



# Steroid Resistant CD8<sup>+</sup>CD28<sup>null</sup> NKT-Like Pro-inflammatory Cytotoxic Cells in Chronic Obstructive Pulmonary Disease

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Corticosteroid resistance is a major barrier to effective treatment in chronic obstructive pulmonary disease (COPD), and failure to suppress systemic inflammation in these patients may result in increased comorbidity. Although much of the research to date has focused on the role of macrophages and neutrophils involved in inflammation in the airways in COPD, recent evidence suggests that CD8<sup>+</sup> T cells may be central regulators of the inflammatory network in this disease. CD8<sup>+</sup> cytotoxic pro-inflammatory T cells have been shown to be increased in the peripheral blood and airways in patients with COPD, whereas smokers that have not progressed to COPD only show an increase in the lungs. Although the mechanisms underlying steroid resistance in these lymphocytes is largely unknown, new research has identified a role for cytotoxic pro-inflammatory CD8<sup>+</sup> T-cells and CD8<sup>+</sup> natural killer T-like (NKT-like) cells. Increased numbers of these cells and their significant loss of the co-stimulatory molecule CD28 have been shown in COPD, consistent with findings in the elderly and in clinical conditions involving chronic activation of the immune system. In COPD, these senescent cells expressed increased levels of the cytotoxic mediators, perforin and granzyme b, and the pro-inflammatory cytokines, IFN $\gamma$  and TNF $\alpha$ . They also demonstrated increased cytotoxicity toward lung epithelial cells and importantly were resistant to immunosuppression by corticosteroids compared with their CD28<sup>+</sup> counterparts. Further research has shown these cells evade the immunosuppressive effects of steroids *via* multiple mechanisms. This mini review will focus on cytotoxic pro-inflammatory CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells involved in COPD and novel approaches to reverse steroid resistance in these cells.

**Keywords:** CD8<sup>+</sup> NKT-like cell, steroid resistance, chronic obstructive pulmonary disease, CD28, IFN $\gamma$  and TNF $\alpha$ , Pgp, HDAC2, Hsp90

## CD8<sup>+</sup> NATURAL KILLER T-LIKE (NKT-LIKE) CELLS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

Natural killer T-like cells comprise a unique subgroup of lymphocytes that express features of both T cells and natural killer (NK) cells. NKT-like cells co-express T-cell receptors and CD4 or CD8 (or CD4<sup>-</sup>/CD8<sup>-</sup>), together with markers associated with NK cells, such as CD56 (**Figure 1C**) and/or CD16 or CD161. Acquisition of CD11b represents an early event in CD8<sup>+</sup> T-cell differentiation, which may allow extravasation to peripheral tissues (1, 2). These cells are a small but important

subset of lymphocytes that represent a bridge between innate and adaptive immunity.

There has been conflicting evidence regarding changes in NKT-like cell numbers in COPD. Numbers of these cells have been reported to be decreased in the peripheral blood of patients with COPD (3). One study showed numbers to be unchanged (4), while a third reported increased numbers (5). However, further characterization into CD4<sup>+</sup> or CD8<sup>+</sup> NKT-like cells was not performed in any of these reports. NKT-like cells have also been reported to be increased in induced sputum and bronchoalveolar lavage (BAL) of COPD patients and, importantly, have been shown to be cytotoxic to autologous lung cells (3, 4, 6).

