Identification of tumor-intrinsic drivers of immune exclusion in acral melanoma

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ABSTRACT

Acral melanoma (AM) has distinct characteristics compared with cutaneous melanoma and exhibits poor response to immune checkpoint inhibitors (ICIs). Tumor

intrinsic mechanisms of immune exclusion have been identified in many cancers but less studied in AM. We characterized clinically annotated tumors from patients diagnosed with AM at our institution in correlation with ICI response using whole transcriptome RNAseq, whole exome sequencing, CD8 immunohistochemistry, and multispectral immunofluorescence imaging. A defined interferon-γ-associated T cell-inflamed gene signature was used to categorize tumors into non-T cell-inflamed and T cell-inflamed phenotypes. In combination with AM tumors from two published studies, we systematically assessed the immune landscape of AM and detected differential gene expression and pathway activation in a non-T cell-inflamed tumor microenvironment (TME). Two single-cell(sc) RNAseq AM cohorts and 11 bulk RNAseq cohorts of various tumor types were used for independent validation on pathways associated with lack of ICI response. In total, 892 specimens were included in this study. 72.5% of AM tumors showed low expression of the T cell-inflamed gene signature, with 23.9% of total tumors categorized as the non-T cell-inflamed phenotype. Patients of low CD3+CD8+PD1+ intratumoral T cell density showed poor prognosis. We identified 11 oncogenic pathways significantly upregulated in non-T cell-inflamed relative to T cell-inflamed TME shared across all three acral cohorts (MYC, HGF, MET, VEGF, EGF, SP1, ERBB2, TFEB, SREBF1, SOX2, and CCND1). scRNAseq analysis revealed that tumor cell-expressing pathway scores were significantly higher in low versus high T cell-inflamed AM tumors. We further demonstrated that the 11 pathways were enriched in ICI non-responders compared with responders across cancers, including AM, cutaneous melanoma, triple-negative breast cancer, and non-small cell lung cancer. Pathway activation was associated with low expression of interferon stimulated genes, suggesting suppression of antigen presentation. Across the 11 pathways, fatty acid synthase and CXCL8 were upregulating downstream target molecules suggesting potential nodes for therapeutic intervention. A unique set of pathways is associated with immune exclusion and ICI resistance in AM. These data may inform immunotherapy combinations for immediate clinical translation.

BACKGROUND

Acral melanoma (AM) is a unique subtype portending poor prognosis as compared with cutaneous melanoma (CM).1,2 Acral tumors arise in sun-shielded locations (eg, palms/soles), often leading to delayed medical attention and advanced stage at diagnosis. While UV radiation-induced CM demonstrates high tumor mutational burden (TMB), neoantigenicity, and frequency of BRAF mutations, AM has lower TMB and rarely demonstrates BRAF mutations.3 AM is also characterized by lower CD8+ T cell infiltration and Programmed Death Ligand-1 (PD-L1) expression, hallmarks of a non-T cell-inflamed tumor microenvironment (TME). Whereas CM is associated with an approximately 40% objective response rate in the treatment naïve metastatic setting to anti-PD1 monotherapy, reports of response in AM are quoted as less than 20%.3 A single-cell expression analysis of AM tumors demonstrated the expression of certain inhibitory checkpoints but primarily highlighted the relative lack of immune cell infiltrates.4

An interferon-γ (IFNg) activated TME is strongly associated with response to immune checkpoint inhibitors (ICIs),5 yet most clinical biomarkers (PD-L1, TMB, tumor infiltrating lymphocyte (TIL) levels) fail to completely encompass this inflammatory phenotype. A more comprehensive T cell-inflamed gene signature has been shown to strongly correlate with IFNg signaling and ICI response.6 Additionally, this signature provides a model whereby ICI resistance mechanisms can be identified by comparing non-T cell-inflamed versus T cell-inflamed tumors.7 In CM, the Wnt/β-catenin pathway was identified as a driver of immune exclusion in non-T cell-inflamed tumors.8 This approach has further nominated other pathways (eg, MYC activation and PTEN loss) associated with suppression of antigen
presentation and cytolytic activity across human cancers.7

To inform combination immunotherapy drug development in AM, we sought to identify tumor-intrinsic drivers of immune exclusion. To this end, we collected a set of acral tumors from individuals exposed to ICI and investigated molecular mediators of non-T cell-inflamed AM. Through this approach, we have nominated a series of molecular targets as potential combination strategies to enhance immune checkpoint blockade in AM.

