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Expression of the nuclear factor-κB inhibitor A20 is altered in the cystic fibrosis epithelium

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ABSTRACT: A20 is a lipopolysaccharide (LPS)-inducible, cytoplasmic zinc finger protein, which inhibits Toll-like receptor-activated nuclear factor (NF)-κB signalling by deubiquitinating tumour necrosis factor receptor-associated factor (TRAF)-6. The action of A20 is facilitated by complex formation with ring finger protein (RNF)-11, Itch and TAX-1 binding protein-1 (TAX1BP1). This study investigated whether the expression of A20 is altered in the chronically inflamed cystic fibrosis (CF) airway epithelium.

Nasal epithelial cells from CF patients (F508del homozygous), non-CF controls and immortalised epithelial cells (16HBE14o- and CFBE41o-) were stimulated with LPS. Cytoplasmic expression of A20 and expression of NF-κB subunits were analysed. Formation of the A20 ubiquitin editing complex was also investigated.

In CFBE41o-, peak LPS-induced A20 expression was delayed compared with 16HBE14o- and fell significantly below basal levels 12–24 h after LPS stimulation. This was confirmed in primary CF airway cells. Additionally, a significant inverse relationship between A20 and p65 expression was observed. Inhibitor studies showed that A20 does not undergo proteasomal degradation in CFBE41o-. A20 interacted with TAX1BP1, RNF11 and TRAF6 in 16HBE14o- cells, but these interactions were not observed in CFBE41o-.

The expression of A20 is significantly altered in CF, and important interactions with complex members and target proteins are lost, which may contribute to the state of chronic NF-κB-driven inflammation.

KEYWORDS: A20 protein, airway epithelial cells, chronic inflammation, cystic fibrosis, nuclear factor-κB

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A20 (encoded by the gene *TNFAIP3*) is an inducible, cytoplasmic zinc finger protein that negatively regulates nuclear factor (NF)-κB signalling. Constitutive expression of A20 is low in most cells, but is rapidly induced in response to a host of different stimuli including tumour necrosis factor (TNF)-α and bacterial products such as lipopolysaccharide (LPS) [1]. The *TNFAIP3* locus at 6q23 has been associated with multiple autoimmune and inflammatory diseases. A20 is an important molecular regulator of inflammation in rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes, multiple sclerosis, psoriasis and inflammatory bowel disease (IBD), despite varied pathogeneses [2]. In type 1 diabetes the intra-islet release of inflammatory mediators by activated immune cells is NF-κB-driven [3, 4], and single-nucleotide polymorphism analyses have identified A20 as a susceptibility gene for type 1 diabetes [5]. Furthermore, mice genetically deficient in A20 develop severe intestinal inflammation [6], suggesting that A20 is essential for intestinal homeostasis and suppression of NF-κB-dependent inflammation. In line with this, a recent genome-wide association study of seven common inflammatory diseases in the UK population identified A20 as a susceptibility gene for Crohn's disease [7]. An earlier independent study on 260 IBD-affected pairs from 139 families also highlighted A20 as a candidate gene for the development of IBD [8].

A20 is relatively unique in that it can exert ubiquitinating and deubiquitinating effects on target proteins [9]. This dual function is divided

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between the N-terminal domain, which acts as a deubiquitinating protein, and the zinc finger (ZnF)-containing C-terminal domain, which acts as an E3 ubiquitin ligase [9]. The dual functionality of A20 facilitates effective inhibition of NF-κB by firstly deubiquitinating target proteins to render them inactive, and subsequently ubiquitinating target proteins for proteasomal degradation [9, 10]. The relationship between A20 and NF-κB is complex and influenced by a variety of mediators and stimuli. A20 terminates the translocation of the NF-κB subunit p65 to the nucleus and terminates inflammatory signalling in healthy salivary gland epithelial cells. Conversely, silencing of A20 in these cells increases p65 activation and potentiates the inflammatory response [11]. This is consistent with the finding that p65 binds to an A20 promoter specific NF-κB sequence [12]. Furthermore, the ability of A20 to inhibit NF-κB activation is dependent on the formation of an ''A20 ubiquitin editing complex'' with the E3 ligases ring finger protein (RNF)-11 and Itch, and an adapter protein called TAX-1 binding protein-1 (TAX1BP1) [13, 14]. In the absence of any complex member, A20 cannot bind and act upon target proteins and, therefore, cannot prevent NF-κB activation [13]. In epithelial and other immune cells, LPS stimulation triggers Toll-like receptor (TLR)-4 mediated activation of the NF-κB pathway. A20 terminates this process by inhibiting the polyubiquitination and activation of TNF receptor-associated factor (TRAF)-6 early in the pathway [15].

Although widely studied as a tumour suppressor gene and highlighted as a potential biomarker for the development of chronic inflammatory disorders, including Crohn's disease and arthritis [16], A20 remains under-investigated in chronic lung diseases. Gon *et al*. [17] showed that A20 is essential in terminating TLR-2 and TLR-4 mediated interleukin (IL)-8 release from primary airway epithelial cells, while Tiesset *et al*. [18] found A20 to be rapidly inducible in the lungs of healthy mice challenged with *Pseudomonas aeruginosa*. However, to our knowledge, A20 has not been investigated in chronic airways diseases, such as cystic fibrosis (CF) [19]. CF is caused by mutations in the CF transmembrane conductance regulator (*CFTR*, with the F508del mutation being most common) gene located on the apical surface of the epithelia. The effects of these mutations are most evident in the lung, where an imbalance in chloride and sodium transport leads to dehydration of the epithelial surfaces and reduced mucociliary clearance of invading pathogens, creating a cycle of chronic infection and inflammation. A modelling-derived hypothesis suggests that desensitised pathogen recognition in chronic lung diseases such as CF may be secondary to hyperactive TRAF6 signalling [20]. Indeed, persistent NF-κB activation is a hallmark of CF airways inflammation, suggesting that this tightly regulated signalling pathway may be distorted. However, a potential role for A20 in chronic CF airways inflammation has not been investigated. The aim of this study was to determine if the expression of A20 was altered in the CF epithelium based on the hypothesis that loss or variation of normal A20 function may contribute to persistent and uncontrolled inflammation in the CF airways.

