Corticosteroid modulation of immunoglobulin expression and B-cell function in COPD

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ABSTRACT We investigated changes in gene expression that occur in chronic obstructive pulmonary disease (COPD) after corticosteroid treatment and sought to identify the mechanisms that regulate these changes. Biopsy samples were taken from patients with COPD (Global Initiative for Chronic Obstructive Lung Disease stage I to II) before and after treatment with fluticasone propionate (FP)/salmeterol (SM) (50/500, 4 wk). Gene expression was measured by microarray and was confirmed by real-time reverse transcription–quantitative PCR (RT-qPCR). The effect of FP on IgG expression and B-cell proliferation in the presence of oxidative stress was also studied. FP/SM significantly increased the expression of 180 genes while repressing 343 genes. The top 5 down-regulated genes were associated with immunoglobulin production, whereas the immunomodulatory FK506 binding protein (FK506BP) was up-regulated. Genes including IL6, IL8, and TBF-encoding TBX21 were unaffected. FP reduced IgG protein and mRNA expression and proliferation of human B cells through the dephosphorylation of ERK-1/2 via increased DUSP1 (dual-specificity protein phosphatase 1) expression. Consistent with in vivo data, oxidative stress did not prevent FP-induced suppression of IgG expression in human B cells in vitro. Changes in expression were validated by RT-qPCR and by gene set enrichment analysis in distinct COPD cohorts. FP may reduce the adaptive immune response in COPD and may be more effective in patients with an increased B-cell/antibody response induced by high autoantibody titers.—Lee, J., Machin, M., Russell, K. E., Pavlidis, S., Zhu, J., Barnes, P. J., Chung, K. F., Adcock, I. M., Durham, A. L. Corticosteroid modulation of immunoglobulin expression and B-cell function in COPD. FASEB J. 30, 2014–2026 (2016). www.fasebj.org

Key Words: DUSP1 • ERK • fluticasone propionate • LABA

Chronic obstructive pulmonary disease (COPD) is an incurable, progressive, and ultimately fatal inflammatory lung disease (1). Inflammation in the lungs is driven initially by cigarette smoke and subsequently continues after smoking cessation. The lung inflammation involves a number of cell types, including increased macrophages, neutrophils, epithelial cells, CD4+ and CD8+ T cells, and B cells, particularly patients with more severe disease (2, 3).

Alveolar destruction in COPD is irreversible, and in contrast to other inflammatory diseases, such as asthma, there is a lack of effective anti-inflammatory treatments available (4). Current treatments are based on reducing further damage and managing symptoms. The main treatments include smoking cessation, long-acting bronchodilators, oxygen therapy, and lung volume reduction surgery (5). It is recommended to treat patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage III or IV disease with long-acting β2 agonists in combination with an inhaled corticosteroid (ICS) to improve lung function and prevent exacerbations (6). The TORCH (Toward a Revolution in COPD Health) study (7) showed that the combination of fluticasone propionate (FP) and salmeterol (SM) reduced the relative mortality risk in a cohort of patients with COPD by 17.5% over 3 yr compared to placebo and reduced the relative decline in lung function (forced expiratory volume in 1 s, FEV1) (8).

Long-acting β agonists (LABA), when combined with ICS, are more effective at improving lung function, reducing symptoms, and preventing exacerbation in asthma (9) and COPD (10). This may be due to complementary actions of the 2 drugs enhancing corticosteroid efficacy, as this has been shown in several cell types (9). Hattotuwa et al. (11) showed that 3 mo of FP did not reduce inflammatory cell marker end points such as CD8+ cells, CD68+ cells, or neutrophils in biopsy samples, although there was a change in CD4:CD8 ratio. However, 13 wk of FP/SM significantly reduced the numbers of CD45+ cells, CD4+ and CD8+ T cells, and neutrophils, but not CD68+ macrophages in bronchial biopsy samples of patients with COPD (12). SM enhances the impaired translocation of glucocorticoid receptors in airway cells from patients with COPD, providing a molecular basis for the enhanced effects of ICS/LABA combination therapy in COPD (13).

The role of ICS in the chronic treatment of COPD remains controversial, although recent evidence suggests

Abbreviations: ACOS, asthma-COPD overlap syndrome; BrdU, bromodeoxyuridine; COPD, chronic obstructive pulmonary disease; DUSP1, dual-specificity protein phosphatase 1; FCS, fetal calf serum; FEV1, forced expiratory volume in 1 s; FP, fluticasone propionate; GLUCOLD, Groningen and Leiden Universities’ Study of Corticosteroids in Obstructive Lung

(continued on next page)
that COPD with increased blood eosinophils may respond better (14). Gene array analysis of bronchial biopsy samples and brushings indicate that there is a set of COPD-associated genes that correspond to COPD-related injury to the small airways and lung parenchyma (15, 16). Importantly, many of the genes in this COPD signature are altered in response to ICS treatment (15, 16). While these patients did not have a clinical history of asthma, it was not possible to determine whether these patients were similar to patients with asthma-COPD overlap syndrome (ACOS) who have a high type 2 T helper (T\(_h\)2) cell signature without a clinical history of asthma but with increased blood eosinophils whose disease responds to corticosteroids (17). However, a post hoc analysis of T\(_h\)2-related genes did not show any increase in the patients with COPD compared to controls, indicating that ACOS was an unlikely explanation for the molecular response to ICS in these patients.

