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Cytotoxic potential of lung CD8+ T cells increases with COPD severity and with in vitro stimulation by IL-18 or IL-15

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Abstract

Lung CD8+ T cells might contribute to progression of chronic obstructive pulmonary disease (COPD) indirectly via IFN-γ production or directly via cytolysis but evidence for either mechanism is largely circumstantial. To gain insights into these potential mechanisms, we analyzed clinically-indicated lung resections from three human cohorts, correlating findings with spirometrically-defined disease severity. Expression by lung CD8+ T cells of IL-18R and CD69 correlated with severity, as did mRNA transcripts for perforin and granzyme B, but not Fas ligand. These correlations persisted after correction for age, smoking history, presence of lung cancer, recent respiratory infection, or inhaled corticosteroid use. Analysis of transcripts for KLRG1, IL-7 receptor and CD57 implied that lung CD8+ T cells in COPD do not belong to the terminally-differentiated effector populations associated with chronic infections or extreme age. In vitro stimulation of lung CD8+ T cells with IL-18 plus IL-12 markedly increased production of IFN-γ and TNF-α, whereas IL-15 stimulation induced increased intracellular perforin expression. Both IL-15 and IL-18 protein expression could be measured in whole lung tissue homogenates, but neither correlated in concentration with spirometric severity. Although lung CD8+ T cell expression of mRNA for both T-bet and GATA-3 (but not ROR-γ or ROR–α) increased with spirometric severity, stimulation of lung CD8+ T cells via CD3ε induced secretion of IFN-γ, TNF-α and GM-CSF, but not IL-5, IL-13, IL-17A. These findings suggest that the production of pro-

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inflammatory cytokines and cytotoxic molecules by lung resident CD8+ T cells contributes to COPD pathogenesis.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD), t he 4th leading cause of death in the Unites States [1] is a progressive, debilitating disease that is rapidly increasing in worldwide prevalence. Current therapies have very limited impact on disease progression, making greater understanding of pathogenesis crucial. COPD is an inflammatory condition triggered by oxidant stress, notably tobacco smoke-exposure and, in the developing world, indoor biomass fuel combustion. CD8+ T cells have been implicated in the development of COPD because their numbers in lung parenchyma and small airways correlate inversely with lung function [2–5]. We [6] and others [7–9] have demonstrated that CD8+ T cells isolated from lung parenchyma in COPD are largely Tc1 cells. Indeed, we previously showed that mRNA transcripts for IFN-γ from unstimulated lung CD8+ T cell correlated directly with disease severity, whereas IL-4 transcripts were essentially undetectable [6]. However, two groups who studied cells isolated from the alveolar spaces found evidence of a significant Tc2 component [10, 11], implying that there may be anatomic compartmentalization of the CD8 T cell phenotype in COPD.

Whether and how lung CD8+ T cells actually contribute to COPD pathogenesis, however, remains undefined. One possibility is that T cell production of IFN-γ fosters lung destruction. Evidence supporting this possibility comes from an inducible transgenic murine system in which local over-expression of IFN-γ led to lung inflammation and emphysema associated with induction of matrix metalloproteinase 12 [12]. Another possibility is that CD8+ T cells directly kill lung parenchymal cells that they recognize as altered-self or infected, via perforin plus granyzmes, or Fas ligand (FasL). The oxidant injury induced by smoking could plausibly lead to antigenic modification that would be recognized by CD8 T cells in the context of class I MHC. A correlation has been shown between numbers of lung CD8+ T cells and apoptotic cells of all types identified in microscopic sections [13], but to date, no studies have directly proven that CD8+ T cells are responsible for parenchymal cell apoptosis in emphysema. Considerations such as these argue for investigation of how the cytotoxic potential of lung CD8 T cells correlates with COPD progression.

Although the effector functions of CD8+ T cells are typically assayed after TCR stimulation, multiple recent findings suggest that TCR-independent mechanisms merit special examination in COPD. IL-18, a member of the IL-1 cytokine superfamily is, in combination with IL-12, an important mediator of antigen-independent IFN-γ production by T cells [14, 15]. IL-18 is strongly expressed by alveolar macrophages (AMø) of patients with severe COPD [16] and is increased in the peripheral blood of COPD patients relative to controls [17, 18]. In mice, treatment with recombinant IL-18 and IL-12 drives pulmonary inflammation and lung injury [19]. Cigarette smoke-exposed wild-type mice had increased levels of IL-18 mRNA and protein that localized to AMø, and cigarette smoke-induced emphysema was decreased by a null mutation of the IL-18Rα chain [17]. IFN-γ production by CD8+ T cells has also been reported to be stimulated by IL-15, a key cytokine for the development and maintenance of CD8+ T cell memory (20, 21). Additionally, TCR-independent activation of CD8+ T cell cytotoxicity is potentially relevant in COPD. Short-term cytokine stimulation can induce TCR-independent, non-MHC-restricted cytotoxicity in highly purified human CD8+ T cells (22). Tobacco smoke-induced up-regulation on pulmonary epithelium of ligands for the cytotoxic T cell-activating receptor NKG2D (CD314) has recently been implicated in COPD pathogenenesis, based on a combination of murine and human data (23).

To investigate the capacity of lung CD8+ T cells to participate in COPD pathogenesis, we correlated their phenotype and in vitro function with disease severity, with special attention to expression of IL-18R. For efficient use of limited human tissues, we employed three available patient cohorts, chosen to provide different types of samples (e.g., viable cells, mRNA from purified lung CD8 T cells, preserved lung tissue) for specific purposes. Our results show that although lung CD8+ T cells from all COPD subjects have a predominantly effector-memory T (T_{EM}) cell phenotype, regardless of disease severity as defined by pulmonary function, their production of mRNA transcripts for perforin a nd granzyme B and their expression of IL-18R protein increases as the disease progresses. We further show that in vitro stimulation with IL-18 (plus IL-12) in the absence of TCR stimulation very markedly increases production of IFN-γ and TNF-α, whereas IL-15 stimulation increases intracellular perforin expression.

MATERIALS AND METHODS

Specimens and subject populations

Different types of samples were obtained from three separate cohorts of human subjects undergoing clinically-indicated resection procedures for pulmonary nodules, lung volume reduction surgery, or lung transplantation. Studies and consent procedures were performed in compliance with the principals expressed in the Helsinki Declaration, and were approved by appropriate Institutional Review Boards. Only non-neoplastic lung tissue remote from the nodules and lacking post-obstructive changes was collected.

Cohort A comprised 47 subjects recruited pre-operatively at the University of Michigan Healthcare System and the VA Ann Arbor Healthsystem to a study registered with ClinicalTrials.gov as NCT00281229. The lung tissue from these subjects was used immediately for flow cytometric analyses and CD8+ T cell culture. All subjects underwent preoperative spirometric tests, collection of a variety of clinical data including medications and history of recent (6 weeks) respiratory infections, and full evaluation by a pulmonologist. To categorize subjects in this cohort based on disease severity, we used the 2008 classification system of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (24). The GOLD classification is based on the forced expiratory volume in 1 second (FEV_1) of the predicted value in combination with the ratio of FEV_1 to forced vital capacity (FVC). GOLD stage 1 represents subjects with mild COPD whereas stage 4 represents subjects with the most severe cases of COPD. Consistent with the most recent GOLD criteria, only subjects who had an FEV_1/FVC ratio of < 0.70 were included in GOLD stages 1 through 4. Subjects (n = 11) with a history of smoking, an FEV_1/FVC ratio > 0.70, normal spirometry, and no clinical diagnosis of COPD represent our smoking controls (S). Subjects with no history of smoking and normal spirometry (n=3), are non-smoking controls (NS). Demographics, smoking histories, spirometry and quantitative emphysema distribution of all subjects in Cohort A used in this study have recently been published [25] but are also listed in Table I.

