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# The pattern recognition receptor Nod1 activates CCAAT/enhancer binding protein β signalling in lung epithelial cells

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**ABSTRACT: The innate immune receptor nucleotide-binding oligomerisation domain protein 1 (Nod1) recognises peptidoglycan containing *meso*-diaminopimelic acid found in all Gram-negative and some Gram-positive bacteria. Nod1 has been shown to activate nuclear factor (NF)-κB. The aim of the present study was to examine the expression of Nod1 in the lung, particularly in lung epithelial cells, and to investigate the activation of CCAAT/enhancer binding protein (C/EBP) transcription factors downstream of the Nod1 receptor in these cells.**

**The expression of Nod1 in mouse lung was examined using immunohistochemistry. A tissue array was used to determine the expression pattern in the human lung. Signalling downstream of Nod1 was examined in the human lung epithelial cell type, BEAS-2B, by electrophoretic mobility shift assay and reporter gene activation.**

**Nod1 expression was seen in various cell types in the lung, including epithelial cells. Activation of Nod1 in these cells resulted in modest activation of NF-κB, together with strong activation of the C/EBP transcription factors, particularly C/EBPβ. This activation appears to be independent of *de novo* protein synthesis.**

**The present study showed that nucleotide-binding oligomerisation domain protein 1 is expressed in lung epithelial cells. The results demonstrate a novel pathway downstream of the nucleotide-binding oligomerisation domain protein 1 receptor in these cells and suggest that C/EBPβ may play a role in immune responses to *meso*-diaminopimelic acid-containing bacteria in the lung.**

**KEYWORDS: CCAAT/enhancer binding protein, innate immune system, intracellular signalling, lung epithelium, nucleotide-binding oligomerisation domain protein 1 receptor, transcription factors**

In the lung, epithelial cells provide a first line of defence to protect the host against invading pathogens. A second line of defence is provided by the host's pattern recognition receptors, which sense pathogen-associated molecular patterns (PAMPs) and activate signals that lead to the destruction of the invading bacteria. PAMPs from bacteria and viruses are detected by toll-like receptors (TLR) [1]. It has recently been demonstrated that bacterial peptidoglycan (PGN) is detected by the intracellular nucleotide-binding oligomerisation domain protein (Nod)1 [2, 3] and Nod2 [4] receptors, rather than by TLR as was previously thought [5]. Nod1 recognises PGN-related molecules that contain *meso*-diaminopimelic acid (*meso*-DAP) [2, 3], which are found in all Gram-negative and some Gram-positive bacteria, whereas Nod2 recognises muramyldipeptide MurNAc-L-Ala-D-*iso*-Gln (MDP) [4], which is found in the PGN of virtually all bacteria.

In response to pathogen recognition, epithelial cells activate signalling pathways that lead to the production of cytokines, chemokines and antimicrobial peptides, which participate in pathogen removal [6]. *Streptococcus pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Mycobacterium tuberculosis* and *Moraxella catarrhalis*, which are all important pulmonary pathogens, have been shown to result in the production of chemotactic mediators from lung epithelial cells [7–11]. It has been demonstrated that many of these pathogens are recognised by the Nod1 receptor [12–14]. Epithelial-derived chemotactic mediators are central to the recruitment of neutrophils to the airways, and thus play a key role in the pulmonary innate immune response.

Following activation, the Nod1 receptor has been shown to activate the transcription factor nuclear factor (NF)-κB [15, 16] resulting in transcription

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STATEMENT OF INTEREST
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of inflammatory genes. However, CCAAT/enhancer binding protein (C/EBP)β and C/EBPδ transcription factors, which are expressed in airway epithelial and alveolar type-II cells [17, 18], are also known to play a role in expression of inflammatory genes [19]. The current authors hypothesised that C/EBPs are activated downstream of the Nod1 receptor and, therefore, could play a role in innate immune responses to bacteria in the lung. The present study demonstrates that Nod1 is expressed in lung epithelial cells and that C/EBPβ and, to a lesser extent, C/EBPδ are activated by this receptor.

## METHODS

### Constructs and reagents

The rabbit anti-human Nod1 antibody (Nod11-S) was obtained from Alpha Diagnostics (San Antonio, TX, USA). Peroxidase-labelled goat anti-rabbit antibody for immunohistochemistry was supplied by Vector Laboratories (Burlingame, CA, USA). Peroxidase-labelled goat anti-rabbit antibody was used for Western blotting (Amersham Biosciences, Little Chalfont, UK). The anti-C/EBPβ (sc-150 x) and anti-C/EBPδ (sc-636 x) were both obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The lung tissue array (LUD151) was purchased from Pantomics (San Francisco, CA, USA). A cDNA clone containing the entire human Nod1 cDNA from the I.M.A.G.E. Consortium [LLNL] clone 4338930 was obtained *via* HGMP, Cambridge, UK. The C/EBP-firefly luciferase reporter constructs have been previously described [20]. Lipopolysaccharide (LPS; serotype 026:B6) was purchased from Sigma-Aldrich (Stockholm, Sweden). The synthetic tripeptide, L-Ala-γ-D-Glu-*meso*-DAP (TriDAP), has been described previously [21].

### Immunohistochemistry

C57BL6 mice were killed by cervical dislocation and lungs were fixed by intratracheal inflation with 4% paraformaldehyde as previously described [22]. Antigens were retrieved by microwaving in 10 mM citrate buffer at pH 6.0. After washing in PBS, samples were incubated for 30 min with 0.3% $H_2O_2$ in PBS. Following a 1-h incubation in buffer A (PBS containing 0.3% Tween and 5% goat serum), samples were incubated overnight at 4°C in buffer A containing a 1:500 dilution of the rabbit anti-human Nod1 antibody. The samples were washed for 1 h in PBS containing 0.3% Tween, and then incubated for 2 h in buffer A containing a 1:200 dilution of peroxidase-labelled goat anti-rabbit immunoglobulin G. After washing, antigen–antibody complexes were detected with the Vectastain ABC-kit (Vector Laboratories, Burlingame, CA, USA) and counterstained with haematoxylin. Staining was performed on lung tissue from four different mice. The lung tissue array was stained as above with the exception that the primary rabbit anti-human Nod1 antibody was used at a 1:1,000 dilution.

