Function of Human Tumor-Infiltrating Lymphocytes in Early-Stage Non–Small Cell Lung Cancer

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Abstract

Cancer progression is marked by dysfunctional tumor-infiltrating lymphocytes (TIL) with high inhibitory receptor (IR) expression. Because IR blockade has led to clinical responses in some patients with non–small cell lung cancer (NSCLC), we investigated how IRs influenced CD8+ TIL function from freshly digested early-stage NSCLC tissues using a killing assay and intracellular cytokine staining after in vitro T-cell restimulation. Early-stage lung cancer TIL function was heterogeneous with only about one third of patients showing decrements in cytokine production and lytic function. TIL hypofunction did not correlate with clinical factors, coexisting immune cells (macrophages, neutrophils, or CD4+ T regulatory cells), nor with PD-1, TIGIT, TIM-3, CD39, or CTLA-4 expression. Instead, we found that the presence of the integrin αβ7 (CD103), characteristic of tissue-resident memory cells (T RM), was positively associated with cytokine production, whereas expression of the transcription factor Eomesodermin (Eomes) was negatively associated with TIL function. These data suggest that the functionality of CD8+ TILs from early-stage NSCLCs may be influenced by competition between an antitumor CD103+ T RM program and an exhaustion program marked by Eomes expression. Understanding the mechanisms of T-cell function in the progression of lung cancer may have clinical implications for immunotherapy.

Introduction

Non–small cell lung cancer (NSCLC) is a lethal cancer. However, inhibitory receptor (IR) blockade, specifically with PD-1 and PD-L1 antibodies, has demonstrated antitumor efficacy in a subset of patients with advanced NSCLC (1). Successful anti–PD-1 therapy has triggered interest in other potential IRs or actionable immune targets on tumor-infiltrating lymphocytes (TIL). IRs identified on NSCLC TIL subsets include Cytotoxic T-Lymphocyte-Associated protein-4 (CTLA-4), T-cell Immuno-globulin, Mucin-domain containing-3 (TIM-3), CD39, and T-cell immunoreceptor with Ig and ITIM domains (TIGIT); refs. 2–6).

The integrin αβ7 (known as CD103), which marks tissue-resident memory cells (T RM), is expressed on lung cancer TILs and is positively correlated with clinical outcome (7–9). Despite this increasingly detailed insight into the immune phenotype of NSCLC TILs, relatively little work has focused on the interplay of specific surface receptors and TIL function. Many of these IRs can also be markers of T-cell activation. For example, PD-1 is upregulated on recently activated CD8+ T cells (10) and marks a set of neoantigen-specific melanoma TILs (11). Tumor T RM8, which are reactive T cells, also overexpress PD-1 constitutively (7, 12, 13).

To study early-stage NSCLC TIL function, we performed flow cytometric single-cell analyses after suboptimal T-cell receptor (TCR) stimulation. We found that early-stage NSCLC TILs had heterogeneous cytokine production and cytolytic activity, and that function was not correlated with IR expression. However, T-cell function was positively associated with CD103 expression and negatively associated with transcription factor Eomesodermin (Eomes) expression.

Materials and Methods

Patient selection

The Perelman School of Medicine Institutional Review Board, in accordance with the U.S. Common Rule, approved this study, and all patients signed informed consent. Patient samples (from 2014 to 2017) were obtained from: (i) 44 patients with early-stage NSCLC (stages I–II) undergoing potentially curative surgery, (ii) 8 patients with advanced-stage NSCLC (stage IV) via fine-needle aspirates (FNA), and (iii) 5 patients with malignant pleural...
effusions (MPE) from advanced NSCLC. Whenever possible, peripheral blood mononuclear cells (PBMC) and uninvolved lung tissue from the same lung lobe (as distal from the tumor as possible) were collected. Healthy appearing lung tissues obtained from 21 nontransplantable organ transplant donors (due to focal areas of pneumonia or donor hypoxemia) were processed. Table 1 presents details.

Tissue acquisition and processing

After resection, tissues, MPEs, and PBMCs were digested within 2 hours using validated approaches to minimize cell surface marker cleavage (14). For transplant donors, the median time between removal of the lung until tissue processing was 7 hours. All digest and samples were analyzed immediately, without freezing or overnight “rest.”

FNAs

Late-stage NSCLC tumor tissue was obtained via 21 g or 22 g endobronchial ultrasound transbronchial aspiration needle (15). Samples were flushed into tissue culture media and immediately processed as above.

