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Lymphocyte senescence in COPD is associated with decreased sirtuin 1 expression in steroid resistant pro-inflammatory lymphocytes

Greg Hodge, Hai B. Tran, Paul N. Reynolds, Hubertus Jersmann and Sandra Hodge

Abstract

Background: The class III NAD-dependent histone deacetylase (HDAC) sirtuin 1 (SIRT1) is an important regulator of senescence, aging, and inflammation. SIRT1de-acetylates chromatin histones, thereby silencing inflammatory gene transcription. We have reported increased steroid-resistant senescent pro-inflammatory CD28nullCD8+ T cells in patients with chronic obstructive pulmonary disease (COPD). We hypothesized that SIRT1 is reduced in these cells in COPD, and that treatment with SIRT1 activators (resveratrol, curcumin) and agents preventing NAD depletion (theophylline) would upregulate SIRT1 and reduce pro-inflammatory cytokine expression in these steroid-resistant cells.

Methods: Blood was collected from $n = 10$ COPD and $n = 10$ aged-matched controls. Expression of CD28, SIRT1, and pro-inflammatory cytokines was determined in CD8+ and CD8– T and natural killer T (NKT)-like cells cultured in the presence of ±1 μM prednisolone, ±5 mg/L theophylline, ±1 μM curcumin, ±25 μM resveratrol, using flow cytometry and immunofluorescence.

Results: There was an increase in the percentage of CD28nullCD8+ T and NKT-like cells in COPD patients compared with controls. Decreased SIRT1 expression was identified in CD28nullCD8+T and NKT-like cells compared with CD28+ counterparts from both patients and controls (e.g. CD28null 11 ± 3% *versus* CD28+ 57 ± 9%). Loss of SIRT1 was associated with increased production of IFNγ and TNFα, steroid resistance, and disease severity. SIRT1 expression was upregulated in the presence of all drugs and was associated with a decrease in steroid resistance and IFNγ and TNFα production by CD28nullCD8+T and NKT-like cells. The presence of the SIRT1 inhibitor, EX-527 negated [by 92 ± 12% (median ± SEM)] the effect of the SIRT1 activator SRT720 on the percentage of CD8+ T cells producing IFNγ and TNFα.

Conclusions: Steroid resistance in pro-inflammatory CD28nullCD8+ T and NKT-like cells is associated with decreased SIRT1 expression. Treatment with prednisolone, in combination with theophylline, curcumin or resveratrol increases SIRT1 expression, restores steroid sensitivity, and inhibits pro-inflammatory cytokine production from these cells and may reduce systemic inflammation in COPD.

The reviews of this paper are available via the supplemental material section.

Keywords: CD28nullCD8+ T and NKT-like cells, COPD, IFNγ and TNFα, lymphocyte senescence, SIRT1

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Introduction

Current treatments such as corticosteroids, used to treat chronic obstructive pulmonary disease (COPD), a significant cause of death in the world, do not modify the disease.[1] The exact mechanisms involved in lymphocyte resistance to

corticosteroids are incompletely known.[2] Our previous research has identified increased expression of pro-inflammatory cytokines and cytotoxic mediators, granzyme b and perforin, in CD8+ T lymphocytes in peripheral blood and airways of ex-current-smoker COPD patients compared with healthy smokers and subjects that have never smoked cigarettes.[3,4]

We have identified the lymphocyte subset/s resistant to current available treatments, and have identified several novel discoveries. Numbers of CD28nullCD8+ senescent cells are increased in the peripheral blood of both ex-smoker and current smoker COPD patients. These senescent cells express more cytotoxic mediators and pro-inflammatory cytokines than their CD8+CD28+ counterparts and are resistant to standard therapeutic dose of prednisolone.[5,6] The percentages of natural killer (NK) and natural killer T (NKT)-like lymphocytes are increased in bronchoalveolar lavage (BAL) of COPD patients, and both cell types show increased cytotoxicity.[7] In other inflammatory lung diseases, CD8+CD28null NKT-like cells are more cytotoxic/pro-inflammatory than CD8+CD28+ NKT-like cells.[8]

Recently, we showed that the glucocorticoid receptor (GCR) was reduced in CD28nullCD8+ T-cells.[9] We hypothesized there may also be other mechanisms that potentiate their pathogenic influence.

Sirtuin 1 (SIRT1) is a class III NAD-dependent histone deacetylase (HDAC) and an important regulator of senescence, inflammation, and aging, as well as de-acetylating chromatin histones halting inflammatory gene transcription.[10] SIRT1 is reduced in macrophages and the lungs of patients with COPD and in peripheral blood mononuclear cells[11–13]; however, to our knowledge, reports of SIRT1 levels in peripheral blood lymphocytes in these patients are lacking. CD8+ T cells have been reported to be central regulators of the inflammatory network in patients with COPD.[14] When exposed to long-term cigarette smoke, CD8+ T-cell deficient mice showed reduced inflammation and did not develop emphysema.[14]

Resveratrol and curcumin are naturally occurring polyphenols have been shown to activate SIRT1 and suppress inflammation *via* inhibiting NF-κB signaling.[15] SIRT1 was shown to physically interact with GCR, enhancing GCR-induced transcriptional activity on glucocorticoid response genes. These effects were attenuated by SIRT1 knockdown.[16]

We hypothesized decreased levels of SIRT1 in steroid-resistant peripheral blood pro-inflammatory CD28nullCD8+ T and NKT-like lymphocyte subsets in patients with COPD, and that treatment with SIRT1 activators would decrease pro-inflammatory cytokine production and reduce steroid resistance in these cells.