METHODS
A full description is supplied in online supplemental methods. In brief: formalin-fixed, paraffin-embedded (FFPE) baseline tumor tissues along with demographic, histopathological, and treatment data were collected from the melanoma biospecimen bank of UPMC Hillman Cancer Center (UPMC) (20 patients, 14 with ICI response evaluable; online supplemental table S1), according to an institutional review board-approved protocol (No. 20090109). Bulk RNAseq, whole exome sequencing (WES), immunohistochemistry (IHC), and multispectral immunofluorescence (mIF) imaging with PhenomImager HT (Akoya Biosciences) were performed for each sample. Baseline AM tumors from two additional published studies (Northwestern University (NW) and Melanoma Institute of Australia (MIA) cohorts), two scRNAseq AM cohorts (Li et al and Zhang et al cohorts), and 11 tumor cohorts from ICI-treated patients with bulk RNAseq and clinical data available were also analyzed (online supplemental table S2). In total, 892 specimens were analyzed across all cohorts. Acral samples were clustered into T cell-inflamed cohorts using a defined T cell-inflamed gene signature6; these clusters were used for differential gene expression detection by limma voom with precision weights and pathway activation prediction by Ingenuity causal network. For scRNAseq cohorts, high and low T cell-infiltrated tumors were defined by fraction of T cells over all cells profiled in TME. Statistical analysis was performed using R (V.4.1.2) and Bioconductor (release V.3.14), with significance level at 0.10. Benjamini-Hochberg Procedure was used to adjust multiple comparisons.

RESULTS
Characterization of the AM immune landscape
We assembled baseline tumor RNAseq data from 109 patients in three independent AM cohorts, including UPMC (n=20), Northwestern University (NW; n=22) (Shi et al, online supplemental table S2), and MIA (n=67) (Newell et al, online supplemental table S2). We generated RNAseq and WES data as well as clinical annotation for specimens from 20 patients in the UPMC cohort and validated our findings in 89 patients from 2 publicly available AM datasets (NW and MIA). Demographic, histopathological, treatment, and ICI response variables for the UPMC cohort are listed in online supplemental table S1.

To investigate the immune landscape of AM, we used the T cell-inflamed gene expression signature3 to categorize tumors into T cell-inflamed and non-T cell-inflamed groups, with the rest as intermediate. The overall analysis workflow is provided in online supplemental figure S1. Since each dataset was generated at a different institution with variations in experimental approaches, strong technical batch effect exists which cannot be fully corrected by computational methods. Therefore, we took an ensemble approach to analyze each dataset independently and then cross-reference results to identify consistent patterns relevant to the biological phenotypes. We detected high expression of T cell signature genes (T cell-inflamed) in 20%, 22.7%, and 31.3% tumors from UPMC, NW, and MIA cohorts, respectively, consistent with T cell infiltration data from AM single-cell cohorts.4 9 Non-T cell-inflamed tumors were identified in 20% (UPMC), 22.7% (NW), and 25.4% (MIA) samples (figure 1A). The T cell-inflamed tumors showed upregulated CD8A gene expression as well as a higher TMB (UPMC, 121.7±27.8; MIA, 121.6±37.0) compared with the non-T cell-inflamed tumors (UPMC, 51.5±6.5; MIA, 100.4±17.3) (figure 1A). The NW cohort does not have TMB data available. Digital immune deconvolution of RNAseq data revealed significantly elevated CD8+ T cell populations in T cell-inflamed versus non-T cell-inflamed TME in all three cohorts (p<0.05) (figure 1B). We detected no significant correlation between the T cell-inflamed gene expression and typical driver mutations of melanoma (BRAF, NRAS, NFI) (online supplemental figure S2A).

