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Supplementary Materials for

MHC proteins confer differential sensitivity to CTLA-4 and PD-1 blockade in untreated metastatic melanoma

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at www.sciencetranslationalmedicine.org/cgi/content/full/10/450/eaar3342/DC1)

Materials and Methods

Clinical Trials

All biopsy samples in the CheckMate 064 trial were collected during the screening period (28 days before the first dose of study drug). The majority of pretreatment specimens were from lesions from metastatic sites (80.9%). The breakdown by metastatic site was as follows: 8.6% lung, 23.7% liver, 33.3% skin/soft tissue, 18.3% lymph nodes, 15.1% other, and 1.1% not reported. In the CheckMate 069 trial, there was no requirement for tumor tissue to be obtained during the screening period before treatment. Instead, tumor tissue was obtained in the metastatic setting or from an unresectable site of disease. The majority of samples with evaluable tissue (98%; 121 of 123) were obtained from metastatic sites.

Immunohistochemistry evaluation and scoring

For major histocompatibility complex (MHC) class I, MHC class II, and beta-2-microglobulin (β2M) immunohistochemistry (IHC), non-neoplastic immune cells, endothelial cells, and stromal cells served as positive internal controls for staining. Stained cases were evaluated separately by two pathologists and then subsequently reviewed together to tabulate a final consensus score (*42*). Individual pathologist IHC scores classifying tumors as above or below the eventually established thresholds were highly concordant (92% to 98%), and consistent with a prior study (*42*).

RNA and DNA sequencing of fresh frozen tissues

RNA concentration and purity were measured on NanoDrop and the RNA integrity was analyzed on Agilent Bioanalyzer. RNA sequencing was performed by Q2 Solutions/EA Genomics using a minimum of 250 ng of total RNA input using the TruSeq Stranded mRNA

with 50 million paired-end reads on Illumina HiSeq. Raw FASTQ files were aligned to Human Ensembl Genome GRCh37 using the STAR aligner using default settings. Raw counts were quantified using HTseq (*51*). Counts were normalized with the calcNormFactors package of the R/Bionconductor package edgeR (*52, 53*) using the weighted trimmed mean of M-values (TMM) method (*54*). Transcripts from the γδ T-cell constant region were quantified using Salmon (*55*) with Gencode v26.

Alignment of exome reads was performed using our Genome Modeling System (GMS) processing-profile (*56*). This pipeline uses BWA (version 0.5.9) for alignment with default parameters except for the following: '-t 4 -q 5' (*57*). All alignments were against GRCh37-lite-build37 of the human reference genome and were merged and subsequently de-duplicated with Picard (version 1.46).

Development of gene set scores

Differential gene analyses were performed using the *limma* package (version 3.28.21) (*58*). The 10 gene IFN-γ pathway signature was previously validated and published as predictive of response to pembrolizumab in a variety of tumor types (*23*). The top 25 differentially expressed genes in cohort A are indicated and had uncorrected p-values (range: 0.0002-0.01) and false discovery rates (FDRs; range: 0.14-0.269). The IFN-γ gene set score was calculated using the R/Bioconductor package GSVA (*59*) and was based on a manually curated set of 13 genes (out of the top 25 genes, blue highlighted genes in Fig. 3) that were found to be directly related to IFN-γ biological activity with literature review. The empirically-derived 25 gene set showed partial overlap with the previously published 10 gene IFN-γ pathway signature (6 genes).

NK-cell gene score was calculated using GSVA and was based on a manually curated set of 20 genes determined to be NK cell-specific with literature review: *B3GAT1*, *FCGR3A*, *KIR2DL1*, *KIR2DL3*, *KIR2DL4*, *KIR2DS4*, *KIR3DL1*, *KIR3DL2*, *KIR3DX1*, *KLRA1P*, *KLRB1*, *KLRC3*, *KLRC4*, *KLRC4-KLRK1*, *KLRF1*, *KLRF2*, *KLRG1*, *KLRG2*, *KLRK1*, and *SIGLEC7*. A one-sided nonparametric Wilcoxon test was used to derive a p-value between groups of GSVA scores.

