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Tumor Mutation Burden and Structural Chromosomal Aberrations Are Not Associated with T-cell Density or Patient Survival in Acral, Mucosal, and Cutaneous Melanomas

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ABSTRACT

Tumor mutation burden (TMB) has been proposed as a key determinant of immunogenicity in several cancers, including melanoma. The evidence presented thus far, however, is often contradictory and based mostly on RNA-sequencing data for the quantification of immune cell phenotypes. Few studies have investigated TMB across acral, mucosal, and cutaneous melanoma subtypes, which are known to have different TMB. It is also unknown whether chromosomal structural mutations [structural variant (SV) mutations] contribute to the immunogenicity in acral and mucosal melanomas where such aberrations are common. We stained 151 cutaneous and 35 acral and mucosal melanoma patient samples using quantitative IHC and correlated immune infiltrate phenotypes with TMB and other genomic profiles. TMB and SVs did not correlate with the densities of $CD8^+$ lymphocytes, $CD103^+$ tumor-resident T cells (Trm), $CD45RO^+$ cells, and other innate and adaptive immune cell subsets in cutaneous and acral/mucosal melanoma tumors, respectively, including in analyses restricted to the site of disease and in a validation cohort. In 43 patients with stage III treatment-naïve cutaneous melanoma, we found that the density of immune cells, particularly Trm, was significantly associated with patient survival, but not with TMB. Overall, TMB and chromosomal structural aberrations are not associated with protective antitumor immunity in treatment-naïve melanoma.

Introduction

Tumor-infiltrating lymphocytes (TIL), particularly cytotoxic $CD8^+$ T cells, have long been recognized as a prognostic factor for patient overall survival in multiple cancers types (1) and are also associated with response to anti–PD-1 and anti–CTLA-4 immunotherapies (2–4).

Patient tumors with a higher tumor mutation burden (TMB), the result of increased single-nucleotide variants (SNV), are thought to create a proportionally higher number of neoantigens. These neoantigens are recognized as foreign by the immune system, such that an antitumor response can be generated; hence, the greater the number of neoantigens, the greater the immune infiltrate within the tumor (5, 6). This theory is based on (i) the observation that tumor types displaying a high TMB are generally more immunogenic (able to generate an immune response and have greater TILs) than low TMB tumor types (7), (ii) tumors with higher TMB are generally more responsive to anti–PD-1 checkpoint inhibitor immunotherapy (5, 8, 9), and (iii) neoantigen-specific T cells exist in tumors and are capable of causing tumor regression (10, 11). Despite these observations, data demonstrating a direct relationship between TMB and immune infiltration within specific cancer types are limited, weak, and often contradictory. First, most, if not all, of the studies exploring this relationship have relied on genomic/RNA-sequencing data, in which immune gene expression has been used as a surrogate for measuring immune infiltration (12–16). Second, even within genomic studies, there are mixed results in the literature regarding the relationship between TMB and immune cell presence. Some studies have reported increased immune gene expression in high TMB patients within certain cancer types (13, 17), whereas other studies found no correlation in the majority of cancers (15), including melanoma (14). Yet, some studies reported weak correlations ($r < 0.16$) to describe the relationship between TMB and TIL densities (12). Some studies have also reported higher immune gene expression within low TMB patients across multiple cancer types (16). Although it is unclear what is the exact contribution TMB and neoantigen numbers have toward the magnitude and type of an antitumor immune response, it is widely assumed

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that higher TMB is positively correlated with a higher immune infiltrate in tumors of the same cancer type.

In addition to mutations arising from SNV changes, several cancer subtypes harbor other types of mutations. In melanoma, for example, it has been shown that acral and mucosal melanomas contain more chromosomal structural rearrangement mutations, also termed as structural variant (SV) mutations, than cutaneous melanoma (18). Whether or not these types of mutations are immunogenic or correlate with an increased immune infiltrate in acral and mucosal melanoma tumors is unknown. In this study, we explored the relationship between TMB, SVs, and tumor-infiltrating immune cell phenotypes, including tumor-resident $CD8^+$ T cells, in 151 cutaneous and 35 acral and mucosal melanoma tumors using quantitative, multiplex immunofluorescence assays. We also validated our findings in a separate cohort of 67 patients with melanoma. We found that TMB and SVs were not associated with the presence of a broad range of immune cell phenotypes in melanoma tumors, including tumor-resident $CD8^+$ T cells. Although the presence of $CD8^+$ T cells and tumor-resident $CD8^+$ T cells was positively associated with patient overall survival, this was independent of TMB. Our data suggest that TMB and SVs are not determining factors for the presence or absence of immune infiltrates within melanoma tumors.

Materials and Methods

Patients

All patient biopsies were treatment naïve at the time of collection. Fresh-frozen tumor tissues from 186 patients and 67 patients were used in the primary and validation cohorts, respectively, for the generation of genomic data, as described previously (18). Patients with corresponding formalin-fixed, paraffin-embedded (FFPE) tumor tissue were included in the study, and FFPE tumor tissue for each of the patient specimens was stored at room temperature until use for the generation of multiplex IHC data. Patients included in survival analyses did not receive any systemic immune checkpoint or targeted therapy for the duration of their clinical follow-up. The study was conducted in accordance with the National Health and Medical Research Council of Australia's National Statement on Ethical Conduct in Human Research. The study was undertaken with institutional Human Ethics Review Committee approval and patient's written informed consent.

