Cytokine 120 (2019) 176–185

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Mono- and poly-functional T cells in nontuberculous mycobacteria lung disease patients: Implications in analyzing risk of disease progression

Chin-Chung Shu[a], Lawrence Shih-Hsin Wu[b], Ming-Fang Wu[c,d], Hsin-Chih Lai[e,f,g], Ping-Huai Wang[h,i,*], Shih-Lung Cheng[h,j], Jann-Yuan Wang[a], Chong-Jen Yu[a]

[a] Department of Internal Medicine, National Taiwan University Hospital, Taiwan
[b] Graduate Institute of Biomedical Sciences, China Medical University, Taiwan
[c] Institute of Statistical Science, Academia Sinica, Taipei, Taiwan
[d] Graduate Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan
[e] Department of Medical Biotechnology and Laboratory Science, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan
[f] Research Center for Chinese Herbal Medicine and Research Center for Food and Cosmetic Safety, College of Human Ecology, Chang Gung University of Science and Technology, Tao-Yuan, Taiwan
[g] Department of Laboratory Medicine, Xiamen Chang Gung Memorial Hospital, Xiamen, Fujian, China
[h] Division of Chest Medicine, Department of Internal Medicine, Far Eastern Memorial Hospital, Taiwan
[i] Department of Nursing, Oriental Institute of Technology, Taiwan
[j] Department of Chemical Engineering and Materials Science, Yuan-Ze University, Taiwan

ARTICLE INFO

Keywords:
Lung disease
Mycobacterium avium complex
Mycobacterium abscessus
Nontuberculous mycobacteria
Poly-functional T cells

ABSTRACT

Summary at a Glance: The diagnosis and progression of nontuberculous mycobacteria lung disease (NTN-LD) are important for clinical judgement but cannot easily be predicted. The immunological response of mono- and poly-functional T cells, a representative of host reactivity to NTM, could be a surrogate biomarker for disease and progression prediction.

Background: Mycobacterium avium complex (MAC) and *M. abscessus* (MAB) induced lung disease (LD) have become a clinical concern. Predicting clinical disease relevance and progression is important, but suitable biomarkers are lacking. The host immune response of mono- and poly-functional T cells might aid in clinical judgement.

Methods: We enrolled 140 participants, including 42 MAC-LD, 25 MAB-LD, 31 MAC airway colonization (MAC-Co), 15 MAB-Co patients, and 27 healthy controls. Their blood mono- and poly-functional T cells were measured and analyzed after in-vitro stimulation.

Results: Patients with MAC-LD generally had lower total IFN-γ+, total TNF-α+ and triple-positive T cells but higher mono-IL-2+ expression than the controls and MAC-Co group. The MAB-LD group had lower total IL-2 and triple positive cells than the controls and colonization group. Multivariate analysis revealed that body mass index (BMI), mono-IL2+ CD4+ and triple positive-CD8+ cells (PMA stimulation) significantly predicted MAC-LD from the controls. By contrast, male gender and triple positive-CD4+ cells predicted MAC-LD from colonization. On the other hand, the triple positive-CD4+ cells (PMA stimulation) alone or together with the mock/MAB ratio of IL-2+/TNF-α+ CD4 cells could predict MAB-LD in the MAB-Co group or the controls. Among MAC/MAB-LD patients without anti-mycobacterial treatment, MAC-specific mono-IFN-γ+ CD4+ cells and PMA-induced triple positive-CD4+ cells were correlated with progression, with an area under the ROC curve of 0.875.

Conclusions: The patients with MAC/MAB-LD had attenuated poly-functional T cells. The triple-positive CD4+ cells could be useful in diagnosing disease from colonization. MAC-specific mono-IFN-γ+ CD4+ cells and triple positive-CD4+ might predict radiographic progression, which could be useful in making treatment decisions.

* Corresponding author at: Division of Chest Medicine, Department of Internal Medicine, Far Eastern Memorial Hospital, No. 21, Sec. 2, Nanya S. Rd., Banciao Dist., New Taipei City 220, Taiwan.
E-mail address: phwang@mail.femh.org.tw (P.-H. Wang).

https://doi.org/10.1016/j.cyto.2019.05.001
Received 5 January 2019; Received in revised form 29 April 2019; Accepted 1 May 2019
Available online 12 May 2019

1. Introduction

Nontuberculous mycobacteria induced lung infection (NTM-LD) has become a clinical concern [1,2] because the prevalence of NTM-LD has increased over the last two decades [3–5]. Among the NTM pulmonary infections, *Mycobacterium avium* complex (MAC) and *M. abscessus* (MAB) are the most frequently isolated pathogens responsible for lung disease in Southeast Asia and North America [3,6,7]. According to diagnosis guidelines established by the American Thoracic Society (ATS), NTM-LD is diagnosed according to clinical findings and radiographic findings as well as the microbiology of respiratory specimens [1]. Among the microbiology criteria, two or more sputum positive results for the same NTM within one year are required, but the culture is time-consuming, taking weeks [8]. In fact, NTM is ubiquitous in the environment, and the presences of MAC and MAB only have clinical relevance of around 35–42% [9,10] and 33%, respectively [10]. However, early diagnosis of NTM infection is important because NTM-LD can be a lethal infection in intensive care units or in patients without early proper treatment [11].

In addition to ATS guideline, immunological response might be helpful for diagnosis of NTM-LD. In previous studies, the detection of glycopeptidolipid antibody was MAC-specific for diagnosis of MAC-LD [12,13]. This finding indicates that a specific immune response, such as mycobacteria-induced T-cell response, may be a good candidate. Mycobacteria-induced Th1 type CD4+ cells and CD8+ cytotoxic T lymphocytes are essential for protective immunity in tuberculosis [14], and they are differentially impacted by disease stage and mycobacterial load [15,16], suggesting that certain subsets may serve as biomarkers of disease activity and pathogen burden [16]. However, the evidence of NTM-specific mono- and poly-functional T cells has rarely been studied in NTM-LD. Therefore, we conducted the present study to examine the roles of mono- and poly-functional T cells in the diagnosis and prognosis of NTM-LD.

2. Materials and methods

2.1. Patient enrollment

This prospective study was conducted at National Taiwan University Hospital and Far Eastern Memorial Hospital from January 2017 to August 2018 under the approval of the hospitals' Research Ethics Committees (IRB No.: 201703051RINC and 106134-F). Patients aged ≥20 years were recruited consecutively when they visited our chest or infection clinics if they were diagnosed as having MAC-LD or MAB-LD according to the diagnostic guidelines suggested by the American Thoracic Society (ATS) [1] (see supplement file). We classified patients as MAC or MAB airway colonization if they were sputum positive for MAC or MAB but did not fulfill the ATS diagnosis criteria. In addition, we recruited a healthy control group of individuals having negative chest radiographic images. All enrolled participants provided written informed consent. Patients with human immunodeficiency virus infection, chemotherapy treatment, active cancer or concomitant bacterial pulmonary infection were excluded.

2.2. Flow cytometry for peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells were isolated using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Sweden) and were then suspended in medium containing RPMI-1640 (Life Technologies; USA), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Life Technologies, USA) in 48-well plates. Medium only (mock stimulation) or heat-killed *Mycobacterium avium* subspecies *avium* (MAC stimulation) (ATCC 25291) at multiplicity of infection (MOI) of 100 or heat-killed *Mycobacterium abscessus* (MAB stimulation) (ATCC 19977) at MOI of 100 were added for co-culturing for 48 h as bacilli stimulation. Because the response to the heat killed antigen alone was low (Table S2 in supplement file), we used CD3 (5 μg/ml, plate-coated, clone: OKT3) and CD28 (1 μg/ml, soluble, clone: CD28.2) antibodies (eBioscience, San Diego, CA) in the mock, MAC and MAB stimulations to amplify the reaction in the last 16 h of the co-culture. One more condition, stimulation of Phorbol 12-myristate 13-acetate (50 ng/ml, TOCRIS, USA) plus ionomycin calcium salt (1 μM/ml, Sigma-Aldrich, USA) (PMA stimulation) for 16 h, was also checked. Protein transport inhibitor (BD Bioscience, USA) was added to the co-culture 12 h before we retrieved the stimulation.