## LOSS OF CD28 ON SENESCENT LYMPHOCYTES IN COPD

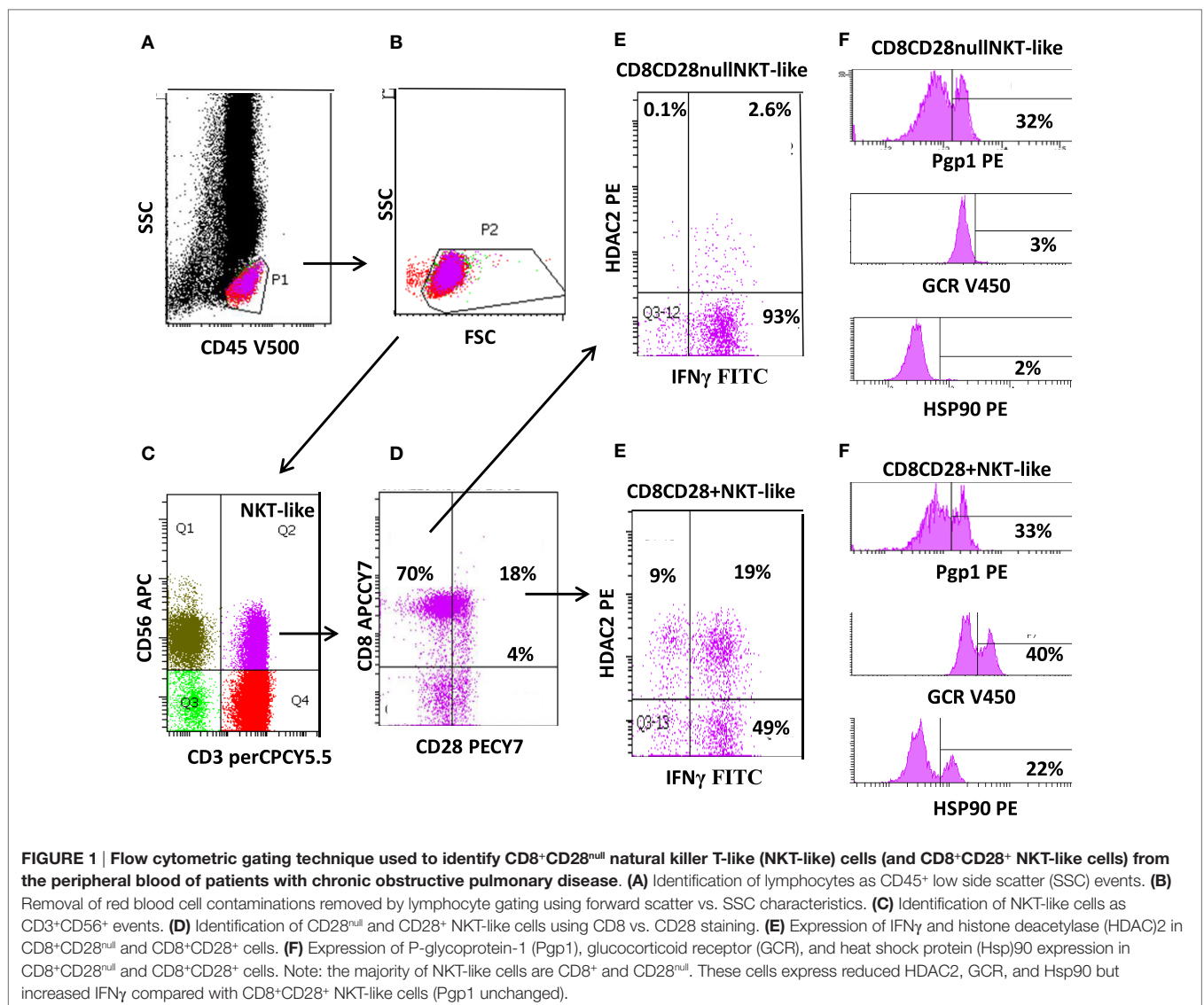
Following persistent antigenic stimulation, NKT-like cells can lose co-stimulatory molecules, undergo telomere shortening, and

exhibit defective IL-2 production; changes that define the state of replicative senescence. The majority of these “effector senescent” lymphocytes are CD8<sup>+</sup>, CD45RA<sup>+</sup>, CD11a<sup>bright</sup>, CD28<sup>null</sup> (Figure 1D), CD62L<sup>-</sup>, and CCR7<sup>-</sup>. Expansion of these cells are found in the elderly and in other clinical conditions involving chronic activation of the immune system such as viral infections, rheumatic, and autoimmune diseases (7). Increased numbers have also been reported in chronic inflammatory lung diseases including COPD and in patients following lung transplantation (8, 9).

