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FACULTAD DE CIENCIAS

Identificación de los cambios en el perfil de fosforilación de proteínas en macrófagos humanos tras la infección con *Mycobacterium sp.*

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“No Man is an Island, entire of itself” - John Donne

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“His face wore a leaden hue; the eyes were utterly lustreless; and the emaciation was so extreme that the skin had been broken through by the cheek-bones. His expectoration was excessive. The pulse was barely perceptible...”

On a tuberculosis dying patient. - The Facts in the Case of M Valdemar - E. A. Poe

“… So they’re going to stay home, drink fluids and get plenty of bedrest, and then they’re going to die. Before they do, they’re going to infect everyone who comes into the same room with them.”

Stephen King

“I see them dying all across the globe
Their greatest foe is but a small microbe”

Ayreon

Contents

Common abbreviations:

1. CD: Cluster of differentiation
2. *M.bovis* BCG: *Mycobacterium bovis* Bacillus Calmette-Guérin
3. *M. tuberculosis* H37Rv: *Mycobacterium tuberculosis* H37Rv strain
4. HIV/AIDS: Human immunodeficiency virus infection and acquired immune deficiency syndrome
5. LAM: Lipoarabinomannan
6. LM: Lipomannan
7. MDRTB: Multidrug resistance tuberculosis
8. MTB / *M. tuberculosis*: *Mycobacterium tuberculosis*
9. SSA: Secretaría de Salud
10. TB: Tuberculosis
11. TLR: Toll-Like receptor
12. WHO: World Health Organization
13. XDR-TB: Extensively drug resistance tuberculosis

14.

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Abstract

Tuberculosis (TB) is an infectious disease caused by the rounded, non-self-motile airborne bacilli *Mycobacterium tuberculosis* that mainly affects lungs. However not every infected person might develop the disease, instead a latent chronic infection can be present in those who do not show symptoms. It is estimated that a third of the world population is infected with *M. tuberculosis* and is considered among the top 10 causes of deaths around the globe. MTB uses the lower respiratory system alveolar macrophages as the primary host, which hold an important role in eliminating parasites due to the recognition of Pathogen Associated Molecular Patterns (PAMP's) by receptors such as Pattern Recognition Receptors (PRR's), CD14 and TLR's that, after interacting with its ligands, activate a signaling cascade regulated by the phosphorylation of proteins, controlling the union of transcription factors and thus effectively modulating gene expression. The mitogen-activated protein kinases (MAPK) control different cellular responses to signals as infection, activating different cellular programs as proliferation, differentiation, development, transformation or apoptosis. Three different families have been

identified: extracellular signal-regulated kinase (ERK), C-Jun terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 kinase.

In order to determine the changes in the activation or inactivation of cellular pathways and changes in the phosphorylation state of proteins in the human immune response against *Mycobacterium sp*, MDM's of healthy donors, were infected with the tuberculosis vaccine as well as a pathogenic strain (*M. tuberculosis* H37Rv), afterwards an analysis of the total phosphoproteins, their band profile, and the activation of the MAPK was carried out. Quantification of total phosphoproteins showed an increase under infection conditions with the highest level corresponding to the virulent strain. The profile analysis demonstrate that the majority of phosphoprotein are shared between all conditions (infections and non-infection) within a subject, but profiles can vary greatly between subjects; no unique phosphoprotein was shared between all subjects for a given condition. The MAPK pathway showed an increased activation under the infection with *M. tuberculosis* but a diminished activation when infecting with *M. bovis* BCG. Only p38 seem to be downregulated when infected with *M. bovis* BCG, on the contrary neither JNK nor ERK ½ showed any significate difference under any treatment.

Resumen

Tuberculosis es una enfermedad de etiología infecciosa causada por el bacilo *Mycobacterium tuberculosis* que se transmite por ví aérea. EL cuadro sintomático incluye debilidad, fiebre, pérdida de peso y sudoración nocturna, aunque el cuadro clínico puede variar de acuerdo a los órganos afectados, por ejemplo, la infección en pulmones causa tos, dolor en el pecho y hemoptisis, que significa toser sangre o mucosa con sangre. También puede infectar riñones, médula espinal y cerebro.

Aspectos socio-económicos como la hambruna y guerras, junto con un uso irracional de antibióticos y baja calidad en los tratamientos contra la tuberculosis han contribuido a que *Mycobacterium tuberculosis* haya

desarrollado resistencia contra antibióticos de primera línea y así mismo favorecen la emergencia de cepas multidrogo resistentes y, en algunos casos, de drogo-resistencia extendida, micobacterias resistentes tanto a la primera como a la segunda línea de antibióticos contra tuberculosis.

La probabilidad de ser infectado por *Mycobacterium tuberculosis* dependen de la frecuencia, proximidad y tiempo de exposición al bacilo, bajo condiciones de calor y humedad las bacterias pueden quedar suspendidas en el aire durante horas. Los pacientes que han desarrollado el síndrome de inmunodeficiencia adquirida por la infección con el virus de inmunodeficiencia humana , los niños menores de cinco años, personas recientemente infectadas o pobremente tratadas por *Mycobacterium tuberculosis* y aquellos bajo terapia inmunosupresora, entre otros, son más susceptibles de desarrollar tuberculosis. Sin embargo, no todas las personas que son infectadas desarrollan la enfermedad, generando dos condiciones: una de latencia y una patogénica.

Durante los siglos 17 y 18 la tuberculosis tuvo una mortalidad cercana al 25 % y fue también conocida como la peste blanca. Datos de la Organización Mundial de la Slaud señalan que en el 2015 10.4 millones de personas desarrollaron tuberculosis, y que 1.4 millones perecieron por dicha enfermedad, 95% de esos decesos ocurrieron en en países en vías de desarrollo. Es considerada la enfermedad infecciosa que provoca más muertes en el mundo, encontrándose entre las diez causas de muerte más comunes en el mundo. La co-infección de *Mycobacterium tuberculosis* con VIH es responsable por 400 000 muertes anualmente.

En México existen quince mil casos nuevos reportados al año, de acuerdo al sistema nacional de vigilancia epidemiológica, con una mortalidad de 2000 personas al año. Usualmente el diagnóstico es confirmado por cultivo, estudios histopatológicos, inmunológicos y pruebas moleculares. De acuerdo a la Secretaría de Salud los grupos más vulnerables para el desarrollo de tuberculosis incluyen a aquellos individuos que sufren otras enfermedades como diabetes, malnutrición, SIDA y alcoholismo, así como los adultos entre

25 y 44 años de edad. Los estados con mayor prevalencia son Baja California, Guerrero, Sinaloa, Sonora y Tamaulipas, presumiblemente por los movimientos migratorios a lo largo de la región.

Mycobacterium tuberculosis es una bacteria redondeada incapaz de motilidad propia que mide de 2 a 4 micrómetros de largo. Es un parásito aerobio intracelular obligado que se transmite entre individuos. Sutiempo de división varía de 15 a 20 horas. Fue descrita por primera vez como causa etiológica de la tuberculosis por Robert Koch en 1882, lo cual llevó al descubrimiento de la tuberculina y su empleo como técnica diagnóstica y, subsecuentemente, al desarrollo de un vacuna basada en el bacilo de Calmette Guérin (*M. bovis* BCG) junto con el coadyuvante completo de Freund, agente inmunogénico que promueve la producción de Interferón gamma por células T CD4+ y CD8+.

Mycobacterium tuberculosis pertenece al género *Mycobacterium*, que incluye a otras especias patogénicas como M. leprae, la causa etiológica de la lepra; M. avium, causante de una enfermedad parecida a la tuberculosis en pacientes con SIDA; *M. bovis*; *M africanum*; *M. microti*; *M. caprae*; *M. pinnipedii*; *M. canetti* y *M. mungi* Este grupo comparte un grupo de antígenos conservados, como PPE38, una proteína de membrana que tiene la capacidad de modular la respuesta inmune al interactuar con receptores TLR.

La membrana de *Mycobacterium tuberculosis* posee una fracción hidrofóbica y una hidrofílica, la primera incluye moléculas relacionadas con señalización e infección, mientras que la parte hidrofóbica contiene moléculas relacionadas con el mantenimiento de la viabilidad.

Antígenos de la pared celular de micobacterias virulentas como los peptidoglicanos, arabino galactosas, ácidos micólicos, manosas fosfatidil-inositol, lipomananas y lipoarabinomananas y lipoarabinomananas con manosas, juegan un papel importante en la activación de células del sistema inmune, como los macrófagos, interactuando con receptores como NOD2, TLR's y de tipo Scavenger. Juntos pared y membrana proveen de un ambiente altamente impermeable al ambiente que le permite a la bacteria sobrevivir en ambientes hostiles modulando la respuesta inmune del hospedero, y

generando resistencia a antibióticos, moléculas del sistema de complemento, compuestos ácidos y alcalinos o factores oxidantes, como la toxicidad mediada por cobre.

El sistema inmune es responsable de la defensa contra cualquier agente efector, los cuales pueden o no ser de origen biológico. Está compuesto por un grupo de mecanismos altamente regulados. Una deficiencia en la respuesta lleva a la incapacidad de eliminar patógenos incrementando la vulnerabilidad frente bacterias, hongos, virus y otros parásitos; mientras que una sobre activación en intensidad o duración puede acarrear graves consecuencias dañando tejidos o incluso provocando la muerte. Podemos estudiar al sistema inmune en dos partes: la innata y la adaptativa, la última de éstas se refiere a la defensa contra antígenos específicos y toma días en desarrollarse, la parte innata es una respuesta altamente conservada entre los vertebrados e incluye factores listos para una reacción inmediata frente a la presencia de patógenos, puede estudiarse como diferentes barreras:

Barreras físicas, que bloquean la entrada de patógenos, como los epitelios, mucosas y la superficie de glándulas exócrinas.

Barreras químicas, son substancias solubles con actividad microbicida, como el sistema de complemento.

Barreras celulares, son una línea de defensa empleada cuando las dos anteriores han sido superadas. La respuesta celular se da cuando hayuna interacción entre receptores membranales o intracitoplasmáticos con componentes moleculares conservados entre los parásitos. Algunas células como los neutrófilos y los macrófagos pueden destruir partículas invasoras mediante fagocitosis. El reconocimiento de patógenos provoca una activación de vías celulares que median la liberación de citosinas y otras moléculas pro-inflamatorias, las cuales incrementan la actividad antibacterial y el reclutamiento de otros factores y células del sistema inmune como los granulocitos polimorfonucleares, células másticas, plaquetas, células endoteliales, macrófagos, células dendríticas y células natural killer.

Los monocitos se derivan de precursores de médula ósea tras la exposición a factores estimulantes de colonias de macrófagos e interleucina 3, la cual induce la expresión de receptores de membrana específicos; posteriormente migran al torrente sanguíneo donde sobreviven hasta 70 horas donde constituyen de 5 a 10% de los leucocitos totales. Existen dos tipos de monocitos: aquellos relacionados con la inflamación y aquellos que desempeñan un papel de inmuno-vigilancia. Mientras viajan por el torrente sanguíneo son expuestos a factores de diferenciación y crecimiento lo cual provoca una diferenciación a macrófagos, los cuales incrementan sus organelos en número y complejidad, adquiriendo nuevas funciones como la capacidad de liberar mediadores citotóxicos y proinflamatorios, incrementan los seudopodios en número y tamaño, y desarrollan los medios necesarios para fagocitar microbios y otras partículas extrañas, así como células apoptóticas.

El principal papel de los macrófagos es engullir partículas extrañas y eliminar residuos celulares y apoptóticos del cuerpo. El primer paso para la iniciación de una respuesta inmune celular es el reconocimiento de patrones de patógenos por receptores tlr o scavenger, los cuales permiten la fagocitosis de las partículas patogénicas para su posterior degradación y procesamiento antigénico y la posterior presentación mediante el complejo de histocompatibilidad II a otras células del sistema inmune adaptativo, las cuales producen anticuerpos específicos.

El sistema inmune tiene la capacidad de mediar la comunicación celular utilizando instrucciones químicas de naturaleza proteica llamadas quimiocinas. Son producidas por un grupo específico de células en condiciones particulares, a su vez pueden ser recibidas por otras células mediante la interacción con receptores de manera autócrina, parácrina regulando su transcripción génica. En el caso de los macrófagos, IFN-gamma es uno de los agentes de activación celular más conocidos y es secretado por células T del sistema inmune provocando la taxia hacia sitios particulares. Dentro del repertorio de citocinas propias del macrófago podemos encontrar:

TNF-alpha (tumor necrosis factor- alpha), estimula la respuesta inmune y la transvasación de monocitos al incrementar la permeabilidad vascular, incrementar la adhesión celular, facilitar la diapédesis y recluta a los monocitos Il-1 (Interleucina-1 alpha o 1 beta y 1Ra) es una señal temprana proinflamatoria que promueve la liberación de histamina y prostaglandina, quimio-atrae granulocitos y favorece la expansión clonal y diferenciación de células T CD 4. Al ser estimulado su receptor, Il-1R, se provoca la expresión de los genes que la producen. Está cercanamente relacionada con la orquestación de la autofagia y la formación de cuerpos apoptóticos. Actúa sobre la vía de NF-kappa beta.

Il-6 Puede tener efectos pro o anti inflamatorios dependiendo al receptor al que se une. Promueve la diferenciación de células B hacia células plasmáticas y activa células T citotóxicas, recluta monocitos, mantiene la población de células Th17 e inhibe la formación de células T reguladoras (Tregs) así como la apoptosis celular.

Il-12 Formado por dos subunidades, la p35 y la p40, promueve la respuesta inmune mediada por células al promover la citotoxicidad de células NK y T CD8. Es un factor anti-angiogénico. Se cree que puede ser secretado junto con TNF.

Il-18 Actúa de manera sinérgica con Il-12 para activar células T y NK. Tiene repercusión sobre la vía de las MAPK.

Il-23 Comparte la subunidad p40 con Il-12 y favorece la activación. Incrementa la producción de Il-10 a induce la síntesis de Il-17.

Il-27 Es un miembro de la familia de Il-12 al compartir la subunidad p28 y el gen 3 de inducción por virus de Epstein-barr. Es secretado de manera temprana y favorece la diferenciación de células Th1 e inhibe la formación de células Th17. Puede tener efectos anti y rpinflamatorios.

Il-10 Suprime la activación de macrófagos y la producción de otras citosinas proinflmatorias así como la presentación de antígenos, induce la expresión de IgG’s en células B. Disminuye la capacidad antimicrobiana de los macrófagos.

TGF-beta (Transforming growth factor beta), disminuye los efectos proinflamatorios de otras citosinas, suprime células Th1 y Th2 y fomenta la formación de Tregs.

