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# *Burkholderia cenocepacia* ET12 strain activates TNFR1 signalling in cystic fibrosis airway epithelial cells

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## Summary

***Burkholderia cenocepacia* is an important pulmonary pathogen in individuals with cystic fibrosis (CF). Infection is often associated with severe pulmonary inflammation, and some patients develop a fatal necrotizing pneumonia and sepsis ('cepacia syndrome'). The mechanisms by which this species causes severe pulmonary inflammation are poorly understood. Here, we demonstrate that *B. cenocepacia* BC7, a potentially virulent representative of the epidemic ET12 lineage, binds to tumour necrosis factor receptor 1 (TNFR1) and activates TNFR1-related signalling pathway similar to TNF-α, a natural ligand for TNFR1. This interaction participates in stimulating a robust IL-8 production from CF airway epithelial cells. In contrast, BC45, a less virulent ET12 representative, and ATCC 25416, an environmental *B. cepacia* strain, do not bind to TNFR1 and stimulate only minimal IL-8 production from CF cells. Further, TNFR1 expression is increased in CF airway epithelial cells compared with non-CF cells. We also show that *B. cenocepacia* ET12 strain colocaizes with TNFR1 *in vitro* and in the lungs of CF patients who died due to infection with *B. cenocepacia,* ET12 strain. Together, these results suggest that interaction of *B. cenocepacia*, ET12 strain with TNFR1 may contribute to robust inflammatory responses elicited by this organism.**

## Introduction

Cystic fibrosis (CF) is caused by mutations in the gene encoding the CF transmembrane conductance regulator (Riordan *et al*., 1989; Rommens *et al*., 1989). Chronic lung disease accounts for most of the morbidity and mortality in CF, and is characterized by airway obstruction, chronic bacterial infection and vigorous inflammation that results in bronchiectasis and eventual pulmonary failure. Although *Pseudomonas aeruginosa* is the most common respiratory pathogen in CF, *Burkholderia cenocepacia*, a member of *B. cepacia* complex (Bcc), is as an important opportunistic pathogen associated with increased rates of morbidity and mortality. *B. cenocepacia*-infected patients show an unpredictable and variable clinical course, ranging from asymptomatic carriage to fatal necrotizing pneumonia and sepsis ('cepacia syndrome') (Isles *et al*., 1984). Among the epidemic lineages of *B. cenocepacia*, the ET12 lineage appears to be disproportionately associated with cepacia syndrome-related deaths (Ledson *et al*., 2002), and patients infected with this strain are at especially high risk of poor outcome after lung transplantation (De Soyza *et al*., 2001; 2004a).

Although pulmonary inflammation is a critical element of host defence, it also contributes significantly to the lung damage that is a hallmark of CF. Pathogens that are capable of inducing an intense and sustained chemokine response from the airway epithelium likely add to the severity of lung disease in CF. Bacterial products from both *P. aeruginosa* and members of the Bcc stimulate an excessive and prolonged pro-inflammatory response in epithelial cells and/or monocytes; however, products from Bcc species are more potent inducers of this response than *P. aeruginosa* (Shaw *et al*., 1995; Zughaier *et al*., 1999; Kube *et al*., 2001).

Epithelial cells lining the airway mucosa express several cell surface receptors, including evolutionarily conserved toll-like receptors (TLRs). These receptors recognize particular molecular patterns of invading pathogens and signal cells to respond appropriately by expressing neutrophil-attracting C-X-C chemokines, including IL-8, which is required for effective clearance of pathogens (Bals and Hiemstra, 2004). *P. aeruginosa*-induced stimulation of IL-8 from CF airway epithelial cells involves interaction of whole bacteria or bacterial components (pili, flagella or exoenzyme) with asialo-GM1 and TLRs 2, 4 and 5 (Bryan *et al*., 1998; Adamo *et al*., 2004; Epelman *et al*., 2004; Soong *et al*., 2004). Similarly, members of the Bcc interact with TLRs 4 and 5 and activate downstream signalling pathways, leading to IL-8

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production (Reddi *et al*., 2003; Urban *et al*., 2004). Bacterial ligands responsible for this interaction were identified as lipopolysaccharide (LPS) and flagella, which are common to all members of the Bcc. We and others have shown that *B. cenocepacia* stimulates a greater pro-inflammatory response than the other species of Bcc in airway epithelial cells (Palfreyman *et al*., 1997; Fink *et al*., 2003; Sajjan *et al*., 2004). More recently, the lipid A moiety from LPS, a key determinant of pro-inflammatory potential, was shown to vary among *B. cenocepacia* isolates, with the lipid A from *B. cenocepacia* strain BC7 being a relatively weak cytokine inducer in monocytic cell lines (De Soyza *et al*., 2004b). BC7 was originally isolated from a patient who died of 'cepacia syndrome', and is capable of stimulating relatively high IL-8 production from CF bronchial epithelial cells and causing intense inflammation in infected CF mice (Sajjan *et al*., 2001a; 2004). These observations suggest that, although the interaction of *B. cenocepacia* components (LPS, lipid A or flagella) with TLRs 4 and 5 plays a significant role in stimulating chemokine responses in monocytes and pneumocytes, it may not explain the robust inflammatory response elicited by *B. cenocepacia* BC7 in CF bronchial epithelial cells. Therefore, we hypothesized that *B. cenocepacia* BC7 binds to yet other undefined receptors on CF bronchial epithelial cells to activate downstream signalling events that leads to the production of IL-8, a potent neutrophil chemoattractant. To test this hypothesis, a cell surface receptor that interacts particularly with *B. cenocepacia* BC7 was identified and the contribution of this interaction to the pro-inflammatory response in CF airway epithelial cells was investigated.

## Results

### Burkholderia cenoceapcia *BC7 binds to tumour necrosis factor (TNF) receptor 1 (TNFR1)*

Previously, we have shown that *B. cenocepacia* BC7 stimulates greater IL-8 production in well-differentiated CF primary airway epithelial cells than in similarly grown non-CF cells (Sajjan *et al*., 2004). To identify the cell surface receptor that mediates this response, plasma membrane proteins corresponding to 25 μg of total protein from CF epithelial cells were resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with $^{35}$S-labelled BC7, and bound bacteria were detected by autoradiography. ATCC 25416, an environmental *B. cepacia* strain, and BC45, an ET12 strain obtained from a patient with no apparent change in disease severity, were used as negative controls, because these strains stimulated only minimal IL-8 production from CF airway epithelial cells. A major protein band at 55 kDa was observed in membranes incubated with BC7, along with a few minor bands (Fig. 1A). Under these experimental conditions, neither BC45 nor ATCC 25416, which stimulate only minimal IL-8 production from CF airway epithelial cells (Sajjan *et al*., 2004), bound to the 55 kDa protein. The differences in bacterial binding could not be attributed to differential protein loading, as Ponceau staining of the membrane before incubation with bacteria showed equal amounts of protein in each lane (data not shown). To examine whether denaturing conditions prevent the identification of other receptor proteins, we carried out a similar binding experiment after resolving the plasma membrane proteins under native conditions. Only the blot incubated with BC7 showed a smear at the top, indicating retention of membrane proteins in the stacking gel (data not shown). These results suggest that denaturing conditions do not prevent bacteria from binding to its receptor and, in fact, facilitates identification of specific receptors. We then sought to determine the identity of 55 kDa protein that specifically interacts with BC7. Protein bands at the 55 kDa position were cut out from an identical gel prepared from plasma membrane protein fraction (which was not processed for bacterial binding) and subjected to in-gel trypsin digestion followed by tandem mass spectrometry (MS/MS) analysis. We obtained the amino acid sequence for seven peptides, three of which matched the amino acid sequence of TNFR1, suggesting that TNFR1 is one of the putative cellular receptors for BC7.

To confirm the receptor identity, plasma membrane blots were pre-incubated with a monoclonal antibody to TNFR1 or normal mouse IgG (negative control), followed by $^{35}$S-labelled BC7, and the bound bacteria were detected by autoradiography. Both normal mouse IgG and TNFR1 antibody increased the binding of BC7 to the 55 kDa receptor, probably due to non-specific interactions (data not shown). Similar results were obtained when a polyclonal antibody to TNFR1 was used instead of a monoclonal antibody; thus it was not possible to determine the inhibitory effect of TNFR1 antibody on the binding of BC7 to the 55 kDa receptor. Next, we examined the effects of recombinant non-glycosylated TNFR1 and a TNFR1/Fc chimera, in which Fc portion is glycosylated on the binding of bacteria to TNFR1. Both recombinant molecules failed to inhibit the binding of BC7 to natural TNFR1 (data not shown). These results suggest that glycosylation of TNFR1 (and not on the Fc carrier molecule) may be required for bacteria to recognize its receptor. These results prompted us to examine the inhibitory effect of TNF-α, which is a natural ligand for TNFR1.

