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Research Article

Nortriptyline overcomes corticosteroid resistance in NK and NKT-like cells from peripheral blood of patients with chronic obstructive pulmonary disease

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Abstract

Introduction: An antidepressant nortriptyline potentiates glucocorticoid (GC) action with synergistic suppression of inflammatory mediator release, but the precise molecular mechanism is unknown.

Materials and methods: Peripheral blood cells from patients with chronic obstructive pulmonary disease (COPD) (n = 21) were incubated with nortriptyline (1 μM or 10 μM), budesonide (10 nM), or their combinations, followed by stimulation with phorbol myristate acetate (PMA) and ionomycin. Cytokine production, glucocorticoid receptor β (GRβ), histone deacetylase 2 (HDAC2) and histone H4 acetylation of K8 (HAT) expression, p65 NF-kB and p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation in NK (CD3-CD56+) and NKT-like (CD3+CD56+) cells were analyzed by flow cytometry.

Results: We observed that nortriptyline (10 μM) significantly attenuated the effects of PMA/ionomycin on the synthesis of interferon γ (IFNγ), interleukin 4 (IL-4), and IL-8, expression of GRβ and HAT, as well as p65 NF-kB and p38 MAPK phosphorylation in NK and NKT-like cells, whereas nortriptyline (1 μM) only inhibited IL-4 production by NK and NKT-like cells.

Discussion: The combination of nortriptyline (10 μM) and budesonide decreased IFNγ, tumor necrosis factor α, IL-4, IL-8, and GRβ expression, as well as phosphorylated p38 MAPK and p65 NF-κB levels by NK and NKT-like cells above that of budesonide alone. Furthermore, the same association of drugs enhanced HDAC2 expression in NK and NKT-like cells.

Conclusion: Collectively, our results show that nortriptyline might enhance GC function through modulation of HAT, HDAC2, GRβ, phospho-p38 MAPK expression. These data provide a strong rationale for combining nortriptyline with budesonide to treat COPD.

Keywords

COPD, HDAC2, nortriptyline, p38 MAPK, p65 NF-kB, NK cells, NKT-like cells, steroid resistance.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic airflow limitation that progressively rises and accompanied by inflammatory response in the airways. Globally, this disease affects 384 million people, while about 65 million patients have moderate to severe COPD (Vogelmeier et al. 2017). The burden of COPD is expected to increase over the coming decades due to the unceasing exposure to risk factors and population aging.

Cigarette smoking is recognized as the main environmental risk factor for COPD. Smoking cessation is an effective approach to slow down the disease progression and reduce COPD-related mortality (Tashkin 2015). However, resolution of inflammation is perceived not to occur even after elimination of this risk factor (Willemse et al. 2005; Ström et al. 2018). Cigarette smoking induces oxidative stress and subsequent activation of the transcription factor NF-κB. This transcription factor stimulates the expression and production of proinflammatory cytokines and chemokines by activated macrophages, airway epithelial cells, neutrophils and lymphocytes (Kadushkin and Taganovich 2016).

Glucocorticoids (GCs), drugs with anti-inflammatory mechanism of action, are widely used to treat COPD, however their therapeutic benefits are unassertive (Leung and Sin 2018). Reduced steroid responsiveness is a feature of COPD and a barrier to its effective treatment. Molecular mechanisms contributing to steroid resistance include altered expression of glucocorticoid receptor (GR) β, macrophage migration inhibitory factor, histone deacetylase 2 (HDAC2), p38 mitogen-activated protein kinase (p38 MAPK) as a consequence of oxidative stress (Kadushkin and Taganovich 2016). In patients with COPD, a reduced steroid sensitivity of natural killer (NK) and natural killer T (NKT) cells has been reported (Hodge and Hodge 2019). The percentages of these lymphocytes are increased in the airways of COPD patients (Hodge et al. 2013).

NK cells are effector cells of the innate immune system and exhibit their effects indirectly through interaction with dendritic cells, macrophages, T-lymphocytes and endothelial cells. NK cells induce apoptosis of autologous lung epithelial cells via the secretion of perforin and granzyme B (Freeman et al. 2014; Abel et al. 2018). In patients with COPD, there is enhanced cytotoxicity of NK cells in the airways, as well as increased expression of granzyme B by NK cells in blood and bronchoalveolar lavage fluid (Hodge et al. 2013). Moreover, these lymphocytes are an important source of cytokines and chemokines such as interferon γ (IFNγ), tumor necrosis factor α (TNFα), interleukin 4 (IL-4), IL-8, and others (Abel et al. 2018).

Similar to NK cells, NKT cells are among the first cells, which respond to antigens entering the body. They regulate immune response by altering the activity of T helper cells and cytotoxic T lymphocytes, NK cells, regulatory T lymphocytes, dendritic and myeloid cells (Krijgsman et al. 2018). These cells synthesize T helper type 1 (Th1)-specific (TNFα and IFNγ) and Th2-associated (IL-4 and IL-13) cytokines, as well as other cytokines and chemokines (Krijgsman et al. 2018). In patients with COPD, an increased natural cytotoxicity of NKT cells against autologous structural lung cells was revealed (Freeman et al. 2014), indicating their involvement in the pathogenesis of COPD.

Steroid resistance in patients with COPD prompted clinicians and scientists to search for the drugs that can enhance the anti-inflammatory effects of GCs. Experimental studies made possible to assume that nortriptyline is able to overcome steroid insensitivity (Mercado et al. 2011). Nortriptyline is a tricyclic antidepressant and the main active metabolite of amitriptyline, which is also a tricyclic antidepressant. Nortriptyline is used for the treatment of depression and anxiety as well as for smoking cessation in patients with COPD (Tselebis et al. 2016; Howes et al. 2020). Two independent studies have demonstrated a synergy between GCs and nortriptyline in reducing proinflammatory cytokine release from peripheral blood mononuclear cells (PBMCs) of healthy subjects and human monocytic U937 cells (Lehár et al. 2009; Mercado et al. 2011). However, the effects of nortriptyline have not been evaluated in cells of COPD patients, and the molecular mechanism of nortriptyline action on cells treated with corticosteroids is still not fully clear.

