University of Nevada, Reno

Tuberculosis: The Opportunistic Disease

A thesis submitted in partial fulfillment

of the requirements for the degree of

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by

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Table of Contents

Chapter 1: A Background of Tuberculosis

Abstract

Tuberculosis is an ancient disease caused by one of the most virulent and fatal biological agents; *Mycobacterium tuberculosis* (*M. tuberculosis*). Active pulmonary tuberculosis (TB) infection, which was nearly eradicated a generation ago, started to reemerge in a concerning manner in the late 1980's and the dawn of the 21st century. Different categories of TB (latent, active, and extrapulmonary) infections were explored in depth in this study. Each of the three types exhibits specific pathophysiology, characteristic clinical features, and epidemiological patterns. The synergistic role of co-infection with human immunodeficiency virus (HIV) in activating TB infections was addressed in the context of diagnosis and treatment. Additionally, the complex correlation between a rather lengthy, diverse, and oftentimes inadequate case-management and the subsequent emergence of drug-resistant and *M. tuberculosis* was highlighted.

Clinical Features

The clinical course of tuberculosis (TB) usually comes in two steps: latent TB infection (LTBI) and active disease. LTBI is an inactive form of tuberculosis caused by the pathogen *M. tuberculosis*. TB is a stepwise process as 1 out of every 10 individuals that have LTBI go on to develop an active TB disease (World Health Organization, 2014).

Initially, an individual has LTBI when she/he is infected with *M. tuberculosis* but has no symptoms of the disease. About one-third of the world's population is already infected with LTBI (World Health Organization, 2014). However, during its latent stage, LTBI is not

contagious and cannot be transmitted from one person to another (Virginia Department of Health, 2011). Many of those infected do not seek medical care for LTBI because they have no symptoms and may not know about their illness. Nevertheless, according to the National Centers for Disease Control and Prevention (CDC), there are six main categories for individuals who should be tested for TB: individuals who have spent time around active TB cases; individuals who have human immunodeficiency virus (HIV) or are otherwise immunocompromised; those that have TB symptoms such as fever, coughing, and night sweats; individuals living in Latin America, Africa, Asia, and Eastern Europe; Americans that live in high risk neighborhoods such as homeless shelters or nursing homes; and individuals who use illegal substances (CDC, Testing, 2014). The Mantoux tuberculin skin test (TST) and the QuantiFERON-TB Gold-In Tube (QTF-GIT) blood assay are the two main laboratory tests used to screen for TB infections. Both tests have inherent limitations including high rates of false positive for the skin test and the need for phlebotomy to perform QTF-GIT. However, QTF-GIT is considered more reliable for detecting TB (Cağlayan et al, 2011). Izoniazid is the antibiotic most widely used to treat patients diagnosed with LTBI; however, unlike most antibiotic therapy, LTBI management requires three to nine months of treatment (CDC, Treatment, 2014).

According to the World Health Organization (WHO), 10 percent of all LTBI patients who did not receive adequate treatment will advance to develop active TB (WHO, 2014). The progression to active TB disease is usually enhanced and promoted after an LTBI patient becomes immunocompromised due to contracting an immunocompromising disease such as HIV, developing type II Diabetes Mellitus, or as a side effect of receiving long-term treatment with immunosuppressing medications. Immunocompromised patients are at a greater risk for developing active TB because they have fewer white blood cells to fight the bacteria (CDC,

Basic TB Facts, 2014). Once the disease is active, TB infection usually attacks the lung tissues causing characteristic TB lesions. Active TB patients usually seek medical care because of fatigue, chest pain, and possible blood in their sputum. (CDC, Diagnosis, 2014) Although active TB is frequently contained within the lungs, it can spread to other parts of the body. The progression of active TB disease is called extrapulmonary TB. *M. tuberculosis* can attack the meninges, lymphatic system, and bones; the spread of TB infection to other organs makes it more serious and more lethal (Houston et al, 2014).

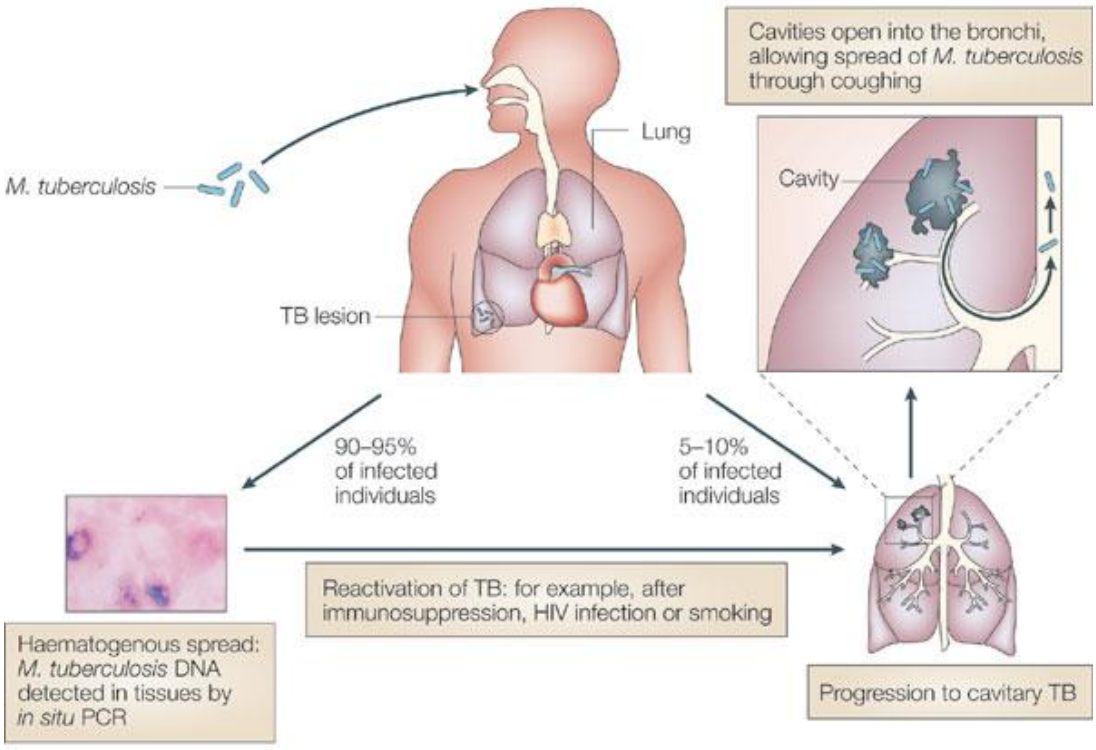

Figure 1. Illustrates the airborne mode of transmission for *M. tuberculosis* (Rook et al, 2005)

Patients diagnosed with active pulmonary TB disease or an extrapulmonary TB, must be treated with a combination of four antibiotic drugs: Isoniazid, Rifampicin, Ethambutol and

Pyrazinamiden. Similar to LTBI, active TB disease management requires at least six months of treatment (CDC, Treatment, 2014).

Pathophysiology/Pathogenesis

As illustrated in Figure 1, TB is an airborne disease and susceptible individuals can contract the bacteria and become infected with *M. tuberculosis* through inhaling infected small droplets that contain this pathogen. Such droplets are suspended in air after an individual who is already infected with active TB coughs or sneezes. Once inhaled, these droplets are deposited into the distal alveoli and become ingested by alveolar macrophages, neutrophils, or dendritic cells (Philips et al, 2012). Although the macrophages engulf the *M. tuberculosis* with the intention of destroying it, the bacteria survive and travel to the interstitium of lungs where it can establish a base for infection (Orme et al, 2014). This mechanism of survival is not very well understood, but the general consensus is that *M. tuberculosis* takes residence in the endosomes and cytosol of the macrophage and survives the leukocytes acidic environment. Then after that it causes the macrophage to become necrotic and lyse (rupture), which allows the bacteria to spread to the interstitium and activate the disease (Behar et al, 2010). The bacteria can stay at the site of the interstitial spaces of the lungs or spread to different parts of the body via the lymphatic system.

Compared to the majority of people that are exposed to *M. tuberculosis*, only a small minority actually develop active TB. This is due to the inhalation of greater concentrations of the bacteria instead of the minuscule amounts of the pathogen that most people breathe (Orme et al, 2014). After contracting the infection, *M. tuberculosis* multiply in the lungs without activating a massive inflammatory response. However, once the cell-mediated immunity develops, the

bacteria are eventually engulfed by monocytes and macrophages. These white blood cells become more destructive to the mycobacteria, causing the formation of the characteristic granulomas of the disease. A granuloma is an aggregation of lymphatic and necrotic cells that have killed most of the bacteria and stopped the infection. However, some of the individuals who previously were able to arrest the progress could develop an immunosuppressive event such as that associated with acquired immune deficiency syndrome (AIDS) or a prolonged treatment with corticosteroids that would inhibit the cell-mediated immunity. Subsequently, the formerly sealed TB can flare up and lead to active TB (Crowley, 2009).

Active TB disease can exhibit several forms; the most common and most understood of those is active pulmonary TB infection, which is associated with granulomas in the upper lobes of the lungs. However, the two most dangerous kinds of tuberculosis are miliary TB and tuberculosis pneumonia. The former is when the mycobacterium spreads to blood vessels, which in turn causes the disease to distribute large amounts of *M. tuberculosis* throughout the body. The latter mainly occurs among immunosuppressed individuals when the disease attacks larger portions or another lobe of the lung causing unusually severe symptoms.

Either through miliary TB or lymphatic TB, extrapulmonary TB disseminates when the infection spreads to body organs and tissues other than the lungs. Secondary TB infections can be spread to the bones, kidneys, uterus, and other reproductive organs. Such extrapulmonary infections could be life-threatening and may persist even after the original active TB is healed (Crowley, 2009).

