Large-Scale Flow Cytometry Analysis of Tumor Tissues

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INTRODUCTION

The foundation of the future of biomedical research requires access to highly-annotated primary human biospecimens. The logistical barriers of acquiring fresh tissue remain an impediment to advances in medicine, requiring the coordination of not only the tissue collection but also the downstream applications in the laboratory. Dissociation and cryopreservation of solid tissue provide a solution to this problem. These single cell suspensions remain viable following cryopreservation and ease the demands on large-scale experimental assays. Furthermore, these cells provide the ability to screen new biomarkers and therapeutic targets as they are uncovered without the need to source new fresh tissue. Using this model, we have analyzed viably cryopreserved single cell suspensions generated from over 400 unique patients across 11 oncology indications by flow cytometry. Flow cytometry allows for the identification of cell surface marker expression on the single cell level and provides in-depth characterization of the cellular composition of the tumor microenvironment. This large-scale characterization revealed indication-specific trends to the tumor composition, which are vitally important considerations as the next-generation of therapeutic interventions are developed.

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RESULTS

Indication-Specific Trends in Tumor and Infiltrating Immune Cells in Dissociated Tumor Cells

Dissociated tumor cells (DTCs) were generated from solid tumors via enzymatic and mechanical dissociation and represent a viable, single cell suspension of the cellular components of the tumor microenvironment. To determine the composition of the tumor microenvironment, flow cytometric analysis was performed on over 400 unique patient samples across 11 different indications (**Table 1**). Epithelial cell adhesion molecule, or EpCAM, is a well-established marker of tumor cells and is overexpressed in numerous oncology indications[1]. When EpCAM expression was examined on dissociated tissue from a normal ovary, benign ovary, and malignant ovarian tumor, few EpCAM+ cells were present in normal or benign ovarian tissue, while malignant ovarian tumors had abundant EpCAM+ cells (**Figure 1A**). EpCAM+ cells were present in dissociated tumor cells across 10 different indications at higher percentages than observed in normal or benign tissue (**Figure 1B**). Interestingly, within each indication there was significant variability in the percentage of EpCAM+ tumor cells, highlighting patient-specific cellular heterogeneity within the tumor microenvironment. However, across indications, clear trends were observed with regard to the percentage of EpCAM+ cells. In general, dissociated tumor cells from bladder, breast, colorectal, endometrial, and ovarian tumors had a high percentage of EpCAM+ tumor cells (>40%), while gastric, head and neck, kidney, lung, and prostate tumors had lower percentages of EpCAM+ tumor cells (<20%).

