	The MAV_2941 gene fused to the GAL4 DNA- binding domain	This study
pGBKT7:AP3B1	The bait vector containing the 540–960aa coding sequence of β 3A subunit of the adaptor-related protein complex 3	This study
pGBKT7:STX8	The bait vector containing the 1–236aa coding sequence of syntaxin-8	This study
pGBKT7:ARCN1	The bait vector containing the 45–423aa coding sequence of archain 1	This study
pLenti6.3/V5-TOPO	The TOPO®- adapted ViraPower™ HiPerform™ lentiviral expression vector	Life Technologies
pLenti/HIS:RFP: MAV_2941	The lentiviral expression vector containing the MAV_2941 gene	This study
pLenti/HIS:RFP: MAV_2941 mutated	The lentiviral expression vector containing the MAV_2941 gene with mutations at 2aa and 73aa location and with deletion in 16–21aa coding sequence	[13] This study
pLenti/HIS:RFP	The lentiviral expression vector containing RFP	This study
pLenti/HIS:PIK3C3	The lentiviral expression vector containing 300–575aa coding sequence of an accessory domain of phosphatidylinositol 3-kinase	This study
pLenti/HIS:PIK3C3(–)	The lentiviral expression vector containing 1–275aa coding sequence of C2 catalytic domain of phosphatidylinositol 3-kinase	This study
pLenti/V5:AP3B1	The lentiviral expression vector containing 540–960aa coding sequence of β 3A subunit of the adaptor-related protein complex 3	This study
pLenti/V5:SXT8	The lentiviral expression vector containing 1–236aa coding sequence of syntaxin-8	This study
pLenti/V5:ARCN1	The lentiviral expression vector containing 45–423aa coding sequence of archain 1	This study
pET28b TAT v1	The protein overexpressing vector containig HIS-tag and TAT peptide/protein transducing domain	[35]
pET/TAT: MAV 2941	The MAV_2941 gene fused with TAT peptide/protein transducing domain	This study

Table 2Co-localization percent of the LAMP-1 protein in fluorescein-labeled *M. avium* phagosomes

Infection	LAMP-1	Rab5
(200 bacteria analyzed) ^a		
Live <i>M. avium</i> 104 infection	17.5 ± 4	90±4
Heat-killed <i>M. avium</i> 104 infection	91 ± 3 **	94±3
Heat-killed <i>M. avium</i> 104 infection and MAV_2941 protein treatment	28 ± 4 *	93±2
Heat-killed <i>M. avium</i> 104 infection in AP3B1 knockout macrophages	39.5 ± 3 *	92±2

^aMean of two different experiments**
p < 0.01 compared with live *M. avium* 104 infection*
p < 0.05 compared with heat-killed *M. avium* 104 infection