Mycobacterium abscessus and *M. avium* Trigger Toll-Like Receptor 2 and Distinct Cytokine Response in Human Cells

Elizabeth P. Sampaio[1,2], Houda Z. Elloumi[1], Adrian Zelazny[1], Li Ding[1], Michelle L. Paulson[1], Alan Sher[3], Andre L. Bafica[3]*, Yvonne R. Shea[4], and Steven M. Holland[1]

[1]Laboratory of Clinical Infectious Diseases, [3]Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases; and [4]Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland; and [2]Leprosy Laboratory, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil

Mycobacterium avium **(MAV) and** ***M. abscessus*** **(MAB) are ubiquitous environmental organisms increasingly recognized to cause chronic lung disease in patients with apparently normal immune function. Little is yet known about their human pathophysiology. Our objective was to examine cytokine and chemokine responses (protein and gene expression) and signaling pathways triggered by reference and clinical isolates of MAB and MAV in human peripheral blood mononuclear cells, monocytes, and murine bone marrow–derived macrophages *in vitro*. MAB-induced TNF-α production was higher than that induced by MAV. IFN-γ, IL-1β, and the chemokines macrophage inflammatory protein-1α and regulated on activation, normal T cell expressed and secreted were equally up-regulated. Differences between MAB and MAV do not require replication and are heat stable. We found no differential effect due to rough or smooth colonies within the same species. Similar to MAV, MAB triggered mitogen-activated protein kinase (MAPK) signaling and nuclear factor-κB translocation. Induction of TNF-α was dependent on MAPK pathways, since pre-incubation of cells with signaling inhibitors led to more than 85% reduction in cytokine secretion. MAB also triggered a Toll-like receptor 2 (TLR2)-mediated response that led to TNF-α production by human monocytes. Accordingly, stimulation of murine TLR2- or myeloid differentiation factor 88–deficient bone marrow–derived macrophages did not elicit TNF-α, reinforcing a critical role for TLR2 in MAB-induced cell activation. We concluded that MAB signals human cells through MAPK and TLR2 pathways and triggers more pronounced pro-inflammatory cytokines and chemokines than MAV.**

Keywords: tumor necrosis factor-α; *M. abscessus*; *M. avium*; chemokines; Toll-like receptor 2

Mycobacteria can be categorized into the *M. tuberculosis* (MTB) complex and nontuberculous mycobacteria (NTM). NTM can be further classified by growth rate. *M. avium* (MAV), a slow-growing mycobacterium (SGM), is the most common agent of NTM lung disease, while *M. abscessus* (MAB), an emerging pulmonary pathogen, is responsible for the majority of lung infections due to rapid growers (RGM) (1, 2). In the United States, MAB is the third leading cause of NTM lung infection, is responsible for approximately 80% of RGM lung disease, and is associated with significant morbidity and mortality (3, 4).

NTM cause disseminated disease mainly in those with primary or acquired immune deficiencies (3–5). In contrast, lung disease is predominantly unassociated with recognized immune defects, but is seen in other chronic lung diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis. In addition, NTM lung infection has been increasingly recognized to occur in otherwise apparently normal individuals (5, 6). Despite *in vitro* susceptibilities, MAB lung disease is clinically resistant to most antibiotics and rarely cured, while MAB skin and soft tissue infections are relatively treatable (2, 7). Both TNF-α and IFN-γ play critical roles in protective immunity to mycobacterial infections and immunopathology. The relevance of these cytokines and pathways is reinforced by naturally occurring human mutations in the genes of the IFN-γ/IL-12 axis (8, 9), nuclear factor-κB (NF-κB) essential modulator (NEMO), and the increased susceptibility to mycobacterial infections seen with therapeutic TNF-α antagonists (10, 11).

Mycobacteria trigger signaling pathways, such as mitogen-activated protein kinase (MAPK) and NF-κB, involved in cytokine response and inflammation (12). These responses are linked to engagement of Toll-like receptor 2 (TLR2) and the myeloid differentiation factor 88 (MyD88), as demonstrated for MAV and MTB (13, 14). However, very little information is available on human cellular responses to MAB (15).

It has been postulated that pathogenic mycobacteria successfully reside within macrophages by inhibiting several host processes. Variability among strains is also related to colony morphology, as NTM have long been recognized to have rough and smooth colony phenotypes (16). Because lung disease due to MAB and MAV are inexplicably different, with significant clinical implications, we sought to characterize in the human system the similarities and differences between these two major pathogens. Therefore, we investigated the cytokine and transcriptional responses induced by clinical and reference strains of MAB and MAV, as well as smooth and rough colony morphotypes.

CLINICAL RELEVANCE

This article demonstrates for the first time fundamental differences in the innate immunogenicity between *Mycobacterium abscessus* and *M. avium* in human cells, which may play roles in clinical prevalence and disease manifestations, and may impact treatment.

MATERIALS AND METHODS

Additional detail on the methodology is provided in the online supplement.

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* Present affiliation: Division of Immunology, Department of Microbiology and Parasitology, Federal University of Santa Catarina, Florianopolis, Brazil

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Corresponding author: Dr. Elizabeth Pereira Sampaio, M.D., Ph.D., Laboratory of Clinical Infectious Diseases, Immunopathogenesis Section, National Institutes of Health, NIAID, CRC B3-4141 MSC 1684, Bethesda, MD 20892-1684. E-mail: sampaioe@niaid.nih.gov

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Mycobacteria Cultures

Mycobacteria were grown to logarithmic phase in suspension, at which time aliquots were frozen and stored at −70°C until use. For confirmation of bacterial numbers, representative vials were thawed and enumerated for viable colony-forming units (CFU). NTM reference strains were MAB (ATCC 19977; ATCC, Rockville, MD), *M. avium avium* (MAV; ATCC 35717), *M. intracellulare* (MAI; ATCC 13950) and the nonpathogenic *M. smegmatis* (MSMg; ATCC 14468). Clinical strains were isolated from blood (disseminated; $n = 4$) or sputum (pulmonary; $n = 11$), distributed as follows: MAB, $n = 5$; MAV, $n = 5$; MAI, $n = 2$; and the two new species belonging to the *M. abscessus/M. chelonae* group, *M. massiliense* and *M. bolletii* (17, 18). Mycobacteria samples were also identified as rough ($n = 7$) or smooth ($n = 8$) isolates. For experiments using dead mycobacteria, MAV and MAB were heat-killed (80°C, 30 min) and mycobacteria was found to be greater than 99% nonviable as determined by CFU counts.

Staining of Mycobacteria

For visualization of acid-fast bacilli (AFB) in infected cultures, cells seeded on coverslips were Kinyoun stained and examined by light microscopy. For selected experiments, SYTO9-labeled (BacLight viability staining kit; Molecular Probes, Eugene, OR) live mycobacteria were used to infect the cells, which allowed their detection by flow cytometry or confocal microscopy.

Cell Isolation and Culture

Human peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque gradient centrifugation, and elutriated monocytes were isolated from heparinized venous blood of healthy volunteers (Dept. of Transfusion Medicine, National Institutes of Health, Bethesda, MD) in accordance with approved protocols by the Institutional Review Boards of the National Institutes of Health. Cells were seeded in RPMI 1640 and infected with single-cell suspensions of each mycobacterium for different periods of time. Supernatants were harvested and assayed for detection of cytokines through a multiplex bead-based assay (Bio-Rad Laboratories, Hercules, CA), and the cells processed for RNA isolation.