Immunophenotyping/Flow cytometry

After digestion, samples were stained and analyzed using standard flow cytometry approaches. Single-cell suspensions from all samples were stained with Live Dead Blue (Invitrogen, 1:400 in PBS) for 10 minutes at 4°C, washed with FACS Buffer (1% FBS in PBS), and then stained with cell surface marker antibodies for 45 minutes at 4°C. For Ki-67 and Foxp3 staining, cells were fixed with eBioscience Fix/Perm for 1 hour at 4°C and washed two times with eBioscience Perm/Wash Buffer. Cells were stained in the presence of Perm Wash for 45 minutes at 4°C. Antibodies used were anti-human CD3 (UCHT1, SK7), CD4 (OKT4), CD69 (F430), CD103 (Ber-Act8), PD-1 (EH12-H7), TIM-3 (F38-2E2), CD62L (DREG-56), CD45RO (UCHL1), CD39 (A1), Foxp3 (206D), CD45RA (H100), PD-L1 (M1H1), CD14 (M5E2), and CD15 (H9P8) from Biologend; Anti-human CD8 (RPA-T8) and CTLA-4 (BN13) from BD Bioscience; and anti-human TIGIT (MBAS43), Eomes (W91228), and Ki-67 (20Raj1) from ebioscience.

Intracellular cytokine staining was performed as detailed previously (16). Cytokine antibodies used were directly conjugated from eBioscience. For concurrent transcription factor and cytokine staining, the cells were treated by the eBioscience FoxP3 Protocol Kit (4S.B3), TNFα (MAb11), and IL2 (MQ1-C14) from BD Bioscience; and anti-human CD8 (OKT4), CD69 (FN50), CD103 (Ber-Act8), PD-1 (EH12-H7), TIM-3 (F38-2E2), CD62L (DREG-56), CD45RO (UCHL1), CD39 (A1), Foxp3 (206D), CD45RA (H100), PD-L1 (M1H1), CD14 (M5E2), and CD15 (H9P8) from Biologend; Anti-human CD8 (RPA-T8) and CTLA-4 (BN13) from BD Bioscience; and anti-human TIGIT (MBAS43), Eomes (W91228), and Ki-67 (20Raj1) from ebioscience.

Statistical analysis

Descriptive statistics were computed for all variables. Because most of our data did not deviate from a normal distribution (using Shapiro–Wilks test and histograms), parametric tests were applied. Comparison between two independent samples was based on two-sample t test or paired t tests when appropriate. One-way ANOVA was used to compare multiple groups, followed by ad hoc pairwise comparison using Tukey correction if the overall F test was significant. Pearson χ2 test was used to compare hypofunction proportions between independent groups of patients or McNemar test for paired samples. All tests were two-sided, and P < 0.05 was considered statistically significant.

Heatmap data

Data (non–log-transformed) were visualized in a heatmap format using the Morpheus software (https://software.broadinstitute.org/morpheus/).

Results

Patient and sample characteristics

We studied T-cell function from (i) tumor-free lung lymphocytes (TFL), (ii) lung tissue adjacent to resected lung cancers (distant lung-associated lymphocytes or DLAL), (iii) tumor-infiltrating lymphocytes from early-stage resected lung
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Table 1. Patient clinical and demographic data

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| Cancers (TIL). (iv) MPEs from patients with stage IV NSCLC, and (v) TILs from advanced-stage lung cancers obtained by FNAs (see Methods). Selected patient characteristics are summarized in Table 1.

Early-stage lung cancer TIL heterogeneity
To test CD8+ T-cell baseline function, we cultured tissue digests overnight with monensin and brefeldin and performed intracellular cytokine staining. The CD8+ TFLLs, DLALs, and TILs showed little spontaneous intracellular IFNy production (Supplementary Fig. S1A) consistent with previous reports (5).

We thus designed an assay to stimulate cytokine secretion by suboptimal TCR stimulation determined by titration of plate-bound anti-CD3 using a dose of 0.5 μg/mL (Supplementary Fig. S1B). To control for intra-assay variability with non-batched patient samples, we stimulated the T cells overnight with PMA/I. This resulted in a robust IFNy production with 85% to 88% of CD8+ cells making cytokine (Supplementary Fig. S1C). We confirmed that TCR stimulation with overnight monensin and brefeldin did not alter IR surface expression (Supplementary Fig. S2). We only analyzed freshly processed samples, as thawing of cryopreserved tumor digests resulted in increased TIL function (Supplementary Fig. S3A).

Using this TCR stimulation assay, we found an average of 33.4% of the CD8+ TFLLs produced IFNy (Fig. 1A) and a similar percentage (36%) by the CD8+ DLALs. However, the TILs were significantly less functional (P < 0.05), with an average of 25.3% of CD8+ T cells producing IFNy. The percentage of cells producing IFNy and TNFα (Fig. 1B) in the TILs was concordant (Spearman ρ = 0.69; P < 0.0001). IFNy+TNFα IL2+ frequencies were also similar among the tissue digests (Supplementary Fig. S3B and S3C). We therefore used IFNy as our primary cytokine readout.

