TITLE: Cytotoxic T cells in PD-L1–positive malignant pleural mesotheliomas are counterbalanced by distinct immunosuppressive factors

RUNNING TITLE: Immunoprofiling of mesothelioma by flow cytometry

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D.A.B. has a consulting role, employment, and stock ownership in NofOne.

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S.J.R. receives honoraria from Perkin Elmer and Bristol-Myers Squibb; has a consulting role with AstraZeneca and Perkin Elmer; receives research funding from Bristol-Myers Squibb; receives travel expenses from Roche; has a patent pending for use of Anti-galectin1 antibodies for diagnostic use.

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ABSTRACT

PD-L1 immunohistochemical staining does not always predict whether a cancer will respond to treatment with PD-1 inhibitors. We sought to characterize immune cell infiltrates and expression of T-cell inhibitor markers in PD-L1–positive and PD-L1–negative malignant pleural mesothelioma samples. We developed a method for immune cell phenotyping using flow cytometry on solid tumors that have been dissociated into single cell suspensions and applied this technique to analyze 43 resected malignant pleural mesothelioma specimens. Compared to PD-L1–negative tumors, PD-L1–positive tumors had significantly more infiltrating CD45+ immune cells, a significantly higher proportion of infiltrating CD3+ T cells, and a significantly higher percentage of CD3+ cells displaying the activated HLA-DR+/CD38+ phenotype. PD-L1–positive tumors also had a significantly higher proportion of proliferating CD8+ T cells, a higher fraction of FOXP3+/CD4+ Tregs, and increased expression of PD-1 and TIM-3 on CD4+ and CD8+ T cells. Double positive PD-1+/TIM-3+ CD8+ T cells were more commonly found on PD-L1–positive tumors. Compared to epithelioid tumors, sarcomatoid and biphasic mesothelioma samples were significantly more likely to be PD-L1 positive, and showed more infiltration with CD3+ T cells and PD-1+/TIM-3+ CD8+ T cells. Immunologic phenotypes in mesothelioma differ based on PD-L1 status and histologic subtype. Successful incorporation of comprehensive immune profiling by flow cytometry into prospective clinical trials could refine our ability to predict which patients will respond to specific immune checkpoint blockade strategies.
INTRODUCTION

The use of immune checkpoint inhibitors to engage the immune system in the fight against cancer has revolutionized the management of an increasing number of tumor types in recent years and offers the opportunity of achieving durable responses for patients.\(^{(1-5)}\) In certain cancer types, such as non-small cell lung cancer, renal cell carcinoma, and bladder cancer, only a minority of patients respond to immune checkpoint inhibitors, and because these drugs can be associated with significant immune-related toxicities, identification of reliable predictive biomarkers for these treatments is imperative. In some cancers, expression of PD-L1 appears to correlate, in part, with response to PD-1 or PD-L1 inhibitors; however, some PD-L1–positive tumors do not respond to these agents, and, in contrast, some PD-L1–negative tumors do respond to these drugs \(^{(6-8)}\).

PD-L1 expression is not likely the sole determinant of tumor immune evasion, and the efficacy of PD-1 pathway blockade may be impacted by expression of other inhibitory regulators of the immune response, such as cytotoxic T lymphocyte–associated antigen 4 (CTLA-4), T-cell immunoglobulin and mucin domain 3 (TIM-3), and lymphocyte-activation gene 3 (LAG-3), among others \(^{(9-13)}\). In order to identify additional biomarkers of immunotherapy response besides PD-L1 immunohistochemical (IHC) expression, several techniques are currently under development to more comprehensively and quantitatively characterize the tumor immune microenvironment, at the RNA, protein, and cellular level \(^{(14-18)}\).

Herein, we present a method for comprehensive immune profiling of solid tumors using flow cytometry, and we describe our initial experience in a cohort of 43 patients
with malignant pleural mesothelioma, a cancer arising from the mesothelial surfaces of the pleural cavity. The majority of mesothelioma patients are not candidates for surgical resection, and first-line chemotherapy with platinum and pemetrexed has remained the standard of care for more than a decade (19). Beyond the first-line setting, no treatments have been associated with a survival benefit, and response rates to single-agent drugs like gemcitabine or vinorelbine are low (20, 21).

Recent data have emerged to suggest that immune checkpoint inhibition may be a promising strategy for treating mesothelioma. PD-L1 expression occurs in 20-40% of mesotheliomas, particularly in the sarcomatoid subtype, and may be an adverse prognostic marker for overall survival (22, 23). In a study of 29 patients with mesothelioma, treatment with the CTLA-4 inhibitor tremelimumab resulted in durable partial responses in two patients (7%) (24). In the KEYNOTE-028 phase 1b trial, 6 of 25 (24%) patients with PD-L1–positive mesothelioma achieved an objective response (25), consistent with response rates in other solid tumors in which anti-PD-1 therapies have been approved by the FDA (6-8, 26-28).

