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McKeon, S., McClean, S. & Callaghan, M. (2010). Macrophage Responses To CF Pathogens: JNKMAP Kinase Signaling By Burkholderia Cepacia Complex Lipopolysaccharide. *FEMS Immunology and Medical Microbiology*, vol. 60, pg. 36–43. doi:10.1111/j.1574-695X.2010.00712.x

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RESEARCH ARTICLE

Macrophage responses to CF pathogens: JNK MAP kinase signaling by *Burkholderia cepacia* complex lipopolysaccharide

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Received 24 February 2010; revised 12 May 2010; accepted 12 May 2010.
Final version published online 29 June 2010.

DOI:10.1111/j.1574-695X.2010.00712.x

Editor: Nicholas Carbonetti

Keywords
U937; macrophage; *Burkholderia multivorans*; *cenocepacia*; *Pseudomonas*; mitogen-activated protein kinase.

Abstract

Chronic bacterial colonization of the airways with opportunistic pathogens is the primary cause of morbidity and mortality in cystic fibrosis (CF) patients. *Burkholderia cepacia* complex (Bcc) organisms pose a particular challenge in CF lung disease, due in part to their ability to trigger a fulminant pneumonia. This study compares the U937 macrophage response to two Bcc species, *B. cenocepacia* and *Burkholderia multivorans*, against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The two Bcc strains demonstrated higher levels of U937 macrophage internalization compared with both *P. aeruginosa* and *S. aureus*. Both the Bcc strains also stimulated significantly greater levels of tumor necrosis factor-α and interleukin-1β from macrophages when compared with *P. aeruginosa*. Further examination of the macrophage response to *B. multivorans* demonstrated that the lipopolysaccharide component of these bacteria was a potent inducer of proinflammatory cytokines and was shown to signal predominantly through the c-Jun N-terminal kinase mitogen-activated protein kinase pathway. These studies further characterize the host response to Bcc and in particular *B. multivorans*, now the predominant Bcc species in many CF populations.

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Riordan *et al.*, 1989). The CFTR dysfunction results in impaired transepithelial ion flow, increased viscosity of submucosal gland secretions and reduced mucociliary clearance of inhaled pathogens (Gibson *et al.*, 2003). Bacterial infection commences soon after birth, with evidence of inflammation in bronchoalveolar fluid. Subsequent persistent or recurrent infections lead to chronic inflammation and significant lung damage in later life. *Staphylococcus aureus* is frequently the first organism isolated from CF infants (Armstrong *et al.*, 1997). However, the predominant CF pathogen is *Pseudomonas aeruginosa* and is associated with CF lung infections in all age groups. The group of pathogens known as *Burkholderia cepacia* complex (Bcc), which comprises at least 17 species (Vanlaere *et al.*, 2008, 2009), is particularly significant as they are highly antibiotic resistant and many strains are transmissible (Mahenthiralingam *et al.*, 2001). In addition, these pathogens have been associated with a high fever and bacteremia, which progresses to a severe necrotizing and fatal pneumonia (Isles *et al.*, 1984). Bacterial uptake and killing by macrophages plays an important role in pathogen clearance. Some members of the Bcc are capable of surviving and persisting within macrophages (Chu *et al.*, 2004) due to a significant delay in the fusion of the bacteria-containing vacuole with lysosomes (Lamothe *et al.*, 2007) and their resistance to oxidative damage (Saini *et al.*, 1999). The macrophage–bacterial interaction is characterized by the secretion of proinflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), facilitating the further recruitment of phagocytic cells to the airway epithelium (Bonfield *et al.*, 1995). In CF where chronic infection is a feature, this response is often prolonged and the persistent stimulation of host cells can result in a state of chronic activation leading to progressive

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pulmonary disease and premature mortality (Ramis *et al.*, 2000).