For analytic purposes, we defined T cells as hypofunctional if the percentage of IFNy-secreting CD8+ T cells was less than one SD from the TFLL mean (19.3%) and confirmed that TFL function was not influenced by ischemia time (Supplementary Fig. S4A). Using this definition, hypofunctional CD8+ T cells constituted 19% (4/21) in TFLLs, 20% (9/44) in DLALs, and 36% (16/44) in TILs, with statistically significant differences between the DLALs and TILs (P = 0.03).

Because the hypofunctional TIL frequency was low in early-stage patients, we examined TILs obtained from 8 advanced lung cancer cases and from 5 malignant lung cancer effusions. The late-stage TIL and MPE samples (Fig. 1A) were more hypofunctional, making significantly (P = 0.01) less IFNy than the early-stage patients.

We also tested a subset of TIL digests (n = 14) for cytolytic activity using a bispecific antibody assay (see Methods; Fig. 1C). Under our experimental conditions, the average lytic activity by hypofunctional TILs was 13%, which was significantly lower (P = 0.02) than in functional TIL digests (23.8%).

TIL functional heterogeneity is not associated with clinical factors
Because only about one third of the patients with early-stage NSCLC had hypofunctional T-cell function, we could examine clinical factors that were potentially associated with functionality. Using our 19.3% cutoff to define hypofunction, we found that patient gender, smoking history, histology, tumor stage, age, and...
tumor size had little influence on CD8+ TIL function (Supplementary Fig. S4B–S4G). However, using correlation analyses, we observed trends toward hypofunction in patients who were older (P = 0.08) or with larger tumors (P = 0.14; Supplementary Fig. S4H and S4I).

We also studied the relationship of T-cell function with the tumor mutational status. In the 29 patients with available data, 9 (31%) had EGFR mutations, 8 (28%) had KRAS mutations, and 16 (55%) had p53 mutations. Seventy-five percent of patients with KRAS mutations also had p53 mutations, and none had concomitant STK11 mutations. We calculated the percentage of IFNγ-secreting cells for each mutation (Supplementary Table S1A) and found that patients with KRAS mutations had more functional T cells than those with wild-type KRAS (P < 0.05).

TIL functional heterogeneity is not associated with other cell types
TILs contained a significantly higher frequency of FoxP3+ regulatory T cells (Treg; 10.5%) in comparison with TFLls (2.2%), DLALs (3.4%), and PBMCs (3%; P < 0.0001; Fig. 2A, left plot). However, the CD4+ Treg percentages in the functional versus hypofunctional patients were not significantly different (Fig. 2A, right plot).

In a subset of patients, we characterized frequencies of CD14+ myeloid cells (tissue monocytes and macrophages) and CD15+ cells (neutrophils; Fig. 2B and C). We observed similar frequencies in all tissue types and no differences between functional and hypofunctional cases. We also performed correlation analyses for tumor microenvironment (TME)–associated cell types (Supplementary Fig. S5A–S5C) and saw no significant relationship with IFNγ production from tumor digests.

TIL functional heterogeneity correlates with CD8+ TIL proliferation
As shown in Fig. 2D (left plot), the CD8+ cell frequency was lowest in TFLls (mean 5.8%), intermediate in the tumors (mean 11.9%), and highest in distant lung tissue (15.2%). Each group was statistically different (P < 0.05) from the other. CD8+ TIL frequency was not different between functional and hypofunctional cases (Fig. 2D, right plot; Supplementary Fig. S5D).
Human TIL Function Is Influenced by CD103 and Eomes Activity

Figure 2.
TIL functional heterogeneity is not associated with TME cell types or T-cell differentiation state, but with proliferation. A, Left: Percentage of CD8+ PD-1+ cells in TFLLs (n = 19), DLALs (n = 32), TILs (n = 31), and PMBCs (n = 36). Right: Representative flow plots of controls, TFLLs, DLALs, TILs, and PMBCs for CD8+ and CD45RO on CD8+ T cells. B, Percentage of live CD3+ PD-L1+ from adjacent distant lung (n = 27) and tumor digest (n = 27). Right: Percentage of CD3+ PD-L1+ cells in functional vs. hypofunctional TILs (n = 27). C, Percentage of CD8+ TIGIT+ in TFLLs (n = 18), DLALs (n = 25), TILs (n = 27), and PMBCs (n = 21). D, Percentage of CD8+ TIM-3+ in TFLLs (n = 14), DLALs (n = 34), TILs (n = 34), and PMBCs (n = 30). E, Percentage of CD8+ CD39+ in TFLLs (n = 15), DLALs (n = 27), TILs (n = 27), and PMBCs (n = 24). F, Percentage of CD8+ cells expressing intracellular CTLA-4 in TFLLs (n = 11), DLALs (n = 13), TILs (n = 15), and PMBCs (n = 15). Parenthesis represents independent flow staining repeats. *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001.

