trapped promoter. The poly (A) signal downstream of the *lox*P plays a role in concentrating the targeted integration event through the poly (A) trap strategy. Since the selection marker gene in a targeting vector does not have a poly (A) signal, only upon targeted integration does the selection marker gene fuse to the poly (A) signal on the trap vector, thereby making the cells drug-resistant.

Although the pU8 vector works quite well, the truncated protein may be produced when the vector is integrated downstream of the endogenous gene. This truncated protein may exert unexpected effects on phenotypes. In addition, recombination may occur between *lox*71 and *lox*P, because both carry the same spacer sequences, resulting in the deletion of a gene of interest. To overcome these problems, we improved and created the new trap vector, pU17. First, we try to produce a promoter trap vector, so that we can make a null allele in the first step. For this purpose, we inserted the three stop codons in splice acceptor, which is located upstream of the ATG of the reporter gene, β-geo, and did not use the IRES sequence. If this vector was inserted into the downstream of endogenous gene, translation would stop at the stop codon. As the β-geo gene does not have IRES sequence, it would not be translated independently. Thus, ES clones will not become drug-resistant and such clones will not be isolated. Second, we took double *lox* strategy to reduce the possibility of recombination as mentioned above. In the double *lox* strategy[38] or recombinase mediated cassette exchange[39,40] a different type of mutant lox site was used. We used heterospecific lox sites that have mutation(s) in the 8 bp spacer region. The principle is that recombination does not occur between two lox sites differing in the spacer region, whereas lox sites having the identical spacer region recombine efficiently.[41] Several groups have used lox511 (Fig. 1d), which contains a single base substitution, and demonstrated successful gene replacement in which a genomic DNA segment flanked by lox511 and loxP was replaced with another cassette flanked by lox511 and loxP located on a transfected plasmid vector.[39,42] Lee and Saito[43] developed heterospecific mutant lox sites with two base substitutions, such as lox2272 (Fig. 1d), and showed that they never recombined with the wild-type loxP site, while lox511 can recombine with loxP at low frequency using an *in vitro* system. Successful selection marker-free replacement using lox2272 and loxP was demonstrated in ES cells by Kolb[44] (Fig. 1c). So, we examined the best combination of mutant lox sites to give a high recombination efficiency and stability. We found that the frequency of site-specific integration was highest and reached a maximum of 35% when we used the combination of the LE/RE mutant and lox2272.[45] Based on these findings, we have constructed the pU17 suitable for production of a null trap allele in the first step, production of the replaced allele in the second step, and the conditionally inactivated allele in the third step by mating recombinase expressing transgenic mice[46] (Fig. 2). This is termed as the exchangeable gene trap method. The new vector, pU-17, carries the intron-*lox*71-splicing acceptor (SA)-β*geo*-*lox*P-pA-*lox*2272-pSP73-*lox*511 (Fig. 2). The SA contains three stop codons in-frame with the ATG of β-*geo* that can function as promoter trappings. We found that the trap vector was highly selective for integrations near the exon containing the start codon (Fig. 3), leading to the null mutation of trapped endogenous gene as expected. Thus, using this pU-17 trap vector, we can initially carry out random mutagenesis. Among 432 trap clones, the trapped DNAs comprise 53% (229) known coding region, 13.4% (58 clones) new or unknown genes, 12% (52 clones) known coding region but in reverse orientation, 8.8% (38 clones) no known coding region, and 12.7% (55 clones) undetermined (TransGenic Inc. Kumamoto, Japan, unpublished data, 2006) (Fig. 4). It is of interest that about 12% of clones show the insertion of a trap vector in a reverse orientation, suggesting that non-coding RNA genes may exist in these

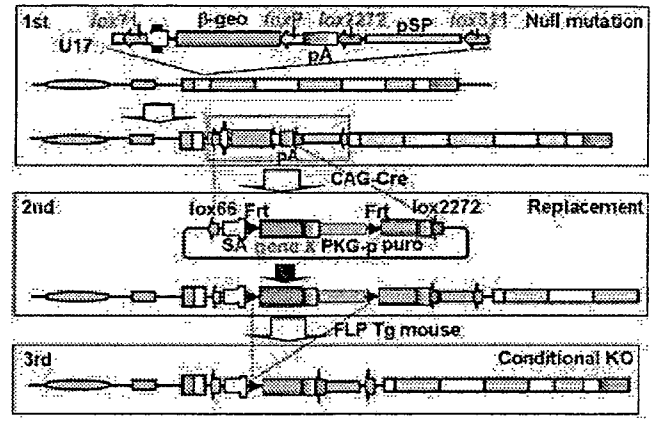

Fig. 2. Exchangeable gene trap method. Production of the null trap allele in the first step, the replaced allele in the second, and the conditionally inactivated allele in the third step is shown.

regions. Furthermore, we successfully integrated the *cre* gene into the mutant lox sites by Cre-mediated recombination. The inserted *cre* gene was stably transmitted through the germline. This cre knock-in system using the LE/RE-*lox*2272 combination should be useful for the production of various cre mice for gene targeting or gene trapping.