In a small pilot study, we found that 28 d of treatment with FP/SM modulated the expression of 523 genes (180 increased, 343 decreased) in patients with COPD despite having no effect on spirometry or inflammation in bronchial biopsy samples. Immunoglobulin encoding genes were the most significantly down-regulated genes; this effect was confirmed in the human B-cell line CLNH1.4 in vitro, where FP down-regulated proliferation and IgG production even in the presence of oxidative stress. We therefore suggest that one of the benefits of ICS/LABA combination in patients with COPD in vivo may be through the reduction of IgG production from plasma cells. This would reduce the effects of autoimmunity in the latter stages of COPD.

MATERIALS AND METHODS

Patients

Six patients with COPD (GOLD stage I or II) were recruited for this study (Table 1). Some results have been previously reported (18). The severity of the airflow obstruction was staged according to GOLD criteria. COPD and chronic bronchitis were defined according to international guidelines as follows: COPD, presence of postbronchodilator FEV\(_1\)/forced vital capacity ratio <70%; and chronic bronchitis, presence of cough and sputum production for at least 3 mo in each of 2 consecutive years. Patients with COPD were stable with no previous exacerbation in the 6 mo before bronchoscopy. None of the subjects was treated with theophylline, antibiotics, antioxidants, mucolytics, and/or glucocorticoids in the month before the study began.

The study conformed to the Declaration of Helsinki and was approved by the ethics committee of the Royal Brompton Hospital. Written informed consent was obtained from each subject, and bronchial biopsies were performed according to the local ethics committee’s guidelines. Pulmonary function tests were performed as previously described (19) according to published guidelines (20). Reversibility of airflow obstruction and postbronchodilator functional values was measured 20 min after the inhalation of 0.4 mg of salbutamol.

Study design

Bronchial biopsy samples were obtained from all subjects at baseline and after 28 d of treatment with FP/SM (Seretide 50/500; GlaxoSmithKline, Brentford, United Kingdom). The second biopsy was performed 24 h after the final dose after repeat spirometry.

Power calculation

Power calculations were conducted using online software (21). Significant results were expected to show at least a 2-fold change in gene expression (\(\mu_0(1) = 1, \mu_1(1) = 2\)). Standard deviation was assumed to be 0.8, the alpha value was 0.05, and the power was 0.08, which gave a sample size of 6.

Fiber-optic bronchoscopy, collection, and processing of bronchial biopsy samples

Fiber-optic bronchoscopy was performed as previously described (22). A fiber-optic bronchoscope (BF10 Key-Med; Olympus, Southend-on-Sea, United Kingdom) was passed through the nasal passages into the trachea, and 4 bronchial mucosal biopsy specimens were taken from the segmental and subsegmental airways of the right lower and upper lobes with size 19 cupped forceps. Biopsy samples for immunohistochemistry were mounted on cork with OCT embedding matrix (CellPath, Newtown, United Kingdom) and frozen at −80°C until analyzed. Bronchial biopsy samples for gene expression profiling analysis were immediately snap frozen in liquid nitrogen and stored at −80°C until processed.

Immunohistochemistry

Lung tissue processing and immunohistochemistry were performed as described previously (22). The total numbers of inflammatory cells (macrophages, neutrophils, mast cells, and T lymphocytes) were counted in 20 nonconsecutive fields (magnification \(\times 40\)). Normal nonspecific IgG from the animals in which the primary antibodies were raised was used for negative controls (Santa Cruz Biotechnology, Santa Cruz, CA, USA). All staining and cell counting was performed in a blinded manner. The number of positively stained cells was expressed as a percentage of the total cells counted. The following antibodies were used for detection: mouse anti-CD4 antigen (lymphocytes; Dako, Ely, United Kingdom), anti-CD8 antigen (lymphocytes; Dako), anti-CD68 antigen (macrophages; Dako), anti-neutrophil elastase antigen (neutrophils; Dako), and anti-tryptase (mast cells; Abcam, Cambridge, MA, USA).

DNA microarray

DNA microarray was performed as previously described (23). Total RNA was extracted from frozen lung tissue with Trizol reagent (Invitrogen, Paisley, United Kingdom). RNA was
further purified with mirVana columns (Ambion, Austin, TX, USA), and DNA was removed while on the column using DNase (Qiagen, Manchester, United Kingdom). RNA quantitation and quality assessment were performed on a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), an Agilent 2100 bioanalyzer, and a RNA 6000 Labchip kit (Agilent Technologies, Santa Clara, CA, USA), and DNA was removed while on the column using the manufacturer’s instructions, including Ambion Brain and Universal Human Reference as standards. The cDNA was purified quantified and integrity measured. Finally, 5 μg of cDNA progressed to enzymic and chemical fragmentation, biotin end labeling, and hybridization to human U133_2 Affymetrix chips. Gene expression profiles were examined by ingenuity pathway analysis (Ingenuity Systems, Redwood City, CA, USA). The microarrays were analyzed for gender and compared to the gender of the biopsy donor as part of the quality assurance protocol.

