Reduced nuclear factor- κ B repressing factor: a link toward systemic inflammation in COPD

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ABSTRACT: Chronic systemic inflammation is implicated in the systemic manifestations and, probably, the excess mortality risk of chronic obstructive pulmonary disease (COPD). The role of nuclear factor (NF)- κ B repressing factor (NRF), a DNA-binding, protein-inhibiting NF- κ B response gene, in human diseases has not been explored. We hypothesised that the NRF-negative regulatory mechanism is impaired in COPD peripheral blood mononuclear cells (PBMCs) leading to excessive interleukin (IL)-8/CXCL8 production.

NRF expression, NF- κ B activation, IL-8/CXCL8 release and intracellular oxidative stress were assessed in PBMCs of normal subjects and stable COPD patients. Primary PBMCs with NRF overexpression, NRF knockdown and exposure to H₂O₂ were used to elucidate the mechanisms.

Stable COPD patients, especially those with severe COPD, showed decreased NRF expression, enhanced NF- κ B activation and increased IL-8/CXCL8 release in PBMCs compared with normal subjects. This was associated with reduced NRF and increased RNA polymerase II occupancy at the IL-8/CXCL8 promoter. NRF knockdown enhanced IL-8/CXCL8 production in normal PBMCs, whilst NRF overexpression attenuated IL-8/CXCL8 production. Intracellular oxidative stress was increased in COPD PBMCs. H₂O₂-decreased NRF expression and -enhanced IL-8/CXCL8 production was augmented in COPD PBMCs.

NRF expression is reduced in PBMCs of stable COPD patients, probably through oxidative stress, leading to increased production of IL-8/CXCL8 and potentially chronic systemic inflammation.

KEYWORDS: Interleukin-8/CXCL8, oxidative stress, peripheral blood mononuclear cell, RNA polymerase II

Chronic obstructive pulmonary disease (COPD) is characterised by an abnormal inflammatory response of the lung to noxious particles or gases, in which neutrophils, alveolar macrophages and CD8⁺ T-cells are all implicated [1, 2]. In addition to the local inflammation, consistent evidence reveals that circulating inflammatory mediators, such as interleukin (IL)-6, tumour necrosis factor (TNF)- α , IL-8/CXCL8 and C-reactive protein (CRP), and leukocytes are increased or activated in stable disease conditions [3, 4]. This is further enhanced either when the disease is severe or during exacerbations [5, 6]. Such systemic inflammation is increasingly recognised as a manifestation of COPD and has been implicated in systemic effects and probably

the excess mortality of COPD [7, 8], especially from cardiovascular diseases or cancer [9]. The precise mechanisms mediating systemic inflammation remain unclear.

Nuclear factor (NF)- κ B repressing factor (NRF) is a transcriptional repressor that is constitutively expressed in all human tissues tested [10]. Endogenous NRF has been implicated in the basal silencing of specific NF- κ B targeting genes, including interferon- β , IL-8/CXCL8 and inducible nitric oxide synthase [10–12] *via* the negative regulatory elements (NREs) in the promoters [10]. NRF seems to mediate full induction of IL-8/CXCL8 by IL-1 β [11]. Nevertheless, our previous reports indicate that NRF is inducible and still plays a repressive

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role in response to stimulation of TNF- α [13], an elevation of which is consistently reported in the serum of COPD patients [3, 4]. Whilst most of the knowledge about NRF is established with *in vitro* models using cell lines, its role in human diseases has not been elucidated.

IL-8/CXCL8, a potent chemokine for neutrophil and monocyte, has long been known to contribute to the pathogenesis of COPD through recruiting these leukocytes into the lung [14]. Recent reports also suggest that IL-8/CXCL8 is associated with small airway remodelling in smokers [15], and that it might regulate mucin gene expression [16] and inhibit lung fibroblast proliferation [17]. Systemically, IL-8/CXCL8 in the serum is correlated with muscle weakness in hospitalised and in stable patients with COPD [18] and might be implicated in the pathogenesis of cardiovascular disease [19], a major comorbidity of COPD. Targeting IL-8/CXCL8 is therefore of potential therapeutic benefit in COPD [20]. This may be achieved through understanding the mechanism whereby IL-8/CXCL8 is controlled and dysregulated in COPD. As IL-8/CXCL8 has been comprehensively studied as an NRF-controlled gene, we therefore used this chemokine as the end-point for our investigation.

