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Tumor CD155 expression is associated with resistance to anti-PD1 immunotherapy in metastatic melanoma

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Running title: CD155 and resistance to immune checkpoint blockade

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Conflict of Interest

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Abstract

Purpose: Resistance to anti-PD1 based immune checkpoint blockade (ICB) remains a problem for the treatment of metastatic melanoma. Tumor cells as well as host myeloid cells can express the immune checkpoint ligand CD155 to regulate immune cell function. However, the effect of tumor CD155 on the immune context of human melanoma has not been well described. This observational study characterizes tumor CD155 ligand expression by metastatic melanoma tumors and correlates results with differences in immune cell features and response to ICB.

Design: Pre-treatment tumor specimens, from 155 metastatic melanoma patients treated with ICB and from 50 patients treated with BRAF/MEK-directed-targeted therapy, were assessed for CD155 expression by immunohistochemistry. Intratumor T cell features were analysed using multiplex-immunohistofluorescence for CD8, PD1 and SOX10. Correlations were made between CD155 tumor level and bulk tumor RNA-seq results, as well as clinical RECIST response and progression-free survival.

Results: High pretreatment CD155 tumor levels correlated with high parenchymal $PD1^{+}CD8^{+}/CD8^{+}$ T cell ratios ($PD1^{tR}$) and poor response to anti-PD1 therapy. In PD-L1 negative tumors, high CD155 tumor expression was associated with patients who had poor response to combination anti-PD1/CTLA4 therapy.

Conclusion: Our findings are the first to suggest that tumor CD155 supports an increase in the fraction of $PD1^{+}CD8^{+}$ T cells in anti-PD1 refractory melanoma tumors

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and, further, that targeting the CD155 pathway might improve response to anti-PD1 therapy for metastatic melanoma patients.

Translational relevance

This is the first study to demonstrate that high tumor CD155 expression affects response to anti-PD1 therapy in metastatic melanoma patients. CD155 promotes anti-PD1 resistance and increased PD1 expression on $CD8^+$ T cells within melanoma tumor parenchyma.

Key words

CD155, immunotherapy, metastatic melanoma, immunotherapy resistance, PD1, CTLA4, CD96, TIGIT.

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Introduction

Tumor cells can upregulate immune checkpoint ligands to suppress T cell function (1-3). Immune checkpoint blockade (ICB) can control tumor growth and sometimes leads to regression of metastatic disease. ICB therapy targeting the inhibitory receptor PD1 (anti-PD1) is the most effective single agent ICB treatment to date, while combination ICB targeting both PD1 and CTLA4 (anti-PD1/CTLA4) further increases response rates, however with increased immune-related toxicity (4-7). In contrast to BRAF-directed targeted therapy (BRAFi; BRAF or combination BRAF/MEK inhibition), ICB therapies can induce durable long-term anti-tumor immune responses. These therapies have materially changed prognosis for patients with metastatic melanoma. Nevertheless, primary resistance to ICB is common, and one-third of ICB-treated melanoma patients who have an initial response will subsequently progress (8). Further, existing biomarkers to predict response to ICB therapy are controversial and limited by an incomplete understanding of the pretreatment immunological features of the tumor microenvironment. Resistance to ICB can involve a number of factors or causes, including the upregulation of other inhibitory checkpoints (9,10). As such, significant efforts are underway to understand which factors in the tumor microenvironment modify sensitivity to current ICB therapies in order to define alternative or ancillary immune checkpoint targets that improve outcomes and minimize toxicity.

A novel ICB target is the adhesion molecule CD155, which additionally functions as an immune checkpoint ligand expressed by tumor cells and tumor-associated myeloid cells (11). CD155 modifies lymphocyte function through multiple cognate

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immune receptors; TIGIT, CD96, and CD226 (DNAM-1), expressed by T cells and NK cells. CD155 is upregulated on tumor cells across multiple solid cancer types, including melanoma, and has been shown to be advantageous to tumor growth and tumor survival (12). Recently, using mouse tumor models in which CD155 was knocked-out ($CD155^{KO}$), we demonstrated that loss of tumor CD155 increased sensitivity to combination anti-PD1/CTLA4 treatment *in vivo* (11), suggesting that co-targeting CD155 may complement current ICB therapies. It is unclear in humans to what extent tumor CD155 impacts the immune infiltrate contexture or if expression of CD155 in human cancers affects sensitivity to immunotherapies. In this study, we characterize CD155 expression in human metastatic melanoma and provide evidence for a significant correlation between high CD155 and decreased sensitivity to ICB therapies. In PD-L1 negative tumors, high tumor CD155 identified those patients who did not respond to combination anti-PD1/anti-CTLA4 therapy. We demonstrated that tumor CD155 correlated with an increase in the ratio of $PD1^{+}CD8^{+}$ T cells infiltrating melanoma tumor parenchyma, but not among T cells localized within the tumor stroma. Further, a high intratumor $PD1^{+}CD8^{+}/CD8^{+}$ T cell ratio ($PD1^{tR}$) was a predictor of poor response to PD1-based ICB therapy.

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Results

High tumor CD155 limits response of metastatic melanoma patients to αPD1-combitherapy

Following from our preclinical studies using mouse tumor models (11), we wanted to understand the impact of CD155 in human cancer. We characterized tumor CD155 protein levels using immunohistochemistry (IHC) in pretreatment surgical specimens from metastatic melanoma patients who were treated with ICB (either anti-PD1 alone [αPD1-monotherapy] or in combination with anti-CTLA4 [αPD1-combitherapy]) or BRAFi therapy (Figure 1A). In pretreatment tumor specimens (*n* = 155 ICB treated; *n* = 50 BRAFi therapy), intra-tumor membrane staining for CD155 was highly constitutive/homogeneous. This is in contrast to the high intra-tumor heterogeneity and low-level expression observed for PDL1 in metastatic melanomas (13). However, considerable variation in the level of intra-patient CD155 membrane staining intensity was observed. Briefly, 5% (10/205) of melanoma pretreatment specimens were negative for CD155 (score 0+), 22% (45/205) were classified as membrane score 1+, 37% (75/205) were score 2+, and 37% (75/205) were score 3+ (Table 1). CD155 score 3+ tumors were characterized by strong circumferential membrane staining. Overall, this indicates that expression of CD155 by tumor cells is common in metastatic melanoma (>95%) but that significant inter-patient variability exists at the level of CD155 membrane expression.

To appreciate if tumor CD155 might influence response to ICB, we compared RECIST response categories (14) with pretreatment CD155 H-scores from patients treated with either αPD1-combitherapy or αPD1-monotherapy. We found that αPD1-combitherapy patients whose pretreatment tumors were CD155 score 3+ ($CD155^{high}$)

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demonstrated higher rates of stable disease (SD) or progressive disease (PD) as their best ICB response, and lower rates of complete response (CR) or partial response (PR), compared to patients who had CD155 low tumors (score 0+, 1+, 2+ [$CD155^{low}$]; *P* = 0.042, Fisher Exact Probability Test [FEPT; Figure 1B). Further, a significant association between disease progression within 6 months of ICB commencement and high CD155 tumor levels in αPD1-combitherapy treated melanoma patients was observed (*P* = 0.007, FEPT; Figure 1C). Interestingly, in melanoma patients treated with BRAFi therapy, $CD155^{high}$ tumors had a better RECIST response profile (*P* = 0.002, FEPT; Figure 1B), but not better or worse PFS (Figure 1C).