Culture of human B-cell line

The human B-lymphocyte hybridoma cell line CLNH11.4 (LGC Standards, Teddington, United Kingdom), which produce IgG1 monoclonal antibodies constitutively, were grown and maintained in RPMI medium supplemented with L-glutamine and 10% fetal calf serum (FCS). Cells were synchronized by growing overnight in RPMI medium supplemented with L-glutamine but not FCS before being centrifuged, counted, and resuspended in fresh medium before experiments were initiated. Cells were treated with FP and SM (both Sigma-Aldrich, Dorset, United Kingdom) using DMSO as a vehicle. DMSO had no effect on any assays at the concentrations used (≤1:1000 dilution; data not shown).

Cell proliferation

Cell proliferation was measured using the BrdU assay (Roche, Burgess Hill, United Kingdom) following the manufacturer’s instructions. The synthetic nucleoside bromodeoxyuridine (BrdU) is incorporated into nascent DNA during synthesis and detected using a specific anti-BrdU antibody that correlates with the amount of DNA synthesized.

Cell viability

Cell viability was measured using the MTT assay, which measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan due to mitochondrial activity. It was assumed that reduction in mitochondrial activity correlated with cell death.

IgG/M antibody assay

IgG and IgM levels were measured using the IgG or IgM antibody assay kits (Easy-Titer; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions. These assays measure agglutination of antibody sensitized microbeads in order to measure the concentration of antibody present.

Measurement of gene expression

Relative gene expression was measured by real-time reverse transcription–quantitative PCR (RT-qPCR). Initially, the cells were lysed in RLT buffer; mRNA was extracted using the RNeasy kit following the manufacturer’s instructions (Qiagen). Subsequently cDNA was generated using MMLV reverse transcriptase and random primers (Life Technologies, Paisley United Kingdom). cDNA levels were quantified using the Corbett LightCycler and SYBR Green (Qiagen) using the following cycle: 95°C, 15 min, followed by 50 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The following primers were used: 8S forward 5’-CTTACGGGACAAAGTGGGC-3’, 18S reverse 5’-AAGCTTGACCGCATCAGTTGA-3’, IGH forward 5’-GTTCTATCCAGGAGATC-3, IGH reverse 5’-GGAGGAGCTTCTTTGTTG-3, IGKC forward 5’-GGACCAAGGTGAAAATCAAA-3, IGKC reverse 5’-TTGGCCCTCCTGGGATAAGA-3, and DUSP1 (dual-specificity protein phosphatase 1) forward 5’-ACCACCGTGTTTACACTTT-3’ DUSP1 reverse 5’-AAGGCTGTAATGGGGCTCGT-3’. Alternatively, cDNA was quantified using TaqMan RT-qPCR (Applied Biosystems, Foster City, CA, USA) using commercially available probes and again standardized against 18S

### Table 1. Patient data before and after treatment

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
<th>Before FP/SM</th>
<th>After FP/SM</th>
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<tr>
<td>Gender (M/F)</td>
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<tr>
<td>Age (yr)</td>
<td>55 ± 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking history (pack-yr)</td>
<td>37 ± 4</td>
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<tr>
<td>Smoking status</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ex-smoker</td>
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</tr>
<tr>
<td>Smoker</td>
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</tbody>
</table>

Sample

- FEV1 (% predicted) 78 ± 5
- Macrophages (CD68+)/mm² 279 ± 53
- Neutrophils/mm² 268 ± 45
- Mast cells (MCT+)/mm² 277 ± 47
- CD4⁺ cells/mm² 125 ± 35
- CD8⁺ cells/mm² 276 ± 39

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<td>Smoking status</td>
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**Significance** was measured between pre- and posttreatment samples with Wilcoxon matched pair signed rank test. Patients with FEV1 >70% had an FEV1/forced vital capacity ratio of <0.7 (FEV1 minus % predicted forced expiratory volume in 1 s). Cell counts are per mm² of subepithelium. Data are expressed as means ± SEM.
gene expression. All gene expression data were compared by the \( \Delta \Delta C_t \) method (24).

**MAPK activation**

The phosphorylation of MAPKs was measured with the PhosphoTracer ELISA assay (Abcam) following the manufacturer’s instructions.

**Flow cytometry**

After treatment, cells were washed with cold PBS and permeabilized by incubation with ice-cold ethanol for 30 min. Subsequently, cells were stained with propidium iodide (PI) and ribonuclease. PI incorporation was measured by flow cytometry, and data were expressed as proportion of cells within the gated population.

**Gene set variation analysis**

Gene set variation analysis (GSVA) was implemented within the R statistical environment with the R Bioconductor GSVA package (25). This analytical approach estimates the variation in the activity of a gene set over a sample population in an unsupervised manner, producing an enrichment score per subject level. Statistical comparison of enrichment scores was performed utilizing a linear model and Tukey’s test for identification of significantly different means.

**Statistical analysis**

Changes in gene expression from the microarray data was considered to be significant with a false-discovery-rate-adjusted value of \( P < 0.01 \). Significant data were subsequently analyzed by ingenuity pathway analysis. Unless otherwise stated, the data were analyzed by Kruskal-Wallis ANOVA with Dunn’s posttest analysis. Statistical significance was considered to be \( P < 0.05 \).

