Digital spatial proteomic profiling reveals immune checkpoints as biomarkers in lymphoid aggregates and tumor microenvironment of desmoplastic melanoma


ABSTRACT

Background Desmoplastic melanoma (DM) is a rare melanoma subtype characterized by dense fibrous stroma, a propensity for local recurrence, and a high response rate to programmed cell death protein 1 (PD-1) blockade. Occult sentinel lymph node positivity is significantly lower in both pure and mixed DM than in conventional melanoma, underscoring the need for better prognostic biomarkers to inform therapeutic strategies.

Methods We assembled a tissue microarray comprising various cores of tumor, stroma, and lymphoid aggregates from 45 patients with histologically confirmed DM diagnosed between 1989 and 2018. Using a panel of 62 validated immune-oncology markers, we performed digital spatial profiling using the NanoString GeoMx platform and quantified expression in three tissue compartments defined by fluorescence colocalization (tumor (S100+/PMEL+/SYTO+), leukocytes (CD45+/SYTO+), and non-immune stroma (S100−/PMEL−/CD45−/SYTO−)).

Results We observed higher expression of immune checkpoints (lymphocyte-activation gene 3 [LAG-3] and cytotoxic T-lymphocyte associated protein-4 [CTLA-4]) and cancer-associated fibroblast (CAF) markers (smooth muscle actin (SMA)) in the tumor compartments of pure DMs than mixed DMs. When comparing lymphoid aggregates (LA) to non-DM tumor cores, LAs were more enriched with CD20+ B cells, but non-LA intratumoral leukocytes were more enriched with macrophage/monocytic markers (CD163, CD68, CD14) and had higher LAG-3 and CTLA-4 expression levels. Higher intratumoral PD-1 and LA-based LAG-3 expression appear to be associated with worse survival. CTLA-4 and cancer-associated fibroblast (CAF) markers (smooth muscle actin (SMA)) in the tumor compartments of pure DMs than mixed DMs. When comparing lymphoid aggregates (LA) to non-DM tumor cores, LAs were more enriched with CD20+ B cells, but non-LA intratumoral leukocytes were more enriched with macrophage/monocytic markers (CD163, CD68, CD14) and had higher LAG-3 and CTLA-4 expression levels. Higher intratumoral PD-1 and LA-based LAG-3 expression appear to be associated with worse survival.

Conclusions Our proteomic analysis reveals an intra-tumoral population of SMA+ CAFs enriched in pure DM. Additionally, increased expressions of immune checkpoints (LAG-3 and PD-1) in LA and within tumor were associated with poorer prognosis. These findings might have therapeutic implications and help guide treatment selection in addition to informing potential prognostic significance.
INTRODUCTION

Desmoplastic melanoma (DM) represents approximately 4% of all cutaneous melanoma diagnoses. Characterized by invasive spindle-shaped cells within a collagen-dense matrix, DM predominantly affects men in their 60s, commonly manifesting in sun-exposed areas, especially the head and neck region. Compared with traditional melanoma, DM tends to have increased Breslow thickness, has a higher rate of local recurrence, and more frequently displays neurotropism. Around 7%–44% of patients with DM will develop distant metastatic disease, and up to 26% of patients who do not succumb to their disease have a local recurrence.\(^2\)–\(^4\)

DM exhibits one of the highest response rates to single-agent programmed cell death protein 1 (PD-1) blockade therapy of any cancer with objective tumor response rates\(^2\) in up to 70% of patients and complete response in 32% of patients.\(^6\) DMs also have a higher tumor mutation burden than non-DM, which might explain the sensitivity to immunotherapy.\(^7\) However, despite the high mutational load, a recent whole exome sequencing study revealed that DMs do not typically harbor \(BR\)AF \(V600E\) or \(NR\)AS mutations.\(^8\) Instead, they most often present with NF-1 mutations, a UV mutation signature, and less frequently TP53 and ARID2 mutations.\(^7,8\)

Histologically, DM is characterized by fusiform melanocytes dispersed within the collagenous stroma and is conventionally categorized into two subtypes: pure and mixed. While pure DM consists almost entirely (≥90%) of desmoplasia and stromal fibrosis, mixed DM features regions of densely cellular foci with minimal stroma fibrosis (10–90%).\(^9\) Histologic features and immunophenotypes also differentiate DM into spindle cell types and desmoplastic types. Spindle cell melanoma typically has <10% collagen content, mixed spindle/DM has 10–90% collagen, and DM has >90% collagen.\(^10\) Importantly, the clinical implications of various histologic subtypes also vary; mixed DM demonstrates more aggressiveness and a less favorable prognosis. Furthermore, mixed DM is associated with a fourfold increase in mortality or metastatic risk relative to pure DM.\(^11\) The influence of tumor thickness on the risk of locoregional recurrence is not consistent across the literature, which is likely due to the unique interplay of fibrous tissue and neurotropism.\(^12\)