### Cell culture and transfection

BEAS-2B cells (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% foetal bovine serum, 100 IU·mL$^{-1}$ penicillin and 0.1 mg·mL$^{-1}$ streptomycin (Gibco, Paisley, UK). When preparing nuclear extracts from transfected cells, the cells were plated 24 h prior to transfection on 75 cm$^2$ flasks. Cells were transfected with Fugene 6 (Roche, Penzberg, Germany) according to the manufacturer's instructions using a Fugene 6/DNA ratio of 3:1, and left for 24 h prior to preparation of nuclear extracts. Typically, transfection efficiencies were 10–20%, as determined by control transfection with a green fluorescent protein (GFP)-expressing construct.

### Preparation of nuclear extracts and electrophoretic mobility shift assays

The preparation of nuclear extracts has been previously described [17]. Double-stranded synthetic oligonucleotides harbouring a consensus C/EBP- (underlined; 5′ CGG GAT CCA TTG CGC AAT GGA TCC 3′) or NF-κB- (underlined; 5′ AGT TGA GGG GAC TTT CCC AGG 3′) binding site were used as probes. The probes were end-labelled using [γ-$^{32}$P] adenosine triphosphate and T4 polynucleotide kinase (Amersham Biosciences). Electrophoretic mobility shift assays (EMSA) were performed as previously described [17] using 1–6 μg of nuclear extracts. Where appropriate, 1 μL of polyclonal antibodies, anti-C/EBPβ (sc-150 x) or anti-C/EBPδ (sc-636 x; both from Santa Cruz Biotechnology) were included. EMSA were carried out using nuclear extracts from a minimum of three experiments and representative gels are shown below.

### Western blotting

For Western blotting, nuclear proteins (3–15 μg) were resolved on 12% SDS-PAGE gels and then transferred to Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). To verify equal loading of samples and even transfer to the membrane, the membrane was stained with Ponceau S stain. Following this, the membrane was blocked in Tris-buffered saline with 0.1% Tween (TBST) and 5% nonfat dried milk powder for 1 h at room temperature. Membranes were incubated overnight at 4°C with 1:10,000 dilution of primary polyclonal antibody to C/EBPβ or C/EBPδ, in TBST with 5% nonfat dried milk powder. Following washing in TBST, primary antibodies were detected using anti-rabbit peroxidase-labelled antibody diluted 1:10,000 in TBST containing 5% nonfat dried milk powder for 2 h at room temperature. After washing, the secondary antibody was detected using enhanced chemiluminescence Western Blotting detection reagent (Amersham Biosciences). Western blots were carried out using nuclear extracts from a minimum of three experiments and representative blots are shown below.

### C/EBP reporter gene assays

BEAS-2B cells were plated at $12 \times 10^4$ per well of a 24-well plate in 300 μL medium. Cells were transfected with 1.2 μg DNA using Fugene 6 (Roche) according to the manufacturer's instructions. The ratio of Fugene 6/DNA was 3:1. In the case of co-transfections, the ratio of reporter gene to expression plasmid was titred to 9:1 in order to avoid squelching effects. The Fugene/DNA mix was incubated with the cells for 24 h, following which the cells were washed twice with PBS and then lysed with 45 μL passive lysis buffer (Biothema, Turku, Finland). The level of expression of luciferase was measured in 30-μL samples in a 96-well plate using the GenGlow Luciferin kit (BioThema). The samples were read on an Orion II luminometer (Berthold, Bad Wildbad, Germany) with a 2-s delay and 10-s read time. Data represent three independent experiments each performed in triplicate. Data were normalised to the basal level seen in unstimulated cells transfected with the reporter gene construct containing an intact C/EBP-site.

## RESULTS

### *Nod1 is expressed in mouse and human lung epithelial cells*

Nod1 is expressed in multiple tissues, including human and mouse adult lung [16, 23]. However, the cellular expression pattern in this tissue has not been extensively studied. The present study examined the expression in mouse adult lung by immunohistochemistry using an antibody to Nod1. Nod1 expression was found in airway epithelial cells and in some alveolar type II cells (fig. 1). In the airways, expression was seen all the way down to the bronchoalveolar duct junction and expression was observed in both ciliated and Clara cells. Expression was also detected in alveolar macrophages and in the endothelium (fig. 1). The current authors also examined Nod1 expression in embryonic mouse lung tissue. Nod1 was expressed in this tissue from as early as 14.5 days post-coital (data not shown). The Nod1 antibody was then used on a tissue array to examine expression in human adult lung tissue. The Nod1 expression-pattern in human lung was similar to that observed in mice, with prominent expression in airway epithelial cells (fig. 2). As in the murine lung, expression was seen in endothelial cells, some alveolar type II cells and alveolar macrophages. Therefore, Nod1-expression is observed in resident lung cells with a known role in the early innate responses in the lung [6]. In the array, lung tissue from a patient with chronic bronchitis was included and when Nod1-expression was investigated in the present study, decreased Nod1-staining was observed in the airway epithelium (fig. 2d).

