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Impaired Expression of Toll-like Receptor 2 in Nontuberculous Mycobacterial Lung Disease

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

We used the reverse transcriptase–polymerase chain reaction to analyze TLR2 mRNA expression in peripheral blood monocytes from 17 NTM patients and 10 healthy controls. We also determined the mRNA and protein secretion levels for the cytokines interleukin (IL)-12p40 and tumor necrosis factor (TNF)-α.

After stimulation with *M. avium* or lipoteichoic acid, expression of TLR2 mRNA by peripheral blood monocytes was lower in NTM patients than in healthy controls. IL-12p40 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 lipoteichoic acid-induced IL-12p40 and TNF-α production in healthy controls, but not in NTM patients.

Our results suggest that the downregulation of TLR2 and the resulting decreased production of IL-12p40 and TNF-α following *M. avium* or lipoteichoic acid stimulation may contribute to host susceptibility to NTM lung disease.

KEYWORDS: Atypical mycobacteria, Disease susceptibility, Lung diseases, Toll-like receptor 2

INTRODUCTION

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

NTM are ubiquitous environmental organisms. Because 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 likely have specific susceptibility factors that lead to NTM infections [7].

Toll-like receptors are important pattern-recognition receptors that function in host innate defense against invading pathogens, such as *Mycobacterium tuberculosis*

and NTM [8, 9]. Signaling through these receptors leads to transcription and translation of a variety of cytokines/mediators [10, 11]. Toll-like receptor-2 (TLR2) is required for NTM signaling [12, 13], and increased susceptibility to *Mycobacterium avium* infection was recently reported in TLR2-knockout mice [14]. Furthermore, mice deficient in myeloid differentiation factor 88 (MyD88) 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.

Despite various studies on animals with TLR2 deficiency, and cell-culture studies examining the role of TLR2 in NTM infection and immunity, whether the results reliably reflect the mechanisms of pathogenesis and immunity in humans is unclear. In the present study, we investigated the expression of TLR2 on the peripheral blood monocytes from patients with NTM lung disease and from healthy controls. We also compared the responses of these monocytes to the TLR2 agonists *M. avium* and lipoteichoic acid (LTA) from *Staphylococcus aureus*. We hypothesized that TLR2 expression is lower in patients with NTM lung disease, and thus we expected that the response of peripheral blood monocytes to TLR2 agonists would be suppressed in NTM patients.

PATIENTS AND METHODS

Study population

Seventeen patients with the nodular bronchiectatic form of NTM lung disease and ten healthy volunteers were included in this study (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 CT 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, and written informed consent was obtained from all participants.

Preparation and stimulation of peripheral monocytes

Peripheral blood mononuclear cells were separated from heparinized whole

blood using standard density gradient centrifugation with Ficoll-Hypaque (Sigma, St. Louis, MO). The cells were cultured for 2 h and washed with DPBS (Sigma, St. Louis, MO) to remove nonadherent cells. To ascertain whether adherent cells were monocytes, the cells were labeled with anti-CD14-fluorescein isothiocyanate (FITC) and examined by flow cytometry. For flow cytometric analysis, FITC-labeled anti-CD14 were purchased from eBioscience (San Diego, CA) and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA). Monocytes (1×10^6) were then stimulated with *M. avium* (ATCC 25291; 1×10^6 CFU per well) or LTA from *S. aureus* (10 μg/ml; InvivoGen, San Diego, CA) for 0, 2, 4, 6, or 24 h at 37 °C.

Total RNA isolation, cDNA synthesis, and RT-PCR

Total RNA was isolated using RNAzol™ B (Invitrogen Life Technologies, San Diego, CA), and cDNA synthesis and reverse transcriptase–polymerase chain reaction (RT–PCR) were performed using the AccessQuick™ RNA-PCR system (Promega, Madison, WI). The first-strand cDNA was synthesized from the total RNA at 45 °C for 45 min in a DNA thermal cycler (Hybaid, Teddinton, 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 analyzed 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)-12p40, and tumor necrosis factor (TNF)-α cDNAs are shown in table 2. RT-PCR was performed before stimulation with the TLR2 agonists and again at 2, 4, 6, or 24 h after stimulation.