The fibroblastic tumor microenvironment (TME) of DM has unique properties that may inform cancer progression or responsiveness to therapy. One area of interest has been lymphoid aggregates (LA), which frequently house tertiary lymphoid structures (TLS) and are prevalent intratumorally in DM.\(^13\) The presence of TLSs has been associated with improved disease-free survival and a better clinical response to anti-PD-1 antibody therapy in melanoma and other cancers.\(^15\) Such structures can potentially facilitate the expansion of tumor-reactive T cells and B cells, leading to positive prognostic outcomes. The therapeutic effectiveness of PD-1 blockade in DM may also be attributed to its robust tumor-infiltrating lymphocytes (TILs). A higher percentage of programmed cell death protein ligand 1 (PD-L1) positive cells and a greater density of CD8+ T cells reside in the tumor parenchyma,\(^8\) and separately, the presence of CD8+ cytotoxic TILs within DMs is shown to correlate with elevated tumor PD-L1 expression levels.\(^17\) These findings along with the high tumor mutational burden collectively may help explain the profound response to anti-PD-1 targeting agents. Currently, there’s a phase II clinical trial evaluating pembrolizumab (anti-PD-1 inhibitor) in patients with unresectable DM (NCT02775851).

The prognosis and treatment approach for melanoma typically depend on factors such as the thickness of the primary tumor, the presence of ulceration, and the spread to sentinel lymph nodes. However, these conventional prognostic factors do not always apply to DM due to its unique TME and molecular drivers. The utility of sentinel lymph node biopsy (SLNB) in DM remains controversial. Several studies have reported SLNB positivity rates as minimal as 0–4.9% in pure DM and 8–16% in its mixed variant, whereas, in conventional melanoma, the frequency of nodal metastasis in SLNB was 20% in intermediate-thickness melanomas and up to 40% in thick melanomas.\(^18\)\(^19\) Most contemporary single-institution studies with greater than 25 patients have reported lymph node metastasis rates for DM ranging from 6% to 17%.\(^20\)

Importantly, the histologic subtype may play a key role in differentiating prognostic markers given that mixed DMs are more associated with a positive SLNB status and have been shown to be significantly correlated with a worse recurrence-free survival (RFS), suggesting that this could be of clinical importance in selecting patients for adjuvant treatment.\(^21\)

Toward the goal of identifying biomarkers for delineating subtypes and prognosis, we used a new spatially informed method known as digital spatial profiling (DSP). Using the GeoMx system (NanoString Technologies), DSP allows for the quantification of multiplexed proteins or RNAs within predetermined cellular regions while conserving their spatial arrangement.\(^22\)\(^23\) This technique employs oligonucleotide barcodes attached to either antibodies or RNA probes with a photocleavable linker. These are cleaved by designated regions of interest at a near single-cell resolution and collected for quantitative assessment. Importantly, DSP can be conducted on formalin-fixed, paraffin-embedded (FFPE) tissue sections, extending its applicability to tissue microarrays (TMA) as seen in previous work. This compatibility permits a streamlined high-throughput process and the utilization of archived specimens.

The aim of our study is to characterize the TME of DM, the significance of LA and the differences between pure and mixed DM. In addition, we identified several differentially expressed proteins and prognostic markers within the tumor that may improve classification accuracy and potentially inform the suitability of sentinel node biopsy and response to checkpoint inhibitor therapy.
METHODS

Patient cohort and tissue microarrays

We retrospectively collected and analyzed cases of invasive DM from the Melanoma Tumor Registry. With the approval of the Yale University Institutional Review Board, FFPE tissue blocks, and clinical information were procured from the Yale University Department of Pathology Archives. The TMA was constructed as previously described.24–26

Yale TMA-519 (YTMA-519) consists of 0.6 mm tissue cores selected from 45 distinct cases of DM, which were resected between 1989 and 2018 (figure 1A,B). For each tissue specimen, different histological regions were selected and marked by an experienced pathologist, including stroma, LAs, desmoplastic-type, spindle cell-type, and conventional-type melanoma. The majority of cores were of stroma (45), LA (42), and DM (37), whereas spindle cell melanoma (9) and conventional melanoma (7) made up a smaller portion of cores (online supplemental table 2). These areas were previously reviewed and selected by two independent pathologists. No replicates were included in this array. An associated clinical database was constructed for YTMA-519, including tumor pathological characteristics, clinical follow-up data up on which survival and recurrence were based, and systemic treatment regimens for patients who received therapy.