In this study, the aim was to examine anti-inflammatory efficacy of nortriptyline, alone and in combination with GC budesonide, on the production of cytokines by blood NK and NKT-like cells of patients with COPD and to investigate possible molecular mechanisms underlying the combine action of these drugs.

Materials and methods

Study participants

A total of 21 patients with COPD were recruited in the study (table 1). COPD was defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (Vogelmeier et al. 2017). Participants were former or current smokers with at least a 10-year smoking history, a ratio of forced expiratory volume in 1 second to forced vital capacity (FEV_1/FVC) < 0.70, and FEV_1% predicted < 80%. We defined former smokers as those subjects who had quit smoking for more than 6 months. Patients were excluded if they had a clinical diagnosis of asthma or other active lung disease, allergy, blood coagulation disorders, or an exacerbation of COPD within 6 weeks of study entry. We also excluded subjects taking regular oral corticosteroids. Approval for this study was obtained from the ethics committee of Belarusian State Medical University, Minsk, Belarus (Protocol no. 8, January 21, 2019). All subjects gave their written informed consent.

Cell stimulation

Heparinized peripheral blood was collected from all study subjects and samples were processed less than 1 hour after venipuncture. Whole blood was mixed in sterile tubes with equal volume of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS, Capricorn Scientific,

Ebsdorfergrund, Germany). Cells were then treated in the presence or absence of nortriptyline (1 and 10 µM, Sigma-Aldrich, St. Louis, MO, USA) and budesonide (10 nM, Glentham Life Sciences Ltd, Corsham, Wiltshire, UK), or with their combinations for 1 hour. After drug incubations, leukocytes were stimulated with phorbol myristate acetate (PMA, 50 ng/mL) (Cayman Chemical, Ann Arbor, Michigan, USA) and ionomycin (1 µg/mL) (Cayman Chemical, Israel), as previously outlined (Kadushkin et al. 2021). Brefeldin A (10 µg/mL) (Cayman Chemical, Israel) was added to inhibit cytokine secretion. Cells were co-incubated with the drugs and stimulants in a humidified 5% CO_2/95% air atmosphere at 37 °C for 6 hours. To remove adherent cells, 100 µL of ice-cold 20 mM EDTA was then added to the culture tubes for 10 minutes.

Table 1. Summary of patient characteristics, smoking history, and spirometry

Characteristics of participants	COPD patients
Patients, n	21
Sex ratio, male/female	17/4
Age, years	65.1±1.5
Body mass index, kg·m^{-2}	26.7±1.2
Smoking status (active/former)	10/11
Smoking, pack-years	32.5±2.4
Post-bronchodilator FEV_1 (% predicted)	52.4±4.1
Post-bronchodilator FEV_1/FVC ratio (%)	57.0±2.5

Note: Data are presented as n or mean ± SEM. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity.

Cytokine and HDAC2 expression in NK and NKT-like cells

Following stimulation as described above, cells were stained with anti-human CD3 PE-DyLight 594, CD56 PE-Cy7 and CD45 APC-Cy7 monoclonal antibodies (Exbio, Prague, Czech Republic) for 15 min in the dark at room temperature. Cells were centrifuged at 500×g for 5 min, supernatant discarded, 3 mL of wash buffer (5% FCS in CellWASH (BD, Poland)) was then added and the tubes centrifuged. After decanting supernatant, leukocytes were fixed using 100 µL of Reagent A (Fixation Medium from FIX & PERM Cell Fixation & Cell Permeabilization Kit, Life Technologies, Bleiswijk, Netherlands). Following washing (as above), the cells were permeabilized using 100 µL of Reagent B (Permeabilization Medium from FIX & PERM Cell Fixation & Cell Permeabilization Kit, Life Technologies). After blocking Fc receptors with human Fc block (BD Pharmingen, San Jose, CA, USA) for 10 min in the dark at room temperature, monoclonal antibodies to IL-4 PE, IFNγ PE, TNFα PE (all from Beckman Coulter), IL-8 FITC (R&D systems Europe, Abingdon, UK), or HDAC2 Alexa Fluor 488 (Abcam, Cambridge, UK) were added to the cells. Appropriate isotype-matched controls were used in all experiments. Cells were incubated with intracellular antibodies in the dark for 15 minutes at room temperature, followed by washing and fixation in 1% paraformaldehyde (500 µl). Fixed preparations were stored at 4 °C in the dark and analyzed within 12 hours. Cells were examined using Navios flow cytometer (Beckman Coulter, Brea, CA, USA) and data were analyzed using Kaluza Analysis software.

NK cells were identified as CD3-CD56+ events. Additionally, we referred CD3+CD56+ cells as NKT-like cells. This designation of CD3+CD56+ cells is due to the feature of T lymphocytes to express CD56, marker of NK and NKT cells (Krijgsman et al. 2018). Unfortunately, we did not evaluate, whether CD3+CD56+ cells were CD1d-restricted, although it is required for assignment of these cells to the population of true NKT cells.

GR, GRβ and histone acetyltransferase (HAT) expression in NK and NKT-like cells

To determine expression of GRβ and acetylated lysine antibody in NK and NKT-like cells, aliquots of blood were stimulated and fixed with Reagent A (Fixation Medium from FIX & PERM Cell Fixation & Cell Permeabilization Kit) as described above. After washing in wash buffer, cell cytoplasmic membrane was permeabilised using Reagent B (Permeabilization Medium from FIX & PERM Cell Fixation & Cell Permeabilization Kit), and anti-GRβ polyclonal antibody (GeneTex, Irvine, CA, USA) or anti-Histone H4 (acetyl K8) monoclonal antibody (Abcam, Cambridge, UK) were added to the tubes. Cells were incubated with antibodies in the dark at room temperature for 15 minutes, and were further washed and stained with goat anti-rabbit IgG H&L Alexa Fluor 488 (Thermo Fisher Scientific, Eugene, Oregon, USA) for 15 minutes in the dark at room temperature. To determine expression of GR, cells were stained with appropriately diluted anti-GR mAb (Abcam) for 15 minutes, followed by washing and staining with goat anti-mouse IgG H&L Alexa Fluor 488 (Abcam) for 15 minutes in the dark at room temperature. After the final wash, cells were resuspended in 1% paraformaldehyde and data were acquired as described above.

