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Glucocorticoids Enhance or Spare Innate Immunity: Effects in Airway Epithelium Are Mediated by CCAAT/Enhancer Binding Proteins[1]

Ning Zhang[2], **Quynh Ai Truong-Tran**[2], **Brian Tancowny**, **Kathleen E. Harris**, and **Robert P. Schleimer**[3]
Northwestern University Feinberg School of Medicine, Chicago, IL 60611

Abstract

Although it is widely accepted that glucocorticoids (GC) are a mainstay of the treatment of diseases characterized by airway inflammation, little is known about the effects of GC on local innate immunity. In this article, we report that respiratory epithelial cells manifested a local "acute phase response" after stimulation with TLR activation and TNF-α and that GC spared or enhanced the epithelial expression of molecules that are involved in host defense, including complement, collectins, and other antimicrobial proteins. As expected, GC inhibited the expression of molecules responsible for inflammation such as cytokines (IFNβ and GM-CSF) and chemokines (RANTES and IL-8). Studies using Western blotting, EMSA, and functional analysis indicated that the selective effects of GC are mediated through activation of the transcription factor C/EBP. Knockdown of C/EBPβ by small interfering RNA blocked the enhancement by GC of host defense molecule expression but had no effect on inflammatory gene expression. These results suggest that GC spare or enhance local innate host defense responses in addition to exerting anti-inflammatory actions. It is possible that the known ability of GC to reduce the exacerbation of diseases in which infectious organisms serve as triggering factors (e.g., asthma, allergic bronchopulmonary aspergillosis, and chronic obstructive pulmonary disease) may result in part from enhanced innate immune responses in airway mucosa.

In response to infections and injury, a spectrum of molecules constituting the acute phase response (APR)[4] is mobilized with the purpose of combating the infection and preventing tissue damage (1). Acute phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) are released by the liver during both adaptive and innate immune responses and have proven to be useful as markers of systemic disease activity or severity (2,3). The airway epithelium is exposed to a large number of potentially pathogenic airborne particles such as microorganisms, allergens, and pollutants and has been shown to produce APR proteins such as C2, C3, C4, C5, factor B (4,5), CRP, and SAA (6,7). Based on these studies and the fact that hepatocytes and the airway epithelium come from the same germinal layer, it appears that

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3 Address correspondence and reprint requests to Dr. Robert P. Schleimer, Allergy-Immunology Division, Northwestern University Feinberg School of Medicine, Mc-Gaw Pavilion M-318, 240 East Huron Street, Chicago, IL 60611. E-mail address: rpschleimer@northwestern.edu.
[2]N.Z. and Q.A.T.-T. contributed equally to this work.

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[4]Abbreviations used in this paper: APR, acute phase response; CRP, C-reactive protein; Ct, cycle threshold; FP, fluticasone propionate; GC, glucocorticoid; HBD, human β-defensin; LAP, liver activating protein; LIP, liver inhibiting protein; MASP, mannose-binding lectin-associated serine protease; MBL, mannose-binding lectin; PBEC, primary bronchial epithelial cell; poly(I:C), polyinosinic acid:cytidylic acid; SAA, serum amyloid A; siRNA, small interfering RNA; SLPI, secretory leukocyte protease inhibitor; Sp, surfactant protein.

airway epithelium can manifest a local version of the acute phase response following stimulation.

Glucocorticoids (GC) are a mainstay of the treatment of diseases characterized by airway inflammation including asthma, rhinitis, and chronic rhinosinusitis. Although it is widely accepted that their success directly reflects their anti-inflammatory effects, such as inhibition of the expression of the cytokines and chemokines that activate endothelial cells, leukocytes, and lymphocytes, the precise mechanisms by which steroids improve lung function remain unclear (8). During preliminary microarray studies, we found that epithelial expression of some of the acute phase proteins involved in innate immunity, including C3 and SAA, was not inhibited by GC and in some cases was actually increased (9). Because GC can enhance the hepatic acute phase response, we reasoned that they may also enhance the manifestations of an APR in airway epithelial cells. In support of this possibility, previous reports indicate that GC enhanced the expression of mRNA for C3 and secretory leukocyte protease inhibitor (SLPI) in lung epithelial cells (10,11). Evidence thus exists to suggest that GC spare or even enhance several innate immune responses of airway epithelial cells while they inhibit inflammatory responses (12).

The APR in the liver is known to be mediated to a great extent by activation of the transcription factor C/EBP, which belongs to the basic region-leucine zipper transcription factor family (13). Six members (C/EBPα-C/EBPζ) have been characterized in mammals; these proteins have been shown to play pivotal roles in numerous cellular responses, including the APR (14,15). C/EBPβ and C/EBPδ are rapidly and dramatically induced by LPS or cytokines in the liver and in turn activate the expression of various genes involved in the APR (13,16,17). Most of the proteins produced in the liver during the APR contain C/EBP binding sites in their promoters and their induction is regulated by C/EBPβ and C/EBPδ (16,18). C/EBP proteins have been detected in the lung (19), and recently the role of C/EBP in lung physiology and pathophysiology has become a topic of great interest (20,21). C/EBPα, C/EBPβ, and C/EBPδ were found to be expressed in lung epithelial cells and involved in the regulation of expression of surfactant protein (Sp) A, SpD, the Clara cell secretory protein, and the P450 enzyme CYP2B1 during development (22).

GC are known to enhance the hepatic APR by a mechanism that involves C/EBP proteins (23). We report that: 1) epithelial cells exhibit a response reminiscent of the hepatic APR; 2) this response is potentiated by GC; and 3) C/EBPβ is an important mediator of the epithelial APR to inflammatory stimuli and GC.

Materials and Methods

Cell culture

Human primary bronchial epithelial cells (PBEC)—PBEC were harvested from human lungs that had been rejected for transplantation purposes (obtained from the National Disease Research Interchange, Philadelphia, PA) using a modification of a previously described protocol (24) approved by the Northwestern University Institutional Review Board. Cells were seeded in LHC-9 medium (BioSource International) and changed to LHC-8 medium to eliminate any effects of hydrocortisone 48 h before GC treatment. Cells were used only during their primary passage.

BEAS-2B cells—An adenovirus 12-SV40 hybrid virus-transformed human bronchial epithelial cell line, BEAS-2B (a gift from Dr. C. Harris, National Cancer Institute, Bethesda, MD), was cultured in DMEM/F12 (Invitrogen Life Technologies) supplemented with 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were used between

passages 38 and 45 and cultured for 48 h in DMEM/F12 without FBS before use in experiments with GC.

A549 cells—A549 cells, a tumor cell line with properties of type II alveolar epithelial cells, were cultured in DMEM as described above.

dsRNA stimulation and GC treatment of cultured cells

Cells were used for experiments when they reached 80% confluence. Cells were treated with synthetic dsRNA (polyinosinic acid:cytidylic acid (poly(I:C)); Amersham Biosciences) at 25 μg/ml or TNF-α (R&D Systems) at 100 ng/ml for 18 h (except in time course studies). Cells were treated with fluticasone propionate (FP) (Sigma-Aldrich) at 100 nmol/L (except in dose-response studies) for 2 h before stimulation with dsRNA. FP was stored as a 0.1 M stock in DMSO. Control cells were treated with an equivalent amount of DMSO diluent.

RNA isolation, generation of cDNA, and real-time PCR

Total RNA was isolated using the RNeasy kit with DNase treatment (Qiagen,). In brief, 500 ng of total RNA was reverse transcribed to cDNA with random hexamers using the TaqMan reverse transcription reagents (Applied Biosystems) and 4 μl of a 1/10 dilution of the resulting cDNA was used for each real-time PCR on an Applied Biosystems Prism 7500 sequence detection system. GAPDH was used as a housekeeping gene for normalization and a “no template” sample was used as a negative control. Validation experiments testing the amplification efficiencies of target genes and a reference gene were performed to ascertain that the efficiency was close to unity to enable the quantification of gene expression relative to a control sample using the cycle threshold ($\Delta\Delta$Ct) method. Primers and probes targeting the genes of interest were either designed using Applied Biosystems Primer Express software (Table I) or obtained as an Assays-on-Demand system from Applied Biosystems.