In support of the in silico findings, we performed IHC on 17 tumor specimens available from the UPMC cohort, demonstrating that higher CD8+ TIL percentage is significantly correlated with a T cell-inflamed phenotype (Spearman’s correlation r=0.51, p=0.039) (online supplemental figure S2B; representative images shown in figure 1C). A clear trend was also demonstrated by mIF imaging for increased intratumoral CD3+CD8+PD1+ T cell infiltrates in the T cell-inflamed versus non-T cell-inflamed (p=0.11) or intermediate (p=0.20) UPMC samples (figure 1D), further validating the immunophenotypic groups used for downstream analysis. Representative non-T cell-inflamed and T cell-inflamed mIF images are shown in figure 1E. Overall, mIF data indicate these tumors are primarily ‘immune excluded’ with a significantly higher proportion of CD3+CD8+PD1+ T cells residing in the tumor edge (online supplemental figure S2CD). Furthermore, whether measured in the intratumoral, tumor edge, or stromal space, a higher proportion of CD3+CD8+PD1+ T cells correlated with improved overall survival (OS) (online supplemental figure S2E).

We and other groups have previously demonstrated that the T cell-inflamed gene signature predicts response to ICI across human solid tumors.3 Within the UPMC AM dataset, we observed 100% of tumors from patients receiving clinical benefit fell in the upper 50th percentile
of T cell-inflamed gene expression. Seventeen patients had survival data available and were included for assessing the association of the T cell-inflamed gene expression profile with outcome. Patients harboring T cell-inflamed or intermediate tumors also experienced improved OS versus patients with non-T cell-inflamed tumors (p=0.10, by log-rank test; **figure 1F**). T cell-inflamed gene expression as a continuous variable was significantly associated with longer OS (p=0.048, HR=0.49, by Cox PH univariate model), and remained significant after adjustment for age, gender, or TMB (p=0.041, HR=0.32, by Cox PH multivariable model; online supplemental figure S2F).
Tumor-intrinsic oncogenic transcriptional programs are associated with immune exclusion across three AM datasets

To investigate the transcriptional programs that potentially drive the non-T cell-inflamed phenotype, we analyzed tumors from the UPMC, NW, and MIA cohorts. We took an unbiased approach by comparing the whole transcriptome expression of non-T cell-inflamed versus T cell-inflamed tumors using bulk RNAseq. Considering batch effect, we first performed the comparison within individual cohorts independently and then reported pathways that were common to all three cohorts. There were 2963, 1555, and 2965 differentially expressed genes (DEGs) between the two tumor groups in UPMC, NW, and MIA cohorts, respectively (p<0.05, fold change ≥1.5 or ≤−1.5).

While common DEGs exist among all three cohorts (online supplemental table S3), we sought to identify pathways that may reflect the actual biologic phenotype relevant for translational targeting rather than individual genes. To gain insights into the functional mechanisms collectively driven by DEGs, we predicted the activation of upstream regulators (pathways) based on the aggregate change of expression from downstream target molecules (encoded by DEGs), using causal network analysis with Ingenuity Knowledge Base (Qiagen) (online supplemental tables S4–S6). We identified 393, 149, and 70 pathways predicted to be activated in non-T cell-inflamed versus T cell-inflamed tumors from the UPMC, NW, and MIA cohorts, respectively (z-score ≥0.9, p<0.05) (figure 2A). Among those, we found known drivers of immune exclusion such as TGFβ1, HIF1A, and CTNNB1 (β-catenin). On cross-referencing results across all three cohorts, we identified 11 activated pathways consistently detected in the non-T cell-inflamed TME, including MYC, HGF, MITF, VEGF, EGFR, SP1, ERBB2, TFEB, SREBF1, SOX2, and CCND1 (figure 2A). Including the intermediate T cell-inflamed tumors in an additional investigation, we demonstrated that the pathway expression scores of all 11 pathways were significantly and inversely correlated with the T cell-inflamed signature in a continuous manner (figure 2B).

