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IMMUNOLOGICAL ASPECTS

# A novel role of Yin-Yang-1 in pulmonary tuberculosis through the regulation of the chemokine CCL4

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SUMMARY

*Mycobacterium tuberculosis (M. tb)* is the etiological agent of pulmonary tuberculosis (TB); this disease remains a worldwide health problem. Yin-Yang-1 (YY1) plays a major role in the maintenance and progression of some pulmonary diseases, including pulmonary fibrosis. However, the role of YY1 in TB remains unknown. The aim of this study was to elucidate the role of YY1 in the regulation of CCL4 and its implication in TB. We determined whether YY1 regulates CCL4 using reporter plasmids, ChIP and siRNA assays. Immunohistochemistry and digital pathology were used to measure the expression of YY1 and CCL4 in a mouse model of TB. A retrospective comparison of patients with TB and control subjects was used to measure the expression of YY1 and CCL4 using tissue microarrays. Our results showed that YY1 regulates the transcription of CCL4; moreover, YY1, CCL4 and TGF-β were overexpressed in the lung tissues of mice with TB during the late stages of the disease and the tissues of TB patients. The expression of CCL4 and TGF-β correlated with YY1 expression. In conclusion, YY1 regulates CCL4 transcription; moreover, YY1 is overexpressed in experimental and human TB and is positively correlated with CCL4 and TGF-β expression. Therefore, treatments that decrease YY1 expression may be a new therapeutic strategy against TB.



## 1. Introduction

*Mycobacterium tuberculosis (M. tb)* is a highly efficient intracellular pathogen that requires specific cell-mediated Th1 immune responses for host protection. In fact, protective immunity against *M. tb* relies not only on a complex but organized series of interactions among mycobacteria, antigen-presenting cells and lymphocytes but also on the coordinated production of chemokines and cytokines. An important aspect associated with the production of cytokines in *M. tb* infection is the activation of macrophages in response to IFN-γ and TNF-α signaling [1]. Several cytokines, such as IL-12, IL-17 and IL-23, contribute to the host's response to mycobacteria, which directly and/or indirectly induces the development of Th1 cells [2]. However, *M. tb* has developed mechanisms that interact with and modulate the host's immune response. *M. tb* expresses surface antigens that can induce the production of IL-10 and IL-4 [3]. IL-10 inhibits the production of pro-inflammatory cytokines and the activity of antigen-presenting cells, blocking the expression of the MHC class II molecule [4]. IL-4 is the prototypical cytokine of the so-called Th2 lymphocyte phenotype; it regulates cellular proliferation and apoptosis, as well as the expression of numerous genes in various cell types [5]. The role of Th2 cytokines in human tuberculosis (TB) is controversial; however, this issue is important because IL-4 and IL-13 are able to inhibit the mechanisms that control *M. tb* growth, such as cytotoxic

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**Abbreviation list**

| | |
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| BCG | Bacillus Calmete-Guérin |
| CCL4 | Chemokine CC Ligand 4 |
| ChIP | Chromatin Immunoprecipitation |
| GFP | Green Fluorescent Protein |
| ICC | Immunocytochemistry |
| IHC | Immunohistochemistry |
| *M. tb* | *Mycobacterium tuberculosis* |
| MHC | Major Histocompatibility Complex |
| PBMC | Peripheral Blood Mononuclear Cell |
| siRNA | Small Interfering RNA |
| TB | Pulmonary Tuberculosis |
| TESS | Transcription Element Search System |
| TMA | Tissue Microarray |
| WB | Western Blot |
| WHO | World Health Organization |
| YY1 | Yin-Yang-1 |

lymphocytes [CTL], apoptosis, autophagy, and macrophage activation, while also promoting pulmonary fibrosis [6–9]. In the mouse model, the role of IL-4 depends on the mouse strain and the dose of bacilli used to infect the animals. IL-4 is not expressed at increased levels in C57Bl mice infected with a low dose of *M. tb* via aerosol, and the number of mice that are resistant to the infection do not increase following the knockout (KO) of the genes encoding IL-4 and IL-13 [10]. In contrast, if BALB/c mice are infected with a high dose, whether by instillation into the airways or by i.v. injection, the IL-4 expression increased to high levels during the late stages of the disease [11,12]. Moreover, the infected IL-4 KO BALB/c mice [11] or the neutralization of IL-4 with antibodies attenuated the disease after a high-dose challenge, demonstrating that IL-4 plays a significant detrimental role [13]. Nevertheless, these results were obtained in BALB/c mice, which are genetically inclined towards this type of response. Interestingly, high levels of IL-4 in tuberculosis patients are most often reported in developing countries [14], including countries in Latin America [15].

