

COMPARATIVE ANALYSIS OF MACROPHAGE INTERACTIONS WITH ANTIGENS OF TUBERCULOUS AND NON-TUBERCULOUS MYCOBACTERIA *IN VITRO*

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ABSTRACT

Background. Ongoing research is investigating the interaction between mycobacteria and macrophages. Upon infection with mycobacteria, macrophages release cytokines. These include IFN γ , TNF, IL-1, IL-6, and IL-12p40, among others. These substances play a critical role in the host response to tuberculosis and other mycobacterial infections.

Aim. To investigate and compare the cytokine patterns produced by human macrophages in response to interaction with antigens from both pathogenic and conditionally pathogenic mycobacterial strains *in vitro*.

Methods. Blood monocytes were cultured for five days in the presence of 10 % active human serum. The resulting macrophages were then cultured with mycobacterial antigens from *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Mycobacterium kansasii* for 24 hours. Changes in macrophage gene expression profiles and the production of pro-inflammatory and anti-inflammatory cytokines were then assessed. A control group of "naive" macrophages cultured without any mycobacterial antigen was also used.

Results. Stimulation of human macrophages with antigens from different mycobacterial species resulted in the formation of distinct cell phenotypes: protective (*M. tuberculosis*), characterized by the expression of IL6, IFN γ , and TNF; pro-inflammatory (*M. kansasii*), with active expression of IL6, IL1 β , and TNF; and pro-inflammatory (*M. avium*), expressing IL6 and IL12. The cytokine production findings were fully consistent with the gene expression data.

Conclusion. These findings on macrophage-mycobacterial antigen interactions provide insight into the mechanisms by which both tuberculous and nontuberculous mycobacterial infections establish chronic macrophage infection.

Keywords: macrophages, cytokines, chemokines, *M. tuberculosis*, *M. avium*, *M. kansasii*, *in vitro* research

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СРАВНИТЕЛЬНЫЙ АНАЛИЗ ОСОБЕННОСТЕЙ ВЗАИМОДЕЙСТВИЯ МАКРОФАГОВ С АНТИГЕНАМИ ТУБЕРКУЛЕЗНЫХ И НЕТУБЕРКУЛЕЗНЫХ МИКОБАКТЕРИЙ *IN VITRO*

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РЕЗЮМЕ

Обоснование. В настоящее время активно продолжают исследования, связанные с изучением взаимодействия микобактерий и инфицированных ими макрофагов человека и животных. В ответ на инфекцию макрофаги производят специфические молекулы, известные как цитокины, среди которых выделяются IFN γ , TNF, IL-1, IL-6, IL-12p40 и IL-12p70. Эти вещества играют ключевую роль в патогенезе туберкулёза и других заболеваний, вызванных микобактериями. В этой связи, **целью нашей работы** было сравнительное исследование паттернов цитокинов, вырабатываемых макрофагами человека в результате взаимодействия с антигенами патогенных и слабопатогенных микобактерий *in vitro*.

Методы. Из моноцитов крови здоровых добровольцев получали культуру макрофагов. Макрофаги культивировали с микобактериальными антигенами *Mycobacterium tuberculosis*, *Mycobacterium avium* и *Mycobacterium kansasii*. Через 24 часа проводили исследование изменения профиля экспрессии генов макрофагов (экспрессионный анализ) и продукции этими клетками про- и противовоспалительных факторов. В качестве отрицательного контроля использовали культуру макрофагов, культивируемых без антигенов микобактерий.

Результаты. Показано что, стимуляция макрофагов антигенами разных микобактерий приводит к формированию различных фенотипов клеток: про-тективного (*M. tuberculosis*) – с экспрессией генов IL6, IFN γ и TNF; провоспалительного (*M. kansasii*) – с активной экспрессией генов IL6, IL1 β и TNF и амбивалентного (*M. avium*) – с экспрессией генов IL6, IL12. Результаты исследования продукции цитокинов макрофагами человека в ответ на антигены различных микобактерий полностью подтвердили данные, полученные с помощью экспрессионного анализа.