To investigate this hypothesis, we determined whether peripheral blood CD28null T cells (particularly CD8+) and NKT-like cells from COPD patients express reduced levels of SIRT1 and whether loss of SIRT1 is associated with concurrent loss of GCR and increased expression of pro-inflammatory cytokines and steroid resistance. We also investigated the effect of theophylline and polyphenols resveratrol and curcumin, SIRT1 activator SRT1720 and SIRT inhibitor, EX-527, in combination with the corticosteroid, prednisolone, on SIRT1 expression and associated pro-inflammatory cytokine expression by lymphocyte subsets.[17]

Materials and methods

Patient and control groups

COPD volunteers were specifically recruited for the study and informed consent obtained. Patients experienced no exacerbation of COPD for 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, and the experiments were conducted with the understanding and the written consent of each participant. COPD was diagnosed using the GOLD criteria with clinical correlation [mild COPD: forced expiratory volume in 1 s (FEV1)/ forced vital capacity (FVC) $<70\%$ but FEV1 $\geqslant 80\%$ predicted; moderate COPD FEV1 $50\% \leqslant 80\%$ predicted, severe COPD FEV1 $30\% \leqslant 50\%$ predicted, very severe COPD FEV1 $<30\%$].[18] Blood was collected from 10 subjects diagnosed with COPD (Table 1). All COPD subjects were ex-smokers (at least 1 year) with an average of 39 pack years. No patients were receiving oral GCS.

Blood was also obtained from 10 healthy aged-matched nonsmoking volunteers (Table 1) with

Table 1. Demographic details of the COPD and control group.

Participants	Controls	COPD
No. of subjects	10	10
Age (years)	56 (±9)	58 (±16)
FEV1, % pred	106.6 (±11)	60.1 (±20)*
FEV1, % FVC	97 (±11)	58 (±15)*
Male/Female	5/5	6/4

Data showing mean ± SEM.
COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.
*$p < 0.05$ compared with controls.

normal lung function and no history of airways disease. All subjects underwent spirometry as part of their routine clinical assessment. Venous blood was collected into 10 U/ml preservative free sodium heparin (DBL, Sydney, Australia), and maintained at 4°C until processing. All patients were submitted to the same protocol and analysis performed retrospectively.

SIRT1 and intracellular cytokine expression in T and NKT-like cell subsets

To determine co-expression of SIRT1 and intracellular cytokine production in CD8+ and CD8– T and NKT-like cells, aliquots of blood were stimulated as previously reported,[3] 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°C. Preliminary experiments showed stimulation of cells was required for detection of SIRT1 expression in lymphocyte subsets. The addition of brefeldin A had no effect on SIRT1 expression in these experiments. At 16 h, cells were treated as previously reported,[19] and appropriately diluted monoclonal antibodies to SIRT1 Alexa-Fluor 488 (Abcam ab157401, Melbourne, Australia), IFNγ PE (BD, Sydney, Australia), CD3 perCP.CY5.5 (BD), CD28 PECY7 (BD), CD56 APC (Beckman Coulter, Sydney, Australia), CD8 APC.CY7 (BD), TNFα V450 (BD) and CD45 V500 (BD) were added for 15 min in the dark at room temperature. Appropriate IgG-negative and fluorescence-minus-one control (FMO) controls were used to set all quadrant markers. Cells were washed and after decanting, 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 3.5×10^5 low SSC events was acquired in list-mode format for analysis. T cells were identified as CD3+CD56– CD45+ and NKT-like cells identified as CD3+CD56+CD45+ low FSC/SSC events as previously reported.[19]

GCR and SIRT1 combined staining with intracellular cytokine expression in T and NKT-like cell subsets

To determine whether GCR and SIRT1 are associated with intracellular cytokine production in CD8+ and CD8– T and NKT-like cells, aliquots of blood were stimulated and treated as described above. Following washing of permeabilized cells, appropriately diluted GCR antibody (MCA2469, Bio-Rad, Sydney, Australia) was added to cells for 15 min. Cells were further washed and stained with anti-mouse IgG1 V450 secondary antibody for 15 min. Cells were then washed and stained with SIRT1 Alexa-Fluor 488, IFNγ PE (BD), TNFα PE (BD), CD3 perCP.CY5.5 (BD, Sydney, Australia), CD28 PECY7 (BD), CD56 APC (Beckman Coulter, Sydney, Australia), CD8 APC.CY7 (BD), and CD45 V500 (BD) for 15 min in the dark at room temperature. Appropriate controls and cells were analyzed as above.