Clinicopathologic variables were retrospectively collected and analyzed. Perineural invasion was defined by the presence of tumor cells in the perineurium or

![Figure 1](A) Histologic section illustrating characteristic features of desmoplastic melanoma for the TMA. (B) Representative spots created by fluorescent colocalization using GeoMx DSP comparing a desmoplastic (1), lymphoid aggregate (2), and stromal core (3). Conventional melanoma (CM) is displayed. Tumor compartment is denoted by S100/PMEL (green), the immune compartment by CD45 (red), and the nuclear stain by SYTO13 (blue). Scale bar: 100 µm. DM, desmoplastic melanoma; DSP, digital spatial profiling; TMA, tissue microarray.
surrounding cutaneous nerves. Mitoses were binarized into two groups: high (≥ 5 per mm²) and low (<4 per mm²). Treatment data is summarized in online supplemental table 1.

**Digital spatial profiling**

DSP was conducted using the GeoMx instrument (NanoString) based on the manufacturer’s guidelines and methods previously outlined.23 27 In brief, slides were first deparaffinized and subjected to antigen retrieval procedures. Subsequently, we stained 5μm TMA sections with three fluorescently labeled antibodies to delineate three different cellular compartments: CD45/SYTO for leukocytes, S100/PMEL/SYTO cocktail for melanoma tumor cells, and CD45 negativity/tumor negativity/SYTO13 positivity for stroma cells. Individual regions of interest (ROI) were drawn around each histospot excluding obvious artifacts.

The TMAs were profiled with 62 immuno-oncology markers, as well as three housekeeping proteins (GADPH, histone H3, and ribosomal protein S6) and three negative controls (mouse IgG1, mouse IgG2a, and rabbit IgG). These markers were identified using photocleavable oligonucleotide-conjugated antibodies, all of which have been validated by NanoString.

Digital barcode counts within each compartment were quantitated by the nCounter platform (NanoString) and used for downstream analysis. Quality control checks were performed as advised by the manufacturer. Initial normalization of digital counts was implemented against internal spike-in controls, as per the External RNA Control Consortium. Subsequent normalization was relative to the geometric mean of two housekeeping proteins (histone H3 and ribosomal protein S6) within each compartment for a specific ROI. The expression levels of the three cellular-molecular within each compartment affirmed the compartmentalization.

**Immunohistochemistry**

Slides were incubated with anti-PD-1 antibody (Cell Marque, clone: NAT105, 1:100) at 4°C overnight. The slides were washed thrice in 1× TBS/0.05% Tween 20 and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Envision; Dako) for 2 hours. After three TBS-T washes, develop with DAB substrate (Vector) at room temperature. Slides were counterstained with hematoxylin, mounted, and imaged. All tissue cores were reviewed independently using the S100/HMB-45 DSP tumor mask as a reference. Staining was regarded as positive if the percentage of positively stained cells ≥ 5% and the intensity on the membrane of tumor cells was ≥2+. (using a semiquantitative scale of 0–3: 0 for no staining, 1+ for weak staining, 2+ for moderate staining, and 3+ for strong staining).

**Statistical analysis**

For statistical analysis, the quantified protein counts within each compartment generated by the nCounter platform were used for all downstream analysis. For differential protein expression analysis, Mann-Whitney tests with Benjamini-Hochberg correction were used to calculate the false-discovery rate. Matched-pair analysis was performed using the mean normalized marker count and non-parametric Wilcoxon signed-rank test. Univariate and multivariable Cox proportional hazards analyses were performed using the mean normalized marker count. Overall survival (OS) and RFS analyses used median levels of a marker within a tumor type and compartment as the cut-point. Kaplan-Meier plots were generated, and log-rank tests were performed. Pearson’s correlation (R²) was computed for regression within GeoMx DSP. All statistical testing was performed with a two-sided p<0.05 considered significant. All data sets were analyzed and visualized using GraphPad Prism V.9 for Mac (GraphPad Software, La Jolla, California, USA) or R Studio V.2023.03.1+446.