Cytoplasmic and nuclear protein extraction and Western blotting

Cytoplasmic and nuclear extracts were prepared using the NER-PER kit (Pierce) with protease and phosphatase inhibitors. Twenty-five micrograms of nuclear protein or 75 μg of cytoplasmic protein were subjected to electrophoresis with a 10% SDS-polyacrylamide gel (Bio-Rad) and then transferred to a polyvinylidene difluoride membrane. The membranes were incubated with mouse anti-human β-tubulin and rabbit anti-human C/EBPβ, NF-κB p65, or a GC receptor (Santa Cruz Biotechnology) at a 1/1000 dilution for 1 h at room temperature after the addition of blocking buffer (LI-COR). Membranes were then washed and probed with goat anti-mouse or anti-rabbit secondary Abs conjugated to Alexa Fluor 680 (Molecular Probes) or IRDye 800 (Rockland Immunochemicals) at a 1/5000 dilution for another hour. Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR).

Luciferase reporter assay

BEAS-2B cells were grown to 60–70% confluence in 6-well plates in DMEM/F12 without any antibiotics before transient transfection with 1 μg/well C/EBPβ luciferase reporter plasmid or empty control plasmid (Panomics) performed in duplicate wells for each treatment condition using FuGENE 6 (Roche). The sequence of the *cis*-acting DNA binding element that is recognized by C/EBP is ATTGCGCAATATTGCGCAATATTGCGCAAT. Luciferase activity was measured using the Promega luciferase assay system. The C/EBPβ luciferase activity was normalized to the control luciferase activity and the protein concentration of the lysates. The normalized relative luciferase activity was expressed as the fold induction of luciferase activity above the control condition, which was given a value of 1.

EMSA

EMSA was performed with IRDye-labeled double-stranded oligonucleotides containing a C/EBPβ consensus site (5′-TGCAGATTGCGCAATCTGCA-3′) using the Odyssey infrared imaging system (LI-COR). The DNA binding reactions were set up by incubating 10 μg of the nuclear protein and 50 fmol of oligonucleotides for 30 min at room temperature in 10 μl of binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM DTT, and 1% Tween 20). Orange G loading dye (1 μl at 10×) was added, the gels were scanned, and the signals were quantified using the Odyssey infrared imaging system (LI-COR).

RNA interference

After BEAS-2B cells reached 60% confluence in 12-well plates, the medium was replaced with small interfering RNA (siRNA) transfection medium (Santa Cruz Biotechnology) at half volume. C/EBPβ siRNA (3.6 μl at 10 μM) and siRNA transfection reagent (2.4 μl) were mixed in 80 μl of transfection medium and incubated at room temperature for 20 min according to the manufacturer's instructions. The cells in each well were then transfected with this mixture. After 6 h, the medium volume was restored with normal medium and after a further 18-h incubation the cells were replaced with fresh medium before stimulation. The sequences of the C/EBPβ siRNA duplex from Santa Cruz Biotechnology (Si1) were 5′-GAGACAGGCUUCUACUACGAGGCGGACUUTT-3′ (sense) and 5′-AAGUCCGCCUCGUAGUAGAAGCCUGUCUCTT-3′ (antisense). Some of the cells were transfected with C/EBP siRNA from Qiagen targeted to nonoverlapping sequences with those above (Hs_C/EBPB_5 HP Validated siRNA (Qiagen); SI02777292) (Si2) or negative control RNA at 5 nM using HiPerFect transfection reagent (Qiagen) following the manufacturer's instruction.

ELISA and cytometric bead array

The concentration of C3 in culture supernatants was assayed by a previously described ELISA (25) with 1.67 μg/ml detection Ab. ELISA kits from R&D Systems and Tri-Delta Diagnostics were used to quantitate SLPI and SAA. The detection limit of ELISA for C3, SLPI, and SAA is 1 ng/ml, 25 pg/ml, and 0.3 ng/ml, respectively.

Levels of IL-8 and RANTES in supernatants were detected using the human chemokine cytometric bead array kit (BD Pharmingen) in a BD FACSArray instrument.

Statistical Analysis

Data are expressed as the mean value ± SEM from the number of replicate experiments indicated in the text. Statistical significance of differences was determined with a Student's *t* test. Differences were considered statistically significant at $p < 0.05$.

Results

DsRNA induced a local APR in respiratory epithelial cells

We first investigated whether the respiratory epithelium could manifest a response resembling an APR and a host defense response upon stimulation with the TLR3 ligand dsRNA, which we found was the most effective epithelial activator among TLR ligands in previous work (7). Human PBEC, the transformed bronchial epithelial cell line BEAS-2B, and the alveolar type II epithelial cell line A549 were treated with the TLR3 ligand dsRNA (25 μg/ml) or saline control for 18 h. RNA was extracted and real-time PCR was performed to compare the mRNA levels of host defense molecules including antimicrobial proteins, collectins, complement proteins, and coagulation molecules together with inflammatory molecules (Table II). Values in the table represent mean raw Ct from two to seven independent experiments; the value on

the left for each gene is the basal level, the value on the right represents the stimulated level, and the value in parentheses is the approximate mean fold stimulation. Higher Ct values represent lower levels of expression and a value of >37 indicates the gene was undetected. Genes induced by dsRNA at least 6-fold are indicated by bold text.

PBEC were found to express a broad spectrum of host defense molecules including human β defensins (HBD)1, 2, and 4, lysozyme, lactoferrin, SLPI, SpD, SAA, C3, factor B, factor H, plasminogen activator inhibitor, and MIP-3$_\alpha$. A549 cells expressed most of these same genes with the exception of lactoferrin. Unlike the PBEC and BEAS-2B, the A549 cells also expressed the LPS binding protein (LBP) and fibrinogen. BEAS-2B cells expressed fewer antimicrobial genes than PBEC. The constitutive expression of some of these genes, such as HBD1, HBD2, HBD4, and SpD, may be lost as a result of transformation of these cells by the adenovirus 12-SV40 hybrid virus. Lysozyme and SLPI were expressed at higher levels in PBEC than in BEAS-2B cells. Bactericidal/permeability-increasing protein, lectin-associated serine protease (MASP) 1, MASP2, and SpA were not expressed by any of the three types of cells. Stimulation with dsRNA increased the expression of the genes studied in the PBEC and to a lesser extent in the immortalized cell lines (see bold values in Table II; ≥ 6-fold or 2.5 Ct change). The stimulus had no effect on levels of the housekeeping gene *GAPDH*.

These results, together with our former study on the presence and function of TLRs in airway epithelial cells (7), indicate that a broad spectrum of inflammatory and host defense acute phase genes is expressed by respiratory epithelial cells and can be induced by TLR3 activation.

Selective effects of GC on host defense and inflammatory genes

As demonstrated in Table II, dsRNA induced many host defense and inflammatory genes in epithelial cells. We next determined the effects of the potent GC FP on these dsRNA-stimulated responses. FP (100nmol/L) was added 2 h before 18 h of stimulation with dsRNA (25 μg/ml). Data in Fig. 1 show a selective effect of FP on gene expression by TLR3 stimulation in PBEC (Fig. 1A) and BEAS-2B cells (Fig. 1B). FP efficiently inhibited the expression of inflammatory genes (IFNβ, GM-CSF, RANTES, and IL-8) but enhanced the expression of C3, SAA, CRP, and MIP-3$_\alpha$ and failed to inhibit the expression of HBD1 and factor B. Most of the genes enhanced or spared by FP are host defense molecules. We use the term “host defense genes” to refer to those proteins that can directly interact with and/or kill microorganisms, recognizing that some of these genes can also provoke the signs of inflammation. Most of the genes inhibited by FP were inflammatory genes and not host defense molecules (with two exceptions, HBD2 and HBD4). As shown in Fig. 1B, BEAS-2B cells showed a similar pattern. The only difference was that FP alone directly induced the expression of C3, SAA, CRP, and mannose-binding lectin (MBL) from 3- to 25-fold in BEAS-2B cells. The pattern of GC “sparing” innate immune response genes was also observed in TNF-α-stimulated cells (Fig. 1C), which indicates that the effects of steroids are not stimulus specific.

