In vivo signal transduction: mitogen activated protein kinases in inflammation
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chapter 9

p42/44 MAPK mediates the inflammatory response to *Streptococcus pneumoniae* in vitro and in vivo

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Abstract

Intracellular transduction of inflammatory signals is largely dependent on members of the Mitogen Activated Protein Kinases (MAPK) family: p42/44 MAPK, p38 MAPK and JNK. p42/44 MAPK is classically known for its involvement in growth and differentiation. Interestingly, in vitro studies have indicated a role for this kinase in pathogen-induced cytokine production. However, the role of p42/44 MAPK in the immune response to Streptococcus pneumoniae in vitro and in vivo is unknown. We found that p42/44 MAPK is an important regulator of S. pneumoniae-induced cytokine production in vitro and during experimental pneumonia in vivo. Heat killed S. pneumoniae-induced TNFα and IL-6 production in macrophages was dose-dependently reduced by PD98059, a specific p42/44 MAPK inhibitor. Furthermore, inhibition of p42/44 MAPK reduced inflammatory cytokine levels in the lungs, 24 hours after induction of pneumococcal pneumonia. Interestingly, inhibition of p42/44 MAPK increased inflammatory cytokine levels at 48 hours after induction of disease, paralleling an increased bacterial burden in the lungs. Additional experiments revealed that at this time point functional p42/44 MAPK is critical for monocyte and granulocyte phagocytic capacity. Thus p42/44 MAPK is a principal regulator of the host response to S. pneumoniae.
**Introduction**

Recent years have seen remarkable progress with respect to the understanding of the molecular machinery that controls the cellular physiology. Especially the Mitogen Activated Protein Kinase (MAPK) family of protein kinases has emerged as a cardinal regulator of a variety of biological responses in a variety of in vitro systems, transducing a myriad of extracellular signals into cell responses\(^7\). Three major MAPK pathways have been described; the p38 MAPK, p42/44 MAPK and c-Jun N-terminal Kinase (JNK) pathway\(^{11,12,17,97,113,228}\). p38 MAPK and JNK are well known for mediating stress signals: they are activated in response to inflammatory stimuli (e.g. lipopolysaccharide (LPS), Tumor Necrosis Factor (TNF)\(\alpha\), Interleukin (IL)-1\(\beta\)) and cellular stresses (e.g. UV, osmotic shock) and regulate (pro-) inflammatory cytokine release and related events such as neutrophil activation in vitro and in vivo\(^7,39,40,96\). p42/44 (MAPK) is activated by stimuli such as growth factors, phorbol esters and insulin and is involved in cell growth, differentiation and survival\(^{17,18,229}\). Remarkably, however, still little insight exists as to how these in vitro events relate to in vivo (patho)physiology. Especially, the importance of MAPKs in complex processes that involve intricate interactions between multiple cell types, like infectious disease, remain largely obscure.

Some insight exists into the role of p38 MAPK and JNK in the control of infection and inflammation. Studies using pharmacological inhibitors both in experimental rodents and human volunteers have shown that p38 MAPK has a critical role in the production of inflammatory cytokines, its inhibition leading to enhanced cytokine production in pneumococcal pneumonia while showing potent anti-inflammatory action in human endotoxemia\(^{98,152}\). An important function of p38 MAPK in host defense is evidenced by the decreased clearing of pneumococci in p38 MAPK-inhibited mice in a pneumonia model\(^{98}\). In vitro JNK is an important regulator of inflammatory cytokine release in monocytes and T cells\(^{30}\), while recently we showed strong anti-inflammatory action of JNK inhibition in patients with steroid-refractory Crohn’s disease\(^{230}\).

Interestingly, p42/44 MAPK was also reported to be involved in the control of inflammatory cytokines in vitro. Activated p42/44 MAPK was found to be necessary for LPS induced TNF\(\alpha\) and IL-1\(\beta\) production\(^{23,24}\), and LPS-induced TNF\(\alpha\) production in selected macrophage
Furthermore, p42/44 MAPK was activated in astrocytes by pneumococcal cell wall components\textsuperscript{92}. On the other hand, p42/44 MAPK was not involved in (heat killed) \textit{Staphylococcus aureus} induced TNF production\textsuperscript{93}. These data suggest that p42/44 MAPK may be involved in inflammatory cytokine release upon stimulation with pathogenic bacteria. However, the role of p42/44 MAPK in mediating the host response to \textit{Streptococcus pneumoniae} in vitro and in vivo is unknown.

