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Abstract

M. avium is an opportunistic pathogen that primarily infects macrophages. In order to survive within the macrophage, *M. avium* secretes proteins into the host cell cytoplasm to inhibit specific functions such as phagosome acidification, altering pathways as well as initiating apoptosis. However, little is known about how those secreted proteins are exported into the host cell. The goal of this study was to identify and characterize the mechanism that allows M. avium to transport proteins into the macrophage cytosol. Magnetic labeling technology was used to isolate phagosomes containing *M. avium*. Through mass spectroscopy, we were able to identify a potential channel, voltage dependent anion channel 1 protein (VDAC-1), on the membrane of the phagosome and also bound to *M. avium* surface. Cyclosporine A (CsA), an inhibitor of Ca^{2+} dependent pores, was used to inhibit VDAC-1. Growth of bacteria in CsA–treated macrophages at 1d, 2d and 3d was significantly lower compared to bacteria in untreated macrophages. Similar results were observed using small interfering RNA (siRNA) to knockdown the expression of VDAC-1 in macrophages. The observed bacterial growth at 1d was significantly decreased compared to bacteria in untreated and in siRNA control groups. Differences between siRNA treated group and control groups on 2d and 3d continued to

be significant. The observations above suggested that functional VDAC-1 has an important role for the survival of intracellular *M. avium*.

Key Words: *M. avium*, phagosome, voltage dependent anion channel 1 protein, cyclosporine A, siRNA

Corresponding e-mail address: phamtuan@onid.oregonstated.edu

Interaction of the human Voltage-Dependent Anion Channel 1 protein (VDAC-1) with *Mycobacterium avium* and its role in bacterial survival within phagocytic cells

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APPROVED:

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Mentor, representing Biomedical Sciences

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Committee member, representing Biomedical Sciences

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Committee member, representing Biochemistry and Biophysics

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Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Tuan Pham, Author

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Introduction

Mycobacterium avium subsp. hominissuis (M. avium) is a widespread environmental bacterium that can be found in soil and water. *M. avium* forms biofilms which allow it to thrive in diverse water sources, including pipes, hospital water supplies, bathtub inlets, faucets, showerheads, and swimming pools (Feazel, et al. 2009, Nishiuchi, et al. 2007, Nishiuchi, et al. 2009, Whiley, et al. 2012). It is also the causative agent of avian tuberculosis (Schaefer, et al. 1973). As an opportunistic pathogenic bacterium, *M. avium* infects through the intestinal tract or the respiratory tract of immune-compromised individuals such as those with HIV, cystic fibrosis, or patients with pre-existing pulmonary diseases such as bronchiectasis and pneumoconiosis (Brodt, et al. 1997, Field, et al. 2004, Muller, et al. 2006). During advanced stages of AIDS in particular, when blood CD4+ T cell counts are lower than 50 per mm^3, patients have increased risk of *M. avium* infection (Appelberg 2006). In healthy individuals, *M. avium* infection has been reported, although with less frequency. Unlike pulmonary tuberculosis which is caused by *M. avium* relative, *Mycobacterium tuberculosis* (*M. tuberculosis*), *M. avium* infection is believed not to be contagious (Field, et al. 2004).

Although *M. avium* infects many host cell types, macrophage is the cell target where *M. avium* establishes unique survival niches for replication and persistence. Macrophages are a part of the immune system and contain a wide array of antimicrobial mechanisms to kill pathogens. These mechanisms can include respiratory burst, and nitric oxide production (Nathan and Xie 1994), as well as the secretion of inflammatory cytokines to recruit other immune cells to the site of infection. In order to capture pathogens, macrophages engulf and form a vesicle around the pathogen known as phagosome

(Stossel 1999). Once a pathogen is within a phagosome, the vesicle undergoes a maturation process and eventually fuses with a lysosome where the pathogen is degraded by proteolytic enzymes.

M. avium, like other members of mycobacteria, is inherently resistant to a number of compounds and drugs. This is partly due to a complex array of parallel hydrocarbon chains which contributes to the impermeability of the cell wall (Inderlied, et al. 1993). Mycobacteria envelope consists of the inner plasma membrane and a unique cell wall composed of peptidoglycan (PG) and arabinogalactan (AG) (Brennan and Nikaido 1995) and the fatty acids known as mycolic acids (MA) which surround the cell wall (Hoffmann, et al. 2008). Previous research have shown that PG from mycobacteria has a few differences such as the amidation of L-Glu and the peptide chain L-alanyl-D-*iso*-glutaminyl-*meso*-diaminopimelic acid (DAP) in PG which allows for resistance against hydrolysis catalyzed by endopeptidases (Mahapatra, et al. 2005).