We next checked the concentration response and time course of FP on the expression of several of these target genes in resting BEAS-2B cells (Fig. 2). These studies confirmed the pattern of suppression of inflammatory genes and the enhancement or sparing of host defense genes. Both the IC_{50} of FP for the inhibition of expression of inflammatory genes and the EC_{50} for the induction of host defense molecules were ~0.5 nmol/L. Twenty-four hours of treatment with FP had the maximum effect. To further confirm these selective effects of GC, we performed ELISA and a cytometric bead array assay to detect protein levels of C3, SAA, SLPI, pentraxin 3, RANTES, and IL-8 (Fig. 3). The protein data further confirmed the results from studies of mRNA. FP reduced the expression of the chemokines RANTES and IL-8 that were synthesized and secreted by PBEC upon dsRNA stimulation while it spared or enhanced the release of C3, SAA, and pentraxin 3. Consistent with the mRNA data, levels of SLPI did not significantly change. These data suggest that GC have selective effects on epithelial gene expression, sparing

several of those involved in host defense and inhibiting those responsible for inflammation (Figs. 1–3).

GC increase C/EBPβ and C/EBPδ mRNA and protein levels

To investigate the role of C/EBP proteins in the regulation of gene expression by GC, we first determined whether mRNA of C/EBPα~ε were expressed in respiratory epithelial cells. We detected mRNA for all C/EBP with the exception of C/EBPε in primary cells and the BEAS-2B cell line (Fig. 4). At the base of each graph the basal Ct is shown. According to the basal Ct, C/EBPβ and C/EBPδ were expressed at a relatively high level. dsRNA induced C/EBPβ and C/EBPδ mRNA in both primary cells and the BEAS-2B cell line. FP alone significantly increased the mRNA for C/EBPβ and the C/EBPδ mRNA in BEAS-2B cells, and the combination of dsRNA and FP produced the highest levels of C/EBPβ and C/EBPδ in both cell types. In contrast to C/EBPβ and C/EBPδ, C/EBPα expression was inhibited by dsRNA in primary cells and FP had no effect. C/EBPγ mRNA levels did not change in any of the conditions examined, and the levels of C/EBPε were undetectable in all conditions (not shown).

We next performed Western blot analysis to determine the effects of dsRNA and FP on levels of C/EBPβ proteins (Fig. 5). Results were complicated as we detected five isoforms of C/EBPβ: bands of 40 kDa (possibly liver activating protein (LAP*)), 35 kDa (LAP), 25 kDa (liver inhibiting protein (LIP)), 50 kDa, and 46 kDa. Using the ratio of LAP/LIP as an indicator of activated C/EBPβ (26), we found that the activated C/EBPβ protein was induced by stimulation with dsRNA and GC, the same pattern that was observed in the analysis of C/EBPβ mRNA (Figs. 4 and 5B). Together, these results suggest that both the TLR3 stimulus dsRNA and the GC FP increase the amount of C/EBPβ and C/EBPδ in airway epithelial cells. Separate experiments indicated that the response was concentration and time dependent (maximal effects at 10^{-10} M FP, and at 4–12 h; data not shown).

Effects of GC on the function of C/EBPβ

To test whether GC can regulate the function of C/EBPβ in addition to increasing its expression, C/EBPβ binding activity in BEAS-2B cells was studied using an EMSA. GC treatment resulted in an increased binding to a C/EBPβ consensus binding site in nuclear extracts as shown in a representative gel (Fig. 6A) and in a densitometric evaluation of three experiments (Fig. 6B). The effect of GC on C/EBPβ transactivation was studied using transfection of a luciferase reporter gene into the BEAS-2B cells. A small but reproducible effect was observed. After treatment with 10^{-7} M FP for 18 h, reporter gene activity was increased 1.7-fold. FP and dsRNA had an additive or possibly synergistic effect; the reporter gene activity increased 2.3-fold in the presence of the combination of these two stimuli (Fig. 6C). One experiment with primary cells confirmed that FP spared the expression and function of C/EBP in primary cells. These results indicate that GC increase the DNA binding activity and the transactivational function of C/EBPβ in epithelial cells.

C/EBP siRNA blocked the enhancement by GC of host defense molecule expression but had no effect on inflammatory gene expression

To determine whether the selective effects of GC on host defense and inflammatory responses are mediated by the transcription factor C/EBP, we transiently transfected subconfluent BEAS-2B cells with C/EBPβ siRNA for 24 h and then repeated the experiments assessing the effects of dsRNA stimulation and FP treatment. After the blockade of C/EBPβ expression, the induced expression of mRNA for CRP, SAA, MBL, and factor H was significantly but not completely reduced. In contrast, the inhibition by the GC of the expression of the inflammatory genes *RANTES*, *GM-CSF*, and *IL-8* was not affected (Fig. 7). The same trend was observed with C3 as well as other host defense molecules. Interestingly, transfection with siRNA for C/EBPδ instead of C/EBPβ blocked the induction of C3 by GC, indicating that C/EBPδ rather

than C/EBPβ may be important for the effects of GC on C3. Western blot analysis confirmed that both of the siRNA that we used (Qiagen and Santa Cruz Biotechnology) reduced (but did not eliminate) C/EBPβ protein compared with scrambled control siRNA (data not shown). Both of the C/EBP siRNA we used failed to induce IFNβ. Taken together, these data indicate that enhancement of the expression of host defense genes in epithelial cells by GC may be mediated by the transcription factor C/EBP.

Discussion

The innate immune response to an infectious challenge is partly mediated by the production of APR proteins by the liver. For this article, we studied the manifestation of a local APR by airway epithelium, a response that may play a role in host defense in the airways. The key findings that we have made are: 1) TLR stimulation of epithelial cells induces the expression of numerous host defense molecules; 2) GC spare or enhance this response; and 3) the epithelial APR and GC responses are mediated by C/EBP. The immune and inflammatory proteins that we found to be induced by dsRNA included complement proteins, small cationic antimicrobial peptides (β-defensins), larger antimicrobial proteins (lysozyme, lactoferrin, and SLPI), collectins (MBL and SpD), cytokines (IFNβ and GM-CSF), and chemokines (RANTES and IL-8). Many of these epithelial-expressed molecules are classical acute phase proteins and display microbicidal activity or inhibit the growth of microorganisms.

The collectin family proteins SpA and SpD induce aggregation and opsonization and can directly cause bacterial lysis by per-meabilizing membranes of a broad spectrum of pathogens (27,28). Another collectin, MBL, and its two associated proteases, MASP1 and MASP2, recognize and bind to mannose and other carbohydrates on bacteria, yeast, and viruses and initiate complement activation (29). Deficiencies of MBL are associated with susceptibility to bacterial and viral infections (30). We observed a trend toward the induction of SpD by dsRNA, FP, and the combination of dsRNA and FP. MBL mRNA was induced by dsRNA and GC in the immortalized BEAS-2B cells but not in primary epithelial cells. Although the explanation for this difference is not clear, the results with SpD and MBL suggest that epithelial collectin expression is inducible by TLR pathways and that this response is not inhibited by GC.

Complement is a centrally important component of the APR and is essential in immunity. Its main physiologic activities include opsonization, chemotaxis, leukocyte activation, direct lysis of bacteria and infected cells, and augmentation of Ab responses (31). Although hepatocytes are the main source of complement biosynthesis, epithelial cells including keratinocytes (32) and renal tubular epithelial cells (33) have been shown to be capable of synthesizing complement proteins. We observed that the basal level of C3 in airway epithelial cells is relatively high at the mRNA level (the raw Ct was 22–25 whereas that of GAPDH was 18–21) and protein level (20 ng/1 × 10^6cells). The production of C3 in airways may be important for host defense at a major pathogen entry site. Upon stimulation with dsRNA, the mRNA level of C3 increased 9-fold while protein levels increased almost 3-fold. This magnitude of change is much higher than the pattern in blood after a moderate inflammatory stimulus (34). The other complement factors we studied in airway include factor B and factor H, two important components of the alternative pathway. When factor B binds to C3b it is activated by factor D and forms the C3 convertase enzyme C3bBb, which then initiates the cleavage of C3. Factor H is a dominant complement control protein that initiates the inactivation of C3 by factor I. Local production of these regulatory molecules may help regulate C3 function. Our results indicate that expression of these important complement proteins by airway epithelial cells can be induced by TLR activation and enhanced by GC, an effect that may boost innate immunity in patients treated with GC.