Bone Marrow–Derived Macrophage Isolation and Culture

Bone marrow–derived macrophages (BMM) were obtained from 6- to 8-week-old C57BL/6, TLR2-, TLR4-, or MyD88-deficient mice as previously described (19, 20). Cells were then cultured in the presence of the mycobacteria for 1, 4, and 20 h, when supernatants and cellular RNA were harvested for further use.

RNA Isolation and Real-Time PCR

Total RNA was extracted from the cultured cells with the RNeasy mini kit (Qiagen, Valencia, CA), reverse transcribed, and the cDNA amplified by PCR in the ABI 7500 Sequence detector using Taqman expression assays (Applied Biosystems, Foster City, CA).

Signaling Inhibition Assays

PBMCs or elutriated monocytes (10^6 cells/well) were pre-treated with the p38 signaling inhibitor SB203580 or the ERK1/2-selective inhibitor U0126 (10 μM) for 30 to 60 minutes before infection and then infected with MAB or MAV. In parallel experiments, cells were pre-incubated (1 h) with anti-TLR2 mAb (4 μg/ml) and stimulated further with the TLR2 agonist Pam3Cys (2 μg/ml) or the mycobacteria. Culture supernatants were harvested and assayed for cytokine activity.

p38 Assay

After stimulation with the mycobacteria, monocytes or PBMCs (3×10^6 cells) were harvested, lysed, and processed for detection of phospho-p38 MAPK using the phospho-p38a enzyme-linked immunosorbent assay (ELISA) as directed by the manufacturer (Cell Signaling, Danvers, MA). For Western blot analysis, samples were separated on 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA), and immunoblotted with the antibodies anti–phospho-p38, anti–total p38 (Cell Signaling), and anti–β-actin (Abcam, Cambridge, MA). Reaction was detected using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ).

Immunofluorescence

To evaluate NF-κB translocation, monocytes (2×10^6 cells) settled on glass coverslips were stimulated with the mycobacteria, the wells were washed, and the cells fixed in PF 3.7%. Cells were permeabilized with PBS-0.5% triton X-100, labeled with a primary antibody against phospho-p65 (Cell Signaling), followed by incubation with the secondary antibody (Chemicon International, Temecula, CA); the nuclei were stained with DAPI and images acquired on a confocal microscope.

Confocal Microscopy

Visualization by confocal microscopy was performed on a Leica SP5 confocal microscope (Leica Microsystems, Exton, PA) using ×20 and ×40 oil immersion objectives NA 1.4. Differential interference contrast (DIC) images were collected simultaneously with the fluorescence images using a transmitted light detector. Images were processed using Leica LAS-AF version 1.6.3 and Adobe Photoshop.

Statistical Analysis

Values are expressed as mean ± SD. Differences between groups were assessed by the paired two-tailed Student's t test and the Mann-Whitney U test, using InStat/Prism software (GraphPad Software, San Diego, CA) with level of significance $P < 0.05$.

RESULTS

MAB Induces More Inflammatory Cytokines than MAV *In Vitro*

To determine the ability of MAB to stimulate cytokine production in primary human cells, PBMCs ($n = 15$) were infected *in vitro* (multiplicity of infection [MOI] 2.5), and the response after 20 hours of culture was compared with that of the well-characterized MAV. MAB induced significantly more TNF-α than *M. avium* ($P = 0.014$, Figure 1A) or MAI ($P = 0.01$, $n = 8$). IFN-γ ($P < 0.01$, Figure 1B) and IL-6 ($P < 0.05$, Figure 1C) were also more strongly induced by MAB than MAV or MAI. Similar profiles were also seen for IL-1β (MAB 4,550 ± 416 pg/ml, MAV 1,870 ± 640 pg/ml, MAI 1,345 ± 310 pg/ml; $P = 0.01$), granulocyte macrophage colony-stimulating factor ($P < 0.05$), and granulocyte colony-stimulating factor ($P < 0.03$). In contrast, production of the anti-inflammatory cytokine TGF-β was negligible during the whole culture period, whereas for IL-10, significant differences between MAB and MAV were noted ($P < 0.05$).

The differential response observed at the protein level was confirmed at the transcriptional level, as well. As shown in Figure 1D, TNF-α, IFN-γ ($P < 0.01$), and IL-6 ($P < 0.05$) mRNA expression in PBMCs was higher for MAB 4 hours after stimulation.

Similar patterns of mycobacteria-induced cytokines were seen regardless of whether purified elutriated monocytes or monocyte-derived macrophages (MDM) were used. The responses of monocytes to increasing bacterial ratios reached a plateau at MOI 5 to 10, with declining TNF-α production at higher MOIs. MAB induced consistently and significantly more TNF-α at all MOI tested (Figure 1E; differences were marginally significant at MOI 50, $P = 0.049$). Human PBMCs behaved similarly to monocytes (data not shown). Even though total cytokine production was much lower in the MDM cultures, the differential cytokine response was maintained (*see* Figure E1 in the online supplement). Interestingly, heat-killed MAB persistently elicited more response than heat-killed MAV both at 4 ($P < 0.05$) or 20 hours culture (Figure 1F, $P = 0.008$).

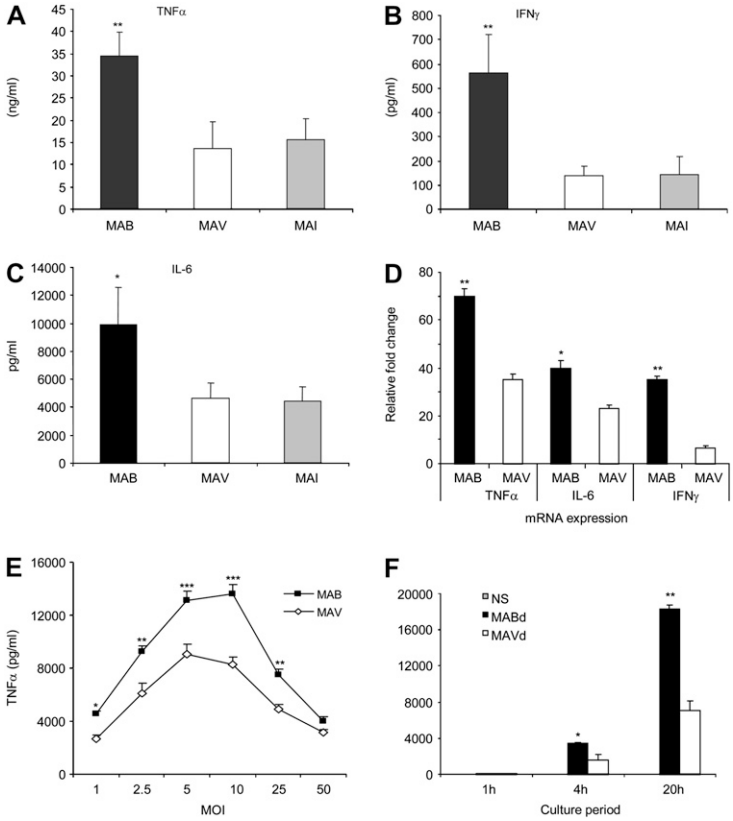

Figure 1. *Mycobacterium abscessus* (MAB) induces higher cytokine response than *M. avium* (MAV). (*A*) TNF-α, (*B*) IFN-γ, (*C*) IL-6 production was assayed in cultured PBMC obtained from normal donors ($n = 15$) after 20 hours of stimulation with MAB, MAV, and *M. intracellulare* (MAI) (mulitplicity of infection [MOI] 2.5). Results represent mean pg/ml ± SD of all experiments performed. NS, nonstimulated cells. (*D*) Differential expression of cytokine mRNA assessed by quantitative RT-PCR in PBMC cultures 4 hours after stimulation with the mycobacteria. Values are mean fold induction (± SD) relative to the NS wells for 10 different donors. GAPDH was used as normalization control. (*E*) TNF-α values were measured in the 20-hour monocytes infected with MAB (*squares*) or MAV (*diamonds*) at different MOI. Cytokine values are presented already diminished from the amounts found in the NS cells ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significant differences when compared with MAV- or MAI-stimulated wells. (*F*) TNF-α assayed in the cultured monocytes ($n = 5$) after 20 hours of stimulation with dead (heat-killed) MAB (MABd) and MAV (MAVd) (MOI 5). *, ** Significant differences when compared with MAVd.

