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Cellular Microbiology (2007) 9(2), 382–396

doi:10.1111/j.1462-5822.2006.00797.x
First published online 22 August 2006

Protein kinase C zeta plays an essential role for *Mycobacterium tuberculosis*-induced extracellular signal-regulated kinase 1/2 activation in monocytes/macrophages via Toll-like receptor 2

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Summary

This study characterized the upstream signalling molecules involved in extracellular signal-regulated kinase (ERK) 1/2 activation and determined their effects on differential tumour necrosis factor (TNF)-α expression by monocytes/macrophages infected with virulent or avirulent mycobacteria. The avirulent *Mycobacterium tuberculosis* (MTB) strain H37Ra (MTBRa) induced higher levels of activation of ERK 1/2 and the upstream MAPK kinase (MEK)1 and, subsequently, higher levels of TNF-α expression in human primary monocytes and monocyte-derived macrophages, as compared with MTB strain H37Rv (MTBRv). The MTB-induced activation of ERK 1/2 was not dependent on Ras or Raf. However, inhibition of the activity of atypical protein kinase C (PKC) ζ decreased the *in vitro* phosphorylation of MEK, ERK 1/2 activation and subsequent TNF-α induction caused by MTBRv or MTBRa. Toll-like receptor (TLR) 2 was found to play a major role in MTB-induced TNF-α expression and PKCζ phosphorylation. Co-immunoprecipitation experiments showed that PKCζ interacts physically with TLR2 after MTB stimulation. Moreover, PKCζ phosphorylation was increased more in macrophages following MTBRa, versus MTBRv, infection. This is the first demonstration that PKCζ interacts with TLR2 to play an essential role in MTB-induced ERK 1/2 activation and subsequent TNF-α expression in monocytes/macrophages.

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Introduction

Mycobacterium tuberculosis (MTB) is the causative agent of pulmonary tuberculosis (TB) and is an intracellular pathogen capable of infecting and surviving within the host's mononuclear cells. Co-ordinated responses of the innate and adaptive immune systems are required for an effective host defence against TB upon recognition of the pathogen-associated molecular patterns of mycobacteria by pattern-recognition receptors (PRRs) (van Crevel *et al.*, 2002). The innate response serves as the first line of defence and is initiated following activation of PRRs such as the Toll-like receptors (TLRs) (Aderem and Ulevitch, 2000). TLR signal transduction leads to the subsequent expression of cytokines and chemokines, which induces further activation and cytokine production in a complex process of regulation and cross-regulation (Means *et al.*, 2000; van Crevel *et al.*, 2002). Among these cytokines, tumour necrosis factor (TNF)-α is known as a master regulator of the signals responsible for directing cells to the site of infection (Fenton, 1998; Algood *et al.*, 2005). Previous studies suggest that several protein kinases, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K), are activated, at least partly, via a TLR signalling cascade (Means *et al.*, 2000). An increasing awareness of the significance of signalling mechanisms in mycobacterial infection should lead to promising new strategies in anti-mycobacterial treatment.

Mycobacteria and their components independently or simultaneously activate the subfamilies of MAPKs: p42 and p44 extracellular signal-regulated kinase (ERK) 1/2, p46 and p54 stress-activated protein kinase/c-Jun NH2-terminal kinase 1/2, and p38 MAPK (Reiling *et al.*, 2001;

Bhattacharyya *et al.*, 2002; Blumenthal *et al.*, 2002; Roach and Schorey, 2002; Tse *et al.*, 2002; Jung *et al.*, 2006). MAPK activation is essential for the mycobacteria-induced production of proinflammatory cytokines, including TNF-α, interleukin (IL)-1β and chemokines (Foey *et al.*, 1998; Blumenthal *et al.*, 2002; Fietta *et al.*, 2002; Roach and Schorey, 2002; Schorey and Cooper, 2003). Previous studies have indicated that pathogenic mycobacteria actively limit the macrophage response and consistently induce less TNF-α, IL-1β and nitric oxide synthase (NOS2) than the less virulent mycobacteria (Falcone *et al.*, 1994; Beltan *et al.*, 2000). In addition, arabinosylated lipoarabinomannan (LAM), a major cell wall-associated glycolipid produced by the avirulent MTB H37Ra (MTBRa) strain, induced significantly more TNF-α than did mannose-capped LAM of the closely related virulent (Erdman) MTB strain (Roach *et al.*, 1993). The ability of the more virulent mycobacteria to limit the production of inflammatory mediators suggests that pathogenic mycobacteria initiate an active process for manipulating macrophage signal transduction pathways. The ability of pathogenic mycobacteria to limit MAPK activity has been suggested as an important virulence mechanism (Schorey and Cooper, 2003; Yadav *et al.*, 2004). However, few studies have addressed how pathogenic and non-pathogenic mycobacteria differentially activate a macrophage signalling pathway or how this affects the secretion of important inflammatory mediators. In addition, little work has examined MAPK upstream signalling induced by intact mycobacteria, although this is the most likely interaction between the mycobacteria and macrophage during an *in vivo* infection.

In this study, we focused primarily on pathways upstream from MTB-induced ERK activation and their effects on subsequent TNF-α expression. The best-described pathway leading to ERK activation is the Ras-Raf-1-MAPK kinase (MEK) 1-ERK kinase cascade (Zhang *et al.*, 1993). The classic Ras-mediated pathway involves the binding of Raf-1 and subsequent phosphorylation at Ser338 by several kinases (Marshall, 1996), which in turn activates ERK 1/2 (Zhang *et al.*, 1993) and phosphorylates target proteins, including transcription factors and protein kinases (Whitmarsh and Davis, 1998). Another possible activator of MEK is the protein kinase C (PKC) family of serine/threonine kinases. PKC proteins have been divided into three distinct groups: conventional calcium-dependent, phospholipid-dependent and diacylglycerol-dependent PKC isozymes (cPKCα, β_I, β_{II}, γ); novel calcium-independent PKC isozymes (nPKCδ, ε, η, θ); and atypical PKC isozymes (aPKCζ, ι, λ), which are calcium-, phospholipid- and diacylglycerol-independent (Hug and Sarre, 1993; Toker, 1998). Several PKC isoforms have been reported to be involved in the activation of ERK 1/2 signalling through Ras-dependent and Ras-independent mechanisms (Cacace *et al.*, 1996; Ueffing *et al.*, 1997; Takeda *et al.*, 1999). Of the PKCs, atypical PKCζ plays a central role in lipopolysaccharide (LPS)-induced activation of the ERK 1/2 signalling pathways in macrophages (Monick *et al.*, 2000).

Our data suggest that the PKCζ-MEK-ERK pathway, but not the Ras-Raf-MEK-ERK pathway, is essential for MTB-induced TNF-α expression in various monocytes/macrophages, such as human primary monocytes, monocyte-derived macrophages (MDMs) and murine primary macrophages. We also show that PKCζ is associated with TLR2 after stimulation with MTB H37Rv (MTBRv) or MTB H37Ra (MTBRa). The differential capacities of MTBRv and MTBRa to induce PKCζ phosphorylation may contribute to the differences in the abilities of virulent and avirulent mycobacteria to activate MEK and ERK 1/2 and to induce TNF-α secretion.

Results

Avirulent MTBRa induces higher levels of TNF-α mRNA and protein expression than does MTBRv

When we evaluated the intracellular growth of the two strains, the mean number of colony-forming units (cfu) of H37Rv was significantly greater compared with H37Ra ($P < 0.01$; data not shown), suggesting that the MTBRv strain used in our study has virulence characteristics. Then, we examined the kinetics of TNF-α mRNA and protein expression in human monocytes and MDMs that were infected with MTBRv or MTBRa. The monocytes were infected at a multiplicity of infection (moi) of 1 for various periods of time. Peak levels of TNF-α mRNA and protein expression were detected 3 and 6 h post infection with MTBRv and MTBRa respectively. The attenuated MTBRa strain induced significantly more TNF-α mRNA and protein expression in human primary monocytes than did MTBRv in the period from 3 to 18 h post infection (Fig. 1). Similarly, MTBRa induced higher levels of TNF-α secretion by MDMs than did MTBRv, at 6 h post infection (data not shown).

Avirulent MTBRa induces higher levels and longer duration of ERK 1/2 phosphorylation than does MTBRv

We also examined the relationship between mycobacterial virulence and ERK 1/2 phosphorylation in human primary monocytes. As shown in Fig. 2A, monocytes infected with MTBRa showed higher levels and longer duration of ERK 1/2 activation than those infected with MTBRv. The elevated phosphorylation level was maintained for up to 48 h in monocytes infected with MTBRa, compared with those infected with MTBRv (Fig. 2A). Of

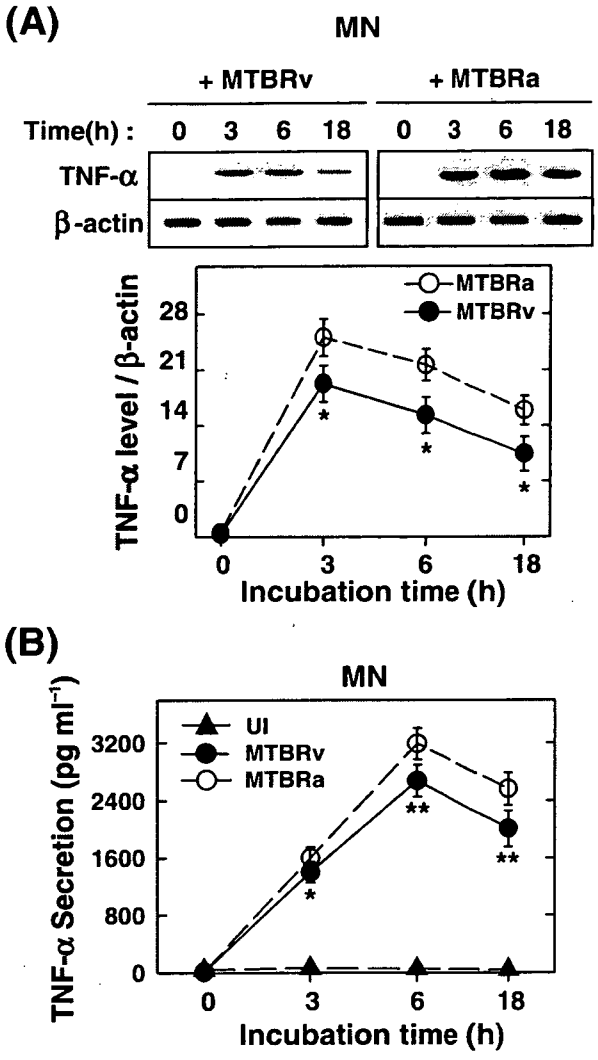

Fig. 1. MTB-induced TNF-α expression from human monocytes. Human monocytes were infected with MTBRv or MTBRa (moi = 1). Cell lysate and supernatants were harvested. Total RNA was assessed by PCR for TNF mRNA (A), and the protein expression was measured by ELISA (B). Data are the mean ± SD of three experiments. Statistical differences (*$P < 0.05$; **$P < 0.01$) compared with cultures infected with MTBRa. MN, monocytes.

note, when the same blot was stripped and reprobed for phospho-p38, there were no significant differences in the levels of p38 activation induced by MTBRv and MTBRa (Fig. 2B). At mois ranging from 0.1 to 50, the ERK 1/2 phosphorylation levels were higher in MTBRa-infected monocytes than in MTBRv-infected monocytes (data not shown).

We also investigated the MEK activities of monocytes that were infected with MTBRv or MTBRa. The kinase activity assays showed that both mycobacterial strains caused substantial activation of MEK, with MTBRa giving higher levels of MEK activation over time (Fig. 2C). These results are consistent with the differential activation of ERK 1/2 by the two mycobacterial strains.