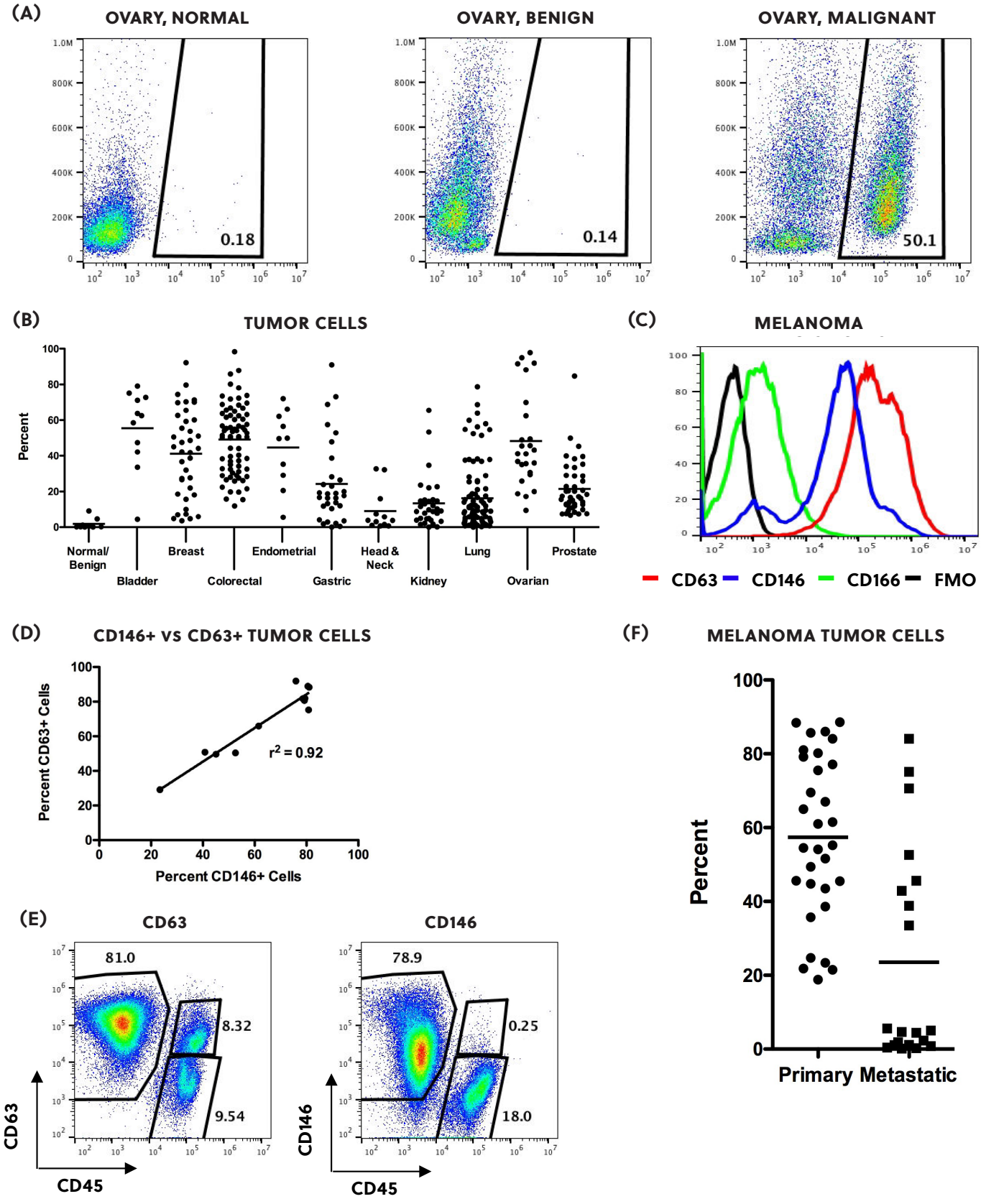
While EpCAM is a particularly good marker of tumor cells in many indications, melanoma cells do not express EpCAM[2]. However, three other cell surface markers – CD63, CD146, and CD166 – have been reported to be expressed on melanoma cells[3]. When the non-immune cell (defined as CD45-) and non-red blood cell (defined as GlyA-) fraction of melanoma dissociated tumor cells was examined, all three of these markers were expressed on melanoma tumor cells (**Figure 1C**). While CD166 expression was low, there was high expression of both CD146 and CD63, and there was a high correlation in the

TABLE 1

Number of unique patient samples analyzed from each indication

INDICATION	SAMPLES
Normal/Benign	9
Bladder	11
Breast	38
Colorectal	72
Endometrial	8
Gastric	31
Head and Neck	12
Kidney	32
Lung	95
Melanoma (Primary)	31
Melanoma (Metastatic)	20
Ovarian	25
Prostate	44
Total	428

FIGURE 1. Analysis of tumor cells with dissociated tumor cells. (Legend on page 4)



percentage of tumor cells identified by CD146 and CD63 (**Figure 1D**). When CD63 and CD146 expression was examined on CD45+ immune cells, however, a subset of CD45+ immune cells was identified that express CD63, consistent with previous reports[4, 5] (**Figure 1E**). In contrast, very few CD45+ immune cells express CD146, and this marker was utilized for further studies on melanoma samples. Dissociated tumor cells from primary melanoma tumors had an average of 57% CD146+ tumor cells (**Figure 1F**). Furthermore, CD146+ melanoma tumor cells were also present in metastatic melanoma tumors from the lymph nodes. Collectively, these data demonstrate that cryopreserved human tumor samples contain viable tumor cells across numerous indications that are ready for potential downstream applications.