MAB *in vitro* production of the chemokines macrophage inflammatory protein (MIP)-1α and regulated on activation, normal T cell expressed and secreted (RANTES) in culture supernatants was significantly higher than MAV (Figure 2A, $P < 0.01$). In contrast, MIP-1β and monocyte chemoattractant protein-1 (MCP-1) were similarly strongly induced by the different NTM (Figure E2).

MAB- and MAV-induced TNF-α and IFN-γ secretion in PBMC cultures was assessed at 1, 4, 12, and 20 hours (Figure 2B). Although low cytokine levels were noted 1 hour after stimulation (MAB TNF-α 30 ± 47 pg/ml; IFN-γ 4 ± 5.7 pg/ml), by 3 to 4 hours cytokine values were easily detectable. Cytokine induction by MAB was consistently higher and was well established by 4 hours. IL-10 and IL-1β protein levels were detected in the culture supernatants mainly after 20 hours (not shown). This profile was sustained at the mRNA level as well: TNF-α message peaked at 4 hours for both mycobacteria (Figure 2C, *left panel*); for IL-10, peak of response occurred at 12 hours (not shown), whereas for IFN-γ, up-regulation of mRNA was still detected after 20 hours (Figure 2C, *right panel*).

Mycobacteria:Monocyte Association

Cultured monocytes on coverslips were fixed with methanol and Kinyoun stained for determination of rate of AFB infection (Figure 3A). At 4 to 6 hours of culture, bacteria were associated with 15 to 18% of cells (total 200–300 cells counted per slide), increasing to 35 to 40% at 20 hours. After adding the mycobacteria to the PBMC cultures, small aggregates of cells were already seen after 1 to 4 hours. These aggregates progressively increased in number and size after 18 to 24 hours (Figure 3B). Labeling mycobacteria with a fluorescent dye before infection followed by confocal microscopy confirmed the bacteria to be mainly inside the cells and within the cell aggregates (Figures 3C and 3D). The total percentage of CD14+ cells containing labeled bacteria at 20 hours in culture was confirmed by flow cytometer (38.4 ± 6.4% for MAB and 36.6 ± 5.5% for MAV).

MAB and MAV Clinical Isolates Show the Same Profiles as Their Respective Reference Strains

To determine whether the differences observed between the two mycobacterial species were maintained among clinical isolates, monocytes were cultured ($n = 10$) with different clinical samples. Clinical isolates from MAB (clinAB1, clinAB2, clinAB3) and MAV (clinAV1, clinAV2, clinAV3) were assayed in parallel. TNF-α protein and message, and IFN-γ secretion (assessed at 4 and 20 h), confirmed among clinical isolates the significant differences observed between the reference strains (Figures 4A and 4B). In addition, two clinical isolates of MAI (MAIclin1, TNF-α 17,497 ± 1,050 and MAIclin2 15,184 ± 3,995 pg/ml) and of the recently characterized MAB-related rapid growers, *M. massiliense* (*M. mass*.1, 42,176 ± 4,900; *M. mass.*2, 28,147 ± 2,550) and *M. bolletii* (34,303 ± 950 pg/ml), showed similar strain-related differences in TNF production in the infected cells as did reference MAB and MAV (Figure 4C).

To explore further the clinical isolates compared with the reference strains, we grouped mycobacteria according to whether their culture morphotype was rough or smooth. We found no significant differences ($P > 0.05$) in induced TNF-α levels by rough (rg) or smooth (sm) isolates within the same group (MAB or MAV) (MABrg, 38,595 ± 4,557; MABsm, 47,750 ± 7,783; MAVrg, 15,654 ± 3,840; MAVsm, 18,244 ± 6,414pg/ml) (Figure 4D). Accordingly, differences between the reference strains (smooth morphotype) and between MAB and MAV clinical isolates were maintained despite their characteristic morphotypes ($P < 0.05$).

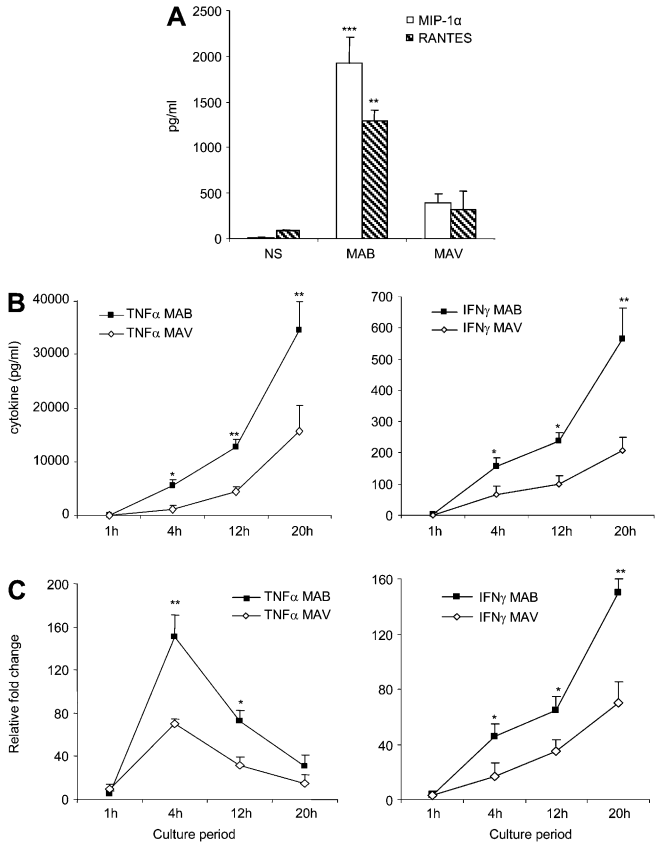

Figure 2. Kinetic cytokine and chemokine induction *in vitro*. (*A*) Chemokines (MIP-1α and RANTES) were evaluated in the 20-hour peripheral blood mononuclear cells (PBMC) cultures after stimulation with MAB or MAV (MOI 2.5). Results are mean (± SD) from five different donors. (*B*) Levels of TNF-α (*left panel*) and IFN-γ (*right panel*) in the PBMC cultures stimulated or not with MAB or MAV were assessed at different time points. Results are averaged values of six independent experiments already subtracted from the NS cells. (*C*) Kinetic evaluation of mRNA expression for TNF-α and IFN-γ in the PBMCs ($n = 6$) after stimulation with both mycobacteria. RNA isolated from the cultures was processed for real time RT-PCR. GAPDH was used as normalization control. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ represent significant differences between MAB and MAV.