We measured the levels of mono- and poly-functional T cells by using flow cytometry (FACSVerse, BD Biosciences, USA). We gated the lymphocyte population by using forward scatter (FSC) and side scatter (SSC). CD4+ and CD8+ T cells were stained using anti-CD4-APC (clone: RPT-T4, Biolegend, USA) and anti-CD8-PE-cy7.0 (clone: RPA-T8, BD Biosciences, CA, USA). Different types of mono- and poly-functional T cells were further stained with anti-interferon-gamma (IFN-γ)-PerCP (clone: 4S.B3), anti-interleukin-2 (IL-2)-PE (clone: MQ1-17H12) and anti-tumor necrosis factor-alpha (TNF-α)-FITC antibodies (clone: MAb11) (Biolegend, USA). We also stained the post-stimulation T cells with anti-CD4-APC, anti-CD8-PE-cy7.0, anti-programmed death-1 (PD-1)-PE (clone: EH12.1) (BD Biosciences, CA, USA), anti-cytotoxic T Lymphocyte Antigen 4 (CTLA-4)-PE/cy7 (clone: L3DD10), and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3)-PerCP/cy5.5 (clone: F38-2E2) (Biolegend, USA). The details of the gating protocol are provided in the supplementary information (Fig. S1). Data were analyzed using BD FACSuite V software (BD, Biosciences, USA).

2.3. Data collection and statistical analysis

Clinical data including age, sex, co-morbidities, radiographic findings and laboratory data at enrollment were recorded in a standardized case report form with default options. The radiographic patterns of the main pulmonary lesions were categorized as fibro-cavitary (FC), nodular-bronchiectasis (NB), and others. The extent of lung lesions was scored as in previous studies [17,18]. 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. If a lesion involved equal to or less than one-third of the area, we gave it one point. If a lesion involved more than one-third but less than two-thirds of the area, we gave it two points. The chest X-ray (CXR) score was the sum of scores in all lung fields. Inter-group differences were analyzed using the Mann-Whitney *U* test for numerical variables and the chi-square test for categorical variables. We performed multivariate logistic regressions to analyze the independent factors for NTM-LD and for radiographic progression. Then we generated the prediction probability with the formula of logits (probability) using all the factors in the corresponding multivariate analysis model. We then applied the probabilities from the multivariate model to receiver operating characteristic (ROC) curves. Statistical significance was set at $p < 0.05$. All analyses were performed in SPSS version 19.0 (Chicago, IL).

3. Results

3.1. Clinical characteristics of enrolled participants

During the study period, we enrolled 140 participants, including 42 MAC-LD, 25 MAB-LD, 31 MAC airway colonization (MAC-Co), and 15 MAB airway colonization (MAB-Co) patients, and 27 healthy controls. The average age, gender ratio and smoking status were similar between the disease group and the controls, and between the disease and colonization groups (Table 1). Patients with MAC-LD had lower BMI than those with colonization and controls, whereas the BMI of patients with MAB-LD was lower than that of controls. We did not include patients with active cancer or HIV or patients receiving chemotherapy. Diabetes mellitus was higher in the controls than in the MAC-LD group.

Table 1
Clinical characteristics according to the status of nontuberculous mycobacteria lung disease.

	MAC-LD n = 42	MAC-Co n = 31	MAB-LD n = 25	MAB-Co n = 15	Healthy subjects n = 27
Age (years)	61.8 [14.8]	63.7 [10.6]	60.6 [17.6]	66.9 [9.6]	56.0 [20.7]
Male sex	11 (26%)	14 (45%)	10 (40%)	8 (53%)	12 (48%)
Current smoker	3 (7%)	1 (7%)	2 (8%)	2 (13%)	4 (15%)
Body mass index, kg/m^2	21.4 [3.7] *,#	23.5 [4.1]	21.0 [3.9] *	23.4 [4.1]	24.4 [3.7]
Diabetes mellitus	1 (2%) *	1 (3%)	2 (8%)	0	5 (20%)
Autoimmune diseases	1 (2%)	2 (7%)	3 (12%)	4 (27%)	0
Prior TB history	3 (7%)	1 (3%)	1 (4%)	0	0
Hemoptysis	12 (29%) *	7 (23%)	7 (28%) *,#	0	0
Sputum study within 1 year					
Max. positive AFS	1.4 [1.6] #	0	1.7 [1.6] #	0.1 [0.5]	–
Proportion of AFS (+)	22 (52%) #	0	15 (60%) #	1 (7%)	
No. of positive cultures	3.5 [3.0]	–	3.0 [1.9]	–	–
Radiological finding					
CXR score #	5.1 [3.2]	–	6.5 [2.9]	–	–
FC pattern	9 (21%)	–	6 (24%)	–	–
NB pattern	27 (63%)	–	15 (60%)	–	–

Abbreviations: AFS, acid-fast smear; Co, colonization; CXR, chest X-ray; FC, fibro-cavitary; LD, lung disease; MAC, *Mycobacterium avium* complex; MAB, *Mycobacterium abscessus*; NB, nodular bronchiectasis; TB, tuberculosis.
Data are no. (%) or mean [standard deviation].

* and # indicate $p < 0.05$ between the LD group and the controls or LD group and Co group, respectively, using the chi square test for categorical variables and the Mann-Whitney *U* test for numerical variables.

CXR score was interpreted by a total score from six lung zones that contained three respective scores [17].

Hemoptysis was significantly higher in the MAC-LD and MAB-LD groups than in the controls. The maximal positive grades of acid fast smear of sputum were 1.4 and 1.7 in the MAC-LD and MAB-LD groups, respectively, and higher than in the colonization groups. The radiological patterns by chest image in the MAC-LD and MAB-LD groups were 21% and 24% of fibro-cavitary, respectively, and 63% and 60% of nodular-bronchiectasis.

3.2. Pattern of poly-functional T cells in enrolled participants

In the total expression of TNFα, IFN-γ or IL-2 on T lymphocytes (Fig. 1), patients with MAC-LD had lower CD4+ TNF-α+ expression after MAC stimulation, CD4+ IFN-γ+ and CD4+ TNFα+ after MAB stimulation and CD4+ IFN-γ+ after PMA stimulation, whereas CD8+ TNFα+ expression was lower in the patients after mock, MAC and MAB stimulation as compared with the controls. The MAC-Co group had higher CD4+ IFN-γ+ after all stimulations, CD4+ TNF-α+ after MAB stimulation, and CD8+ TNF-α+ after all than did the MAC-LD group. For MAB-LD, the patients had lower CD4+ IL-2+ after MAB stimulation and CD8+ IL-2+ after mock, MAC and MAB stimulations as compared with the controls. The comparison between MAC-LD and MAB-LD groups showed that MAC-LD had lower CD4+ IFN-γ+ after MAC or MAB stimulation, lower CD8+ TNF-α+ after all, and higher CD8+ IL-2+ after mock and MAC stimulation than those in the MAB-LD group.

In the isolated mono-cytokine expression (Fig. 2), TNF-α on CD8 lymphocytes was lower in MAC-LD patients than in controls after mock and MAB stimulation. Levels of mono-IL-2+ CD4+ and IL-2+ CD8+ cells during PMA stimulation were higher in MAC-LD patients than in controls. CD4+ IFN-γ+ upon MAB stimulation was lower but CD8+ IL2+ by PMA stimulation was higher in MAC-LD patients than in the MAC-Co group. CD4+ IFN-γ+ was higher in MAB-LD patients than in the controls and in MAC-LD patients after mock and MAC stimulation.