### *Activation of transcription factors downstream of Nod1*

As Nod1 expression was prominent in both human and mouse airway epithelium, the human BEAS-2B bronchial epithelial cell line was used to explore the role of Nod1 in the airways. These cells have been reported to express Nod1 [24] and, therefore, would be expected to express the signalling components required for Nod1 signalling. The cells were transiently transfected with a Nod1-expression clone and 24 h after transfection, nuclear activation of the transcription factors NF-κB and C/EBP was assessed by EMSA. It has previously

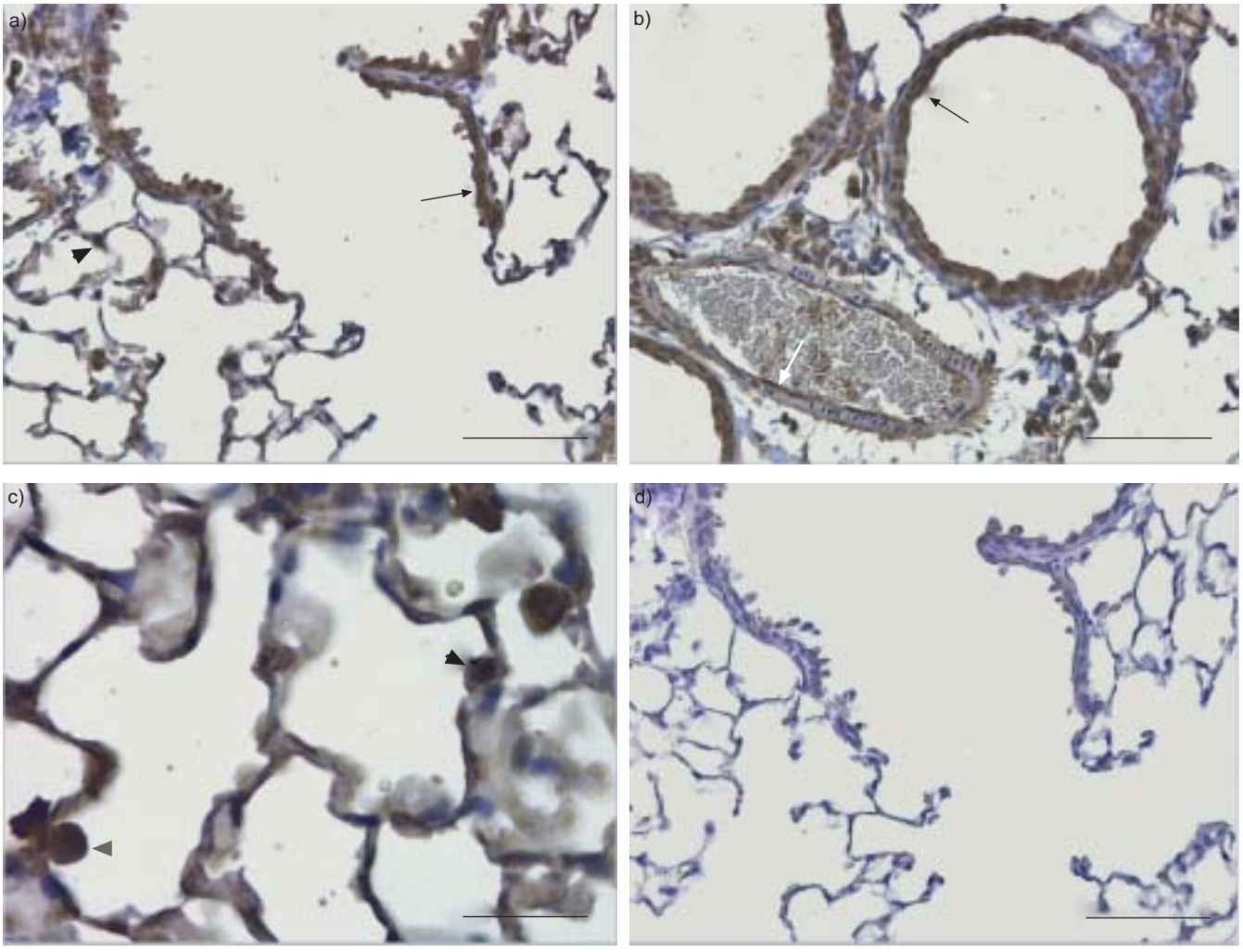


**FIGURE 1.** a-c) Expression pattern of nucleotide-binding oligomerisation domain protein 1 (Nod1) in mouse lung. Immunohistochemical staining for Nod1 in adult mouse lung (brown stain). Sections were counter-stained with haematoxylin. d) A control in which the primary antibody was excluded. Arrows point to expression of Nod1 in airway epithelial cells and arrowheads indicate expression in alveolar type II-cells. The white arrow indicates expression in endothelial cells and the grey arrowhead indicates Nod1 expression in alveolar macrophages. Scale bars: a, b, d) 200 μm, c) 100 μm.