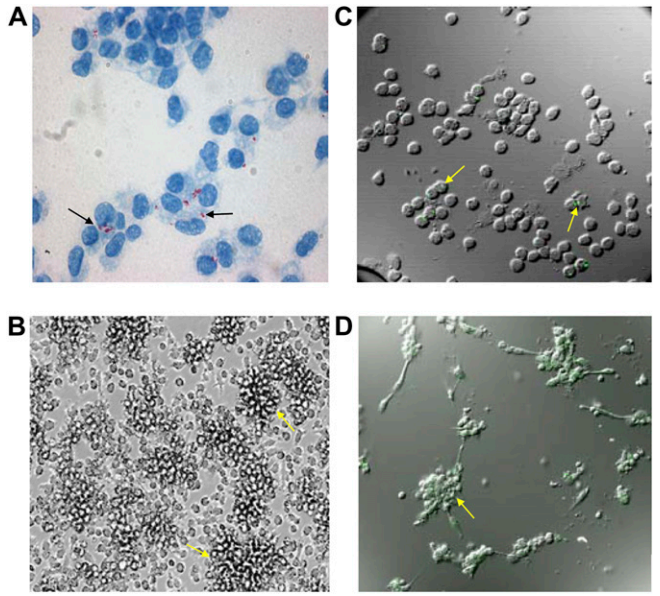

Figure 3. Mycobacteria–cell interaction. (*A*) Kinyoun staining of cultured cells infected with MAB (MOI 5) for 4 hours. Monocytes cultured on coverslips were examined under light microscopy to determine percentage of cells with associated bacteria. *Arrows* indicate acid-fast bacilli (×1,000 magnification with oil immersion). (*B*) Inverted microscopy of the PBMC cultures infected with MAB for 20 hours. Formation of cell aggregates (*arrow*) can be seen already after 4 hours of culture and increased in size with time (×200 magnification). (*C*) Confocal microscopy of monocyte and (*D*) PBMC cultures infected with viable labeled mycobacteria (×40 and ×20 oil immersion, respectively). Merged images show MAB (*arrows*) to be mainly inside the cells and within the cell aggregates (*D*).

To compare MAV and MAB responses to those of nonpathogenic mycobacteria, PBMCs (or monocytes) were challenged with MSMg. MSMg induced significantly more TNF-α than did MAB, MAV, or MAI after 4 (not shown) and 20 hours in culture (Figure E3, $P < 0.05$).

MAB-Induced Response in Human Cells Is Dependent on p38 and ERK1/2

We investigated p38 MAPK activation in primary human monocytes after mycobacterial stimulation *in vitro*. A kinetic analysis of p38 phosphorylation evaluated by an enzymatic binding assay was performed in cultured cells infected with either MAB or MAV (MOI 5) with cell lysates harvested at 2, 10, and 30 minutes, and at 1, 2, 12, and 20 hours. Both MAB and MAV (Figure 5A) triggered p38 MAPK phosphorylation by 2 minutes, which persisted up to 20 hours, without major differences between them. MAPK phosphorylation by ELISA correlated with the expression pattern seen on immunoblot (Figure 5B, also shown by densitometry). Both reference strains and clinical isolates of MAB and MAV induced rapid and sustained p38 MAPK phosphorylation (Figure E4).

To establish whether MAPK activation was necessary for mycobacteria-induced monocytes or PBMC TNF-α production, cells were pre-treated with the p38 MAPK inhibitor SB203580 or the ERK inhibitor UO126 and then challenged with viable mycobacteria. Neither cell viability (trypan blue exclusion) nor extent of infection was affected by the inhibitors (not shown). Pre-treatment of cultures with the p38 MAPK inhibitor SB203580 reduced TNF-α release induced by either MAB and MAV or their corresponding clinical isolates (data not shown) to less than 5% of vehicle control (Figure 5C and Table 1). Results were similar at both MOI 5 and 2.5.

Blocking the ERK 1/2 pathway also reduced TNF-α to less than 20% of vehicle in response to MAB or MAV (Table 1). Both inhibitors showed the same effect whether mycobacteria colony morphotype was rough or smooth (not shown). However, the inhibitors had different impacts on the production of other monocyte-secreted cytokines. In the presence of the p38 inhibitor, IL-10 was greatly reduced (> 90%), whereas IL-6 was only moderately affected; on the other hand, the ERK1/2 inhibitor interfered only partially with IL-6 and IL-10 (35–45% inhibition, Table 1).

MAB Induces NF-κB Translocation in Macrophages

Mycobacteria are known to trigger the NF-κB pathway, which is also involved in cytokine response. We assayed the ability of MAB to induce NF-κB translocation in monocytes or MDM cultures by confocal analysis. Specific staining for phosphorylated p65 showed MAB-induced activation of NF-κB in cultured cells to the same extent as seen with MAV (Figure 6).