In addition to tumor cells, the tumor microenvironment consists of additional cellular populations (often grouped together as tumor stromal cells) including immune cells, fibroblasts, and endothelial cells[6]. Immune cells represent a large proportion of the stromal cell element within the tumor microenvironment and can have both tumor-promoting and tumor-eradicating functions[7]. CD45+ immune cells were readily identified in dissociated tumor cells (**Figure 2A-B**). Similar to tumor cells, there was patient-specific cellular heterogeneity amongst the different indications tested, with the percentage of CD45+ immune cells ranging from <1% to 99% of the dissociated tumor cells. Overall, high percentage of CD45+ immune cells were observed in kidney, lung, and prostate tumors, while lower percentage of CD45+ were largely observed in bladder, melanoma, and ovarian tumor samples

FIGURE 1. Legend

(A) EpCAM+ cells were analyzed in dissociated single cell suspensions from normal ovary, benign ovary, and malignant ovarian tissue.

(B) Quantification of EpCAM+ cells from normal/benign tissue and 10 different oncology indications.

(C) Surface expression of CD63, CD146, and CD166 on CD45- GlyA- cells in dissociated single cell suspensions from primary melanoma tissue. The black line represents the fluorescence-minus-one (FMO) control.

(D) Correlation of CD63+ and CD146+ tumor cells identified from dissociated primary melanoma cells.

(E) CD63/CD146 and CD45 surface expression was analyzed on dissociated primary melanoma cells.

(F) Quantification of CD146+ cells from dissociated primary melanoma cells and dissociated metastatic melanoma cells from lymph nodes. Data from A, B, F are representative of and compiled from 428 unique patient samples (see Table 1). Data from C,D, E are representative of and compiled from 12 unique patient samples.

(**Figure 2B**). These results correlate with previous reports analyzing the immune cell component of the tumor microenvironment using genomic methods[8, 9]. Cancer-associated fibroblasts and vascular endothelial cells also contribute to the tumor microenvironment. Cancer-associated fibroblasts often have an activated phenotype and aid in tumor progression[10], while endothelial cells constitute the blood vessels that provide nutrients to the tumor[11], and both fibroblasts and CD31+ endothelial cells were present in dissociated tumor cells (**Figure 2C**). Collectively, these observations demonstrate that cryopreserved single cell suspensions generated from numerous oncological indications recapitulates the cellular composition of the tumor microenvironment.

FIGURE 2.