Double and triple cytokine expressions were all weaker in the MAC-LD group than in controls for MAC stimulation. The MAC-Co group had higher CD4+ TNF-α+ IFN-γ+ cells after mock, MAB, and MAC stimulation and higher triple positive cells after MAB and PMA stimulation. In the MAB-LD group, almost all IL-2 expressed double or triple cytokine cells were lower than those in the controls. The MAB-Co group had higher triple positive cells in CD4 after PMA stimulation and in CD8 after mock, MAB and PMA stimulation.

We checked the data of PD-1/CTLA-4/Tim-3 on CD4+ or CD8+ T cells after different kinds of stimulation. However, the results for different kinds of stimulation (Table S1 in the supplemental file) were similar between MAC-LD, MAB-LD and controls.

We compared the mono-, double and triple cytokine producing T cells between mock stimulation (CD3/CD28) and MAC or MAB, respectively. The mono- and poly-functional T cells became attenuated significantly under MAC or MAB stimulation in comparison with mock stimulation with CD3/CD28 (Table S3 in the supplement file), except that mono-IFN-γ+ on CD4+ cells increased significantly and was not influential on mono-IL-2+ (CD4+ or CD8+), total IL-2+ (CD8+ by MAB), mono-IFN-γ+ on CD8 T cells, and IL-2+/IFN-γ+, IL-2+/TNF-α+ & Triple positive CD4+ cells (by MAC stimulation). When we used the ratio of response to mock stimulation by dividing the mock stimulation by antigen stimulation, MAC-LD patients had significantly higher ratios of total IL-2+ (CD4+, mock/MAB stimulation), mono-IFN-γ+ (CD4+, mock/MAC stimulation), IL-2+/IFN-γ+ (CD8+, mock/MAC stimulation), and IL-2+/IFN-γ+ and IL-2+/TNF-α+ (CD4+, mock/MAB stimulation) but lower IFN-γ+/TNF-α+ (CD8+, mock/MAC stimulation) as compared to controls. In addition, the ratios for MAB-LD patients were significantly higher for total IL-2+ (CD4+, mock/MAB stimulation), mono-IL-2+ (CD8+, mock/MAC stimulation) and IL-2+/TNF-α+ (CD4, mock/MAB stimulation) as compared to controls (Figs. 1 and 2).

3.3. Prediction for NTM-LD from the controls or the colonization group

To discriminate MAC-LD and controls, we used logistic regression with age, sex, BMI, and the data of poly-functional T cells. In multivariate analysis, BMI (OR 0.836 [0.714–0.977] per 1 kg/m^2 increment, p = 0.024), mono-IL2+ CD4+ cells (OR 1.095 [1.003–1.196] per 1% increment, p = 0.044) and triple positive cytokine CD8 cells for PMA stimulation (OR 0.623 [0.435–0.893] per 1% increment, p = 0.010) were significant factors correlated with MAC-LD. The probability using the three factors could predict or exclude MAC-LD well with AUC of 0.862 ($p < 0.001$) (Fig. 3A). For discriminating MAB-LD from the controls, IL-2+/TNF-α+ CD4 cell ratio (mock/MAB) (OR 1.455 [1.025–2.067], p = 0.036) and triple positive cytokine CD4 cells by PMA stimulation (OR 0.687 [0.502–0.939], p = 0.019) were

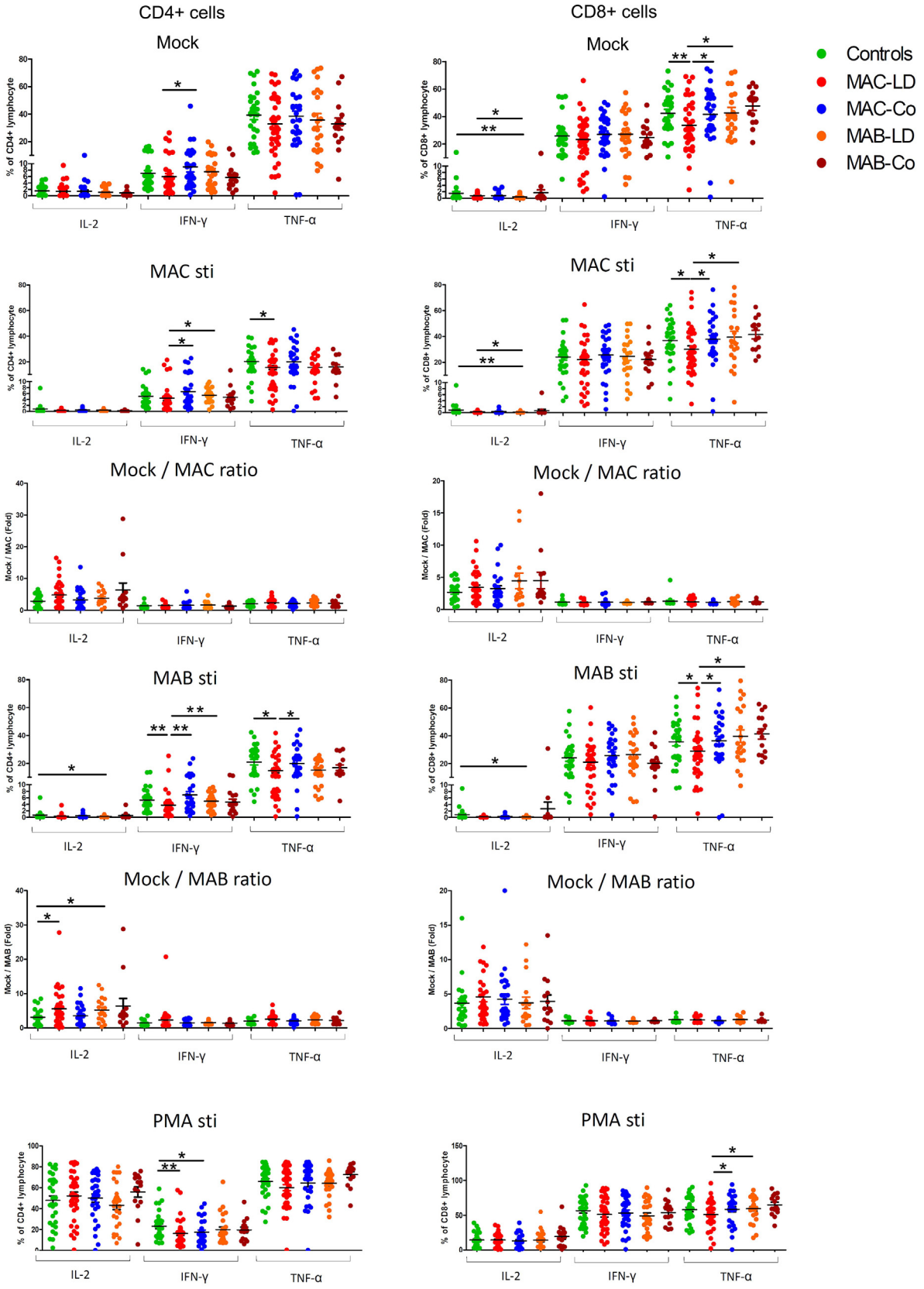

Fig. 1. Poly-functional T cells of total cytokine expression are shown for different stimulations according to disease status. Human peripheral blood mononuclear cells were co-cultured with mock stimulation of medium, *Mycobacterium avium* complex (MAC) (multiplicity of infection [MOI]: 100), or *Mycobacterium abscessus* (MAB) (MOI: 100) for 48 h and CD3 (5 μg/ml)/CD28 (1 μg/ml) antibodies were added for the co-culture in the last 16 h. Phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and ionomycin calcium salt (1 μM/ml) were used for 16 hr of stimulation. The cytokine expressions were measured for total interleukin-2 [IL-2], tumor necrosis factor-alpha [TNF-α], and interferon-gamma [IFN-γ] expression on CD4+ and CD8+ lymphocytes, respectively, by flow cytometry. The ratios are the same cytokine expressed T cells in mock stimulation divided by MAC or MAB stimulation. LD, lung disease; Co, colonization. $^{*}0.01 \leq p < 0.05$; $^{**}0.001 < p < 0.01$.