**RESULTS**

**Patient selection**

Patients represented in the DM TMA are described (table 1; online supplemental table 1). 75% of patients were men, and the mean age at diagnosis was 73 years. We profiled a total of 139 ROIs from 45 unique patients with invasive DM. After processing and normalization steps, 33/42 cores of LA (nearly all intratumoral), 23/45 stromal cores, 26/36 desmoplastic cores, 9/9 spindle cores, and 5/7 conventional melanoma cores were deemed sufficient for downstream analysis based on GeoMx quality control thresholds and abundance of normalized barcode and cell counts (online supplemental table 2). Of all specimens, 81% had pure histology, while 19% had mixed histology. 80% of primary tumors were in the head and neck region. Approximately half of the patients underwent an SLNB (51%), of which 3 (15%) were found to have melanoma. Nearly all patients were diagnosed initially without metastases (96%). Of all patient samples, 27% had perineural invasion, 17% had ulceration, and 13% had greater than 5 mitoses. In terms of outcomes, 44% of patients had recurrence with over half being local recurrence (45%) and in-transit melanomas (5%) and the other half distant metastases (50%) (online supplemental table 1).

**Region-specific heterogeneity between desmoplastic melanoma tumors and microenvironment**

DM is markedly a morphologically heterogeneous malignancy. To assess intratumoral proteomic heterogeneity, up to four regionally distinct cores or ROIs were chosen per tumor specimen to represent a range of histologic regions, including tumor pathologies (desmoplastic, spindle cell, conventional melanoma), stroma, and LA (figure 1A). Fluorescent images of representative spots from histologic subtypes are shown in figure 1B.

Unsupervised hierarchical clustering was used to investigate the relatedness of the protein expression profiles...
across different histologic core types for each compartment (figure 2A–C). This analysis illustrates the proteomic heterogeneity seen across intratumoral core types and its TME for each compartment. Principal component analysis showed a relative divergence between the CD45+ compartments of the spindle and that of desmoplastic ROIs (online supplemental figure 1A). In contrast, the tumor and stromal compartments showed a closer relation across a variety of histologic core types (online supplemental figure 1B,C). When comparing pairs of compartments (immune, tumor, and stroma), a broad spectrum of differential marker expression was observed in all DMs and pure DM alone (online supplemental figure 2). Together, these data show that DM is comprised of multiple regionally distinct protein expression profiles, highlighting its intrinsic heterogeneity.

Identification of differentially expressed proteins in pure versus mixed desmoplastic melanoma

We performed differential expression analysis between pure and mixed histologic patterns for all protein analytes across compartments. All marker counts were previously normalized to the geometric mean of two housekeeping markers (histone H3 and ribosomal protein S6) within each compartment for a given ROI. As shown in the volcano plot (figure 2D), we observed several immune-related and stroma-related protein targets in the S100/PMEL enriched compartment. Smooth muscle actin (SMA) levels were higher in pure DM samples compared with those in mixed DM samples. Similarly, the expression levels of cytotoxic T-lymphocyte associated protein-4 (CTLA-4) and lymphocyte-activation gene 3 (LAG-3) were found to be higher in pure than mixed DMs, (p=0.016, p=0.026, respectively; figure 2E). No markers were differentially expressed in the CD45+ and stroma compartments, as shown in online supplemental figure 3.

Differential expression patterns across distinct clinicopathologic characteristics

Next, we conducted a comparative analysis of known relevant clinicopathologic variables for DM. We first compared groups with and without perineural invasion across the three main compartments (CD45, stromal, and S100/PMEL). Within the tumor (S100/PMEL)-enriched compartment, Ki-67 and GZMB exhibited higher relative levels of expression within samples exhibiting perineural invasion (figure 3A). Given the discordant behaviors and the differences in histologic content between pure and mixed DM and the over-representation of pure DM in our sample set, we performed the same analysis excluding mixed DM (figure 3B), which notably did not affect the markers’ significance.