MATERIALS AND METHODS

Please see online supplementary material for full details.

Cell culture and stimulations

Bronchial epithelial cell lines 16HBE14o- and CFBE41o- (homozygous for F508del) were obtained from D. Gruenert (University of California San Fransisco, San Fransisco, CA, USA) and grown in submersion. All cell line experiments were conducted when cells were approximately 80% confluent. Primary nasal epithelial cells (NEC) were obtained from CF patients homozygous for F508del and matched control volunteers as previously described [21] and were fully differentiated at the air–liquid interface (ALI). The Research Ethics Committee of Northern Ireland approved the study and all participants provided informed consent (07/NIR02/23).

Flow cytometry

Cytoplasmic A20 expression was quantified by flow cytometry in permeabilised 16HBE14o- and CFBE41o- cells. The NF-κB heterodimer of p50 and p65 is abundant in most cell types [22]. Given the described relationship between p65 and A20, epithelial cells positive for the nuclear p50/p65 heterodimer subunits of NF-κB were investigated using an adapted flow cytometric method [23] as previously described [24]. Data are expressed as mean fluorescence intensity to evaluate the shift in fluorescence intensity of cytoplasmic A20, or nuclear p50/p65 positive cells compared to isotype controls. Data acquisition and analyses were performed on a flow cytometer (Epics XL; Beckman Coulter, High Wycombe, UK).

Determination of IL-8

The concentration of IL-8 in cell line supernatants and apical and basolateral washings from primary NECs was measured by commercially available ELISA (PeproTech EC Ltd, London, UK). Combined IL-8 release from apical and basolateral washings of NECs are presented.

Inhibitor studies

Prior to stimulation with LPS, CFBE41o- cells were pretreated for 1 h with a selective p65 inhibitor (JSH-23, 30 μM), or for 4 h with inhibitors of E1 ubiquitin activating enzymes (PYR-41, 25 μM) and the 26S proteasome (MG-132, 10 μM). All inhibitors were obtained from Merck KGaA (Darmstadt, Germany).

Quantitative PCR

Total RNA was extracted from cells (RNeasy Micro kit; Qiagen, Crawley, UK) and quantified (NanoDrop; Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of RNA were reverse transcribed into cDNA (Sensiscript Reverse Transcription Kit; Qiagen). Primer sequences, gene accession numbers and product sizes are given in online supplementary table S2. Relative expression to β-actin was calculated using the ΔΔCt method.

Immunoprecipitations and Western blotting

For immunoprecipitation experiments, protein lysates were precipitated with an antibody against full-length A20 (sc-52910, Santa Cruz Biotechnology, Dallas, TX, USA) using a Direct IP Kit (Thermo Fisher Scientific). Cell lysates were diluted in nuclease free water and Laemmli loading buffer, loaded onto Tris-HCl polyacrylamide gels (Thermo Fisher Scientific), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Membranes were incubated with primary