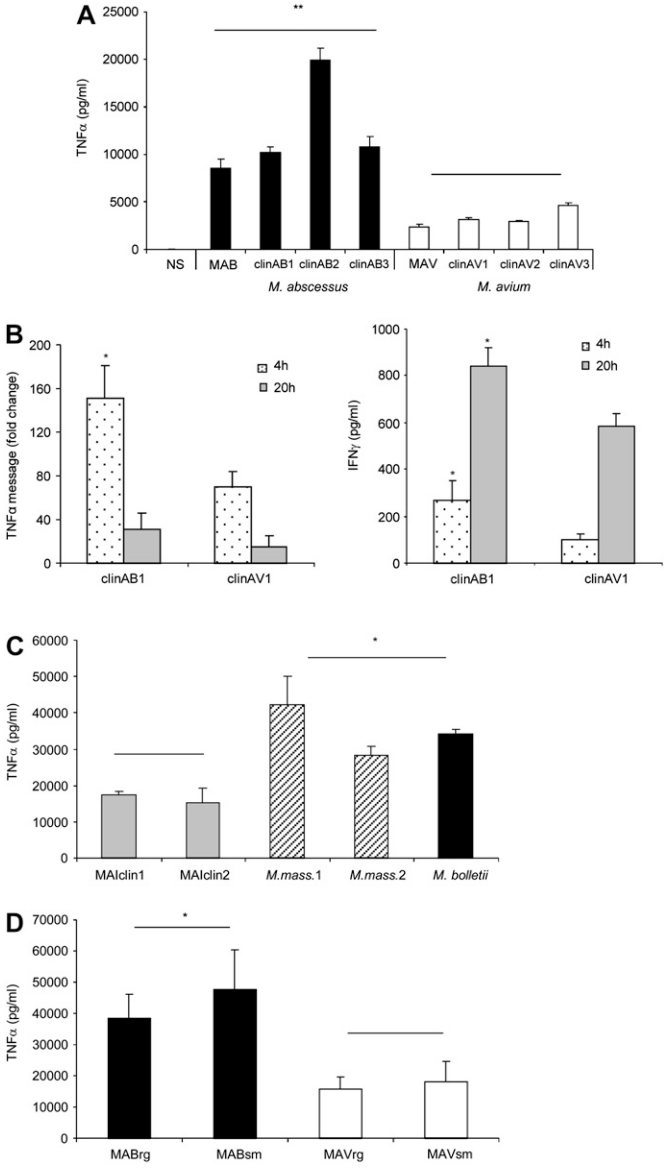

Figure 4. Clinical isolates of MAB and MAV confirm the induced differential cytokine responses from human cells. (*A*) TNF-α values were measured in the 20-hour monocyte cultures infected with the reference strains (MOI 2.5) and the clinical isolates of MAB (clinAB1, clinAB2, clinAB3) and MAV (clinAV1, clinAV2, clinAV3). Results are mean (± SD) of 10 individual experiments. (*B*) TNF-α mRNA expression and IFN-γ release from PBMC in response to the clinical isolates clinAB1 and clinAV1 were assessed 4 and 20 hours after cell stimulation ($n = 6$). *$P < 0.05$, **$P < 0.01$ when compared with MAV isolates. (*C*) Clinical isolates of MAI (MAI clin1 and MAI clin2), *M. massiliense* (*M.mass*.1 and *M.mass*.2), and *M. bolletii* induce differential response in PBMC cultures. TNF-α levels are presented after subtraction of the amounts found in the NS wells. (*D*) MAB or MAV rough (rg) or smooth (sm) were used to infect PBMCs *in vitro* for 20 hours, and supernatants were assayed for TNF-α. Results are average values from five individual experiments. *$P < 0.05$ when compared with MAV or MAI.

TLR2 and MyD88 Mediate MAB and MAV Cytokine Induction

Both p38 and NF-κB signaling pathways are activated by engagement of TLRs. To evaluate whether MAB triggered TLR2 signaling, human monocytes were infected in the presence or absence of an anti-human TLR2 monoclonal antibody. Mycobacteria-induced TNF-α production was reduced by 52 ± 12% in treated cultures (Figure 7A), whereas TNF-α induction by the specific TLR2 agonist Pam3Cys was completely abrogated.

To further confirm the importance of TLR2 signaling for MAB responses, murine BMM cultures from TLR2-deficient and wild-type (WT) C57BL/6 mice were established and infected with either MAB or MAV (MOI 5). After 1, 4, or 20 hours, supernatants were harvested and assayed for cytokines by specific ELISA. TNF-α induction by the two NTM species in the WT BMM cultures, although not statistically significant, showed a trend for higher values in response to MAV earlier in culture (1 and 4 h), and in response to MAB only after 20 hours of stimulation (Figure 7B). Moreover, cells from TLR2- or MyD88-deficient (Figure 7C), but not TLR4−/− mice (not shown), showed no TNF-α protein release or mRNA (Figure 7D) expression after exposure to either NTM. Control experiments showed the response of BMM cells to LPS to be abolished in the TLR4−/− and MyD88−/− mice (Figure E5).

Murine bone marrow–derived dendritic cells (BMDCs) were also generated from both WT and TLR2−/− mice as previously described (20). In contrast to WT BMDC cultures, neither TNF-α nor IL-12p40 were induced at 4 or 20 hours after challenge of TLR2−/−BMDCs with mycobacteria (not shown), confirming an essential role for TLR2 signaling in MAB and MAV interaction with macrophages and dendritic cells.

DISCUSSION

M. abscessus is a significant pulmonary pathogen in patients with bronchiectasis. However, despite its relative importance, very little has been learned about its human pathophysiology. We challenged freshly isolated primary human PBMCs and monocytes with a number of reference and clinical bacterial isolates *in vitro*. Both reference and clinical strains of MAB were more potent inducers of cytokine and chemokine secretion from human mononuclear cells than MAV. This property was somewhat specific, with increased production of TNF-α, IFN-γ, IL-1β, RANTES, and MIP-1α, but not MIP-1β or MCP-1. The prompt enhanced production of both TNF-α and IFN-γ implies a prominent immediate T cell response induced by MAB. To our knowledge, this is the first description of the profile and kinetics of MAB-induced response in primary human cells.

The differential ability of species of NTM (e.g., MAV, MSMg) and MTB to induce TNF-α production seems to be inversely related to their virulence, as assessed in murine models and human macrophages (21–24). Studies in the monocytic cell line THP1 showed similar profiles when comparing BCG, MTB, and *M. leprae* (12, 23, 24). It has been suggested that "virulent" strains elicit less activation of cellular effector mechanisms, thereby escaping early elimination and allowing establishment of infection. In line with this, MAV is a much more common pulmonary and disseminated pathogen than MAB. The additional observation that MSMg induced even higher cytokine response than MAB reinforces this hypothesis.

The dual roles of TNF-α in protection and pathology are well recognized (25). Our observation that MAB induces more inflammatory response than MAV could be associated with the limited number of patients within this infection population compared with MAV, and may explain the devastating lesions induced by MAB when infection is established, which is also consistent with the clinical observation of MAB infection in patients receiving TNF-inhibitor therapies (11).

TNF-α and IFN-γ are central to granuloma formation, directly and through the regulation of chemokines. RANTES and MIP-1α are chemotactic for macrophages, neutrophils, and lymphocytes; are up-regulated during inflammation in pulmo-

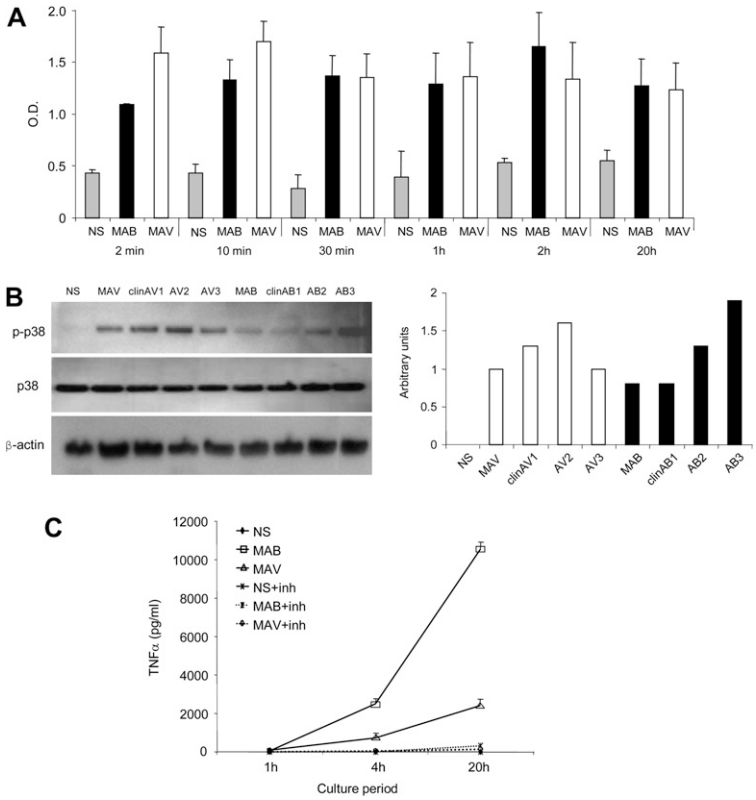

Figure 5. MAV and MAB isolates induce MAPK activation *in vitro*. (*A*) Cell lysates of elutriated purified monocytes (3×10^6) were recovered from the stimulated (MAB and MAV, MOI 5) and nonstimulated (NS) cultures and assayed for p38 MAPK phosphorylation. Up-regulation of p38 MAPK activity was detected by 2 minutes and persisted up to 20 hours. (*B*) Immunoblot analysis of p38 phosphorylation (p-p38) performed on monocytes in the presence of the reference strains and their corresponding clinical isolates (clinAB1, AB2, AB3, and clinAV1, AV2, and AV3) for 10 minutes. Blots were stripped and reprobed with antibodies against total p38 or β-actin to control for protein loaded per lane. Relative densities of the p38 bands were analyzed by densitometry. Results are representative of four independent experiments. (*C*) NTM-induced TNF-α release is dependent on p38 MAPK activity. Monocytes pretreated with the p38 inhibitor SB203580 or DMSO vehicle control were infected with the mycobacteria for the indicated periods. Values are average from five different experiments. NS, cultures treated only with DMSO.

nary alveolar macrophages; and play a role in the pathogenesis of COPD (25–27). It is therefore tempting to hypothesize that in the setting of NTM lung disease MAB-induced up-regulation of RANTES and MIP-1α could exacerbate the clinical picture. *In vitro* models allow study of the expression of these molecules in granulomas (28). The cellular aggregates found in the infected culture wells may be surrogate makers for cell interaction, as reported previously (29) and may well represent the actual site at which the response occurs.