Signs/Symptoms

Tuberculosis infections are characterized in two parts: asymptomatic LTBI and symptomatic active TB disease. There are many important symptoms that can prompt an individual with active TB to seek medical care. The classic and most obvious symptoms of active TB disease include night sweats, fatigue, chest pain, chronic cough, and blood-stained sputum. However, since TB is not a stationary disease, there is a wide variety of symptoms that *M. tuberculosis* can cause.

Pulmonary TB is usually categorized by its six cardinal symptoms: chronic cough, fever, weight loss, fatigue, chest pain/discomfort, sweating, and blood in sputum. On the other hand, these main, nonspecific symptoms can commonly be attributed to other less serious illnesses such as the common cold and influenza infection (Craig et al, 2014). More advanced signs of active pulmonary TB may include scarring of the lungs, hydrothorax (fluids in the lungs), and possible hemorrhaging from a Rasmussen's aneurism (pulmonary artery aneurism), which may lead to massive hemoptysis (Crowley, 2009).

Extrapulmonary TB can exhibit a wide variety of signs and symptoms that are directly related to its infection site. The main extrapulmonary TB infections may include the central nervous system (CNS), the genitourinary tracts, the bones and joints, and the lymphatic system. Most of the CNS tuberculosis infections are associated with severe signs and symptoms of bacterial meningitis. Tuberculous meningitis (TB meningitis) may rapidly exacerbate from an initial stage with no clear symptoms to the specific signs of meningitis including stiff neck, lethargy, and cranial nerve palsies. The final stage of TB meningitis is characterized with severe clinical manifestations of encephalitis such as clouding of consciousness, coma, paralysis and death (Jaypee, 2006).

Genitourinary TB is mainly characterized with progressive and diurnal enuresis (loss of urinary control), dysuria (painful urination), chronic scrotal dull pain and induration due to epididymitis in males, and lower abdominal pain with amenorrhea in females (2006).

Osteoarticular TB is mainly categorized with arthralgia (joint pain) and atrophy around the joints known as tubercular arthritis. It may also cause bone deformities causing exaggerated kyphosis and oftentimes lordosis which may result in paralysis due to a persistent compression of the spinal cord (2006).

A relatively rare form of extrapulmonary TB infection is the peripheral tuberculous lymphadenitis which includes adenopathy (swollen lymph nodes) and a large painless cervical mass (neck mass) known as the *cold abscess* (2006).

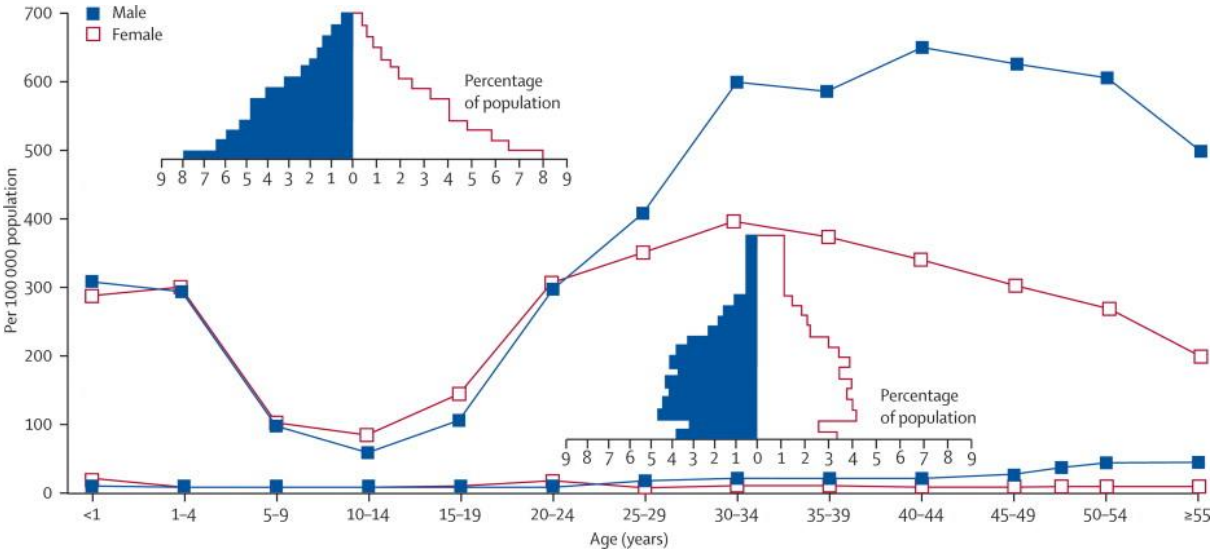

Figure 2. Illustrates the correlation of age and sex with TB infectivity (Donald et al, 2010)

Epidemiology

The epidemiology of tuberculosis (TB) is characterized by several crucial variables such as age, sex, race/ethnicity, geographic location, and environmental and socioeconomic risk

factors. Figure 2 illustrates the trends of active TB infections by age and sex. Children less than 4 years of age are at higher risk for developing a primary TB infection than their older siblings. Additionally, in such young infants, TB infections have a much higher chance of disseminating to different systems and organs in the body.

Among all age groups, children age 5 to 19 years of both genders have the lowest chances of contracting a TB infection. However, from that point on, the risk of developing TB infections starts to increase reflecting significant sex-related differences. Adult males age 20 and older exhibit higher incidence rate and subsequently are at higher risk to contract TB compared to adult females in the same age group. (Donald et al, 2010).

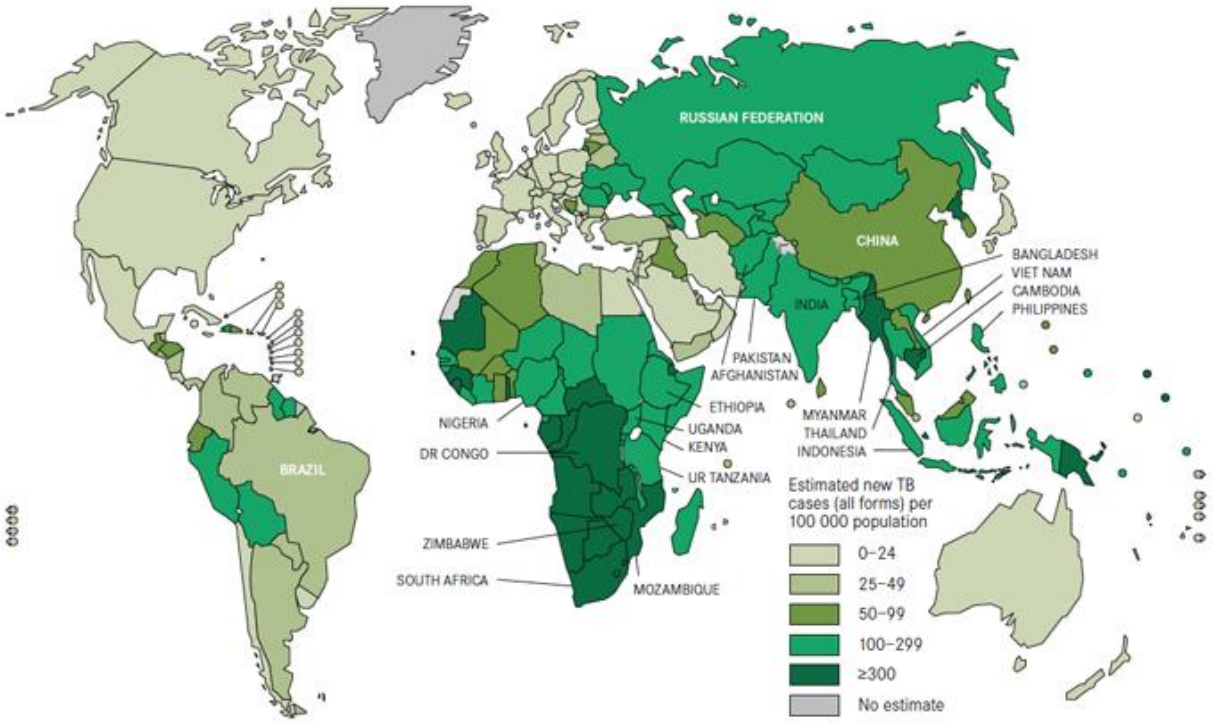

Figure 3. 2011 - New cases of TB by Country of Residence (Feleke et al, 2012)

Risk factors for contracting TB may include race/ethnicity and the geographic location or country of residence. Figure 3 illustrates the high frequency of TB infections observed in

developing countries around the world. Populations of African countries especially sub-Saharan Africa, and residents of developing countries in Asia are most likely to become infected with TB. Additionally, the incidence and distribution of active TB around the world seems to mirror that of the HIV infection. African countries have the highest rates of HIV which synergizes the infectivity of *M. tuberculosis* and increases the spread of disease around the continent. After one year of acquiring HIV, the risk of developing an active TB infection increases by more than 100%. High rates of the HIV infection in Africa, mediocre health care systems, and large population densities that live in poverty provide optimistic conditions for the continuous spread of TB among African and Asian countries (Feleke et al, 2012).

Environmental and Socioeconomic Factors

TB infections are associated with poverty, pollution, malnutrition, and overcrowding. Such environmental injustices and other socioeconomic risk factors are the main reasons that developing countries in the sub-Saharan Africa and East Asia have the highest rates of TB cases. HIV and several other infectious and parasitic debilitating diseases that are kept under control in North America and West Europe contribute heavily to these alarming rates of the active TB disease in Africa. Tobacco use and alcohol abuse are additional behavioral risk factors that may increase an individual's risk to contract TB infections.

Although the treatment for nations in Africa and East Asia are lackluster, a focus on reducing such risk factors could lead to a decline in TB infections, and a decrease in the mortality rate. Currently TB infections kill more adults worldwide than all other infectious diseases combined (WHO, 2014). Scarce public health and healthcare resources, inadequate housing, and a severe lack of funding for education, training, and environmental hygiene must be

promptly addressed in order to reverse the alarming trends of this reemerging disease (Schmidt, 2008).