**RESULTS**

**Spirometry and immunohistochemistry**

Lung spirometry did not differ from baseline after 28 d of treatment with FP/SM (Table 1). At least 2 bronchial biopsy samples from each patient with COPD were sectioned and stained at each time point. Hematoxylin and eosin staining of the samples indicated a wide variety of cell types present in each sample, including epithelial cells, ciliate, airway smooth muscle, goblet cells, and macrophages. Additionally, the extracellular matrix and vascular tissue could be identified. Treatment with FP/SM for 28 d did not significantly affect the staining intensity or the number of inflammatory cells detected (Table 1).

The B-cell numbers were not considered to be a primary end point before the microarray analysis, so the numbers of B cells were not counted; nor were we able to directly measure immunoglobulin levels in the lung mucosa. Unfortunately, insufficient samples remained to retrospectively count the cells in the samples used for this study.

**Effect of FP/SM treatment on gene expression profiles**

RNA was extracted from the bronchial biopsy samples, and changes in gene expression profile due to FP/SM were measured. RNA expression profiles were measured in 16 biopsy samples obtained at baseline and 11 biopsy samples obtained after 28 d of treatment. Significant changes in gene expression were detected in 523 of 17,657 mRNA targets tested (\( P < 0.01 \); 343 were down-regulated and 180 were up-regulated.

The classic steroid inducible immunophilin gene FKBP5/FK506BP was the second most up-regulated gene after FP/SM treatment. Ingenuity pathway analysis indicated that the highest number of significant changes in canonical pathways was in the glucocorticoid signaling pathway, with 277 genes changing. The top networks affected by FP/SM treatment are summarized in Table 2.

However, examination of individual genes indicated little or no effect on many of the genes associated with COPD inflammation, including cytokines such as IL6 and IL8. In contrast, there were significant changes in the expression of genes associated with B-cell development and antibody production. Eight of the 10 most strongly down-regulated genes encoded immunoglobulins, including the top 5 (IGHG3, IGKC, IGKV1,IGHA1,IGHG4)(Table 3). In total, 25 of the 71 probes for immunoglobulin genes showed significant levels of down-regulation, and only one showed a marginal increase in expression (1.08× expression).

The transcription factor GATA3, which is important for the development of T\(_h\)2 cells, activation of mast cells (26), and type 2 innate lymphoid cells (27), as well as the epithelial–mesenchymal transition in breast epithelium (28), was also significantly down-regulated, although the transcription factor TBET (responsible for T\(_{h1}\) development) remained unchanged. Additionally, the TNFSF13 gene, which is involved in B-cell development, was also down-regulated. Full data can be found online (http://dx.doi.org/10.6084/m9.figshare.698230).

In order to validate the gene expression arrays, we performed RT-qPCR on the mRNA isolated from the biopsy samples to determine the expression of the immunoglobulin G heavy chain and the \( \kappa \) light chain encoding genes.

**TABLE 2. Top networks identified by ingenuity pathway analysis of changes in gene expression due to combination ICS/LABA treatment**

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA replication, recombination, and repair, cellular growth and proliferation, cardiac dysfunction</td>
</tr>
<tr>
<td>2</td>
<td>Cell death and survival, cellular growth and proliferation, behavior</td>
</tr>
<tr>
<td>3</td>
<td>Cell cycle, cell death and survival, hematological system development and function</td>
</tr>
<tr>
<td>4</td>
<td>Cellular assembly and organization, cellular compromise, cell cycle</td>
</tr>
<tr>
<td>5</td>
<td>Gene expression, cancer, cellular development</td>
</tr>
</tbody>
</table>
**TABLE 3. Top 50 microarray results by (significant) negative fold change**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene description</th>
<th>Fold change</th>
<th>P</th>
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</thead>
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<td>IGHV1-3</td>
<td>Immunoglobulin heavy variable 1-3</td>
<td>-29.5358</td>
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</tr>
<tr>
<td>IGHD_V0</td>
<td>Immunoglobulin heavy constant δ</td>
<td>-16.63055</td>
<td>0.02888</td>
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<td>IGHG2-V0</td>
<td>Immunoglobulin heavy constant γ 3</td>
<td>-14.2187</td>
<td>0.0039</td>
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<td>IGHM_V0</td>
<td>Immunoglobulin heavy constant μ</td>
<td>-11.89571</td>
<td>0.01051</td>
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<td>IGTV3-I</td>
<td>Immunoglobulin λ variable 3-1</td>
<td>-11.17632</td>
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<tr>
<td>ITLIN1_V0</td>
<td>Intelectin 1 (galactofuranose binding)</td>
<td>-9.40995</td>
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<td>IGKC_V0</td>
<td>Immunoglobulin κ constant</td>
<td>-8.48886</td>
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<td>GALNAC-V0</td>
<td>Glutamate decarboxylase-like 1</td>
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<td>Immunoglobulin heavy constant μ</td>
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<tr>
<td>RERG_V0</td>
<td>RERG/RAS-like</td>
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<td>Immunoglobulin heavy constant μ</td>
<td>-6.63575</td>
<td>0.01581</td>
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<tr>
<td>IGHG4_V0</td>
<td>Immunoglobulin heavy constant γ 4</td>
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<tr>
<td>SLCO5A1-V0</td>
<td>Solute carrier organic anion transporter family, member 5A1</td>
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<td>PTCHD1_V0</td>
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<td>CXCL11-V0</td>
<td>Chemokine (C-X-C motif) ligand 11</td>
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<td>ARB-A_V0</td>
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<td>PPP1R1B_V0</td>
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<td>Actinin, α2</td>
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<td>Immunoglobulin κ constant</td>
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Immunoglobulin and nonimmunoglobulin encoding genes. Full data are available online (http://dx.doi.org/10.6084/m9.figshare.698230).