Figure 3.
Tissue and PBMC lymphocyte inhibitory receptor expression is heterogeneous. A, Left: Percentage of CD8+ PD-1+ cells in TFLLs (n = 19), DLALs (n = 32), TILs (n = 31), and PMBCs (n = 36). Right: Representative flow plots of controls, TFLLs, DLALs, TILs, and PMBCs for PD-1+ and CD45RO on CD8+ T cells. B, Percentage of live CD3+ PD-L1+ from adjacent distant lung (n = 27) and tumor digest (n = 27). Right: Percentage of CD3+ PD-L1+ cells in functional vs. hypofunctional TILs (n = 27). C, Percentage of CD8+ TIGIT+ in TFLLs (n = 18), DLALs (n = 25), TILs (n = 27), and PMBCs (n = 21). D, Percentage of CD8+ TIM-3+ in TFLLs (n = 14), DLALs (n = 34), TILs (n = 34), and PMBCs (n = 30). E, Percentage of CD8+ CD39+ in TFLLs (n = 15), DLALs (n = 27), TILs (n = 27), and PMBCs (n = 24). F, Percentage of CD8+ cells expressing intracellular CTLA-4 in TFLLs (n = 11), DLALs (n = 13), TILs (n = 15), and PMBCs (n = 15). Parenthesis represents independent flow staining repeats. *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001.
Figure 4.

TIL function is not associated with inhibitory receptor expression. A, Heatmap associations of relative expression of IFN\(\gamma\) production and inhibitory receptor frequencies (rows) for individual patient CD8\(^+\) TILs (columns). Red, maximum; blue, minimum; and grey, no values. Data were not transformed. B, The percentage of CD8\(^+\) TILs expressing PD-1, TIGIT, CD39, TIM-3, and intracellular CTLA-4 was compared in functional (>19.3%) and hypofunctional (<19.3%) cases. C, The percentage of CD8\(^+\) TILs expressing PD-1\(^-\)TIGIT\(^+\), PD-1\(^-\)TIM-3\(^+\), and PD-1\(^-\)CTLA-4\(^+\) combinations was compared in functional and hypofunctional cases. D, Representative single-cell analyses showing IFN\(\gamma\) expression (x axis) versus PD-1 expression (y axis) for two cases. The boxes depict the percentage of PD-1\(^+\) IFN\(\gamma\)\(^+\) (top) or PD-1\(^-\) IFN\(\gamma\)\(^+\) (bottom). E, Representative single-cell analyses showing IFN\(\gamma\) expression (x axis) versus TIGIT, TIM-3, and CD39 expression (y axis) for four cases. The boxes depict the percentage of TIGIT\(^-\) IFN\(\gamma\)\(^+\), TIM-3\(^-\) IFN\(\gamma\)\(^+\), or CD39\(^-\) IFN\(\gamma\)\(^+\) cells. F, The percentage of CD8\(^+\) TILs cells producing IFN\(\gamma\) based on their IR expression is plotted. Flow staining was performed individually for the TFLLs, and concurrently for the PBMCs, DLALS, and TILs from each respective patient. * \(P < 0.05\) and ** \(P < 0.001\).
Figure 5. CD103+ TILs are the main source of IFNγ. A. Left top plot: Tm5 were identified (top right quadrants) as CD8+CD103+RO+ cells in TFLs, DLLs, TILs, and PBMCs. Right: The Tm5 frequency is plotted for TFLs (n = 8), DLLs (n = 25), TILs (n = 26), and PBMCs (n = 18). Left bottom plot: Heatmap associations of the relative expression of CD8+ TIL frequency, CD103, and IRs (rows) expression on individual CD8+ TILs (columns). Red, maximum; blue, minimum; and grey, no values. Data are not transformed. B. IR expression (CD39, IcTLA-4, TIM-3, PD-1, and TIGIT) on CD8+CD103+ versus CD8+CD103-/C0 TILs. C. Percentage of CD8+CD45RO+ cells expressing CD103 in functional vs. hypofunctional TILs. D. Restimulated CD103+CD8+ were compared with CD103-CD8+ TILs for their IFNγ production. E, Baseline Ki-67 expression on CD103+CD8+ was compared with CD103-/CD8+ TILs. F, By single-cell analysis, the percentage of CD8+ TILs cells producing IFNγ based on their IR expression and CD103 status (CD103+ (black) and CD103-/C0 (gray)) is plotted. One-way ANOVA test compared CD103+ IR+ vs. CD103+ IR- and were nonsignificant. *, P < 0.05; **, P < 0.01; ***, P < 0.001, and ****, P < 0.0001.
We characterized CD8⁺ T-cell proliferation (Ki-67%) and observed that CD8⁺ TILs had significantly more proliferation (26%) compared with PBMCs (3.4%), TFLLs (6.5%), and the DLALs (9.5%; Fig. 2E, left). The functional cases had significantly increased Ki-67 activity versus the hypofunctional cases (30% vs. 12%, P = 0.002; Fig. 2E, right plot; Supplementary Fig. S5E).