Exchangeable gene trap a tool for cancer research

Using the exchangeable gene trap method, we established more than 300 ES trap mouse lines and these lines are cryopreserved. The database of these lines is publicly available at the Database for the Exchangeable Gene Trap Clones (EGTC) website (http://egtc.jp/view/index). Among these, we analyzed six lines in detail, termed as Ayu3-112, Ayu8-021, Ayu8-025, Ayu8-104, Ayu8-108, and Ayu21-127, to examine whether these genes had any relation with cancer. In these lines, the integration of a trap vector resulted in the disruption of *Crebbp* (cyclic AMP response element-binding protein) gene[47,48] *Skt* (Sickle) gene[49] *Abhd2* (α/β hydrolase domain containing 2) gene[50] *c-crk* gene[51] *Impβ* (*Importin β*)/*Kpnb1* (*karyopherin β1*) gene[52] and *Lgr4* (leucine-rich repeat domain containing G protein-coupled receptor 4) gene,[53] respectively. These strains are termed as $Crebbp^{GtAyu3-112Imeg}$, $Skt^{GtAyu8-021Imeg}$, $Abhd2^{GtAyu8-025Imeg}$, $c\text{-}crk^{GtAyu8-104Imeg}$, $Impb^{GtAyu8-108Imeg}$, and $Lgr4^{GtAyu21-127Imeg}$, respectively. In the present review we will focus on three strains: $Skt^{GtAyu8-021Imeg}$, $Abhd2^{GtAyu8-025Imeg}$, and $Lgr4^{GtAyu21-127Imeg}$. These genes were initially thought to be unrelated to cancer and therefore, no-one had chosen these genes as targets for cancer research. This is because most cancer studies have been carried out using a hypothesis-driven approach. In this approach, researchers need information that suggests a relationship exists between the gene and cancer to commence work. On the contrary, the gene trap approach is a Baconian science, like natural history. This is also called an 'ignorance-driven' approach.[54] The seventeenth-century philosopher Francis Bacon suggested a system for understanding the world that begun with the accumulation of facts, based on observation. This kind of approach does not include bald theories or sudden leaps of understanding. It does not attract the same level of recognition as in hypothesis-driven types of research. Thus, many people are doubtful about this type of approach in science. However, through the gene trap approach, we can find the genes that are paid little attention in cancer research, but are actually related to cancer. Next we will briefly introduce these three lines and explain how they are related to cancer.

We established the mutant mouse line, B6; CB-$Skt^{GtAyu8021IMEG}$ (Skt^{tf}), through gene-trap mutagenesis in embryonic stem cells.[49]

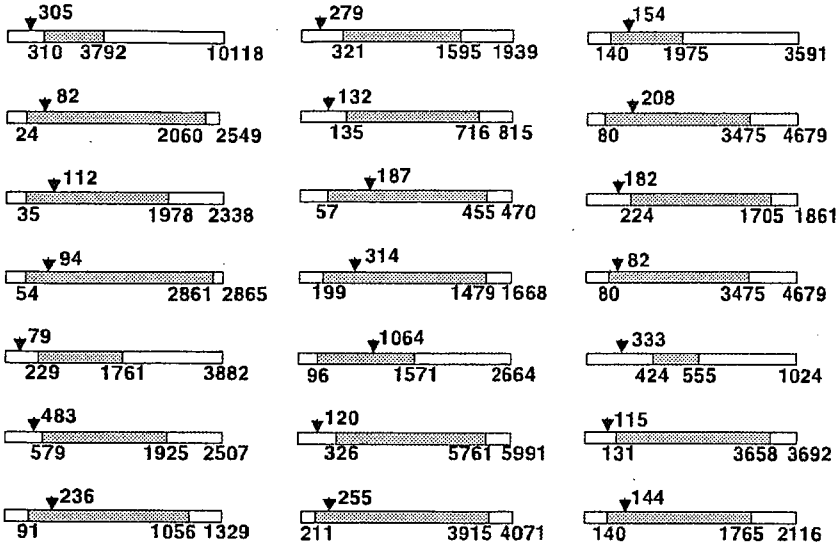

Fig. 3. Insertion sites in known genes using the pU17 vector. Black bars indicate coding region and blank boxes indicate 5′ or 3′ untranslated regions. Arrows indicate the nucleotide numbers of insertion sites. Nucleotide numbers of translation initiation site, translation termination site, and transcription termination site are shown below the genes.

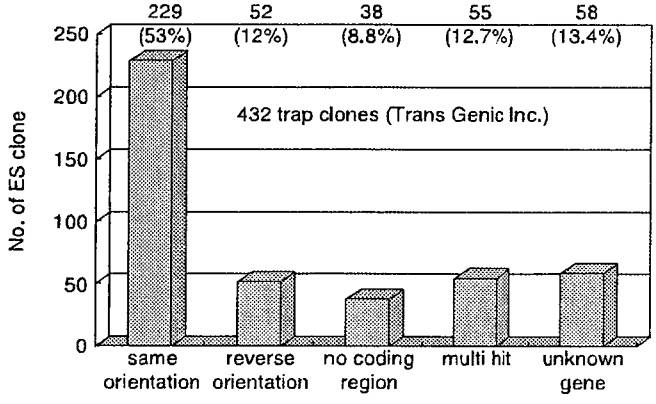

Fig. 4. Characterization of trapped DNA. In 12% of clones, the trap vector is inserted into the known coding region but in reverse orientation. In 8.8% of clones there is no known coding region around the insertion site.