We hypothesized that flow cytometric analysis of tumor immune infiltrates, when coupled with PD-L1 immunohistochemistry, would more accurately reveal distinct and heterogenous immunologic phenotypes across mesothelioma tumor samples. A more detailed understanding of the immunosuppressive factors within mesothelioma tumors may uncover alternative or combination immunotherapeutic strategies for the treatment of this disease.

**MATERIALS AND METHODS**
All patients in this study were consented to a tissue collection protocol which was approved by the institutional review board (IRB) at Brigham and Women’s Hospital and the Dana-Farber Cancer Institute.

Next Generation Sequencing

For patients in this study who had consented to our institutional tumor genomics protocol, next generation sequencing (results available upon request) was performed on DNA isolated from formalin-fixed paraffin-embedded tissue using previously described methods (1, 2).

Tissue Dissociation

Fresh patient tumor samples which were surgically resected were placed in RPMI containing 10% fetal bovine serum (FBS). Approximately 50 mg of each sample was fixed in 10% buffered formalin phosphate for immunohistochemical (IHC) analysis. The remaining tissue was mechanically minced using scissors and subsequently incubated for 45 minutes at 37°C in dissociation buffer: RPMI containing 10% FBS, 100U/mL collagenase type IV (Life Technologies), and 50 ug/mL DNase I (Roche). The resulting cell suspension was strained through a 70 µm nylon mesh to remove larger aggregates of tissue. Cells were treated with RBC Lysis Buffer (BioLegend) for five minutes at 37°C to remove red blood cells and then washed in FACS buffer: DPBS containing 2% FBS.

Staining Procedure

The nonfixed cell suspension was stained using LIVE/DEAD® Fixable Yellow Dead Cell Stain Kit (Life Technologies) diluted 1:500 in FACS buffer and incubated for 10 minutes in the dark at room temperature. After two washes, the cell pellet was
incubated in a 1:100 dilution of FcR blocking reagent (Miltenyi Biotec) in FACS buffer for 15 minutes in the dark on ice. After a single wash, 100 µL of cell suspension was aliquoted into multiple wells in a 96-well round-bottom plate for subsequent tagging using fluorescently-conjugated antibodies. Pre-made antibody cocktails, each containing five to seven different surface-staining antibodies, were added to their respective wells and incubated for 20 minutes in the dark on ice. For wells containing a complete set of seven surface staining antibodies, cells were fixed using a 1% buffered formalin phosphate solution in FACS buffer. Samples to be permeated to tag intracellular antigens were fixed in Fix/Perm buffer following the FOXP3 staining kit protocol (eBioscience). Antibodies targeting intracellular antigens were diluted 1:20 in Permeabilization buffer (eBioscience) and incubated for 30 minutes in the dark on ice. After two washes in Permeabilization buffer, cells were resuspended in FACS buffer and stored at 4°C until flow analysis.

**Antibodies**

Single cell suspensions were stained using mouse-anti-human antibodies. Surface antibodies against CD3 (HIT3a; UCHT1), CD8 (RPA-T8), CD14 (M5E2; MphiP9), CD45 (HI30), CD56 (B159), CD279 (EH12.1) and its isotype control (MOPC-21), and HLA-DR (G46-6) were purchased from BD Biosciences. Surface antibodies against CD4 (RPA-T4), CD16 (3G8), CD19 (HIB19), CD33 (WM53), CD66b (G10F5), CD123 (6H6), and TIM-3 (F38-2E2) and its isotype control (MOPC-21) were purchased from BioLegend. The surface antibody against CD45 (2D1) was purchased from eBioscience and the surface antibody against LAG-3 (polyclonal) and its isotype control (polyclonal) were purchased from R&D Systems. The intracellular antibody FOXP3
(236A/E7) was purchased from eBioscience. For CD3 (HIT3a), LAG-3, and its isotype control, and HLA-DR, 8 µL of antibody was added to each well. For all other surface antibodies, 2.5 µL was added to each well. Sample volume was brought up to a total of 100 µL using FACS buffer, resulting in a 1:12.5 or a 1:40 dilution, respectively. For all intracellular antibodies, 2.5 µL of antibody was added to 50 µL of FACS buffer for a 1:20 dilution.

**Flow Analysis**

Cells were analyzed using flow cytometry within 72 hours of fixation. Prior to analysis, cells were filtered through 35 µm nylon mesh. Samples were acquired on a BD FACSCanto II HTS cell analyzer with FACSDiva software v8.0.1 (BD Biosciences) and analyzed using FlowJo software v10.

**Immunohistochemistry**

For immunohistochemistry, 4µm thick paraffin-embedded sections were baked in a 60°C oven for one hour. Heat-induced antigen retrieval was performed using ER2 solution (pH8) (Leica Biosystems) for 20-30 minutes.

