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# Immunotherapy of mycobacterial infections: a cell and molecular model

## Introduction

*Mycobacterium tuberculosis* is a major cause of morbidity and mortality worldwide, causing an estimated 1.5 to 2 million deaths each year. It remains a major public health problem, despite effective antimicrobial therapy and development of preventive vaccines. One third of the world's population is infected with *M tuberculosis* as suggested by tuberculin skin testing. The recent resurgence in tuberculosis is attributable partly to the AIDS epidemic and the emergence of multidrug-resistant strains.<sup>1</sup> The number of new cases of active *M tuberculosis* has been projected to reach 11.9 million annually by 2006. Each year there are approximately 6000 new cases of *M tuberculosis* in Hong Kong.

Many fundamental questions about the mechanisms of *M tuberculosis* pathogenesis, persistence and reactivation remain unanswered. The most important questions are: (1) What are the major immune responses triggered by *M tuberculosis*? (2) How do the bacilli evade host defences to ensure their survival? Following infection of the host by *M tuberculosis*, induction of cytokines is a major defence mechanism to limit pathogen invasion. Interferon (IFN)-inducible, double-stranded (ds) RNA-activated protein kinase (PKR) has been conventionally known to regulate the induction of cytokine expression in response to virus infection through nuclear factor-kappa B (NF-κB).

Interferon-gamma is a key cytokine in the immune response against *M tuberculosis*; IFN-gamma knockout mice are deficient in macrophage activation and more susceptible to *M tuberculosis*. The biological actions of IFNs are mediated by multiple pathways resulting in mRNA degradation by ribonuclease L and inhibition of translation by a dsRNA-activated kinase PKR. We showed that viral RNA activates PKR to provide transducing signals for IFN expression.<sup>2</sup> The activities attributed to PKR include a role in (1) mediating the antiviral and anti-proliferative activities of IFN, (2) cellular response to physiological stress, and (3) regulation of cell growth.

In contrast, interleukin (IL)-10 is a potent immunosuppressive cytokine for dampening host immune responses.<sup>3</sup> Intracellular pathogens (including mycobacteria and HIV) specifically target macrophages to induce IL-10 that inhibits the immune response including the expression of IL-2, IFN, GM-CSF, tumour necrosis factor (TNF) and major histocompatibility complex class II antigens. We previously showed that HIV is capable of perturbing IFN and related cytokine systems, which contribute to its immune evasion strategy.<sup>4</sup> While *M tuberculosis* has been known to induce IL-10 for immune evasion, the mechanism of IL-10 dysregulation is not well understood.

## Methods

This study was conducted from February 2005 to January 2007.

To study the pathogenesis of *M tuberculosis* infection and develop the scientific rationale for new treatment strategies, we used Bacillus Calmette-Guerin (BCG) and human primary blood monocytes (PBMs). The BCG vaccine strain 1077 from Aventis Pasteur and Danish strain 1331 were used in all experiments. These vaccine samples have been shown to be free from any virulent mycobacteria and conform to the World Health Organization standards.

## Key Messages

1. RNA-activated protein kinase serves as an important molecule in an innate defence against mycobacteria.
2. Exogenous interferon- $\gamma$  can reverse the immunosuppressive role of interleukin-10 prevailing in the presence of mycobacterial infection.
3. This study provides new therapeutic strategies for the global tuberculosis pandemic, in particular for AIDS patients with multidrug-resistant mycobacteria.

