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Cytotoxic Potential of Lung CD8+ T Cells Increases with Chronic Obstructive Pulmonary Disease Severity and with In Vitro Stimulation by IL-18 or IL-15

Christine M. Freeman,∗† Mei Lan K. Han,‡ Fernando J. Martinez,† Susan Murray,‡ Lyrica X. Liu,‡ Stephen W. Chensue,§,*‡ Timothy J. Polak,§ Joanne Sonstein,‡ Jill C. Todt,§ Theresa M. Ames,‡ Douglas A. Arenberg,‡ Catherine A. Meldrum,‡ Christi Getty,§ Lisa McCloskey,‡ and Jeffrey L. Curtis∗†,‡,‖

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 killer cell lectin-like receptor G1, IL-7R, 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 and TNF-α markedly increased production of IFN-γ and IL-12, respectively. Cytotoxic Potential of Lung CD8+ T Cells Increases with Chronic Obstructive Pulmonary Disease Severity and with In Vitro Stimulation by IL-18 or IL-15. The Journal of Immunology, 2010, 184: 000–000.

Abbreviations used in this paper: AMø, alveolar macrophage; COPD, chronic obstructive pulmonary disease; DC, dendritic cell; F, female; FasL, Fas ligand; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; GATA-3, GATA-binding protein 3; GOLD, Global Initiative for Chronic Obstructive Lung Disease; KLRL1, killer cell lectin-like receptor G1; LTRC, Lung Research Tissue Consortium; M, male; No stil, no stimulation; NS, nonsmoker; ROR, retinoic acid receptor-related orphan receptor; S, smoker with normal lung function; T-bet, T-box expressed in T cells; TEM, T effector memory cell.

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Chronic obstructive pulmonary disease (COPD), the fourth leading cause of death in the United 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 lung CD8+ T cells isolated from lung parenchyma in COPD are largely TEM, T effector memory cell.
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 Ag-independent IFN-γ production by T cells (14, 15). IL-18 is strongly expressed by alveolar macrophages (AMs) of patients with severe COPD (16) and is increased in the peripheral blood of patients with COPD relative to controls (17, 18). In mice, treatment with rIL-18 and rIL-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 AMs, and cigarette smoke-induced emphysema was decreased by a null mutation of the IL-18R protein that localized to AMs, and cigarette smoke-induced emphysema was decreased by a null mutation of the IL-18R (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 upregulation on pulmonary epithelium of ligands for the cytotoxic T cell-activating receptor NKG2D (CD244) has recently been implicated in COPD pathogenesis 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 cell (TEM) phenotype, regardless of disease severity as defined by pulmonary function, their production of mRNA transcripts for perforin and 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 nonneoplastic lung tissue remote from the nodules and lacking postobstructive changes was collected.

Cohort A comprised 47 subjects recruited preoperatively 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 wk) 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 s (FEV₁) of the predicted value in combination with the ratio of FEV₁ 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 FEV₁/FVC ratio of <0.70 were included in GOLD stages 1–4. Subjects (n = 11) with a history of smoking, an FEV₁/FVC ratio >0.70, normal spirometry, and no clinical diagnosis of COPD represent our smoking controls. Subjects with no history of smoking and normal spirometry (n = 3) are nonsmoking controls. 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 deidentified lung tissue was obtained from the Tissue Procurement Cores at the University of Michigan Healthcare System and the VA Ann Arbor Healthcare System (Ann Arbor, MI). 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 LungTissueResearchConsortium (LTRC). This National Heart, Lung, and Blood Institute-sponsored tissue bank (www.ltrcpublic.com/) preoperatively collects extensive demographic, physiological, and radiographic information, which is then deidentified 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