Заключение. Выявленные в работе особенности реакции макрофагов на антигены различных видов микобактерий позволяют более детально охарактеризовать механизмы персистенции туберкулёзных и нетуберкулёзных микобактерий в макрофагах.

Ключевые слова: макрофаги, цитокины, хемокины, *M. tuberculosis*, *M. avium*, *M. kansasii*, исследование *in vitro*

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INTRODUCTION

Although *Mycobacterium tuberculosis* is often regarded as a classical bacterial pathogen, a deeper understanding of its biology requires a more comprehensive investigation of its adaptation to its primary host – humans. Furthermore, it is important to consider that in the majority of individuals, *M. tuberculosis* infection may be asymptomatic, which may contribute to its transmission, including in regions with a high tuberculosis burden [1]. Upon reaching the alveoli, airborne bacteria are phagocytosed by macrophages, which become their primary niche. Mycobacteria can survive and replicate within these cells, thereby evading the immune system and migrating through the epithelium into the lung interstitium [2, 3]. The precise mechanisms underlying these processes remain hypothetical. However, observations in animal models and experiments using *ex vivo* systems suggest that early proliferation of *M. tuberculosis*-infected macrophages leads to the formation of cellular aggregates. These aggregates recruit monocytes and neutrophils, creating new cellular targets for *M. tuberculosis* dissemination [4].

Nontuberculous mycobacteria (NTM) are environmentally widespread pathogens that can cause severe disease, particularly in immunocompromised individuals, but also pose a threat to immunocompetent hosts [5]. The most common NTM species are the *Mycobacterium avium* complex (MAC) [6, 7]. The global incidence of NTM-associated diseases is rapidly increasing. Following inhalation or aspiration of NTM into the lower respiratory tract, the first cells to encounter them are airway epithelial cells, macrophages, and dendritic cells. All three cell types are capable of presenting bacterial antigens on major histocompatibility complex class II (MHC II) molecules and activating adaptive immune responses [8]. The immunopathogenesis of MAC involves a complex interplay between the bacteria and the human immune system. MAC survive and replicate within macrophages by preventing phagosome-lysosome fusion. Through their enzymatic machinery, mycobacteria are able to neutralize reactive oxygen and nitrogen species produced by macrophages. Furthermore, MAC modulate cytokine production, enabling them to suppress or regulate the host immune response [9, 10]. The second most common cause of NTM infections is *Mycobacterium kansasii*. Diseases caused by *M. kansasii* are frequently observed in immunocompromised individuals, such as HIV-infected patients [11]. Phylogenetic analysis places *M. kansasii* within the group of mycobacteria most closely related to *M. tuberculosis* [12]. However, *M. kansasii* is considered an opportunistic pathogen.

It is hypothesized that compared to NTM, *M. tuberculosis* exhibits higher virulence due to its enhanced ability to suppress the host immune response. However, the characterization of *M. tuberculosis* in the context of macrophage infection and the analysis of its effector

functions in comparison with various NTM species remain incomplete.

Currently, the complex interactions between mycobacteria and macrophages, characterized by the induction and production of various cytokines such as IFN- γ , TNF, IL-1, IL-6, IL-12p40, and IL-12p70, remain insufficiently studied. It has been well established that cytokines exert a significant influence on the pathogenesis and protective immunity to tuberculosis and mycobacteriosis [13, 14].

AIM

To investigate and compare the cytokine patterns produced by human macrophages in response to antigens of pathogenic and weakly pathogenic mycobacteria *in vitro*.