Immunohistochemical staining of CD3 (polyclonal rabbit anti-human, Dako) and PD-L1 (monoclonal rabbit anti-human, E1L3N, Cell Signaling Technologies) was performed using an automated staining system (Bond III, Leica Biosystems, Buffalo Grove, IL) following the manufacturer’s protocols. CD3 immunostaining was performed with 1:250 dilution using Bond Primary Antibody Diluent (Leica) for 30 minutes at room temperature. PD-L1 immunostaining was performed with 1:200 dilution using Bond Primary Antibody Diluent (Leica) for 2 hours at room temperature. Detection and development of the primary antibody was performed using the Bond Polymer Refine
detection kit (Leica Biosystems). Slides were counterstained with hematoxylin, dehydrated and mounted. Positive and negative controls were included in each panel of staining for both markers.

PD-L1 expression in tumor cells was considered positive if ≥1% of tumor cells had distinct membranous staining. The intensity (0, negative; 1, weak; 2, moderate; 3, intense) and the percentage of positive stained tumor cells were recorded. For CD3, three representative hot spots on each slide were chosen to count the positive cells under 400X high power field view, and the average number was recorded. All the slides were evaluated and scored blinded to clinical data.

**t-SNE and Statistical Analyses**

The unsupervised non-linear dimension reduction method t-distribution based stochastic non-linear embedding (t-SNE)(3) was applied to the flow cytometry data to investigate in reduced dimension space how samples from all mesothelioma tumor samples and 7 paired normal controls were located in relation to each other. t-SNE minimizes the divergence of neighborhood closeness moving from high dimensions to low dimensions. Data were first Z-score normalized and the perplexity parameter input to t-SNE was 3. The following flow cytometry parameters were used for embedding: percent CD45+ cells, percent CD3+ cells, percent CD19+ cells, percent CD56+ cells, percent CD3+CD56+ cells, percent CD33+ cells, percent CD66b+ cells, percent CD123+ cells, percent CD16+ cells, percent CD4+ cells, percent CD8+ cells, CD8:CD4 ratio, percent TIM3−PD1−CD4+ cells, percent TIM3−PD1−CD4+ cells, percent TIM3+PD1−CD4+ cells, percent TIM3−PD1−CD4+ cells, percent TIM3−PD1−CD4+ cells, percent LAG3−PD1−CD4+ cells, percent LAG3−PD1−CD4+ cells, percent LAG3−PD1−CD4+ cells,
percent TIM3+CD4+ cells, percent PD1+CD4+ cells, percent LAG3+CD4+ cells, percent FOXP3+CD4+ cells, percent TIM3-PD1+CD8+ cells, percent TIM3+PD1+CD8+ cells, percent TIM3+PD1−CD8+ cells, percent TIM3−PD1−CD8+ cells, percent LAG3−PD1+CD8+ cells, percent LAG3+PD1−CD8+ cells, percent LAG3−PD1−CD8+ cells, percent TIM3−CD8+ cells, percent PD1−CD8+ cells, percent PD1+CD8+ cells, percent TIM3−CD8− cells, percent LAG3−CD8+ cells, percent CD14+CD16− cells, percent CD14+CD16+ cells, and percent CD14+HLADR− cells.

The clinical data such as histology and IHC PD-L1 staining are mapped to the tSNE1~tSNE2 scatter plot to visualize the distribution of patient cases. Subsequently k-means clustering were applied to identify the two major clusters and Fisher’s exact tests and Wilcoxon rank sum tests were applied for clusters associated statistics. Linear regression was used to identify the association between a tumor’s immune marker expression and the distance from the normal cluster. Overall survival was determined using the Kaplan-Meier method.

RESULTS

Sample characteristics

Patients with malignant pleural mesothelioma who were undergoing surgical resection during routine clinical care at our institution were offered participation in a correlative tissue collection protocol. Clinical, pathologic, and genomic characteristics of the first 43 consecutive mesothelioma samples analyzed in this study are shown in Table 1. The histologic subtypes among the 43 cases were: 29 (67%) epithelioid, 4 (9%) sarcomatoid, and 10 (23%) biphasic. Three-quarters of the samples came from male patients and nearly all 43 of the patients were Caucasian. Of the 39
mesothelioma cases for which next generation sequencing was completed, 20 (57%) had a $BAP1$ mutation and 13 (37%) had an $NF2$ mutation. Prior to surgical resection, 6 (14%) patients received neoadjuvant chemotherapy, and the remaining 86% of patients did not receive chemotherapy before surgery.

Sufficient tissue was available for PD-L1 immunohistochemistry (IHC) for 39 (91%) of the cases. We defined PD-L1 positivity using the criteria for the mesothelioma cohort on the KEYNOTE-028 pembrolizumab study: any intensity of PD-L1 membranous expression in at least 1% of tumor cells. Among the 39 samples, 18 (46%) cases were PD-L1 positive and 21 (54%) were PD-L1–negative. By histologic subtype, non-epithelioid (sarcomatoid and biphasic) tumors were significantly more likely ($P = 0.01$) to be PD-L1 positive (10 of 13 cases, 77%) compared to epithelioid tumors (8 of 26 cases, 31%).