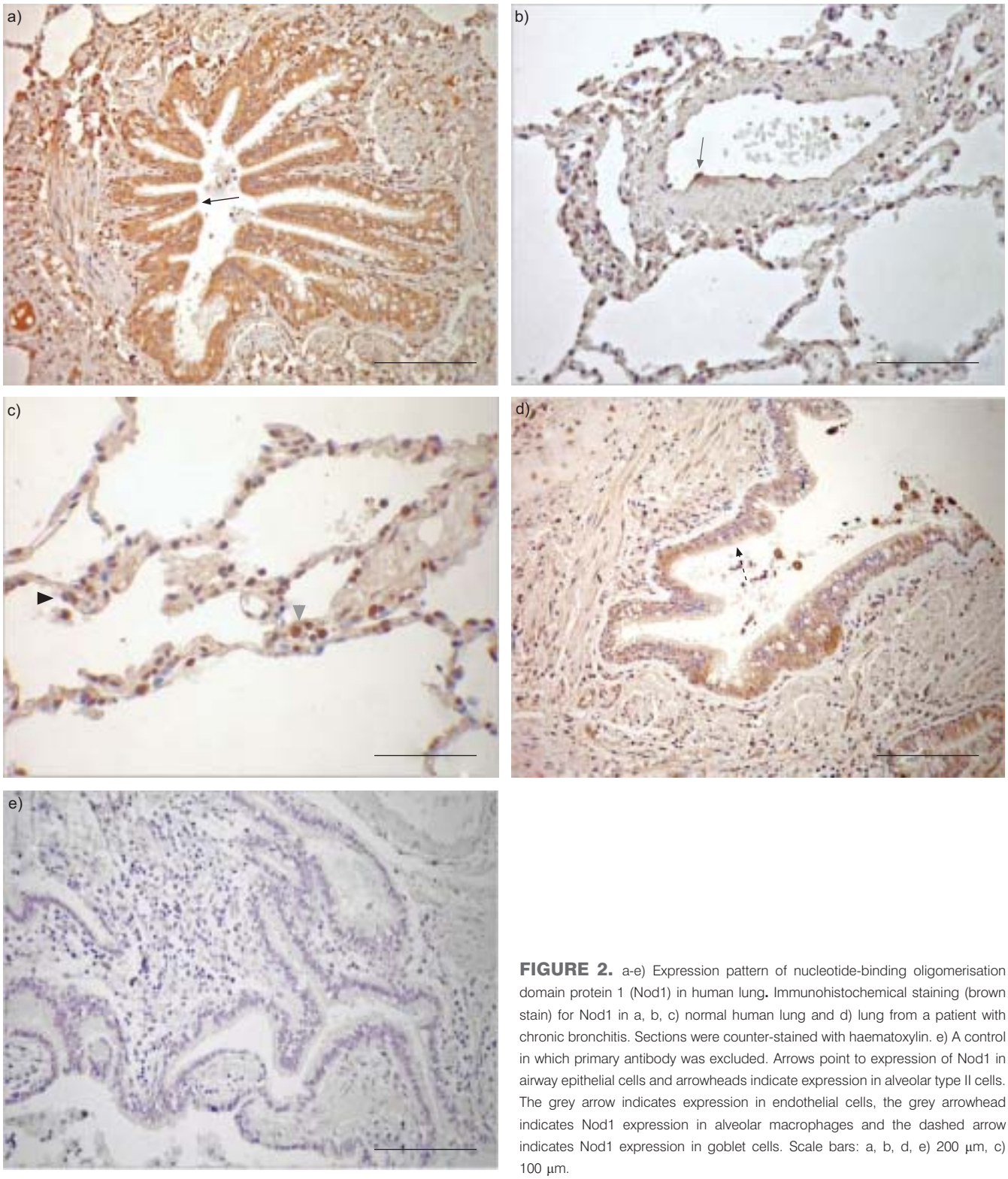


**FIGURE 2.** a-e) Expression pattern of nucleotide-binding oligomerisation domain protein 1 (Nod1) in human lung**.** Immunohistochemical staining (brown stain) for Nod1 in a, b, c) normal human lung and d) lung from a patient with chronic bronchitis. Sections were counter-stained with haematoxylin. e) A control in which primary antibody was excluded. Arrows point to expression of Nod1 in airway epithelial cells and arrowheads indicate expression in alveolar type II cells. The grey arrow indicates expression in endothelial cells, the grey arrowhead indicates Nod1 expression in alveolar macrophages and the dashed arrow indicates Nod1 expression in goblet cells. Scale bars: a, b, d, e) 200 μm, c) 100 μm.

been reported that overexpression of Nod1 is sufficient to activate the receptor independently of ligand binding [15, 16]. Nuclear extracts from transfected cells were incubated with radiolabelled probes containing either NF-κB or C/EBP consensus sites, following which EMSAs were performed. Untransfected BEAS-2B cells and GFP-transfected cells possess some C/EBP and NF-κB activity (data not shown). Activation of Nod1 has previously been described to lead to activation of NF-κB [15, 16]. As can be seen in figure 3a, transfection of cells with a Nod1-expression construct results in a modest increase in NF-κB binding activity. In contrast, a strong increase in C/EBP binding activity in cells transfected with Nod1 was

observed compared with control transfected cells. These data demonstrate that activation of the Nod1 receptor leads to activation of C/EBPs. As a control for activation of NF-κB, cells were stimulated with LPS, which activates the TLR4 receptor and results in NF-κB binding activity [1]. As expected, 30 min after LPS stimulation an increase in NF-κB binding activity was observed (fig. 3b). However, following LPS stimulation, no significant change in C/EBP binding was seen.

### *C/EBPβ is the main C/EBP family member activated by Nod1*

There is a striking overlap, both in human and mouse lung, in the cellular expression pattern of Nod1 (as observed in the present study) and the C/EBP-transcription factors [17, 18, 25]. Therefore, the activation of C/EBP family members downstream of Nod1 was further investigated by EMSA using antibodies to C/EBPβ and C/EBPδ. Inclusion of these antibodies revealed that the majority of the C/EBP-shift in Nod1-transfected cells is due to C/EBPβ (fig. 4a). C/EBPβ also comprised the majority of the C/EBP-shift in nontransfected cells. However, C/EBPδ is also activated in Nod1-transfected cells (fig. 4a), although not as strongly as C/EBPβ. To determine whether the increase in C/EBPβ binding activity was due to an increase in the levels of C/EBPβ protein, the amount of C/EBPβ protein in nuclear extracts from Nod1-transfected cells was compared with those from GFP- and pCMV-SPORT6-transfected cells by Western blotting. The level of C/EBPβ in Nod1-transfected cells was similar to the level in the control-transfected cells (fig. 4b), suggesting that the increase in C/EBPβ binding activity is not due to an increase in C/EBPβ protein levels. However, there did appear to be an increase in the smaller forms of C/EBPβ protein.