Además de las citosinas los macrófagos cuentan también con un repertorio de quimiocinas que son un grupo de citosinas que promueven la taxia celular, reclutando células del sistema inmune al sitio donde son requeridas, así mismo pueden tener un papel en la diferenciación celular y la respuesta inflamatoria. Las principales quimiocinas de los macrófagos son:

CXCL1 y CXCL2 conocindos también como MIP o proteína inflamatoria de macrófago. Recluta neutrófilos y células del sistema hematopoietico. Tienen propiedades angiogénicas.

CCL5 quimioatrae células T, basófilos, eosinófilos y células dentríticas, asimismo tiene un papel en la activación de células NK.

CXCL8 (Il-8) Utilizado para reclutar neutrófilos y su degranulación.

CXLCL9 Potente reclutador de células T, es necesario para la inflamación y la reparación de tejidos. Expresión es inducida por interferón gamma.

CXCL10 (IP-10) Quimioatrae células T, NK y dentríticas. Es inducido por interferón gamma.

CXCL11 (IP-9) con funciones similares a CXCL9 y CXCL10 peor con efectos más potentes.

La secreción de citosinas por parte del macrófago dependerá de la manera en la que fue activado, de la manera clásica (M1) o alternativa (M2a, M2b o M2c). Las señales que determinarán el tipo de activación pueden ser hormonas, citosinas, complejos inmunes o la presencia de células apoptóticas. Los macrófagos M1 tienen una alta capacidad bactericidad mediante la producción de radicales libres, mientras que los macrófagos M2 interactúan con células Th2, eosinófilos y basófilos

Mycobacterium tuberculosis tiene como principal nicho los macrófagos alveolares del sistema respiratorio inferior, para llegar a ellos emplea una

estrategia evasiva que se basa en la infección por gotas con pocas bacterias las cuales tienen un tamaño aproximado de 5 micrómetros, atravesando boca, nariz y el resto del aparato respiratorio superior y bronquios hasta llegar a los alveolos pulmonares también tiene la capacidad de evitar el reconocimiento por lípidos. Los macrófagos alveolares introducen al patógeno mediante receptores de fagocitosis, por lo cual la bacteria reside en una vacuola de membrana llamado fagosoma dentro de la cual previene su degradación y correcta presentación evitando la unión de lisosomas a los endosomas, mecanismo común para eliminación microbiana de partículas fagocitadas, en donde se ha observado la presencia de antígeno temprano de endosomas, pero el proceso de maduración es interrumpido, la bacteria patógena también es capaz de evitar la maduración de autofagosomas. Obtiene de su célula hospedera los recursos necesarios para su supervivencia, incluido el uso de colesterol del macrófago como fuente de carbono. El macrófago responde a la infección produciendo intermediarios de oxígeno reactivo (ROI) e intermediarios reactivos de nitrógeno (RNI) como agentes bactericidas, así como moléculas de adhesión celular y quimosinas que reclutan otras células del sistema inmune. A su vez la célula hospedera puede puede sufrir 3 destinos:

- Necrosis, en el cual se pierde la integridad de la membrana, dicho fenómeno se observa más en cepas virulentas como en la H37Rv.

- Apoptosis, en donde se conserva la membrana y se reduce la viabilidad bacteriana. Las formas más virulentas de tuberculosis inhiben la apoptosis, uno de los mecanismos por los que lo logran es interactuando con el retículo endoplásmico (ER) bloqueando la salida de Ca^{2+} impidiendo una correcta homeostasis del colesterol por medio de un cambio en la expresión de EST-1 y AMRP, la apoptosis puede ser activada por la actividad un factor de virulencia bacteriano llamado ESX1 tipo VII que forma un complejo de secreción, o mediante la estimulación de células T por medio de la vía de CD95 y se puede desarrollar por vía extrínseca o intrínseca.

- La supervivencia del macrófago permitiendo la proliferación de la bacteria.

Las micobacterias inducen la formación de granulomas que tienen condiciones anóxicas y ácidas en un núcleo necrótico. Dichos cuerpos tuberculosos pueden infiltrarse a vasos sanguíneos donde pueden diseminarse causando tuberculosis extrapulmonar, también pueden ser objeto de respuestas de hipersensibilidad en donde las lesiones primarias se vuelven fibróticas y se calcifican formando cuerpos de Ghon los cuales son visibles en rayos X. MTB explota la formación de granulomas al infectar macrófagos nuevos que son reclutados de macrófagos que están muriendo. Posteriormente son liberados cuando un individuo afectado tose y los granulomas se rompen en los bronquios liberando a los bacilos. El bacilo de la TB tiene la capacidad de conservarse en un estado inactivado durante años en condiciones pobres en nutrientes e hipóxicas dentro de los granulomas, estableciendo una infección crónica.

Cuando las micobacterias interactúan con los receptores de los macrófagos, estos modulan su expresión génica de acuerdo a la activación de vías de señalización regulando la unión de factores de transcripción mediante fosforilaciones. Una vez que un ligando ha interactuado con su receptor la señal se transduce hacia el interior celular mediante proteínas adaptadoras. Las fosforilaciones no sólo pueden generar un cambio en la conformación en la proteína que es fosforilada, también son capaces de permitir la unión con otras proteínas a través de los dominios de fosfotirosina, alterando su localización dentro de la célula y evitando o promoviendo su degradación.

Una de las vías más importantes en la comunicación celular es la de las MAPK, las cuales controlan diferentes respuestas celulares frente a diversas señales como feromonas, ayuno, alta osmolaridad, shock hipotónico, depravación de carbono/nitrógeno, infección, etc, y promueven una respuesta celular acorde activando diversos programas celulares como la proliferación, diferenciación,

desarrllo, transofrmación o apoptosis. Existen 3 familias identificadas dentro de las MAPK: JNK, ERK (1 y2) y p38

Este trabajo tiene como objetivo Determinar la activación o inactivación de vías celulares analizando cambios en la fosforilación de proteínas de macrófagos humanos infectados con *Mycobacterium sp*. Para lo cual macrófagos humanos derivados de monocitos fueron infectados como *M. tb o M. bovis* a una MOI de 5:1, por 24 horas, posteriormente las células fueron lisadas y se recuperó la porción hidrosoluble. Fue realizado un análisis de fosfoproteínas totales, activación de la vía de las MAPK estudiando las formas fosforiladas de JNK, P38 y ERK ½ , y un análisis del perfil de bandas de proteínas fosforiladas. Fue posible observar un incremento en la fosforilación de proteínas en condiciones de infección en todos los casos, en 3 de ellos el mayor incremento se da en macrófagos que fueron infectados con la cepa virulenta H37Rv. Un incremento en la cantidad de fosfoproteínas totales es un reflejo del aumento de vías de señalización celular que están siendo activadas, podemos ver que las células responden a la infección.

Las densitometrías y posterior análisis mediante el uso de modelos lineares mixtos generalizados señalan que ni ERK en sus dos isoformas 1 y 2 o JNK parecen sufrir alteraciones bajo ninguna condición de infección, sin embargo, es importante mencionar que los datos presentaron una alta dispersión. Por lo contrario P38 se encuentra menos fosforilado en la infección con BCG. Al analizar todas las proteínas es posible ver que las células responden incrementando la activación de la vía de las MAPK cuando se encuentran infectadas con cepas virulentas, de modo contrario, hay una menor activación bajo infección con BCG comparando contra el estado basal y la infección con *M. tb*.

Con el objetivo de encontrar patrones coincidentes tanto en la aparición como en la desaparición de bandas comparamos las condiciones basales con sus respectivas infecciones, encontramos que existe una gran variación en la respuesta entre los individuos, tanto en el número total de bandas como en

los patrones. Realizamos un análisis densitométrico de los western blots Una prueba de t arrojó que no existen diferencias significativas.

Introduction

Tuberculosis:

Tuberculosis (TB) is an infectious disease caused by the airborne bacilli *Mycobacterium tuberculosis*. Symptoms may include weakness, fever, weight loss, sweating while sleeping, although the clinical picture might change according to the affected organs, e.g., lung infection causes coughing, chest pain and hemoptysis (coughing up blood or bloody mucus). It can also infect kidneys, spinal cord and brain [1].

Social and economic issues such as war and famine; in addition to irrational antibiotic use and poor quality TB treatments contribute to the development of MTB resistance against first-line antituberculosis (anti-TB) drugs [2], favour the emergence of multi-drug resistant tuberculosis strains (MDR-TB), and in some cases extensively drug resistance (XDR-TB) which are MTB resistant to both first and second line of anti-TB drugs [3].

Chances of an MTB infection depend on the frequency, proximity and time of exposure to the bacilli [1], in some cases humidity and heat allow the bacteria to be suspended in the air for many hours. HIV/AIDS patients, children younger than five years, people recently infected or poorly treated for MTB and those under immunosuppressing therapy, among others, are most likely to develop TB, [4] however not every infected person might develop the disease, this interaction between patient and bacteria creates two possible conditions: one of latency and other one pathogenic [5].

History and epidemiology:

During the 17th and 18th century tuberculosis had a mortality prevalence of up to 25% and was known as the white plague [6]. Data from the World Health Organization (WHO) shows that in 2015 10.4 million people worldwide developed TB and 1.4 mills passed away [4], 95% of those fatalities happened in developing countries. It is the single infectious disease that causes more fatalities worldwide [7] and it is now considered among the top 10 causes of death around the globe. The coinfection with HIV/AIDS is responsible for 400 000 deaths annually [8].

In Mexico, there are 15 thousand new cases reported every year, according to the National System of Epidemiological Vigilance, with a mortality of 2 thousand each year. There's a legal regulation about the treatment standardization for tuberculosis patients, which is described in the NOM-006-SSA2-1993. Usually diagnostic must be confirmed by cultures, tissue and immunological studies, and molecular tests. As reported by the Government Secretariat of Health (SSA), there are groups of people more vulnerable to the development of TB such as those who already suffer from another disease like diabetes, malnutrition, HIV/AIDS, and alcoholism; as well as adults ranging from 25 to 44 years old [9]. The states with the higher incidence of tuberculosis are Baja California, Guerrero, Sinaloa, Sonora, and Tamaulipas; presumably, this is due to migration movements along the region [10], Figure 1.

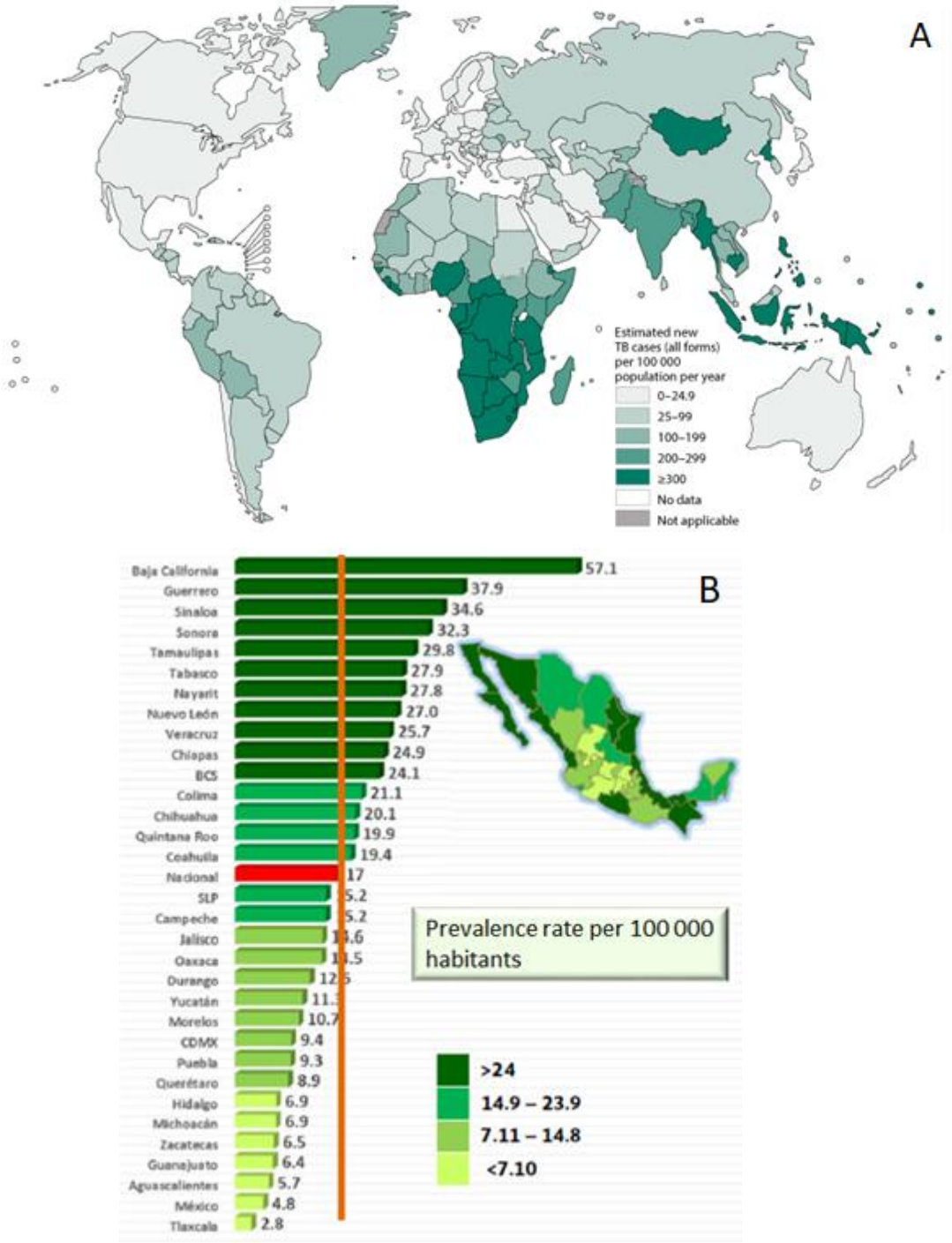


Figure 1.- The incidence rate of TB during 2015. (A) The map is showing the worldwide incidence of TB per 100000 persons [11]. (B) The map of TB incidence in Mexico with a list of incidence by state [10].

Mycobacterium tuberculosis

Mycobacterium tuberculosis (MTB) is a rounded, non-self-motile bacterium that measures 2 to 4 micrometers in length (Fig. 2), it is an obligate intracellular aerobe parasite which is transmitted between subjects (Fig. 3), with a division time of 15 up to 20 hours [9]. It was described as the cause of tuberculosis by Robert Koch in 1882 which led to the discovery of the tuberculin as a diagnostic test, and subsequently to the development of a vaccine based on the bacilli Calmette Guérin (*M. bovis* BCG) [5], along with the complete Freund adjuvant, which has immunogenic agents that elicit INF γ production by T CD4+ and CD8+ cells [12].