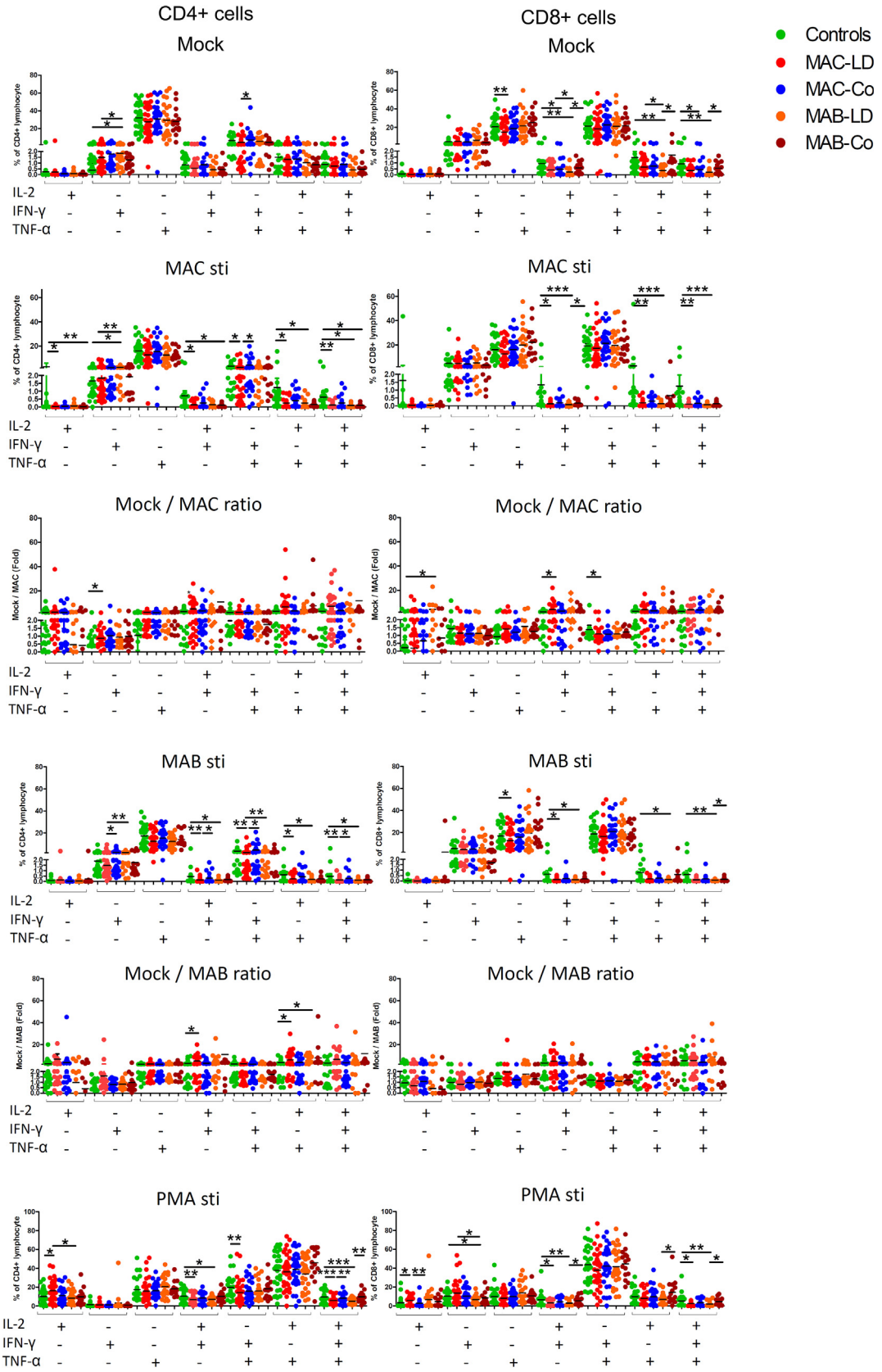

Fig. 2. Poly-functional T cells of mono-, double or triple cytokine expression are shown for different stimulations according to disease status. Peripheral blood mononuclear cells were stimulated by mock stimulation with medium, *Mycobacterium avium* complex (MAC), or *Mycobacterium abscessus* (MAB) (multiplicity of infection: 100) for 48 h and CD3/CD28 antibodies were added for the co-culture in the last 16 h. Phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and ionomycin calcium salt (1 μM/ml) were used for 16 h of stimulation. The cytokine expressions were measured for interleukin-2 [IL-2], tumor necrosis factor-alpha [TNF-α], and interferon-gamma [IFN-γ] expression on CD4 + and CD8 + lymphocytes, respectively. The ratios are the same cytokine expressed T cells in mock stimulation divided by MAC or MAB stimulation. LD, lung disease; Co, colonization. $^{*}0.01 \leq p < 0.05$; $^{**}0.001 < p < 0.01$; $^{***}p < 0.001$.