MTB-induced TNF-α expression is dependent on ERK 1/2 pathway activation

To define the role of ERK 1/2 activation in the secretion of TNF-α, the monocytes and MDMs were pretreated with MEK inhibitor (PD98059 or U0126) for 45 min before infection with MTBRv or MTBRa, and then assayed for ERK 1/2 phosphorylation and cytokine production. The dose-dependent inhibition of ERK 1/2, but not p38 MAPK, phosphorylation by U0126 following infection with MTB was confirmed by Western blotting (Fig. 3A). In addition, the expression of DN-MEK1 significantly reduced TNF-α production in THP-1 cells infected with MTBRv or MTBRa (Fig. 3B). The expression levels of TNF-α mRNA (Fig. 3C) and protein (Fig. 3D) in the MTB-treated monocytes were reduced by U0126 in a dose-dependent manner. Similar inhibitory patterns were noted for MTBRv- and MTBRa-induced TNF-α secretion in MDMs that were pretreated with PD98059 or U0126 (Fig. 3E). These results demonstrate that ERK 1/2 signalling pathways participate in MTBRv- and MTBRa-induced expression of TNF-α.

Ras and Raf-1 are not responsible for ERK activation and TNF-α expression in human monocytes/macrophages following infection with MTB

The upstream signalling pathways that lead to ERK activation were investigated further using specific inhibitors. Ras is one of the upstream kinases that activate the MEK/ERK pathway, and the Ras-Raf-1-MEK-ERK kinase cascade is one of the important pathways for ERK activation (Zhang *et al.*, 1993). Primary human monocytes were pretreated with the Ras inhibitor manumycin A or the Raf-1 inhibitor GW5074 for 45 min before adding MTB. No loss of ERK 1/2 activation was observed in macrophages that were infected for 30 min with MTBRv following treatment with the Ras or Raf-1 inhibitor (Fig. 4A and B, Ras and Raf-1 inhibition respectively). A similar finding was observed for MTBRa-treated human monocytes (data not shown). ERK 1/2 activation remained unaffected after the inhibition of Ras or Raf-1 in MTB-infected human monocytes at 4 h post infection (data not shown).

Based on the Western blot results, neither manumycin A nor GW5074 had any significant effect on TNF-α mRNA or protein production in the MTBRv- or MTBRa-induced human monocytes and MDMs (Fig. 4C and D). We attempted to confirm these findings using Ras-deficient cells. Adenoviral transduction of primary human monocytes with either Ad-Ras DN or control virus had no effect on MTB-induced TNF-α production (Fig. 4E). These data

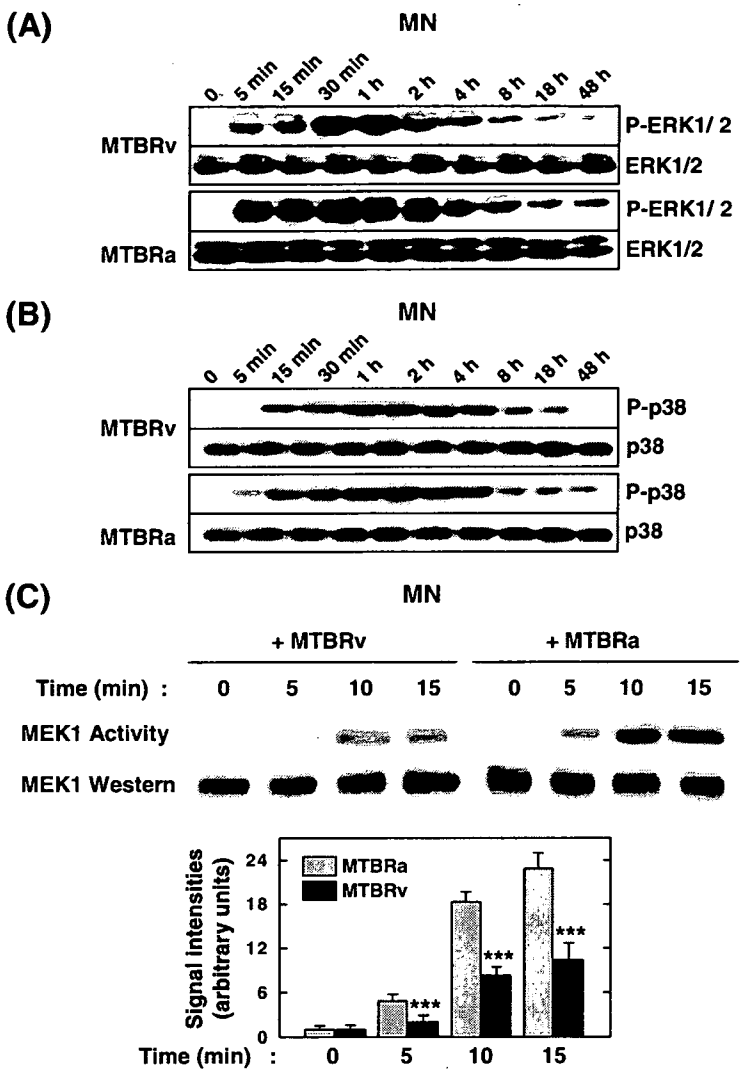

Fig. 2. MTB-mediated activation of MAPKs and MEK1 in human primary monocytes.
A and B. Human monocytes were infected by MTBRv or MTBRa (moi = 1) for the times indicated. The cells were harvested and subjected to Western blot analysis for phosphorylated ERK1/2 (A) and p38 (B). The same blots were washed and blotted for total ERK1/2 and p38 as the loading controls. Data are representative of five independent experiments with similar results.
C. Human monocytes were infected by MTBRv and MTBRa for the times indicated. Whole cell protein was obtained, and MEK1 was immunoprecipitated from 200 μg of the lysates. Kinase activity assays were performed using MBP (10 μg) as a substrate. Data are representative of three independent experiments with similar results. MN, monocytes.

suggest that neither Ras nor Raf-1 mediates the processes of ERK 1/2 activation and TNF-α induction following infection with MTBRv or MTBRa.

The PKC pathways modulate ERK phosphorylation induced by MTB

Previous studies using human macrophages have described the release of TNF-α in response to stimulation with phorbol ester or LPS, in a process mediated by ERK 1/2 via upstream PKC activation (Foey and Brennan, 2004). Therefore, we investigated the association between the PKC pathway and ERK 1/2 phosphorylation induced by MTB. The strategy of use of PKC inhibitors was referred from the previous studies (Contreras *et al.*, 2004). As shown in Fig. 5A, treatment of monocytes with bisindolylmaleimide I, Ro 31-8220, or Gö6983 significantly reduced MTBRv-induced ERK 1/2 activation, confirming the involvement of PKC. However, Gö6976 treatment had no effect on MTB-induced activation in ERK 1/2 phosphorylation. The phospho-p38 or phospho-JNK protein levels after pretreatment with inhibitors remained similar to those of the untreated controls (Fig. 5A). A similar finding was observed for MTBRa-treated human monocytes (data not shown).

In addition, the specific inhibitors of the PKC pathways (Ro31-8220, bisindolylmaleimide and Gö6983) severely reduced the MTB-induced expression of TNF-α mRNA in human monocytes in a dose-dependent manner, whereas treatment with Gö6976 did not (Fig. 5B). These results were confirmed at the protein level using human MDMs (Fig. 5C). Therefore, MTB-induced ERK 1/2 activation and TNF-α expression appear to be modulated by a PKC-

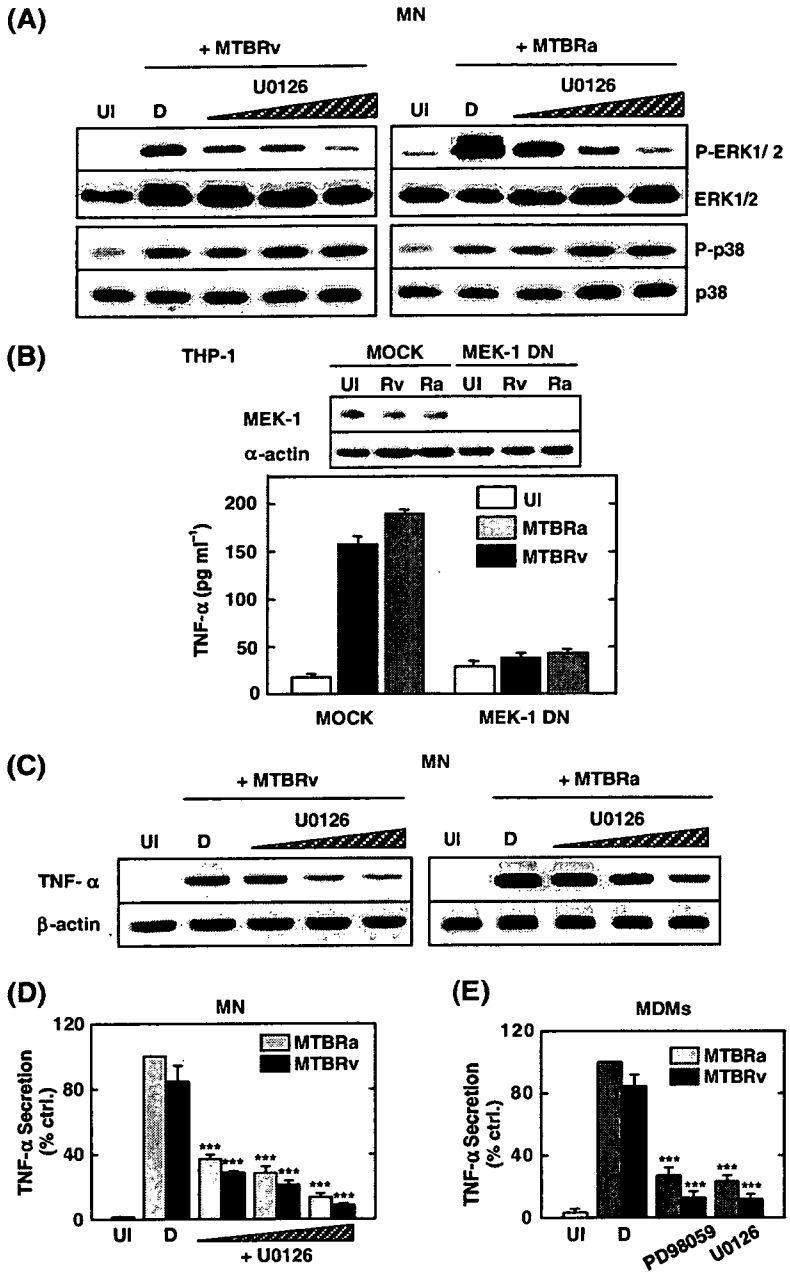

Fig. 3. MTB stimulates the TNF-α expression of human monocytes or MDMs via the MEK–ERK pathway.
A. The MEK inhibitor U0126 was added to monocytes at concentrations ranging from 5 to 20 μM at 45 min before infection with MTBRv or MTBRa (moi = 1) and culture continued for a further 30 min. The cells were harvested and subjected to Western blot analysis for phosphorylated ERK1/2 (upper) and p38 (lower). The same blots were washed and blotted for total ERK1/2 and p38 as the loading controls. Data are representative of three independent experiments with similar results.
B. THP-1 cells were transfected with DN-MEK1, or empty vector. After a 24 h incubation in normal culture medium, the transfected cells were stimulated for 6 h with MTBRa or MTBRv (at moi = 1). After harvest of supernatants, TNF-α ELISA was determined. In the upper panel, lysates of 5×10^5 cell equivalent from each transfectant were immunoblotted with anti-MEK1 Ab. The same blots were stripped and reprobed with anti-β-actin mAb.
C–E. The experimental conditions follow the same pattern as outlined in A. Total RNA was purified at 3 h, and semi-quantitative RT-PCR analysis of TNF-α was performed. A representative gel of three independent replicates with similar results is shown (C). The supernatants from monocytes (D) and MDMs (E, 10 μM for both inhibitors) were harvested after 6 h for cytokine assessment using ELISA for TNF-α. Data are the mean ± SD of five experiments. The mean levels of TNF-α following infection with MTBRa were set to 100, and the relative loss of cytokine production in the presence of inhibitor is shown. Statistical differences (***$P < 0.001$) compared with cultures in an absence of inhibitors. D, the solvent control of 0.1% DMSO; UI, uninfected; MN, monocytes.

dependent pathway, although classical PKC isoforms α, β_I and novel PKC isoform μ could be excluded by treatment of the cells with Gö6976.