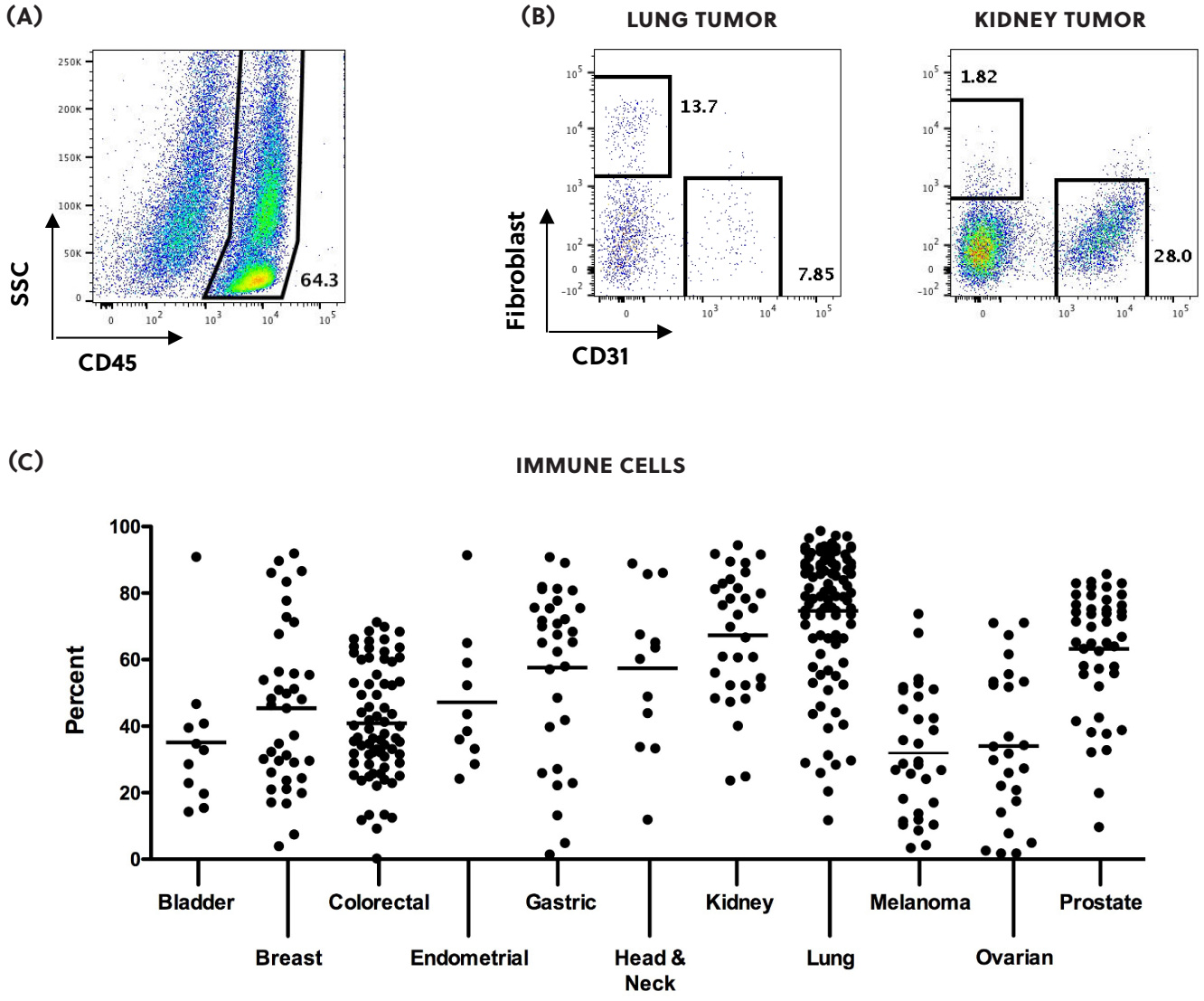
Tumor stromal cells are present in dissociated tumor cells.

(A) CD45+ cells were analyzed in dissociated kidney cancer cells.

(B) Quantification of CD45+ immune cells from 11 different oncology indications.

(C) Fibroblasts and CD31+ endothelial cells were analyzed in dissociated kidney and lung cancer cells.

Data from A, B are representative of and compiled from 419 unique patient samples (see Table 1). Data from C is representative of 9 unique patient samples.



Large Scale Analysis of the Lymphocyte Population within the Tumor Microenvironment

The immune cell compartment in the tumor microenvironment is composed of numerous innate and adaptive immune cell subsets. Numerous reports have highlighted the critical role for T cells within the tumor microenvironment in both promoting and inhibiting tumor progression[7]. Indeed, certain subsets of CD4+ T cells, such as Th17 and T regulatory cells, have been implicated in tumor progression[12, 13]. Conversely, CD8+ T cells can directly recognize and lyse tumor cells, making them attractive candidates for immunotherapeutic intervention[14]. T cells represented the largest component of the CD45+ immune cell population in dissociated tumor cells, with CD4+ T cells, CD8+ T cells, and γδ T cells observed (**Figure 3A**). Surprisingly, across all the indications tested, the average percentage of CD4+ T cells was largely similar, ranging from an average of 13% of CD45+ immune cells in ovarian DTCs to 35% of CD45+ cells in breast cancer DTCs (**Figure 3B**). In contrast to CD4+ T cells, CD8+ T cells had a greater variability both within, and across, indications (**Figure 3C**). The lowest average percentage of CD8+ T cells was observed in ovarian DTCs, while CD8+ T cells represented nearly half of the CD45+ T cells in prostate DTCs, consistent with a previous report that utilized a combination of flow cytometry and immunohistochemistry to demonstrate that CD8+ T cells were the majority population of tumor infiltrating immune cells in prostate cancer[15].

