COPD is associated with increased pro-inflammatory CD28null CD8 T and NKT-like cells in the small airways

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Abstract

We previously showed increased steroid resistant CD28null CD8+ senescent lymphocyte subsets in peripheral blood from COPD patients. These cells expressed decreased levels of the glucocorticoid receptor (GCR), suggesting their contribution to the steroid resistant property of these cells. COPD is a disease of the small airways. We therefore hypothesized that there would be a further increase in these steroid resistant lymphocytes in the lung, particularly in the small airways. We further hypothesized that the pro-inflammatory/cytotoxic potential of these cells could be negated using prednisolone with low-dose cyclosporin A.

Blood, bronchoalveolar lavage, large proximal and small distal airway brushings were collected from 11 COPD patients and 10 healthy aged-matched controls. The cytotoxic mediator granzyme b, pro-inflammatory cytokines IFNγ/TNFα, and GCR were determined in lymphocytes subsets before and after their exposure to 1μM prednisolone and/or 2.5ng/mL cyclosporin A.

Particularly in the small airways, COPD subjects showed an increased percentage of CD28null CD8 T-cells and NKT-like cells, with increased expression of granzyme b, IFNγ and TNFα and a loss of GCR, compared with controls. Significant negative correlations between small airway GCR expression and IFNγ/TNFα production by T and NKT-like cells (eg, T-cell IFNγ R= -.834, p=.031) and with FEV_1 (R= -890) were shown. Cyclosporine A and prednisolone synergistically increased GCR expression and inhibited pro-inflammatory cytokine production by CD28null CD8- T and NKT-like cells.

COPD is associated with increased pro-inflammatory CD28null CD8+ T and NKT-like cells in the small airways. Treatments that increase GCR in these lymphocyte subsets may improve morbidity in COPD patients.

Abstract 250 words

Abbreviations

COPD: chronic obstructive pulmonary disease

GCR: glucocorticoid receptor

BAL: bronchoalveolar lavage

IFNγ: interferon gamma

TNFα: tumour necrosis factor alpha

NKT-like: natural killer T-like

Key words: COPD**;** pro-inflammatory CD28null CD8 T and NKT-like cells; small airways; GCR

Background

T cells are a major inflammatory cell type present in the lung in COPD patients [1]. We previously investigated intracellular cytokine production in lymphocytes from the blood, bronchoalveolar lavage (BAL) and intraepithelial compartment obtained by bronchial brushings in COPD patients and control groups [2]. COPD patients exhibited systemic inflammation as evidenced by increased IFNγ and TNFα in blood, BAL and bronchial intraepithelial cell CD8+T cells compared with healthy controls. There was a negative correlation between forced expiratory volume in 1 sec (FEV_1) and the percentage of BAL and bronchial intraepithelial CD8+T cells producing TNFα [2]. We have also shown an increase in CD28null CD8+ T and NKT-like senescent cells in the peripheral blood of COPD patients [3,4,5]. These senescent cells expressed increased levels of cytotoxic mediators perforin and granzyme B, with increased production of pro-inflammatory cytokines IFNγ and TNFα, compared to their CD28+ counterparts [3,4]. Reduced responsiveness to the anti-inflammatory effects of corticosteroids is a major barrier to effective management of the majority of patients with COPD [6]. CD8+ T cells have been described as the central regulator of the inflammatory network in COPD [7]; thus targeting the inflammatory nature of these cells could be vital to reduce inflammation in patients with COPD. Our research to identify the mechanism/s of lack of steroid responsiveness in COPD recently showed that COPD was associated with loss of glucocorticoid receptor (GCR) from these pro-inflammatory senescent CD28null CD8+ T and NKT-like cells [4].

Small airway disease is a cardinal feature of COPD [8] and it has been suggested that small airway disease must be targeted to attenuate the progression of COPD [8]. It is

unknown whether these cytotoxic pro-inflammatory CD28null CD8+ T and NKT-like cells particularly target the small airways in COPD.