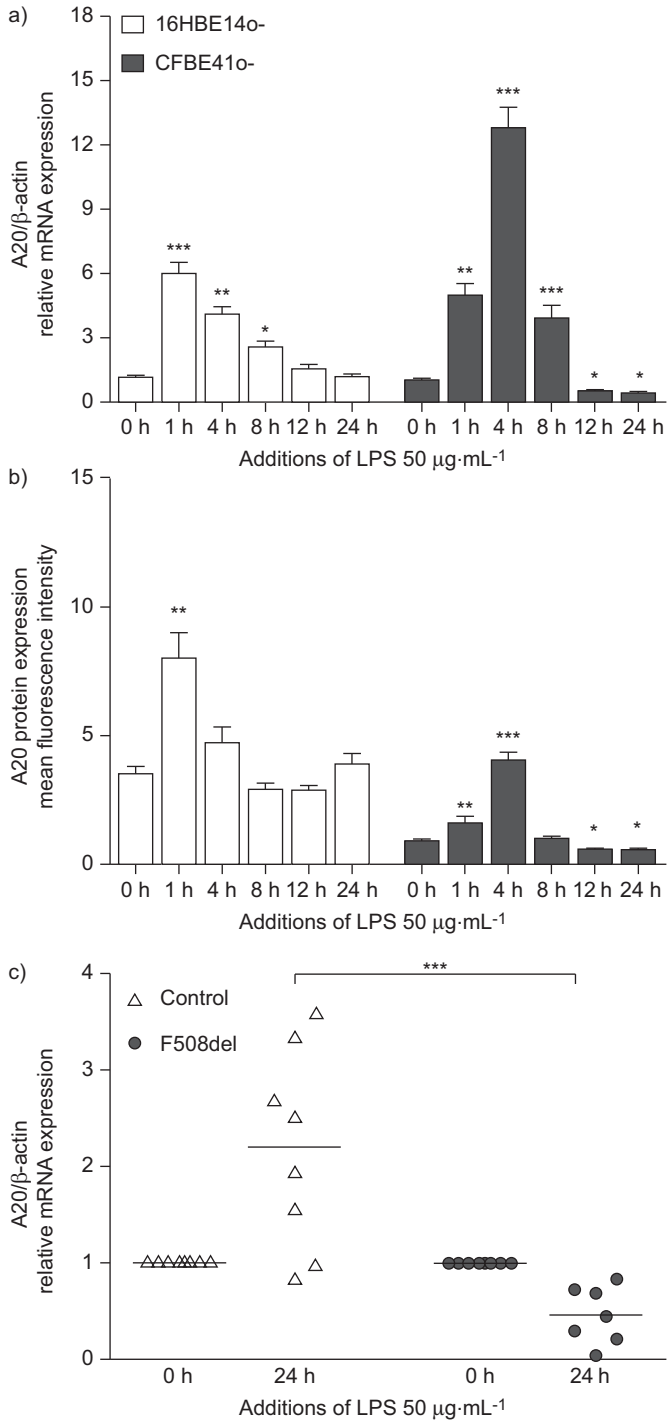

FIGURE 1. A20 expression in airway epithelial cells. A20 a) mRNA and b) protein expression was measured in 16HBE14o- and CFBE41o- cells by quantitative PCR and flow cytometry. Cells were stimulated with 50 μg·mL^{-1} lipopolysaccharide (LPS). Data are presented as mean±SEM. n=6 for quantitative PCR experiments; n=5 for flow cytometry experiments. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ compared with corresponding untreated control (*i.e.* 0 h) for each individual cell line. c) mRNA expression of A20 was also assessed by quantitative PCR in primary nasal epithelial cells from patients homozygous for F508del and from age- and sex-matched controls. Expression was calculated after 24 h of simulation with LPS and data are presented as mean±SEM; n=8. ***: $p<0.001$ compared with age- and sex-matched controls.

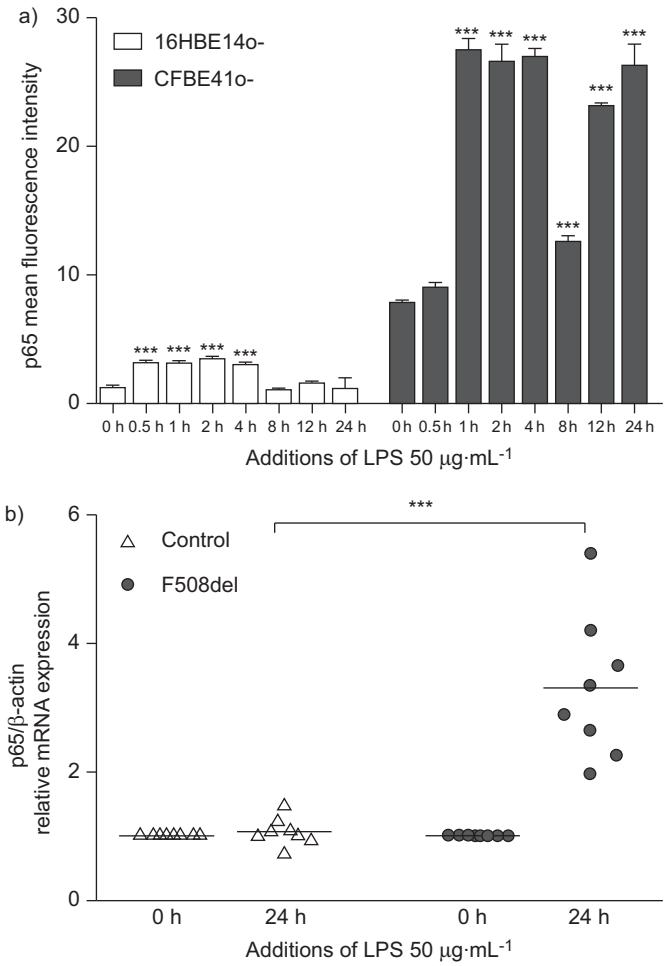

FIGURE 2. Nuclear factor (NF)-κB (p65) expression in airway epithelial cells. 16HBE14o- and CFBE41o- cells were exposed to lipopolysaccharide (LPS) for 0, 12 and 24 h and a) the percentage of cells positive for the nuclear expression of the p65 subunit of NF-κB was determined using an adapted published flow cytometric method [25]. Data are presented as mean±SEM; n=5. ***: $p<0.001$ compared with corresponding untreated (*i.e.* 0 h) control for each cell line. b) mRNA expression of p65 was assessed in primary nasal epithelial cells from patients homozygous for F508del and from age- and sex-matched controls by quantitative PCR. Expression was calculated after 24 h simulation with LPS and data are presented as mean±SEM; n=8. ***: $p<0.001$ compared with age- and sex-matched controls.

antibodies (see online supplementary material), washed, incubated with horseradish peroxidase-conjugated secondary antibodies (Thermo Fisher Scientific, Hemel Hempstead, UK) and visualised on a Bio-Rad Chemi Doc XRS system (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK).

Statistical analysis

All data are presented as mean±SEM. Differences between groups were analysed using the Kruskal–Wallis nonparametric ANOVA with Dunn's post-test and were considered to be significant if $p<0.05$.