PKCζ is critical for the activation of ERK and MEK, and TNF-α expression in human monocytes, MDMs and monocytic cell lines following infection with MTB

Activation of ERK signalling by atypical PKC isoform PKCζ is reported to be mediated by Ras-independent mechanisms (Takeda *et al.*, 1999). To define the specific role of PKCζ in the activation of ERK 1/2 and MEK, we treated human monocytes with inhibitory peptides for PKCαβ and PKCζ. The ERK 1/2 activation in monocytes following infection with MTBRv or MTBRa was strongly inhibited by pretreatment with inhibitory peptides for PKCζ, but not by treatment with inhibitory peptides for PKCαβ (Fig. 6A). By contrast, the MTB-induced phospho-SAPK/JNK and phospho-p38 MAPK levels were not affected in the presence of inhibitory peptides for PKCζ,

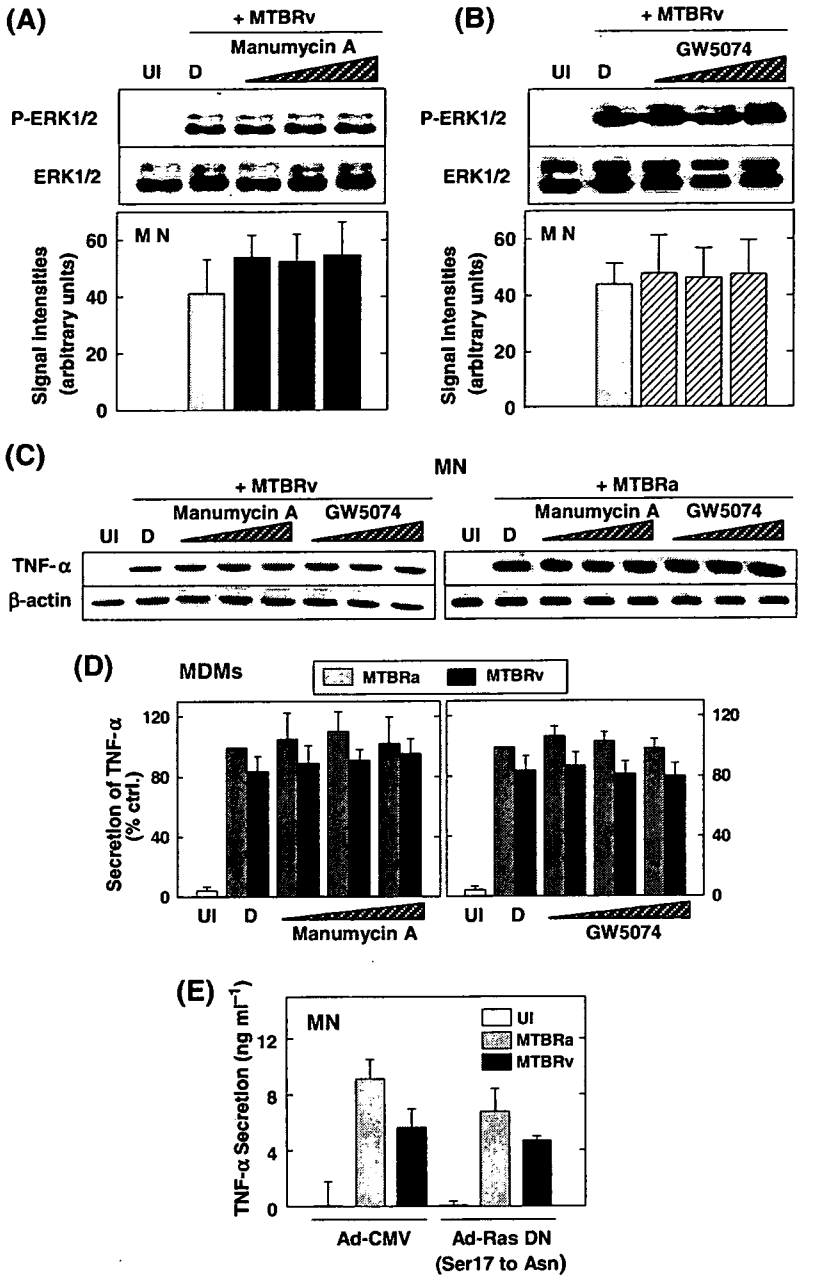

Fig. 4. Effects of inhibition of Ras or Raf-1 on the MTB-mediated ERK-1 phosphorylation and TNF-α expression in human monocytes or MDMs.
A–D. The Ras inhibitor manumycin A (0.1, 1, 10 μM) or Raf-1 inhibitor GW5074 (0.1, 1, 10 nM) were added to monocytes (for A–C) or MDMs (for D) at 45 min before infection with MTBRv or MTBRa (moi = 1) and culture continued for a further 30 min. The cells were harvested and subjected to Western blot analysis for phosphorylated ERK1/2 and the same blots were washed and blotted for total ERK1/2 as the loading control. All densitometry values of the p-ERK 1/2 band were normalized to the total ERK 1/2 protein. The densitometry values are depicted as means ± SD of five independent experiments (A and B, Bottom). Human monocytes (for C) and MDMs (for D) were preincubated with manumycin A or GW5074 for 30 min before infection with MTBRv or MTBRa. Total RNA was purified at 3 h, and semi-quantitative RT-PCR analysis of TNF-α was performed. A representative gel of three independent replicates with similar results is shown (C). The supernatants were harvested after 6 h for cytokine assessment using ELISA for TNF-α. The mean levels of TNF-α following infection with MTBRa were set to 100, and the relative loss of cytokine production in the presence of inhibitor is shown. Data are the mean ± SD of five experiments (D).
E. Human monocytes were infected with Ad-Ras DN, or control adenovirus (Ad-CMV) at 100 pfu cell^{-1}. After 24 h, the cells were further incubated with MTBRa or MTBRv, and then TNF-α production was determined by ELISA at 6 h. Data are expressed as the means ± SD of values for three separate experiments. D, the solvent control of 0.1% DMSO; UI, uninfected; MN, monocytes.

suggesting that the ERK1/2 activation is specifically related to PKCζ after stimulation with MTB (Fig. 6A). We also showed that the PKCζ-specific peptide essentially blocked MTBRv- or MTBRa-induced MEK activation, whereas the α- and β-specific peptides had no effect on MEK activation (Fig. 6B).

We also examined whether a peptide specific for PKCζ blocked the induction of TNF-α mRNA and protein in MTB-infected monocytes and MDMs. As shown in Fig. 6C and D, the PKCζ-specific peptide essentially blocked the formation of TNF-α mRNA and protein, whereas the α- and β-specific peptides had no effect on TNF-α expression. In addition, the expression of DN-PKCζ constructs mimicked the effects of the pharmacological inhibitors. As shown in Fig. 6E, transfection of U937 and THP-1 cell lines with the DN-PKCζ construct resulted in marked inhibition of the MTB-induced production of TNF-α, as compared with transfection with the empty vector. In sharp contrast, the PKCζ overexpression using the WT-PKCζ plasmid resulted in

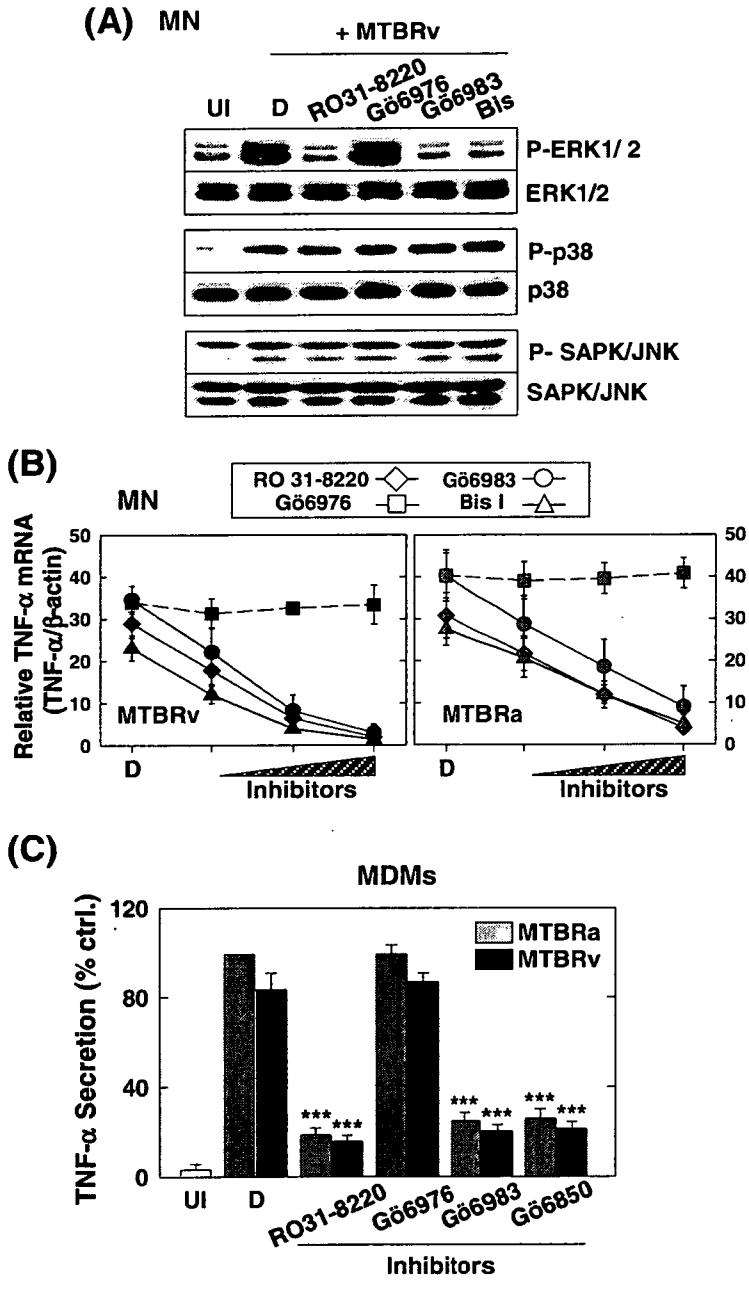

Fig. 5. Effects of inhibition of PKC on the MTB-induced phosphorylation of ERK1/2 and TNF-α expression in human monocytes.
A. Human monocytes were preincubated with the PKC inhibitors (RO 31-8220, 5 μM; Gö6976, 20 nM; Gö6983, 10 μM; bisindolylmaleimide I, 10 μM). The cells were harvested and subjected to Western blot analysis for phosphorylated ERK1/2, p38 and SAPK/JNK. The same blots were washed and blotted for total ERK1/2, p38 and SAPK/JNK as the loading controls. Data are representative of five independent experiments with similar results. Bis I, bisindolylmaleimide I.
B. Human monocytes were preincubated with PKC inhibitors [RO 31-8220 (1, 5, 10 μM); Gö6976 (10, 20, 40 nM); Gö6983 (5, 10, 20 μM); bisindolylmaleimide I (5, 10, 20 μM)] for 30 min before infection with MTBRv or MTBRa. Total RNA was purified at 3 h, and semi-quantitative RT-PCR analysis of TNF-α was performed. Densitometric analysis of data for 3 donors (means ± SE) is shown. The densitometry values for TNF-α mRNA levels were normalized to the β-actin levels.
C. The experimental conditions follow the same pattern as outlined in A. The supernatants were harvested after 6 h for cytokine assessment using ELISA for TNF-α. The mean levels of TNF-α following infection with MTBRa were set to 100, and the relative loss of cytokine production in the presence of inhibitor is shown. Data are the mean ± SD of three experiments. Significant differences (***$P < 0.001$) compared with cultures in an absence of inhibitors. D, the solvent control of 0.1% DMSO; UI, uninfected; MN, monocytes.

significantly enhanced production of TNF-α in human monocyte cell lines. These data confirm that PKCζ, but not Ras, is essential for TNF-α production by human monocytes infected with MTB.