p38 MAPK and p65 NF-κB phosphorylation in NK and NKT-like cells

After incubation of blood cells with budesonide and nortriptyline for 1 hour as described above, appropriately diluted CD3 PE-DyLight 594, CD56 PE-Cy7 and CD45 APC-Cy7 monoclonal antibodies (Exbio) were added for 15 minutes in the dark at room temperature. Blood cultures were then stimulated with PMA (250 ng/mL) and ionomycin (1 µg/mL) for 15 minutes at 37 °C. To lyse red blood cells and fix leukocytes, 200 µl of blood were mixed with 4 mL of pre-warmed to 37 °C BD Phosflow Lyse/Fix Buffer (BD Biosciences, San Diego, CA, USA). The tubes were then incubated in a 37 °C water bath for 10 minutes. Leukocytes were spun down at 500×g for 5 minutes and washed once with CellWASH, followed by permeabilization with pre-cooled at -20 °C 1 mL of BD Phosflow Perm Buffer III (BD Biosciences, San Jose, CA, USA) for 30 minutes on ice. After washing twice with BD Pharmingen Stain Buffer (BD Biosciences), the cells were stained with PE mouse anti-p38 MAPK (pT180/pY182) and PE mouse anti-NF-

κB p65 (pS529) (BD Biosciences) for 1 hour in the dark at room temperature. Isotype controls were run with each set of samples. Following a final wash, cells were resuspended in 500 µl of BD Pharmingen Stain Buffer and analyzed within 2 hours on a Navios flow cytometer using Kaluza Analysis software (Beckman Coulter, Brea, CA, USA).

Statistical analysis

Data were analyzed using GraphPad Prism 7.00 (GraphPad Software Inc., San Diego, CA, USA) and expressed as mean ± standard error of mean (SEM). Results were assessed for a Gaussian distribution using Shapiro-Wilk normality test. Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Differences were considered significant at the 95% confidence level.

Results

Anti-inflammatory properties of nortriptyline and budesonide on NK and NKT-like cells

While there was negligible baseline synthesis of cytokines (data not shown), substantial percentage of NK and NKT-like cells treated with PMA and ionomycin in the presence of brefeldin A produced IL-4, IL-8, IFNγ, and TNFα (Figs 1, 2). Initial experiments showed that the majority of the NKT-like cells stimulated with PMA and ionomycin expressed IFNγ

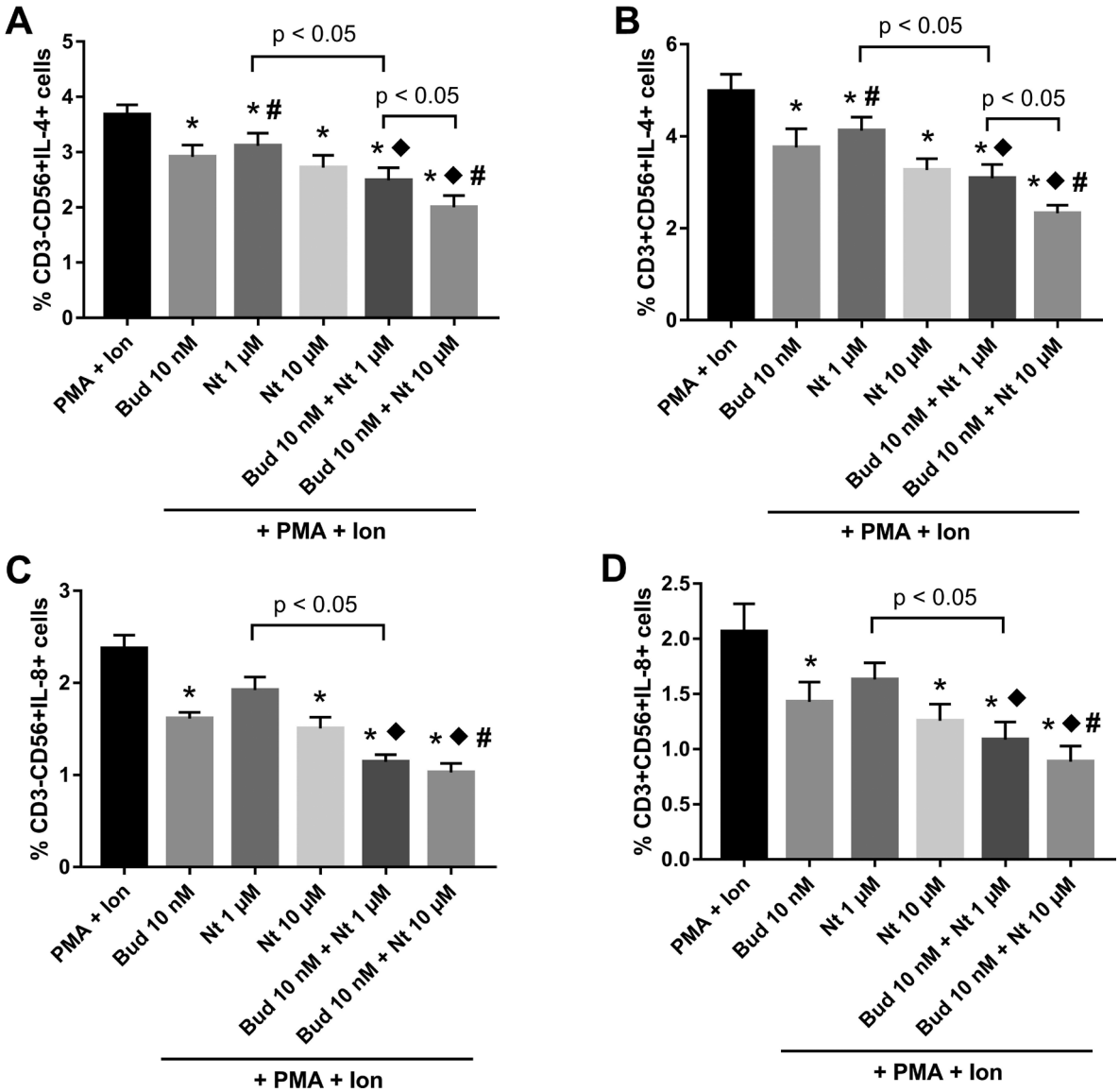

Figure 1. Percentage of NK (**A** and **C**) and NKT-like (**B** and **D**) cells from peripheral blood of COPD patients producing IL-4 and IL-8 after treatment with budesonide and nortriptyline. **Note:** Peripheral blood cells from patients with chronic obstructive pulmonary disease (COPD, n = 6) were incubated with nortriptyline (Nt, 1 µM or 10 µM), budesonide (Bud, 10 nM), or their combinations for 1 hour, followed by stimulation with phorbol myristate acetate (PMA, 50 ng/mL) and ionomycin (Ion, 1 µg/mL) for 6 hours. Production of interleukin 4 (IL-4, A and B) and IL-8 (C and D) in NK and NKT-like cells was examined by flow cytometry. Data are presented as mean ± SEM. One-way ANOVA followed by post hoc Tukey test: * – P<0.05 versus PMA + Ion control; ♦ – P<0.05 versus Bud 10 nM; # – P<0.05 versus Nt 10 µM.