## STERIOD RESISTANCE IN CD8<sup>+</sup>CD28<sup>null</sup> NKT-LIKE CELLS IN COPD

### Steroid Resistant CD8<sup>+</sup> T Cells in COPD

Patients with COPD have been shown to be resistant to the immunosuppressant effects or glucocorticoids (10). Most of the investigations into steroid resistance in this disease have focused on the role of the airway macrophages and neutrophils (10); however,



the mechanisms underlying steroid resistance in lymphocytes in patients with COPD until recently has been largely unknown. The role of T-cells is likely to be important in this regard, as their increased numbers have been reported in the lungs of patients with COPD. A study by Maeno et al. demonstrated an important requirement for CD8<sup>+</sup> T cells in the development of cigarette smoke-induced emphysema. They suggested a unifying pathway whereby CD8<sup>+</sup> T cells are the central regulators of the inflammation network in COPD (11). Inhaled corticosteroids have been shown to reduce exacerbation rates and improve health status in patients with COPD but can also increase the risk of pneumonia (12, 13). The numbers of bronchial CD8<sup>+</sup> T-cells were reduced following long-term treatment with inhaled corticosteroids in ex-smoker COPD patients only but not persistent COPD smokers (12, 13).

There have been reports of increased numbers of CD8<sup>+</sup> T cells in the peripheral blood, BAL, and lung parenchyma from COPD smoker and ex-smoker patients compared with healthy smokers and control subjects (14, 15). This indicates the systemic involvement of these cells in COPD. The production of the pro-inflammatory cytokines, IFN $\gamma$  and TNF $\alpha$ , by CD8<sup>+</sup> T cells was increased from peripheral blood, BAL, and intraepithelial compartments in patients with COPD. This was regardless of whether patients were receiving inhaled corticosteroids (14) indicating the lack of effectiveness of steroids at reducing pro-inflammatory cytokines by these cells. However, further lymphocyte subtyping with NKT-like cell markers was not performed. Steroid resistance was further shown *in vitro* by assessing the production of IFN $\gamma$  by follicular CD8<sup>+</sup> T cells in the presence of 0.1–1 $\mu$ M dexamethasone (16), although further subtyping of NKT-like subsets was not performed in the study. Recently, steroid resistant CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells were reported to be increased in number and to express increased levels of the cytotoxic mediators, perforin and granzyme b. Pro-inflammatory cytokines, IFN $\gamma$  and TNF $\alpha$  (8), were also increased in the peripheral blood of patients with COPD, confirming the important role of these lymphocytes in steroid resistance.

### P-glycoprotein-1 (Pgp1) in CD8<sup>+</sup>CD28<sup>null</sup> NKT-Like Cells

P-glycoprotein is a transmembrane efflux pump well-characterized in drug-resistant cancer cells (17) and also thought to play a role in the function of steroid resistant lymphocytes in COPD. Pgp1 expression has been shown to be increased in T, NKT, and NK cells that also co-express IFN $\gamma$ , TNF $\alpha$ , and granzyme b, in peripheral blood from COPD patients compared with healthy controls (Figure 1). However, further differentiation of NKT-like cells into CD4<sup>+</sup> and CD8<sup>+</sup> subsets was not performed (18).

Recent further investigations by the same authors comparing COPD patients with healthy age-matched controls showed no difference in Pgp1 expression between CD8<sup>+</sup>CD28<sup>null</sup> NKT-like and CD28<sup>+</sup>CD8<sup>+</sup> NKT-like subsets. However, the percentages of CD8<sup>+</sup>Pgp1<sup>+</sup>CD28<sup>null</sup> NKT-like and CD8<sup>+</sup>Pgp1<sup>+</sup>CD28<sup>+</sup> NKT-like cells were both increased in the COPD group (8) (Figure 2A). Treatment with very low-dose cyclosporine A (CsA), a Pgp1 inhibitor (2.5 ng/ml; approximately 25 times less than that used for transplant rejection therapy), combined with standard dose

corticosteroid [1 $\mu$ M prednisolone (pred)] resulted in synergistic inhibition of pro-inflammatory cytokines in CD8<sup>+</sup>Pgp1<sup>+</sup>CD28<sup>null</sup> NKT-like cells (18) (Figure 2B). These data indicate that these agents may be an effective add-on therapy to standard steroid treatment.