Effect of therapies on SIRT1, GCR, and intracellular IFNγ expression in T and NKT-like cell subsets

Our aim was to investigate the effect of standard therapeutic dose of prednisolone (1 µM), theophylline (5 µg/ml) (prevents NAD+ depletion) and polyphenols,[20] resveratrol (25 µM),[21] curcumin (2 µM),[22] SIRT activator STR720 (1 µM),[23] and SIRT inhibitor, EX-527 (1 µM),[17] on SIRT1, GCR expression and production of IFNγ and TNFα by CD8+ and CD8– T and NKT-like cells. Exposure to theophylline and polyphenols used concentrations previously shown not to cause significant side effects.[20–23] Aliquots of blood were mixed in 10 ml sterile tubes with equal volumes of RPMI medium with 10% fetal calf serum (FCS) and incubated with treatments (and combinations) and the tubes

incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for 24 h. Blood cultures were then stimulated as for intracellular cytokine production as described above for 16 h. SIRT1, GCR, IFNγ, and TNFα expression in blood was assessed as described above.

SIRT1 expression in CD28+ and CD28null T cells by fluorescent microscopy

CD28+ and CD28 null T cells (1×10^3 cells) were sorted as described above, and centrifuged at 500 *g* for 5 min in a Cytospin 4 cytocentrifuge (ThermoFisher Scientific, Scorseby, Victoria, Australia). Slides were treated as previously reported and incubated at 4°C with 1/25 diluted SIRT1 rabbit monoclonal antibody (Serotec, Abacus ALS, Brisbane, Australia), then 1 h with AF594-conjugated donkey IgG F(ab')2 fragment polyclonal antibody to rabbit IgG (Abcam, Sapphire Bioscience, Waterloo, NSW, Australia). 4′,6-Diamidino-2-phenylindole (DAPI) was used as a counterstain (Sigma-Aldrich). Immunofluorescence was detected and imaged with an Olympus IX73 fluorescence microscope (Olympus, Notting Hill, VIC, Australia). For quantitative analysis, cells from each cytospin were photographed under a 40× objective in eight optical fields, selected in the DAPI channel for bias prevention. The mean fluorescence intensities were then measured in the AF594 channel using ImageJ software (NIH, Bethesda, MD, USA) as described previously.[9,24]

Statistical analysis

Statistical analyses were performed using the Friedman test with Wilcoxon sign rank test for *post hoc* pairwise comparisons. For T-cell subsets CD28null/CD8+/CD3+/CD56–/CD45+/TNFα+/IFNγ+), a sample size of $n = 10$ allowed a power of 98–99.5% for analysis. Variance was estimated from our previous studies.[3–6] Correlations were performed using Spearman Rho correlation tests. SPSS software was applied and differences between groups of $p < 0.05$ considered significant.

Results

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

There was a significant increase in CD28null CD8+ T cells in patients with COPD compared with healthy controls, but no change in CD28nullCD8– T cells (CD28nullCD8+ T cells: COPD 57% ± 8.4% *versus* controls 33% ± 8.5%); CD28nullCD8– T cells: COPD 7.1% ± 3.1% *versus* controls 5.9% ± 4.2% (median ± SEM) consistent with our previous findings for CD28null T cells.[5] CD28nullCD8+ were significantly increased in NKT-like cells from patients with COPD compared with healthy controls, but no changes were noted in CD28nullCD8– NKT-like cells (CD28nullCD8+ NKT-like cells: COPD 39% ± 5.9% *versus* controls 22% ± 6.1%; CD28nullCD8– T cells: COPD 8.8% ± 3.6% *versus* controls 7.8% ± 3.3%; median ± SEM).

SIRT1 expression by CD28+ and CD28null T and NKT-like cells

A reduced percentage of CD28nullCD8+ T and NKT-like cells expressed SIRT1 in both COPD groups and controls was noted, compared with CD28+ T and NKT-like cells (data for T cell and NKT-like cell subsets from COPD group shown in Figure 1). SIRT1 expression in CD28nullCD8+ T and NKT-like cells in controls was unchanged compared with the COPD group for example, control SIRT1+CD28null CD8+T cells 10% ± 2.7%; SIRT1+CD28+ CD8+T cells 57% ± 7.8%; SIRT1+CD28null CD8+NKT-like cells 8% ± 2.3%; SIRT1+CD28 +CD8+NKT-like cells 54% ± 6.9%.

SIRT1, IFNγ, and TNFα production by CD28+, CD28null T, and NKT-like cells

A significant increase in the percentage of CD28nullCD8+ T and NKT-like cells producing IFNγ and TNFα, compared with CD28+CD8+ T and NKT-like cells, was noted in COPD patients and control groups (data for CD28null and CD28+ CD8+ and CD8– T and NKT-like cells producing IFNγ for the COPD group shown in Figure 2 and data for IFNγ and TNFα production for the control group and TNFα production by the COPD group not shown). There were no significant correlations between SIRT1 expression and the percentage of pro-inflammatory cytokine producing T cells or NKT-like cells in COPD or control groups (data not shown). A significant negative correlation was shown between loss of SIRT1 expression by CD28nullCD8+ T cells and the percentage of these cells producing IFNγ (Figure 3) and TNFα in the COPD group but not the control group