The novel gene identified, called *Sickle tail* (*Skt*), is composed of 19 exons and encodes a protein of 1352 amino acids with a proline-rich region and a coiled-coil domain. Expression of a reporter gene (*β-geo*) was detected in the notochord during embryogenesis and in its derivative, the nucleus pulposus, in adult mice. Compression of some of the nuclei pulposi in the center of intervertebral discs appeared at embryonic day (E) 17.5, resulting in a kinky-tail phenotype in $Skt^{Gt(Gt}$ adult mice. Chordoma is a malignant tumor, but is characterized by slow growth, with local destruction of the bone and extension into the adjacent soft tissue. Very rarely, distant metastases are encountered. Chordomas are thought to arise from primitive notochordal remnants along the axial skeleton. The distribution of tumors matches the distribution of notochordal remnants. The Skt could be a marker for differential diagnosis of chordoma and chondrosarcoma. This differential diagnosis may be important to decide the extent of surgical dissection.

We also isolated a clone, Ayu8025, in which the trap vector was inserted into the fifth intron of α/β hydrolase domain containing 2 (Abhd2).[50] The α/β hydrolase fold family consists of hydrolytic enzymes of widely differing phylogenetic origin. Each of these family members has the same protein fold, termed as the α/β hydrolase fold.[55] The core of each enzyme is similar: an α/β-sheet of five to eight β-sheets connected by α-helices to form an α/β/α sandwich. They all have a nucleophile–histidine–acid catalytic triad, which is the best-conserved structural feature in the fold. In mice, three cDNA encoding proteins containing an α/β hydrolase fold were cloned from lung cDNA.[56] These are now termed as abhydrolase domain containing (*Abhd*) 1, 2 or 3. Reverse transcription-polymerase chain reaction (RT-PCR) analyses showed that these three genes are widely, but differentially expressed in many tissues. The expression of *Abhd1* and *Abhd3* was highest in the liver and lowest in the spleen, whereas the expression of *Abhd2* was high in the testis and the spleen. All three Abhd proteins are shown to have a single predicted amino-terminus transmembrane domain. Although proteins in this family generally act as enzymes such as acetylcholinesterases, or lipases, the specific functions of the three Abhd proteins are unknown. To obtain some clue for Abhd2 function, we first analyzed the expression pattern during embryonic development using X-gal staining. Interestingly, *Abhd2* was expressed in the vitelline vessels of yolk sacs at embryonic day (E) 12.5 and expression of Abhd2 switched from endothelial cells to vascular smooth muscle cells (SMCs) during further embryonic development. In adult, Abhd2 was expressed in many tissues including vascular and non-vascular SMCs such as intestinal SMCs. Although homozygous mutant mice were apparently normal, enhanced SMC migration in the explants SMCs culture and marked intimal hyperplasia after cuff placement were observed in homozygous mice. Our results show that *Abhd2* is involved in SMC migration and neo-intimal thickening on vascular SMCs. When we started to analyze this gene in 2002, there was no report to suggest a relationship between this gene and cancer. Using microarray analysis, Johansson *et al.*[57,58] reported that the expression of Abhd2 was enhanced in a mouse brain tumor model induced by intracerebral injection of a recombinant Moloney murine leukemia virus encoding the platelet-derived growth factor B-chain (MMLV/PDGFB) into newborn mice. In addition, using microarray analysis again, Chen *et al.*[59] reported that ABHD2 expression was increased in MCF-7 cells transfected with human Sp5, a member of the Sp transcription factor family, suggesting that ABHD2 is a downstream target of Sp5, and that ABHD2 is associated with tumorigenesis. At the moment, the role of ABHD2 in tumorigenesis is not known yet.

Another gene trap line is $Lgr4^{Gt(Ayu21\text{-}127)Imeg}$ ($Lgr4^{Gt}$) in which the trap vector was integrated into the 2nd intron of the *Lgr4* gene.[53] Northern blot analysis and quantitative RT-PCR showed

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that the $Lgr4^{Gt/Gt}$ mice had 10% mRNA expression of the *Lgr4* gene, suggesting that the $Lgr4^{Gt/Gt}$ mouse is a hypomorphic mutant. Leucine-rich repeat domain containing G-protein coupled receptor (GPCR) 4 (LGR4) family members are characterized by an extracellular domain with multiple leucine-rich repeats (LRRs).[60,61] The presence of a large extra-cellular domain is a remarkable feature that separates the LGR family members from the other GPCRs. Studies of LGRs from different species suggest that LGRs can be classified into three subtypes (A, B and C). Type A LGRs include the follicle-stimulating hormone receptor (FSHR), the lutenizing hormone receptor (LHR), and the thyroid-stimulating hormone receptor (TSHR), in which the ligands are glycoprotein hormones.[62] Type B LGR comprises three members: LGR4, also known as GPR48, LGR5, and LGR6. Type B LGR remains an orphan GPCR and its physiological functions have not yet been determined. Type C LGRs, including LGR7 and LGR8, were recently described as relaxin receptors.[63] Following the identification of LGR7 and LGR8 as relaxin receptors, the closely related relaxin3 and INSL3 have been shown to function as selective agonists for LGR7 and LGR8, respectively. The homozygous male was infertile showing morphological abnormalities in both the testes and the epididymides. In the testes, luminal swelling, loss of germinal epithelium in the seminiferous tubules, and rete testis dilation were observed. Rete testis dilation was due to a water reabsorption failure caused by a decreased expression of ESR1 and SLC9A3 in the efferent ducts. The epididymis contained short and dilated tubules and completely lacked its initial segment. Interestingly, we observed multilamination and distortion of the basement membranes with an accumulation of laminin in caput epidydimidis. These results indicate that *Lgr4* has pivotal roles for the control of duct elongation through basement membrane remodeling, and the regional differentiation of the caput epididymidis. On the other hand, Gao *et al.*[64] reported that LGR4 expression is upregulated in p27-deficient HCT116 cells using microarray analysis. Forced expression of LGR4 increased both *in vitro* invasive activity and lung metastasis potency of HCT116 cells. In contrast, the depletion of endogenous LGR4 by RNA interference reduced the invasive potential of HeLa and Lewis lung carcinoma cells not only *in vitro* but also *in vivo*. Moreover, GPR48 expression was significantly associated with lymph node metastasis and inversely correlated with p27 expression in human colon carcinomas. As a reduced expression level of the cyclin-dependent kinase inhibitor p27Kip1 is associated with increased tumor malignancy and poor prognosis in individuals with various types of cancer, LGR4 may play an important role in invasiveness and metastasis of carcinoma and might therefore represent a potential prognostic marker or therapeutic target. This is consistent with our hypothesis that Lgr4 is involved in the remodeling of BM.