The suppression of T cell responses to mycobacterial antigens in peripheral blood mononuclear cells (PBMCs) is a consistent feature of TB [16], and *in vitro* observations have indicated that TGF-β participates in these effects [17]. TGF-β suppresses the major histocompatibility complex (MHC) at multiple levels [18], blocking lymphocyte proliferation and function (CD-4-positive cells are particularly susceptible), suppressing IL-2 production, IFN-γ receptor and MHC-II expression [19,20], and IFN-γ-induced macrophage activation [21], thus inhibiting IL-1, TNF-α and iNOS production [22]. In the late 1990s, the work of Toossi and Ellner suggested that TGF-β is an important factor in the pathophysiology of TB. Their work with peripheral blood mononuclear cells from TB patients demonstrated that their deficient T cell responses, IFN-γ production and antigen-driven blastogenesis could be restored by TGF-β neutralizing agents [23]. These observations prompted the authors to propose the possibility of using TGF-β inhibitors as adjuvants to anti-tuberculous chemotherapy [24]. Simultaneously, our work with BALB/c mice infected with high doses of *M. tb* supported this notion by showing a clear correlation between the TGF-β levels and TB progression [25], and blocking TGF-β activity with soluble receptor III or betaglycan significantly decreased the bacilli burdens, confirming the contribution of TGF-β to TB progression [26]. Interestingly, IL-4 and TGF-β are regulated by the transcription factor YY1 *in vitro* and *in vivo*, particularly in some pulmonary diseases, including asthma and fibrosis [27–29].

The chemokine CCL4 (first named Macrophage Inflammatory Protein-1 beta or MIP-1β) is a member of the CC chemokine family. It is an 8 kDa acidic protein that is upregulated in T cells, monocytes [30], and lymphocytes and is involved in the major migration of Th2 cells [31]. Furthermore, CCL4 overexpression has been related to inflammatory diseases [31,32]. CCL4 has been recently proposed as a possible biomarker for the detection of pulmonary TB in serum and saliva samples [33]; however, the mechanism by which CCL4 expression is transcriptionally regulated and whether this regulation plays an important role in the immunopathogenesis of pulmonary TB remain unclear. Thus, we hypothesized that YY1 regulates the expression of CCL4 because this transcription factor and its target genes, including CCL4 and TGF-β, are produced at high levels in the lungs during the progression of pulmonary TB in a mouse model and in patients with pulmonary TB.

## 2. Materials and methods

### 2.1. Bacterial strains

*M.tb* H37Rv was obtained from the ATCC (Rockville, MD, USA). As soon as the culture reached mid-log phase, the bacilli were harvested, suspended in PBS, adjusted to $2.5 \times 10^5$ bacteria in 100 μl PBS, aliquoted and maintained at −70 °C until further use [34].

### 2.2. Experimental mouse model of pulmonary TB

Briefly, male BALB/c mice from 6 to 8 weeks of age were used. To induce progressive pulmonary tuberculosis, the mice were anesthetized and intratracheally inoculated with $2.5 \times 10^5$ CFUs of *M. tuberculosis* H37Rv. After infection, the mice were kept in a vertical position until the effects of the anesthesia had passed. The animals were maintained in groups of five in cages fitted with microisolators connected to negative pressure. The animals from each group were sacrificed by exsanguination at 1, 3, 7, 14, 21, 28 and 60 days after infection. One lung lobe, right or left, was perfused with 10% formaldehyde dissolved in PBS, and prepared for histopathological studies. The histopathological analysis showed that inflammatory infiltrate that formed in the alveolar-capillary interstitium and around small blood vessels and bronchial walls 1–2 weeks after the infection was predominantly composed of lymphocytes and activated macrophages, with well-formed granulomas at 14 days after the infection (early phase). At 28 days postinfection, H37Rv induced significant pneumonia, involving approximately 30% of the lung surface. Pneumonia, necrosis and fibrosis are well established at day 60, inducing the death of the infected mice (late phase). All procedures were performed in a biosafety level III animal facility, and all experimental work was performed in accordance with the Experimental Animals Committee of the National Institute of Medical Sciences and Nutrition (register number CINVA 190) and with the Mexican regulations regarding animal care and experimentation [34].

### 2.3. Human autopsy material and tissue microarray (TMA) construction

Post-mortem lung tissue was obtained from the Pathology Department of the General Hospital of Mexico “Eduardo Liceaga”, México City, Mexico. The Institutional Review Board approved this protocol. Paraffin-embedded lung tissue from 44 individuals with pulmonary TB and from 25 control subjects was analyzed using a tissue microarray, as previously described [35]. TB was confirmed through clinical data (sputum culture, PPD, and chest X-ray), and all of the cases were positive for Ziehl-Neelsen staining. The control