MATERIALS AND METHODS

Generation of Human Macrophage Cultures

Human macrophages were differentiated from peripheral blood mononuclear cells (PBMCs) obtained from healthy donors, as previously described [15]. Briefly, PBMCs were isolated from whole venous blood of healthy donors by density gradient centrifugation on Ficoll (density 1.077 g/mL, PanEco, Russia). Cells were then washed and incubated for 3 hours in DMEM medium at 37°C and 5 % CO₂ (PanEco, Russia). Adherent monocytes were differentiated into macrophages in the presence of 10 % “active” human serum. Serum was obtained either from the same donors or from donors with identical ABO and Rh blood groups. Macrophage enrichment was assessed by expression of the macrophage marker CD163.

Healthy donors included in the study provided written informed consent for participation in the scientific study prior to its initiation. All studies involving human biological material complied with the Declaration of Helsinki [16] and were approved by the local ethics committee of the Central Research Institute of Tuberculosis (Protocol No. 1/2 dated January 17, 2025). Biological material (whole venous blood) was obtained from healthy donors after January 2025.

Mycobacterial Antigens

Culture filtrate (CF) proteins of *M. tuberculosis* H37Rv, *M. avium*, and *M. kansasii* from the collection of the Central Research Institute of Tuberculosis were used as antigens in this study, prepared as previously described [17].

CF proteins of *M. tuberculosis*, *M. avium*, and *M. kansasii* were used as mycobacterial antigens in *in vitro* experiments at a dose of 10 μ g/mL.

Macrophage Culture

Activation of macrophages in response to mycobacterial antigens of *M. tuberculosis*, *M. avium*, and *M. kansasii* was compared using an *in vitro* human macrophage

model. Blood monocyte cultures were maintained for 5 days in the presence of 10 % active human serum. The resulting M0 macrophages were then cultured with mycobacterial antigens. After 24 hours of culture, changes in the gene expression profile of M0 macrophages and the production of pro- and anti-inflammatory factors by these cells were examined. Untreated macrophages cultured without mycobacterial antigens served as the negative control.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from human macrophage cultures using TRIzol (Thermo Fisher Scientific (Invitrogen), USA) according to the manufacturer's instructions, without modifications. Complementary DNA (cDNA) was synthesized using the SuperScript™ III Reverse Transcriptase kit (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. One microgram of total RNA was used as a template for reverse transcription.

Gene expression levels were determined by semi-quantitative real-time PCR (TaqMan®) using TaqMan™ Gene Expression Assay kits (Thermo Fisher Scientific (ABI), USA). GAPDH was used as a housekeeping gene.

Protein Concentration Determination

Cytokine and chemokine concentrations in cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using Human DuoSet ELISA kits (R&D Systems, USA) according to the manufacturer's instructions, without modifications.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software, USA) with multiple

t-tests (Student's *t*-test) corrected for multiple comparisons using the Holm-Šidák method. Normality of distribution was assessed using the Shapiro – Wilk test.

RESULTS

It was demonstrated that stimulation of macrophages with *M. tuberculosis* antigens significantly up-regulated the expression of the cytokine genes *IL6*, *TNF*, and *IFNG* (Fig. 1A, D and Fig. 2A). *M. avium* antigens induced increased expression of *IL6*, *NF-IL6*, and *IL12* (Fig. 1A, B and (Fig. 2B). For *IL12*, expression was significantly higher than upon macrophage exposure to *M. tuberculosis* antigens (Fig. 2B). The effect of *M. kansasii* antigens on the macrophage expression profile was characterized by upregulation of *IL6*, *NF-IL6*, *IL1B*, *TNF*, and *IL12* (Fig. 1 and Fig. 25).

Notably, in the case of macrophage interaction with *M. kansasii* antigens, expression of *IL6* and *IL1B* was significantly higher than with *M. avium* and *M. tuberculosis* (Fig. 1A, C). Expression of *NF-IL6* was significantly higher with *M. avium* and *M. kansasii* compared to *M. tuberculosis*, with no difference between the two (Fig. 1B). The highest *TNF* expression was stimulated by *M. kansasii* and *M. tuberculosis* antigens (Fig. 1D).