M. tuberculosis belongs to the genus *Mycobacterium* that includes other pathogenic species such as *M. leprae*, the etiological cause of Leprosy; *M. avium* causative of a tuberculosis-like disease among HIV patients; *M. bovis; M. africanum; M. microti; M. caprae; M. pinnipedii; M. canetti and M. mungi*[1]. This group shares highly conserved antigens [13], as PPE38, a membrane protein, that can modulate the immune response by interacting with TLR's [14]. MTB membrane has both hydrophilic and hydrophobic fractions, the first one including signaling and infection-related molecules, whereas the second holds viability-related molecules [15].

Cell wall antigens of virulent mycobacteria like peptidoglycans, arabinose-galactose, mycolic acids, phosphatidylmyo-inositol mannosides, phthiocerol, Lipomannan (LM) and Lipoarabinomannan (LAM) [12] and ManLAM [16] (LAM with attached mannoses oligosaccharides) play an essential role in the activation of immune cells (such as macrophages) by interacting with receptors like NOD2 (Nucleotide Binding Oligomerization Domain Containing 2) [17] and TLRs [15], altering the phagocytic capabilities of antigen-presenting cells [16].

Altogether cell wall and membrane provide a highly impenetrable environment that allows bacteria to survive within hostile environments [18,19] by modulating the immune response of the host and by generating resistance against antibiotics, complement system molecules, acidic and alkaline compounds, or oxidizing factors [20] such as copper-mediated toxicity [18].

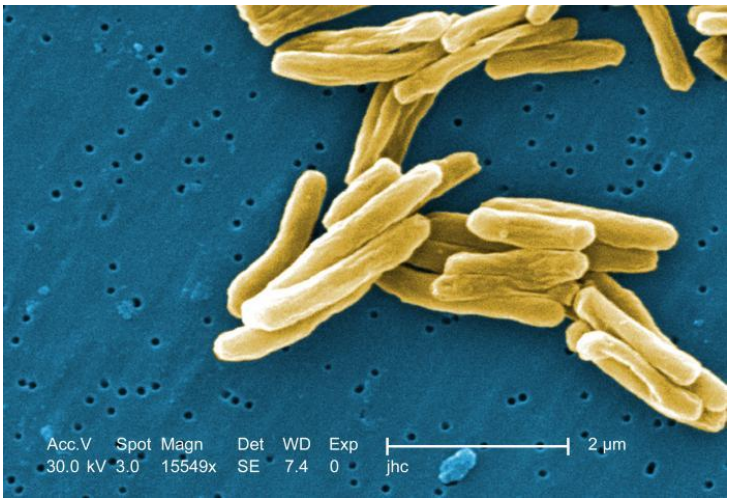


Fig 2.- A microphotograph of MTB as seen on a scanning transmission electron microscope. 15549X. Showing the bacilli shape [6].

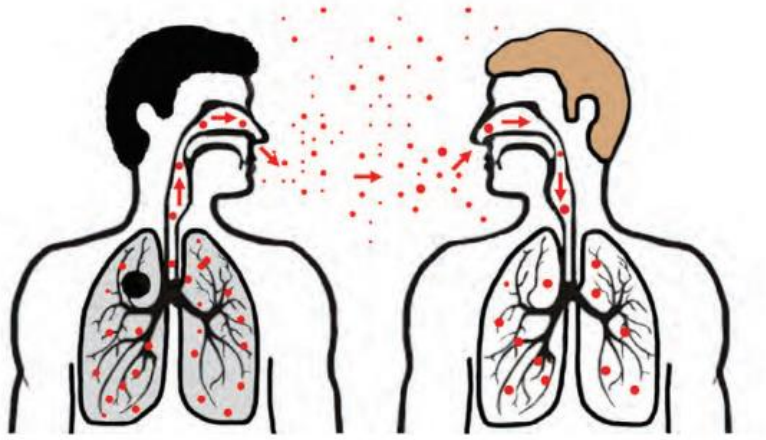


Fig 3.- Representation of the infection by MTB. Red dots represent the bacteria embed within tiny particles. The TB patient (left) has a productive pulmonary lesion in lungs [1].

Innate immunity

The immune system is responsible for the defense against any effector agent, which can or cannot be of biological origin. It compromises a set of highly regulated mechanisms where a deficiency on its response leads to the incapacity of eliminating pathogens thus increasing the vulnerability against the virus, bacteria, fungi, and parasites; whereas an over-activation by intensity or duration hold important consequences by damaging tissues and even causing death. We can study immune response in two parts, innate and adaptive; the latter compromises defence against specific antigens and takes days to be developed; the former, innate immunity, is a highly conserved response among vertebrates and includes several factors ready for an immediate reaction against a pathogen, it can be divided in different barriers [21], Figure 4:

1. Physical barriers: Block the entrance of pathogens to the host.
    1. Epithelium.
    2. Mucus.
    3. The surface of exocrine glands.

2. Chemical barriers: soluble substances with antimicrobial activity, e.g., complement system.

3. Cellular barriers: A defense line used when pathogens have gone through the first two barriers. Cell response is activated by the interaction of membrane or intracytoplasmatic receptors with parasites conserved molecular components. Some cells as neutrophils and macrophages can destroy invasive particles or cells by phagocytosis. The recognition of pathogens elicits the activation of cellular pathways that mediate the liberation of cytokines and other proinflammatory molecules. These molecules increase antibacterial activity and the recruitment of other immune cells and factors such as

polymorphonuclear granulocytes, mastic cells, platelets, endothelial cells, macrophages, dendritic cells (DC's) and natural killer cells (NK's) [22].

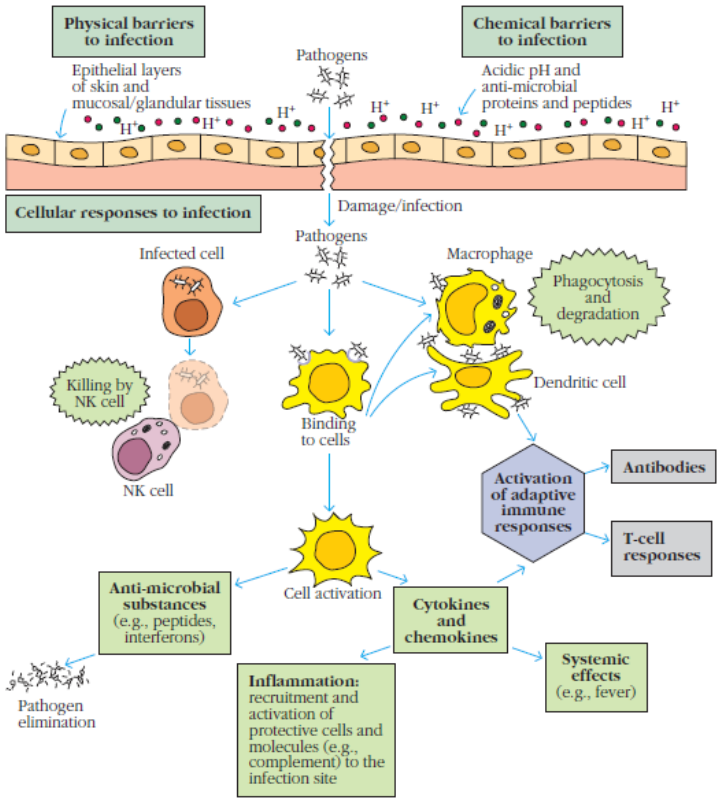


Figure 4. Resume of the different innate system barriers.

Since the lung is constantly exposed to a hazardous environment, there's been an evolutionary response towards a constant vigilance and protection from immune cells. Macrophages are equipped with the necessary machinery to recognize and respond to different threats. [16]

Monocyte - macrophage

Monocytes differentiate from bone marrow precursors after the exposure to macrophage colony stimulating factor (GM-CSF) and interleukin-3 (Il3) that induces the expression of particular membrane receptors, then they migrate to the bloodstream and stay there for an average of 70 hours. There are two types of monocytes: those related to inflammation and those in charge of immune surveillance in blood, constituting from 5% to 10% of the total leukocytes. While traveling through the bloodstream, they are exposed to differentiation and growth effectors causing phenotypic changes that lead to the differentiation into macrophages. The number and complexity of its organelles increase; they acquire new functions such as the capability to release cytotoxic and proinflammatory mediators; increment pseudopodia in size and number; and develop the necessary means to phagocyte microbes, other foreign particles and apoptotic cells [23].

Some monocytes migrate to different tissues where they differentiate into resident macrophages (Figure 5), responsible for the maintenance of tissue homeostasis and the elimination of infectious agents. Macrophages living in alveoli are named alveolar. Moreover, another two kinds of interstitial macrophages can be found with different immunophenotypes [24]. Mature macrophages can be identified by the immunophenotype $CD11c^{+}$ $CD11b^{lo}$ $CD64^{+}$ but can also be identified by SIRPα $(CD172α)^{+}$, $MAR\text{-}1^{+}$, $Siglec\text{-}F^{+}$ and $F4\text{-}80^{hi}$ [16].

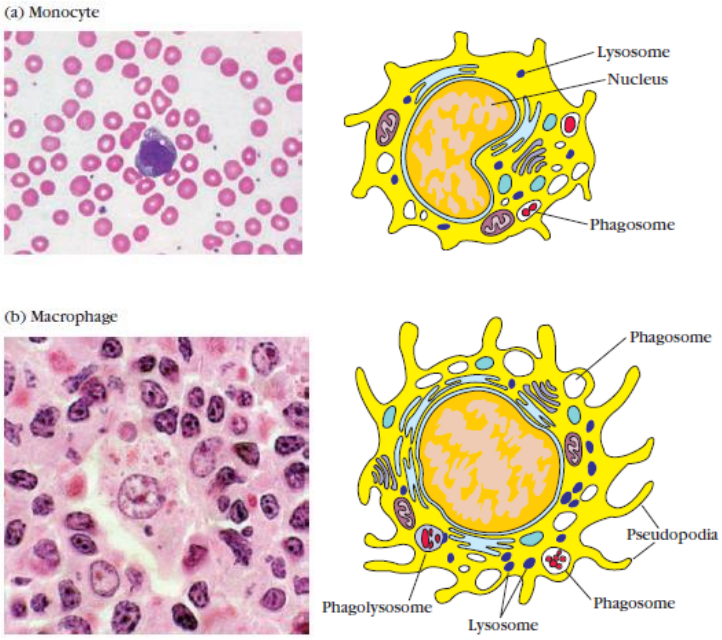


Figure 5.- Cytological differences between monocytes and derived macrophages. First column staining with H.E. Modified from [25].

Immunological function of macrophages

The main role of macrophages is to engulf foreign particles from a biological or non-biological origin, eliminate apoptotic cells of the body and waste products from tissues. The recognition of pathogen patterns by toll-like and scavenger receptors trigger a phagocytic response, which can be increased by cytokines as IFN; this constitutes a first step in initiating an immune response. They also mediate a transition to an adaptive response, by expressing the major histocompatibility complex II (MHCII) that is used in antigen presentation to lymphocytes [26], later on, specific antibodies produced by B cells bind to the invading microbes which result in a more avidly phagocytosis by macrophages [23]. Alveolar macrophages hold a central involvement in the clearance of many viral and bacterial infections, being the sentinels of the organisms given their exposed position, strong phagocytosis

capabilities, and the expression of PRR's. Their absence is related to an exacerbated inflammation [16].

Cytokines and chemokine's

The immune system is capable of cellular communication using proteic instructions named cytokines, produced by few specialized cells under particular conditions; those signals can be received in an autocrine or paracrine way by other cells by interacting with their receptors, modulating their genetic transcription. The distinctive set of macrophages' cytokines and chemokine's is resumed in tables 1 and 2.

Macrophages are usually activated by IFN-gamma, produced and secreted by T cells, causing migration to particular tissues or sites of infection. In vitro and ex vivo models has demonstrated that macrophages are also capable of secreting IFN-gamma themselves after stimulation with Il-12 and Il-18 [27].

Table 1.- Cytokines (left) and their effect (right) over a tissue or cell.

Cytokines	Effect
TNF-alpha (tumor necrosis factor-alpha)	Stimulates immune response and transendothelial monocyte migration (leukocyte extravasation), by increasing vascular permeability, cell adhesion, facilitating diapedesis and monocyte recruiting.
IL-1 (Interleukin-1 alpha or 1 beta or 1Ra).	Induce the early proinflammatory signaling that promotes the liberation of histamine and prostaglandin. Chemo-attracting granulocytes. Favouring clonal expansion and differentiation of T CD4+ cells. Stimulating its receptor, IL-1R, cells respond by expressing genes that produce IL-1. Closely related with autophagy and the formation of apoptotic bodies. Intervenes in the NF-kappa beta pathway.

IL-6	Promotes differentiation of B cells into plasmatic cells and activates cytotoxic T cells, recruits monocytes and maintains a Th17 cells population, inhibits the formation of regulatory T cells Tregs and cellular apoptosis.
IL-12	Assembled by two subunits: p35 and p40. Promotes cellular-mediated-immune response by stimulating cytotoxic systems of NK and T CD8+ cells. It also functions as an anti-angiogenesis factor. Might be secreted along with TNF-α.
IL-18	Works synergistically with IL-12 activating T and NK cells. Influences MAPK pathways.
IL-23	Shares a subunit with p40 that favor its activation. Increases IL-10 production and IL-17 synthesis.
IL-27	Since it shares the subunit p28 and the Epstein-Barr virus-induced gene 3 it is considered a member of the IL-12 protein family. Usually secreted in an early stage and favors Th1 cell differentiation while inhibiting the Th17 cell formation. May act as pro- or anti-inflammatory.
IL-10	Suppresses activation, production of proinflammatory chemokine's, and antigen presentation in macrophages reducing its antibacterial capabilities. Produce diminished expression of IgG's in B cells.
TGF-β	(Transforming growth factor beta), diminishes proinflammatory cytokines effects, suppress Th1 and Th2 cells. TGF-β promotes Treg's differentiation.

Besides cytokines macrophages hold a repertoire of chemokines, which are a group of cellular taxis promoting cytokines that recruit cells of innate immune system wherever they are required. May also have a role in cellular differentiation and inflammatory response [25].

Table 2.- Chemokines (left) and their effect (right) over a tissue or cell.

Chemokine	Effect
CXCL1 and CXCL2	Also known as MIP or Macrophage inflammatory protein. Recruits neutrophils and cells from the hematopoietic system. Promote angiogenesis.
CCL5	Chemo attractant of basophils, eosinophils, T and dendritic cells. Activators of NK cells.
CXCL8 (Il-8)	Recruits neutrophils and promotes their degranulation.