Genomic data

Genomic data were generated previously by Hayward and colleagues (18). SNVs and SVs were defined and analyzed from whole-genome sequencing data as described previously (18). All SNVs represented protein-coding mutations using methods described previously (18). All somatic variants for this study have been deposited in the International Cancer Genome Consortium Data Coordination Centre and are publicly available at https://dcc.icgc.org, under project ID MELA-AU. The BAM files have been deposited in the European Genome-phenome Archive (https://www.ebi.ac.uk/ega/) with accession number EGAS00001001552. Where appropriate, neoantigen load was calculated using the pVAC-Seq (v4.0.10) pipeline (19), whereas the NetMHCpan algorithm (20) was used to estimate binding affinity to HLA genotypes.

Multiplexed immunofluorescence staining

Immunofluorescence staining was carried out on 4-μm thick sections using an Autostainer Plus (Dako – Agilent Technologies) and Opal Multiplex IHC Assay Kit (Akoya Biosciences) with appropriate positive and negative controls, as reported previously (21). Briefly, FFPE tumor specimens were deparaffinized and rehydrated by xylene and an Ethanol gradient (100%, 95%, and 70%; Sigma-Aldrich). Heat-induced antigen retrieval (AR) was performed at 95°C for 20 minutes in pH 9 AR Buffer (Akoya Biosciences). Sections were then cooled and incubated with 3% Hydrogen Peroxide (Sigma-Aldrich) for 10 minutes at room temperature, followed by incubation with a single primary antibody against CD8 (1:500; SP16, Cell Marque), CD103 (1:800; EPR4166, ref. 2, Abcam), PD-L1 (1:2,000; E13LN, Cell Signaling Therapeutics), CD45RO (1:1,000; UCHL-1, Dako), CD11c (1:1,000; EP1347Y, Abcam), CD68 (1:500; Kp-1, Cell Marque), CD4 (1:500; 4B12, Biocare Medical), CD20 (1:250; L26, Biocare Medical), or SOX10 (1:800; BC34, Biocare Medical) for 30 minutes at room temperature. Following this, samples were either incubated with Opal Polymer HRP (Akoya Biosciences) for 30 minutes (CD103) or incubated with the MACH3 Probe/HRP-Polymer Kit (Biocare Medical) for 10 minutes (CD8, PD-L1, CD45RO, and SOX10). Finally, sections were incubated with opal fluorophores at 1:100 dilution made up in Tyramide Signal Amplification Reagent (Opal 7-Color IHC, Akoya Biosciences). The AR step was repeated for subsequent stains on the same slide. On the last staining run, DAPI was added to the sample for 5 minutes. All samples were cover slipped using Vectashield (H-1400) and left overnight to dry at 4°C.

IHC staining

Melanoma paraffin-embedded tissue sections were cut and prepared for staining as described above, with appropriate positive and negative controls. Sections were incubated with a single primary antibody against CD8 (1:200; SP16, Cell Marque) or PD-L1 (1:200; E13LN, Cell Signaling Therapeutics) for 45 minutes at room temperature. Sections were then incubated with a probe antibody (MACH3 Probe, Biocare Medical) specific to the species of the primary antibody for 20 minutes, washed, and then incubated further for 20 minutes with a horseradish peroxidase (HRP)-conjugated antibody (MACH3-HRP, Biocare Medical) specific to the probe. Melanoma sections were then stained with 3,3′-diaminobenzidine (Biocare Medical) for 5 minutes and counterstained with hematoxylin for 5 minutes. All samples were cover slipped with xylene and left to air dry at room temperature.

Imaging and staining quantification

The Vectra 3 multispectral slide scanner was used in conjunction with Vectra 3.3 and Phenochart 1.0.4 Software (Akoya Biosciences) to image immunofluorescence staining. Images were then unmixed and analyzed using inForm 2.3.0 Software (Akoya Biosciences). Quantitative analysis was then conducted using the TIBCO Spotfire 3.3.1 from TIBCO. Lymphocyte/leukocyte densities and PD-L1 expression was assessed within tumor only (defined by the presence of SOX10-positive staining). This was performed by a trained pathologist (P.M. Ferguson). In nodal metastases, any residual lymph node tissue, associated structures, and/or cells were annotated out and excluded from downstream analyses. Tumor-resident $CD8^+$ T cells were quantified using the colocalization of CD103 on $CD8^+$ cells. $CD4^+$ T cells were quantified as $CD68^-$ $CD11c^-$ $CD4^+$ cells. For IHC staining quantification, a trained pathologist (P.M. Ferguson) reviewed each individual case using a conventional upright brightfield microscope and assigned a score to each based on the density of intratumoral CD8 and PD-L1 staining. CD8 staining was quantified using a score from 0 to 3, where 0, absent; 1, sparse (<25%); 2, moderate (25%–75%); and 3, dense

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(>75%). PD-L1 positivity was determined as the percentage of total cells within the tumor microenvironment (tumor and immune cells, predominantly macrophages) positive for PD-L1 (≥1%) in both immunofluorescence and conventional IHC staining using a continuum scale from 0% to 100%.