**Immune cell subsets in PD-L1–positive and PD-L1–negative tumors**

Tumors from each of the 43 mesothelioma cases were dissociated into single cell suspensions and analyzed by flow cytometry. A sufficient number of viable cells was available for comprehensive analysis by flow cytometry in 38 of the 43 cases (88%). Across cases, there was considerable variability in the percentage of live cells that consisted of CD45$^+$ leukocytes, from 17.6% to 99.8%, and we noted that the percentage of infiltrating immune cells appeared to correlate with PD-L1 status (**Fig. 1A**). Of all the live cells isolated from each tumor, PD-L1–positive tumors had a significantly higher percentage of CD45$^+$ immune cells (median 87.7%) than PD-L1–negative tumors (median 68.2%, $P = 0.05$, **Fig. 1B**).
When analyzed by histologic subtype, non-epithelioid (sarcomatoid or biphasic) tumors were also significantly more likely to have a higher fraction of infiltrating immune cells (median 91.4%) than epithelioid tumors (median 64.1%, \( P < 0.0001 \), Fig. S1B). Among CD45\(^+\) immune cells, non-epithelioid tumors were also significantly more likely to have a higher fraction of CD3\(^+\) T cells than epithelioid tumors (\( P = 0.004 \), Supplemental Fig. S1). There was no significant difference in the proportion of CD45\(^+\) live cells in tumors from patients who received neoadjuvant chemotherapy compared to tumors from patients who received no treatment before surgery (Supplemental Fig. S2).

In order to determine which immune cell subtype contributed to the increase in CD45\(^+\) cells in PD-L1-positive tumors, we next determined the relative fraction of T cells, B cells, monocytes, granulocytes, dendritic cells, and natural killer cells within each sample (Fig. 1C). Again, there was considerable variability in the immune cell composition across tumors, and we noted that the percentage of T-cell infiltration also appeared to correlate with PD-L1 status. CD3\(^+\) T cells were commonly identified in tumor samples (range 5.2% to 81.2% of CD45\(^+\) cells), and there were significantly more CD3\(^+\) T cells in PD-L1-positive tumors than in PD-L1-negative tumors (median 30.4% vs 19.3%, \( P < 0.05 \), Fig. 1D). There was no significant difference in the number of CD19\(^+\) B cells in PD-L1-negative vs. PD-L1-positive tumors (median ~3%, \( P = 0.50 \), although some samples showed markedly high levels of B-cell infiltrates (up to 51.8% of CD45\(^+\) cells). Comparing PD-L1-positive tumors to PD-L1-negative tumors, there were no significant differences in the fraction of CD66b\(^+\) neutrophils (median ~6%, \( P = 0.05 \), CD123\(^+\) dendritic cells (median ~0.4%, \( P = 0.82 \), CD3- CD56\(^+\) natural killer (NK) cells (median ~4%, \( P = 0.88 \), CD3\(^+\) CD56\(^+\) natural killer T (NKT) cells (median ~0.6%, \( P =
0.27) as shown in Fig. 1D, or CD16⁺ cytotoxic natural killer cells (median ~37% of CD56⁺ NK cells, \( P = 0.17 \), not shown). CD33⁺ monocytes were commonly found in most tumor samples, representing ~42% of CD45⁺ cells (range 5.7% to 86.1%), but there was no significant difference between PD-L1–positive and PD-L1–negative tumors \( (P = 0.65) \). Among CD33⁺ cells, there was no significant difference in the proportion of CD14\(^{high}\)/HLA-DR\(^{low}\) monocytic myeloid derived suppressor cells (MDSCs) in PD-L1–positive and negative tumors (range 0.6% to 31% of CD33⁺ cells, \( P = 0.47 \)) and no differences in CD14⁺/CD16⁻ or CD14⁺/CD16⁺ monocytes by PD-L1 status (Supplemental Fig. S3).

To validate flow cytometry as an accurate tool for quantifying immune cell subsets, we also determined the number of infiltrating CD3⁺ T cells in each tumor using immunohistochemistry (IHC). Similar to the flow cytometry results, PD-L1–positive mesothelioma specimens had a significantly higher number of infiltrating T cells compared to PD-L1–negative tumors (median 176 vs 59 cells/hpf, \( P = 0.006 \), Fig. 2A). As with flow cytometric analysis, some PD-L1–negative and PD-L1–positive tumors showed a relative paucity of infiltrating CD3⁺ T cells, whereas others showed an abundance of infiltrating CD3⁺ cells (range 3-549 cells/hpf, Fig. 2A). Representative CD3 IHC images with paired flow cytometry results from four cases are shown in Fig. 2B.

