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Mycobacterium tuberculosis $H_{37}R_v$ Induces Gene Expression of PDE4A and PDE7A in Human Macrophages

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Abstract

The World Health Organization reported that one-fourth of the global population is infected with *Mycobacterium tuberculosis (M.tb),* the causative agent of an airborne infectious disease known as tuberculosis (TB). In 2017, TB alone caused 1.6 million deaths. *M.tb* is an intracellular pathogen equipped with specialized evolutionary traits to evade immune mechanisms. Upon inhalation, macrophages phagocytose *M.tb* and become a niche due to their inability to resolve the infection. The intracellular growth of *M.tb* is influenced, in part, by host transcription factors and immunosuppressive second messengers like cyclic adenosine monophosphate (cAMP). The importance of cAMP as an inflammatory response mediator derives from its ability to suppress innate immunity functions in macrophages, monocytes, and neutrophils by limiting pro-inflammatory cytokine release. Despite its known effects, the mechanisms underlying cAMP activation in response to *M.tb* are incompletely understood, particularly in human macrophages. Preliminary data indicate that cAMP levels are increased in human monocyte-derived macrophages (hMDMs) following infection with virulent *M.tb* $H_{37}R_v$ and attenuated *M.tb* $H_{37}R_a$. Phosphodiesterases (PDEs) comprise a group of enzymes that degrade cAMP to regulate signal transduction. We hypothesize that elevated cAMP levels induce gene expression of certain PDEs as a host response mechanism to degrade *M.tb*-induced cAMP. Gene expression studies demonstrated that transcription of PDE4A and PDE7A increased 48 and 72 h after infection, whereas PDE3A and PDE5A remained unaltered. These data suggest that human macrophages up-regulate PDE expression to limit *M.tb* from dampening the immune response via high cAMP levels. Further studies will demonstrate the clinical feasibility of cAMP degradation as a novel host-directed therapy to reduce *M.tb* pathogenesis.

Keywords: Host-directed therapy, macrophages, *Mycobacterium tuberculosis*, pathogenesis, signal transduction, cAMP, PDE, gene regulation.

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1. Introduction

Mycobacterium tuberculosis is primarily a pulmonary pathogen transmitted airborne through droplet nuclei from active TB patients. The available two-phased chemotherapy regimen has failed to eradicate TB due to the intrinsic obstacles related to sustaining treatment for at least six consecutive months [1]. The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains poses a threat to humanity as core treatments have been rendered inefficacious. The therapeutic approach for drug-resistant TB is cumbersome due to less-efficient, expensive, and toxic substitutes to the first-line agents [2]. Adjunctive host-directed therapies (HDTs) have emerged as novel alternatives to attenuate *M.tb* pathogenesis. The principle of HDTs is to augment the host's immune mechanisms and limit excessive tissue pathology; therefore, decreasing treatment duration while reducing morbidity and mortality [3]. *M.tb* can affect all compartments of the respiratory tract (i.e., nose, sinuses, pharynx, larynx, trachea, bronchi, bronchioles, and lungs); however, it remains unclear if the disease manifestations arise due to primary infection or reactivation either locally or in secondary tissues [4]. The *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is a live attenuated vaccine, which has been effective in mitigating severe disseminated forms of TB in children but remains unsuccessful in diminishing primary infections or the reactivation of latent TB [5]. There are no environmental reservoirs for *M. tuberculosis*; consequently, it acts as both a pathogen and a symbiont in humans [6]. It is crucial to elucidate the host-pathogen interactions to aid in the eradication of TB.

Alveolar macrophages (AMs) are tissue-resident lung phagocytes responsible for maintaining lung homeostasis, clearing cellular debris, and defending against pathogenic invasion [7]. AMs phagocytose *M.tb* after the intracellular pathogen has traversed several infection points along the respiratory tract. However, the vast repertoire of AMs is insufficient to resolve the infection. *M.tb* manipulates a myriad of processes in a multipronged effort to establish a permissive environment and subvert the host immune response. As for evasion mechanisms, *M. tuberculosis* can inhibit phagolysosomal maturation, manipulate host cellular trafficking, neutralize the host's oxidative bursts, as well as inhibit apoptosis and autophagy pathways [8-10]. *M.tb* immunopathology is also influenced, in part, by eicosanoid signaling pathways, transcription factors, and immunosuppressive second messengers like cyclic adenosine monophosphate (cAMP).