RESULTS

Peak induction of A20 is delayed in CFBE41o- cells

Peak induction of A20 was observed 1 h after LPS stimulation in 16HBE41o- cells. Thereafter, A20 expression returned to

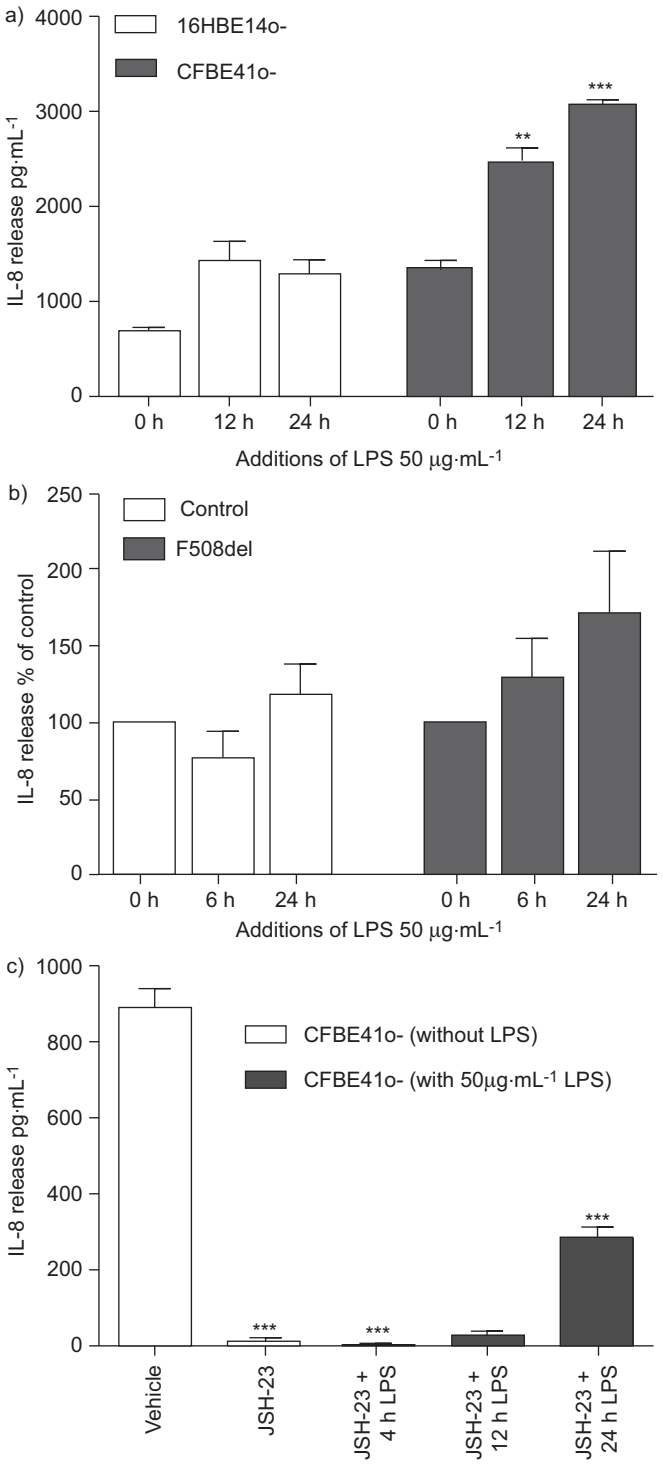

FIGURE 3. Regulation of interleukin (IL)-8 processing. Persistent expression of p65 was also accompanied by a time-dependent increase in IL-8 release in a) CFBE14o- cells (n=5) and b) cystic fibrosis nasal epithelial cells (n=4), which was determined by ELISA. c) to determine if p65 selectively regulates the processing of IL-8, CFBE41o- cells were pretreated with a commercially available and selective p65 inhibitor (JSH-23) for 1 h prior to challenge with LPS. IL-8 release into cell supernatants was subsequently measured by ELISA (n=3). Data are presented as mean ± SEM with **: $p<0.01$; ***: $p<0.001$ compared with corresponding untreated (*i.e.* 0 h) or vehicle control for each cell line.

basal levels in these cells. However, in CFBE41o- cells A20 expression was significantly induced 1 h after LPS stimulation, with peak expression observed 4 h post-stimulation ($p<0.001$). Although A20 protein expression returned to basal levels 8 h post-stimulation, expression fell significantly ($p<0.05$) below basal levels 12 and 24 h after treatment with LPS. These findings were consistent for A20 mRNA (fig. 1a) and protein levels (1b). A20 mRNA expression was also shown to fall below basal levels in primary NECs from CF patients homozygous for F508del (fig. 1c).

Reduced A20 expression is associated with heightened inflammatory signalling in CF cells

We examined the nuclear expression of the p50 and p65 subunits of NF-κB in 16HBE14o- and CFBE41o- cells. Although basal p50 expression was higher in CFBE41o- than 16HBE14o- cells, the overall pattern of p50 induction in response to LPS stimulation did not differ between the two cell lines (online supplementary fig. S2A). Nuclear p65 expression was rapidly induced in both 16HBE14o- and CFBE41o- cells following 1 h LPS stimulation. However, p65 expression returned to basal levels in 16HBE14o- cells by 8 h post-stimulation but remained persistently upregulated ($p<0.001$) in CFBE41o- cells at all time-points examined (fig. 2a). These findings were confirmed in primary NECs from CF patients homozygous for F508del at the mRNA level (fig. 2b). Additionally, a significant ($r= -0.709$, $n=13$, $p=0.007$) inverse relationship between A20 and p65 levels was observed (online supplementary fig. S3).

p65 regulates IL-8 induction in CFBE41o- cells

Following LPS stimulation, a time-dependent increase in IL-8 release was observed in CFBE41o- cells only (fig. 3a). Similar trends were observed in CF NECs, but these did not reach significance (fig. 3b), a commonly observed phenomenon with ALI cultures. Previous work has shown that p65 selectively regulates IL-8 processing in gingival epithelial cells [26], but the relationship in CF has not been investigated. We sought to determine if persistent nuclear expression of p65 in CFBE41o- cells was responsible for IL-8 production. CFBE41o- cells pretreated with a selective p65 inhibitor (JSH-23) showed significant reductions ($p<0.001$) in unstimulated and LPS-induced IL-8 release (fig. 3c). The inhibitory action of JSH-23 was found to be specific for p65 as p50 expression was not affected (online supplementary fig. S2B).