Diagnosis

The diagnosis of TB is a multi-step process, and it should start with screening for LTBI. The two main tests to determine if an individual has LTBI are the tuberculin skin test (TST) and the QuantiFERON-Gold blood test. Up until the late 2000's, the TST, also known as the Mantoux test, was the main screening/diagnostic tool to determine if an individual had LTBI.

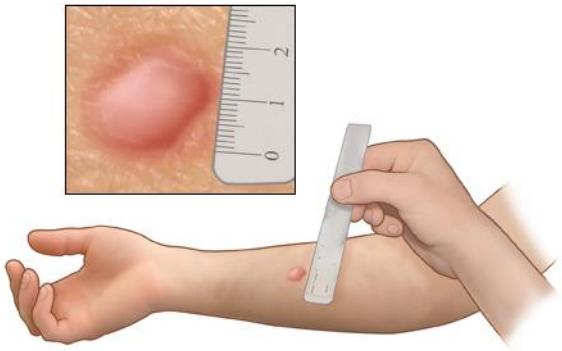

Figure 4. Result of a tuberculin skin test is measured by its induration not it erythema (WebMD, 2013)

This skin test is performed by injecting 5 units of purified protein derivative (PPD) intradermally into a patients forearm. Then it should be read 2-3 days later and be measured by the length of its induration (hardness) not its erythema (redness). Figure 4 illustrates how the test is read by a medical professional. The key issue with the skin test is it may bring about false positive results especially if the patient has a mycobacterium other than *M. tuberculosis* or if the patient had a history of receiving the Bacillus Calmette-Guerin (BCG) vaccine. The skin test might also reveal false negative results, which is when a patient actually has TB but the TST does not correctly detect the infection. False negative results are relatively high among

immunocompromised or malnourished patient, since they cannot mount a strong immune response to the PPDs. Because HIV attacks the body defenses and destroys the white blood cells, the TST may show up as a false negative even if the patient does in fact have TB (Knechel, 2009).

The next major test to detect LTBI is the QuantiFERON-Gold blood assay for the cell-mediated reactivity to the mycobacterium by measuring the amount of interferon gamma (IFNγ) that is released from leukocytes. The QuantiFERON-Gold blood test provides results in 24 hours, and in addition to LTBI, it can also detect active tuberculosis. Additionally, the CDC now recommends that QuantiFERON-Gold should replace the TST for TB detection (Knechel, 2009).

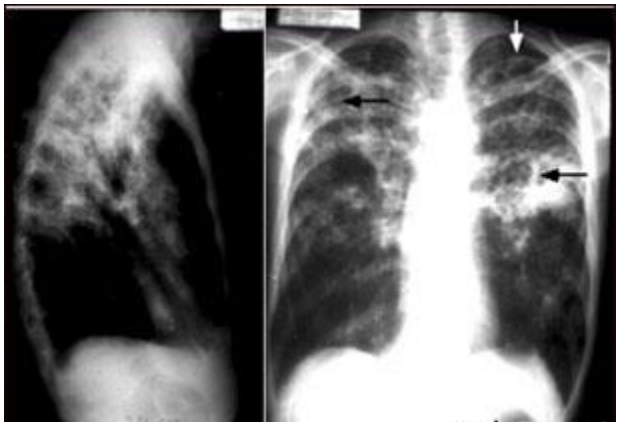

Figure 5. X-Ray of patient with progressive pulmonary tuberculosis (Medline Plus, 2012)

While the QuantiFERON-Gold blood test can detect active TB, radiology imaging as represented in Figure 5 can pinpoints the exact anatomic location and the extent of TB infection in the lungs. Figure 5 illustrates a patient with advanced pulmonary TB. The light areas or opacities are mainly located in the upper lobe. These opacities are due to granuloma formation, which contain the mycobacteria. However, it takes a whole series of tests to diagnose TB because other

pulmonary diseases may very look similar to TB (Medline Plus, 2012). Other tests that are used to diagnose active TB are sputum smears and sputum cultures, CT scans, and PET scans. Sputum smears are generally the first laboratory test to detect active TB because *M. tuberculosis* is present only in the sputum of a patient with active TB. Sputum smear requires sputum to be spread on a slide then stained and treated with alcohol. If any mycobacteria are present, it will remain stained. Also, much like the other tests for TB, the sputum smear can read false positives by staining another species of mycobacterium that are not *M. tuberculosis* (Knechel, 2009). Sputum culture and antibiogram can be a lengthy process and are used to further characterize the biological agent and test its *in-vitro* sensitivity for TB medications.

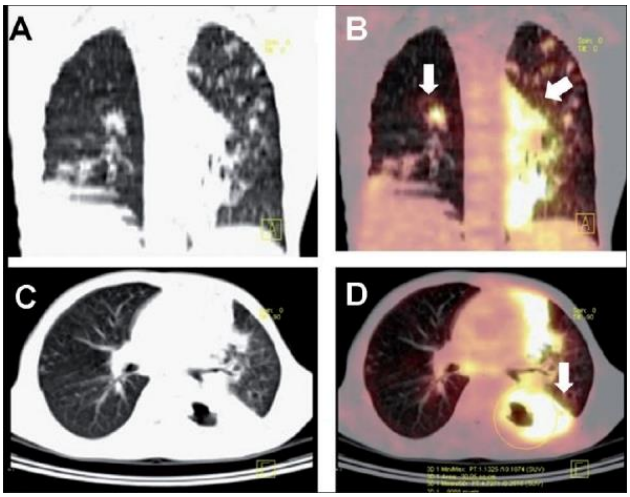

Figure 6. (A) coronal plane CT scan and (B) coronal plane FDG PET/CT scan of the same patient with progressive pulmonary tuberculosis, (C) transverse plane CT scan and (D) transverse plane FDG PET/CT (Harkirat et al, 2008)

The newest technique for identifying TB comes in the form of CT and PET Scans. These scans play an important role because they can detect TB even in patients who had inconclusive X-Ray imaging. The left side of Figure 6 shows a CT scan of a patient with pulmonary tuberculosis, while, the right shows a fludeoxyglucose (FDG) PET/CT image of that same patient. The PET/CT scan illustrates the difference between a cancerous lung and one that is

infected with TB. By injecting a patient with FDG, a TB lesion stains about 10 times brighter than a cancerous legion. Furthermore, the FDG PET/CT scan can also detect extrapulmonary TB strands by measuring the stain intensity of the scanned areas (Karkirat et al, 2008).

Treatment

After a diagnosis of TB, the treatment depends on the type of infection. LTBI can be treated with one of three antibiotics (Isoniazid, Rifapentine, and Rifampin). Such treatment should be initiated only after the possibility of active TB disease has been excluded. Treatment regimens for LTBI may vary in duration ranging from 3 months to 9 month. While patients with LTBI do not have any symptoms and may not like to undergo an extensive treatment plan, it is important to understand that treatment at such an early stage is essential to prevent the development of active TB and stop any further spread of the infection.

Active TB can be treated with 4 antibiotics: Isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA). Table 1 shows the 3 main regimens for treatment of active TB. Each treatment regimen has an initial phase and a continuation phase. The most common regimen requires a total of 26 weeks. Moreover, it is essential for TB patients to comply with the prescribed daily medication in order to prevent antibiotic resistance, which is an ever growing problem in the realm of medicine (CDC, Treatment, 2014). The emergence of drug resistant biological agent can be associated with the overuse and misuse of antibiotics. Multiple drug resistant tuberculosis (MDR-TB) occurs when *M. tuberculosis* becomes resistant to the two main antibiotic drugs that treat TB, which are Isoniazid and Rifampin. Drug resistance can also be the result of interrupted, erratic, and meager treatment plans. Since TB treatment guidelines are

not universally respected, certain strains of extremely drug-resistant TB (XDR-TB) have emerged making it exceedingly difficult to treat this reemerging disease (TB Alliance, 2014). Multi drug resistance acquired by dangerous biological agents such as *M. tuberculosis* is causing serious complications and is increasing the cost of treatment as well as the lethality due to TB.

Table 1. Basic TB Disease Treatment Regimens (CDC, Treatment, 2014)

Preferred Regimen	**Alternative Regimen**	**Alternative Regimen**
Initial Phase Daily INH, RIF, PZA, and EMB* for 56 doses (8 weeks)	Initial Phase Daily INH, RIF, PZA, and EMB* for 14 doses (2 weeks), then twice weekly for 12 doses (6 weeks)	Initial Phase Thrice-weekly INH, RIF, PZA, and EMB* for 24 doses (8 weeks)
Continuation Phase Daily INH and RIF for 126 doses (18 weeks) or Twice-weekly INH and RIF for 36 doses (18 weeks)	Continuation Phase Twice-weekly INH and RIF for 36 doses (18 weeks)	Continuation Phase Thrice-weekly INH and RIF for 54 doses (18 weeks)

Conclusion

M. tuberculosis is a protean and extremely infectious pathogen that is difficult to treat and easily spread. TB can reside in the alveoli of the lungs and lie dormant in its latent stage. Once active, it has the ability to spread through cough and small droplets in the air until it finds a new susceptible host. Diagnosing TB is a difficult task because the symptoms, laboratory tests, and radiography results are rather vague and oftentimes nonspecific. Additionally, testing for TB reveals a fairly high rate of false positives and false negatives results. Furthermore, once TB is diagnosed, the patient must be given a comprehensive and rather rigid treatment regime that requires at least 6 months of multiple antibiotics which may have unpleasant, adverse effects.

Inefficient treatment regimens allow the mycobacterium to mutate and acquire extra resistance to medications. If treatment plans are not properly followed, stronger and more virulent strains of TB may develop rendering the serious illness even more challenging to manage and treat.