Even though the cell wall acts as a protective barrier, it is insufficient in protecting against the wide array of macrophage antimicrobial mechanisms. In order to survive within the phagosome, *M. avium* has to use different strategies, such as to secrete proteins across the phagosomal membrane and into the host cytoplasm to interfere with macrophage functions. Secreted proteins have a range of functions including, inhibiting phagosome acidification, and blocking signaling pathways (Abdallah, et al. 2008). Studies have demonstrated that many PE/PPE proteins found in *M. avium* are secreted (Abdallah, et al. 2006, Abdallah, et al. 2009) and the disruption of PE/PPE family genes is linked to bacterial attenuation (Li, et al. 2005). Furthermore, research has shown that *M. avium* is able to inhibit the recruitment of proton-ATPase to the vacuole which allows

the bacterium to inhibit the acidification of the phagosome (Sturgill-Koszycki, et al. 1994), an important stage in the maturation process. In addition, *M. avium* is capable of preventing the fusion of the phagosome to the lysosome to stop a key step in the pathogen elimination. Malik and colleagues (2001) have previously suggested that the prevention of phagosome-lysosome fusion by *M. avium* is linked to the inhibition of Ca^{2+} signaling.

The mechanisms which secreted proteins are exported by the bacterium are still largely unknown. Bacteria of other species have many secretion systems in order to translocate proteins across the membranes. Gram-negative bacteria such as *Salmonella typhimurium* has Type III secretion system which involves an injectisome and is used to deliver proteins across membranes and into the host cells (Cornelis 2006, Grant, et al. 2006). Other secretion system such as type IV is also widely used by Gram-negative bacteria to secrete proteins across membranes into the host cell.

Although protein secretion systems such as type III, IV, and VI are common in other species of bacteria, *M. avium* does not have such secretion systems. However, the general secretion (Sec) and twin arginine transporter (Tat) pathways dependent secretion systems do exist in *M. avium.* In mycobacteria and all other bacteria, the Sec-dependent pathway serves as a way to secrete unfolded proteins that contain N-terminal signal sequences across the cytosolic membrane (Champion and Cox 2007). In addition, two homologs of SecA, SecA1 and SecA2 are important in the secretion of proteins by mycobacteria. Previous studies in *Mycobacterium smegmatis* have shown that growth inhibition and decreased Sec export is the result of SecA1 depletion (Guo, et al. 2007, Rigel, et al. 2012). In addition to the essential SecA1, mycobacteria also contain the accessory SecA2. A secA2 mutant in *M. tuberculosis* is attenuated for growth in

macrophages (Braunstein, et al. 2003, Kurtz, et al. 2006). Previous studies suggested that SecA2 functions as a 'proofreader' and deliver proteins that are normally overlooked by SecA1 (Feltcher, et al. 2010).

Similar to the Sec pathway, twin arginine transporter (Tat) also exports proteins with N-terminal signal peptides (Natale, Bruser and Driessen 2008). Unlike the Sec pathway, the Tat signal peptides contain a pair of arginine residues next to hydrophobic residues (Stanley, et al. 2000). Under standard laboratory conditions, the Tat export system is crucial for the growth of *M. tuberculosis*, as knocking out the genes encoding for *tatA*, *tatB*, or *tatC* result in *M. tuberculosis* inability to survive (Saint-Joanis, et al. 2006).

In addition to the Sec and Tat dependent pathways, studies have shown that a major pathway for secreting proteins to the cell surface or the environment is mediated by the Type VII secretion (T7SS) pathway or the ESX pathway in mycobacteria. There are currently 5 ESX systems (ESX-1-5) that have been described with *M. avium* containing only 4 ESX systems (ESX 2-5) and ESX-5 being important in mycobacteria pathogenesis (Abdalla, et al. 2008). Previous studies have shown that disruption of the ESX-5 locus inhibits the ability of the bacteria to modulate the macrophage response. Additionally, the ESX-5 secretion system is required to secrete PE/PPE proteins during culture *in vitro* and during intracellular growth in macrophages (Abdallah, et al. 2009).

Since *M. avium* is bound in phagosomes and the mechanism for which secreted proteins are exported across the phagosomal membrane into the host cytosol is still unknown, our aim in this study is to identify possible phagosomal proteins that are employed by *M. avium* to translocate proteins into the host cell cytoplasm.

Methods and Materials

Macrophage: RAW 264.7 (a mouse macrophage cell line) were cultured in Roswell Park Memorial Institute medium (RPMI; Corning) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Corning) in 75 cm^3 flasks, to a confluence of ~60%.

Bacteria: *Mycobacterium avium* strain 104 was originally isolated from the blood of AIDS patients with disseminated infection. Bacteria were cultured on MiddleBrook 7H10 agar (BD Biosciences) supplemented with 10% (vol/vol) olei acid-albumin-dextrose-catalase (OADC; Hardy Diagonstics) at 37^{o}C for 7-10 days until bacterial colonies were present. Individual colonies were restreaked and used for experiments.