Statistical analyses

Statistical analyses were performed using Prism version 6.0f (GraphPad Software) or TIBCO Spotfire 3.3.1. Patient characteristics were summarized using frequencies and percentages. *P* values were determined using a nonparametric Dunn multiple comparisons test for comparisons between melanoma subtypes, nonparametric log-rank test for survival data, or Spearman rho test for correlations, where appropriate. *P* values less than 0.05 were considered significant. Variability in genomic and immune cell density data was expressed in terms of ± 1 SEM. Correlation plots between genomic (including TMB and SVs) and IHC staining data were performed in Prism using a Spearman correlation test. Clinical outcomes analyzed included melanoma-specific survival (MSS). MSS time was calculated as the time from the date of surgical resection of stage III melanoma specimen to date of last follow-up or the date of death from melanoma. Survival curves were generated using Kaplan–Meier method and stratified by expression of a marker using the median staining intensity value (below and above the median as low and high groups, respectively).

Results

Cutaneous melanoma has higher TMB and immune cell infiltration relative to other subtypes

A total of 186 melanoma samples from 186 patients (Supplementary Table S1), including cutaneous ($n = 151$), acral ($n = 30$), and mucosal ($n = 5$) melanomas, of which 51% were nodal metastases, were stained for CD8, CD103, CD45RO, CD4, CD20, CD68, CD11c, and PD-L1 by multiplex immunofluorescence-IHC (mIHC). The number of $CD8^+$ lymphocytes per mm^2 within the intratumoral region was significantly higher in cutaneous melanoma ($395 \pm 50/mm^2$) compared with acral melanoma ($164 \pm 61/mm^2$; $P < 0.01$) and also showed trends for being higher relative to mucosal melanoma ($137 \pm 43/mm^2$), although this difference was not statistically significant (**Fig. 1A** and **B**). The densities of tumor-resident $CD8^+$ T cells, a subset of $CD8^+$ T cells expressing the E-cadherin ligand, CD103, and are thought to be enriched for T-cell clones specific to tumor antigens, were also significantly higher in cutaneous melanomas ($92 \pm 17/mm^2$) compared with acral melanomas ($23 \pm 9/mm^2$; $P < 0.01$; **Fig. 1C**). The proportion of tumor-resident cells formed a small subset of the total $CD8^+$ T-cell populations across all melanoma subtypes, cutaneous (26% ± 2%), acral (13% ± 3%), and mucosal (20% ± 5%; Supplementary Fig. S1A). Acral melanoma, but not mucosal melanoma, tended to harbor a smaller proportion of resident $CD8^+$ T cells compared with cutaneous melanoma, which approached significance ($P = 0.054$; Supplementary Fig. S1A). CD45RO, another marker associated with memory T cells, and PD-L1, the ligand that binds to PD-1 on T cells and is upregulated by IFNγ, were expressed highest in cutaneous melanomas (**Fig. 1D**; Supplementary Fig. S1B), along with CD68 (Supplementary Fig. S1E). CD4, CD20, and CD11c displayed modest trends for higher expression in cutaneous melanoma (Supplementary Fig. S1C, S1D, and S1F), together indicating that acral and mucosal melanomas are less immunogenic than cutaneous melanomas and contain fewer immune populations associated with antitumor immunity.

No correlation between immune cells and SNV counts in cutaneous or acral/mucosal melanomas

TMB in cutaneous melanomas had higher protein-coding SNV counts (1,281 ± 136) compared with noncutaneous melanomas (acral, 11 ± 74 and mucosal, 59 ± 12; **Fig. 1E**). However, when we analyzed the effect of TMB on TIL densities in cutaneous melanomas only, no correlative relationship between SNV counts and $CD8^+$ T-cell densities (Spearman $r = -0.10$; **Fig. 2A**) or $CD103^+CD8^+$ tumor-resident T cells (Spearman $r = 0.04$) was observed (**Fig. 2B**), despite increasing evidence that $CD103^+CD8^+$ tumor-resident T cells (Trm) represent a subset of T cells specific for tumor antigens (21, 22). We also found no correlation between $PD\text{-}L1^+$, $CD45RO^+$, $CD4^+$, $CD20^+$, $CD68^+$, or $CD11c^+$ cells and SNV counts (Supplementary Fig. S1G–S1L). Correlative relationships between immune infiltrates and TMB did not improve when we focused our analysis on the site of disease (Supplementary Table S2). To avoid any bias on representative regions of tissue microarrays, we also stained a subset of cutaneous ($n = 17$) samples for CD8 and PD-L1 on whole-slide sections and quantified $CD8^+$ T-cell densities and PD-L1 expression in each tumor. Consistent with our main findings, no correlation was observed between $CD8^+$ T-cell density and SNV counts or PD-L1 expression and SNV counts (Supplementary Fig. S2A). Cutaneous tumors with relatively high SNV counts could also simultaneously lack immune infiltration, whereas cutaneous tumors with relatively low SNV counts could show immune infiltrate presence (Supplementary Fig. S2B). We also investigated the effect of TMB and immune infiltrates in acral/mucosal melanomas ($n = 32$). However, we found no correlative relationship between SNV counts and $CD8^+$ T-cell densities (Spearman $r = -0.15$), $CD103^+CD8^+$ TILs (Spearman $r = -0.05$), PD-L1 expression (Spearman $r = -0.03$), CD45RO-positive cells [Spearman $r = -0.08$, not significant (ns)], or other immune cells (Supplementary Fig. S3). Finally, we sought to determine whether any of the known mutation melanoma subtypes (i.e., *BRAF, NF1*, and *NRAS*) were associated with an immune infiltrate presence in cutaneous or acral/mucosal melanomas. No significant trends were found, despite *NF1*-positive cutaneous tumors showing a higher average SNV count compared with other known melanoma subtypes (Supplementary Fig. S4).