We hypothesised that NRF-negative regulatory mechanism is impaired in circulating inflammatory cells from COPD patients, leading to enhanced release of IL-8/CXCL8. In addition, it is appreciated that oxidative stress contributes to several of the systemic manifestations in COPD [21], and is an important regulator of IL-8/CXCL8 production [22, 23]. We also studied the hypothesis that oxidative stress is involved in the impairment of the NRF-negative regulatory mechanism.

MATERIAL AND METHODS

Study population

We studied 20 patients with stable COPD. Diagnosis and severity grading were according to the guidelines of the Global Initiative for Obstructive Lung Disease (GOLD) [24]. Patients were in a stable condition and were without acute exacerbations in the previous 3 months, defined as no requirement for antibiotic or oral corticosteroid therapy and no change in respiratory symptoms. Patients were excluded if they had known asthma, bronchiectasis, tuberculosis, pneumoconiosis, heart disease, malignancy, or other infectious, inflammatory or metabolic conditions. We also recruited 10 nonsmoker control (NSC) subjects and 10 smoker control (SC) subjects whose forced expiratory volume in 1 s (FEV₁) values were >80% of predicted and FEV₁/forced vital capacity ratios were >75%. This study was approved by the institutional review board of Chang Gung Memorial Hospital (Taipei, Taiwan). All patients and control subjects signed an informed consent document before study inclusion.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs), isolated by Ficoll–Hypaque density gradient centrifugation [25], were incubated in 24-well culture plates at 1 × 10⁶ PSMCs per mL for 18 h. The cultured supernatants were collected for determination of IL-8/CXCL8.

Western blotting

NRF in whole-cell extracts was detected by Western blotting with an antibody specific for NRF as described previously [13].

Immunostaining and confocal laser microscopic analysis

PBMCs were plated onto 24-mm diameter round coverslips in six-well plates. NRF was immunostained and images were acquired with a confocal laser-scanning microscope (Leica, Exton, PA, USA) as described previously [13]. Hoechst dye (Sigma Chemical Co., St. Louis, MO, USA) was used as nuclear stain.

RT-PCR and quantitative real time PCR

RT-PCR and quantitative real time PCR (qPCR) were performed as previously described [13]. qPCR was performed in a LightCycler® 2.0 System (Roche Applied Science, Indianapolis, IN, USA) using LightCycler® DNA Master SYBR Green I (Roche Applied Science).

ELISA

IL-8/CXCL8 protein concentrations in the supernatants, diluted 50-fold, were measured by a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Determination of oxidative stress

Oxidative stress in PBMCs was determined by using the redox-sensitive dye dichlorodihydrofluorescein (DCFH) as a probe. Fluorescence from 2',7'-dichlorofluorescein, a green fluorescent oxidation product of DCFH, was analysed with a FACScan flow cytometer (Becton Dickinson, Bohemia, NY, USA) (excitation 488 nm and emission 610 nm).

TABLE 1 Characteristics of the study subjects				
Characteristic	Controls		COPD	
	NSCs	SCs	GOLD I or II	GOLD III or IV
Subjects	10	10	10	10
Age yrs	65.4 ± 3.0	60.7 ± 2.3	65.1 ± 2.1	70.8 ± 1.6
Sex M/F	7/3	9/1	6/4	10/0
Smoking pack-yrs	0	53.8 ± 5.3**	51.7 ± 8.4**	59.0 ± 13.3**
FEV ₁ /FVC %	83.6 ± 1.8	82.4 ± 1.4	64.2 ± 3.0**.#	56.3 ± 4.2***.###
FEV ₁ % pred	94.0 ± 3.6	95.0 ± 3.1	63.6 ± 2.5*.#	35.8 ± 3.4***.###
BMI kg·m ⁻²	24.2 ± 1.3	24.3 ± 1.0	24.5 ± 1.2	22.3 ± 0.8
Medications				
Oral corticosteroid	0	0	0	1
ICS	0	0	5	8
Theophylline	0	0	0	6
Data are expressed as n or mean ± SEM. COPD: chronic obstructive pulmonary disease; NSC: nonsmoker control; SC: smoker control; GOLD: Global Initiative for Chronic Obstructive Lung Disease; M: male; F: female; FEV ₁ : forced expiratory volume in 1 s; FVC: forced vital capacity; % pred: % predicted; BMI: body mass index; ICS: inhaled corticosteroid. *: p<0.05; **: p<0.01; ***: p<0.001 compared with NSCs; #: p<0.05; ###: p<0.001 compared with SCs.				

