

Checkpoint Inhibitor Therapy Utilization and PD1/PDL1 Expression Across a Global Clinical Network

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ABSTRACT

Recent breakthroughs in checkpoint inhibitor^{1,2} and CAR-T cell therapies³ have accelerated research endeavors to refine current immunotherapies and develop the next-generation immunotherapeutic interventions. Checkpoint inhibitor immunotherapies seek to directly modulate T cell function and enhance T-cell-mediated tumor cell killing by targeting inhibitory receptors expressed on T cells and their ligands expressed on tumor cells, among other intratumoral cell populations. Specifically, current therapies are targeting PD1 and CTLA4, inhibitory receptors that dampen T cell responses when they interact with their cognate ligands (CD80/CD86 or PDL1/PDL2, respectively) to suppress the immune response.^{4,5} In particular, the PD1/PDL1 pathway has been targeted by multiple different antibody-based immunotherapies, including Keytruda (pembrolizumab, anti-PD1), Opdivo (nivolumab, anti-PD1), Imfinzi (durvalumab, anti-PDL1), and Tecentriq (atezolizumab, anti-PDL1). Since the initial approval of Keytruda in the

treatment of advanced melanoma in 2014, numerous oncological indications, including lung cancer, kidney cancer, head and neck cancer, and urothelial cancer, have been approved for anti-PD1 and anti-PDL1 therapies.⁶ More recently, Opdivo has been approved for microsatellite instability-high (MSI-high) or MMR-deficient colorectal cancer patients, as these commonly exhibit high levels of tumor neoantigens and tumor-infiltrating lymphocytes.⁷ As the use of these current immunotherapies grows and next-generation therapies are developed, it is fundamentally important to understand patient responses to therapies as well as the mechanisms of action, including expression patterns and functionality, within the tumor microenvironment. Discovery Life Sciences (DLS) manages a global clinical network of oncology practices that supply primary patient samples in multiple formats as well as real-time information on immunotherapeutic uses and responses.

PD1/PDL1-TREATED PATIENTS WITHIN THE DISCOVERY LIFE SCIENCES CLINICAL NETWORK

Discovery Life Sciences manages a global clinical network of local hospitals and oncology practices. The Discovery Clinical Network provides access to electronic medical records (EMR) and detailed physician notes, providing the opportunity to assess the use and effectiveness of anti-PD1/anti-PDL1 therapies on patients from various oncological indications. Treatment with targeted checkpoint inhibitor therapy is based on clinical guidelines set forth by the drug manufacturers and the FDA (**Table I**). Accordingly, some patients are targeted prior to any other therapeutic intervention, while some are targeted only following disease progression, and not all patients within the Discovery Clinical Network are afforded the opportunity to receive these treatments. Physicians within the Discovery Clinical Network closely monitor patient responses to checkpoint inhibitor therapy to assess response or nonresponse to therapy. Two categories of disease progression are observed and closely evaluated: pseudoprogression and true progression. Pseudoprogression occurs when tumor burden increases after receiving therapy, possibly due to increased inflammation within the tumor as a result of activation of tumor-infiltrating lymphocytes, prior to tumor shrinkage.⁸ Often, Discovery clinicians opt for a “wait and see” approach before discontinuing checkpoint inhibitor therapy to determine if the increased tumor burden is pseudoprogression or true progression. In true progression, the patient does not respond to checkpoint therapy, with increased tumor burden and number of distant lesions observed. Upon identification of true clinical progression, patients are either switched to a different checkpoint inhibitor therapy or transitioned to a conventional chemotherapy regimen.⁹ Finally, adverse reactions to checkpoint inhibitor therapies are observed and are sometimes serious enough to require immediate medical attention. For these cases, checkpoint

inhibitor therapy may be discontinued in favor of a different treatment option. All patient responses to these therapies are detailed within the EMR and physician notes.