Associations between CD155 and therapy response were further evaluated using progression-free survival (PFS) and Kaplan-Meier-Cox proportional hazard modeling. For melanoma patients treated with αPD1-combitherapy, median PFS was shorter in those patients with $CD155^{high}$ tumors compared with $CD155^{low}$ tumors (HR = 2.886 [1.11 - 7.504]; *P* = 0.007; Figure 1D). For αPD1-monotherapy-treated patients, a similar trend in PFS was observed but it did not reach statistical significance (Figure 1D). For patients treated with BRAFi therapy, no difference in PFS by CD155 tumor expression levels was observed (Figure 1D) even though $CD155^{high}$ tumors responded better to BRAFi therapy by RECIST criteria (Figure 1B). A comparison of PFS between αPD1-monotherapy and αPD1-combitherapy demonstrated improved outcome in $CD155^{low}$ tumors treated with αPD1-combitherapy (HR = 2.0 [1.1 – 3.8]; *P* = 0.032), but no significant benefit was seen in $CD155^{high}$ patients by the addition of anti-CTLA4 therapy (HR = 1.0 [0.5 – 2.1]; *P* = 0.933; Figure S1A).

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CD155^{high}/PD-L1^{negative} melanomas respond poorly to anti-PD1 therapy and show decreased expression of critical genes involved in T cell function.

We next compared tumor CD155 with immunotherapy outcome for PDL1 negative and PD-L1 positive melanomas. Patients whose tumors were PDL1 negative and CD155^{high} had poor RECIST response (no PR or CR in this group; Figure 2A), regardless of whether they received αPD1-combitherapy (P = 0.0009) or αPD1-monotherapy (P = 0.05). Patients treated with αPD1-combitherapy whose tumors were PD-L1 negative and CD155^{high} also had worse 6-month PFS rates (P = 0.05; Figure 2B), and shorter median PFS (HR = 6.12 [1.3 – 29.8]; P < 0.0001; Figure 2C). In contrast, patients with PD-L1 negative tumors whose CD155 expression was low had better PFS outcome, similar to those patients whose tumors were PD-L1 positive.

We next wanted to understand how the level of CD155 protein expressed by tumor cells affected the immune cell contexture in human tumors. Gene expression data generated from pretreatment tumor specimens from 41 metastatic melanoma patients were analyzed and compared to CD155 scores from surgically matched archival-FFPE tumor specimens. Using principal component analysis, no association between PC1 or PC2 and CD155 IHC score was determined, suggesting that CD155 was not associated with features underlying the basic biology of these tumors (Figure S1B). CD155 score by IHC correlated significantly with *PVR* (*CD155*) gene expression (r = 0.604; P < 0.001; Figure S1C). Next, we determined differentially expressed (DE) genes between CD155 score 1+ versus score 3+ tumors to represent low and high levels of CD155 protein expression (n = 867 genes, p-value <

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0.01; Table S1). Reactome pathway analyses were used to identify significantly enriched biological processes. Pathways significantly down-regulated in CD155 score 3+ tumors included interferon-gamma signaling among other T cell related pathways (Figure 2D, Figure S1D), which suggested an association between CD155 and reduced $CD8^+$ T cell function. Genes in the interferon signaling pathway DE between CD155 score 1+ vs 3+ are shown in the heatmap clustered by CD155 score and PD-L1 status (Figure 2E). Out of 52 DE genes, 51 were downregulated in $CD155^{high}$ tumors. Among score 3+ tumors, there were outlier patients who had a PFS response > 6 months and these tumors were often PD-L1 positive and showed higher expression of IFN-related genes. Together, these data suggest that CD155 plays a critical role in promoting tumor immune suppression in the context of anti-PD1 therapy but that this suppression might be overcome by a robust pre-existing pretreatment immune response.

Tumor CD155 correlates with increased PD1 expression on tumor-infiltrating $CD8^+$ T cells

RNA-seq analysis of pretreatment melanoma specimens suggested that tumor CD155 might influence the phenotype and function of tumor-infiltrating $CD8^+$ T cells. Here, we used multiplex-immunohistofluorescence (multiplex-IF) to examine the expression of the key inhibitory receptor PD1 on $CD8^+$ T cells (Figure 3A). Melanoma tissue arrays or whole slide tissue sections from archival specimens were used and multiplex-IF data were matched to CD155 H-scores. Samples were enriched for high lymphocyte content selected by a pathologist using morphologically stained (H&E) slides. Tumor parenchyma ($SOX10^+$) and stromal regions ($SOX10^-$) were separately analyzed (Figure 3B). Parenchymal $CD8^+$ T cell counts did not

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correlate with tumor CD155 score indicating that T cell infiltration is independent of tumor CD155 score (Figure 3C). In contrast, the number of parenchymal $PD1^{+}CD8^{+}$ T cells significantly correlated with increasing CD155 score (r = 0.248; *P* = 0.015; Figure 3C). Notably, the ratio of parenchymal $PD1^{+}CD8^{+}$ T cells to total $CD8^{+}$ T cells ($PD1^{tR}$) significantly correlated with tumor CD155 score (r = 0.359; *P* = 0.0001; Figure 3C). These correlations were validated in an independent cohort of pretreatment melanoma specimens from patients who received targeted therapy only (Figure 3D). Interestingly, no correlation was found for the same ratio calculated for stromal-localized $PD1^{+}CD8^{+}$ T cells (data not shown). In summary, our data support the notion that tumor CD155 protein expression is associated with increased PD1 expression on tumor-infiltrating $CD8^{+}$ T cells specifically within a $PD1^{high}$ $CD8^{+}$ T cell phenotype, but does not affect the recruitment of T cells into the melanoma parenchyma.

Increased $PD1^{tR}$ is associated with progressive disease and early disease progression

We evaluated the impact of high $PD1^{tR}$ on RECIST categories and PFS outcome. Compared to CD155 score, a high $PD1^{tR}$ was more significantly associated with good RECIST categories and with higher rates of PD and SD in αPD1-combitherapy and αPD1-monotherapy treated melanoma patients (*P* = 0.001 and 0.007 respectively, FEPT; Figure 4A). A significant association between disease progression within 6 months of ICB commencement and high $PD1^{tR}$ in αPD1-combitherapy and αPD1-monotherapy treated melanoma patients was observed (*P* = 0.008 and 0.007 respectively, FEPT; Figure 4A). Shorter PFS correlated with high $PD1^{tR}$ in αPD1-combitherapy treated melanoma patients (HR = 7.301 [1.808 –

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29.49]; *P* = 0.0032) and in αPD1-monotherapy treated patients (HR = 1.905 [0.7571 – 4.793]; *P* = 0.094; Figure 4C). We next compared the predictive value of $PD1^{tR}$ with therapy-specific outcome. As expected, αPD1-combitherapy resulted in better PFS for melanoma patients with low $PD1^{tR}$, compared to αPD1-monotherapy (HR = 5.658 [2.431 - 13.17]; *P* = 0.007; Figure S1A). However, for melanoma patients with high $PD1^{tR}$, αPD1-combination therapy did not lead to improved PFS over that seen for patients treated with αPD1-monotherapy (HR = 1.582 [0.6108– 4.1]; *P* = 0.345; Figure S1A). We additionally wanted to understand if the effect of $PD1^{tR}$ on outcome was specific to immunotherapy treated patients. In *BRAF*-mutated melanoma patients who did not receive anti-PD1 therapy, but received BRAFi therapy, there was no association between RECIST response or PFS outcome with $PD1^{tR}$ (Figure 4C).