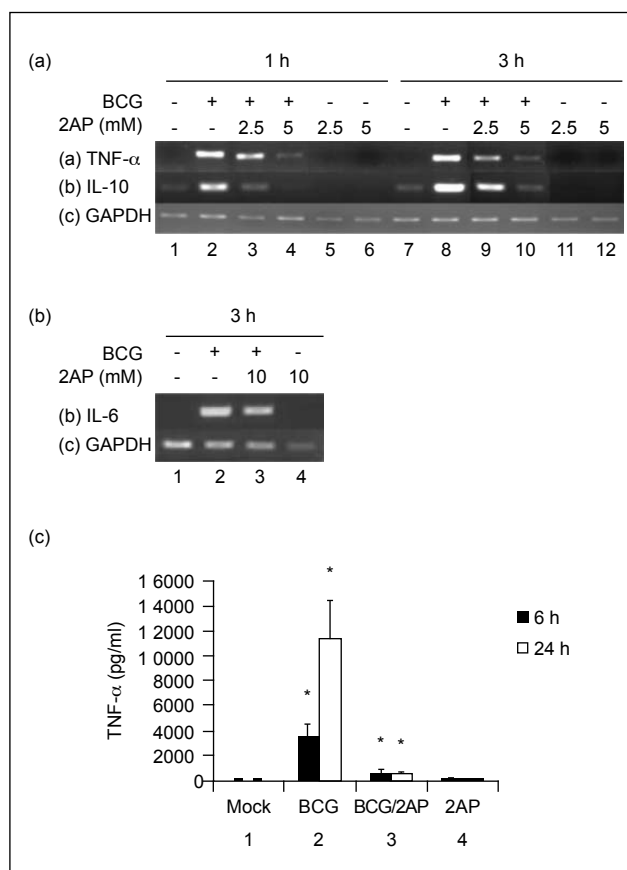
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**Fig 1. Bacillus Calmette-Guerin (BCG) induction of cytokine expression is dependent on RNA-dependent protein kinase activity in primary human monocytes (Copyright 2005 The American Association of Immunologists)**

(a) Human monocytes were incubated with medium alone or with 2.5 mM or 5 mM 2-AP for 1 h. BCG was added and the cells were incubated for an additional 1 or 3 h before harvest. Cytokine mRNA levels were assayed by RT-PCR. (b) Human monocytes were treated as in (a) except that the concentration of 2-AP was 10 mM and BCG was added for 3h. (c) Release of TNF- $\alpha$  from human monocytes after stimulation with BCG and/or 2-AP for 6 and 24 h. Mock, diluent for BCG; BCG, BCG (5 CFU/cell); BCG/2AP, BCG (5 CFU/cell) with 2-AP at 5 mM; 2AP, 5 mM 2-AP alone. The data for RT-PCR are representative of three independent experiments and ELISA results are shown as average and standard deviation (error bars) of three different donors

\*  $P < 0.05$

Human peripheral blood monocytes were isolated from buffy coats of healthy blood donors by Ficoll-Paque density gradient centrifugation.

To examine cytokine expression, cells were treated with various doses of BCG and different kinds of inhibitors for indicated time periods. Total RNA extraction was performed using TRIzol reagent; cDNA generation was performed either by reverse transcription-polymerase chain reaction (RT-PCR) or real time RT-PCR analysis so as to measure the mRNA expression level of IL-6, IL-10 and TNF- $\alpha$ . The levels of cytokine production in the culture supernatant were measured by ELISA.

To delineate the mechanism involved, we determined the phosphorylation levels of different kinases and the activation status of transcription factors by Western blot analysis and electrophoretic mobility shift assay (EMSA), respectively. Equal amount of protein was separated by 10% SDS-PAGE, electroblotted onto nitrocellulose membranes, and followed by probing with specific antibodies. The bands were detected using the Enhanced Chemiluminescence System as per the manufacturer's instructions. For the EMSA, the radioactively labelled NF- $\kappa$ B oligodeoxynucleotides were incubated with nuclear proteins to allow for the formation of DNA-protein complex. The DNA-protein complex was resolved in 5% non-denaturing polyacrylamide gel and further dried in a gel dryer and exposed to x-ray film.

All data presented in this study were statistically analysed by two-tailed, paired  $t$  tests. A  $P$  value of less than 0.05 was considered significant.

