



Impaired expression of Toll-like receptor 2 in nontuberculous mycobacterial lung disease

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ABSTRACT: The aims of the present study were to investigate the expression of Toll-like receptor (TLR)2 on the peripheral blood monocytes of patients with nontuberculous mycobacterial (NTM) lung disease and healthy controls, and to assess the responses of these monocytes to TLR2 agonists such as *Mycobacterium avium* and lipoteichoic acid (LTA).

Reverse transcriptase–PCR was used to analyse TLR2 mRNA expression in peripheral blood monocytes from 17 NTM patients and 10 healthy controls. mRNA and protein secretion levels were also determined for the cytokines interleukin (IL)-12 p40 and tumour necrosis factor (TNF)- α .

Expression of TLR2 mRNA by peripheral blood monocytes after stimulation with *M. avium* or LTA was lower in NTM patients than in healthy controls. IL-12 p40 and TNF- α mRNA and cytokine secretion levels were also lower in patients than in healthy controls. Treatment with anti-TLR antibody decreased *M. avium*- and LTA-induced IL-12 p40 and TNF- α production in healthy controls, but not in NTM patients.

The present results suggest that the downregulation of Toll-like receptor 2 and the resulting decreased production of interleukin-12 p40 and tumour necrosis factor- α following *Mycobacterium avium* or lipoteichoic acid stimulation may contribute to host susceptibility to nontuberculous mycobacterial lung disease.

KEYWORDS: Atypical mycobacteria, disease susceptibility, lung diseases, Toll-like receptor 2

The incidence of lung disease caused by nontuberculous mycobacteria (NTM) appears to be rising worldwide, but it is unclear whether this is due to enhanced detection or to actual increases in the burden of infection [1–4]. Lung disease due to NTM commonly occurs in structural lung disease, such as chronic obstructive pulmonary disease, tuberculosis and pneumoconiosis. NTM lung disease also occurs in females without clearly recognised predisposing factors [5, 6]. Although bronchiectasis and NTM infection often coexist in these patients (nodular bronchiectasis), it remains controversial whether bronchiectasis is truly caused by NTM infection or is a predisposing condition favouring NTM infection [1, 2].

NTM are ubiquitous environmental organisms. Since exposure to these organisms is universal and the occurrence of disease is uncommon, normal host defense mechanisms must be effective enough to prevent the infection [5]. Therefore, otherwise healthy individuals who develop NTM lung disease are likely to have

specific susceptibility factors that lead to NTM infections [7].

Toll-like receptors (TLRs) are important pattern-recognition receptors that function in the host's innate defence mechanism against invading pathogens, such as *Mycobacterium tuberculosis* and NTM [8, 9]. Signalling through these receptors leads to transcription and translation of a variety of cytokines/mediators [10, 11]. TLR2 is required for NTM signalling [12, 13] and increased susceptibility to *M. avium* infection was recently reported in TLR2-knockout mice [14]. Furthermore, mice deficient in myeloid differentiation factor (MyD) 88 were more sensitive to *M. avium* infection [15]. These results suggest that infection with opportunistic *M. avium* species is controlled in a TLR2- and MyD88-dependent manner.

It remains unclear whether the results reliably reflect the mechanisms of pathogenesis and immunity in humans, despite various studies on animals with TLR2 deficiency and cell-culture studies examining the role of TLR2 in NTM

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infection and immunity. In the present study, the expression of TLR2 was investigated on the peripheral blood monocytes of patients with NTM lung disease and healthy controls. The responses of these monocytes to the TLR2 agonists *M. avium* and lipoteichoic acid (LTA) from *Staphylococcus aureus* were also compared. It was hypothesised that TLR2 expression is lower in patients with NTM lung disease, and thus it was expected that the response of peripheral blood monocytes to TLR2 agonists would be suppressed in NTM patients.

PATIENTS AND METHODS

Study population

The present study included 17 patients with the nodular bronchiectatic form of NTM lung disease and 10 healthy volunteers (table 1). Of the 17 patients, eight exhibited *M. avium-intracellulare* complex infection and nine exhibited *M. abscessus* infection. The diagnosis of NTM lung disease was made according to the diagnostic criteria published by the American Thoracic Society [1], and all patients had characteristic findings on high-resolution computed tomography scans, such as bilateral bronchiectasis combined with multiple small nodules and branching linear structures [16]. Peripheral blood samples of 30 mL were obtained from both groups before antibiotic therapy was initiated. The study was approved by the institutional review board of the Samsung Medical Centre

(Seoul, Republic of Korea), and written informed consent was obtained from all participants.

