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# Interleukin 23/interleukin 17 axis activated by *Mycobacterium avium* complex (MAC) is attenuated in patients with MAC-lung disease

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ABSTRACT

*Background:* *Mycobacterium avium* complex (MAC)-lung disease (LD) is increasing in patients without human immunodeficiency virus infection. However, data on host vulnerability to MAC-related immune responses, and in particular the interleukin (IL)-23/IL-17 axis, are lacking.

*Methods:* We enrolled 50 patients with MAC-LD, 25 age-matched patients with tuberculosis (TB) and 25 controls. We measured levels of plasma cytokines, and studied IL-12/IL-17 responses in macrophage and lymphocyte activation to MAC.

*Results:* The plasma level of IL-17 in the MAC group was higher than in the TB and control groups. In in-vitro macrophage stimulation, the expression of IL-23 in macrophages was similar in the patients with MAC-LD and controls, although the expression of IL-12 p40 was lower in the patients with MAC-LD. In assays of lymphocyte activation, IL-17 was induced by MAC-primed macrophages, but its level was lower in the patients with MAC-LD and TB than in the controls. The expression of programmed death (PD)-1 receptor was higher in $CD4^+IL17A^+$ lymphocytes in the patients with MAC-LD, and the production of IL-17 was significantly increased by blockade of PD-1 and PD-ligand 1.

*Conclusions:* MAC induced a similar expression of IL-23 from macrophages in the patients with MAC-LD compared to the controls, but a lower expression of IL-17 from lymphocytes, which may be through an increased expression of PD-1. The macrophage response of IL-12 p40 was stronger than that of IL-12 p70, and higher in the controls during MAC disease, which may suggest another kind of MAC-related immune evasion.

## 1. Introduction

Nontuberculous mycobacteria-lung disease (NTM-LD) is an increasing medical concern [1,2] because of the increasing prevalence of NTM infections in patients without human immunodeficiency virus infection [3–5]. *Mycobacterium avium* complex (MAC) is one of the most common species responsible for NTM-LD [3,6,7]. MAC exists ubiquitously in the environment, and it can be isolated in sputum by possibly contamination [1]. Patients with true NTM-LD are only identified in 35–42% of cases with sputum positive for NTM [8,9]. The low prevalence of MAC pulmonary infections (16.8/100,000 in 2012) [10] indicates that individual vulnerability may be a key factor [11].

In the pathogenesis of mycobacterial infections, macrophages are the first line of defense when bacilli enter through the airway, and innate immunity plays a pivotal role in protecting against MAC infection [12]. Human studies have demonstrated that type 1 cytokines from macrophages, including interleukin (IL)-12, play an important role in the development of cell-mediated immune responses to intracellular infections [13,14]. In addition, IL-23 from antigen presenting cells is a major cytokine inducing T helper 17 (Th17) pathway in addition to IL-6 and IL-1beta [15–17]. Th17 cells are an important kind of lymphocyte that play a role in establishing protective immunity against mycobacterial infections in addition to Th1 [18]. The response of Th17 cells including IL-17 can recruit and activate neutrophils [19], acting early

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bacteria clearance. The function of IL-17 can thus help controlling pulmonary tuberculosis [20], MAC-LD [21] and chronic lung infection induced by bacteria [22]. Furthermore, a decreased level of IL-23 or IL-17 has been shown to attenuate granulopoiesis [23]. Recently, deficiency of IL-17 production has been reported in *M. abscessus* pulmonary infection [24]. The axis of IL-23 and IL-17 has yet to be elucidated in patients with MAC-LD, however this is an important issue due to the underlying immune dysfunction. Therefore, we conducted this study to investigate the IL-23 and IL-17 axis in patients with MAC-LD.

## 2. Materials and methods

### 2.1. THP-1 cells and M. avium subspecies

We obtained THP-1 cells, a human acute monocytic leukemia cell line, from American Type Culture Collection (ATCC), and cultured the cells in RPMI 1640 medium with 25 mM HEPES and L-glutamine (LONZA, USA) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (PAA Laboratories, Austria), penicillin and streptomycin (Gibco, USA) at 37 °C with 5% CO2 in a humidified incubator. *Mycobacterium avium* subspecies *avium* (ATCC 25291) dead bacilli was used as the bacilli antigen and prepared in phosphate-buffered saline (PBS, Ambion, USA). We used a dry bath incubator to heat-kill the bacilli at 80 °C for 30 min.

### 2.2. Stimulation assay of THP-1 derived macrophages

THP-1 cells were differentiated into adhered macrophages by culture for 24 h in medium supplemented with 20 ng/ml of phorbol 12-myristate 13-acetate (TOCRIS, UK) [25] and allowed to rest for 2 days prior to stimulation. THP-1-derived macrophages were challenged with MAC at the indicated multiplicity of infection (MOI) for 6 and 24 h, after which the cells were harvested for mRNA isolation and supernatants, respectively. Lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4; Sigma-Aldrich, USA) 5 μg/ml was used as a positive control for stimulation.