Initially, we examined whether TNF-α binds to the denatured TNFR1 by overlay assay. Blots of plasma membrane protein fraction and recombinant TNFR1/Fc chimera were either subjected to Western blot analysis with a monoclonal antibody to TNFR1, or incubated with

 

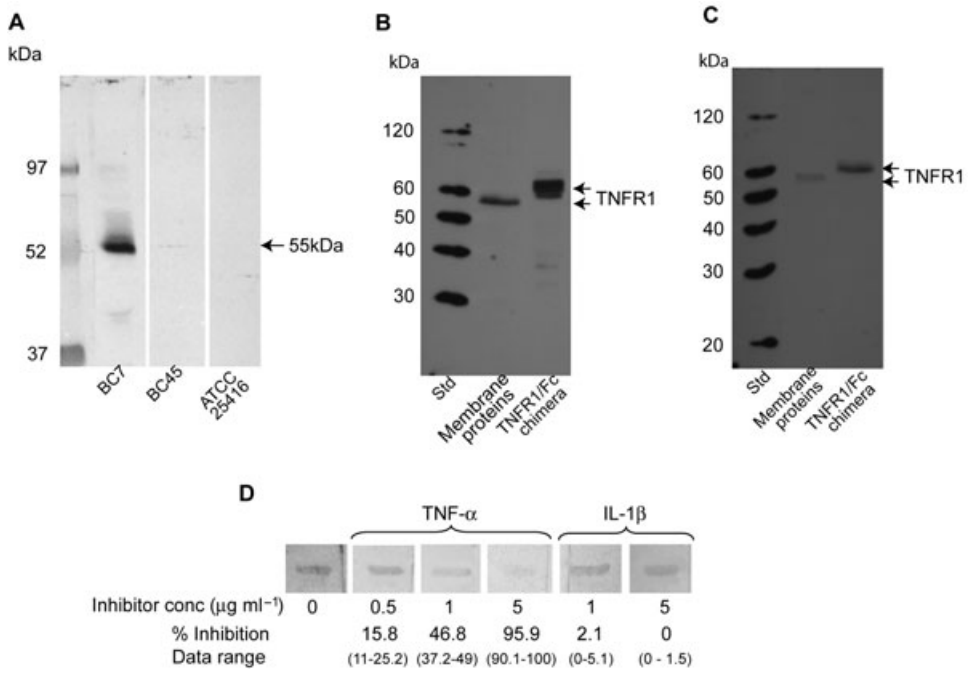


**Fig. 1.** Identification of cellular receptor for *B. cenocepacia*.
A. Binding of bacteria to 55 kDa protein. A fraction enriched in membrane proteins prepared from CF airway epithelial cells differentiated into a mucociliary phenotype was subjected to SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, blocked with gelatin, incubated with $^{35}$S-labelled bacteria, and bound bacteria were detected by autoradiography.
B and C. Binding of TNF-α to denatured TNFR1. Denatured plasma membrane fraction or TNFR1/Fc chimera was subjected to SDS-PAGE, and proteins were transferred to nitrocellulose. Blots were either subjected to Western blot analysis with monoclonal antibody to TNFR1 (B), or incubated with TNF-α, and the bound protein was detected by using polyclonal antibody to TNF-α (C).
D. Inhibition of BC7 binding to 55 kDa protein by TNF-α. Blots of plasma membrane proteins were pre-incubated with TNF-α or IL-1β, followed by incubation with $^{35}$S-labelled bacteria. Bound bacteria were detected by autoradiography and semiquantified by densitometry.

TNF-α, and the bound TNF-α was detected by using a polyclonal antibody to TNF-α. Antibody to TNFR1 detected a band at 55 kDa in the plasma membrane protein fraction. Recombinant TNFR1/Fc chimera was detected at a slightly higher position (Fig. 1B). Although the molecular weight of TNFR1/Fc chimera is 48 kDa, the recombinant protein migrates as a 60 kDa protein as a result of glycosylation in the Fc portion, according to the manufacturer's information sheet (R&D Systems). Nearly identical bands were observed in blots incubated with TNF-α, confirming that TNF-α binds to denatured TNFR1 and could be used to inhibit the binding of BC7 to TNFR1 (Fig. 1C). TNF-α also binds to TNFR2, which has a molecular weight of 75 kDa. Absence of a positive band at this position in plasma membrane protein fraction was not surprising, because airway epithelial cells do not express TNFR2 (Gomez *et al.*, 2004).

To assess the effect of TNF-α on the binding of BC7 to TNFR1, blots of epithelial cell plasma membrane fraction were pre-incubated with TNF-α or IL-1β, incubated with $^{35}$S-labelled BC7, and the bound bacteria detected by autoradiography. TNF-α inhibited the binding of BC7 to TNFR1 in a dose-dependent manner and almost completely abrogated the binding at a concentration of 5 μg ml$^{-1}$ (Fig. 1D). In contrast, IL-1β had no effect on the binding of BC7 to TNFR1 even at the highest concentration used. These results suggest that TNF-α receptor (TNFR1) may function as a receptor for BC7.

### *Tumour necrosis factor receptor 1 (TNFR1) expression is increased in primary cultures of CF airway epithelial cells*

Airway epithelial cells obtained from two CF and two non-CF individuals were cultured at an air/liquid interface. Total protein extracts from these cells were subjected to immunoblot analysis using TNFR1 antibody (Fig. 2A). Compared with non-CF cells, CF airway epithelial cells showed more intense 55 kDa immunoreactive bands, although intensity of β-actin bands was similar in each lane, an indication of increased expression of TNFR1 in CF cells. To determine the distribution of TNFR1, paraffin sections of CF and non-CF cultures were immunostained with antibody to TNFR1. Both CF and non-CF cells expressed TNFR1, but there was a difference in distribution of this receptor in these cell types. CF cells appear to have more TNFR1 on the cell membrane (Fig. 2B and C). Sections incubated with normal mouse IgG (negative

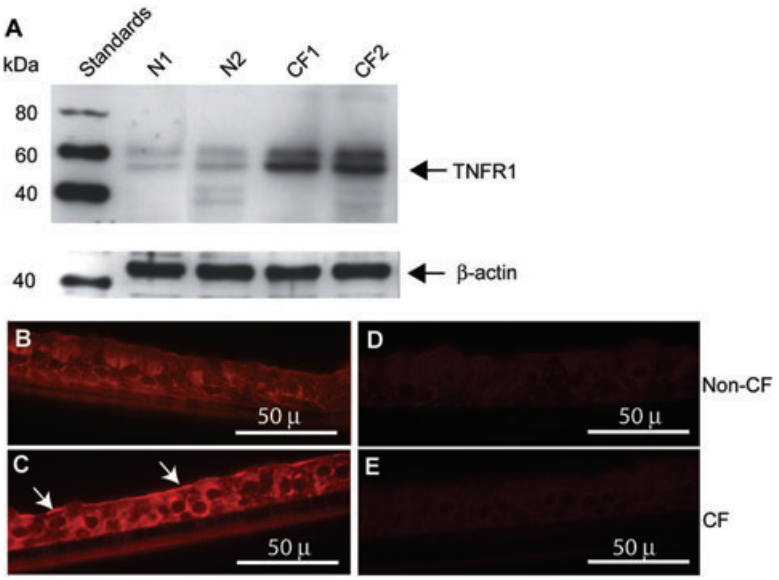


**Fig. 2.** Expression of TNFR1 in CF and non-CF airway epithelial cells.
A. Western blot analysis. Cell lysates from CF and non-CF primary airway epithelial cells differentiated into a mucociliary phenotype were probed with monoclonal antibody to TNFR1. N1 and N2 are normal airway epithelial cells from two different donors; CF1 and CF2 are airway epithelial cells from two different CF patients.
B–E. Immunofluorescence detection of TNFR1. Paraffin-embedded sections prepared from CF (C) and non-CF (B) mucociliary cultures were stained with monoclonal antibody to TNFR1, and the bound antibody was detected by anti-mouse IgG conjugated with Alexa Fluor-598. Arrowheads represent TNFR1 expression on the apical surface. Sections stained with normal IgG are shown in D and E.

control) instead of TNFR1 antibody were negative, indicating the specificity of the antibody (Fig. 2D and E).