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*Data are presented as average (SD) unless otherwise indicated.
F, female; M, male.
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 Healthcare System 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 ~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/ml 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 Abs against CD45 (H330), CD8 (RPA-T8), CD27 (O323), CD69 (FN50), IL-18R (H44) (eBioscience, San Diego, CA), and CD62L (Dreg 56) (BD Biosciences, San Jose, CA) were used. Appropriate isotype-matched controls were used in all experiments. Abs were conjugated to either FITC, PE, or PE-cyanine 5 (PE-Cy5). Cells were incubated in the dark with Abs 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/well with lymphocyte culture media (10% FBS, 1 mM sodium pyruvate, 0.5 mM 2-ME, 1 mM HEPES, 100 μ/mL penicillin, 100 μ/mL streptomycin, and 0.292 mg/mL γ-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-min incubation in Permeabilization Buffer (eBioscience) plus Ab (Δ G9; BD Biosciences). 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 Biosciences) equipped with 488-nm blue, 405-nm violet, and 633-nm red lasers. Data were collected on an HP XW4300 Workstation (Hewlett-Packard, Palo Alto, CA) using FlowJo software (BD Biosciences) with automatic compensation and were analyzed using FlowJo software (Tree Star, 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-box expressed in T cells (T-bet) (Hs00203436_m1), GATA-binding protein 3 (GATA-3) (Hs00233682_m1), retinoic acid receptor-related orphan receptor (ROR) γ (Hs01076112_m1), RORα (Hs00395959_m1), IL-17F (Hs00233682_m1), FasL (Hs00181225_m1), killer cell lectin-like receptor G1 (KLRG1) (Hs00915153_m1), CD57 (Hs00218629_m1), and IL-7R (Hs00233682_m1) were purchased commercially (Applied Biosystems, Foster City, CA). Transcript levels were 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 PBS 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, Austin, TX), protein levels for IL-15 and IL-18 were determined by Biosource Multiplex Assays (Invitrogen, Carlsbad, CA). Total lung protein concentration was determined using a Micro BCA protein assay kit (Pierce, Rockford, 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, La Jolla, CA). Nonparametric (Spearman) correlation analysis was used to determine the correlation coefficient, rS. 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 used for analyses of data that did not meet assumptions for normality. PROC GLM with Tukey’s method for multiple comparisons was employed to contrast patient groups using SAS 9.1 statistical software (SAS Institute, Cary, NC). A similar approach was used 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 FEV1 percent 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 lung 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 determine 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 CD69. Regardless of disease severity, the majority (~75% at all stages) of lung CD8+ T cells were 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 (rS = 0.43, p = 0.006) (Fig. 1C, 1D) or FEV1 percent predicted (rS = −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 with GOLD stage (rS = −0.47, p = 0.009; data not shown) with decreasing diffusion lung capacity for carbon monoxide (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 RORγt, transcription factors that drive Tc1, Tc2, and Tc17 responses, respectively. T-bet transcripts were significantly inversely correlated with FEV1 ($r_S = -0.60, p = 0.004$) (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 (C.M. Freeman, J.L. Curtis, S.W. Chensue, unpublished observations) by CD8+ T cells in this cohort, GATA-3 transcripts were produced by CD8+ T cells and also showed a trend to increase as FEV1 decreased ($r_S = -0.44, p = 0.049$) (Fig. 1F). By contrast, lung

![Image 1](http://www.jimmunol.org/)
CD8+ T cells showed little to no RORγ (Fig. 1G) or RORα (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: KLRG1, IL-7R (CD127), and CD57. Driven by elevated levels of T-bet, especially during chronic viral infections, CD8+ T cells can become terminally differentiated, KLRG1high, IL-7Rlow 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 TEM cells capable of immaturely derived cells typically associated with chronic infections or damaged effector cells (26, 27). CD57-positivity identifies oligoclonally derived cells typically associated with chronic infections or extreme age. CD8+ CD57+ T cells are TEM cells capable of terminally differentiated, KLRG1 high, IL-7R low short-lived effector cells (26, 27). CD57-positivity identifies oligoclonally derived cells typically associated with chronic infections or extreme age. 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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, 4B) or as FEV1 percent predicted \((r_S = -0.30, p = 0.04; \text{data not shown})\). IL-18R expression did not show any correlation with pack-years, diffusion lung capacity for carbon monoxide, or percent emphysema, but there was a very strong correlation in individual subjects between expression of IL-18R and CD69 \((r_S = 0.83, p = 0.0001; \text{data not shown})\). 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 because 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 overexpressed 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, without 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 was very significantly increased \((≥800\text{-fold})\) (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 proinflammatory 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 Fig. 1 that lung CD8+ T cells display transcripts for the Tc2 transcription factor, GATA-3, we next asked whether TCR stimulation, via anti-CD3e 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-CD3e. All cytokines were below the limit of detection in unstimulated wells. Anti-CD3e 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 that 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, stimulation 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 with the unstimulated control CD8+ lung T cells (Fig. 7A). IL-15 has been shown to induce the synthesis of perforin in T cells in vitro (39) and in vivo in a model of acute lung injury (40). We measured intracellular expression of perforin to avoid the technical pitfalls of in vivo or in vitro cleavage of the extracellular domain of perforin, which can affect detection of intracellular perforin by flow cytometry.