FIGURE 1. Expression of nuclear factor-κB repressing factor (NRF) in peripheral blood mononuclear cells (PBMCs) of nonsmoker control (NSC) subjects, smoker control (SC) subjects and patients with chronic obstructive pulmonary disease (COPD). **a)** Immunoblot analysis of NRF expression in PBMC lysates of subjects with and without COPD. The upper panel shows a representative blot. The lower panel shows the quantification of NRF in PBMCs from NSC subjects ($n=10$), SC subjects ($n=10$) and patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage I-II ($n=10$) and GOLD stage III-IV ($n=10$) COPD by densitometry analysis of immunoblot normalised to β-actin. **b)** RT-PCR analysis of NRF mRNA expression in PBMCs from normal subjects and patients with COPD. The upper panel shows a representative picture. The lower panel shows the quantification of NRF mRNA in NSC subjects ($n=9$), SC subjects ($n=9$) and patients with GOLD stage I-II ($n=6$) and GOLD stage III-IV ($n=7$) COPD by densitometry analysis of RT-PCR normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data in **a)** and **b)** are represented as mean $\pm$ SEM. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. **c-h)** Confocal images of NRF expression in PBMCs from normal subjects and from COPD patients. PBMCs were stained with pre-immune immunoglobulin (IgG) (Cy3; **c** and **f**) or an antibody specific for NRF (Cy3; **d**, **e**, **g** and **h**, and magnified inserts). Hoechst dye was used as nuclear stain (**f-h** and magnified inserts). Images from nuclear stain (**f-h**) and IgG (**f**) or NRF (**g** and **h**) were merged to show the subcellular localisation of NRF. Data shown are representative of three normal subjects and three COPD patients (GOLD stage III-IV). **c-h)** Scale bars=25 μ m; **d**, **e**, **g** and **h** magnified inserts) scale bars=10 μ m.

Chromatin immunoprecipitation assay

Occupancy of NRF and RNA polymerase II at the *IL-8/CXCL8* promoter was determined by chromatin immunoprecipitation (ChIP) assays as described previously [13, 26].

NRF overexpression and RNA interference

Small interfering RNA (siRNA) (si-NRF, siCONTROL) and plasmid DNA (a pCMV based NRF expression construct) were introduced into PBMCs by nucleofection using monocyte Nucleofector kits (Amaxa Inc., Gaithersburg, MD, USA) as previously described [13].

Statistics

Data were analysed with Prism 5.01 (GraphPad Software, San Diego, CA, USA). Data are presented as the mean $\pm$ SEM. ANOVA was performed using the Kruskal–Wallis test with the Dunn's multiple comparison test to compare all pairs of variances. A post-test for linear trend was also performed. Spearman's correlation coefficients were performed to evaluate the relationships of NRF expression and IL-8/CXCL8 production, of NRF and RNA polymerase II occupancy at the *IL-8/CXCL8* promoter, of oxidative stress and NRF expression and of oxidative stress and IL-8/CXCL8 production. For the *in vitro* experiments, the one-way ANOVA with Dunnett's multiple comparison test was performed to compare all groups *versus* the control and paired t-test was used to compare the same group before and after treatments. Differences were considered significant when the p-value was <0.05 .

RESULTS

Expression of NRF in PBMCs of normal subjects and COPD patients

Table 1 shows the demographic characteristics of the study subjects. Western blotting revealed a decline in the expression NRF in PBMCs of patients with COPD compared with those of normal subjects (fig. 1a). NRF expression in PBMCs was 0.90-fold in GOLD stage I–II COPD and 0.64-fold ($p<0.01$) in GOLD stage III–IV COPD as compared with that in NSC subjects, whereas it was 0.81-fold and 0.57-fold ($p<0.001$) that of SC subjects, respectively (fig. 1a, lower panel). Post-test for linear trend revealed a significant severity-associated decline of NRF in COPD compared with NSC subjects ($p<0.0001$) and SC subject ($p<0.0001$). The decline in the expression of NRF was not seen in SC subjects compared with NSC subjects, supporting a relationship with COPD rather than an effect of smoking. The ratio of monocytes to lymphocytes in PBMCs was similar among all study groups (fig. S1), precluding a decline of NRF simply due to an increase of one subset of these cells in stage III–IV COPD patients. Confocal microscopy studies confirmed the nuclear predominant localisation of NRF and its reduction in individual cells (fig. 1c). Using samples from part of the study subjects enough for mRNA determination, it was demonstrated that the amount of NRF mRNA was decreased in PBMCs of GOLD stage III–IV COPD (0.66-fold) compared with those of NSC subjects ($p<0.05$) (fig. 1b).