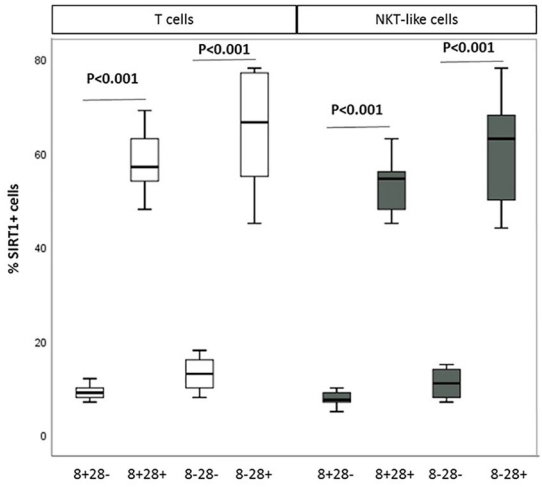

Figure 1. The percentage of CD28null and CD28+ CD8+ and CD8– T cells (clear bars) and NKT-like cells (grey bars) expressing SIRT1 in patients with COPD. Data presented as box plots. There was a significant decrease in the percentage of CD28null and CD28+ CD8+ and CD8– T and NKT-like cells expressing SIRT1 compared with CD28+CD8+ and CD28+CD8– T and NKT-like cells.
NKT, natural killer T; SIRT1, Sirtuin 1.

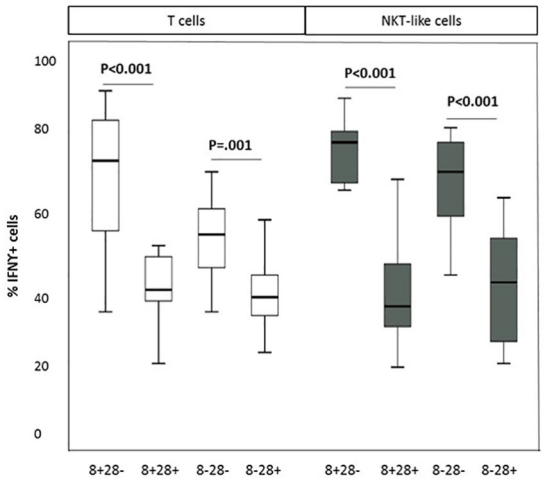

Figure 2. The percentage of CD28null and CD28+ CD8+ and CD8– T (clear bars) and NKT-like cells (grey bars) producing IFNγ in patients with COPD. Data presented as box plots. There was a significant decrease in the percentage of CD28null and CD28+ CD8+ and CD8– T and NKT-like cells producing IFNγ compared with CD28+CD8– and CD28+CD8+ T and NKT-like cells.
IFNγ, interferon gamma; NKT, natural killer T.

(data not shown). There was a significant negative correlation between loss of SIRT1 expression by CD28nullCD8+ NKT-like cells and the percentage of these cells producing IFNγ ($R=-0.627$, $p=0.036$) and TNFα ($R=-0.541$, $p=0.039$) in the COPD group.

SIRT1 expression in CD28+ and CD28null T cells by fluorescent microscopy

Sorted CD28+ and CD28null T cells were stained for SIRT1 expression. There was significant positive staining for SIRT1 in CD28+ T cells compared with CD28null T cells. SIRT1 staining was found to be located in the cytoplasm and cell nucleus (Figure 4).

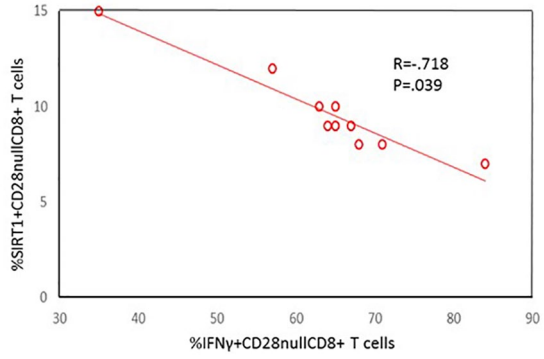

Figure 3. Significant negative correlation between the percentage of CD28nullCD8+ T cells expressing SIRT1 and producing IFNγ in COPD subjects.
COPD, chronic obstructive pulmonary disease; IFNγ, interferon gamma; SIRT1, Sirtuin 1.

Correlation between SIRT1 and GCR by CD8+ T and NKT-like cells

There has been a report that SIRT1 and GCR colocalize in HeLa cells[15]; therefore, we investigated the possibility that these two molecules are co-expressed in CD8+ T and NKT-like cells.

There was a correlation between SIRT1 and GCR expression by CD28nullCD8+ T and CD28+CD8+ T and NKT-like cells from both the COPD group and control group (data for COPD group are shown in Figure 5(a) and (b); data for control group not shown). Representative dot plots showing SIRT1 and GCR expression in CD28null and CD28+ CD8+ T cells from a COPD patient are shown in Figure 6.