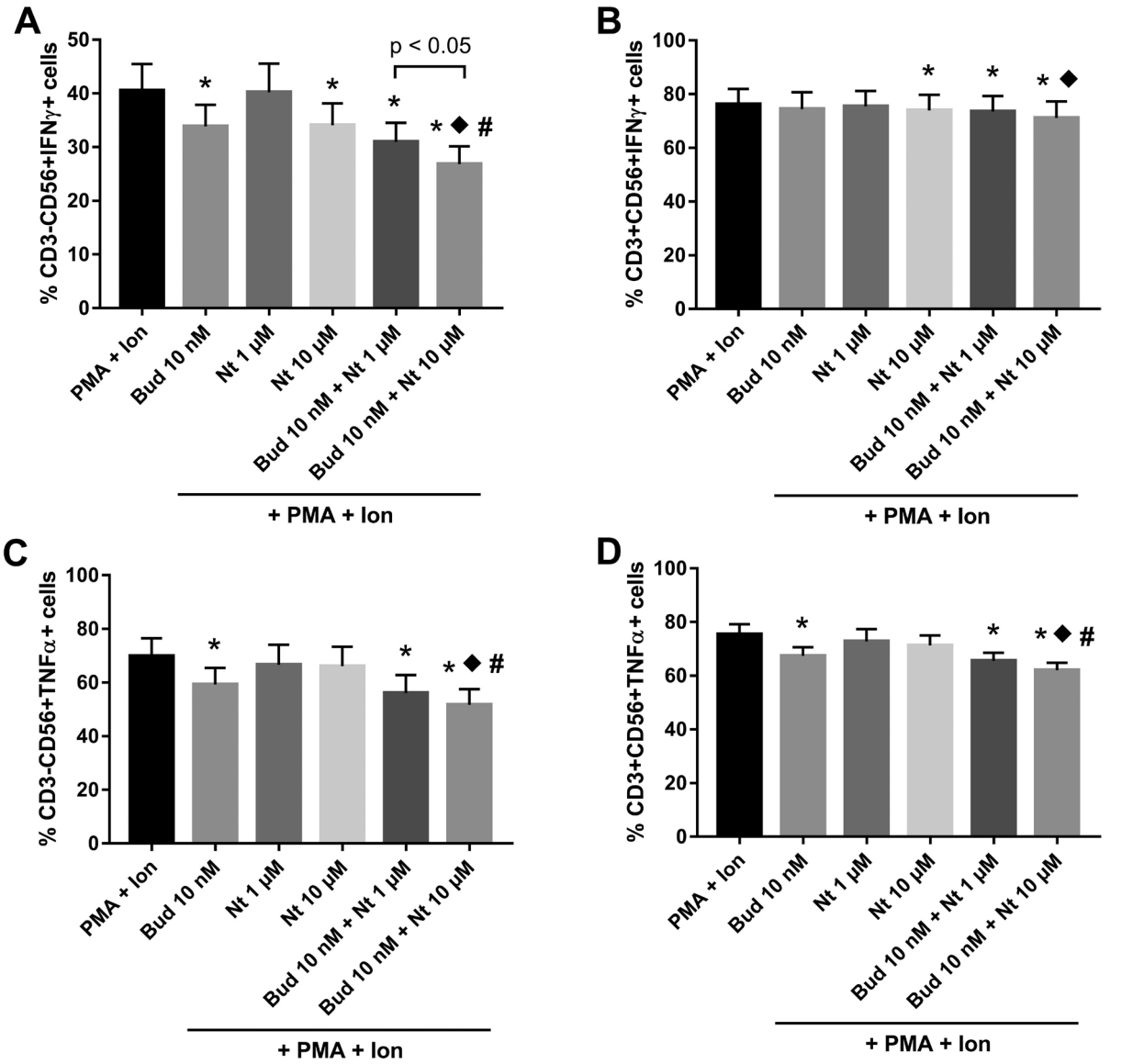

Figure 2. Percentage of NK (**A** and **C**) and NKT-like (**B** and **D**) cells from peripheral blood of COPD patients producing IFNγ and TNFα after treatment with budesonide and nortriptyline. **Note:** Peripheral blood cells from patients with chronic obstructive pulmonary disease (COPD, n = 6) were incubated with nortriptyline (Nt, 1 μM or 10 μM), budesonide (Bud, 10 nM), or their combinations for 1 hour, followed by stimulation with phorbol myristate acetate (PMA, 50 ng/mL) and ionomycin (Ion, 1 μg/mL) for 6 hours. Production of interferon γ (IFNγ, A and B) and tumor necrosis factor α (TNFα, C and D) in NK and NKT-like cells was examined by flow cytometry. Data are presented as mean ± SEM. One-way ANOVA followed by post hoc Tukey test: *– P<0.05 versus PMA + Ion control; ♦ – P<0.05 versus Bud 10 nM; # – P<0.05 versus Nt 10 μM.

and TNFα, while fewer cells produced IL-4 and IL-8. More than half of NK cells were also found to express TNFα.