We therefore investigated intracellular granzyme b, IFNγ and TNFα pro-inflammatory cytokine production by CD28± CD8+ and CD8- (CD4+) T and NKT-like lymphocyte subsets, and GCR expression in cultured peripheral blood, bronchoalveolar lavage (BAL), bronchial and small airway brushings from COPD patients and healthy controls using multiparameter flow cytometry. We hypothesized that CD28null CD8+ T and NKT-like cells, and their production of pro-inflammatory cytokines, would be increased, and GCR decreased, in intraepithelial lymphocytes from the small distal airways in patients with COPD.

Based on our previous findings of upregulation of heat shock protein by blood CD28null T and NKT-like cells in the presence of 2.5ng/mL cyclosporine A [9] we also investigated the effect of low dose cyclosporine A and standard dose prednisolone on GCR expression and IFNγ and TNFα pro-inflammatory cytokine production by these cells.

MATERIALS AND METHODS

Patient and control groups

COPD subjects were specifically recruited for the study and informed written consent obtained. All had no exacerbation of COPD disease for at least 6 weeks prior to this study. Subjects with other co-existing lung disease or malignancy or aged greater than 75 years were excluded. Ethics approval was obtained from the Royal Adelaide Hospital Human Ethics Committee. COPD was diagnosed using the GOLD criteria with clinical correlation [Stage I COPD: forced expiratory volume in 1s (FEV_1)/ forced vital capacity (FVC) <70% but FEV_1 ≥80% of predicted; Stage II COPD FEV_1 50%-79% of predicted, Stage III COPD FEV_1 30-49% of predicted; Stage IV <30% of predicted] [10]. COPD subjects were ex-smokers (> 1 year) (n=10) with an average of 39 pack years. No patients were receiving oral GCS. Healthy age-matched non-smoking volunteers (n=11) with normal lung function and no history of lung disease were recruited as controls. All subjects underwent spirometry as part of their routine clinical assessment. BAL, proximal and distal brushings were collected as previously reported [11]. Demographic details of patient and control groups are shown in Table 1. Venous blood was collected into 10 U/mL preservative heparin (DBL, Sydney, Australia), and all samples were maintained at 4^0C until processing. All subjects were submitted to the same protocol and analysis performed retrospectively.

Leucocyte Counts

Full blood counts, including white cell differential counts, were determined on blood specimens using a CELL-DYN 4000 (Abbot Diagnostics, Sydney, Australia). Blood

films and BAL cytospins were stained by the May-Grunwald-Giemsa method and leucocyte differential counts checked by morphological assessment microscopically.

CD28± CD8± T, NKT-like cell subsets

Aliquots of blood were added to FACS tubes and red blood cells were lysed using FACSLyse (BD Biosciences, Sydney, Australia) as described previously [2-5].

BAL, and proximal and distal brushings samples, were centrifuged at 300 ×g for 5 min. After decanting, cells were re-suspended in 2 mL of RPMI completed with 10%FCS (R10, Sigma, Sydney, Australia). Macrophages from BAL were adhered to plastic dishes incubated for 60 min in a humidified 5% CO_2/95% air atmosphere at 37^0C. Non-adherent cells were transferred to 10 mL sterile centrifuge tubes and cells, centrifuged at 300 ×g for 5 min and cells re-suspended in 2 mL of R10. All cells were permeabilized using FACSPerm (BD) as previously reported [2] then washed with wash buffer (0.5% BSA in Isoflow (Beckman Coulter, Sydney, Australia)). Appropriately diluted monoclonal antibodies to CD3 perCP.CY5.5 (BD, Sydney, Australia), CD28 PECY7 (BD), CD56 APC (Beckman Coulter, Sydney, Australia), CD8 APC.CY7 (BD), and CD45 V500 (BD) were added for 15 min in the dark at RT. After further washing, cells were analyzed within 1 h on a FACSCanto II flow cytometer using FACSDiva software (BD). Samples were analyzed by gating lymphocytes using CD45 staining versus side scatter (SSC) as reported [2-5]. A minimum of 350,000 low SSC events were acquired in list-mode format for analysis. T cells were identified as CD3+CD56-CD45+; CD8 and CD4 T cell subsets were then identified by CD8+ and CD8- staining, NKT-like cells identified as CD3+CD56+CD45+ low FSC/SSC events [12].