Thus, stimulation of macrophages with antigens from different mycobacterial species leads to the formation of distinct cellular phenotypes:

- protective (*M. tuberculosis*), characterized by expression of *IL6*, *IFNG*, and *TNF*;
- pro-inflammatory (*M. kansasii*), with active expression of *IL6*, *IL1B*, and *TNF*;

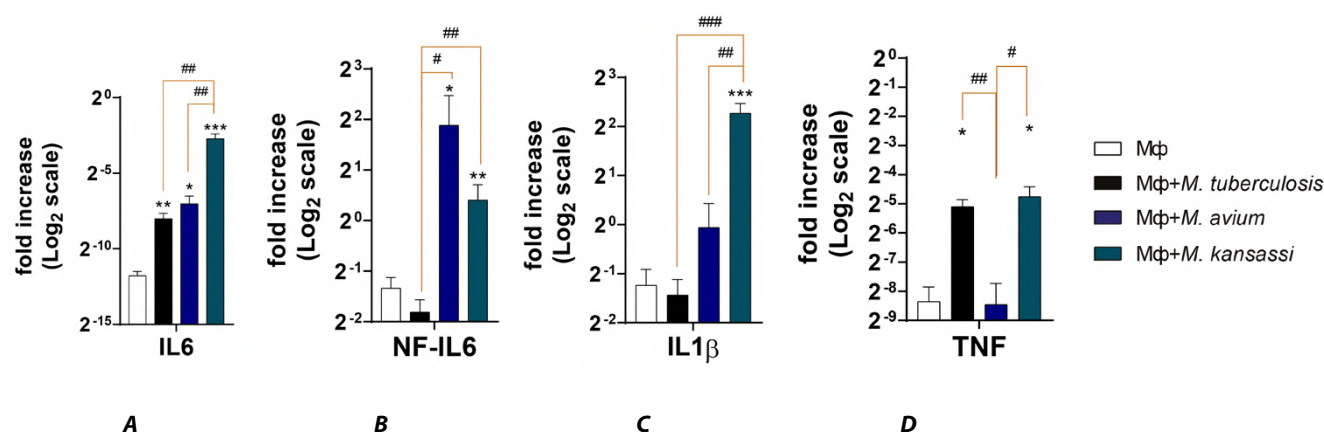


FIG. 1.

Changes in expression of *IL6* (A), *NF-IL6* (B), *IL1B* (C), and *TNF* (D) in macrophages upon stimulation with *M. tuberculosis*, *M. avium*, and *M. kansasii* antigens

Data are presented as mean ± SEM (n = 3 biological replicates per group). * – significant difference from the control group (macrophages cultured without mycobacterial antigens): * – *p* < 0.05; ** – *p* < 0.01; *** – *p* < 0.001; # – significant differences between groups: # – *p* < 0.05; ## – *p* < 0.01; ### – *p* < 0.001.

• ambivalent (*M. avium*), expressing *IL6* and *IL12*.
The findings from the study of cytokine production by human macrophages in response to antigens of various

mycobacterial species were consistent with the expression profile data. It was demonstrated that macrophages cultured with *M. tuberculosis* antigens were characterized by production of *IL-6*, *IL-1β*, *TNF*, *IFN-γ*, and both forms of *IL-12* (*IL-12p70* and *IL-12p40*) (Fig. 3 and 4).

Macrophages cultured with *M. avium* antigens produced *IL-6*, *IL-1β*, *TNF*, *IFN-γ*, and *IL-12p40*, with *IFN-γ* production being significantly higher upon culture of macrophages with *M. tuberculosis* culture filtrate compared to the other groups (Fig. 3 and 4).

Cells cultured with *M. kansasii* antigens were characterized by production of *IL-6*, *IL-1β*, *IFN-γ*, and *IL-12p40* (Fig. 3 and 4). Notably, macrophages stimulated with *M. kansasii* culture filtrate secreted significantly higher levels of *IL-6*, *IL-1β*, and *IL-12p40* compared to the other experimental groups (Fig. 3, 4).

