Intracellular network of phosphatidylinositol 3-kinase, mammalian target of the rapamycin/70 kDa ribosomal S6 kinase 1, and mitogen-activated protein kinases pathways for regulating mycobacteria-induced IL-23 expression in human macrophages

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Summary
We previously demonstrated that Mycobacterium tuberculosis (M. tbc)-induced interleukin (IL)-12 expression is negatively regulated by the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) 1/2 pathways downstream from PI3K in M. tbc-induced IL-23 expression in human MDMs. To extend these studies, we examined the nature of the involvement of toll-like receptors (TLRs) and intracellular signaling pathways downstream from PI3K in M. tbc-induced IL-23 expression in human MDMs. M. tbc-induced Akt activation and IL-23 expression were essentially dependent on TLR2. Blockade of the mammalian targets of rapamycin (mTOR)/70 kDa ribosomal S6 kinase 1 (S6K1) pathway by the specific inhibitor rapamycin greatly enhanced M. tbc-induced IL-12/IL-23 p40 (p40) and IL-23 p19 (p19) mRNA and IL-23 protein expression. In sharp contrast, p38 mitogen-activated protein kinase (MAPK) inhibition abrogated the p40 and p19 mRNA and IL-23 protein expression induced by M. tbc. Furthermore, the inhibition of PI3K-Akt, but not ERK 1/2 pathway, attenuated M. tbc-induced S6K1 phosphorylation, whereas PI3K inhibition enhanced p38 phosphorylation and apoptosis signal-regulating kinase 1 activity during exposure to M. tbc. Although the negative or positive regulation of IL-23 was not reversed by neutralization of IL-10, it was significantly modulated by blocking TLR2. Collectively, these findings provide new insight into the homeostatic mechanism controlling type 1 immune responses during mycobacterial infection involving the intracellular network of PI3K, S6K1, ERK 1/2 and p38 MAPK pathways in a TLR2-dependent manner.

Introduction
Mycobacterium tuberculosis (M. tbc), responsible for at least 1.5 million deaths per year worldwide, is a slow-growing acid-fast bacillus transmitted primarily by the respiratory route. Binding and uptake of pathogens by macrophages and dendritic cells via innate ‘pattern recognition receptors’ such as toll-like receptor (TLR) family members is the first step in the encounter between the human host immune system and invading bacteria (Ottenhoff et al., 2005). The TLR stimulation increased production of interleukins (IL)-12 and 23, leading to antigen-presenting cells (APCs) with enhanced and sustained T helper type 1-polarizing capacity (Napolitani et al., 2005). Mycobacteria are such strong IL-12 and IL-23 inducers that mycobacterial infection can skew the response to a secondary antigen (Ag) towards a Th1 phenotype (Verreck et al., 2004). IL-12 is a heterodimeric cytokine (p70) comprising disulphide-linked p40 and p35 subunits (Ma and Trinchieri, 2001) and favours the differentiation of Th1 cells (Trinchieri, 2003). In addition, a novel, four-α helix molecule, p19, has been identified that is structurally similar to p35 and forms a disulphide-bonded heterodimer with the p40 subunit (Oppmann et al., 2000); the p19/p40 heterodimer is termed IL-23. Similar to IL-12, IL-23 induces interferon (IFN)-γ secretion from T cells (Hickman et al., 2002) and is involved in type 1 immune defence against mycobacteria (Cooper et al., 2002; Ottenhoff et al., 2002). Both IL-12 and IL-23 play an important role in bridging innate and adaptive responses (Langrish et al., 2004). An increasing awareness of the significance of signal
transduction mechanisms in mycobacterial infection has given rise to develop potentially promising new strategies in antimycobacterial treatment. Mycobacteria triggers several intracellular signalling cascades, such as phosphatidylinositol 3-kinase (PI3K) (Maiti et al., 2001) and mitogen-activated protein kinases (MAPKs) cascades, including extracellular signal-regulated kinase (ERK) 1/2, p38 kinase, and stress-activated protein kinase/c-Jun NH2-terminal kinase 1/2 (Chan et al., 2001; Jones et al., 2001). Previous studies show that PI3K is an endogenous suppressor of IL-12 production triggered by TLR signalling and limits excessive Th1 polarization (Fukao and Koyasu, 2003). Although previous studies suggest a various signalling enzyme cascades following mycobacterial infection, little is known about the early, receptor proximal signalling mechanisms underlying mycobacteria-mediated induction of IL-23.