TLR2, but not phagocytosis or TLR4, plays a major role in TNF-α expression induced by MTB

To determine the mechanism by which MTB-induced TNF-α secretion is modulated, human MDMs were preincubated with cytochalasin D, which inhibits actin polymerization, before adding the inhibitors and MTB. Pretreatment with cytochalasin D completely blocked the phagocytic activities of MDMs (data not shown), whereas it did not affect MTB-induced TNF-α secretion (Fig. 7A). Moreover, no significant differences were observed in TNF-α levels between cultures that were pretreated with specific inhibitors (for Ras) or inhibitory peptides (for PKCαβ or PKCζ) in the presence or absence of cytochalasin D (Fig. 7A).

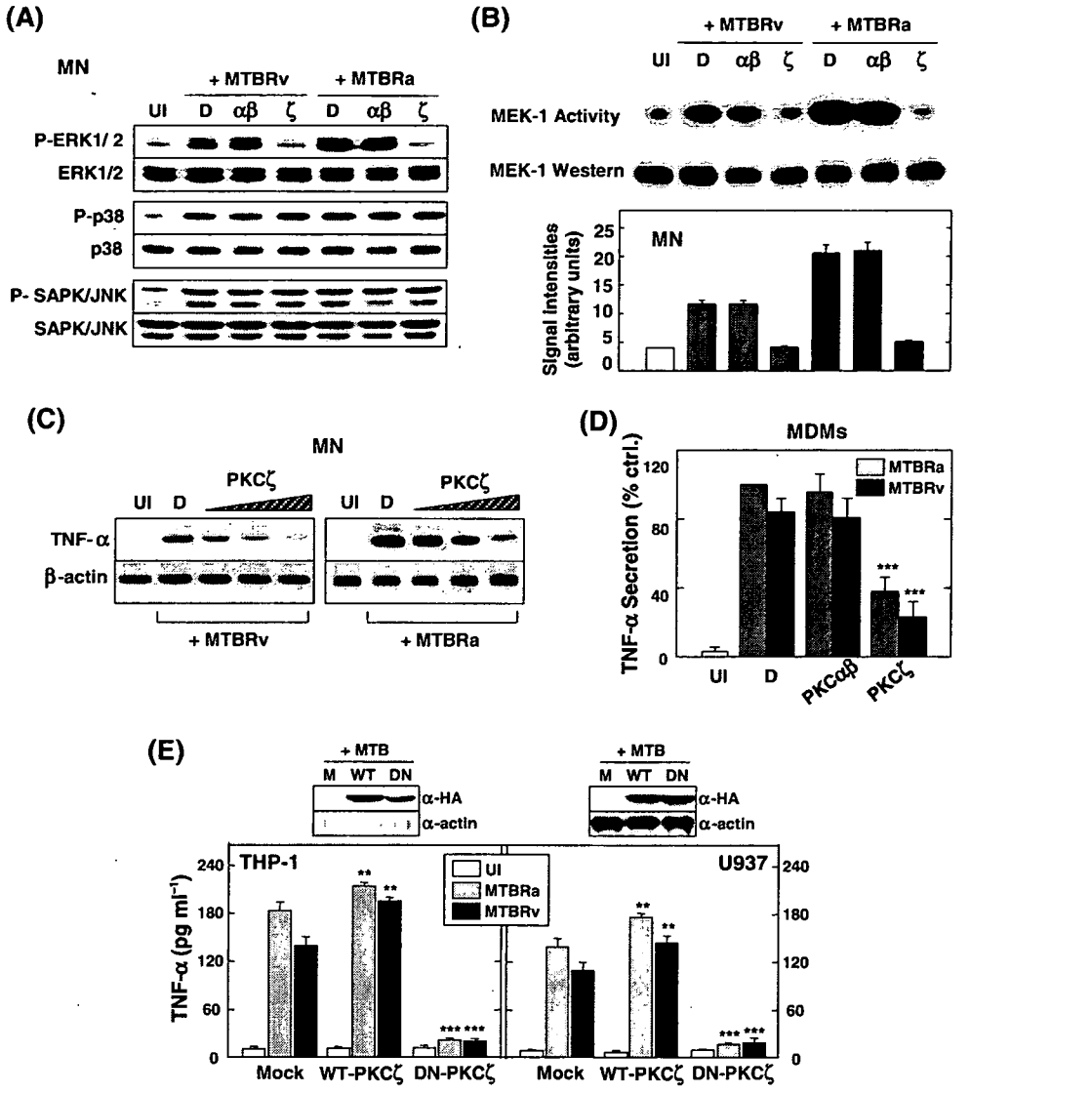

Fig. 6. PKCζ plays an essential role on MTB-mediated ERK-1 and MEK activation, and TNF-α expression.

A. The PKCαβ- or PKCζ-specific peptides (20 μM) were added to monocytes 30 min before infection with MTBRv or MTBRa (moi = 1). The cells were harvested and subjected to Western blot analysis for phosphorylated ERK1/2, p38 and SAPK/JNK. The same blots were washed and blotted for total ERK1/2, p38 and SAPK/JNK as the loading controls. Data are representative of five independent experiments with similar results.

B. Human monocytes were treated with PKCαβ- or PKCζ-specific peptides (20 μM) for 30 min followed by MTBRv or MTBRa for 15 min. Whole cell protein was obtained, and MEK1 was immunoprecipitated from 200 μg of the lysates. Kinase activity assays were performed using MBP (10 μg) as a substrate. The resulting phosphorylated proteins (MBP) were separated on 12% SDS-PAGE gels. The gels were dried and autoradiography was performed. A representative experiment of three independent replicates with similar results is shown.

C and D. Human monocytes (for C) and MDMs (for D) were preincubated with a PKCζ-specific (5, 20, 40 μM) or a PKCαβ-specific peptide (20 μM, for both peptides) as described, before *M. tuberculosis* stimulation. Total RNA was purified at 3 h, and semi-quantitative RT-PCR analysis of TNF-α was performed. A representative gel of three independent replicates with similar results is shown. MN, monocytes (C). The supernatants were harvested after 6 h for TNF-α assessment using ELISA. The mean levels of TNF-α following infection with MTBRa were set to 100, and the relative loss of cytokine production in the presence of inhibitory peptide is shown. Data are the mean ± SD of three experiments. Significant differences (***$P < 0.001$) compared with cultures in an absence of inhibitory peptides. D, the solvent control of 0.1% DMSO; UI, uninfected (D).

E. THP-1 or U937 cells were transfected with WT-PKCζ, DN-PKCζ, or empty vector. After a 24 h incubation in normal culture medium, the transfected cells were stimulated for 6 h with MTBRa or MTBRv (at moi = 1). After harvest of supernatants, TNF-α ELISA was determined. In the upper panel, lysates of 5×10^5 cell equivalent from each transfectant (M, mock; WT, WT-PKCζ; DN, DN-PKCζ) were immunoblotted with anti-HA Ab. The same blots were stripped and reprobed with anti-β-actin mAb. Data are the mean ± SD of three experiments. Significant differences (***$P < 0.001$; **$P < 0.01$) compared with cultures of empty vector control.

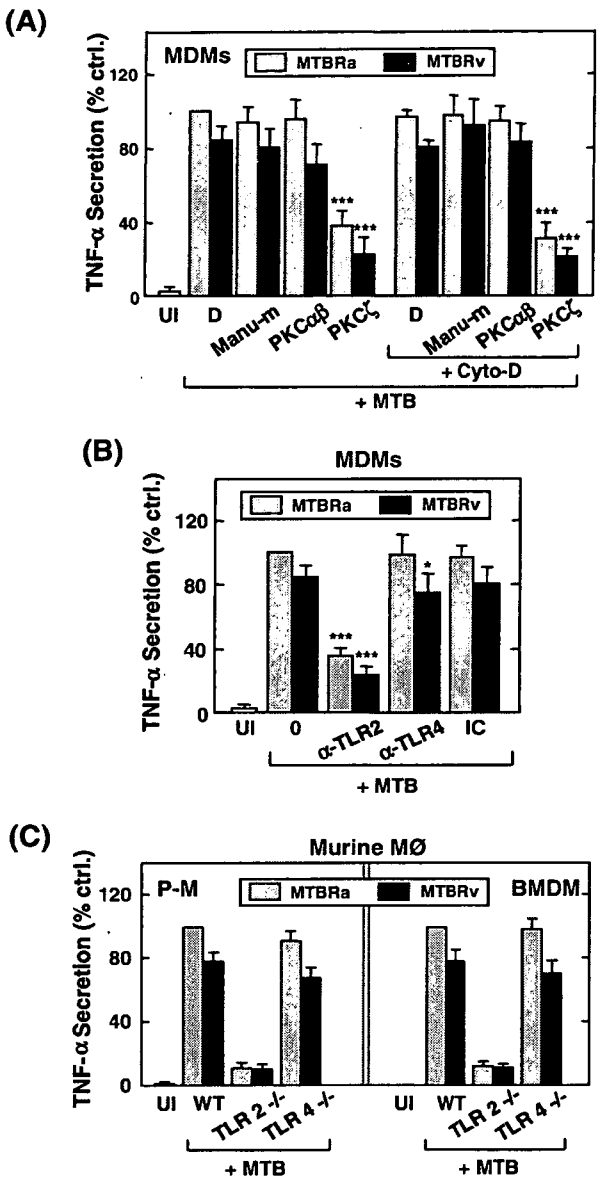

Fig. 7. TLR2, but not phagocytosis or TLR4, plays a major role for TNF-α expression by MTB.
A. Human MDMs were preincubated with or without cytochalasin D (Cyto-D; 10 μM) in the presence or absence of manumycin M (Manu-m; 1 μM), PKCζ-specific or a PKCαβ-specific peptide (20 μM), or 0.1% DMSO control (D) at 45 min before adding MTBRa or MTBRv (moi = 1). Cell free supernatants were then collected after 6 h, and TNF-α levels were determined by ELISA. Significant differences (***$P < 0.001$; **$P < 0.01$) compared with cultures in an absence of inhibitors or inhibitory peptides. Data are expressed as the means ± SD of values for three separate experiments using cells from different five donors.
B. Human MDMs were preincubated with anti-TLR2, anti-TLR4 mAbs, or IC Ab (all, 10 μg ml^{-1}) at 45 min before adding MTB (moi = 1). The supernatants were then collected after 6 h, and the levels of TNF-α were determined by ELISA. Significant differences (***$P < 0.001$; *$P < 0.05$) compared with cultures of IC control. Data are expressed as the means ± SD of values for two separate experiments using cells from different four donors.
C. Peritoneal macrophages (P-M) and BMDM from TLR2$^{-/-}$, TLR4$^{-/-}$ and control mice were isolated and cultured with MTBRv or MTBRa (moi = 1). Cell free supernatants were then collected after 6 h, and the levels of TNF-α were determined by ELISA. Data are the mean ± SD of two experiments. UI, uninfected; Murine MØ, murine macrophages.