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Discussion

Development of alternative checkpoint therapies to compliment or substitute anti-PD1 and anti-CTLA4 are the subject of intensive academic and industrial pharmaceutical studies. In the current study we found that in metastatic melanoma, pretreatment CD155 tumor levels affected response to αPD1-combination therapy suggesting that targeting the CD155 pathway might be beneficial in anti-PD1 refractory melanoma patients. A possible mechanism underpinning this observation is tumor CD155 signaling through the immune checkpoint receptors CD96 and TIGIT to promote $CD8^+$ T cell inhibition. Elevated CD155 tumor expression was found to correlate with an increase in the ratio of $PD1^+CD8^+$ T cells within the tumor parenchyma ($PD1^{tR}$), and this increase was associated with progressive disease and early time to progression among αPD1-combination treated melanoma patients. The detrimental effect of high CD155 expression was evident but was not statistically significant in αPD1-monotherapy treated patients. One explanation for this might be the greater effectiveness of αPD1-combination therapy (15), resulting in more impressive RECIST response and survival outcomes for patients with favorable tumor CD155 expression. Further, CD155 suppression of therapeutic response was specific to ICB-treated patients as it was not observed in melanoma patients treated with BRAFi therapy, indicating that tumor CD155 specifically limits sensitivity to ICB therapy in metastatic melanoma. Indeed, in BRAFi-treated patients a high tumor CD155 predicted better RECIST response albeit with no difference in PFS compared to patients whose tumors were CD155 low. Given these data, we hypothesize that co-targeting the CD155 pathway (CD96/TIGIT co-blockade) in combination with anti-PD1 therapy might increase response rates in anti-PD1 refractory patients.

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Analysis of the spatial distribution of lymphocyte subsets in tumors can be predictive and reveal aspects of tumor biology not apparent in gross T cell estimations typically achieved in flow cytometry data (16-18). We found a positive correlation between CD155 tumor levels and $PD1^{tR}$ when lymphocytes were counted within the tumor parenchyma, but not for lymphocytes counted within the tumor stroma. This suggests that $PD1^{tR}$ might partially be driven by the interaction of the CD155 ligand on tumor cells with its cognate T cell receptors (TIGIT and CD96). Further this parenchymal interaction of tumor CD155 and T cells could be affecting sensitivity to anti-PD1 therapy (11). Better PFS outcome was observed in $CD155^{high}$ tumors which had a favorable pretreatment PD-L1 status and IFN gene signatures, reflecting an active pretreatment anti-tumor immune response (19-22). However, no apparent statistical benefit from combination therapy coupled with the inferior survival outcomes in melanoma patients with $CD155^{high}$ or high $PD1^{tR}$ tumors suggests that additional or alternative treatment approaches are required for those patients, possibly including blockade of CD155 interactions with TIGIT and CD96. The immunosuppressive effect of CD155 on response to anti-PD1 therapy was most apparent in PD-L1 negative tumors that scored 3+ for CD155, regardless of ICB therapy type, and this group of patients showed no complete or partial RECIST responses.

Interestingly, CD155 negative tumors consistently demonstrated excellent outcome to ICB therapy, however, RNA-seq of CD155 negative melanoma tumors indicated a diminished IFN gene signature and were often PD-L1 negative. This suggests that tumors of this type do not contain a pre-treatment immune response and this is

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particularly interesting given that PD-L1 negative tumors with limited IFN gene signatures are generally thought to respond poorly to immunotherapy (19-22). However, CD155 has an intrinsic role in mediating tumor cell growth and invasion and these tumors may represent a less aggressive melanoma tumor type (11). Nevertheless, given the positive response of CD155 negative tumors to ICB, the absence of an existing intra-tumor immune response before treatment must not preclude the development of one on treatment, at least not in CD155 negative melanomas. A caveat is that the natural history of CD155 negative tumors is unknown and this may be an intrinsically positive prognostic factor independent of ICB treatment. In any case CD155 negative melanomas are rare (<5%) and the biology of CD155 negative tumors deserves greater scrutiny.

We have previously shown in mice that expression of CD155 in the tumor microenvironment limits the efficacy of tumor growth control by T and NK cells (11,23,24). Recently, it has been shown that $PD1^{hi}CD8^{+}$ T cells in chronic infection and cancer are dysfunctional and in fact epigenetically repressed and terminally differentiated, as such, this population is insensitive to anti-PD1 therapy (25,26). Given that multiplex-IF is less sensitive than conventional flow cytometry, it may be reasonable to assume that $PD1^{+}CD8^{+}$ T cells as detected by multiplex-IF in patient samples are those expressing the highest levels of PD1 ($PD1^{+}CD8^{+}$ = $PD1^{hi}CD8^{+}$) and therefore likely to have a dysfunctional phenotype. While we have not demonstrated that the $PD1^{+}CD8^{+}$ T cell population observed by multiplex IHC in pretreatment human melanoma specimens was dysfunctional *per se*, the combination of our observations made by IHC and bulk tumor RNA-seq supports a model in which tumor cell CD155 expression correlates with high $PD1^{tR}$ levels

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thought to be associated with a dysfunctional $CD8^+$ T cell phenotype. Importantly, varied mechanisms of resistance to anti-PD1 therapy can affect response rates (9,27-30), nevertheless, CD155 is commonly expressed in metastatic melanoma lesions (>95%) indicating the CD155 pathway is an attractive immunotherapy co-target in anti-PD1 ICB therapy.

$CD8^+$ T cells infiltrating human melanoma also express TIGIT and CD96 in addition to PD1 (24,31,32). Therefore, it may be reasonable to propose that the inhibitory signaling mediated not only through PD1, but also CD96 and TIGIT, drives T cell dysfunction. Indeed, co-blockade of PD1 and CD96 in established CT26 colon adenocarcinomas increased IFNγ production above that observed for anti-PD1 blockade, suggesting that these cell populations may be amenable to functional reinvigoration (23). Further, therapeutic blockade of TIGIT and CD96 had superior activity in controlling primary tumor growth compared to αPD1-monotherapy in B16F10 melanoma tumor models (23). In mouse tumor models using CD155-deficient mice, it was previously shown that both tumor and host CD155 were critical for tumor growth, so therapeutic targeting of TIGIT and CD96 must take this into consideration (11). The contribution of CD155-dependent signaling could potentially be assessed in early-phase clinical trials of anti-TIGIT as monotherapy or in combination with anti-PD1 (NCT02964013), and in other future randomized controlled trials targeting members of this pathway.