DISCUSSION

Understanding the distinct responses of macrophages to antigens of different mycobacterial species may aid in the identification of novel therapeutic targets. In our study, we compared the response of macrophages to complex antigens of *M. tuberculosis* H37Rv and two NTM species in terms of their ability to produce a panel of key cytokines *in vitro*. The results revealed an interesting pattern of differential cytokine expression and secretion by macrophages in response to stimulation with antigens of virulent and weakly virulent mycobacteria (Fig. 1–4). Stimulation with *M. tuberculosis* antigens resulted predominantly in *IFN-γ* and *TNF* production, whereas NTM antigens preferentially induced *IL-1*, *IL-6*, and *IL-12*. Importantly, this

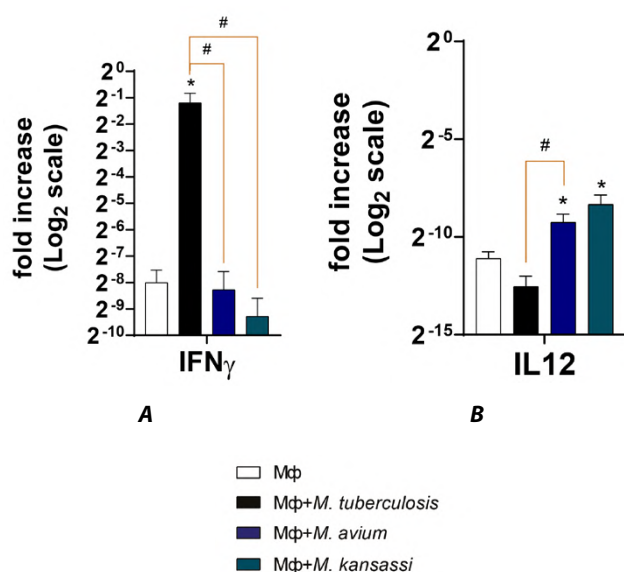


FIG. 2. Changes in expression of *IFNγ* (A) and *IL12* (B) in macrophages upon stimulation with *M. tuberculosis*, *M. avium*, and *M. kansasii* antigens. Data are presented as mean ± SEM (n = 3 biological replicates per group). * – significant difference from the control group (macrophages cultured without mycobacterial antigens); * – p < 0.05. # – significant differences between groups: # – p < 0.05.