Cohort B comprised 22 subjects whose de-identified lung tissue was obtained from the Tissue Procurement Cores at the University of Michigan Healthcare System and the VA Ann Arbor Healthsystem. The lung tissue from these subjects was used immediately to isolate CD8+ T cells for subsequent real-time PCR analyses. The clinical data available for this cohort was restricted to age, gender, smoking history, and pulmonary function tests, and has been described previously [6], although at that time we categorized subjects based on the 2004 GOLD classification system. Clinical data for this cohort based on the 2008 GOLD guidelines [24] is shown in Table I.

Cohort C comprised 95 subjects whose tissue and clinical data were obtained from the Lung Tissue Research Consortium (LTRC). This NHLBI-sponsored tissue bank (http://www.ltrcpublic.com/) preoperatively collects extensive demographic, physiological and radiographic information, which is then de-identified and available along with various types of preserved tissue samples to qualified investigators. The lung tissue from these subjects was obtained from the LTRC Tissue Core as frozen tissue sections, which we used to extract total protein for cytokine analysis. Table I shows the number of subjects, gender ratio, and ranges of age and smoking history for each subject group in Cohort C. Importantly, because the University of Michigan Healthcare System and the VA Ann Arbor Healthsystem are collectively one of four contributing sites to the LTRC, it is highly likely that some subjects in this cohort are also represented in Cohort A; however, no attempt was made to correlate subjects in the two cohorts.

Sample preparation for flow cytometric analysis and in vitro stimulation

Lung sections from Cohort A weighing approximately 3 g were homogenized using a Waring blender without enzyme treatments, which we have previously shown efficiently produces single cell suspensions of high viability [6]. Cells were filtered through a 70 μm strainer to remove debris and then were used immediately in two types of experiments.

For flow cytometry, cells were resuspended at 10×10^6 cells per ml of staining buffer (2% FBS in PBS), incubated at 4°C for 10 min and then were added in a volume of 100 μl to each flow tube. Monoclonal anti-human antibodies against CD45 (HI30), CD8 (RPA-T8), CD27 (O323), CD69 (FN50), IL-18R (H44) (eBioscience, San Diego, CA), and CD62L (Dreg 56) (BD Bioscience, San Jose, CA) were used. Appropriate isotype-matched controls were used in all experiments. Antibodies were conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE), or phycoerythrin-cyanine 5 (PE-Cy5). Cells were incubated in the dark with antibodies for 25 min at room temperature and then washed. Cells were fixed and stored in staining buffer plus 2% paraformaldehyde prior to being analyzed on the flow cytometer.

To obtain viable CD8+ T cells for in vitro experiments, the homogenized lung tissue was incubated with CD8 magnetic beads (Miltenyi Biotec, Auburn, CA) for 15 min at 4°C and isolated using MACS LS columns (Miltenyi Biotec). CD8+ T cells were cultured in 96-well plates as a density of 50,000 cells per well with lymphocyte culture media (10% FBS, 1 mM sodium pyruvate, 0.5 mM 2-Mercaptoethanol, 1 mM HEPES, 100 u/ml penicillin, 100 u/ml streptomycin, 0.292 mg/ml L-Glutamine). Cells were stimulated with either IL-18 (10 ng/ml) alone, IL-12 (10 ng/ml) alone, IL-18 and IL-12 combined at 10 ng/ml each, IL-15 (0.1 ng/ml), or plate-bound anti-CD3ε (5 μg/ml). After 48 h, supernatants and cells were collected for analysis. For intracellular staining of perforin, cells were incubated in Fixation Buffer (eBioscience) for 20 min, followed by a second 20 minute incubation in Permeabilization Buffer (eBioscience) plus antibody (δ G9, BD Bioscience). Cells were washed with Permeabilization Buffer between incubations, and were analyzed immediately by flow cytometry.

Flow cytometry

Cells were analyzed on an LSR II flow cytometer (BD Bioscience) equipped with 488 nm blue, 405 nm violet and 633 nm red lasers. Data were collected on an HP XW4300 Workstation using FACS Diva software with automatic compensation, and were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR) on an iMac computer. A minimum of 10,000 CD45+ events were collected per sample.

Real-time RT-PCR

Analysis of mRNA transcripts was performed on cDNA previously obtained from Cohort B, as described [(6)]. Analysis of the transcripts was performed by real-time PCR using the Mx3000P QPCR System (Stratagene, La Jolla, CA). Human GAPDH, which acted as the endogenous reference, and primer-probe sets for T-bet (Hs00203436_m1), GATA-3 (Hs00231122_m1), ROR-γ (Hs01076112_m1), ROR-α (Hs00931959_m1), IL-17F (Hs00369400_m1), IL-22 (Hs01574154), Perforin (Hs00168473_m1), Granzyme B (Hs01554355_m1), FasL (Hs00181225_m1), KLRG1 (Hs00195153_m1), CD57 (Hs00218629_m1), and IL-7R (Hs00233682_m1) were purchased commercially (Applied Biosystems, Foster City, CA). Transcipt levels are expressed as arbitrary units and were calculated using the comparative threshold cycle method.

Protein Analysis of lung homogenates and culture supernatants

Frozen lung sections from Cohort C were resuspended in 2 ml of phosphate-buffered saline and homogenized using a tissue homogenizer. Samples were centrifuged at 300 × *g* for 20 min. Supernatants were collected and stored at −80°C. Using the Luminex 200 system (Luminex Corporation, Austin, TX), protein levels for IL-15 and IL-18 were determined by Biosource Multiplex Assays (Invitrogen). Total lung protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce Biotechnology, Roc kford, IL) and cytokine levels were normalized to milligrams of total lung protein.

Similarly, to measure protein levels from supernatants of cultured cells, IFN-γ, TNF-α, GM-CSF, IL-5, IL-13, and IL-17A Biosource Multiplex Assays (Invitrogen) were used according to the manufacturer’s instructions.

Statistical analyses

Initial statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Nonparametric (Spearman) correlation analysis was used to determine the correlation coefficient, r_S. Paired t tests and one-way ANOVA, with Dunn's post-hoc testing, were used to determine statistical differences between treatment groups of in vitro experiments. A two-tailed p value of < 0.05 was considered to indicate significance. Log transformation was utilized for analyses of data that did not meet assumptions for normality. PROC GLM with Tukey's method for multiple comparisons were employed to contrast patient groups using SAS 9.1 statistical software (Cary, NC). A similar approach was utilized to examine difference between smoking-status groups (never-smoker, ex-smoker, active smoker). Regression analysis was used to examine the relationship of age, gender, percent emphysema, smoking status, smoking exposure (pack-years, time-since-quitting), presence versus absence of lung cancer as the indication for surgery, and recent infections to the relationship between FEV_1 % predicted (continuous or categorically defined) and expression of various markers.

RESULTS

Description of clinical cohorts from which tissue was derived

To overcome the inherent limitations of working with human l ung tissue samples that are usually small and yield relatively few cells, different samples were obtained from three separate cohorts of human subjects undergoing clinically-indicated resection procedures for pulmonary nodules, lung volume reduction surgery, or lung transplantation. The lung tissue from Cohort A (n = 47) was used immediately for flow cytometric analyses and CD8+ T cell culture. Cohort B (n = 22) lung tissue was used immediately to isolate CD8+ T cells for subsequent real-time PCR analyses. Lung tissue from Cohort C (n =95) was obtained from the Lung Tissue Research Consortium (LTRC) as frozen tissue sections, which we used to extract total protein for cytokine analysis. Table I shows the number of subjects, gender ratio, and ranges of age and smoking history for each subject group in all three cohorts. Because each cohort provided only one type of sample, results of different assays necessarily derive from different subjects, precluding some direct comparisons. It can be seen, however, that the three cohorts are quite comparable in distributions of age, gender, smoking history and pulmonary function.