Isolation of intact phagosomes: *M. avium* 104 was grown in Middlebrook 7H9 growth medium till mid-log phase. Bacterial pellet was washed with Hank's Balanced Salt Solution (HBSS; Corning), resuspended in 1ml HBSS and then passed through a 27-gauge needle to ensure a single cell suspension. *M. avium* was incubated with EZ-Link sulfo-NHS- LC biotin (Thermoscientific) for 30 minutes. The reaction was stopped by washing with HBSS containing 0.1 M glycine, pH 7.2. Cells were washed with HBSS with .05% Tween-80 to remove unbound biotin. Biotinylated *M. avium* was incubated with streptavidin-coated microbeads (Miltenyi Biotech) for 20 minutes.

RAW 264.7 macrophages were seeded at 80% confluency in T-200 flasks. Cells were then infected with biotin-labeled *M. avium* at MOI of 10:1. After 4h and 24h incubation at 37^{o}C, 5% CO_2, macrophages were removed by scrapping and resuspended in homogenization buffer (1M HEPES; Life Technologies, 1M sucrose) containing protease inhibitors cocktail (Sigma-Aldrich). RAW 264.7 cells were then mechanically lysed by

multiple passages through a 23-gauge needle. Phagosomes were selected through a MiniMACS column on a magnetic selector obtained from Miltenyi Biotech. Isolated intact phagosomes were incubated with Alexa Fluor 488- conjugated Annexin V (Invitrogen) and visualized on a Leica DM4000B.

Isolation of *M. avium* surface bound phagosomal proteins: Proteins were isolated from phagosomes according to Garin et al. 2001. Phagosomes were lysed overnight with 1% Tergitol (Sigma-Aldrich) with 20 mM HEPES (Life Technologies) and protease inhibitor cocktail purchased from Sigma Aldrich. Intracellular *M. avium 104* was obtained as previously described (Danelishvili, et al. 2007). Digested phagosomes were combined with the intracellular *M. avium* and incubated at 4^{o}C. After 24h, bacterial pellet was centrifuged at 3,500 rpm for 20 min, washed three times with PBS and resuspended in the extraction buffer (20 mM Octyl β-D-glucopyranoside; Sigma-Aldrich, 25 mM EDTA; Sigma-Aldrich) to elute any phagosomal protein from the surface of the intracellular *M. avium*. After 24h incubation, bacteria were pelleted down and supernatant were collected. Buffer exchange was performed with 25 mM ammonium bicarbonate using 3 kDa filters. Eluted phagosomal proteins were trypsin digested at 37^{o}C for 5hrs and samples were processed for sequencing in the Environmental Health Science Center's (EHSC) Mass Spectrometry Facility at Oregon State University (Corvallis, OR).

***In vitro* testing of Cyclosporine A inhibition effects on *M. avium*:** Cyclosporine A (CsA; Novartis) is an inhibitor of CA^{2+}- dependent pores such as VDAC-1 (Montero, et al. 2004, Yuqi, et al. 2009). To determine that CsA does not produce harmful effect to *M. avium,* bacteria cultures were incubated with 16 μM of CSA and turbidity was measured with OD600 at several time points (4h, 1d, 2d, and 3d).

Inhibition of VDAC-1: Approximately, 10^5 macrophages were seeded in 24-well plates and pre-treated with 16μM of CsA, a blocker of VDAC-1, for 4h. Cells were then infected with *M. avium* 104 for 1h at MOI of 10, washed 3 times to remove extracellular bacteria with HBSS, and lysed with 0.1% Triton X-100. The Colony Forming Units (CFUs) were recorded at 4h, days 1, 2 and 3 post-infection.

Inactivation of VDAC-1 by siRNA: RAW 264.7 macrophages were seeded at 60% confluence in 6-well plates and, 24 hours prior infection, transfected with control (scrambled sequences) and experimental (VDAC-1) siRNAs purchased from Santa Cruz Biotechnology. Briefly, siRNAs were diluted in DMEM without serum at a final concentration of 25nM and 3μl of Continuum^{TM} transfection reagent (Gemini) was added into diluted siRNA. The transfection mixture was supplemented drop-wise to monolayers and then incubated at 37^0C in presence of 0.5% CO_2. After 24hr, cells were infected with *M. avium* for several time points and CFUs were recorded on Middlebrook 7H10 agar plates. The VDAC-1 and β-actin protein levels from control and experimental wells were analyzed by Western blotting.

