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

Shaun M. O’Brien1, Astero Klampatsa1, Jeffrey C. Thompson1, Marina C. Martinez1, Wei-Ting Hwang2, Abhishek S. Rao3, Jason E. Standalick3, Soyeon Kim1, Edward Cantu4, Leslie A. Litzky5, Sunil Singhal3, Evgeniy B. Eruslanov5, Edmund K. Moon1, and Steven M. Albelda1

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 α5β1 (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 subpopulation 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 Immunoglobulin, Mucin-domain containing-3 (TIM-3), CD39, and T-cell immunoreceptor with Ig and ITIM domains (TIGIT; refs. 2–6).

The integrin α5β1 (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 RM, 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–III) 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 digests 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 (FN50), CD103 (Ber-Act8), PD-1 (EH12-H7), TIM-3 (F38-2E2), CD62L (DREG-56), CD45RO (UCHL1), CD39 (A1), Foxp3 (206D), CD45RA (H100), PD-1 (M1H1), CD14 (M5E2), and CD15 (H9P8) from Biolegend; Anti-human CD8 (RPA-T8) and CTLA-4 (BN13) from BD Bioscience; and anti-human TIGIT (MBAS43), Eomes (Wd1928), and Ki-67 (20Raj1) from eBioscience.

Intracellular cytokine staining was performed as detailed previously (16). Cytokine antibodies used were directly conjugated based on two-sample t test or paired t test (using Shapiro–Wilk test and histograms), parametric tests were applied. Comparison between two independent samples was based on nonparametric tests. 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

Human TIL Function Is Influenced by CD103 and Eomes Activity

To measure cytotoxicity, we employed a bispecific T-cell engager (BiTE) killing assay similar to that recently reported (4). A lentiviral expression vector (ref. 17; graciously provided by Dr. Yang Bing Zhao, UPenn) was constructed to produce a BiTE by fusing the scFV from blinatumomab (that recognizes CD3) with the scFV from SS1 that recognizes mesothelin. 293T cells were transduced by the vector, and supernatant was collected. We also generated a human mesothelioma cell line that expressed high levels of mesothelin and luciferase (EMMESO, ref. 18). The killing assay was performed by plating EMMESO target tumor cells at 5,000/well in a 96-well plate and allowing them to adhere over a 4- to 6-hour period. A volume of digested patient tumor cell suspension was then added to the EMMESO cells based on live CD8+ T-cell frequency (as measured by flow cytometry) to achieve a ratio of approximately 10 CD8+ T cells to 1 EMMESO tumor cell. 30 µL of BiTE supernatant or control media were added to some wells of those cocultures. After 18 hours of coculture, the supernatant from the wells was aspirated, and the remaining tumor cells were washed and then lysed. Luminescence from the remaining tumor cells was measured using a Glomax Luminometer (Promega, Inc.). Percent lysis was measured using the formula % killing = 100 – [(luminescence of tumor alone well – luminescence of control wells)/luminescence of tumor alone well] × 100. BiTE-induced killing was the difference in percent lysis measured in BiTE + digest + tumor wells and digest + tumor wells. As a control, we used the EMParental cell line (a mesothelin-negative mesothelioma cell line).

Statistical analysis
Descriptive statistics were computed for all variables. Because most of our data did not deviate from a normal distribution (using Shapiro–Wilk test and histograms), parametric tests were applied. Comparison between two independent samples was based on nonparametric tests. 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