In addition to T cells, two additional lymphocyte populations, B cells and NK cells, are present in the tumor microenvironment[7]. B cells have the ability to generate anti-tumor antibody responses, leading to targeting of tumor cells by macrophages and NK cells[16,17]. Unfortunately, a subset of B cells, known as B regulatory cells, has been identified that suppress anti-tumor immune responses[18]. NK cells, on the other hand, have potent cytotoxic potential, but are often dysregulated within the tumor microenvironment[19, 20]. Both B cells (CD19+ CD20+) and NK cells (CD3- CD56+) are present in dissociated tumor cells (**Figure 4A,B**) and were present at lower percentages than T cells. Across the indications, B cells were very rare in endometrial and ovarian DTCs, but were present at higher percentages in colorectal, gastric, and lung dissociated tumor cells (**Figure 4C**). These results are consistent with a previous study analyzing B cell content via genomic methods from the TCGA database[21]. NK cells, on the other hand, were very rare across all the indications analyzed (**Figure 4D**), being only 1-2% of the CD45+ immune cells. Previous studies demonstrated that NK cells represent a substantial percentage of infiltrating immune cells in kidney cancer[22]. Indeed, dissociated tumor cells from kidney tumors, as well as ovarian tumors, had numerous patients where NK cells represented greater than 10% of the CD45+ immune cell compartment. In total, these results highlight that all major lymphocyte subsets are present in cryopreserved dissociated tumor cells and provide insight into indication-specific trends that are crucial for the next-generation of immunotherapies.

FIGURE 3.

T cells are the major immune cell population in dissociated tumor cells.

(A) CD45+ CD3+ cells from dissociated lung cancer cells were analyzed for CD4+ T cells (CD4+ CD3+), CD8+ T cells (CD8+ CD3+) and γδ T cells (γδ TCR+ CD3+).

(B-C) Quantification of CD4+ T cells and CD8+ T cells from 11 different oncology indications. Data are representative of and compiled from 419 unique patient samples (see Table 1), except for γδ T cells, which are representative of 12 unique patients.