In figure 3C,D, we examined cases of recurrence. In contrast to those without recurrence, individuals with recurrence demonstrated elevated PD-1 levels within the tumor compartment (S100+/PMEL+). Additionally, within the group of individuals with recurrence, CTLA-4 expression was higher in the CD45-enriched subset of LAs. There were no statistically significant differences in marker expression in tumors with perineural invasion or tumors from patients with recurrence in the

<table>
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<th>Table 1 Patient demographics and tumor characteristics</th>
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<td>AJCC, American Joint Committee on Cancer; LN, lymph node; SLNB, sentinel lymph node biopsy .</td>
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Figure 2  (A–C) Heatmap of digital spatial profiling protein z-scores for log2(count) for all markers across different histologic core types by S100/PMEL (A) CD45 (B) and stroma (C) compartments. (D) Differential marker expression of pure versus mixed DMs. Volcano plot depictions of the log2(fold-change) in marker expression on the x-axis and the log10(p value) on the y-axis for the relevant markers for each tissue compartment. Fold-change values and statistical testing were performed using Mann-Whitney tests with Benjamini-Hochberg correction. Markers with fold-change cut-off >1 are depicted with green dots, markers with p value cut-off >0.05 are represented with blue dots, and markers exceeding both thresholds are depicted with red dots. Sample numbers are found in parentheses. (E) Box and whisker plots displaying logarithm base two-normalized counts of significant markers, comparing pure and mixed DM. DM, desmoplastic melanoma; SMA, smooth muscle actin.
Figure 3  Differential marker expression by clinicopathologic variables. Volcano plot depictions of the log2(fold-change) in marker expression on the x-axis and the log10(p value) on the y-axis for the relevant markers for each clinical characteristic: (A) perineural invasion; (B) perineural invasion without mixed DM; (C) recurrence; (D) recurrence without mixed DM. Fold-change values and statistical testing were performed using Mann-Whitney tests with Benjamini-Hochberg correction. Markers with fold-change cut-off >1 are depicted with green dots, markers with p value cut-off >0.05 are represented with blue dots, and markers exceeding both thresholds are depicted with red dots. Sample numbers are found in parentheses. DM, desmoplastic melanoma; SMA, smooth muscle actin.
stromal compartment. Due to the small sample size, pure DM could not be directly compared with conventional melanoma.

Interestingly, cases characterized by low mitotic activity exhibited a paradoxically higher expression of SMA. Additionally, in cases with a higher number of mitoses, the expression of CD163, a marker associated with tumor-associated macrophages (TAMs), was found to be significantly elevated (online supplemental figure 4A,B). Notably, the presence of ulceration was associated with a high expression of CD66+ neutrophils, while the absence of ulceration correlated with elevated expression levels of many markers (online supplemental figure 4C,D). These findings underscore the intricate relationships between specific pathologic attributes and molecular markers within the DM microenvironment, revealing potential insights into the interplay between cellular and immune components.

Given the poor prognostic outcomes observed in male patients with DM, we examined gender-based differences in marker expression. We observed a non-significant trend of men having worse RFS and OS in our cohort as compared with women (p=0.19, p=0.27, respectively; online supplemental figure 5A). Male patients exhibited higher levels of PD-L2 in the immune compartment (p=0.007) and CTLA-4 levels in the stromal compartment (p=0.049), compared with female patients (online supplemental figure 5B,C).

**Marker expression differences between lymphoid aggregates and intratumoral leukocytes**

LA are a common histologic characteristic in DM and are theorized to hold biological significance in patients who respond to immunotherapy. To study how the expression profiles of LAs differ from isolated immune cells in the tumor, we performed pairwise analyses comparing the CD45+ compartment in LA cores and the CD45+ compartment of tumor cores (non-LA). CD20+B-cell marker was higher in LA, whereas markers of monocytic lineage (CD163, CD68, and CD14) were found to be elevated in intratumoral leukocytes outside of LA (figure 4A–C,E). In addition, immune checkpoint molecules such as LAG-3 and CTLA-4 were downregulated within LA (figure 4D,F). Since LAs are often sites of immune “hotspots” akin to TLS, downregulating checkpoints may allow for robust immune priming and activation as well as downstream effector functions. Intratumoral leukocytes may encounter a more immunosuppressive TME that triggers the upregulation of checkpoint molecules as a means of tumor immune evasion. There were no major differences in the markers of T cells (CD3, CD4, CD8),

![Figure 4](http://jitc.bmj.com/)

**Figure 4** Pairwise comparisons in marker expression between lymphoid aggregates (LA) and CD45+leukocytes from intratumoral cores for CD163 (A) CD68 (B) CD14 (C) LAG-3 (D) CD20 (E) and CTLA-4 (F). Comparisons were performed with non-parametric pairwise analyses. Cytotoxic T-lymphocyte associated protein 4, CTLA-4; lymphocyte-activation gene 3, LAG-3.
DISCUSSION