Human TIL Function Is Influenced by CD103 and Eomes Activity

To measure cytotoxicity, we employed a bispecific T-cell engager (BiTE) killing assay similar to that recently reported (4). A lentiviral expression vector (ref. 17; graciously provided by Dr. Yang Bing Zhao, UPenn) was constructed to produce a BiTE by fusing the scFV from blinatumomab (that recognizes CD3) with the scFV from SS1 that recognizes mesothelin. 293T cells were transduced by the vector, and supernatant was collected. We also generated a human mesothelioma cell line that expressed high levels of mesothelin and luciferase (EMMESO, ref. 18). The killing assay was performed by plating EMMESO target tumor cells at 5,000/well in a 96-well plate and allowing them to adhere over a 4- to 6-hour period. A volume of digested patient tumor cell suspension was then added to the EMMESO cells based on live CD8+ T-cell frequency (as measured by flow cytometry) to achieve a ratio of approximately 10 CD8+ T cells to 1 EMMESO tumor cell. 30 µL of BiTE supernatant or control media were added to some wells of those cocultures. After 18 hours of coculture, the supernatant from the wells was aspirated, and the remaining tumor cells were washed and then lysed. Luminescence from the remaining tumor cells was measured using a Glomax Luminometer (Promega, Inc.). Percent lysis was measured using the formula % killing = 100 – [(luminescence of tumor alone well – luminescence of control wells)/luminescence of tumor alone well] × 100. BiTE-induced killing was the difference in percent lysis measured in BiTE + digest + tumor wells and digest + tumor wells. As a control, we used the EMParental cell line (a mesothelin-negative mesothelioma cell line).

Statistical analysis
Descriptive statistics were computed for all variables. Because most of our data did not deviate from a normal distribution (using Shapiro–Wilk test and histograms), parametric tests were applied. Comparison between two independent samples was based on nonparametric tests. 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
O’Brien et al.

Table 1. Patient clinical and demographic data

<table>
<thead>
<tr>
<th>Table 1. Patient clinical and demographic data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with NSCLC (n = 44)</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>Median age (range)</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Race</td>
</tr>
<tr>
<td>White or other</td>
</tr>
<tr>
<td>Black</td>
</tr>
<tr>
<td>Smoking Current</td>
</tr>
<tr>
<td>Former</td>
</tr>
<tr>
<td>Never</td>
</tr>
<tr>
<td>Histology</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Squamous</td>
</tr>
<tr>
<td>Large cell neuroendocrine</td>
</tr>
<tr>
<td>Epithelioid</td>
</tr>
<tr>
<td>Pleomorphic</td>
</tr>
<tr>
<td>Disease stage</td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>IV</td>
</tr>
<tr>
<td>Tumor size</td>
</tr>
<tr>
<td>&lt;3 cm</td>
</tr>
<tr>
<td>≥3 cm</td>
</tr>
<tr>
<td>Cause of death</td>
</tr>
<tr>
<td>CVA (incl. stroke)</td>
</tr>
<tr>
<td>Anoxia</td>
</tr>
<tr>
<td>Head trauma</td>
</tr>
<tr>
<td>Ischemia time</td>
</tr>
</tbody>
</table>

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+ TFFLs, DLALs, and TILs showed little spontaneous intracellular IFNγ production (Supplementary Fig. S1A) consistent with previous reports (5).

We thus designed an assay to stimulate cytokine secretion by suboptimal TCR stimulation 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, to prove the cells were capable of 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 digest resulted in increased TIL function (Supplementary Fig. S3A).

Using this TCR stimulation assay, we found an average of 33.4% of the CD8+ TFFLs produced IFNγ (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 IFNγ. The percentage of cells producing IFNγ and TNFα (Fig. 1B) in the TILs was concordant (Spearman ρ = 0.69; P < 0.0001). IFNγ+TNFα+IL2+ frequencies were also similar among the tissue digests (Supplementary Fig. S3B and S3C). We therefore used IFNγ as our primary cytokine readout.