Western Blot: Samples were mixed with an equal volume of 2X Laemmli sample buffer (Bio-Rad), resolved onto 12% SDS-PAGE gel (Bio-Rad) and transferred to nitrocellulose membrane (Bio-Rad). Membrane was blocked with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) overnight. After, the membrane was incubated with primary antibody at 1:250 dilution overnight. Membrane was probed with corresponding IRDye secondary antibody (Li-Cor Biosciences, Inc) at a dilution of 1:5000 for 1h. Proteins were visualized using Odyssey Imager (Li-Cor).

Statistical analysis: All data are presented as ± SD and comparison of variables is performed using the unpaired Student's *t* test. Statistical significance was set at $P < 0.05$.

Results

Magnetic Selection of Phagosomes

Phagosomes labeled with microbeads were magnetically isolated at 4h and 24h time points. To show the integrity of intact phagosomes, fluorescence microscopy was used. Annexin V can be used as a marker for phagosomes due to the fact that it binds to phosphatidylserine (Garin, et al. 2001), a lipid that is normally present in the outer leaflet of the phagosomal membrane (Figure 1A and 1B).

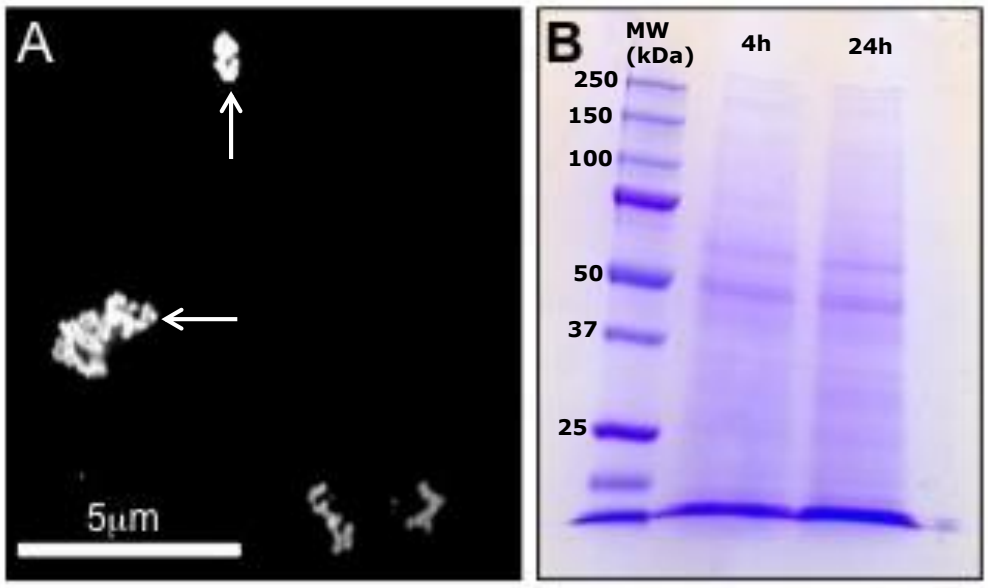

Figure 1. (A) Visualization of intact phagosomes. Intact phagosomes were visualized using Alexa Fluor 488- conjugated Annexin V. Phagosomes are indicated by arrows. **(B) Phagosomal proteins visualized on SDS-PAGE**. Phagosomal proteins were lysed with 1% Tergitol in 20 mM HEPES containing protease inhibitor cocktail and visualized on SDS-PAGE with Coomassie staining.