SAA and CRP are acute phase proteins whose serum concentrations can increase by as much as 100- to 1000-fold in disease (35). Recently, Raynes and coworkers discovered that SAA binds rapidly and firmly to a surprisingly large number of Gram-negative bacteria through the outer member protein A (36). Stimulation of polymorphonuclear cells with SAA increases the secretion of lactoferrin and enhances polymorphonuclear cell phagocytic activity against *Candida albicans* (37), suggesting that SAA is an important molecule in innate immunity. We observed an impressive increase in the expression of SAA by dsRNA and FP individually, and the combination of dsRNA and FP induced the greatest increase of SAA expression by epithelial cells. Once again, these results are compatible with the hypothesis that GC enhance local innate immune responses.

CRP is the prototypical member of the pentraxin family. It has been used as a clinical marker of inflammatory processes for a many years, although the roles of CRP in host defense and disease are not completely understood. CRP transgenic mice are resistant to experimental pneumococcal sepsis and display reduced bacteremia following an i.p. inoculation of pneumococci due to the high level of CRP expression (38). Gould and Weiser found that CRP was present in secretions of the human respiratory tract (6). When the secretions were pretreated to remove CRP, the complement-dependent bactericidal activity of normal nasal airway surface fluid and sputum against *Haemophilus influenzae* was abolished (6). The protective effect of CRP is thought to be mediated by binding to phosphorylcholine, a constituent of the membrane of major bacterial pathogens of the human respiratory tract, and initiating the classical complement pathway through interaction with C1q (39). We observed a consistent increase of CRP in epithelial cells exposed to the glucocorticoid FP and found enhanced CRP expression by cells exposed to FP and dsRNA.

As has been published by us and many other groups, GC are excellent inhibitors of the expression of chemokines by airway epithelial cells and other airway cell types (12,40,41). Indeed, we found that FP inhibited the expression of all of the chemokines studied with the exception of MIP-3α. MIP-3α is the unique ligand for CCR6, a receptor with a restricted distribution. In addition to being a potent chemoattractant for immature dendritic cells and T cells, MIP-3α also possesses antibacterial activity of greater potency against *Escherichia coli* and *Staphylococcus aureus* than HBD1 and HBD2, which have also been found to bind and activate CCR6 (42). Thus, MIP–3α appears to respond to GC in our in vitro system more like the other host defense molecules than the chemokines and other inflammatory genes.

Another exception to the pattern that we have observed (i.e., sparing of host defense genes and suppression of inflammatory genes) is the findings with the defensins. HBD are small (3–5 kDa) protease-resistant antimicrobial peptides that permeabilize the membranes of bacteria, fungi, and enveloped viruses. β-Defensin-deficient mice had delayed clearance of *H. influenzae* from the lung (43) and inhibition of the synthesis of HBD1 resulted in decreased antimicrobial activity of airway surface fluid (44). It is of note that GC failed to inhibit the expression of HBD1 but suppressed HBD2 and HBD4. HBD1 is constitutively expressed in enteric epithelial cells unlike HBD2–4, which are inducible (45). These results may indicate that HBD1 plays a more important role in resistance to commensal organisms. Interestingly, we found that HBD1 was inducible by TLR3 activation in our studies. HBD2 has been suggested to have a pathophysiological role as a proinflammatory mediator (46). It is not clear why HBD2 and HBD4 are regulated differently from the other antimicrobial proteins. The HBD1 promoter contains a NF-IL-6 (C/EBPβ) site but no NF-κB recognition sites whereas four consensus sequences for NF-κB are found within the promoter region of the HBD2 (47), which may offer a mechanistic explanation for the difference between them in regulation by GC.

We conclude that the respiratory epithelium manifests a local equivalent of the APR that may play an important role in innate immunity. The results also indicate that the expression of genes expected to play a direct role in pathogen destruction, including collectins, pentraxins, complement proteins, and some antimicrobial peptides, is not blunted by GC and in many cases is enhanced. This enhancement of host defense molecule expression was accompanied by GC inhibition of the expression of inflammatory molecules such as cytokines (IFNβ and GM-CSF) and chemokines (RANTES, IL-8, etc.). This pattern of selective effects of GC was observed in both primary cells and an immortalized epithelial cell line and was observed at the level of mRNA and protein. These findings support the view that GC suppress inflammation but spare innate immune response in the airways. Several reports in the literature support this hypothesis. In addition to SAA and CRP mentioned previously, SpA has been found to be increased 2-fold in rats following treatment with dexamethasone (48), and maternal treatment with dexamethasone led to increased levels of SpA in newborn rats (49). Hepatocyte expression of MBL was found to be induced by GC (50). In endothelial cells, GC enhanced expression of C3, factor B (51), and factor H (52). Gene profiling of human PBMC revealed enhancing actions of GC on innate immune-related genes, including complement family members, scavengers, and Toll-like receptors (53).

Our results suggest that the ability of GC to reduce exacerbations of airway inflammatory diseases in which infectious organisms serve as triggering and exacerbating factors, including asthma, chronic obstructive pulmonary disease, and chronic rhinosinusitis, may result not only from their anti-inflammatory effects but also from their ability to spare or enhance local innate host defense responses.

To study the possible mechanism of the effect of GC on epithelial host defense gene expression, we tested whether C/EBP, which has been implicated in the regulation of a number of acute phase genes in the liver, was involved. We first found that dsRNA stimulation increased C/EBPβ and C/EBPδ mRNA expression and that GC had a synergistic effect in both primary cells and cell lines. In BEAS-2B cells, FP alone significantly induced C/EBPβ and C/EBPδ mRNA expression, which was in agreement with the ability of the GC to directly induce host defense gene expression (see Fig. 2). Our results indicate that C/EBPβ and C/EBPδ are induced by inflammatory stimuli, whereas C/EBPα is inhibited. In the process of cell differentiation and proliferation, C/EBPβ and C/EBPδ drive cell cycle progression while antiproliferative transcription factors C/EBPα and C/EBPε lead to cell differentiation (54,55). We detected by Western blotting five isoforms of C/EBPβ having the following molecular weights: 40 kDa (LAP*), 35 kDa (LAP), 25 kDa (LIP), 50 kDa, and 46 kDa. The first three have been described in the literature (15,56). A 50-kDa band was described in the C/EBPβ Ab product manual (Santa Cruz Biotechnology). The 46-kDa band appears to be an isoform that is not commonly seen in studies of other cell types. However, Jamaluddin et al. reported that respiratory syncytial virus infecting human type II pulmonary alveolar epithelial cells synthesized a single 45.7-kDa isoform of C/EBPβ (19). Th respiratory syncytial virus is a negative-sense RNA virus that produces dsRNA during replication. Although we do not know the exact structure of this isoform, we suspect it may also function like LAP based on its molecular size. The heterogeneity of the sizes of C/EBP proteins has been shown to result from multiple translation initiation codons in the same mRNA molecule (57). LAP contains both the activation and the basic region-leucine zipper domain, whereas only the latter is present in LIP (56). Therefore, by forming nonfunctional heterodimers with the other members, LIP can act as a dominant negative inhibitor of C/EBP function. We observed that the activating C/EBPβ LAP isoforms were induced to a greater extent by dsRNA stimulation and GC treatment than LIP. Expression and functional studies both indicate that the amount of active C/EBPβ and C/EBPδ is increased in airway epithelial cells after treatment with both the TLR3 activator and GC and that the combination is most effective. The functional activity of the induced proteins and the

conclusion that both dsRNA and GC activate them are supported by the results of EMSA and luciferase reporter assays.