Budesonide suppressed PMA/ionomycin-induced production of IL-4, IL-8, and TNFα in NK and NKT-like cells, as well as IFNγ synthesis in NK cells. However, this drug did not affect IFNγ expression by NKT-like cells. Nortriptyline 10 μM reduced synthesis of IL-4, IL-8, and IFNγ in NK and NKT-like cells from COPD patients. In contrast, nortriptyline 1 μM only attenuated the increase of IL-4 by NK and NKT-like cells. Treating PMA/ionomycin-stimulated whole blood cells with combination of nortriptyline 10 μM and budesonide significantly decreased IL-4, IL-8, IFNγ, and TNFα expression by NK and NKT-like cells above that of budesonide alone. The association of nortriptyline 1 μM and budesonide showed additive effects, reducing IL-4 and IL-8 production in NK and NKT-like cells from COPD patients.

Effects of nortriptyline and budesonide on p38 MAPK and p65 NF-κB phosphorylation

To assess the impact of nortriptyline on molecular pathways leading to steroid resistance, we examined its effect on the phosphorylation status of p38 MAPK and p65 NF-κB. Phosphorylation (and thus activation) of p38 MAPK and p65 NF-κB in NK and NKT-like cells was increased in response to PMA and ionomycin (Suppl. material 1: Table S1). Budesonide was unable to reverse phosphorylated p38 MAPK and p65 NF-κB levels induced by PMA/ionomycin (Table 2). In contrast, nortriptyline 10 μM reduced

Table 2. Mean fluorescence intensity (MFI) of GR, GRβ, phosphorylated p38 MAPK and p65 NF-κB staining in NK and NKT-like blood cells from COPD patients

Cell type	PMA + Ion	PMA + Ion + Bud 10 nM	PMA + Ion + Nt 1 μM	PMA + Ion + Nt 10 μM	PMA + Ion + Bud 10 nM + Nt 1 μM	PMA + Ion + Bud 10 nM + Nt 10 μM
MFI GR						
NK cells	30.87±2.26	27.73±2.30	33.43±1.14	32.92±1.34	32.40±1.05	31.80±1.91
NKT-like cells	40.12±6.97	36.00±7.50	40.42 ± 6.27	39.75±6.95	38.96±6.63	40.01±8.59
MFI GRβ						
NK cells	3.56±0.25	3.43±2.24	3.30±0.25	3.17±0.23*♦	3.16±0.23*♦	2.99±0.19*♦
NKT-like cells	3.34±0.18	3.13±0.18	3.12±0.19	2.94±0.20*♦§	2.91±0.20*♦§	2.78±0.19*♦
MFI p-p38 MAPK						
NK cells	2.56±0.23	2.50±0.26	2.47±0.22	2.37±0.24*	2.28±0.25*♦	2.24±0.22*♦
NKT-like cells	3.97±0.35	3.82±0.37	3.79±0.33	3.72±0.34*	3.64±0.36*♦	3.61±0.36*♦
MFI p-p65 NF-κB						
NK cells	1.78±0.20	1.73±0.19	1.69±0.16	1.58±0.17*	1.60±0.21*	1.57±0.21*♦
NKT-like cells	3.32±0.43	3.20±0.41	3.14±0.40	2.93±0.40*	2.92±0.41*♦	2.81±0.41*♦

Note: The values shown are mean ± SEM of 6 experiments. * – P<0.05 versus PMA + Ion; ♦ – P<0.05 versus PMA + Ion + Bud 10 nM; § –P<0.05 versus PMA + Ion + Nt 1 μM. Bud, budesonide; COPD, chronic obstructive pulmonary disease; GR, glucocorticoid receptor; Ion, ionomycin; NK cells, natural killer cells; NKT-like cells, natural killer T-like cells; Nt, nortriptyline; PMA, phorbol myristate acetate; p-p38 MAPK, phosphorylated p38 mitogen-activated protein kinase; p-p65 NF-κB, phosphorylated p65 nuclear factor-κB.

phosphorylation of p38 MAPK and p65 NF-κB in NK and NKT-like cells from COPD patients. Importantly, NK and NKT-like cells of COPD patients that were pretreated with nortriptyline became significantly more responsive to the suppressive effects of GCs. As shown by flow cytometry, preincubation of whole peripheral blood from COPD patients with nortriptyline resulted in a significantly greater budesonide-mediated suppression of PMA/ionomycin-induced levels of phosphorylated p38 MAPK and p65 NF-κB.

Impact of nortriptyline and budesonide on GR and GRβ expression

The expression of GR was not modified under any experimental condition (Table 2). In particular, GR expression was not modified by PMA/ionomycin stimulation in the presence of brefeldin A (Suppl. material 1: Table S1) and following nortriptyline or budesonide exposure.

The human GR gene encodes two splicing variants, GRα and GRβ. While GRα mediates most of the known GC functions, GRβ may have dominant negative effect on GRα, leading to steroid resistance (Kino et al. 2009). Therefore, we next evaluated whether nortriptyline, alone and in combination with GCs, is able to modulate GRβ expression by NK and NKT-like cells. Treatment of whole blood cells with budesonide or nortriptyline 1 μM showed no effect on GRβ expression. Nortriptyline 10 μM effectively decreased GRβ expression in the presence of PMA and ionomycin. Moreover, nortriptyline (1 μM to 10 μM) combined with budesonide markedly suppressed GRβ expression by NK and NKT-like cells.

Effects of nortriptyline and budesonide on HDAC2 and histone acetyltransferase (HAT) expression

In other experiments, we assessed the expression of HDAC2, which suppresses activated inflammatory genes. Reduced activity and expression of HDAC2 leads to the resistance of corticosteroids to the anti-inflammatory actions (Barnes 2011). At the same time, histone H4 (acetyl K8) was evaluated, as it gives evidence for the activity of HAT, which upregulates pro-inflammatory gene transcription. Budesonide was without significant effect on any of these two molecules in lymphocyte subsets (Fig. 3A, B). Nt alone was unable to reverse the downregulation of HDAC2 expression induced by PMA/ionomycin in CD3-CD56+ and CD3+CD56+ cells. In contrast, the combination of Nt (1 μM to 10 μM) with budesonide at 10 nM enhanced the percentage of NK and NKT-like cells expressing HDAC2 from COPD patients (Fig. 4).

We also observed that nortriptyline (10 μM) significantly attenuated the effects of PMA/ionomycin on histone H4 (acetyl K8) expression in NK and NKT-like cells (Fig. 3C, D). Furthermore, the association of Nt (1 μM to 10 μM) with budesonide at 10 nM reduced the expression of histone H4 (acetyl K8) in CD3-CD56+ and CD3+CD56+ cells.