Preparation and stimulation of peripheral monocytes

Peripheral blood mononuclear cells were separated from heparinised whole blood using standard density gradient centrifugation with Ficoll-Hypaque (Sigma, St. Louis, MO, USA). The cells were cultured for 2 h and washed with Dulbecco's PBS (Sigma) to remove nonadherent cells. To ascertain whether adherent cells were monocytes, the cells were labelled with anti-CD14-fluorescein isothiocyanate (FITC) and examined by flow cytometry. For flow cytometric analysis, FITC-labelled anti-CD14 was purchased from eBioscience (San Diego, CA, USA) and analysed by flow cytometry using a FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA, USA). Monocytes (1×10^6 cells·mL⁻¹) were then stimulated with *M. avium* (American Type Culture Collection 25291; 1×10^6 colony-forming units·well⁻¹) or LTA from *S. aureus* ($10 \mu\text{g}\cdot\text{mL}^{-1}$; InvivoGen, San Diego, CA, USA) for 0, 2, 4, 6 and 24 h at 37°C.

Total RNA isolation, cDNA synthesis and RT-PCR

Total RNA was isolated using RNeasyTM B (Invitrogen Life Technologies, San Diego, CA, USA), and cDNA synthesis and reverse transcriptase (RT)-PCR were performed using the AccessQuickTM RNA-PCR system (Promega, Madison, WI, USA). The first-strand cDNA was synthesised from the total RNA at 45°C for 45 min in a DNA thermal cycler (Hybaid, Teddington, UK). Forty cycles of PCR amplification were subsequently performed. The amplified PCR product was confirmed on a 2% agarose gel, and the gel bands were analysed quantitatively using a PhosphorImager with Bio-1D V.97 software for Windows 95 (Vilber Lourmat, Marne-la-Vallée, France). The primers for amplification of TLR2, interleukin (IL)-12 p40 and tumour necrosis factor (TNF)- α cDNAs are shown in table 2. RT-PCR was performed before stimulation with TLR2 agonists and again at 2, 4, 6 and 24 h after stimulation.

TABLE 1 Clinical characteristics of patients with nontuberculous mycobacterial lung disease

Characteristics	
Patients n	17
Age yrs	59 (52–64)
Sex	
Male	2 (12)
Female	15 (88)
Body mass index kg·m⁻²	20.4 (19.2–21.0)
Aetiology	
<i>M. avium-intracellulare</i> complex	8 (47)
<i>M. abscessus</i>	9 (53)
Sputum AFB smear	
Negative	9 (53)
Positive	8 (47)
Smoking history	
Nonsmoker	17 (100)
Presenting symptoms	
Cough	15 (88)
Sputum	12 (71)
Haemoptysis	7 (41)
Underlying diseases	
Bronchiectasis	17 (100)
Sinusitis	4 (24)
History of tuberculosis	3 (18)
Diabetes mellitus	3 (18)

Data are presented as n (%) or median (interquartile range), unless otherwise stated. *M. avium*: *Mycobacterium avium*; *M. abscessus*: *Mycobacterium abscessus*; AFB: acid-fast bacilli.

TABLE 2 Primer sequences used for reverse transcriptase-PCR analysis of mRNA expression levels for β -actin, Toll-like receptor (TLR)2, interleukin (IL)-12 p40 and tumour necrosis factor (TNF)- α

Gene	Primer sequence (5'→3')
β-actin	
Sense	ATG GAG AAA ATC TGG CAC CA
Antisense	AAT GGT GAT GAC CTG GCC CT
TLR2	
Sense	ACC TTA TGG TCC AGG AGC TG
Antisense	TGC ACC ACT CAC TCT TCA CA
IL-12 p40	
Sense	GAT GGA ATT TGG TCC ACT AA
Antisense	CGG CAT GGA CCA TGA CCT CA
TNF-α	
Sense	CCA TGA GCA CTG AAA GCA TG
Antisense	TCA CAG GGC AAT GAT CCC AA