Correlation between SIRT1 expression by CD28nullCD8+ T cells and FEV1

There was a significant correlation between SIRT1 expression by CD28nullCD8+ T cells and FEV1

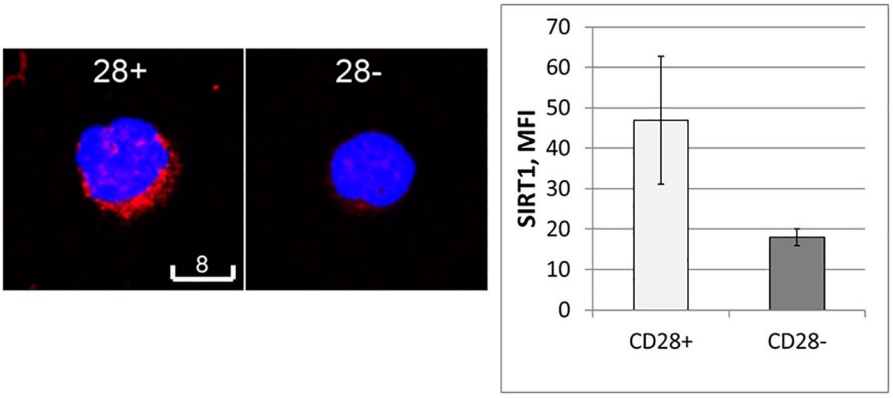

Figure 4. Representative laser confocal images of SIRT1 staining (red) in FACS-sorted CD28null (right) and CD28+ T cells (left). Blue is DAPI counterstaining. Scale bars are 8 μm. The bar graph shows results of quantitative analysis by ImageJ. Experiments were repeated three times, showing similar results. ***$p < 0.05$.
DAPI, 4′,6-Diamidino-2-phenylindole; FACS, fluorescence-activated *cell sorting*; SIRT1, Sirtuin 1.

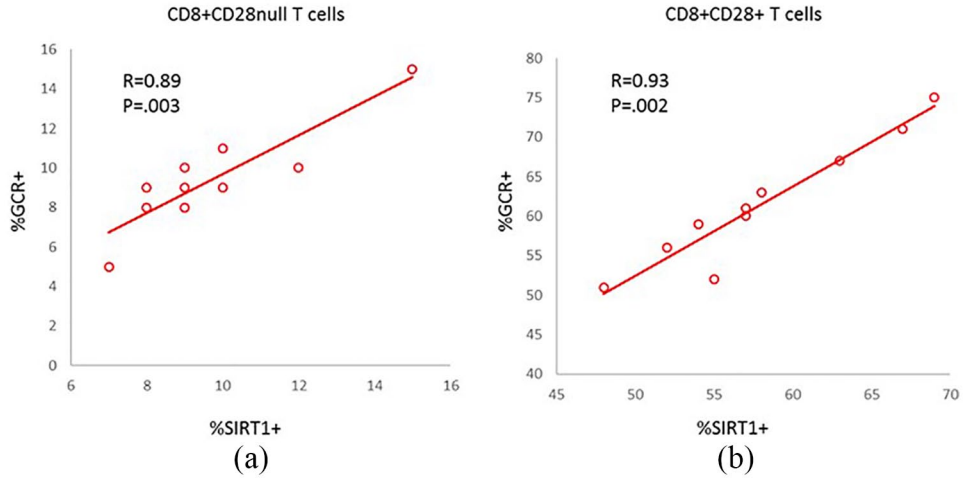

Figure 5. Significant correlation between SIRT1 and GCR expression by CD28nullCD8+ T (a) and CD28+CD8+ T (b) from the COPD group.
COPD, chronic obstructive pulmonary disease; GCR, glucocorticoid receptor; SIRT1, Sirtuin 1.

(% predicted) in COPD subjects (Figure 7). No correlations between SIRT1 expression by any other lymphocyte subset and FEV1 were observed (data not shown).

Effect of drugs on SIRT1, GCR, and intracellular cytokine expression by CD28null CD8+ T and NKT-like cells in COPD patients

There was significant decrease in the percentage of CD28nullCD8+T cells producing IFNγ (Figure 8a), and a corresponding increase expressing SIRT1 (Figure 8b) and GCR (Figure 8c) in the presence of prednisolone, curcumin, resveratrol, or combination of drugs ($p < 0.05$ for all). There was an additive increase in the percentage of CD28nullCD8+T cells expressing SIRT1 and GCR in the presence of all drugs. Similar results were obtained for upregulation of SIRT1 and GCR and inhibition of IFNγ production by CD28+CD8+ and CD8– T cells and CD28+ and CD28null CD8+ and CD8– NKT-like cells (i.e. results were similar for all T and NKT-like subsets). The presence of the SIRT1 inhibitor, EX-527 (1 μM),[16] negated [by 92 ± 12% (median ± SEM)] the effect of the SIRT1 activator SRT720 on the percentage of CD8+ T cells producing IFNγ and TNFα. The percentage of