**TIL functional heterogeneity is not associated with differentiation status**

The differentiation status of CD8⁺ TFFs, DLALs, and TILs was similar, each consisting primarily of effector memory T cells (CD45RO⁺CD62L⁻; mean 58%–70.4%) and effector cells (CD45RO⁺CD62L⁺; mean 18.2%–30.3%; Fig. 2F and G). TIL effector and effector memory CD8⁺ T-cell frequencies had little influence on TIL functional status (Fig. 2H; Supplementary Fig. S5F and S5G). In comparison, PBMCs showed more naive cells and fewer memory T cells (all P < 0.0001; Supplementary Fig. S6A–S6H). We also determined that TIL CD8⁺RO⁻ cells (mostly effector cells) and CD8⁺RO⁺ cells (mostly effector memory T cells) had similar IFNγ production (Supplementary Fig. S6I).

**Heterogeneous expression of IRs on tissue lymphocytes and PBMC**

With regard to T-cell IR expression, we observed minimal Lag3 and BTLA. In contrast, PD-1 expression was high, but very heterogeneous, being expressed significantly more on CD8⁺ TFFs (57.9%), TFLLs (66.6%), and DLALs (42%) compared with PBMCs (21.3%; all P < 0.001; Fig. 3A, left and middle plots).

Tumor PD-L1 assessment in a subset of patients (Fig. 3B, left plot) revealed that PD-L1 expression on CD3⁺ cells (tumor cells and macrophages) was relatively low in the DLALs (11%) and TILs (15.8%). PD-L1 expression did not differ between functional and hypofunctional patient cohorts (Fig. 3B, right plot; Supplementary Fig. S7A).

The IR TIGIT was highly expressed on DLALs (55.4%), TILs (61%), and PBMCs (54%), but significantly less expressed on the TFFs (33.4%; P < 0.001; Fig. 3C). TIGIT expression was highest on TILs (16.4%) in comparison with DLALs (8.8%) and PBMC (7.9%), with the TILs showing significantly less expression (3.7%) than TILs (P < 0.01; Fig. 3D). The TILs expressed significantly more TIM-3 than the DLALs (P < 0.001).

CD39 expression was significantly (P < 0.0001) higher on TILs than the other groups (Fig. 3E) similar to other reports (6, 8, 19). Intracellular CTLA-4 expression was low on TFFs (13.6%), DLALs (10.5%), and PBMCs (1.9%), with significantly higher expression on the TILs (37.4%; P = 0.001 compared with DLALs and PBMC and P < 0.01 compared with TFFs; Fig. 3F).

**TIL hypofunction is not correlated with expression of IRs on CD8⁺ TILs**

To assess the association between IR expression and overall T-cell function, we plotted from each individual patient their respective percentage of CD8⁺ T cells expressing IFNγ against their specific IR⁺ CD8⁺ frequency in heatmap format (Fig. 4A). The percentage of IRs on CD8⁺ TILs from functional versus hypofunctional cases was compared (Fig. 4B). We observed no associations in the heatmap and no statistically significant differences in PD-1, TIGIT, CD39, TIM-3, and iCTLA-4 expression among the functional versus hypofunctional patients. Combined PD-1 expression with other IRs (TIGIT, TIM-3, or CTLA-4) also had no influence on TIL function (Fig. 4C).

Correlation analyses detailed no significant associations with IR frequency and percentage of patient’s TILs making IFNγ (Supplementary Fig. S7B–S7F).

Utilizing intracellular cytokine-based flow cytometry, we measured IFNγ production by specific CD8⁺ IR⁺ TIL population for each patient. As example, in Fig. 4D, the expression of IFNγ (x axis) is plotted versus the expression of PD-1 (y axis) from two patients’ CD8⁺ TILs. The boxes represent the frequency of PD-1⁺ or PD-1⁻ cells that produced IFNγ. In Patient LC393, the PD-1⁺ cells (top quadrants) made more IFNγ in comparison with the PD-1⁻ cells (bottom quadrants), whereas in Patient 354, PD-1⁻ and PD-1⁺ cells made similar amounts of IFNγ. Examples of TIGIT, TIM-3, and CD39 expression versus IFNγ production are shown in Fig. 4E.