Reduced A20 expression in CFBE41o- does not result from proteasomal degradation

We hypothesised that A20 may undergo increased proteasomal degradation in CF and that this would explain why A20 expression fell below basal levels in CF cells following LPS stimulation. To test this, CFBE41o- cells were treated with commercially available inhibitors of E1 ubiquitin-activating enzymes (PYR-41) and proteasomal degradation (MG-132) prior to stimulation with LPS, and the expression of A20 investigated by quantitative PCR and Western blot (fig. 3). However, pretreatment with either inhibitor had no effect on A20 mRNA expression, which remained significantly ($p<0.05$) below basal unstimulated levels 24 h after LPS treatment (fig. 4a). Similar findings were observed at the protein level (fig. 4b).

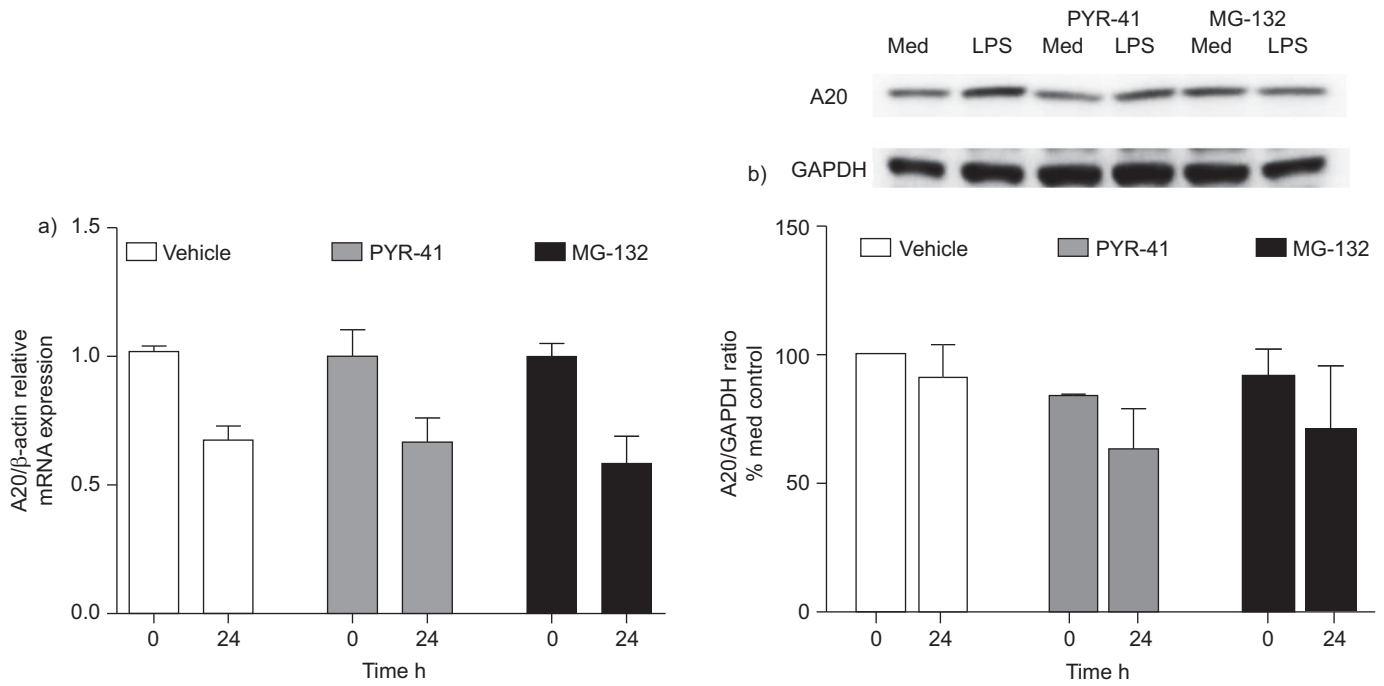

FIGURE 4. Effects of PYR-41 (ubiquitin inhibitor) and MG-132 (26S proteasome inhibitor) on A20 expression. 16HBE14o- and CFBE41o- cells were pretreated with commercially available inhibitors of E1 ubiquitin activating enzymes (PYR-41) and the 26S proteasome (MG-132) for 4 h, prior to challenge with lipopolysaccharide (LPS) for a further 24 h. a) A20 mRNA expression was then examined by quantitative PCR (n=3). Data are presented as mean±SEM. *: $p<0.05$ compared with corresponding untreated control (*i.e.* 0 h) for each inhibitor or vehicle control. b) A20 protein expression was also determined by Western blot (n=3). A representative image of three independent experiments is shown. Med: unstimulated medium control.