Granzyme b expression in CD8+ and CD8- T, NKT-like cell subsets

Aliquots of blood, BAL, proximal and distal brushings were treated as above. Appropriately diluted monoclonal antibodies to CD3 perCP.CY5.5 (BD), CD28 PECY7 (BD), CD56 APC (Beckman Coulter), CD8 APC.CY7 (BD), granzyme B V450 (BD) and CD45 V500 (BD) were added for 15 min in the dark at RT. After further washing, cells were analyzed within 1 h on a FACSCanto II flow cytometer using FACSDiva software (BD), and analyzed as above.

GCR and intracellular cytokine expression in CD28±CD8±T and NKT-like cells

Blood, BAL, proximal and distal brushings were treated as above. To determine expression of GCR and intracellular cytokine production in CD28± CD8+ and CD8- T and NKT-like cells, aliquots were stimulated as previously reported with phorbol myristate (25 ng/mL) (Sigma, Sydney, Australia) and ionomycin (1 μg/mL) (Sigma) in the presence of brefeldin A (1 μg/mL) (Sigma) and the tubes incubated in a humidified 5% CO_2/95% air atmosphere at 37^0C. At 16 h 100 μL of 20 mM EDTA / PBS was added to the culture tubes which were vortexed vigorously for 20 sec to remove adherent cells. Red blood cells in blood were lysed and cells from all specimens permeabilized as described previously [2-6]. Two mL of 0.5% bovine serum albumin (Sigma/Aldrich, Sydney, Australia) / Isoflow (Beckman Coulter, Sydney, Australia) was then added and the tubes centrifuged at 300 ×g for 5 min. After decanting supernatant, Fc receptors were blocked with 10 μL of human immunoglobulin for 10 min in the dark at RT. After centrifuging and decanting supernatant, 5 μL of appropriately diluted anti-GCR (clone

5E4, Serotec, Sydney, Australia; raised against a conserved sequence of the regulatory part of the receptor) as previously reported [4] was added for 15 min in the dark at RT. After a further wash, 5 uL of appropriately diluted secondary antibody rat-anti-mouse V450 (BD) was added for 15 min. After washing, appropriately diluted monoclonal antibodies to IFNγ FITC, TNFα PE, CD3 perCP.CY5.5, CD28 PE.CY7 (BD, Sydney, Australia), CD56 APC (Beckman Coulter, Sydney, Australia), CD8 APC.CY7 (BD) and CD45 V500 (BD) were added for 15 min in the dark at RT. After washing, cells were analyzed within 1 h on a FACSCanto II flow cytometer using FACSDiva software (BD). Samples were analyzed by gating lymphocytes using CD45 staining versus side scatter (SSC). A minimum of 350,000 low SSC events were acquired in list-mode format for analysis. T cell and NKT-like cell subsets were identified as reported above.

Effect of drugs on GCR and IFNγ/TNFα expression by in CD28± CD8±T and NKT-like cells

We have previously shown upregulation of heat shock protein 90 and a decrease in pro-inflammatory cytokine production by CD28null CD8+ T and NKT-like cells in the presence of 2.5ng/mL cyclosporine A. We wanted to determine the effect of this very low dose cyclosporin A in combination with corticosteroid prednisolone on GCR expression by these steroid resistant pro-inflammatory cells in blood and the small airways. This was performed using small airway lymphocytes from 3 COPD subjects only, due to restrictions of cell numbers in these samples. Aliquots of blood and distal brushings were incubated with ±1 μM prednisolone ± 2.5 ng/mL cylosporin A in 10 mL sterile tubes in a humidified 5% CO_2/95 % air atmosphere at 37 ^oC for 24 h. These cultures were then

stimulated as for intracellular cytokine production then processed as for intracellular cytokines and GCR, IFNγ and TNFα expression as described above.