### *Tumour necrosis factor receptor 1 functions as a receptor for BC7 in airway epithelial cells*

To determine the role of TNFR1 as a receptor for *B. cenocepacia* in intact cells, we used immortalized CF (IB3) cells, because these cells retain the characteristics of CF cells and, at the same time, do not produce mucus, which might hinder the accessibility of cell surface molecules to potential inhibitors. TNFR1 expression on the surface of IB3 cells was confirmed by fluorescence-assisted cell sorting (FACS) analysis. Non-permeabilized IB3 cells incubated with a TNFR1 antibody showed increased fluorescence as indicated by shift in the histogram to the right (Fig. 3A) compared with normal IgG-treated cells (control), suggesting that IB3 cells express abundant amounts of TNFR1 on their surface. Bacterial binding to cells was then determined by incubating IB3

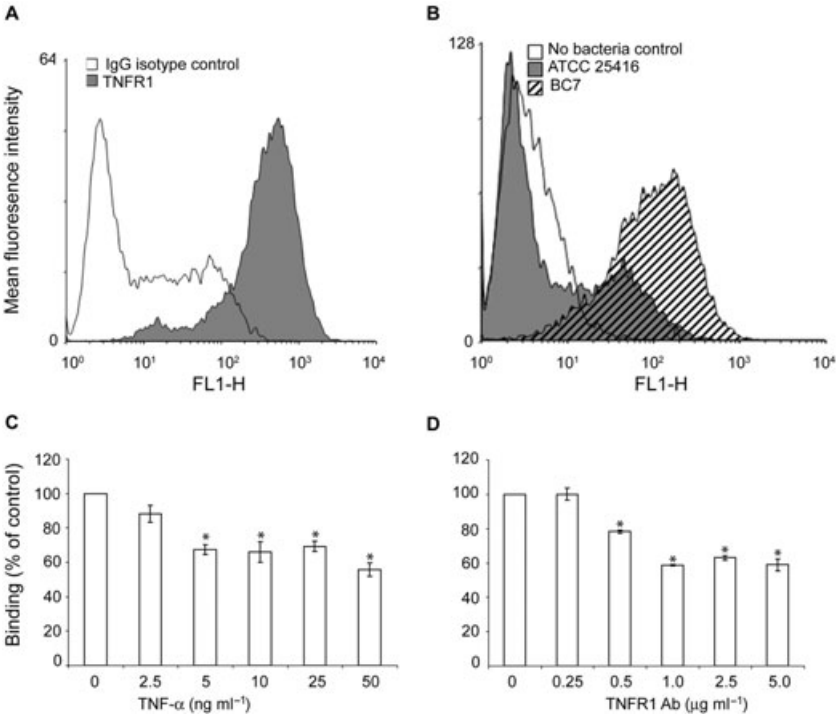


**Fig. 3.** Binding of bacteria to TNFR1 in intact cells.
A. Expression of TNFR1: non-permeabilized IB3 cells were incubated with monoclonal antibody to TNFR1, and bound antibody was detected with anti-mouse IgG conjugated with Alexa Fluor-488 and analysed by FACS. Data presented are representative of three independent experiments.
B. Binding of bacteria to cells: IB3 cells were lightly fixed with 0.5% paraformaldehyde, washed and incubated with FITC-labelled BC7 or ATCC 25416. Cells were harvested after removal of unbound bacteria and analysed by FACS. Data presented are representative of three independent experiments.
C and D. Effect of TNF-α or TNFR1 antibody on binding of bacteria to IB3 cells: lightly fixed IB3 cells were incubated with TNF-α or TNFR1 antibody for 30 min, followed by incubation with FITC-labelled BC7. Bacterial binding was then assessed by FACS. Data represent mean ± SEM of four independent experiments carried out in triplicates; $^*P < 0.05$, ANOVA.

 

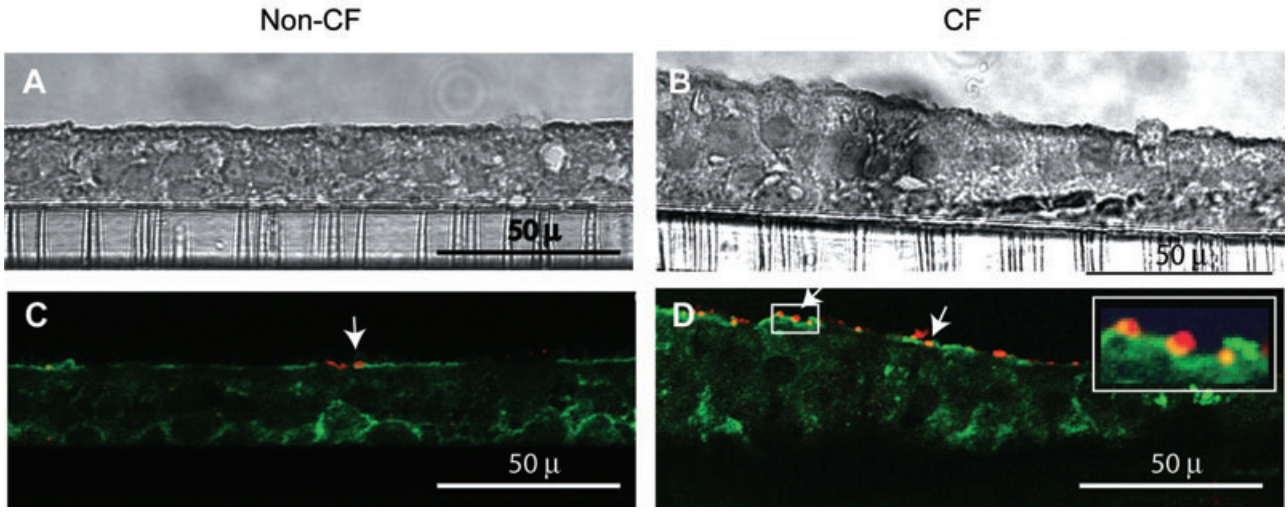


**Fig. 4.** Colocalization of *B. cenocepacia* BC7 with TNFR1 in non-CF and CF cell cultures: Paraffin sections of non-CF (A and C) and CF (B and D) primary cultures infected with BC7 were incubated with antibodies to TNFR1 and *B. cenocepacia*, and the bound antibodies were detected by Alexa Fluor-488 (for detection of TNFR1) and anti-rabbit Alexa Fluor-598 (for detection of bacteria). Arrowheads in C and D indicate colocalization of bacteria with TNFR1. A and B are phase-contrast micrographs and correspond to C and D respectively. The inset in D is a digital magnification of an area marked in rectangle to show colocalization of bacteria with TNFR1 (yellow).

cells with fluorescein isothiocyanate (FITC)-labelled BC7 or ATCC 25416, followed by FACS analysis. Only cells incubated with BC7 showed a shift in the histogram, indicating that BC7, but not ATCC 25416, binds to IB3 cells (Fig. 3B). Pre-incubation of IB3 cells with TNF-α, a natural ligand for TNFR1 (Fig. 3C), and antibody to TNFR1 (Fig. 3D), each inhibited the binding of BC7 to IB3 cells in a dose-dependent manner, with maximum reductions of 43% and 45% respectively, indicating that TNFR1 may serve as a cellular receptor for BC7. IL-1β or normal IgG, which were used as negative controls, had no effect on BC7 binding to IB3 cells (data not shown). The partial inhibition of BC7 binding to epithelial cells by TNF-α contrasts with previous results, in which TNF-α completely inhibited BC7 binding to TNFR1 (Fig. 1D), because in this experiment, the binding of BC7 to isolated TNFR1 was being determined rather than the intact cells. Taken together, these data suggest that in intact cells, BC7 may also bind to other cellular receptors in addition to TNFR1.

### *BC7 colocalizes with TNFR1 in cell cultures differentiated into mucociliary phenotype*

Well-differentiated CF and normal cell cultures were incubated with BC7 and incubated for 2 h. Cell cultures were washed to remove unbound bacteria, fixed and embedded in paraffin. Thin sections from these cultures were immunostained with antibodies to TNFR1 and *B. cenocepacia* and visualized by confocal fluorescence microscopy, taking optical sections at 0.2 μ intervals. In general, CF cell cultures showed more bound bacteria than the similarly treated normal cells, as observed previously (Sajjan *et al*., 2004). Further, at least 40–50% of the bound bacteria colocalized with TNFR1 in both CF and normal cells (Fig. 4C and D).