In this study, we examined the expression of 62 immuno-oncology-related markers in the leukocyte (CD45+), tumor (S100+/PMEL+), and stromal (CD45−/tumor−) compartments of tissue specimens on a TMA representing various histologic regions of DM. Within the multifaceted molecular landscape of DM, we revealed several noteworthy observations that underscore the unique characteristics of pure DM, when compared with mixed DM, with potential clinical implications. First, we observed a higher expression of specific immune checkpoints and cytolytic markers in pure DMs. The pronounced expression of CTLA-4 and LAG-3 in the regions of pure DM containing CD45-enriched LA suggests an immunosuppressive TME that might facilitate tumor evasion from host immunity. Furthermore, we observed higher expression levels of LAG-3 and CTLA-4 in intratumoral leukocytes outside LAs as compared with those within LA. T-cell lymphocytes exhibiting these markers are often indicative of an exhausted or dysfunctional phenotype. Numerous clinical trials have combined anti-CTLA-4 and recently anti-LAG-3 with PD-1 blockade in the treatment of advanced melanoma.26–30 The recent Relativity-047 trial showed that dual inhibition of LAG-3 and PD-1 improves progression-free survival more than single-agent PD-1 blockade in patients with unresectable or metastatic melanoma, suggesting there may be an advantageous benefit to dual LAG-3 and PD-1 inhibition in treating patients unresponsive to PD-1 monotherapy.31 Finding differences in expression patterns of immune checkpoints such as CTLA-4 and LAG-3 in different DM histologies and intratumoral features may be critical to understanding DM and may have clinical implications in guiding responses to various immunotherapeutic strategies. Since our study centered on primary DM, further studies are needed to determine whether these characteristics of LAs are preserved in DM metastases.

The hallmark of DMs is the proliferation of fibrous connective tissue, which may help explain the concomitant elevations in alpha SMA and fibronectin. Our study found that the relative protein expression of SMA may be higher in pure than mixed DM, a marker previously shown to be histologically characteristic of reactive myofibroblasts.31 Many cancers such as cholangiocarcinomas and pancreatic ductal adenocarcinomas induce desmoplastic reactions, marked by an accumulation of SAMP-positive cancer-associated fibroblasts (CAFs), producing more extracellular matrix proteins (eg, fibronectin) and pro-invasive growth factors as a means to cultivate an immunosuppressive TME and augment malignant behavior and therapeutic resistance.32–34 CAFs have been shown to drive local tumor progression and metastasis by modulating the local TME.35 Of note, the fibroblast activation protein (FAP)-associated CAF population, which functions through fibrogenesis and remodeling of the extracellular matrix, was notably not associated with either histologic subtype. In DM, a recent preclinical study is employing a nanoemulsion to deliver an anti-tumor vaccine fraxinella, targeting both fibrosis-inducing CAFs and cancer cells.36 Combining this vaccine with the tyrosine-kinase inhibitor sunitinib reduces collagen, inhibits CAFs, enhances drug permeability, and reduces immunosuppressive cells, suggesting that modifications of the TME may improve therapeutic efficacy and drug delivery.36 The elevated expression of SMA may suggest that pure DMs are more susceptible to this or other strategies targeting CAFs.