The extent to which TLRs are involved in MTB-induced TNF-α responses was examined in human MDMs using specific monoclonal antibodies (mAbs) for TLR2 and TLR4. As shown in Fig. 7B, the anti-TLR2 mAb strongly inhibited (~70% inhibition for both bacteria) the MTB-induced TNF-α production. Although MTBRa-induced TNF-α production was not changed by pretreatment with the anti-TLR4 mAb, MTBRv-induced TNF-α secretion was partially inhibited by TLR4 blockade ($P < 0.05$). The role of TLR2 in MTB-induced TNF-α synthesis was further investigated using peritoneal macrophages and bone marrow-derived macrophages (BMDM) from TLR2$^{-/-}$, TLR4$^{-/-}$ and control mice. The MTB-stimulated production of TNF-α was significantly attenuated in the peritoneal macrophages and BMDM from TLR2$^{-/-}$ mice stimulated with MTBRv or MTBRa (Fig. 7C). These results indicate that the MTB-induced expression of TNF-α is mainly mediated via TLR2.

TLR2 is essential for MTB-induced PKCζ activation, and PKCζ associates with TLR2 after MTB stimulation

As the MTB-mediated TNF-α release was abrogated in TLR2$^{-/-}$ cells, we tested the hypothesis that rapid PKCζ activation is linked to TLR2 signalling caused by MTB stimulation. To determine whether TLR2 affects MTB-induced PKCζ activation, we measured the MTB-induced activation of PKCζ in BMDM from TLR2$^{-/-}$, TLR4$^{-/-}$ and control mice using the antiphospho-(Thr410)-PKCζ Ab. As shown in Fig. 8A, PKCζ activity was enhanced markedly in BMDM at 1 min after MTBRa stimulation, whereas weak PKCζ activation was observed after stimulation with MTBRv. Longer incubation with MTB increased the phosphorylation of PKCζ in a time-dependent manner; this effect was maximal at 5 min in cells infected with MTBRv or MTBRa. Nevertheless, infection with MTBRv induced less activation of PKCζ in BMDM than did MTBRa infection, at all time points. The PKCζ phosphorylation was totally abrogated in TLR2$^{-/-}$ BMDM, but not TLR4$^{-/-}$ BMDM, after MTBRa or MTBRv stimulation, demonstrating that TLR2 critically participates in MTB-induced PKCζ activation.

We also examined the potential association between PKCζ and TLR2 in human monocytes *in vivo*. Total protein extracts were prepared from MTB-treated primary human monocytes and used to immunoprecipitate TLR2 protein.

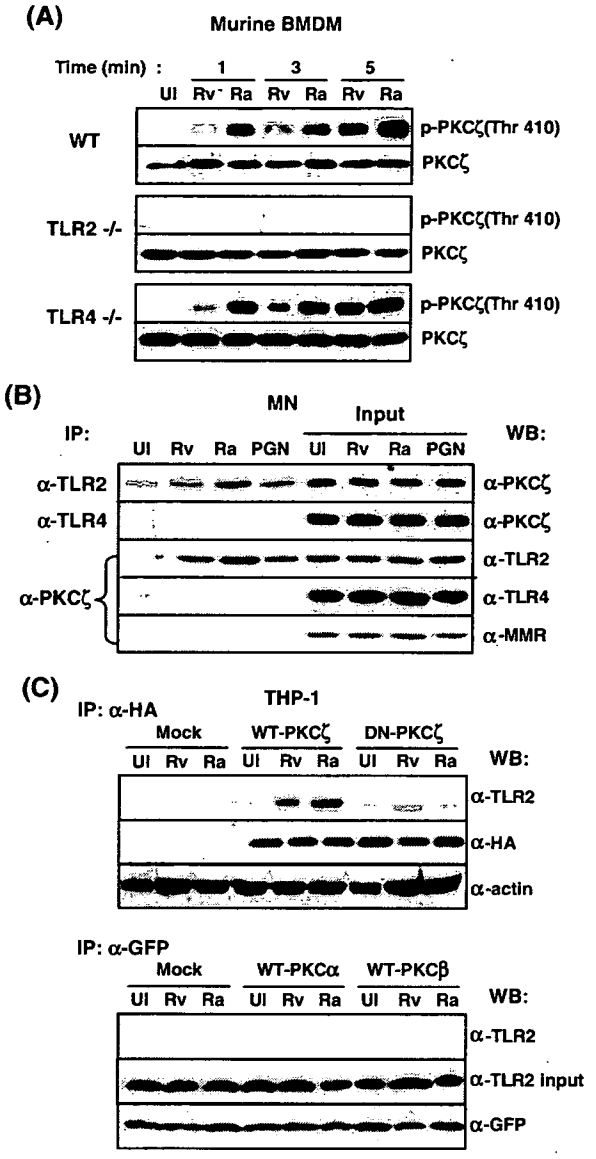

Fig. 8. TLR2 is essential for MTB-induced PKCζ activation, and the PKCζ associates with TLR2 after MTB stimulation.
A. BMDMs from WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were stimulated with MTBRv (Rv) or MTBRa (Ra; moi = 1, for both strains) for the times indicated. The cells were harvested and subjected to Western blot analysis for phosphorylated PKCζ using antiphospho-(Thr410)-PKCζ Ab. The same blots were washed and blotted for total PKCζ as the loading control.
B. Human primary monocytes were stimulated with MTBRv (Rv), MTBRa (Ra; moi = 1, for both strains) or peptidoglycan (PGN, 10 μg ml^{-1}) for 5 min, and subjected to immunoprecipitation with anti-TLR2, anti-TLR4, or anti-PKCζ Abs. Total cell lysate (input controls for TLR2, TLR4, or MR) and immunoprecipitated proteins were detected by immunoblotting with anti-PKCζ, anti-TLR2, anti-TLR4, or anti-MR Abs.
C. THP-1 cells transfected with various plasmids for WT- or DN-PKCζ (for the upper panel), and WT-PKCα or WT-PKCβ (for the lower panel) were stimulated with MTBRv (Rv) or MTBRa (Ra; moi = 1, for both strains) for 5 min. Then, each lysate was subjected to immunoprecipitation with anti-HA or anti-GFP Ab. Western blot analysis was carried out with anti-TLR2, anti-HA, or anti-GFP Abs. Data (A–C) are representative of three independent experiments with similar results. UI, uninfected.

As shown in Fig. 8B, endogenous PKCζ was detected in the immunoprecipitated TLR2, but not TLR4, complex. Similarly, TLR2 protein was observed in the PKCζ immunoprecipitated from human primary monocytes following stimulation with MTBRv, MTBRa, or peptidoglycan (Fig. 8B). When the membrane containing the proteins from the immunoprecipitation was stripped and reprobed with anti-TLR4 or antimannose receptor (MR) Abs, neither TLR4 nor MR was recognized in the immunoprecipitates, indicating that TLR2 can bind to PKCζ specifically after stimulation with MTB.

To complement these results, we next transfected human monocytic THP-1 cells with the WT- or DN-PKCζ plasmid and challenged the cells with MTBRv or MTBRa. When the cell lysates were immunoprecipitated with anti-HA Ab and immunoblotted for the presence of TLR2, an association between PKCζ and TLR2 was detected for MTB-stimulated THP-1 cells transfected with the WT-PKCζ plasmid, although a stronger association was detected in transfectants stimulated with MTBRa than with MTBRv (Fig. 8C). In marked contrast, cells transfected with the DN-PKCζ plasmid exhibited no TLR2 expression in the presence or absence of MTBRv or MTBRa stimulation, which confirms that TLR2 physically interacts with PKCζ. Further, no interaction between TLR2 and PKCα/β was found in THP-1 cells transfected with GFP-tagged PKCα or β after stimulation with MTB (Fig. 8C, lower). Combined, these results suggest that MTB-induced PKCζ activation occurs through the specific interaction of PKCζ with TLR2 after MTB stimulation.

Discussion

The MAPKs play important roles in the macrophage response to mycobacterial infection. However, there is less information regarding MAPK and its upstream signalling by intact mycobacteria in monocytes/macrophages. Given the described ability of pathogens to modulate MAPK activation in host cells (Song *et al.*, 2003), we wanted to more clearly define the role of MAPKs and their upstream mediator(s) responsible for differential macrophage activation between virulent and avirulent strains of MTB.

Previous studies have demonstrated the importance of ERK 1/2 during the early induced macrophage response, primarily its ability to upregulate monocyte production of proinflammatory cytokine TNF-α, which mediates the activation of chemokines and recruitment of lymphocytes involved in combating an infection (Mulligan *et al.*, 1993;

Roach *et al.*, 2002; Algood *et al.*, 2005). We show that the differences in ERK activation at very early time points are important to the subsequent levels of TNF expression and production. Our data concurred with the previous findings that the inhibition of ERK resulted in decreased TNF-α synthesis following *Mycobacterium avium* infection (Tse *et al.*, 2002). Whereas p38 and ERK1/2 are of critical importance for the release of proinflammatory cytokines such as TNF-α and IL-1 (Lee *et al.*, 1994), only activated p38, but not ERK 1/2, is necessary for IL-10 formation in response to LPS (Foey *et al.*, 1998). In human MDMs, the activation of ERK1/2, but not of p38, was essential for *M. avium*-induced TNF-α formation (Reiling *et al.*, 2001). Further, TNF-α production was significantly lower during infection with virulent MTB than with BCG, and this differential response was associated with a reduction in the extent and duration of phosphorylation of ERK1/2 by the virulent strain (Hasan *et al.*, 2003). Combined, these studies indicate that the induction of the ERK 1/2 pathway during exposure to bacteria is instrumental in determining the eventual fate of the bacteria and host responses.

In fact, our finding that MTBRa induces more TNF-α than does MTBRv in both mature and immature macrophages contrasts with other reports (Balcewicz-Sablinska *et al.*, 1998; Silver *et al.*, 1998). This discrepancy might be attributable to the different strain types and the culture status in our study. Our studies focused on the bacilli in late log-phase cultures of MTB. The bacilli in late log-phase cultures probably contain various mycobacterial components, including LAM, which may be important in stimulating PRRs in mononuclear phagocytes (Chatterjee *et al.*, 1992). We observed that MTBRa-induced TNF-α mRNA and protein levels were significantly higher than those induced by MTBRv in primary human monocytes, MDMs and murine macrophages. Subsequently, other investigators reported that the attenuated mutant MTB strain induced stronger expression of pulmonary mRNA messages for TNF-α (Copenhaver *et al.*, 2004). Further studies will be necessary to clarify the exact component(s) of MTB responsible for modulating macrophage defence mechanisms associated with virulence *per se*.

Many upstream signalling pathways in macrophages converge on ERK 1/2. However, limited information is available on the upstream pathways of ERK 1/2 signalling in mycobacteria. The classic Ras-mediated pathway involves the binding of Raf-1 and the subsequent phosphorylation of Raf-1 at Ser-338 by several kinases (Zang *et al.*, 2002), which in turn activates ERK (Zhang *et al.*, 1993) and consequently phosphorylates many transcription factors and protein kinases (Whitmarsh and Davis, 1998). A previous study showed that *M. avium* triggers TLR2-dependent signalling, leading to the phosphorylation of ERK and p38 MAPK, which are necessary for *COX-2* gene transcription and PGE2 production (Pathak *et al.*, 2004). The same study reported that Ras associates with TLR2 and activates the ERK pathways in *M. avium*-challenged RAW264.7 macrophages (Pathak *et al.*, 2004). The discrepancy between the previous study (Pathak *et al.*, 2004) and ours might be attributable to the different cell types used, indicating that the involvement of Ras in signalling processes is cell-type-dependent. However, we observed that the ERK 1/2 phosphorylation was significantly abrogated in primary murine macrophages following pretreatment with inhibitory peptides for PKCζ, but not with specific inhibitors for Ras and Raf (data not shown). Therefore, our data demonstrate that Ras is not primarily involved in MTB-induced ERK 1/2 activation and TNF-α expression in different types of macrophages.