Cyclic AMP remains the archetypal second messenger due to its ability to regulate a wide range of essential cellular events such as gene expression, cell division, metabolism, phagocytosis, and the immune response [11]. The signal transduction pathway initiates when an extracellular messenger (i.e., neurotransmitter, chemokine, lipid mediator, hormone, or drug) binds to the G protein-coupled receptor (GPCR), which is architecturally composed of seven transmembrane helices [12]. Ligand binding to the GPCR promotes the exchange of GDP/GTP in the G protein α subunit (Gαs) to facilitate the βγ complex dissociation [13]. The free Gαs stimulates the enzyme adenylyl cyclase (AC) to catalyze the cyclization of ATP and yield cAMP, as well as

pyrophosphate [14]. Elevations in intracellular cAMP suppress the innate immunity functions of macrophages, which account for 95% of the leukocytes present in the lower respiratory tract under steady-state conditions [15].

In the AMs, cAMP exerts inhibitory effects by modulating the production of cytokines, chemokines, and lipids [16]. Additionally, a variety of cell activation components such as phagocytosis, reactive oxygen intermediate (ROI) generation, and the production of inflammatory mediators can be inhibited [17]. For instance, the pro-inflammatory cytokines TNF-α, MIP-1α, and leukotriene B4 are inhibited by cAMP-dependent pathways; in contrast, the production of the anti-inflammatory cytokine IL-10 and the suppressor of cytokine signaling-3 (SOCS3) is enhanced [18]. The role of downstream cAMP-activated effector complexes such as Protein Kinase A (PKA) and Epac1 remains poorly understood. The virulence arsenal of *M.tb* can manipulate the cAMP-PKA system to inhibit the phagosomal assembly of actin; thus, promoting its intracellular survival [19]. The secretion of interferon-gamma (IFN-γ) by type 1 T-helper cells (Th1) activates macrophages in cell-mediated immunity during *M.tb* infection. However, patients with advanced TB disease exhibit an inhibition of IFN-γ production due to the enzymatic activity of type I PKA [20].

The delicate intracellular balance of cAMP is alternatively regulated by a family of enzymes known as cyclic nucleotide phosphodiesterases (PDEs). PDEs hydrolyze cyclic nucleotide second messengers such as cAMP and cyclic guanosine monophosphate (cGMP) to regulate signal transduction pathways, as well as physiological processes [21]. There are 11 gene-related families named PDE1 to PDE11 [14]. Although the members of each PDE family display a considerable divergence of amino acid sequences, the functional relationship remains the same according to their substrate, inhibitor specificities, and enzymatic activity regulation [22]. PDEs hold promising therapeutic potential due to their unique tissue distribution and gene expression patterns. For instance, PDE5 inhibitors are available for treating male erectile dysfunction and pulmonary hypertension; in contrast, PDE3 inhibitors target short-term dilated cardiomyopathies [23,24]. Adjunct HDTs such as the pharmacological inhibition of PDE4 augment the bactericidal activity of isoniazid while reducing pulmonary fibrosis in *M.tb*-infected mouse and rabbit models [25,26]. The regulation of anti- and pro-inflammatory cytokines can be translated into a marked reduction of *M.tb*'s granulomatous pathology, which is associated with clinical persistence. The success of innovative HDTs has extrapolated the interest in PDEs to various research disciplines.

In the present study, we employed the human monocyte-derived macrophage (hMDM) model to determine cAMP fluctuations and the gene expression signature of PDEs in response to *M.tb* infection. We preliminarily discovered that cAMP levels are increased in hMDMs following infection with attenuated *M.tb* $H_{37}R_a$ and virulent *M.tb* $H_{37}R_v$. Gene expression studies via qRT-PCR demonstrated that transcription of PDE4A and PDE7A is induced by the *M.tb* strain $H_{37}R_v$ in hMDMs. In contrast, the gene expression of PDE3A and PDE5A is not significantly altered. Thus, our research identifies new potential targets

to modulate *M.tb*-induced cAMP in macrophages, and further investigation is required to determine the feasibility of cAMP regulation as a novel HDT.