To determine how currently approved anti-PD1/anti-PDL1 therapies are being utilized, we examined the percentage of patients within the Discovery Clinical Network that are currently receiving checkpoint inhibitor therapy. Among the indications approved for checkpoint inhibitor therapy, we observe a high percentage of lung cancer (45%) and melanoma (65%) patients in the Discovery Clinical Network on anti-PD1/anti-PDL1 therapies (**Table II**). Within these treated populations, Keytruda is the predominant therapy prescribed to lung cancer patients; however, both Opdivo and Imfinzi are also prescribed. For melanoma patients, Opdivo is the predominantly prescribed therapy, both as a single agent or in combination with Yervoy (ipilimumab, anti-CTLA4). Additionally, a small percentage of melanoma patients are currently prescribed Keytruda. Patients with kidney cancer, head and neck cancer, and urothelial cancer are also treated with anti-PD1/anti-PDL1 therapies within the Discovery Clinical Network, although at percentages lower than observed for both lung cancer and melanoma. Kidney cancer patients are currently only being treated with Opdivo. Half of HNSCC and urothelial cancer patients currently on checkpoint inhibitor therapies are prescribed Keytruda, with the other half being prescribed Opdivo or Tecentriq, respectively. Therefore, we observe substantial usage of checkpoint inhibitor therapies within the Discovery Clinical Network across multiple indications and utilizing different therapies, providing opportunities to track patient responses, particularly in terms of response or nonresponse to therapy, as well as adverse side effects.

TABLE 1. Current Checkpoint Inhibitor Usage by Indications.

Drug	Indication	Usage
Keytruda	Melanoma	Unresectable or metastatic melanoma
	NSCLC	Metastatic, nonsquamous NSCLC with no EGFR or ALK mutations, in combination with Alimta Metastatic NSCLC with tumor PD-L1 expression of $\geq 50\%$ and no EGFR or ALK mutations Second line therapy after disease progression on platinum-based chemotherapy for metastatic NSCLC with tumor PD-L1 expression $\geq 1\%$
	HNSCC	Recurrent or metastatic HNSCC with disease progression on or after platinum-based chemotherapy
	Urothelial Carcinoma	Locally advanced or metastatic urothelial carcinoma not eligible for platinum-based chemotherapy or with disease progression during or following platinum-based chemotherapy
Opdivo	Melanoma	Unresectable or metastatic melanoma, as a single agent or in combination with Yervoy Adjuvant therapy for patients with melanoma who have undergone complete resection
	NSCLC	Metastatic NSCLC with progression on or after platinum-based chemotherapy Metastatic NSCLC with EGFR or ALK mutations after progression on or after receiving mutation-specific targeted therapy
	SCLC	Metastatic SCLC with progression after platinum-based chemotherapy and at least one other line of therapy
	RCC	Advanced RCC after prior anti-angiogenic therapy Previously untreated RCC in combination with Yervoy
	HNSCC	Recurrent or metastatic HNSCC with disease progression on or after platinum-based chemotherapy
	Urothelial Carcinoma	Locally advanced or metastatic urothelial carcinoma not eligible for platinum-based chemotherapy or with disease progression during or following platinum-based chemotherapy
Imfinzi	NSCLC	Locally advanced, unresectable NSCLC with no disease progression following treatment with platinum-based chemotherapy
	Urothelial Carcinoma	Urothelial carcinoma with disease progression during or following platinum-based chemotherapy or within 12 months of neoadjuvant or adjuvant treatment with platinum-based chemotherapy
Tecentriq	NSCLC	Metastatic NSCLC with progression on or after platinum-based chemotherapy Metastatic NSCLC with EGFR or ALK mutations after progression on or after receiving mutation-specific targeted therapy
	Urothelial Carcinoma	Locally advanced or metastatic urothelial carcinoma not eligible for platinum-based chemotherapy or with disease progression during or following platinum-based chemotherapy

The FDA-approved usages for Keytruda, Opdivo, Imfinzi, and Tecentriq, grouped by indication.

TABLE 2. Checkpoint Inhibitor Therapy Usage within the Discovery Clinical Network.

Indication	Percent of Patients within DLS Network on I/O Therapies	Treatment	Percent of Patients within DLS Network on Therapy
Lung Cancer	45%	Keytruda	50%
		Opdivo	30%
		Imfinzi	20%
Melanoma	65%	Keytruda	20%
		Opdivo	70%
		Opdivo + Yervoy	10%
Kidney Cancer	30%	Opdivo	100%
Head and Neck Squamous Cell Carcinoma	10%	Keytruda	50%
		Opdivo	50%
Urothelial Cancer	10%	Keytruda	50%
		Tecentriq	50%

The total number of patients in each indication who received immunotherapy was calculated and divided by the total number of patients in the Discovery Clinical Network for that indication.