The already heavy medical, epidemiological, financial, and societal burden of TB in developing countries with poor medical care and dense populations continues to exacerbate. Effective methods of prevention, early detection, screening, contact tracing, and control of the spread of *M. tuberculosis* have been utilized in countries like the United States. Nevertheless, it is necessary to utilize similar approaches in developing countries in order to control and ultimately eliminate this pathogen before it becomes even more resistant.

Since much of the pathophysiology of TB is the subject of complex ongoing research and studies, there is promise that a better understanding of its mechanisms will result in more efficient community-based interventions, treatment therapies, and case management that will control the infection and eventually eliminate this opportunistic pathogen.

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Chapter 2: Immunopathogenesis of *M. tuberculosis* due to inhibition of macrophage plasma membrane repair and mitochondrial degradation

Abstract

The key component of a virulent strand of *Mycobacterium tuberculosis (Mtb)* is related to its ability to induce macrophage necrosis in place of cell mediated apoptosis. Macrophage (Mφ) necrosis depends mainly on the *Mtb* infection leading to plasma membrane micro-disruptions (micro-lesions) and interference with the plasma membrane repair mechanism. Membrane repair depends on the translocation of vesicles derived from lysosomes and the Golgi apparatus which is dependent on prostaglandin E_2 (PGE_2). Once a cell membrane is restored, it undergoes negative feedback, and lipid mediators release lipoxin A_4 (LXA_4) to block PGE_2 and stop cell membrane repair. The virulence of *Mtb* is reliant on its ability to induce LXA_4 production and block PGE_2 biosynthesis. Without proper repair mechanisms, the Mφ undergoes cell necrosis, and *Mtb* effectively evades a host innate defenses. Additionally, *Mtb* has been shown to negatively affect the mitochondrial outer membrane (MOM) and the mitochondrial transmembrane potential ($\Delta\psi_m$). Some virulent strands of *Mtb* have illustrated a formidable ability to disrupt the MOM and cause substantial loss of $\Delta\psi_m$, which leads to the destruction of the mitochondria and cell necrosis. *Mtb's* combination of inhibiting plasma membrane repair and degrading the mitochondria effectively induces Mφ necrosis leading to Mφ lysis, which releases the *Mtb* into the surrounding tissue; thus, infecting more cells and repeating the cycle.

Introduction

M. tuberculosis (Mtb) is known to be one of the most infectious and destructive facultative bacterial parasites. It kills 1.5 million people each year and effects 33 percent of the

world's population. Humans are easily infected by *Mtb* because it is an airborne pathogen (WHO, 2014). After inhalation of the *Mtb,* the body's innate immunity uses pulmonary macrophages (Mφ) to phagocytize the foreign invaders in an effort to destroy the pathogen. When the pathogen is avirulent or attenuated, the Mφ is freely allowed to undergo plasma membrane repair and eventual apoptosis to rid itself of the mycobacterium (Chen et al, 2006).

Plasma membrane repair is enhanced by the release of PGE_2, which protects against necrosis and regulates the Ca^{2+} dependent sensors: synaptotagmin 7 (Syt-7) for lysosome and neuronal calcium sensor 1 (NCS-1) for the Golgi apparatus. Both Syt-7 and NCS-1 are involved in vesicle transport that brings modified proteins to the cell membrane to fix old or injured membrane component. Although membrane repair is crucial for a cells survival, it is especially important for Mφ that are infected with *Mtb* because this pathogenic agent is equipped with a specialized protein secretion system known as ESX 1, which is thought to poke holes in the Mφ membrane. While a Mφ has the ability to reseal lesions of its cell membrane, virulent strains of *Mtb* have the ability to induce the production of LXA_4 which shuts down PGE_2 synthesis (Divangahi et al, 2009).

In addition to negatively impacting plasma membrane repair, *Mtb* also affects the Mφ mitochondria. Virulent strains of *Mtb* have been shown to disrupt the mitochondrial outer membrane (MOM). This disruption of the MOM leads to a substantial loss of mitochondrial transmembrane potential ($\Delta\psi_m$), which causes mitochondrial degradation and eventual cell necrosis (Chen, 2006).

It is important to emphasize that cell apoptosis defends the body against *Mtb,* while cell necrosis caused by *Mtb* facilitates the spread of this dangerous bacteria into the interstium.

Methods

Fluorescence Activated Cell Sorting (FACS) analysis measured the amount of the expressed protein and the cells which expressed such protein. FACS analysis was used to test for the presence of proteins that facilitate the repair of the plasma membrane and the $\Delta\psi_m$ in Mφ. In order to ensure valid and reliable test results, FACS analysis used cells of the H37Rv and H37Ra that are stained with FDX, LAMP-1, and Mannosidase II (Mannos II) in vitro, and it deployed a control staining uninfected cells with FDX (Divangahi et al, 2009). In the case of the $\Delta\psi_m$, the Mφ was stained with 3, 3′-dihexyloxycarbocyanine iodide ($DiOC_6$), which determined if the mitochondria changed in permeability (Chen et al, 2006).

Immunoblot (Western Blot) analysis with siRNA transfection. Transfection provides an important tool in molecular and cellular biology studies. Through the process of transfection, genes can be manipulated and silenced allowing for the analysis of gene function, the determination of disease pathways, and the identification of potential drug targets. This research method demonstrated the effects of the silenced gene with stained murine antibodies that detect Syt-7 and NCS-1 by latching on to the molecule. The final image is resolved on an SDS-PAGE. As a control, the expression of β-actin is measured with and without the siRNA transfection on the SDS-PAGE gel (Divangahi et al, 2009).

Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR) - was used to magnify the western blot analysis by using lung tissue Mφ cultures and transcribing those into cDNA. The cDNA was then denatured under high temperatures (95^{o}C) and amplified with Max3000p Stragenen cyclerboth β-actin, the Syt-7, and NCS-1 (Divangahi et al, 2009)

Cell Death Detection Enzyme-linked Immunosorbent Assay Plus (ELISA^{Plus}) - The Cell Death Detection ELISA^{PLUS} photometric enzyme immunoassay was used to determine

whether the death of a Mφ was due to apoptosis or necrosis by analyzing the histone-associated DNA fragments after induced cell death (Divangahi et al, 2009)

Magnetic Activated Cell Sorting System (MACS) Column Purification **-** The MACS method allowed cells to be separated by identifying cells that get attached to magnetic nanoparticles coated with murine cell antibodies. After running these cells through a column, cells that were coated with murine antigens would ultimately attach to the column, while the other cells would flow through. This method can purify and support the Western blot analysis results (Divangahi et al, 2009).

Confocal Fluorescence Microscopy **–** This method used rhodamine-2, a Ca^{2+}-sensitive fluorescent dye, and MitoTracker Green, to label the mitochondria. In order to create a confocal image, cultured cells with red and green dyes were excited with light at wavelengths (λ) of 488 and 543 nm (for the green dye) and 516 and 570 (for the red die) (Chen et al, 2006).

Results

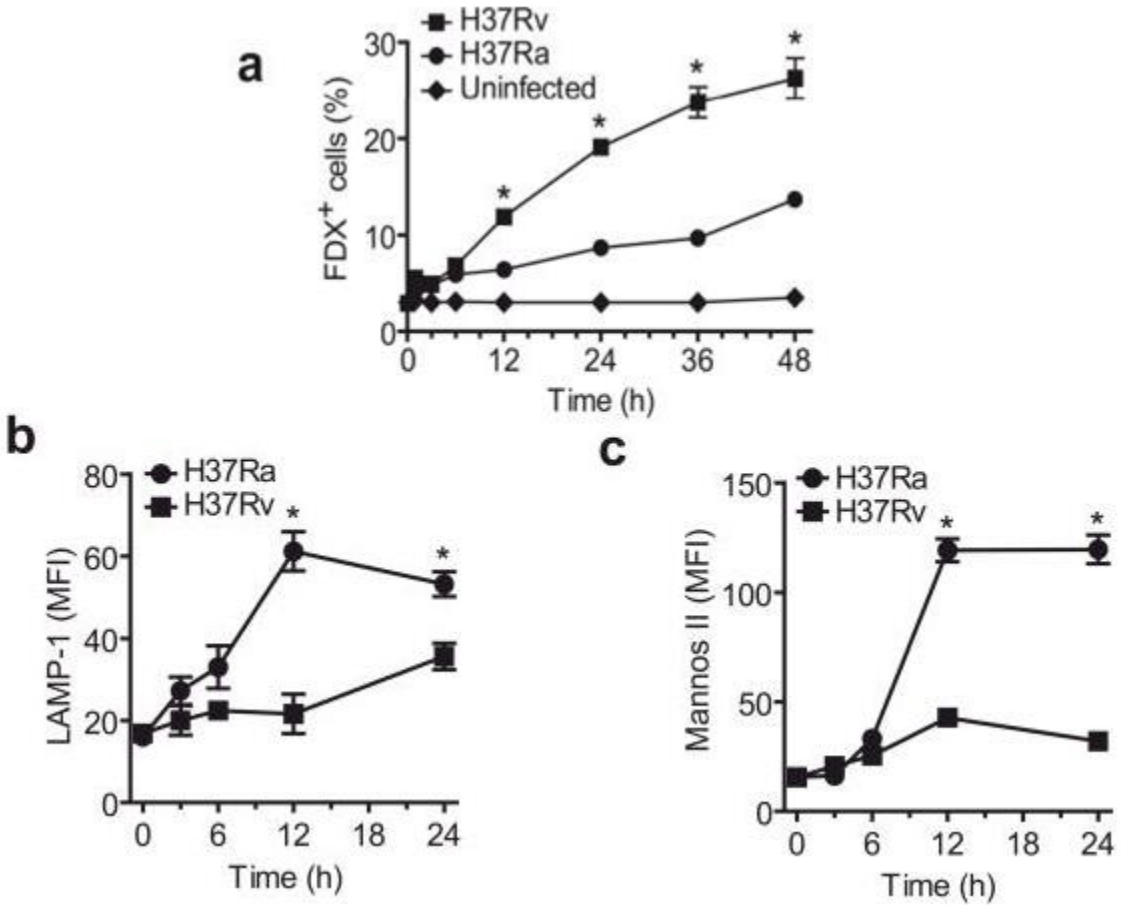

Figure 1. (A) FACS analysis showing the amount of FDX^+ cells from uninfected, H37Rv, or H37Ra infected cells. (B and C) FACS analysis of LAMP-1 and MFI in H37Ra and H37Rv cells (Divangahi et al, 2009).