Time course experiments showed that C/EBPβ mRNA was induced by FP as early as 2 h and that the peak time for expression in the nucleus was 8 h. By 12 h, C/EBPβ had disappeared from the nucleus. Accordingly, the enhancing or inhibiting effects of dsRNA and GC appeared at 8 h and peaked at 24 h. When we used RNA interference to block the function of C/EBPβ, the enhancement of CRP, SAA, and MBL by GC was blocked while the inhibition of the expression of inflammatory genes was not affected. The promoters of both CRP and SAA genes contain *cis*-acting C/EBP elements (58,59), while there is no direct evidence showing that C/EBPβ is important for MBL gene transcription. Our data suggest that the enhancement by GC of the expression of the host defense molecules CRP, SAA, and MBL is mediated by the activation of C/EBPβ. The fact that FP still increases the expression of some genes in siRNA transfected cells indicates that the knock-down of C/EBP is incomplete or that there may be C/EBP-independent genes (such as C3). We suspect that C/EBPδ rather than C/EBPβ is involved in C3 gene expression. Two papers indicating that C/EBPδ is the major transcription factor responsible for regulating the expression of the human C3 gene in brain and liver support this opinion (60,61). The up-regulation by GC of MIP-3α was not inhibited by C/EBPβ siRNA (data not shown). The report that microbe-induced MIP-3α production was blocked by the protein kinase C inhibitor Ro31–8220 and a protein kinase C pseudosubstrate (62) suggests that the induction of MIP-3α may involve protein kinase C and NF-κB pathways instead of C/EBPβ. The mechanism of the enhancement of MIP-3α by FP is not clear at this time.

Although the promoter of the inflammatory gene IL-8 also contains a C/EBP binding site (63), the knockdown of C/EBPβ by siRNA did not abolish IL-8 gene repression by GC. The suppressive effects of GC on IL-8 expression have been shown to be mediated by the effects on NF-κB (64). Our results support the concept that the transcription factors that play the predominant role in target gene expression determine the way a gene responds to GC treatment. C/EBP is essential for acute phase protein expression whereas others, such as NF-κB and AP-1, are more important for the expression of inflammatory genes and are thus GC sensitive. We hasten to point out that many effects of GC are exerted post-transcriptionally and there may be selective effects of GC on these mechanisms as well (65).

Taken together, the results of the present study demonstrate that TLR activation triggers the respiratory epithelium to manifest a local APR that is likely to contribute to the defense against pathogens in the airways. GC inhibit the concomitant inflammatory response while sparing or enhancing the local innate host defense response. These selective effects of GC are partially mediated through activation of the transcription factor C/EBP by GC. The results presented here are worthy of consideration during ongoing efforts to develop improved GC for the treatment of airway inflammatory diseases.

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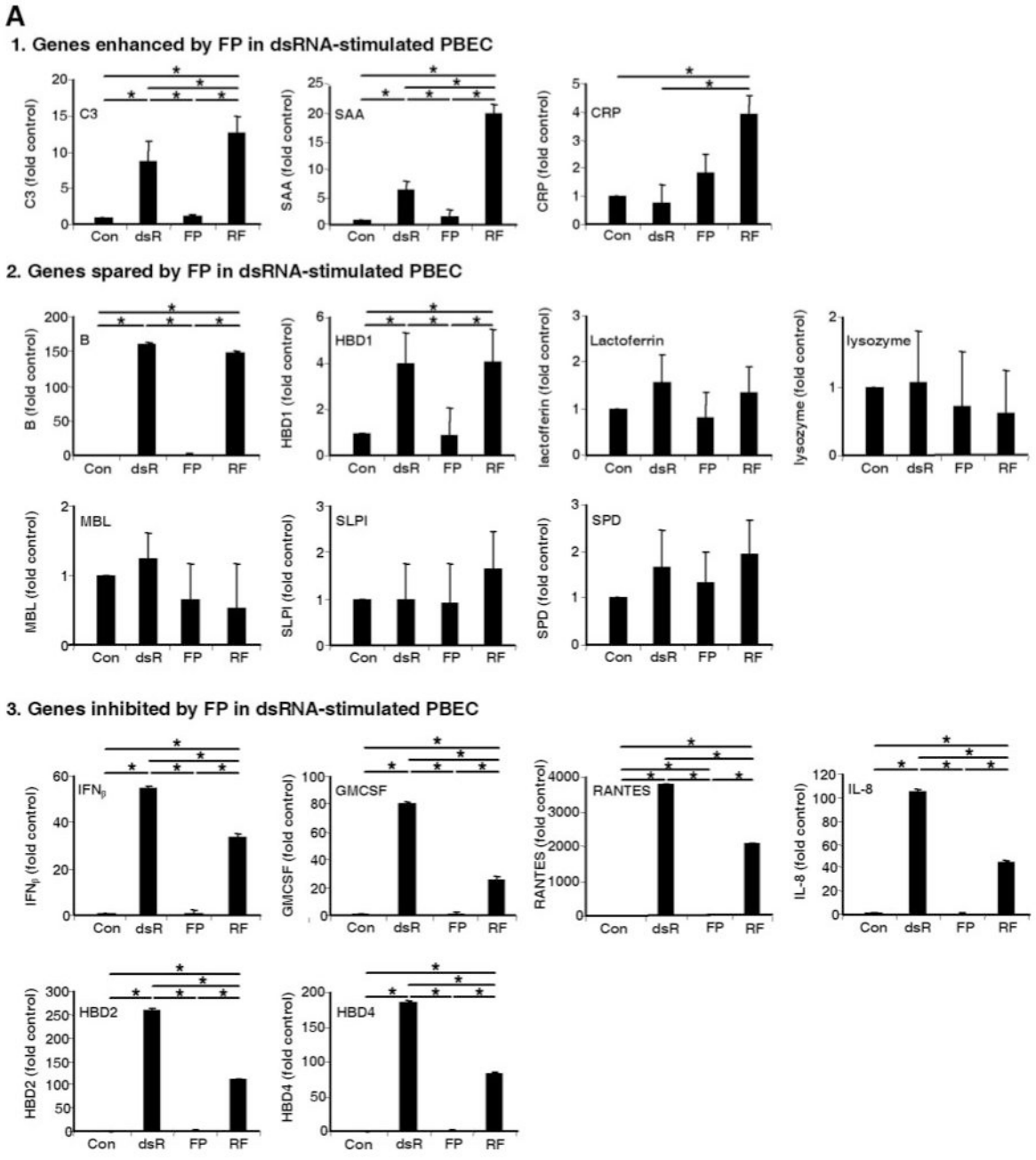