### *Colocalization of* B. cenocepacia *with TNFR1 in CF and normal lung sections*

Paraffin sections prepared from lungs of a CF patient who died due to infection with *B. cenocepacia* ET12, were immunostained with antibodies to TNFR1 and *B. cenocepacia* (Figs 5C–F). Lung sections obtained from a normal donor served as a negative control (Fig. 5A and B). CF lung sections showed increased expression of TNFR1 compared with normal lung sections. As expected, normal lung sections did not show *B. cenocepacia* (Fig. 5B). On the other hand, CF sections showed *B. cenocepacia* organisms in both the airways and parenchyma, which frequently colocalized with TNFR1 in bronchial and alveolar epithelial cells (Fig. 5D and F). The *B. cenocepacia* isolate (BCM132) obtained from this patient belongs to ET12 lineage as determined by PFGE analysis. It showed binding to 55 kDa TNFR1 and also stimulated IL-8 from CF cells similar to BC7 (Fig. 6A and B). These observations suggest that *B. cenocepacia* ET12 may interact with TNFR1 in the lungs of infected CF patients.

### Burkholderia cenocepacia *BC7 activates TNFR1-related signalling pathway*

We sought to examine the pro-inflammatory signalling pathways activated by BC7 in CF airway epithelial cells.

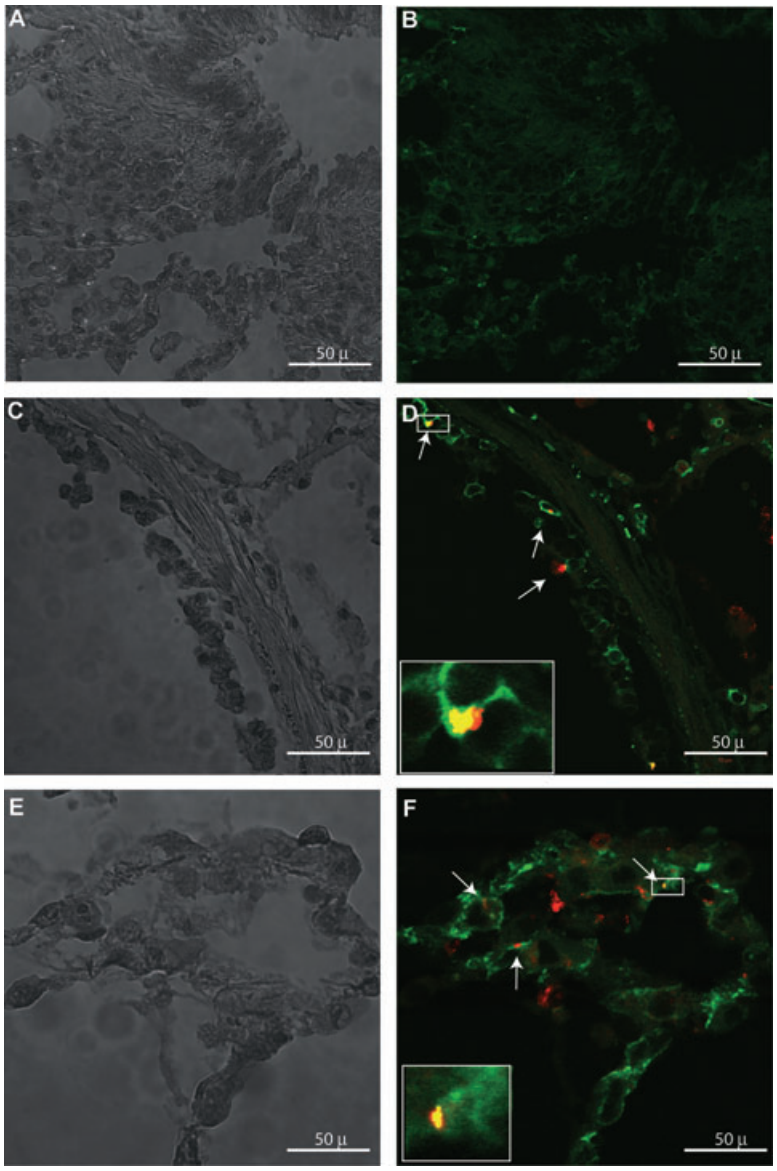


**Fig. 5.** Colocalization of *B. cenocepacia* with TNFR1 in lungs of a CF patient. Lung sections from normal donor (A and B) or a CF patient who died due to *B. cenocepacia* infection (C–F) were incubated with antibodies to TNFR1 and *B. cenocepacia*, and the bound antibody was detected as described in Fig. 4. A, C and E are phase-contrast micrographs and correspond to B, D and F respectively. Arrowheads indicate colocalization of bacteria with TNFR1. The inset in D and F is a digital magnification of an area marked in rectangle to demonstrate colocalization of bacteria with TNFR1 (yellow) in the lung sections.

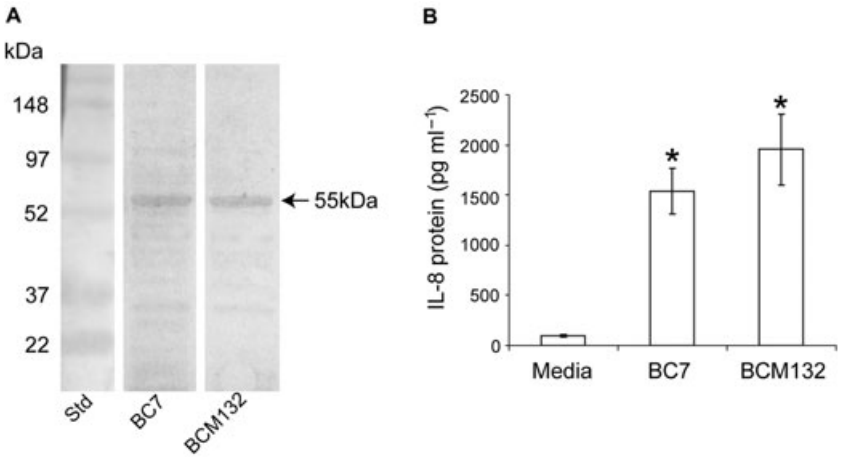


**Fig. 6.** A. Binding of BCM132 to 55 kDa TNFR1. Western blots of plasma membrane proteins were either incubated with $^{35}$S-labelled BCM132 or BC7. Bound bacteria were detected by autoradiography.
B. Stimulation of IL-8 response in IB3 cells by BCM132. IB3 cells were incubated with BCM132 or BC7 at a moi of 0.1 for 24 h, and the IL-8 in cell culture media was measured by ELISA. *$P < 0.05$, ANOVA.

 

IB3 cells were utilized in this study, because these cells can be transfected readily compared with primary cells and also we have established that TNFR1 in IB3 cells functions as one of the receptors for BC7. To assess whether IB3 cells respond to BC7 infection similar to primary cells (Sajjan *et al*., 2001a; 2004), IB3 cells were incubated with BC7 or ATCC 25416, and IL-8 protein measured by ELISA, at 1–24 h post infection. IB3 cells incubated with BC7 produced more IL-8 protein than cells incubated with ATCC 25416 at all time points (Fig. 7A), and IL-8 protein level corresponded to number of bacteria associated with cells (Fig. 7B). UV- or heat-killed BC7 stimulated significantly less IL-8 production than viable bacteria (Fig. 7C), indicating that infection of cells by bacteria is required for maximum IL-8 production.

Because airway epithelial cells may release TNF-α in response to infection, we tested whether BC7-stimulated IL-8 production in IB3 cells was mediated by TNF-α. IB3 cells were incubated with bacteria in the presence of TNF-α neutralizing antibody or normal IgG (negative control), and IL-8 protein was again measured. To confirm the neutralizing activity of the TNF-α antibody, TNF-α was used as a positive control. TNF-α neutralizing antibody completely inhibited TNF-α-stimulated IL-8 production, at a concentration as low as 50 ng $ml^{-1}$ (Fig. 7D). In contrast, BC7-stimulated IL-8 expression was not affected by TNF-α neutralizing antibody, even at 1 μg $ml^{-1}$ (Fig. 7E). Normal IgG did not affect the expression of IL-8 stimulated by either TNF-α or BC7, indicating the specificity of TNF-α neutralizing antibody. Together, these results suggest that BC7-stimulated IL-8 production in IB3 cells is not mediated by TNF-α, but rather occurs as a result of direct interaction between BC7 and TNFR1.