Conclusion

In summary, we show here that exchangeable gene trap is a powerful tool for finding genes that are related to cancer, but are paid little attention in cancer research. By combining microarray analysis and gene trap mutagenesis, we will be able to analyze function of such genes in more systematic ways. In addition, we show that we can replace the reporter gene with a gene of interest using the Cre-loxP system. We can apply this system to the vectors for homologous recombination, thus we can make the multipurpose allele at any gene locus. These methods can be applied for detailed analysis of gene function in cancer research.

Acknowledgments

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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

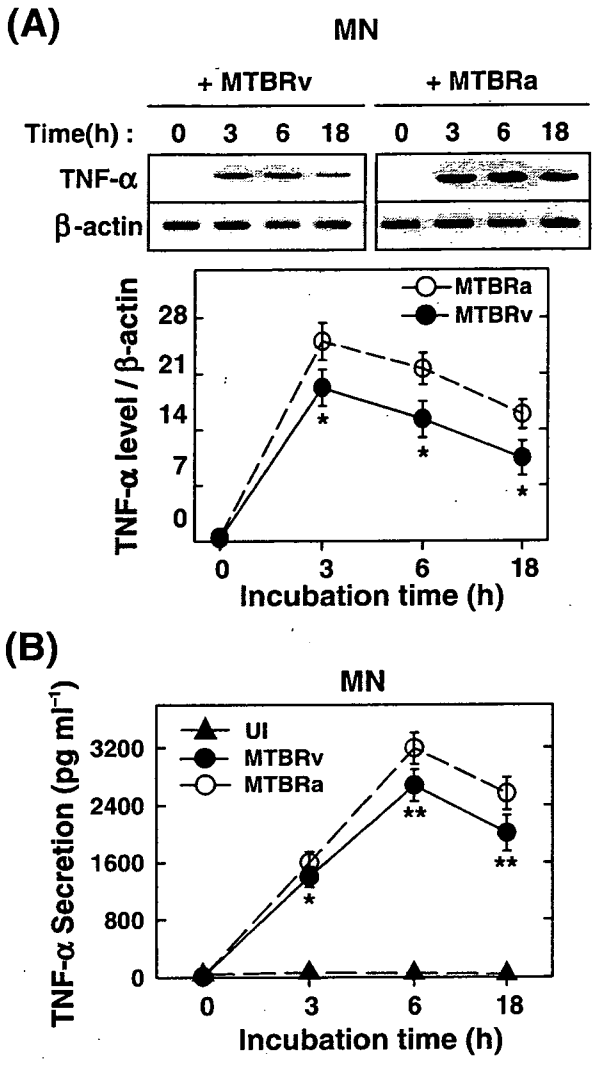

Fig. 1. MTB-induced TNF-α expression from human monocytes. Human monocytes were infected with MTBRv or MTBRa (moi = 1). Cell lysate and supernatants were harvested. Total RNA was assessed by PCR for TNF mRNA (A), and the protein expression was measured by ELISA (B). Data are the mean ± SD of three experiments.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