Altered expression of RNF11, Itch and TAX1BP1 in CFBE41o- cells

A20 must form a complex with RNF11, Itch and TAX1BP1 in order to prevent NF-κB activation [13]. We therefore examined the expression of these complex members using quantitative PCR. Expression levels following 24 h LPS treatment are shown in figure 5, and changes in expression over a full time-course of LPS treatments are presented in online supplementary figure S4. Data are presented relative to the untreated control (basal level normalised to 1) for each cell line or individual patient sample. In 16HBE14o- cells, the expression of RNF11 (fig. 5a), Itch (fig. 5b) and TAX1BP1 (fig. 5c) was significantly induced ($p<0.01$-0.001) following 24 h of LPS stimulation. Peak RNF11 and TAX1BP1 expression was observed 24 h after LPS stimulation, while peak Itch expression was obtained 1 h after treatment and remained significantly above basal levels until 4 h post-stimulation, before falling back towards basal levels (see online supplementary fig. S4). However, expression of RNF11 and Itch remained unchanged in CFBE41o- cells, whilst TAX1BP1 expression fell significantly ($p<0.05$) below basal levels after stimulation. These findings were confirmed (and found to be more pronounced) in primary NECs, where RNF11 (fig. 5d), Itch (fig. 5e) and TAX1BP1 (fig. 5f) were induced in control NECs and fell significantly ($p<0.001$) below basal levels in CF NECs.

A20 does not interact with RNF11 or TRAF6 in CFBE41o- cells

To assess the formation of the A20 ubiquitin editing complex, 16HBE14o- and CFBE41o- cells were immunoprecipitated with an antibody against full length A20. A20 strongly associated with RNF11 1 h after LPS stimulation in 16HBE14o- cells (fig. 6a), which coincided with peak A20 expression in this cell line (fig. 1). This interaction appeared to be transient and was observed again 6 and 8 h after LPS treatment. Furthermore, A20 was found to interact with TRAF6 in 16HBE14o- cells at all time-points examined, and was most pronounced 1 h after LPS stimulation. Our initial experiments did not reveal an interaction between A20 and Itch or TAX1BP1 in either 16HBE14o- or CFBE14o- cells 1–24 h after LPS treatment. Since these interactions are known to occur rapidly and transiently [14], we performed an additional LPS treatment after 15 min (fig. 6b). A20 interaction with TAX1BP1 was observed after 15 min in 16HBE14o- cells, but interaction with Itch was still not evident. None of the described interactions were found in CFBE41o- cells at any time-point examined (fig. 6).

DISCUSSION

The zinc finger protein A20 has been studied as a tumour suppressor gene and has been implicated in the progression of autoimmune and inflammatory diseases such as Crohn's disease and arthritis [16]. However, this study is the first to investigate A20 expression in chronic inflammatory airways disease. CF lung disease is characterised by chronic bacterial infection (in particular with Gram-negative *P. aeruginosa*) and exaggerated NF-κB-driven inflammation, leading to tissue damage and loss of lung function. A20-deficient mice display severe multi-organ inflammation, increased numbers of activated inflammatory cells and rarely survive beyond 1 week of age, suggesting a role for A20 in preventing spontaneous