For analytic purposes, we defined T cells as hypofunctional if the percentage of IFNγ-secreting CD8+ T cells was less than one SD from the TFFL mean (19.3%) and confirmed that TFFL function was not influenced by ischemia time (Supplementary Fig. S4A). Using this definition, hypofunctional CD8+ T cells constituted 19% (4/21) in TFFLs, 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 IFNγ 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 IFN-γ-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⁺ CD4⁺ regulatory T cells (Treg; 10.5%) in comparison with TFLs (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 IFN-γ 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 TFLs (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).
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 PBMCs (n = 27). Right: Representative flow plots of controls, TFLLs, DLALs, TILs, and PBMCs for PD-1 and CD45RO on CD8\(^{+}\) T cells. B, Percentage of live CD3\(^{-}\)PD-L1\(^{+}\) from adjacent distant lung (n = 27) and tumor digests (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 PBMCs (n = 21). D, Percentage of CD8\(^{+}\)TIM-3\(^{-}\) in TFLLs (n = 14), DLALs (n = 34), TILs (n = 34), and PBMCs (n = 30). E, Percentage of CD8\(^{+}\)CD39\(^{-}\) in TFLLs (n = 13), DLALs (n = 27), TILs (n = 27), and PBMCs (n = 24). F, Percentage of CD8\(^{+}\) T cells expressing intracellular CTLA-4 in TFLLs (n = 11), DLALs (n = 15), TILs (n = 15), and PBMCs (n = 13). Parenthesis represents independent flow staining repeats. ***, P < 0.001; ****, 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 = 32), and PBMCs (n = 27). Right: Representative flow plots of controls, TFLLs, DLALs, TILs, and PBMCs for PD-1 and CD45RO on CD8\(^{+}\) T cells. B, Percentage of live CD3\(^{-}\)PD-L1\(^{+}\) from adjacent distant lung (n = 27) and tumor digests (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 PBMCs (n = 21). D, Percentage of CD8\(^{+}\)TIM-3\(^{-}\) in TFLLs (n = 14), DLALs (n = 34), TILs (n = 34), and PBMCs (n = 30). E, Percentage of CD8\(^{+}\)CD39\(^{-}\) in TFLLs (n = 13), DLALs (n = 27), TILs (n = 27), and PBMCs (n = 24). F, Percentage of CD8\(^{+}\) T cells expressing intracellular CTLA-4 in TFLLs (n = 11), DLALs (n = 15), TILs (n = 15), and PBMCs (n = 13). Parenthesis represents independent flow staining repeats. ***, P < 0.001; ****, P < 0.0001; and n.s., not significant.
Figure 4.
TIL function is not associated with inhibitory receptor expression. A, Heatmap associations of relative expression of IFNγ 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⁺ ICTLA-4⁺ combinations was compared in functional and hypofunctional cases. D, Representative single-cell analyses showing IFNγ expression (x axis) versus PD-1 expression (y axis) for two cases. The boxes depict the percentage of PD-1⁺ IFNγ⁺ (top) or PD-1⁻ IFNγ⁺ (bottom). E, Representative single-cell analyses showing IFNγ expression (x axis) versus TIGIT, TIM-3, and CD39 expression (y axis) for four cases. The boxes depict the percentage of TIGIT⁺ IFNγ⁺, TIM-3⁺ IFNγ⁺, or CD39⁺ IFNγ⁺ cells. F, The percentage of CD8⁺ TILs cells producing IFNγ 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⁺ CD45RO⁺ CD8⁺ TILs are the main source of IFNγ. A, Left top plot: TRMs were identified (top right quadrants) as CD8⁺ CD103⁺ CD45RO⁺ cells in TFLLs, DLALs, TILs, and PBMCs. Right: The TRM frequency is plotted for TFLLs (n = 8), DLALs (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, IC39, PD-1, and TIM-3) on CD8⁺ CD103⁺ versus CD8⁺ CD103⁻ TILs. C, Percentage of CD8⁺ CD45RO⁺ TILs expressing CD103 in functional vs. hypofunctional TILs. D, Restimulated CD103⁺ CD8⁺ were compared with CD103⁻ CD8⁺ TILs for their IFNγ production. E, Baseline K67 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⁻ (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 (5.4%), 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⁺ TILs, TFLLs, 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 values < 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⁺ TILs (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 TFLLs (33.4%; P < 0.001; Fig. 3C). TIM-3 expression was highest on TILs (16.4%) in comparison with DLALs (8.8%) and PBMC (7.9%), with the TFLLs showing significantly less expression (5.7%) 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, 18, 19). Intracellular CTLA-4 expression was lower on TFLLs (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 TFLLs; 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⁺ TDLRm (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 TDLRm 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⁺ TDLRm cells were enriched with high CD8⁺ TIL density, consistent with another report (7). TDLRm cells have been reported to have high IR expression (7–9, 12), and we also noted...
Eomes expression in CD103\(^+\)T\(_{\text{RM}}\) cells is associated with loss of TIL function