**IGH1G and IGKC**, respectively. The levels of IGH1G and IGKC were both significantly reduced in the biopsy samples taken from patients with COPD (IGH1G COPD median expression = 9.4 × 10^{-6} relative to 18S vs. 1.8 × 10^{-6} after Seretide; IGKC COPD median = 1.5 × 10^{-5} relative to 18S vs. 2.7 × 10^{-6} after Seretide, $P = 0.0046$ and 0.0037, respectively, by Kruskal-Wallis test followed by Mann-Whitney test) (Fig. 1A, B).

The gene expression of the patients with COPD was compared to controls to determine whether it showed evidence of an increased T1,2 cell gene signature, as identified by Christenson et al. (17). The expression of genes comparing patients with COPD to smokers did not show any significant changes due to disease status, indicating that the patients with COPD did not have an underlying T1,2-driven inflammation: POSTN ($-1.12138 \times, P = 0.69398$), SERPINB2 (1.37392\times, $P = 0.37043$), IGHG3 (1.74835\times, $P = 0.26401$), and IGKC (1.1579\times, $P = 0.48029$).

**GSVA**

GSVA of IGHG3, IGKC, IGKV1, IGHAI, and IGH4G was carried out using published data from the Groningen and Leiden Universities’ study of Corticosteroids in Obstructive Lung Disease (GLUCOLD) study (16). Patients in the GLUCOLD study were enrolled in 3 arms, with placebo alone for 30 mo, FP alone for 6 mo followed by placebo for 24 mo, or a combination of FP and SM for 30 mo.
GSVA of the GLUCOLD data indicated, in agreement with our study, that immunoglobulin gene expression was increased in patients with COPD compared to smoking and nonsmoking controls (Fig. 1C) and that FP alone and in combination with SM reduced immunoglobulin gene expression compared to placebo (Fig. 1D). Patients at the end of the 6 mo of FP had significantly lower gene set expression compared to placebo (0.28× expression, \( P = 0.03 \)), as was the gene set after combination treatment versus placebo (0.31× expression, \( P = 0.02 \)). The effect of treatment was lost after the 24 mo FP withdrawal (FP followed by placebo vs. FP, \( P = -0.47, P < 10^{-4} \), FP followed by placebo vs. FP and SM 0.50, \( P < 10^{-4} \)).

**Effect of FP on antibody production in vitro**

CLNH11.4 cells constitutively produce (IgG1) antibodies, and we tested whether treatment of the cells with FP, SM, or a combination would affect antibody production. Cells were grown overnight in 0% FCS medium in order to reduce any effects of proliferation, after which the cells were resuspended in fresh medium and treated for 24 h. We detected no significant IgM production at 24 h (data not shown) under any conditions. In contrast, the high basal levels of IgG that were detected in the supernatant after 24 h were significantly reduced by FP in a concentration-dependent manner, with a maximal reduction of 61.6% of basal IgG production detected at 10^{-8} M (Fig. 2A). In contrast, SM alone had no significant effect on IgG levels. The combination of FP and SM significantly reduced IgG levels with a maximal effect (57% of basal level) reported at 10^{-6} M of both drugs (Fig. 2A). However, overall, there was no significant difference in efficacy or potency between the FP and the FP/SM treatment groups (Fig. 2B). There was no effect of any of the drugs used on cell viability at 24 h except for a small, albeit significant, effect of FP at 10^{-5} M (data not shown).

FP down-regulates IgG gene expression in human B cells

The effect of FP and SM (both 10^{-8} M) alone and in combination on the expression of *IGH1G* and *IGKC*, the IgG encoding genes expressed in the CLNH11.4 cell line,
was examined by RT-qPCR. The cells were incubated overnight in 0% FCS medium and gene expression measured at baseline and at 2, 4, 6, and 24 h after treatment. FP and combination treatment showed a significant reduction in both IGHL1 (Fig. 2B) and IGKC (Fig. 2C) mRNA expression at 24 h. The expression of these genes was not affected at earlier time points (data not shown).

**Effect of FP on human B-cell proliferation in vitro**

CLNH11.4 cells were incubated with a range of concentrations of FP and SM either alone or in combination for 48 h before BrdU was added to the cells. After an additional 24 h incubation, BrdU incorporation was measured. B-cell proliferation was significantly repressed by FP in a concentration-dependent manner with a maximal effect at $10^{-7}$ M (Fig. 3A). Although SM alone had no effect on proliferation (Fig. 3B), FP/SM-treated cells had a similar response to that seen in cells exposed to FP alone (Fig. 3C). PI staining in the nucleus corresponds to DNA copy number and is used as a marker of cell cycle phase. A representative fluorescence-activated cell sorting plot is shown in Fig. 3D. FP and the FP/SM combination had no effect on the number of cells in G1 (Fig. 3E), but both decreased the proportion of cells in the S (Fig. 3F) and G2/M (Fig. 3G) phases of the cell cycle. In contrast, SM had no effect on the cell cycle in human B cells.