The contribution of bacterial lipopolysaccharide to the inflammatory response from host macrophages is well described. In Bcc organisms, both the lipid A moiety, which plays a key role in the proinflammatory response, and the adjacent inner core oligosaccharide differ from that of other Gram-negative bacteria (Silipo *et al.*, 2007). The outer O-antigens, which determine the chemotype of the lipopolysaccharide, rough or smooth, may be less significant as they do not correlate with the severity of infection and are not distributed according to species (Vinion-Dubiel & Goldberg, 2003). The unusual structural features of Bcc lipopolysaccharide particularly in the lipid A core oligosaccharide region may impact on the activation of cell signaling cascades and play a role in the potent cytokine response triggered by Bcc organisms compared with that of *P. aeruginosa* lipopolysaccharide.

The aims of this study were to establish whether the macrophage interaction between members of the Bcc differs from that of *P. aeruginosa* and *S. aureus* and to determine which macrophage signaling pathway(s) is activated by the lipopolysaccharide component of *Burkholderia multivorans*, the most prevalent member of the Bcc in many CF populations.

Materials and methods

Bacterial strains and growth

All the bacterial strains are presented in Table 1. *Staphylococcus aureus* and *P. aeruginosa* isolates were purchased from the American Type Culture Collection (ATCC). *Burkholderia multivorans* and *B. cenocepacia* strains were obtained from the BCCM/LMG, University of Ghent, Belgium. All strains were routinely grown in Luria–Bertani (LB) broth (Sigma-Aldrich, Ireland) at 37 °C. The growth curves of each strain were determined by growing the bacteria at 37 °C in LB broth (pH 7.0) with shaking (200 r.p.m.). Aliquots were taken at particular time intervals, the $OD_{600\,nm}$ was recorded and CFU mL^{-1} of cultures was determined by plating serial dilutions onto LB agar.

Cell line

The U937 human monocytic myeloid-derived cell line was purchased from the ATCC and maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (Sigma-Aldrich). U937 monocytes were differentiated into macrophage-like cells by treatment with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich).

Table 1. Bacterial strains used in this study

Species	Strain	Source
Staphylococcus aureus	ATCC 25923	Clinical
Pseudomonas aeruginosa	PAO1	Clinical
Burkholderia multivorans	LMG 13010	CF, Belgium
Burkholderia cenocepacia	J2315	CF, ET12, UK

Antibiotic susceptibility of bacterial isolates

The minimum bactericidal concentration (MBC) of each strain was determined for amikacin and ceftazidime using a modified version of the method described by Ge *et al.* (1999). Briefly, each test bacterium was grown to a mid-logarithmic phase of growth at 37 °C in LB broth ($OD_{600\,nm}$ of 0.6). 1×10^6 cells mL^{-1} were added in duplicate to LB broth containing serial dilutions of amikacin or ceftazidime in a 96-well plate (Corning Life Sciences, VWR International Ltd, Ireland). The plates were incubated at 37 °C for 24 h, after which 50 μL from each well was spread onto LB agar plates. Plates were incubated for 48 h to determine the MBC of each antibiotic. Each plate contained the relevant positive and negative controls.

Internalization of pathogens by U937 macrophages

Internalization assays were carried out on PMA-stimulated monocytes using an adaptation of the gentamicin protection assay (Isberg & Falkow, 1985) carried out by Martin & Mohr (2000). A combination of 1 mg mL^{-1} amikacin and 1 mg mL^{-1} ceftazidime (both from Sigma-Aldrich) was shown to effectively kill all isolates examined (data not shown). PMA-stimulated monocytes seeded (5×10^5 cells per well) in 24-well plates (Corning Life Sciences, VWR International Ltd) were infected with *S. aureus*, *P. aeruginosa*, *B. multivorans* and *B. cenocepacia* at various multiplicities of infection (MOIs). The plates were centrifuged for 5 min at 700 ***g*** to facilitate bacterial entry and incubated for 2 h at 37 °C in the presence of 5% CO_2. Monolayers were washed three times with phosphate-buffered saline (PBS) (pH 7.0) and treated with a combination of amikacin/ceftazidime (1 mg mL^{-1}, each) for 2 h to kill the extracellular bacteria. After antibiotic treatment, the cells were washed a further three times with PBS and the absence of viable extracellular bacteria was confirmed by plating these washes onto LB agar. The intracellular bacteria were released using 10 mM EDTA (Sigma-Aldrich) and lysed with 0.25% Triton X-100 (Sigma-Aldrich). Intracellular bacteria were quantified by plating serial dilutions of the lysate onto LB agar. The percentage of internalization was determined as follows:

$$\left(\frac{\text{CFU mL}^{-1}\text{ recovered from macrophages}}{\text{CFU mL}^{-1}\text{ applied to macrophages}}\right) \times 100$$