### 2.3. Participant enrollment

This prospective study was conducted at National Taiwan University Hospital in Taiwan from January 2015 to June 2016. The hospital's Research Ethics Committee approved the study (No. 201505069RINC), and all participants provided written informed consent. We used residual blood cells from previous studies on NTM immunity (IRB201312043RINB and IRB201407079RIND) which were conducted from October 2014 to March 2016, after approval from the hospital's Research Ethics Committee. Patients aged ≥20 years who had respiratory samples that were culture-positive for MAC were identified. Of these patients, we consecutively enrolled those with MAC-LD according to the ATS diagnostic guidelines [1]. Patients with human immunodeficiency virus (HIV) infection, and those co-infected with non-MAC NTM-LD were excluded. Patients with pulmonary TB were enrolled for comparison. Participants were enrolled as controls if they had no major systemic diseases (cancer, autoimmune disease, liver cirrhosis, or renal failure) and normal chest radiographs. For those with mildly increased infiltrates on chest radiographs, we enrolled them as controls after sputum mycobacterial cultures had been confirmed to be negative.

### 2.4. Isolation of peripheral blood mononuclear cells and macrophage differentiation

Peripheral blood samples (10 ml) were collected from the enrolled patients before mycobacterial treatment for MAC-LD or TB and stored in heparin-containing tubes. Peripheral blood mononuclear cells (PBMCs) were immediately isolated using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Sweden), and a MACS system (Miltneyi Biotec Inc., CA) was used to select CD14-positive monocytes from the PBMCs. Human $CD14^+$ monocytes ($5 \times 10^5$ cells/ml) were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 ng/ml human macrophage colony-stimulating factor (M-CSF, R&D Systems, MN) for 6 days to allow for macrophage differentiation [26]. At day 7, macrophages were detached for 90 min using cell detachment solution (Accutase, Millipore, USA).

### 2.5. Macrophage stimulation assay and lymphocyte activation

We co-cultured the macrophages with heat-killed MAC bacilli for 6 h for RNA isolation or for 1 day to collect the reaction supernatant. The reaction supernatants were collected, and the macrophages were co-cultured with CD14-negative cells from the same subjects (1:10) for 5 days. We then collected the supernatants for further cytokine assays, and added CD3 and CD28 antibodies (eBioscience, San Diego, CA) to re-stimulate the cells for 24 h. We added protein transport inhibitor (BD Bioscience, USA) for the second half of the co-culture. We measured $CD4^+IL17^+$ lymphocytes and the expression of programmed cell death (PD)-1 using anti-CD4-APC, anti-IL-17 A-PE and anti-PD-1-PerCP/Cy5.5 (BD Pharmingen, San Diego, CA) by flow cytometry. For the samples from the patients with MAC-LD, we blocked the lymphocyte activation assay using antagonistic PD-1 (10 μg/ml, eBioscience, San Diego, CA) antibodies for CD14-negative cells and PD-L1 (10 μg/mL, eBioscience, San Diego, CA) antibodies for macrophages for 1 h before co-culture.

### 2.6. Cytokine measurement

The patients' plasma and reaction supernatants were tested for tumor necrosis factor-alpha (TNF-α) and IL-12 p40, IL-12 p70, and IL-23 for pro-inflammatory responses from antigen-presenting cells, and interferon-gamma (IFN-γ) and IL-17 A (Cat: DY317) for lymphocyte function using an enzyme-linked immunosorbent assay (ELISA) system (R&D Systems, MN).

### 2.7. RNA isolation and real-time polymerase chain reaction

Total cellular RNA from THP-1-derived macrophages and human macrophages was extracted using a Direct-zol™ RNA MiniPrep kit (Zymo Research, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using an iScript™ cDNA Synthesis kit (Life Science Research, USA). A real-time polymerase chain reaction (PCR) with iQ SYBR Green Supermix (BIO-RAD, Singapore) was performed using a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System and analyzed using a Bio-Rad iQ5 Optical System 2.0 (Bio-Rad, CA). PCR mixtures were denatured at 95 °C for 3 min, followed by 40 cycles of 10 s at 95 °C, and 30 s at 58 °C. The mRNA expression level of each target gene was normalized to the respective GAPDH expression. The sequences of sense and antisense primers are shown in Table 1.

### 2.8. Data collection and statistical analysis

Clinical data including age, sex, body mass index (BMI), smoking status, co-morbidities, past pulmonary TB, symptoms, and radiographic findings upon enrollment were recorded. A radiographic score was used to measure the extent of lung lesions as reported in previous studies [27,28]. In brief, we divided each lung field into three zones according to two horizontal lines located at the distal end of the lobar pulmonary artery. We rated each zone from 0 to 3 points and summed the total score for radiographic extent. The radiographic patterns of the main pulmonary lesions were categorized as fibro-cavitary, nodular-bronchiectasis, and others. We performed follow-up chest radiographs after 1 year. If the radiographic score had increased, we categorized it as radiographic progression.

Data were presented as mean (standard deviation/standard error)