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 analyzed for cytokine release. The supernatants were stored in aliquots at –70°C until used in experiments. Concentrations of IL-12p40 and TNF-α were determined using commercially available ELISA kits (Biosource, Camarillo, CA).

Addition of anti-human TLR2 antibody

To examine whether a TLR2 signaling blockade would reduce TLR2 agonist-

induced cytokine production, we performed a blocking experiment with anti-human TLR2 antibody. Before *M. avium* or LTA stimulation, peripheral blood monocytes were pre-incubated with anti-TLR2 antibody (10 μg/ml; eBioscience, San Diego, CA) 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-12p40 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 analyzed using nonparametric analysis. Differences in each variable between the patients and controls were evaluated with Mann–Whitney U test. P values < 0.05 were considered statistically significant.

RESULTS

Expression of TLR2 mRNA in peripheral blood monocytes

We 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).

The unstimulated monocytes from NTM patients exhibited decreased TLR2 mRNA levels compared to 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 to 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-12p40 and TNF-α mRNA

Next, we performed a comparative analysis of *M. avium*-induced cytokine gene expression in NTM patients and healthy controls. In healthy controls, *M. avium* stimulation caused levels of mRNA for both IL-12p40 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-12p40 and TNF-α secretion

We also compared the IL-12p40 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-12p40 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-12p40 and TNF-α production

Finally, we stimulated monocytes 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-12p40 production in healthy controls (50% inhibition), a level of inhibition far greater than that observed in patients (8%). Antibody treatment also reduced *M. avium*-induced TNF-α synthesis to a greater extent in controls (51%) than in patients (24%). In addition, post-treatment cytokine secretion was significantly lower (six-fold lower for IL-12p40; two-fold for TNF-α) for monocytes from healthy controls than for those from patients. However, antibody treatment did not significantly change the IL-12p40 or TNF-α secretion responses of patient monocytes to stimulation with *M. avium* or LTA.

DISCUSSION

To our knowledge, this study is the first to examine TLR2 mRNA expression and its relationship to cytokine responses in patients with NTM lung disease. Our data demonstrate that both *M. avium* and LTA induce TLR2 mRNA expression in peripheral blood monocytes from healthy controls and subsequently upregulate IL-12p40 and TNF-α expression and production. In peripheral blood monocytes from NTM patients,

however, the induction of TLR2 mRNA expression (and of expression and production of IL-12p40 and TNF-α) occurred to a significantly lesser extent. Thus, our 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.

Because NTM are ubiquitous organisms, most animals are resistant to NTM infection unless their defense mechanisms have undergone iatrogenic alteration. However, the nature of the precise immune dysfunction that predisposes otherwise healthy persons 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 Th1-type 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 immunologic 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 responsible for *M. avium*-induced upregulation of pro-inflammatory Th1 cytokines in mouse macrophages [12, 13, 19]. Furthermore, TLR2 plays an important roll in controlling NTM infection in animal studies. For example, control of *M. bovis bacillus* Calmette–Guerin in mice after intraperitoneal 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 to 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, we found that *M. avium* and LTA downregulated TLR2 mRNA expression and IL-12p40 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-12p40 and TNF-α are mediated primarily by TLR2.

IL-12 is a major stimulus for IFN-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], our data suggest that downregulation of TLR2 may play a key role in mediating the impairment of host defense, 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 (GT) 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 immunoreactivity of airway cells, but immune response of peripheral blood monocytes may be confounded because of lack of the local immunoregulatory mechanisms present in the lung [29, 30]. Therefore, further studies involving TLR2 mRNA expression and the subsequent cytokine immune responses using bronchoalveolar lavage cells or lung tissue are needed.

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

In conclusion, TLR2 mRNA expression in response to stimulation with *M. avium* or LTA is lower in peripheral blood monocytes from patients with NTM lung disease than in those from healthy controls. LTA- and *M. avium*-induced mRNA

expression and IL-12p40 and TNF-α production were also lower in patients than in controls. Thus, our findings suggest that the downregulation of TLR2 and the subsequent decrease in IL-12p40 and TNF-α production following *M. avium* infection may contribute to host susceptibility to NTM lung disease. This disease is associated with 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 TLR2 and the associated decrease in IL-12p40 and TNF-α production may facilitate the development of immunomodulatory strategies to treat or prevent NTM lung disease.

