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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,§* Tim 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 cytotoxicity, 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-18 and IL-69 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 in concentration with spirometric severity. Although lung CD8+ T cell expression of mRNA for both T-box transcription factor 1 and GATA-binding protein 3; GOLD, Global Initiative for Chronic Obstructive Lung Disease; KLRG1, killer cell lectin-like receptor G1; LTRC, Lung Research Tissue Consortium; M, male; No stim, 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; Tc2, T effector memory cell.

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Abbreviations used in this paper: AMoE, 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; KLRG1, killer cell lectin-like receptor G1; LTRC, Lung Research Tissue Consortium; M, male; No stim, 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; Tc2, T effector memory cell.

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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 (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 (CD314) 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 Lung Tissue Research Consortium (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

### Table I. Summary of cohort A, B, and C demographics, smoking histories, and spirometry

<table>
<thead>
<tr>
<th>Group</th>
<th>Cohort Subjects (n)</th>
<th>Smoking History (packs/yr)</th>
<th>FEV1 (% Predicted)</th>
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<tr>
<td></td>
<td>Sex Ratio (M/F)</td>
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<td></td>
<td>Age (y)</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>NS</td>
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<td>0/3</td>
<td>59 (6)</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>5 4/1</td>
<td>71 (10)</td>
<td>0 (0) 83 (18)</td>
</tr>
<tr>
<td>S</td>
<td>A 11</td>
<td>8/3</td>
<td>68 (11) 43 (54)</td>
</tr>
<tr>
<td>B</td>
<td>8 8/0</td>
<td>65 (12)</td>
<td>42 (16) 75 (14)</td>
</tr>
<tr>
<td>C</td>
<td>25 8/17</td>
<td>63 (10)</td>
<td>51 (35) 91 (20)</td>
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<td>GOLD stage</td>
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<td>6 3/3</td>
<td>63 (8) 33 (130)</td>
</tr>
<tr>
<td></td>
<td>A 2</td>
<td>2/0</td>
<td>68 (6) 50 (0)</td>
</tr>
<tr>
<td>B</td>
<td>7 6/1</td>
<td>73 (8)</td>
<td>35 (26) 90 (8)</td>
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<td></td>
<td>A 12</td>
<td>10/2</td>
<td>61 (5) 56 (25)</td>
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<tr>
<td></td>
<td>B 4</td>
<td>3/1</td>
<td>67 (4) 43 (6)</td>
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<td></td>
<td>C 17</td>
<td>10/7</td>
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</tr>
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<td></td>
<td>A 6</td>
<td>5/1</td>
<td>61 (13) 50 (23)</td>
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<tr>
<td>B</td>
<td>6 5/1</td>
<td>58 (7)</td>
<td>40 (13) 40 (4)</td>
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<tr>
<td>C</td>
<td>11 9/2</td>
<td>59 (12)</td>
<td>52 (24) 58 (6)</td>
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<tr>
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<tr>
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<td>2 1/1</td>
<td>69 (0)</td>
<td>52 (3) 24 (4)</td>
</tr>
<tr>
<td>C</td>
<td>19 8/11</td>
<td>59 (7)</td>
<td>57 (36) 21 (6)</td>
</tr>
</tbody>
</table>

aData 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 x 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-cyamine 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 5 x 10^5 cells/well in αMEM containing 10% FBS, 1 µM β-mercaptoethanol, 100 µM streptomycin, and 0.292 µg/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-CD3e (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 (6 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 XW4900 Workstation (Hewlett-Packard, Palo Alto, CA) using FACSDiva 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 Maxx00P PCR 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) (Hs00231122_m1), retinoic acid receptor-related orphan receptor (ROR) γ (Hs01076612_m1), RORα (Hs00931959_m1), IL-17F (Hs00369400_m1), IL-22 (Hs01574355_m1), IL-18R (Hs00181225_m1), killer cell lectin-like receptor G1 (KLRG1) (Hs00195153_m1), CD57 (Hs00218629_m1), and IL-7R (Hs00233682_m1) were purchased commercially (Applied Biosystems, Foster City, CA). Transcript 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 PBS and homogenized using a tissue homogenizer. Samples were centrifuged at 300 x 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, r_s. Paired t tests and one-way ANOVA, with Dunn’s post hoc testing, were used to determine statistical differences between treatment groups 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 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 (~75% at all stages) of lung CD8+ T cells were CD62L+CD27+, implying that they are TEM cells (Fig. 1A, 1B). 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 CD45RA (6). By contrast, CD8+ T cells from peripheral blood of patients with COPD are largely T central memory cells (i.e., CD2L+,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, 1D) or FEV₁ percent 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 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 ROR\(_g\), transcription factors that 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-\(\gamma\) 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 FEV\(_1\) decreased (\(r_S = -0.44, p = 0.049\)) (Fig. 1F). By contrast, lung