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Cytokine induction from U937 macrophages

U937 macrophages were stimulated with *S. aureus*, *P. aeruginosa*, *B. multivorans* and *B. cenocepacia* at an MOI of 50 : 1 for 24 h. Cytokine responses were determined at a range of MOIs, with no significant difference in the levels of cytokine secreted up to an MOI of 1000 : 1 (data not shown). We therefore chose a mid-range MOI of 50 : 1 for the cytokine induction studies. Culture supernatants were analyzed for TNF-α and IL-1β production by enzyme-linked immunosorbent assay (ELISA) (eBioscience, UK).

Isolation of rough lipopolysaccharide from *B. multivorans*

Burkholderia multivorans rough lipopolysaccharide extraction was carried out as described previously by Galanos *et al.* (1969). All reagents were supplied from Sigma-Aldrich. After harvesting, cell pellets were washed twice with distilled water and treated with ethanol, acetone and ether. Fifty grams of dried bacteria were homogenized with 200 mL of extraction buffer (liquid phenol, chloroform and petroleum ether at a ratio of 2 : 5 : 8), centrifuged (10 000 ***g*** for 15 min) and filtered. This process was repeated two more times and the filtrates were pooled. Petroleum ether and chloroform were evaporated and the phenol/lipopolysaccharide solution was transferred into a glass tube and water was added dropwise until the lipopolysaccharide precipitated. Precipitated lipopolysaccharide was centrifuged (4000 ***g*** for 10 min), and washed three times with 80% phenol and three times with ether. The precipitate was dried *in vacuo*, resuspended in distilled water and centrifuged (100 000 ***g*** for 4 h). After centrifugation, the supernatant was decanted and the lipopolysaccharide pellet was dissolved in Limulus amebocyte lysate water, lyophilized and weighed. The quality of lipopolysaccharide was analyzed by SDS-PAGE and silver stained (Fig. 1).

Mitogen-activated protein (MAP) kinase inhibition studies

U937 macrophages preincubated with 10 μM of SP600125 [c-Jun N-terminal kinase (JNK) inhibitor], SB203580 (P38 inhibitor) or PD98059 (ERK P42/44 inhibitor), individually or in combination, for 1 h at 37 °C were stimulated with 100 $\mu g\,mL^{-1}$ of *B. multivorans* lipopolysaccharide. All MAP kinase inhibitors were purchased from Sigma-Aldrich. For gene expression studies, total RNA was extracted after 2 and 4 h and analyzed for TNF-α and IL-1β gene expression as described below. Culture supernatants were collected after 24 h and TNF-α and IL-1β secretion was detected by ELISA (eBioscience).

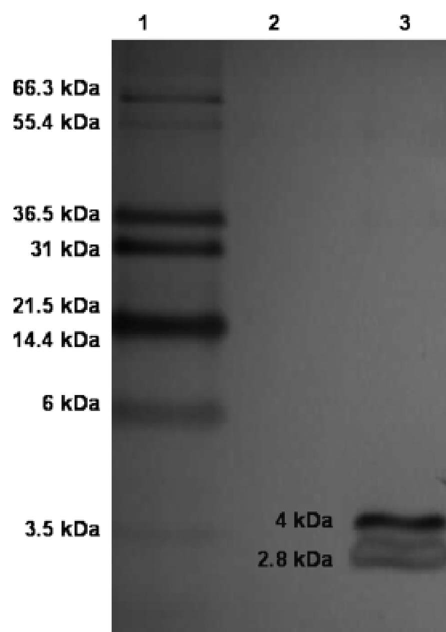

Fig. 1. Identification of *Burkholderia multivorans* LMG 13010 rough lipopolysaccharide. Lane 1, Mark12™ unstained standard MW marker (Invitrogen, Bio Sciences, Ireland); lane 3, *B. multivorans* LMG 13010 lipopolysaccharide sample (5 $mg\,mL^{-1}$).