### *Activation of C/EBPs by the Nod1 ligand TriDAP*

Nod1 detects muropeptides from PGN that have a terminal *meso*-DAP residue (M-TriDAP) but, as the sugar group is not important for its recognition, Nod1 can detect the TriDAP with equal affinity [21]. To determine whether activation of the endogenous Nod1 receptor by its ligand leads to activation of C/EBP, and also to determine the kinetics of the Nod1-induced C/EBP activation, BEAS-2B cells were stimulated with a synthetic TriDAP molecule and EMSA was performed. Although the Nod1 receptor is located intracellularly there is evidence that addition of the Nod1 ligand to the culture medium of cells is sufficient to activate the receptor [21]. As seen in figure 5a, C/EBPβ is activated following addition of the TriDAP ligand to the culture media of the cells. Activation of C/EBPβ occurs within 30 min of addition of the ligand and can still be seen after 3 h. However, after 24-h stimulation with the ligand, the levels of active C/EBP proteins have returned to normal, if not lower levels. Activation of NF-κB by TriDAP was also investigated. NF-κB was activated within 30 min of stimulation but, unlike C/EBP, remained activated after 24 h of stimulation (fig. 5b).

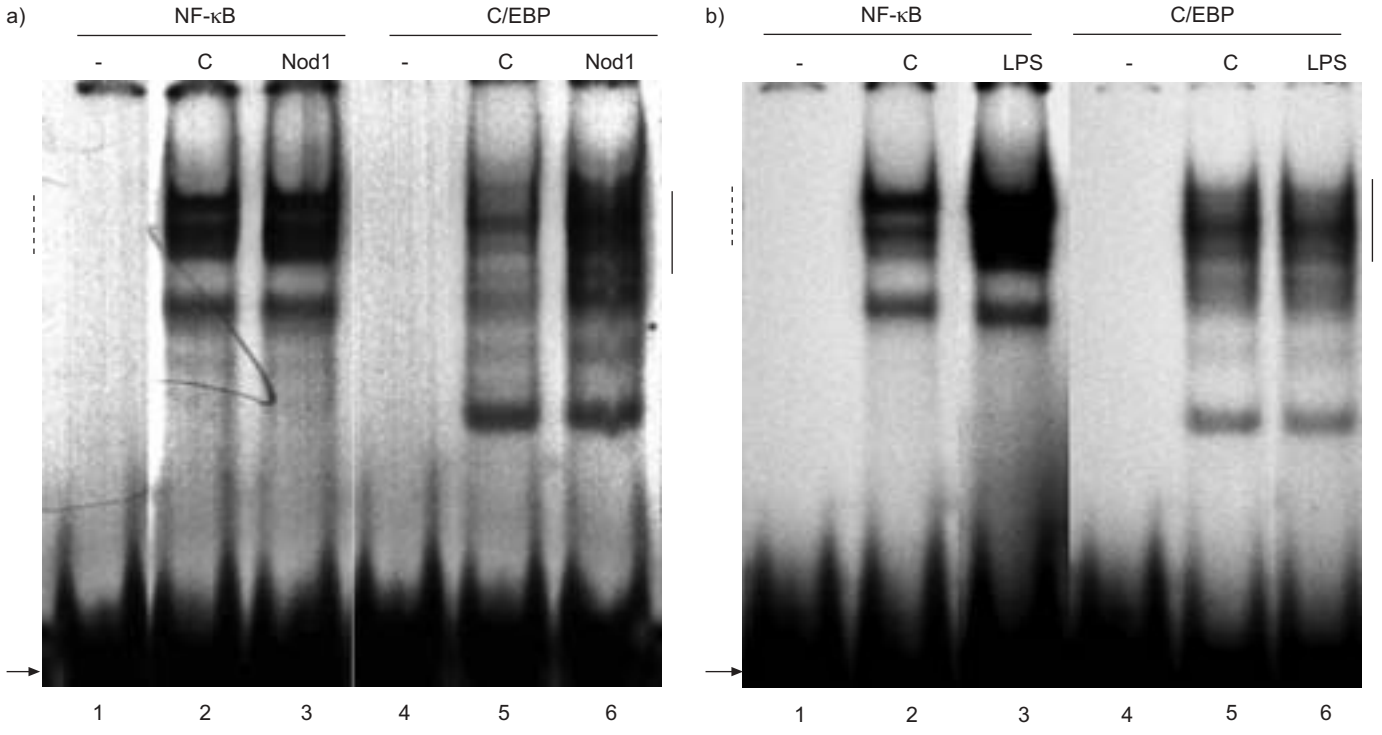


**FIGURE 3.** a) Nuclear extracts from BEAS-2B cells transfected with a nucleotide-binding oligomerisation domain protein 1 (Nod1) expression plasmid (lanes 3 and 6) or empty expression vector as control (C; lanes 2 and 5) were analysed by electrophoretic mobility shift assay (EMSA) using a nuclear factor (NF)-κB (lanes 1–3) or a CCAAT/enhancer binding protein (C/EBP; lanes 4–6) consensus oligonucleotide probe. Lanes 1 and 4 contain free probe only. Shifted complexes are indicated with dashed or solid lines for NF-κB and C/EBP, respectively. The arrow indicates migration of free probe. b) Nuclear extracts from BEAS-2B cells untreated (lanes 2 and 5) or treated with 100 ng·mL$^{-1}$ lipopolysaccharide (LPS; lanes 3 and 6) were analysed by EMSA using a NF-κB (lanes 1–3) or a C/EBP (lanes 4–6) consensus oligonucleotide probe. Lanes 1 and 4 contain free probe only. Shifted complexes are indicated with dashed or solid lines for NF-κB and C/EBP, respectively. The arrow indicates migration of free unshifted probe.