Discussion

GC insensitivity is a significant barrier to successful COPD treatment. Our current study demonstrates that a tricyclic antidepressant nortriptyline exhibits anti-inflammatory effects, alone and in combination with GCs, on cytokine production by NK and NKT-like cells from COPD patients. Combination therapy with corticosteroids and nortriptyline affords the advantage over GCs alone, as the degree of cytokine inhibition was enhanced when budesonide was used in association with nortriptyline. Moreover, we found that nortriptyline is able to overcome molecular mechanisms that can contribute to reduced responsiveness of NK and NKT-like cells to corticosteroids. The data obtained convincingly prove the feasibility of

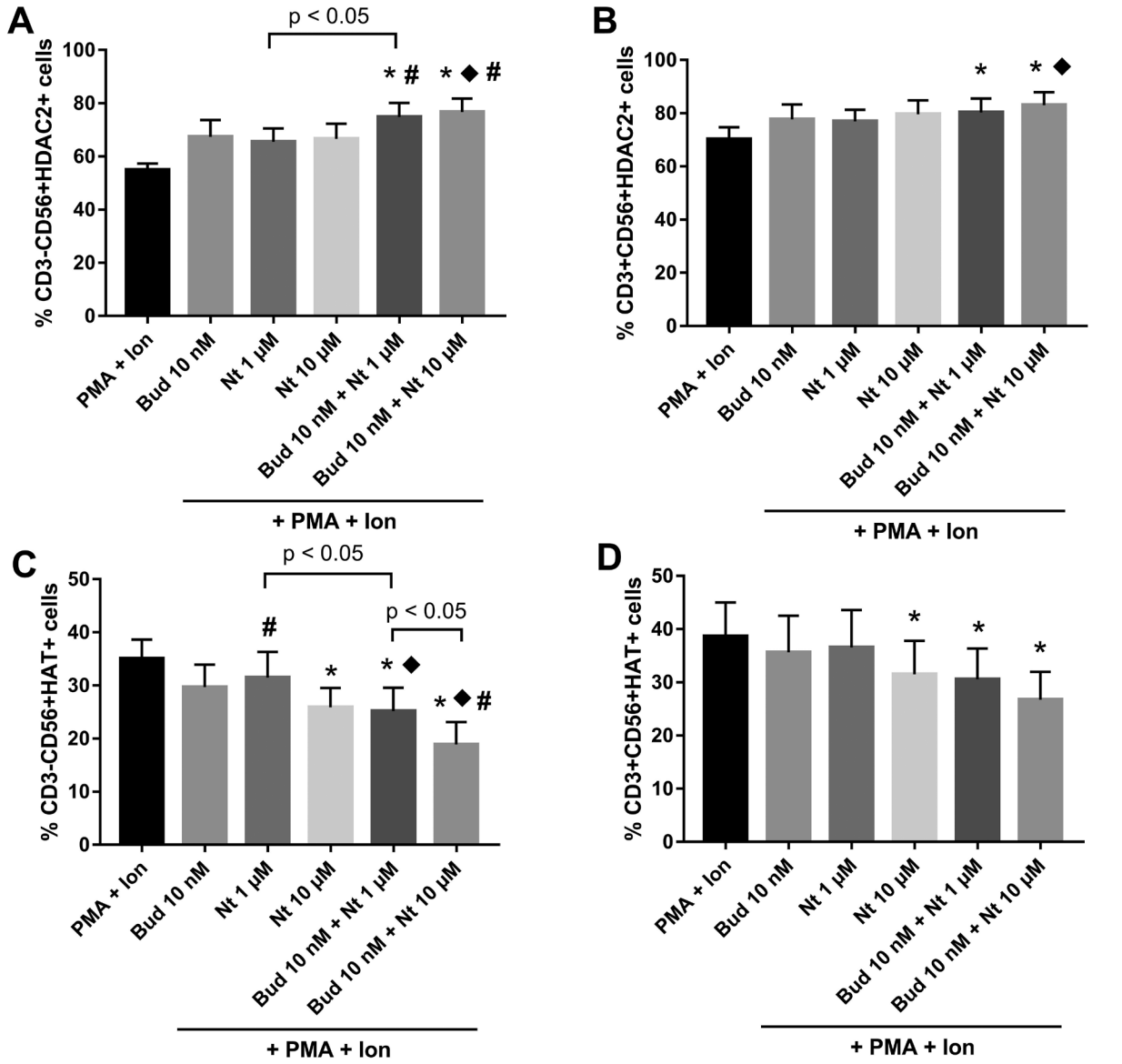

Figure 3. Effects of nortriptyline and budesonide on histone deacetylase 2 (HDAC2) and histone H4 (acetyl K8) expression in NK (**A** and **C**) and NKT-like (**B** and **D**) blood cells from COPD patients. **Note:** Peripheral blood cells from patients with chronic obstructive pulmonary disease (COPD, n = 6) were incubated with nortriptyline (Nt, 1 µM or 10 µM), budesonide (Bud, 10 nM), or their combinations for 1 hour and stimulated with phorbol myristate acetate (PMA, 50 ng/mL) and ionomycin (Ion, 1 µg/mL) for 6 hours. Expression of histone deacetylase 2 (HDAC2, A and B) and histone H4 acetylation of K8 (HAT, C and D) in NK and NKT-like cells was detected by flow cytometry. Data are presented as mean ± SEM. One-way ANOVA followed by post hoc Tukey test: * – P<0.05 versus PMA + Ion control; ♦ – P<0.05 versus Bud 10 nM; # – P<0.05 versus Nt 10 µM.

a combined use of nortriptyline and corticosteroids for suppressing the inflammatory process in COPD patients.