**Analysis of T-cell subsets in mesothelioma samples**

Given that both flow cytometry and IHC demonstrated that T cells were more abundant in PD-L1–positive tumors than in PD-L1–negative tumors, we next analyzed
T-cell subsets within mesothelioma samples using flow cytometry. We found no significant differences in the proportion of $\text{CD}4^+$ ($P = 0.26$) or $\text{CD}8^+$ ($P = 0.11$) live cells in PD-L1–negative tumors, as compared to PD-L1–positive tumors, (Fig. 3A) and no significant difference in the CD8:CD4 ratio (Fig. 3B).

We then analyzed $\text{CD}4^+$ and $\text{CD}8^+$ T-cell lineage subpopulations by flow cytometry within each tumor sample. For $\text{CD}4^+$ T cells, we observed no significant differences between PD-L1–negative and PD-L1–positive tumors in the proportion of $\text{CD}4\text{5RA}^+\text{CCR7}^+$ naïve ($P = 0.88$), $\text{CD}4\text{5RO}^+\text{CCR7}^+$ central memory ($P = 0.16$), $\text{CD}4\text{5RO}^+\text{CCR7}^-$ effector memory ($P = 0.88$), or $\text{CD}4\text{5RO}^+$ total memory ($P = 0.54$) T cells, but there were significantly fewer $\text{CD}4\text{5RA}^+\text{CCR7}^+$ effector $\text{CD}4^+$ cells in PD-L1–positive tumors compared to PD-L1–negative tumors ($P = 0.01$) (Fig. 3C). Among $\text{CD}8^+$ T cells, however, there was a significantly higher proportion of $\text{CD}8^+$ memory T cells ($P = 0.007$) with an increase in $\text{CD}8^+$ effector memory T cells ($P = 0.03$) and a lower proportion of $\text{CD}8^+$ effector T cells ($P = 0.001$) in PD-L1–positive tumors compared to PD-L1–negative tumors. We detected no differences between PD-L1–negative and PD-L1–positive tumors in the proportion of $\text{CD}8^+$ naïve ($P = 0.39$) or central memory ($P = 0.90$) T cells (Fig. 3D).

**Analysis of T-cell activation, proliferation, and inhibition**

We also used flow cytometry to interrogate the functional status of infiltrating T cells with respect to markers of T-cell activation, proliferation, and inhibition. $\text{CD}3^+$ T cells in PD-L1–positive tumors were significantly more likely to display the activated HLA-DR$^+$ CD38$^+$ phenotype than T cells in PD-L1–negative tumors ($P = 0.001$, Fig. 4A).
While there was no significant difference in the proportion of proliferating Ki67+ CD4+ T cells in PD-L1–positive vs. PD-L1–negative tumors (P = 0.15), CD4+ T cells in PD-L1–positive tumors were more likely to express the regulatory T-cell (T_{reg}) marker FOXP3+ (P = 0.005). In terms of T-cell inhibitory markers, CD4+ T cells in PD-L1–positive tumors were also significantly more likely to be TIM-3+ (P = 0.002) as well as PD-1+ (P = 0.01) than CD4+ T cells in PD-L1–negative tumors. LAG-3 was infrequently detected on CD4+ T cells and there was no significant difference based on PD-L1 status (P = 0.58, Fig. 4B).

In PD-L1–positive tumors, CD8+ T cells were significantly more likely to be proliferating than those in PD-L1–negative tumors (P = 0.02, Fig. 4C). As with the CD4+ T-cell analysis, CD8+ T cells in PD-L1–positive tumors were also more likely to express the inhibitory markers TIM-3 (P = 0.003) and PD-1 (P = 0.008) than CD8+ T cells isolated from PD-L1–negative tumors (Fig. 4C). LAG-3 was also rarely seen on CD8+ T cells, with no difference between PD-L1–positive and negative samples (P = 0.43, Fig. 4C).

We next used flow cytometry to examine co-expression patterns of TIM-3 and PD-1 inhibitory receptors on T cells. PD-L1–positive tumors had higher levels of TIM-3+/PD-1+ “double positive” CD4+ (P = 0.002) and CD8+ (P = 0.005) T cells and lower levels of TIM-3-/PD-1- “double negative” CD4+ (P = 0.01) and CD8+ (P = 0.005) T cells as shown in Fig. 5A and Fig. 5B. About 50% of CD4+ T cells and 30% of CD8+ T cells expressed PD-1 in the absence of TIM-3 (“PD-1 single positives”), but expression of TIM-3 in the absence of PD-1 was rarely observed (“TIM-3 single positives”, Fig. 5A, 5B).
Analysis by Tumor Genotype

In addition to comparing mesothelioma samples according to PD-L1 status and histologic subtype, we also compared immune infiltrates by tumor genotype. We found no significant differences in the degree of immune cell infiltration in BAP1 wild-type vs. BAP1-mutant mesothelioma (Supplemental Fig. S4A), and no difference in NF2 wild-type vs. NF2-mutant samples (Supplemental Fig. S4B). Most PD-L1–negative tumors harbored BAP1 mutations (11 out of 12 samples) whereas roughly half of PD-L1–positive tumors were BAP1-mutant (7 out of 15, P = 0.02). NF2 mutation status did not correlate with PD-L1 expression (not shown). Germline DNA from patients was not available in this study to discriminate germline vs. somatic tumor mutations.