Statistical Analysis

Statistical analysis was performed using the non-parametric Kruskal-Wallis test. When $p<0.05$, post hoc analysis was performed using the Mann-Whitney test. For post hoc analyses, all groups were compared with healthy control subjects for all parameters. Pearson's correlation tests were performed with SPSS software (SPSS Inc. IBM Chicago, USA) and differences between groups of $p<0.05$ considered significant.

RESULTS

Percentages of blood lymphocyte subsets

We noted a significant decrease in peripheral blood T cells in patients with COPD compared with control group (COPD 63.6% of total lymphocytes (31-86) and control 84.0% (71-89), (median (range)). A significant increase in peripheral blood NKT-like cells in patients with COPD compared with control group was identified (COPD 21.3% of total lymphocytes (6-39) and control 6.0% of total lymphocytes (1-26)). There was no change in the percentage of peripheral blood NK cells between groups (data not shown).

An increase in CD8+ and a decrease in CD4+ T cells in COPD compared with control group (CD8: COPD 75.0% of CD3+ T cells (52-78) and control 48.4% of CD3+ T cells (27-66)) was noted but no changes in any other lymphocyte subsets between groups ($p>0.05$ for all).

Percentages of BAL lymphocyte subsets

A decrease in the percentage of BAL T cells and CD4+ T cells in COPD patients compared with control subjects was identified. (CD3+ T cells as a % of total leukocytes: COPD 71% (53-90), control 78% (51-93); CD4+: COPD 34% (31-34), control 66% (12-86)). There was an increase in CD8+ T cells, NKT-like cells and NK cells in COPD patients compared with control subjects (CD8+T COPD 63% (58-71) control 33% (13-68); NKT-like: COPD 12% (3-14), control 3% (1-8); NK: COPD 15% (3-21), control 1% (1-4)) (median (range)).

Percentages of large airway brushing intraepithelial lymphocyte subsets

There was no change in the percentage of large (proximal) airway brushing CD8+ or CD4+ intraepithelial lymphocytes between COPD compared with controls (CD8+ T cells: COPD 74% (56-88), control 67% (31-88) (median (range)); (CD4+ T cells: COPD 22% (15-42) control 31% (15-65) (median (range)). There were no other changes in the percentages of intraepithelial lymphocyte subsets in the large airways between groups ($p>0.05$ for all).

Percentages of small airway brushing intraepithelial lymphocyte subsets

A significant decrease in the percentage of CD3+ T cells and CD4+ T cells and an increase in CD8+ T cells, NK and NKT-like cells in small (distal) airway intraepithelial lymphocytes from patients with COPD compared with control group. (CD3+ T cells: COPD 41% (33-83), control 79% (66-93); CD4+ T cells: COPD 23% (16-31) control 41% (32-56); CD8+ T cells: COPD 75% (66-87), control 52% (41-69); NK cells: COPD 19% (1-23), control 3% (1-10); NKT-like cells: COPD 19% (5-23), control 5% (2-19) (median(range)).

Increased CD28null CD8 +T and NKT-like cells in COPD

We noted a significant increase in the percentage of peripheral blood CD28null CD8+ T cells and CD28null CD8+ NKT-like cells in COPD patients compared with the healthy control group (Table 2). There was no change in the percentage of peripheral blood CD28null CD8- T cells or NKT cells between COPD and control group (Table 2). Similarly these was a significant increase in the percentage of BAL, large and small airway CD28null CD8+ T cells and CD28null CD8+ NKT-like cells in COPD patients

compared with the healthy control group (Table 2) but no change in CD28null CD8- (CD4+) T cells or NKT cells between COPD and control group (Table 2).

Percentages of granzyme b positive CD28±CD8±T and NKT-like subsets

There was a significant increase in the percentage of granzyme b positive blood CD28null CD8+ T and NKT-like cells in COPD patients compared with control subjects (Table 3). Similarly there was an increase in the percentage of granzyme b positive CD28null CD8+T and NKT-like lymphocyte subsets from BAL, large airways and small airways in COPD patients compared with control group ($p<0.05$ for all) (Table 3).