Again, the current authors determined the amount of C/EBPβ protein in the nuclear extracts of the stimulated cells. After 30 min of stimulation, the density of the upper band appeared to be similar to that of the unstimulated cells, even though a significant increase in C/EBPβ DNA binding was observed, and only a slight increase was seen after 3 h of stimulation (fig. 5c). The more defined double band in figure 5c compared with figure 4b may be due to cytoplasmic contamination of the nuclear extracts. The rapid increase in C/EBPβ DNA binding after addition of the Nod1 ligand makes it unlikely that the increased binding is mediated *via* effects on transcription or protein synthesis, and suggests that post-translational modification of the C/EBPβ protein underlies this increase. Post-translational modifications have been shown to regulate the activity of C/EBPs, with phosphorylation of C/EBPβ being the best characterised [26].

### *Activation of Nod1 activates C/EBP-dependent transcription*

To determine whether activation of Nod1 results in C/EBP-dependent transcription, the present authors co-transfected cells with the Nod1-expression construct and either a C/EBP–luciferase reporter construct in which the luciferase reporter gene was under the control of a consensus C/EBP binding site (fig. 6a), or the same construct with a mutated C/EBP site. As shown in figure 6b, expression of Nod1 resulted in an increase in C/EBP-dependent transcription. This was confirmed by mutation of the C/EBP site, which abolished the effect. The experiment was repeated in cells where the endogenous Nod1 receptor was activated with the TriDAP ligand. As shown in figure 6c, addition of TriDAP to the medium of the cells resulted in an increase in C/EBP-dependent transcription, and again this increase was prevented by mutation of the C/EBP consensus site in the reporter construct. These data demonstrate that the increase in C/EBP activity after activation of the Nod1 receptor is functional with regard to transcriptional activation and, therefore, suggests that C/EBP acts downstream of Nod1.

## DISCUSSION

The present study has demonstrated the presence of Nod1 in multiple cells types of mouse and human lung tissue, including airway epithelial cells. The expression pattern of C/EBPs in the lung has recently been described [17, 18, 25]. C/EBPβ is expressed in airway epithelial cells, as well as alveolar type-II cells and alveolar macrophages, and C/EBPδ is found in the airway epithelium and alveolar type-II cells. Thus, Nod1 and the relevant C/EBPs are co-expressed *in vivo* in resident cell types with a known role in the early innate responses of the lung [6]. In the human lung tissue array, decreased Nod1 staining intensity was observed in the chronic bronchitic airway epithelium. Decreased C/EBPβ activity has also been noted in the airway epithelium of patients with chronic bronchitis, and has been implicated in the pathogenesis of this common disease [25]. Together with the activation of C/EBPβ downstream of Nod1 seen in the current study, these findings demonstrate a correlation that hints at a potential role for these signalling molecules in chronic bronchitis pathogenesis. However, in light of the limited data on the Nod1 expression in chronic bronchitis in the current study, this correlation should be interpreted with care until it is confirmed in future studies.

The role of Nod1 in activating transcription factors of the C/EBP family in a human airway epithelial cell line, BEAS-2B, was examined. Strong activation of C/EBPβ was detected together with a more modest activation of C/EBPδ following overexpression of the Nod1 receptor. Overexpression of Nod1 has been shown to result in oligomerisation of Nod1 through its nucleotide-binding domain and this is thought to activate signalling by bringing the downstream signalling molecules into closer proximity with one another [27]. The current study also confirmed activation of C/EBPs downstream of Nod1

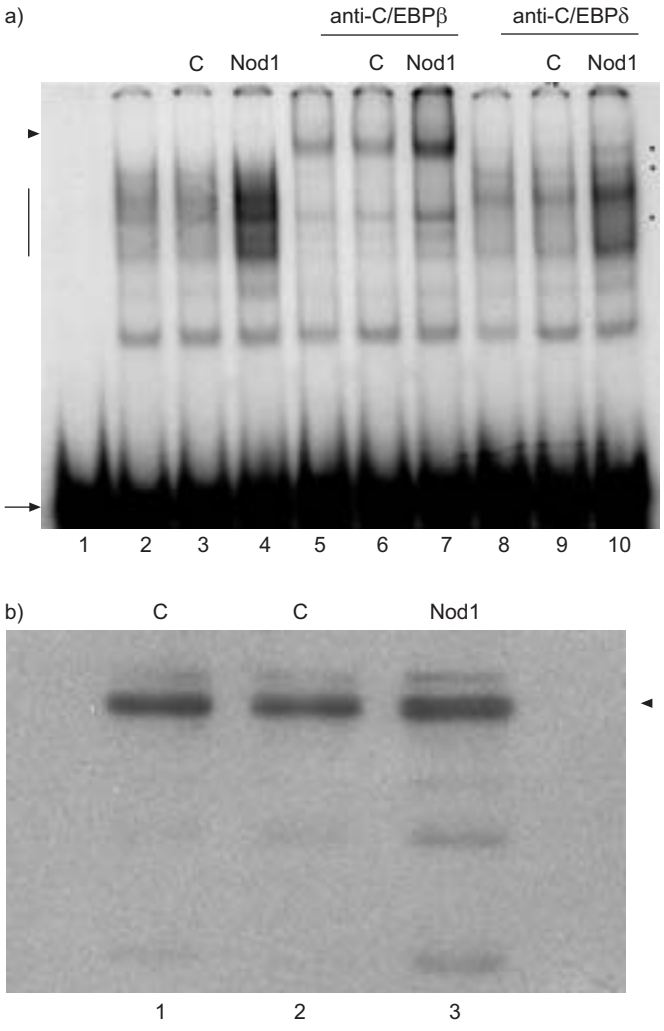


**FIGURE 4.** a) CCAAT/enhancer binding protein (C/EBP)β and C/EBPδ DNA-binding activity in nuclear extracts from BEAS-2B cells transfected with: a nucleotide-binding oligomerisation domain protein 1 (Nod1) expression plasmid (lanes 4, 7 and 10); controls (C), an empty expression vector, (lanes 3, 6 and 9); or a green fluorescent protein expression plasmid (lanes 2, 5 and 8), were analysed by electrophoretic mobility shift assay using a C/EBP consensus oligonucleotide probe and antibodies against C/EBPβ (lanes 5–7) or C/EBPδ (lanes 8–10). The solid line indicates the position of the shifted complex. The arrowhead indicates a supershift upon inclusion of antibody, and the arrow shows migration of free unshifted probe. b) C/EBPβ protein levels in the same extracts as (a) were analysed by Western blotting using a C/EBPβ antibody. The arrowhead indicates the p46/49 isoform of C/EBPβ, the main isoform expressed in human cell lines [26]. C: control.