CXLCL9	Powerful recruiter of T cells, necessary for inflammation and tissue repair. Interferon γ induced.
CXCL10 (IP-10)	Interferon γ induced. Attracts T, NK, and dendritic cells.
CXCL11 (IP-9)	Interferon γ induced. Similar functions as CXCL9 and CXCL10 with an enhanced effect.

Hormones, cytokines, immune complexes and the presence of apoptotic cells determine the type of activation macrophages will exhibit, might be either classic M1 or alternative M2a, M2b or M2c. In turn, each particular phenotype shall determine which specific set of cytokines the macrophages will produce. M1 Macrophages have an increased bactericide activity by producing free radicals whereas M2 cells interact with Th2 cells, eosinophils and basophils [23], exhibiting anti-inflammatory functions. However, macrophages can switch from one activation state to another in response to changes in their microenvironment [16]. Figure 6.

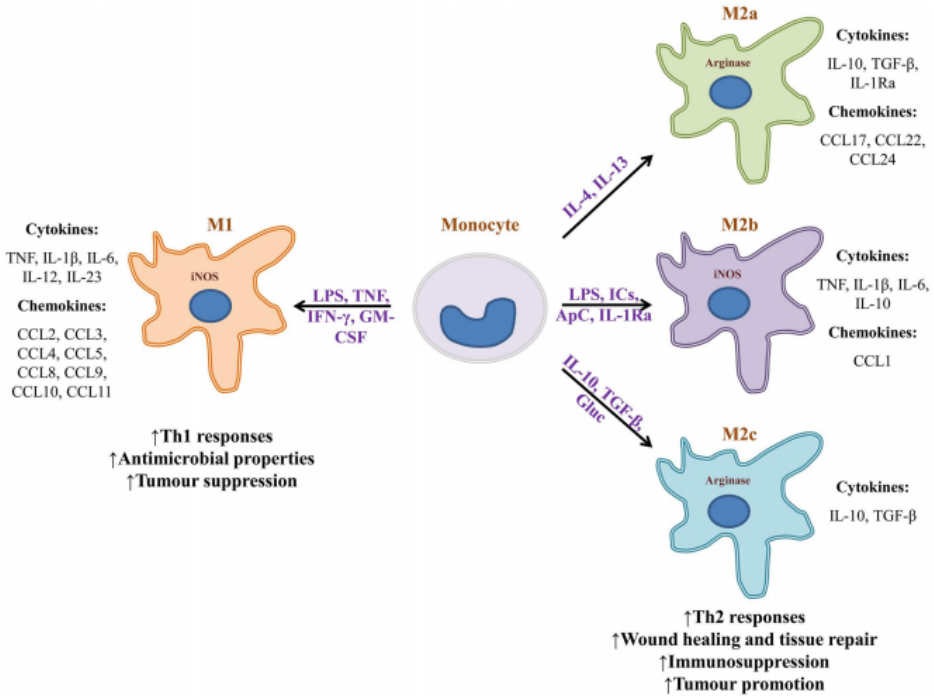


Figure 6.- Chemo and cytokines that lead to the classic (M1) or alternative (M2, M2b or M2c) macrophage activation way, and the products that this cells shall produce [23].

Infection

MTB uses the lower respiratory system alveolar macrophages as the primary host, and to reach them it uses an evasive strategy based on small droplets containing few bacteria (with a size of approximately 5 micrometers) going through the upper respiratory system to the pulmonary alveolus [1], figure 7.

Alveolar macrophages introduce pathogens that interact with phagocytosis receptors within a vacuole named phagosome which usually matures by fusion with lysosomes, a common mechanism used to eliminate pathogen bacteria; but when mycobacteria are internalized they interfere with the correct phagosome maturation [28], and so it prevents its degradation and correct immune presentation [16,17,29] (see macrophage-MTB interaction).

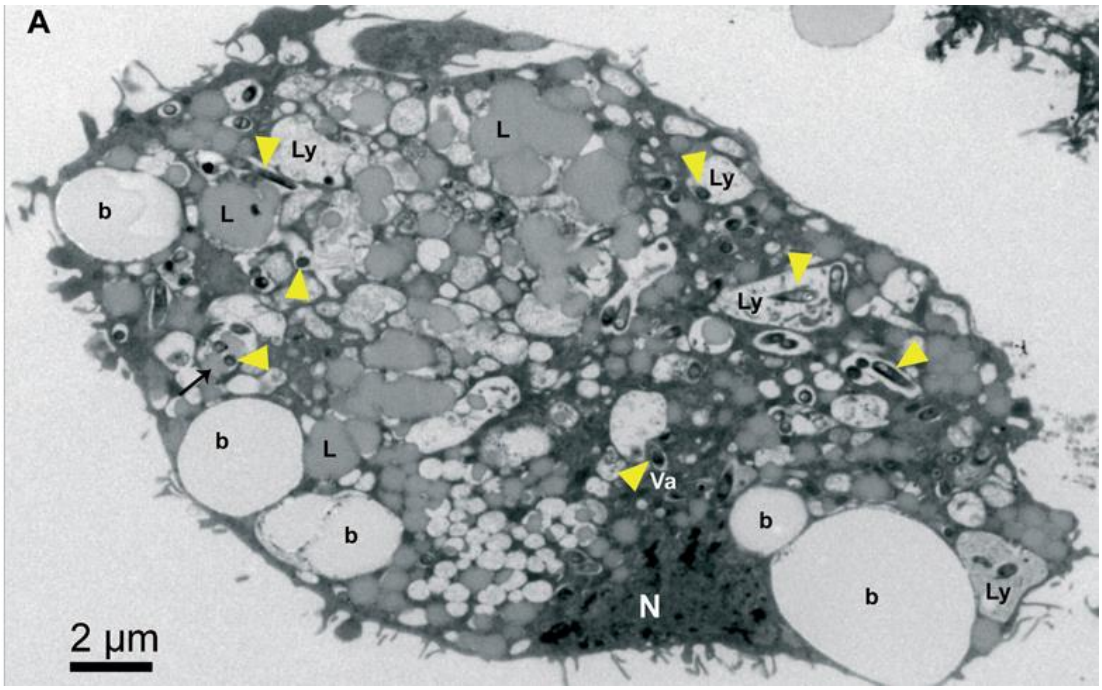


Figure.- 7 Monocyte-derived Macrophage (MDM) from peripheral blood. Arrows: MTB inside vacuoles. Ly: Lysosomes; b: beads; L: Lipid droplets; N: Nucleus. Observed through an electron microscope. Modified from [30].

The bacteria obtain the necessary resources to survive from their host. E.g., the use of cholesterol as a carbon source metabolizing it using a cytochrome from the 9450 family, cyp125 [31].

It is considered that the first day of infection is the most critical one: in this period the bacteria are either eliminated or starts its proliferation [28,32]. If the bacteria survive then the host cells might endure three fates:

1. Necrosis: characterized by the loss of membrane integrity and liberation of the content of the cell in an unorganized way. It is more commonly found with virulent strains such as *M. tuberculo*sis H37Rv [29].

2. Apoptosis: Membrane is conserved meanwhile bacterial viability is reduced. More virulent strains of MTB inhibit apoptosis, one of the mechanisms involved in such phenomena its mediated by the interaction with the endoplasmic reticulum (ER), blocking the Ca^{2+} trafficking, and preventing a correct cholesterol homeostasis by altering the expression of EST-1 and AMRP [20]. In some cases, apoptosis can be activated by virulence factors such as ESX1 type VII or by the stimulation of T cells by the CD95 pathway either as intrinsic or extrinsic ways [33].

3. Survival of the macrophage, allowing bacterial proliferation [34].

Correct immune response relies on the recognition of Pathogen Associated Molecular Patterns (PAMP's) by receptors such as Pattern Recognition Receptors (PRR's), CD14 and TLR's. This interaction induces proinflammatory cytokines expression as Il-12 subunits, INF, IL-1 and TNF [35] (critical for the infection outcome), activates the signaling pathway of NF-κβ [32], and liberates exosomes with mycobacteria specific antigens for the recognition of other immune system cells [36]. In turn, macrophages respond to the infection

increasing antibacterial response by producing reactive oxygen intermediaries (ROI), reactive nitrogen intermediaries (RNI) [34], cell adhesion molecules and cytokines to recruit more immune cells [33].

If an infection is established, tuberculosis bodies are expelled from the body when infected person coughs, which liberates the bacteria [33] it can also infiltrate blood vessels causing extra-pulmonary tuberculosis. MTB may also be the responsible for a hypersensitivity response where primary tissue damage become fibrotic and get calcified forming Ghon's bodies which are visible under X rays [34]. These granulomas hold hypoxic and acidic conditions on its necrotic core [29], where the bacilli persist in a quiescent state for years in weak nutrient and oxygen conditions. MTB reduces protein synthesis and upregulates the expression of specific genes as part of an integral strategy of metabolic adaptation which allows the bacteria to establish a chronic infection [22].

The first adaptive cell responses appear between 2 and 12 weeks after the initial infection, after that APC's will activate T lymphocytes in lymph nodules by the Major Histocompatibility Complex II (MHCII) eliciting a Th1 response that generates CD4+ and CD8+ effector cells [5]. However, MTB is capable of evading clearance by both innate and adaptive systems [37].

Inadequate antibiotic choices, the pharmacodynamics of the host, rapid adaptation, DNA repair capabilities, mutations and the development of membrane pumps may have contributed to the development of multidrug resistance against treatments (MDR MTB) that makes the epidemic even harder to control [38].

Macrophage – MTB interaction

Macrophages have a broad range of autonomous antimicrobial mechanisms, but coevolution of MTB with their hosts has armed the parasite with sophisticated strategies to evade the immune defenses [39,40]. It has the capability of avoiding lipidic recognition [41], modulates macrophage gene expression when there's interaction with the bacterial lipids or lipoproteins [21], and delaying the immune response of the body by stimulating the production of anti-inflammatory cytokines [32] and reducing macrophage's MHC II gene expression [42].

One that MTB has reached the alveolus it can enter into macrophages through different mechanisms [39]:

1. Type I phagocytosis: Requires the activation of FcγRs receptors, which then activate pro-inflammatory mediators. Involves the recognition of IgG-coated bacteria and the formation of pseudopodia
2. Type II phagocytosis: The union of the receptor CR3 with C3bi coated bacteria. There's neither pseudopodia formation nor production of inflammatory mediators.

3. Type I phagocytosis (CR3-mediated): it is a non-opsonic phagocytic mechanism elicited after the interaction of MTB with TLR, CD14, scavenger receptors, cytosolic DNA-sensors, and Mannose receptor which is key for the phagosome-lysosome fusion.

4. Macropinocytosis: Is the internalization of a large amount of fluids and solutes, it is not specific and does occur in the absence of specific molecular markers.

5. Normally phagosomes undergo a process of maturation that includes the interaction of phagosomes with endosomes and lysosomes which result in the acidification of the phagolysosome that digest engulfed bacteria, however MTB has the capability of avoiding these killing mechanisms by preventing phagosome acidification and blocking the fusion of lysosomes with the phagosomes, even some bacteria can escape the phagosome milieu [42]. It has been demonstrated that the phagolysosome maturation process is inhibited by vacuole traffic blocking due to changes in phosphorylation of macrophage proteins by mycobacteria's tyrosine-phosphatases [14], which also prevents $Ca^{2+ \text{ y } 3+}$ signaling, Rab family GTPases traffic and suppresses the activity of Rab8b by inhibiting the activity of TBK-1 [43].

Under infection with MTB, macrophages produce NO and reactive oxygen species (ROS) that can kill tuberculosis bacilli, but tuberculosis can counteract the production of ROS by modulating its generation; it is also capable of neutralizing NOX2. Macrophages can also activate the apoptotic cell program to limit the growth of pathogens, in turn, MTB modulates cell death to avoid innate host defense by increasing necrosis and suppressing the apoptosis with the help of several proteins as Rv3364c, the serine/threonine kinases PknE [39] and the phosphatases PtpA and SapM [42].

When exhibiting a polarized Th1 response, there's an increase in NO and TNF production, which is essential for the formation of the granuloma, a mechanism that MTB also exploits [16]. When a macrophage is dying it recruits more macrophages that the mycobacteria infect, this mechanism is orchestrated by an excretion system called ESX-1 that pumps out the protein ESAT-6 [41].

Cell activation / Protein phosphorylation

Cells have the capacity of modulating their gene expression by regulating cell pathways that control the union of transcription factors by phosphorylating proteins [44], a post-translational modification that is needed to induce conformational changes on other proteins allowing the interaction with other molecules, Figure 8. For example, the phosphatidic inositol bisphosphate (PIP2) of the cell membrane is the substrate of the kinase PI3 (PI3K), which after being activated by membrane receptors, phosphorylates its target protein resulting in the formation of PIP3, an anchor site needed for protein transport from the cytosol to the membrane.

Once a ligand has interacted with its membrane receptor, signaling is then transmitted to the inner cell by adapting proteins, commonly mediated by a tyrosine-kinase phosphorylation that in turn activate other families of kinases such as Src. Phosphorylation does not only alters molecular conformation but also prevents or promotes degradation, promotes a particular cell localization or allow protein-protein unions thanks to phosphotyrosine domains. Tyrosine phosphorylation is commonly observed upstream in cellular pathways, whereas serine phosphorylation is more likely to happen downstream [25].

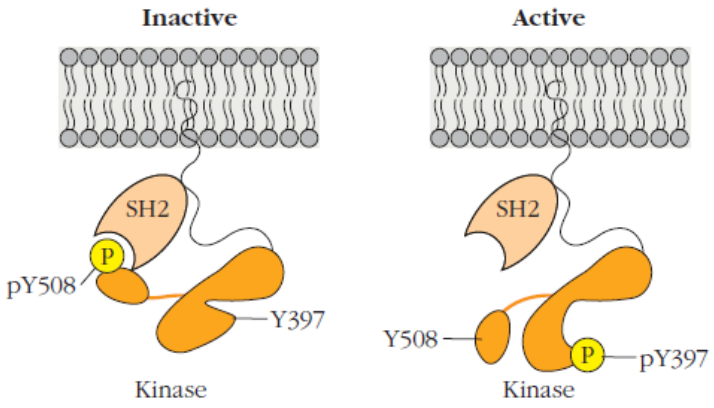


Figure 8.- A change in the phosphorylation state rapidly changes the three-dimensional structure of proteins giving them the capability to interact with other molecules. Modified from [25].