In summary, we have shown that high expression of CD155 in metastatic melanoma correlates with an increase in the intratumor ratio of $PD1^+CD8^+/CD8^+$ T cells, abbreviated here as $PD1^{tR}$, and reduced sensitivity to αPD1-combitherapy. It is likely

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that $PD1^{+}CD8^{+}$ T cells detected by multiplex-IF are of a $PD1^{hi}$ phenotype and thus dysfunctional and resistant to PD1-based ICB reinvigoration. Further CD155 combined with PD-L1 might be a useful predictor of a group of patients who do not respond to anti-PD1 ICB. Our findings give impetus for validation in a prospective cohort the utility of CD155 plus PD-L1 as a predictive biomarker, and for clinical trials to assess therapies blocking CD96/TIGIT or CD155 in combination with anti-PD1 therapy.

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Methods

Patients and specimens

The study was approved by the QIMR Berghofer Human Research Ethics Committee (HREC). Research involving human subjects was also approved by HRECs at each clinical site and was conducted according to the Declaration of Helsinki. Where prospective biospecimen collection was undertaken, informed consent was granted by study participants. For retrospective annotated specimens a waiver of consent was gained by the site HREC. Retrospective archival-FFPE tissue specimens were obtained for patients with radiologically confirmed stage IV melanoma (AJCC) from four institutional sites; Melanoma Institute Australia [MIA], Royal Brisbane Women's Hospital [RBWH], Istituto Nazionale Tumori - IRCCS [INT-IRCCS], and Papa Giovanna XXIII Hospital [PG-XXIII]. Presence of tumor cells was monitored by H&E staining. Patient demographics, primary tumor characteristics and therapy details are listed in Table-1. Fresh tumor specimens for RNA-seq analysis were collected through the MIA tumor biobank. REMARK guidelines (33) were followed where data were available from the contributing institute.

Immunohistochemistry

Archival-FFPE tumors were sectioned at 3 µm on superfrost+ slides. Slides were dehydrated at 65^{o}C for 20 min, deparaffinized in xylene and rehydrated in graded ethanol. Antigen retrieval was performed in EDTA buffer (pH 9) in a Decloaking Chamber (Biocare Medical) at 100ºC for 20 min. IHC was performed on an Autostainer-Plus (DAKO). Primary antibodies against CD155 (D3G7H; CST#13544) or PDL1 (E1L3N; CST#13684) were incubated for 45 minutes at room temperature using a 1:100 dilution for CD155 or 1:150 for PDL1. Staining was visualized using a

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Rabbit-HRP-polymer detection system (Biocare; M3R531) and DAB Chromogen Kit (Biocare; BDB2004) and counterstained with diluted hematoxylin. IHC was evaluated for two representative high-power fields of view noting the percentage of membrane positive tumor cells and the maximum intensity of immunohistochemical signal (0+ to 3+). CD155 score was assigned using the blinded H-score method and categorized as follows; score 0+ (negative), score 1+ (0 - 99), score 2+ (100 - 199), or score 3+ (200 - 300). PDL1 score was assigned as the combined percentage of PDL1 positive tumor and inflammatory cells per representative whole specimen slide.

Multiplex immunohistofluorescence

Archival-FFPE tissue specimens were sectioned at 3 µm onto Superfrost+ slides. Slides were then deparaffinized, rehydrated, and washed in tris-buffered saline with 0.01% Tween-20 (TBS-T). Antigen retrieval was performed in modified citrate buffer pH 6.1 (Agilent; S169984-2) at 100^{o}C for 20 minutes. All multiplex steps were performed using an Autostainer Plus (Dako, Agilent Technologies) with two TBS-T washes between each step. Tissue sections were blocked with 3% hydrogen peroxide in TBS-T for 5 min and background sniper for 10 min (Biocare Medical). Sequential staining was performed using the Opal method (PerkinElmer) with antibody stripping steps performed in Tris/EDTA buffer (Agilent; S236784-2) at 100^{o}C for 20min. Primary antibodies incubated for 30 minutes, followed by two-step polymer-HRP detection (Biocare; Mach3) and then labeled with TSA-based fluorophores (PerkinElmer; Opal Reagent Pack). The following primary antibodies/clones were used sequentially in the order listed; PD1/NAT105 (1:500;Opal520), CD8/144b (1:7500; Opal570), and SOX10/BC34 (1:600; Opal690). Slides were counterstained with DAPI and cover-slipped (DAKO; S3023).

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Multiplex-IF Image acquisition and analysis

Images were obtained using the Vectra 3.0 slide scanner (PerkinElmer) under the appropriate fluorescent filters. A fluorescent whole slide scan at 4x was produced and visualized in Phenochart (v1.0.4), followed by multispectral image acquisition of each TMA core or selected high power regions at 20x. Multispectral images were spectrally unmixed followed by tissue and cell segmentation using InForm analysis software (v2.2.1). Nuclear expression of SOX10 by tumor cells was used to segment melanoma tumor parenchyma and stroma tissue regions. Merged data files from InForm were pre-processed and fluorescence thresholds were set using Spotfire image-mapping tools for each marker ($PD1^+$, $CD8^+$, and $SOX10^+$; Tibco Spotfire Analyst, v7.6.1) followed by segmented cell counting using Spotfire and tabulation in Microsoft Excel.

RNA-seq preparation, data processing, and differential expression analysis

RNA-seq was performed on pretreatment tumor specimens from 41 metastatic melanoma patients treated with immunotherapy at Melanoma Institute Australia. Briefly, total RNA was isolated from fresh frozen tissue sections using the AllPrepDNA/RNA/miRNA Universal kit (Qiagen) according to the manufacturer's instructions (34,35). RNA quantity was assessed on Qubit, and RNA integrity was assessed using the RNA 6000 Nano kit and run on the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA synthesis and library construction were performed using the TruSeq RNA Library Prep Kit (Illumina) and paired-end 100bp sequencing, with each sample yielding 40-50 million read. Sequencing was performed on the Illumina Hiseq 2500 platforms at the Australian Genome Research Facility in

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Melbourne. Fastq data were downloaded and sequence reads were trimmed for adapter sequences using Cutadapt (version 1.9)(36) and aligned using STAR (version 2.5.2A) to the GRCh37 human reference genome assembly using the gene, transcript, and exon features model of Ensembl (release 70). Quality control metrics were computed using RNA-SeQC (version 1.1.8) and transcript abundances were quantified using RSEM (version 1.2.30). Further analysis of the RNA-seq data was carried out in R (version 3.5.1). Protein-coding genes with < 3 counts per million in fewer than 5 samples were removed from down-stream analyses. Trimmed mean of M-values (TMM) normalization and differential gene expression analysis were performed using the edgeR package (37). The `prcomp` function in R was used to perform principal component analysis on gene-wise centered and scaled values of TMM normalized expression data. To perform pathway analysis, the `clusterProfiler::bitr` function (38) was used to convert gene IDs from Ensembl to Entrez, then consequently passed to the `ReactomePA::enrichPathway` function (39), before plotting the results with the `clusterProfiler::dotplot` function (38). Heatmaps were produced using `ComplexHeatmap` R package (40,41) using gene-wise centered, scaled, log2 values of TMM normalized expression data, and "Pearson" distance with "ward.D" criteria to cluster the rows. Cytolytic activity was calculated as the geometric mean of GZMA and PRF1 (as expressed in TPM) as per (40). RNA-seq data analyzed in this study have been published (42) and deposited in the European Genome-phenome Archive (EGA) under dataset accession EGAD00001005501 and study accession EGAS00001001552.