TUMOR-INFILTRATING LYMPHOCYTES ARE CHARACTERIZED BY HIGH PD1 EXPRESSION

Understanding the mechanism by which checkpoint inhibitor therapies work will be fundamental in not only improving their overall efficacy, but also in determining which patients will respond favorably, or unfavorably, to these treatment regimens. We have established dissociated tumor cells (DTCs) as a viable alternative to the logistical demands required when sourcing and utilizing fresh tumor tissue.^{10, 11} DTCs are mechanically and enzymatically digested to single cell suspensions with high viability and representation of the cellular components of the tumor microenvironment. The best-studied functions of PD1 have been elucidated on CD4+ and CD8+ T cells, where ligation of PD1 by its ligands PDL1 and PDL2 acts to dampen T cell responses.⁴ Consistent with previous reports,^{12, 13} we observed high percentages of

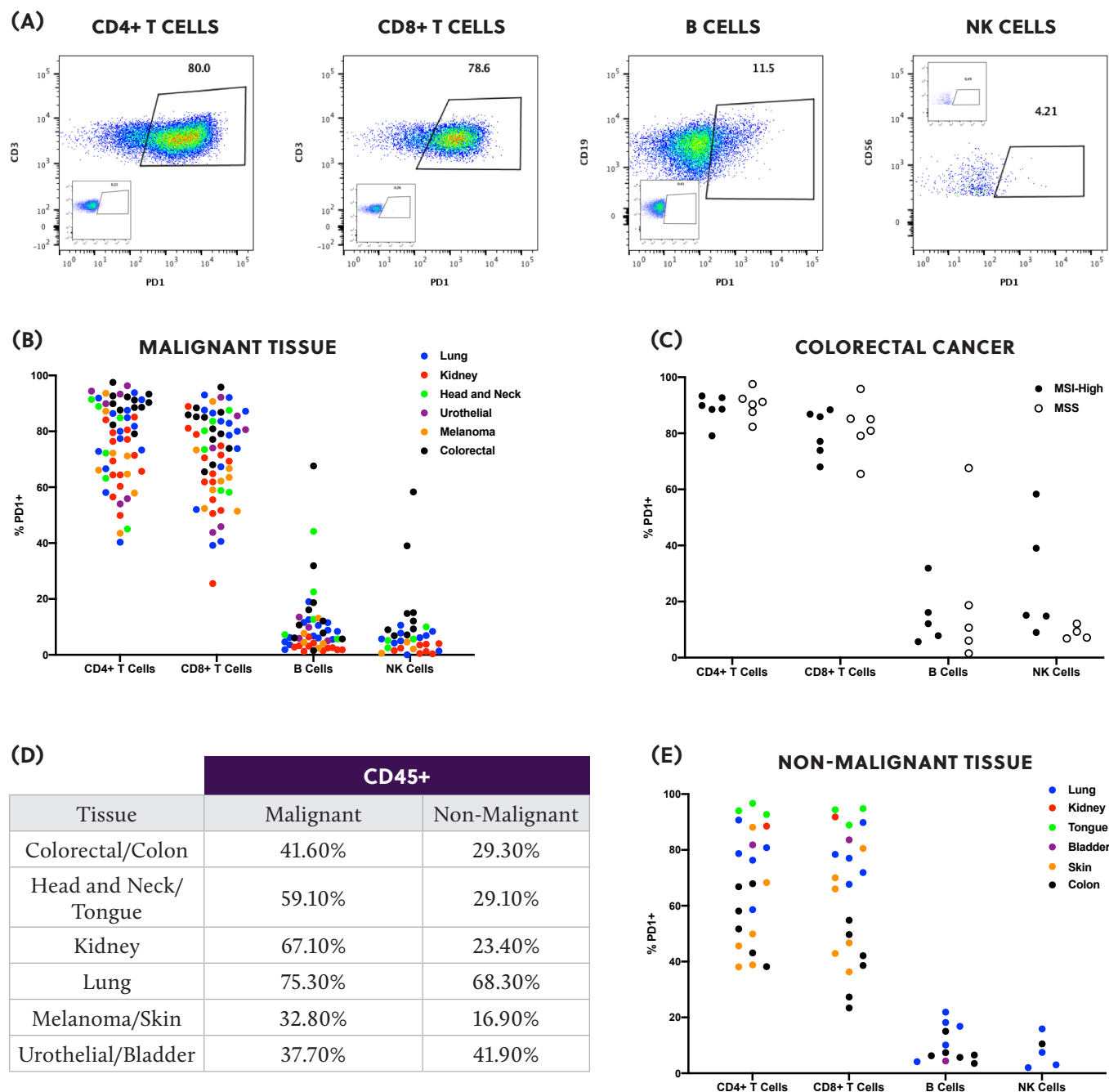
PD1+ CD4+ and CD8+ T cells from DTCs generated from lung cancer, kidney cancer, head and neck cancer, urothelial cancer, colorectal cancer, and melanoma (**Figure 1A and 1B**), ranging from 25% to 97% of cells. For colorectal cancer, Opdivo has been approved for patients with either MSI-high or dMMR colorectal cancer. We characterized MSI status of the colorectal cancer DTCs via PCR to evaluate potential differences in PD1 expression between MSI-high and MSS colorectal cancer samples. Overall, there was no difference in the percentage of PD1+ CD4+ and CD8+ T cells from MSI-high and MSS colorectal cancer DTCs (**Figure 1C**). Collectively, these data demonstrate that tumor-infiltrating T cells have uniformly high PD1 expression, ultimately dampening their anti-tumorigenic potential.