LA similarly play a role in desmoplasia and response to therapy. Stowman et al noted that a vast majority of LA...
In lymphoid aggregates, higher expression of LAG-3 is associated with worse overall survival and higher expressions of CD8, PTEN, OX40L, and SMA portend a worse recurrence-free survival. Multivariable Cox proportional hazards regression analyses for significant factors identified by univariable analysis for CD45+ compartment of lymphoid aggregates using: (A) OS after diagnosis; (C) RFS using all samples; and (G) RFS (using only pure desmoplastic melanoma samples). (B, D, E, F, H) Kaplan-Meier plots of RFS and OS after diagnosis, with the corresponding HRs and log-rank p values, based on dichotomized (by median) expression of the indicated marker in lymphoid aggregates. All plots were cut off at 200 months for visualization purposes, with no meaningful changes to the data. CTLA-4, Cytotoxic T-lymphocyte associated protein 4; LAG-3, lymphocyte-activation gene 3; OS, overall survival; RFS, recurrence-free survival; SMA, smooth muscle actin.
Higher expression of intratumoral PD-1 marker portends a worse recurrence-free survival. Multivariable Cox proportional hazards regression analyses for RFS using significant factors identified by univariable analysis for: (A) S100+/PMEL+ compartment of all tumor cores; (C) S100+/PMEL+ compartment of only desmoplastic tumor cores; and (E) S100+/PMEL+ compartment of only pure desmoplastic melanoma. (B, D, F) Kaplan-Meier plots of RFS, with the corresponding HRs and log-rank p values, based on dichotomized (by median) expression of the indicated marker. All plots were cut off at 200 months for visualization purposes, with no meaningful changes to the data. PD-1, programmed cell death protein 1; RFS, recurrence-free survival.
in pure DM represent TLS. In contrast to conventional melanoma subtypes, where TLS remain predominantly in the peritumoral areas, TLS in DM are typically found intratumorally. We observed several notable differences when comparing the molecular makeup of LA to that of intratumoral leukocytes outside of LAs. While LA were enriched with CD20 expressing B-cell component, intratumoral leukocytes outside of LAs displayed higher levels of CTLA-4 and LAG-3, potentially indicating a shift toward a state of T-cell exhaustion. This same leukocytic compartment showed elevations in CD68+ TAMs, CD14+ monocytes, and CD163+M2-phenotype-specific macrophage markers, which are often associated with recurrence and poor survival in melanoma as well as immunosuppression, tissue repair, and tumor promotion. Hence, the contrasting profiles paint a complex balance in the immune landscape in DM. On the one hand, LA house a higher density of B-cell lymphocytes to serve as potential hotspots of immune activity, while on the other, the markers within intratumoral leukocytes externally hint at a tumor environment striving to dampen the immune response and potentially promote tumor progression. This dual nature may underscore the interaction of DM tumors with the immune system and highlight the possibility that DMs could be reactivated with therapies like immune checkpoint inhibitors, where the delicate balance is often interrupted to favor antitumor immunity. An interesting next step would be to compare these data to non-DM to assess differences between LA in DM when compared with peritumoral TLSs in conventional melanoma.

Owing to the broad range of treatment dates, only eight patients received immunotherapies, including six individuals treated with immune checkpoint blockade. Remarkably, among these, five of six patients exhibited either a partial or complete response. However, due to the constraints of a limited sample size, a comprehensive analysis could not be performed (online supplemental table 2). Drawing comparisons with cohorts subjected to immunotherapy could aid in the identification of patients who could derive advantages from checkpoint inhibitor therapy, whether in the context of adjuvant treatment or for those facing distant metastatic disease. This holds particular significance as recent studies show that up to 70% of patients with metastatic DM exhibit a positive response to PD-1 blockade.

The findings from digital spatial profiling are subject to technological constraints, specifically the absence of single-cell resolution using GeoMx. The assessment of protein expression was restricted to predefined compartments based on morphology markers. This limitation prevented in-depth analysis of spatial heterogeneity within LA and intratumoral subsets. Future investigations achieving a more detailed subcellular perspective would be beneficial. This is especially pertinent since LA are known to host a heterogeneous array of immune cells and activation markers, a level of granularity that is not visualizable when regions are analyzed in aggregate. Altogether, DSP had the advantage of using archived FFPE tissue, allowing us to examine 45 unique DM specimens in a high-throughput manner. Additional limitations of our study merit attention. The rarity of DM and restrictions in tissue accessibility constrained our analyses of subgroups such as mixed DMs and female patients. In addition, while different regions from each tumor section were cored to account for intratumoral heterogeneity, the TMA did not contain replicates from each representative region to address the known intraregional heterogeneity within melanoma. Lastly, mutational data among patients were not accessible for conducting subgroup analyses and necessitates further exploration.

To date, efforts to uncover predictive biomarkers of therapy response such as CD8+ T-cell infiltration, PD-L1 immunostaining, and tumor mutational load (TMB) have led to limited results. In many cancers, proteins secreted to the extracellular matrix by tumor cells, CAF, and myeloid-derived suppressor cells modulate the tumor-host relationship and the capacity of malignant cells to survive and migrate out to regional and distant sites. While generally a favorable prognostic marker in many types of melanomas, higher densities of CD8+ T cells and granzyme B-expressing cells in the LA were associated with worse RFS in DM on multivariable analyses. This can be seen in some immunogenic cancers including renal cell carcinoma. We were unable to assess the colorization of markers such as PD-1 or PD-L1 within the CD8 positive cells with this technology.