Outcome analysis

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Univariate survival analysis was carried out by fitting Cox proportional hazard models to dichotomize patient groups according to respective variables and the survival variable. Cut-off points for the dichotomized variable were calculated as the arithmetic mean of the two successive values which gave the most significant log-rank split using cut-off finder version 2.0(43). Hazard ratios (HRs) including 95% confidence intervals are calculated and log-rank *P* values given. PFS was defined as time from commencement of therapy to documented disease progression (PFS). Response to ICB was assessed by site investigators using timepoint RECIST version 1.1 (i.e. best response in time-point fashion).

Statistical methods and data availability

Correlations between categorical clinical variables and experimental variables (CD155, $PD1^{tR}$) were calculated using a two-tailed Fisher Exact Probability Test (FEPT). Correlations of CD155 immunohistochemistry with immune cell counts by multiplex-IF were performed using Pearson r method. Statistical analyses listed in figure legends were performed using PRISM. All data for multiplex-IF and chromogenic IHC supporting the findings of this study are available from the corresponding author upon reasonable request. RNA-seq data have been deposited in the European Genome-Phenome Archive (accession number: EGAD00001005501).

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Figure Legends

Figure 1. High tumor CD155 expression limits response of metastatic melanoma patients to αPD1-combitherapy and predicts better RECIST response to BRAFi-targeted therapy.

(A) Representative immunohistochemistry images of CD155 H-scores (0+, 1+, 2+, 3+) from metastatic melanoma tumor specimens. **(B)** Histograms for CD155 H-scores by RECIST category (Complete Response [CR], Partial Response [PR], Stable Disease [SD], Progressive Disease [PD]) in pretreatment tumor specimens from metastatic melanoma patients treated with either αPD1-combitherapy (*n* = 64 patients), αPD1-monotherapy (*n* = 87 patients), or BRAFi therapy (*n* = 41 patients). Fisher Exact Probability Test (FEPT) by CR+PR *vs* SD+PD and $CD155^{low}$ (score 0+,1+,2+) *vs* $CD155^{high}$ (score 3+). *P < 0.05; **P < 0.01; n.s. not significant. **(C)** The fraction of patients with progression free response to therapy greater than 6 months. FEPT by response > 6 months and response < 6 months *vs* $CD155^{low}$ (score 0+, 1+, 2+) *vs* $CD155^{high}$ (score 3+). **P < 0.01; n.s. not significant **(D)** Association of pretreatment tumor CD155 H-scores, $CD155^{low}$ (0+,1+,2+) vs $CD155^{high}$ (3+) with progression-free survival (PFS) evaluated using the Kaplan-Meier method and Cox proportional hazard modeling in melanoma patients treated with αPD1-monotherapy (*n* = 57, $CD155^{low}$; *n* = 34, $CD155^{high}$; HR = 1.377; *P* = 0.26), αPD1-combitherapy (*n* = 45, $CD155^{low}$; *n* = 19, $CD155^{high}$; HR = 2.886; *P* = 0.007), or BRAFi therapy (*n* = 25, $CD155^{low}$; *n* = 16, $CD155^{high}$; HR = 1.037; *P* = 0.9176).

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Figure 2. Anti-PD1 therapy is ineffective in PDL1 negative melanomas that are high for CD155 expression and CD155 high tumors show decreased expression of critical genes involved in T cell function.

(A) Histograms of RECIST response categories (Complete Response [CR], Partial Response [PR], Stable Disease [SD], Progressive Disease [PD]) in metastatic melanoma by PDL1 status and CD155 tumor expression ($CD155^{high}$ [3+] *vs* $CD155^{low}$ [0+, 1+, 2+]) in pretreatment tumor specimens from metastatic melanoma patients treated with either αPD1-combitherapy (*n* = 38 patients) or αPD1-monotherapy (*n* = 44 patients). Fisher Exact Probability Test (FEPT) by CR+PR *vs* SD+PD and $PDL1^{negative}/CD155^{high}$ *vs* other scores. *P < 0.05; ***P < 0.001. **(B)** The fraction of patients with response > 6 months by PDL1 status and CD155 tumor expression. FEPT by response > 6 months *vs* response < 6 months and $PDL1^{negative}/CD155^{high}$ *vs* other scores. **P < 0.01; n.s. not significant. **(C)** Progression free survival (PFS) of metastatic melanoma patients categorized by PDL1 status and CD155 tumor expression. Association between $PDL1^{negative}/CD155^{high}$ *vs* other scores evaluated using Kaplan-Meier method. Patients were treated with either αPD1-monotherapy (*n* = 11, $CD155^{low}/PDL1^{positive}$; *n* = 12, $CD155^{high}/PDL1^{positive}$; *n* = 5, $CD155^{high}/PDL1^{negative}$; *n* = 17, $CD155^{low}/PDL1^{negative}$; HR = 2.391; *P* = 0.0584) or αPD1-combitherapy (*n* = 11, $CD155^{low}/PDL1^{positive}$; *n* = 3, $CD155^{high}/PDL1^{positive}$; *n* = 7, $CD155^{high}/PDL1^{negative}$; *n* = 17, $CD155^{low}/PDL1^{negative}$; HR = 6.117; *P* < 0.0001). **(D)** The top 20 enriched Reactome pathways from differential gene expression analysis (p-value < 0.01) of CD155 H-score 1+ vs 3+ melanomas. (**E)** Heatmap of genes which contribute to the Reactome pathway “Interferon signaling” (R-HSA-913531). Patients are clustered

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according to CD155 H-score (from left to right: 0+, 1+, 2+, 3+) followed by PDL1 status (*n* = 41 patients).

Figure 3. Intratumor ratio of PD1$^+$CD8$^+$ T cells to total CD8$^+$ T cells correlates with tumor CD155 levels.

(A) Representative multiplex-IF composite images of melanoma tumors with increasing CD155 score stained for CD8$^+$ (red), PD1$^+$ (green), and SOX10$^+$ (light grey nuclei). White arrows indicate PD1$^+$CD8$^+$ lymphocytes. **(B)** Representative images for computational image analysis defining tissue category regions showing tumor parenchyma and tumor stroma with CD8$^+$ T cells in tumor stroma indicated by red arrows and CD8$^+$ T cells in tumor parenchyma indicated by white arrows. **(C)** Data summaries of total counts for tumor-infiltrating CD8$^+$ lymphocytes (R = 0.130; *P* = 0.206), total PD1$^+$CD8$^+$ lymphocytes (R = 0.248; *P* = 0.015), and the ratio of PD1$^+$CD8$^+$ to total CD8$^+$ lymphocytes (PD1^{tR}; R = 0.359; *P* < 0.0001), against CD155 H-score in pretreatment specimens from immunotherapy treated metastatic melanoma patients (*n* = 106 patients). **(D)** Validation of data in (C) using an independent cohort of patients treated with BRAFi therapy (*n* = 48 patients). Data summaries of total counts for tumor-infiltrating CD8$^+$ lymphocytes (R = 0.095; *P* = 0.521), total PD1$^+$CD8$^+$ lymphocytes (R = 0.302; *P* = 0.037), and PD1^{tR} (R = 0.382; *P* < 0.007). Pearson correlation coefficient analyses were used (C and D) to assess the relationship between CD155 H-score and CD8$^+$ T cells, PD1$^+$CD8$^+$ T cells, and PD1^{tR}. Exact *P* values and R coefficients have been included in each chart.