Analysis of TNF-α and IL-1β gene expression by real-time reverse transcriptase (RT)-PCR

Total RNA was isolated from lipopolysaccharide-stimulated macrophages using RNAqueous® (Ambion, Ireland) and converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, UK). Cytokine gene expression was carried out using TNF-α and IL-1β primer/probe sets (Applied Biosystems) by real-time RT-PCR (7500 Real-Time PCR system, Applied Biosystems). A glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems) probe was used as an endogenous control. Each RT-PCR was carried out in a 20 μL final volume containing 1 μL primers/probe set, 10 μL universal PCR master mix (Applied Biosystems), 2 μL of cDNA sample and 7 μL RNAse-free water. Each cycle consisted of an initial holding temperature of 50 °C for 2 min, a denaturation step of 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Statistical analysis

Results are expressed as mean values ± SEM. Statistical analysis was performed using ANOVA at a 95% confidence level, and a P value of ≤ 0.05 was considered significant.

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Results and discussion

Internalization of bacterial species within U937 macrophages

Bcc macrophage internalization was compared with that of *P. aeruginosa* and *S. aureus* using a variation of the gentamicin protection assay (Martin & Mohr, 2000) at five different MOIs ranging from 5 : 1 to 500 : 1. The highest level of uptake occurred at an MOI of 5 : 1 for all strains examined (Fig. 2), indicating that at least for these organisms, there is a specific and potentially saturable bacterial–macrophage interaction involved. The reduced internalization of *Burkholderia* strains at higher MOIs was reported previously in epithelial cells and macrophages (Burns *et al*., 1996; Martin & Mohr, 2000). We compared the internalization of Bcc organisms with that of *P. aeruginosa* and *S. aureus*. *Burkholderia cenocepacia* demonstrated a significantly greater level of internalization by U937 macrophages compared with the two non-Bcc species at each MOI examined ($P = 0.002$). *Burkholderia multivorans* was also internalized in higher numbers compared with the non-Bcc species at three of the MOIs analyzed in this study. Uptake by macrophages, although ultimately detrimental to bacteria, may facilitate the triggering of a more potent immune response while the bacteria are protected from antimicrobial therapy. Reports of delayed clearance by macrophages of Bcc organisms (Saini *et al*., 1999; Chu *et al*., 2004; Lamothe *et al*., 2007) indicate that internalization may be an important survival strategy for at least some Bcc organisms in the lung. Previous literature demonstrated that alveolar macrophages were not competent to phagocytose unopsonized *P. aeruginosa in vitro* (Cheung *et al*., 2000), which may explain the low rate of internalization of *P. aeruginosa* by U937 cells *in vitro* reported here. Whether the uptake in these experiments was receptor mediated, as a result of phagocytosis or a combination of both was not determined, but under the same *in vitro* conditions, a significant difference in the macrophage internalization of these Bcc vs. non-Bcc species was observed. This enhanced uptake, combined with the previously reported delayed clearance by the macrophage, may be an important survival or virulence strategy with the potential to contribute to the rapid decline in lung function associated with some strains from the Bcc group of pathogens.