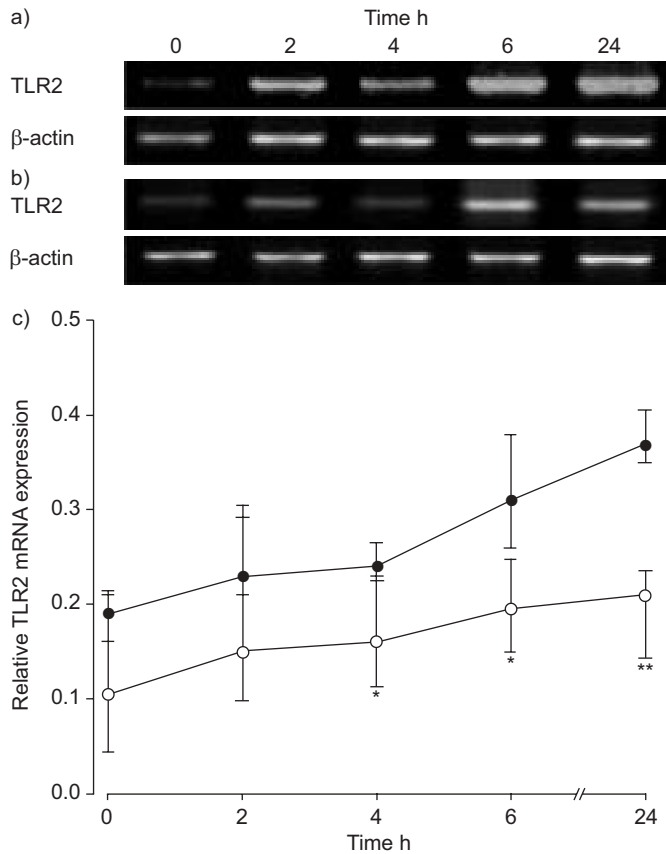


FIGURE 1. *Mycobacterium avium*-induced Toll-like receptor (TLR)2 mRNA expression in peripheral blood monocytes from patients with nontuberculous mycobacteria (NTM) lung disease and healthy controls. Peripheral blood monocytes were plated in a 24-well dish at 1×10^6 cells-well⁻¹ and then treated with *M. avium* American Type Culture Collection 25291 (10^6 colony-forming units) for the indicated lengths of time. a, b) TLR2 mRNA expression was assessed by reverse transcriptase-PCR analysis of isolated total RNA (600 ng) using β -actin as a control. Data shown represent: a) NTM lung disease patients ($n=17$) and b) independent healthy controls ($n=7$). c) TLR2 mRNA expression levels of patient and control monocytes, presented as median (25th–75th interquartile range). TLR2 mRNA expression was significantly lower in patients (○) than in controls (●) after 4 h of stimulation with *M. Avium*. *: $p<0.05$; **: $p<0.01$.

Cytokine assays

At 0, 2, 4, 6 and 24 h after stimulation of monocytes with either *M. avium* or LTA, cell-free (cleared) supernatant fractions were collected and analysed for cytokine release. The supernatants were stored in aliquots at -70°C until used in experiments. Concentrations of IL-12 p40 and TNF- α were determined using commercially available ELISA kits (Biosource, Camarillo, CA, USA).

Addition of anti-human TLR2 antibody

A blocking experiment with anti-human TLR2 antibody was performed to examine whether a TLR2 signalling blockade would reduce TLR2 agonist-induced cytokine production. Before *M. avium* or LTA stimulation, peripheral blood monocytes were pre-incubated with anti-TLR2 antibody ($10 \mu\text{g}\cdot\text{mL}^{-1}$; eBioscience) at room temperature for 30 min. The cells were then stimulated with *M. avium* or LTA for 24 h. RT-PCR and cytokine assays were performed to determine

IL-12 p40 and TNF- α mRNA and protein secretion levels, respectively.

Statistical analysis

The majority of data were not normally distributed. Therefore, all values were expressed as medians and interquartile range (25th and 75th percentiles) and the data were analysed using nonparametric analysis. Differences in each variable between patients and controls were evaluated with Mann–Whitney U-tests. A p -value <0.05 was considered statistically significant.