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Figure 4. PD1^{tR} correlates with response to immunotherapy but not targeted therapy treated metastatic melanoma.

(A) Histogram of immunotherapy RECIST response categories (Complete Response (CR), Partial Response (PR), Stable Disease (SD), Progressive Disease (PD)) from αPD1-combitherapy (*n* = 39), αPD1-monotherapy (*n* = 64), and BRAFi therapy (*n* = 39) treated metastatic melanoma patients. Fisher Exact Probability Test (FEPT) by CR+PR *vs* SD+PD and PD1^{tR}low *vs* PD1^{tR}high. **P < 0.01; ***P < 0.001; *n.s.* not significant. **(B)** The fraction of patients with therapy response greater than 6 months by PD1^{tR} measured in pretreatment tumor specimens. FEPT by response > 6 months vs response < 6 months and PD1^{tR}low vs PD1^{tR}high. **P < 0.01; n.s. not significant. **(C)** Association of pretreatment tumor PD1^{tR} with progression free survival (PFS) evaluated using the Kaplan-Meier method and Cox proportional hazard modeling in αPD1-combitherapy (*n* = 39; HR = 7.301; *P* = 0.0032), αPD1-monotherapy (*n* = 66; HR = 1.905; *P* = 0.094), and BRAFi therapy (*n* = 41; HR = 1.007; *P* = 0.9848), treated metastatic melanoma patients.

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Declaration of Interests

Disclosures: MJS has research agreements with Bristol Myers Squibb, Aduro Biotech and Tizona Pharmaceuticals. MWLT has received speaker's honorarium from Roche, MSD, and BMS. WCD has received research funding from Bristol Myers Squibb and is a consultant for Omeros Corporation and Cascadia Drug Development Group. T.B. has research agreements with Bristol Myers Squibb and Ena Therapeutics and is member of the Scientific Advisory Board of Oncomyx. EA has speaker's honorarium from Amgen Australia, Conference/meeting travel/accommodation expenses or sponsorship from MSD, BMS, Roche, Amgen. GVL is consultant advisor to Aduro, Amgen, BMS, Merck MSD, Novartis, Roche. PAA is consultant advisor to BMS, Roche-Genentech, MSD, Array, Novartis, Merck Serono, Pierre Fabre, Incyte, Genmab, Newlink Genetics, Medimmune, AstraZeneca, Syndax, Sun Pharma, Sanofi, Idera, Ultimovacs, Sandoz, Immunocore, 4SC, Research Funds from BMS, Roche-Genentech, Array, Travel support from MSD. RAS has served on advisory boards for Merck Sharp Dohme, Glaxo Smith Kline Australia, Bristol Myers Squibb, Novartis Pharmaceuticals Australia, Myriad, Amgen, and NeraCare, unrelated to the content of this work. All other authors declare that they have no conflict of interest.