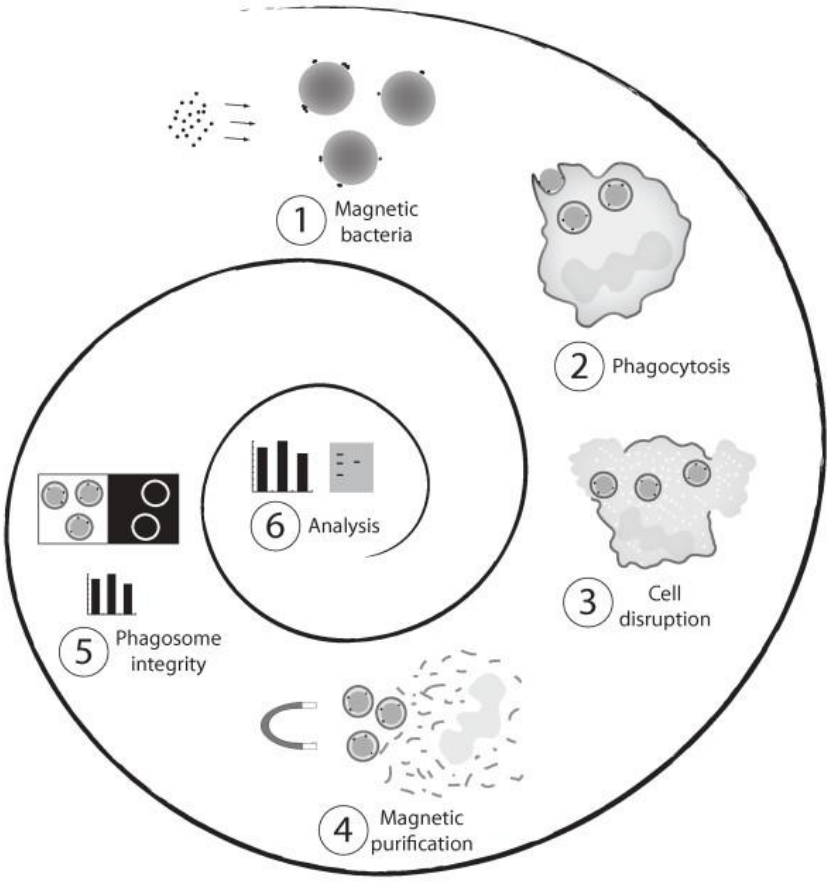

Figure 2: Overview of phagosome isolation. 1. *M. avium* was covalently labeled with magnetic beads. 2. Macrophages were seeded at 80% confluency and were infected with *M. avium* at MOI of 10. After 4h and 24h incubation at 37ºC, 5% CO_2 cells were resuspended in homogenization buffer protease inhibitors. Cells were mechanically lysed after incubation periods. 4. Phagosomes were magnetically selected. 5. Phagosome integrity was determined using Annexin V fluorescent antibody as a marker for intact phagosomes. 6. Phagosomal proteins were incubated with intracellular *M. avium*. Bound phagosomal proteins were eluted and analyzed using mass spectrometry. Figure adapted from Garin, et al. 2001.

Protein Identification by Mass Spectrometry

Mass spectrometry identified some previously described phagosomal proteins including ATP synthase, prohibitin, voltage-dependent anion channel 1 (VDAC-1) and vimetin. VDAC-1 is a type 1 porin molecule and forms dimers, trimers, tetramers, and higher oligomers (Shoshan-Barmatz, et al. 2013, Keinan, et al. 2013) on the outer mitochondrial membrane. This protein is also present on endosomes, as identified by immunofluorescence and immunogold electron microscopy (Reymann, et al. 1998), and is localized on the plasma membrane, as well (Baker, et al. 2003). In addition, proteomic profiling of phagosomes of *Mycobacterium bovis* containing cells demonstrated VDAC-1 presence on mycobacterium containing phagosomes (Lee, et al. 2010).

Table 1: *M. avium* surface bound phagosomal proteins identified by mass spectrometric sequencing.

#	Identified Proteins	Accession Number	MW (kDa)	Numbers of Peptides	
				24h	4h
1	Cluster of Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4 (VIME_HUMAN)	VIME_HUMAN [3]	54	36	26
2	Prelamin-A/C OS=Homo sapiens GN=LMNA PE=1 SV=1	LMNA_HUMAN (+1)	74	26	24
3	ATP synthase subunit beta, mitochondrial OS=Homo sapiens GN=ATP5B PE=1 SV=3	ATPB_HUMAN	57	20	19
4	ATP synthase subunit alpha, mitochondrial OS=Homo sapiens GN=ATP5A1 PE=1 SV=1	ATPA_HUMAN	60	12	11
5	Prohibitin OS=Homo sapiens GN=PHB PE=1 SV=1	PHB_HUMAN	30	10	12
6	Cluster of ADP/ATP translocase 2 OS=Homo sapiens GN=SLC25A5 PE=1 SV=7 (ADT2_HUMAN)	ADT2_HUMAN	33	12	7
7	Heterogeneous nuclear ribonucleoprotein A3 OS=Homo sapiens GN=HNRNPA3 PE=1 SV=2	ROA3_HUMAN	40	8	11
8	Cluster of Histone H2A (Fragment) OS=Homo sapiens GN=H2AFJ PE=2 SV=1 (H0YFX9_HUMAN)	H0YFX9_HUMAN [12]	10	10	10
9	U5 small nuclear ribonucleoprotein 200 kDa helicase OS=Homo sapiens GN=SNRNP200 PE=1 SV=2	U520_HUMAN	245	7	11
10	Annexin A5 OS=Homo sapiens GN=ANXA5 PE=1 SV=2	ANXA5_HUMAN (+1)	36	9	8
11	ATP-dependent RNA helicase A OS=Homo sapiens GN=DHX9 PE=1 SV=4	DHX9_HUMAN	141	8	6
12	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6	K2C1_HUMAN	66	13	7
13	Splicing factor 3B subunit 3 OS=Homo sapiens GN=SF3B3 PE=1 SV=4	SF3B3_HUMAN	136	6	8
14	**Voltage-dependent anion-selective channel protein 1 OS=Homo sapiens GN=VDAC1 PE=1 SV=2**	**VDAC1_HUMAN**	**31**	**6**	**11**
15	60S acidic ribosomal protein P2 OS=Homo sapiens GN=RPLP2 PE=1 SV=1	RLA2_HUMAN	12	8	7
16	Cluster of Histone H2B OS=Homo sapiens GN=HIST2H2BF PE=2 SV=1 (B4DR52_HUMAN)	B4DR52_HUMAN [11]	18	6	6
17	Heterogeneous nuclear ribonucleoprotein M OS=Homo sapiens GN=HNRNPM PE=1 SV=3	HNRPM_HUMAN	78	3	7
18	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6	K1C10_HUMAN	59	5	7
19	Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2	H4_HUMAN	11	6	7
20	Prohibitin-2 OS=Homo sapiens GN=PHB2 PE=4 SV=1	J3KPX7_HUMAN (+1)	33	4	7