The cumulative results of this single-cell analysis (Fig. 4F) show considerable heterogeneity, but no significant association of individual or combination of IR expression with IFNγ production. That is, on average, TILs lacking IR expression (IRs = “zero”) made similar amounts of IFNγ as those expressing any single or multiple IR combination.

**CD103⁺ CD45RO⁺ CD8⁺ T cells are the main source of IFNγ in TILs**

Multiple reports have suggested that patients with NSCLC with higher CD8⁺ TDM (CD103⁺CD69⁺) had a better prognosis (7, 9, 20). We observed high concordance for CD103 and CD69 expression (21) in our patient TILs (Supplementary Fig. S7G) and thus used CD103 as our TDM marker. Our data showed a significantly higher frequency of CD103⁺CD45RO⁺CD8⁺ TILs (60.2%; Fig. 5A, top and right plots) in comparison with TFLLs (36.8%, P = 0.006) and DLALs (48.7%, P = 0.0015) and negligible expression in PBMCs. When arranged by increasing CD8⁺ TIL frequency in a heatmap format (Fig. 5A, bottom left plot), the tumor CD103⁺ TDM cells were enriched with high CD8⁺ TIL density, consistent with another report (7). TDM cells have been reported to have high IR expression (7–9, 12), and we also noted

**Figure 6.**

Eomes expression in TIL. CD103⁺ TDM is associated with hypofunction. A, The percentage of CD8⁺ TILs producing IFNγ versus their EOMES expression was plotted, and a negative correlation was found. B, The CD8⁺ “Eomes⁺” percentage was compared between functional and hypofunctional cases. C, Representative flow plots of Eomes and CD69 expression on CD103⁺ CD45RO⁺ CD8⁺ TDM for a functional and hypofunctional case illustrating higher Eomes expression with hypofunction. D, For each patient, the percentage of CD8⁺ TILs producing IFNγ versus the percentage of CD103⁺ CD8⁺ TILs expressing EOMES was plotted. A negative correlation was found. E, The Eomes percentage in CD103⁺ CD8⁺ TILs was compared between functional and hypofunctional cases. F, Left: Representative tracing of CD103 versus EOMES expression. Right: The graph represents the Eomes percentage by CD103 Eomes⁺, CD103 Eomes⁻, and CD103 Eomes⁺ CD8⁺ TILs from left plot (n = 12). One-way ANOVA test was applied. G, CD8⁺ T-cell ViSNE analysis of a functional TIL case. H, IFNγ (left plot) and TNFα (right plot) expressions by each cell (red, high; blue, low expression) were superimposed on CD8 map revealing “cytokine producing” (red circle) and “cytokine non-producing” (blue circle) regions. I, CD103 expression (red, high; blue, low) is overlaid. J, Eomes expression (red, high; blue, low) is overlaid. *, P < 0.05 and **, P < 0.01.
Eomes expression in CD103+ TRM cells is associated with loss of TIL function

Despite the CD103+ TILs being a primary IFNγ source in the tumor, this IFNγ production varied (Fig. 5D and F), suggesting additional factors might influence this heterogeneity. Expression of the transcription factor Eomes is associated with exhausted T cells (23-28), and our intracellular flow cytometric assessment of Eomes revealed high expression in the CD8+ TILs, with a significant negative correlation (r = -0.67, P < 0.0001) to IFNγ production (Fig. 6A). Functional TILs had a significantly lower Eomes expression (43.4%) than hypofunctional TILs (66.7%, P < 0.0001; Fig. 6B).

TILs from normal tissues have low Eomes expression (12, 13). We therefore focused on Eomes in the TIL TRM cells. Increased Eomes expression was observed in the CD103+ TRM cells of hypofunctional cases (right plot, Fig. 6C) compared with functional TILs (Fig. 6C, left plot). The percentage of patients whose CD103+ TILs expressed Eomes was significantly negatively correlated (r = -0.52, P < 0.0001) to overall IFNγ production (Fig. 6D). Patients with functional TILs had a significantly lower percentage of Eomes expression in the CD103+ cells (31.7%) than in hypofunctional TILs (59%, P < 0.001; Fig. 6E). Using single-cell analysis (Fig. 6F), we observed that CD103+Eomes+ CD8+ TILs produced significantly (P < 0.05) more cytokine than CD103+Eomes-, CD103+Eomes+, and CD103+Eomes-CD8+ T cells. Thus, CD103+ TRM cell hypofunction in early NSCLC tumors was associated with Eomes expression.