Several proteins or enzymes have been reported to play a role downstream PI3K signalling. Among them, the 70 kDa ribosomal S6 kinase 1 (S6K1) is an important effector for a growth factor- and nutrient-regulated signalling network containing the PI3K and target of rapamycin (TOR) (Burnett et al., 1998; Pullen et al., 1998). Although the general role played by S6K1 has been investigated (Huang and Houghton, 2003; Atkins et al., 2004), its specific role in monocytes/macrophages is largely unknown. Previous reports propose a role of p38 MAPK, because PI3K inhibition results in an increase in the activity of p38 MAPK (Fukao et al., 2002). In addition, apoptosis signal-regulating kinase 1 (ASK1) is an upstream activator of p38 MAPK during exposure to oxidative stress and other stressful stimuli. TLR2 ligands have capabilities to induce ASK1-dependent signalling pathways which regulate p38 MAPK activation, leading to activation of NF-κB and AP-1 (Into and Shibata, 2005).

In a previous study, we demonstrated that M. tbc- or mycobacterial Ag-induced IL-12 expression is negatively regulated by PI3K and ERK 1/2 pathways in human monocytes. We also found that PI3K activity is required for the M. tbc-induced phosphorylation of ERK 1/2 activation (Yang et al., 2006). In this study, we further investigated the intracellular regulatory network of the PI3K-Akt, mammalian targets of rapamycin (mTOR)/S6K1, ERK and p38 MAPK signalling pathways for the M. tbc-induced expression of IL-23 (p40/p19) in human monocyte-derived macrophages (MDMs). Here we generated the M1 type macrophages, which promote type 1 cellular immunity in antimycobacterial host defence, whereas M2 are poor APC for supporting type 1 immunity (Verreck et al., 2004). Our data demonstrate the differential role of S6K1 and p38 MAPK pathways downstream of PI3K pathway in M. tbc-induced IL-23 synthesis. Further, the nature of the involvement of TLRs in M. tbc-induced IL-23, and its regulation are investigated in human MDMs.

Results

M. tbc-induced expression of IL-12/IL-23 p40 (p40) and IL-23 (p40/p19) is dependent on TLR2, but not TLR4

We recently reported that M. tbc H37Rv-induced p40 and p35 mRNA expressions are negatively regulated by PI 3-K and ERK 1/2 pathways (Yang et al., 2006). These findings raised an issue of whether IL-23 is also induced by human MDMs with M. tbc stimulation and regulated by PI3K signalling. We first examined the p19 and p40 mRNA expression by human MDMs after treatment with M. tbc (moi = 1) for various time-courses. The highest peak of M. tbc-induced p40 mRNA expression was observed between 3 and 6 h, as described previously (Yang et al., 2006). Similarly, we observed that p19 mRNA expression peaked between 3 and 6 h after M. tbc treatment (Fig. 1A). As similar to the previous data with IL-12 kinetics (Yang et al., 2006), the IL-23 protein peaked at 18 h after M. tbc stimulation (data not shown). When human MDMs (n = 5) were incubated with M. tbc in ratios of 0.1, 1 and 10 bacteria per cell, moi-dependent increases in p19 gene expression and p40 production were noted in human MDMs (Fig. 1B). In subsequent experiments, we collected the mRNA and supernatants at 6 and 18 h, respectively, after treatment of M. tbc at a moi of 1.

To examine the extent to which TLRs are responsible for the M. tbc-induced cytokine responses, we incubated MDMs with or without anti-TLR2 monoclonal antibody (mAb), anti-TLR4 mAb or isotype-matched control (IC) mAb for 30 min before treatment with M. tbc. As shown in Fig. 1C, the M. tbc-induced p40 protein secretion was strongly inhibited (68.06%) with anti-TLR2 mAb treatment, whereas it was not changed by pretreatment with anti-TLR4 mAb or IC mAb. Because p40 can pair with either p35 or p19 to form IL-12 (p40/p35) and IL-23 (p40/p19) respectively, we studied the capacity of MDMs to produce IL-23 using soluble IL-23-specific enzyme-linked immunosorbent assay (ELISA). In a manner similar to that of p40 regulation, the M. tbc-induced IL-23 protein production was significantly inhibited (68.06%) with anti-TLR2 mAb treatment, whereas it was not changed by pretreatment with anti-TLR4 mAb or IC mAb. Because p40 can pair with either p35 or p19 to form IL-12 (p40/p35) and IL-23 (p40/p19) respectively, we studied the capacity of MDMs to produce IL-23 using soluble IL-23-specific enzyme-linked immunosorbent assay (ELISA). In a manner similar to that of p40 regulation, the M. tbc-induced IL-23 protein production was significantly inhibited (68.06%) with anti-TLR2 mAb, but not by anti-TLR4 mAb (Fig. 1C, right panel). In addition, M. tbc-induced p40 and p19 mRNA expression was prevented by blocking TLR2, but not TLR4 (Fig. 1D). These results indicate that the M. tbc-induced IL-23 expression is mainly mediated via TLR2.