Increased CD28± CD8± T and NKT-like lymphocyte subsets producing IFNγ and TNFα pro-inflammatory cytokines in COPD

There was an increase in the percentage of CD28null CD8+T and NKT-like blood lymphocyte subsets producing IFNγ and TNFα pro-inflammatory cytokines in COPD patients compared with control group ($p<0.05$ for all) (Table 4)

Similarly there was an increase in the percentage of CD28null CD8+T and NKT-like lymphocyte subsets from BAL, large airway and small airway producing IFNγ and TNFα in COPD patients compared with control group ($p<0.05$ for all). (Table 4). There was a significant increase in the percentage of CD28null CD8+ T and NKT-like cells producing IFNγ/TNFα in small airways compared with large airways, BAL and blood in patients with COPD (Table 4) ($p<0.05$ for all).

Percentages of CD28±CD8±T and NKT-like blood lymphocyte subsets expressing GCR

There was no difference in the percentage of CD28null CD8+T and NKT-like lymphocyte subsets expressing GCR in COPD patients compared with control group ($p<0.05$ for all) in either blood, BAL, large or small airways ie., all CD28null CD8+T and NKT-like lymphocytes express reduced GCR regardless of subject.

Correlation between small airway CD28null CD8+GCR+T cells producing IFNγ/TNFα and patient FEV_1

There was a negative correlation between the percentage of blood and small airway CD28null CD8+ intraepithelial T cells expressing GCR and producing IFNγ and TNFα in patients with COPD. Data for small airway GCR and IFNγ and TNFα are shown in Figure 1a and 1b respectively.

A negative correlation was identified between FEV_1 and the percentage of blood and small airway CD28null CD8+ T cells expressing GCR in patients with COPD. Data for small airway FEV_1 and GCR are shown in Figure 2. There was no correlation between any other lymphocyte subset and FEV_1 ($p>0.05$ for all).

Effect of 1μM prednisolone ±2.5ng/mL cyclosporin A on IFNγ/TNFα production by CD28null CD8+ T cells

Based on our previous findings of upregulation of heat shock protein by blood CD28null T and NKT-like cells in the presence of 2.5ng/mL cyclosporine A [9], we also investigated the effect of low dose cyclosporine A and standard dose prednisolone (1

μM) on GCR expression and IFNγ and TNFα pro-inflammatory cytokine production by these cells *in vitro*. In small airway brushings there were insufficient lymphocytes to investigate the effect of these two immunosuppressants individually. We therefore investigated the combined effects of 1μM prednisolone + 2.5ng/mL cyclosporine A in small airway derived lymphocytes, and in whole blood. Addition of 1μM prednisolone and/or 2.5ng/mL cyclosporine A resulted in the upregulation GCR and decreased production of IFNγ/TNFα production by blood CD28null CD8+ T and NKT-like cells. The effect was synergistic when both immunosuppressants were used. Addition of 1μM prednisolone and 2.5ng/mL cyclosporine A resulted in the upregulation GCR and decreased production of IFNγ/TNFα production by small airway intraepithelial CD28null T and NKT-like cells. Representative plots from a COPD patient showing the combined effect of 1μM prednisolone and 2.5ng/mL cyclosporine A on the upregulation of GCR and decreased production of IFNγ/TNFα production by small airway CD28null CD8+ T is shown if Figure 3.

There was no difference in the combined effect of 1μM prednisolone and 2.5ng/mL cyclosporine A on upregulation GCR and decreased production of IFNγ/TNFα production by CD28null T and NKT-like cells from blood or small airway lymphocytes ($p>0.05$ for all). The effect of ±1μM prednisolone ±2.5ng/mL cyclosporine A on the percentage decrease in IFNγ production, decrease in TNFα production and increase in GCR expression by blood CD28null T cells from all patients with COPD is shown in Figure 4a,b,c respectively.