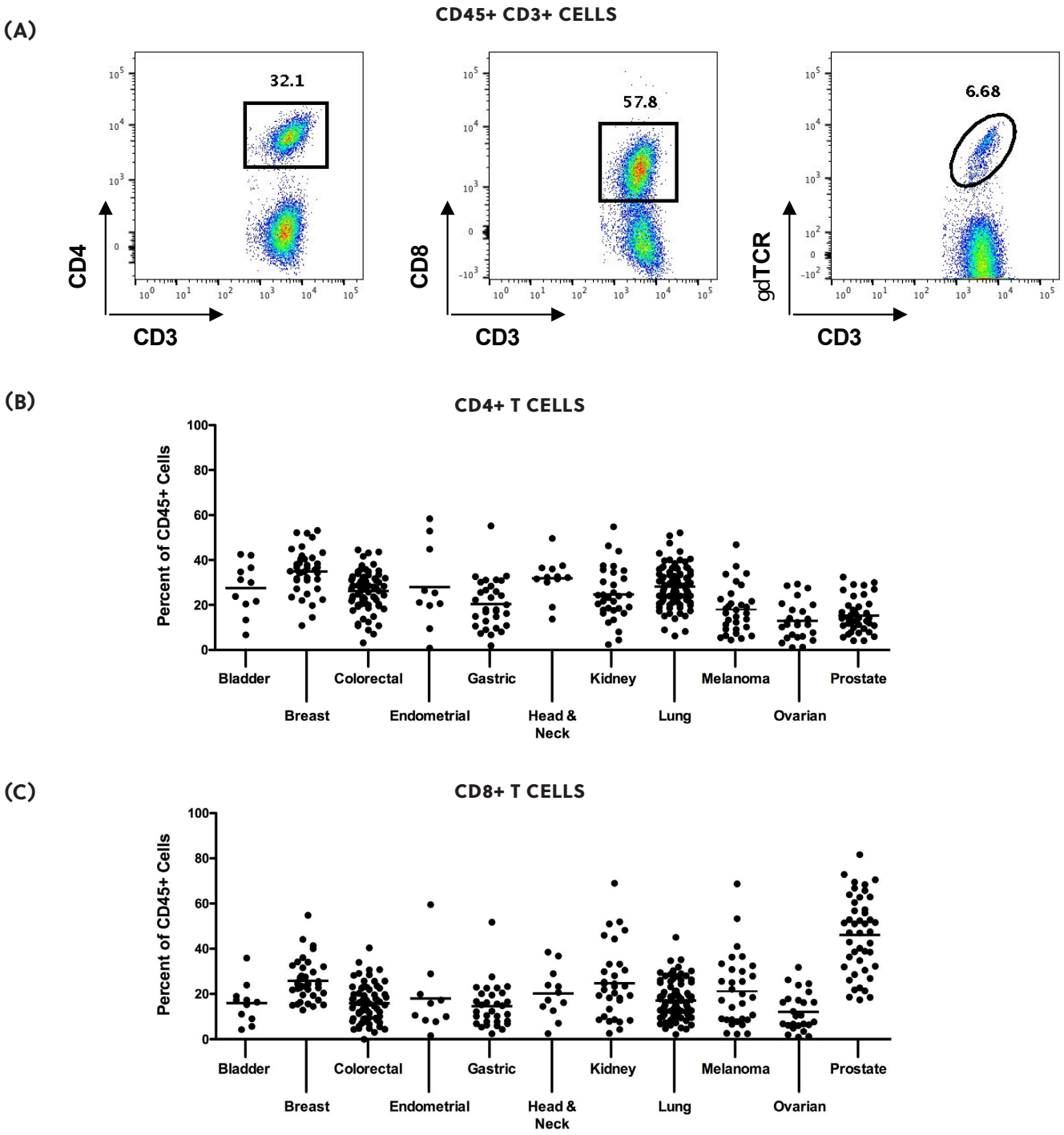


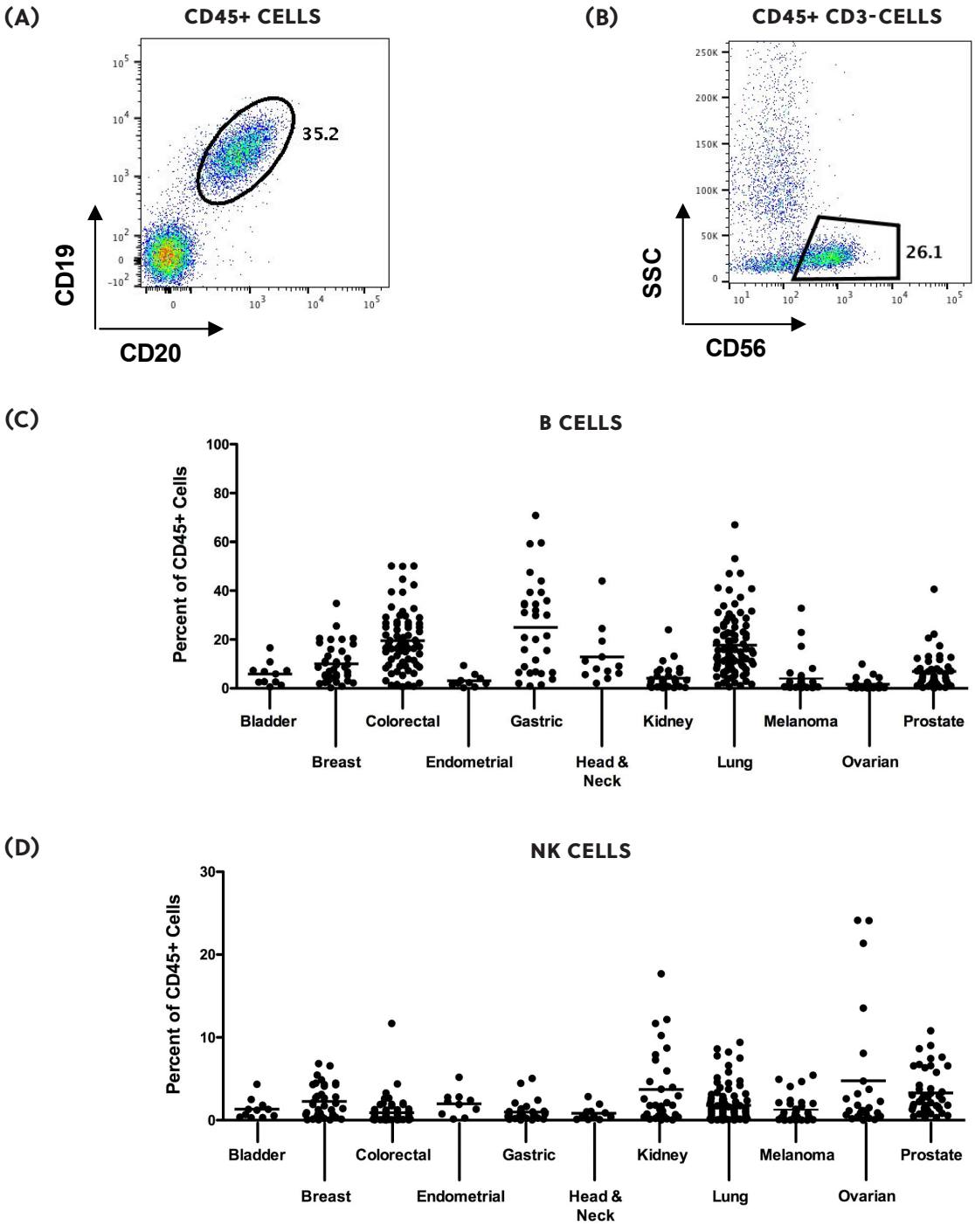
FIGURE 4.