Figure 1a highlights the effects of the virulent strand (H37Rv) and the attenuated strand (H37Ra) on a cells plasma membrane by measuring the percent of FDX, which can only cross a membrane if there are lesions. As illustrated in this top figure, the uninfected cells (control) do not show any increase in the amount of FDX in the cell over the 48 hour time span.

Whereas, both the *Mtb* strains of H37Rv and H37Ra show an increase in the percent of FDX throughout the 48 hours. As expected, the virulent strand H37Rv exhibits a significantly higher

percent of FDX in its cell than that of the attenuated strand H37Ra; confirming that H37Rv is more destructive than H37Ra to the plasma membrane (Divangahi et al, 2009).

Figure 1b and 1c illustrate the ability of the plasma membrane to repair itself after being infected with the two strains. To test membrane repair, cells were stained with either LAMP-1 or Mannos II, and translocation to the cell membrane was measured. LAMP-1 was used as a specific marker for lysosomal membrane repair, while Mannos II as a specific marker for Golgi membrane repair. In both cases, H37Ra shows a greater translocation of lysosomal and Golgi content to the cell membrane proposing that virulent *Mtb* inhibits membrane repair to a far greater extent than attenuated *Mtb* (2009).

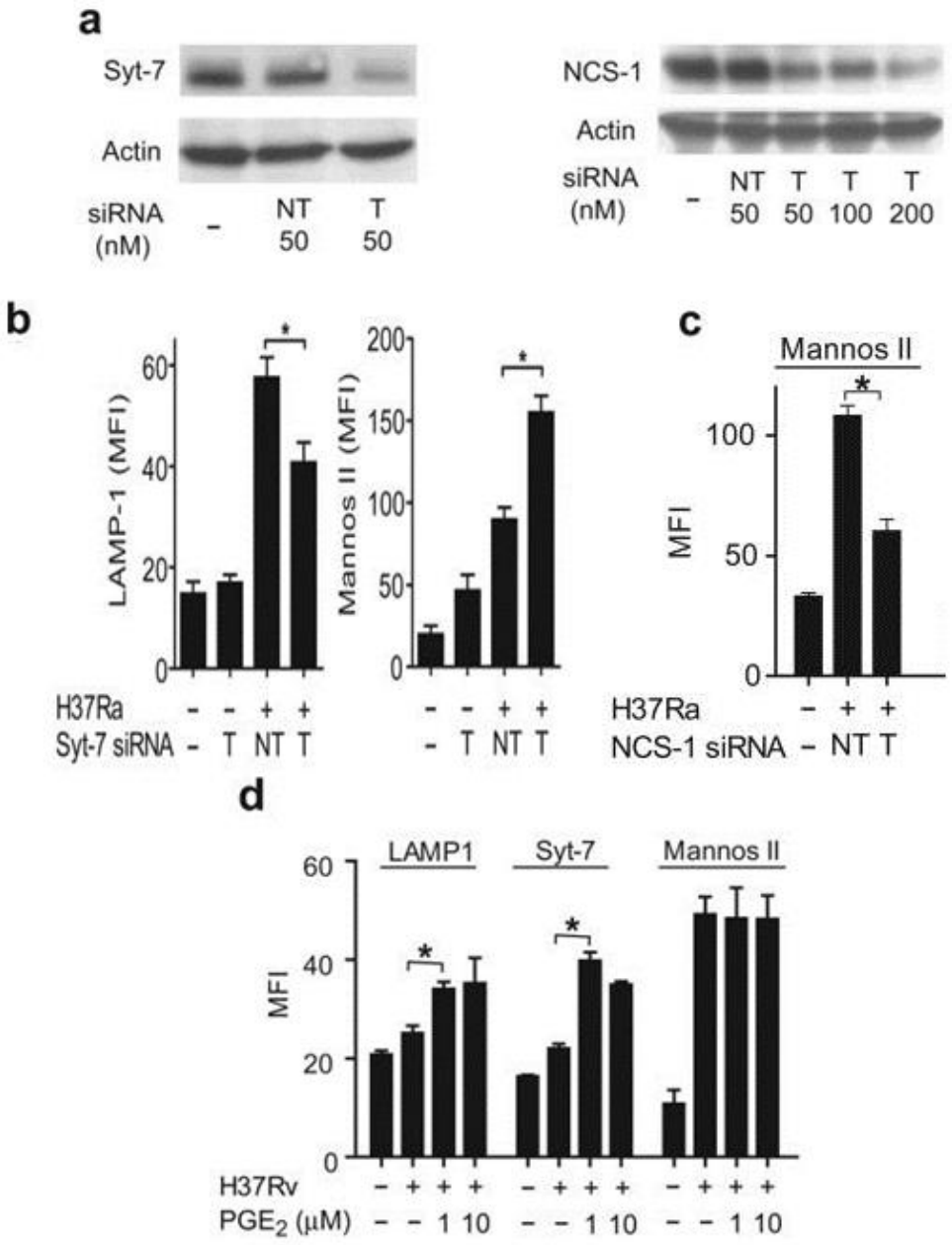

Figure 2. (A) Immunoblot analysis of Ca^{2+} sensors Syt-1 and NCS-1 after siRNA transfection (NT = Not Targeted, T = Targeted) (B) Translocation of LAMP-1 and Mannose II with Syt-7 gene silenced and H37Ra infection Present (+) or Not present (−) (C) Translocation of Mannose II with NCS-1 gene silenced and H37Ra infection (D) Translocation of LAMP-1, Syt-7, and Mannos II with H37Rv and PGE_2 being either present or not present (Divangahi et al, 2009).

Figure 2a illustrates the ability to silence genes using siRNA transfection by demonstrating that when the gene to Syt-7 is silenced there is little Syt-7 in the cell, and the same

rules apply for NCS-1. Figures 2b and 2c apply gene silencing to show siRNA targeting Syp-7, which decreases LAMP-1 translocation, and siRNA targeting CNS-1, which decreases Mannos II translocation to the plasma membrane. Figure 2d clearly demonstrates the extent to which a virulent strain of *Mtb* affects a Mφ membrane. At the LAMP-1 section, the graph shows that when H37Rv is present without the PGE_2 inserted; LAMP-1's ability to translocate to cell membrane is hindered. Additionally, the Syt-7 section shows that its translocation is hindered with H37Rv present without PGE_2. Lastly, the Mannos II region is of value because it shows no signs of inhibition even with H37Rv. This suggests that only the lysosomal membrane repair with the use of Syt-7 Ca^{2+} receptors is inhibited by virulent *Mtb* (Divangahi et al, 2009).

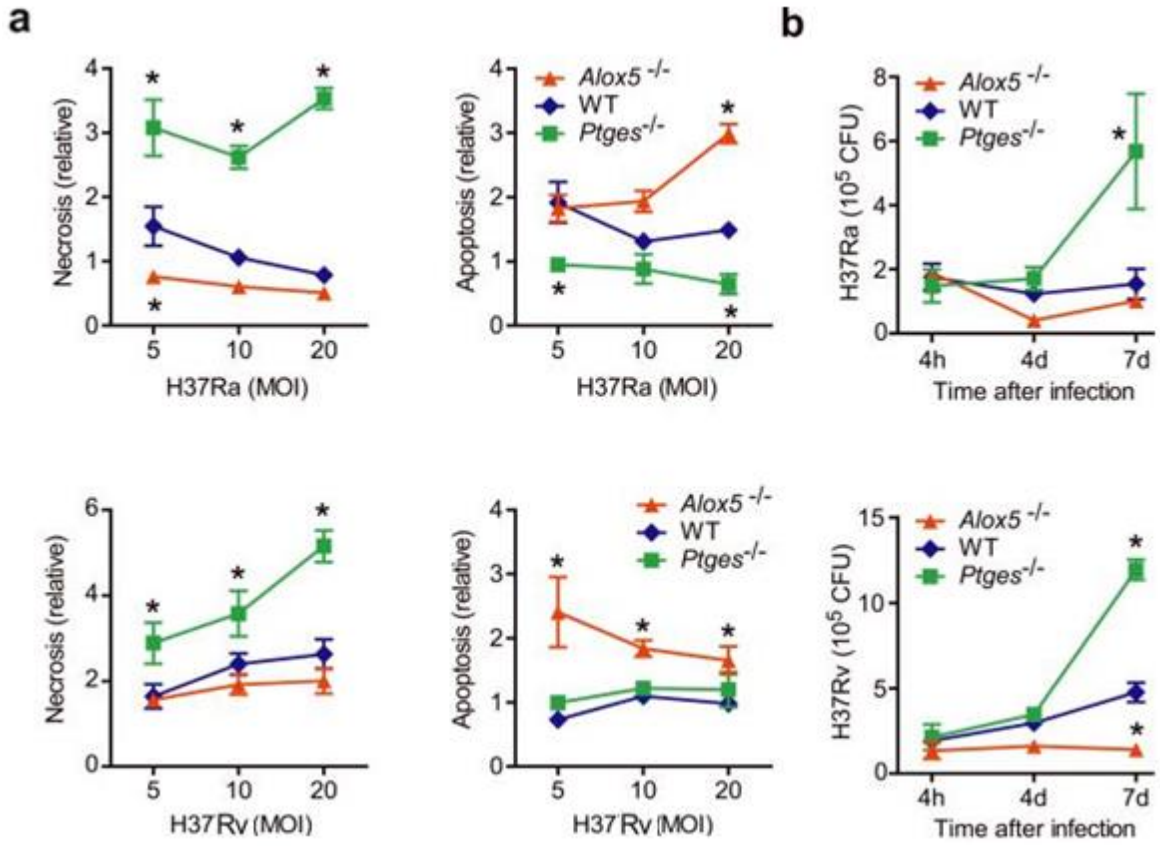

Figure 3. (A) Relates the Necrosis and Apoptosis of a Mφ based off multiplicities of infection (MOI) of $Alox5^{-/-}$, WT, and $Ptges^{-/-}$ as well as if $Ptges^{-/-}$ is infected with H37Ra (top) or H37Rv (bottom) (B) Shows the number of Colony Forming Units (CFU) of the $Alox5^{-/-}$, WT, and $Ptges^{-/-}$ with H37Rv or H37Ra over 7 days (Divangahi et al, 2009).