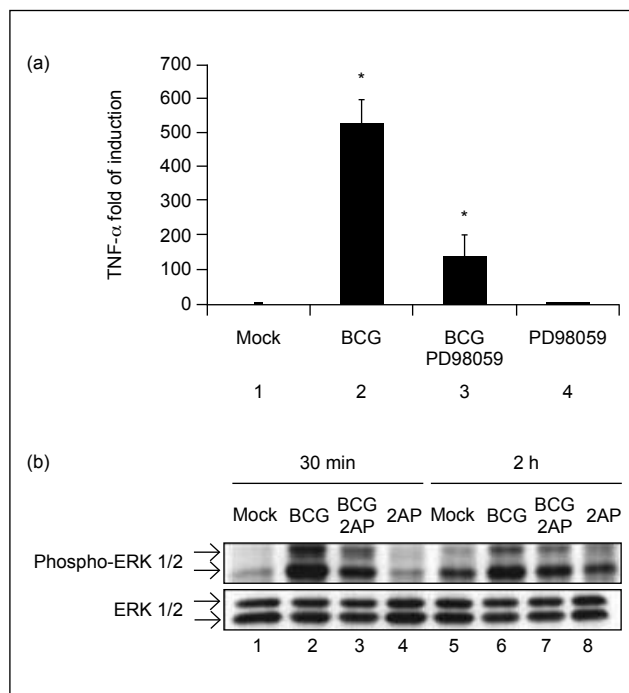
## Results

We examined the role of signalling kinases in the regulation of the immune response against *M tuberculosis*, using BCG and PBMs as a cellular model. Our results showed that 3 hours of BCG incubation with PBMs stimulates the induction of cytokine expression including TNF- $\alpha$ , IL-6, and IL-10. With the suppression of PKR using the PKR-mutant gene or 2-aminopurine as a PKR inhibitor, we showed that BCG-induced cytokine expression in human monocytes is regulated by activation of PKR (Fig 1).

To further investigate the involvement of PKR in cytokine induction in BCG-treated monocytes, the expression and kinase activity of PKR were monitored using RT-PCR and Western blot analysis. Our results showed that the mRNA levels of PKR did not change over a period of 24 h in the presence of BCG, suggesting that BCG does not affect PKR transcription. Nonetheless, BCG stimulates the phosphorylation and activation of PKR very rapidly.

To investigate the involvement of mitogen-activated protein kinase (MAPK) in BCG-induced cytokine expression, PD98059 (a specific inhibitor of ERK1/2) was used. The results showed that BCG-induced TNF transcription can be suppressed by pretreatment of the cells with 5  $\mu$ M PD98059 for 1 h (Fig 2a: lanes 2 and 3). To further determine the role of PKR in MAPK activation upon mycobacterial infection, PBMs were treated with BCG and resulted in a strong phosphorylation of ERK1/2 within 30 min, indicative of kinase activity. When PKR activation was inhibited by 2-AP, the levels of phospho-ERK1/2 were significantly reduced (Fig 2b: lanes 2, 3, 6, and 7). This suggests that ERK1/2 phosphorylation is mediated by PKR activation.

Next, to elucidate the participation of NF- $\kappa$ B in the BCG-induced signalling leading to cytokine expression, an inhibitor of NF- $\kappa$ B translocation, CAPE, was used. The



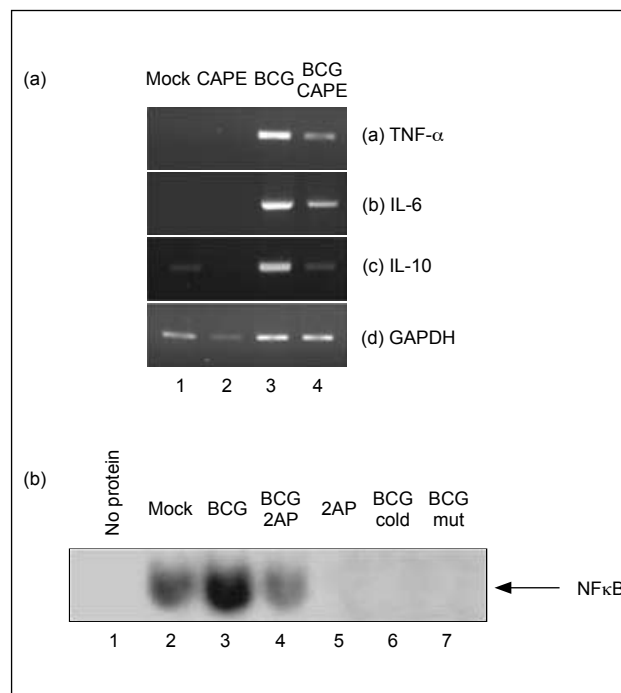
**Fig 2. Bacillus Calmette-Guerin (BCG)-induced activation of ERK1/2 phosphorylation is inhibited by 2-AP (Copyright 2005 The American Association of Immunologists)**