DISCUSSION

This is the first study to show an increase in intra-epithelial cytotoxic/pro-inflammatory lymphocyte subsets in the small airways compared with blood, BAL and large airways in patients with COPD. A previous study showed that CD8+T cells are required for inflammation and lung destruction in cigarette smoke-induced emphysema in mice [7], indicating CD8+ T cells are a central regulator of the inflammatory network in COPD. Senescent CD28null T and NKT-like cells have been shown to be more pro-inflammatory and cytotoxic than their CD28 positive counterparts, show cytotoxicity to lung epithelial cells [13], and exhibit relative resistance to corticosteroids [4,9,12,14-17]. We had previously shown CD8+ T cells from COPD patients produce increased IFNγ and TNFα from blood, BAL and bronchial brushings compared with aged-matched controls [2] and our current study takes this further to show that CD28null CD8+ T and NKT-like cells are the most pro-inflammatory lymphocyte subsets in COPD. Their increase in the COPD lung supports the emerging concept of accelerated aging and accumulation of aged cells in the lung in COPD [18]. Furthermore, in line with the notion of COPD as a disease of small airways, these senescent cells are in greatest numbers in the small distal airways compared with blood and other areas of the lungs.

We showed that the percentage of T cells expressing GCR in the small airways is reduced, likely resulting in a reduced capacity to respond to corticosteroids, the mainstay of anti-inflammatory medication in COPD. Importantly, the loss of GCR by the increased numbers of senescent CD28null CD8+ T and NKT-like cells was shown to correlate with COPD disease severity. These findings go a long way in explaining why current anti-inflammatory treatments with glucocorticoids are ineffective in the treatment of patients

with COPD [6] particularly in the small airways. There have been several theories suggested for the poor response of COPD patients to the anti-inflammatory effects of glucocorticoids [6]. For glucocorticoids to be therapeutically effective they must first enter the cell and bind to the GCR. This complex is taken into the nucleus by various chaperones such as HSP90 and sirtuin-1 where HDAC2 is engaged to switch off pro-inflammatory cytokine production. We have shown that many steps are involved in the reduced responsiveness of senescent CD28nullCD8+T and NKT-like cells to steroids. The drug efflux pump Pgp1 is increased in peripheral blood T, NKT-like and NK cells from subjects with COPD [14]. However, there was no difference in Pgp-1 levels between CD28+ and CD28null T and NKT-like cells [14] suggesting this is not involved in GC resistance by these cells in COPD. We however did show that there are reduced levels of Hsp90 [9], sirtuin-1 [16] and HDAC2 [17] in CD28null CD8+ T and NKT-like cells compared with their CD28+ counterparts. Interestingly there were no differences between levels of these proteins in CD28null and CD28+ lymphocytes between COPD patients and healthy control subjects although the percentages of CD28null lymphocytes were significantly greater in COPD subjects. Unfortunately, lymphocyte cell numbers from the small airway brushings were insufficient to investigate all patients with COPD and further mediators of steroid resistance including HSP90, sirtuin-1 and HDAC2. However, given the similar results in the blood of COPD patients from our previous studies, it is highly likely these other mechanisms of steroid resistant are also present in these lymphocyte subsets in the small airways. Our finding that addition of very low dose cyclosporine A resulted in increased expression of GCR in senescent CD28nullCD8+T and NKT-like cells is very significant given our previous findings of decreased PGP-1

[14] expression and increased HSP90 [9], sirtuin-1[16] and HDAC2 [17] also in the presence of this drug. The very low dose of cyclosporine A (2.5ng/mL) is unlikely to cause any significant side effects known to be associated with the much higher levels of this drug used to prevent lung transplant rejection (80-250ng/mL). Our findings argue for a larger clinical randomized control trial using low dose cyclosporine ± standard glucocorticoid dose examining pro-inflammatory cytokine production and levels of GCR, PGP-1, HSP-90, sirtuin-1 and HDAC2 in these cells. One could also speculate that these CD28null lymphocytes may be the precursor to other systemic GC resistant diseases such as CVD, autoimmune disease, arthritis, IBD, aging and aging associated with COPD [19-24]. Others have shown increased CD8+ T cells in the small airways of patients with severe COPD compared with mild COPD consistent with our findings [25].