Supplementary Figures

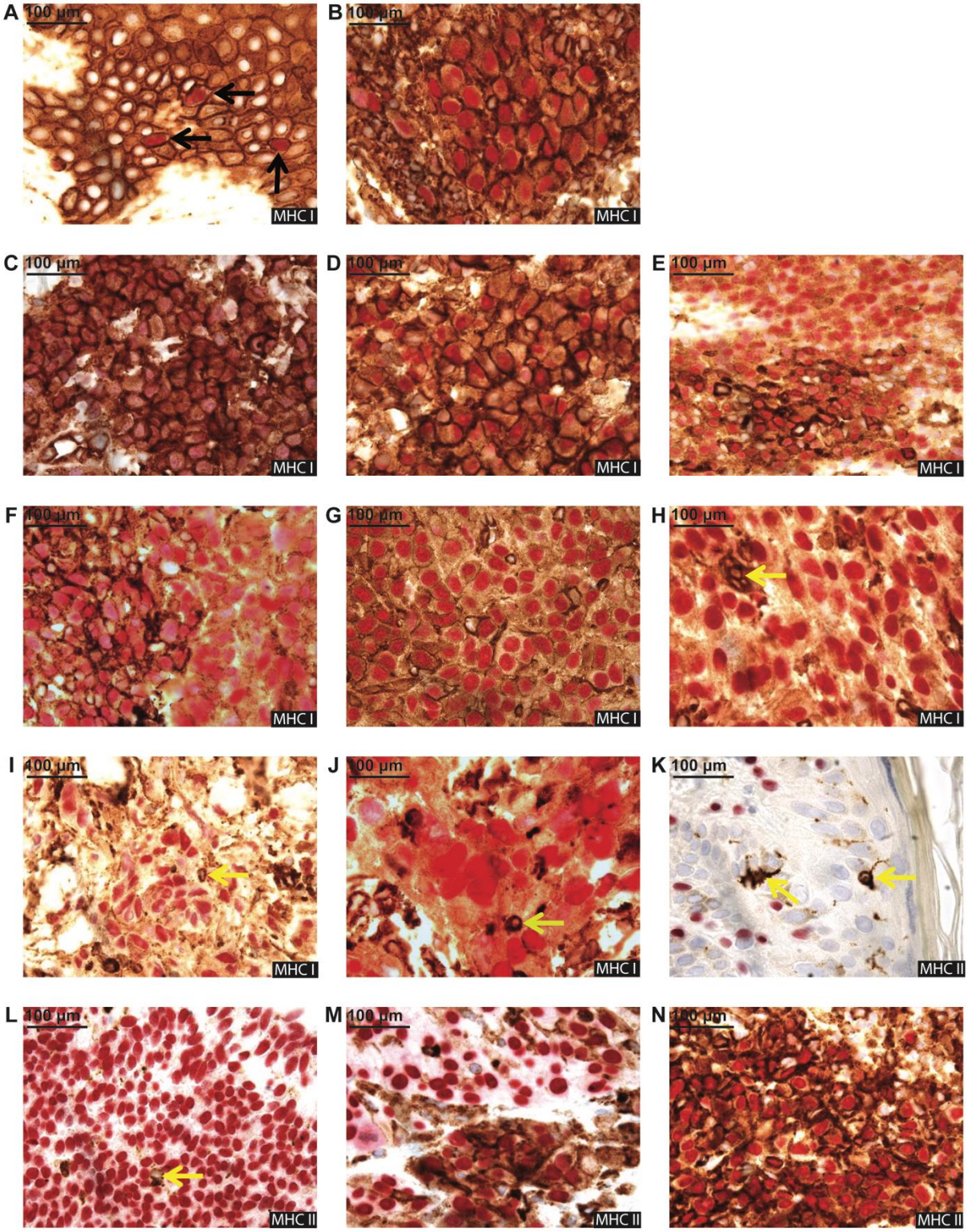

A
100 µm
MHC I
B
100 µm
MHC I
C
100 µm
MHC I
D
100 µm
MHC I
E
100 µm
MHC I
F
100 µm
MHC I
G
100 µm
MHC I
H
100 µm
MHC I
I
100 µm
MHC I
J
100 µm
MHC I
K
100 µm
MHC II
L
100 µm
MHC II
M
100 µm
MHC II
N
100 µm
MHC II

Fig. S1. Representative IHC images. Dual chromogenic IHC for SOX10 (red) and MHC class I (brown) performed on (**A**) normal skin and (**B** to **J**) 181 individual melanomas showing representative (**A**) positive membrane staining of SOX10-negative keratinocytes and positive membrane staining of SOX10-positive melanocytes (black arrows), (**B** to **D**) positive membrane staining of all SOX10-positive melanoma cells, (**E** to **G**) positive membrane staining of a subset of melanoma cells (range 30-50%), and (**H** to **J**) positive membrane staining of no melanoma cells (positive membrane staining of SOX10-negative inflammatory cells serve as an internal control, yellow arrows). Dual chromogenic IHC for SOX10 (red) and MHC class II (brown) performed on (**K**) normal skin and (**L** to **N**) 181 individual melanomas showing representative (**K**) positive membrane staining of SOX10-negative cells with long cytoplasmic processes morphologically consistent with Langerhans cells (yellow arrows), but no staining of SOX10-positive melanocytes, (**L**) no membrane staining of SOX10-positive melanoma cells (positive membrane staining of SOX10-negative inflammatory cells serve as an internal control, yellow arrow), (**M**) positive membrane staining of a subset of melanoma cells (20%), and (**N**) positive membrane staining of nearly all melanoma cells (90%). IHC, immunohistochemistry; MHC, major histocompatibility complex.