(a) Primary human monocytes were treated with medium alone or with 5 μM PD98059 for 1 h. Cells were incubated in the presence or absence of BCG for an additional 6 h. Total RNA was extracted and real-time RT-PCR for TNF-α was performed as described. Mock, diluent for BCG; BCG, BCG (5 CFU/cell); BCG/PD98059, BCG (5 CFU/cell) with 5 μM PD98059; PD98059, 5 μM PD98059 alone. Results are shown as average and standard deviation (error bars) of cells from three different donors. (b) Human monocytes were pretreated with 5 mM 2-AP or medium alone for 1 h. Then BCG (5 CFU/cell) was added for an additional 30 min or 2 h before proteins were harvested. Phosphorylated and total ERK1/2 were detected by Western blotting. Mock, diluent for BCG; BCG, BCG (5 CFU/cell); BCG/2AP, BCG (5 CFU/cell) with 2-AP at 5 mM; 2AP, 5 mM 2-AP alone. The data are representative of three independent experiments

\* P<0.05

PBMs were pretreated with CAPE for 1 h, and then BCG was added to the cells (5 CFU/cell). While the inhibitor itself did not affect any cytokine level, the expression of cytokines was significantly reduced by CAPE pretreatment (Fig. 3a: lanes 3 and 4). This suggests that BCG-induced cytokine expression involved NF-κB activation. We also determined the DNA binding activity of NF-κB in the BCG-induced PBMs by EMSA. There was a significant activation of NF-κB binding detected in BCG-stimulated monocytes (Fig 3b: lanes 2 and 3), which can be inhibited by 2-AP treatment (Fig 3b: lane 4). Taken together, PKR may serve as an important molecule in the innate defence against mycobacteria.

The results described above have revealed the signal transduction events of mycobacterial activation leading to expression of the immunosuppressive IL-10, which



**Fig 3. Transcription factor nuclear factor-kappa B (NF-κB) is involved in the induction of cytokine expression through the activation of RNA-dependent protein kinase (Copyright 2005 The American Association of Immunologists)**

(a) Human monocytes were incubated with medium alone or with 15 μg/mL CAPE (NF-κB inhibitor) for 1 h. BCG was added, and the cells were incubated for an additional 6 h. Cells were lysed and RT-PCR was performed. (b) Human monocytes were incubated with medium alone or with 5 mM 2-AP for 1 h. BCG was added and the cells were incubated for an additional 1 h. Cells were lysed and then assayed for NF-κB activation by EMSA as described. 'Cold' represents the additional use of unlabelled NF-κB oligodeoxynucleotides, whereas 'mut' represents the use of mutated NF-κB oligodeoxynucleotides. The data are representative of three independent experiments

interferes with IFN-γ signalling. To delineate whether exogenous IFN-γ can mitigate the inhibitory effects of IL-10 on IFN-γ signalling, we first demonstrated that IL-10 inhibited IFN-γ-induced human leukocyte antigen (HLA)-DR and TNF-α expression. With pretreatment of PBMs with exogenous IFN-γ, we showed that the IL-10-inhibited HLA-DR and TNF-α expression can be restored. Our results suggested that exogenous IFN-γ can reverse the immunosuppressive role of IL-10.

To delineate mycobacterial pathogenesis, we examined the effects of Th-1 cytokine pretreatment on BCG-induced IL-10 production. The PBMs were pretreated with IL-12 or IFN-γ prior to the addition of BCG. Our result showed that the BCG-induced IL-10 was suppressed by the Th-1 cytokines in a dose-dependent manner. In contrast, production of the proinflammatory cytokine TNF-α induced by BCG was unaffected by the Th1 cytokine pretreatment. The data also demonstrated that the mechanism involved

in the IL-12- and IFN- $\gamma$ -mediated IL-10 suppression did not entail changes in glycogen synthase kinase-3 (GSK3) activity. In addition, we showed that there were no changes in the stability of mRNA for TNF- $\alpha$  and IL-10.