The arrest of cell division was further confirmed by TaqMan RT-qPCR measuring the expression of the cyclin encoding genes CCND1 (Fig. 3H), CCNA2 (Fig. 3I), and CCNB2 (Fig. 3J). The expression of cyclin D encoding CCND1, which would correspond with cell cycle arrest in the G0 phase of the cell cycle, was significantly decreased after treatment with FP and with the FP/SM combination (Fig. 3H–J). SM alone had no effect on cyclin gene expression.

**MAPK activation**

MAPKs have been implicated in the control of cell cycle proliferation and checkpoint control (29). MAPKs, particularly ERKs, are also critical for B-cell development (30). MAPKs are also involved in the reciprocal modulation of corticosteroid function (31), so we investigated the effect of FP and SM, alone and in combination, on MAPK activation using PhosphoTracer after 24 h treatment to monitor kinase activity (Fig. 4). Treatment with FP ($10^{-8}$ M) significantly reduced the levels of phosphorylated ERK-1/2 in human B cells at 24 h (Fig. 4A) without affecting p38 MAPK (Fig. 4B) or JNK phosphorylation (Fig. 4C). SM had no effect on MAPK phosphorylation, and the combination of FP and SM had similar effects on ERK activation (Fig. 4A).

MAPK phosphorylation status is regulated by the activity of dual-specificity protein phosphatases such as DUSP1 (also known as MKP1), which dephosphorylates MAPK proteins (MAPK14, MAPK1, and MAPK8) (32) and has been associated with the anti-inflammatory effects of glucocorticoids (33). We therefore tested if DUSP1 expression was raised in the model cell line after treatment with FP, DUSP1 mRNA expression was increased after 30 min incubation with $10^{-8}$ M FP (Fig. 4D). These elevated levels were maintained for 120 min before decreasing by 240 min (Fig. 4D) and returned to baseline after 24 h (data not shown).

**Effect of oxidative stress on CLNH11.4 cell function**

COPD is associated with increased oxidative stress, which can also reduce corticosteroid efficacy (34). Oxidative stress using hydrogen peroxide ($\text{H}_2\text{O}_2$) did not have a significant effect on cell viability up to a concentration of 3.1 $\mu$M (Fig. 5A), which was used as the maximal concentration in subsequent experiments. $\text{H}_2\text{O}_2$ did not significantly alter immunoglobulin release (Fig. 5A) and IGH1G (Fig. 5B) and IGKC (Fig. 5C) gene expression in CLNH11.4 cells. Cells were pretreated with 1.6 and 3.1 $\mu$M $\text{H}_2\text{O}_2$ for 30 min, after which they were treated with FP for 24 h. Pretreatment with $\text{H}_2\text{O}_2$ did not significantly alter the effects of FP on either immunoglobulin release (Fig. 5E) or on IGH1G (Fig. 5F) and IGKC (Fig. 5G) gene expression.
DISCUSSION

We demonstrated in a pilot study that 4 wk of treatment with FP/SM had a profound effect on the mRNA expression profile in bronchial biopsy samples without any effect on inflammatory cell numbers within the samples. Among the 523 genes with altered expression, many were associated with B-cell function and antibody production. Using in vitro experiments in the CLNH11.4 human B-cell line, we were able to demonstrate that FP alone, but not SM, reduced IgG production at the protein and mRNA level and attenuated B-cell proliferation in a concentration-dependent manner. A similar degree of inhibition was seen in cells cotreated with FP and SM. This effect was associated with changes in ERK MAPK activity and occurred even in the presence of exogenous oxidative stress. Overall, the data suggest that a corticosteroid is able to alter gene expression profiles in COPD biopsy samples even over a short course of treatment and that this was particularly evident against genes involved in antibody production.

While remaining best clinical practice for many patients, prescription of ICS/LABA combinations have been noted for their lack of efficacy in controlling the underlying chronic inflammation in COPD, especially compared to other chronic inflammatory diseases of the lung such as asthma (12, 35–37). Nevertheless, ICS/LABA combinations have significant benefits for patients with COPD, including improving quality of life and reducing exacerbations (8). The relative lack of efficacy of combination therapies and ICS monotherapy has been suggested to be due to the presence of oxidative stress caused by cigarette smoking (38). There is still much debate about the role of ICS in the chronic treatment of COPD, although it is becoming evident that some patients respond more favorably to these drugs (39).