We have previously used the technique of comparing pro-inflammatory intra-epithelial T cells between trachea and bronchi to identify COPD severity consistent with our current study [26]. An important addition to our current study would be the addition of a non-COPD smoker group and a COPD-smoker group to identify changes that may be attributed to smoking alone. A COPD group receiving inhaled steroids would also allow determination of any effects of steroids on pro-inflammatory cytokine production by the various lymphocyte subsets.

In this regard, we have previously shown an increase in cytotoxic/pro-inflammatory CD8+ T cells in the blood of patients diagnosed with another small airways disease, bronchiolitis obliterans syndrome (BOS) following lung transplantation [27]. Importantly, these changes were noted up to 18 months preceding a fall in lung function of these patients suggesting phenotypic analysis of these cells in blood may be predictive

of pending BOS [28, 29]. A similar study determining granzyme b, GCR expression and IFNγ/TNFα production in CD28null CD8+T and NKT-like cells in the small airways and blood in smokers who have not progressed to COPD would be important. There are currently no tests available that we are aware of to determine if smokers will develop COPD before a change in airway resistance or fall in lung function [29]. There is a need to specifically target small airway disease to attenuate the progression of COPD [8, 29].

In conclusion, COPD is associated with increased intra-epithelial cytotoxic/pro-inflammatory CD28null CD8+ T and NKT-like in the blood, BAL, large and small airways, the latter being the largest site of these cell accumulation.. Treatments that increase GCR in these lymphocyte subsets in the blood and airways in COPD patients may improve patient morbidity.

Declarations

Ethics approval and consent to participate

COPD subjects were specifically recruited for the study and informed written consent obtained. Ethics approval was obtained from the Royal Adelaide Hospital Human Ethics Committee.

Availability of data and materials

All data are present in the manuscript.

Competing interests

The authors declare they have no competing interests.

Autor's contribuations

GH performed the concept and design of experiments, analysis and interpretation of data and manuscript preparation; HJ supplied and characterized patient specimens and helped draft the manuscript; HT helped with experiments and helped draft the manuscript; PFA helped with experiments and helped draft the manuscript; MJ helped with experiments and helped draft the manuscript; PNR supplied and characterized patient specimens and helped draft the manuscript; MH supplied and characterized patient specimens and helped draft the manuscript;. SH: helped with study design, statistical analyses and helped draft the manuscript. All authors read and approved the final manuscript.

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Table 1. Demographic details of the study participants

Subjects	Control	COPD
Number	11	10
Age (years)	56 (44-68)#	54 (39-69)
FEV_1 % pred	104.2 (95.2-103.1)	52 (43-82)*
FEV_1% FVC	68 (± 12)	51 (36-66)*
Male/female	5/6	5/5

median (range)

* significantly decreased compared with control ($p<0.05$ for all). (Wilcoxon, SPSS software).

FEV_1, forced expiratory volume in 1 second; % pred, percent predicted; FVC, forced vital capacity.

Table 2. Percentage of CD28null CD8+ T and NKT-like cells in blood, bronchoalveolar lavage (BAL), small airways (SA) and large airways (LA) from COPD patients and control subjects. There was a significant increase in CD28null CD8+ T and NKT-like cells in blood, BAL, SA and LA from COPD patients compared with control subjects.

There was a significant increase in CD28null CD8+ T and NKT-like cells in SA compared with blood, BAL and LA from COPD patients.

COPD	T CELLS		NKT-like CELLS	
	CD28null CD8+	CD28nullCD4+	CD28nullCD8+	CD28nullCD4+
BLOOD	*55 (38-63)#	8 (3-12)	*61 (36-71)	6 (2-9)
BAL	*62 (42-76)	9 (4-14)	*67 (48-91)	7 (2-10)
LA	*72 (54-92)	7 (2-12)	*74 (42-79)	8 (1-14)
SA	*86 (69-98)@	12 (3-18)	*88 (71-99)	11 (2-16)

CONTROL	T CELLS		NKT-like CELLS	
	CD28null CD8+	CD28nullCD4+	CD28nullCD8+	CD28nullCD4+
BLOOD	34 (18-42)	6 (3-12)	36 (23-44)	5 (2-8)
BAL	33 (13-46)	6 (2-13)	36 (18-53)	6 (1-10)
LA	31 (12-43)	9 (3-16)	33 (16-47)	7 (2-14)
SA	38 (22-53)	11 (2-19)	42 (25-62)	10 (1-16)

median (range)

* significantly increased compared with control ($p<0.05$ for all).