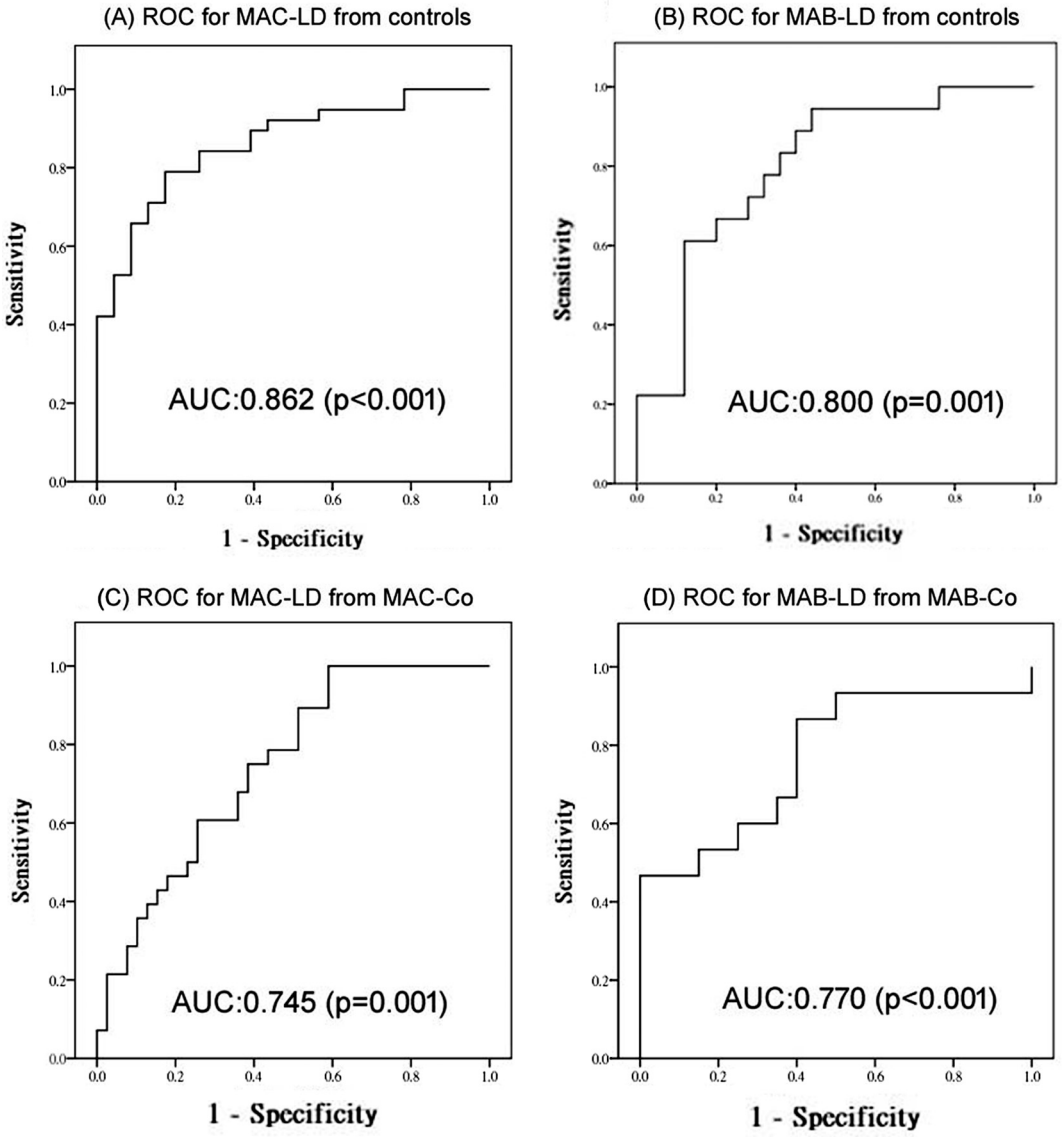

Fig. 3. Receiver operating characteristic (ROC) curves for discriminating (A) patients with *Mycobacterium avium* complex (MAC)-lung disease(LD) from the controls; (B) patients with *Mycobacterium abscessus* (MAB)-LD from the controls; (C) patients with MAC-LD from those with MAC airway colonization (MAC-Co); and (D) patients with MAB-LD from the MAB-Co group. The factors used in the ROC curves were those included in the final model by multivariate analyses.

independent factors. The probability using two-factor analysis had an AUC of 0.800 (p = 0.001) (Fig. 3B). The equations for generating the probabilities are listed in the supplementary information.

For discriminating NTM-LD from NTM-Co, multivariate logistic regression revealed that gender (OR: 0.268 [0.084–0.858], if male, p = 0.027) and triple positive cytokine CD4 cells by PMA stimulation (OR: 0.813 [0.701–0.943] per 1% increment p = 0.006) were independent factors for MAC-LD. The triple positive cytokine CD4 cells for PMA stimulation were a significant predictor for MAB-LD (OR:0.779 [0.629–0.966] per 1% increment, p = 0.023). The AUROCs by the probability generated by the multivariate model were 0.745 [p = 0.001] and 0.770 [p < 0.001] for MAC-LD and MAB-LD, respectively (Fig. 3C and D).

3.4. The radiographic pattern of NTM-LD vs. poly-functional T cells

Those with MAC-LD and MAB-LD were classified as the FC group (n = 15), NB group (n = 42) and others (n = 10). Those with FC had weaker CD4+ IFN-γ+ (mock and PMA stimulation), CD8+ IFN-γ+ (PMA), CD4+ TNF-α+ and CD8+ TNF-α+ after all stimulations (Fig. 4). The double positive and triple positive data had similar trends of lower IFN-γ+ and TNF-α+ expression, but single IL-2 expressions on CD4 (MAB stimulation) and on CD8 (mock and MAC stimulation) were higher in those with the FC pattern. The other details of single, double and triple cytokine expression are provided in the supplementary information (Fig. S1). The changes in ratio from mock to MAC or MAB stimulation were not significantly different between those with radiographic FC and NB pattern.

3.5. Prediction for NTM-LD with radiographic progression

At 1-year follow-up, 62 (92.5%) received chest imaging, and 11 (17.7%) had radiographic progression. CD4+ IFN-γ+ (MAC and PMA stimulation) and CD8+ IFN-γ+ (all stimulation) were higher but the CD8+ TNF-α+ ratio (mock/MAB) was lower in those with radiological progression than in those without progression (Fig. 4). After adjustment for age, sex, BMI, AFS grade, anti-NTM treatment and initial CXR score, multivariate logistic analysis showed CD4+ IFN-γ+ on PMA

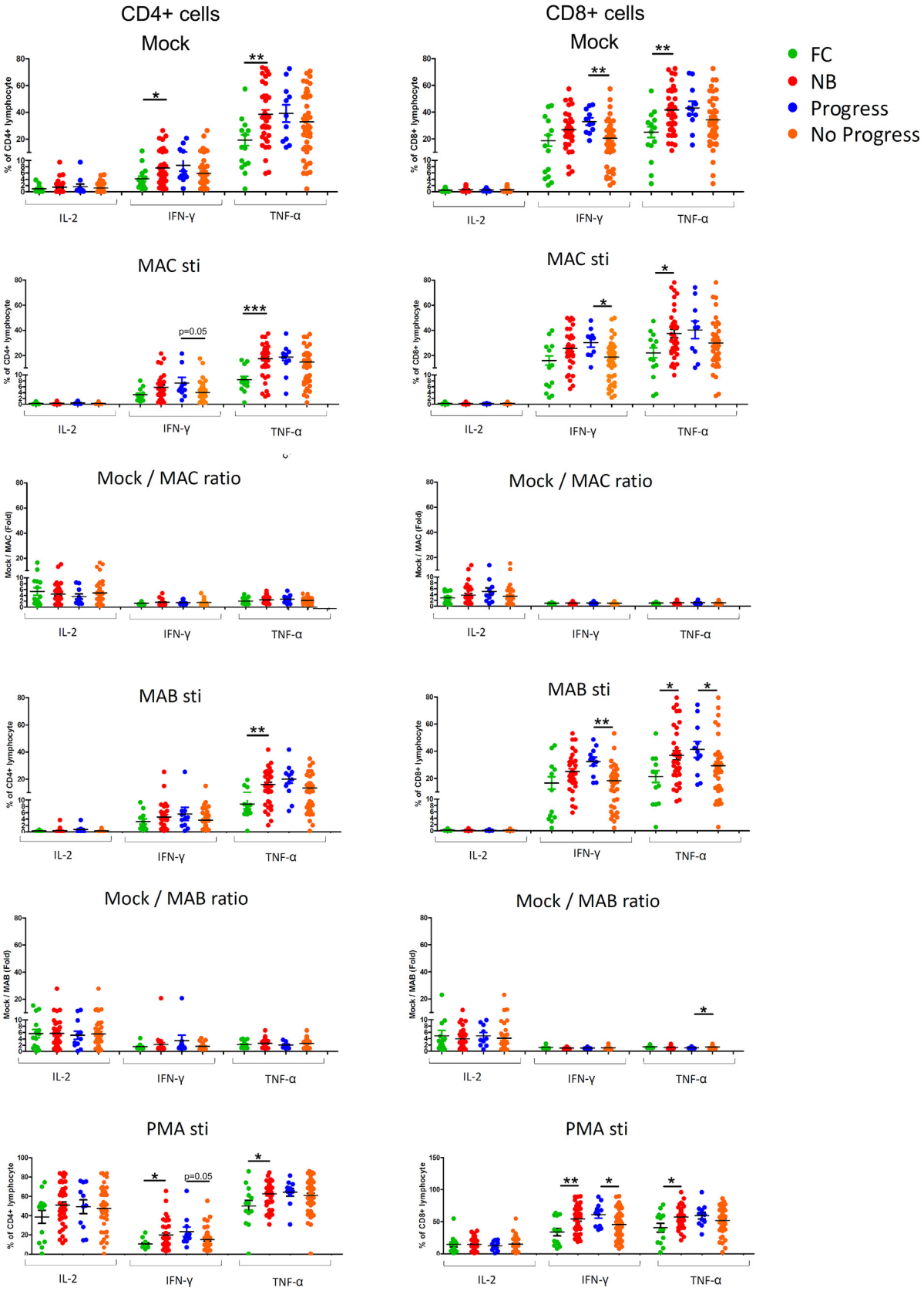

Fig. 4. Poly-functional T cells of total cytokine expression in patients with nontuberculous mycobacteria lung disease (NTM-LD) according to radiographic pattern and progression under different stimulation. Peripheral blood mononuclear cells were co-cultured with mock stimulation with medium, *Mycobacterium avium* complex (MAC), or *Mycobacterium abscessus* (MAB) (multiplicity of infection:100) for 48 h and added CD3/CD28 antibodies for the last 16 h. In comparison, phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and ionomycin calcium salt (1 μM/ml) were used for 16 h of stimulation. The cytokine expressions were measured for total interleukin-2 [IL-2], tumor necrosis factor-alpha [TNF-α], and interferon-gamma [IFN-γ] expression on CD4+ and CD8+ lymphocytes, respectively. The ratios are the same cytokine expressed T cells in mock stimulation divided by MAC or MAB stimulation. Co, colonization; FC, fibro-cavitary; LD, lung disease; NB, nodular-bronchiectasis; NP, non-progression; P, progression $^{*}0.01 \leq p < 0.05$; $^{**}0.001 < p < 0.01$; $^{***}p < 0.001$.