To further explore the associations of CD103, Eomes, and IRs with CD8+ TIL function, we applied viSNE analysis (29) to a functional TIL case (Fig. 6C-K). After generating a 2D map of CD8+ T-cell regions (Fig. 6G), we then applied a heatmap to determine IFNγ- and TNFε-enriched regions. We identified the regions that did (red circle) or did not (blue circle) produce cytokines (Fig. 6H). CD103+ cells almost exclusively colocalized with cytokine-producing cells (Fig. 6I). The cytokine-enriched region (red circle) expressed little Eomes in comparison with the cytokine-negative area (blue circle; Fig. 6J). One Eomes+ node in the CD103+ region overlapped with the non-cytokine-producing region, demonstrating how Eomes abrogates TRM cytokine production. PD-1 analysis (Fig. 6K) showed diffuse expression, consistent with its poor association with cytokine expression and TIL hypofunction.

Discussion

The goal of this study was to evaluate the functional status of early-stage lung cancer TILs primarily by measuring intracellular cytokine staining by flow cytometry. Because we detected minimal intracellular cytokine production in unstimulated T cells from fresh tumor samples, we submaximally stimulated T cells in fresh tumor digests overnight with a plate-bound anti-CD3 that cross-links the TCR. Using our assay, we observed that advanced lung cancer TILs were consistently hypofunctional, as might be expected (30-33). In contrast, we found that early-stage lung cancer TIL function was quite heterogeneous and that only about one third of the patients had TILs with hypofunction. Lung TIL function has not been studied extensively; however, functional heterogeneity in early-stage lung cancer TILs after TCR stimulation has been observed by other investigators who measured: (i) cytokine secretion measured by ELISA (3, 4), (ii) intracellular calcium concentrations (32), and (iii) a bispecific antibody (anti-CD3-anti-Folate receptor alpha) killing assay (4). Our data were consistent with most previous studies with human and mouse TILs in that the defects we observed were in the initial part of the TCR signaling pathway and could be overcome by direct stimulation of the distal part of the signaling pathway by PMA/ ionomycin (31, 34, 35).

The observed TIL functional heterogeneity provided an opportunity to determine what factors were associated with T-cell hypofunction. Function did not correlate with clinical features or with the presence of Tregs, neutrophils, macrophages, or PD-L1 expression. We also saw no correlations of function with CD8+ T-cell numbers or differentiation status. We did note that TILs were more proliferative (as measured by percent Ki-67+ T cells) than other types of lymphocytes and that functionality was associated with increased proliferation.

Given previous studies, we explored the link between hypofunction and expression of IRs that are presumed to define “exhausted” T cells. Our IR expression data were similar to previous reports (2, 3, 5, 7), however, the expression of most IRs was not specific to TILs, as we saw a similar phenotype in the lymphocytes from lung tissue adjacent to the tumor and from tumor-free lungs harvested from transplant donors. However, compared with the other lung tissue-resident lymphocytes, TILs expressed more CD39, as observed by others (6, 8, 19).

Although a hallmark of dysfunctional T cells in tumors is the overexpression of IRs, the actual contribution of these IRs to the dysfunctional state is controversial (33, 36, 37). Work from some groups (3, 4) suggests that the activation and effector function of lung cancer CD8+ TILs correlates with coexpression of multiple immune checkpoints, leading to the suggestion that PD-1hi T-cell frequency may be useful as a surrogate marker for TIL functionality upon TCR activation. A report on PD-1hi TILs in NSCLC describes this phenotype (38). In contrast, increasing evidence suggests that IR expression is contextual and may depend just as much on differentiation status as exhaustion in human CD8+ T cells (10, 39, 40). For example, TILs, despite being fully functional, express high amounts of PD-1 and other IRs (7, 8, 19). In our study, IR expression on TILs did not seem to predict their functionality. This was true when we compared IR expression on patients with functional versus hypofunctional TILs, as well as when we examined the cytokines secreted by individual CD8+ T cells with or without single or multiple IRs. Our data thus support

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the idea that using PD-1 as a marker of TIL dysfunction may be misleading; one reason may be because the functional T_{RM} in the tumors expresses high levels of PD-1. T_{RM} cells are a subset of effector CD8\(^+\) T cells marked by expression of the integrin \(\alpha_\beta_3\) (CD103) and, in lung, the surface marker CD69 (21, 41, 42). These T_{RM} cells have a genetic program that enables them to react rapidly to potential infectious agents (12, 13) despite expression of IRs (7, 8, 19). They also express a set of transcription factors that includes high expression of Notch and low expression of Eomes (12). Their presence has been reported as a positive prognostic factor for survival in lung cancer (7, 20) and other tumors (8, 9). Our data are consistent with these findings. We observed that the CD103\(^+\) cells marked more functional TILs that produced more cytokines and proliferated better compared with CD103\(^-\) cells, regardless of which IRs were expressed.