We were unable to demonstrate any effects of FP/SM on spirometry or immune cell numbers in the bronchial biopsy samples of these subjects. These latter results are in contrast to previous studies; for example, Barnes et al. (12) showed significant reduction in T cells and neutrophils, although that was over a longer study duration (13 wk compared to 4 wk) and in a larger study population. In addition, expression profiling shows no significant change in the genes normally associated with COPD inflammation such as the interleukins IL6 and IL8 after 4 wk of FP/SM treatment. In contrast, many genes associated with the B-cell/T2-driven immune response were down-regulated. Of particular note, GATA3, encoding the GATA3 transcription factor, which is a key driver of naive T cell

Figure 3. Regulation of human B-cell proliferation and cell cycle by FP. A–C) Concentration-dependent effect of FP (A), SM (B), and their combination (both at same concentration) (C) on proliferation of CLNH11.4 B cells was measured by BrdU incorporation after 24 h. Results are expressed as means ± SEM of 3–7 independent experiments. D) DNA staining with PI enables detection of cell cycle phases according to copies of DNA present and is shown with representative flow cytometry plot. E–G) Neither FP nor SM (both at 10⁻⁸ M) affected G1 phase (E), but FP suppressed percentage of cells in both S phase (F) and G2/M phase (G), whereas SM was without effect. Results are expressed as means ± SEM of ≥5 independent experiments. H–J) FP, SM, and FP-SM significantly reduced expression of cyclin D1 (H) but not of A2 (I) and B2 (J) compared to control unstimulated cells. All proliferation experiments were carried out in medium containing 10% FCS. *P < 0.05, **P < 0.01.

MODULATION OF IMMUNOGLOBULIN IN COPD 2021
Figure 4. Effect of FP and SM on activation of MAPKs in human B cells. A) FP (10^{-8} M) alone and in combination with SM (10^{-8} M) significantly reduced phosphorylation of ERK-1/2 MAPK at 24 h, as determined by PhosphoTracer. B, C) FP had no effect on p38 MAPK (B) or JNK (C) activation. SM alone had no effect on phosphorylation of any MAPK. Phosphorylation data were normalized to control treatment of 10% FCS. D) FP (10^{-8} M) induced rapid time-dependent induction of DUSP1 gene mRNA expression that was maximal at 30 min and remained elevated until 120 min. Results are expressed as means ± SEM of 3 independent experiments. *P < 0.05.

differentiation into T_{h}2 cells, was down-regulated (1.9×) after combination treatment. The effect on GATA3 is consistent with previously published work on the effects of glucocorticoids (40, 41). In contrast to the down-regulation of GATA3, the T_{h}1 cell transcription factor T-Bet and associated T_{h}1 cell cytokines were unaltered after combination steroid treatment. These data, taken together, may indicate a selective effect of combination treatment on T_{h}2 cell development. Whether the lack of effect on the T_{h}1 cell response in the patients with COPD is due to the oxidative stress caused by cigarette smoking or epigenetic changes, such as reduction in HDAC2 in the lungs (38), and whether these mechanisms still play a role in the T_{h}2/B-cell adaptive immune response are beyond the scope of this study.

Recent gene expression analysis has indicated that ICS and combination therapy can modulate a COPD-associated gene signature in bronchial biopsy samples and brushings of patients with COPD (15, 16) and has also been reported in patients with ACOS (17). The post hoc analysis of T_{h}2 cell gene expression signatures carried out, combined with the fact that our patients had no clinical history of asthma, means that our results’ being due to ACOS is unlikely, although this cannot be ruled out.

In this study, FP/SM treatment significantly down-regulated the levels of genes associated with mature B cells, such as immunoglobulin encoding genes (e.g., IGH3, IGKC, IGKV1, IGHA1, and IGHG4) in bronchial biopsy samples.

Unfortunately, we were unable to confirm these effects directly on B-cell counts within our patients; however, previous studies have indicated the corticosteroids can reduce B-cell proliferation, for example in healthy volunteers (42). Similarly, a reduction in plasma cell numbers was found in the GLUCOLD study, and, conforming to our in vitro data, this response was mediated by FP but not enhanced by concurrent SM treatment (43).

The reproducibility of our results were confirmed by testing previous data released as part of the GLUCOLD study (16) using GSEA (25). Analysis of the combined expression profile of the top immunoglobulin genes identified in our study showed a trend to increased expression in patients with COPD, although the small data set prevents this from reaching significance. As with our study, the GLUCOLD cohort showed decreased immunoglobulin expression after FP treatment, alone and in combination with SM, and this reduction was reversed when the treatment was withdrawn. These data, taken together with ours, indicate that reduced immunoglobulin production occurs in COPD as a result of glucocorticoid treatment. Further experiments would be required to determine whether this effect occurs only in the lungs or is systemic.

The production of autoimmune antibodies in the latter stages of COPD has been previously reported (44–46) and may arise as a result of oxidative stress modifying “self”-proteins, allowing their recognition by the immune system. If autoantibody production is a key driver of the latter stages of COPD, helping to explain the continuance of the disease after smoking cessation, prevention of the development of or in the control of autoimmunity may explain some of the clinical effects of combination therapy seen in patients with COPD (47). Corticosteroid use increases the risk of lung infection, including pneumonia (48). This increased risk may be due to the reduction in the levels of protective immunoglobulins. For example, patients with reduced immunoglobulin due to common variable immunodeficiency disease have an increased risk of pneumonia, which can be reduced through intravenous immunoglobulin treatment (49). It is possible that the reduced immunoglobulin level could either allow new
did it alter the response to FP. While this result re
from CLNH11.4 cells at the concentrations tested; nor
m
3.1
tive stress did not signi
any, oxidative stress would have on our model. Oxida-
types (52), we attempted to determine what effect, if
MTT assay, was only signi
major events such as activation and
proliferation (54), it is unclear whether combination
proliferation (54), it is unclear whether combination
treatment is having a direct effect on B-cell gene ex-
proliferation in vivo. Studies using T-cell-deficient nude mice in models of COPD may be able to resolve this issue.