2. Materials and Methods

2.1 Monocyte isolation and culture of human monocyte-derived macrophages (hMDMs)

The MDM preparation process is described elsewhere [27,28]. Briefly, peripheral blood samples were collected from healthy donors following approved protocols from the Institutional Review Board and Texas Biomedical Research Institute. All donors were informed and provided written consent. Peripheral blood mononuclear cells (PBMCs: 80% monocytes and 20% lymphocytes) were isolated from heparinized blood with Ficoll-Paque separation media (GE Healthcare, Uppsala, Sweden). The PBMCs were cultured with RPMI (Invitrogen, Carlsbad, CA) as well as 20% autologous serum in Teflon wells (Savillex, Eden Prairie, MN) for five days at 37 ˚C in 5% CO_2. PBMCs were harvested and transferred to tissue culture plates supplemented with RPMI and 10% autologous serum. After 2 h, lymphocytes were removed by washing, while hMDMs adhered to the plates to form a monolayer. The hMDMs were incubated overnight prior to infection.

2.2 Bacterial strains

The lyophilized *M.tb* strain $H_{37}R_v$ was obtained from the American Tissue Culture Collection (ATCC, Manassas, VA). Single cell suspensions were prepared as described previously [29,30]. The Petroff-Hausser Chamber aided in the determination of clumping degree (<10%) as well as bacterial concentration. The CFU assays confirmed bacterial viability (≥90%). *M.tb* infections of hMDMs were conducted in the BSL3 laboratory of the Texas Biomedical Research Institute following approved facility and safety guidelines.

2.3 M.tb infection of hMDMs

Single cell suspensions of *M.tb* in RHH (0.1% human serum albumin [CSL Behring, King of Prussia, PA], RPMI, and 10mM HEPES [Life Technologies]) were added to the hMDMs at a Multiplicity of Infection (MOI) 5 or the equivalent of 5 bacteria per macrophage. The cells were incubated for 2 h at 37°C to facilitate bacterial phagocytosis, with the first 30 min on a platform shaker [31]. hMDMs were then washed to remove extracellular bacteria and incubated in RPMI with 2% autologous serum. hMDM monolayer integrity was assessed during the indicated time points by cell viability. Pictures were captured with 10X and 40X objective lenses (Olympus CKX41).

2.4 RNA isolation and quantification

hMDMs in triplicate wells were lysed with TRIzol (Invitrogen) according to the manufacturer's recommendations. The RNA samples were centrifuged at 4˚C for 15 min under a g-force of 14,000 after the addition of chloroform (0.2 mL). The upper aqueous phase was isolated, and 500 µL of isopropanol added. The samples were centrifuged at 14,000 x g for 10 min. The pellet was washed with 500 µL of 75% ethanol and then centrifuged for 5 min at 14,000 x g. The precipitate was dissolved in 35 µL of Ultrapure distilled water (Invitrogen). Confirmatory readings were conducted with the NanoDrop 1000 (ThermoFisher Scientific) to quantify the RNA and assess its purity.

2.5 Gene expression studies via qRT-PCR

The RNA samples were reverse transcribed into cDNA with SuperScript III Reverse Transcriptase (Invitrogen), dNTPs, and random primers following the manufacturer's guidelines. Gene

expression was determined by quantitative real-time PCR (qRT-PCR) via TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) and a 7500 Real-Time Thermocycler (Applied Biosystems). The gene expression values were analyzed by the ΔΔCt method, in which β-actin served as a housekeeping gene for normalization controls.

2.6 cAMP ELISA

Supernatants were collected from *M.tb*-infected hMDMs at the indicated time points, and cAMP was measured by competitive binding ELISA (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's recommendations.

2.7 Statistical Analysis

PDE gene expression results are presented as the fold change ± SEM unless otherwise indicated in the figure legends. The trend remained constant for each donor; however, biological replicates displayed a distinct magnitude of change. Therefore, the data were normalized to an internal control. A one-way ANOVA was performed, followed by Dunnett's multiple comparisons test. The cAMP results are reported as mean ± SD with $P < 0.05$ considered significant. All data were analyzed and plotted using GraphPad Prism 8.

3. Results

***M.tb* strains $H_{37}R_a$ and $H_{37}R_v$ induce cAMP expression in hMDMs**

Cyclic nucleotide second messengers partly mediate the immunopathology of hMDMs during *M.tb* infection. *M.tb* induces an acute increase in cAMP intramacrophage concentrations as a virulence factor to inhibit inflammatory mediators such as cytokines, chemokines, and lipids [16]. We hypothesized that elevated cAMP levels induce the gene expression of certain PDEs as a host response mechanism to restore cellular homeostasis. We infected hMDMs with the *M.tb* strain $H_{37}R_v$ at MOI 5. This multiplicity ratio ensures that approximately 30% of MDMs will be infected with the bacteria. Figure 1 shows the intact MDM monolayer in uninfected (1A) and infected (1B) cells at 48 h post-infection.