M. tbc-induced Akt phosphorylation is TLR2-dependent

Further, we sought to assess whether M. tbc-induced Akt activation acts through TLR2 or TLR4 signalling. The M. tbc-induced Akt phosphorylation was detected within 5–15 min, and the peak activation of Akt occurred within 15 min of stimulation with M. tbc (Fig. 2A), in agreement with previous report (Yang et al., 2006). To examine the extent to which TLRs are responsible for the M. tbc-
induced Akt phosphorylation, MDMs were incubated with or without anti-TLR2 mAb, anti-TLR4 mAb or IC mAb for 30 min before treatment with M. tbc, and then further incubated for 15 min. As shown in Fig. 2B, the M. tbc-mediated Akt phosphorylation was dose-dependently inhibited in MDMs by pretreatment with anti-TLR2 mAb, but not IC Ab. In addition, M. tbc-mediated Akt phosphorylation was not changed by anti-TLR4 mAb (data not shown).
To further determine which TLRs may play a specific role in the response to *M. tbc*, we chose to examine the *M. tbc*-induced Akt phosphorylation in human embryonic kidney (HEK) 293 cells stably transfected with TLR2, or TLR4. In the representative experiments (a total of three) shown in Fig. 2C, the *M. tbc*-induced Akt phosphorylation was significantly inhibited by specific mAbs in a dose-dependent manner in HEK/TLR2, whereas it was not changed in HEK/TLR4 cells. This implies that blockade of Akt phosphorylation by *M. tbc* is highly specific for TLR2, but not for TLR4. Taken together, these results indicate that the *M. tbc*-induced Akt phosphorylation is mainly mediated via TLR2, and not TLR4.

*M. tbc*-induced S6K1 phosphorylation negatively regulates p40 and p19 expression downstream from the PI3K pathway

We previously found that both PI3K and ERK 1/2 pathways are essential for the *M. tbc*-induced p40 expression, and that ERK 1/2 plays a role downstream PI3K signalling (Yang et al., 2006). In the subsequent work, we were to further address the detailed intracellular network underlying the regulation of IL-23 expression in response to *M. tbc* stimulation. Based on the findings presented in our recently published work (Yang et al., 2006), the working model that we propose for the mechanism involved in *M. tbc*-induced IL-12/IL-23 expression is outlined in Fig. 3A. As widely described, S6K1 is one of the critical effectors of PI3K and TOR (Burnett et al., 1998; Pullen et al., 1998). We first examined whether the S6K1 pathway is activated by *M. tbc* in human MDMs. *M. tbc* induced the phosphorylation of S6K1 at Ser421/Thr424 and Thr-389 within 15 min of stimulation (Fig. 3B). The peak activation of S6K1 at Thr-389, and at Ser421/Thr424 in human MDMs occurred within 15–30 min of stimulation with *M. tbc*, slightly later than Akt phosphorylation (Fig. 3B).

To confirm that the S6K1 activation is specifically induced by MDMs after stimulation with *M. tbc*, we next examined whether the *M. tbc*-induced S6K1 phosphorylation is blocked by pretreatment with specific pharmaco-
logic inhibitor, rapamycin, because this compound is routinely used for specific inhibition of the molecule mTOR/S6K1 (Abraham and Wiederrecht, 1996). As expected, treatment with rapamycin completely inhibited S6K1 phosphorylation (Fig. 3C).

To define the role of mTOR/S6K1 in \( M. \text{tbc} \)-induced cytokine synthesis, \( p40 \) and \( p19 \) mRNA expression were determined in cultures pretreated with rapamycin using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and ELISA assay. Fig. 3D–F shows a strong...
dose-dependent enhancement of IL-23 (p40/p19) mRNA and protein production in *M. tbc*-treated MDMs after pre-treatment with rapamycin, whereas expression of an independent housekeeping gene, β-actin was not affected.