Acknowledgments

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Table 1. Clinical characteristics of patients with nontuberculos mycobacterial lung disease.

Characteristics		Patients (n=17)
Age (years)		59 (52-64)
Sex	Men	2 (12%)
	Women	15 (88%)
Body mass index (kg/m^2)		20.4 (19.2-21.0)
Etiology	*M. avium-intracellulare* complex	8 (47%)
	M. abscessus	9 (53%)
Sputum AFB smear	Negative	9 (53%)
	Positive	8 (47%)
Smoking history	Non-smoker	17 (100%)
Presenting symptoms	Cough	15 (88%)
	Sputum	12 (71%)
	Hemoptysis	7 (41%)
Underlying diseases	Bronchiectasis	17 (100%)
	Sinusitis	4 (24%)
	History of tuberculosis	3 (18%)
	Diabetes mellitus	3 (18%)

The data are presented as the number (%) or median (interquartile range [IQR]).

AFB: acid-fast bacilli.

Table 2. Primer sequences used for RT–PCR analysis of mRNA expression levels for *β*-actin, TLR2, IL-12p40, and 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-12p40	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

TLR2: Toll-like receptor-2; IL: interleukin; TNF: tumor necrosis factor.

Figure Legends

Figure 1. *Mycobacterium avium*-induced TLR2 mRNA expression in peripheral blood monocytes from patients with NTM lung disease and from healthy controls. Peripheral blood monocytes were plated in a 24-well dish at 1×10^6 cells/well and then treated with *M. avium* ATCC 25291 (10^6 CFU) for the indicated lengths of time. (A) TLR2 mRNA expression was assessed by RT–PCR analysis of isolated total RNA (600 ng), with β-actin as a control. The data shown represent 17 NTM lung disease patients and 7 independent healthy controls. (B) The TLR2 mRNA expression levels of patient and control monocytes are shown at the bottom of the figure (median and 25th-75th interquartile range). TLR2 mRNA expression was significantly lower in patients than in controls after 4 h of stimulation with *M. avium* (*$p < 0.05$; **$p < 0.01$). TLR2 = Toll-like receptor-2; NTM = nontuberculous mycobacteria.