FIGURE 1. PD1 Expression on Lymphocyte Subsets in Malignant and Non-Malignant Tissue.

Single cell suspension were generated from malignant and non-malignant tissue, and lymphocyte subsets were analyzed for PD1 expression by flow cytometry.

(A) Representative flow cytometry plots of PD1 surface expression of CD4+ T cells, CD8+ T cells, B cells, and NK cells from a lung cancer DTC sample. Insets represent isotype controls. (B) CD4+ T cells, CD8+ T cells, B cells, and NK cells from DTCs from each indication were analyzed for PD1 expression. Each dot represents a unique patient sample.

(C) FMSI status was analyzed on colorectal DTCs, and MSI-H and MSS samples were analyzed for PD1 expression. (D) The average percentage of CD45+ immune cells in malignant vs non-malignant dissociated cells. (E) CD4+ T cells, CD8+ T cells, B cells, and NK cells from DTCs from each non-malignant tissue were analyzed for PD1 expression. Each dot represents a unique patient sample.



In addition to PD1+ T cells, tumor-infiltrating PD1+ B cells and PD1+ NK cells have been previously described. PD1+ B cells represent regulatory B cell subsets, which have been reported to contribute to the immunosuppressive environment within the tumor.¹⁴ We observed PD1+ B cells from DTCs generated from all relevant indications, with the sole exception of kidney cancer, where PD1+ B cells were rare (**Figure 1A and 1B**). Surprisingly, we did identify two colorectal cancer and two head and neck cancer DTC samples where the percentage of PD1+ B cells approached the percentage observed in the T cell compartment (**Figure 1B**). PD1 expression on NK cells has been postulated to inhibit anti-tumor NK cell activity, similar to what has been observed with CD8+ T cells.¹⁵ We identified PD1+ NK cells in colorectal cancer, lung cancer, and head and neck cancer DTCs (**Figure 1A and 1B**). NK cells were rare in the urothelial cancer DTCs analyzed, while melanoma and kidney cancer DTCs had very low percentages of PD1+ NK cells. However, similar to PD1+ B cells, there were two colorectal cancer DTC samples that had substantial percentages of PD1+ NK cells (**Figure 1B**). Therefore, all major lymphocyte subsets present in the tumor microenvironment express PD1 and could be influenced by checkpoint inhibitory therapy.