B cells and NK cells contribute to the immune cell compartment in dissociated tumor cells.

(A) CD45+ immune cells from dissociated lung cancer cells were analyzed for B cells (CD19+ CD20+).

(B) CD45+ CD3- cells from dissociated kidney cancer cells were analyzed for NK cells (CD56+).

(C-D) Quantification of B cells **(C)** and NK cells **(D)** from 11 different oncology indications. Data are representative of and compiled from 419 unique patient samples (see **Table 1**).



Myeloid Cells Are Highly Enriched in Kidney, Melanoma, and Ovarian Tumors

Myeloid cells represent a critical component of the tumor microenvironment and aid in tumor progression and eradication[7]. Myeloid cells, identified by surface expression of CD11b, were present in dissociated tumor cells (**Figure 5A left panel**). Myeloid cells can be further subdivided in CD14+ monocytic and CD15+ granulocytic cells (**Figure 5A right panel**). Monocytes and macrophages have important roles in tumor progression through regulation of the anti-tumor immune response, angiogenesis, and tumor metastasis[23]. CD14+ monocytic cells were identified in all indications (**Figure 5B**). In general, the highest percentage of CD14+ monocytes were observed in kidney cancer, melanoma, and ovarian cancer dissociated tumor cells. Interestingly, increased percentages of monocytes are correlated with poor survival prognosis in all three of these indications[24, 25, 26]. Conversely, monocytes were rare, but present, in gastric, head and neck, and prostate cancer dissociated tumor cells. CD15+ granulocytes have the capacity to lyse tumor cells, but growing evidence suggests that these cells have crucial roles in tumor progression[27]. Granulocytes were present in viable cryopreserved dissociated tumor cells, although at lower percentages than observed for monocytic cells (**Figure 5C**). There were significant populations of CD15+ granulocytic cells present in colorectal, gastric, lung, and melanoma dissociated tumor cells, consistent with previous reports analyzing the functions of these cells in these specific indications[28, 29, 30]. Taken together, these results demonstrate that, like their lymphocyte counterparts, myeloid cells represent a significant portion of the tumor microenvironment, and given their role in mediating the immunosuppressive environment within the tumor, highlight the need to understand the cellular composition of the tumor when deciding on the appropriate immunotherapy.