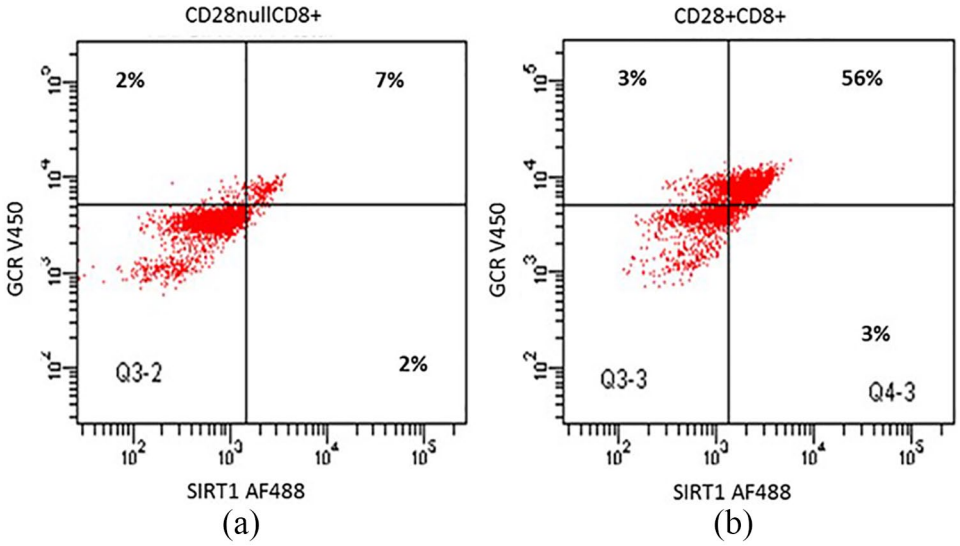

Figure 6. Representative dot plots showing SIRT1 and GCR expression in CD28null (a) and CD28+ CD8+ T cells (b) from a COPD patient.
COPD, chronic obstructive pulmonary disease; GCR, glucocorticoid receptor; SIRT1, Sirtuin 1.

IFNγ+ CD8+ T cells (72 ± 9%) and TNFα (75 ± 11%); in the presence of SRT720 (IFNγ: 42 ± 5% and TNFα: 35 ± 6%) and in the presence of EX-527 (IFNγ: 70 ± 10% and TNFα: 71 ± 13%). Representative dot plots showing the combined effect of 1 μM prednisolone + 5 mg/ml theophylline + 1 μM curcumin + 25 μM resveratrol on the percentage of CD28nullCD8+ expressing SIRT1 and IFNγ compared with control (no drugs) is shown in Figure 9.

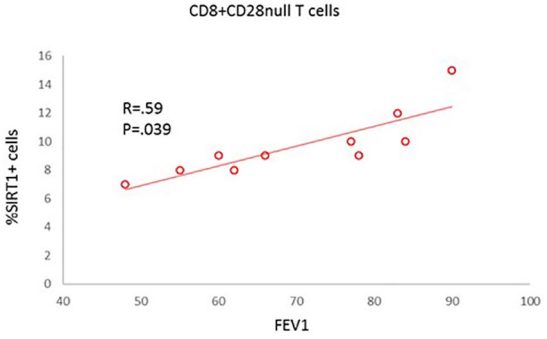

Figure 7. Correlation between SIRT1 expression by CD28nullCD8+ T cells and FEV1 from the COPD group.
COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s; SIRT1, Sirtuin 1.

Discussion

Loss of SIRT1 from senescent CD8+CD28null T and NKT-like cells has not previously been reported in the literature. Loss of SIRT1 correlated with the potential pro-inflammatory ability of these cells, and, importantly, the severity of the disease in these patients.

COPD is associated with significant chronic inflammation, and we have frequently reported increased CD8+ pro-inflammatory T lymphocytes in lungs and peripheral blood,[3] an increase in NK and NKT-like lymphocytes in the lungs,[6] and increased CD28nullCD8+ cells producing increased pro-inflammatory cytokines (TNFα and IFNγ) in ex-smoker and current COPD subjects.[5] Chronic inflammation and cellular senescence are intertwined with disease-related premature aging, and are considered important contributing factors driving the pathogenesis of COPD.[2]

SIRT1 is a key modulator of inflammation, with anti-inflammatory effects including inhibiting the activity of transcription factors such as NF-κB and AP-1 that are involved in the production of inflammatory cytokines.[25] There has been a report of reduced SIRT1 in macrophages and the lungs of patients with COPD[11]; however, to our knowledge, there have been no reports of SIRT1 levels in CD8+ peripheral blood lymphocytes in