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Figure 1

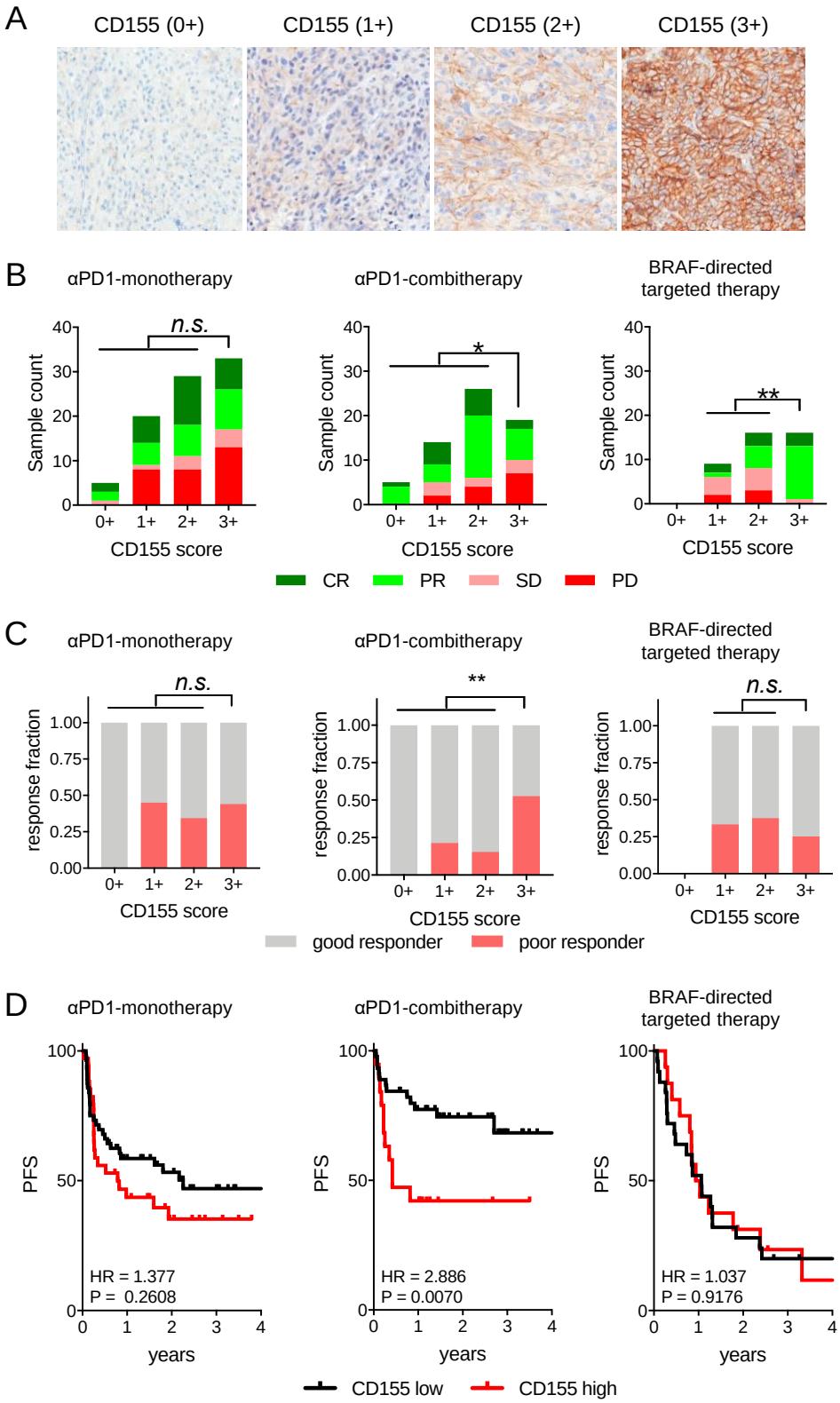

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Figure 2

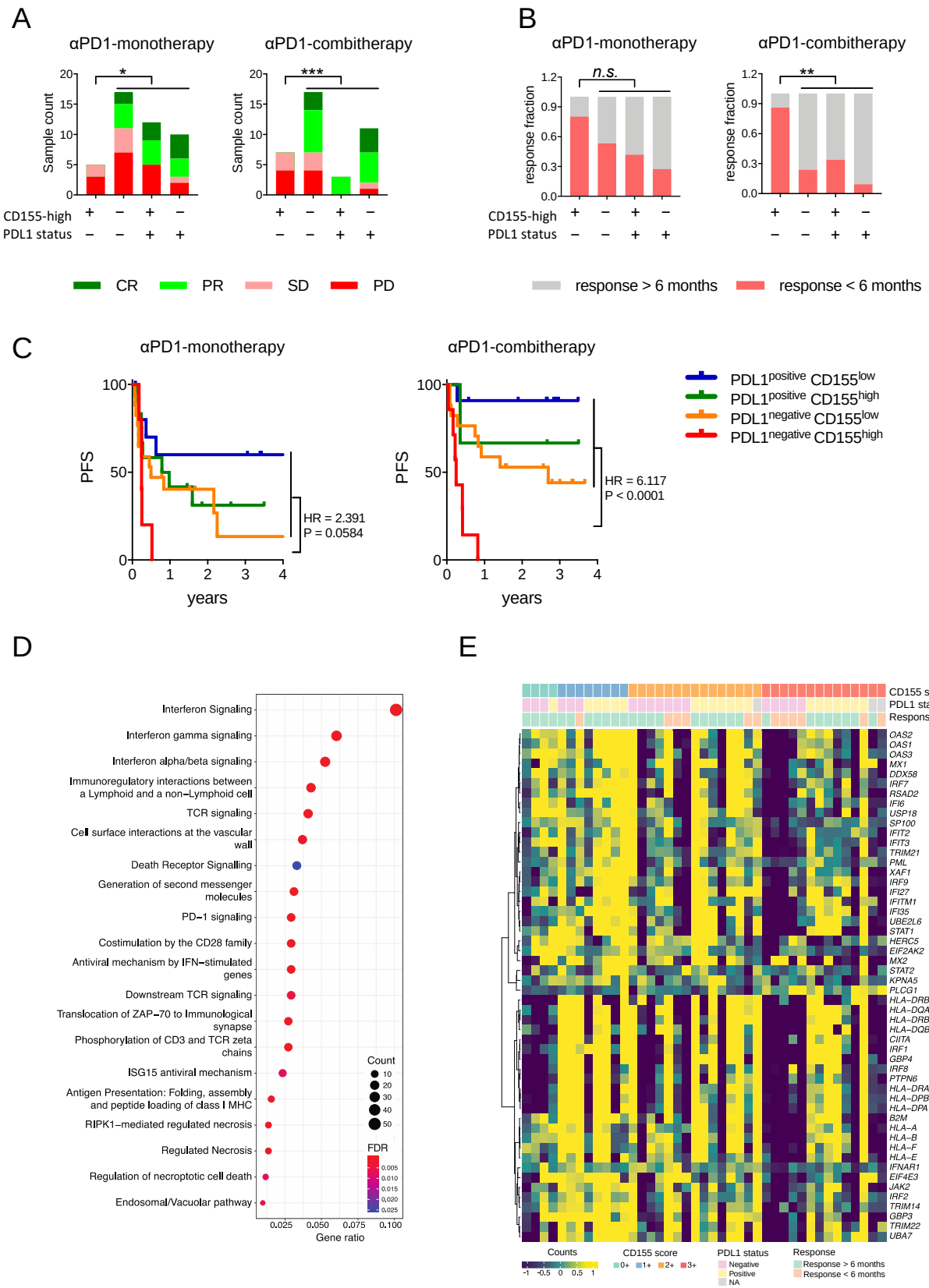

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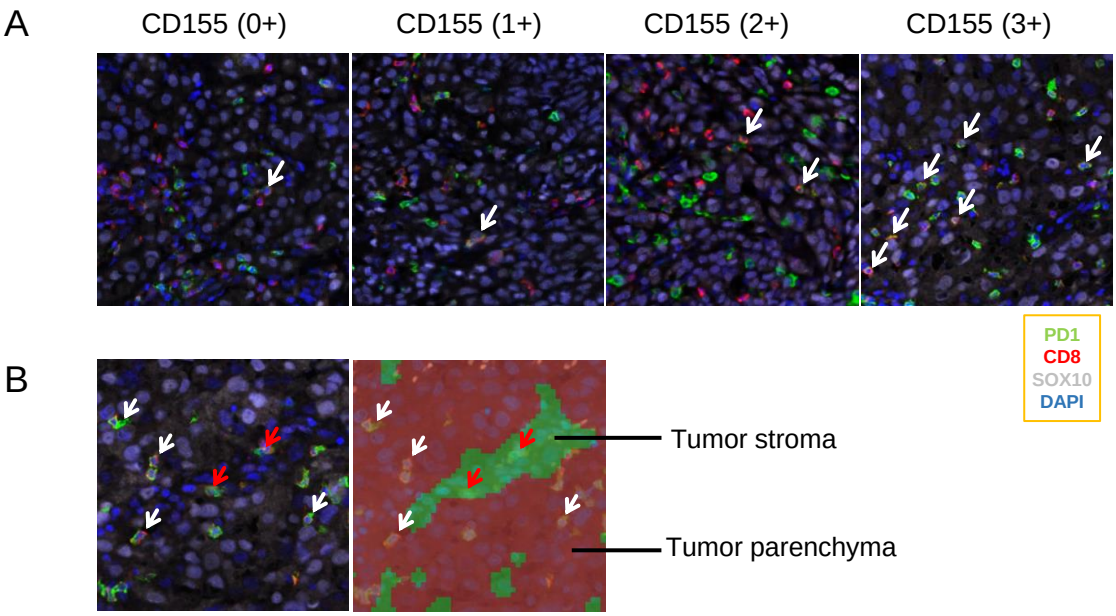
Figure 3