Global Analysis of Flow Cytometry Data

In order to better visualize immunologic differences among tumor samples, we used t-stochastic neighbor embedding (t-SNE) to incorporate 38 flow cytometry parameters into a two-dimensional scatter plot, where close proximity between any two samples on the t-SNE map represents their immunophenotypic similarity.(29) In addition to the mesothelioma tumor samples, flow cytometric analysis was also performed on adjacent pieces of normal tissue in 7 cases.

Two main immunologic groups were identified through t-SNE analysis, termed “Normal Cluster,” which contained all the paired normal tissue samples, and “Tumor Cluster” which was significantly enriched for tumor samples (Fig. 6A, P < 0.0001). Sarcomatoid and biphasic tumors were more immunologically distant from the Normal Cluster than epithelioid tumors (P = 0.03, Fig. 6A and Supplemental Fig. S5A).
greater distance from the Normal Cluster was associated with an increasing fraction of infiltrating CD3$^+$ T cells ($P = 0.0001$, Fig. 6B and Supplemental Fig. S5B), along with a decreasing fraction of CD66b$^+$ granulocytes ($P < 0.0001$, Fig. 6C and Supplemental Fig. S5C). Distance from the Normal Cluster was also associated with PD-L1 positivity ($P = 0.001$, Fig. 6D and Supplemental Fig. S5D), increasing proportions of TIM-3$^+$/PD-1$^+$/CD8$^+$ T cells ($P < 0.0001$, Fig. 6E and Supplemental Fig. S5E), as well as an increasing expression of FOXP3 on CD4$^+$ T cells ($P < 0.0001$, Fig. 6F and Supplemental Fig. S5F).

**Survival Analysis**

Overall survival data were collected on all patients in this study. We found that patients with non-epithelioid mesothelioma had a significantly worse prognosis compared to patients with epithelioid tumors ($P = 0.02$, Supplemental Fig. S6A). There was no significant difference in overall survival among patients with PD-L1–positive tumors vs. PD-L1–negative tumors ($P = 0.15$, Supplemental Fig. S6B). There was also no significant difference in survival among tumors with high vs. low CD3 infiltrates, whether assess by flow cytometry ($P = 0.90$, Supplemental Fig. S6C) or immunohistochemistry ($P = 0.89$, Supplemental Fig. S6D).

**DISCUSSION**

Chronic inflammation in response to inhaled asbestos fibers has long been recognized as a contributing factor for the development of malignant pleural mesothelioma.(30, 31) Dissecting the properties of these inflammatory cells within
tumors will provide greater insights into the immunologic mechanisms of response and resistance to immunotherapy in this disease. Here, we used flow cytometry to characterize 43 resected malignant pleural mesothelioma specimens and uncovered distinct immunologic phenotypes in PD-L1–positive tumors as compared to PD-L1–negative tumors, and in sarcomatoid/biphasic tumors as compared to epithelioid tumors. We found that PD-L1–positive and sarcomatoid/biphasic tumors have a significantly greater proportion of infiltrating T cells than PD-L1–negative and epithelioid tumors, respectively. PD-L1–positive tumors also show significant increases in T-cell proliferation and activation, along with significant increases in regulatory T cells and expression of T-cell inhibitory markers.

Among PD-L1–positive tumors, we observed considerable immunophenotypic variability across samples, which may explain why only a minority of PD-L1–positive mesotheliomas responded to the PD-1 inhibitor pembrolizumab in the KEYNOTE-028 study (25). Factors other than PD-L1 expression that may modulate the efficacy of PD-1 inhibitors include: 1) the relative abundance of infiltrating lymphocytes, 2) co-expression of multiple inhibitory receptors on T cells, and 3) the influence of myeloid derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs) (32, 33). In the present study, we demonstrate that tumor immune profiling with flow cytometry enables deeper investigation into these factors.

We found that some PD-L1–positive samples contained only a small proportion of infiltrating lymphocytes whereas others were associated with dense immune infiltrates. While the presence of tumor infiltrating lymphocytes (TILs) may be a favorable prognostic factor in some cancers (34, 35), it is not clear if TILs are predictive
of a response to immune checkpoint blockade. One study of 41 patients with different
tumor types showed no association of TILs with response to nivolumab, but the vast
majority of samples analyzed in that study were not obtained immediately prior to
initiating treatment with nivolumab (36). We also observed considerable variation in the
proportion of infiltrating monocytic MDSCs across tumor samples, which in some cases
may have a substantial role in creating an immunosuppressive microenvironment and
promoting tumor growth and metastasis (32). There also appeared to be an inverse
correlation between T-cell and neutrophil infiltrates in our study. The interaction
between T cells and tumor-associated neutrophils (TANs) is becoming increasingly
recognized as an important modulator of cancer control and progression (37-39) and
warrants further study in mesothelioma.