In summary, our study used digital spatial profiling of immuno-oncology-related proteins to examine the molecular nuances in DM, underscoring both the proteomic heterogeneity of the tumor environment and the spatial distribution of checkpoint marker expressions. We found that compared with mixed DMs, pure DMs have higher expressions of CTLA-4 and LAG-3 as well as SMA, which may indicate a prognostically important SMA+CAF population. We also found that intratumoral leukocytes outside of LA carry higher expressions of CTLA-4 and LAG-3 than immune cells within LA. Additionally, a higher expression of LAG-3 within LAs was associated with worse OS, and a higher expression of intratumoral PD-1 predicts a shorter RFS in DMs. These findings may have potential prognostic implications and provide new avenues for therapeutic combinations, including targeted therapies and immunomodulation. Further studies are warranted to both validate the preliminary biomarker findings in larger cohorts and to elucidate the functional implications and mechanisms of these markers in DM.

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Supplementary Figure 1. Proteomic overlap among cores by category. PCA plots show the distribution of cores retrieved by their compartment: immune (A), tumor (B), and stroma (C). Cases are shown as colored circles: conventional melanoma (orange), desmoplastic melanoma (yellow), cores of lymphoid aggregates (green), spindle cell melanoma (blue), and stromal cores (pink).
Supplementary Figure 2. Differential marker expression by compartment. Volcano plot depictions of the log$_2$(fold-change) in marker expression on the x-axis and the log$_{10}$(P-value) on the y-axis for the relevant markers: (A) Comparisons among pairs of CD45, S100/PMEL, and stroma compartments in all DM samples; (B) Comparisons among pairs of CD45, S100/PMEL, and stroma compartments in only pure DM. Fold-change values and statistical testing were performed using Mann-Whitney tests with Benjamini-Hochberg correction. Markers with fold-change cutoff > 1 are depicted with green dots, markers with p-value cutoff > 0.05 are represented with blue dots, and markers exceeding both thresholds are depicted with red dots.
Supplementary Figure 3. Differential marker expression of pure and mixed DM by CD45+ and stromal compartments. Volcano plot depictions of the log₂(fold-change) in marker expression on the x-axis and the log₁₀(P-value) on the y-axis for the relevant markers. Fold-change values and statistical testing were performed using Mann-Whitney tests with Benjamini-Hochberg correction. Markers with fold-change cutoff > 1 are depicted with green dots, markers with p-value cutoff > 0.05 are represented with blue dots, and markers exceeding both thresholds are depicted with red dots. Sample numbers found in parentheses.
Supplementary Figure 4. Differential marker expression by clinicopathologic variable. Volcano plot depictions of the \( \log_2 \) (fold-change) in marker expression on the x-axis and the \( -\log_{10} \) (P-value) on the y-axis for the relevant markers for each clinical characteristic: (A) binarized mitotic rate; (B) binarized mitotic rate (without mixed DM); (C) ulceration; and (D) ulceration (without mixed DM). Fold-change values and statistical testing were performed using Mann-Whitney tests with Benjamini-Hochberg correction. Markers with fold-change cutoff > 1 are depicted with green dots, markers with p-value cutoff > 0.05 are represented with blue dots, and markers exceeding both thresholds are depicted with red dots. Sample numbers found in parentheses.
Supplementary Figure 5. Differential marker expression by sex. (A) Kaplan-Meier plots of OS and RFS dichotomized by sex with corresponding log-rank p values (B) Marker expression by sex depicted by volcano plot with the log_{2}(fold-change) in marker expression on the x-axis and the log_{10}(P-value) on the y-axis for the relevant markers. Mann-Whitney tests with Benjamini-Hochberg correction was performed. Markers with fold-change cutoff > 1 are depicted with green dots, markers with p-value cutoff > 0.05 are represented with blue dots, and markers exceeding both thresholds are depicted with red dots. Sample numbers found in parentheses. (C) Significant markers from (B) were compared using Mann-Whitney tests.
Supplementary Figure 6. Marker expression between lymphoid aggregates (LA) and intra-tumoral CD45+ leukocytes outside the lymphoid aggregates.
**Supplementary Figure 7.** Expression patterns of PD-1 using immunohistochemistry (IHC) staining intensities of protein levels. PD-1 expression is measured within the tumor mask (S100+/PMEL+) generated by DSP. (A) Representative images of a histospot with strong and weak PD-1 staining are shown. Scale bar: 50 µm. (B) Stacked bar chart highlighting differences in number of subjects with PD-1 positive versus negative cases by IHC ($r = 0.55, p = 0.006$). (C) Kaplan-Meier plots of RFS and OS with corresponding log-rank p values, using PD-1 expression dichotomized by median.