A B

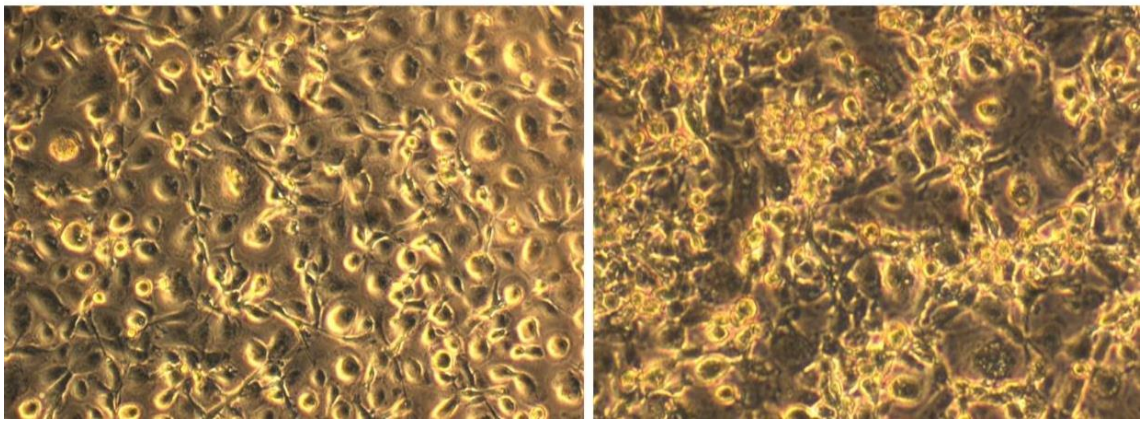

Figure 1. *M.tb* infection of hMDMs. Uninfected control (A) compared to hMDMs infected with $H_{37}R_v$ at MOI 5 (B). The pictures exhibit monolayer integrity and confluency at 48 h.

The kinetic rates of cAMP secretion are unclear in *M.tb*-infected hMDMs. Therefore, we measured cAMP concentrations over a time-course to identify physiologically relevant fluctuations. *M.tb* infections of hMDMs were also conducted with the attenuated strain $H_{37}R_a$ to assess any effects of

virulence on cAMP production. Supernatants were collected from triplicate wells for uninfected hMDMs (Fig. 1A) and *M.tb*-infected macrophages at MOI 5 (Fig. 1B). The levels of cAMP remained unaltered 30 min, 1 h, and 3 h upon $H_{37}R_a$ and $H_{37}R_v$ infection (Fig. 2A). In contrast, marked increases were observed at 24, 48, and 72 h after *M.tb* infection (Fig. 2B). The results demonstrate that *M.tb* infection of hMDMs elicits cAMP production in a time-dependent fashion. We noted a considerable increase in cAMP at 72 h post-infection with the attenuated $H_{37}R_a$ strain compared to the virulent $H_{37}R_v$ strain.

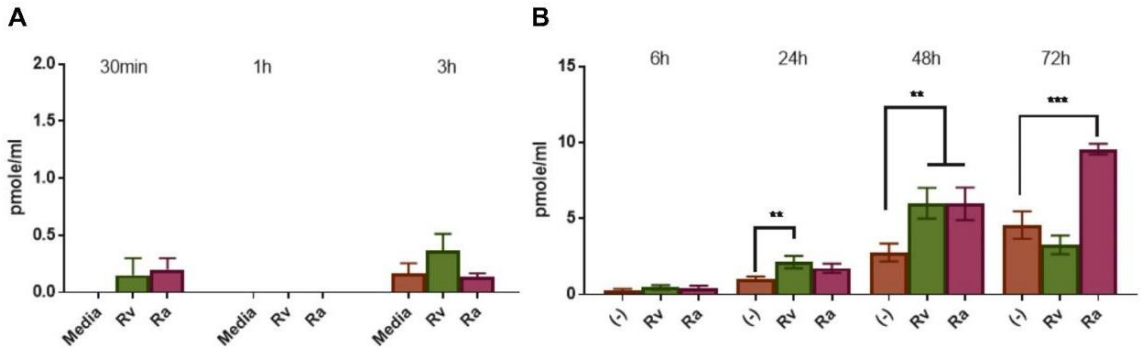

Figure 2. *M.tb* induces cAMP at 24, 48, and 72 h post-infection. The hMDMs were infected with *M.tb* $H_{37}R_v$ or *M.tb* $H_{37}R_a$ (MOI 5). Supernatants were collected in triplicate at various time points, and cAMP levels were measured via a competitive binding ELISA. The results are represented as mean ± SD. *n=1*; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Gene expression of PDE4A and PDE7A is increased in hMDMs infected with $H_{37}R_v$.