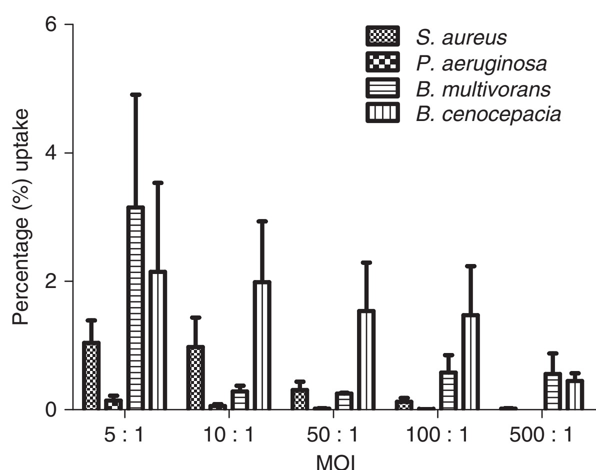

Fig. 2. Percentage uptake (%) of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia multivorans* and *Burkholderia cenocepacia* pathogens by U937 macrophages after 2 h at various MOIs. Values represent % uptake ± SEM of three independent assays.

Cytokine induction from U937 macrophages in response to pathogens

The proinflammatory cytokine response of U937 macrophages to Bcc species and to *P. aeruginosa* and *S. aureus* was compared. *Burkholderia multivorans* or *B. cenocepacia* induced significantly higher levels of TNF-α than the non-Bcc species examined, with *B. cenocepacia* inducing over 25 times more TNF-α than *P. aeruginosa* (Fig. 3b). Zughaier *et al*. (1999) demonstrated that unspeciated Bcc strains were more potent TNF-α stimulators than both *P. aeruginosa* and *Stenotrophomonas maltophilia* in human MonoMac6 macrophages (Zughaier *et al*., 1999). Our findings, using two clinically important Bcc species, establish that macrophage exposure to these species can contribute to the pronounced inflammation with potential deterioration of the airway epithelia associated with this group of organisms. Furthermore, IL-1β induction from the macrophages was most pronounced in response to *B. cenocepacia* exposure when compared with the other pathogens (Fig. 3a). In contrast to TNF-α secretion, significantly less IL-1β was induced by *B. multivorans* compared with *B. cenocepacia*. Of the four species examined, *S. aureus* induced the second highest concentration of IL-1β, which suggests that the initial influx of IL-1β triggered by *S. aureus* in the early stages of CF may cause sufficient epithelial damage to the local environment, facilitating further colonization by Bcc or *P. aeruginosa* organisms. In a comparative study of the potency within the Bcc, De Soyza *et al*. (2004), using whole-cell lysates, demonstrated increased potency of *B. cenocepacia* compared with other Bcc species in TNF-α, but not IL-1β induction from U937 monocytic cells (De Soyza *et al*., 2004). Of the two Bcc species examined in our studies, *B. cenocepacia* induced higher levels of both of these proinflammatory cytokines compared with the *B. multivorans* strain. IL-1β cytokine is a significant mediator of the acute-phase response, involved in a variety of cellular activities implicated in lung damage (Laskin & Pendino, 1995), and may be significant in the poor clinical outcome associated with the J2315 strain of *B. cenocepacia*.

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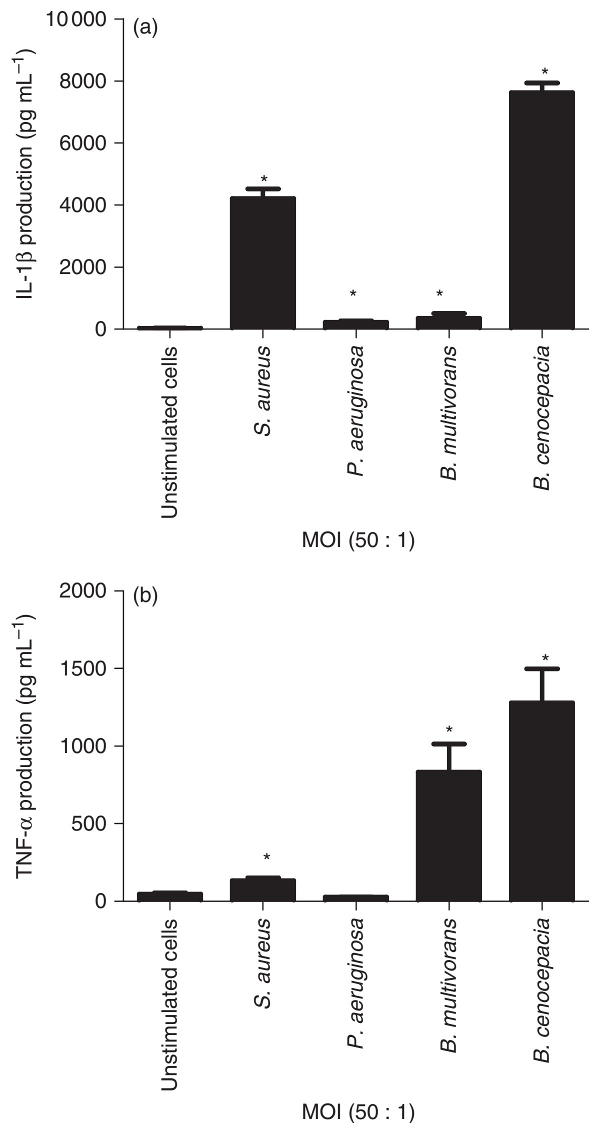