We set out to investigate the role of p42/44 MAPK in the cell’s cytokine response to (Heat killed) \textit{S. pneumoniae}, and in a murine model for streptococcal pneumonia. We observed that p42/44 MAPK is involved in TNF and IL-6 production in macrophages in vitro and in mice in vivo. Inhibition of p42/44 affected the phagocytic capacity of monocytes and neutrophils in an ex vivo assay, and diminished the bacterial clearing capacity in vivo. Thus our results define a principal role for p42/p44 MAPK in the regulation of lung host defense in vivo.

\section*{Methods}

\subsection*{Cells and reagents}

L929 fibrosarcoma cells were cultured in DMEM supplemented with 10\% fetal calf serum, 2 mM L-glutamin, and antibiotic-antimyotic (GibcoBRL Life Technologies, Grand Island, NY). Murine macrophages, 4-4 clone\textsuperscript{224,225}, were cultured in RPMI (Bio-Whittaker Europe, Verviers, Belgium) supplemented with 10\% fetal calf serum, 2 mM L-glutamin, and antibiotic-antimyotic. PD98059 (Alexis Biochemicals Leiden, The Netherlands), dissolved in DMSO, was used at 1-10\mu M in cell culture; the DMSO concentration was never higher than 0.05\%. Cells were incubated with PD98059 for 1.5 h before stimulation. Stimulation was done with either Heat Killed \textit{S. pneumoniae} (HKSP; 2x10\textsuperscript{7} bacteria/ml), LPS (\textit{Escheria coli} 0111:B4 Sigma, St Louis, MO; various concentrations), or 50ng/ml recombinant murine TNF for 8 or 16 hours. Supernatants were collected and stored at \textdegree C for later cytokine sampling with ELISA.
Murine model of pneumococcal pneumonia

The animals used were 8-week-old female BALB/c mice. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center. Experimental groups consisted of 8 mice. Mice were subjected to 1mMol PD98059 per kilogram body weight by intraperitoneal injection in 1 ml sterile saline or 1μl solvent control (DMSO) in 1ml sterile saline for controls. 1.5 h before inducing disease PD98059 was administered, then given once daily. No apparent toxicity was observed.

Pneumococcal pneumonia was induced as described previously. 24 and 48 hours after induction of disease, blood and whole lungs were obtained. Blood was collected from the vena cava inferior. Whole lungs were harvested and homogenized. Serial 10-fold dilutions in sterile isotonic saline were made of these homogenates, and 50μl volumes were plated onto sheep-blood agar plates and incubated at 37 °C and 5% CO2. Colony Forming Units (CFU) were counted after 16 hours. Homogenates of lungs were then diluted 1:1 with lysisbuffer (150mM NaCl, 15mM Tris, 1mM MgCl, 1mM CaCl2, 1% Triton (X-100) and protease inhibitors) for 30 minutes at 4 °C. Homogenates were then spun at 1500 x g at 4 °C for 15 minutes, to remove cell debris, after which the supernatants were stored at –20 °C until cytokine measurements.

Cytokine determination

Cytokines were measured by using commercially available ELISAs according to the manufacturers recommendations: murine TNFα (Genzyme, Cambridge, MA), IL-6 (Pharmingen, San Diego, CA), Interferon (IFNγ) and IL-1β (R&D Systems, Minneapolis, MN).

Phagocytosis assay

Measurement of phagocytosis was done using the Phagotest kit (Orpegen, Heidelberg, Germany). Aliquots of 100 μl heparinized murine whole blood, obtained at 48 hours, were mixed with 2 × 10⁷ fluorescein isothiocyanate (FITC)-labeled E. coli cells at 0°C and
subsequently incubated in a 37°C horizontal shaking water bath for 30 minutes or at 0°C as a control. Monocytes and granulocytes were gated according to their light scatter profiles and phagocytosed fluorescent particles were measured using FACScan analysis. Phagocytic capacity per cell was calculated as the mean fluorescence intensity (MFI) at 37°C minus MFI at 0°C (adherence control).

Statistical Analysis

Data were statistically analyzed by the Student's t test. All data are given as the mean +/- SEM.