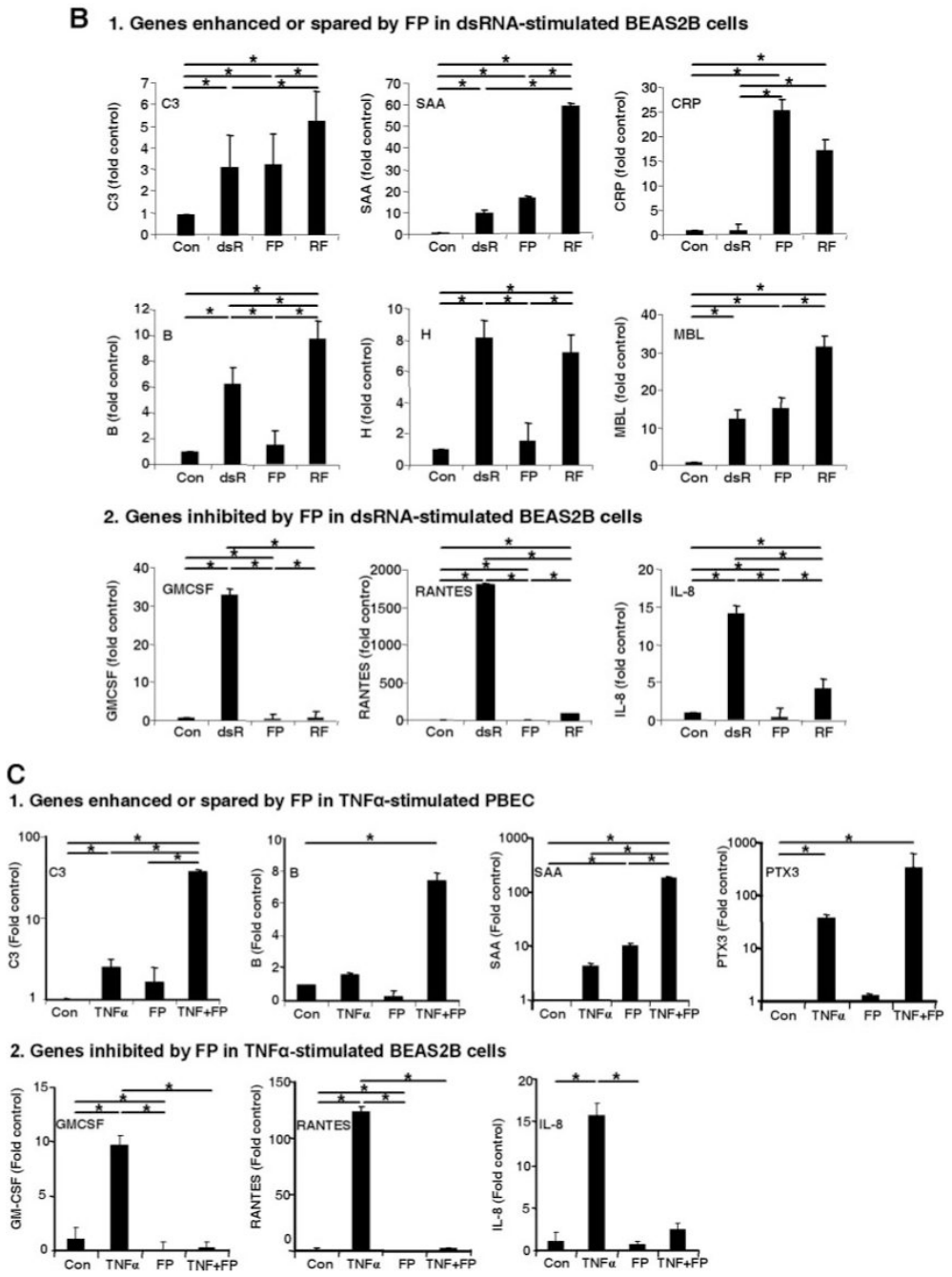

FIGURE 1.

PBEC (*A*) and BEAS-2B cells (*B*) were treated with diluent (Con, control), 25 μg/ml dsRNA (dsR; poly(I:C)), 100 nmol/L FP, or the combination of dsRNA and FP (RF) for 18 h. In the RF group, cells were treated with FP for 2 h before stimulation with dsRNA. The cells were lysed, RNA was extracted, and gene expression was evaluated by real-time PCR. In some experiments, TNF-α was used as the stimulus instead of dsRNA under the same other conditions (*C*). Gene expression is expressed relative to the diluent control group; $n = 4–7$. *, $p < 0.05$.

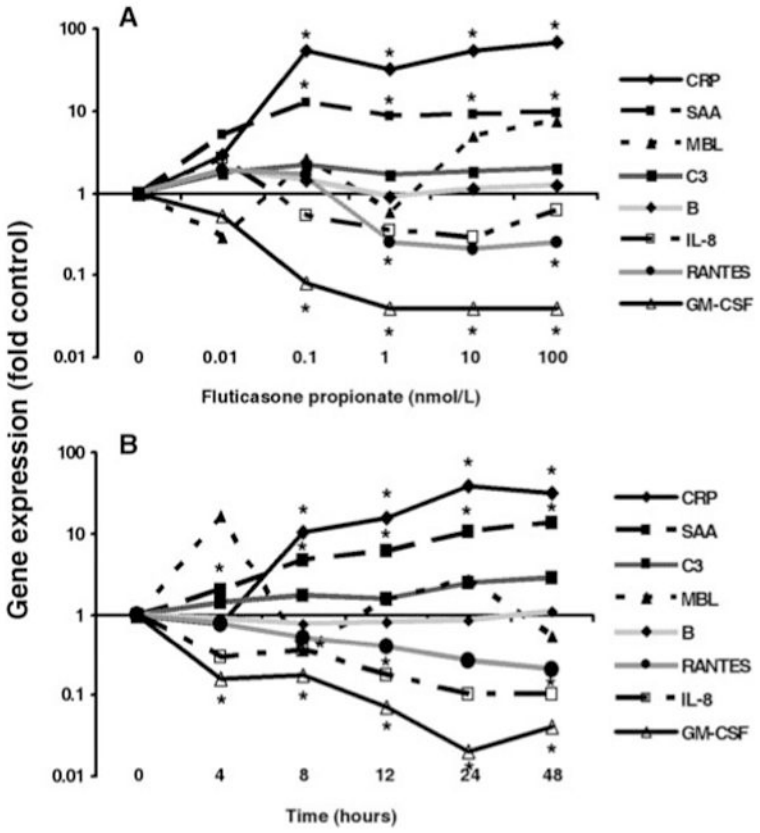

FIGURE 2.

Resting BEAS-2B cells were treated with FP for 18 h at the indicated concentration (*A*) or 100 nmol/L FP for the indicated times (*B*). The levels of selected host defense and inflammatory molecules were evaluated by real-time PCR. Gene expression is expressed relative to the respective control groups. *, $p < 0.05$. The values results shown are the mean of three independent experiments.

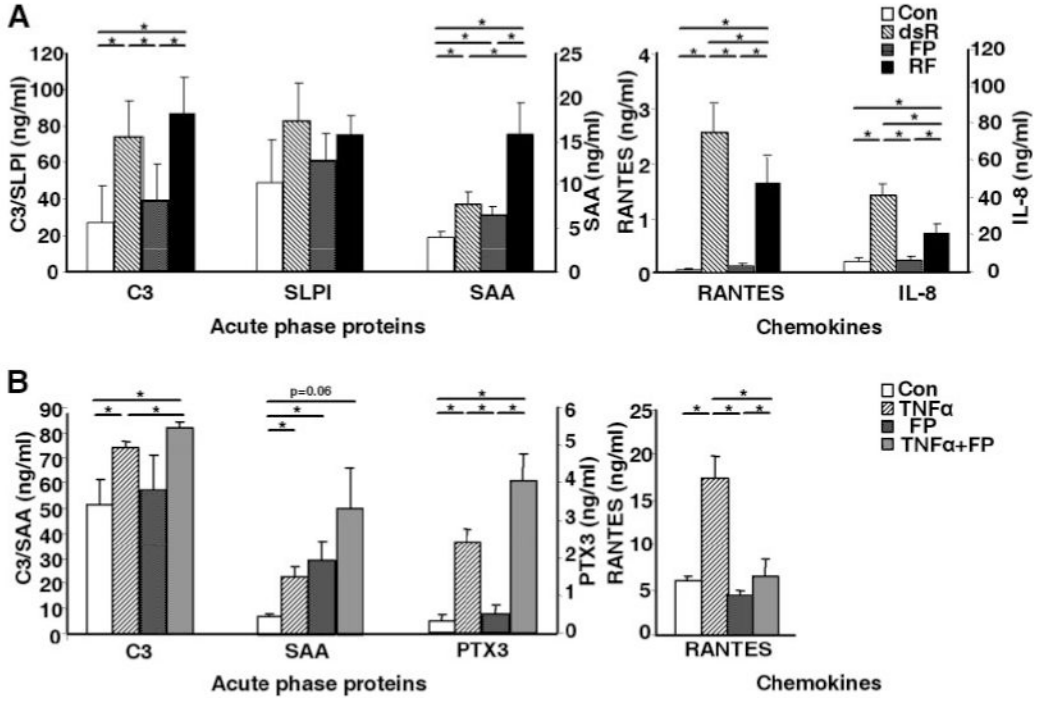

FIGURE 3.

PBEC were treated with diluent (Con, control), 25 μg/ml dsRNA (dsR; poly(I:C)) (*A*) or 100 ng/ml TNF-α (*B*), 100 nmol/L FP, or the combination of dsRNA and FP (RF) (*A*) or TNF-α and FP (TNFα+FP) (*B*) for 18 h. In the RF group, cells were treated with FP for 2 h before stimulation with dsRNA. Supernatants were collected and proteins were detected using ELISA (C3, SLPI, and SAA) or cytometric bead array assay (RANTES and IL-8); $n = 4$–6. *, $p < 0.05$.