Fig. 3. (a) IL-1β and (b) TNF-α production from U937 macrophages stimulated with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia multivorans* and *Burkholderia cenocepacia* (MOI 50 : 1) for 24 h compared with unstimulated cells. Values represent cytokine levels (pg mL^{-1}) ± SEM. *Statistically different from the unstimulated control cells ($P < 0.05$).

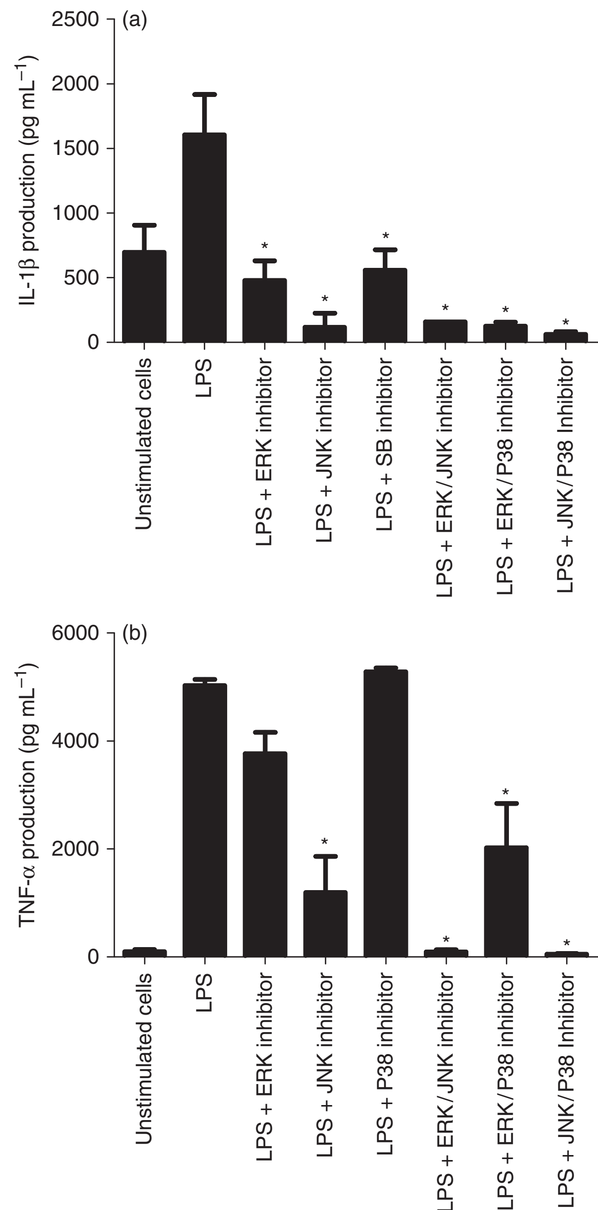

Fig. 4. (a) IL-1β and (b) TNF-α production from U937 macrophages stimulated with 100 μg mL^{-1} of *Burkholderia multivorans* LMG 13010 lipopolysaccharide for 24 h. Cells were preincubated (1 h) with individual or combination MAP kinase inhibitors. Values represent cytokine levels (pg mL^{-1}) ± SEM. *Statistically different from the lipopolysaccharide-stimulated control cells ($P < 0.05$).