CD69 expression on lung CD8+ T cells correlates with disease severity

To gain insights into the potential function of human lung CD8+ T cells in COPD, we first used flow cytometry on single-cell lung suspensions from Cohort A to analyze their expression of CD62L and CD27. Regardless of disease severity, the majority (approximately 75% at all stages) of lung CD8+ T cells were CD62L-CD27-, implying that they are T_{EM} cells (Fig. 1A, B). These data extend our previous report of a highly significant correlation between COPD severity and expression by lung CD8+ T cells of the memory cell marker CD45A [(6)]. By contrast, CD8+ T cells from peripheral blood of COPD patients are largely T central memory cells, i.e., CD62L+, CD27+ (data not shown). Interestingly, when CD69, a marker of acute activation, was analyzed in the same manner, the fraction of lung CD8+ T cells expressing CD69 increased significantly with disease severity, expressed either as

GOLD stage (r_S = 0.43, p = 0.006) (Fig. 1C, D) or FEV1 % predicted (r_S = −0.35, p = 0.02) (data not shown). Further analyses suggested that the predominant differences were between normal nonsmokers and the COPD groups. CD69 expression also correlated (r_S = −0.47, p = 0.009, data not shown) with decreasing DLCO (n = 32), but did not correlate with percent emphysema determined by analysis of high resolution computed tomographs. Similarly the frequency of CD8+ T cells failed to relate to percent emphysema. Additional analyses showed no significant correlation between CD8+ CD69+ lung T cells and age, gender, pack-years, smoking status, duration since cessation of smoking, presence versus absence of lung cancer as the final diagnosis from the surgery, recent respiratory infections, or use of inhaled corticosteroids.

We next analyzed RNA transcripts from isolated CD8+ T cells from Cohort B for T-bet, GATA-3, and ROR-γ, transcription factors which drive Tc1, Tc2, and Tc17 responses, respectively. T-bet transcripts were significantly inversely correlated with FEV_1 (r_S = −0.60, p = 0.004) (Fig. 1E), in accord with the strong correlation of disease severity with IFN-γ mRNA transcripts that we have previously reported in this cohort [6]. Surprisingly, even though we were previously unable to detect transcripts for IL-4 [6], IL-5, or IL-13 (unpublished observation) by CD8+ T cells in this cohort, GATA-3 transcripts were produced by CD8+ T cells and also showed a trend to increase as FEV_1 decreased (r_S = −0.44, p = 0.049) (Fig. 1F). By contrast, lung CD8+ T cells showed little to no ROR-γ (Fig. 1G) or ROR-α (not shown) transcript expression at any disease severity. Purified lung CD8+ T cells also did not express transcripts for IL-17A, IL-17F, or IL-22. Collectively, these data are consistent with the widely-held view that lung CD8+ T cells in COPD principally display a Tc1 phenotype, but imply that some might exhibit Tc2 characteristics under appropriate stimulation.

We also examined mRNA transcripts for three receptors that have been used to identify subsets of human CD8+ effector T cells: killer cell lectin-like receptor G1 (KLRG1); IL-7 receptor (IL-7R) (CD127); and CD57. Driven by elevated levels of T-bet, especially during chronic viral infections, CD8+ T cells can become terminally-differentiated, $KLRG1^{high}$, $IL\text{-}7R^{low}$ short-lived effector cells [26, 27]. CD57-positivity identifies oligoclonally-derived cells typically associated with chronic infections or extreme age. CD8+ CD57+ T cells are T_{EM} cells capable of immediate functional activity, producing IFN-γ and TNF-α in response to antigen challenge [28, 29]. We found that a large fraction of subjects at all GOLD stages has no detectable mRNA transcripts for any of these receptors. We did not detect any consistent relationship to disease severity (Fig. 2); however, we did detect a correlation between KLRG1 and IL-7R (r_S = 0.46, p = 0.03) (data not shown).

Perforin and granzyme B, but not FasL, transcripts from lung CD8+ T cells correlate with decreasing FEV_1

We next analyzed transcript expression from isolated lung CD8+ T cells from Cohort A for the cytotoxic molecules perforin, granzyme B and FasL. These pre-formed, apoptosis-inducing molecules represent two pathways by which CD8+ T cells kill target cells, typically those which are virally-infected or damaged. We found a significant inverse correlation between mRNA expression of perforin (r_S = −0.69, p = 0.001) and of granzyme

B (r_S = −0.50, p = 0.02) and pulmonary function, as measured by FEV_1 (% predicted) (Fig. 3A–B). Furthermore, expression of transcripts for perforin and granzyme B within individual subjects was strongly correlated (r_S = 0.85, p < 0.0001) (data not shown). The strength of the correlation did not vary when adjusted for age and gender. Based on the known hierarchy of cytotoxic effector molecules expression [30, 31], these results, together with those presented above, imply that the CD8+ T cells that increasingly accumulate within the lungs during progression of COPD are short-term T_{EM} cells, rather than terminally-differentiated effectors. By contrast, FasL transcript expression did not correlate with FEV_1 (Fig. 3C). None of these molecules showed any correlation with smoking history, as measured by pack-years (data not shown). Because CT scans are not available for this cohort of patients, correlations between the cytotoxic molecules and percent of emphysema cannot be assessed.

IL-18R expression on lung CD8+ T cells correlates with worsening pulmonary function

Recent human and animal studies have implicated e levated IL-18 levels and IL-18R signaling in the pathogenesis of pulmonary emphysema and COPD [16, 17]. Therefore, we used flow cytometry to analyze the expression of IL-18R on human lung CD8+ T cells from Cohort A. We found that IL-18R expression showed a significant correlation with disease severity, whether expressed as GOLD stage (r_S = 0.36, p = 0.02) (Fig. 4A, B) or as FEV1 % predicted (r_S = −0.30, p = 0.04) (data not shown). IL-18R expression did not show any correlation with pack-years, DLCO, or percent emphysema, but there was a very strong correlation in individual subjects between expression of IL-18R and CD69 (r_S = 0.83, p = 0.0001) (data not shown). Although we did not simultaneously measure the two receptors on individual CD8+ T cells, these data suggest that IL-18R is likely expressed on CD69+ lung CD8+ T cells. The correlation persisted after adjustment for age, gender, duration since cessation of smoking, presence versus absence of lung cancer and history of recent respiratory infections.

To determine whether IL-18 is present in the lungs in COPD, we homogenized frozen lung tissue from 95 subjects from Cohort C and analyzed expression of the mature, bioactive form of IL-18 (Fig. 4C). Although IL-18 levels did not correlate with COPD severity, even when adjusted for the same variables as analyzed for IL-18R expression, these data show that IL-18 is present in human lung tissue collected during periods of clinical stability.

IL-18 stimulation induces lung CD8+ T cells to upregulate production of IFN-γ

IL-18R expression by lung CD8+ T cells might contribute to COPD pathogenesis by inducing IFN-γ production [14, 15] and thereby activating macrophage production of matrix metalloproteinase-12, which is essential for development of emphysema in a murine model [32], and which some [33, 34] but not all [35, 36] studies have found to be over-expressed in COPD. To test this possibility, isolated lung CD8+ T cells from five individual lung samples from Cohort A with GOLD stages of 2, 3, or 4 were cultured for 48 h in the presence of various recombinant cytokines, including IL-18, IL-12, and IL-15, but importantly, w ithout any stimulation via TCR. By themselves, none of these cytokines had any effect on IFN-γ production, as assessed by protein concentrations in culture supernatants. However, when IL-18 and IL-12 were used simultaneously, IFN-γ production

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was very significantly increased (>800-fold) (Fig. 5A). These data extend to the human lungs the previous observation that this cytokine combination can induce IFN-γ in CD8+ T cells [37, 38], and are particularly interesting for the magnitude of the synergistic effect.