IL-8/CXCL8 release and its relationship with NRF in PBMCs of controls and COPD patients

Figure 2a demonstrates that PBMCs release more IL-8/CXCL8, albeit not significant, in GOLD I–II COPD patients

(551.6 ± 81.2 pg·mL⁻¹). This was remarkably augmented in GOLD III–IV COPD (761.1 ± 107.0 pg·mL⁻¹) compared with that in NSC subjects (288.9 ± 27.7 pg·mL⁻¹, $p<0.01$) and in SC subjects (351.2 ± 51.0 , $p<0.05$). A significant COPD severity-associated increase in IL-8/CXCL8 was also demonstrated by post-test for linear trend compared with NSC subjects ($p<0.001$) and with SC subjects ($p<0.01$). Correlation analysis demonstrated negative relationships between NRF and IL-8/CXCL8 (fig. 2b; Spearman $r=-0.5680$, $p<0.001$). The relationship holds when the analysis was performed on COPD patients only (Spearman $r=-0.4980$; $p<0.05$).

FIGURE 2. Release of interleukin (IL-8/CXCL8) from peripheral blood mononuclear cells (PBMCs) and its relationship with nuclear factor- κ B repressing factor (NRF) expression. a) Washed PBMCs from nonsmoker control (NSC) subjects (n=10), smoker control (SC) subjects (n=7) and patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage I–II (n=10) and GOLD stage III–IV (n=10) chronic obstructive lung disease (COPD) were incubated in complete medium for 18 h. The concentration of IL-8/CXCL8 in the supernatants was determined by ELISA. Data represented as mean $\pm$ SEM. *: $p<0.05$; **: $p<0.01$. b) Spearman correlation analysis of NRF expression and IL-8/CXCL8 release of PBMCs from NSC subjects (n=10), SC subjects (n=7) and patients with GOLD stage I–II (n=10) and GOLD stage III–IV (n=10) COPD. The solid line represents the dose–response relationship based on simple linear models of NRF expression *versus* IL-8/CXCL8 release. Dotted lines represent the 95% confidence interval.

FIGURE 3. Activation of nuclear factor (NF)-κB in peripheral blood mononuclear cells (PBMCs) of nonsmoker control (NSC) subjects and patients with chronic obstructive pulmonary disease (COPD). a) Western blotting analysis of inhibitor of NF-κB (IκBα) phosphorylation in PBMC from normal subjects and Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage III–IV COPD patients (upper panel) and the quantification by densitometry (lower panel). b–g) Confocal images of p65 in PBMCs from normal subjects and from COPD patients. PBMCs were stained with pre-immune immunoglobulin (IgG) (Cy3; b and e) or an antibody specific for p65 NF-κB (Cy3; c, d, f and g, and magnified inserts). Hoechst dye was used as nuclear stain (e–g and magnified inserts). Images from nuclear stain (e–g) and IgG (e) or NRF (f and g) were merged to show the subcellular localisation of NF-κB repressing factor. Data shown are representative of three normal subjects and three COPD patients (GOLD III–IV). h) p65 TransAM analysis of NF-κB DNA binding activity in nuclear extracts of PBMCs from NSC subjects (n=5), smoker control (SC) subjects (n=6), GOLD stage I–II COPD patients (n=6) and GOLD stage III–IV COPD patients (n=5). Data are represented as mean ± SEM in arbitrary units. i) Chromatin immunoprecipitation (IP) analysis of p65 NF-κB binding at the native interleukin IL-8/CXCL8 promoter. Data are represented as mean ± SEM of the ratio to the input control from at least three subjects in each group. j) ELISA analysis of IL-8/CXCL8 release in the cultured supernatants of PBMCs from patients with GOLD stage III–IV COPD for 18 h in the absence or presence of pyrrolidine dithiocarbamate (PDTC; 30 μM) pre-treatment. Data are mean ± SEM from three independent experiments using cells from different subjects. b–g) Scale bar=25 μm. c, d, f and g magnified inserts) scale bars=10 μm. *: p<0.05; **: p<0.001.