RESULTS

Expression of TLR2 mRNA in peripheral blood monocytes

The present authors first examined the kinetics of expression of TLR2 mRNA in peripheral blood monocytes from healthy controls in response to two different TLR2 agonists, *M. avium* and LTA. The level of TLR2 mRNA increased within 2 h post-infection with *M. avium*. RT-PCR analysis showed a time-dependent increase in TLR2 mRNA expression in healthy controls (fig. 1). The extent of TLR2 mRNA induction caused by LTA in these control monocytes was similar to that caused by *M. avium* (data not shown).

Unstimulated monocytes from NTM patients exhibited decreased TLR2 mRNA levels compared with those of controls, although this difference was not statistically significant. After stimulation with *M. avium*, TLR2 mRNA levels increased slightly, with a gentle slope. Compared with controls, TLR2 mRNA levels of patients were significantly lower at 4 h post-infection ($p<0.05$ at 4, 6 and 24 h; fig. 1). LTA stimulation affected TLR2 mRNA expression similarly to *M. avium* infection (data not shown).

Expression of IL-12 p40 and TNF- α mRNA

A comparative analysis of *M. avium*-induced cytokine gene expression was performed in NTM patients and healthy controls. In healthy controls, *M. avium* stimulation caused levels of mRNA for both IL-12 p40 and TNF- α to increase in a time-dependent manner. Expression in unstimulated and *M. avium*-stimulated monocytes was significantly lower in patients than in healthy controls ($p<0.05$ at all time-points; fig. 2). LTA stimulation had effects similar to those of *M. avium* infection (data not shown).

IL-12 p40 and TNF- α secretion

The present authors also compared IL-12 p40 and TNF- α production response of patient and control monocytes to stimulation with *M. avium* or LTA, using an ELISA kit. The concentrations of IL-12 p40 and TNF- α in unstimulated and *M. avium*-stimulated monocytes were significantly lower in patients than in controls ($p<0.05$ at all time-points except TNF- α levels at 4 h; fig. 3). LTA stimulation yielded similar results (data not shown).

Inhibitory effect of anti-TLR2 antibody on IL-12 p40 and TNF- α production

Finally, monocytes were stimulated with *M. avium* or LTA in the presence or absence of anti-TLR2 antibody. As shown in figure 4, antibody treatment substantially inhibited *M. avium*-induced IL-12 p40 production in healthy controls, a level of inhibition far greater than that observed in patients (50 versus 8% inhibition). Antibody treatment also reduced