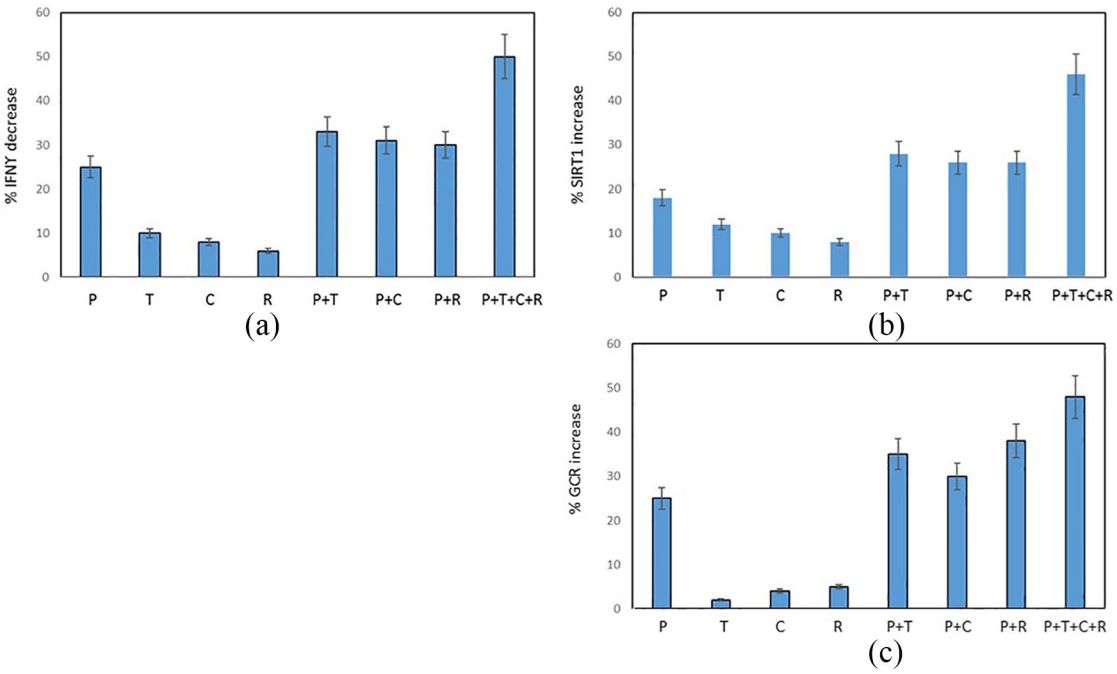

Figure 8. Graphs showing the effect of ±1 μM prednisolone (P), ±5 mg/l theophylline (T), ±1 μM curcumin (C), ±25 μM resveratrol (R) on (a) the inhibition of IFNγ production by CD28nullCD8+ T cells and (b) upregulation of SIRT1. There was significant increase in the percentage of CD28nullCD8+T cells expressing SIRT1 and a decrease in IFNγ production in the presence of prednisolone, theophylline, curcumin, resveratrol, or combination of drugs ($p < 0.05$ for all). There was an additive increase in the percentage of CD28nullCD8+T cells expressing SIRT1 in the presence of all drugs.
IFNγ, interferon gamma; SIRT1, Sirtuin 1.

patients with COPD. Our findings of reduced expression of SIRT1 by these pro-inflammatory lymphocytes is therefore likely to be of clinical significance in the control of inflammatory responses in COPD. We and others have also shown that a further potential contributor to the chronic inflammation in COPD is the resistance of pro-inflammatory lymphocytes to the effects of corticosteroids.[9,24] In this regard, we further reported reduced GCR expression by CD8+ CD28null T and NKT-like lymphocyte subsets in COPD.[9] SIRT1 acts as a transcriptional enhancer of the GCR, possibly by functioning as a scaffold for the transcriptional complex.[13] Further to this, SIRT1 and GCR colocalize in HeLa cells.[15] Our new findings of a loss of SIRT1 and GCR in the same cell therefore suggest that therapeutics aimed at increasing both SIRT1 and GCR may be required to improve steroid sensitivity in these pro-inflammatory lymphocytes in COPD. Interestingly, prednisolone increased both GCR and SIRT1 expression,[9] suggesting a previously unidentified important mode of action for this commonly used corticosteroid.

We showed a reduction in a further mediator linked to steroid resistance, histone deacetylase, HDAC2, in senescent CD8+ T and NKT-like cells.[24] Prednisolone upregulated HDAC2 in these cells, suggesting yet another mode of action of this drug.[24] The upregulation was synergistically enhanced when prednisolone was used in combination with theophylline. The combination also decreased pro-inflammatory cytokine production by these steroid-resistant lymphocyte subsets. Importantly, these effects were noted at a low dose of theophylline shown to have no adverse side effects in patients.[25]

Taken together with other studies showing that theophylline can restore HDAC2 in lung macrophages, a further major cell type found increased in the lungs of patients with COPD, our findings suggest that theophylline may restore multiple deficiencies found in several cell types in COPD.[25] The involvement of specific cell types in cellular senescence and their regulation by SIRT1 in the lung is still relatively unclear.[26]