Binding of TNF-α to TNFR1 induces receptor trimerization and recruitment of the TNF receptor-associated death domain protein (TRADD). TRADD in turn acts as a platform and recruits TNF receptor activating factor 2 (TRAF2) and receptor interacting protein 1 (RIP1). This process then activates NF-κB via mitogen-activated protein kinases p38 and c-jun N-terminal kinases 1 and 2, ultimately leading to IL-8 production (Baud and Karin, 2001; Gomez *et al*., 2004). As a next step, we examined whether binding of BC7 to TNFR1 activates TNFR1-related signalling pathway, similar to TNF-α in CF airway epithelial cells. IB3 cells were incubated with TNF-α (3 ng $ml^{-1}$), BC7 or ATCC 25416 [each at a multiplicity of infection (moi) of 10] for 5, 15 or 30 min. Cell lysates were immunoprecipitated with a TNFR1 antibody , and the immunoprecipitates were subjected to Western blot analysis with antibody to TRADD or RIP1. Control cells or cells incubated with ATCC 25416 did not show receptor complex formation (Fig. 8A). In contrast, both TNF-α and BC7 induced recruitment of TRADD and RIP1 within 5 min, and it persisted up to 30 min. The binding of TNF-α or BC7 to TNFR1 also activated mitogen-activated protein kinases p38 and Jun amino-terminal kinase 1/2 (JNK1/2) in IB3 cells as indicated by phosphorylation of these two kinases (Fig. 8B and C). Cells incubated with ATCC 25416 or control cells did not show phosphorylation of either p38 or JNK1/2. Incubation of cells with UV- or heat-killed BC7 did not cause phosphorylation of p38 or JNK1/2 (Fig. 8D), indicating that either these treatments destroyed the bacterial ligand or infection with live bacteria is necessary for binding and activation of TNFR1 signalling pathway. These results suggest that BC7 activates a TNFR1-associated signalling pathway, similar to TNF-α in CF cells.

### *Tumour necrosis factor receptor 1 (TNFR1) siRNA partially inhibits transactivation of NF-κB by* B. cenocepacia *BC7*

IB3 cells were co-transfected with cDNAs encoding NF-κB-luciferase and renilla luciferase, and incubated with BC7 or ATCC 25416. TNF-α-treated cells served as positive controls and showed increased NF-κB transactivation as expected. Cells incubated with BC7 showed 5.9-fold mean increases in NF-κB transactivation, a significantly greater response than control cells or cells incubated with ATCC 25416 (Fig. 9A). These results suggested that BC7-stimulated IL-8 production in IB3 cells is accompanied by NF-κB transactivation.

To assess the contribution of TNFR1 in BC7-stimulated NF-κB transactivation, we used a mononoclonal antibody to TNFR1, which significantly inhibited the binding of BC7 to TNFR1 (Fig. 4) or TNFR1 siRNA to decrease or abolish the expression of TNFR1. IB3 cells transfected with NF-κB-luciferase and renilla luciferase were pre-incubated with a TNFR1 antibody or normal IgG, and *B. cenocepcia*-stimulated NF-κB transactivation was measured as before. TNFR1 antibody, by itself, caused NF-κB transactivation (data not shown) probably by ligating to TNFR1 and activating the downstream signalling pathway. Hence, TNFR1 antibody could not be used to determine the contribution of TNFR1 in BC7-induced transactivation of NF-κB.

IB3 cells were transfected with TNFR1 or non-targeting siRNA at 0, 20 or 40 pmol, and the expression of TNFR1 was determined by Western blot analysis. TNFR1-specific, but not non-targeting siRNA, reduced the expression of TNFR1 at both concentrations to a similar extent (Fig. 9B). Based on these results, we used 20 pmol of siRNA in subsequent experiments. IB3 cells were co-transfected with TNFR1 or non-targeting siRNA, NF-κB-luciferase and renilla luciferase, and BC7-stimulated NF-κB transactivation was measured as before. TNF-α and ATCC 25416 were used as positive and negative controls respectively. TNFR1-specific siRNA inhibited TNFα-stimulated NF-kB transactivation by

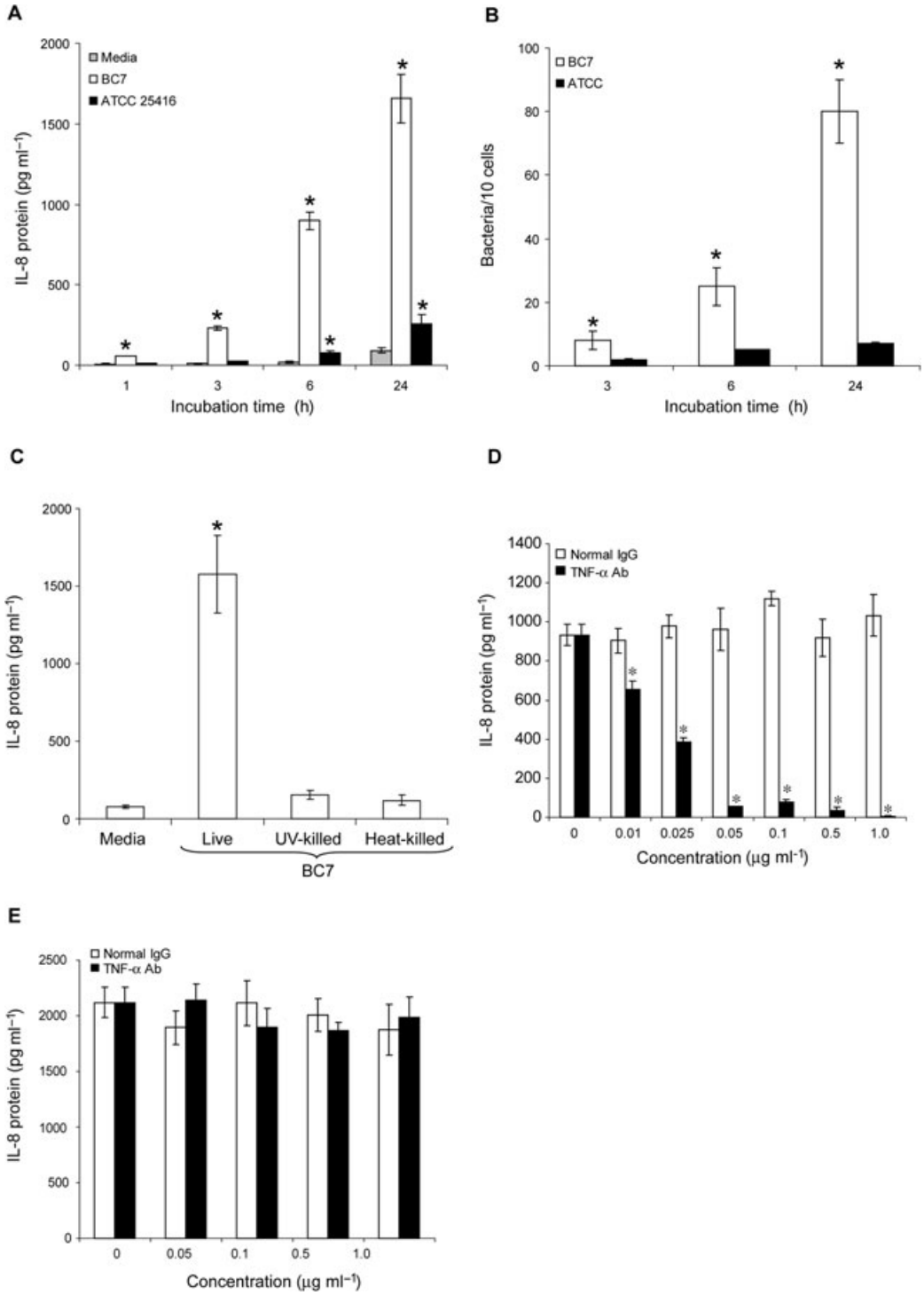


**Fig. 7.** Effect of TNF-α neutralizing antibody on *B. cenocepacia*-stimulated IL-8 response in IB3 cells.
A. IL-8 response: IB3 cells were incubated with *B. cenocepacia* BC7 or ATCC 25416 for 1, 3, 6 or 24 h, and IL-8 in the cell culture media was quantified by ELISA.
B. Binding of bacteria to cells. Cells incubated with bacteria for 3, 6 or 24 h were fixed, and bacteria associated were immunostained with Bcc antibody and quantified by immunofluorescence microscopy.
C. IL-8 response to UV- or heat-killed BC7: IB3 cells were incubated with live, UV-killed or heat-killed BC7 for 24 h, and IL-8 in the cell culture media was quantified by ELISA.
D and E. Effect of TNF-α neutralizing antibody: IB3 cells were incubated with 3 ng of TNF-α (D) or *B. cenocepacia* BC7 at a moi of 0.1 (E) in the presence or absence of varying concentrations of TNF-α neutralizing antibody or normal IgG for 24 h. IL-8 in cell culture supernatants was quantified by ELISA. Bars represent mean ± SEM of four independent experiments carried out in triplicate; $*P < 0.05$, ANOVA.