From a precision immuno-oncology perspective, we sought to investigate the spectrum of pathway activation in individual patient’s tumors, and calculated an activation score for each pathway using previously described methods. Our result demonstrated that the 11 pathways operate in a relatively homogeneous manner, with activation of each pathway detected in at least 77% of non-T cell-inflamed tumors, compared with 23% or less of the T cell-inflamed tumors (figure 2C). Given the high degree of pathway coactivation in individual tumors (figure 2D), we analyzed for shared downstream mechanisms across the 11 gene pathways. Both CXCL8 (also known as interleukin-8; IL-8) and FASN (fatty acid synthase) were observed to be among the top target molecules shared across pathways (figure 2E), altogether forming a functional network of associated proteins regulating cell cycle, fatty acid biosynthesis, and cytokine production (figure 2F).

Single cell analysis of two AM cohorts confirms tumor cell-intrinsic expression of immunosuppressive pathways with significant upregulation in low T cell-infiltrated tumors

To assess the cellular derivation of the 11 shared pathways, we analyzed a cohort of AM scRNAseq tumors (figure 3A) (Li et al, online supplemental table S2). Eight of 11 pathways (MYC, MITF, EGFR, SP1, ERBB2, SREBF1, SOX2, and CCND1) were included for downstream analysis after removing redundant target molecules and requiring at least five unique molecules per pathway. Tumor cells were observed as the predominant source of expression for all pathways (figure 3B). The expression of each pathway was significantly elevated in tumor cells of low T cell-infiltrated relative to those of high T cell-infiltrated samples, including a combined score of all pathways (figure 3C). Additionally, tumor cells of low T cell-infiltrated samples showed a significant downregulation in the expression of 108 DEGs enriched in IFN stimulating functions, consistent with suppression of type I and II IFN signaling (figure 3D–3E; online supplemental table S7). An additional validation cohort of five scRNAseq AM samples (Zhang et al, online supplemental table S2) similarly showed a predominance of pathway expression from tumor cells. More specifically, significant upregulation of SP1, ERBB2, SOX2, and CCND1 pathways in low T cell-infiltrated relative to high T cell-infiltrated samples (the latter exhibiting tumor clusters enriched for type I and II IFN signaling) was observed (online supplemental figure S3A–D). Altogether, these data highlight a tumor-intrinsic source of these immunosuppressive pathways leading to a TME state associated with suppressed antigen presentation.

Common pathways are upregulated in ICI-resistant tumor cohorts

Expression of the 11 oncogenic pathways associated with a non-T cell-inflamed phenotype was increased within the AM tumors of UPMC patients who had no clinical benefit to ICI (figure 4A). Given the paucity of harmonized secretion and ICI-response data in AM, we analyzed these pathways in two larger CM ICI datasets (online supplemental table S2). Ten of 11 pathways within Riaz et al (MYC, HGF, MITF, VEGF, EGFR, SP1, ERBB2, TFEB, SOX2, and CCND1; figure 4B upper panel), and 8 of 11 pathways within Liu et al (MYC, HGF, VEGF, EGFR, SP1, ERBB2, SREBF1, SOX2; figure 4B bottom panel) were found to be upregulated in baseline tumors from patients who did not have clinical benefit to ICI (false discovery rate (FDR)-adjusted p<0.10). For further validation, we compared the expression of these common pathways with both ICI response and the T cell-inflamed gene signature across three additional CM datasets, one triple-negative breast cancer cohort, one head and neck squamous cell carcinoma cohort, two non-small cell lung cancer (NSCLC) datasets, one renal cell carcinoma cohort, and one urothelial carcinoma cohort, where tissue transcriptomics and ICI clinical data were publicly available (online supplemental table S2). As depicted in figure 4C,
Figure 2  Activation of 11 oncogenic pathways in non-T cell-inflamed versus T cell-inflamed acral tumors across three cohorts (UPMC, NW, MIA). (A) Pathways activated in non-T cell-inflamed versus inflamed UPMC tumors and one or both of the NW and MIA cohorts. A total of 102 pathways with a z-score of at least 0.9 at p<0.05 in at least one cohort are shown. Eleven shared pathways are identified across the three cohorts (shown on top, solid side bar). Each row represents one pathway (top to bottom). Pathways were sorted by z-score high to low in the UPMC cohort. (B) Correlation between pathway expression scores and the T cell-inflamed gene signature. All samples are shown; n=20 in UPMC, n=22 in NW, and n=67 in MIA. Each data point represents one tumor; color denotes the T cell-inflamed (red), non-T cell-inflamed (blue), and intermediate groups (gray). Spearman’s correlation coefficient ρ and FDR-adjusted p values are shown for each pathway. Linear regression was shown with 95% confidence bands. (C) The proportion of tumors harboring activation of each pathway in non-T cell-inflamed and T cell-inflamed groups. The x-axis shows the number of tumors with all three acral cohorts combined. (D) Correlation between the T cell-inflamed gene signature and activation of each pathway at a continuous scale. (E) Downstream target molecules (shared across at least 4 pathways) and upstream regulators (pathways) with at least one connection from STRING functional protein association networks (confidence score>0.4; active interaction sources as ‘experiments’, ‘databases’, and ‘coexpression’). Line thickness indicates the strength of data support. Nodes were clustered by graph-based Markov Cluster Algorithm (MCL) (inflation parameter=2.5), with color indicating each cluster and dotted lines indicating edges between clusters. P values were computed by Spearman’s correlation in B, with denotation: *P<0.05, **P<0.01, ***P<0.001 after FDR adjustment for multiple comparisons. MIA, Melanoma Institute of Australia; FDR, false discovery rate.
these pathways are consistently upregulated in baseline tumors from patients who did not have clinical benefit to ICI and inversely correlated with the T cell-inflamed gene signature across several cancers.