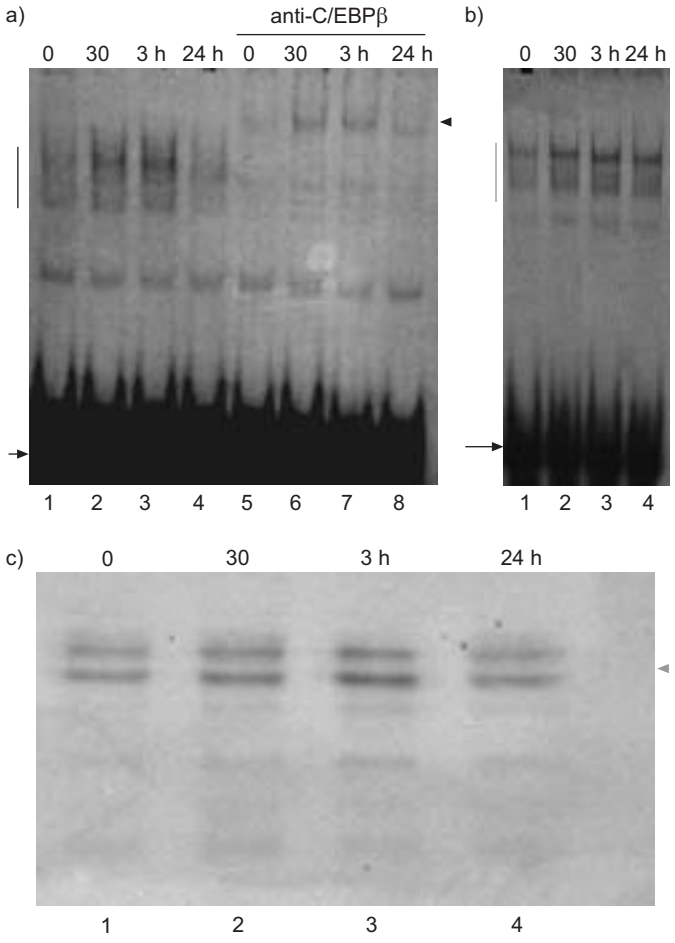


**FIGURE 5.** a) Nuclear extracts from BEAS-2B cells untreated (0) or treated with 200 nM tripeptide L-Ala-γ-D-Glu-*meso*-DAP (TriDAP) for between 30 min and 24 h were analysed by electrophoretic mobility shift assay (EMSA) using a CCAAT/enhancer binding protein (C/EBP) consensus oligonucleotide probe and antibodies against C/EBPβ (lanes 5–8). b) Nuclear extracts from BEAS-2B cells untreated (0) or treated with 200 nM TriDAP for between 30 min and 24 h were analysed by EMSA using a nuclear factor-κB consensus oligonucleotide probe. c) C/EBPβ protein levels in the same extracts as a) were analysed by Western blotting using a C/EBPβ antibody. The solid line indicates the position of the shifted complex and the arrow indicates the free unshifted probe. The black arrowhead indicates a supershift upon inclusion of antibody. The grey arrowhead indicates the p46/49 isoform of C/EBPβ, the main isoform expressed in human cell lines [26].