Figure 3a illustrates the process virulent strands deploy in order to survive against the body's innate immune defenses. It shows how H37Ra strains induce apoptosis and H37Rv strains induce necrosis. It demonstrates the effect of a normal, wild type strain (WT), and the effects of the attenuated and virulent strains. To demonstrate this process, the $Ptges^{-/-}$ gene was infected with either H37Ra or H37Rv strains revealing that the more infection sites there are in a Mφ the more likely the cell will either undergo apoptosis, for H37Ra, or necrosis, for H37Rv's. Since the $ALOX^{-/-}$ silences the biosynthesis of LXA4, $ALOX^{-/-}$ precipitated the least amount of colony forming unit, as illustrated in Figure 3b, suggesting that LXA4 is crucial for an *Mtb's* - ability to form colonies, which will eventually disperse into other cells (Divangahi et al, 2009).

The above information addressed the process of plasma membrane repair and focused on the mechanism *Mtb* uses to inhibit the repair process. The next sections will outline the process Mtb uses to degrade the mitochondria.

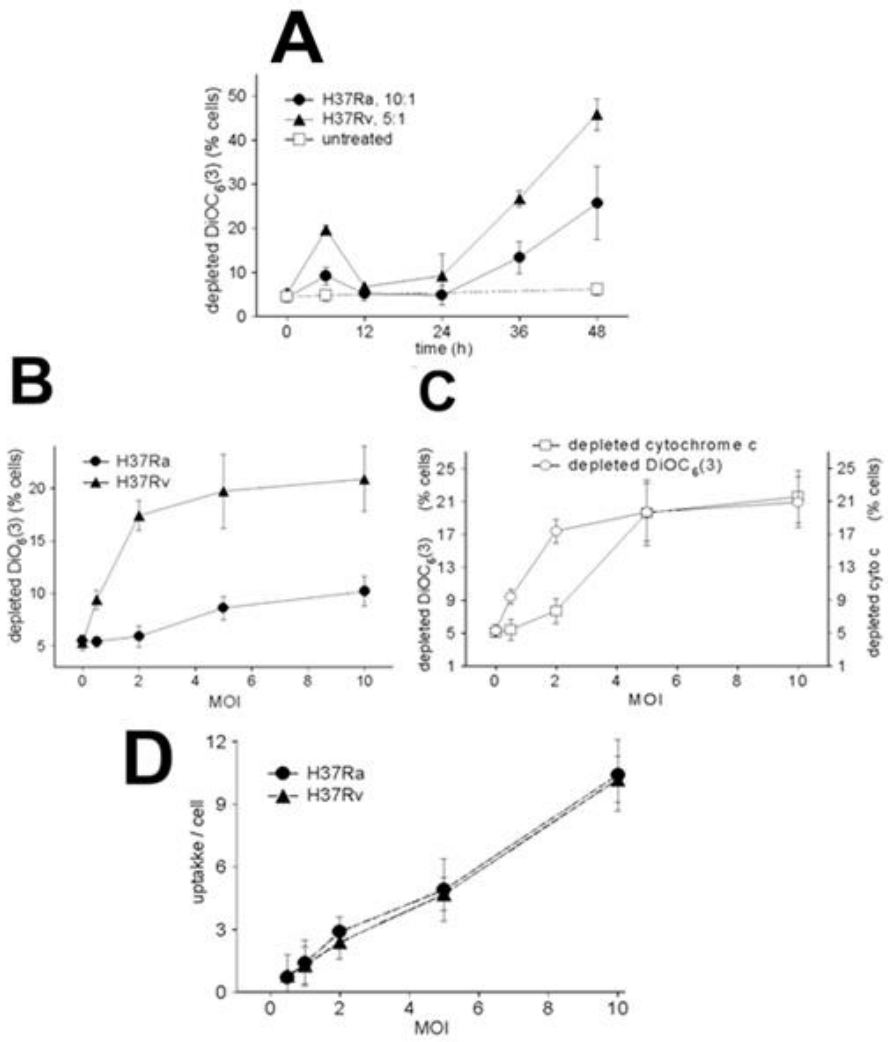

Figure 4. (A) Compares H37Ra with H37Rv's ability to deplete $DiOC_6$ in Mφ (B) MOI ability to deplete $DiOC_6$ (C) Correlates $DiOC_6$ with cytochrome c depletion (D) Control showing how much H37Ra is phagocytized compared to H37Rv (Chen et al, 2006).

To test for the change in the mitochondrial inner membrane potential ($\Delta\psi_m$), $DiOC_6$ was used as a marker in order to determine if the membrane exchanged ions. In Figure 4a, both H37Rv and H37Ra showed a depletion of $DiOC_6$ with the control showing no significant change in $DiOC_6$. This depletion illustrates the dissipation of $\Delta\psi_m$, which disrupts the mitochondria's respiratory (electron transport) chain in metabolism. H37Rv has about double the amount of

$DiOC_6$ depleted compared to H37Ra. Figure 4b shows $DiOC_6$ depletion based on the number of MOIs. The more infection sites there are the greater the dissipation of the $\Delta\psi_m$ by H37Rv compared to H37Ra. Figure 4c's data relates the depletion of $DiOC_6$ with the depletion of cytochrome c. Depletion of cytochrome c is a marker for Mφ apoptosis. Figure 4d shows that Mφ phagocytizes similar amounts of H37Rv and H37Ra strains (Chen et al, 2006).

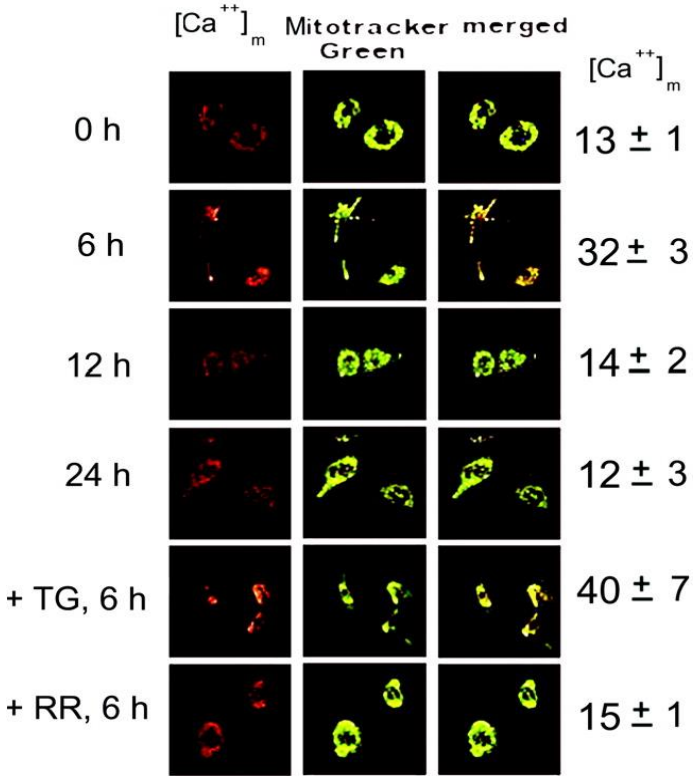

Figure 5. Confocal image with the green dye labeling the mitochondria and red dye labeling Ca^{2+} ions of H37Ra Mφ. Thapsigargin (TG) and Ruthenium Red (RR) effects on Ca^{2+} entering the mitochondria are shown (Chen et al, 2006).

Figure 5 demonstrates that an *Mtb* infection leads to an influx of Ca^{2+} in the mitochondria. This image reveals an increase in the concentration of Ca^{2+} at 6 hours suggesting a possible mechanism of $\Delta\psi_m$ dissipation. To illustrate the effects of virulent *Mtb*, TG which increases Ca^{2+} ability to enter the mitochondria was added leading to a greater concentration in

the mitochondria. Furthermore, RR which abrogates Ca^{2+} uptake into the mitochondria was added as well (Chen et al, 2006).

Discussion

These experiments demonstrated that Mtb interacts with a cell causing cell necrosis in two different ways: inhibiting the plasma membrane repair process and degrading the mitochondria. However, the severity of this dangerous infection is directly related to two factors. The first is the virulence of the strain inhaled demonstrated with H37Rv, which induced stronger tendencies to cause Mφ necrosis than H37Ra, which more frequently induced apoptosis. The second factor is the concentration (dose) of the *Mtb* inhaled and the extent of exposure, which is illustrated by the correlation of MOI and necrosis. The more infection sites there are in a Mφ, the more likely that cell will undergo necrosis (Divangahi et al, 2009).

The first mechanism that *Mtb* goes through to stimulate Mφ necrosis is by disrupting the Mφ plasma membrane repair machinery, especially when a virulent strain of *Mtb* is engulfed into a Mφ by phagocytosis. Once inside, the mycobacterium uses it ESX-1 protein secretion system to cause micro-disruptions in the Mφ plasma membrane.