CONCLUSIONS

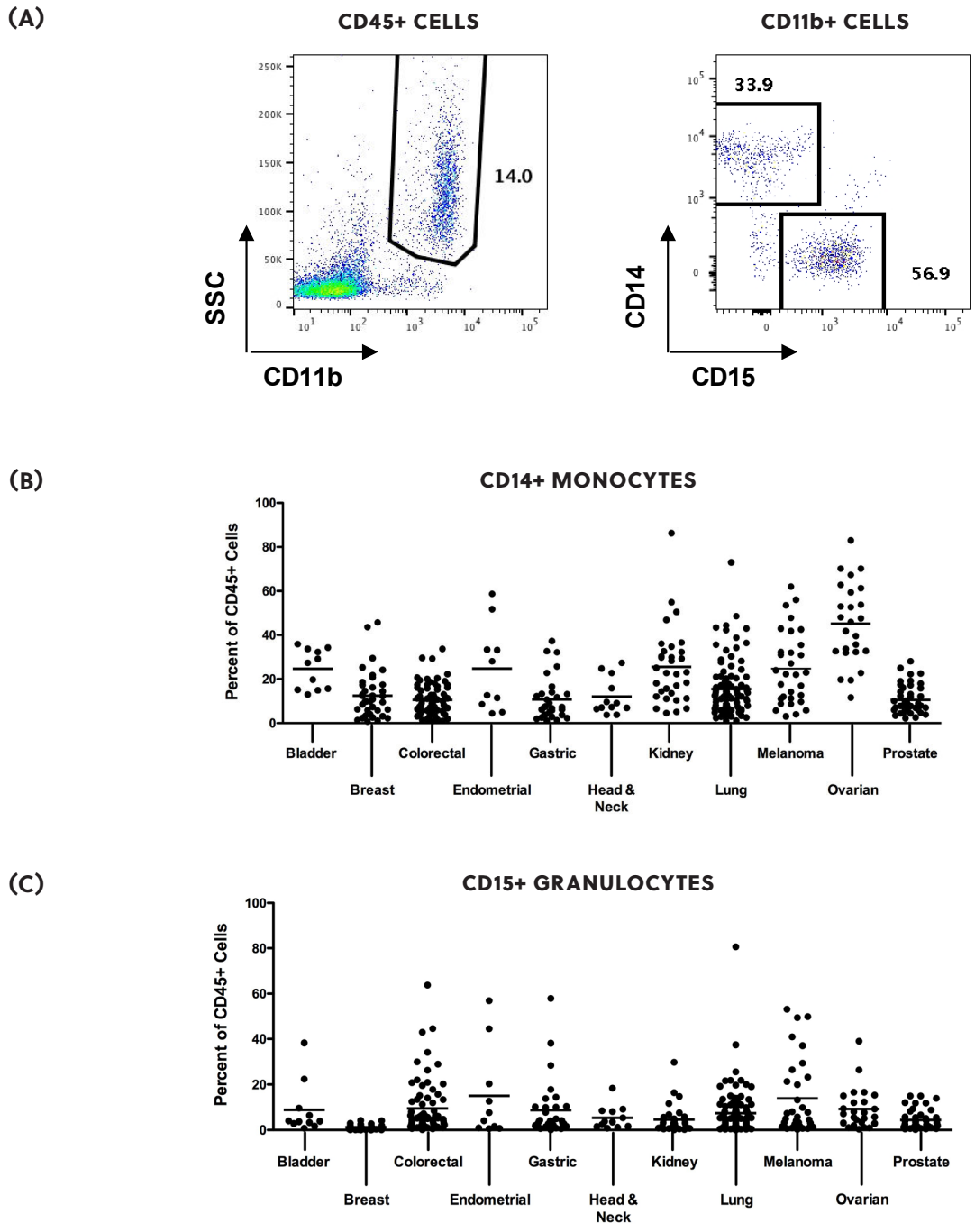
Viably cryopreserved single cell suspensions from solid tissues provide numerous benefits to the logistical demands of sourcing fresh tissue. Using solid tumor indications as a model, we have demonstrated the utility of cryopreserved dissociated tumor cells to understand and screen the cellular composition of the tumor microenvironment. In particular, these results highlight the patient-specific heterogeneity of the tumor microenvironment, but also demonstrate indication-specific trends that are crucial when developing future immunotherapies.

FIGURE 5.

Myeloid cells constitute a significant proportion of immune cells in dissociated tumor cells.

(A) CD45+ cells from dissociated bladder cancer cells were analyzed for myeloid cells (CD11b+). CD11b+ cells were subsequently analyzed for CD14+ monocytic and CD15+ granulocytic cells.

(B-C) Quantification of CD14+ monocytes **(B)** and CD15+ granulocytes **(C)** from 11 different oncology indications. Data are representative of and compiled from 419 unique patient samples (see **Table 1**).



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