Activation of NF-κB in PBMCs of normal subjects and COPD patients

Activation of NF-κB was increased in stage III–IV COPD PBMCs, demonstrated by Western blotting analysis of inhibitor of NF-κB (IκBα) phosphorylation (fig. 3a) and confocal microscopy analysis of p65 NF-κB nuclear translocation (fig. 3b–g). The latter was also confirmed by quantification analysis using p65 TransAM, a NF-κB DNA binding ELISA kit (fig. 3h, p<0.01), and by ChIP analysis of p65 occupancy at the native *IL-8/CXCL8* promoter (fig. 3i). The functional role of NF-κB is supported by the observation that the NF-κB inhibitor pyrrolidine dithiocarbamate

(PDTC, 30 μM) attenuated IL-8/CXCL8 release from PBMCs in these patients (fig. 3j).

NRF suppresses IL-8/CXCL8 production in primary PBMCs in vitro

Knockdown of NRF (fig. 4a) by siRNA targeting NRF (si-NRF) significantly increased the release of IL-8/CXCL8 compared with that from nontargeting siRNA (scrambled, fig. 4c). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays showed similar cellular viability between si-NRF and scrambled siRNA transfected cells (data not shown). Those

FIGURE 4. Functional effects of nuclear factor- κ B repressing factor (NRF) on interleukin (IL)-8/CXCL8 production. a, c) Effects of NRF knockdown on IL-8/CXCL8 production. Primary peripheral blood mononuclear cells (PBMCs) from normal subjects were transfected with siCONTROL (Dharmacon Inc., Chicago, IL, USA) nontargeting small interfering RNA (siRNA) (scrambled) or siRNA targeting NRF (si-NRF). After 48 h, the level of NRF in cell lysates was determined by Western blotting using the antibody specific for NRF (a). In parallel experiments, transfected cells were collected and were incubated in fresh complete medium for 18 h. The concentration of IL-8/CXCL8 in the supernatants was determined by ELISA. Data represented as mean $\pm$ SEM from four normal subjects in independent experiments (c). b, d) Effects of NRF overexpression on IL-8/CXCL8 production. Primary PBMCs from normal subjects and from chronic obstructive pulmonary disease (COPD) stage III–IV Global Initiative for Chronic Obstructive Lung Disease (GOLD) patients were transfected with the empty vector (pCMV) or the NRF expression plasmid (pNRF). After 24 h, the level of NRF in cell lysates was determined by Western blotting using the antibody specific for NRF (b). In parallel experiments, transfected cells were collected and were incubated in fresh complete medium for 18 h. The concentration of IL-8/CXCL8 in the supernatants was determined by ELISA. Data represented as mean $\pm$ SEM from six normal subjects and from four patients with GOLD stage III–IV COPD in three independent experiments (d). *: p<0.05.

data indicate that endogenous NRF suppresses basal production of IL-8/CXCL8 in PBMCs and support that down-regulation of NRF in COPD PBMCs causatively enhances the production of IL-8/CXCL8.

In contrast, PBMCs transfected with pNRF (fig. 4b) produced less IL-8/CXCL8 in patients with stage III–IV COPD (p<0.05) and in normal subjects (p<0.05) compared with those transfected with the empty vector pCMV, respectively (fig. 4d). It is noteworthy that NRF overexpression reduced IL-8/CXCL8 release from stage III–IV COPD PBMCs to a level similar to that from the empty vector controls of normal subjects (fig. 4d). These observations indicate that restoring the amount of NRF can ameliorate the enhanced production of IL-8/CXCL8 in COPD.

NRF suppresses the IL-8/CXCL8 promoter in PBMCs

Both NRF knockdown in COPD PBMCs (fig. S2A) and overexpression in normal PBMCs (fig. S2b) did not affect the DNA binding activity of NF- κ B, supporting a suppressive mechanism at a level after NF- κ B DNA binding [10]. ChIP assays revealed that NRF overexpression induced a remarkable amount of NRF binding to the IL-8/CXCL8 promoter (fig. S2c) and led to removal

of RNA polymerase II, the major component of the transcriptional machinery, from the promoter (fig. 5a). These experiments provided direct evidence that NRF functionally suppresses the IL-8/CXCL8 promoter in human PBMCs. Further ChIP assays using PBMCs from normal subjects (n=3) and stage III–IV COPD patients (n=5) revealed that occupancy of NRF at the IL-8/CXCL8 promoter in COPD was 0.45-fold that of normal subjects (fig. 5b, p<0.05). In contrast, RNA polymerase II occupancy at the promoter was 2.5-fold in COPD compared with that in normal subjects (fig. 5c, p<0.05). Correlation analysis demonstrated negative relationships between NRF and RNA polymerase II (fig. 5d, Spearman $r = -0.8095$; p<0.05), supporting that down-regulation of NRF in COPD PBMCs results in de-repression of the promoter and therefore enhancement in transcriptional initiation.