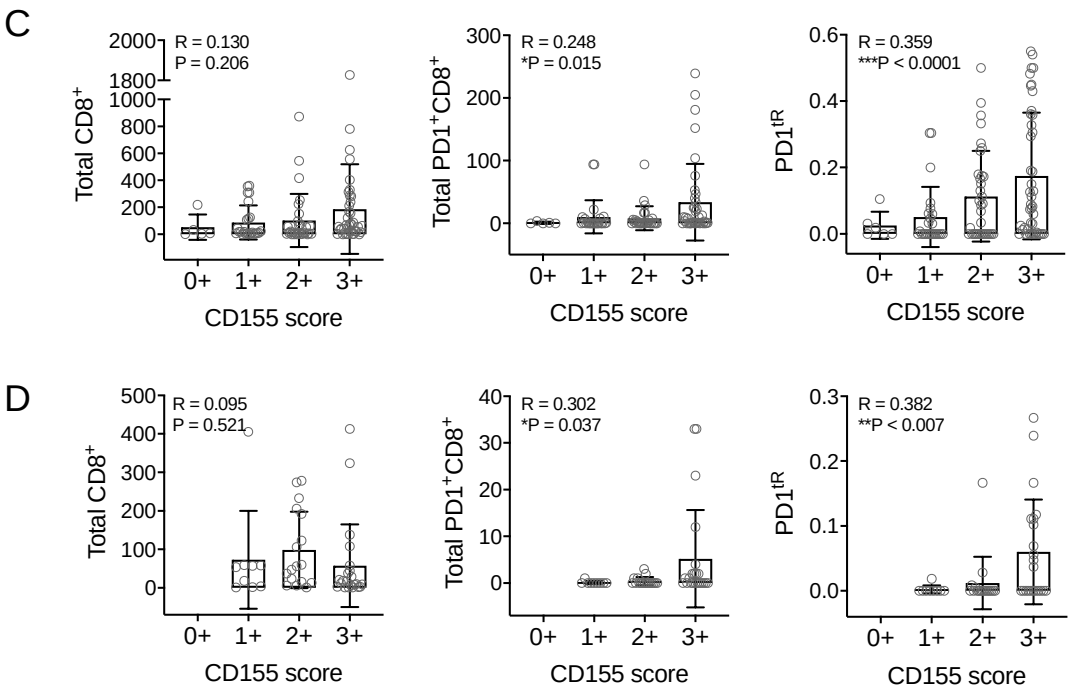


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Figure 4

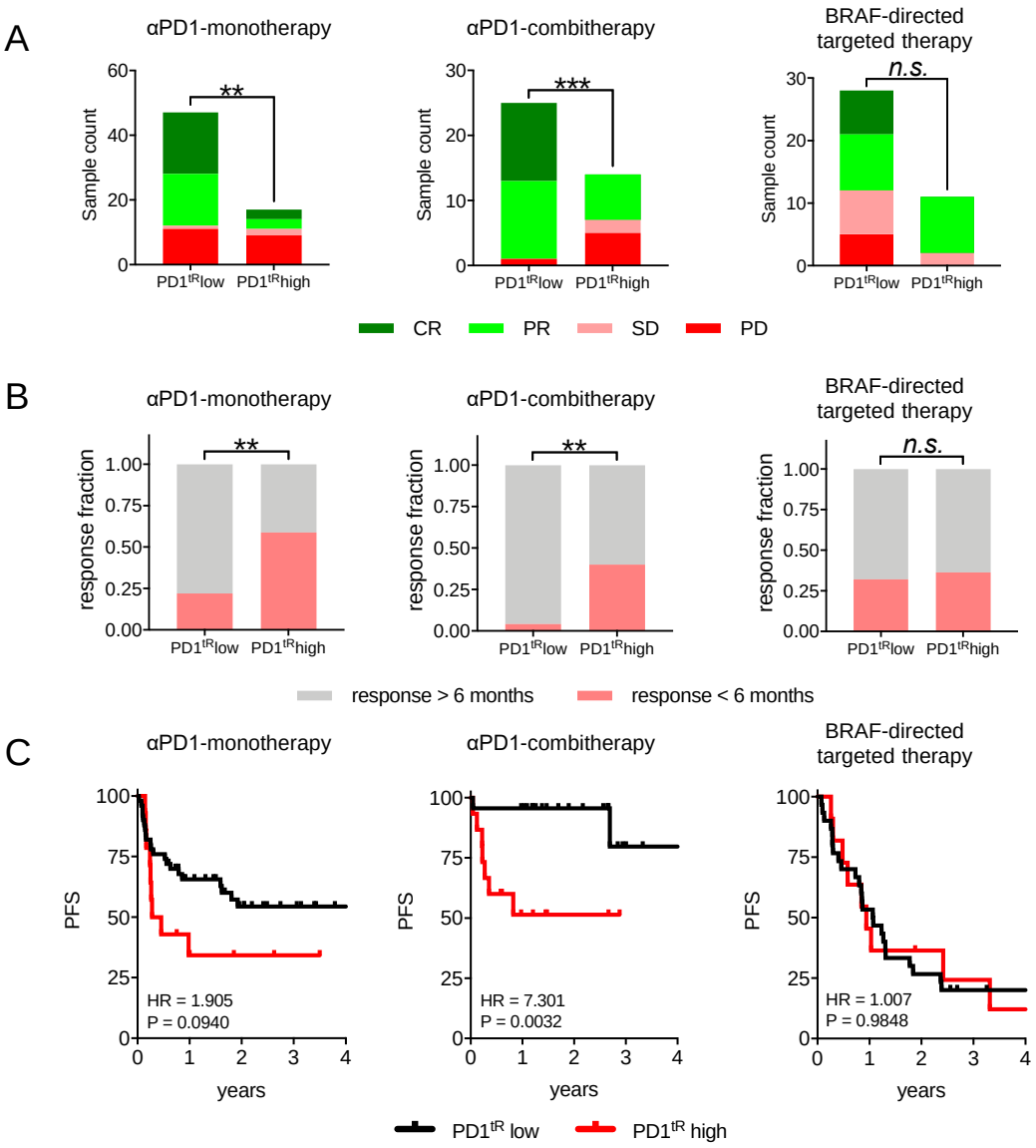

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Table 1 – Specimen cohort details

	[MIA] melanoma	[RBWH] melanoma	[INT-IRCCS] melanoma	[PG-XXIII] melanoma
Specimen details				
n =	101	33	21	50
Sex				
female	24	8	9	23
male	62	25	12	27
unknown	15			
Median age (start of IO)	67	68	64	54
Surgery type				
excision	101	25	21	50
biopsy		8		
Multiplex-IF data	yes	yes	no	yes
Tissue microarray	yes	yes	no	yes
Therapy details				
Nivolumab	16		7	
Pembrolizumab	33	29	3	
Ipi + Nivo	18	4	8	
Ipi + Pembro	34		3	
1st line BRAFi targeted therapy	7	6	2	41
Clinically reported variables				
BRAF mutant	22	7	6	50
BRAF wild type	60	26	15	
LDH elevated	16		10	
LDH normal	63		10	
RECIST response				
PD	22	10	10	9
PR	42	6	3	16
SD	6	4	7	8
CR	28	11	1	8
no data	3	2		
Progression summary				
# censored subjects	64	16	3	8
# events (progression)	37	17	18	33
Median PFS (years)	4.83	0.82	0.42	1.03
Median follow-up	1.38	0.63	0.41	1.56
Survival summary				
# censored subjects	75	19	5	10
# events (death)	26	14	16	31
Median OS (years)	undefined	undefined	0.833	1.85
Median follow-up	1.68	1.44	0.82	2.14
CD155 H-Score				
0+ (0)	7	0	3	0
1+ (1 - 99)	23	7	4	11
2+ (100 - 199)	35	14	9	17
3+ (200 - 300)	36	12	5	22

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