As our previous studies (Yang et al., 2006) demonstrated, *M. tbc*-induced ERK 1/2 phosphorylation was inhibited in the presence of LY294002 or wortmannin. Therefore, we examined whether *M. tbc*-induced S6K1 activation is modulated by the activity of PI3K. As shown in Fig. 4, the inhibition of PI3K, but not ERK 1/2, significantly attenuated the *M. tbc*-induced S6K1 phosphorylation (at Ser421/Thr424 and at Thr-389; 55–70% at 10 µM LY294002; Fig. 4B) in human MDMs, indicating that PI3K activity is required for the *M. tbc*-induced phosphorylation of S6K1 and ERK 1/2 activation.

**M. tbc**-induced p38 MAPK activation is essential for p40 and p19 expression

As the p38 MAPK pathway is pivotal in TNF-α formation...
induced by \textit{M. tbc} or PPD Ag (Song \textit{et al.}, 2003; Jung \textit{et al.}, 2005), we next investigated whether \textit{M. tbc}-induced IL-23 expression requires p38 MAPK activity in MDMs. Similar to S6K1 phosphorylation, the peak activation of p38 MAPK in MDMs occurred within 15–30 min of stimulation (the time range was donor-dependent) with \textit{M. tbc} (Fig. 5A).

To examine the role of p38 MAPK in the \textit{M. tbc}-induced IL-23 expression, MDMs were pretreated with SB203580, a specific pharmacologic inhibitor of p38, for 45 min before adding \textit{M. tbc} to the cultures, and cytokine mRNA and protein expression were assayed at 6 and 18 h respectively. Fig. 5B–D shows that p40 and p19 mRNA and IL-23 protein production in \textit{M. tbc}-treated MDMs were significantly inhibited by the specific p38 MAPK inhibitor SB203580 in a dose-dependent manner. These results show that IL-23 expression by \textit{M. tbc} is essentially dependent on the p38 MAPK pathway at both the transcriptional and translational levels.

\textit{PI3K inhibition enhances \textit{M. tbc}-induced p38 MAPK phosphorylation, and ASK1 activity in human MDMs}

We also assessed the effect of PI3K on p38 MAPK activation. As shown in Fig. 6, the inhibition of PI3K significantly increased the \textit{M. tbc}-induced p38 MAPK phosphorylation in human MDMs in a dose-dependent manner (Fig. 6A). Previous studies have shown that mycoplasmal lipoproteins and staphylococcal peptidoglycans can induce ASK1-dependent signalling pathways, which regulate p38 MAPK activation via TLR2 (Into and Shibata, 2005). Therefore, we also investigated whether ASK1 activity is regulated by PI3K inhibition in our system. As shown in Fig. 6B, using an ASK1 activity assay, we demonstrated that PI3K inhibition dose-dependently enhanced ASK1 activation in human MDMs. These results indicate that PI3K inhibition negatively modulates p38 MAPK and its upstream kinase ASK1.
The role of PI3K activity in modulating or 0.3 pre-incubated with LY294002 (1, 5 or 10 \( \mu \)M) for 45 min before adding \( M. \) tbc. Whole-cell lysates were prepared at 30 min, and assessed for the phosphorylation states for p38 MAPK by immunoblotting (panel A). Whole-cell protein was obtained at 10 min, and assessed for ASK1 kinase activity using ASK1 kinase assay (panel B). A representative experiment of three independent replicates with similar results is shown in panels A and B. UI, uninfected; D, DMSO control.

Role of IL-10 in PI3K, ERK 1/2 or S6K1-mediated enhancement of p40 synthesis by \( M. \) tbc-treated MDMs

As IL-10 negatively affects the production of inflammatory cytokines, including IL-12, by lipopolysaccharide (LPS)-stimulated monocytes (Snijders et al., 1996), we investigated whether enhanced p40 production induced by PI3K, S6K1 or MEK1 inhibition was the result of suppressed IL-10 production by \( M. \) tbc-infected MDMs. To investigate this hypothesis, we first examined the effect of specific inhibitors on IL-10 mRNA expression in \( M. \) tbc-treated MDMs. As shown in Fig. 7A, IL-10 mRNA expression was significantly lowered by the specific inhibitors of PI3K (LY294002) in a dose-dependent manner. However, there were no significant changes in \( M. \) tbc-induced IL-10 mRNA expression by pretreatment with specific inhibitors of MEK (PD98059) or mTOR/S6K1 (rapamycin; Fig. 7A). These data were also confirmed at protein levels (Fig. 7B).