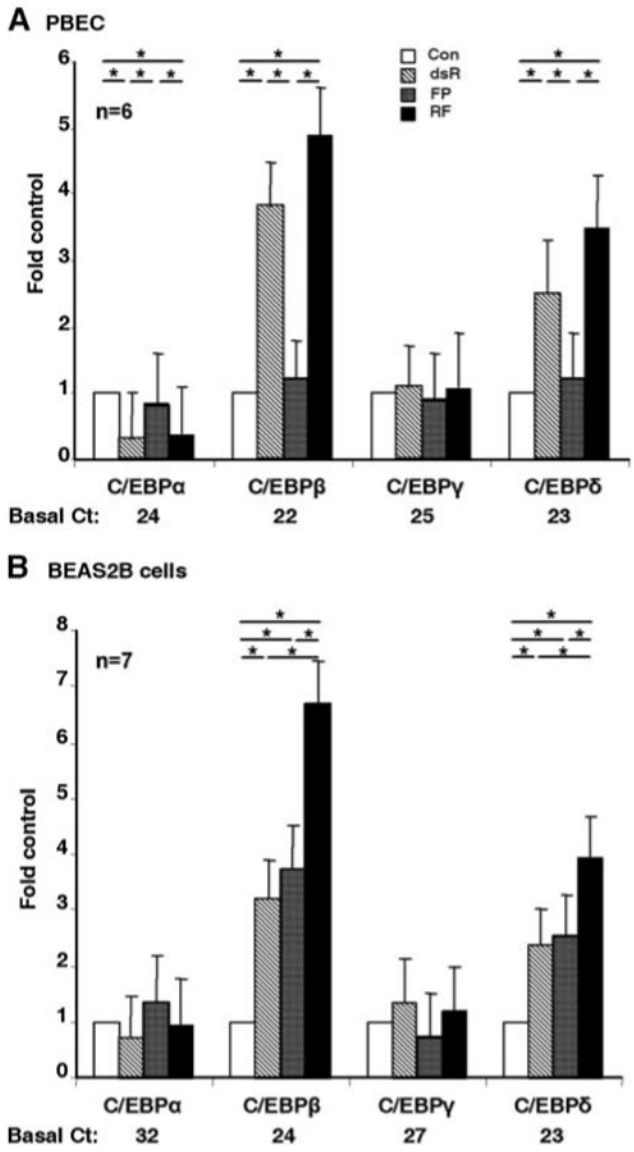

FIGURE 4.

PBEC (*A*) and BEAS-2B (*B*) cells expressed mRNA for C/EBP*α*, C/EBP*β*, C/EBP*γ*, and C/EBP*δ* but not C/EBP*ε*. Basal Ct is indicated below the *x*-axis (higher numbers indicates lower basal levels; 40 = undetected). Levels of C/EBP*ε* were 40 (data not shown). dsR, dsRNA; Values are mean ± SEM of expression relative to the diluent control (Con). *, $p < 0.05$.

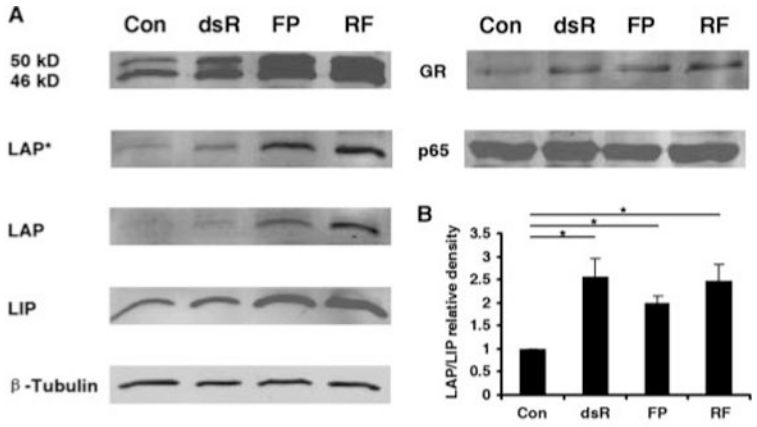

FIGURE 5.

Assessment of the expression of C/EBP*β* in BEAS-2B cells treated as indicated for 18 h by using Western blotting. Bands at 50 kDa, 46 kDa, 40 kDa (LAP*), 35 kDa (LAP), and 25 kDa (LIP) were detected. NF-*κ*B p65 and GC receptor (GR) were assessed as controls. *A*, One representative experiment of three independent experiments is shown. *B*, The mean ± SEM relative density of LAP/LIP from three independent experiments is shown. *, $p < 0.05$. Con, control; dsR, dsRNA; RF, dsRNA plus FP.

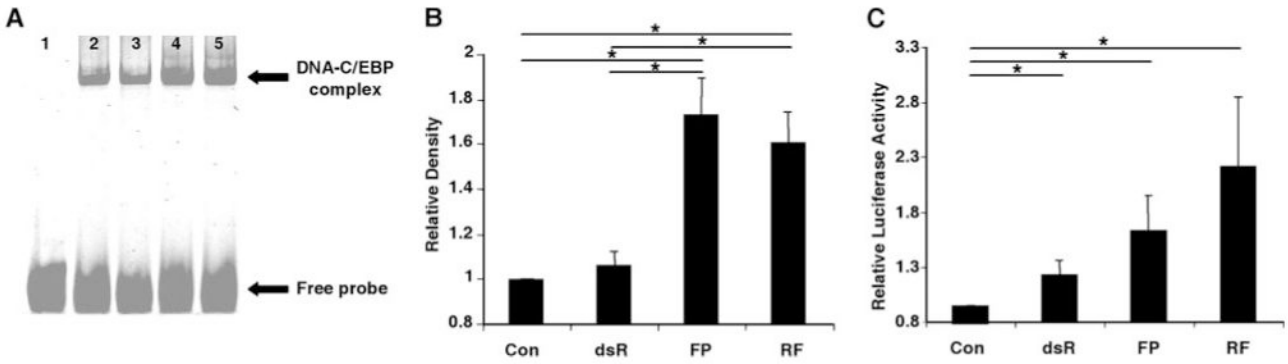

FIGURE 6.

Analysis of the effects of FP and dsRNA (dsR) on the function of C/EBP*β* using electrophoresis mobility shift assay (*A* and *B*; n = 3) and C/EBP*β* luciferase reporter assay (*C*; *n* = 3). *A*, Representative C/EBP*β* EMSA using the infrared Odyssey detection system. The C/EBP*β* consensus probe was labeled with IRDye 800. *Lane 1*, negative control (no protein); *lane 2*, control (Con); *lane 3*, dsRNA; *lane 4*, FP; *lane 5*, dsRNA plus FP (RF). *B* and *C*, Values shown are mean ± SEM for relative DNA binding and relative luciferase activity, respectively. *, $p < 0.05$.