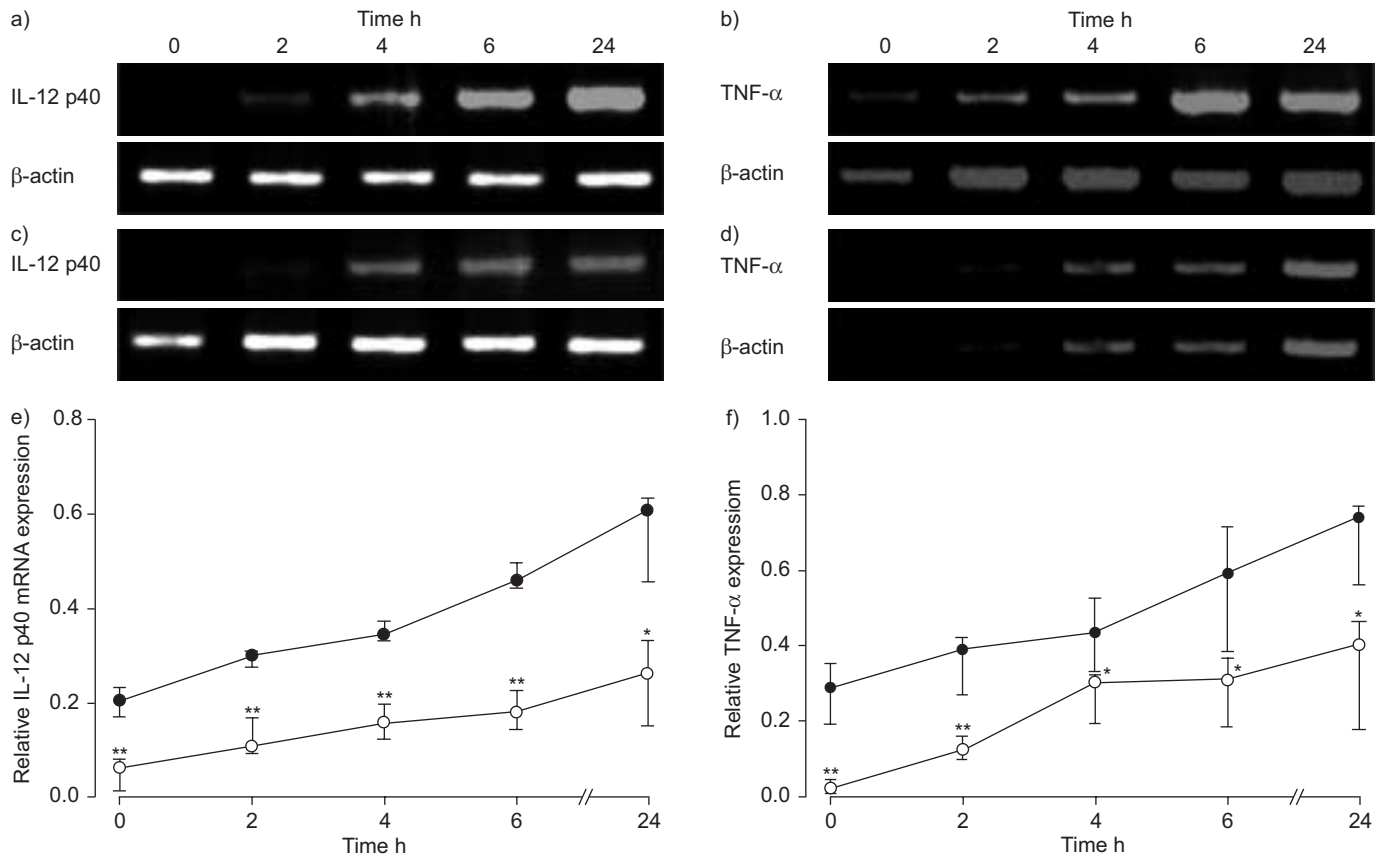


FIGURE 2. *Mycobacterium avium*-induced interleukin (IL)-12 p40 and tumour necrosis factor (TNF)- α mRNA expression in peripheral blood monocytes from nontuberculous mycobacteria (NTM) patients and from healthy controls. Peripheral blood monocytes were infected with *M. avium* American Type Culture Collection 25291 (10^6 colony-forming units) for the indicated lengths of time. Expression of a, c) IL-12 p40 and b, d) TNF- α mRNA was assessed by reverse transcriptase PCR analysis using β -actin as a control. Data shown represent 12 patients (c, d) and four controls (a, b). e) IL-12 p40 and f) TNF- α mRNA expression levels are presented as median (25th–75th interquartile range). Expression of these mRNAs was significantly lower in unstimulated and *M. avium*-stimulated monocytes of patients than in those of controls. *: $p < 0.05$; **: $p < 0.01$.

M. avium-induced TNF- α synthesis to a greater extent in controls than in patients (51 versus 24%). In addition, post-treatment cytokine secretion was significantly lower (six- and two-fold lower for IL-12 p40 and TNF- α , respectively) for monocytes from healthy controls than for those from patients. However, antibody treatment did not significantly change IL-12 p40 or TNF- α secretion responses of patient monocytes to stimulation with *M. avium* or LTA.

DISCUSSION

To the present authors' knowledge, the current study is the first to examine TLR2 mRNA expression and its relationship to cytokine responses in patients with NTM lung disease. The present data demonstrate that both *M. avium* and LTA induce TLR2 mRNA expression in peripheral blood monocytes from healthy controls and, subsequently, upregulate IL-12 p40 and TNF- α expression and production. In peripheral blood monocytes from NTM patients, however, the induction of TLR2 mRNA expression (and of IL-12 p40 and TNF- α expression and production) occurred to a significantly lesser extent. Thus, the current findings suggest that impaired induction of TLR2 expression in response to *M. avium* or LTA stimulation may contribute to host susceptibility to NTM lung disease.