 

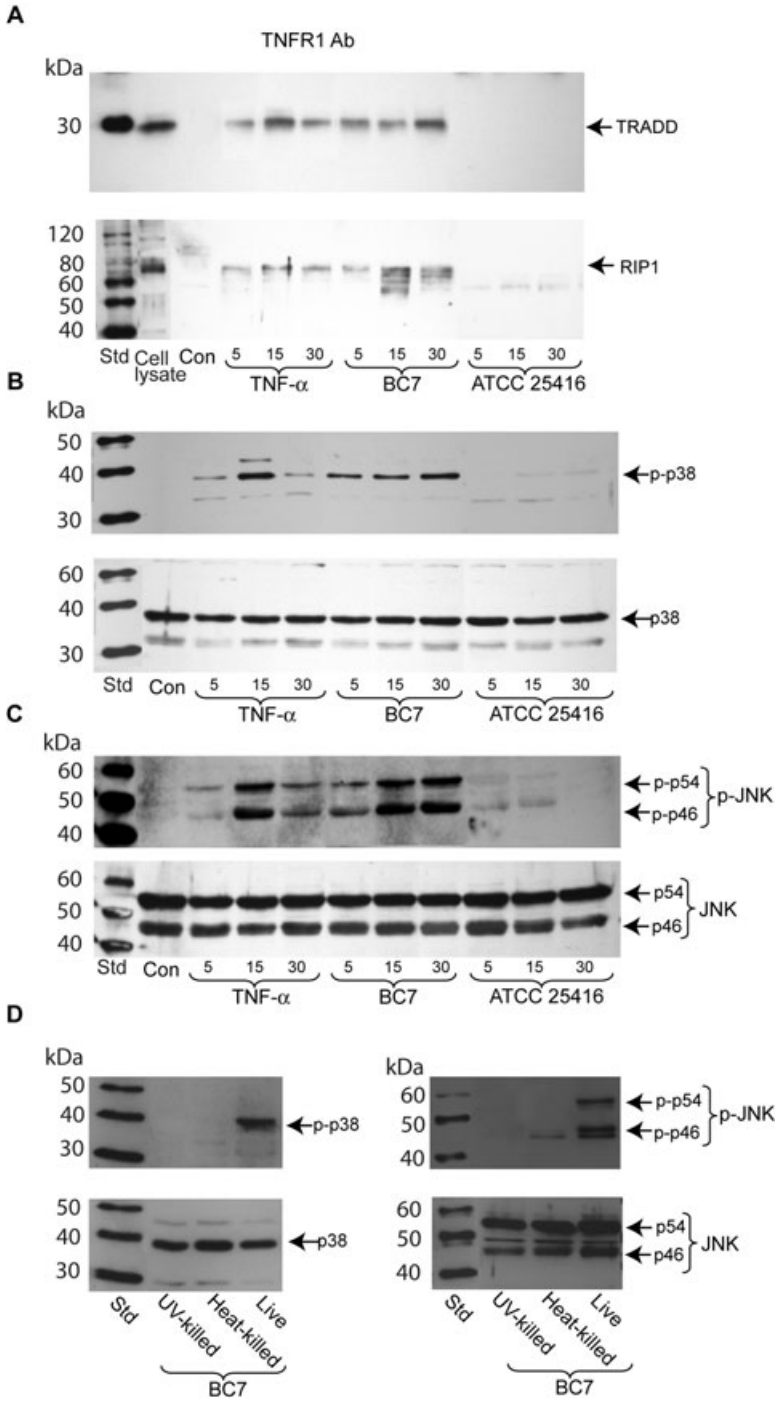


**Fig. 8.** Activation of TNFR1-related signalling pathway by *B. cenocepacia*.
A. Western blot analysis of immunoprecipitates: IB3 cells were incubated with either TNF-α (3 ng ml$^{-1}$) or *B. cenocepacia*, BC7 or ATCC 25416 at a moi of 10 for 5, 15 or 30 min and lysed. Cell lysates were immunoprecipitated with a TNFR1 antibody and subjected to Western blot analysis with either TRADD or RIP1 antibody.
B and C. Phosphorylation of p38 and JNK: lysates from the same experiment were subjected to immunoblot analysis with antibodies to phosho-p38, or total p38 (B) phospho-JNK1/2, or total JNK1/2 (C).
D. Phosphorylation of p38 and JNK in IB3 cells incubated with heat-killed or UV-killed BC7: cell lysates from IB3 cells incubated with live, UV- or heat-killed bacteria for 30 min were subjected to immunoblot analysis with antibodies to phosho-p38, or total p38 phospho-JNK1/2, or total JNK1/2 (D). Data presented are representative of three independent experiments. Std, molecular mass standards; cell lysate: total-cell lysate was used to confirm the band positions of TRADD or RIP1 observed in immunoprecipitates; Con, untreated IB3 cells.

almost 90%, indicating that TNF-α-driven NF-κB transactivation is dependent on TNF-α ligation to TNFR1 under the present experimental conditions. BC7-stimulated NF-κB transactivation was partially inhibited by TNFR1-specific siRNA (Fig. 9C), suggesting that binding of BC7 to TNFR1 is required for maximal BC7-stimulated cell activation. Non-targeting siRNA did not affect NF-κB transactivation stimulated by either TNF-α or BC7, indicating the specificity of TNFR1-siRNA. ATCC 25416-induced transactivation of NF-κB was not affected by either TNFR1-siRNA or non-targeting siRNA. Taken together, these results suggest that TNFR1 may function as one of the cellular receptors for potentially virulent ET12 strains of *B. cenocepacia*, and this binding interaction contributes to the robust IL-8 response from CF airway epithelial cells.

 

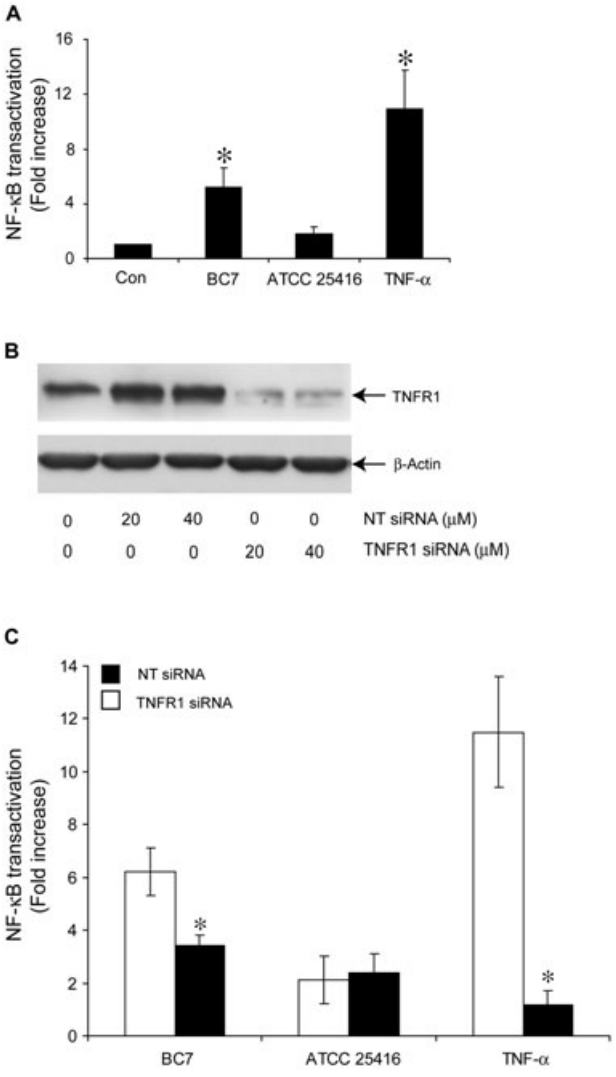


**Fig. 9.** Effect of TNFR1 siRNA on transactivation of NF-κB by *B. cenocepacia* BC7.
A. Transactivation of NF-κB by bacteria: IB3 cells were co-transfected with NF-κB luciferase and renilla luciferase, and then incubated with BC7, ATCC 25146 at a moi of 0.1 or TNF-α (3 ng ml$^{-1}$) for 24 h. Cells were lysed, and luciferase activity was measured and expressed as fold increase over control.
B. Western blot analysis: IB3 cells were transfected with TNFR1-specific or non-targeting siRNA, and incubated for 72 h. Cell lysates were subjected to Western blot analysis with a TNFR1 antibody .
C. Inhibition of transactivation of NF-κB by TNFR1 siRNA: IB3 cells were co-transfected with TNFR1-specific or non-targeting siRNA, NF-κB luciferase and renilla luciferase, and incubated for 24 h. Cells were serum starved overnight and then incubated with bacteria at a moi of 0.1 or TNF-α (3 ng ml$^{-1}$) for 24 h, and luciferase activity was measured as before. Bars represent mean ± SEM of six independent experiments carried out in duplicates; *$P < 0.05$, ANOVA.