Macrophage activation:

When an antigen interacts with a macrophage TLR, a signaling cascade is then activated which in turn elicits a cellular response mediated by MAPK (See MAPK). The activation of MAP3K is a complex process that can be mediated by phosphorylation of upstream kinases or interactions with signaling adaptors and small GTPases.

The same kinase can activate two different cascades. For example, TGFB-activated kinase 1 (TAK1) functions as a direct MAP3K for both the p38 and JNK pathways. However, also indirectly facilitates tumor progression locus 2 (TPL2) activation, which is a MAP3K for the extracellular signal-regulated kinase 1 (ERK1) and ERK2 pathway, promoting lkB kinase (IKK)-induced proteolysis of the Nf-Kb subunit precursor protein p105. It has been described that scaffolding proteins can link different tiers of the MAPK signaling pathway into multienzyme complexes forming signalosomes gathering multiple components and facilitating their interactions [48] which ensures signal specificity. They also regulate signal amplitude and duration [49]. Figure 9.

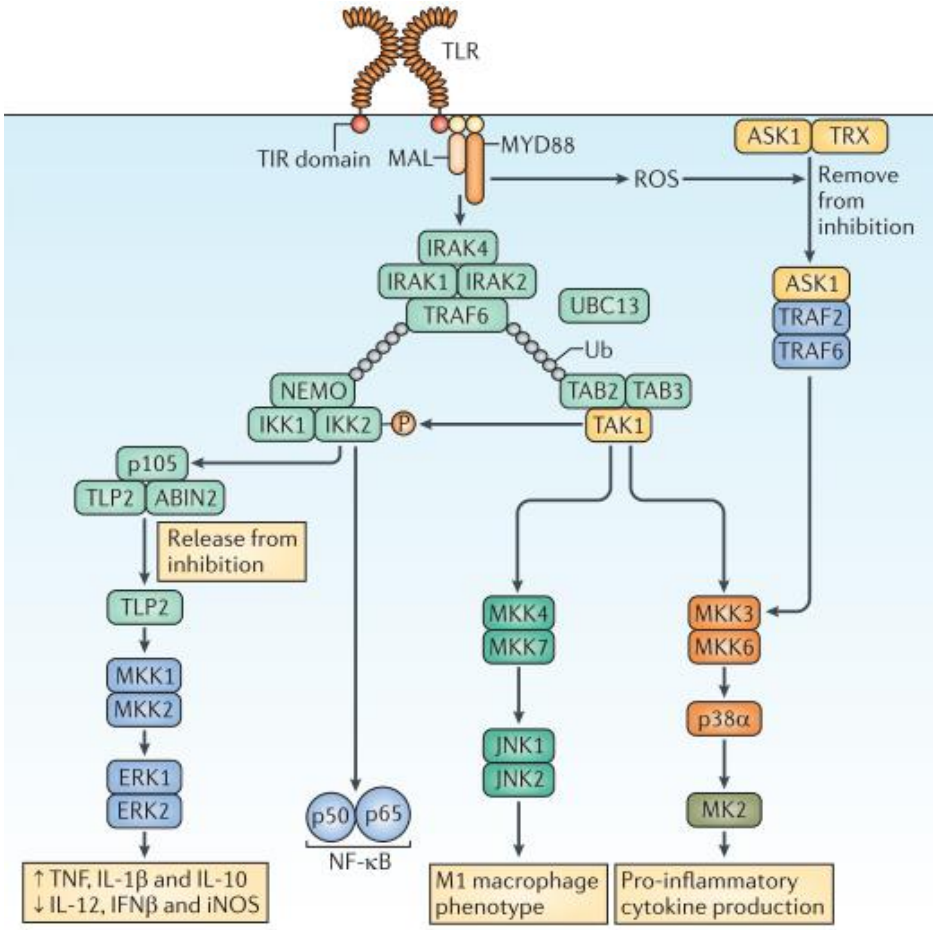


Figure 9.- Activation of MAPK after the interaction of a receptor with its ligand, the transduction of an extracellular signal is initialized by the recruitment of MYD88 to the TIR domain of the receptors; an adaptor protein, MAL, is required for the activation of TLR2 and TLR4. Later, MYD88 interacts with IRAK4 (serine/threonine kinase IL-1 receptor-associated kinase 4) which assembles an activation complex with IRAK1, IRAK2, TRAF6; the latter will then catalyze the formation of K63-linked polyubiquitin chains on itself and IRAK1. Then TAB 2/3 (TAK binding proteins) recruits TAK1 (TG-β-activated kinase 1), which activates the p38α and Jun N-terminal kinase (JNK) pathway. On the other hand, IRAK4 also recruits NEMO (NF-κB essential modulator) and interacts with TAK1 on its IKK2 domain that induces the proteolytic degradation of p105, allowing TLP2 (tumor progression locus 2) to be activated, subsequently activating ERK1 and ERK2.

MAPK

There is also a significant difference in the activation of MAPK when different kinds of macrophages are studied: TLR-induced MAPK activation and cytokine production are independent of both TAB2 and TAB3 in human tissular macrophages. However, TAK1 is fundamental for the activation of JNK followed by stimulation with LPS, but not necessary for the activation of IKK, ERK1 or ERK2 in bone marrow-derived macrophages, suggesting the involvement of another MAP3K. Whereas the knockdown of Mekk3 reduces LPS-mediated activation of p38a, ERK1, and ERK2 in RAW264.7 cells. The activation of ERK1 and ERK2 by all TLRs in primary macrophages is mediated by the MAP3K TPL2 [49]

The mitogen-activated protein kinases (MAPK) control different cellular responses to signals and conditions such as pheromones, starvation, high osmolality, hypotonic shock, carbon/nitrogen deprivation, infection, etc., [45]; and elicit a cellular response accordingly, activating different cellular programs as proliferation, differentiation, development, transformation or apoptosis. Three different families have been identified: extracellular signal-regulated kinase (ERK), C-Jun terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 kinase [46]. MAPK pathways involve a highly conserved three tier-cascade of kinases. It starts with an extracellular stimulus that activates a receptor tyrosine kinase (RTK), followed by the activation of a MAPK kinase kinase (MAP3K or MAPKKK), a MAPK kinase (MAP2K or MAPKK) and finally a MAPK [47], Figure 10.

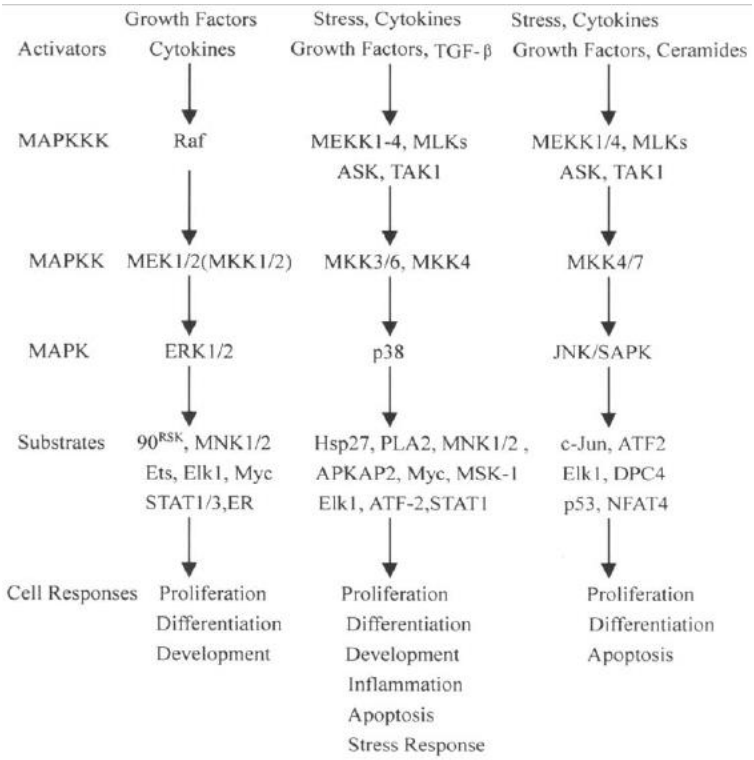


Fig.- 10 Major MAPK cascades in mammalian cells [46]

Phosphorylation of a MAP2K on a MAP kinase causes a conformational change on a phosphorylation lip promoting dimerization and binding of its substrates [45], Figure 11.

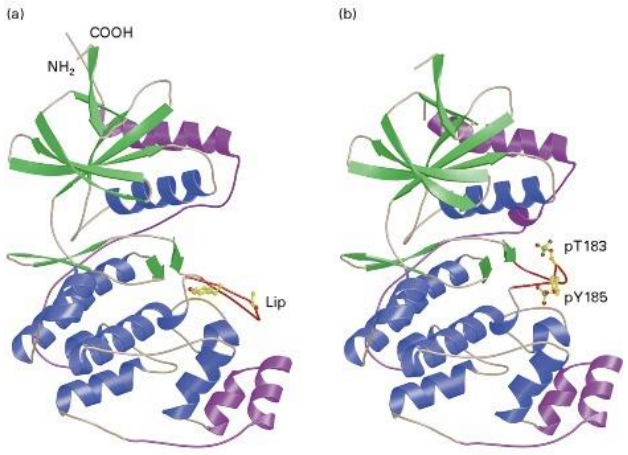
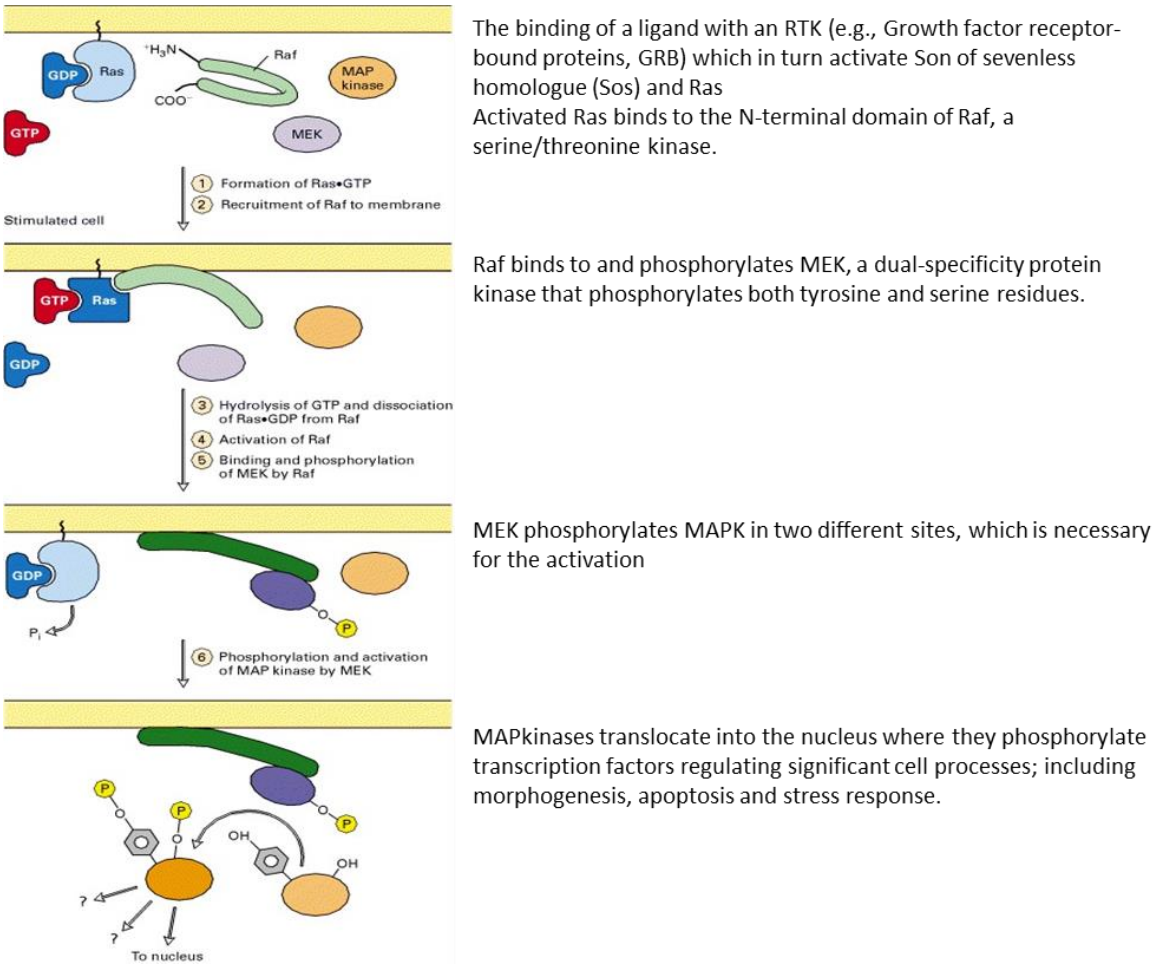


Fig.- 11. Phosphorylation of MAPK happens when MEK, a MAP2K, destabilizes the structure of its

substrate exposing a Tyrosine that is then phosphorylated, followed by a neighboring Threonine; both residues interact with other amino acids near them changing the configuration of the lip. Inactive form (a), active phosphorylated form (b) [45].

The canonical process of activation of MAPK is resumed in figure 12:



The binding of a ligand with an RTK (e.g., Growth factor receptor-bound proteins, GRB) which in turn activate Son of sevenless homologue (Sos) and Ras
Activated Ras binds to the N-terminal domain of Raf, a serine/threonine kinase.

Raf binds to and phosphorylates MEK, a dual-specificity protein kinase that phosphorylates both tyrosine and serine residues.

MEK phosphorylates MAPK in two different sites, which is necessary for the activation

MAPkinases translocate into the nucleus where they phosphorylate transcription factors regulating significant cell processes; including morphogenesis, apoptosis and stress response.

Figure.- 12 Kinase cascade that transmits signals downstream from activated Ras protein. [45]

Justification

Tuberculosis persists today as the deadliest infectious disease in the world. The natural development of evasion mechanisms and drug resistance increases the difficulty of developing an effective treatment, which creates the need to continue expanding the knowledge on the interaction between MTB and its host. Therefore, a study that reflects the macrophage dynamism to stand against the infection with MTB is crucial, making imperative the understanding of the associated components as well as the cellular pathways that are being activated or deactivated in the presence of mycobacteria.

Research hypothesis:

If MAPK are involved in the elimination of bacteria, but *M. tuberculosis* is able to modulate the immune response, then we will find differences in the level of activation of MAPK if macrophages are infected according to the virulence of the strain. Also, there should be differences between the infection with MTB and the infection with *M. bovis* BCG.