21	60S ribosomal protein L4 OS=Homo sapiens GN=RPL4 PE=1 SV=5	RL4_HUMAN	48	7	5
22	Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Homo sapiens GN=HNRNPA2B1 PE=1 SV=2	ROA2_HUMAN	37	6	5
23	Splicing factor 3B subunit 1 OS=Homo sapiens GN=SF3B1 PE=1 SV=3	SF3B1_HUMAN	146	4	6
24	Cluster of Heterogeneous nuclear ribonucleoprotein L OS=Homo sapiens GN=HNRNPL PE=1 SV=2 (HNRPL_HUMAN)	HNRPL_HUMAN [2]	64	5	6
25	Pre-mRNA-processing-splicing factor 8 OS=Homo sapiens GN=PRPF8 PE=1 SV=2	PRP8_HUMAN	274	3	7
26	Heterogeneous nuclear ribonucleoprotein A1 (Fragment) OS=Homo sapiens GN=HNRNPA1 PE=2 SV=1	F8VZ49_HUMAN (+2)	26	4	5
27	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	K1C9_HUMAN	62	7	4
28	116 kDa U5 small nuclear ribonucleoprotein component OS=Homo sapiens GN=EFTUD2 PE=4 SV=1	K7EJ81_HUMAN (+1)	108	4	5
29	60S ribosomal protein L9 (Fragment) OS=Homo sapiens GN=RPL9 PE=2 SV=1	D6RAN4_HUMAN (+2)	21	3	6
30	Cluster of 60S acidic ribosomal protein P0 (Fragment) OS=Homo sapiens GN=RPLP0 PE=2 SV=1 (F8VU65_HUMAN)	F8VU65_HUMAN [3]	27	6	4
31	rRNA 2'-O-methyltransferase fibrillarin OS=Homo sapiens GN=FBL PE=1 SV=2	FBRL_HUMAN	34	2	5
32	60S ribosomal protein L10a OS=Homo sapiens GN=RPL10A PE=1 SV=2	RL10A_HUMAN	25	5	5
33	Histone H1.2 OS=Homo sapiens GN=HIST1H1C PE=1 SV=2	H12_HUMAN (+2)	21	4	4
34	Cluster of Heterogeneous nuclear ribonucleoprotein H2 OS=Homo sapiens GN=HNRNPH2 PE=1 SV=1 (HNRH2_HUMAN)	HNRH2_HUMAN [2]	49	4	3
35	Polypyrimidine tract-binding protein 1 OS=Homo sapiens GN=PTBP1 PE=1 SV=1	PTBP1_HUMAN	57	3	5
36	Cluster of RNA-binding motif protein, X-linked-like-3 OS=Homo sapiens GN=RBMXL3 PE=2 SV=2 (RMXL3_HUMAN)	RMXL3_HUMAN [6]	115	3	3
37	Nucleolar GTP-binding protein 1 OS=Homo sapiens GN=GTPBP4 PE=2 SV=1	B7Z7A3_HUMAN (+1)	68	5	0
38	Mitochondrial inner membrane protein OS=Homo sapiens GN=IMMT PE=2 SV=2	B9A067_HUMAN (+1)	79	3	4
39	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2	K22E_HUMAN	65	5	4
40	Nucleolin OS=Homo sapiens GN=NCL PE=1 SV=3	NUCL_HUMAN	77	4	3

VDAC-1 inhibition by Cyclosporine A (CsA)

Before CsA was used to inhibit VDAC-1 protein in macrophages, *M. avium* was incubated with 16 μM CsA in 7H9 broth to determine that CsA does not slow the growth of *M. avium* in culture. Figure 3A showed no difference between the OD_{600} of treated and untreated groups, indicating that CsA does not affect bacterial growth.