In contrast, we observed that expression of the transcription factor Eomes was associated with T-cell hypofunction. In murine chronic viral infection models, T-cell exhaustion is characterized by T cells that are Eomes\(^{hi}\) and T-Bet\(^{lo}\) (23). However, the functions of Eomes and T-bet are complex, interdependent, and likely context dependent (24, 43, 44). The actual role of Eomes in driving the exhaustion program thus remains uncertain.

There is relatively little data exploring Eomes function in human T-cell exhaustion, with most of that data obtained from human blood in patients with HIV (24, 25). A report in NSCLC TILs (28) detailed increased numbers of Eomes\(^{hi}\)T-Bet\(^{lo}\) cells, although there were no functional assessments. In our patients with early-stage lung cancer, there was a negative correlation (\(r^2 = 0.67\)) between Eomes expression and IFN\(\gamma\) production. Whether Eomes is driving TIL hypofunction or denotes a larger exhaustion program remains uncertain.

Although this study identified TIL functionality within early-stage tumors, it did not address three questions that deserve future study. First, we did not establish the clinical relevance of T-cell function by showing that T-cell hypofunction predicted recurrence rate in these patients. Because of the long observation periods needed to see recurrence, it will take a number of additional years to answer this question. However, we were able to identify 28 patients with at least 2 years of follow-up (Supplementary Table S1B). Of these patients, disease has recurred in 9 (32%). The 11 patients with hypofunctional TILs had a recurrence rate of 46%, whereas those 17 patients with functional TILs had a recurrence rate of only 24%, despite the fact that the hypofunctional patients included a higher percentage of stage I patients. The average percentage of TILs making IFN\(\gamma\) was 29.3% in patients without recurrence versus 21% in patients with recurrence. None of these differences are yet statistically significant, but as we have longer follow-up times, a more definitive conclusion can be reached.

Second, we did not attempt to determine which of the TILs were tumor-reactive. Most studies suggest that only a small percentage of TILs recognize tumors (19, 45). Reports suggest that these tumor-reactive TILs may be enriched in CD103\(^+\) and CD39\(^+\) T-cell populations (8, 19). We did not study the function of CD103\(^+\)CD39\(^+\) cells, but we saw no increased function in the CD39\(^+\) cells versus CD39\(^-\) cells, in contrast to the increased functionality of CD103\(^+\) cells.

Third, we did not determine how checkpoint blockade (with anti–PD-1 or anti–PD-L1) related to our findings. We observed that IR expression did not predict T-cell function (exhaustion) after our TCR stimulation assay; however, this assay was performed without testing IR interactions with tumor ligands. In our stimulation assay, we had low endogenous PD-L1 expression on tumors and leukocytes. It is thus possible that we would have seen some additional hypofunction on IR-expressing T cells if these ligands were engaged.

Checkpoint blockade may have its best effects in patients who retain tumor-reactive TILs without severe T-cell hypofunction. In mice, these severely hypofunctional cells are proposed to express both high PD-1 (33) and Eomes (23). Our data also suggest that Eomes expression marks a hypofunctional group of human TILs. On the other hand, one study suggests that PD-1\(^{hi}\) cells might be the most tumor reactive and have the potential for reinvigoration with anti–PD-1 therapy (38). To determine which T cells would respond to checkpoint blockade, prospective studies on TIL training markers on TILs (such as CD103, CD39, and Eomes), as well as TIL functional assays with PD-L1 signals provided in the presence and absence of anti–PD-1, would be needed.

In summary, our findings suggest a model in which early-stage lung cancer TILs are regulated by an antitumoral T_{RM} program competing with a protumoral exhaustion program. Expression of IR ligands could also play a role when the appropriate ligands are present. We speculate that early in tumor formation, circulating effector or effector memory cells encounter antigens within the tumor (perhaps neoantigens being the most reactive) and are converted to CD103\(^+\) T_{RM} cells which then exert some antitumor activity. However, due to a variety of factors associated with tumor growth and the TME, the tumors are not eliminated. These factors, likely in addition to resulting chronic antigen stimulation, then trigger initiation of an exhauston program characterized by (and possibly driven by) increased Eomes (23) and CD39 expression (8, 19). Presence of B7H4 on tumors or other TME cells might function to upregulate Eomes in T cells (46). As the tumors grow, the exhaustion program wins out in the T_{RM} cells, resulting in more and more TILs becoming hypofunctional.

Disclosure of Potential Conflicts of Interest
E. Cantu is a consultant/advisory board member for LignaMed, Inc. S.M. Albelda reports receiving commercial research grant from Janssen Pharmaceutical. No potential conflicts of interest were disclosed by the other authors.

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Function of Human Tumor-Infiltrating Lymphocytes in Early-Stage Non–Small Cell Lung Cancer

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