IL-4 is involved in several aspects of airway inflammation and remodeling, such as differentiation of naive CD4+ T cells into Th2 cells, alternative macrophage activation, and collagen synthesis in fibroblasts (Junttila 2018). IL-4 stimulates airway epithelial cells to produce and release chemokine CCL26/eotaxin-3 that recruit eosinophils into the inflamed lung through CCR3 receptors (Abonyo et al. 2005). It has been reported that nearly a third of patients with stable COPD have evidence of eosinophilic airway inflammation (Tashkin and Wechsler 2018), while higher blood eosinophil counts have been associated with an increased risk of exacerbations among individuals with COPD (Vedel-Krogh et al. 2016). In this work, we observed that exposure of NK and NKT-like cells to PMA and ionomycin increased IL-4 production and this was inhibited by pretreatment with either budesonide or nortriptyline (1 µM and 10 µM). More importantly, the combination of budesonide and nortriptyline (1 µM to 10 µM) was more potent at reducing IL-4 synthesis in NK and NKT-like cells when compared with budesonide alone, implying that nortriptyline, even at low concentrations, has the potential to enhance GC sensitivity in COPD.

IL-8 is known for its ability to attract neutrophils to the sites of inflammation in the lung tissue. There is an

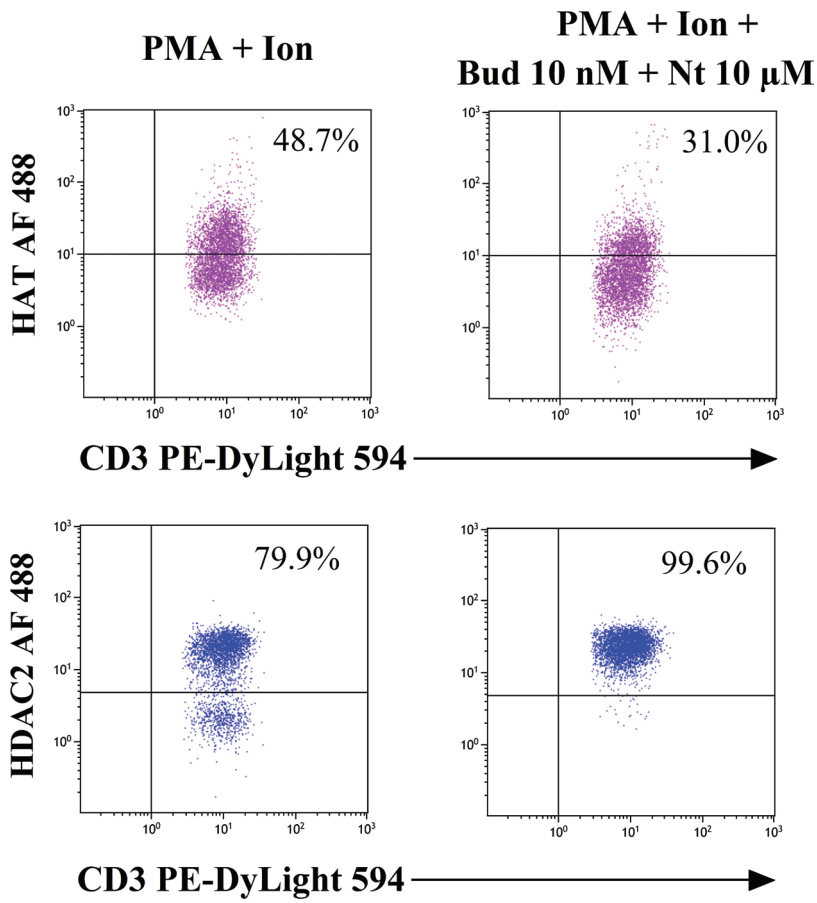

Figure 4. Representative dot plots showing the combined effect of 10 nM budesonide (Bud) and 10 μM nortriptyline (Nt) on the percentage of NKT-like cells expressing histone acetyltransferase (HAT) and histone deacetylase 2 (HDAC2) from a patient with chronic obstructive pulmonary disease.

increase in serum IL-8 level in patients with stable COPD, with a further rise during an acute exacerbation (Zhang and Bai 2018). Higher blood IL-8 level in COPD patients was associated with emphysema progression over 5 years after measuring cytokine level (Bradford et al. 2017). In this regard, reducing blood IL-8 level seems logical and may be effective in COPD. In the current study, the percentage of IL-8-producing NK and NKT-like cells was significantly reduced after budesonide and nortriptyline 10 μM treatment. We also found that inhibitory effect of budesonide on IL-8 synthesis in NK and NKT-like cells was increased in the presence of nortriptyline (1 μM and 10 μM). In addition, the synergistic action of GCs and nortriptyline to decrease the release of IL-8 from stimulated human monocytic U937 cells has been reported previously (Mercado et al. 2011). Therefore, IL-8 suppression using nortriptyline combined with GCs may be a good therapeutic approach in COPD where this cytokine plays an important role.

IFNγ induces the expression of MIG, IP-10 and I-TAC by bronchial epithelial cells and macrophages, which serve as ligands for the chemokine receptor CXCR3. This receptor is expressed on lymphocytes, including NK and NKT cells, and involved in their recruitment in the peripheral airways of COPD patients (Hughes and Nibbs 2018). Stimulation of macrophages with IFNγ and lipopolysaccharide leads to their polarization towards the M1 phenotype, which is characterized by the production of pro-inflammatory cytokines, including TNFα and IL-1β (Smith et al. 2016). Moreover, IFNγ contributes to the reduced sensitivity of alveolar macrophages of COPD patients to GCs through the activation of the transcription factor STAT1 (Southworth et al. 2012). In this work, budesonide decreased production of IFNγ in NK cells, but did not alter IFNγ synthesis in NKT-like cells. Interestingly, a previous study detected a greater inhibitory effect of other GC, methylprednisolone, on IFNγ production by NK cells compared with the effect of this drug on IFNγ synthesis by NKT-like cells from COPD patients (Hodge et al. 2014). Moreover, we observed that combination of nortriptyline (10 μM) and budesonide had additive effects, inhibiting IFNγ expression by both NK and NKT-like cells.