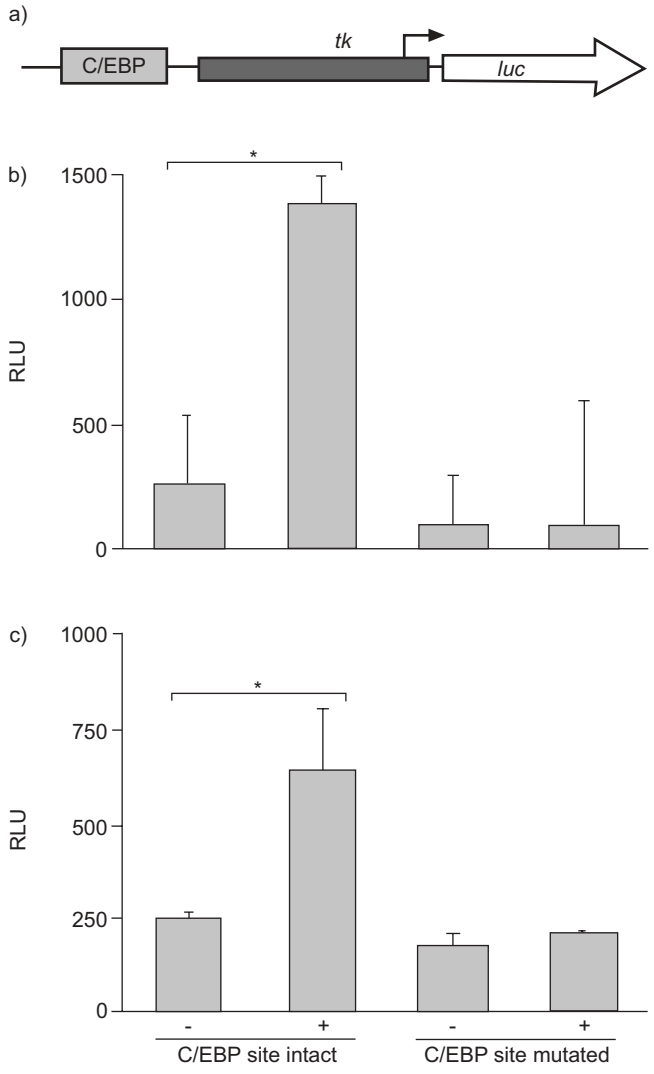


**FIGURE 6.** a) Schematic representation of the CCAAT/enhancer binding protein (C/EBP) reporter gene. The luciferase reporter gene (luc) is under the control of a consensus C/EBP binding site in front of a minimal herpes simplex virus thymidine kinase (tk) promoter. b) Activation of C/EBP-driven reporter gene activity in BEAS-2B cells by co-transfection of a Nod1 expression plasmid (+) or empty expression vector as control (-). The reporter gene construct contains either a C/EBP-binding site or a C/EBP-binding site that has been inactivated by point mutation. c) C/EBP-driven reporter gene activity in BEAS-2B cells untreated (-) or treated (+) with 200 nM tripeptide L-Ala-γ-D-Glu-*meso*-DAP. The reporter gene construct contains either a C/EBP-binding site or a C/EBP-binding site that has been inactivated by point mutation. RLU: relative luciferase units. Error bars indicate SD. *: $p<0.05$, unpaired t-test; n=3.

using a Nod1 ligand. To the current authors knowledge, this is the first report of Nod1 receptor signalling activating members of the C/EBP-family. One previous study examining the signalling pathways activated by murabutide (MB), a clinically accepted synthetic derivative of MDP that is now known to be a ligand for Nod2, demonstrated activation of C/EBPβ [28]. Similar to the present results from stimulation of Nod1, high C/EBPβ activation but weaker and transient activation of NF-κB was observed in MonoMac6 cells activated by MB. Similarly, PGN-polysaccharides from *Streptococcus pneumoniae*, which are recognised by Nod2 [24], activate NF-κB, C/EBPβ and activator protein-1 in human bronchial epithelial cells, and lead to the production of interleukin (IL)-8 [29]. Nod1 and Nod2 share common signalling pathways, as both have been shown to physically interact with RIP-like interacting CLARP kinase (RICK) *via* a caspase recruitment domain (CARD)-CARD interaction [15, 16], and signalling downstream of these receptors has been shown to be defective in RICK-deficient cells [30, 31]. Activation of RICK has been shown to lead to activation of Jun N-terminal kinase, p38 and extracellular signal-regulated kinase signalling pathways [30, 31]. Therefore, it is possible that C/EBPs are also activated downstream of the Nod2 receptor.

To begin to address the mechanism by which Nod1 stimulation leads to activation of C/EBPβ the levels of C/EBPβ protein in BEAS-2B cell nuclei were examined. Cells in which Nod1 was activated by over-expressing the Nod1 receptor appeared to contain similar amounts of C/EBPβ protein to those transfected with empty vector. However, there did appear to be a slight increase in the levels of the truncated form of C/EBPβ in these cells. The truncated form of C/EBPβ behaves as a transcriptional repressor as it lacks the amino-terminal activation domains [32]. However, as Nod1 over-expression still increased C/EBP-dependent transcription in the reporter gene assay, this does not seem to affect the overall C/EBP-transactivation potential. The current authors also stimulated cells with the TriDAP ligand and examined the levels of C/EBPβ in the cell nuclei. There appears to be a slight increase in C/EBPβ protein at 3 h post-stimulation, a time-point when increased C/EBPβ DNA-binding activity was detected. This increase in protein levels is marginal compared with the striking increase in DNA-binding activity, indicating a mechanism other than increased synthesis of C/EBPβ protein. The speed with which C/EBPβ is activated following stimulation of Nod1 also suggests another mechanism. It is possible that, as a result of Nod1 signalling, post-translational modification of C/EBPβ occurs, resulting in increased DNA binding of this protein. Phosphorylation of C/EBPβ has been reported to alter its binding ability [26] and since Nod1 can activate several protein kinases, including JNK and p38 mitogen-activated protein kinase [33, 34], it is possible that they play a role in regulating the activity of C/EBPβ. Further work is needed to determine the mechanism of C/EBPβ activation by Nod1 and the pathways involved.

A role for C/EBPβ downstream of Nod1 activation by bacterial PAMPs is easy to understand. The C/EBPβ protein was first identified as a protein capable of inducing expression of IL-1 and IL-6. Subsequently C/EBP binding sites have been identified in the regulatory regions of many genes involved in the innate and inflammatory responses [19]. C/EBP transcription factors are expressed in lung epithelial cells [17, 18] and these cells have been shown to express the Nod1 receptor. It has recently been shown [11, 14] that *Moraxella catarrhalis* invades lung epithelial cells and is recognised by Nod1, resulting in production of IL-8, a cytokine whose expression is controlled by C/EBPβ [35]. C/EBPβ knockout mice are highly susceptible to *Listeria monocytogenes* infection [36], a Gram-positive bacteria that is recognised by Nod1 [34]. Mice deficient in RICK, a component of Nod1-signalling, also have a reduced ability to defend against *Listeria monocytogenes* [30]. Both RICK and C/EBPβ knockout mice have impaired T-helper 1 responses [30, 37]. Together, these findings make investigation of the Nod1 response in the lungs of C/EBPβ knockout mice, and the immune responses to pathogens recognised by Nod1 in these animals, important future studies.

In conclusion, the current authors have demonstrated that the nucleotide-binding oligomerisation domain protein 1 receptor is expressed in mouse and human lung cells, including lung epithelial cells. Activation of nucleotide-binding oligomerisation domain protein 1 in the lung epithelial cell line BEAS-2B results in the activation of CCAAT/enhancer binding protein and nuclear factor-κB transcription factors, both of which control expression of inflammatory cytokine genes. The involvement of CCAAT/enhancer binding protein factors represent a novel pathway downstream of nucleotide-binding oligomerisation domain protein 1, highlighting the need for further investigations of the role that the CCAAT/enhancer binding proteins play in response to bacterial infection in the lung.

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