Despite the CD103\(^+\) TILs being a primary IFN\(_\gamma\) source in the tumor, this IFN\(_\gamma\) 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 IFN\(_\gamma\) production (Fig. 6A). Functional TILs had a significantly lower Eomes expression (43.4\%) than hypofunctional TILs (66.7\%, \(P < 0.001;\) Fig. 6B).

T\(_{\text{RM}}\) cells from normal tissues have low Eomes expression (12, 13). We therefore focused on Eomes on the TIL T\(_{\text{RM}}\) cells. Increased Eomes expression was observed in the CD103\(^+\) T\(_{\text{RM}}\) 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 IFN\(_\gamma\) 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\(^-\)CD8\(^+\) TILs. Thus, CD103\(^+\)T\(_{\text{RM}}\) cell dysfunction 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. 6G–K). After generating a 2D map of CD8\(^+\) T-cell regions (Fig. 6G), we then applied a heatmap to determine IFN\(_\gamma\)- and TNF\(_\alpha\)-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. 6I). One Eomes\(^+\) node in the CD103\(^+\) region overlapped with the non–cytokine-producing region, demonstrating how Eomes abrogates T\(_{\text{RM}}\) 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 by 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\(^+\)) 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-1\(^+\) 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, T\(_{\text{RM}}\), 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...
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 $\alpha_4 \beta_7$ (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 is a question for future research.

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 increase in 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 utilizing 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 antitumor TRM 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$^+$ TRM cells which then elicit 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 exhaustion 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 TRM 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.

Authors’ Contributions

Conception and design: S.M. O’Brien, E.K. Moon, S.M. Albelda


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. O’Brien, J.C. Thompson, M.C. Martinez, A.S. Rao, J.E. Standallick, S. Kim, E. Cantu, L.A. Lidley, S. Singhal, E.B. Eruslanov, E.K. Moon


Writing, review, and/or revision of the manuscript: S.M. O’Brien, A. Klampatsa, J.C. Thompson, W.-T. Hwang, S. Kim, S. Singhal, E.K. Moon, S.M. Albelda

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.M. O’Brien, A. Klampatsa, J.C. Thompson, E. Cantu, S. Singhal, E.K. Moon, S.M. Albelda

Study supervision: S.M. O’Brien, E.K. Moon, S.M. Albelda
Acknowledgments

We acknowledge the Donor Lung Acquisition team, Naomi St. Jean, and Mike Annunziata for experimental assistance.

S.M. O’Brien was funded by T32-CA009140. A.S. Rao, M. Annunziata, J. Standalick, and E. Eruslanov were funded by DOD-ICU140199, W81XWH-15-1-0717, and NIH/NCI CA187392. E. Cantu was funded by HL116636 and HL1135227. Support was also provided by the Translational Center of Excellence in Lung Cancer Immunology (Abramson Cancer Center) and a grant from Janssen Pharmaceuticals.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

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

Shaun M. O'Brien, Astero Klampatsa, Jeffrey C. Thompson, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-18-0713

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2019/04/24/2326-6066.CIR-18-0713.DC1

Cited articles
This article cites 46 articles, 11 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/7/6/896.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerimmunolres.aacrjournals.org/content/7/6/896.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerimmunolres.aacrjournals.org/content/7/6/896.
Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.