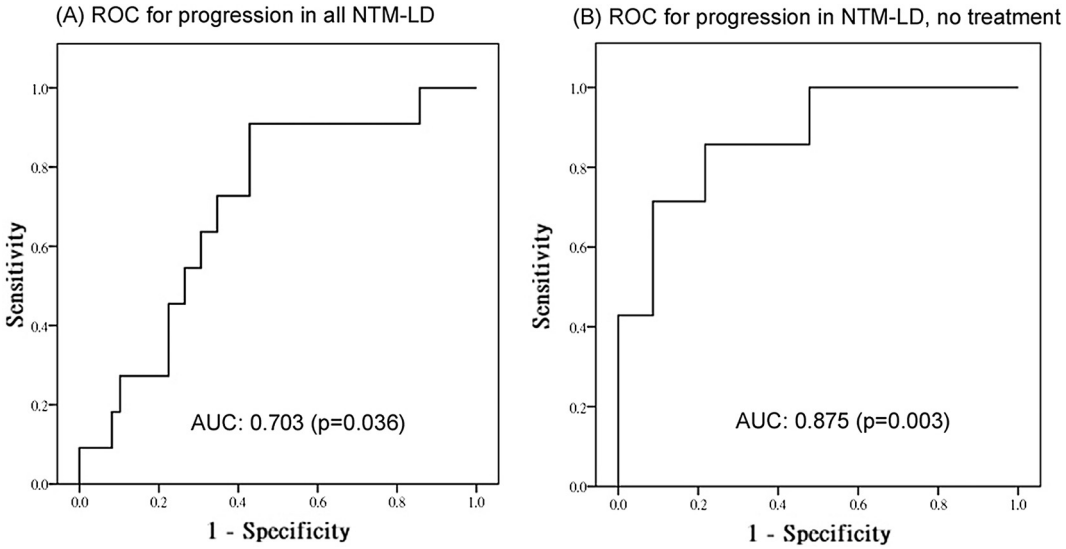

Fig. 5. Receiver operating characteristic (ROC) curves for radiographic progression. (A) To discriminate patients with all nontuberculous mycobacteria lung disease (NTM-LD) and radiographic progression from those without progression. (B) To discriminate NTM-LD patients without anti-NTM treatment but radiographic progression (+) from those without progression. The factors used in the ROC curves were those included in the final model by multivariate analyses.

stimulation was correlated with radiographic progression (OR: 1.132 [1.021–1.255], p = 0.019). The AUROC was 0.703 (p = 0.036) (Fig. 5A). In regard to those without anti-NTM therapy (n = 38), seven patients (18%) had radiographic progression, which was correlated with mono-IFN-γ+ on CD4 cells on MAC stimulation (OR: 1.672 [0.993–2.814], p = 0.053) and triple cytokine (+) expressed CD4 on PMA stimulation (OR: 1.400 [0.998–1.963], p = 0.051) by multivariate logistic analysis. The equation for generating the probability of the two factors was as *Logit* (Probability) = −4.832 + 0.514 × mono-IFNγ+ CD4+ during MAC stimulation (%) + 0.336 × Triple cytokine (+) on CD4 during PMA stimulation (%). Please see the details in the supplementary file for an explanation and example of the probability calculation. The AUROC by the probability generated from the two factors was 0.875 (p = 0.003) (Fig. 5B). According to the Youden index, when the probability was 0.297, the sensitivity was 86% and specificity was 78.3%.

4. Discussion

In the present study, we observed that the patients with MAC-LD generally had lower expression of triple-positive poly-functional T cells but higher single IL-2+ expression than the controls and MAC-Co group. On the other hand, the MAB-LD group had lower IL-2 expression, mostly on CD8+ cells, and lower triple positive cells than the controls and MAB-Co group. The triple positive CD4 cells (by PMA stimulation) could predict MAC-LD and MAB-LD from the corresponding colonization groups. For those with NTM-LD and not receiving anti-NTM treatment, mono-IFN-γ+ on CD4 cells (MAC stimulation) and triple positive CD4+ (PMA) were correlated with progression, with a good AUROC of 0.875.

The status of mono- and poly-functional T cells in MAC-LD and MAB-LD patients might be impaired and thus responsible for the underlying immune problem leading to pulmonary infection. Functional CD4+ and CD8+ T-cell subsets have been defined based on the cytokine signatures of IFN-γ, IL-2, and TNF-α. For example, TNF-α-only and dual IFN-γ/TNF-α expression were greater in patients with active tuberculosis than in those with latent tuberculosis infection and controls [16]. In the present study, lower triple-positive CD8+ cells and higher mono-IL-2+ CD4+ cells (PMA stimulation) together with lower BMI favored MAC-LD. This finding is compatible with previous studies showing that PBMCs from MAC-LD patients had lower response of cytokine production [19] and that anti-TNF biologic agents might be correlated with increased NTM infection [20,21]. The reason for the loss of polyfunctionality in the NTM-LD group of T cells as compared to healthy controls is very important, but it remains unclear and will require further investigation.

The higher response of mono-IL-2+ expression was the only positive predictor of MAC-LD from the controls and airway colonization. Traditionally, IL-2 is synthesized by T cells during the early stages of the immune response and has a pivotal role in cell expansion and effector functions after primary antigen challenge [22]. IL-2 is a marker of polyfunctional T-cells, which are crucial for protection against tuberculosis [23]. In this study, the increased IL-2-expressing lymphocytes might be compensating for reduced response and lower IFNγ/TNF-α during MAC-LD. By contrast, for MAB-LD, weakened IL-2 expression was noted in MAB-specific poly-functional T cells, similar to a previous study in cystic fibrosis [24]. It may indicate differences in the immune deficiency of IL-2+ poly-functional T cells between MAB-LD and MAC-LD. But the attenuated triple positive poly-functional T cells were the same in the two NTM-LD.

On the other hand, CD4+ IFN-γ+ was lower in the MAC-LD group than in the MAB-LD group. Although we reviewed the literature showing decreased Th1 response, such as IFN-γ level, in both the MAC-LD [19,25] and the MAB-LD groups [26], the literature directly comparing Th1 response between MAC-LD and MAB-LD is scarce. We speculated that the attenuation of CD4+ IFN-γ+ in MAC-LD might be due to the disease-related T cell dysregulation because in a previous study, the IFN-γ response was improved after MAC treatment [19]. Although PD-1, Tim-3, and CTLA-4 on CD4 and CD8 T cells were not significantly increased in MAC-LD patients after stimulation in the present study, other immunomodulators might be responsible factors, like immunosuppressive macrophages and regulatory T cells [27]. By contrast, *M. abscessus*, a rapidly growing mycobacteria causing lung disease not as indolent as MAC, might lead to lower immune exhaustion than MAC-LD. However, this speculation needs to be studied in the future.

For differentiation of MAC-LD and MAB-LD from MAC-Co and MAB-Co, triple-positive CD4+ cells could provide a good diagnostic indicator if 1 or 2 sets of sputum yield MAC. Poly-functional triple-positive CD4+ T cells play a critical role in the control of chronic bacterial and viral infections [28], but only limited data on NTM-LD are available. Airway NTM colonization sometimes cannot be easily

differentiated by the clinical criteria recommended by the ATS [1]. The triple-positive CD4+ T cells could be a new diagnostic factor for clinical practice. Once poly-functional functional T-lymphocyte responses are attenuated, MAC or MAB bacilli could be considered.