In animal cancer models, co-expression of additional inhibitory receptors on
immune cells may also blunt responses to PD-1 inhibition. For example, TIM-3, a
negative regulator of T-cells, is frequently co-expressed with PD-1 on TILs in mice with
solid tumors or leukemia, and targeting both PD-1 and TIM-3 can be more effective at
controlling tumor growth than targeting each pathway alone (40, 41). We also found
frequent co-expression of PD-1 and TIM-3 on CD8+ T cells, suggesting that combined
inhibition of the PD-1 and TIM-3 pathways might be an effective therapeutic strategy in
mesothelioma. High expression of PD-1 and TIM-3 on CD8+ T cells has previously
been shown to identify clonally-expanded, mutation-specific, tumor-reactive immune
cells in melanoma (13). The high proportion of effector memory CD8+ cells that we
observed in PD-L1–positive tumors compared to PD-L1–negative tumors may also
reflect chronically-stimulated T cells with high-affinity T-cell receptors (TCRs) that
recognize tumor antigen (42). In our tumors with the highest proportion of TIM-3+/PD-1+/CD8+ T cells, we also saw high PD-L1 expression on tumor cells as well as increased FOXP3 expression on CD4+ T cells. This finding is in keeping with the concept of adaptive immune resistance (43), and recent work in melanoma has similarly shown that CD8+ T cells within a tumor can both upregulate expression of PD-L1 and indoleamine-2,3-dioxygenase (IDO) through interferon-γ signaling and also recruit FOXP3+ Tregs by cytokine-mediated CCR4 signaling in the tumor microenvironment (44, 45).

Immune profiling by flow cytometry in solid tumors has several potential advantages over traditional immunohistochemistry. Analysis of immune infiltrates by flow cytometry can be performed rapidly and is quantitative, objective, and largely automated, whereas IHC tends to be semi-quantitative and relies on subjective interpretation by a pathologist. Flow cytometry allows for multiplexed analysis of several markers simultaneously as well as phenotyping of specific immune cell types. By contrast, IHC has historically been restricted to looking at one marker per slide, making it difficult to analyze subpopulations of immune cells and challenging to determine co-expression of markers at single cell resolution, though newer multiplex techniques are under investigation for examining multiple markers per slide (46, 47).

One disadvantage of flow cytometry compared to immunohistochemistry is the loss of spatial relationships between tumor cells and immune cells, which may be an important determinant of the response to immune checkpoint blockade (48). However, this limitation may be overcome by combining data from IHC and flow cytometry on clinical samples.
Along with PD-1, we found frequent co-expression of TIM-3 in several samples. This may partially explain why only a minority of PD-L1–positive mesothelioma patients responded to the PD-1 inhibitor pembrolizumab (25). Comprehensive immunoprofiling by flow cytometry will hopefully improve our understanding of the factors that determine response and resistance to immunotherapy and lead to rationally designed immunotherapy combination trials.
REFERENCES


<table>
<thead>
<tr>
<th>Sample Characteristic</th>
<th>Number (%)</th>
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<tbody>
<tr>
<td>Total mesothelioma cases</td>
<td>43 (100%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33 (77%)</td>
</tr>
<tr>
<td>Female</td>
<td>10 (23%)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>42 (98%)</td>
</tr>
<tr>
<td>Asian</td>
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</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Epithelioid</td>
<td>29 (67%)</td>
</tr>
<tr>
<td>Biphasic</td>
<td>10 (23%)</td>
</tr>
<tr>
<td>Sarcomatoid</td>
<td>4 (9%)</td>
</tr>
<tr>
<td>PD-L1 immunohistochemistry completed</td>
<td>39 (91%)</td>
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<tr>
<td>PD-L1 positive</td>
<td>18 (46%)</td>
</tr>
<tr>
<td>PD-L1 negative</td>
<td>21 (54%)</td>
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<td>Full flow cytometry data available</td>
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<td>35 (81%)</td>
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<tr>
<td>NF2 mutation</td>
<td>13 (37%)</td>
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<tr>
<td>Neoadjuvant chemotherapy</td>
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<tr>
<td>Treated with neoadjuvant chemotherapy</td>
<td>6 (14%)</td>
</tr>
<tr>
<td>Did not receive neoadjuvant chemotherapy</td>
<td>37 (86%)</td>
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FIGURE LEGENDS

Figure 1. Analysis of immune cell infiltrates in mesothelioma samples. (A) Mesothelioma cases are ordered from left to right by increasing percentage of live cells that were CD45+. PD-L1 status and histologic subtypes are shown. (B) The percentage of live cells that were CD45+ is shown for PD-L1–negative (−) and positive (+) samples (top panel) and for epithelioid and non-epithelioid (sarcomatoid or biphasic) samples (bottom panel). Immune cell subtypes, shown as a fraction of CD45+ cells, are shown in (C). (D) For PD-L1–negative and PD-L1–positive tumors, the percentage of CD45 cells that were B cells, T cells, natural killer (NK) cells, natural killer T (NKT) cells, monocytes (mono), neutrophils (neut), and dendritic cells (DC) are shown. For the scatter plots in (B) and (D), the median and interquartile range are shown.