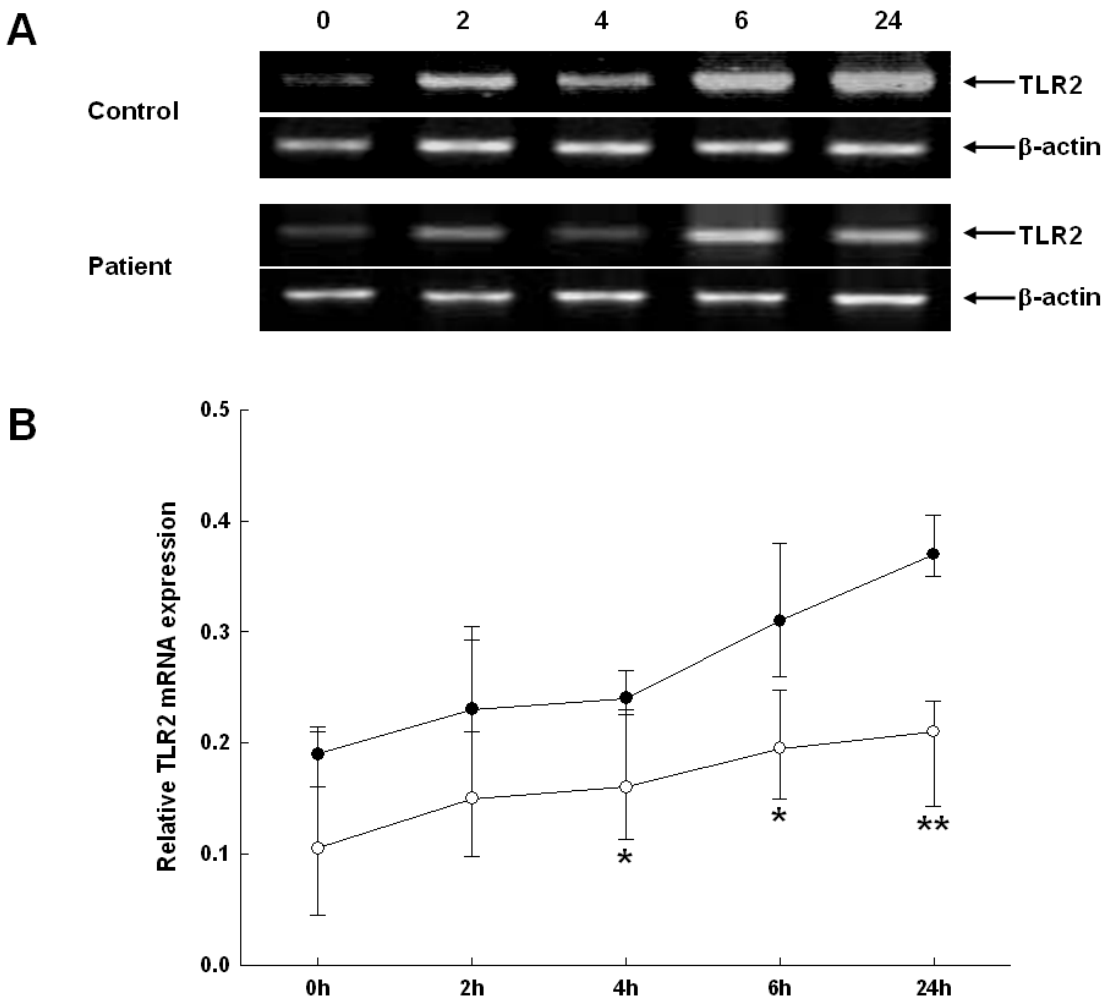

A
0
2
4
6
24
Control
TLR2
β-actin
Patient
TLR2
β-actin
B
Relative TLR2 mRNA expression
0.5
0.4
0.3
0.2
0.1
0.0
0h
2h
4h
6h
24h
*
*
**

Figure 2. *M. avium*-induced IL-12p40 and TNF-α mRNA expression in peripheral blood monocytes from NTM patients and from healthy controls. Peripheral blood monocytes were infected with *M. avium* ATCC 25291 (10^6 CFU) for the indicated lengths of time. (A) Expression of IL-12p40 and TNF-α mRNA was assessed by RT–PCR analysis using β-actin as a control. The data shown represent 12 patients and 4 controls. (B) The IL-12p40 and TNF-α mRNA expression levels are shown at the bottom of the figure (median and 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 (*$p < 0.05$; **$p < 0.01$). IL = interleukin; TNF = tumor necrosis factor; NTM = nontuberculous mycobacteria.