### Loss of Glucocorticoid Receptor (GCR) in CD8<sup>+</sup>CD28<sup>null</sup> NKT-Like Cells in COPD

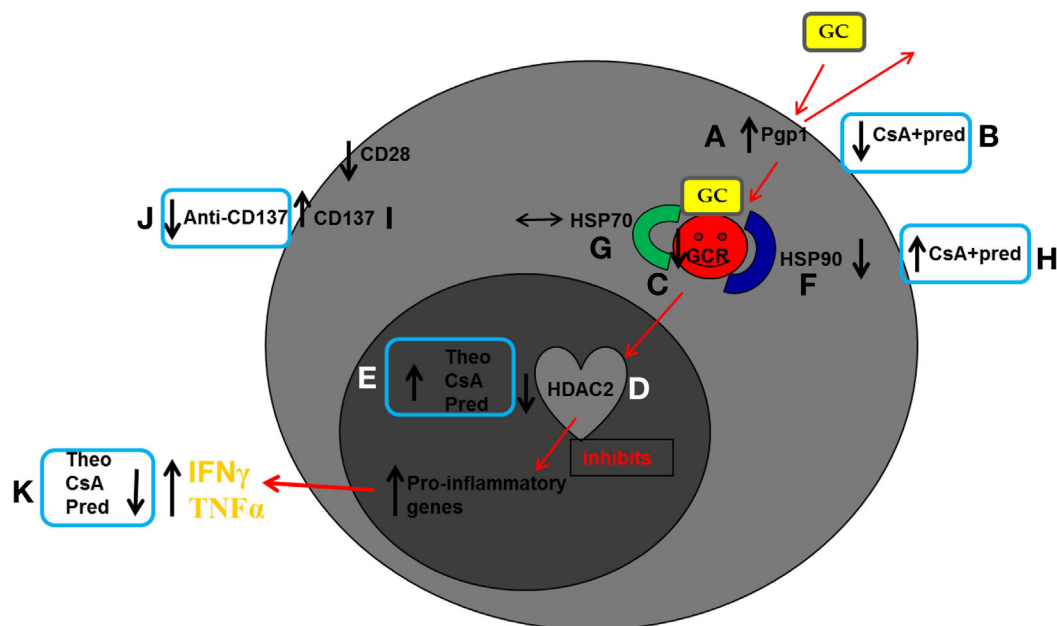
Glucocorticoids must bind to the GCR in the cytoplasm of a cell before being transported to the nucleus. A recent study examined the expression of GCR in pro-inflammatory NKT-like cells in the peripheral blood of patients with COPD (8). COPD was associated with increased percentage of CD28<sup>null</sup> NKT-like cells compared with healthy controls. Loss of CD28 was associated with an increase in percentage of NKT-like cells producing IFN $\gamma$  and TNF $\alpha$  and importantly, with a loss of GCR (8) (Figure 2C). A significant loss of GCR in CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells was noted in both COPD patients and controls compared with CD8<sup>+</sup>CD28<sup>+</sup> NKT-like cells (mean  $\pm$  SEM: 9  $\pm$  4% CD8<sup>+</sup>GCR<sup>+</sup>CD28<sup>null</sup> NKT-like cells vs. 39  $\pm$  7% CD8<sup>+</sup>GCR<sup>+</sup>CD28<sup>+</sup> NKT-like cells in COPD). There was a significant correlation between GCR expression and IFN $\gamma$  and TNF $\alpha$  production by CD8<sup>+</sup> NKT-like cells. Taken together, these data show a loss of GCR in senescent CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells and suggest that alternate treatment options to glucocorticoids are required to suppress pro-inflammatory cytokine production in patients with COPD.