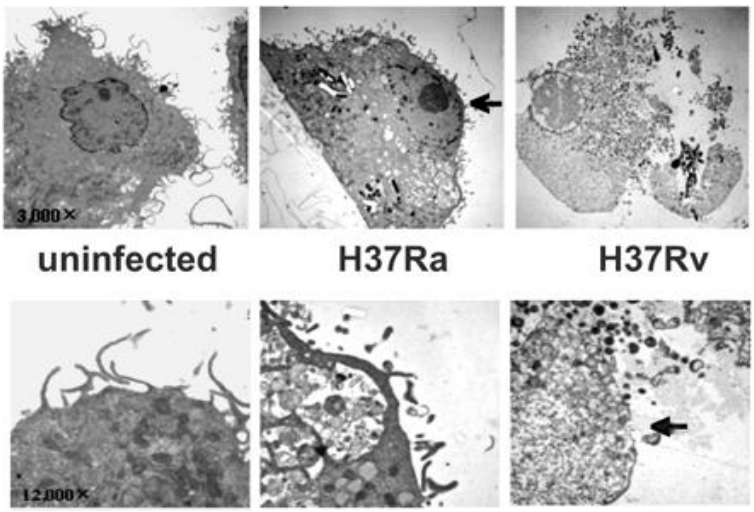

Figure 6. Morphology of Mφ infected with H37Ra and H37Rv. Uninfected Mφ acts as a control. H37Ra induces apoptosis which is shown by an intact cell membrane. H37Rv induces necrosis shown by a cell membrane (Chen et al, 2006).

As a result of these micro lesions in the membrane, the Mφ secretes PGE_2 stimulating lysosomes to release membrane vesicles aimed to repair the damage. It is unclear how PGE_2 facilitates the membrane repair. However, it is well established that a virulent strain of *Mtb* inhibits the synthesis of PGE_2 by activating the Mφ synthesis of LXA_4. Although the exact process used by *Mtb to* activate LXA_4 synthesis in Mφ is not fully understood, it could be a topic for future research. Nevertheless, LXA_4 inhibits PGE_2 by down regulating cyclooxygenase-2 (COX2) mRNA accumulation. Once PGE2 is shut down, lysosomes can no longer aid in plasma membrane repair. Without the Mφ ability to repair itself, the Mφ degrades and necrosis ensues. While lysosomes are directly influenced by PGE_2 activation, the Golgi apparatus' membrane repair mechanism is not dependent on PGE_2 stimulation suggesting that Mφ may have additional mechanisms to repair its cell membrane (Divangahi et al, 2009).

The experimental research also revealed that *Mtb* causes mitochondrial degradation and necrosis. H37Rv's ability to shuttle Ca^{2+} into the mitochondria was demonstrated through the depletion of $DiOC_6$ in a Mφ causing the dissipation of $\Delta\psi_m$ on the inner mitochondrial

membrane. Once the gradient between the intermembrane space and the mitochondrial matrix is broken, the proton gradient that fuels the respiratory (electron transport) chain will soon get destroyed (Chen et al, 2006). The Mφ loses its ATP making machine and the uncoupling of the respiratory chain lead to hyperproduction of superoxide anions, disruption of mitochondrial biogenesis, and the release of soluble intermembrane proteins. This negative cascade leads to a bioenergetic catastrophe that culminates in the disruption of the plasma membrane eventually causing necrosis. However, mitochondrial degradation also leads to the release of specific proteins such as cytochrome c and other apoptosis-inducing factors, which may cause a Mφ to undergo apoptosis. What determines the fate of the cell is the relative rate between bioenergetic catastrophes and apoptosis-inducing factor activation (Kroemer et al, 1998).

Although inhibiting membrane repair and the degradation of mitochondria are two separate processes, it is possible that both have a synergistic negative effect of causing cytotoxicity within an Mφ.

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Chapter 3: Case Study

Patient Intro

Name: Raul Meza
Age – 48
Sex: Male
Race: Hispanic

Why he came into the ER complaint:

"I woke up to have congestion and a deep cough, which I have had almost every morning for about 2 months. However, this time I coughed a lot of blood. Also, my muscles and joints have felt very weak for over a month, and just had very little energy throughout the day.

Patient history

History of Present Illness

Raul has had a chronic cough for over 2 months and has recently started seeing small amounts of blood in his sputum. He feels chest congestion and chest pain throughout the day but mostly when waking up in the morning. Additionally, he frequently has night sweats, and he feels fatigue throughout the day without any strenuous activities. He says he has had little to no appetite most days, which has led to a 20lb weight loss over 2 months.

Past Medical History

Raul has a history of hypertension and type II diabetes. He says he is on a healthy diet and exercises regularly to control his diabetes and hypertension. He is on Vasotec to control his blood pressure and Metformin for diabetes. Raul has no history of allergies, surgeries, or hospitalizations.

<u>Immunization Record:</u>

MMR: 01/15/1968 and 02/03/1973
DTaP: 08/23/1972
Tdap: 9/15/2013
Varicella: 04/17/1996

IPV: 04/17/1972
Hep A: 06/09/1995, 07/15/1995
Hep B: 06/09/1995, 07/15/1995, 08/27/1995
Yellow Fever: 11/19/1979
BCG: none

Raul does not get his annual influenza vaccine

Social History

He was born in Guadalupe, Mexico and migrated to the United States in 2008. Currently, he owns his lawn care business as a gardener/handyman. He does not smoke, and denies using illicit drugs or abusing alcohol. He is sexually active with his girlfriend.

Family History

Both parents are alive; his mother, Lucia, was a nurse, and his father, Fernando, was a middle school teacher in Mexico. Neither of his parents is a smoker. His mother's side of the family has no history of diabetes, hypertension, heart disease, liver disease, or kidney disease, but his grandmother (on his mother's side) developed breast cancer around 50 years of age. His dad's side has a history of diabetes and hypertension, but nothing too dramatic. His father has type II diabetes but watches his diet and has mild hypertension.

Review of Systems (ROS)

Constitutional Symptoms: unexplained weight loss, night sweats, fatigue, dizziness, nausea, malaise, fever, and increased urination

Eyes: Blurred vision and headaches

Cardiovascular: Chest pain and shortness of breath while at work

Respiratory: Coughing especially in the morning and hemoptysis

GI: diarrhea/constipation and recent, unintentional, and significant weight loss

Physical Examination

Vital signs recorded by the nurse before the physical examination:

Height: 5'8" Weight: 195 lbs BP: 123/84 mmHg Temp: 98.6 ºF HR: 75 bpm

Respiration Rate: 25 breathes/min O_2 saturation: 81%

General: Raul is an overweight individual but still fit. Appears agitated and nervous.

Skin: Good skin color and tone. No rashes.

HEENT:

Head: Unremarkable, no tenderness

Eyes: PERRLA, OS, 20/30 OD on the Snellen Chart,

Ears: Light reflex and tympanic membranes are grey and intact

Nose: No nasal discharge or mucosal swelling

Mouth: Gingivitis and poor dentition.

Face: Symmetrical.

Neck: Thyroid has normal size, lymph nodes normal and no bruits sounds of the carotid arteries

Chest/Lungs: Rales (Crackles) auscultation heard in the upper lobes of the lungs during inspiration. Deep breaths are not labored

Heart: 1/5 systolic murmur. RRR. S1 and S2 heard, no rub or gallop.

Abdomen: Soft, no pain during palpitation, non-distended. No organomegally. Normal bowel sounds

Musculoskeletal: Slight edema of feet, but muscle tone appears normal. No skeletal deformities

Neurological: Patient has mild loss of sensation in both feet. Otherwise neurologically intact.

Laboratory Findings:

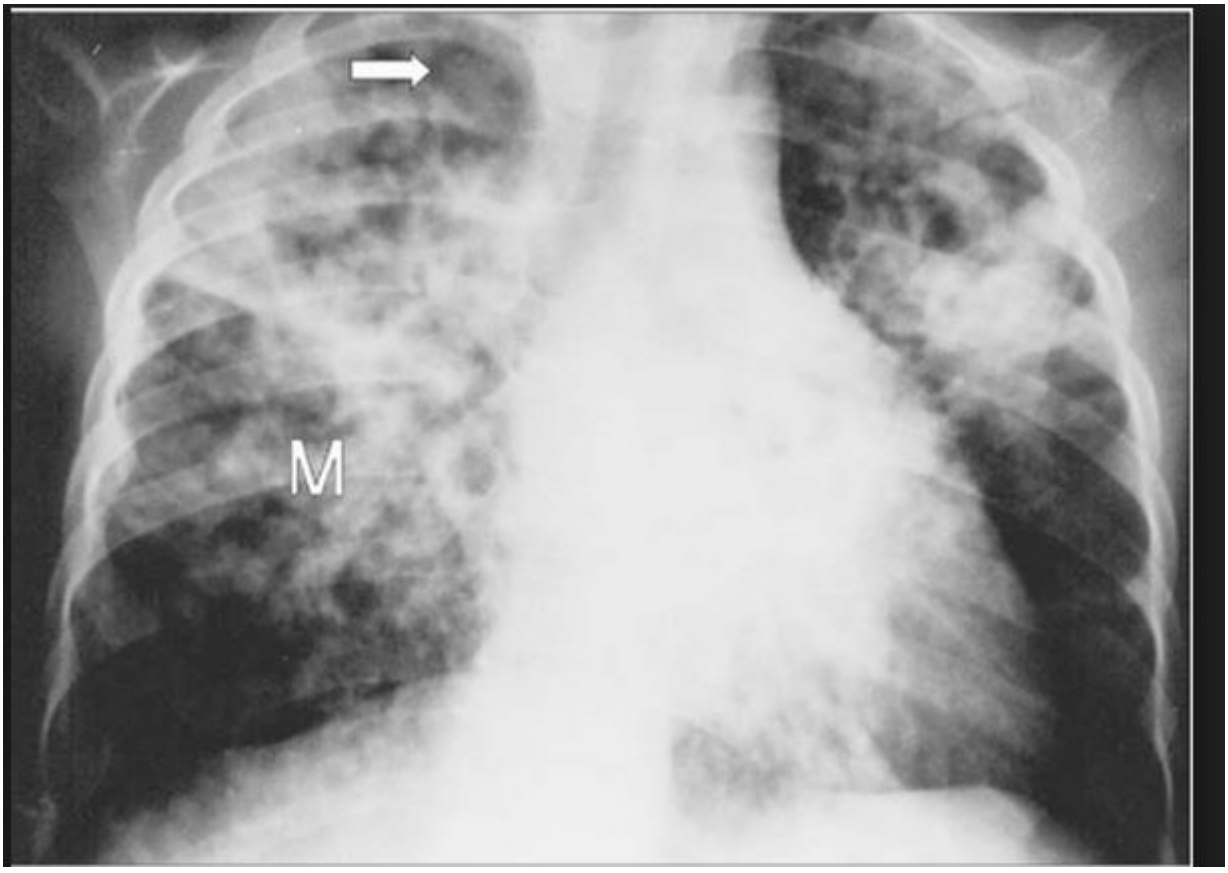
Test	Reference Value	Result
CBC		
RBC Count	**4.2-5.9 x 10^6/ µl**	**4.1 x 10^6 /µl**
Hemoglobin	14-17 g/dL	14 g/dL
Hematocrit	**41-51%**	**40.5%**
MCV	80-100 fL	99.4 fL
MCH	**28-32 pg**	**32.1 pg**
MCHC	32-36 g/dL	32.2 g/dL