As well as a different regulation of the MAPK pathway, other cell signaling cascades must be altered; therefore phosphoprotein profiles should show a characteristic pattern when infected with *M. tuberculosis* H37Rv or *M. bovis* BCG.

Aim:

The goal of this research study is to increase the knowledge of the host response to the infection and to try to determine if there are proteins in which their phosphorylation state only occurs in a particular condition of infection (*M. bovis* BCG or *M. tuberculosis* H37Rv) and then establish the cellular pathways of the macrophage that tuberculosis alters.

Objectives

General: Determine the changes in the activation or inactivation of cellular pathways analyzing changes in protein phosphorylation of human monocyte-derived macrophages infected with *Mycobacterium sp*.

Particulars:

1. Obtain human monocyte-derived macrophages from peripheral blood.
2. Infection MDM with *Mycobacterium bovis* BCG *Mycobacterium tuberculosis* H37Rv.
3. Evaluation of the MAPK pathway activation in response to mycobacterial infection.
4. Determination of macrophage phosphorylation profile in response to virulent and non-virulent mycobacterial infection.

Methods

Cells acquirement.

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats from healthy donors donated by the blood bank of the 20 de Noviembre Hospital. Blood and Histopaque 1077 where poured into 50 mL conic tubes in a 2:1 ratio as shown in figure 13, the mix was then centrifuged at 400 r-c-f. for 30 minutes.

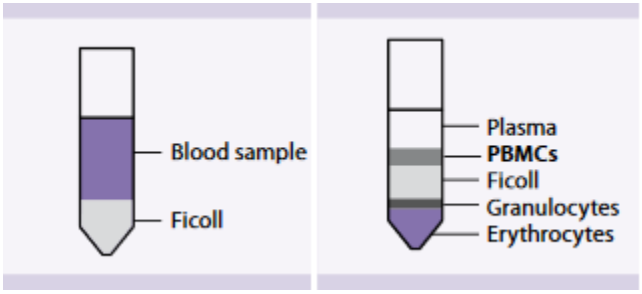


Figure 13.- Density separation of PBMC's by ficoll protocol. Left: preparation of tube with blood and Histopaque 1077. Right: Resultant gradients after the first centrifugation.

The PBMC band was recovered using 4 mL sterile serological pipettes, depositing the contents in other 50 mL tubes with 40 mL RPMI medium. Cells were then washed with centrifugation at 1200 r.p.m. for 10 minutes, the supernatant was discarded and the pellet recovered, which was then again re-suspended in 40 mL of RPMI medium. This wash was repeated 2 times.

Cells were counted using a Neubauer chamber and seed 10 million in 100 mm plates which were left at 37°C with 5% CO_2 for 2 hours; afterward, plates were washed with RPMI medium twice to eliminate non-adherent cells and then left the monocytes to differentiate with 10% heat-inactivated-FBS-enriched RPMI medium for 14 days.

Infection:

Cells were infected at an MOI of 5:1 with *Mycobacterium tuberculosis* H37Rv or *Mycobacterium bovis* BCG, after two hours plates were washed three times with RPMI medium to eliminate non-phagocyted bacteria. Then we left the cells incubating for 24 hours at the same conditions as the growing. Cells were then lysate with a lysis buffer (see protein extraction).

Protein extraction:

Twenty-four hours post-infection, the plates were washed three times with physiological solution (NaCl 0.9%) then 1 mL of lysis buffer (Tris 20 mM, NaCl 1 mM, EGTA 1 mM, NaEDTA 1 mM and tryton at 1% at a pH of 7.5) containing PMSF 1 mM, Roche's proteases inhibitor cOmplete 1X and PhosStop 1X. Cell lysates were recovered using a sterile plastic scrapper and poured into 1.5 mL tubes. Samples were stored at -80°C until their use. For membrane separation, tubes were sonicated for 30 seconds and then centrifuged at 10 000 r.c.f. for 30 minutes at 4°C. The supernatant was recovered and filtered through 0.22 µm membranes to eliminate bacteria from the samples, which were then aliquoted. Total protein concentration was determined by the Bradford method.

Protein quantification (Bradford protocol):

Protein concentration was determined by micro Bradford assay. Briefly, samples were diluted 1:50 in microtitration plates by a triplicate (1 µL of each sample plus 49 µL of water). An standard curve was established as follows: a triplicate of BSA at a concentration of 1mg/m ranging from 0 to 7 µg was pipetted into the plate and the volume of each well was completed to 50 µl with water. Afterwards, 200 µl of Bradford reagent was added to each well and then the plates to incubate while being shaken in the dark for 10 minutes. Finally, the absorbance was measured with a spectrophotometer at a wavelength of 595 nm. A linear regression was then fitted to the readings of the standard

curve and the resulting equation was used to determine the concentration of the samples.

Whole lysate protein and phosphoprotein analysis:

To analyze the proteins of cell's lysates, an SDS-PAGE was performed using 8%–15% gradient gels of 10 centimeters long, that were then stained with ProQ diamond to evaluate phosphorylated proteins and with Sypro to observe total proteins. Gel images were obtained with a ChemiDoc by a BioRad reader, and were processed and analyzed using the Image-Lab ver 5.2.1 suite on a high sensitivity setting.

Phosphoprotein quantification:

To quantify the total phosphoproteins present in the samples, the Thermo Phosphoprotein Phosphate Estimation Assay Kit #23270 was used. Briefly: on a microtritation plate we compared the samples of 5 different subjects at a concentration of 100 µl/ml against a phosvitin standard curve. Then, 50 µl of NaOH 2N was poured, and the plates were incubated at 65 °C for 30 minutes in agitation and darkness. Then 50 µl of HCl 4.7N were added, a 30 seconds cycle of agitation was followed by the addition of 50 µl of phosphates reactive. Finally, plates were left for 30 minutes at room temperature to read the absorbance with a spectrophotometer at 650 nm wavelength.

Determination of the phosphorylation levels of p38, ERK, and JNK by Western Blot:

Using Bio-Rad mini gels, proteins were separated using a 15% SDS-PAGE assay and then transferred to nitrocellulose membranes with current intensity depending on the area (3 mA/cm^2) on a semi-humid chamber at 25 Volts max for 25 minutes. Transference was then evaluated by staining with Ponceau Red. The blocking step was made with 3% BSA at 4 °C overnight. Primary antibodies were used at a concentration of 1:5000 for two hours at 37 °C followed by three PBS-Tween washes and two PBS washes, each one of 5

minutes. Secondary antibody was incubated 37 °C for two hours at room temperature, washes were repeated. To reveal the membranes by the chemo-luminescent reaction, Luminata Forte by Millipore was used, reaction intensity was measured with a ChemiDoc reader and data was processed with the suite Image-Lab version 5.2.1.

CD14 expression:

Plates of MDM cells were washed with PBS and discarded the most of it, then158 µl of PBS and 150 µl of 0.25% trypsin were added and the plates were incubated at 37°C were added for 5 minutes. Once the cells appeared detached 10 volumes of medium were poured, and the cells were then recovered into tubes and centrifuged at 1200 r.p.m. for 10 minutes, the pellet was then resuspended in 100 µl of PBS with 5 µl of anti-CD14 antibody and left for 5 minutes in darkness. Finally, 300 µl of FACS-Flow were added and then read at a FACSCalibur cytometer.

Statistics:

Data analysis was made with RStudio ver 3.3.3. using generalized linear mixed models. Response variables were established as for whether the cells were infected with either *M. bovis* BCG or *M. tuberculosis* H37Rv, considered as fixed effects. The variation of individual immunological response to the infection (due to age or immunological status at the time of blood extraction) of the test subjects may not remain as a fixed effect between samples. Thus individuality was considered as a random effect. Data was fitted to a Poisson distribution, which only handles discrete values. Since R might interpret absorbance values as continuous, the normalized values of the readings were multiplied by 100 and then rounded to the nearest whole number. Graphics were made in Excel 2013, GraphPad Prism and Statistica.

Results:

Monocytes showed differentiation into monocyte-derived macrophages after 14 days as analyzed under light microscopy (figure 14), and the expression of CD14 evaluated by flow cytometry.

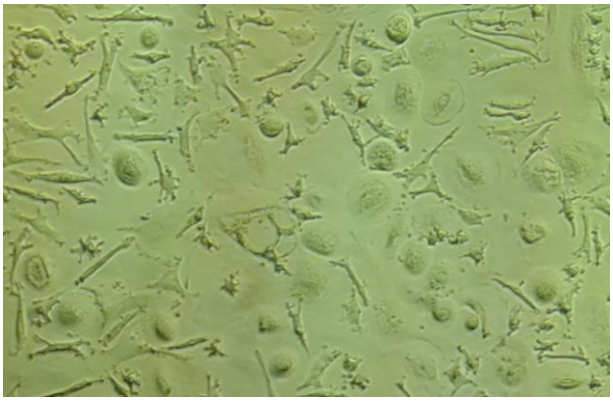


Figure 14.- Monocyte-derived macrophages after 14 days of incubation. It is possible to observe the highly activated transcriptional machinery of the cells with a conspicuous nucleus.

Quantitative changes of total phosphoproteins in the macrophages under infection conditions were determined. It was possible to measure an increment of the phosphorylated proteins under either one of the infections, *M. bovis* BCG or *M. tuberculosis* H37Rv. On three out five cases the most significant increase corresponds to the infection with the virulent strain, figure 15.

Results confirm the response of the cells to the presence of bacteria. However, it is unclear if there are either more cell pathways activated or the same cell signaling components have been more phosphorylated. To address this issue, the MAP kinase pathway was analyzed to reveal the presence of the phosphorylated forms of Erk ½, Jnk and p38 by chemiluminescent western blotting, figure 16.

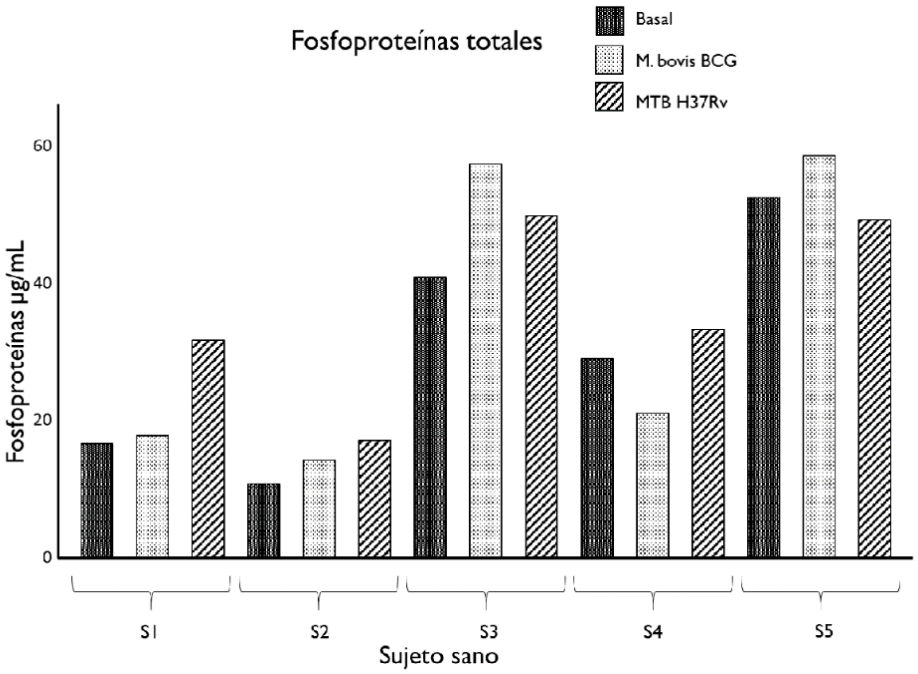


Figure 15.- Total phosphoprotein of the lysates of macrophages of 5 subjects under three conditions: non-infected (basal), infected with *M. bovis* BCG or infected with *M. tuberculosis* H37Rv. Infection conditions always showed more phosphoproteins compared to non-infected (basal) cells.

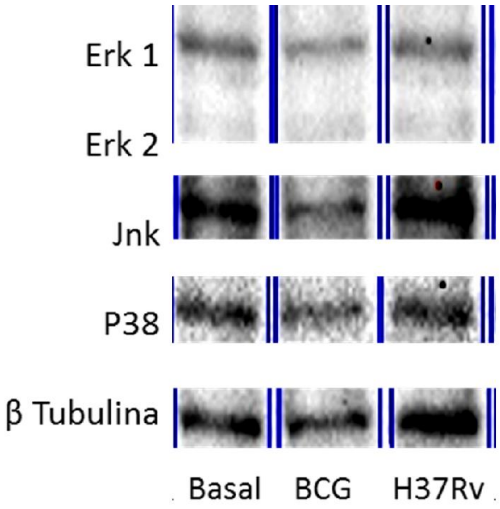


Figure 16. - Western blot of phospho-ERK ½ (Erk 1 and Erk 2), phospho-JNK (Jnk) and phospho p38 (P38). *β* tubulin was used as a control.

To determine if the MAPK, as a cell pathway, was differentially activated, an analysis on a scramble of the four phosphoproteins was made, showing that there is a statistical difference between all conditions (basal- *M. bovis* BCG, *M. bovis* BCG – *M. tuberculosis* H37Rv, and basal - *M. tuberculosis* H37Rv), figure 17. Macrophage response to a 24-hour infection with a virulent strain of tuberculosis induces an over-activation of the MAPK pathway, whereas there is a biological tendency to reduce the activation while interacting with *M. bovis* BCG.