**DISCUSSION**

Tumor-intrinsic mechanisms of immune exclusion have been proposed to be drivers of the non-T cell-inflamed phenotype, leading to resistance to ICI. Whereas...
Figure 4  Validation of immunosuppressive oncogenic pathways in ICI-treated cohorts. Baseline (pre-ICI treatment) tumors were used in all analyses. (A) Comparison of 11 pathways in acral melanoma (AM) samples from patients who showed clinical benefit (CB) or not (NCB) to ICI. n=14 shown, UPMC AM cohort. Patients in NW or MIA AM cohort did not receive ICI, hence not shown. (B) Comparison of 11 pathways in cutaneous melanoma (CM) samples from patients who received ICI (Riaz et al and Liu et al). (C) Pan-cancer analysis of pathways across ICI-treated patient cohorts. (left panel) The fold change of pathway scores in non-T cell-inflamed relative to T cell-inflamed tumors in each cohort; A label 'X' was added to the grids when significant upregulation (log2(fold change) >0) in NCB versus CB or NR versus R was detected at FDR-adjusted P<0.10. (right panel) The correlation between pathway scores and T cell-inflamed gene expression; A label ‘X’ were added when significant inverse correlation (Spearman’s ρ<0) was detected at FDR-adjusted P<0.10. Eleven ICI datasets with gene expression data and deidentified clinical data publicly available were included in analysis, consisting of five cutaneous melanoma studies (Riaz et al, Liu et al, Hugo et al, Gide et al, and Checkmate-064 from Campbell et al), one triple negative breast cancer study (TNBC; Blenman et al), one head and neck squamous carcinoma study (HN; Foy et al), two non-small cell lung cancer studies (NSCLC; Ravi et al, Foy et al), one renal cell carcinoma study (RCC; Miao et al), and one urothelial carcinoma study (Rose et al). P-values were computed by two-sided two-sample t-test in A–C upper panel, Spearman’s correlation in C bottom panel. ICI, immune checkpoint inhibitor; MIA, Melanoma Institute of Australia; FDR, false discovery rate.
SOX2, and CCND1). Analysis of single-cell ATAC-seq data from three independent AM cohorts, we identified 11 pathways as significantly associated with immune exclusion (MYC, HGF, MITF, VEGF, EGFR, SP1, ERBB2, TFEB, SREBF1, SOX2, and CCND1). Analysis of single-cell AM cohorts suggests that these immunosuppressive pathways are primarily expressed from the tumor cells and not other cells of the TME. Moreover, the shared downstream target molecules across these pathways, fatty acid synthase and CXCL8 (alias IL-8), may represent high priority targets for therapeutic intervention in non-T cell-inflamed AM.