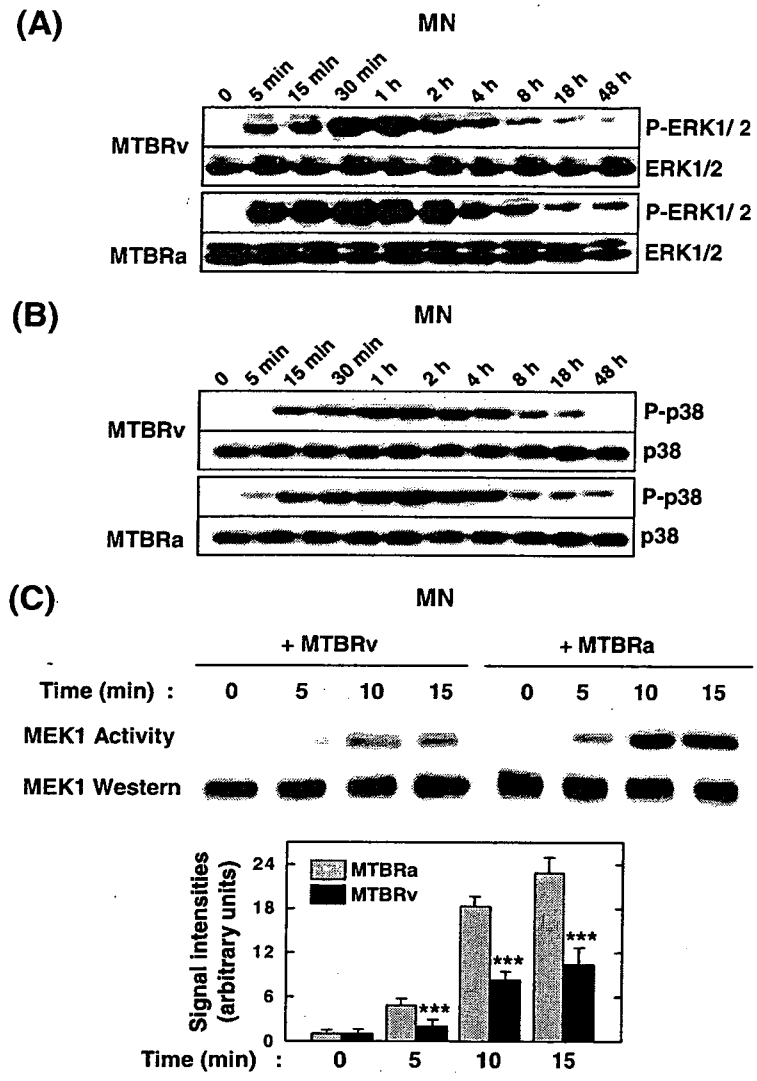

Fig. 2. MTB-mediated activation of MAPKs and MEK1 in human primary monocytes.
A and B. Human monocytes were infected by MTBRv or MTBRa (moi = 1) for the times indicated. The cells were harvested and subjected to Western blot analysis for phosphorylated ERK1/2 (A) and p38 (B). The same blots were washed 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-

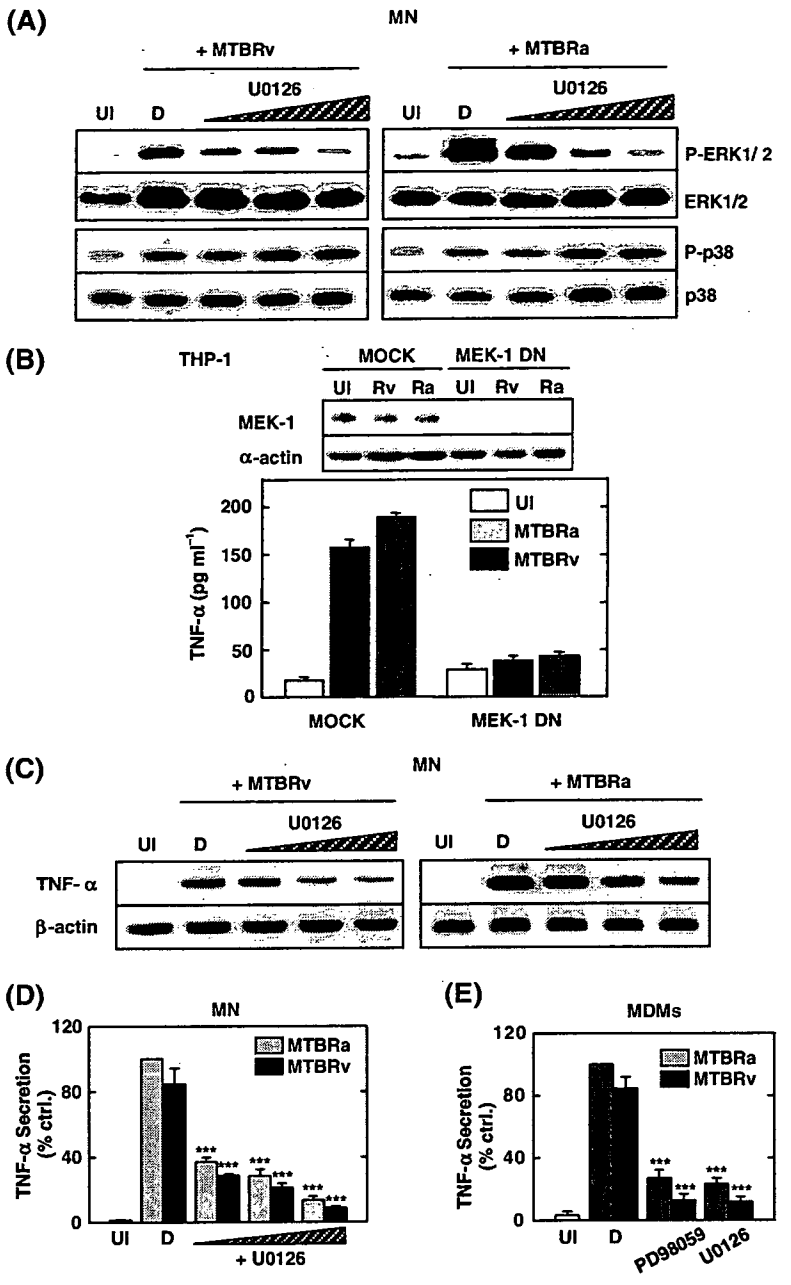

Fig. 3. MTB stimulates the TNF-α expression of human monocytes or MDMs via the MEK–ERK pathway.
A. The MEK inhibitor U0126 was added to monocytes at concentrations ranging from 5 to 20 μM at 45 min before infection with MTBRv or MTBRa (moi = 1) and culture continued for a further 30 min. The cells were 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,