Inspired by our findings, we proceeded to test the PDE transcription profile of *M.tb*-infected hMDMs in comparison to the increased intracellular cAMP levels. Quantitative real-time PCR (qRT-PCR) analyses revealed that hMDMs transcribe the PDE4A and PDE7A genes 48 and 72 h post-infection (Fig. 3A-B). In contrast, gene expression studies did not detect transcriptional alterations for PDE3 and PDE5 (data not shown). The mechanism regarding *M.tb* induction of cAMP remains in question; however, it is notable that the small decrease in cAMP observed 72 h following infection with the virulent strain $H_{37}R_v$ (Fig. 2B) coincides with the increased gene expression of PDE4A and PDE7A at 48 and 72 h. The results suggest hMDMs increase the gene expression of PDE4A and 7A as a host response to degrade *M.tb*-induced cAMP levels.

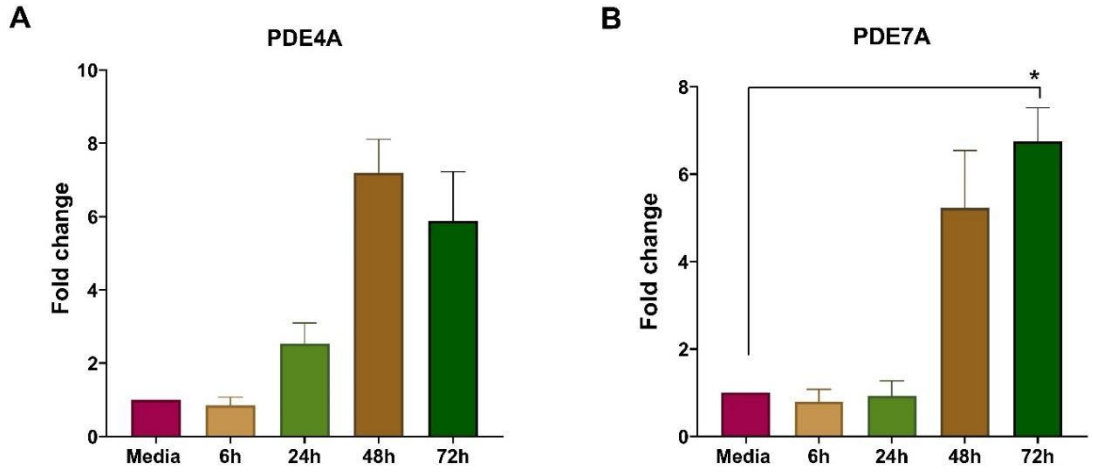

Figure 3. Gene expression of PDE4A and PDE7A increases upon *M.tb* infection of hMDMs. The hMDMs were infected with $H_{37}R_v$ (MOI 5) in triplicate. RNA was isolated at the indicated time points, and qRT-PCR analyses were performed to determine the gene expression of PDE4A and PDE7A. Results were normalized to β-actin and reported as the fold change compared to uninfected. *n=3*; *$P <$ 0.05.

4. Discussion

M.tb has numerous interaction points along the respiratory tract until AMs phagocytose the pathogen. The virulence arsenal of *M.tb* subverts the host immune response to establish a multipronged permissive environment. Immunosuppressive cyclic nucleotide second messengers partly influence the immunopathology and intramacrophage growth of *M.tb*. Preliminary studies quantifying cAMP in hMDMs revealed that concentrations increase 24, and 48 h following infection with $H_{37}R_v$, while $H_{37}R_a$ induces cAMP 48, and 72 h post-infection (Fig. 2B). The results demonstrate that *M.tb* infection of hMDMs elicits cAMP production in a time-dependent interval. The observed heterogeneity in cAMP concentrations is potentially attributable to the pathogenicity differences of the strains. Gene expression analyses 6, 24, 48, and 72 h after infection revealed the expression of specific PDEs. The transcription of PDE4A and PDE7A is induced 48 and 72 h after infection with $H_{37}R_v$ (Fig. 3A-B). These results are possibly linked to the cAMP decrease exhibited by the virulent strain at 72 h post-infection. We speculate that hMDMs upregulate the gene expression of PDE4A and 7A to degrade cAMP levels and prevent $H_{37}R_v$-mediated dampening of the immune response.