In considering the 11 immune exclusion pathways identified, all 11 have been previously described to drive innate immune dysfunction or limit CD8+ T cell cytotoxicity. Noting that MYC and VEGF are well established mediators of immunosuppression, knockdown of MITF has been shown to upregulate type I IFN signaling in melanocyte stem cells. HGF is associated with M2 macrophage polarization, SP1 promotes Treg activity via DcR3, and TFEB has been shown to regulate tumor-associated macrophages in breast cancer. Additionally, SOX2 desensitizes melanoma cells to CD8+ cytotoxicity, CCND1 promotes immunosuppressive features through epithelial-mesenchymal transition (EMT), and SREBF1-mediated fatty acid production is associated with M2 polarization and ICI resistance. Though ERBB1/EGFR-mutant NSCLC is a known predictor of ICI resistance, the role of ERBB2/HER2 in modulating the immune milieu of the TME has been rarely studied outside the context of HER2-directed therapies. Prior studies have, however, associated HER2-mediated fatty acid production and protumor cytokine secretion (eg, CXCL8) with tumor progression and immune dysfunction in breast cancer models.

While functional validation of specific pathways is a future priority, we observe that expression of each pathway was associated with suppression of interferon-stimulated genes. A deficit of antigen presentation leading to resistance to ICI would be broadly consistent with what has been described for tumor-intrinsic mechanisms of immune exclusion in other contexts. For example, STK11 mutation strongly predicts lack of response to ICI in lung cancer where epigenetic silencing of STING is observed. Similarly, activation of MYC is associated with immunosuppressive tumor hypoxia as well as lack of response to ICI.

Both fatty acid synthase and CXCL8 (alias IL-8) were found to be downstream target genes across nine out of the 11 pathways in our study. Notably, inhibitors of both the fatty acid synthase and IL-8 axes have entered clinical trials in various solid tumors in combination with ICI. Lipid and cholesterol metabolism have been linked to systemic immunosuppression and reduced rates of response to ICI. Specifically, FASN\textsuperscript{high} ovarian cancer cells have been shown to inhibit tumor-infiltrating dendritic cells via lipid accumulation, and FASN expression is associated with poor ICI efficacy and survival in bladder cancer.

Elevated levels of circulating IL-8 have also been associated with poor outcomes to ICI in refractory advanced solid tumors. The immune inhibitory functions of IL-8 drive several mechanisms including but not limited to neutrophil extracellular traps and myeloid-derived suppressor cell accumulation, EMT, angiogenesis, and proliferation of cancer stem cells, among others. Breast cancer cells coexpressing HER2 and HER3 have been found to upregulate IL8 expression with resultant tumor invasion, findings reversed with anti-IL8 antibodies. Another study involving breast cancer mammospheres showed that increasing levels of IL8 exposure were associated with both EGFR and HER2 signaling pathways and tumor growth; inhibiting CXCR1/2 (IL8 cognate receptor) reversed this activity. An anti-IL8 antibody (BMS-986253) in combination with ICI has delivered responses in clinical trials of patients with immunotherapy refractory melanoma, including patients with AM. Our group has further identified IL-8 as a mediator of resistance to stereotactic body radiotherapy plus immunotherapy combinations. Leveraging these observations in identifying IL-8 as a mediator of resistance, we have launched a phase I/II study of SBRT with nivolumab and BMS-986253 (NCT04572451) which will investigate the safety and efficacy of this combination to overcome the non-T cell-inflamed TME in melanoma.

In conclusion, we have identified an AM-specific group of tumor-intrinsic pathways tied to immune exclusion and ICI resistance with validation across several tumor cohorts. Given the lack of effective treatment options for patients with AM, we propose further investigation into targeting these pathways to improve immunotherapy outcomes in AM.
Hillman Cancer Center CCSG award (P30CA047904), and by part in The University of Pittsburgh Center for Research Computing through the resources provided, specifically the HTC high-performance computing cluster supported by NIH award number S1000D28483.