**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 stained and analyzed by flow cytometry (A–D) or isolated using positive selection magnetic beads and processed for real-time RT-PCR (E–G). A, Representative histograms showing staining for CD27 (top panel) and CD62L (bottom panel) on gated lung CD8\(^+\) T cells of a patient with COPD. Shaded profiles, isotype control; open profiles, specific staining. B, Percentage of CD8\(^+\) T cells that display an effector memory phenotype (CD62L\(^-\) CD27\(^-\)) stratified by subject group. Spearman nonparametric analysis was used to calculate \(r_S\). Circles represent individual patients; bars represent the mean ± SEM (cohort A, \(n = 42\)). C and 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 FEV\(_1\) (percent predicted). E, T-bet. F, GATA-3. G, ROR\(_g\). Results are expressed as arbitrary units. Circles represent individual patients (cohort B, \(n = 22\)). Spearman nonparametric analysis was used to calculate \(r_S\). 1–4, GOLD stages of COPD severity; NS, nonsmoker; S, smoker with normal lung function.
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 immediate functional activity, producing IFN-γ and TNF-α in response to Ag 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 (rS = 0.46, p = 0.03) (data not shown).

**Perforin and granzyme B, but not FasL, transcripts from lung CD8+ T cells correlate with decreasing FEV1**

We next analyzed transcript expression from isolated lung CD8+ T cells from cohort A for the cytotoxic molecules perforin, granzyme B, and FasL. These preformed, 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 (rS = −0.69, p = 0.001) and of granzyme B (rS = −0.50, p = 0.02) and pulmonary function, as measured by FEV1 (percent predicted) (Fig. 3A, 3B). Furthermore, expression of transcripts for perforin and granzyme B within individual subjects was strongly correlated (rS = 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 TEM cells, rather than terminally differentiated effectors. By contrast, FasL transcript expression did not correlate with FEV1 (Fig. 3C). None of these molecules showed any correlation with smoking history, as measured by pack-years (data not shown). Because computed tomography 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 elevated IL-18 levels and IL-18R signaling in the pathogenesis of pulmonary

![FIGURE 2](image-url)  
**FIGURE 2.** Expression of KLRG1, CD57, and 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 FEV1 (percent predicted) on the horizontal axis. Circles represent individual subjects (cohort B, n = 22). Spearman nonparametric analysis was used to calculate rS.

![FIGURE 3](image-url)  
**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. Perforin (A), granzyme B (B), and FasL (C) were measured and are expressed on the vertical axis as arbitrary units versus FEV1 (percent predicted) on the horizontal axis. Circles represent individual patients (cohort B, n = 22). Spearman nonparametric analysis was used to calculate rS.
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; 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; 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-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 TGF-β 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. 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 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 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 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 AMø. 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-γ and TNF-α (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 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.

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 stim, IL-18 alone, IL-12 alone, combined IL-18 and IL-12, or IL-15 alone. Supernatants were collected for protein measurement by Lumixen assay. A, IFN-γ. B, TNF-α. C, GM-CSF. D, IL-5. E, IL-13. F, IL-17. The principal findings of this study indicate that lung CD8+ T cells...
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 shown to protect 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 protective activity for longer than 1 y postrecovery from an acute infection (56), 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 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-18+ cell density between nonsmokers and 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...