Similarly, TNF-α was significantly increased by stimulation by IL-18 plus IL-12 (Fig. 5B), whereas GM-CSF, IL-5, IL-13, and IL-17A showed no changes when stimulated with any of the cytokines (Fig. 5C–F). Taken together, these data demonstrate the capacity of IL-18R+ lung CD8+ T cells to produce and secrete pro-inflammatory Tc1 cytokines in a TCR-independent fashion, and further support the potential relevance of AMø elaboration of IL-18 in COPD.

TCR stimulation of CD8+ T cells does not result in Tc2 cytokine production

Having demonstrated in Figure 1F that lung CD8+ T cells display transcripts for the Tc2 transcription factor, GATA-3, we next asked whether TCR stimulation, via anti-CD3ε exposure, would induce the CD8+ T cells to secrete Tc2 cytokines. Isolated lung CD8+ T cells were cultured for 48 h without or with plate-bound anti-CD3ε. All cytokines were below the limit of detection in unstimulated wells. Anti-CD3ε stimulation caused significant increases in production of IFN-γ, TNF-α, and GM-CSF; however, IL-5, IL-13, and IL-17 showed only very slight changes which did not attain statistical significance (Fig. 6).

IL-15 stimulation induces lung CD8+ T cells to upregulate intracellular perforin production

To investigate whether cytokine stimulation could also increase the cytotoxic potential of lung CD8+ T cells, we measured intracellular perforin following 48 h of in vitro stimulation. Lung tissue from five individual subjects with GOLD stages of 2, 3, or 4 were used in this experiment. In contrast to the effect on cytokine production, s timulation with IL-18 plus IL-12 had no effect on perforin. However, IL-15 stimulation led to a 3-fold increase in intracellular perforin expression, compared to the unstimulated control CD8+ lung T cells (Fig. 7A). IL-15 has been shown to induce the synthesis of effector molecules, such as perforin, in peripheral blood CD8+ T cells [20], but this study is the first to demonstrate this response in lung CD8+ T cells. Our finding that IL-15 stimulation by itself had no effect on IFN-γ protein expression agrees with results of Smeltz [39]. Thus, IL-15 primes CD8+ lung T cells to have greater cytotoxic potential when they encounter a target cell.

Analysis of lung homogenates from Cohort C did not reveal a correlation between IL-15 protein concentrations and disease severity, but showed that IL-15 is present in detectable quantities within the lung (Fig. 7B). Immunohistochemical staining of frozen human lung tissue from Cohort C showed that IL-15 was predominantly expressed by AMø but not by the airway epithelium [40, 41] (Fig. 7C).

DISCUSSION

The principal findings of this study indicate that lung CD8+ T cells in COPD are activated T_{EM} cells of a Tc1 phenotype, whose expression of molecules linked to tissue destruction increases both with worsening disease severity and following in vitro TCR-independent stimulation by cytokines known to be produced by human AMø. Key novel results include: (a) a significant correlation between disease severity and lung CD8+ T cell expression of

CD69 and IL-18R, and of mRNA for perforin, granzyme B, T-bet, and GATA-3; (b) demonstration of the functional significance of IL-18R expression by lung CD8+ T cells in COPD, as shown by very significantly augmented secretion of IFN-γ and TNF-α (but not Tc2 or Tc17 cytokines) on stimulation with IL-18 plus IL-12 in the absence of TCR activation; (c) evidence that IL-15 can prime lung CD8+ T cells for increased cytotoxic potential, as indicated by enhanced intracellular perforin expression. Together with previously published findings [3, 6, 8, 9, 42, 43], these data suggest a model in which the disease stage-dependent recruitment of CD8+ T cells to the lungs under the influence of ligands for CCR5, CXCR3 and CXCR6 and their retention and survival there sets the stage for a bi-directional, potentially TCR-independent, positive-feedback interaction with lung macrophages leading to progressive lung inflammation and destruction.

COPD is an insidious, highly heterogeneous condition that primarily affects the lungs, but which is also associated with significant systemic inflammation [44]. Individuals with COPD variably exhibit increased resistance in the conducting airways due to mucus gland hypertrophy and fibrosis, and increased parenchymal compliance due to emphysema. These apparently independent processes combine to determine the defining feature of COPD, irreversible airflow limitation measured during forced exhalation [45]. Despite important insights derived from animal models [17, 19, 23, 32, 46–49], investigation of human pathological specimens remains essential in understanding this heterogeneity.

The current study is significant because it extends previous seminal studies of CD8+ T cell involvement in COPD [2–5, 7, 8, 13] in several ways: by the size of our primary sample cohorts (cohort A, $n = 42$, cohort C, $n = 95$); by the correlations with disease severity, with logistic regression analysis to exclude potentially confounding variable; and by our complementary use of flow cytometric, real-time PCR, and in vitro stimulation to analyze lung CD8+ T cells. Because the primary site of pathological changes in COPD is the small (< 2 mm diameter) airways [50], we believe that analyses that derive from distal lung parenchyma are more representative of key disease processes than those that depend on sampling of sputum or bronchoalveolar lavage. To overcome the inherent limitations of working with small samples yielding relatively few cells, this study employed several complementary subject cohorts. Hence, results of different assays necessarily derive from different subjects, precluding some direct comparisons.

The findings of increased expression of CD69 and IL-18R on lung CD8+ T cells with advancing disease raises several interesting questions about their life history. CD69 expression is generally taken to signify acute activation, as it is one of the earliest surface molecules to be up-regulated on TCR engagement [51, 52], and it declines when antigenic stimulation is withdrawn [53, 54]. Hence, our data imply any of three possibilities: differing in vivo kinetics of CD69 expression from that seen in vitro; a constant turn-over of recently activated cells recruited from the periphery; or prolonged stimulation of resident lung CD8+ T cells via TCR-dependent or TCR-independent means. The strong correlation between expression of CD69 and IL-18R is unsurprising, as IL-18R is absent from naïve CD8+ T cells and is itself up-regulated by activation. However, expression of IL-18R is potentially important as a mechanism for T cell persistence within the lungs, because its engagement protects CD8+ T cells from activation-induced cell death, in part, by increasing Bcl-2 [55].

Studies on murine spleen-derived cells have shown that virus-specific CD8+ T cells retain high levels of IL-18Rα for longer than one year after recovery from an acute infection [37]. “Persistently activated T cells” with a generally similar surface phenotype to that we observed here have been described in the lungs following viral pneumonitis in humans [56] and in mice [57, 58], and in the latter species, data do not favor a large fraction of recent immigrants. Thus, one possible interpretation of our findings is that the observed phenotype results from the cumulative effect of repeated viral infections, to which COPD patients appear to be particularly susceptible [59], or even perhaps of persistent viral infection(s), as recently suggested [60]. However, the lack of correlation of mRNA for KLGR1, IL-7R and CD57 with GOLD stage do not support this possibility. An alternative possibility is that expression of CD69 and possibly IL-18R is being sustained by recognition of danger signals induced on lung parenchymal cells by oxidant injury [23], via non-clonally-restricted receptors. Such danger signals could be sensed via functional Toll-like receptors, several of which human peripheral blood T cells express [61, 62]. We have preliminary evidence indicating that stimulation via various TLRs can activate lung CD8+ T cells (Freeman and Curtis, manuscript in preparation). The very high fraction of CD69+ lung CD8+ T cells we found in many subjects suggests to us the likelihood of TCR-independent activation, but a definitive answer will require considerable additional research.