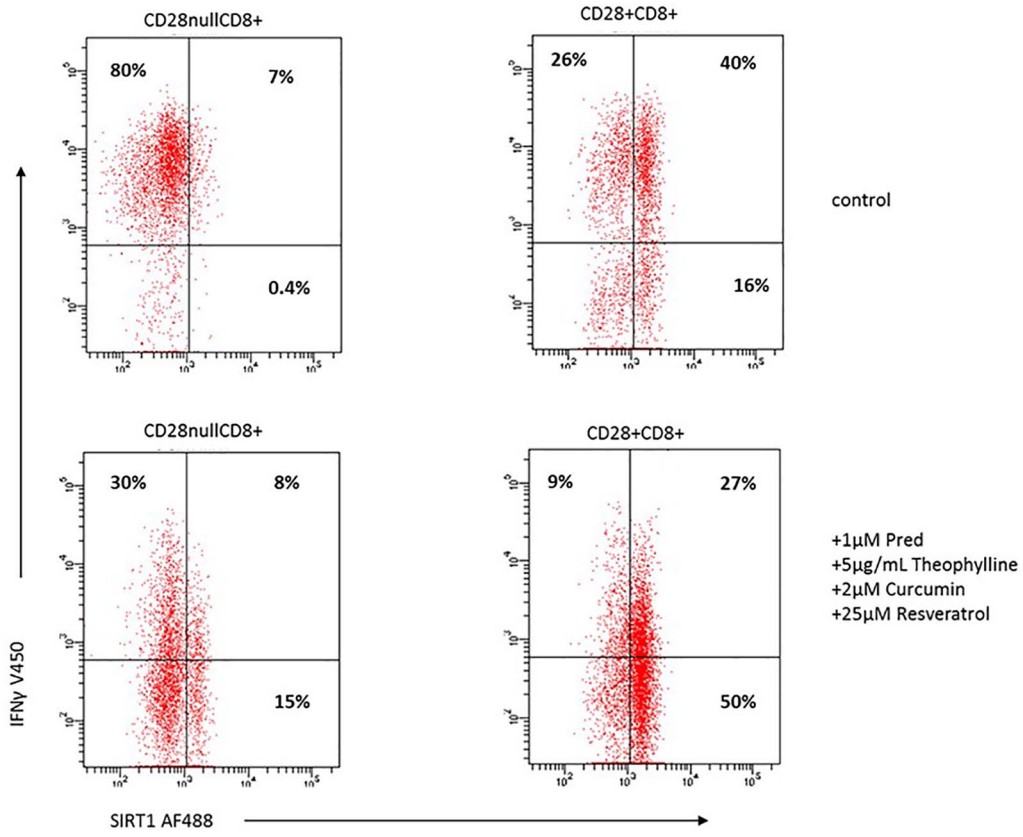

Figure 9. Representative dot plots showing the combined effect of 1 μM prednisolone + 5 mg/ml theophylline + 1 μM curcumin + 25 μM resveratrol on the percentage of CD28nullCD8+ and CD28+CD8+ T cells expressing SIRT1 and IFNγ from a patient with COPD. Note the significant increase in SIRT1 expression and decrease in IFNγ production in both CD28null and CD28+ CD8+ T cells in the presence of the combination of drugs compared with control (no drugs).
COPD, chronic obstructive pulmonary disease; IFNγ, interferon gamma; SIRT1, Sirtuin 1.

In addition to the synergistic effects of theophylline with prednisolone, the present study found an additive increase in the percentage of CD28nullCD8+T cells expressing SIRT1 in the presence of curcumin or resveratrol. The effects of these well-studied anti-inflammatory agents with regard to steroid resistance in lymphocytes have not been previously studied. The phytoalexin resveratrol is found in wine, grapes, and other plant products, and its anti-inflammatory, antioxidant and anti-tumour activities have been reported.[23] Resveratrol has been shown to inhibit several aspects of cell function (cell proliferation, cell-mediated cytotoxicity and cytokine production) partially *via* inhibiting NF-κB activation,[23] and inhibits CD4+ T cell activation by increasing SIRT1 activity.[27] At higher doses, resveratrol has toxic side effects[28]; however, we used doses that were not associated with any adverse side effects that were previously identified for human studies.[21] Curcumin is a naturally occurring polyphenolic phytochemical, and has previously been shown to inhibit COPD-like airway inflammation in mice.[29] Our results indicate combined treatment with prednisolone, theophylline, curcumin, and resveratrol may significantly upregulate SIRT1 and reduce inflammation.

These findings may have clinical relevance for treating and monitoring COPD. A reduction in adverse side effects of GCS, for example, may be achieved by reducing GCS dose due to the additive anti-inflammatory effects of the other agents.[30] SIRT1/GCR deficient lymphocytes enumerated using our ex vivo assays may identify COPD patients that may benefit from these drug combinations and post-therapy lymphocyte phenotyping could identify therapeutic effectiveness. The very recent finding that reduced levels of SIRT1 destabilize the expression and function of

transcription factor FoxO1 to enhance the pro-inflammatory and cytotoxic capacity of CD28nullCD8+ T cells, thereby contributing to immune dysfunctions of this cell type, may explain its pathophysiological role in age-related chronic inflammatory diseases.[31]

Interestingly, we also observe reduced expression of SIRT1 by CD28nullCD8+ T and NKT-like cells from healthy control subjects, although not to the same extent as found in COPD patients. Lymphocyte senescence and resistance to GC occur in several other inflammatory conditions, including cardiovascular disease,[32] autoimmune disease,[33] arthritis,[34] IBD,[35] aging,[36] and inflammaging in COPD.[37] Hence it could speculated that the CD28null lymphocytes may be the precursors to these other inflammatory diseases.

Important forward studies will be to determine whether levels of SIRT1 are changed in lymphocyte subsets in smokers who have not yet developed COPD, and to determine other correlations such as pack years of smokers and SIRT1 expression in COPD patients. Interestingly, these findings may seem counterintuitive to the evidence in the literature that SIRT1 and AMP-activated protein kinase (AMPK) both regulate each other and have similar effects on diverse processes such as inflammation and aging,[38] although reports suggesting SIRT1 abundance and AMPK activation diminish in some mammalian tissues with aging are consistent with the findings of this current study.[38]

In conclusion, lymphocyte senescence in COPD is associated with loss of SIRT1 in CD28nullCD8+ T and NKT-like cells. Loss of SIRT1 is related to disease severity in COPD, and, hence, therapeutics aimed at increasing SIRT1 expression in pro-inflammatory senescent lymphocytes may reduce inflammaging reported in patients with COPD.[10]

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Author contributions

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 performed immunofluorescence staining and helped draft the manuscript; ER performed western blots and helped draft the manuscript; PNR supplied and characterized patient specimens and helped draft the manuscript; SH helped with study design, statistical analysis, and helped draft the manuscript. All authors read and approved the final manuscript.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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Supplemental material

Supplemental material for this article is available online.

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