### Decreased Histone Deacetylase (HDAC)2 in CD8<sup>+</sup>CD28<sup>null</sup> NKT-Like Cells in COPD

Histone acetyltransferases and HDAC are enzymes that upregulate and downregulate pro-inflammatory gene transcription, respectively. HDAC2 is required by corticosteroids to switch off activated inflammatory genes and is reduced in lung macrophages in COPD (10). A recent study showed that HDAC2 expression was suppressed in pro-inflammatory CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells in patients with COPD (19) and negatively correlated with the percentage of CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells producing IFN $\gamma$  or TNF $\alpha$  in all subjects (e.g., COPD:  $R = -0.789$ ,  $p < 0.001$  for CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells producing IFN $\gamma$ ) (Figure 2D). Theophylline is an activator of HDAC and enhances the anti-inflammatory effects of corticosteroids in alveolar macrophages in COPD patients (20). Addition of theophylline has recently been shown to increase the anti-inflammatory effects of steroids in senescent lymphocytes from COPD patients (18). Addition of low-dose theophylline (5 mg/l) induced a synergistic upregulation of HDAC2 in CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells in the presence of 1 $\mu$ M pred and 2.5 ng/ml CsA (Figure 2E). This was associated with a decrease in pro-inflammatory cytokine production by these cells. These findings suggest this form of therapy may enhance the anti-inflammatory effects of steroids and thus reduce inflammation caused by these cells in COPD.

### Decreased Heat Shock Protein (Hsp)90 in CD8<sup>+</sup>CD28<sup>null</sup> NKT-Like Cells in COPD

Glucocorticoid receptor must be bound to molecular chaperones Hsp70 and Hsp90 to acquire a high-affinity steroid binding



**FIGURE 2 | Schematic diagram summarizing reported findings in peripheral blood CD8<sup>+</sup>CD28<sup>null</sup> natural killer T-like (NKT-like) cells in chronic obstructive pulmonary disease (COPD).** Glucocorticoids enter cells by overcoming membrane drug efflux pump P-glycoprotein-1 (Pgp1) and binding to the glucocorticoid receptor (GCR) in the cytoplasm. GCR must be bound to the molecular chaperones heat shock protein (Hsp)70 and Hsp90 to acquire a high-affinity steroid binding conformation, and traffic to the nucleus where engagement of histone deacetylases (HDACs), particularly HDAC2, results in reduction of pro-inflammatory gene activation. In COPD compared with age-matched healthy control subjects: **(A)** Pgp1<sup>+</sup> NKT-like cells are increased in COPD, reducing intracellular levels of GC. Expression of GCR **(C)**, Hsp90 **(F)**, and HDAC2 **(D)** are decreased in CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells (no change in Hsp70) **(G)** reducing steroid effectiveness. **(I)** The percentage of steroid resistant CD8<sup>+</sup>CD28<sup>null</sup>CD137<sup>+</sup> NKT-like cells is increased. Possible therapeutic targeting to overcome steroid resistance CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells in COPD: **(B)** Pgp1 is synergistically decreased in the presence of 2.5 ng/ml cyclosporine A (CsA) and 1 $\mu$ M prednisolone (pred). **(H)** Hsp90 expression is increased in the presence of 2.5 ng/ml CsA and 1 $\mu$ M pred. **(E)** HDAC2 expression is increased in the presence of 5 mg/ml theophylline, 2.5 ng/ml CsA, and 1 $\mu$ M pred. **(J)** Blocking CD137 expression with anti-CD137 antibody. **(K)** This targeting results in decreased IFN $\gamma$  and TNF $\alpha$  pro-inflammatory cytokine expression.

conformation and traffic to the nucleus (21). A recent study examined expression of Hsp70/90 in CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells from the peripheral blood of patients with COPD (22). Loss of expression of Hsp90 and GCR from the CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells in COPD was noted (Figure 2F), whereas expression of Hsp70 was unchanged (Figure 2G). The loss of Hsp90 was shown to correlate with the cytotoxic/pro-inflammatory potential of these cells and importantly, degree of airflow limitation in patients with COPD. The immunosuppressant, CsA, binds to the GCR–Hsp90 complex, but not Hsp70 (23), and was shown to upregulate Hsp90 with an associated decrease in pro-inflammatory cytokine production by CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells when combined with 1 $\mu$ M pred (Figure 2H). The concentration of CsA (2.5 ng/ml) used in these *in vitro* experiments was 50 times less than that used for patients following lung transplant to prevent graft rejection. Hence, these low concentrations are not likely to be associated with any of the side effects reported with higher doses of this drug.