To investigate if VDAC-1 protein was involved in the transport of secreted mycobacterial proteins, first, we examined if inhibition of this phagosomal pore would lead to the suppression of *M. avium* growth inside the phagocyte. Macrophages treated with one treatment of 16 μM CsA for 4h were infected with *M. avium* for several time-points and bacterial growth rates were recorded by CFU counts. Figure 3B shows that bacterial invasion at 4hr between treated group and untreated groups were relatively similar, however, at 1d, 2d and 3d post-infection treated group was significantly lower than untreated group.

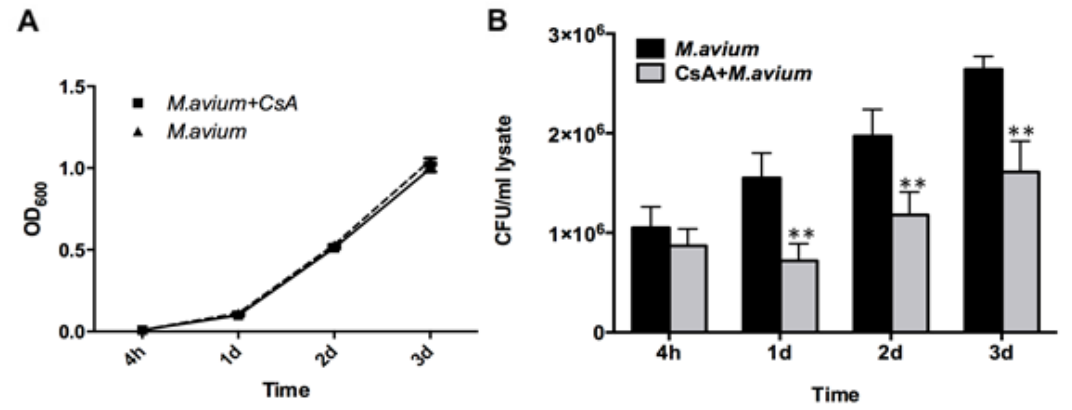

Figure 3. (A) *In vitro* testing of CsA effect on *M. avium* growth. *M. avium* cultures were treated with CsA and compared to control at different time points **(B) VDAC-1 inhibition by CsA.** Inhibition of VDAC-1 by CsA. Macrophages were pre-treated with 16 μM CsA and infected with *M. avium* for 1h. *M. avium* growths were compared at different time points. Results represent mean ± standard error of the mean of three independent experiments. **, $p < 0.01$, the significance of differences between CsA treated and control groups at the corresponding time points.

Knockdown of VDAC-1 gene by siRNA

Using small interfering RNA (siRNA), we were able to knockdown the expression of VDAC-1. Western blot result showed that VDAC-1 expression was significantly reduced compared to control siRNA (scramble) and untreated cells (Figure 4A). The result of siRNA knockdown was similar to that of CsA inhibition of VDAC-1. At 4h, the treated group showed a reduction in viability. At 1d, the difference observed was statistically significant. *M. avium* was able to recover on 2d and 3d, however, bacterial growth in the treated group continued to lag behind when compared with siRNA control and untreated groups at the same time points.