Since nontuberculous mycobacteria are ubiquitous organisms, most animals are resistant to NTM infection unless their defence mechanisms have undergone iatrogenic alteration. However, the nature of the precise immune dysfunction that predisposes otherwise healthy subjects to NTM lung disease is not clear. A few studies that have compared the immune activation of peripheral blood mononuclear cells from NTM patients and healthy controls [17, 18] have shown that patients produce lower concentrations of IL-12 and TNF- α and higher concentrations of IL-10 in response to various antigens. These results suggest that the T-helper cell (Th) type 1 immune response in NTM patients is suppressed, whereas the Th2-type reaction is augmented. Therefore, deficiencies in the type-1 cytokine cascade might enhance susceptibility to NTM infection. However, the mechanisms responsible for this immunological imbalance remain unknown.

TLR2 is critical to the immune response to NTM infection and it is required for induction of IL-12, which plays a major role in promoting Th1 responses [8–11]. Infection of murine macrophages with *M. avium* was previously shown to upregulate TLR2 mRNA expression [12, 13], and TLR2 activation induces early production of IL-12 and TNF- α from specific phagocyte subsets. Previous *in vitro* studies showed that TLR2 stimulation is

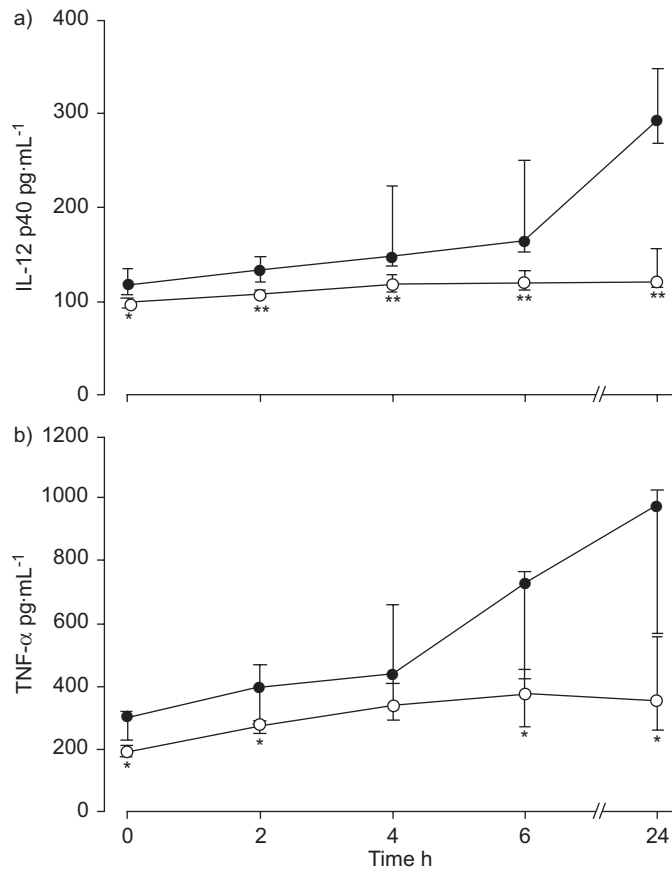


FIGURE 3. *Mycobacterium avium*-induced a) interleukin (IL)-12 p40 and b) tumour necrosis factor (TNF)- α production in peripheral blood monocytes from nontuberculous mycobacteria patients (○; n=17) and healthy controls (●; n=7). Peripheral blood monocytes were infected with *M. avium* American Type Culture Collection 25291 (10^6 colony-forming units) for the indicated lengths of time. Cell-free supernatants, cleared by centrifugation, were harvested at the indicated times and secretion of IL-12 p40 and TNF- α cytokines was assessed using ELISA. Data are presented as median (25th–75th interquartile range). *: p<0.05; **: p<0.01.

responsible for *M. avium*-induced upregulation of pro-inflammatory Th1 cytokines in mouse macrophages [12, 13, 19]. Furthermore, TLR2 plays an important role in controlling NTM infection in animal studies. For example, control of *M. bovis bacillus Calmette–Guerin* in mice after *i.p.* infection was dependent on TLR2 [20] and FENG *et al.* [15] found that mice deficient in TLR2 had increased bacterial load and increased susceptibility to *M. avium* infection compared with wild-type mice.

Although these findings strongly suggest that regulation of TLR2 expression is an important determinant of NTM lung disease susceptibility, the relationship between NTM lung disease and TLR2 and cytokine expression has not been studied previously. In the present study, it was found that *M. avium* and LTA downregulated TLR2 mRNA expression, and IL-12 p40 and TNF- α production in peripheral blood monocytes of NTM patients. In addition, treatment with anti-TLR2 antibody blocked *M. avium*- and LTA-induced cytokine gene production and subsequent cytokine secretion in monocytes of

healthy controls, but had only a minimal effect in those of patients. These results indicate that *M. avium*- and LTA-induced activation of IL-12 p40 and TNF- α are mediated primarily by TLR2.