## Discussion

Previously, we demonstrated that the interaction of BC7 with cytokeratin 13 (CK13) influenced the pro-inflammatory response from squamous epithelial cells (Sajjan *et al*., 2000a; 2002). While CK13 is normally expressed in nasal mucosa and trachea, expression is increased in the lower airway epithelium after repeated injury, which is common in CF lungs (Moll *et al*., 1982; Nagle *et al*., 1985; Sajjan *et al*., 2000b). However, we also showed that *B. cenocepacia* BC7 can stimulate intense and sustained IL-8 production in CF airway epithelial cells differentiated into a mucociliary phenotype, which do not express CK13 on their apical surface (Sajjan *et al*., 2004). We further observed that *B. cenocepacia* interacts with alveolar epithelial cells in the lungs of CF patients, where CK13 expression was not evident (Sajjan *et al*., 2000b; 2001b). TLR4 and TLR5 have been shown to be potential receptors for *B. cenocepacia* LPS and flagella respectively (Reddi *et al*., 2003; Urban *et al*., 2004). However, in preliminary studies, we observed that HEK293 cells, which lack TLRs, responded to infection with BC7 by producing IL-8 (data not shown). Together, these observations suggested that BC7 interacts with a receptor other than CK13 or TLRs in CF airway epithelial cells.

In this study, we demonstrated that BC7, one of the potentially virulent ET12 strains of *B. cenocepacia*, binds to TNFR1 and this binding interaction induces association of TNFR1 with TRADD and RIP, as well as activation of p38 and JNK, similar to the receptor's natural ligand TNF-α. Further, we found that TNFR1 siRNA partially inhibited NF-κB transactivation stimulated by BC7. Confocal microscopy revealed that *B. cenocepacia*, BC7 and isolate BCM132 colocalize with TNFR1 in primary human mucociliary-differentiated airway epithelial cells, and in the lungs of a CF patient who died due to *B. cenocepacia* infection respectively. Taken together, these data suggest that interaction of potentially virulent ET12 strains with TNFR1 may contribute to the intense inflammation observed in infected CF patients.

Tumour necrosis factor receptor 1 (TNFR1) belongs to a superfamily of TNF receptors and plays an important role in innate host defence (Pfeffer *et al*., 1993; Castanos-Velez *et al*., 1998; Gomez *et al*., 2004; Hehlgans and Pfeffer, 2005). Binding of TNF-α to TNFR1 activates the NF-κB and JNK pathways, leading to transcriptional activation of genes encoding pro-inflammatory proteins, including IL-8 (Stanger *et al*., 1995; Hsu *et al*., 1996a; Wright *et al*., 2004). Binding of TNF-α to TNFR1 may also activate the caspase cascade, ultimately inducing apoptosis (Hsu *et al*., 1996b). The inflammatory pathway deviates from the apoptotic pathway very early in the signalling process. Binding of TNF-α to TNFR1 induces trimerization and recruitment of TRADD, which then recruits either RIP1 and TRAF2 or FAS-associated death domain, activating inflammatory or apoptotic pathways respectively (Baud and Karin, 2001; Hehlgans and Pfeffer, 2005). We observed that binding of *B. cenocepacia* to CF airway epithelial cells did not induce apoptosis (data not shown);

rather, this interaction stimulated increased production of IL-8. This effect was preceded by the recruitment of TRADD and RIP1 to TNFR1 and phosphorylation of p38 and JNK, similar to cells treated with TNF-α, a natural ligand for TNFR1. Neutralizing antibody to TNF-α did not inhibit the production of IL-8 from IB3 cells incubated with BC7, indicating that the observed effect was not mediated by TNF-α. On the other hand, knockdown of TNFR1 expression by specific siRNA significantly attenuated BC7-induced NF-κB transactivation. These observations suggest that direct binding of BC7 to TNFR1 in CF cells is required to activate a TNFR1-stimulated inflammatory signalling pathway.

The IL-8 response stimulated by *B. cenocepacia* may not depend solely on the interaction of bacteria with TNFR1. We and others have demonstrated that *B. cenocepacia* also binds to other cellular receptors (Sajjan *et al*., 2002; Reddi *et al*., 2003; Urban *et al*., 2004). In the present study, our cell culture studies demonstrated that approximately half of the bound bacteria colocalized with TNFR1 receptor in both CF and non-CF cells. Although TNF-α completely abrogated the binding of BC7 to isolated TNFR1 *in vitro*, it only partially inhibited the binding of BC7 to CF cells even at the highest concentration used. Similarly, blocking of TNFR1 expression by TNFR1-specific siRNA partially repressed the NF-κB transactivation associated with IL-8 production. Thus, the capacity of BC7 to interact with TNFR1 may increase its potential to stimulate pro-inflammatory response from CF cells, but is not sufficient for the maximal response.

Although activation of TNFR1 plays a critical role in host defence, pathogenic bacteria have evolved to utilize and subvert this pathway to their advantage in susceptible hosts. As we have shown for *B. cenocepacia*, *Staphylococcus aureus* also has been shown to bind to TNFR1 in airway epithelial cells and induce a TNFR1-related signalling pathway, leading to NF-κB activation and IL-8 production (Gomez *et al*., 2004). Protein A, a major surface protein present in almost all strains of *S. aureus*, was identified as a bacterial ligand. In a murine model of lung infection, the interaction of protein A with TNFR1 resulted in the mobilization of PMNs to airways, leading to severe inflammation. Although recruitment of PMNs to airways is a critical component of host defence, excessive and repeated infiltration of PMNs can cause lung damage.

Previously we have shown that cable pili and the associated 22 kDa adhesin are required to stimulate maximum IL-8 production from squamous-differentiated bronchial epithelial cells. Preliminary studies indicated that isogenic mutants of BC7 lacking either cable pili or adhesin retained their capacity to bind to TNFR1 (data not shown), suggesting a role for yet another undefined bacterial ligand. Identification and characterization of this bacterial ligand is in progress.

Gene array analysis of CF airway epithelial cells infected with *P. aeruginosa* points to increased expression of TNF receptor and other related genes (Eidelman *et al*., 2001). In the present study, we showed that expression of TNFR1 is increased in cultured CF airway epithelial cells, as well as in the lungs of CF patients. These observations implicate that chronic respiratory infection with *P. aeruginosa* may, in fact, increase the expression of TNFR1 in the lungs of CF patients. Thus, *B. cenocepacia* ET12 strain, capable of binding to TNFR1 and activating downstream signalling pathway may cause severe inflammation in infected CF patients.

## Experimental procedures

### *Bacterial strains and growth conditions*

*Burkholderia cenocepacia* isolates BC7 and BC45 were obtained from CF patients, and have been described previously (Sajjan *et al*., 1992; 2002). Both BC7 and BC45 belong to ET12 lineage and are indistinguishable by PFGE analysis and ribotyping, but differ phenotypically. BC7, which was obtained from a patient who died of 'cepacia syndrome', binds to mucin glycopeptides and CK13, induces cell damage and a robust IL-8 response from primary CF airway epithelial cells, and causes severe lung inflammation and necrotizing pneumonia in CF mice. In contrast, BC45, which was obtained from a patient with no apparent change in disease severity, does not bind to mucin or cytokeratin 13, stimulates low amounts of IL-8 from CF cells, and causes relatively mild cell damage *in vitro* (Sajjan *et al*., 1992; 2000a; 2001a; 2002; 2004). BCM132 was isolated from post-mortem lungs of a CF patient who died of cepacia syndrome, and also belongs to ET12 lineage. ATCC 25416 is an environmental strain purchased from the American Tissue Culture Collection (Manassas, VA). Frozen stocks of BC7, BC45 and ATCC 25416 were subcultured on BHI agar and incubated at 37°C for 24–36 h. A single colony was inoculated into 10 ml tryptic soy broth and grown at 37°C for 12–16 h in the presence or absence of $^{35}$S-Translabel (GE Healthcare, Mississauga, ON, Canada). Bacteria were harvested by centrifugation, washed with PBS, and suspended in serum-free cell culture media or PBS containing 1% bovine serum albumin (BSA) to a desired concentration by measuring the optical density at 600 nm (1 unit at $OD_{600}$ corresponds to $\sim 1 \times 10^9$ cfu ml$^{-1}$). In some experiments, bacteria were labelled with FITC (Pierce Biotechnology, Rockford, IL) as described previously (Sajjan *et al*., 2000b).