### INHIBITING CD137 EXPRESSION IN CD8<sup>+</sup>CD28<sup>null</sup> NKT-LIKE CELLS IN COPD

The loss of CD28 on CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells from COPD subjects has been reported to be associated with an upregulation

of the “alternate” co-stimulatory molecule CD137 (4-1BB) (24) (Figure 2I). Targeting CD137 has been shown to be effective in treatment of rheumatoid arthritis and may thus be effective in other diseases associated with increased expression of this co-stimulatory molecule, including COPD (25). *In vitro* studies showed that blocking CD137 with an anti-CD137 antibody following PHA stimulation of PBMC from COPD patients resulted in a decrease in the percentage of CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells producing IFN $\gamma$ , TNF $\alpha$ , and granzyme b production (26) compared with CD8<sup>+</sup>CD28<sup>+</sup> NKT-like cells (Figure 2J), whereas stimulatory CD137 antibody increased production of these molecules. This indicates that targeting CD137 with anti-CD137 antibody may have novel therapeutic options for reducing inflammation in patients with COPD.

### DOES OXIDATIVE STRESS PLAY A ROLE IN STEROID RESISTANCE IN NKT-LIKE CELLS?

There is increasing evidence that oxidative stress is important in the pathogenesis of COPD (27, 28). Oxidative stress occurs due to an increase of reactive oxygen species (ROS) causing damage to lipids, proteins, and DNA. Increased burden of oxidants from

cigarette smoke and air pollutants and from ROS and reactive nitrogen species (RNS) released from inflammatory neutrophils, eosinophils, macrophages, and epithelial cells occurs in the lungs of COPD patients (27–29). The aging process is associated with a decrease in the antioxidant defense mechanisms in the lung resulting in increased ROS and RNS (30). Although there is a causal link between ROS, COPD, and aging in cellular senescence in many cells in the lung, sensitivity of individual lymphocyte subsets to oxidative stress and how this process affects disease progression remains largely unknown (30). While one study showed an association between ROS and cellular senescence in lymphocytes, some markers of oxidative stress were decreased (31). Increasing concentrations of ROS has been shown to suppress Th1 cells and increase Th2 cells, findings at odds with ours and many others in patients with COPD (30). Furthermore, it has been shown that neutrophils in the inflamed lung produce large amounts of ROS, which suppress T cells, while macrophages secrete cysteine and thioredoxin, which increase oxidation resistance of T cells (32). Although oxidative stress has been shown to inhibit expression of GCRs in total blood leukocytes, the effect on T and NKT-like cells was not determined (33). It is clear further research is needed specifically on the effect of ROS on T cell and NKT-like cell biology (32).

## FUTURE THERAPY FOR COPD

Lymphocyte senescence and glucocorticoid resistance have been described in several other inflammatory conditions such as cardiovascular disease (34), autoimmune disease (35), arthritis (36), IBD (37) associated with aging (38), and aging with associated inflammation in COPD (39). Some of these conditions are associated with respiratory muscle dysfunction resulting in

further increases in ROS and oxidative stress (40). CD28<sup>null</sup> T cells have been reported in patients with asthma (41), another inflammatory lung disease also associated with increased ROS and oxidative stress (42). Interestingly, several of these inflammatory diseases are also comorbid conditions associated with COPD (10) and therefore may also be associated with increased cytotoxic/pro-inflammatory CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells. Hence, targeting the pro-inflammatory nature of these cells by decreasing the expression of Pgp1 and/or CD137 and increasing the expression of GCR, HDAC2, and Hsp90 by CD8<sup>+</sup>CD28<sup>null</sup> NKT-like cells may reduce inflammation (**Figure 2K**) associated with a range of steroid resistant diseases including COPD and comorbid conditions associated with COPD. Furthermore, targeting these cytotoxic/pro-inflammatory cells at early onset of COPD may prevent the inevitable spiral of worsening lung function, and associated comorbidity of this progressive debilitating disease, and reduce the associated massive health-care costs (43).

## AUTHOR CONTRIBUTIONS

GH and SH organized, wrote, and edited the manuscript. Figures were drawn by GH and edited by SH.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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