Previous studies have shown that mycobacteria trigger signaling pathways such as MAPK and NF-κB through engagement of TLRs (12, 14, 30–32). Similar to MAV, MAB induces NF-κB nuclear translocation and activates the p38 and ERK1/2 MAPK signaling pathways. Published data have indicated p38 and ERK activation to be required for mycobacteria-induced TNF-α secretion (12, 21, 31). Here we confirmed and demonstrated that these pathways were necessary for appropriate TNF-α response to MAB as well, since specific pharmacologic inhibitors interfered with MAV- and MAB-induced cytokine

TABLE 1. DIFFERENTIAL REQUIREMENTS OF MAPK PATHWAYS FOR NONTUBERCULOUS MYCOBATERIUM–INDUCED CYTOKINE PRODUCTION *IN VITRO*

	P38 inhibitor (% inhibition)					ERK inhibitor (% inhibition)				
Stimuli	1 h		20 h			1 h		20 h		
MAB	TNF	IL-10	TNF	IL-10	IL-6	TNF	IL-10	TNF	IL-10	IL-6
	99	nd	98	93.7	35	75.7	nd	80	44.8	35
MAV	97	nd	98.5	92.1	41.3	69.3	nd	85	40.2	38.7

Monocyte cultures were pre-treated with each of the MAPK inhibitors (SB203580 or UO126) or DMSO for 1 hour and then stimulated either with MAB or MAV (MOI 5). Supernatants were harvested after 20 hours of culture and assayed by multiplex bead assay. Data represent percentage inhibition calculated from the total TNF amount released in the mycobacteria-stimulated pretreated wells versus the mycobacteria-stimulated DMSO-treated wells. Results represent average of five and three independent experiments, respectively; nd = not detected.

Definition of abbreviations: ERK, extracellular signal–regulated kinase; MAB, *Mycobacterium abscessus*; MAPK, mitogen-activated protein kinase; MAV, *M. avium*.

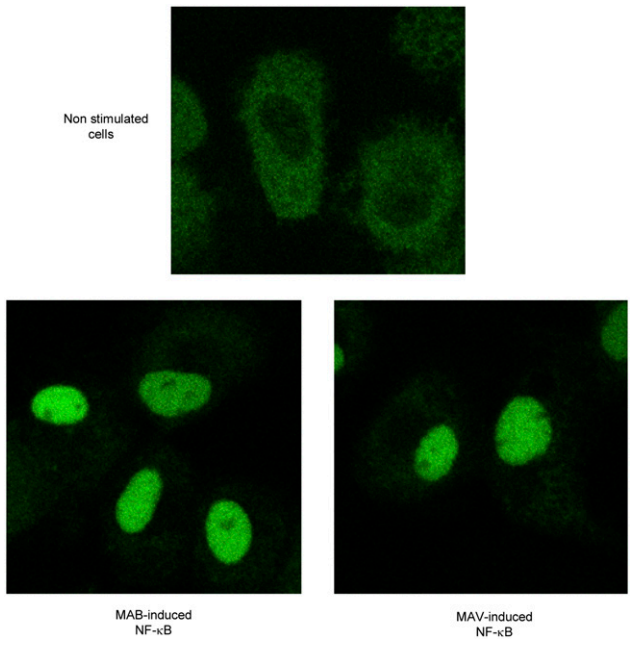

Figure 6. Immunofluorescence staining and confocal microscopy show translocation of NF-κB p65 subunit to the nucleus in MDM cultures after stimulation with MAB and MAV (MOI 5, *lower panels*) as opposed to the nonstimulated cultures (*upper panel*). One representative experiment out of four is presented. Visualization of the cultured cells was performed by confocal microscopy using a ×40 oil immersion objective and a ×4 zoom magnification.

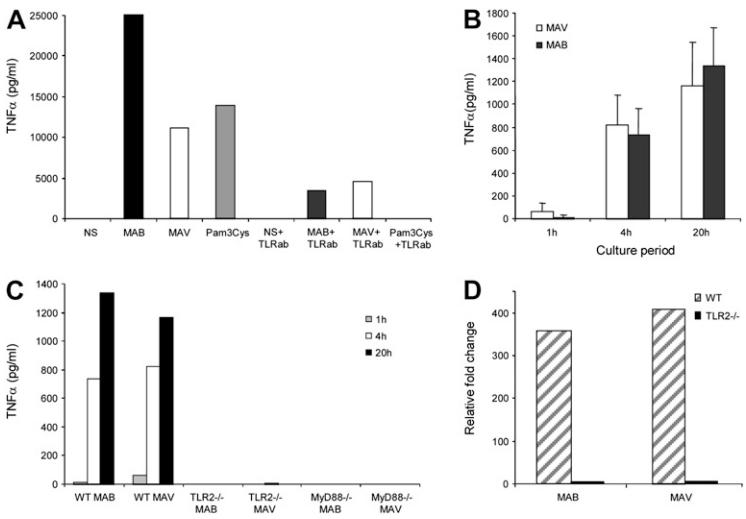

Figure 7. TLR2 mediates MAB-induced cytokine stimulation. (*A*) Cytokine production in response to MAB and MAV (MOI 5) was assessed in cultured human monocytes in the presence or absence of the anti-TLR2 antibody (TLRab, 4 μg/ml). Cells were pre-treated with the antibody and stimulated with the mycobacterial preparations or the TLR2 agonist Pam3Cys (2 μg/ml). The inhibitory effect of TLRab was not observed in cultures treated with the isotype control antibody. Data show one representative experiment out of four. (*B*) Cytokine response induced in C57BL/6 BMM cultures 1, 4, and 20 hours after stimulation with the mycobacteria reference strains (MOI 5). TNF-α measurement (mean pg/ml ± SD) was performed in triplicate. Total of three independent experiments with three mice/group. (*C*) BMM cells were also obtained from WT, TLR2-deficient, or MyD88-deficient mice and infected with MAB or MAV *in vitro*. Cytokine data are subtracted from the NS wells. (*D*) From the same BMM cell cultures obtained from WT and TLR2-deficient mice, TNF-α mRNA expression was assessed at 4 hours by real-time PCR. Results are relative fold change compared with the NS cells. Data show one representative experiment out of three.

secretion. Early signal transduction events induced by NTM also showed differential induction of IL-10 and IL-6. In contrast to a previous study (33), we found induction of those cytokines to be critically dependent on p38 MAPK activity, but only partially dependent on the ERK1/2 pathway. Such differential requirements for the activity of both pathways for TNF and IL-10 release could be related to the type of cell used in the experiments and/or to the bacterial isolates tested, which in the former case were obtained from patients with AIDS (33).