In regard to the clinical characteristics of NTM-LD, the patients with radiographic FC patterns generally had higher bacilli loads [6], which might induce more immune suppression and low cytokine production [19,29]. The present study supported this point by finding lower cytokine expression of IFN-γ and TNF-α on poly-functional T cells in patients with FC patterns. For the clinical course, approximately 22% and 53% of MAC-LD patients reportedly present with radiographic deterioration at follow up after 5 and 10 years, respectively [30]. To predict radiographic progression that requires early anti-MAC treatment, we integrated clinical factors, AFS grade, radiographic pattern and poly-functional T cells into multivariate analysis. Notably, in the NTM-LD patients not receiving anti-NTM treatment, MAC-specific mono-IFN-γ+ CD4 cells and triple-positive CD4+ cells by PMA could predict the radiographic progression with excellent effectiveness (sensitivity of 86% and specificity of 78.3%).

From interpretation of the study results, we found that the CD4+ IFN-γ+ by MAC 24-hour stimulation was compatible between MAC-LD and the controls, which is similar to the results on Sensitin stimulation in a study by Lim et al. [31]. However, Lim et al. showed MAC-LD patients had higher CD4+ IFN-γ+ T cell than the controls after 6-hour Sensitin stimulation, which was not performed in this study. In contrast, the attenuation of CD4+ IFN-γ+ T cells in MAC-LD was detected after MAB and PMA 24-hour stimulation, which was not used by Lim et al. In addition to antigen and stimulation duration, the co-stimulatory antibodies were different (CD28/CD49d vs. CD3/CD28 antibodies respectively), as well as the participants' ethnicity. Therefore, the results should be carefully interpreted and generalized because differences may arise due to different study designs and subject ethnicities.

This study had several limitations. First, the case number was small, so some phenomenon observed in the present study will require validation. Second, a causal relationship could not be confirmed in this cross-sectional study. Third, the mechanism by which the heat-killed NTM antigen induced attenuation of mono- and poly-functional T cells is unclear and will require future investigation. Fourth, the participants were enrolled in Taiwan, so generalization to other areas or ethnicities may not be applicable.

In conclusion, patients with MAC-LD had lower BMI, higher mono-IL2+ CD4+ cells and lower triple positive poly-functional cells (PMA stimulation), whereas patients with MAB-LD had lower triple positive CD4 cells (PMA stimulation) and a higher mock/MAB ratio of IL-2+/TNF-α+ CD4 cells than the controls. The percentage of triple positive CD4 cells (PMA stimulation) could predict MAC-LD or MAB-LD from airway colonization. For those with NTM-LD but no treatment, MAC-specific mono-IFN-γ+ CD4+ cells and triple positive CD4+ (PMA) might predict radiographic progression.

5. Declarations

5.1. Ethics approval and consent to participate

The Research Ethics Committee of National Taiwan University Hospital and Far Eastern Memorial Hospital approved this study (IRB No.: 201703051RINC and 106134-F).

5.2. Consent for publication

Not applicable. The present study did not contain any individual person's data in any form.

Funding

The study was supported in part by grants from the Far Eastern Memorial Hospital National Taiwan University Hospital Joint Research Program (106-FTN07) and the Ministry of Science and Technology Taiwan (MOST 105-2628-B-002 -046 -MY3 and 107-2628-B-002-006). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data availability statement

Please contact author for data requests.

Declaration of Competing Interest

All authors declare no financial, professional or other personal interest of any nature or kind in a related product, service, and/or company.

Acknowledgments

The authors thank the staff of the Second, the Seventh and the Eighth Core Labs of the Department of Medical Research of National Taiwan University Hospital for their technical support.

Author contributions

Drs. Shu CC and Wang PH designed and conducted the study and performed the experiments. Drs. Shu CC, Cheng SL, and Wang PH performed the experiments, and data collection. Drs. Shu CC, Wang JY, Wang PH, Prof. Wu MF, Lai HC and Wu L. SH contributed for data analysis, manuscript writing and revision. Dr. Wang PH and Prof. Yu CJ were responsible for coordination.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.05.001.

References

[1] D.E. Griffith, T. Aksamit, B.A. Brown-Elliott, A. Catanzaro, C. Daley, F. Gordin, S.M. Holland, R. Horsburgh, G. Huitt, M.F. Iademarco, M. Iseman, K. Olivier, S. Ruoss, C.F. von Reyn, R.J. Wallace Jr., K. Winthrop, An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases, Am. J. Respir. Crit. Care Med. 175 (4) (2007) 367–416.

[2] D. Menzies, P. Nahid, Update in tuberculosis and nontuberculous mycobacterial disease 2012, Am. J. Respir. Crit. Care Med. 188 (8) (2013) 923–927.

[3] C.C. Lai, C.K. Tan, C.H. Chou, H.L. Hsu, C.H. Liao, Y.T. Huang, P.C. Yang, K.T. Luh, P.R. Hsueh, Increasing incidence of nontuberculous mycobacteria, Taiwan, 2000–2008, Emerg. Infect Dis. 16 (2) (2010) 294–296.

[4] S.K. Field, R.L. Cowie, Lung disease due to the more common nontuberculous mycobacteria, Chest 129 (6) (2006) 1653–1672.

[5] J. Adjemian, K.N. Olivier, A.E. Seitz, S.M. Holland, D.R. Prevots, Prevalence of nontuberculous mycobacterial lung disease in U.S. Medicare beneficiaries, Am. J. Respir. Crit. Care Med. 185 (8) (2012) 881–886.

[6] C.C. Shu, C.H. Lee, C.L. Hsu, J.T. Wang, J.Y. Wang, C.J. Yu, L.N. Lee, Clinical characteristics and prognosis of nontuberculous mycobacterial lung disease with different radiographic patterns, Lung 189 (6) (2011) 467–474.

[7] W. Hoefsloot, J. van Ingen, C. Andrejak, K. Angeby, R. Bauriaud, P. Bemer, N. Beylis, M.J. Boeree, J. Cacho, V. Chihota, E. Chimara, G. Churchyard, R. Cias, R. Daza, C.L. Daley, P.N. Dekhuijzen, D. Domingo, F. Drobniewski, J. Esteban, M. Fauville-Dufaux, D.B. Folkvardsen, N. Gibbons, E. Gomez-Mampaso, R. Gonzalez, H. Hoffmann, P.R. Hsueh, A. Indra, T. Jagielski, F. Jamieson, M. Jankovic, E. Jong, J. Keane, W.J. Koh, B. Lange, S. Leao, R. Macedo, T. Mannsaker, T.K. Marras, J. Maugein, H.J. Milburn, T. Mlinko, N. Morcillo, K. Morimoto, D. Papaventsis, E. Palenque, M. Paez-Pena, C. Piersimoni, M. Polanova, N. Rastogi, E. Richter, M.J. Ruiz-Serrano, A. Silva, M.P. da Silva, H. Simsek, D. van Soolingen, N. Szabo, R. Thomson, T. Tortola Fernandez, E. Tortoli, S.E. Totten, G. Tyrrell, T. Vasankari, M. Villar, R. Walkiewicz, K.L. Winthrop, D. Wagner, The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples: an NTM-NET collaborative study, Eur. Respir. J. 42 (6) (2013) 1604–1613.

[8] D. Lu, B. Heeren, W.M. Dunne, Comparison of the Automated Mycobacteria Growth Indicator Tube System (BACTEC 960/MGIT) with Lowenstein-Jensen medium for recovery of mycobacteria from clinical specimens, Am. J. Clin. Pathol. 118 (4) (2002) 542–545.