Often checkpoint inhibitor therapy can have adverse effects, presumably due to the reactivation of tissue-resident T cells. To understand PD1 expression in non-malignant tissues, we generated single cell suspensions from lung, kidney, tongue, and bladder tissues from cadaveric donors, skin tissue from breast reduction procedures, and colon tissue from diverticulitis patients. With the exception of urothelial cancer/normal bladder, the immune cell composition of non-malignant tissues was lower than in non-malignant tissues when compared to malignant tissues from the same site (**Figure 1D**). However, despite the lower

percentages of immune cell percentages, CD4+ and CD8+ T cells isolated from non-malignant tissue displayed high expression of PD1, particularly in the case of tongue-resident T cells, which were >90% PD1+ (**Figure 1E**). Interestingly, non-malignant colon and skin tissue had lower percentages of PD1+ CD4+ and CD8+ T cells. As colon tissue is currently sourced from diverticulitis patients, it will be important to evaluate the status of PD1 expression of T cells from non-malignant and non-inflamed colon tissue to fully understand the dynamics of PD1 expression within the colon. While B cells and NK cells were rare in non-malignant tissues, PD1+ B cells and PD1+ NK cells were present in lung and colon tissue (**Figure 1E**). Collectively, these data demonstrate that PD1 is expressed by a majority of CD4+ and CD8+ T cells in both malignant and non-malignant tissues, with small, but distinct, populations of B cells and NK cells also expressing PD1. As PD1 expression is widespread amongst tissue and tumor-resident lymphocyte populations, it is crucial to understand the dynamics of PD1 functions on T cells within these environments when refining current checkpoint inhibitor therapies as well as developing the next generation of immunotherapeutic interventions.

PDL1 IS EXPRESSED BY TUMOR AND IMMUNE CELLS WITHIN THE TUMOR MICROENVIRONMENT

Two ligands for PD1, PDL1 and PDL2, have been identified,¹⁶ and, while Keytruda and Opdivo both target PD1, Imfinzi and Tecentriq specifically target PDL1. Currently, PDL1 expression on tumor cells, as measured by immunohistochemistry, is evaluated on non-small cell lung cancer samples to identify patients to be prescribed Keytruda (Table I). We analyzed PDL1 expression on 143 formalin-fixed, paraffin-embedded tissues from urothelial cancer, head and neck cancer, kidney cancer, lung cancer, and melanoma patients (Table III). The vast majority of these tissues displayed <25% expression of PDL1 on tumor cells. Tumors with >50% expression of PDL1 were largely restricted to lung cancer tissues, with the lone exception being one case of urothelial cancer in which 50% of the tumor cells expressed PDL1. To determine if PDL1 expression could be analyzed on DTCs, we generated DTCs from matched fresh tumor tissue from a subset of these fixed tissues and analyzed these viable cells for PDL1 expression by flow cytometry. We were able to delineate PDL1+ and PDL1- lung cancer tumor samples that had been previously evaluated as 100% and 0% PDL1+ tumor expression by board-certified pathologists via immunohistochemistry (Figure 2A). Furthermore, kidney cancer samples that had 25% and 28% PDL1+ tumor cells by IHC also had clearly identified populations of PDL1+ tumor cells by flow cytometry that matched the percentages observed by IHC. Overall, there was a high correlation between the percentage of PDL1+ tumor cells identified by either flow cytometry on viable, dissociated cells and immunohistochemistry on fixed tissue (Figure 2B). We further analyzed PDL1 expression of DTCs generated from lung cancer, kidney cancer, head and neck cancer, urothelial cancer, colorectal cancer, and melanoma patient samples. Similar to the results observed by IHC, the majority of these samples displayed low expression of PDL1 on tumor cells

within these suspensions (Figure 2C). However, there were multiple DTC samples, particularly those generated from lung cancer patients, that had a substantial percentage of tumor cells expressing PDL1. Therefore, PDL1 expression is low on tumor cells as assessed by both flow cytometry and immunohistochemistry, providing crucial information when determining the mechanism of action of anti-PDL1 therapies.