The reproducibility of the relationships between reference and clinical isolates by species is striking. When we tested different clinical isolates of either MAB or MAV, we repeatedly found differential cytokine response in the clinical isolates similar to what was seen between the reference strains. Such an array of responses induced by NTM is likely due to the diverse lipids in the outer layer of the bacterial cell wall including glycolipids, glycopeptidolipids (GPLs), and phenolglycolipids (PGL). PGL, carried by the hypervirulent MTB W-Beijing and the PGL-1 of *M. leprae*, seem to play a major role in pathogenicity by interfering with the host immune system (34–37). Previous studies of the NTM colony morphotypes (16, 38) implied the rough variant to have different or to lack GPLs and induce more inflammatory response than the smooth morphotype (16, 38–40). Surprisingly, despite differences between species, we did not find any significant differences between rough and smooth morphotypes within the same species in terms of cytokine production or MAPK signaling.

There is also a lack of association between mycobacterial colonial morphotype and clinical features in our patients infected with either MAB or MAV (S. M. Holland, unpublished data). Moreover, mycobacterial isolates from the same patient can grow both rough and smooth morphotypes, even from the same clinical sample (Y. Shea, unpublished data).

The genetic and biochemical bases for the different morphotypes remain unclear. Recently, genome sequencing of two RGM that cause human infections, MAB and *M. chelonae* (41), found that GPL genes are scattered, unlike in MSMg, where they are clustered. Such studies may help to better understand lipid synthesis and regulation by the different mycobacteria.

TLRs are activated by components of pathogens, and more than one TLR molecule may mediate protection from and response to mycobacteria (14, 20). Engagement and activation of TLR2 by MAB was confirmed by experiments with both human and mouse cells, similar to what has been shown for MAV (13, 42). Complete abrogation of TLR response in the human system is difficult to achieve *in vitro*, and may reflect either incomplete TLR2 blockade by existing reagents or the existence of other receptors that lead to cell stimulation in response to NTM. However, experiments using cells from TLR2 knockout and MyD88 knockout mice confirmed the nonredundant roles of TLR2 and the downstream adaptor molecule MyD88 (13, 42) in host resistance and response to MAB and MAV.

IFN-γ, T cell response, and TNF-α are essential for control of *M. abscessus* in a mouse model (43). Our data strongly support these findings and show cellular immune mechanisms involved in the early human response to *M. abscessus*. Innate immune responses induced by these mycobacteria may set the stage for clinical features and outcomes that are specific manifestations of infection with MAB and MAV.

Differences between MAB and MAV are seen early after infection, with MAB causing higher responses than MAV as early as 4 hours after stimulation. Heat-killed MAB still elicit more TNF than heat-killed MAV, indicating that the different inflammatory factors between MAB and MAV do not require replication or metabolism and are heat stable. After TLR activation, it is possible that other components that lead to down-regulation of immune/inflammatory response may be more prominently triggered by MAV, including the activation of phosphatases that will ultimately inactivate MAPKs. Such factors can be induced in macrophages by mycobacterial components, such as lipomannan (44), and offers an attractive feedback loop for modulating mycobacterially induced cell response *in vivo*. Experiments to characterize these activities are underway.

In conclusion, the initiation of marked gene transcription and cytokine/chemokine responses induced by NTM *in vitro* begins within minutes after infection and is already significantly different between species at very early time points. This *in vitro* effect may reflect the inflammation and clinical features caused by MAB, and may help to provide new targets for future clinical intervention in NTM induced lung disease.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