SV counts in acral and mucosal melanomas do not correlate with $CD8^+$ T-cell densities

In our cohort of patient samples, and similar to what has been published previously by our group (18), acral and mucosal melanomas displayed significantly higher SV mutations (acral, 160 ± 3; $P < 0.0001$ and mucosal, 154 ± 5; $P < 0.01$) compared with cutaneous melanomas (75 ± 4; **Fig. 2C**), despite having lower TMB. We, therefore, sought to investigate whether higher SV counts within acral and mucosal melanoma might be correlated with an immune presence. We found no significant correlations between SV counts and densities of $CD8^+$ T lymphocytes (Spearman $r = -0.34$; **Fig. 2D**) or $CD103^+CD8^+$ tumor-resident T cells (Spearman $r = -0.28$; **Fig. 2E**). We also did not found a significant correlation for PD-L1 expression (Spearman $r = -0.22$), $CD4^+$ T cells (Spearman $r = -0.3$), $CD68^+$ cells (Spearman $r = -0.3$), and SV counts (Supplementary Fig. S5A, S5C, and S5E). $CD45RO^+$, $CD11c^+$, and $CD20^+$ immune cells were inversely correlated with SV counts (Supplementary Fig. S5B, S5D, and S5F). We investigated this further for $CD8^+$ T-cell density and PD-L1 expression on whole-slide sections in acral ($n = 21$) and mucosal ($n = 8$) melanoma samples containing a higher range of SV counts (52–1,148). Consistent with our findings, no positive correlation was observed between $CD8^+$ T-cell density and SV counts or PD-L1 expression and SV counts (Supplementary Fig. S6A and S6B), indicating that SV mutations are not a driver of immunity in acral/mucosal melanomas.

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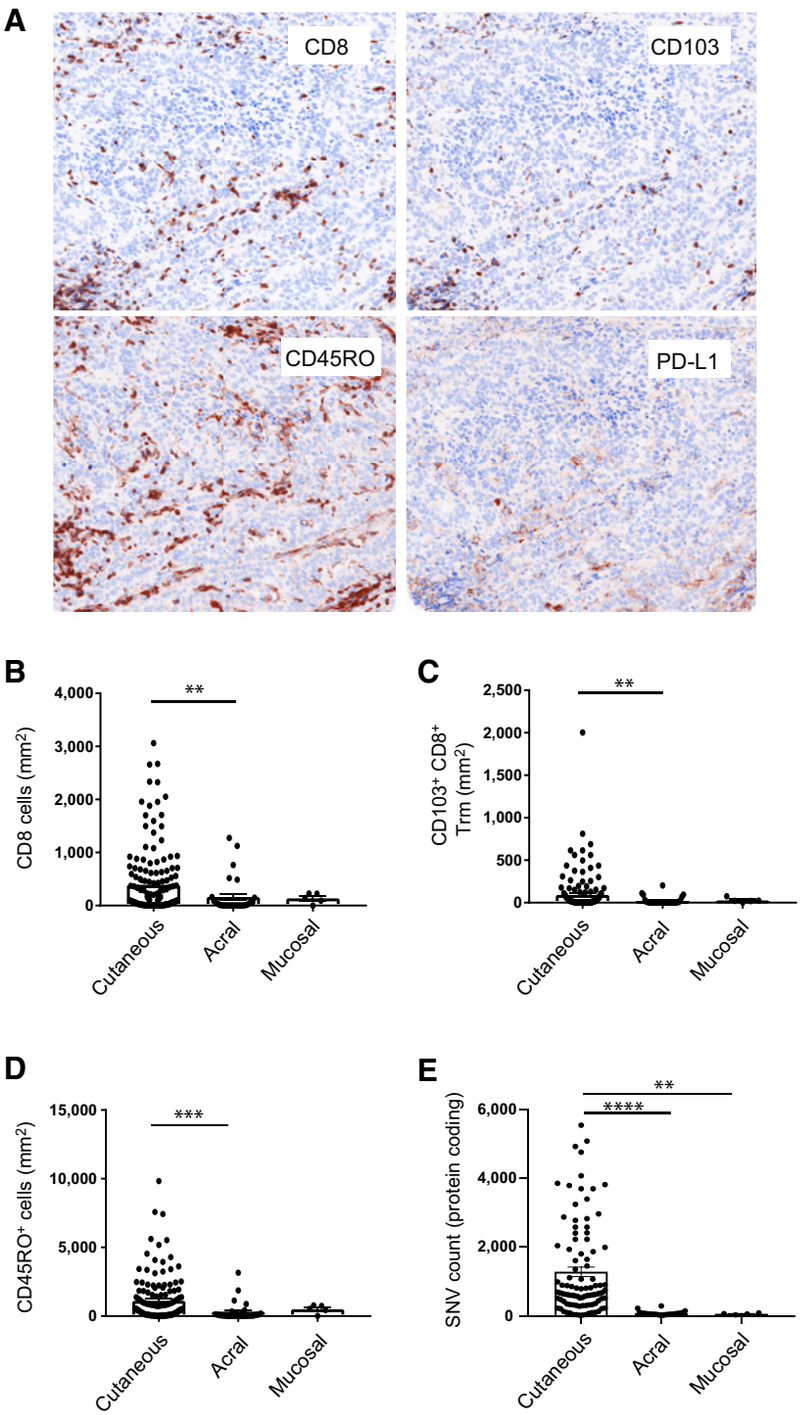

Figure 1.