Figure 2. Correlation between immunohistochemistry (IHC) and flow cytometry for CD3. The absolute number of CD3+ T cells per high-power field (hpf) in PD-L1–negative and positive tumors by IHC analysis is shown in (A), with the median and interquartile range displayed on the scatter plot. (B) Images from CD3 IHC are shown alongside the flow cytometry plot and histogram for four representative mesothelioma cases that had particularly high (top two panels) or low (bottom two panels) CD3+ infiltrates. The quantification of CD3+ cells is reported as a percentage of live cells in each case.

Figure 3. Analysis of CD4+ and CD8+ T-cell lineages in mesothelioma specimens. The proportion of CD45+ cells that were CD4+ or CD8+ in PD-L1–negative
and PD-L1–positive cases is shown in (A) and the CD8/CD4 ratio is shown in (B). As a proportion of CD4⁺ (C) and CD8⁺ (D) T cells, the percentage of naïve, central memory, effector memory, effector, and total memory T cells are plotted. On each graph, the median and interquartile range are displayed.

**Figure 4.** Markers of T-cell activation, proliferation, and inhibition in mesothelioma samples. (A) The fraction of CD3⁺ T cells that displayed the activated HLA-DR⁺/CD38⁺ phenotype is shown. (B) CD4⁺ T-cell expression of Ki67, FOXP3, LAG-3, TIM-3, and PD-1 in PD-L1–negative and PD-L1–positive tumors is shown. (C) CD8⁺ T-cell expression of Ki67, LAG-3, TIM-3, and PD-1 in PD-L1–negative and PD-L1–positive tumors is shown.

**Figure 5.** Co-expression of TIM-3 and PD-1 on CD4⁺ (A) and CD8⁺ (B) T cells is shown. On each graph, the median and interquartile range are shown.

**Figure 6.** Comparison of tumor and normal samples by t-stochastic neighbor embedding (t-SNE) analysis. (A) Samples segregated into two distinct groups, a “Normal Cluster” (black dotted box) and a “Tumor Cluster” (red dotted box). Cases with epithelioid histology are shown in blue; sarcomatoid/biphasic histology is shown in orange; for 7 cases, paired normal tissue was also available for analysis (black). The percentage of CD45⁺ cells that were CD3⁺ T cells is shown in (B) and that were CD66b⁺ granulocytes is shown in (C). In (D), cases are colored according to PD-L1 status with PD-L1–negative cases in blue and PD-L1–positive cases in orange. For two samples,
sufficient tissue was not available (N/A, gray) for PD-L1 IHC. PD-L1 IHC was not performed on adjacent normal tissue (black). Panel (E) shows the percentage of CD8⁺ T cells that express both TIM-3 and PD-1, and panel (F) shows the percentage of CD4⁺ T cells that express FOXP3. In panels (B), (C), (E), and (F), the percentage of positivity is color coded according to the heatmap provided. For each panel, a statistical analysis comparing samples relative to their distance from the Normal Cluster is provided in Supplemental Fig. 5.
Figure 2

(A) **
P = 0.006

(B) PD-L1 Negative: CD3: 85.1%
PD-L1 Positive: CD3: 60.1%
CD3: 10.8%
CD3: 3.7%
Figure 5

A

% of CD4+ cells

<table>
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<tr>
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<th>TIM3- PD1-</th>
<th>TIM3+ PD1-</th>
<th>TIM3- PD1+</th>
<th>TIM3+ PD1+</th>
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<tbody>
<tr>
<td>P</td>
<td>0.01</td>
<td>0.48</td>
<td>0.16</td>
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B

% of CD8+ cells

<table>
<thead>
<tr>
<th>PD-L1 Status</th>
<th>TIM3- PD1-</th>
<th>TIM3+ PD1-</th>
<th>TIM3- PD1+</th>
<th>TIM3+ PD1+</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>0.005</td>
<td>0.004</td>
<td>0.66</td>
<td>0.005</td>
</tr>
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</table>
Figure 6

A. Paired Normal Tissue
- Black: Normal
- Blue: Epithelioid
- Orange: Sarcomatoid/Biphasic

B. % CD3+ (of CD45+ cells)

C. % CD66b+ (of CD45+ cells)

D. PD-L1 neg
- Blue: PD-L1 neg
- Orange: PD-L1 pos
- Gray: N/A

E. % of CD8 cells that are TIM-3+/PD-1+

F. % of CD4 cells that are FOXP3+

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