Oxidative stress in PBMCs of normal subjects and COPD patients

PBMCs from six normal subjects and eight stage III–IV COPD patients, whose blood had been used for analysis of IL-8/CXCL8 production and NRF expression (figs 1 and 2) and was enough for further studies, were used for intracellular oxidative

FIGURE 5. Nuclear factor- κ B repressing factor (NRF) suppresses the interleukin (*IL*)-8/*CXCL8* promoter. a) Chromatin immunoprecipitation (ChIP) assay analysis of primary peripheral blood mononuclear cells (PBMCs) from normal subjects transfected with the empty vector (pCMV) or the NRF expression plasmid (pNRF) using antibodies specific for RNA polymerase II. Quantitative real-time PCR was used to quantify the immunoprecipitated (IP)-DNA with the primer pairs amplifying a region including the negative regulatory element of the *IL*-8/*CXCL8* promoter. Data were normalised by the input control and were expressed as the ratio to the input control. Representative data from two normal subjects in independent experiments with similar results are shown. b and c) ChIP assay analysis of occupancy of NRF and RNA polymerase II at the *IL*-8/*CXCL8* promoter in primary PBMCs from normal subjects and from Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage III-IV chronic obstructive pulmonary disease (COPD) patients. Data are mean $\pm$ SEM of the ratio to the input control from three normal subjects and from five GOLD stage III-IV COPD patients in three independent experiments. d) Spearman correlation analysis of NRF and RNA polymerase II occupancy at the *IL*-8/*CXCL8* promoter using data from b) and c). The solid line represents the dose-response relationship based on simple linear models of NRF *versus* RNA polymerase II. Dotted lines represent the 95% confidence interval. NSC: nonsmoker control. *: $p < 0.05$.

stress analysis (fig. 6a). We found a 2.4-fold increase in oxidative stress in COPD PBMCs compared with that in normal PBMCs (fig. 6b, $p < 0.05$). The intensity of oxidative stress was negatively correlated with the level of NRF (fig. 6c; Spearman $r = -0.8254$, $p < 0.001$), whilst positively correlated with the production of IL-8/*CXCL8* (fig. 6d; Spearman $r = 0.7133$, $p < 0.001$).

Effect of oxidative stress on NRF expression and IL-8/*CXCL8* release and effect of dexamethasone

H_2O_2 at 100 μ M significantly reduced the amount of NRF in stage III-IV COPD PBMCs (fig. 7a, left panels; $p < 0.05$), an effect prevented by 30 min pre-treatment with the oxidant scavenger

N'-acetyl-L-cysteine (10 mM; $p < 0.01$). It is noteworthy that H_2O_2 decreased NRF in normal PBMCs at the higher concentration (fig. 7a, right panels, 300 μ M; $p < 0.001$ *versus* controls). Although H_2O_2 at 100 μ M was enough for full activation of NF- κ B in normal PBMCs (fig. 7b), a significant induction of IL-8/*CXCL8* was only seen at 300 μ M (fig. 7c) (299.3 ± 77.8 *versus* 122.9 ± 34.4 pg·mL $^{-1}$; $p < 0.001$). Similar to the effect on NRF, H_2O_2 at 100 μ M stimulated IL-8/*CXCL8* release from stage III-IV COPD PBMCs (fig. 7c) (986.6 ± 171.4 *versus* 468.5 ± 100.8 pg·mL $^{-1}$, $n = 6$; $p < 0.05$). An involvement of NF- κ B in basal and H_2O_2 -stimulated IL-8/*CXCL8* was confirmed by PDTC attenuation of IL-8/*CXCL8* production (fig. 7d).