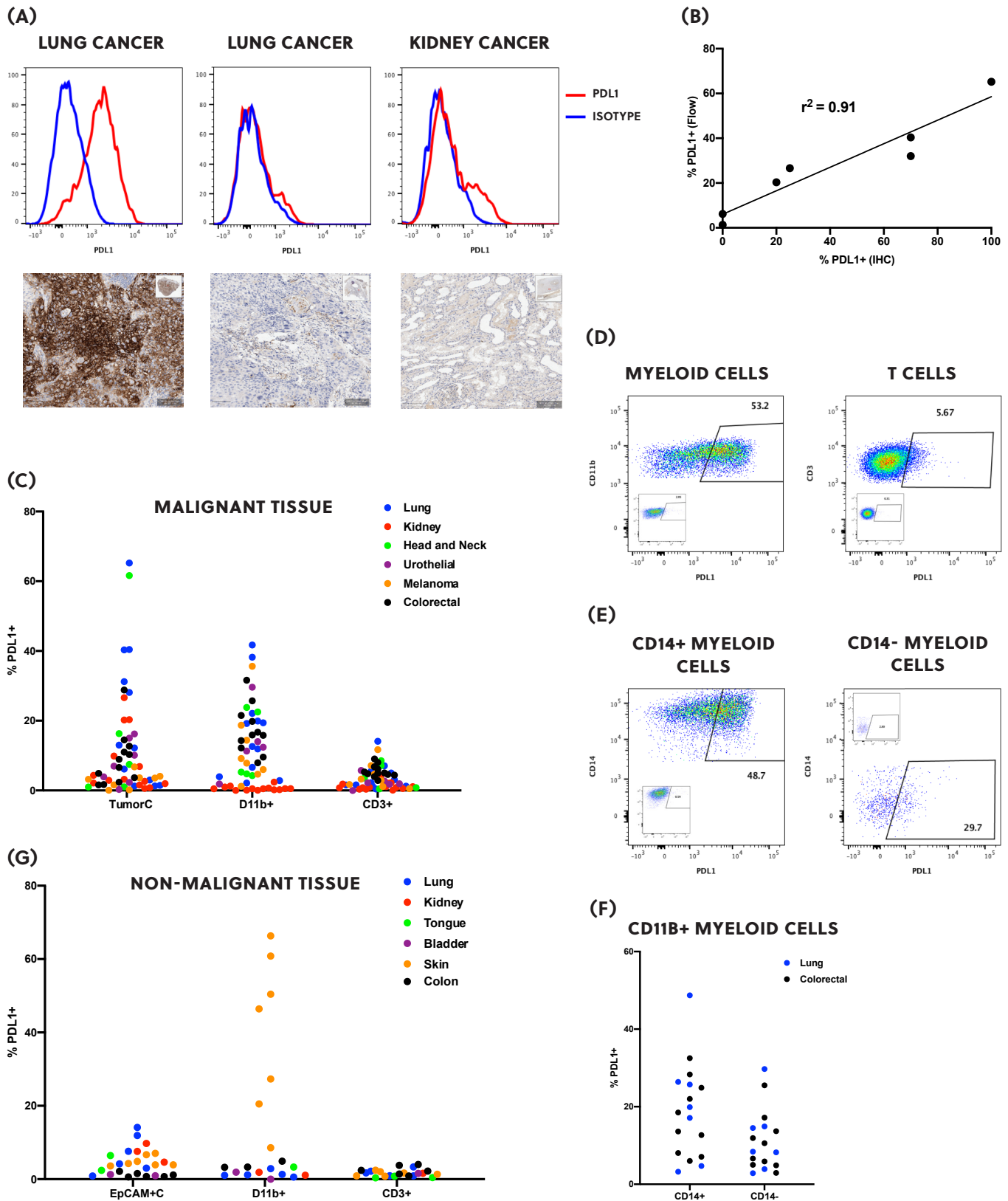
TABLE III. Analysis of PDL1 Expression on Tumor Cells from FFPE Tissue.

Indication	Total Blocks	PDL1 (% of Tumor)			
		0-24	25-49	50-74	75-100
Head and Neck	28	25	3	0	0
Kidney	14	13	1	0	0
Lung	58	38	6	4	10
Melanoma	27	27	0	0	0
Urothelial	16	14	1	1	0
Total	143	117	11	5	10

Slides were cut from FFPE blocks from each indication and stained for PDL1 expression. PDL1 expression on tumor cells were analyzed by board-certified pathologists.