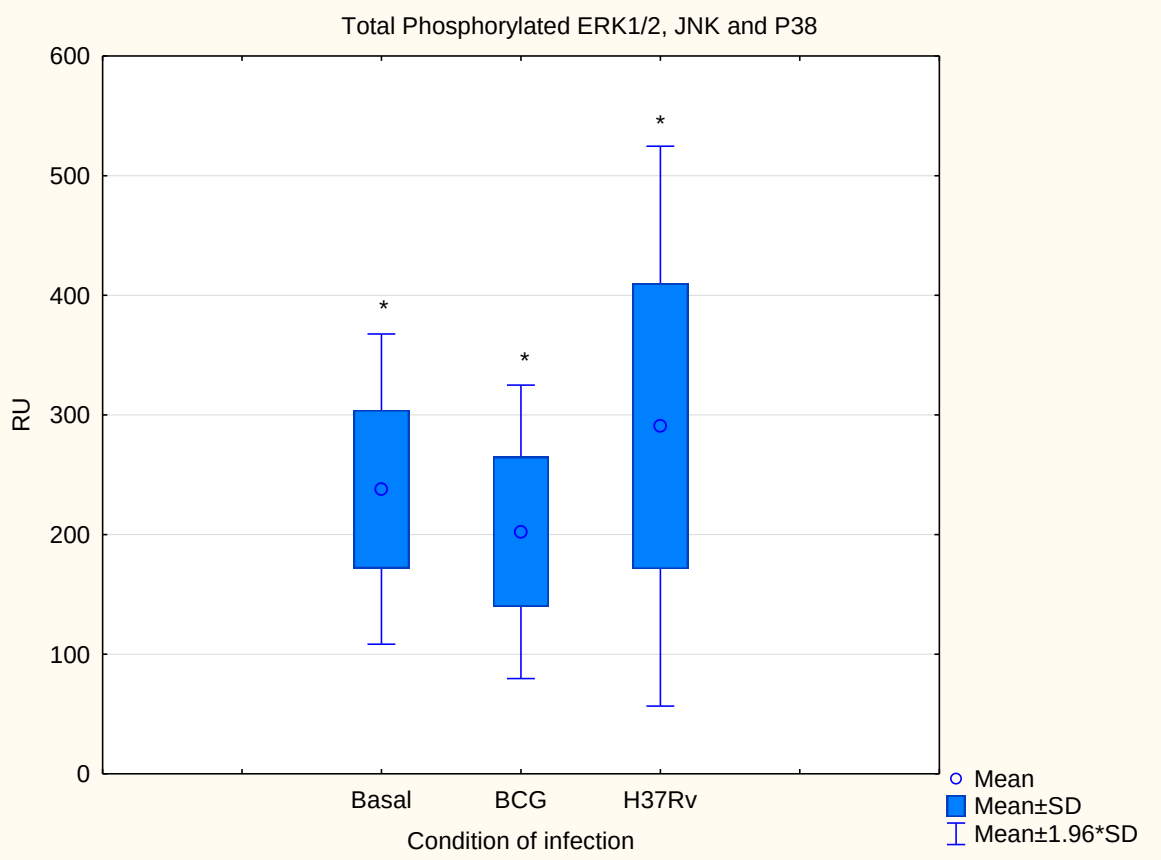


Figure 17.- Activation of the MAPK pathway analyzed by the altogether phosphorylation of ERK1/1, JNK, and P38 under the same three conditions.

To understand if all or only some particular cascades are differentially activated, we analyzed the effect of treatments on each protein, Erk1, Erk2, Jnk, and

p38). It is worth mentioning that when proteins are analysed individually, no infection appears to causes a measurable effect (figs, 18-20) except for the *M. bovis* BCG-infection-effect on phospho-P38, Figure 21.

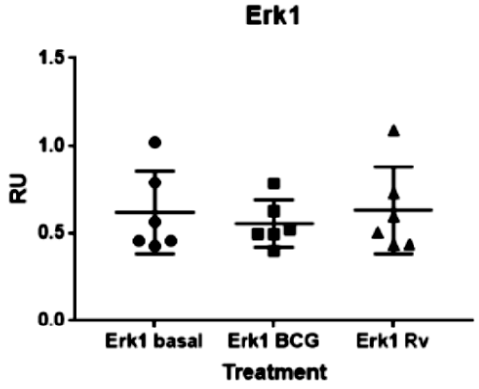


Figure 18.- Densitometric analysis of the phosphorylation level of ERK1 in MDM infected with *M. bovis* BCG and *M. tuberculosis* H37Rv and in a non-infected state (Basal) by western blotting. No differences were found among infections or between any infection and the basal, non-infected, state p = 0.1549.

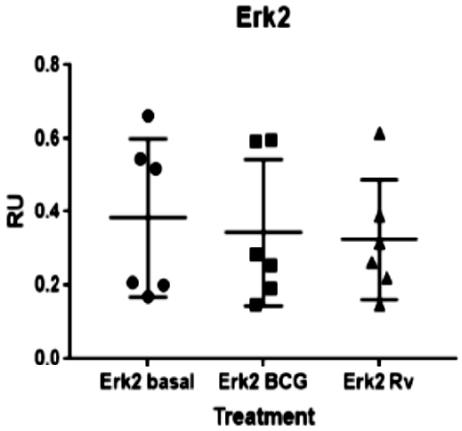


Figure 19.- Densitometric analysis of the phosphorylation level of ERK2 in MDM infected with *M. bovis* BCG and *M. tuberculosis* H37Rv and in a non-infected state

(Basal) by western blotting. No differences were found among infections or between any infection and the basal, non-infected, p = 0.2136.

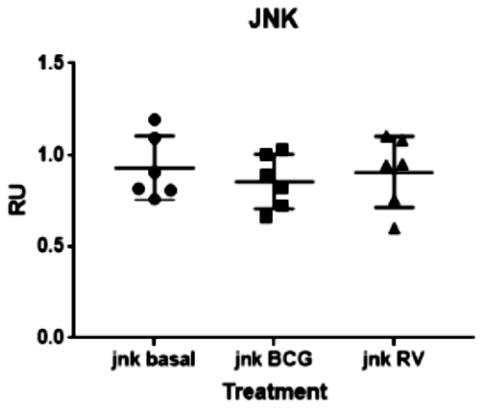


Figure 20.- Densitometric analysis of the phosphorylation level of ERK2 in MDM infected with *M. bovis* BCG and *M. tuberculosis* H37Rv and in a non-infected state (Basal) by western blotting. No differences were found among infections or between infected and the basal, non-infected condition p = 0.2285.

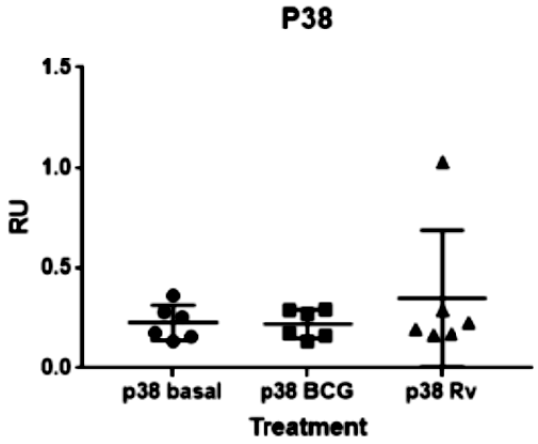


Figure 21.- Phosphorylation level of P38 in MDM infected with *M. bovis* BCG and *M. tuberculosis* H37Rv and in a non-infected state (Basal) that was used as a control ANOVA analysis showed a difference in treatment, Tukey post-hoc

analysis showed a significant difference between BCG and *M. tuberculosis* H37Rv p=0.019.

Table 3.- Number of subjects that showed an increase in the phosphorylation of p38, ERK1, ERK 2 or JNK.

	Infection with *M. bovis* BCG	Infection with *M. tuberculosis* H37Rv
P38	2/6	4/6
ERK1	3/6	3/6
ERK2	2/6	3/6
JNK	1/6	3/6

To make an approximation if other signaling pathways are affected by the infections or the virulence of strain, an analysis of the phosphoprotein bands profile was carried out. SYPRO stained gels showed that proteins suffered no degradation and wells were loaded evenly, as can be seen in figure 22. The same gels where then stained for phosphoproteins, figure 23. A comparison of the phosphoprotein band profile of the basal condition against infections of four subjects showed high variability in the phosphorylation patterns between subjects, Figure 24. A resume of unique, shared and total bands is shown in Table 4

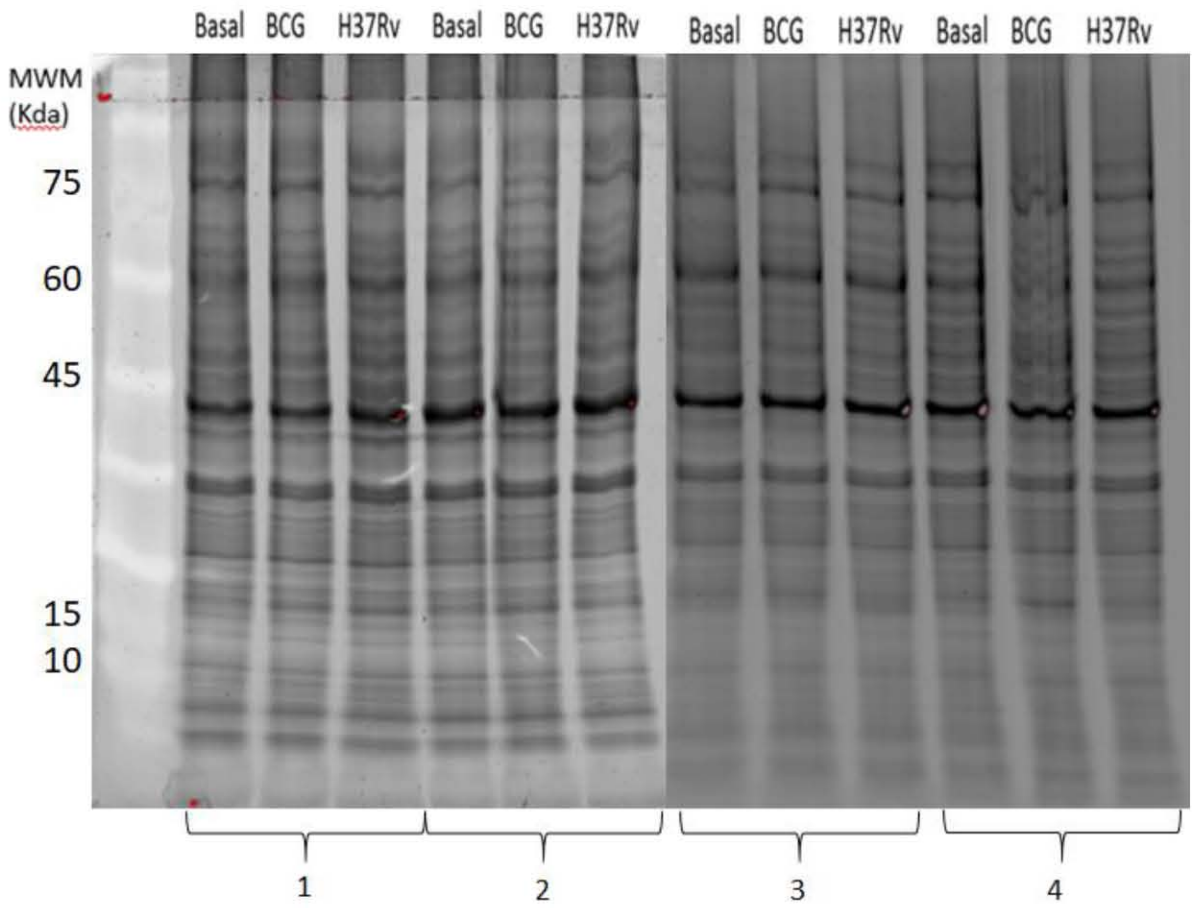


Figure 22.- Total proteins of cell lysates of MDM's of 4 individuals (1-4) under three conditions: non-infected (basal), infected with *M. bovis* BCG (BCG) and infected with *M. tuberculosis* H37Rv (H37Rv) in a gradient (5-14%) SDS-PAGE stained with SYPRO. All lanes show the same band pattern indicating similar band expression patterns.

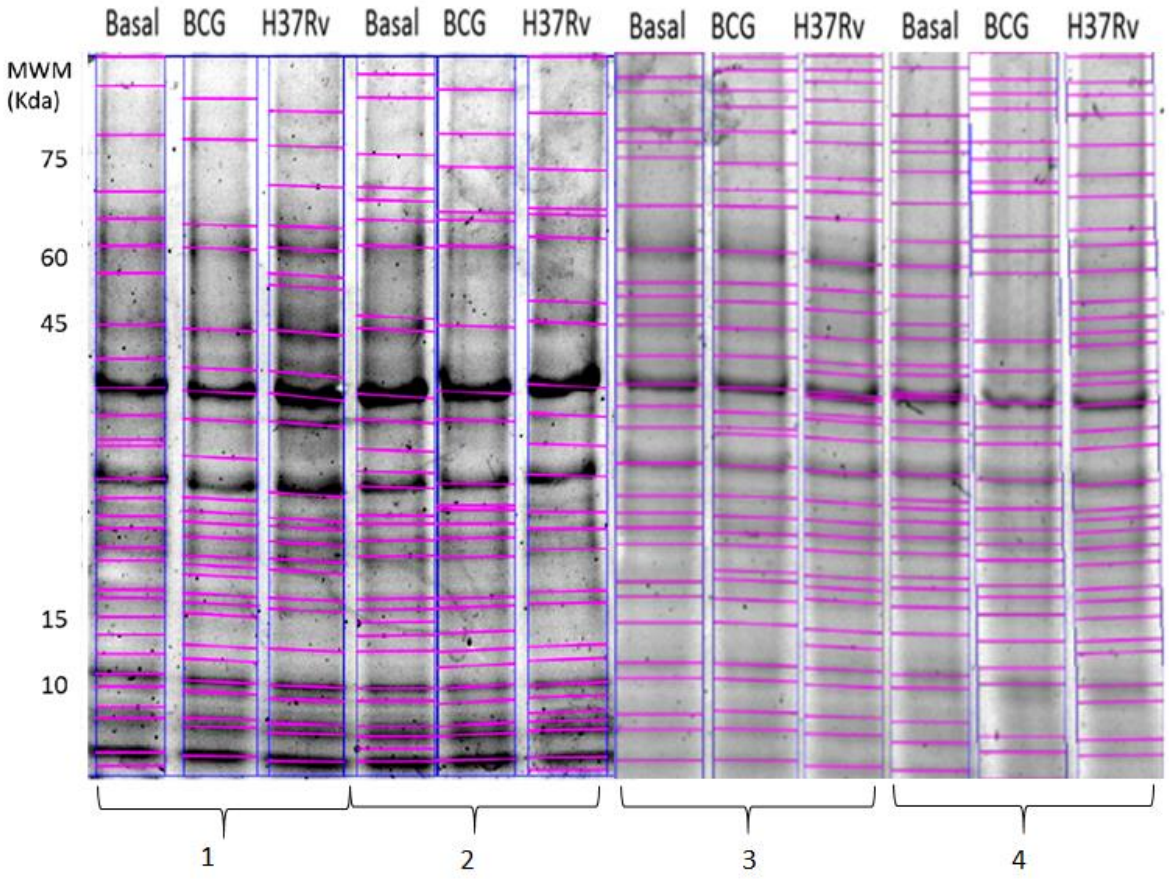


Figure 23.- Same gel as in figure 22 but stained with ProQ diamond showing the phosphoprotein-band profiles: MDM's of 4 subjects (1-4) under three conditions: non-infected (Basal), infected with *Mycobacterium bovis* BCG (BCG) and infected with *Mycobacterium tuberculosis* H37Rv (H37Rv). In purple bands automatically detected by ImageLab. Each one of the treatments shows the existence of unique bands, but also there seem to be bands shared between at least two conditions or bands present despite if there was an infection or not