Density of immune cell infiltration in cutaneous, acral, and mucosal melanoma tumors. **A,** Representative single-color images of mIHC staining for CD8, CD103, PD-L1, and CD45RO immune markers in melanoma tumors (images taken at 20× magnification). **B,** The mean number of $CD8^+$ T cells per mm^2 of tumor in cutaneous ($n = 151$), acral ($n = 30$), and mucosal ($n = 5$) melanomas. **C,** The mean number of $CD103^+CD8^+$ tumor-resident T cells (Trm) per mm^2 in cutaneous ($n = 151$), acral ($n = 30$), and mucosal ($n = 5$) melanomas. **D,** The mean number of $CD45RO^+$ cells per mm^2 in cutaneous ($n = 151$), acral ($n = 30$), and mucosal ($n = 5$) melanomas. **E,** The mean number of SNVs in cutaneous ($n = 97$), acral ($n = 28$), and mucosal ($n = 4$) melanomas. Error bars are ± 1 SEM. *P* values were determined using a nonparametric Dunn multiple comparisons test for comparisons between melanoma subtypes (**, $P \le 0.01$; ***, $P \le 0.001$; ****, $P \le 0.0001$).

$CD8^+$ T-cell densities are independent of TMB in a validation cohort

To validate our findings, we stained whole-slide sections for CD8 and PD-L1 using conventional IHC in an independent cohort of patient samples ($n = 67$; Supplementary Table S3; $n = 56$, cutaneous melanoma and $n = 11$, acral/mucosal melanoma) and correlated staining with genomic factors of mutation burden. When we compared average SNV counts by density of CD8 staining (0–3) in cutaneous melanoma samples, we found no significant changes in SNV count between all four levels of CD8 density (**Fig. 3A**). Cutaneous melanoma tumors with the least (0) and highest (3) $CD8^+$ T-cell infiltration had almost identical average SNV counts (1,647 ± 692 and 1,579 ± 405, respectively). We also explored the predicted neoantigen count from tumor burden in each sample with CD8 density, but found no significant trends (**Fig. 3B**). PD-L1 expression was weakly correlated with SNV counts (Spearman $r = 0.27$; $P = 0.04$; Supplementary Fig. S7A) and with predicted neoantigens (Spearman

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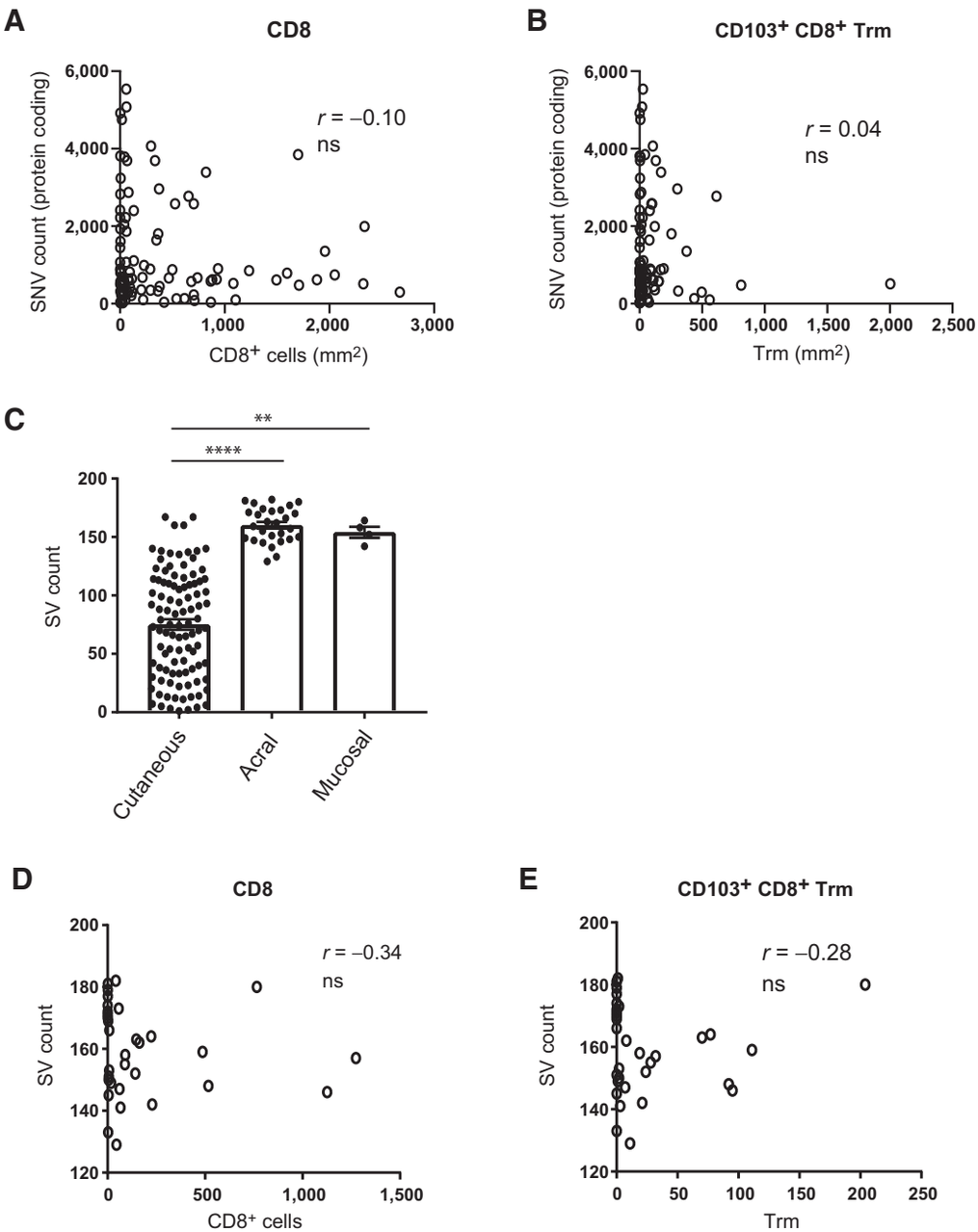

Figure 2.