IL-12 is a major stimulus for interferon- γ production and it plays a pivotal role in forming a major link between innate and adaptive immunity [21, 22]. TNF- α is essential for the development of protective immunity against mycobacterial disease [23]. Taken together with the cytokine profiles of NTM patients [17, 18], the present data suggest that downregulation of TLR2 may play a key role in mediating the impairment of host defence, and support the hypothesis that TLR2 deficiency plays an important role in host susceptibility to NTM lung disease.

Recent studies of TLR2 polymorphisms have shown the importance of TLR in human mycobacterial diseases. The Arg677Trp polymorphism of TLR2 is associated with lepromatous leprosy [24] and pulmonary tuberculosis [25], and the Arg753Gln polymorphism of TLR2 is associated with tuberculosis [26]. In addition, guanine-thymine repeat polymorphisms in intron II of the TLR2 gene are associated with the development of tuberculosis [27]. However, whether these genetic polymorphisms determine the susceptibility to NTM lung disease is unknown [28].

The present study has several important limitations. The gene expression in cells in the peripheral blood may not reflect the gene expression response in the lung, which is the actual site of NTM infection. Peripheral blood monocytes can reflect the immunoreactivity of airway cells, but the immune response of peripheral blood monocytes may be confounded due to a lack of local immunoregulatory mechanisms present in the lung [29, 30]. Therefore, further studies involving TLR2 mRNA expression and subsequent cytokine immune responses using bronchoalveolar lavage cells or lung tissue are needed.

In addition, although downregulation of TLR2 and a resulting decreased production of IL-12 p40 and TNF- α following *M. avium* or LTA stimulation in patients with NTM lung disease was found, the nature of this relationship remains uncertain. The present data are consistent with the downregulation of TLR2 causing or contributing to the development of NTM lung disease *via* decreased production of IL-12 p40 and TNF- α . Alternatively, the downregulation of TLR2 might be a secondary phenomenon. It may also be possible that the NTM infection led to the differences observed in the present study. Further studies including patients after successful treatment of NTM lung disease would be needed.

In conclusion, Toll-like receptor 2 mRNA expression in response to stimulation with *Mycobacterium avium* or lipoteichoic acid is lower in peripheral blood monocytes from patients with nontuberculous mycobacterial lung disease than in those from healthy controls. Lipoteichoic acid- and *Mycobacterium avium*-induced mRNA expression and interleukin-12 p40 and tumour necrosis factor- α production were also lower in patients than in controls. Thus, the present findings suggest that the downregulation of Toll-like receptor 2 and the subsequent decrease in interleukin-12 p40 and tumour necrosis factor- α production following *Mycobacterium avium* infection may contribute to host susceptibility to nontuberculous mycobacterial lung disease. This disease is associated with