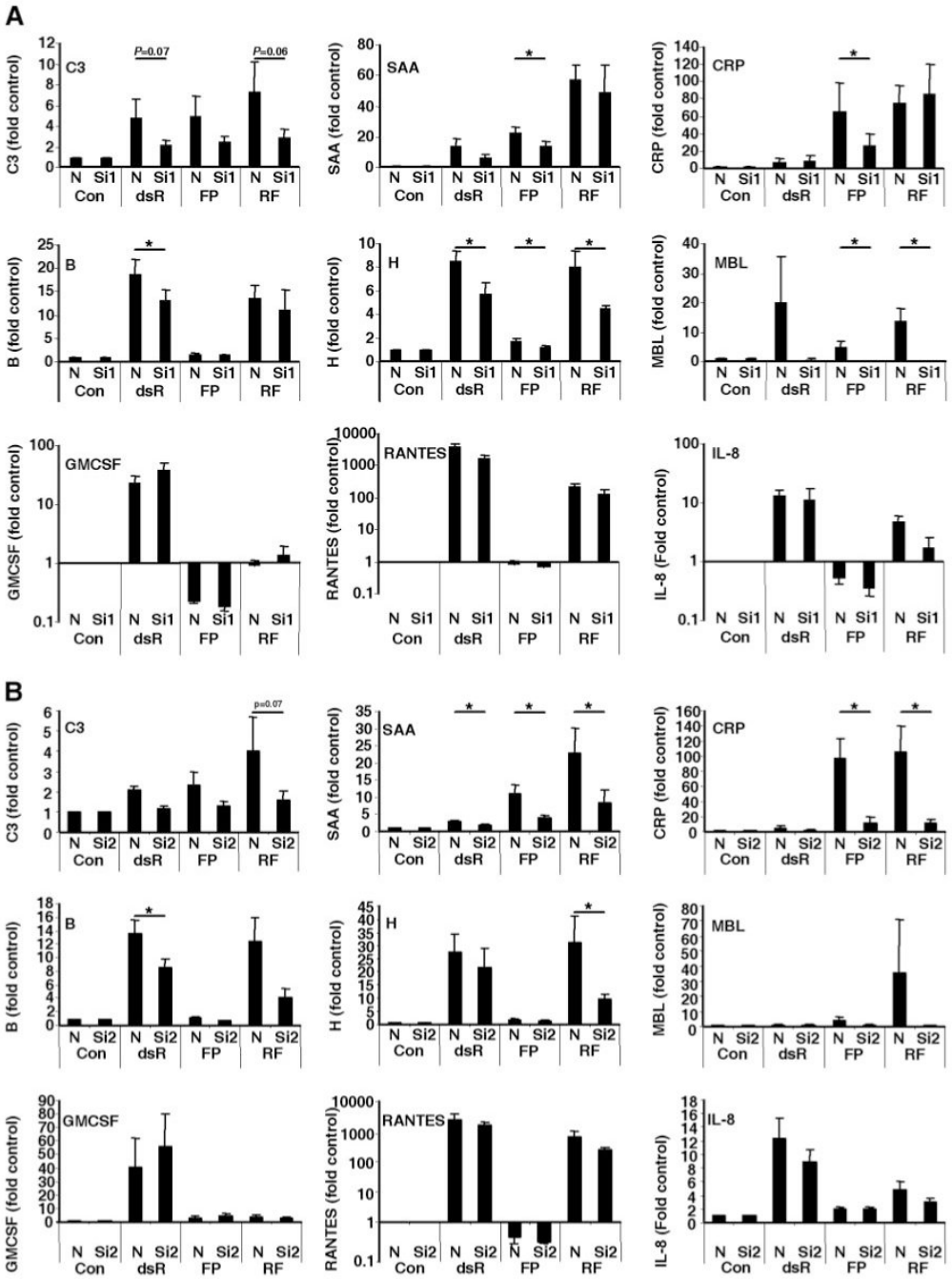

FIGURE 7.

Subconfluent BEAS-2B cells were transiently transfected with negative control siRNA (N) or C/EBP*β* siRNA (*A*, Si1 or *B*, Si2) for 24 h and then stimulated with diluent (Con, control), dsRNA (dsR), FP, or dsRNA plus FP (RF) for an additional 18 h. Values shown are mean ± SEM of levels of gene expression expressed relative to the control from four to nine separate experiments. *, $p < 0.05$, negative control siRNA vs C/EBP*β* siRNA.

Table I

Minor groove-binding (MGB) probes and primers used for quantitative TaqMan real-time polymerase chain reaction analysis of gene expression

Target Gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	MGB Probe (5′ to 3′)
C3	ATCACCGCCAGGTTCCTCTA	GAATGCGCTTGAGGGATTCA	AAGAAAGTGGAGGGAACT
Factor B	GGTTCTGATGAAGATTGTGTATTGCTAC	GACGGCCAGATGGGAGG	ACGTCCCCTGCTCCA
Factor H	GGTTCTGATGAAGATTGTGTATTGCTAC	GACGGCCAGATGGGAGG	ACGTCCCCTGCTCCA
GM-CSF	AGGGCCCCTTGACCATGA	CAAAGGTGATAGTCTGGTTGCA	CAGCACTGCCCTCCAACCCCG
HBD2	TTTTGGTGGTATAGGCGATCCT	GGAGACCACAGGTGCCAATTT	TTTTGCCCTAGAAGGTATAAA
IFNβ	TCTCCACGACAGCTCTTTCCA	ACACTGACAATTGCTGCTTCTTTG	AACTTGCTTGGATTCCT
CXCL8 (IL-8)	CTGGCCGTGGCTCTCTTG	TTGGCAAAACTGTTTAGCACTCC	CAGCCTTCCTGATTTCTGCAGCTCTGTGT
Lactoferrin	CAGACGGGCTCCTGCAAAT	TGCTCGTCGCCAATACACA	TGACCCGAGATCTAAT
Lysosome	AGGAGCAGTTAATGCCTGTCATTTAT	ATGCTCTAATGCCTTGTGGATCA	TTGCTGCAAGATAACAT
MASP1	GACCGCCGCACACTGC	TTTGACGCCGAGATGCTGT	ACCAGTCACTCGATCC
MASP2	ATCACCTTTGTCACAGATGAATCAG	GGAGAAGCTGTCTTTCAGGATGTAT	AAGATCCACTACACGAGCAC
MBL	GGTTCTGATGAAGATTGTGTATTGCTAC	GACGGCCAGATGGGAGG	ACGTCCCCTGCTCCA
MIP-3α	GCTCCTGGCTGCTTTGATG	CAAAGTTGCTTGCTGCTTCTGA	CTGCTACTCCACCTCTGCGGCGA
SAA	CATTTTCTGCTCCCTGGTCCT	TGATTTTTTGTAATTGGCTTCTTTCAT	AGCCAAGGATGGTTAACAT
CCL5 (RANTES)	TCGCTGTCATCCTCATTGCTA	GCACTTGCCACTGGTGTAGAAA	CTGGGACACCACACCCTGCTGC

Table II

Expression of host defense and inflammatory molecules in human bronchial and alveolar epithelial cells

	BEAS-2B (*n* = 4–7)	**A549 (*n* = 2)**	**PBEC (*n* = 4–7)**
Host Defense Molecules			
Complement			
C3	+ 23/21 (3)*[a]	+ 24/23 (2)	**+ 25/20 (9)***
Factor B	**+ 22/18 (6)***	**+ 21/19 (7)**	**+ 25/17 (160)***
Factor H	**+ 33/29 (8)***	+ 24/22 (4)	**+ 27/23 (12)***
Defensins			
HBD1	—	**+ 36/33 (11)**	+ 28/26 (4)*
HBD2	—	**+ 35/32 (9)**	**+ 35/24 (262)***
HBD4	—	+ 31/31 (1)	**+ 35/22 (185)***
Collectins			
MBL	**± 40/34 (12)***	**± 40/33 (226)**	+ 36/36 (1)
MASP1	—	—	—
MASP2	—	—	—
SpA	—	—	± 40/35 (5)*
SpD	—	+ 34/34 (1)	+ 33/31 (2)
Pentraxins			
CRP	—	—	—
Pentraxin3	**+ 25/21 (11)***	+ 31/29 (4)	+ 31/29 (4)*
Other antimicrobial proteins			
Lysozyme	+ 34/33 (1)	+ 30/28 (4)	+ 30/29 (1)
Lactoferrin	+ 34/34 (1)	—	+ 32/31 (2)
SLPI	**+ 29/25 (8)**	+ 23/23 (1)	+ 21/20 (1)
BPI	—	—	—
LBP	—	+ 28/26 (5)	—
SAA	**+ 24/19 (10)***	**+ 30/28 (7)**	**+ 24/17 (6)***
Coagulation proteins			
Fibrinogen	—	+ 24/24 (1)	—
PAI	+ 23/22 (1)	+ 23/23 (1)	+ 23/21 (1)
Inflammatory molecules			
Cytokines			
IFNβ	**+ 33/29 (28)***	**+ 35/32 (13)**	**+ 35/26 (55)***
GM-CSF	**+ 30/21 (33)***	+ 33/31 (4)	**+ 30/21 (81)***
Chemokines			
RANTES	**+ 29/17 (1805)***	**+ 27/23 (13)**	**+ 31/19 (55)***
IL-8	**+ 37/32 (14)***	+ 35/33 (5)	**+ 40/28 (106)***
MIP-3α	**+ 36/26 (29)***	+ 26/24 (4)	**+ 29/24 (36)***
Reference			
GAPDH	+ 19/19 (1)	+ 19/19 (1)	+ 19/19 (1)

[a] Values on the left are mean basal Ct and values on the right are mean Ct after stimulation with dsRNA. Lower values represent higher expression and each unit represents a 2-fold change in expression. Ct > 37 indicates no or minimal detectable expression. —, No expression in either basal or stimulating condition. ±, no expression in basal condition but induced by stimulation; +, Ct ≤ 37 in both conditions. Bold type indicate genes induced by at least 6-fold.

* $p < 0.05$ for the comparison of stimulated to controls. BPI, bactericidal/permeability-increasing protein; PAI, plasminogen activator inhibitor.