## Discussion

Proinflammatory cytokines play a significant role in the pathogenesis of mycobacterial infections, whereas TNF- $\alpha$  is an important antimycobacterial protein that (1) acts in synergy with IFN- $\gamma$  to produce reactive oxygen intermediates and nitrogen oxides in macrophages in order to kill the engulfed mycobacteria, and (2) controls the multiplication and dissemination of the mycobacteria, in particular, during the formation of granulomas.

In our study, macrophages release a huge amount of TNF- $\alpha$  when the cells are infected with *M tuberculosis*. In turn, the proinflammatory cytokine regulates macrophages and other immune cells to induce chemokine expressions. Production of BCG-induced TNF- $\alpha$  is via a PKR-dependent signalling mechanism. This further supports the immune defence activities and proinflammatory properties of PKR. The novel function of PKR in antimycobacterial activities is also implicated. Nevertheless, the mechanisms on how PKR elicits antimycobacterial responses remain unanswered. It is suggested that PKR plays a role in the antiviral response. After viral infection, cells are stimulated with IFN. The induced PKR recognises dsRNA which acts as an intracellular 'virus detector'. Upon interaction with dsRNA, PKR becomes an active protein kinase by dimerisation and autophosphorylation. This activated form then phosphorylates eIF-2 $\alpha$ , which binds to eIF-2B together with GDP to become a stable complex. All these lead to inhibition of translation and antiviral responses and apoptosis of the infected cells.

Our results showed that PKR can mediate transcriptional control of gene expression via the activation of NF- $\kappa$ B. The partial inhibition of cytokine induction by CAPE in response to BCG may be explained by the incomplete inhibition of NF- $\kappa$ B activities or involvement of other transcription factors in the cytokine induction process (NF-IL6 has been implicated in the production of IL-6 in *M tuberculosis* infection). In addition, ERK phosphorylation is regulated by PKR during mycobacterial activation of human monocytes. These findings are in line with previous reports that TNF- $\alpha$  and IL-10 production involves MAPK pathways when cells are infected with *M tuberculosis* H37Rv, and that TNF- $\alpha$  secretion is dependent on ERK1/2 activation in human monocyte-derived macrophages infected with *Mycobacterium avium*. In our study, ERK1/2 acts as a downstream effector of PKR because pretreatment of cells with 2-AP inhibits ERK1/2 phosphorylation after BCG challenge. This implicates that both PKR and MAPK are important signal transducers in cytokine regulation in the innate immune response against mycobacteria. Following BCG-induced phosphorylation of PKR, activation of MAPK

and NF- $\kappa$ B activities appears to be the downstream events. In addition to being an antiviral kinase, PKR is an important signal molecule in innate immunity against mycobacteria.<sup>5</sup>

After delineating the molecular mechanism of BCG-induced cytokines, we further identified that IL-10 could affect *M tuberculosis* infection pathogenesis through the inhibition of IFN- $\gamma$  signalling pathways at the level of Jak2 and Stat1. Interferon- $\gamma$  is a pleiotropic cytokine produced primarily by T lymphocytes and NK cells in response to viral infection. It is an important mediator with multiple biological activities, which include macrophage activation, antimicrobial, antiproliferative, and immunomodulatory effects. It has been shown that IFN- $\gamma$  receptor-deficient mice are significantly impaired in their abilities to resist infection by microbes including viruses, bacteria and protozoa. Central to IFN- $\gamma$ -induced cellular responses are the activation of Jak1 and Jak2 and the consequent phosphorylation of Stat1.

To investigate the potential therapeutic use of Th-1 cytokines on *M tuberculosis* infection, we examined whether Th-1 cytokines could inhibit *M tuberculosis* induced IL-10 production, thus restoring host immune function to fight *M tuberculosis*. Our results showed that treatment with IL-12 or IFN- $\gamma$  could inhibit BCG-induced IL-10 synthesis and shift cytokine production back to the Th-1 profile.

In conclusion, we have provided new insights into the immunopathogenesis of mycobacterial infection and delineated the role of signalling kinases and dysregulation of cytokines. This information may be useful for designing new therapeutic strategies for *M tuberculosis*, in particular for AIDS patients with multidrug-resistant mycobacteria.

## Acknowledgements

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