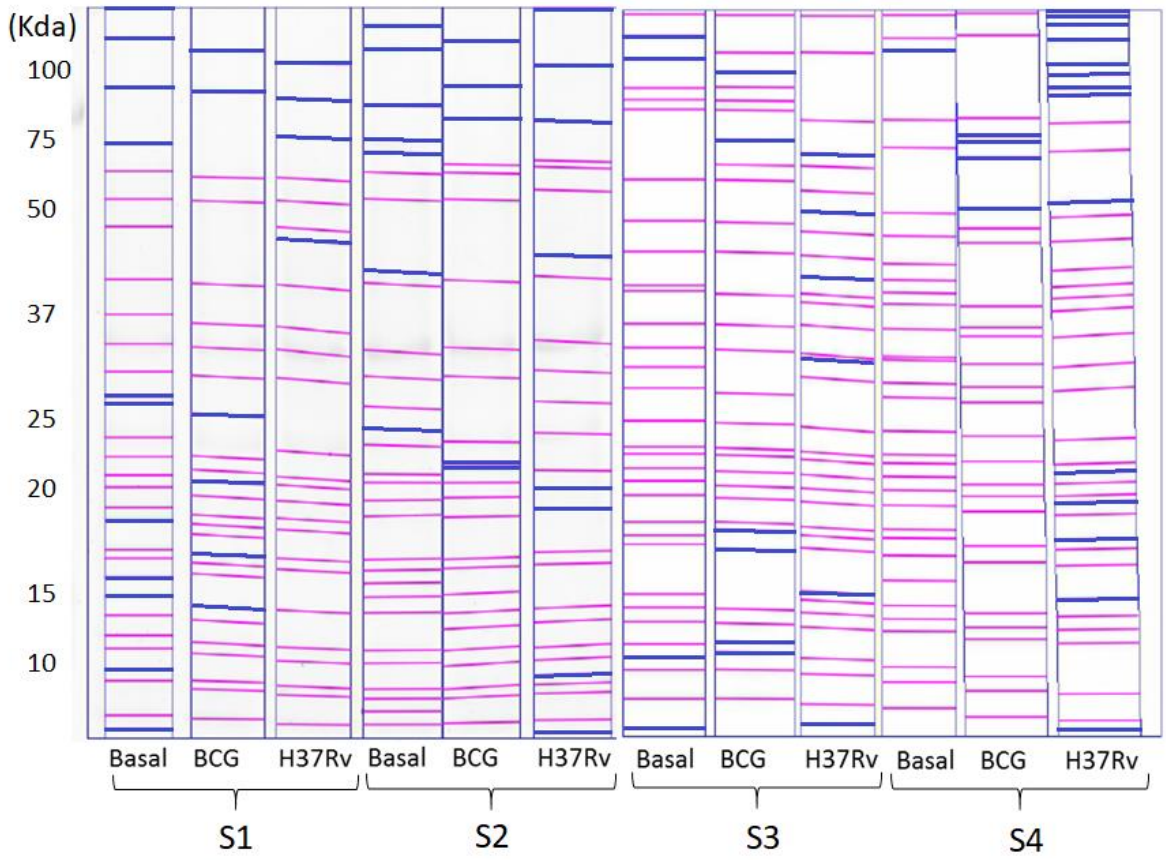


Figure 24.- Phosphoprotein band profiles of MDM's infected with *M. bovis* BCG, *M. tuberculosis* H37Rv or non-infected of 4 subjects (S1 - S4) as found by ImageLab. Image result of the merging of the two gels in figures 24 and 25, with the background reduced to maximum. In purple: bands that are shared between two or three conditions. In blue: unique phosphoprotein bands found for a given treatment of each subject. Unique bands can be found for every particular treatment in every subject studied.

We found phosphoproteins bands that are solely present under a specific condition, but so spoken band might not be present in other subject or could be shared with another subject but under a different treatment. Most of the phosphoproteins of all subjects can be found simultaneously under infection and non-infection conditions, probably reflecting the activation of cell processes necessary for survival.

Table 4.- The table shows the total band counting according to the infection condition, *M. bovis* BCG, *M. tuberculosis* H37Rv or non-infected (basal).

Subject	Condition	X Basal	X BCG	X Rv	Common
1	Basal	11	3	1	15/30
	M. bovis BCG	3	8	1	15/27
	M. tuberculosis H37Rv	1	1	8	15/25
2	Basal	9	4	2	14/29
	M. bovis BCG	4	5	2	14/25
	M. tuberculosis H37Rv	2	2	8	14/26
3	Basal	3	3	7	19/32
	M. bovis BCG	3	6	2	19/30
	M. tuberculosis H37Rv	7	2	5	19/33
4	Basal	6	4	4	18/32
	M. bovis BCG	4	4	2	18/28
	M. tuberculosis H37Rv	4	2	13	18/37

X Basal: bands shared with the basal condition; X BCG: bands shared with the infection with *M. bovis* BCG; and X Rv: bands shared with the infection with *M. tuberculosis* H37Rv.

Except for one band with a molecular weight less than 10 kDa found in three out of four subjects, there's no typical response of macrophages against the infection to MTB. Total band counting shows the high variability between subjects, table 5.

Table 5.- Table shows the total band counting of all subjects analyzed.

	Basal	Infection with *M. bovis* BCG	Infection with *M. tuberculosis* H37Rv
Total bands	29 - 32	25 – 30	25 – 37

Discussion:

M. tuberculosis elicits a highly variable immunological response among the macrophages of different subjects stimulating the activation of different cellular pathways. Individual variation played an important role in this study; some factors that could affect it were:

1. Blood donors are not required to do a PPD or any other test that rules out a previous or an active infection with MTB [50]. Since México is considered to be a country where TB is endemic, it is highly probable that some subjects were previously in contact with the bacteria and either showed no symptoms or a previous infection was positively eliminated. As well the constant exposition to non-tuberculosis mycobacteria, this could modify the innate immune response against a second or subsequent expositions to MTB.

2. Assuming that all of the subjects had no contact with MTB before this study, it is still important to take into consideration their genetic background [51].

3. There is also no possibility to rule out that any subject was under a subclinical infection of any other parasite.

4. Although all subjects were male, their ages were not the same.

Still using MDM's could be a more precise tool to describe the interaction with MTB than cell lines such as THP1 which are based on oncological cells

with different expression of receptors and cytokines [26].

For this experiments, a MOI 5:1 (bacteria/cell) was used. This might generate a lower bacillary load within macrophages compared to other works where MOIs up to 40:1 are used [52]. In previous studies, murine models have been infected with only 1000 CFUs, and the mathematical models reached a total bacterial load of 10^4 after one week [53]. It is therefore more likely that lower MOIs reproduce natural phenomena in a more rigorous way than higher ones, and although an MOI of 5:1 might be producing a cellular response too small to be accurately measured and analyzed, it is an important response nonetheless.

Another important consideration is the time the cells were infected. A 24-hour assay does not allow to determine the dysregulated pathways of the first steps of the infection, and after that time it is possible that *M. bovis* BCG has been already eliminated so it could be too late to address all the changes that the bacteria creates.

In most of the cases there is a major level of protein phosphorylation state under conditions of particular infection increase in the infection with *M. tuberculosis* H37Rv, which could mean that more cellular pathways are activated and/or proteins are gaining more than on phosphate group. This information makes clear that macrophages indeed respond to the infection although the way in which cells respond to the treatments is yet to be determined. Since some mycobacteria phosphatases have been described, it is apparent mycobacteria takes advantage of changing the activation status of some target proteins [29]. However, dynamism and changes of activation along time and among pathways is not yet well established.

To analyze the subsequent results, Generalized Mixed Models (GLMM) were implemented. They also because it allows us to analyze data under not

normal distributions where the results might be affected by random effects such as individual variation that otherwise, studied through other statistic tools, may cause a type II statistics error, a false negative. Although GLMM is a common tool in other biological sciences like ecology and evolution there is little to none documented application of this valuable statistic model in immunology.

To make clear if the MAPK pathway suffered any alterations, the levels of the phosphorylated forms of ERK 1 ERK 2, JNK and P38. These kinases activate cellular programs related to proliferation, apoptosis, cytokine biosynthesis and cytoskeletal reorganization [54].

Both ERK 1 and 2 showed no significant difference, but data dispersion was the greatest among the four analyzed proteins. We found that 3 out of 6 subjects increased their levels of phosphorylation of this pathway under infection with *M. tuberculosis* H37Rv, the small studied n might be hiding a tendency. After 24 hours it is possible that there was no longer need to produce iNOS when infected with *M. bovis* BCG, for it may have been killed, thus showing similar levels to those of basal condition. On the other hand, *M. tuberculosis* H37Rv could be evading the cellular immune response, and so iNOS and other proinflammatory cytokines were not produced.

One of the most important effects of the JNK pathway is the activation of the apoptotic program, but it showed no different activation under any treatment, data dispersion could be masking results. It is interesting that only one of the subjects showed a higher phosphorylation of JNK under *M. bovis* BCG infection, so it is probably that this the least used signaling cascade. Is already being reported that Mycobacteria can modulate cellular apoptosis [55], but this study could not clarify if MAPK holds an important role.

In our study, only phospho-p38 was lowered in MDM infected with *M. bovis*

BCG in comparison to *M. tuberculosis* H37Rv, probably because macrophages were already coping with the aftermath of destroying bacteria and no longer needed an increased inflammatory response. The fact that the difference found was only between the vaccine and the virulent strain and not between *M. bovis* BCG, and basal state shows a biological tendency too subtle for being measured. This response can be understood as a slightly over-activation of MDM under *M. tuberculosis* H37Rv infection, four out six cases showed an increased phosphorylation under this treatment, a down-regulation for the *M. bovis* infection, and only two out of four subjects showed an increased phosphorylation. It is important to mention that this protein showed very little data dispersion.

It is also known that the same particular MAPK pathway can be activated by different effectors and still produce two cellular fates as different as apoptosis and proliferation (Figure 25).

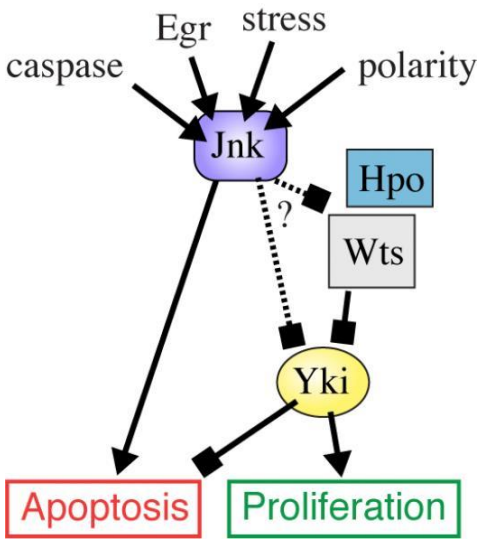


Figure 25.- Different stimuli on a cell can activate the JNK pathway with different outcomes [56]

It is yet to be determined the exact mechanisms that control the result of MAPK activation. Scaffolding proteins could be potential modulators commonly

oversight. Only a few epitopes of TLRs, like LPS, have their signaling cascades widely described, the interaction with live bacteria holds much more complex activations and the effect of mycobacterial phosphatases with human targets can also be dysregulated MAPK. A factor that could affect in-vitro assays is the use of macrophages with different origins because differentially expressed receptors could also mean different activation.

Protein profile analysis showed, even more, variation between subjects, many phosphorylated proteins greatly vary among subjects, and there seems to be no particular pattern whatsoever.

To analyze the bands that could be shared (a core response pathway) or not between conditions, relative front was analyzed. To do so, bands with similar patterns are selected according to a visual inspection of the gel. This introduces a human error with possible subjective results.

On the sharing of bands between the basal – infection with *M. bovis* BCG, BCG – *M. tuberculosis* H37Rv and basal – *M. tuberculosis* H37Rv:

1. Basal - *M. bovis* BCG

Bands shared between the non-infected and the infection with BCG could be the result of macrophages returning to a normal state after eliminating bacteria. Although there's only one protein band under this case that's shared by 2/4 subjects.

2. *M. bovis* BCG – *M. tuberculosis* H37Rv

On the other hand, common bands between *M. bovis* BCG and *M. tuberculosis* H37Rv infections probably reflect common mechanisms of the host cell in response to the interaction with bacteria. Then again, only one band under aforementioned description has been found to be shared by 2/4 subjects.

3. Basal and *M. tuberculosis* H37Rv

Finally, phosphorylated proteins shared between the basal conditions and the infection with *M. tuberculosis* H37Rv could hold the pathways that remain unaltered due to the interaction of the host cell with MTB, where the bacteria modulate the immune response supressing cytotoxic factors and phagosome maturation and any other mechanisms that could jeopardize its viability. Subjects showed an average of 3.5 (S.D. = 1.91) basal – *M. tuberculosis* H37Rv shared bands, whereas basal – *M. bovis* BCG only got an average of 3.25 and *M. bovis* BCG – *M. tuberculosis* H37Rv just 2.5 bands. There was no shared band between the non-infected and *M. tuberculosis* H37Rv infection treatments that was common among subjects which could mean that the same bacteria reacts in a different way when infect macrophages of different persons, suggesting that there must be plenty mechanisms allowing the bacteria to adapt to different environments according to different immune systems. Moreover, human macrophages showed the capability of delivering a huge variability of responses to an infection challenge, not even infections with *M. bovis* BCG provoke a unique way of dealing with the bacteria.

Conclusion:

MTB does change the phosphorylation of macrophages, indicating that their infecting and evasion mechanisms rely on over-activating or under-activating cell pathways. The MAPK pathway, as a whole, seems to be altered with an increased level of phosphorylated proteins under infection with *M. tuberculosis* H37Rv and a decreased level of activation under *M. bovis* BCG infection. When Erk 1, Erk 2, Jnk and p38 activation were analysed no significant difference was found between infections or between any of the infections and the control, showing that the change in the pathway is rather a subtle one. Phosphorylation profile of MDM demonstrates a highly varied response between subjects. Finally, the interaction between bacteria and human macrophages can elicit a broad range of responses.

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"…Disease seems to have spread across the entire country, maybe the entire world, mowing down the righteous and the unrighteous alike… It had even crossed his mind at one point that maybe the disease had killed everyone in the world…"

Stephen King

"And now was acknowledged the presence of the Red Death. He had come like a thief in the night. And one by one dropped the revellers in the blood-bedewed halls of their revel, and died each in the despairing posture of his fall."

Poe