Correlation between $CD8^+$ T cells, $CD103^+CD8^+$ T cells, and TMB and SV counts in cutaneous and acral/mucosal melanomas. **A,** Spearman correlation (*r*) between SNV counts and number of $CD8^+$ T lymphocytes per mm^2 of tumor in ($n = 97$) cutaneous melanomas. **B,** Spearman correlation (*r*) between SNV counts and number of $CD103^+CD8^+$ Trm per mm^2 of tumor in ($n = 97$) cutaneous melanomas. **C,** The mean number of SVs in cutaneous ($n = 97$), acral ($n = 28$), and mucosal ($n = 4$) melanomas. **D,** Spearman correlation (*r*) between SV counts and $CD8^+$ T lymphocytes per mm^2 of tumor in acral and mucosal melanoma tumors ($n = 32$). **E,** Spearman correlation (*r*) between SV counts and $CD103^+CD8^+$ Trm per mm^2 of tumor in acral and mucosal melanoma tumors ($n = 32$). *P* values were determined using a nonparametric Spearman test [**, $P \le 0.01$; ****, $P \le 0.0001$; ns, not significant ($P > 0.05$)].

$r = 0.27$; $P = 0.04$; Supplementary Fig. S7B). Although the number of acral/mucosal samples in this cohort was small ($n = 11$), we found no significant trends between CD8 density and average SV counts (**Fig. 3C**) or PD-L1 expression and SV counts (Supplementary Fig. S7C), consistent with our previous cohort findings.

MSS for patients with stage III cutaneous melanoma is independent of TMB

We next explored the relationship between TMB and immune infiltrates in a subset of 43 patients with treatment-naïve stage III cutaneous melanoma to dissect the role of TMB in patient survival. The median follow-up time in this cohort was 24 months

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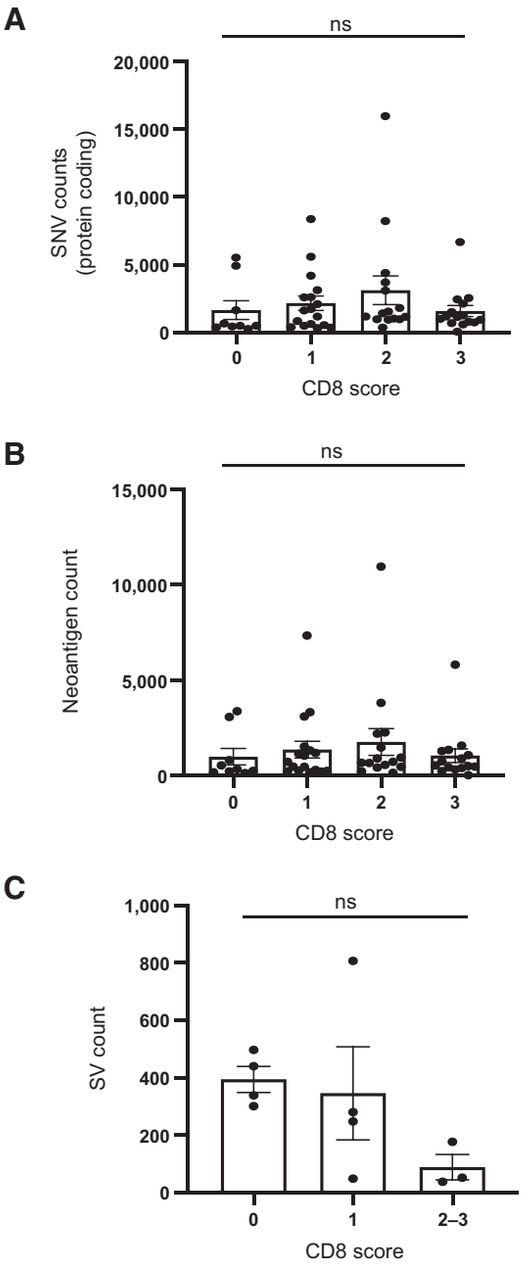

Figure 3.

Validation cohort. **A,** Mean SNVs in cutaneous melanoma tumors ($n = 56$) with varying degrees of intratumoral $CD8^+$ T-lymphocyte infiltration (0–3), where 0, absent CD8 infiltration; 1, sparse CD8 infiltration; 2, moderate CD8 infiltration; and 3, dense CD8 infiltration. **B,** Mean neoantigen counts in cutaneous melanoma tumors ($n = 56$) with varying degrees of $CD8^+$ lymphocyte infiltration (0–3). **C,** Mean SV count in samples with varying CD8 scores (0–2) in acral ($n = 8$) and mucosal ($n = 3$) melanomas. Error bars are ± 1 SEM. *P* values were determined using a Dunn multiple comparisons test (ns, $P > 0.05$ considered not significant).