The mechanism(s) underlying the host-pathogen interaction that provokes cAMP activation remains in question. The enzyme adenylyl cyclase canonically regulates cAMP concentrations. Interestingly, *M.tb* is equipped with 17 adenylyl cyclase genes, and at least one (Rv0386) is involved in the delivery of bacterial cAMP into the macrophage cytoplasm [32]. The disruption of cAMP steady-state concentrations in macrophages by the Mycobacterial species can interfere with endogenous signal transduction pathways. Additionally, *M.tb* is known to induce host cell Prostaglandin E_2 (PGE_2) synthesis in the early stages of infection. The pleiotropic effects of PGE_2 are mediated primarily via the G protein-coupled receptor subtype EP4 in human macrophages [33]. The action of

EP4 traditionally stimulates the G protein α subunit (Gαs), which increases intracellular cAMP concentrations by activating the enzyme adenylyl cyclase [34]. Therefore, it is plausible that *M.tb* infection of hMDMs increases cAMP via bacterial secretion and PGE_2-dependent mechanisms (Fig. 4). The primary role of PGE_2 is counterintuitive and remains controversial in the immune regulation of *M.tb*-infected hMDMs. Further investigation is required to elucidate the contribution of PGE_2, as well as cAMP to the intramacrophage growth of *M.tb*.