Results

P42/44 MAPK mediates cytokine production in response to S. pneumoniae in vitro

p42/44 MAPK is best known for its involvement in proliferation and differentiation\(^{231}\). Its role in mediating stress signals and controlling cytokine production remain largely obscure. We investigated whether p42/44 MAPK is involved in S. pneumoniae induced cytokine production in macrophages. Inhibition of p42/44 decreased HKSP induced TNFα production in a dose dependent fashion (Figure 1A; 1μM and 10 μM PD98059 \(P<0.05\)). A similar effect was seen upon stimulating cells with LPS (Figure 1B 1μM and 10 μM PD98059: \(P<0.05\)). Also HKSP induced IL-6 levels were diminished upon inhibition of p42/44 MAPK, although not significantly at low dosage of PD98059 (Figure 1C; 10 μM PD98059: \(P<0.05\)). Thus p42/44 seems to be involved in S. pneumoniae induced cytokine production in vitro. To investigate whether this effect was cell type dependent, murine L929 cells where stimulated with TNFα, and subsequent IL-6 production was measured. Again inhibition of p42/44 diminished cytokine production, showing that p42/44 MAPK mediates cytokine production under various conditions in a non cell type-dependent manner.
Figure 1. Effects of p42/44 MAPK inhibition on cytokine production in 4-4 macrophages (mφ) and L929 cells (A) Effect of pre-treatment with 1-10μM PD98059 (PD) of 4-4 macrophages on HKSP induced TNFα production, compared to vehicle (DMSO) control. (No Stim: cells not stimulated with HKSP) (B) Effect of pre-treatment with 1-10μM PD98059 of 4-4 macrophages on LPS induced TNFα production. (C) Effect of pre-treatment with 1-10μM PD98059 of 4-4 macrophages on HKSP induced IL-6 production. (D) Effect of pre-treatment with 10μM PD98059 of L929 fibrosarcoma cells on TNFα induced IL-6 production. *P<0.05 vs. DMSO control.

Inhibition of p42/44 MAPK affects cytokine production in response to S. pneumoniae in vivo

The use of MAPK inhibitors for modulating inflammation has been widely advocated, mainly based on in vitro studies. Recently several studies were reported, using p38 MAPK/JNK inhibitors successfully as anti-inflammatory agents in humans. However, earlier we reported that inhibition of p38 MAPK can also lead to increased cytokine levels in pneumococcal pneumonia, suggesting that MAPKs are not suitable anti-inflammatory agents per se. The role of p42/44 MAPK in inflammation in vivo is largely unknown. Our in vitro data suggest that S. pneumoniae induced cytokine response is under control of p42/44 MAPK. We decided to investigate the effect of inhibition of p42/44 MAPK in vivo, in the complex inflammatory model of pneumococcal pneumonia.
Mice were intranasally inoculated with *S. pneumoniae*, and lungs were harvested 24 and 48 hours thereafter. Induction of disease was associated with a marked increase in TNFα levels in lung homogenates, in parallel with a rise in the numbers of CFU in lung homogenates. Inhibition of p42/44 with PD98059 decreased inflammatory cytokine levels, such as TNFα, IL-6, IL-1β and IFNγ in the lungs at 24 hours (Figure 2A, B, C&D; P<0.05), compared to controls. Together with our in vitro data, these data suggest that also in vivo *S. pneumoniae* induced inflammatory cytokine production is, at least in part, under control of p42/44 MAPK. Strikingly however, at 48 hours the levels of pro-inflammatory cytokines such as TNFα and IL-1β were significantly enhanced in the lungs of animals treated with the p42/44 MAPK inhibitor, compared to controls (Figure 2A&C; P<0.05).

![Figure 2](image)

*Figure 2. Effect of p42/44 MAPK inhibition on pulmonary cytokine levels during pneumococcal pneumonia.* TNFα (A), IL-6 (B), IL-1β (C) and IFNγ (D) levels in lung homogenates of p42/44 MAPK-inhibited mice (1μM PD98059) 24 and 48 hours (hr) after induction of pneumococcal pneumonia. *P<0.05 vs. vehicle (DMSO control) treated mice.
The increased levels of inflammatory cytokines in the lung homogenates of p42/44 MAPK inhibited animals at 48 hours were paralleled by the number of CFU in these lungs (Figure 3). Whereas bacterial outgrowth at 24 hours was not different between groups, the number of CFU at 48 hours tended to be higher in animals receiving PD98059, although this did not become significant.

**Figure 3.** Effects of p42/44 MAPK inhibition on bacterial outgrowth in mice with pneumococcal pneumonia. Numbers of CFU in lungs from p42/44 MAPK-inhibited mice (1mM PD98059) mice with pneumococcal pneumonia at 24 hours (hr) and 48 hours after induction of disease, compared to vehicle (DMSO control) treated mice.

**Figure 4.** Effect of PD98059 on phagocytic capacity of monocytes and granulocytes in vivo. At 48 hours after induction of pneumococcal pneumonia, blood was obtained from p42/44 MAPK-inhibited mice (1mM PD98059) and controls (DMSO), and subjected to a phagocytosis assay as described in the methods section. Phagocytic capacity per cell is shown as the mean fluorescence intensity (MFI).