1. Dailloux M, Abalain ML, Laurain C, Loos-Ayav C, Lozniewski A, Maugein J; the French Mycobacteria Study Group. Respiratory infections associated with nontuberculous mycobacteria in non-HIV patients. *Eur Respir J* 2006;28:1211–1215.
2. Petrini B. *Mycobacterium abscessus*: an emerging rapid growing potential pathogen. *APMIS* 2006;114:319–328.
3. Marras TK, Daley CL. Epidemiology of human pulmonary infection with non-tuberculous mycobacteria. *Clin Chest Med* 2002;23:553–567.
4. De Groote MA, Huitt G. Infections due to rapidly growing mycobacteria. *Clin Infect Dis* 2006;42:1756–1763.
5. Field SK, Cowie RL. Lung disease due to the more common nontuberculous mycobacteria. *Clin Microbiol Rev* 2002;129:1653–1672.
6. Henry MT, Inamdar L, O'Riordain D, Schweiger M, Watson JP. Nontuberculous mycobacteria in non-HIV patients: epidemiology, treatment and response. *Eur Respir J* 2004;23:741–746.
7. Uslan DZ, Kowalski TJ, Wengenack NL, Virk A, Wilson JW. Skin and soft tissue infections due to rapidly growing mycobacteria: comparison of clinical features, treatment and susceptibility. *Arch Dermatol* 2006;142:1287–1292.
8. Haverkamp MH, van Dissel JT, Holland SM. Human host genetic factors in nontuberculous mycobacterial infection: lessons from single gene disorders affecting innate and adaptive immunity: lessons from molecular defects in IFNγ dependent signaling. *Microbes Infect* 2006;8:1157–1166.
9. Altare F, Durandy A, Lammas D, Emile JF, Lamhamedi S, Le Deist F, Drysdale P, Jouanguy E, Doffinger R, Bernaudin F, *et al.* Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* 1998;280:1432–1435.
10. McDonald DR, Janssen R, Geha R. Lessons learned from molecular defects in nuclear factor kappa B dependent signaling. *Microbes Infect* 2006;8:1151–1156.
11. Doffinger R, Patel SY, Kumararatne DS. Host genetic factors and mycobacterial infections: lessons from single gene disorders affecting innate and adaptive immunity. *Microbes Infect* 2006;8:1141–1150.
12. Hasan Z, Shah BH, Mahmood A, Young DB, Hussain R. The effect of mycobacterial virulence and viability on MAP kinase signaling and TNFα production by human monocytes. *Tuberculosis (Edinb)* 2003;83:299–309.
13. Feng CG, Scanga CA, Collazo-Custodio CM, Cheever AW, Hieny S, Caspar P, Sher A. Mice lacking myeloid differentiation factor 88 display profound defects in host resistance and immune responses to *Mycobacterium avium* infection not exhibited by Toll-like receptor 2 (TLR2)- and TLR4-deficient animals. *J Immunol* 2003;171:4758–4764.
14. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 2007;7:179–190.
15. Byrd TF, Lyons CR. Preliminary characterization of a *Mycobacterium abscessus* mutant in human and murine models of infection. *Infect Immun* 1999;67:4700–4707.
16. Bhatnagar S, Schorey JS. Elevated mitogen-activated protein kinase signaling and increased macrophage activation in cells infected with a glycopeptidolipid-deficient *Mycobacterium avium. Cell Microbiol* 2006;8:85–96.
17. Simmon KE, Pounder JI, Greene JN, Walsh F, Anderson CM, Cohen S, Petti CA. Identification of an emerging pathogen *Mycobacterium massiliense* by *rpoB* sequencing of clinical isolates collected in the United States. *J Clin Microbiol* 2007;45:1978–1980.
18. Adekambi T, Berger P, Raoult D, Drancourt M. *rpoB* gene sequence-based characterization of emerging non-tuberculous mycobacteria with descriptions of *Mycobacterium bolletii* sp. nov., *Mycobacterium phocaicum* sp. nov. and *Mycobacterium aubagnense* sp. nov. *Int J Syst Evol Microbiol* 2006;56:133–143.
19. Ehrt S, Schnappinger D, Bekiranov S, Drenkow J, Shi S, Gingeras TR, Gaasterland T, Schoolnik G, Nathan C. Reprogramming of the macrophage transcriptome in response to interferon-γ and *Mycobacterium tuberculosis*: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. *J Exp Med* 2001;194:1123–1140.
20. Bafica A, Scanga CA, Feng CG, Leifer C, Cheever A, Sher A. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to *Mycobacterium tuberculosis. J Exp Med* 2005; 202:1715–1724.
21. Roach SK, Schorey JS. Differential regulation of the mitogen-activated protein kinases by pathogenic and non pathogenic mycobacteria. *Infect Immun* 2002;70:3040–3052.
22. Freeman S, Post FA, Bekker L-G, Harbacheuski R, Steyn LM, Ryffel B, Connell ND, Kreiswirth BN, Kaplan G. *Mycobacterium tuberculosis* H37Ra and H37Rv differential growth and cytokine/chemokine induction in murine macrophages in vitro. *J Interferon Cytokine Res* 2006;26:27–33.
23. Manca C, Reed MB, Freeman S, Mathema B, Kreiswirth BN, Barry CE III, Kaplan G. Differential monocyte activation underlies strain-specific *Mycobacterium tuberculosis* pathogenesis. *Infect Immun* 2004;72:5511–5514.
24. Theus SA, Cave MD, Eisenach KD. Intracellular macrophage growth rates and cytokine profiles of *Mycobacterium tuberculosis* strains with different transmission dynamics. *J Infect Dis* 2005;191:453–460.
25. Stenger S. Immunological control of tuberculosis: role of tumour necrosis factor and more. *Ann Rheum Dis* 2005;64:24–28.
26. Shi MM, Godleski JJ, Paulauskis JD. Regulation of macrophage inflammatory protein-1α mRNA by oxidative stress. *J Biol Chem* 1996; 271:5878–5883.
27. Bracke KR, Demedts IK, Joos GF, Brusselle GG. CC-chemokine receptors in chronic obstructive pulmonary disease. *Inflamm Allergy Drug Targets* 2007;6:75–79.
28. Puissegur M-P, Lay G, Gilleron M, Botella L, Nigou J, Marrakchi H, Mari B, Duteyrat J-L, Guerardel Y, Kremer L, *et al.* Mycobacterial lipomannan induces granuloma macrophage fusion via a TLR2-dependent, ADAM9- and β1 integrin-mediated pathway. *J Immunol* 2007;178:3161–3169.
29. Birkness KA, Guarner J, Sable SB, Tripp RA, Kellar KL, Barlett J, Quinn FD. An in vitro model of the leukocyte interactions associated with granuloma formation in *Mycobacterium tuberculosis* infection. *Immunol Cell Biol* 2007;85:160–168.
30. Lee S-B, Schorey JS. Activation and mitogen-activated protein kinase regulation of transcription factors Ets and NF-kB in mycobacterium-infected macrophages and role of these factors in tumor necrosis alpha and nitric oxide synthase 2 promoter function. *Infect Immun* 2005;73:6499–6507.
31. Yadav M, Clark L, Schorey JS. Macrophage's pro-inflammatory response to a mycobacterial infection is dependent on sphingosine kinase-mediated activation of phosphatidylinositol phospholipase C, protein kinase C, ERK1/2, and phosphatidylinositol 3-kinase. *J Immunol* 2006;176:5494–5503.
32. Blumenthal A, Lauber J, Hoffmann R, Ernst M, Keller C, Buer J, Ehlers S, Reiling N. Common and unique gene expression signatures of human macrophages in response to four strains of *Mycobacterium avium* that differ in their growth and persistence characteristics. *Infect Immun* 2005;73:3330–3341.
33. Reiling N, Blumenthal A, Flad HD, Ernst M, Ehlers S. Mycobacteria-induced TNFα and IL-10 formation by human macrophages is differentially regulated at the level of mitogen-activated protein kinase activity. *J Immunol* 2001;167:3339–3345.
34. Krzywinska E, Krzywinski J, Schorey JS. Phylogeny of *Mycobacterium avium* strains inferred from glycopeptidolipid biosynthesis pathway genes. *Microbiology* 2004;150:1699–1706.
35. Reed MB, Domenech P, Manca C, Su H, Barczak AK, Kreiswirth G, Kaplan G, Barry CE III. A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature* 2004;431: 84–87.
36. Krzywinska E, Bhatnagar S, Sweet L, Chatterjee D, Schorey JS. *Mycobacterium avium* 104 deleted of the methyltransferase D gene by allelic replacement lacks serotype-specific glycopeptidolipids and shows attenuated virulence in mice. *Mol Microbiol* 2005;56: 1262–1273.
37. Hashimoto K, Maeda Y, Kimura H, Suzuki K, Masuda A, Matsuoka M, Makino M. *Mycobacterium leprae* infection in monocyte-derived dendritic cells and its influence on antigen-presenting function. *Infect Immun* 2002;70:5167–5176.
38. Pedrosa J, Florido M, Kunze ZM, Castro AG, Portaels F, McFadden J, Silva MT, Appelberg R. Characterization of the virulence of *Mycobacterium avium* complex (MAC) isolates in mice. *Clin Exp Immunol* 1994;98:210–216.
39. Howard ST, Rhoades E, Recht J, Pang X, Aslup A, Kolter R, Lyons CR, Byrd TF. Spontaneous reversion of *Mycobacterium abscessus* from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype. *Microbiology* 2006;152:1581–1590.
40. Catherinot E, Clarissou J, Etienne G, Ripoll F, Emile JF, Daffe M, Perronne C, Soudais C, Gaillard JL, Rottman M. Hypervirulence of a rough variant of the *Mycobacterium abscessus* type strain. *Infect Immun* 2007;75:1055–1058.
41. Ripoll F, Deshayes C, Pasek S, Laval F, Beretti JL, Biet F, Risler JL, Daffe M, Etienne G, Gaillard JL, *et al.* Genomics of glycopeptidolipid biosynthesis in *Mycobacterium abscessus* and *M. chelonae. BMC Genomics* 2007;8:114–122.

42. Sweet L, Schorey JS. Glycopeptidolipids from *Mycobacterium avium* promote macrophage activation in a TLR-2- and MyD88-dependent manner. *J Leukoc Biol* 2006;80:415–423.
43. Rottman M, Catherinot E, Hochedez P, Emile JF, Casanova JL, Gaillard JL, Soudais C. Immune control of the rapid-grower *Mycobacterium abscessus* in C57BL/6 mice: importance of T cells, interferon gamma and tumor necrosis factor. *Infect Immun* 2007;75:5898–5907.
44. Elass E, Coddeville B, Kremer L, Mortuaire M, Mazurier J, Guerardel Y. Mycobacterial lipomannan induces MAP kinase phosphatase-1 expression in macrophages. *FEBS Lett* 2008;582:445–450.