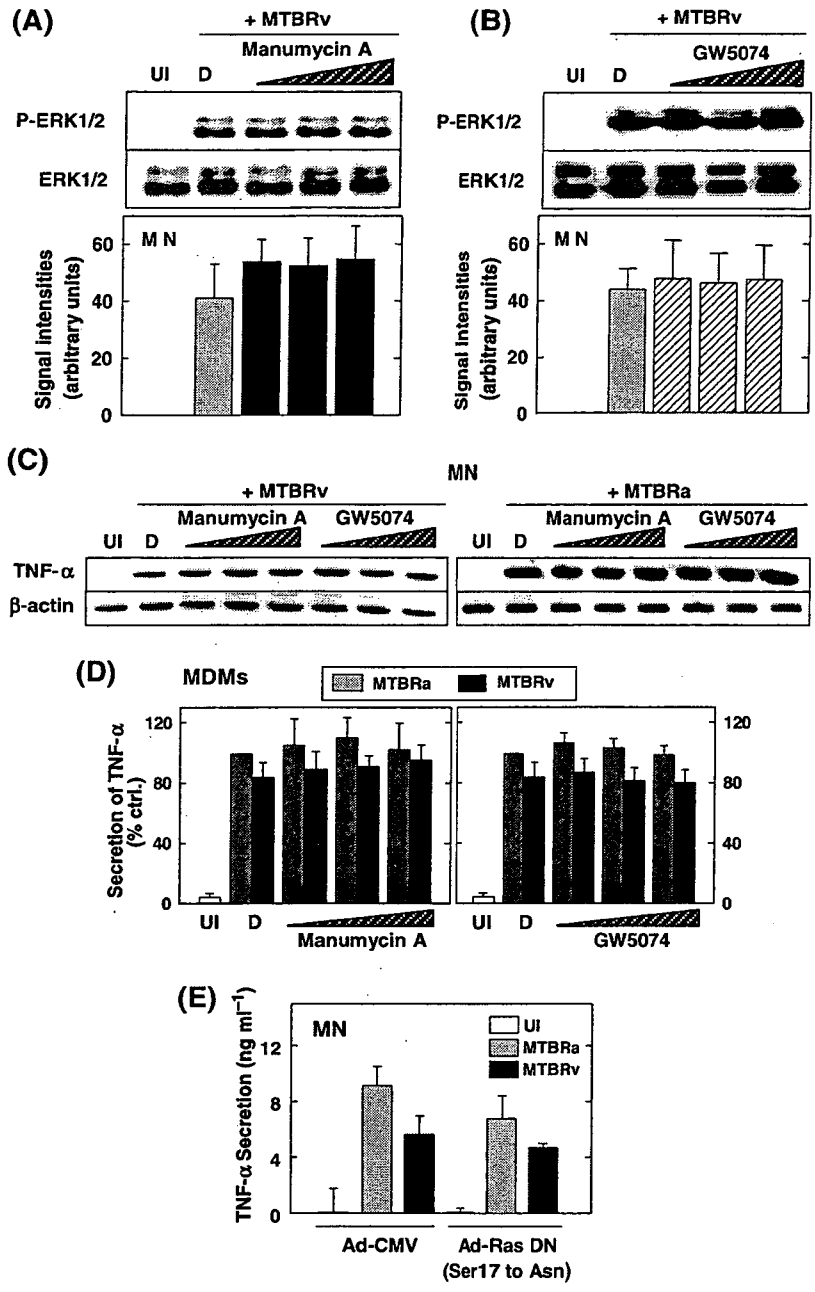

Fig. 4. Effects of inhibition of Ras or Raf-1 on the MTB-mediated ERK-1 phosphorylation and TNF-α expression in human monocytes or MDMs.