**P42/44 MAPK affects phagocytosis capacity of monocytes and granulocytes**

In vitro, p42/44 has been suggested to be involved in regulating phagocytosis. As the bacterial clearance capacity showed a tendency to decrease at 48 hours in animals receiving PD98059, we decided to test whether inhibition of p42/44 MAPK affected the phagocytosis in vivo at this time point. For testing the effect of PD98059 treatment on the phagocytic capacity we evaluated peripheral blood monocytes and granulocytes. Blood was obtained from mice 48 hours after induction of disease, when bacterial clearance showed sign of diminished capacity, as we argued that this was the most relevant time point for measurements on p42/44 MAPK.
mediated effects on phagocytosis in vivo. Both monocytes and granulocytes from p42/44 MAPK inhibited mice showed significantly decreased phagocytic capacity compared to controls (P<0.05; fig 4), whereas binding of the opsonised bacteria was not. (Not shown)

Discussion

Despite potent anti-microbial agents, pneumococcal pneumonia remains an important cause of morbidity and mortality. It accounts for up to 70% of community-acquired pneumonias in hospital, and its incidence seems to be rising85,86. Furthermore, an increase in penicillin-resistant S. pneumoniae was reported, prompting for research into new therapeutic options and a better understanding of host defense87. In recent years, much has been learned about both the cytokine network, that forms an intricate part of the host defense mechanism against bacterial pathogens, as well as of the molecular details of the processes that convert extracellular signals into changes in gene transcription. However, how these intracellular processes are related to host defense remains relatively poorly understood. In the present study we have investigated the role of p42/p44 MAPK in pneumococcal infection. The results indicate a cardinal role for this kinase in the regulation of host responses.

We observed that murine macrophages in vitro require p42/p44 enzymatic activity for the production of inflammatory cytokines in response to bacterial cell wall constituents. In accordance, in animals infected with S. pneumoniae, inhibition of p42/44 MAPK reduced TNFα, IL-1β, IL6 and IFNγ production in lung homogenates at 24 hours, indicating that this kinase is essential for cytokine production in vivo. However, at 48 hours p42/p44 MAPK-inhibited animals displayed higher levels of inflammatory cytokines. As at the same time CFU counts in the lungs of p42/p44 MAPK-inhibited animals were considerably higher than in controls, we assume that these increased cytokine levels are a result of the increased bacterial load in these animals. Thus it appears that p42/p44 MAPK is not required for cytokine production per se, and that very high numbers of bacteria are apparently able to activate signal transduction pathways leading to increased cytokine production that are p42/p44 MAPK independent. In agreement we observed that the LPS-induced TNF production in thioglycolate-stimulated murine peritoneal macrophages was sensitive to p42/p44 MAPK inhibition at low LPS concentrations (< 100 ng/ml), but this effect was not
observed at high LPS concentrations (> 1 μg/ml) (B. van den Blink and M.P. Peppelenbosch, unpublished observations). Thus a function of p42/p44 MAPK in the regulation of cytokine production seems restricted to moderate levels of immune cell stimulation.

Apart from controlling cytokine release, also other aspects of host defense against pneumococcal pneumonia appear to be regulated by p42/p44 MAPK. We observed that monocytes and granulocytes isolated from p42/p44 MAPK-inhibited animals have a markedly reduced capacity to phagocytose bacteria. Strikingly, we did not observe effects on phagocytosis in both monocytes and granulocytes isolated from p38 MAPK-inhibited animals (B. van den Blink and M.P. Peppelenbosch, unpublished observations) nor did we see a decreased oxidative burst in in vivo p42/p44 MAPK-inhibited monocytes or granulocytes (B. van den Blink and M.P. Peppelenbosch, unpublished observations). Thus p42/p44 MAPK seems to have a significant action in phagocytosis. It is of course tempting to speculate that this decrease phagocytic activity is causal in the increased bacterial outgrowth observed in p42/p44 MAPK inhibited animals, but until more specific phagocytosis-inhibiting drugs are tested in this model, other possibilities should be kept in mind.

However, disregarding the exact molecular details by which p42/p44 MAPK influences host defense, the present study has demonstrated an important regulatory role for p42/p44 MAPK in host defense. With the onset of clinical trails employing p42/p44 MAPK inhibitors to combat malignant disease, it may thus be advisable to be aware of the risk to immuno-compromise such patients. Conversely, although conventional anti-microbial drugs will most likely form the mainstay of therapy, modulation of MAPK pathways may form a new angle of therapeutic approach in abrogating a harmful systemic inflammatory response in early disease. However, rational development of such therapy will require systematic evaluation of the activation of various kinases in disease as well as assessment as to the effect of inhibition of particular kinases on family members. Experiments addressing this issue are currently under progress.

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