Although it is entirely consistent with a Tc1 phenotype, the correlation of lung CD8+ T cell expression of IL-18R with COPD severity is intriguing, due to the known variable effect of IL-18 on immune responses depending on cytokines co-expressed with it. First identified as an IFN-γ-inducing factor [15], IL-18 was later shown to enhance both type 1 responses (in the presence of IL-12) and type 2 responses (in the absence of IL-12) (reviewed in [63]). Indeed, when stimulated by IL-18 plus antigen, murine memory T_H1 cells produced not only IFN-γ and TNF-α but also IL-9 and IL-13 in vitro, and induced severe lung inflammation in vivo [64]. Therefore, given the strong GATA-3 expression by lung CD8+ T cells we found in advanced COPD, the lack of Tc2 cytokine production on stimulation with IL-18 (alone or in combination with IL-12) or with anti-CD3ε is noteworthy. Similarly, although transgenic over-expression of IL-18 alone has been shown to induce emphysema associated with increased IFN-γ, IL-5 and IL-13 [65], our data argue that lung CD8+ T cells are not solely re sponsible for such an effect in humans with COPD. It is also possible that IL-18 has other actions that favor lung destruction in COPD, e.g., by facilitating co-localization of CD8+ T cells and lung mononuclear phagocytes. IL-18 has been shown to be a chemoattractant for human CD4+ T_H1 cells [66]; although CD8+ T cells in that study were not responsive to IL-18, they also did not express IL-18R. Additionally, a positive regulatory loop has recently been shown, by which IFN-γ and IL-18 signaling accelerate proliferation of memory murine CD8+ T cells during recall responses to antigen presented by splenic DEC205+ dendritic cells (DC) in vitro [67]. It will be interesting to see whether a similar effect exists with human lung DC subsets. Thus, the lack of correlation between total lung concentrations of IL-18 and disease severity in the current study, a novel finding, should not be taken to exclude a role for that cytokine in disease progression. It is possible that measuring IL-18 in the whole lung homogenate prevents us from detecting more subtle changes in IL-18 expression that are occurring on a microenvironmental level. Hence, our data should not be construed to negate the potentially more sensitive results of Imaoka and

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colleagues, who used morphometric analysis of immunohistochemically stained lung tissue to show a difference in IL-18+ cell density between non-smokers or smokers with normal lung function and COPD patients [16]. However, like our results, they also did not find a correlation between IL-18 expression and spirometric severity within COPD patients. IL-18 is constitutively produced not only by macrophages and DC but by lung epithelium [68] as an inactive pro-peptide, and its processing and secretion is regulated, like that of IL-1, by caspase-1. We considered the possibility that the antibody we used might detect both pro-IL-18 and its processed fragment, but rejected it based on the manufacturer's information about that antibody clone.

Our data demonstrating that CD8+ T cells display increased transcripts for perforin and Granzyme B with worsening pulmonary function agree with and extend previous studies of sputum [69] and bronchoalveolar lavage fluid [70]. Results of several studies that have shown no increase in the expression of cytotoxic enzymes in CD8+ T cells in the peripheral blood of emphysema patients [71, 72] further argues for the local activation of CD8+ T cells within the lungs. Importantly, the study by Hodge et al. [70] found a significant correlation between granzyme B expression and apoptosis of bronchial epithelial cells. The idea that apoptosis of lung structural cells might contribute to emphysema has gained increasing support from basic and clinical data [34, 73]. CD8+ T cells can also use the perforin/Granzyme system for immunoregulatory function, e.g., to lyse DC, thereby preventing them from migrating to lymph nodes and prolonging immune response. Interestingly, memory CD8+ T cells that secrete TNF-α have recently been shown to block this lysis of DC [74], suggesting the possibility of complex regulatory networks within lung parenchyma.

In summary, we found that lung CD8+ T cells demonstrate increased IL-18R and CD69 expression and increased mRNA transcripts for T-bet, perforin, and granyzme B with worsening pulmonary function (increased GOLD stage or decreased FEV_1). Furthermore, expression of IFN-γ, TNF-α, and intracellular perforin by CD8+ T cells was increased by in vitro stimulation with either IL-18 plus IL-12 or IL-15, respectively. Our findings support the concept that CD8+ T cells contribute to COPD progression via production of cytotoxic molecules and pro17 inflammatory cytokines.

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Non-standard abbreviations

AMø	alveolar macrophage(s)
COPD	chronic obstructive pulmonary disease
DC	dendritic cell(s)
FasL	Fas ligand
LTRC	Lung Research Tissue Consortium
T_{EM}	T effector-memory

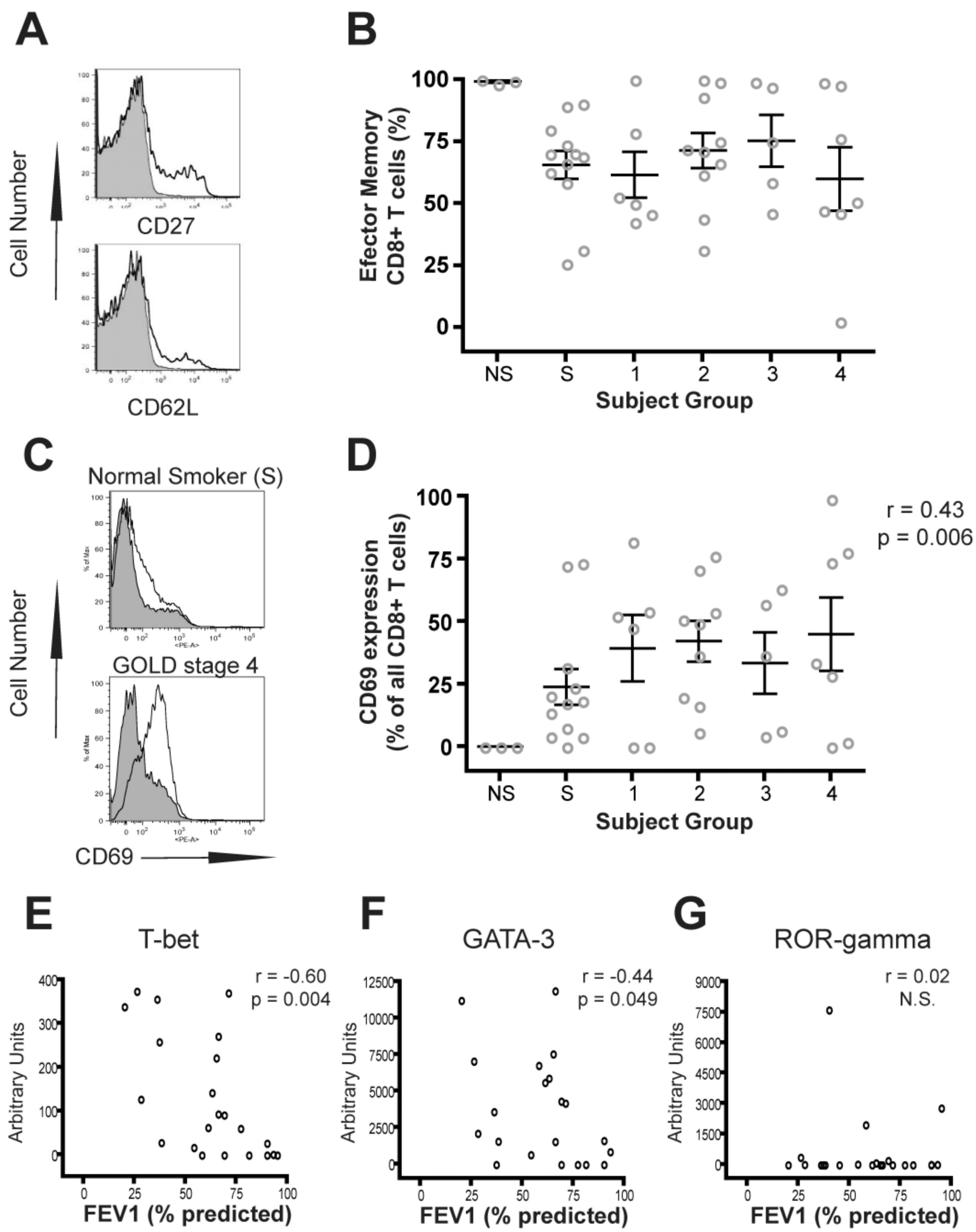

Figure 1. Lung CD8+ T cells in COPD are predominately T_{EM} cells at all disease stages, but their expression of CD69 and T-bet increases as FEV_1 decreases