Perhaps the most intriguing finding in this study was that PKCζ plays an essential role in the MTB-mediated ERK 1/2 activation and TNF-α expression in various monocytes/macrophages, such as primary human monocytes, monocyte cell lines (THP-1 and U937), and primary murine macrophages. A large body of literature suggests that the activation of the MAPK pathway is downstream from PKCζ, although the functional role of PKCζ in primary macrophages for regulating the MTB-induced activation of ERK1/2 and TNF-α release has not been reported. An early report provided evidence for a link between PKCζ and the MAPK pathway using cotransfection experiments in which PKCζ stimulated the activation of both MEK and MAPK (Berra *et al.*, 1995). Previous studies have also shown that Ca^{2+}-dependent PKC activation plays a crucial role in ERK activation and the IKK/IκB/NF-κB cascades, leading to the upregulation of cytokine expression (Matsubara *et al.*, 2005). Furthermore, PKCζ induces MEK-dependent ERK1 activation and ERK1 translocation to the nucleus in human monocytic U937 leukaemic cells (Mansat-De Mas *et al.*, 2002). Thus, the targeting of PKCζ signalling leading to ERK activation in macrophages might play an important role in the production of various effector molecules, including TNF-α, following infection with mycobacteria.

We found that TLR2 plays a pivotal role in MTBRv- and MTBRa-induced TNF-α expression and PKCζ activation. In addition, we are the first to show that PKCζ associates with TLR2 after MTB stimulation of primary human monocytes and monocytic cells. Of the PKCs, PKCζ is activated and associates with RhoA following TLR2 interaction with bacterial products (Teusch *et al.*, 2004). Hu *et al.* (Hu *et al.*, 2002) observed a direct interaction between IRAK1, which is a serine/threonine kinase upstream from TRAF6, and PKCζ in monocytic cells stimulated with LPS. Using a TLR2-dependent stimulus, we did not detect any association between PKCζ and IRAK1 (data not shown). In this study, we used primary monocytes and THP-1 cells to elucidate the interaction of PKCζ with TLR2. Given that

PRRs are predominantly expressed in macrophages where TLR is associated with PKCζ after MTB stimulation, the interaction might involve an entire complex of receptors. Our data provide important insight into the specific interaction between TLR2 and PKCζ, because neither TLR4 nor MR is coprecipitated from cell lysates (Fig. 8B). In addition, note that PKCζ phosphorylation by MTB is essentially dependent on TLR2 (Fig. 8A). Collectively, MTB engagement to TLR2 might cause the direct association and phosphorylation of PKCζ, which is crucial for the downstream MEK1-ERK 1/2 activation and TNF-α release in monocytes/macrophages.

In summary, we have investigated the upstream signalling cascades that lead to ERK activation, through which MTB stimulates primary human monocytes to secrete TNF-α. This is the first demonstration that MTB-induced TNF-α release by monocytes/macrophages occurs in a Ras-independent manner via the TLR2→PKCζ →MEK1→ERK 1/2 signalling pathway. Both MTBRv and MTBRa strains lead to similar activation cascades of signalling pathways. Although the reduction in PKCζ phosphorylation following MTBRv stimulation is accompanied by the decreased activation of ERK 1/2 and TNF-α protein production by this virulent strain, it is difficult to draw conclusion that these findings are attributed to the relative virulence between two strains. Future studies that more thoroughly characterize the overt differences in macrophage defence mechanisms between strains MTBRv and MTBRa will undoubtedly shed light on the bacterial pathogenesis by which a virulent MTB strain subverts macrophage function during mycobacterial infection.

Experimental procedures

Bacteria

MTBRv was kindly provided by Dr Richard L. Friedman (University of Arizona, Tucson). Both MTBRv and MTBRa (ATCC 25177) was grown at 37°C on Middlebrook 7H10 agar (Difco, Detroit, MI) medium supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase; Becton Dickinson) and 0.05% Tween 80 (Sigma). Stock strains were grown in roller bottles, to late log phase in liquid Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween 80 and 10% OADC enrichment. Then, the bacterial culture were divided into 1 ml aliquots in cryovials, and stored at −70°C until needed. Representative vials were thawed, and viable cfu were enumerated on Middlebrook 7H10 agar. Single-cell suspensions of mycobacteria were prepared as described previously (Song *et al.*, 2003). To rule out the influence of LPS in the assays, the bacterial suspensions were tested by the *Limulus* amebocyte lysate assay (Cambrex, QCL1000). The effective LPS concentration was < 50 pg ml^{-1} in experiments with bacteria to cell ratios of 1 : 1.

Human and murine cells, and cell lines

The study was approved by the bioethics committee of Chungnam University Hospital's review board overseeing studies on samples from human subjects. Adherent monocytes were prepared from peripheral blood mononuclear 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 (Yang *et al.*, 2006). 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 μg ml^{-1}) before mycobacterial stimulation. Human monocytic cell line, U937 (ATCC CRL 1593; American Type Culture Collection) and THP-1 (ATCC TIB-202; American Type Culture Collection) were maintained in complete medium [RPMI 1640 (Gibco-BRL) with 10% fetal bovine serum (Gibco-BRL), sodium pyruvate, non-essential amino acids, penicillin G (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1})]. U937 and THP-1 cells were treated with 4 nM phorbol myristate acetate (PMA; Sigma) for 48 h to induce differentiation into macrophage-like cells and washed with PBS three times.

Primary peritoneal macrophages and BMDM from TLR2-/– and TLR4-/– mice of C57BL/6 background and C57BL/6 mice were prepared from these mice, as previously described (Takeuchi *et al.*, 1999). All animals were maintained in a pathogen-free environment. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) in Chungnam National University.

Materials, reagents and antibodies

The specific inhibitors of MEK, PD98059 and U0126, the PKC-specific inhibitors Gö6976, Ro31-8220, bisindolylmaleimide I and Gö6983, the Ras inhibitor manumycin A, the Raf-1 inhibitor GW 5074 and specific inhibitory peptides for PKC-αβ and PKCζ were all purchased from Calbiochem. Dimethyl sulphoxide (DMSO; Sigma) was added to cultures at 0.1% (v/v) as a solvent control. LPS (*Escherichia coli* 026:B6) and peptidoglycan (*Staphylococcus aureus*) were purchased from Sigma and Fluka respectively.

Mouse antihuman TLR2 mAb (clone TL2.1, IgG2a), mouse antihuman TLR4 mAb (clone HTA125, IgG2a), isotype-control (IC) mAb (IgG2a) was purchased from eBioscience. Mouse antihuman MR Ab (ab8918; clone 15–2, IgG1) was from Abcam. Other Abs used were anti-PKCζ Ab (Cell Signaling Technology), anti-HA Ab (InvivoGen), anti-TLR2 Ab (H-175, Santa Cruz Biotechnology), anti-TLR4 Ab (H-80, Santa Cruz Biotechnology) and anti-GFP Ab (clone B-2, Santa Cruz Biotechnology) for immunoprecipitation.

DNA and adenoviral constructs

Expression plasmids encoding WT-PKCζ-HA, and dominant negative (DN)-PKCζ-HA (K281M), in pMT-2 were a generous gift from Dr Ji Won Sho (Inha university, Incheon, Korea). The DN-MEK1 (S218A, S222A) expression vector was a kind gift from Dr Chul-Man Cho (Korean National Institute of Health, Seoul, Korea). For the expression plasmids of WT-PKCα and WT-PKCβ, cDNA encoding the murine PKCα and β were amplified by polymerase chain reaction (PCR) from their parent plasmids [pBL-PKCα and pMTH-PKCβ, kindly provided by Dr J. Frederic Mushinski (National Institute of Health, USA)], and then sub-

cloned into N terminal enhanced GFP vector pEGFP-N1 (BD Biosciences Clontech). The integrity of the inserts was verified by DNA sequencing. Cells were transfected using LipofectAMINE as indicated by the manufacturer (Invitrogen).

The replication-deficient adenovirus vectors carrying dominant-negative Ras (Ad-Ras DN; Ser17 to Asn) were obtained from Dr S. Tanaka. Adenovirus carrying the cytomegalovirus (CMV) promoter (Ad-CMV: control virus) was kindly provided by Dr Byeong-Hwa Jeon (Chungnam National University, Daejeon, Korea). The primary monocytes were plated at 5×10^5 cells per well in 96-well tissue-culture plates and were fed with medium (RPMI1640 + 2% FCS) containing the recombinant adenovirus at a concentration of 100 plaque-forming units (pfu) per cell for Ad-CMV. For Ad-Ras DN, infection was performed according to the method described previously (Song *et al.*, 2004).

Western analysis and enzyme-linked immunosorbent assay

Human primary monocytes and MDMs (at a concentration of 8×10^5 per ml) were treated with MTBRv or MTBRa for the times indicated. Cell lysates were prepared and Western blot analysis was performed with specific primary Abs [ERK1/2, phospho-(Thr202/Tyr204)-ERK1/2, p38, phospho-(Thr180/Tyr182)-p38, phospho-(Thr183/Tyr185)-SAPK/JNK and SAPK/JNK Abs (New England Biolabs, Schwalbach, Germany)]; antiphospho-(Thr410)-PKCζ Ab (Santa Cruz Biotechnology), as described previously (Song *et al.*, 2003). Specific bands were developed by ECL (Amersham Biosciences).

A sandwich enzyme-linked immunosorbent assay (ELISA) was used for detecting TNF-α?(PharMingen) in culture supernatants, as described previously (Song *et al.*, 2003).

Reverse transcriptase PCR

RNA was extracted from the cells using TRIzol (Invitrogen). Complementary DNA (cDNA) was reverse transcribed from 2 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen) and oligo-dT-3′ primer in a total volume of 20 μl. Using PCR, 2 μl of cDNA was amplified. The primers and PCR conditions were as described previously (Yang *et al.*, 2006). The PCR products were resolved on 1% agarose gels and were stained with ethidium bromide.

In vitro *MEK assay*

Confluent monocytes or human monocytic THP-1 cells in 100 mm dishes were infected with MTBRv or MTBRa with or without pretreatment of inhibitory peptides for PKCζ or PKC-αβ. After incubation, the cells were lysed with ice-cold lysis buffer and *in vitro* MEK assay was conducted, as previously described (Monick *et al.*, 2000). Briefly, the cell lysates were incubated with antihMEK-1 Ab (SantaCruz Biotechnology) for 2 h on a rocking platform. Then, 20 μl of protein A sepharose (Amersham Bioscience) slurry were added and rocking continued for another 3 h. The immunoprecipitates were washed twice in lysis buffer and twice in kinase assay buffer (20 mM $MgCl_2$, 25 mM HEPES, 20 mM p-nitrophenylphosphate, 20 mM β-glycerophosphate, 20 mM sodium orthovanadate and 2 mM DTT). The immune complexes were then suspended in 20 μl of kinase buffer, and the following were added: 20 μM ATP, 5 μCi [γ-^{32}P]ATP (BLU 002Z, DuPont/NEN, Boston, MA), and 10 μg of myelin basic protein (MBP, Sigma). The reaction was continued for 30 min at 25°C with gentle agitation and then stopped by the addition of 10 μl of 4 [X] 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 ^{32}P-labelled MBP. Densitometry was performed on films and fold increase calculated as experimental sample/control sample.

Immunoprecipitation

THP-1 cells transiently transfected with indicated HA-PKCζ expression plasmids were harvested and lysed, as described previously (Hu *et al.*, 2002). Extracts with equal amount of proteins were used for the immunoprecipitation. A total of 5 μl of anti-HA mAb (Sigma-Aldrich) was added to 800 μl each of the isolated cell extracts and incubated at 4°C for 3 h on a rotator. A total of 50 μl of a 50% slurry of prewashed protein G agarose beads was then added to each sample, followed by incubation for an additional 2 h at 4°C. The samples were spun briefly in a microcentrifuge and washed for four times in lysis buffer. Samples were subsequently solubilized by SDS sample buffer (80 mM Tris-HCl (pH 6.8), 2% SDS, 50% glycerol, 0.05% bromphenol blue, 0.2 M DTT), separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories), Western blotted with anti-TLR2 mAb, and detected by ECL reagent. A similar procedure, as described above, was used to prepare cell extracts from primary monocytes. Endogenous TLR2 proteins from these cells were immunoprecipitated using polyclonal anti-TLR2 Ab (Santa-Cruz Biotechnology).