[9] W.J. Koh, O.J. Kwon, K. Jeon, T.S. Kim, K.S. Lee, Y.K. Park, G.H. Bai, Clinical significance of nontuberculous mycobacteria isolated from respiratory specimens in Korea, Chest 129 (2) (2006) 341–348.
[10] J. van Ingen, S.A. Bendien, W.C. de Lange, W. Hoefsloot, P.N. Dekhuijzen, M.J. Boeree, D. van Soolingen, Clinical relevance of non-tuberculous mycobacteria isolated in the Nijmegen-Arnhem region, The Netherlands, Thorax 64 (6) (2009) 502–506.
[11] C.C. Shu, C.H. Lee, J.Y. Wang, J.S. Jerng, C.J. Yu, P.R. Hsueh, L.N. Lee, P.C. Yang, Nontuberculous mycobacteria pulmonary infection in medical intensive care unit: the incidence, patient characteristics, and clinical significance, Intensive Care Med. 34 (12) (2008) 2194–2201.
[12] S. Kitada, K. Kobayashi, S. Ichiyama, S. Takakura, M. Sakatani, K. Suzuki, T. Takashima, T. Nagai, I. Sakurabayashi, M. Ito, R. Maekura, Serodiagnosis of Mycobacterium avium-complex pulmonary disease using an enzyme immunoassay kit, Am. J. Respir. Crit. Care Med. 177 (7) (2008) 793–797.
[13] C.C. Shu, M. Ato, J.T. Wang, R. Jou, J.Y. Wang, K. Kobayashi, H.C. Lai, C.J. Yu, L.N. Lee, K.T. Luh, Sero-diagnosis of Mycobacterium avium complex lung disease using serum immunoglobulin A antibody against glycopeptidolipid antigen in Taiwan, PloS One 8 (11) (2013) e80473.
[14] C.H. Ladel, S. Daugelat, S.H. Kaufmann, Immune response to Mycobacterium bovis bacille Calmette Guerin infection in major histocompatibility complex class I- and II-deficient knock-out mice: contribution of CD4 and CD8 T cells to acquired resistance, Eur. J. Immunol. 25 (2) (1995) 377–384.
[15] R. Casey, D. Blumenkrantz, K. Millington, D. Montamat-Sicotte, O.M. Kon, M. Wickremasinghe, S. Bremang, M. Magtoto, S. Sridhar, D. Connell, A. Lalvani, Enumeration of functional T-cell subsets by fluorescence-immunospot defines signatures of pathogen burden in tuberculosis, PloS One 5 (12) (2010) e15619.
[16] C.L. Day, D.A. Abrahams, L. Lerumo, E. Janse van Rensburg, L. Stone, T. O'Rie, B. Pienaar, M. de Kock, G. Kaplan, H. Mahomed, K. Dheda, W.A. Hanekom, Functional capacity of Mycobacterium tuberculosis-specific T cell responses in humans is associated with mycobacterial load, J. Immunol. 187 (5) (2011) 2222–2232.
[17] G.L. Snider, L. Doctor, T.A. Demas, A.R. Shaw, Obstructive airway disease in patients with treated pulmonary tuberculosis, Am. Rev. Respir. Dis. 103 (5) (1971) 625–640.
[18] C.C. Shu, C.H. Lee, M.C. Lee, J.Y. Wang, C.J. Yu, L.N. Lee, Hepatotoxicity due to first-line anti-tuberculosis drugs: a five-year experience in a Taiwan medical centre, Int. J. Tuberc. Lung Dis. 17 (7) (2013) 934–939.
[19] C.C. Shu, J.Y. Wang, M.F. Wu, C.T. Wu, H.C. Lai, L.N. Lee, B.L. Chiang, C.J. Yu, Attenuation of lymphocyte immune responses during Mycobacterium avium complex-induced lung disease due to increasing expression of programmed death-1 on lymphocytes, Sci. Rep. 7 (2017) 42004.
[20] S.K. Brode, F.B. Jamieson, R. Ng, M.A. Campitelli, J.C. Kwong, J.M. Paterson, P. Li, A. Marchand-Austin, C. Bombardier, T.K. Marras, Increased risk of mycobacterial infections associated with anti-rheumatic medications, Thorax 70 (7) (2015) 677–682.
[21] J.W. Yoo, K.W. Jo, B.H. Kang, M.Y. Kim, B. Yoo, C.K. Lee, Y.G. Kim, S.K. Yang, J.S. Byeon, K.J. Kim, B.D. Ye, T.S. Shim, Mycobacterial diseases developed during anti-tumour necrosis factor-alpha therapy, Eur. Respir. J. 44 (5) (2014) 1289–1295.
[22] J.H. Cho, H.O. Kim, K.S. Kim, D.H. Yang, C.D. Surh, J. Sprent, Unique features of naive CD8+ T cell activation by IL-2, J. Immunol. 191 (11) (2013) 5559–5573.
[23] H. Kang, Q. Yuan, H. Ma, Z.D. Hu, D.P. Han, K. Wu, D.B. Lowrie, X.Y. Fan, Enhanced protective efficacy against Mycobacterium tuberculosis afforded by BCG prime-DNA boost regimen in an early challenge mouse model is associated with increased splenic interleukin-2-producing CD4 T-cell frequency post-vaccination, Immunology 143 (4) (2014) 661–669.
[24] M. Steindor, V. Nkwouano, E. Mayatepek, C.R. Mackenzie, D. Schramm, M. Jacobsen, Rapid detection and immune characterization of Mycobacterium abscessus infection in cystic fibrosis patients, PloS One 10 (3) (2015) e0119737.
[25] M. Kartalija, A.R. Ovrutsky, C.L. Bryan, G.B. Pott, G. Fantuzzi, J. Thomas, M.J. Strand, X. Bai, P. Ramamoorthy, M.S. Rothman, V. Nagabhushanam, M. McDermott, A.R. Levin, A. Frazer-Abel, P.C. Giclas, J. Korner, M.D. Iseman, L. Shapiro, E.D. Chan, Patients with nontuberculous mycobacterial lung disease exhibit unique body and immune phenotypes, Am. J. Respir. Crit. Care Med. 187 (2) (2013) 197–205.
[26] S.Y. Kim, W.J. Koh, Y.H. Kim, B.H. Jeong, H.Y. Park, K. Jeon, J.S. Kim, S.N. Cho, S.J. Shin, Importance of reciprocal balance of T cell immunity in Mycobacterium abscessus complex lung disease, PloS One 9 (10) (2014) e109941.
[27] K. Ogasawara, H. Tomioka, T. Shimizu, C. Sano, H. Kawauchi, K. Sato, Profiles of cell-to-cell interaction of Mycobacterium intracellulare-induced immunosuppressive macrophages with target T cells in terms of suppressor signal transmission, Clin. Exp. Immunol. 129 (2) (2002) 272–280.
[28] S. Kannanganat, C. Ibegbu, L. Chennareddi, H.L. Robinson, R.R. Amara, Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells, J. Virol. 81 (16) (2007) 8468–8476.
[29] A. Singh, A. Mohan, A.B. Dey, D.K. Mitra, Inhibiting the programmed death 1 pathway rescues Mycobacterium tuberculosis-specific interferon gamma-producing T cells from apoptosis in patients with pulmonary tuberculosis, J. Infect. Dis. 208 (4) (2013) 603–615.
[30] S. Kitada, T. Uenani, K. Yoshimura, Y. Tateishi, K. Miki, M. Miki, H. Hashimoto, T. Fujikawa, M. Mori, K. Matsuura, M. Kuroyama, R. Maekura, Long-term radiographic outcome of nodular bronchiectatic Mycobacterium avium complex pulmonary disease, Int. J. Tuberc. Lung Dis. 16 (5) (2012) 660–664.
[31] A. Lim, C. Allison, P. Price, G. Waterer, Susceptibility to pulmonary disease due to Mycobacterium avium-intracellulare complex may reflect low IL-17 and high IL-10 responses rather than Th1 deficiency, Clin. Immunol. 137 (2) (2010) 296–302.