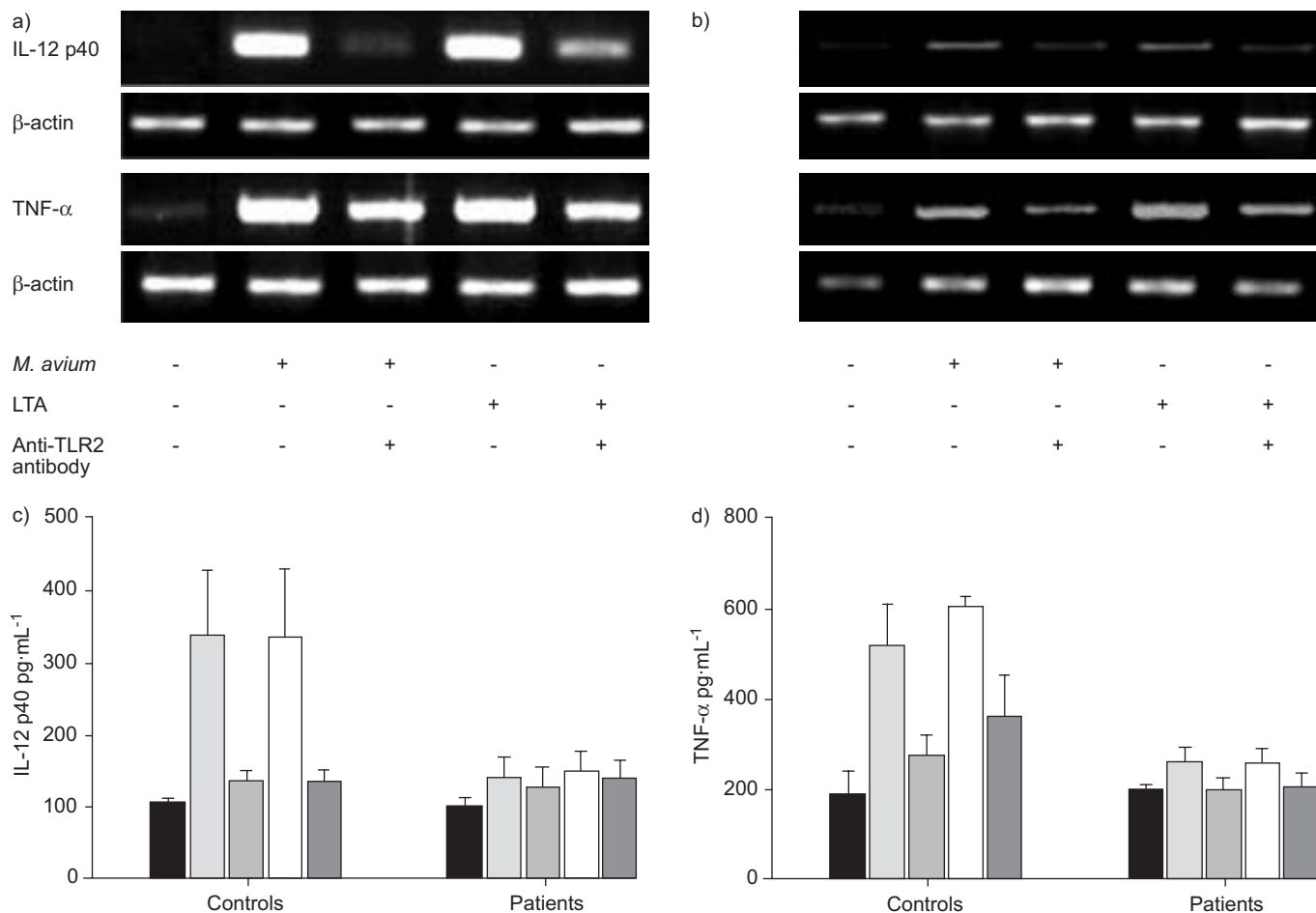


FIGURE 4. Inhibitory effect of anti-Toll-like receptor (TLR)2 antibody on *Mycobacterium avium*-induced interleukin (IL)-12 p40 and tumour necrosis factor (TNF)- α production in peripheral blood monocytes from nontuberculous mycobacteria (NTM) patients and healthy controls. The antibody (10 $\mu\text{g}\cdot\text{mL}^{-1}$) was added to peripheral blood monocytes 30 min before stimulation with *M. avium* American Type Culture Collection 25291 (10^6 colony-forming units) or lipoteichoic acid (LTA) for 24 h. a) Total RNA was isolated and expression of IL-12 p40 and TNF- α mRNA was determined in a) healthy controls and b) NTM patients by reverse transcriptase-PCR analysis using β -actin as a control. Data shown represent four patients and three controls. The culture supernatants were harvested for c) IL-12 p40 and d) TNF- α cytokine assessment using ELISA after *M. avium* or LTA stimulation for 24 h. Data shown represent 11 patients and seven controls. ■: no stimulus; ■: *Mycobacterium avium*; ■: anti-TLR2 antibody and *M. avium*; □: LTA; ■: anti-TLR2 antibody and LTA.

substantial morbidity; current treatment strategies using chronic, multidrug therapy are ineffective and can have a variety of side-effects. Understanding the precise mechanisms responsible for downregulation of Toll-like receptor 2 and the associated decrease in interleukin-12 p40 and tumour necrosis factor- α production may facilitate the development of immunomodulatory strategies to treat or prevent nontuberculous mycobacterial lung disease.

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