Competing interests: RB declares PCT/US15/612657 (Cancer Immunotherapy), PCT/US18/36052 (Microbiome Biomarkers for Anti-PD-1/PD-L1 Responsiveness: Diagnostic, Prognostic and Therapeutic Uses Thereof), PCT/US63/055227 (Methods and Compositions for Treating Autoimmune and Allergic Disorders); JIL declares DSMB: Abbvie, Immune; Scientific Advisory Board: (no stock) 7 Hills, Fstar, Inzen, Reflection, Xilio (stock) Actym, Alphamab Oncology, Arch Oncology, Kanaph, Mavu, Onc-A, Pyxis, Tempex; Consultancy with compensation: Abbvie, Atypical, Avilion, Bayer, Bristol-Myers Squibb, Checkmate, Codial, Crown, Day One, Eisai, EMD Serono, FlareTech, Genentech, Gilead, Hotspot, Kadmon, KSF, Janssen, Ikaria, Immunocore, Incyte, Macrogenics, Merck, Merrusa, Nektar, Novartis, Pfizer, Regeneron, Ribon, Rubius, Signal, Syngene, Synthekine, Thex, Werewolf, Xencor; Research Support: (all for institutional clinical trials unless noted) AbbVie, Agios (IIT), Astellas, AstraZeneca, Bristol-Myers Squibb (IIT & industry), Corvus, Day One, EMD Serono, Fstar, Genmab, Ikena, Immatics, Incyte, Kadmon, KANH, Macrogenics, Merck, Moderna, Nektar, Next Cure, Numab, Pfizer (IIT & industry) Replimmune, Rubius, Scholar Rock, Synge, Takeda, Trishula, Tizona, Xencor; Patents: (both provisional) Serial #15/612,657 (Cancer Immunotherapy), PCT/US18/36052 (Microbiome Biomarkers for Anti-PD-1/PD-L1 Responsiveness: Diagnostic, Prognostic and Therapeutic Uses Thereof), PL declares equity interest in Amgen, DG declares grants/research support (NIH/NCI and Checkmate Pharmaceuticals) and consulting (Checkmate Pharmaceuticals) during the conduct of the study. DD also reports grants/research support (Arcus, CellSight Technologies, Immunocore, Merck Sharp & Dohme, Tesaro/GSK), consulting (Clinical Care Options (CCO), Finch Therapeutics, Gerson Lehrman Group (GLG), Medical Learning Group (MLG), Xilio Therapeutics), speakers’ bureau (Castle Biosciences) and pending provisional patents related to gut microbial signatures of response and toxicity to immune checkpoint blockade (US Patent 63/124,231 and US Patent 63/208,719) outside the submitted work. JMK declares grants/research support (Bristol-Myers Squibb, Amgen) and consulting (Bristol-Myers Squibb, Checkmate Pharmaceuticals, Novartis, Amgen, Checkmate, Castle Biosciences, Immunocore, Incyte, Novartis) outside the submitted work. HMZ declares grants/research support (NIH/NCI and Checkmate Pharmaceuticals) and consulting (Checkmate Pharmaceuticals) during the conduct of the study, grants/research support (NIH/NCI, Bristol-Myers Squibb and GlaxoSmithKline), personal fees (GlaxoSmithKline and Vedanta) and pending provisional patents related to gut microbial signatures of response and toxicity to immune checkpoint blockade (US Patent 63/124,231 and US Patent 63/208,719) outside the submitted work. Correspondence and requests for materials should be addressed to JJJ (lukesji@upmc.edu) and RB (brad@upmc.edu). The remaining authors declare no competing interests.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and the study protocol was approved by The University of Pittsburgh institutional review board (IRB)-approved protocol (Protocol No. 20090109). All samples have written informed patient consent. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as online supplemental information. The gene expression data have been deposited to NCBI GEO repository under accession ID EGE243238. De-identified clinical data is provided in Supplementary Tables. Public datasets have been referenced as appropriate (Supplementary Methods, section “Study Cohorts and Datasets”, and Table S2).

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