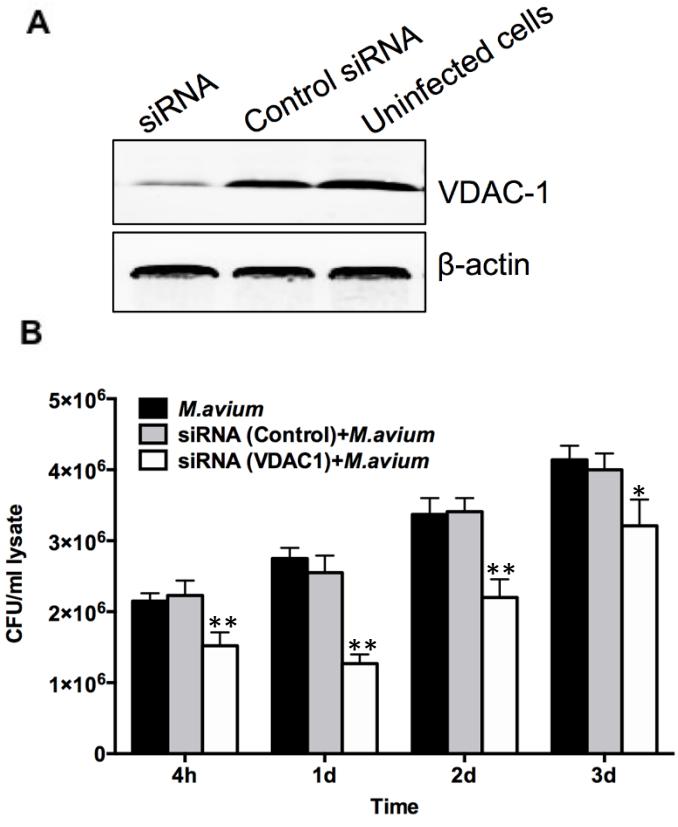

Figure 4. (A) Western blot of VDAC-1. Beta-actin was used as a loading control. **(B) siRNA knockdown of VDAC-1.** *M. avium* survival assay in VDAC-1 knockdown macrophages. Macrophages were transfected with experimental (VDAC-1) siRNA prior to *M. avium* infection. CFUs were recorded at various time points. Results represent means ± standard error of the mean of three independent experiments. **, $p < 0.01$ and *, $p < 0.05$, the significance of differences between VDAC-1 knockout and siRNA control or *M. avium* infection groups.

Discussion

The objective of this study was to identify transport mechanisms for *M. avium* secreted proteins which can be involved in the translocation of effectors into the macrophage cytosol. *M. avium* infection is believed to be initiated through the binding of different receptors such as CR3 complement receptor, fibronectin, and mannose receptor in order to gain entry into macrophages (Bermudez, et al. 1991). Following bacterial phagocytosis, the phagosome recruits proton-ATPase pumps in order to acidify the phagosome. However, studies have shown that *M. avium* is able to inhibit the recruitment of proton-ATPase pumps, effectively inhibiting the process of acidification and stopping the maturation of the phagosome (Sturgill-Koszycki, et al. 1994).

Recent studies have established that many PPE family proteins are exported through the ESX-5 secretion system (Abdallah, et al. 2008, Bottai, et al. 2012) and many of these proteins are involved in the virulence of *M. avium*. Li and colleagues (2005) have demonstrated that by disrupting a PPE gene in *M. avium*, the bacteria was unable to prevent the acidification of the phagosome and resulted in attenuation. Despite all this, little is known about the mechanism that *M. avium* use to export proteins from the vacuole into the host cell cytosol. In this work, we used magnetic labeling technology to isolate phagosomes containing *M.avium* and capture phagosomal proteins bound to *M.avium* surface. Using mass spectrometry, we identified host macrophage proteins from which ATPase, prohibitin, vimetin and VDAC-1 (Table 1) have been previously shown to be on the phagosome (Garin, et al. 2001). In addition, VDAC-1 has also been demonstrated to be on phagosomes containing *Mycobacterium bovis* (Lee, et al. 2010).

On the outer mitochondrial membrane, VDAC-1 monomer is an important channel in which metabolites such as K+, Na+, Ca2+, ATP and NADH can be transported in and out the mitochondria (Colombini 2004). In addition, the transportation of ions and metabolites through the VDAC-1 pore is suggested to be regulated by the N-terminal α-helix region of VDAC-1 (Bayrhuber, et al. 2008, Ujwal, et al. 2008). Furthermore, VDAC-1 can oligomerize into dimers, trimers, tetramers, hexamers, and higher-order oligomers (Shoshan-Barmatz, et al. 2013, Keinan, et al. 2013). Previous studies have suggested that the oligomerization of VDAC-1 allows for the secretion of cytochrome c (Shoshan-Barmatz, et al. 2010) out of the mitochondria to initiate apoptosis. Moreover, Abu-Hamad and colleagues (2010) have shown that the translocation of cytochrome c is controlled by the N-terminal α-helix segment. We predict that oligomerization of VDAC-1 on the phagosome may form a channel that can export *M. avium* secreted proteins from the phagosome into the cytosol of the host cell.

To determine whether VDAC-1 plays a role in the survival of *M. avium*, we inhibited VDAC-1 using CsA, a Ca^{2+} channel blocker. The result of CSA inhibition of VDAC-1 showed that while *M. avium* was able to enter and infect the cell at the same rate as in untreated control, it was not able to survive in the phagosome and a significant decrease in growth during 1d, 2d, and 3d time points was observed compared to control group. A similar trend was observed when siRNA was used to knock down VDAC-1 gene. Our observations suggested that the inhibition of VDAC-1 affects bacterial growth inside the macrophage.

While this study did not directly show that VDAC-1 has a role in the translocation of proteins across the phagosomal membrane, the results of CsA inhibition and siRNA knockdown of VDAC-1 protein in macrophages indicated that VDAC-1 is important to the survival of *M. avium* inside host cell. With VDAC-1 being found on the phagosomal membrane, it is possible for VDAC-1 to be targeted by *M. avium* and used as a mechanism to survive.

The premise of our future studies will concentrate on the characterization of the role of VDAC-1 as a possible export mechanism of *M. avium* secreted proteins. Through functional characterization of VDAC-1, we may be able to identify novel secretion mechanisms used by *M. avium*. Overall, this will enhance our understanding of *M. avium* pathogenesis.

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