We also investigated whether pretreatment with a neutralizing anti-IL-10 mAb would induce a significant increase in the levels of p40 in the absence or presence of specific inhibitors compared with untreated cells. In the absence of inhibitors, the pretreatment with a neutralizing anti-IL-10 mAb significantly increased the \( M. \) tbc-induced p40 protein secretion in MDMs (\( P < 0.05; \) Fig. 7C). However, in the presence of inhibitors, no significant differences were observed in the p40 levels between cultures pretreated with and without a neutralizing anti-IL-10 mAb.

These results reveal that the negative regulation of p40 levels induced in \( M. \) tbc-treated MDMs by the PI3K, ERK 1/2 and mTOR/S6K1 pathways are not solely attributable to the inhibition of IL-10 production.

TLR2 is partially responsible for regulating \( M. \) tbc-induced IL-23 protein secretion

As \( M. \) tbc-induced Akt phosphorylation and IL-23 expression were dependent on TLR2 (Figs 1 and 2), we investigated whether pretreatment with anti-TLR2 mAb modulates the regulation of IL-23 levels in human MDMs. As shown in Fig. 8, the \( M. \) tbc-induced secretion of p40 and IL-23 (p40/p19) was significantly decreased in MDMs in a presence of anti-TLR2 mAb, compared with the cells in an absence of anti-TLR2 mAb (\( P < 0.001 \)). In addition, a significant decrease in the levels of \( M. \) tbc-induced p40 and IL-23 was observed in MDMs pretreated with inhibitors (LY294002, PD98059, rapamycin or SB203580) in a presence of a neutralizing anti-TLR2 mAb compared with that in an absence of anti-TLR2 mAb (\( P < 0.001 \)). However, the negative or positive regulation for IL-23 protein expression was not completely abrogated by pretreatment with anti-TLR2 mAb. These results suggest that the regulation of \( M. \) tbc-induced IL-23 expression by the PI3K, mTOR/S6K1, ERK 1/2 and p38 pathways is modulated in TLR2-dependent and -independent manners.

Discussion

Although previous studies have postulated the existence of various signalling enzyme cascades following mycobacterial infection, little is known of the early, receptor-proximal signalling mechanisms underlying the \( M. \) tbc-mediated induction of IL-23 p40 and p19 expression. In our recent studies, we showed that \( M. \) tbc- and mycobacterial protein-induced IL-12 expressions are negatively regulated by the PI3K and ERK 1/2 pathways in human monocytes (Yang et al., 2006). This study further demonstrated that \( M. \) tbc-induced IL-23 expression is modulated differently by the mTOR/S6K1 and p38 MAPK pathways, in a PI3K-dependent manner.

The requirement of the mTOR/S6K1-signalling pathway for \( M. \) tbc-induced expression of proinflammatory mediators has not been studied previously. Our data suggest that the rapamycin-sensitive signalling pathway acts as a negative feedback cascade in regulating IL-23 production. While most previous studies have focused on the role of rapamycin in T cell functions and the inhibition of IFN-\( \gamma \) production in T cells (Wasowska et al., 2001; Kusaba et al., 2005), our data emphasize the opposite role, i.e. Th1 induction by rapamycin in IL-23 production by human MDMs. Our finding is partially consistent with a previous finding that rapamycin significantly enhances Staphylo-
Fig. 7. Role of IL-10 in PI3K, ERK 1/2 or S6K1-mediated enhancement of IL-12/IL-23 p40 production by M. tbc-treated MDMs.

A. The PI 3-K inhibitor LY294002 (5, 10 or 20 µM), PD98059 (5, 10 or 20 µM) or rapamycin (0.1 1 or 100 pM) was added to MDMs at 45 min before adding M. tbc (moi = 1). Six hours after the addition of M. tbc, total RNA was purified, and semiquantitative RT-PCR analysis of IL-10 was performed. A representative experiment of four independent replicates from six donors is shown.

B. Human MDMs were pre-incubated in the presence of LY294002 (1, 5 or 10 µM), wortmannin (0.1, 0.2 or 0.3 µM), PD98059 (1, 5 or 10 µM), U0126 (1, 5 or 10 µM) or rapamycin (0.1, 1 or 100 pM) at 45 min before adding M. tbc (moi = 1). The supernatants were harvested after 18 h for cytokine assessment using ELISA. All experiments were performed at least three times using cells from different donors, and the qualitative effects described here were reproduced in all individuals (n = 5).