MAP kinase pathways involved in lipopolysaccharide-induced IL-1β and TNF-α induction from macrophages

Currently, *B. multivorans* is the predominant Bcc pathogen present in the lungs of CF patients (Baldwin *et al*., 2008; Mahenthiralingam *et al*., 2008). For this reason, the mechanisms of *B. multivorans* induction of proinflammatory cytokines were further investigated using purified lipopolysaccharide, an important mediator of this response (Shaw *et al*., 1995). The macrophage response to lipopolysaccharide, consistent with the response from whole bacterial cells, demonstrated significant levels of both IL-1β and TNF-α, with over three times the amount of TNF-α induced compared with IL-1β (Fig. 4).

Although *B. multivorans* lipopolysaccharide interacts with toll-like receptor 4 (TLR4), the best-characterized lipopolysaccharide receptor, Shimomura *et al*. (2001) also

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reported that Bcc lipopolysaccharide can signal through various TLRs depending on the lipopolysaccharide preparation (Shimomura *et al.*, 2001). Following TLR engagement, we were interested in the potential role of the three MAP kinase cellular pathways in cytokine induction by *B. multivorans* lipopolysaccharide. Using a variety of MAP kinase inhibitors, the greatest inhibition of both IL-1β and TNF-α secretion in response to lipopolysaccharide occurred with the JNK MAP kinase inhibitor (Fig. 4). Inhibition of the ERK (P42/44) pathway partially inhibited both IL-1β and TNF-α responses to *B. multivorans* lipopolysaccharide. The P38 MAP kinase inhibitor, however, demonstrated contrasting results. Although this inhibitor had no effect on lipopolysaccharide-induced TNF-α, it had a significant impact on the reduction of IL-1β from the U937 macrophages, indicating the potential for the differential activation of these two cytokines. All inhibitor combinations significantly decreased IL-1β and TNF-α production. Inhibition of the ERK and P38 pathways was established as being the least effective in reducing TNF-α production, providing further evidence of a central role for JNK pathway activation in this response (Fig. 4b).

To investigate whether the inhibition observed was occurring at the transcriptional level, real-time PCR was used to examine TNF-α and IL-1β gene expression in the presence of the MAP kinase inhibitors. Lipopolysaccharide-induced TNF-α gene expression was not altered by the P38 inhibitor at either 2 or 4 h (Fig. 5b), consistent with the lack of inhibition on TNF-α secretion at 24 h (Fig. 4b), confirming that this pathway is not central to TNF-α induction by this species. However, despite significantly reducing IL-1β protein secretion at 24 h (Fig. 4a), IL-1β gene expression by Bcc lipopolysaccharide remained unaltered in the presence of the P38 inhibitor (Fig. 5a), suggesting that this pathway may be involved indirectly in regulating IL-1β induction or that there is additional regulation at the translational level. The ERK (P42/44) and JNK MAP kinase inhibitors significantly decreased the TNF-α gene expression response (Fig. 5b), correlating with the reduction seen at the protein level (Fig. 4b). It should be noted that although the ERK inhibitor had a profound effect at the transcriptional level at 2 and 4 h, there was only a partial reduction at the TNF-α protein level at 24 h, suggesting a later reactivation of this pathway. Similar to the protein experiments, IL-1β gene expression induced by *B. multivorans* lipopolysaccharide in the presence of the ERK (P42/44) and JNK inhibitors demonstrated significant abrogation of this response from the inhibited cells, confirming a key role for both of these MAP kinase pathways in *B. multivorans* IL-1β induction (Fig. 5a). A study by Bamford *et al.* (2007), in which the TNF-α response to clinical isolates of Bcc was examined, determined that the ERK (P42/44) and JNK MAPK inhibitors were more important than the P38 pathway (Bamford *et al.*, 2007).