CD8+ T cells from lung tissue were either (A-D) stained and analyzed by flow cytometry or (E-G) isolated using positive selection magnetic beads and processed for real time RT-PCR. (A) representative histograms showing staining for CD27 (top panel) and CD62L (bottom panel) on gated lung CD8+ T cells of a COPD patient. Shaded profiles, isotype control; open profiles, specific staining. (B) the percentage of CD8+ T cells that display an effector memory phenotype (CD62L- CD27-) stratified by subject group. NS, non-smoker, S smokers with normal lung function, 1–4, GOLD stages of COPD severity. Spearman nonparametric analysis was used to calculate r_S. Circles represent individual patients, bars represent the mean ± SEM (Cohort A, *n* = 42). (C, D) CD69 expression. (C) representative histograms from a normal smoker without COPD (top panel) and GOLD stage 4 subject (bottom panel). (D) the percentage of CD69+ CD8+ lung T cells stratified by subject group (Cohort A, *n* = 42). (E-G) mRNA expression by lung CD8+ T cells stratified by FEV_1 (% predicted). G, T-bet. H, GATA-3. I, RORγ. Results are expressed as arbitrary units. Circles represent individual patients (Cohort B, *n* = 22). Spearman nonparametric analysis was used to calculate r_S. N.S., not significant.

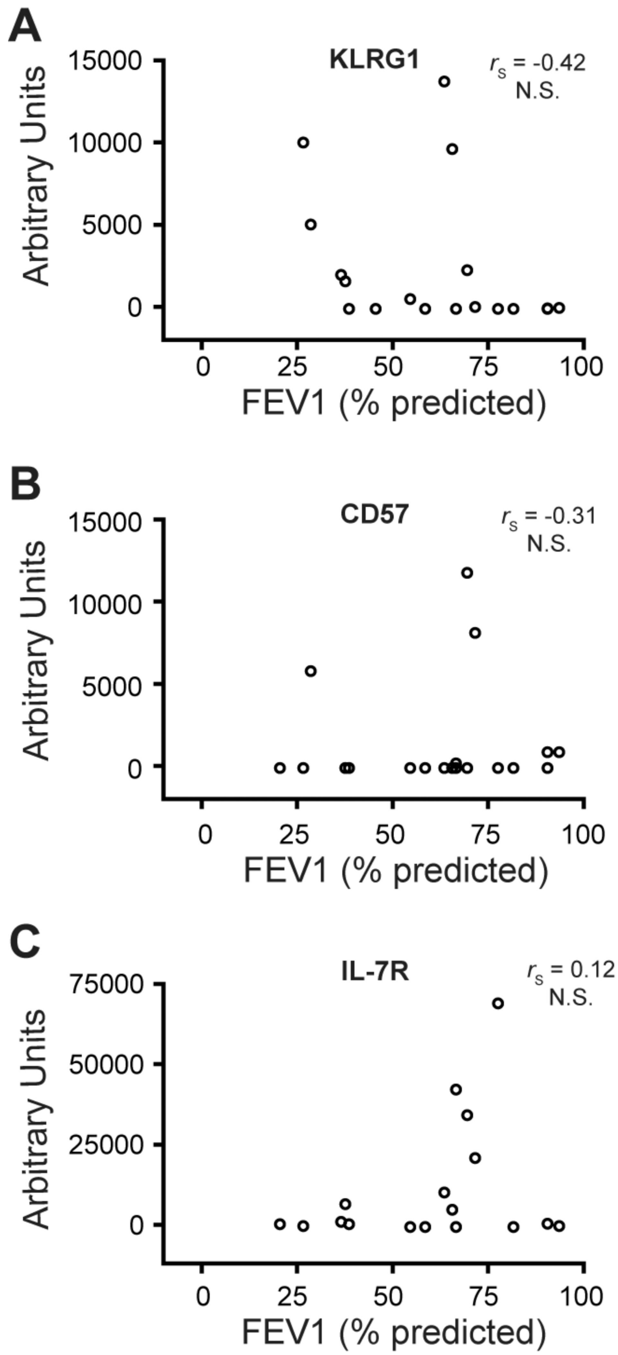

Figure 2. Expression of KLRG1, CD57 & IL-7R by lung CD8+ T cells

Lung CD8+ T cells were isolated for RNA analysis by real-time RT-PCR. A. KLRG1; B. CD57; C. IL-7R. Transcripts are expressed on the vertical axis as arbitrary units versus FEV_1 (% predicted) on the horizontal axis. Circles represent individual subjects (Cohort B, *n* = 22). Spearman nonparametric analysis was used to calculate r_S. N.S., not significant.

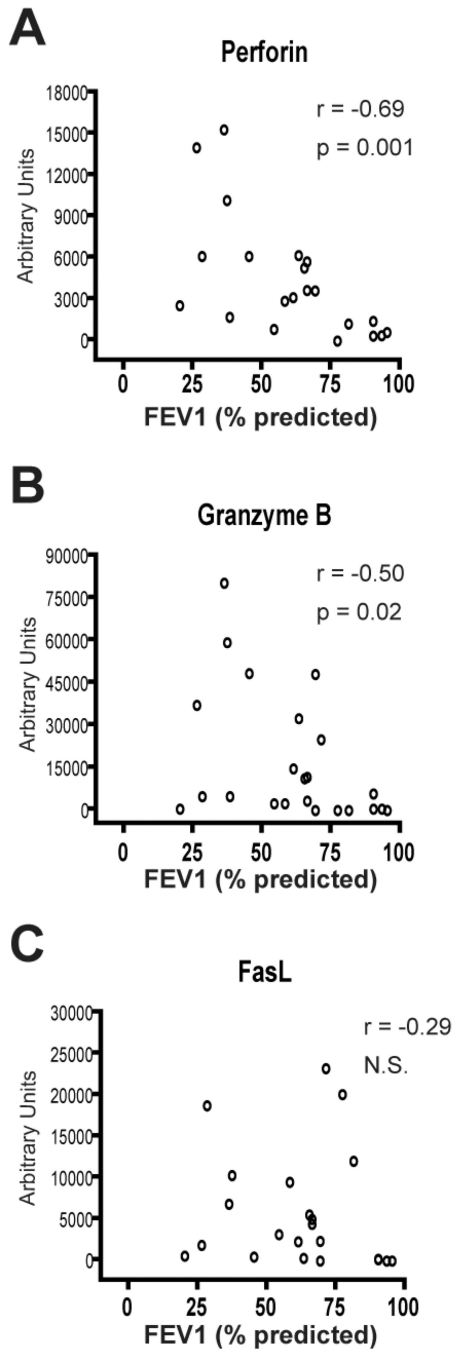

Figure 3. Expression of perforin and granzyme B by lung CD8+ T cells increases with worsening COPD severity

Lung CD8+ T cells were isolated for RNA analysis by real-time RT-PCR. A, perforin, B, granzyme B, and C, FasL were measured and are expressed on the vertical axis as arbitrary units versus FEV_1 (% predicted) on the horizontal axis. Circles represent individual patients (Cohort B, n = 22). Spearman nonparametric analysis was used to calculate r_S. N.S., not significant.