@ significantly increased compared with blood, BAL and LA ($p<0.05$ for all).

Table 3. Percentage of granzyme b positive CD28null CD8+ T and NKT-like cells in blood, bronchoalveolar lavage (BAL), small airways (SA) and large airways (LA) from COPD patients and control subjects. There was a significant increase in granzyme b positive CD28null CD8+ T and NKT-like cells in blood, BAL, SA and LA from COPD patients compared with control subjects.

Granzyme b pos	CD28null CD8+ T CELLS		CD28null CD8+ NKT-like CELLS	
	Control	COPD	Control	COPD
BLOOD	25 (17-28)	*70 (52-83)	80 (38-88)	*92 (66-98)
BAL	20 (8-25)	*50 (36-66)	25 (12-32)	*50 (32-68)
LA	10 (2-15)	*45 (36-61)	15 (6-27)	*75 (61-84)
SA	8 (1-12)	*75 (49-88)	16 (4-23)	*85 (68-96)

median (range)

* significantly increased compared with control ($p<0.05$ for all).

Table 4. Percentage of CD28null CD8+ T and NKT-like cells producing interferon gamma (IFNγ) and tumour necrosis factor alpha (TNF α) in blood, bronchoalveolar lavage (BAL), small airways (SA) and large airways (LA) from COPD patients and control subjects. There was a significant increase in CD28null CD8+ T and NKT-like cells producing IFNγ and TNFα in blood, BAL, SA and LA from COPD patients compared with control subjects. There was a significant increase in CD28null CD8+ T and NKT-like cells producing IFNγ and TNFα in SA compared with blood, BAL and LA from COPD patients.

COPD	T CELLS		NKT-like CELLS	
	CD28null CD8+		CD28nullCD8+	
	IFNγ	TNFα	IFNγ	TNFα
BLOOD	*55 (38-63)#	*58 (42-66)	*61 (36-71)	*66 (38-73)
BAL	*62 (42-76)	*59 (39-73)	*67 (48-91)	*69 (41-78)
LA	*72 (54-92)	*67 (51-88)	*74 (42-79)	*78 (52-84)
SA	*86 (69-98)@	*92 (72-97)@	*88 (71-99)@	*91 (69-96)@

CONTROL	T CELLS		NKT-like CELLS	
	CD28null CD8+		CD28nullCD8+	
	IFNγ	TNFα	IFNγ	TNFα
BLOOD	34 (18-42)	36 (20-45)	36 (23-44)	35 (16-48)
BAL	33 (13-46)	33 (12-33)	36 (18-53)	36 (13-46)
LA	31 (12-43)	39 (14-41)	33 (16-47)	37 (13-51)
SA	38 (22-53)	31 (12-38)	42 (25-62)	30 (11-40)

median (range)

* significantly increased compared with control ($p<0.05$ for all).

@ significantly increased compared with LA ($p<0.05$ for all).

Figure Legends

Figure 1. Negative correlation between the percentage of small airway CD28null CD8+ intraepithelial T cells CD28null CD8+ intraepithelial T cells expressing GCR and producing IFNγ (Fig 1a) and TNFα (Fig 1b) in patients with COPD.

Figure 2. Negative correlation between FEV_1 and the percentage of small airway CD28null CD8+ T cells expressing GCR in patients with COPD.

Figure 3. Representative plots from a COPD patient showing the combined effect of 1μM prednisolone and 2.5ng/mL cyclosporine A on the upregulation of GCR and decreased production of IFNγ/TNFα production by small airway CD28null CD8+ T cells.

Figure 4. Effect of ±1μM prednisolone ±2.5ng/mL cyclosporine A on the percentage decrease in IFNγ production (Fig 4a), decrease in TNFα production (Fig 4b) and increase in GCR expression (Fig 4b) in blood CD28null T cells from all patients with COPD.