**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 \((\text{top panel})\) and from a subject with advanced COPD \((\text{GOLD stage 4})\) \((\text{bottom panel})\). 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 Fig. 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. 1–4, GOLD stages of COPD severity; NS, nonsmoker; S, smoker with normal lung function.
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-\(\gamma\) 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 AM\(\phi\) 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 TEM cells of a Tc1 phenotype, for which the 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 AM\(\phi\). Key novel results include: 1) 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; 2) 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-\(\gamma\) and TNF-\(\alpha\) (but not Tc2 or Tc17 cytokines) on stimulation with IL-18 plus IL-12 in the absence of TCR activation; and 3) 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 bidirectional, 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 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 variables; 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 (\(<2\, \text{mm diameter}\) airway (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.
have shown that virus-specific CD8+ T cells retain high levels of perforin expression by increasing Bcl-2 (55). Studies on murine spleen-derived cells have shown that IL-18R expression is unsurprising, as IL-18R is absent from naive CD8+ T cells and is itself upregulated 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 1 year postrecovery from an acute infection (37). Persistently activated T cells with a generally similar surface phenotype to that observed in this study have been described in the lungs following viral pneumonitis in humans (56) and 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 KLGRI, IL-7R, and CD57 with GOLD stage does 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 nonclonally restricted receptors. Such danger signals could be sensed via functional TLRs, 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 (C.M. Freeman and J.L. 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 coexpressed 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 Ref. 63). Indeed, when stimulated by IL-18 plus Ag, murine memory Th1 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 overexpression 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 responsible 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 colocalization of CD8+ T cells and lung mononuclear phagocytes). IL-18 has been shown to be a chemoattractant for human CD4+ Th1 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 Ag presented by splenic DEC205+ dendritic cells (DCs) 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 colleagues (16), who used morphometric analysis of immunohistochemically stained lung tissue to show a difference in IL-18ε cell density between nonsmokers or smokers with normal lung function and COPD patients. However, like our results, they also did not find a correlation between IL-18 expression and spirometric severity within patients with COPD. IL-18 is constitutively produced not only by macrophages and DC but by lung epithelium (68) as an inactive propeptide, and its processing and secretion is regulated, like that of IL-1, by caspase-1. We considered the possibility that

**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 nonstimulated controls. Bars represent means ± SEM from five individual experiments. 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. "p" < 0.05 compared with all other conditions (one-way ANOVA with Dunn’s post hoc testing). 1–4, GOLD stages of COPD severity; NS, nonsmoker; S, smoker with normal lung function.

The findings of increased expression of CD69 and IL-18R on lung CD8+ T cells with advancing disease raise 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 upregulated 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 and IL-18R is being sustained by recognition of danger signals induced on lung parenchymal cells by oxidant injury (23) via nonclonally restricted receptors. Such danger signals could be sensed via functional TLRs, 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 (C.M. Freeman and J.L. 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.

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The ab we used might detect both pro–IL-18 and its processed fragment, but rejected it based on the manufacturer’s information about that Ab 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 patients with emphysema (71, 72) further argue 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-\(\alpha\) 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 granzyme B with worsening pulmonary function (increased GOLD stage or decreased FEV\(_1\)). Furthermore, expression of IFN-\(\gamma\), TNF-\(\alpha\), and intracellular perforin by CD8\(^+\) \(T\) cells was increased by in vitro stimulation with both IL-12 and IL-15, respectively. Our findings support the concept that CD8\(^+\) \(T\) cells contribute to COPD progression via production of cytotoxic molecules and proinflammatory cytokines.

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Disclosures

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