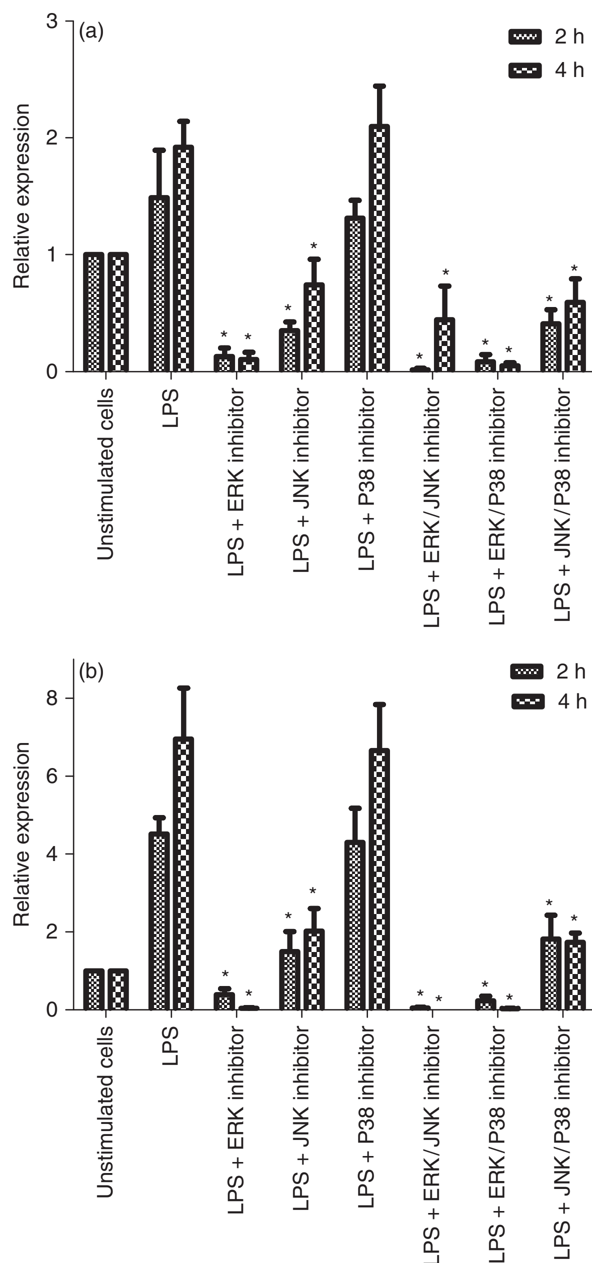

Fig. 5. Relative expression of (a) IL-1β and (b) TNF-α genes from U937 macrophages stimulated with 100 μg mL^{-1} lipopolysaccharide from *Burkholderia multivorans* LMG 13010 for 2 h or 4 h. Cells were preincubated (1 h) with individual or a combination of MAP kinase inhibitors. Values represent relative gene expression ± SEM. *Statistically different from the lipopolysaccharide-stimulated control cells ($P < 0.05$).

Therefore, while a combination of pathways may potentially be activated as a consequence of bacterial–macrophage interaction, in terms of *B. multivorans* cytokine production, the JNK pathway is the most significant, with the ERK and P38 pathways playing lesser roles.

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To conclude, the interaction of U937 macrophages with Bcc strains results in a higher level of internalization and a more potent proinflammatory response compared with other pathogens including *P. aeruginosa*. The MAP kinase pathways and in particular the JNK pathway play an important role in the induction of that proinflammatory response. From a clinical perspective, there are significant differences in the host reaction to Bcc infection compared with that of *P. aeruginosa*. Understanding the pathophysiological basis for those differences will bring us closer toward a more effective management of these infections in CF.

Acknowledgements

This work was partly funded by the Irish Research Council for Science, Engineering and Technology (IRCSET), Embark Postgraduate Research Scholarship Program and by the Programme for Research in Third Level Institutions (PRTLI) Cycles 3 and 4 (supported by the European Union Regional Development Plan, the Irish Government National Development Plan 2007-2013 and administered by the Higher Education Authority in Ireland). All are acknowledged with thanks.

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