(range, 1–156 months). In each case, the median of each variable was used to separate patients into high and low groups for each variable. Survival analysis found that patients with high TMB (average SNV count, 1,494 and median SNV count, 979) had no significant survival advantage compared with patients with low TMB (average SNV count, 282 and median SNV count, 296; $P = 0.34$; **Fig. 4A**). Despite this, patients with high $CD8^+$ T-cell densities had significantly longer median MSS (131 months) compared with patients with low $CD8^+$ T-cell densities [21 months; HR, 2.5 (1.12–5.8); $P = 0.024$; **Fig. 4B**). Tumor-resident $CD8^+$ T cells most associated with survival of all the prognostic markers evaluated [HR, 4.1 (1.6–10.0); $P = 0.001$; **Fig. 4C**]. High PD-L1 expression (>1% cells) also associated with better survival [HR, 2.4 (0.4–1.0); $P = 0.035$] compared with patients with low PD-L1 expression (Supplementary Fig. S8A). We next investigated whether patient groups with high CD8, $CD103^+CD8^+$ Trm, and PD-L1 might have an average increase in TMB compared with CD8, $CD103^+CD8^+$ Trm, and PD-L1-low patient groups. We found no differences in the SNV counts between high and low groups (Supplementary Table S4), indicating that the survival advantage associated with high immune cell densities was independent of TMB.

Discussion

We showed that TMB does not correlate with immune cell infiltration in cutaneous and acral/mucosal melanoma tumors, including for $CD103^+CD8^+$ tumor-resident memory T-cell numbers. These findings remained consistent even when we focused on the site of disease. In melanoma subtypes, where a bias exists for chromosomal structural aberration mutations, we also showed a lack of correlation between these mutation types and the presence of immune cells, likely indicating that these mutations are also not robust elicitors of an antitumor immune response in acral and mucosal melanoma tumors. We also demonstrated that the prognostic effects of immune cell densities, particularly $CD103^+$ tumor-resident $CD8^+$ T cells, are not necessarily associated with TMB in cutaneous melanoma. Although it is possible that increased TMB results in enhanced T-cell receptor diversity and, therefore, improved quality of the immune response, the fact that TMB did not have an impact on melanoma-specific patient survival suggests that it is unlikely to be a key determinant of the protective immunity. Together, our results suggest that immune infiltration in melanomas is independent of TMB, highlighting the importance of other driving factors. Limitations to this study include the moderate sample size of the two patient cohorts, especially the number of acral and mucosal samples with genomic data, and, therefore, it is possible that the study is not powered to detect a significant association between TMB/SVs and immune infiltrates, particularly if those associations are very weak. In addition, no functional characterization of antitumor immunity between high and low TMB tumors was performed. However, despite moderate sample size, known prognostic immune cells/markers, including $CD8^+$ T cells, Trm, and PD-L1, were prognostic in this study, whereas TMB was not. A strength to the study was the use of mIHC, which allowed for an accurate assessment of intratumoral lymphocytes and the characterization of complex phenotypes ($CD103^+CD8^+$ Trm). Overall, our data are in-line with the results of a number of other studies (14, 15), including a pan-cancer analysis utilizing The Cancer Genome Atlas database, where no significant correlations were identified between somatic mutation numbers and $CD8^+$ T-cell infiltration scores in the majority of cancers (15).

There are many reasons why TMB may not directly correlate with the magnitude of an antitumor immune response. (i) It is possible that not all the predicted neoantigens from sequencing data are recognizable to the immune system. For example, tumors that have down-regulated MHC class I expression may limit the recognition of neoantigens. (ii) It is probable that neoantigens are not equally

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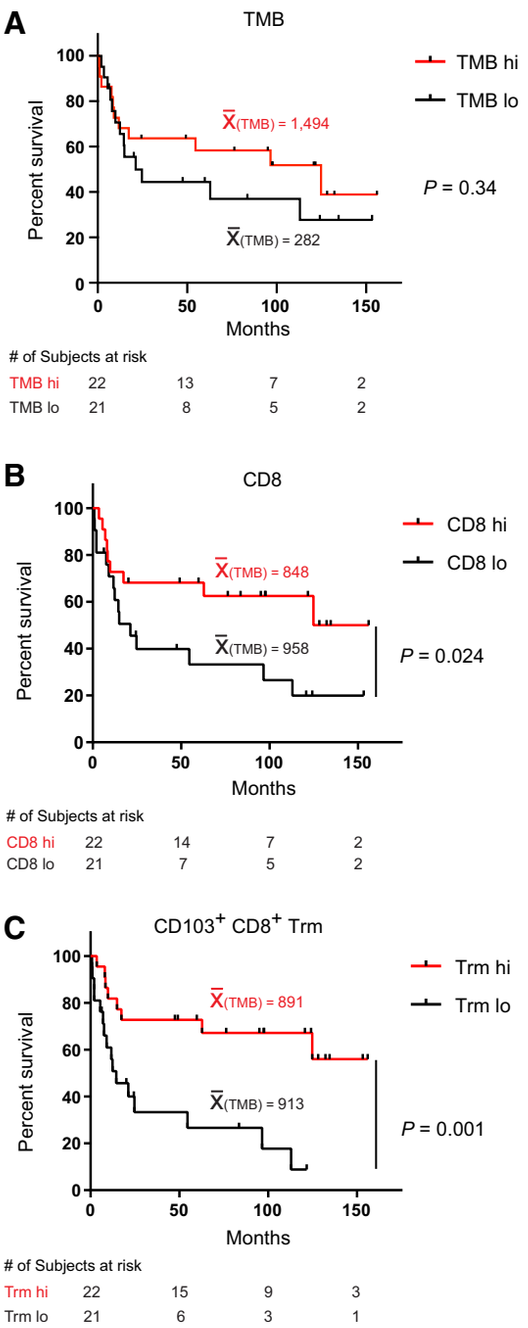

Figure 4.