C. Human MDMs were pre-incubated with a neutralizing mAb to IL-10 (1 µg ml⁻¹) or IC Ab (1 µg ml⁻¹) in the presence or absence of LY294002 (10 µM), wortmannin (0.2 µM), U0126 (10 µM), PD98059 (10 µM), rapamycin (1 pM) or 0.1% DMSO control for 45 min MDMs were then treated with M. tbc (moi = 1) for 18 h. Cell free supernatants were then collected, and the levels of p40 were determined by ELISA. Significant differences (*P < 0.05; **P < 0.01; ***P < 0.001) compared with cultures without anti-IL-10 mAb. Data are expressed as the mean of two separate experiments using cells from different four donors. UI, uninfected; D, DMSO control.
Several studies have reported the essential role of p38 in TLR signalling. The TLR4 agonist *Escherichia coli* LPS and the TLR5 agonist flagellin stimulate Th1 responses via IL-12p70 production dependent on the phosphorylation of p38 and JNK 1/2 (Agrawal et al., 2003). In addition, a major TLR2 agonist, *Plasmodium falciparum* glycosylphosphatidylinositol-induced pro-inflammatory responses are almost completely abrogated by the inhibition of p38 activity by SB203585 or SB202190, indicating that the p38 pathway is crucial for the TLR2-dependent cytokine response (Krishnegowda et al., 2005). Moreover, Lu et al. (1999) showed that macrophages deficient in p38 activity expressed drastically reduced levels of IL-12 in response to LPS, whereas TNF-α levels were normal. Therefore, our findings are consistent with previous findings and can be extended to show a negative regulatory role in *M. tuberculosis*-induced IL-23 expression via the p38 MAPK pathway.

Our data also showed that PI3K inhibition enhanced p38 MAPK activation, and an upstream regulator of p38, ASK1, activity, during exposure to *M. tuberculosis*. Consistent with our observation, the inhibition of PI3K, or a lack of PI3K, upregulated p38 activity in dendritic cells (Fukao et al., 2002). In addition, previous studies found a novel functional interaction between Akt and ASK1, a MAPK kinase. Akt-mediated phosphorylation of ASK1 blocked ASK1 kinase activity, leading to the suppression of MAPK kinase 3 (MKK3) or MKK6, upstream regulators of p38 (Kim et al., 2001). As the activation of Akt is positively regulated by PI3K (Toker and Cantley, 1997), PI3K inhibition upregulates ASK1 and p38 activity in human macrophages. Our findings are also supported by a very recent report that TLR2 ligands, such as mycoplasmal lipoproteins or staphylococcal peptidoglycans, induced sustained p38 phosphorylation, which is modulated by ASK1 activity (Into and Shibata, 2005). In addition, our data further emphasize the role of PI3K as a principal modulator of ASK1 and p38 activity in TLR2 signalling.

Previous studies demonstrate that IL-12 regulation can be controlled by the autocrine or paracrine production of IL-10, which can negatively influence the production of IL-12 p70 (Snijders et al., 1996; Aste-Amezaga et al., 1998). In addition, the inhibition of *Porphyromonas gingivalis* LPS-mediated activation of the PI3K–Akt pathway results in a severe reduction in IL-10 production, with a concurrent augmentation of IL-12 levels (Martin et al., 2003). In our findings, the PI3K inhibition suppressed IL-10 levels; however, neither ablation of mTOR/S6K1 nor ERK 1/2 activity affected IL-10 expression in human MDMs. In addition, the neutralization of IL-10 did not affect the negative regulation of IL-12/IL-23 p40 induced by *M. tuberculosis*. Therefore, our data are unique in indicating that both the S6K1 and ERK 1/2 pathways downstream from the PI3K

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**Fig. 8.** Role of TLR2 in *M. tuberculosis*-induced IL-23 (p40/p19) regulation in human MDMs. Human MDMs were pre-incubated with a neutralizing mAb to TLR2 (10 μg ml⁻¹) or IC Ab (10 μg ml⁻¹) in the presence or absence of LY294002 (10 μM), PD98059 (10 μM), rapamycin (1 μM), SB202190, SB203580 (5 μM) or 0.1% DMSO control at 45 min before adding *M. tuberculosis* (moi = 1). Cell free supernatants were then collected after 18 h, and the levels of p40 and IL-23 (p40/p19) were determined by ELISA. Significant differences (**P < 0.001) compared with cultures in an absence of anti-TLR2 mAb. Data are expressed as the means ± SD of values for three separate experiments using cells from different five donors. UI, uninfected; D, DMSO control.
pathway likely modulate IL-12/IL-23 expression via IL-10-independent mechanisms.