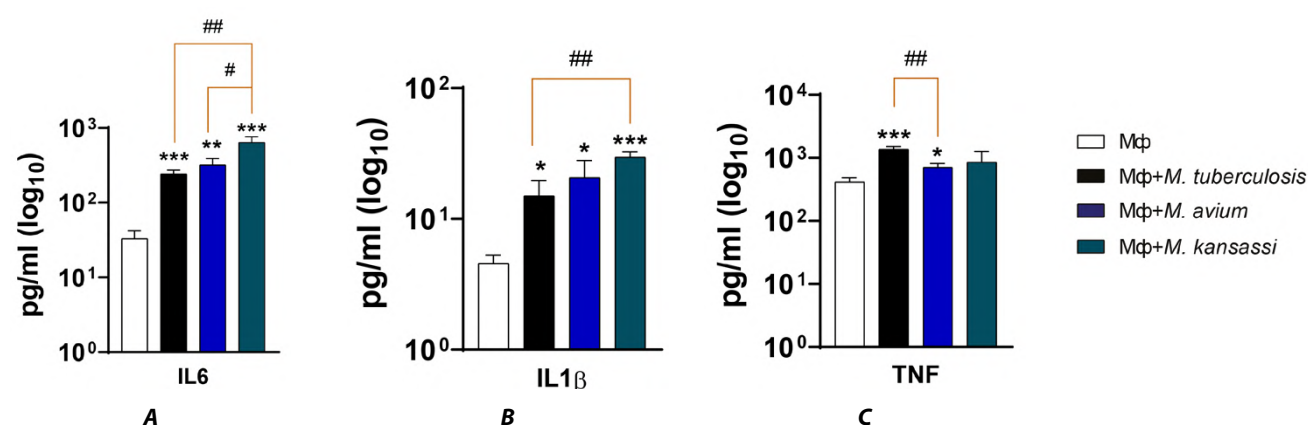


FIG. 3. Changes in production of *IL-6* (A), *IL-1β* (B), and *TNF* (C) in macrophages upon stimulation with *M. tuberculosis*, *M. avium*, and *M. kansasii* antigens. Data are presented as mean ± SEM (n = 3 biological replicates per group). * – significant difference from the control group (macrophages cultured without mycobacterial antigens); * – p < 0.05; ** – p < 0.01; *** – p < 0.001. # – significant differences between groups: # – p < 0.05; ## – p < 0.01.

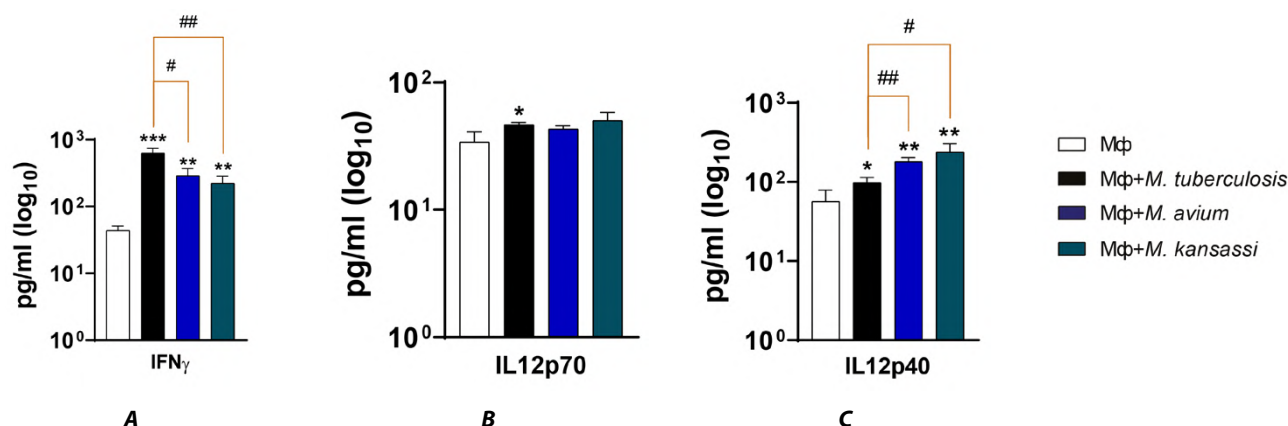


FIG. 4.

Changes in production of IFN- γ (A), IL-12p70 (B), and IL-12p40 (C) in macrophages upon stimulation with *M. tuberculosis*, *M. avium*, and *M. kansasii* antigens

Data are presented as mean $\pm$ SEM ($n = 3$ biological replicates per group). * – significant difference from the control group (macrophages cultured without mycobacterial antigens): * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$. # – significant differences between groups: # – $p < 0.05$; ## – $p < 0.01$.

pattern was consistent at both the transcriptional level and the protein level.

Other researchers have also compared various NTM species with *M. tuberculosis* in macrophage infection models [18–20]. Although, in contrast to our study, the majority of these investigations employed live mycobacterial infection models and quantitative assessment of mycobacterial burden, the considerable differences in observation duration and data analysis still allow for meaningful comparison with our findings.

Mononuclear cells, such as monocytes and macrophages, are professional phagocytes with high efficiency in defending the host against various pathogens, including tuberculous and NTM mycobacteria. Advances in the classification of mononuclear cell subsets, the study of their phenotypic and functional properties, and their modulation in various diseases have prompted research aimed at identifying monocyte-derived biomarkers that could be used for diagnostic and therapeutic purposes.