Prognostic effect of TMB relative to $CD8^+$ T cells and Trm in treatment-naïve melanoma patients. **A,** Kaplan–Meier curve for 43 patients with stage III cutaneous melanoma with high (hi) TMB [greater than the median (76,310); red] and low (lo) TMB (lower than median; black). **B,** Kaplan–Meier curve for 43 patients with stage III cutaneous melanoma with high numbers of $CD8^+$ T cells (greater than the median = 175 cells/mm^2; red) and low numbers of $CD8^+$ T cells (lower than median; black). **C,** Kaplan–Meier curve for 43 patients with stage III cutaneous melanoma with high numbers of $CD103^+CD8^+$ Trm (greater than the median = 8 cells/mm^2; red) and low numbers of $CD103^+CD8^+$ Trm (lower than median; black). For each curve, the average TMB is given for high and low groups. *P* values were determined using a nonparametric log-rank test and are indicated in panels.

immunogenic and, therefore, may elicit stronger or weaker immune responses depending on the quality of the neoantigen. Indeed, there is evidence to show that clonal neoantigens, rather than subclonal neoantigens, are more associated with immunity in tumors (11). However, this is unlikely to be a strong confounding variable in melanoma, where the overwhelming majority of neoantigens is classed as clonal (23). (iii) Nonmutated antigens, such as cancer-testis antigens, which can be expressed in melanomas, may also contribute significantly to antitumor immune responses. (iv) It is possible that TMB is only one of many more dominant factors responsible for recruiting and retaining immune cells in tumors, including the presence of $Batf3^+$ dendritic cells, upregulation of the Wnt signaling pathway (24, 25), and the prevalence of myeloid suppressor cells (26), among others. In conclusion, our data indicate that TMB, SV rearrangements, and known melanoma mutation subtypes are not determining factors for the presence or absence of immune infiltrates within melanoma tumors.

Disclosure of Potential Conflicts of Interest

S.N. Lo reports grants from National Health and Medical Research Council of Australia (program grant) during the conduct of the study. R.P.M. Saw reports personal fees and other from Novartis (advisory board), Bristol-Myers Squibb (speaking honoraria), MSD (advisory board), and QBiotics (advisory board) outside the submitted work. J.F. Thompson reports grants from National Health and Medical Research Council of Australia (program grant) during the conduct of the study, as well as personal fees from GlaxoSmithKline (honoraria and travel support), BMS Australia (honoraria for advisory board participation), MSD Australia (honoraria for advisory board participation), and Provectus Inc. (honoraria and travel support) outside the submitted work. A.M. Menzies reports personal fees from Bristol-Myers Squibb (advisory board), MSD (advisory board), Novartis (advisory board), Roche (advisory board), and Pierre Fabre (advisory board) outside the submitted work. G.V. Long reports personal fees from Aduro (consultant adviser), Amgen (consultant adviser), Bristol-Myers Squibb (consultant adviser), Highlight Therapeutics S.L (consultant adviser), Mass-Array (consultant adviser), Merck (consultant adviser), MSD (consultant adviser), Novartis (consultant adviser), OncoSec Medical (consultant adviser), Pierre Fabre (consultant adviser), Roche (consultant adviser), QBiotics (consultant adviser), Skyline DX (consultant adviser), and Sandoz (consultant adviser) outside the submitted work. J.V. Pearson reports other from genomiQa PTY LTD (genome analysis company; cofounder, equity holder) outside the submitted work. N. Waddell reports grants from National Health and Medical Research Council of Australia (fellowship) during the conduct of the study and other from genomiQa PTY LTD (cofounder, board member, and equity holder) outside the submitted work. N.K. Hayward reports grants from National Health and Medical Research Council (partial funding of work) during the conduct of the study. R.A. Scolyer reports grants from National Health and Medical Research Council of Australia (program grant and fellowship grant) and nonfinancial support from Melanoma Institute Australia during the conduct of the study, as well as personal fees from Royal Prince Alfred Hospital (salary), Qbiotics, Novartis, MSD Sharp & Dohme, NeraCare, Amgen, Bristol-Myers Squibb, Myriad Genetics, and GlaxoSmithKline outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

J. Edwards: Conceptualization, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. **P.M. Ferguson:** Formal analysis, investigation, methodology. **S.N. Lo:** Formal analysis. **I. Pires da Silva:** Visualization, methodology, writing–review and editing. **A.J. Colebatch:** Investigation, methodology, writing–review and editing. **H. Lee:** Methodology. **R.P.M. Saw:** Resources, writing–review and editing. **J.F. Thompson:** Resources, writing–review and editing. **A.M. Menzies:** Resources, writing–review and editing. **G.V. Long:** Conceptualization, resources, supervision, funding acquisition, investigation, visualization, methodology, project administration, writing–review and editing. **F. Newell:** Resources, data curation, writing–review and editing. **J.V. Pearson:** Data curation, writing–review and editing. **N. Waddell:** Data curation, writing–review and editing. **N.K. Hayward:** Resources, data curation. **P.A. Johansson:** Resources, data curation, writing–review and editing. **G.J. Mann:** Resources, data curation. **R.A. Scolyer:** Conceptualization, resources, supervision, funding acquisition, investigation, visualization, methodology, project

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