FIGURE 6. Intracellular oxidative stress in primary peripheral blood mononuclear cells (PBMCs) of normal subjects and chronic obstructive pulmonary disease (COPD) patients and its relationship to nuclear factor- κ B repressing factor (NRF) expression and to interleukin (IL)-8/CXCL8 production. a) Fluorescence-activated cell sorter analysis of intracellular oxidative stress by measuring green fluorescence due to 2',7'-dichlorofluorescein (DCF), the oxidation product of dichlorodihydrofluorescein. A representative histogram of DCF fluorescence intensity in PBMCs from a Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage III COPD patient and from a normal subject is shown. The two peaks of fluorescence intensity for each subject were predominantly from monocytes (the higher intensity one) and lymphocytes (the lower intensity one). b) Mean fluorescence intensity (MFI) of DCF in PBMCs from nonsmoker control (NSC) subjects (n=6) and from GOLD stage III-IV COPD patients (n=8). Data are presented as mean $\pm$ SEM of MFI. Spearman correlation analysis of c) intracellular oxidative stress and NRF expression and d) intracellular oxidative stress and IL-8/CXCL8 release. The solid lines represent the dose-response relationship based on simple linear models of c) DCF MFI versus NRF expression and d) DCF MFI versus IL-8/CXCL8 release. Dotted lines represent 95% confidence interval. *: $p < 0.05$.

Most of the patients with stage III-IV COPD were taking inhaled corticosteroid or oral corticosteroid. The potential effect of corticosteroid on NRF expression in normal PBMCs was also studied. The data revealed that dexamethasone did not reduce the expression of NRF; indeed it slightly increased NRF at higher concentrations, *e.g.* 10^{-6} μ M (fig. 7f).

DISCUSSION

We have demonstrated, for the first time, that the expression of NRF is decreased in PBMCs of patients with stable COPD. The downregulation of NRF was found to be closely related to enhanced IL-8/CXCL8 production from these peripheral blood cells through de-repression of the IL-8/CXCL8 promoter. Oxidative stress is likely to be implicated in the reduction of NRF.

In the present study, the causative role of NRF in modulating IL-8/CXCL8 synthesis is clearly demonstrated by the observations that restoration of NRF in COPD PBMCs by overexpression plasmids attenuated the production of IL-8/CXCL8, whilst NRF knock-down in normal PBMCs augmented the release of IL-8/CXCL8. A reduction of NRF thus contributes to elevated production of IL-8/CXCL8 in stable disease condition. This may be a link to the systemic effects and comorbidities of COPD, such as muscle weakness and cardiovascular diseases. Potentially, reduced NRF may also contribute to the augmentation and self-amplification of neutrophilic inflammation in COPD and to the further enhanced systemic inflammation during acute exacerbation.

Mechanistically, the amount of NRF at the NRE in the native IL-8/CXCL8 promoter was decreased in stage III-IV COPD

FIGURE 7. Effects of H₂O₂ on nuclear factor-κB repressing factor (NRF) expression, nuclear factor (NF)-κB activation and interleukin (IL)-8/CXCL8 production and effects of dexamethasone on NRF expression. Representative immunoblot analysis (upper panels) and densitometry quantification of NRF expression (lower panels) in lysates of primary peripheral blood mononuclear cells (PBMCs) from a) four Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage III-IV chronic obstructive pulmonary disease (COPD) patients and b) three nonsmoker control (NSC) subjects in the presence and absence of 100 μM H₂O₂ (COPD) or H₂O₂ at variable concentrations (NSC, 0–300 μM). N'-acetyl-L-cysteine (NAC, 10 mM) pre-treatment for 30 min was also used for COPD patients. Data are presented as mean ± SEM percentage of control. c) TransAM analysis of NF-κB p65 activation in nuclear extracts of PBMCs from normal subjects in (a) treated with variable amounts of H₂O₂ (0–300 μM) for 1 h. d) ELISA analysis of IL-8/CXCL8 release from PBMCs of NSC subjects (n=6) and from patients with GOLD stage III-IV COPD (n=6) incubated in the presence or absence of H₂O₂ (0–100 μM) for 24 h. Data are presented as mean ± SEM from three independent experiments. e) ELISA analysis of IL-8/CXCL8 release in the cultured supernatants of primary PBMCs from normal subjects treated with variable amounts of H₂O₂ (0–300 μM) for 24 h in the presence or absence of the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC; 30 μM). Data are presented as mean ± SEM from three independent experiments using cells from different subjects. f) Immunoblot analysis of NRF expression (upper panel) in lysates of primary PBMCs from one normal subject incubated for 24 h in the presence of variable amounts of dexamethasone, as indicated. β-actin was used as the loading control. The lower panel shows densitometry analysis of NRF protein expression in primary PBMCs from normal subjects by immunoblot normalised to β-actin. Data are presented as mean ± SEM, in arbitrary units, from three normal subjects in three independent experiments. *: p<0.05; **: p<0.01; ***: p<0.001.