There were several limitations to the clinical part
which the oxidative stress sensitivity in the CLNH11.4
cells reflect those of B cells in vivo are unknown but will be of interest for further research into the mechanisms of antibody-driven immune responses in COPD.

Although we have confirmed previous reports indicating
that corticosteroids can have a direct effect on B cells, particularly on early-stage events such as activation and proliferation (54), it is unclear whether combination treatment is having a direct effect on B-cell gene expression in vivo in patients with COPD or is having an indirect effect by altering the T-helper/T-suppressor cell ratio. Studies using T-cell-deficient nude mice in models of COPD may be able to resolve this issue.

There were several limitations to the clinical part
of this study linked to the small sample size and the lack of patients with COPD treated with placebo or individual monotherapies. This raises the possibility that the changes seen may reflect disease variability over time rather than a true treatment effect. In addition, the biopsies were performed 24 hours after the final treatment and not at the optimal time (4–8 hours) predicted to detect changes in gene expression. However, we were able to detect changes in the expression of known corticosteroid-inducible genes, and the top canonical pathway identified by Ingenuity was glucocorticoid signaling. We have confirmed the effect of FP on gene expression in vitro, and we believe that the data provide valuable insights into the effects of FP/SM in patients with COPD.

Our data suggest that FP alone or in combination with SM leads to the transactivation of DUSP1 and the dephosphorylation of ERK-1/2 in B cells (Fig. 6). This in

Figure 5. Effects of hydrogen peroxide (H$_2$O$_2$) on human B-cell IgG expression and FP actions in human B cells. A–C) H$_2$O$_2$ significantly reduced production of total IgG release (A), IGH1G (B), and IGKC (C) mRNA expression (encoding heavy and light chains, respectively) from human CLNH11.4 B-cell line in concentration-dependent manner. D) Cell viability, as measured by MTT assay, was only significantly affected by H$_2$O$_2$ at highest concentration tested. E–G) Pretreatment of cells with H$_2$O$_2$ (1.5 or 3.1 μM, 2 h) did not affect ability of FP (10^{-8} M) to suppress IgG release (E) or mRNA expression of (F) IGH1G and (G) IGKC without affecting cell viability (data not shown). Results are expressed as means ± SEM of four independent experiments. *P < 0.05 compared to control.

pathogens to colonize the lung or predispose patients
who are already colonized to develop an infection.

In order to investigate the direct effects of FP on B cells, we used the human myeloma cell line CLNH11.4, which expresses surface immunoglobulin (IgG1, κ light chain). Our results show that FP significantly down-regulated both proliferation and IgG production (both mRNA and protein) from these cells. Furthermore, the effects of FP include the inhibition of ERK phosphorylation associated with increased DUSP1 (MKP-1) transcription. While DUSP-1 has previously been shown to be strongly expressed in B cells (50) and ERK MAPK function has been shown to be important for B-cell function (51), for the first time we have identified this pathway as a target of corticosteroids in B cells.

Because of the importance of oxidative stress in COPD and in modulating corticosteroid efficacy in several cell types (52), we attempted to determine what effect, if any, oxidative stress would have on our model. Oxidative stress did not significantly alter IgG production from CLNH11.4 cells at the concentrations tested; nor did it alter the response to FP. While this result reflects our in vivo results in suggesting that B cells remain glucocorticoid sensitive under oxidative stress conditions, these data may also equally reflect the high sensitivity of the cell type to oxidative stress. H$_2$O$_2$-induced corticosteroid insensitivity in epithelial and monocytic cells (e.g., BEAS2B and THP-1 cells, respectively) tends to occur at high concentrations (≥100 μM) (52, 53), and therefore it is possible that B cells do not survive at the levels of oxidative stress required to affect steroid responsiveness. The mechanisms involved and extent to
Figure 6. Glucocorticoid activation of DUSP1 inhibits B-cell proliferation and immunoglobulin gene and protein expression. In absence of glucocorticoids (gray), B-cell activation, for example through tyrosine kinase receptors, leads to activation of signaling pathways such as MAPK cascade, which includes phosphorylation of ERK-1/2 by MEK-1/2. Phosphorylated ERK-1/2 in turn activate various transcription factors such as c-Myc, which leads to transcription of immunoglobulin encoding genes (including IGKC and IGH1G) and cyclin genes that drive cell proliferation (e.g., cyclin D encoding CCND1). Glucocorticoids (GC) enter cell and bind to glucocorticoid receptor (GR). GC:GR complex translocates to nucleus and acts as transcription factor for several genes including DUSP1, which encodes DUSP1 protein. DUSP1 in turn dephosphorylates, and thereby inactivates, ERK-1/2, inhibiting immunoglobulin gene expression and cell proliferation (pathway shown in black).


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