Statistical analysis

For statistical analysis, the data obtained from independent experiments are presented as the mean ± SD; they were analysed using a paired *t*-test with Bonferroni adjustment or ANOVA for multiple comparisons. Differences were considered significant at $P < 0.05$.

Acknowledgements

We thank Dr Hwa-Jung Kim for invaluable assistance and technical suggestions, and we thank Dr Jeong-Kyu Park for critical review of the article. We also thank Dr Chul-Man Cho, Dr J. Frederic Mushinski and Dr Byeong-Hwa Jeon for kind provision of constructs, and Dong-Min Shin for technical assistance. This work was supported by Grant R042 0040001 00220 2004 from the Korea Research Foundation for the 2004 program year and partially supported by a grant of the Korea Health 21 R and D Project, Ministry of Health and Welfare, Republic of Korea (01-PJ10-PG6-01 G03-002).

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Erk pathways negatively regulate matrix mineralization

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Received 5 April 2006; revised 12 July 2006; accepted 21 July 2006
Available online 15 September 2006

Abstract

Skeletal mineralization is an important step regulating the mechanical properties of the calcified tissues, but molecular events underlying mineralization still remain elusive. We examined the role of extracellular signal-regulated kinase (Erk) pathways in matrix mineralization of osteogenic cells both in vitro and in vivo. Matrix mineralization by preosteocytic MLO-A5 cells and osteoblastic MC3T3-E1 cells was increased by either PD98059 Mek inhibitor treatment or adenovirus vector-mediated dominant negative Ras (Ras^{DN}) expression and was suppressed by Erk activation by platelet-derived growth factor (PDGF) treatment or constitutively active Mek1 (Mek^{CA}) expression. Administration of adenovirus vectors carrying Ras^{DN} gene onto the calvaria of 1-day-old mice increased the mineralization of the tissues, while that of the Mek^{CA} adenovirus suppressed it. These results suggest that the Erk pathway is a negative regulator of the matrix mineralization both in vitro and in vivo.

Keywords: Mineralization; Osteocyte; Osteoblast; Erk; Ras; Mek

Introduction

Cells of osteoblast lineage, osteoblasts and osteocytes are derived from mesenchymal stem cells and have functions depending on their differentiation stages [2,9,27]. Bone forming osteoblasts differentiate into either bone lining cells or preosteocytes, which are embedded in the calcified bone matrix. Production of type I collagen is followed by the expression of alkaline phosphatase (ALP) and osteocalcin (OCN), and finally mineralization of the matrix protein occurs [17]. Osteocytes are a terminally differentiated form of osteoblasts and considered to be mechanosensory cells of the skeletal environment [1,19]. Osteoblasts and osteocytes are implicated in the mineralization process of the bone matrix. Mineralization is one of the most important steps to establish the structural integrity and the mechanical strength of the skeletal tissue, however, the molecular mechanism regulating this process remains unclear.

We investigated the intracellular signal transduction pathways regulating the mineralization process using MLO-A5 preosteocytic cell line and MC3T3-E1 osteoblastic cells. Activation of Erk pathways strongly suppressed the matrix mineralization of the cells, while its suppression promoted the process. In vivo injection of Mek^{CA} adenoviruses onto calvaria of newborn mice suppressed, while that of Ras^{DN} viruses promoted the local mineralization. These results suggest that the Erk pathway is a negative regulator of the matrix mineralization of the skeletal tissues.

Materials and methods

Chemicals and antibodies

Alpha-modified Minimum Essential Medium (α-MEM) was purchased from GIBCO BRL Inc. (Rockville, MD, USA). β-glycerophosphate (β-GP), ascorbic acid and recombinant human PDGF-BB were purchased from SIGMA CHEMICAL Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Cell Culture Laboratory (Cleveland, OH, USA). Anti-Erk antibody and anti-phospho-Erk antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Other chemicals and reagents used in this study were of analytical grade.

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doi:10.1016/j.bone.2006.07.024

Cells

The osteoblastic cell line MC3T3-E1, which was established from normal mouse calvaria [24], was purchased from RIKEN Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were cultured in α-MEM supplemented with 10% FBS. They are known to differentiate into mature osteoblasts in the course of the culture and to exhibit matrix mineralization when cultured in the presence of 5 mM β-GP and 100 μg/ml ascorbic acid [24]. MLO-A5 cells are a preosteocytic cell line established from the long bones of 14-day-old osteocalcin promoter-driven T-antigen transgenic mice [14]. MLO-A5 cells mineralize in sheets, not nodules, within 3 days of culture in the presence of β-GP and ascorbic acid and within 7 days even in the absence of these two substances.

Constructs and gene transduction

The recombinant adenovirus vector carrying β-galactosidase gene (AxLacZ) was kindly provided by Dr. Izumu Saito (Institute of Medicine, The University of Tokyo). The recombinant adenovirus vector carrying the dominant negative *ras* gene (Ser17 to Asn, AxRas^{DN}) or constitutively active *mek*1 gene (Ser218 and Ser222 to Glu, AxMek^{CA}) under the control of CAG [cytomegalovirus (CMV) immediate early (IE) enhancer+chicken β-actin promoter+rabbit β-globin poly (A) signal] promoter was provided by Dr. Hideki Katagiri (Tohoku University) [20], and *runx2* gene was by Toshihisa Komori (Nagasaki University) [8]. The efficiency of infection is affected not only by the concentration of viruses and cells, but also by the ratio of the former to the latter, the multiplicity of infection (MOI). Infection of the cells by adenovirus vectors was carried out as described.

Immunoblotting

All the extraction procedures were performed at 4°C or on ice. Cells were washed with PBS and then lysed by adding TNE buffer (1% NP-40, 10 mM Tris–Hcl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF and 10 μg/ml aprotinin). The lysates were prepared by centrifugation at 15,000× rpm for 20 min. An equal amount (15 μg) of proteins was electrophoresed on 10% SDS polyacrylamide gels. After electrophoresis, proteins were electronically transferred onto a nitrocellulose membrane. Immunoblotting with specific antibodies was carried out using ECL Western blotting reagents (Amersham Co., IL, USA) according to the conditions recommended by the supplier.

MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] assay

After viral infection for 2 days, cells were replated on 96 well plates at the density of 5000 cells/well and incubated in 10 μl MTT solution (5 mg/ml in PBS) for 4 h. After incubation, 100 μl of 0.04 M HCl/isopropanol solution was added to melt the formazan crystal. The OD values were read at 570 nm wavelength. The cell proliferation indices were calculated by the OD value in adenovirus wells (cells plus adenoviruses) divided by the OD value in control wells (cells only), and then multiplied by 100.

Histocytochemistry

Cultures were fixed with 99% ethanol and subjected to Alizarin Red staining to assess matrix mineralization. In brief, cells were immersed in Alizarin Red solution (1% at pH 6.4) for 2 min at room temperature, and nonspecific staining was removed by several washes in distilled water.

Total RNA extraction and real time PCR

Total RNA was isolated from cultured cells with ISOGEN (Wako) following the supplier's protocol. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the SUPERSCRIPT II reverse transcriptase kit (Invitrogen, CA, USA). For real time PCR, ABI Prism Sequence Detection System 7000 was used. Primers were designed based on Genbank, and amplicons of 50–250 base pairs with T_m between 55 and 60°C were selected. Aliquots of first-strand cDNA (1 μg) were amplified using QuantiTect SYBER Green PCR Kit (Qiagen, CA, USA) under the following conditions: initial denaturation for 10 min at 94°C followed by 40 cycles of 15 s at 94°C and 1 min at 60°C. Data analysis consisted of fold induction, and expression ratio was calculated from differences in threshold cycles at which an increase in reporter fluorescence above a baseline signal was first detected among three samples and then averaged for duplicate experiments. The primers we utilized in real time PCR to detect ALP, OCN, osteopontin (OPN), bone sialoprotein (BSP), type I collagen (Col I) and β-actin were as follows:

ALP	5′-GCTGATCATTCCCACGTTTT-3′
	5′-CTGGGCCTGGTAGTTGTTGT-3′
OCN	5′-AAGCAGGAGGGCAATAAGGT-3′
	5′-TTTGTAGGCGGTCTTCAAGC-3′
OPN	5′-ACACTTTCACTCCAATCGTCC-3′
	5′-TGCCCTTTCCGTTGTTGTCC-3′
BSP	5′-CAGAGGAGGCAAGCGTCACT-3′
	5′-CTGTCTGGGTGCCAACACTG-3′
Col I	5′-ACGTCCTGGTGAAGTTGGTC-3′
	5′-CAGGGAAGCCTCTTTCTCCT-3′
ANK	5′-CTGCTGCTACAGAGGCAGTG-3′
	5′-ACAAGGCTGGTGATGAGGAC-3′
PC-1	5′-TGAGAAGAGGCTGTCCAGGT-3′
	5′-AGTATGTGCCGGACTTGACC-3′
β-actin	5′-AGATGTGGATCAGCAAGCAG-3′
	5′-GCGCAAGTTAGGTTTTGTCA-3′

In vivo mineralization assay

Fifty microliters of adenovirus vectors containing approximately 2.5×10^7 virus particles or normal saline was injected onto the calvaria of 1-day-old ddY mice using a microinjector. Five days after the injection, the mice were sacrificed and the calvaria were removed, and paraffin-embedded sections of the calvaria were subjected to Alizarin Red staining. To analyze the efficiency of the adenovirus infection, calvaria from AxLacZ-injected mice were subjected to X-gal staining.

Results

MLO-A5 cells were positively stained with Alizarin Red after 3 days of culture in the presence of β-GP and ascorbic acid, and 7 days even in the absence of these reagents, demonstrating the strong mineralization activity of the cells (Fig. 1A) [14]. Mineralization of the cells was strongly promoted by Mek inhibitor PD98059, which dose-dependently suppressed Erk activity in the cells as determined by Western blotting with anti-phospho-Erk antibody (Fig. 1B). PDGF is a member of the PDGF/VEGF (vascular endothelial growth factor) family, which binds to its specific receptor PDGFR belonging to the receptor-type tyrosine kinase family and activates the Ras-Raf-Mek-Erk pathway [25,26]. Treatment of MLO-A5 cells with PDGF-BB dose-dependently stimulated Erk pathways and strongly suppressed mineralization of the cells even in the presence of β-GP and ascorbic acid (Fig. 2A). Mineralization of the cells was restored by the addition of PD98059 together with PDGF, indicating that the inhibitory action of PDGF is mainly mediated through Erk activation (Fig. 2B). We further investigated the role of Erk pathways in mineralization of MLO-A5 cells using the adenovirus vector-mediated gene transduction system. MLO-A5 cells were infected with AxLacZ to determine the efficiency of adenovirus vector-mediated gene transduction. As shown in Fig. 3A, strong

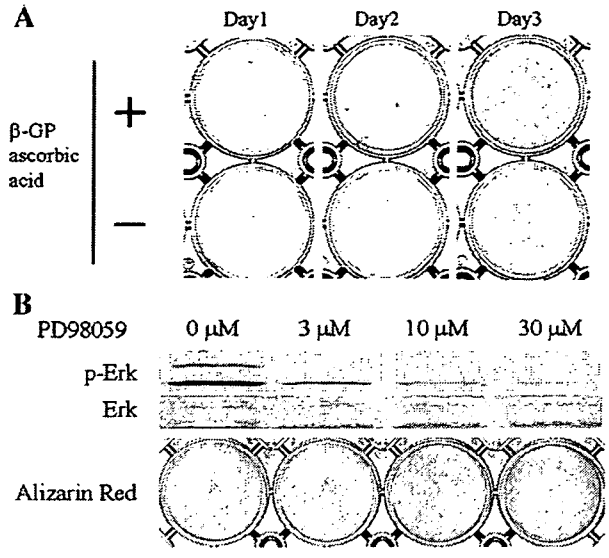

Fig. 1. Effect of matrix mineralization of MLO-A5 cell cultures by Mek inhibitor PD98059. (A) Alizarin Red staining of MLO-A5 preosteocytic cell cultures. In the presence of 5 mM β-GP and 100 μg/ml ascorbic acid, MLO-A5 cells are mineralized in 3 days as determined by Alizarin Red staining (upper column). Slight induction of mineralization was observed even in the absence of β-GP and ascorbic acid. (B) Dose-dependent inhibition of Erk activity as determined by anti-phospho-Erk Western blotting (upper column) and promotion of mineralization (lower column) in MLO-A5 cells by Mek inhibitor PD98059 on the 7th day of culture. No apparent change in the Erk expression was observed (middle column).