Additional cellular populations within the tumor microenvironment, including myeloid cells and T cells, express PDL1, potentially modifying and repressing the immune response against the tumor.¹⁷⁻¹⁸ Indeed, we observed PDL1 expression on both CD11b+ myeloid cells and CD3+ T cells from DTCs (Figure 2D). Overall, there was a higher percentage of CD11b+ myeloid cells that expressed PDL1 compared to CD3+ T cells, and PDL1+ myeloid cells and T cells were identified in most indications that were analyzed. Kidney cancer was the one exception, as very few PDL1+ myeloid and PDL1+ T cells were present. CD11b+ myeloid cells within the tumor microenvironment contain both monocytic and granulocytic subsets, and we had previously identified by CD14+ CD11b+ and CD15+ CD11b+ subsets within DTCs¹¹. PDL1 expression was present on both CD11b+ CD14+ and CD11b+

FIGURE 2. PDL1 Expression on Cellular Subsets in Malignant and Non-Malignant Tissue.
(Legend on page 9)



CD14- subsets (**Figure 2E**), consistent with previous reports.¹⁹ However, there was a higher percentage of PDL1+ cells within the CD14+ fractions compared to the CD14- fraction (**Figure 2F**). Therefore, in addition to tumor cells, myeloid and T cell subsets within the tumor microenvironment express PDL1, contributing to the immunosuppressive environment in the tumor.

The majority of tissue-resident T cells in non-malignant tissues had high expression of PD1 (**Figure 1E**). To evaluate PDL1 expression in non-malignant tissues, we examined the presence of PDL1 on single cell suspensions generated from non-malignant lung, kidney, tongue, bladder, skin, and colon tissue. Unlike PD1 expression, which largely mirrored the expression observed in tumor-infiltrating lymphocytes, there were few PDL1+ epithelial cells identified from any tissue examined (**Figure 2G**). Only two non-malignant lung tissue samples had PDL1 expression on greater than 10% of the EpCAM+ epithelial cells, with the majority of non-malignant tissues displaying PDL1 on less than 5% of EpCAM+ epithelial cells. Furthermore, there were also very few PDL1+ CD11b+ myeloid cells or PDL1+ CD3+ T cells in non-malignant lung, colon, tongue, bladder, and kidney tissue examined. Surprisingly, there was abundant PDL1+ CD11b+ myeloid cells present in normal skin. CD11b+ cells in the skin include Langerhan's cells and dermal dendritic cells, both of which have been reported to express PDL1.²⁰⁻²¹ Therefore, in contrast to PD1 expression of tissue-resident and tumor-infiltrating lymphocytes, PDL1 expression is largely restricted to tumor cells, tumor-infiltrating myeloid cells and tumor-infiltrating T cells, and is rare in epithelial cells and immune cells from non-malignant tissue.

FIGURE 2. Legend

Single cell suspensions were generated from malignant and non-malignant tissue, and cellular subsets were analyzed for PDL1 expression by flow cytometry.

(A) Representative flow cytometry histograms of PDL1 expression on EpCAM+ tumor cells from 2 lung cancer and 1 kidney cancer DTC sample (top panel). Aperio scans of PDL1 expression on slides generated from FFPE blocks from the same donors (bottom panel).

(B) Correlation of PDL1 expression measured by flow cytometry on viable dissociated cells and immunohistochemistry on FFPE tissue.

(C) FEpCAM+ or CD146+ tumor cells, CD11b+ myeloid cells, and CD3+ T cells from DTCs from each indication were analyzed for PDL1 expression. Each dot represents a unique patient sample.

(D) Representative flow cytometry plots for PDL1 expression on CD11b+ myeloid cells and CD3+ T cells from a lung cancer DTC sample. Insets represent isotype controls.

(E) Representative flow cytometry plots for PDL1 expression on CD11b+ CD14+ and CD11b+ CD14- myeloid subsets from a lung cancer DTC sample. Insets represent isotype controls.

(F) CD11b+ CD14+ and CD11b+ CD14- myeloid subsets from each indication were analyzed for PDL1 expression. Each dot represents a unique patient sample.

(G) EpCAM+ epithelial cells, CD11b+ myeloid cells, and CD3+ T cells from DTCs from each non-malignant tissue were analyzed for PDL1 expression. Each dot represents a unique patient sample.

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