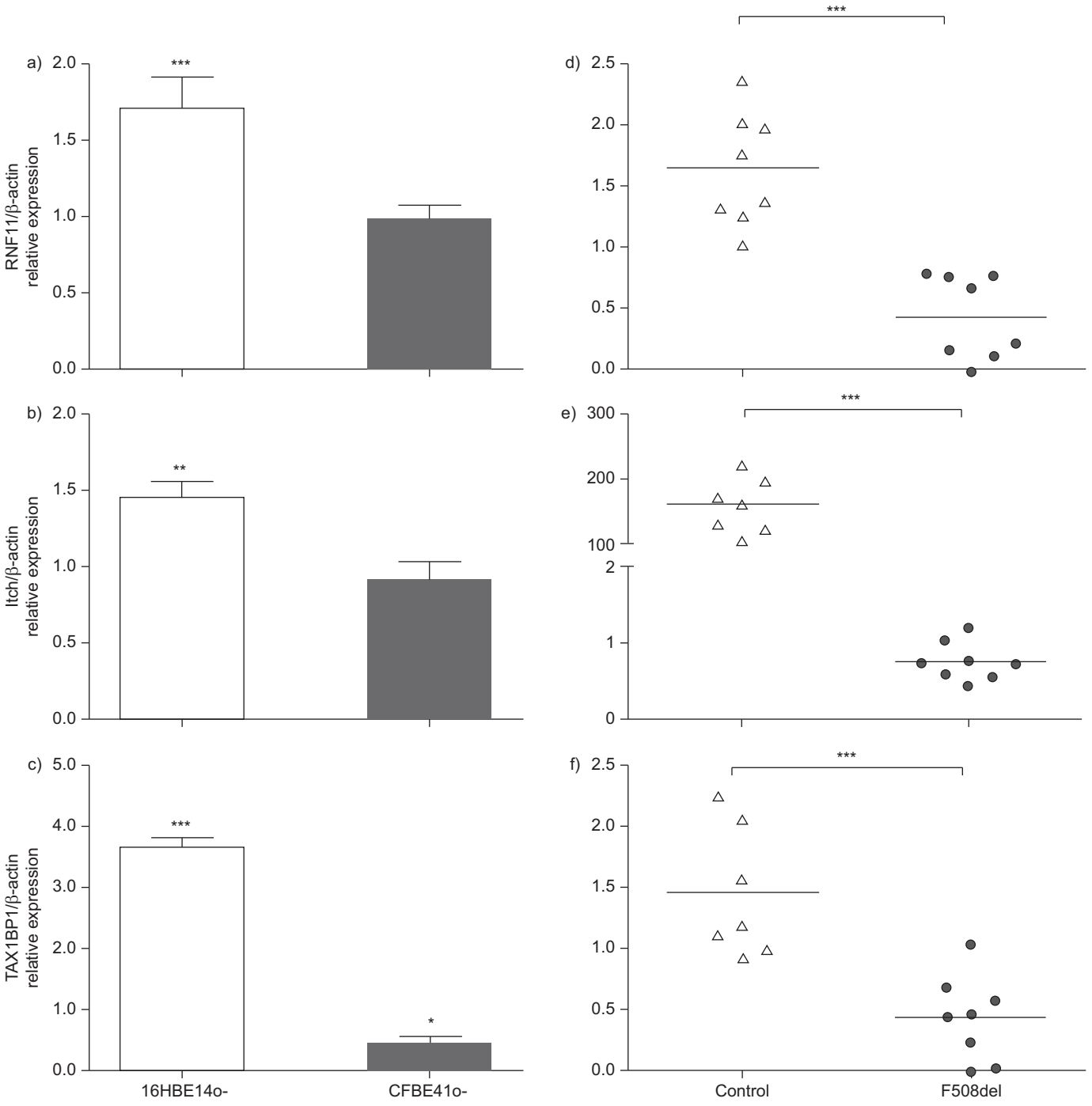

FIGURE 5. Expression of members of the A20 ubiquitin editing complex. The mRNA expression of RNF11 (a and d), Itch (b and e) and TAX1BP1 (c and f) was examined in cell lines (a, b and c) and primary nasal epithelial cells (NECs) (d, e and f) from patients homozygous for F508del and from age- and sex-matched controls by quantitative PCR. Expression was calculated after 24 h of stimulation with lipopolysaccharide. Data are presented as mean±SEM; n=5 for cell lines and n=8 for NECs. a–c) *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ compared with the corresponding untreated control for each individual cell line. d–f) ***: $p<0.001$ compared with age- and sex-matched controls.

innate immune cell-mediated inflammation and tissue destruction [6]. Additionally, these mice are particularly sensitive to exogenous administration of TNF-α and LPS, suggesting that A20 plays a critical role in protection against chronic inflammation [6]. Using epithelial cells derived from patients with CF (F508del homozygous) and non-CF control subjects, our current findings suggest that A20 function is altered in CF and may contribute to uncontrolled inflammation. These findings are consistent with recent reports in rheumatoid arthritis [25] and Sjögren's syndrome [11].

The original work by Opipari *et al*. [1] using a squamous carcinoma cell line (A431), found peak A20 expression 1 h after stimulation with LPS or TNF-α. Consistent with this, we found

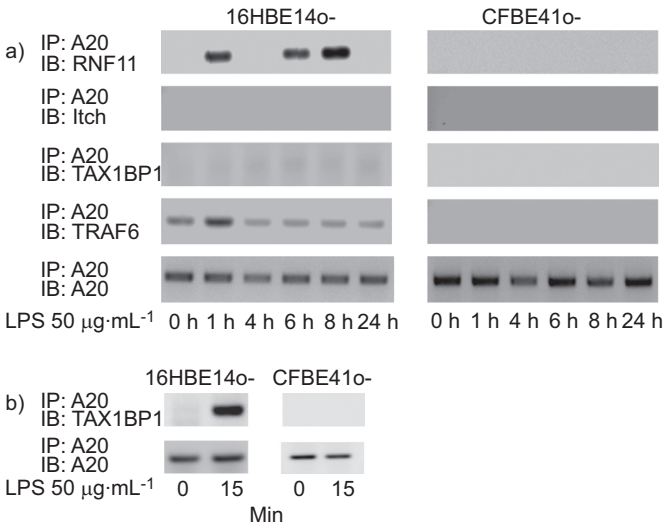

FIGURE 6. Formation of the A20 ubiquitin editing complex. The nuclear factor (NF)-κB inhibitory effect of A20 is dependent on the formation of the A20 ''ubiquitin editing complex''. a) 16HBE14o- and CFBE41o- cells were stimulated with lipopolysaccharide (LPS) for 0–24 h and the complex formed examined by immunoprecipitation (IP). b) Since interactions between A20 and TAX1BP1 are known to occur rapidly and transiently [14] the same experiments shown in (a) were performed after 0 and 15 min LPS. Images representative of two independent experiments are shown. IB: immunoblot; RNF: ring finger protein; TAX1BP1: TAX1 binding protein-1; TRAF: tumour necrosis factor receptor-associated factor.

that peak A20 expression occurred in non-CF cells 1 h after LPS stimulation. However, in CF cells, peak A20 expression was delayed until 4 h post-stimulation and, in contrast to non-CF cells, subsequently fell below basal levels (12–24 h post-stimulation). Furthermore, we found a significant inverse correlation between A20 and p65 mRNA expression in primary CF NECs. Given the array of mediators we sought to examine and the limited amount of material available from our primary ALI cultures, we were not able to confirm increased p65 expression or the correlation with A20 expression at the protein level in primary CF NECs. However, increased nuclear protein expression of p65 was found to account for a time-dependent increase in IL-8 release in CFBE41o- cells. Although peak A20 expression was delayed in CF cells, we still observed significant induction of the protein 1, 4 and 8 h after LPS stimulation. However, this had no obvious negative effect on nuclear NF-κB expression or IL-8 release. These findings posed questions regarding the fate and action of A20 in CF airway epithelial cells.