### *Cell cultures*

Cystic fibrosis and normal primary airway epithelial cells at passage one were cultured at air/liquid interface as previously described (Sajjan *et al*., 2004), and were kindly provided by Dr Keshavjee (University Health Network, Toronto, Ontario, Canada). Immortalized CF bronchial epithelial cells (IB3 cells) (Zeitlin *et al*., 1991) were kindly provided by P. Zeitlin (Johns Hopkins Medical Institute, Baltimore, Maryland). IB3 cells were routinely grown in LHC-8 cell culture medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine, 5% fetal bovine

serum, and 50 U $ml^{-1}$ penicillin and 50 μg $ml^{-1}$ streptomycin at 37°C in 5% $CO_2$.

### *Binding assays*

*Bacterial overlay assay.* Binding of bacteria to isolated plasma membrane proteins was determined by a bacterial overlay assay as described previously (Sajjan, 1993). Briefly, blots of CF airway epithelial cell plasma membrane proteins were blocked with 1% gelatin, washed and incubated with PBS-BSA containing $^{35}$S-labelled BC7 or BC45, or ATCC 25416. Blots were washed with PBS-BSA to remove unbound bacteria, and bound bacteria were detected by autoradiography. In some experiments, blots of plasma membrane proteins were pre-incubated with TNF-α (R&D Systems, Minneapolis, MN) or IL-1β. Autoradiographs were semi-quantified using the ChemiDoc XRS system combined with Quantity One 1-D analysis software (BIO-RAD Laboratories, Hercules, CA).

*Binding of bacteria to epithelial cells.* IB3 cell monolayers grown in six-well plates were lightly fixed with 0.5% paraformaldehyde for 30 min and washed with PBS containing 0.5% BSA. FITC-labelled bacteria were added to cells at a moi of 1, centrifuged at 200 *g* and incubated for 1 h at 37°C. Cells were washed with PBS-BSA to remove unbound bacteria, and cells along with bound bacteria were detached from the plate and analysed with a Becton Dickinson FACSCalibur using CellQuest software. The autofluorescence contributed by cells was subtracted from all experimental samples. In inhibition assays, cells were pre-incubated with potential inhibitors (TNF-α, or antibody to TNFR1) for 1 h, washed with PBS and then incubated with FITC-labelled bacteria. Cells incubated with normal IgG or IL-1β instead of inhibitors served as negative controls.

*Bacterial binding to cells by microscopy.* Binding of bacteria to IB3 cells was determined by quantitative immunofluorescence microscopy as previously described (Sajjan *et al*., 2000a; 2002).

### *Identification of receptor protein*

The plasma membrane-enriched fraction from primary CF airway epithelial cells was subjected to SDS-PAGE and stained with Coomassie blue. Protein bands at 55 kDa were cut out and partially digested with trypsin. Peptides isolated from the gel were subjected to peptide mapping and MS/MS sequencing with Applied Biosystems/MDS Sciex API QSTAR XL MALDI QTOF (Urban *et al*., 2004). Peptide fingerprinting of the in-gel digests was analysed by database searching with ProFound (129.85.19.192/profound_bin/WebProFound.exe), and the peptide sequence obtained by MS/MS spectra was interpreted manually and used to search for protein homologies (http://www.ncbi.nlm.nih.gov/blast).

### *Infection of cell cultures*

IB3 cells were shifted to serum- and antibiotic-free media for at least 16 h prior to infection with bacteria. Bacteria were added to cells at a moi of 0.1, 1 or 10 and centrifuged at 200 *g*, and incubated as indicated in the *Results* section. Medium was collected and centrifuged, and IL-8 present in the supernatants was quantified by ELISA (R&D Systems). In some experiments, cells were incubated with bacteria in the presence of neutralizing antibody to TNF-α (R&D Systems, MN) or normal IgG. Well-differentiated airway epithelial cell cultures were shifted to antibiotic-free media at least 24 h prior to infection. Cultures were incubated with ATCC 25416 or BC7 ($1 \times 10^5$ cfu equivalent to moi of 0.1) apically for 24 h. Apical surface of cell cultures was washed, embedded in agarose and fixed in 10% buffered formalin, and finally embedded in paraffin as described previously (Sajjan *et al*., 2002).

### *Detection of cell surface TNFR1 by flow cytometry*

IB3 cells were incubated with monoclonal antibody specific to TNFR1 or normal mouse IgG (matched isotype control) and analysed by flow cytometry as previously described (Gomez *et al*., 2004; Sajjan *et al*., 2006).

### *Immunoprecipitation and Western blot analysis*

After relevant treatment, epithelial cells were washed and lysed in ice-cold RIPA buffer containing complete protease inhibitors, 1 mM sodium orthovandate and 1 mM sodium fluoride. Cellular proteins were resolved by SDS-PAGE and transferred to an Immobilon™-P membrane (Millipore, Billerica, MA). Membranes were probed with primary antibodies to phosphorylated or total p38, JNK1, JNK2 (Cell Signaling Technology, Danvers, MA), TNFR1, TRADD or RIP1 (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were amplified and visualized with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories) and chemiluminescent substrate (Pierce). Immunoprecipitation of cell lysates with TNFR1 antibody was performed using ExactaCruz™ B immunoprecipitation kit (Santa Cruz Biotechnology) following the manufacturer's instructions. Immunoprecipitates were solubilized in reducing buffer and subjected to Western blotting with either TRADD or RIP1 antibodies. Whole-cell lysates were used to confirm the band position of TRADD and RIP1 observed in the immunoprecipitates.

### *Transfection of cells and measurement of NF-κB activation*

IB3 cells grown in six-well plates were transiently transfected with hNF-κB-luciferase (0.2 μg; Stratagene, La Jolla, CA) and renilla luciferase (10 ng) using Lipofectamine 2000 (Invitrogen). The following day, the cells were shifted to serum-free media and stimulated with either *B. cenocepacia* BC7 at a moi of 0.1 or TNF-α (3 ng $ml^{-1}$). After incubation for 24 h, the cells were harvested and luciferase activity was measured using a luminometer. Changes in promoter activity were normalized for transfection efficiency by dividing luciferase light units by renilla luciferase light units. Results were then reported as fold increase over the empty vector/untreated control. When siRNA was used to inhibit the expression of TNFR1 protein, cells were transfected with 20 or 40 pmol of TNFR1 siRNA (Santa Cruz Biotechnology) along with hNF-κB and renilla luciferase. Cells transfected with non-targeting siRNA instead of TNFR1 siRNA was used as control.

 

### *Immunofluorescence*

Paraffin lung sections from a CF patient who succumbed to cepacia syndrome was provided by Dr E. Tullis (University Health Network, Toronto, Canada). Collection and utilization of lung tissue was approved by the hospital ethics committee. Paraffin section of non-CF lung was obtained from the pathology tissue library at the Hospital for Sick Children, Toronto, Canada. Deparaffinized and hydrated sections of lungs or primary airway epithelial cell cultures were heated in 10 mM citric acid buffer under pressure for 90 s to unmask the antigens and blocked with normal donkey serum. Sections were incubated with a TNFR1 antibody or a mixture of TNFR1 antibody and antibody to *B. cepacia complex* (R418) (Sajjan *et al*., 2001b), and the bound antibodies were detected with anti-goat conjugated with Alexa Fluor-488 (for detection of TNFR1) and anti-rabbit Alexa Flour-598 (for detection of bacteria) (Molecular Probes, Portland, OR). Sections were visualized by confocal fluorescent microscopy with a Zeiss LSM 510 confocal microscope mounted on a Zeiss Axiovert 100 M inverted microscope. Optical sections at 0.2 μ interval were taken to assess the colocalization of bacteria with TNFR1 as described previously (Adamo *et al*., 2004; Gomez *et al*., 2004; 2005; 2007). Sections treated with normal IgG instead of primary antibody served as negative controls.

### *Data analysis*

Statistical significance was assessed by analysis of variance (ANOVA). Differences identified by ANOVA were pinpointed by the Tukey-Kramer test.

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