Host defense against NTM is regulated by macrophages through direct killing of mycobacteria and production of IL-12, which activates natural killer cells or T cells to secrete IFN- γ . IL-12 and IFN- γ mediate cross-talk between innate and adaptive immunity against NTM. Effective Th1 immunity, mediated by IL-12, IFN- γ , and TNF, is essential for the elimination or containment of intracellular NTM infection.

Beltan E. et al. (2000) conducted a study in which human monocyte-derived macrophages were infected with various pathogenic mycobacteria (*M. tuberculosis*,

M. avium, *M. kansasii* or *M. xenopi*) and non-pathogenic mycobacteria (*M. smegmatis*, *M. phlei*), followed by measurement of cytokine induction levels. The study found that non-pathogenic mycobacteria induced higher levels of TNF and GM-CSF, and, to a lesser extent, more IL-6, compared to pathogenic mycobacteria [18].

In a study conducted by Song C.H. et al. (2003), IL-8 levels were measured in human monocytes. It was found that *M. smegmatis* induced significantly higher IL-8 levels compared to cells infected with *M. tuberculosis* H37Rv [19].

Using the THP-1 macrophage cell line, Feng Z. et al. (2020) demonstrated an interesting pattern of differential cytokine and chemokine production in macrophages infected with various mycobacterial species: rapid growers, slow growers, and *M. tuberculosis*. Overall, *M. smegmatis* stimulated higher production of pro-inflammatory cytokines (IL-1 β , IL-6, IL-12, and TNF) as well as IL-10 compared to *M. abscessus*. However, these two rapid-growing species induced comparable amounts of chemokines (IL-8, MCP-1, and MIP-1 α). When comparing *M. tuberculosis* and slow-growing *M. avium*, the latter stimulated higher production of IL-1 β , IL-6, IL-12p70, TNF, and IL-10. At the same time, all three NTM species induced similar levels of IL-8, MCP-1, and MIP-1 α [20].

Zhao S. et al. (2024) investigated plasma cytokine levels in patients with NTM-associated lung disease as markers of pathogenesis and treatment response. Analysis of combinations of various plasma cytokines, particularly IL-1 β , IL-6, and IL-10, revealed their potential utility in the differential diagnosis of patients

with different mycobacterial burdens. IL-6 and IL-10 emerged as promising biomarkers of early treatment response. The combination of IL-6 and IL-1 β demonstrated high discriminatory capacity between patient groups with and without a positive response to anti-bacterial chemotherapy, as well as between patients with NTM infection and those with TB [13].

Using digital spatial profiling of lung tissue from patients with mycobacterial lung disease, Koh J. et al. (2024) demonstrated unique gene expression patterns in T cells and macrophages. Gene set enrichment analysis revealed that pathways related to antigen presentation and processing were predominantly activated in lung macrophages of these patients. Affected lung tissues showed increased numbers of macrophages with elevated expression of M1-associated genes (CD40 and CD80), whereas T cell activation markers remained unchanged [21].

Therefore, the results of our study, together with the findings reviewed here, indicate that macrophages may serve as therapeutic targets for focusing antibacterial immunity. Novel therapeutic strategies for TB, leprosy, and NTM infections that target specific cell types are currently being explored.

CONCLUSION

The present study identified distinct patterns of macrophage responses to antigens of different mycobacterial species. This provided a more comprehensive characterization of the mechanisms through which tuberculous and NTM mycobacteria establish persistent macrophage infection. Further investigation of host-mycobacterium interactions in macrophages and other cell types may lead to the discovery of novel therapeutic targets for combating difficult-to-treat infections.

Conflicts of interest

The authors declare no conflicts of interest.

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The authors declare that their authorship complies with the ICMJE international criteria.

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