To address the issue of why A20 expression fell below basal levels in CF cells only, we first examined whether A20 was ubiquitinated or targeted for proteasomal degradation using commercially available inhibitors. Ubiquitination is catalysed by the sequential action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin protein ligases (E3). PYR-41 is a cell-permeable compound that specifically inhibits the activity of the universal E1 ubiquitin-activating enzyme to prevent further activation of the ubiquitination sequence, thereby blocking ubiquitin-dependent cellular activities, including protein degradation. MG-132 is a potent, reversible and cell-permeable inhibitor of the 26S proteasome. CF cells were pretreated with each inhibitor for 4 h prior to stimulation with LPS. However, A20 expression was not restored following treatment with either inhibitor and remained below basal levels in CF cells, indicating that the reduction in A20 expression after extended LPS stimulation of CF cells does not result from proteasomal degradation. A20 mutation or methylation has been cited as a means of inactivation [27] and our findings that A20 does not undergo increased degradation in CF may point towards an inherent defect in the gene. Alternatively, A20 may be proteolytically cleaved, which may render it inactive. For instance, mucosa-associated lymphoid tissue lymphoma translocation protein (MALT)-1 has recently been described as having protease activity and to cleave A20 [28]. While these alternative mechanisms of A20 inactivation are currently speculative, they will be the subject of future study.

We next examined expression of members of the ''ubiquitin editing complex''. Shembade *et al*. [13] have previously reported that the NF-κB inhibitory action of A20 was dependent upon the formation of a complex with RNF11, Itch and TAX1BP1. Silencing of any member of this complex meant that A20 could not bind to, or act on, its target proteins and could not prevent activation of NF-κB [13]. In addition to facilitating the action of A20, RNF11 was also shown to independently inhibit NF-κB at the inhibitor of κB kinase (IKK) level [29]. Moreover, RNF11 prevents the ubiquitination of TRAF6, which may prove crucial in the inhibition of TLR-induced NF-κB signalling [29]. TAX1BP1, alternatively named TRAF6 binding protein, is an adapter protein critically important in A20 deubiquitination of TRAF6 in response to bacterial LPS [30] while also facilitating downstream inhibition of NF-κB signalling in response to viral stimuli [31]. Here we report differential expression of all complex members between CF and non-CF cells. These differences were more pronounced in primary NECs than in cell lines. Overall, the expression of RNF11, Itch and TAX1BP1 remained unchanged or was upregulated following LPS stimulation in control NECs, but was significantly downregulated in CF NECs. Given the differences in expression patterns, we wondered whether the A20 ubiquitin editing complex was being properly formed in CF. Although we observed interactions between A20 and TRAF6, RNF11 and TAX1BP1 in non-CF cells, A20 was not found to interact with Itch in any cell type, or at any time-point examined. Moreover, despite significant induction of A20 in CF cells 4 h after LPS stimulation, interaction with TRAF6, RNF11 or TAX1BP1 was not observed at this or any other time-point examined.

The interaction of A20 with TRAF6, RNF11 and TAX1BP1 in non-CF control cells and the absence of these interactions in CF cells may have pathological significance. TLR activation of the NF-κB pathway begins with receptor–ligand association at the cell membrane. This recruits adaptor proteins (myeloid differentiation primary response (MyD)-88 and interleukin-1 receptor-associated kinase (IRAK)) and TRAF6 is ubiquitinated. Active A20 halts downstream signalling by deubiquitinating TRAF6 [14]. Prior work has shown that transient A20 interaction with RNF11, Itch and TAX1BP1 governs this process by promoting TRAF6 deubiquitination and disrupting

TRAF6 association with the ubiquitin ligase Ubc13, thereby terminating IKK and NF-κB activation [14]. The A20–TRAF6 axis has been deemed essential in preventing LPS-stimulated NF-κB activation. Silencing of A20 was shown to restore TRAF6 action and NF-κB activation following LPS stimulation [32]. The fact that A20 does not interact with any member of the ubiquitin editing complex and, we would hypothesise, is consequently unable to interact with TRAF6 in CF cells, could partly explain why induction of A20 in these cells appears to have little impact on the activation of NF-κB. Prior work has shown that the anti-inflammatory activity of A20 may be attributed to the C-terminal domain which contains seven ZnF proteins and acts as an E3 ubiquitin ligase. ZnF4 appears to be particularly important since A20 ZnF4 mutants are unable to inhibit TRAF6 ubiquitination [14]. Future studies will investigate the enzymatic activity of the C-terminal domain in CF epithelial cells.

Interest in the action, signalling and, potentially, the therapeutic targeting of A20, has increased significantly in recent years providing novel findings and excellent summaries of A20 function (see HARHAJ and DIXIT [33]). While direct and permanent inhibition of NF-κB may render the immune system unable to respond to pathogens, transient induction of negative regulators such as A20 will help target inflammatory signal transduction pathways. It has been suggested that this approach may provide a more specific and tailored means of treating inflammatory diseases without global suppression of the immune system [34]. A better understanding of these processes, as well as the regulation of A20 itself, will greatly improve the likelihood of A20-driven therapeutics for inflammatory lung diseases. The current study is the first to report deregulation of A20 expression in CF and highlights the potential relevance of this ZnF protein in chronic pulmonary inflammation.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

Conflict of interest information can be found alongside the online version of this article at www.erj.ersjournals.com

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