PBMCs, which was correlated with an increase in the promoter-bound RNA polymerase II. Furthermore, overexpression of NRF in normal PBMCs induced a removal of RNA polymerase II from the promoter without affecting the DNA binding activity of NF-κB. Those observations are in agreement with the previous reports [10] and indicate that NRF functionally suppresses the *IL-8/CXCL8* promoter by binding to and inhibiting the recruitment of the pre-initiation complex to the promoter, thus suppressing the transcriptional initiation. This is the first time

that the fine-tuning role of NRF in modulating IL-8/CXCL8 synthesis in a human disease, an occurrence that may also be implicated in other chronic inflammatory conditions, has been addressed.

The present study revealed that the decline in NRF in PBMCs was COPD severity-associated; a gradual decline of the protein was seen from stage I-II to stage III-IV patients. Smoking did not decrease NRF expression in healthy smokers. A reduction

of NRF corticosteroid was also excluded by *in vitro* studies. In addition, we had enrolled age-matched subjects to exclude the bias of age, which might affect the levels of NRF and IL-8/CXCL8 production (data not shown). Taken together, our observations support the fact that a reduced level of NRF is COPD associated. An interesting finding is that a decline in NRF mRNA was only seen in patients with stage III–IV COPD. It is likely that post-transcriptional mechanisms mediate reduction of NRF in the early stages of the disease, whereas further transcriptional mechanisms develop and mediate clinically significant decline in NRF at the advanced stages of the disease.

We provided evidence that intracellular oxidative stress was increased in PBMCs of severe COPD patients, which was negatively correlated with NRF expression. Oxidative stress in the serum was also increased in the serum of COPD patients ([21] and data not shown). *In vitro* experiments confirmed that H₂O₂ at higher concentrations could reduce NRF expression and that only at this concentration could H₂O₂ enhance IL-8/CXCL8 production. We therefore provided a working model of NRF in COPD (fig. S5). In normal PBMCs, low NF- κ B activity was controlled by NRF, leading to low constitutive production of IL-8/CXCL8. NRF also inhibits the promoter in response to NF- κ B induced by lower concentrations of H₂O₂. In COPD PBMCs, which are similar to normal PBMCs stimulated by higher concentrations of H₂O₂, oxidative stress not only stimulates NF- κ B but also releases the promoter from repression by NRF, and thus augments the production of IL-8/CXCL8. Oxidative stress renders proteins more susceptible to proteolytic degradation by modifying amino acid chains, causing them to form protein aggregates, and cleaving peptide bonds [27]; it also achieves this through nitration [28]. It is possible that a significant impairment in antioxidant protective pathways in advanced COPD patients renders NRF more susceptible to oxidative stress and/or induces further mechanisms, through upstream regulatory pathways or epigenetic regulation, leading to downregulation of NRF mRNA and, thus, a further decline in NRF protein [29]. Counteracting oxidative stress, such as through oxygen supplementation [30], might be a strategy to restore NRF and to control augmented inflammation.

In conclusion, for the first time, the present study indicates the implication of impaired NRF negative regulatory mechanism in COPD systemic inflammation. Reduced NRF in PBMCs releases the IL-8/CXCL8 promoter from repression and leads to augmented production of this inflammatory mediator in response to NF- κ B signalling, which may be activated by oxidative stress or other mechanisms under stable conditions or, probably, during acute exacerbation. Targeting restoration of NRF is therefore a potential therapeutic strategy for controlling COPD systemic inflammation and its related comorbidities. Our study also demonstrates that oxidative stress is a mechanism for reduced NRF expression. Clinical benefits from anti-oxidative stress therapies could be at least in part due to restoration of NRF. Further studies are warranted to explore the regulatory mechanism for NRF by oxidative stress and by other, as yet unknown, factors. In addition, the role of NRF in airway inflammation of COPD and in other chronic inflammatory diseases is worthy of further investigation.

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STATEMENT OF INTEREST

None declared.

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