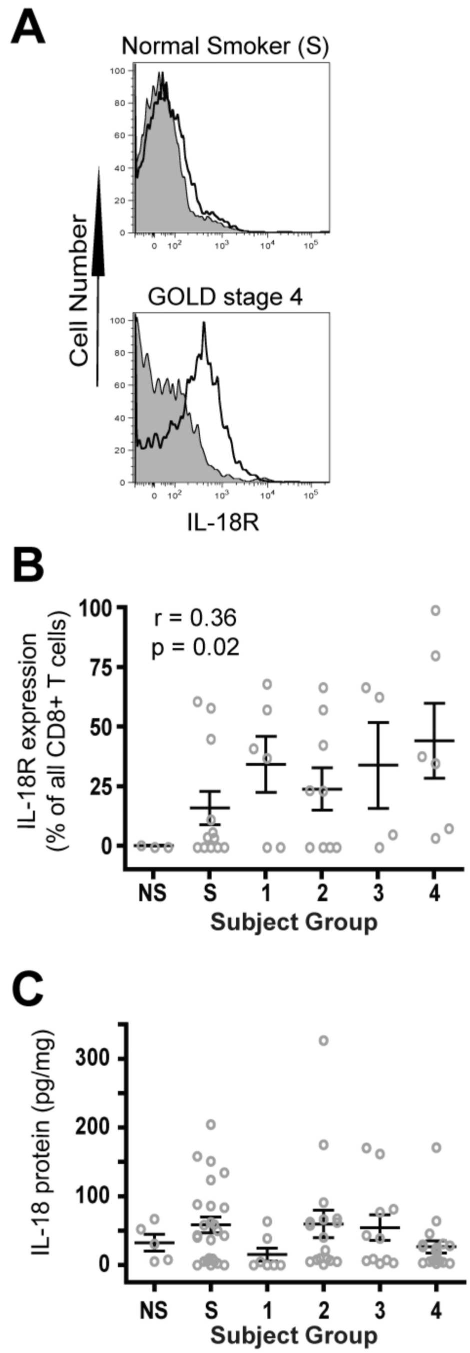

Figure 4. IL-18R expression on lung CD8+ T cells increases with worsening COPD severity

CD8+ T cells from lung tissue were stained and analyzed by flow cytometry. (A) representative histograms showing IL-18R mAb staining of gated CD8+ T cells from a smoker with normal lung function (top panel) and from a subject with advanced COPD (GOLD stage 4) (bottom panel). Shaded profiles, isotype control; open profiles, specific staining. (B) the percentage of CD8+ T cells that express IL-18R stratified by subject group (Cohort A, *n* = 42) as defined in the legend to Figure 1. (C) IL-18 protein levels from human lung tissue, stratified by group (Cohort C, *n* = 95). Spearman nonparametric analysis was used to calculate r_S and p values. Open circles represent individual patients, bars represent the mean ± SEM.

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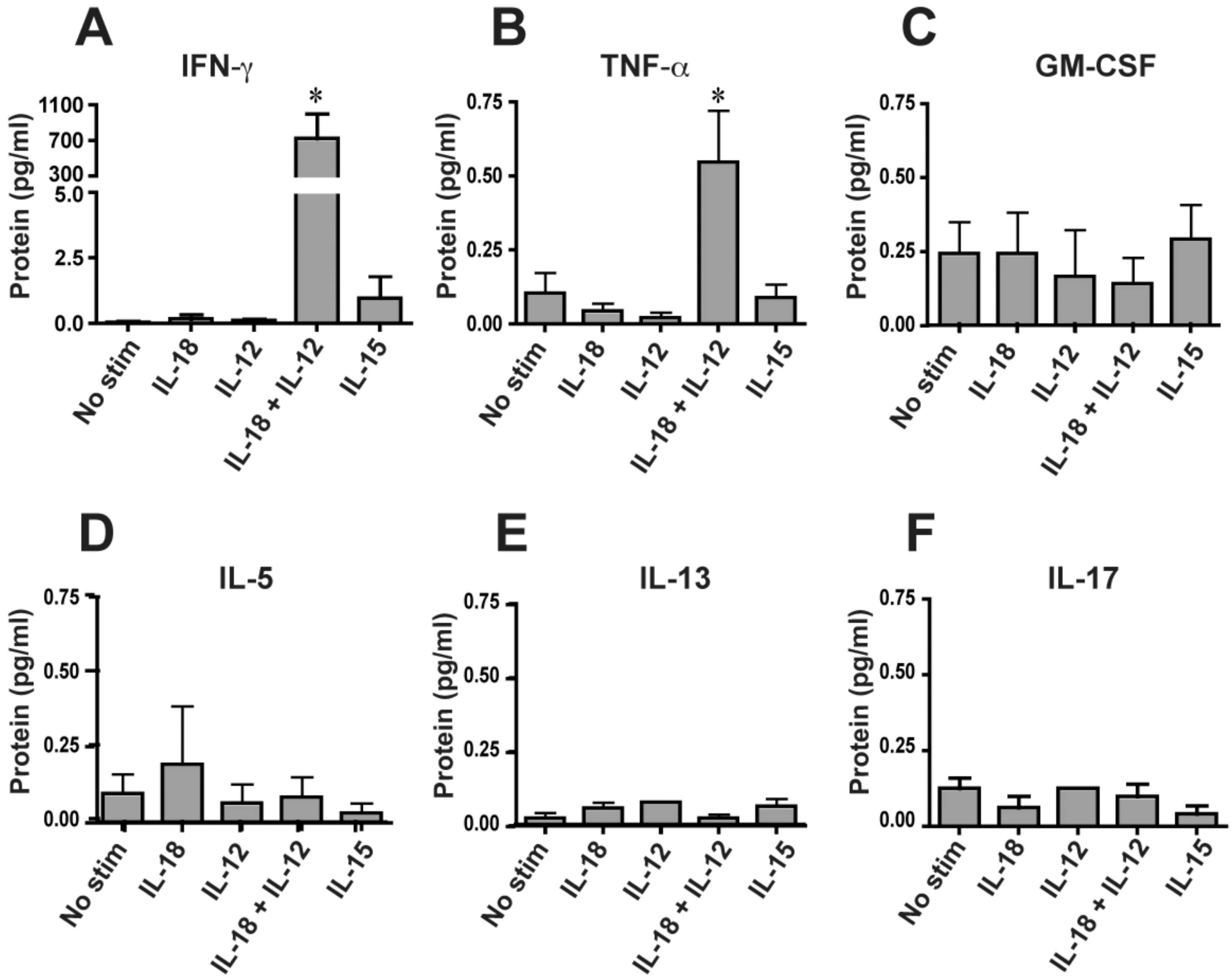

Figure 5. Lung CD8+ T cell production of IFN-γ and TNF-α is increased by stimulation with IL-18 plus IL-12

Isolated lung CD8+ T cells were cultured for 48 h either with no stimulation (no stim), IL-18 alone, IL-12 alone, combined IL-18 and IL-12, or IL-15 alone. Supernatants were collected for protein measurement by Luminex assay. (A) IFN-γ; (B) TNF-α; (C) GM-CSF; (D) IL-5; (E) IL-13, and (F), IL-17; note the difference in scales between panel A and other panels. Bars are means ± SEM from five independent experiments. * $p < 0.05$ as compared to no stimulation (one-way ANOVA with Dunn's post-hoc testing).