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

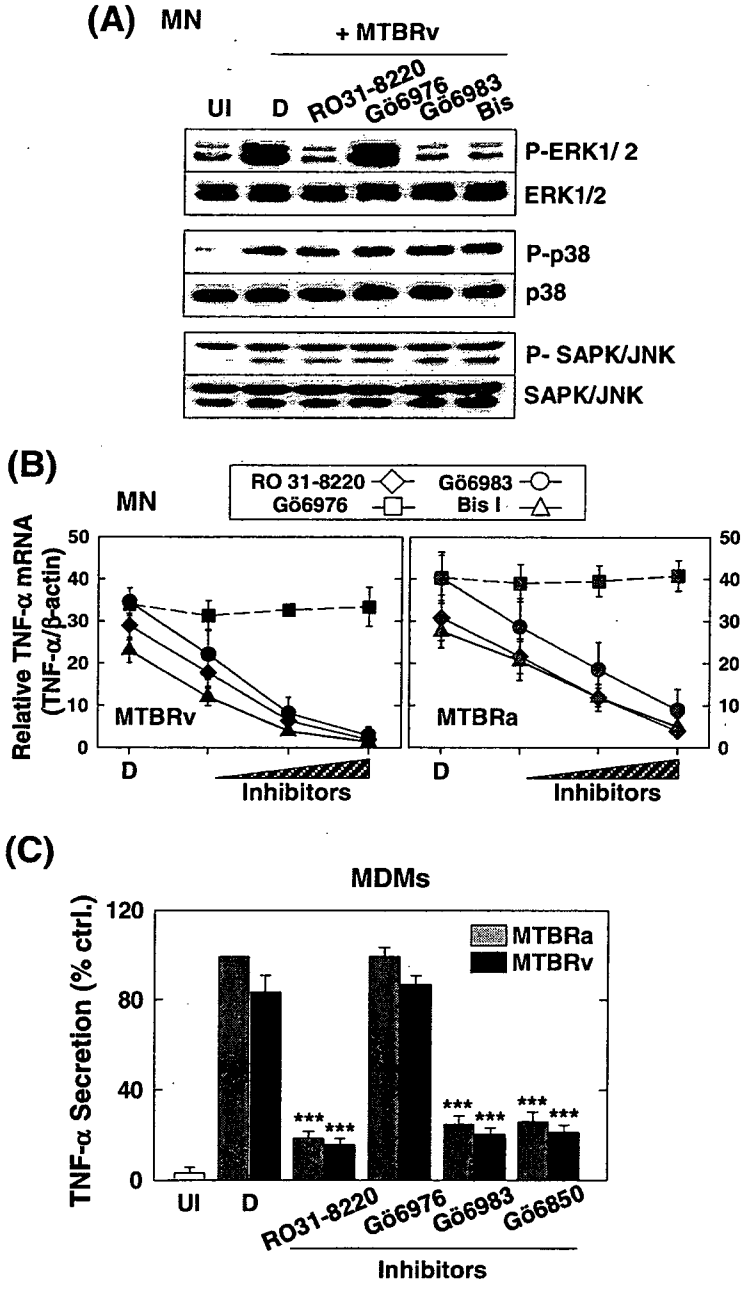

Fig. 5. Effects of inhibition of PKC on the MTB-induced phosphorylation of ERK1/2 and TNF-α expression in human monocytes.
A. Human monocytes were preincubated with the PKC inhibitors (RO 31-8220, 5 μM; Gö6976, 20 nM; Gö6983, 10 μM; bisindolylmaleimide I, 10 μM). The cells were harvested and subjected 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).

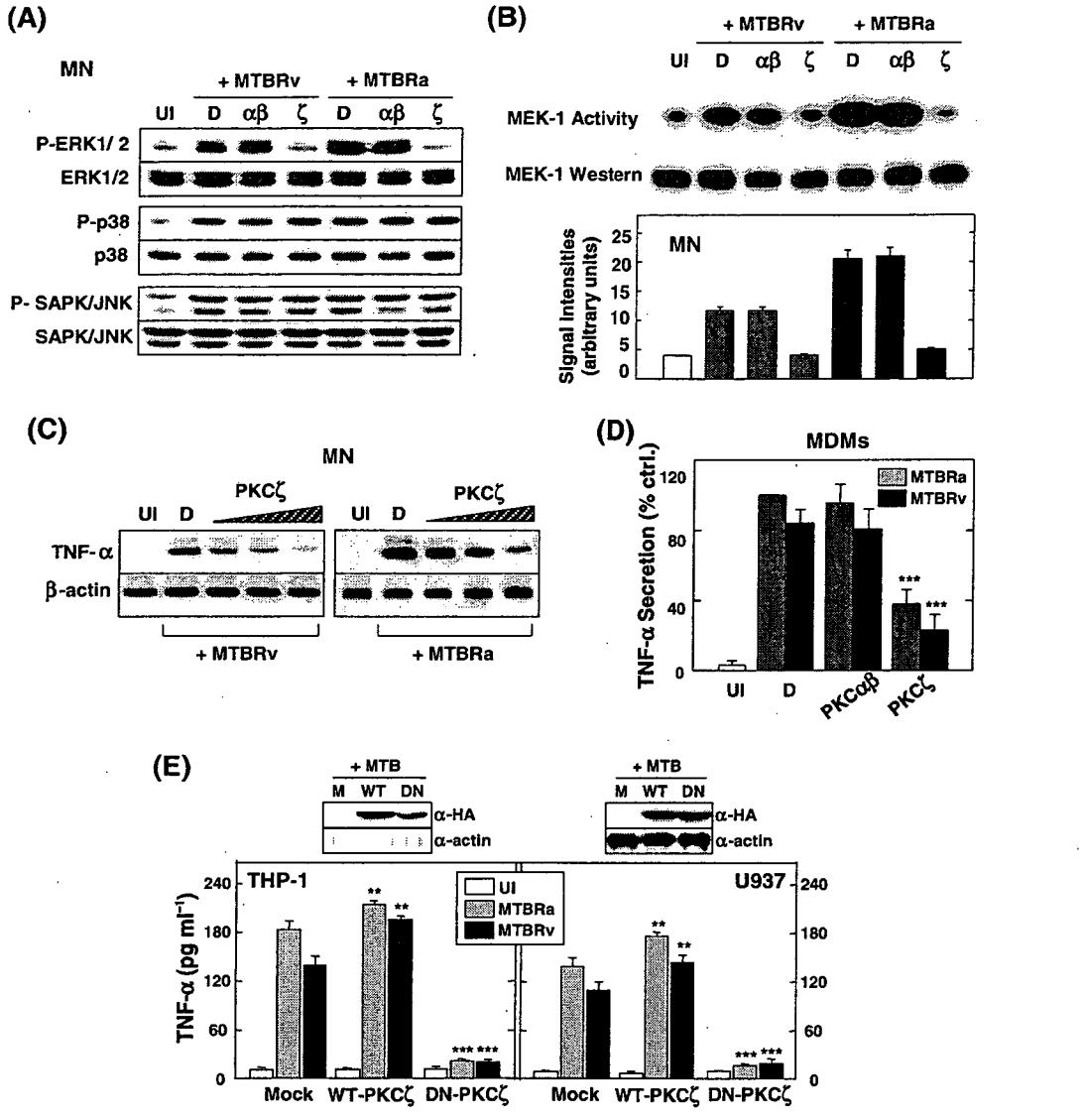

Fig. 6. PKCζ plays an essential role on MTB-mediated ERK-1 and MEK activation, and TNF-α expression.