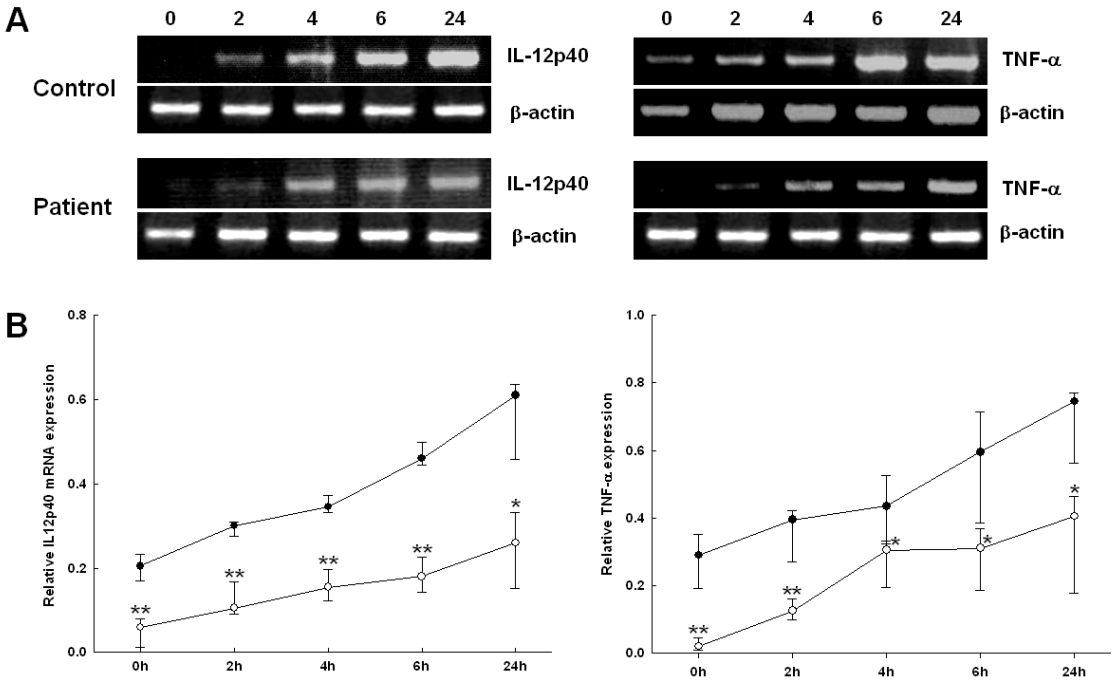


Figure 3. *M. avium*-induced IL-12p40 and TNF-α production in peripheral blood monocytes from NTM patients and healthy controls. Peripheral blood monocytes were infected with *M. avium* ATCC 25291 (10^6 CFU) for the indicated lengths of time. Cell-free supernatants, cleared by centrifugation, were harvested at the indicated times, and secretion of IL-12p40 and TNF-α cytokines was assessed using ELISA (median and 25th-75th interquartile range). Monocytes from 17 patients and 7 healthy controls were compared (*$p < 0.05$; **$p < 0.01$). IL = interleukin; TNF = tumor necrosis factor; NTM = nontuberculous mycobacteria; ELISA = enzyme-linked immunosorbent assay.