In patients with COPD, TNFα activates transcription factors NF-κB and AP-1, which promote the synthesis of proinflammatory mediators (Barnes 2016). TNFα upregulates expression of cytokines (IL-5, IL-6, IL-8, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor), chemokines (CCL5, CCL11, MCP-1), matrix metalloproteinases (MMP-9, MMP-12), adhesion molecules (ICAM-1, VCAM-1, E-selectin), and mucins (MUC1, MUC2, MUC5AC) in inflammatory and structural cells in the lung (Commins et al. 2010). TNFα also contributes to cachexia and skeletal muscle wasting in patients with COPD (Webster et al. 2020). Blood TNFα level was found to be increased in patients with COPD

compared with healthy controls (Yao et al. 2019). Here, addition of budesonide to culture tubes resulted in a decrease of TNFα production in NK and NKT-like cells of COPD patients. Furthermore, the percentage of TNFα-producing NK and NKT-like cells rose significantly upon addition of nortriptyline 10 μM to budesonide compared with cells cultured in the presence of budesonide alone. Our data agrees with another study that reported synergistic attenuation of TNFα secretion from stimulated PBMCs of healthy subjects by the combination of nortriptyline plus GCs (Lehár et al. 2009). This indicates that COPD subjects could benefit from nortriptyline/corticosteroid combination therapy.

To determine molecular basis for combined nortriptyline and budesonide action on NK and NKT-like cells, we tested their effects on known markers of steroid resistance. There is compelling evidence that inflammatory gene repression is mediated by histone deacetylases. HDAC2 has been shown to cause deacetylation of GR, enabling this receptor to bind to the p65 NF-κB-activated complex and subsequently repress inflammatory gene expression (Ito et al. 2006). In patients with COPD, reduced HDAC2 expression was first demonstrated in lung macrophages (Cosio et al. 2004) and was recently found in PBMCs (Tan et al. 2016). Impaired HDAC2 causes hyperacetylation of histones and enhances inflammatory gene expression. Moreover, reduced HDAC2 activity and expression prevents the association between GR and NF-κB, leading to steroid resistant cytokine production (Ito et al. 2006). We report here a rapid suppression of HDAC2 in NK and NKT-like cells following stimulation with PMA plus ionomycin. Budesonide or nortriptyline alone produced no change in HDAC2 expression by NK and NKT-like cells from that seen in control untreated samples. In addition, our study showed beneficial effects of nortriptyline (1 μM and 10 μM) combined with budesonide in enhancing HDAC2 expression by NK and NKT-like cells from COPD patients.

Of note, gene expression is affected by a balance between acetylation and deacetylation of histones. Inflammatory gene activation is associated with the histone acetylation, which is induced by coactivators having intrinsic HAT activity (Barnes 2011). In this work, there was no alteration of PMA/ionomycin-induced histone H4 acetylation of K8 in NK and NKT-like cells following either budesonide or nortriptyline 1 μM treatment. However, higher concentration of nortriptyline (10 μM), as well as the association of nortriptyline (1 μM to 10 μM) and budesonide inhibited histone H4 acetylation status. These results suggest that suppression of HAT activity and induction of HDAC2 expression may be possible mechanism by which nortriptyline regains steroid sensitivity of NK and NKT-like cells from COPD patients.

Previous studies have shown that dominant negative isoform of GR, GRβ, can inhibit hormone binding to GRα, leading to steroid resistance (Kino et al. 2009). Extending those findings, we revealed that budesonide alone had no effect on PMA/ionomycin-stimulated GRβ expression in NK and NKT-like cells from COPD patients. Intriguingly, nortriptyline 10 μM alone reduced percentage of GRβ-expressing NK and NKT-like cells. Similarly, nortriptyline (1 μM to 10 μM) and budesonide combined caused greater GRβ inhibition in NK and NKT-like cells compared with either cultures with no drugs or budesonide alone. Our data imply that nortriptyline is able to repress GRβ, and thus may afford GRa to implement its functions.

Proinflammatory transcription factor NF-κB mediates induction of various proinflammatory genes in innate immune cells and regulates the maturation and effector functions of NK and NKT cells (Lougaris et al. 2017; Stankovic et al. 2011). Heightened NF-κB activation is a hallmark of COPD (Schuliga 2015). Moreover, targeting NF-κB signaling is supposed to increase effectiveness of COPD treatment (Schuliga 2015). In the present study, budesonide had no effect on p65 NF-kB activation in NK and NKT-like cells after stimulation with PMA/ionomycin. In contrast, nortriptyline (10 μM) efficiently reduced p65 NF-kB protein phosphorylation in these cells, suggesting that its anti-inflammatory properties are superior to those of corticosteroids in COPD.

p38 MAPK signaling is increased in COPD and can cause the inefficiency of GCs to suppress inflammation (Renda et al. 2008). Activated p38 MAPK causes GR phosphorylation leading to inhibition of GR nuclear translocation and modification of GR transcriptional activity (Galliher-Beckley and Cidlowski 2009). Our data show that budesonide did not change PMA/ionomycin-induced expression of phospho-p38 MAPK in NK and NKT-like cells of patients with COPD. Additionally, there are other data showing that corticosteroid prednisolone prescribed orally to patients with COPD did not inhibit p38 MAPK activation in whole blood (Singh et al. 2010). We observed that nortriptyline 10 μM decreased budesonide-insensitive expression of phospho-p38 MAPK in NK and NKT-like cells of patients with COPD. Moreover, combination of nortriptyline (1 μM to 10 μM) with budesonide inhibited the p38 MAPK pathway to a greater degree than budesonide alone did. Thus, suppression of p38 MAPK signaling and subsequent inhibition of inflammatory mediators may be achieved by combination treatment with a corticosteroid and nortriptyline.

Collectively, our results show that nortriptyline augments budesonide's anti-inflammatory effects on IL-4, IL-8, TNFα and IFNγ production by NK and NKT-like cells of patients with COPD. Nortriptyline might enhance the function of GCs through modulation of HAT, HDAC2, GRβ, phospho-p38 MAPK expression. These data provide a strong rationale for combining nortriptyline with budesonide to treat COPD. Importantly, nortriptyline may also be beneficial in other steroid-refractory diseases, such as asthma, where GCs are the first-line therapy for controlling airway inflammation, but 5–10% of asthma patients respond unsatisfactorily to steroid-based therapies (Henderson et al. 2020). Future research should demonstrate if this assumption is correct.

Conflict of interest

The authors have declared that no competing interests exist.

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Supplementary material 1

Table S1

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Data type: Table (docx. file)

Explanation note: Basal and stimulated intracellular expression of enzymes, glucocorticoid receptor, phosphorylated p38 MAPK and p65 NF-κB staining in NK and NKT-like cells from patients with chronic obstructive pulmonary disease.

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