Purified mycobacterial Ags, such as 19 kDa lipoprotein, lipoarabinomannan and STF (soluble tuberculosis factor), preferentially interact with TLR2, whereas infection with whole bacilli evokes a more complex activation pattern involving at least TLR2 and TLR4 and leads to the differential activation of antibacterial effector pathways (Means et al., 1999; Bulut et al., 2005). In this study, we found that M. tbc-induced Akt activation and IL-23 expression are dependent on TLR2, but not TLR4. In addition, we demonstrated that the modulation of M. tbc-induced IL-23 expression is partially dependent on TLR2, because the pretreatment with anti-TLR2 mAb significantly, but not completely, affected the regulation of p40 and IL-23 (p40/p19) production in the presence of specific inhibitors of PI3K, ERK 1/2, S6K1 and p38. We observed that this modulation is completely abrogated by combined treatments with anti-TLR2 mAb and cytochalasin D, a reagent that blocks actin polymerization and phagocytosis (C.-S. Yang and E.-K. Jo, unpubl. obs.). The molecular mechanisms controlling the regulation of IL-12/IL-23 expression, which may be governed by both TLR2 and phagocytosis, are now being investigated in more detail.

Collectively, our data are the first to demonstrate the functional importance of the mTOR/S6K1 pathways in the negative regulation of IL-23 by human macrophages stimulated with M. tbc. In addition, our findings suggest that the M. tbc-induced IL-23 expression in the human macrophage compartment is critically regulated by PI3K and its downstream pathways via TLR2. These intracellular signalling networks may contribute to the homeostatic control of the type 1 immune response during mycobacterial infection.

**Experimental procedures**

**Bacteria**

*M. tbc* H37Rv was kindly provided by Dr Richard L. Friedman, University of Arizona, Tucson, M. tbc was grown to late log phase in Middlebrook 7H10 agar (Difco) medium supplement with 10% OADC (oleic acid, albumin, dextrose, catalase; BD Immunomycotmetry) supplemented with 0.05% Tween 80 (Sigma). Batch cultures were aliquoted and stored at −70°C. Representative vials were thawed and enumerated for viable colony-forming unit (cfu) on Middlebrook 7H10 agar (Difco). Single-cell suspensions of mycobacteria were obtained as previously described (Song et al., 2003). To rule out the influence of LPS in the assays, the bacterial suspensions were tested in the *Limulus* amebocyte lysate assay (BioWhittaker). The effective LPS concentration was <0.2 pg ml⁻¹ in experiments with bacterial to cell ratios of 1:1.

**Isolation and cultivation of human MDMs**

Adherent monocytes were collected from peripheral blood mono- nuclear cells donated by healthy subjects, as previously described (Song et al., 2003). Human MDMs were prepared by culturing peripheral blood monocytes for 6 days in the presence of 20 ng ml⁻¹ human macrophage colony-stimulating factor (Sigma) as previously described (Verreck et al., 2004). In order to show that the stimulatory capacity of mycobacteria was not the result of contamination with LPS, experiments were performed that added the specific LPS-inhibiting oligopeptide polymyxin B (10.0 µg ml⁻¹) before mycobacterial stimulation. The study was approved by the bioethics committee of Chungnam University Hospital's review board overseeing studies on samples from human subjects.

**Stable cell culture**

HEK293 cells stably transfected with human TLR2 (HEK/TLR2) or human TLR4 (HEK 293/TLR4) were purchased from Invivogen. The HEK/TLR clones were grown in standard Dulbecco's modified Eagle's medium (DMEM) with 10% FBS supplemented with plasticidin (10.0 µg ml⁻¹) and normocin (100.0 µg ml⁻¹).