X-gal staining was observed in the cells infected with AxLacZ, and more than 95% of the cells were positively stained at MOI 100 with no apparent morphological changes or toxic effects. We next examined the effect of $AxMek^{CA}$ and $AxRas^{DN}$ on the mineralization of MLO-A5 cells. $AxMek^{CA}$ infection induced phospho-Erk expression in the cells and strongly suppressed their mineralization, while $AxRas^{DN}$ infection downregulated Erk phosphorylation and promoted mineralization as shown in Fig. 3B. This was not due to the effect on the cell viability since no significant change in MTT assay was observed by the viruses (Fig. 3C). $AxRas^{DN}$ expression recovered the mineralization of MLO-A5 cells suppressed by PDGF treatment (Fig. 2C).

MC3T3-E1 is a cell line with preosteoblastic phenotypes, which differentiates into mature osteoblasts and exhibits mineralization during culture [24]. MC3T3-E1 cells were also efficiently infected by adenovirus vectors as determined by AxLacZ infection and X-gal staining (Fig. 3A). As shown in Fig. 3D, adenovirus vector-mediated *runx2* gene expression strongly upregulates the mineralization of the cells, and matrix mineralization was suppressed by $AxMek^{CA}$ infection and promoted by $AxRas^{DN}$ infection (Fig. 3D). These results suggest that Erk activation is a negative regulator of the mineralization independent of Runx2.

To investigate the molecular mechanism underlying the effect of Erk on MLO-A5 cells, we analyzed the osteoblastic gene expression in the cells. As shown in Fig. 4, significant increase in OPN gene expression was observed in $AxMek^{CA}$-infected cells compared to AxLacZ-infected cells (1.97-fold), and the gene expression was suppressed by Ras^{DN} expression (0.8-fold). ALP gene expression was significantly increased by $AxRas^{DN}$ infection but was not affected by $AxMek^{CA}$. OCN gene expression was significantly decreased by $AxRas^{DN}$ infection and increased by $AxMek^{CA}$ but with no statistical significance. BSP and Col I gene expression was suppressed by $AxMek^{CA}$ and increased by $AxRas^{DN}$ without statistical significance (Fig. 4). $AxMEK^{CA}$ infection also increased OPN expression in MC3T3-E1 cells expressing *runx2*, while no significant reduction in the expression was observed in $AxRas^{DN}$-infected cells (Fig. 4). We also analyzed the expression of plasma cell membrane glycoprotein 1 (PC-1) and ANK, which are known to regulate the accumulation of extracellular inorganic pyrophosphate. $AxMEK^{CA}$ infection significantly decreased the expression of both PC-1 and ANK in MLO-A5 cells, while no significant change was observed in $AxRas^{DN}$-infected cells (Fig. 4).

We finally examined the effect of Erk activation in the mineralization in vivo. When AxLacZ was injected onto the calvaria of 1-day-old mice, strong X-gal staining was observed after 3 days of the injection, indicating an effective gene transduction by the adenovirus in vivo. Alizarin Red staining

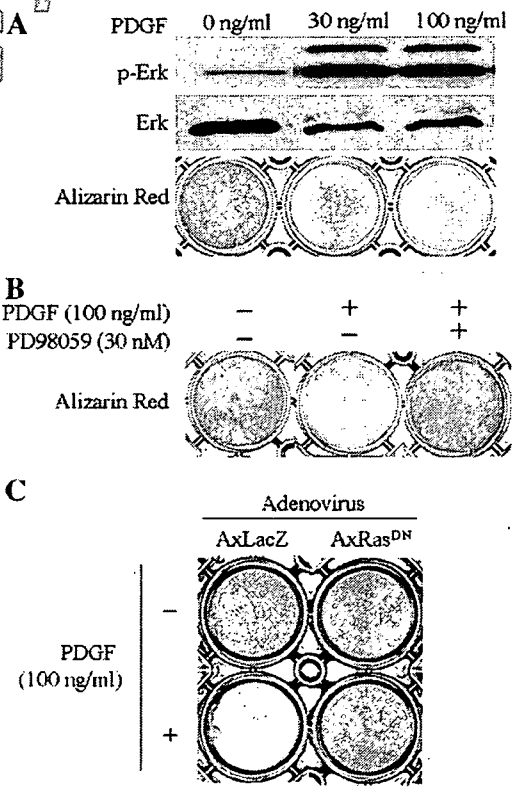

Fig. 2. Effect of PDGF-BB on mineralization of MLO-A5 cells. (A) PDGF-BB dose-dependently increased Erk activity in MLO-A5 cells as determined by phospho-Erk blotting (upper column) and promoted their mineralization (lower column). No apparent change in the Erk expression was observed (middle column). (B) Mek inhibitor PD98059 at 30 μM recovered the mineralization of MLO-A5 cells even in the presence of PDGF-BB on the 7th day of culture. (C) Inhibition of Erk pathways by dominant negative Ras adenovirus ($AxRas^{DN}$) recovered mineralization of MLO-A5 cells suppressed by PDGF-BB.

exhibited a decreased mineralization by AxMek^{CA} and an increased mineralization by AxRas^{DN} 5 days after the injection (Fig. 5).

Discussion

Research on the regulatory mechanism of osteoblast differentiation was remarkably advanced by the recent finding of critical transcription factors such as Runx2 and Osterix [7,9,15,21,27]. Matrix mineralization is a final step of osteoblast differentiation and plays a critical role in maintaining the mechanical integrity of the calcified tissues [2,9,17]. Defective mineralization is observed in various pathological conditions such as rickets and osteomalacia, in which bone strength is greatly reduced [3,18]. However, molecular events leading to the matrix mineralization still remain unclear. Erk pathways are essentially involved in various aspects of physiological and pathological events and regulate proliferation and differentiation of the cells [4,5,23]. In the present study, we tried to elucidate the role of these pathways on the matrix mineralization by osteogenic cells. Several previous studies attempted to clarify the role of Erk pathways on osteoblast differentiation with different and partly contradictory results. Consistent with our results, Higuchi et al. demonstrated that the Erk inhibitor PD98059 promoted the early osteoblastic differentiation and mineralization in C2C12 pluripotent mesenchymal cells treated with BMP-2 and MC3T3-E1 cells [10]. In addition, Chaudhary and Avioli reported that Erk activation by PDGF-BB or fibroblast growth factor-2 suppressed type I collagen expression in MC3T3-E1 cells, and the inhibition of Erk pathways either by specific inhibitors or by culturing the cells in low serum (0.3%) markedly increased it [6]; and Nakayama et al. further reported that Erk activation negatively regulates osteoblast differentiation induced by BMP-2 [22]. On the other hand, Jaiswal reported that osteogenic medium activates Erk pathways in human mesenchymal stem cells and that the inhibition of Erk activity suppressed their osteoblastic differentiation and mineralization, indicating a positive regulatory role for Erk pathways on mineralization [13]. Lai et al. also reported that

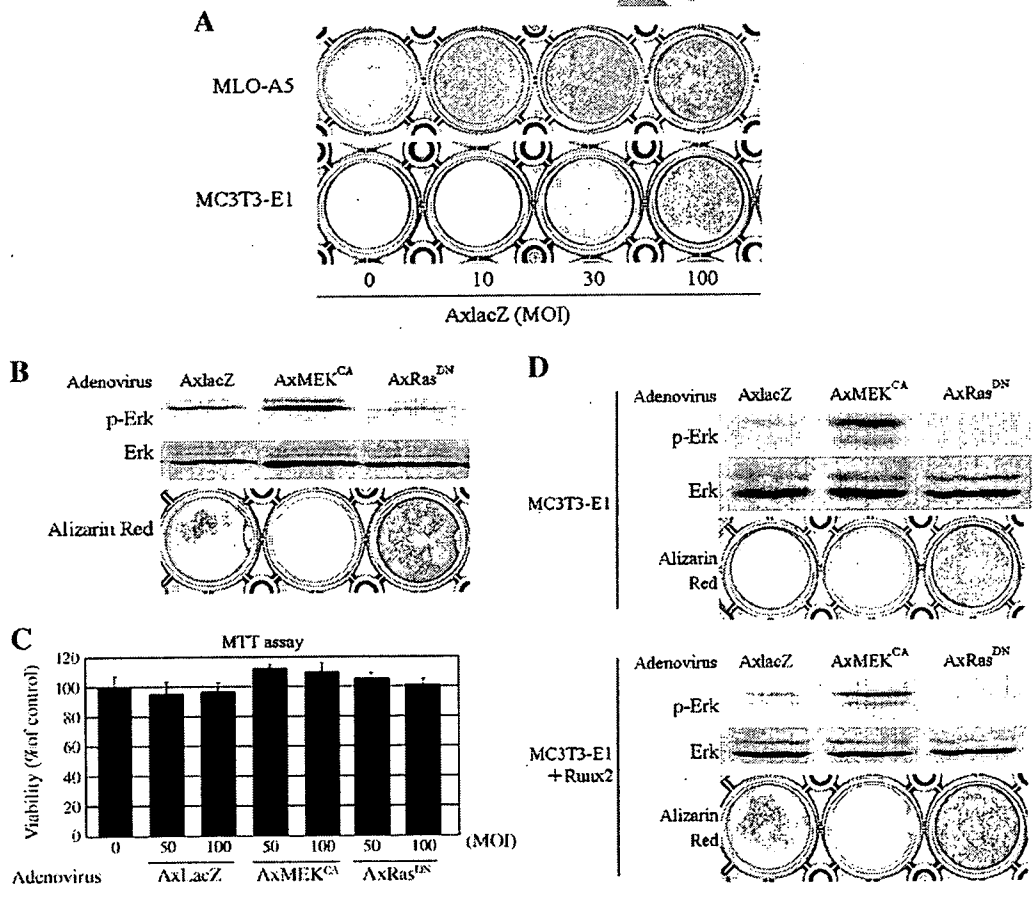

Fig. 3. Regulation of Erk pathways by adenovirus vector-mediated gene transduction system. (A) X-gal staining after 2 days of AxLacZ infection. AxLacZ efficiently and dose-dependently transduced β-galactosidase gene in MLO-A5 cells (upper) and MC3T3-E1 cells (lower). (B) Activation of Erk pathways by AxMek^{CA} infection suppressed and their inhibition by AxRas^{DN} infection promoted the matrix mineralization of MLO-A5 cells on the 7th day of culture. (C) MTT assay. Neither infection of AxMek^{CA} nor AxRas^{DN} affected the viability of MLO-A5 cells. No significant difference was observed between adenovirus-infected cultures and control cultures. (D) Effect of AxMek^{CA} and AxRas^{DN} infection on Erk activation and matrix mineralization of MC3T3-E1 cells (upper) and MC3T3-E1 cells overexpressing Runx2 (lower).