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.

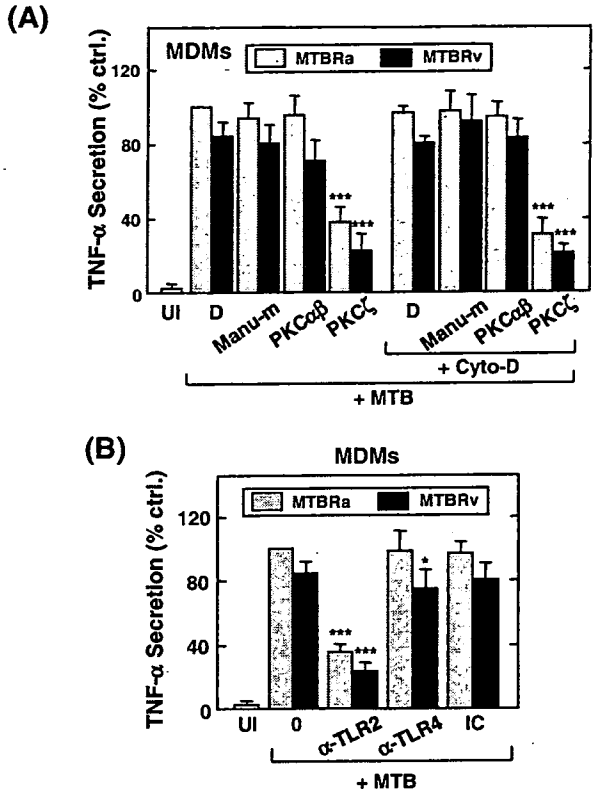

Fig. 7. TLR2, but not phagocytosis or TLR4, plays a major role for TNF-α expression by MTB.
A. Human MDMs were preincubated with or without cytochalasin D (Cyto-D; 10 μM) in the presence or absence of manumycin M (Manu-m; 1 μM), PKCζ-specific or a PKCαβ-specific peptide (20 μM), or 0.1% DMSO control (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.

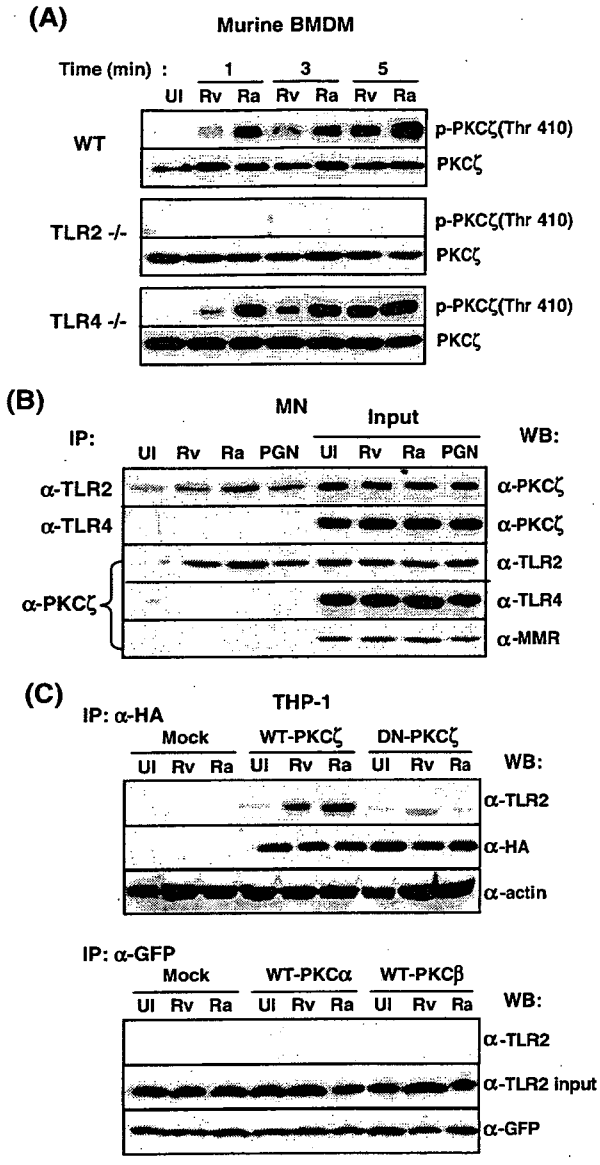

Fig. 8. TLR2 is essential for MTB-induced PKCζ activation, and the PKCζ associates with TLR2 after MTB stimulation.
A. BMDMs from WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were stimulated with MTBRv (Rv) or MTBRa (Ra; moi = 1, for both strains) for the times indicated. The cells were harvested and subjected 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 anti-human 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 $P < 0.05$.

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Erk pathways negatively regulate matrix mineralization

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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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