**Inhibitors and Abs**

Specific inhibitors of MEK (PD98059 and U0126), PI3K (LY294002 and wortmannin), p38 MAPK (SB203580) and mTOR/S6K1 (rapamycin) were purchased from Calbiochem. Dimethyl-sulphoxide (DMSO; Sigma) was added to cultures at 0.1% (v/v) as a solvent control. MDMs were washed with PBS and pretreated with inhibitors for 45 min before treatment with M. tbc. An assessment using Trypan Blue exclusion indicated that monocyte viability was not affected by the presence of the inhibitors (data not shown). Mouse anti-human TLR2 mAb (clone TL2.1, IgG2a), mouse anti-human TLR4 mAb (clone HTA125, IgG2a), IC mAb (IgG2a) were purchased from eBioscience. Mouse anti-human IL-10 neutralizing mAb (clone 23738) was purchased from R and D system.

**Determination of Akt, S6K1, ERK 1/2, and p38 phosphorylation**

Total cell lysates were prepared and Western blot analysis was done with specific primary Abs [phospho-(Thr-308)-Akt, Akt, phospho-(Thr180/Tyr182)-p38 MAPK, total p38 MAPK, phospho-(Thr-389)-p70 S6K1, phospho-(Ser421/Thr424)-p70 S6K1, and total p70 S6K1, phospho-(Thr202/Tyr204)-ERK1/2, unphosphorylated ERK 1/2; Cell Signaling Technology]. Membranes were developed using a chemiluminescence assay (ECL; Pharmacia-Amersham) and subsequently exposed to chemiluminescence film (Pharmacia-Amersham).

**In vitro ASK1 assay**

Confluent MDMs in 100 mm dishes were uninfected or infected with M. tbc. In some cases, specific inhibitors for PI3K/Akt pathway were added 30 min before infection. The cells were lysed with ice-cold lysis buffer for 30 min on ice. Insoluble materials were removed by centrifugation and the supernatant was incubated with anti-hASK1 Ab for 2 h on a rocking platform. Twenty microlitres of protein A sepharose (Amersham Bioscience) slurry

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was added and rocking continued for another 2 h. The immuno-
precipitates were washed twice in lysis buffer and twice in kinase
assay buffer (20 mM MgCl₂, 25 mM Hepes, 20 mM p-nitrophen-
olphosphate, 20 mM β-glycerophosphate, 20 mM sodium ortho-
vanadate and 2 mM DTT). The immune complexes were then
suspended in 20 µl kinase buffer, and the following were added:
20 µM ATP, 5 µCi [γ-32P] ATP (BLU 002Z, DuPont/NEN), and
10 µg myelin basic protein (MBP, Sigma). The reaction was con-
tinued for 30 min at 25°C with gentle agitation and then stopped
by the addition of 10 µl of 4× Laemmli's sample buffer, followed
by boiling of the sample and separation by SDS-PAGE. The gel
was dried and autoradiography was performed to visualize the
32P-labelled MBP. Densitometry was performed on films and fold
increase calculated as experimental sample/control sample.

Cytokine ELISA
Sandwich ELISA was used for detecting p40 and IL-10 (BD
PharMingen) in culture supernatants, as described (Song et al.,
2000). The IL-23 levels in culture supernatants were determined
with commercial kits for soluble human IL-23 ELISA assay
(Bender MedSystems). Assays were performed as recom-
mended by the manufacturers. Cytokine concentrations in the
samples were calculated using standard curves generated from
recombinant cytokines, and the results were expressed in pico-
grams or nanograms per millilitre. The difference between dupli-
icate wells was consistently less than 10% of the mean.

RT-PCR
Total RNA was extracted from human MDMs (1 × 10⁶) using
TRIzol (Invitrogen), and semiquantitative RT-PCR was performed
as previously described (Yang et al., 2006). The IL-23 p19 primer
sequences were 5′-TCGGCACGAGAACAACTGAG and 3′-
TGGGGAACATCATTTGTAGTCT. The PCR products were
resolved on 1% agarose gel and stained with ethidium bromide.
In addition, TaqMan real-time RT-PCR was performed as
described previously (Goodridge et al., 2003). The probes used
were 5′ FAM (6-carboxy-fluorescein; reporter) and 3′ TAMRA (6-
carboxy-tetramethyl rhodamine; quencher). Each PCR amplifica-
tion was performed in triplicate, data were analysed using the
Applied Biosystems Sequence Detection Software (Applied Bio-
systems) and samples were normalized by their reference reporter β-actin.

Statistical analysis
For statistical analysis, the data obtained from independent
experiments are presented as the mean ± SD and were analysed
using paired t-test with Bonferroni adjustment or ANOVA for mul-
tiple comparisons. Differences were considered significant at
P < 0.05.

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