White Cell Count	**4,000-10,000 /µl**	**12,500 / µl**
Neutrophil Count (Absolute)	1,800-7,700 /µl	4930/ / µl
Neutrophil Count (Relative)	35-80%	39.44%
Lymphocyte (Absolute)	**800-4,800 /µl**	**5,630/ µl**
Lymphocyte (Relative)	**18-44%**	**45.04 %**
Monocyte Count (Absolute)	**200-900/µl**	**1,810/ µl**
Monocyte Count (Relative)	**0-10%**	**14.48 %**
Eosinophil Count (Absolute)	0-800/µl	120 / µl
Eosinophil Count (Relative)	0-3%	0.96%
Basophil Count (Absolute)	0-100/µl	10/ µl
Basophil Count (Relative)	0-1%	0.1%
Platelet Count	150,000-350,000/µl	152,000/ µl
MPV	7.4-10.4 fL	10.3 fL
BMP		
Sodium	136-144 mEq/L	142 mEq/L
Potassium	3.7-5.2 mEq/L	5.0 mEq/L
Chloride	101-111 mmol/L	108 mmol/L
CO_2	20-29 mmol/L	24mmol/L
BUN	7-20 mg/dL	14 mg/dL
Creatinine	0.8-1.4 mg/dL	1.3 mg/dL
Glucose	**64-128 mg/dL**	**134 mg/dL**
Cholesterol	**< 200 mg/dL**	**210 mg/dL**
LDL	**65-180 mg/dL**	**191 mg/dL**
HDL	$\geq 35\ mg/dL$	35 mg/dL
Triglycerides	**< 150 mg/dL**	**161 mg/dL**
A1c	**4-5.6%**	**6.3%**
Liver Function Test		
ALT	0.-35 U/L	19 U/L
ALP	36-92 U/L	59 U/L
AST	0-35 U/L	24 U/L
γGT	0-30 U/L	23 U/L
Total Bilirubin	0.3-1.2 U/L	0.8 U/L
Conjugated Bilirubin	0-0.3 mg/dL	0.1 U/L
Albumin	3.5-5.5 g/dL	4.2 g/dL

1. Enzyme Immunoassay (EIA): Negative for HIV antibodies.
2. Tuberculin Skin Test (TST/PPD): 13 mm induration observed, positive test reading. Patient has no history of taking the BCG vaccine, which means it could possibly be LTBI. Further evaluation for active TB is required. Needs a chest X-ray as soon as possible and eventually a sputum smear, culture, and sensitivity to further test for active TB, as well as check if the *mycobacterium* is multidrug resistant.

Radiology

Chest X-Ray:



(Physiopedia,2011)

Arrows shows lung cavities in right upper lobe, while M shows the miliary pattern. X-ray is indicative of active TB.

Laboratory Findings (cont):

3. Sputum Test for Acid-Fast Bacilli (AFB): Positive for *Mycobacterium tuberculosis,* clearly indicative of active TB that is potentially infective. The patient is now isolated, and the ER doctor, who tested negative for TB 7 months ago was tested again as an immediate contact of an active TB case, is placed under observation; watching for any

signs of TB. Additionally, family members and immediate contacts will be brought in for TB testing.

4. QuantiFERON TB Gold In Tube (QFT) test : positive for TB antigens. Test collected as part of blood work. Additional evidence for TB.

Differential Diagnosis

Raul's CBC showed lymphocytosis. Due to a DTaP immunization and a recent history of receiving the Tdap booster, pertussis is unlikely even though his symptoms (e.g. cough and congestion) could be consistent with pertussis. In addition, hemoptysis is an unlikely symptom of pertussis. HIV/AIDS shows similar symptoms such as night sweats, cough, and shortness of breath, and could be a major factor for immunosuppression causing LTBI progression to active TB. But, the EIA showed no antibodies for HIV. Moreover, lymphoma may show similar levels of lymphocytosis, and its metastasis to the lung could lead to symptoms like chronic cough, hemoptysis, and weight loss. However, laboratory blood tests performed did not support such diagnosis. A lack of an abnormal readings on the CXR makes lung cancer improbable.

Additionally, cold and flu like symptoms are being considered, and the patient can have a severe case of influenza on top of his active TB. However, it is highly unlikely that seasonal influenza would cause prolonged respiratory symptoms such as hemoptysis and decreased respiratory function. However, during treatment, Raul should be observed as TB might just overshadow influenza symptoms.

Diagnosis and Supporting Argument

Raul Meza is diagnosed with active pulmonary tuberculosis based on his present symptoms, the physical exam findings, and the positive PPD, CXR, sputum smear, and QFT gold test.

TB is not a very frequent infection as there are less than 100 newly diagnosed active TB cases in Nevada each year (Department of Health, 2015). However, Raul's symptoms are characteristic for active TB, especially his persistent cough, hemoptysis, weight loss, and night sweats. Additionally, he is a diabetic Hispanic male who emigrated from a TB endemic area in Central America. Also, his recent travels to his native country put him at an elevated risk for contracting TB. The physical examination and lung auscultation illustrated that Raul had crackles in his upper lobes that are evident during inspiration. Moreover, these finding were confirmed with skin testing, CXR, sputum smear and blood assay with QFT gold tests.

Treatment Plan

The overall goals for Raul's treatment aim to 1) cure his active TB, 2) prevent the transmission of *M. tuberculosis* to other individuals and 3) minimize the risk of drug resistance. Treatment will start with an initial phase of 8 weeks of antibiotic combination therapy, which include isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA). One dosage of each (300mg INH, 600mg RIF, 800mg EMB, and 1,000mg PZA) will be administered daily for a total of 56 doses. The continuation phase of treatment will be determined based on sputum culture results obtained during the completion of the initial treatment phase. If the sputum culture is negative, there will be an additional 18 weeks of treatment with daily doses (126 doses) of INH, 300 mg, and RIF 600 mg. However, if the sputum culture is positive, there

will 18 to 31 more weeks of treatment, depending on the AFB results after the first 8 weeks of the continuation phase, with daily doses of INH, 300 mg, and RIF, 600 mg. If the AFB becomes negative after 8 weeks of the continuation phase of treatment, the 18 month plan will be followed. If the AFB culture continued to be positive, then the full 31 week plan (maximum plan with both initial and continuation being 9 months) will be administered (CDC, 2003).

Raul will be given patient-centered care which is supervised by the public health department, since a deviation from the plan could cause a threat of disease transmission into the community. This strategy should include a strict adherence to the treatment plan, and the implementation of directly observed therapy (DOT). Raul's management plan will be individualized to ensure adherence to and completion of the drug regimen. This may require direct involvement and support of the county social services and even the provision of incentives and enablers (CDC, 2003).

Intact anti-tuberculosis medications should be administered together and split dosing avoided. Mr. Meza will undergo routine monthly evaluations to identify any possible adverse effects of the medications and assess his adherence to the medication plan until the completion of the treatment (CDC, 2003).

Since Raul is currently taking Vasotec, which has shown to have some drug interactions with INH and RIF. He will be closely monitored to make sure that his hypertension is stabilized and under control. If not, then he will be placed on another more appropriate anti-hypertensive medication (CDC, 2003). Moreover, there is a small risk of exacerbating his peripheral neuropathy due to side effects of INH. If peripheral neuropathy worsens, pyridoxine supplementation will be administered (CDC, 2013). Also, any new medications should be discussed before being taken.

As active TB is infectious, his girlfriend and all his immediate contacts will be tested for TB. They will also be instructed on the symptoms of TB and advised to seek out medical attention if symptoms occur.

Prognosis

Raul is very likely to be cured of TB if he adheres to the treatment regimen. Because of evident cavitation observed in his CXR, he will mostly likely need to be on the full 9 month plan. However, AFB cultures will still be the determining factor for longevity of his continuation phase of treatment. Once he is done with his treatment plan and his sputum cultures are negative, he will be clear of his *M. tuberculosis* but will have to continue to take his Metformin and anti-hypertensive medications. With this medication plan, a relapse of TB symptoms is unlikely, but if symptoms return, a new treatment plan will be considered.

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