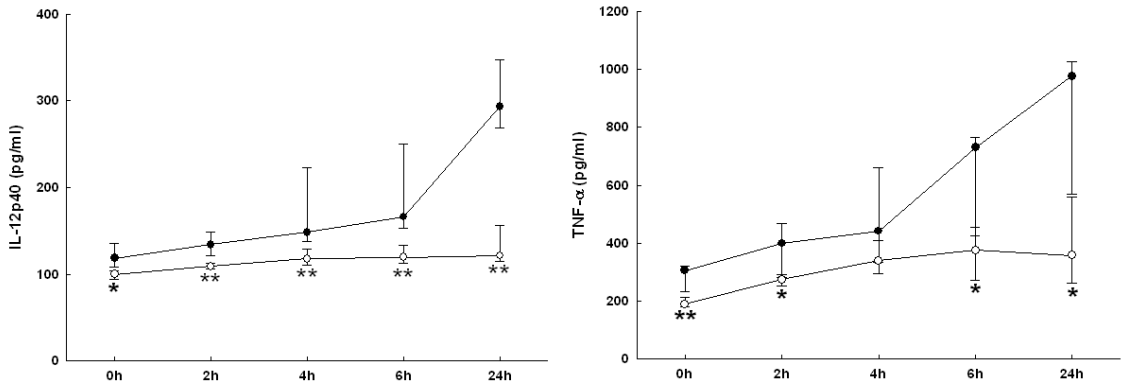
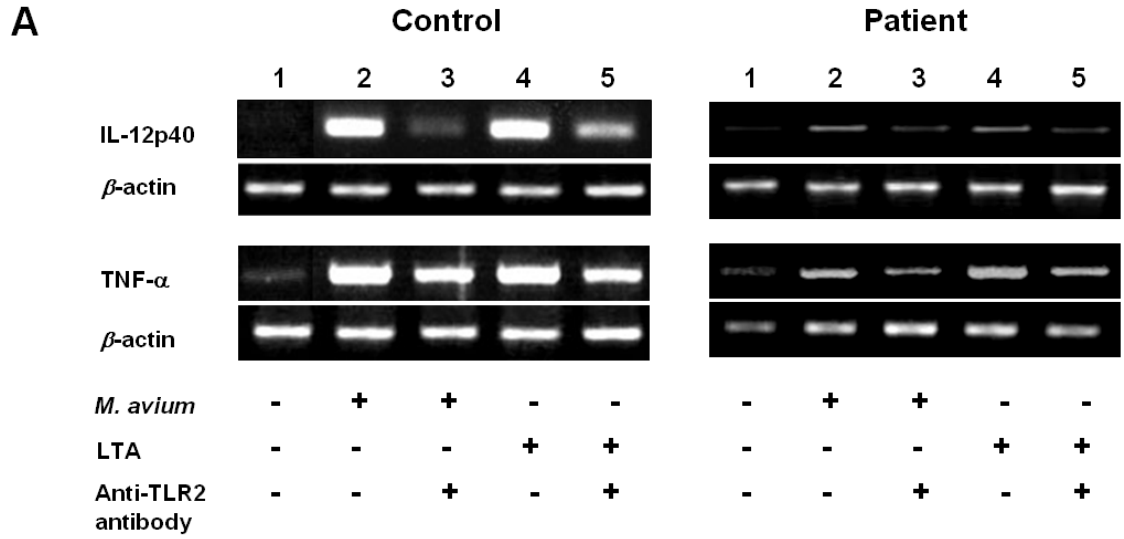
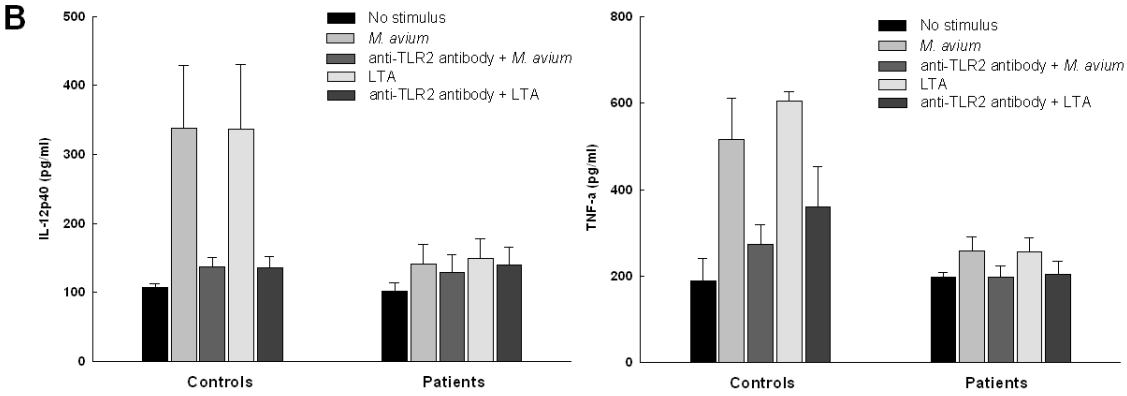


Figure 4. Inhibitory effect of anti-TLR2 antibody on *M. avium*-induced IL-12p40 and TNF-α production in peripheral blood monocytes from NTM patients and healthy controls. The antibody (10 μg/ml) was added to peripheral blood monocytes 30 min before stimulation with *M. avium* ATCC 25291 (10^6 CFU) or LTA for 24 h. (A) Total RNA was isolated, and expression of IL-12p40 and TNF-α mRNA was determined by RT–PCR analysis using β-actin as a control. The data shown represent four patients and three controls. (B) The culture supernatants were harvested for cytokine assessment using ELISA after *M. avium* or LTA stimulation for 24 h. The data shown represent 11 patients and 7 controls. TLR2 = Toll-like receptor-2; IL = interleukin; TNF = tumor necrosis factor; NTM = nontuberculous mycobacteria; LTA = lipoteichoic acid.




B
IL-12p40 (pg/ml)
500
400
300
200
100
0
No stimulus
M. avium
anti-TLR2 antibody + M. avium
LTA
anti-TLR2 antibody + LTA
Controls
Patients
TNF-a (pg/ml)
800
600
400
200
0
No stimulus
M. avium
anti-TLR2 antibody + M. avium
LTA
anti-TLR2 antibody + LTA
Controls
Patients