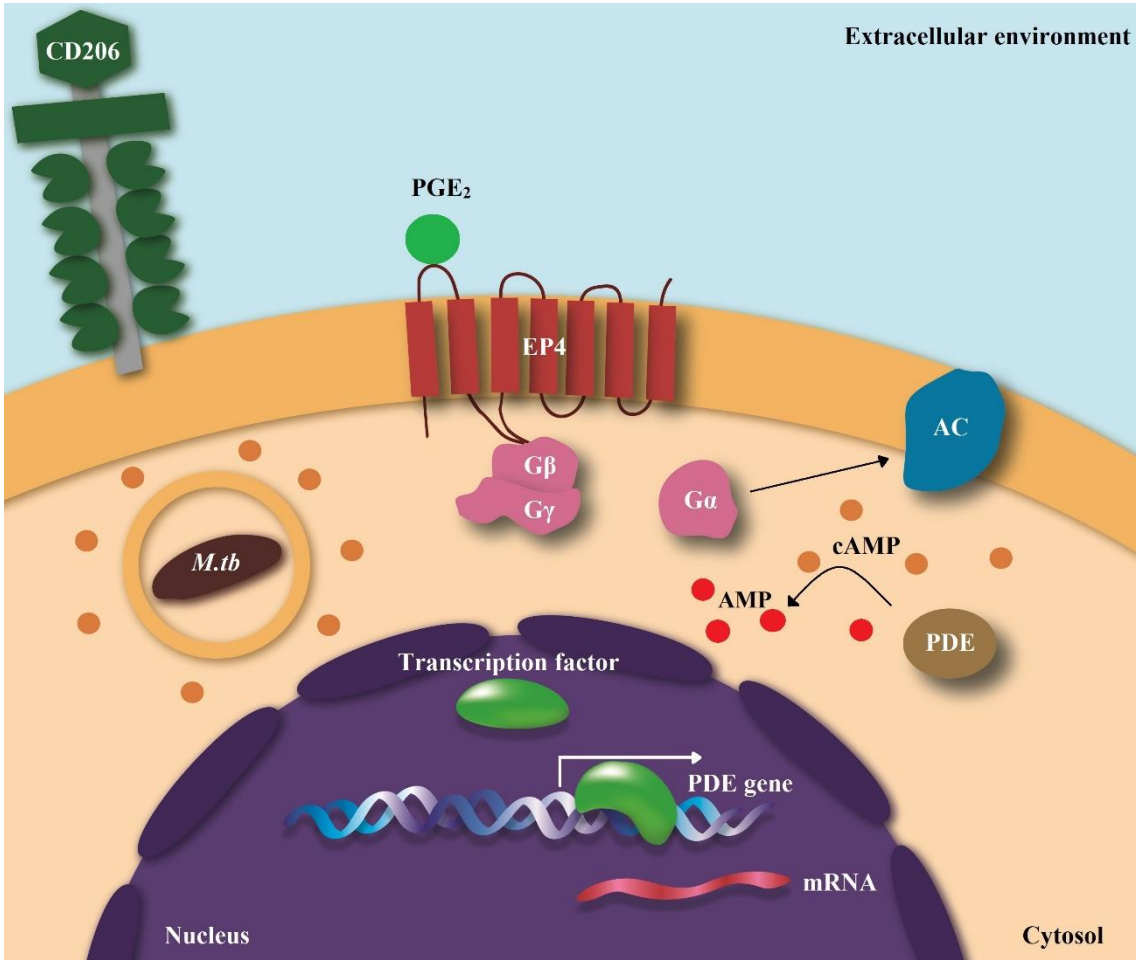

Figure 4. Proposed model. hMDMs phagocytose *M.tb* through complement and pattern recognition receptors.

The host-pathogen interaction provokes an intramacrophage increase in cAMP. Although the signal transduction pathway is incompletely understood, we speculate that PGE_2 acts as an extracellular ligand binding to the GPCR subtype EP4 in hMDMs. Activation of the enzyme adenylyl cyclase by the Gαs promotes the catalytic conversion of ATP into cAMP.

Additionally, the Mycobacterial adenylyl cyclase Rv0386 is associated with cAMP delivery into the cytosol of hMDMs [32]. PDEs regulate signaling events through the hydrolysis of cAMP into AMP. Therefore, PDE gene expression would be induced as a host response mechanism to attenuate *M.tb* pathogenesis.

The hydrolytic activity of PDE4A and PDE7A would reestablish cellular homeostasis by degrading the $H_{37}R_v$-induced cAMP. Marked increases in cAMP levels act as a virulence factor to intoxicate human macrophages and dampen the immune response. Regulation of cAMP would presumably restore the delicate balance of cytokines, chemokines, and lipids in macrophages; thus, enhancing the immune response to *M.tb*. Additionally, downstream cAMP-activated signaling complexes such as PKA will be deactivated. The role of PKA in *M.tb* infection includes the inhibition of phagolysosomal maturation in tissue-resident macrophages and the reduction of IFN-γ secretion in T cells [19,20]. Future studies will characterize all PDE isoforms to elucidate the host-pathogen interaction during *M.tb* infection of human macrophages. The gene expression profile of PDEs in response to avirulent and virulent *M.tb* strains will provide valuable clues to untangle the signaling pathway.

PDEs are clinically significant due to their unique tissue distribution and functional properties. The success of PDE-specific therapies for different diseases can be extrapolated as a novel mechanism to regulate macrophage activation. The design of HDTs can be dependent on PDE activator molecules such as MR-L2 to regulate *M.tb* pathogenesis. MR-L2 is a noncompetitive activator promoting the reversible dimeric stimulation of PDE4 variants through a phenocopy mechanism [35]. The allosteric activation of PDE4 would result in lower cAMP intracellular concentrations due to increased degradation, which would favor the host by reducing *M.tb* pathogenesis.

The effect of HDTs can synergistically enhance pivotal components of the macrophage response to *M.tb*. Activators can reduce immunopathogenesis via critical PDEs, while non-specific reactions such as cGMP hydrolysis can be inhibited. A broad spectrum of macrophage responses can be selectively regulated to enhance the intracellular neutralization of *M.tb*. The immunosuppressive role of PDE4 inhibition can be clinically relevant in patients with advanced TB disease. Granulomas are compact structures that emerge as a pathological hallmark of chronic *M.tb* infection. The granuloma is composed of macrophages, monocytes, dendritic cells, neutrophils, epithelioid cells, multinucleated giant cells, and a solid layer of lymphocytes [36]. The role of granulomas was contemplated as solely protective from a histological standpoint. However, chronic inflammation can negatively impact the host through pathological lesions inflicting collateral lung damage. The pharmacological inhibition of PDE4 augments the bactericidal activity of isoniazid and reduces pulmonary fibrosis [25,26]. In the early stages of *M.tb* infection, PDEs would presumably confer a protective role at the cellular level by degrading cAMP. In contrast, the selective inhibition of PDE4 in chronic TB patients can reduce tissue damage and pulmonary fibrosis. Further investigation is required to demonstrate the efficacy of novel PDE-mediated immunoregulatory HDTs.

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