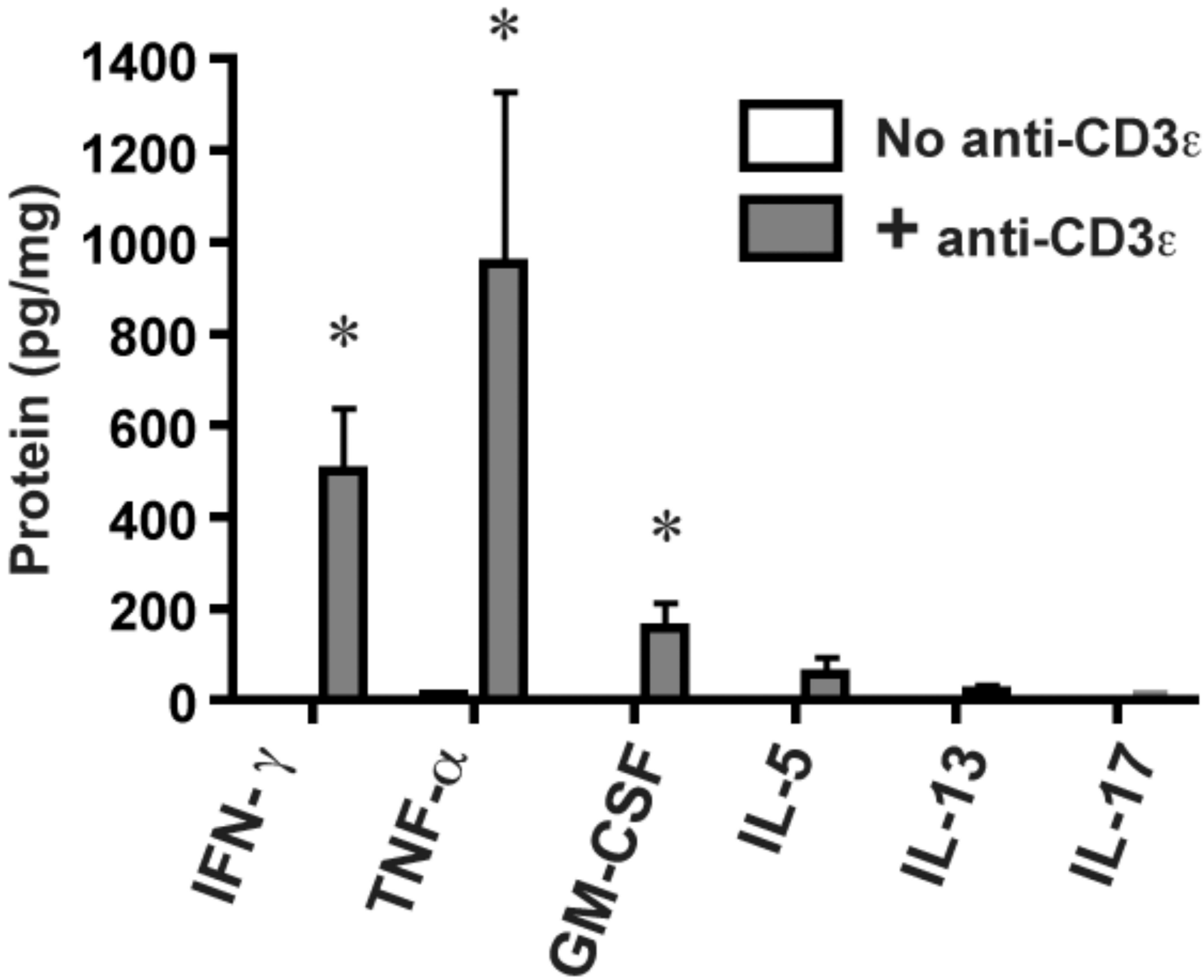

Figure 6. Lung CD8+ T cell production of Tc2 cytokines is not increased by TCR stimulation

Isolated lung CD8+ T cells were cultured for 48 h without (white columns) or with (gray columns) plate-bound anti-CD3ε. Supernatants were collected for protein measurement by Luminex assay. Bars are means ± SEM from five independent experiments. * $p < 0.05$ as compared to no anti-CD3ε stimulation (paired t-test).

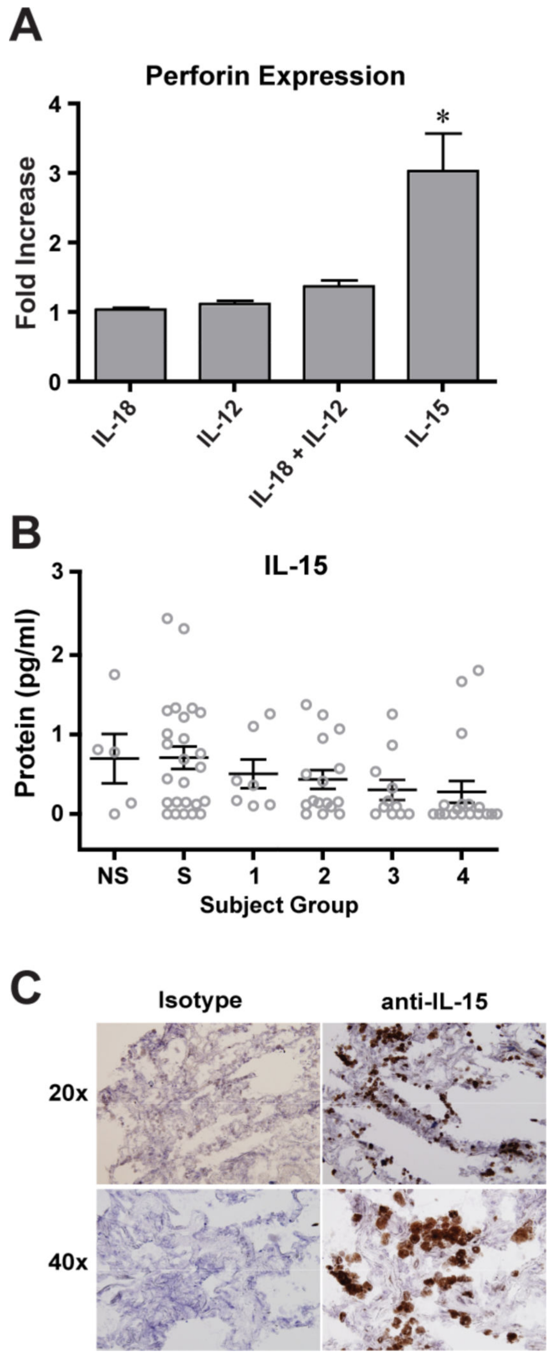

Figure 7. IL-15 stimulation induces increased perforin production by lung CD8+ T cells

A, isolated lung CD8+ T cells were cultured for 48 h with either no stimulation, IL-18 alone, IL-12 alone, combined IL-18 plus IL-12, or IL-15 alone. Intracellular perforin expression by CD8+ T cells was measured by flow cytometry. Results are expressed as the fold-increase over non-stimulated controls. Bars represent means ± SEM from five individual experiments, * $p < 0.05$, compared to all other conditions (One-way ANOVA with Dunn's post-hoc testing). (B) Whole lung IL-15 protein levels were measured by Luminex assay and stratified by subject group (Cohort C, $n = 95$). Circles represent individual patients, bars represent means ± SEM. (C) Immunohistochemical staining for IL-15 on frozen lung tissue from a representative subject (smoker with normal pulmonary function). Left panels, isotype control staining; right panels, IL-15 staining. Top panels, 20× magnification; bottom panels, 40× magnification.

Table I

Summary of Cohort A, B, and C demographics, smoking histories & spirometry

Group	Cohort	Subjects, *n*	Sex ratio, M/F	Age years [1]	Smoking history, pack-years [1]	FEV_1, % pred. [1]
NS	A	3	0/3	59 (6)	0 (0)	91 (5)
	B	0				
	C	5	4/1	71 (10)	0 (0)	83 (18)
S	A	11	8/3	68 (11)	43 (54)	98 (19)
	B	8	8/0	65 (12)	42 (16)	75 (14)
	C	25	8/17	63 (10)	51 (35)	91 (20)
GOLD	Stage					
1	A	6	3/3	63 (8)	33 (130)	89 (12)
	B	2	2/0	68 (6)	50 (0)	86 (6)
	C	7	6/1	73 (8)	35 (26)	90 (8)
2	A	12	10/2	61 (5)	56 (25)	67 (7)
	B	4	3/1	67 (4)	43 (6)	67 (8)
	C	17	10/7	69 (7)	68 (44)	66 (8)
3	A	6	5/1	61 (13)	50 (23)	45 (4)
	B	6	5/1	58 (7)	40 (13)	40 (4)
	C	11	9/2	59 (12)	52 (24)	38 (6)
4	A	9	4/5	55 (6)	45 (19)	22 (6)
	B	2	1/1	69 (0)	52 (3)	24 (4)
	C	19	8/11	59 (7)	57 (36)	21 (6)

[1] Data are presented as average (SD).