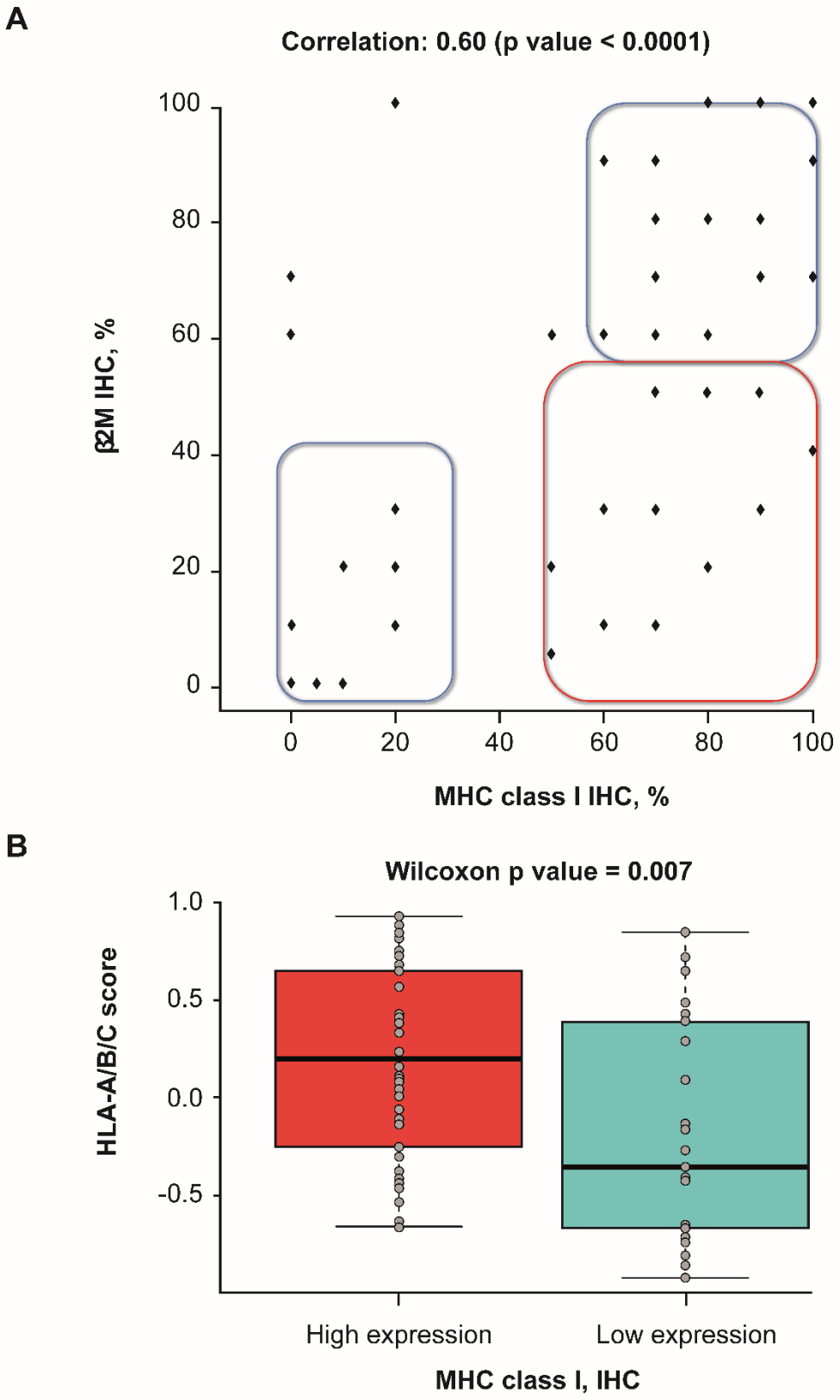

A
Correlation: 0.60 (p value < 0.0001)
β2M IHC, %
100
80
60
40
20
0
0
20
40
60
80
100
MHC class I IHC, %
B
Wilcoxon p value = 0.007
HLA-A/B/C score
1.0
0.5
0.0
-0.5
High expression
Low expression
MHC class I, IHC

Fig. S2. Correlations of MHC class I IHC scores with β2M IHC scores and HLA transcripts. (**A**) The percentage of tumor cells with positive membrane staining for MHC class I and β2M by IHC were positively correlated (Kendall's tau coefficient, 0.60, $p<0.0001$). High and low expression of MHC class I correlated with high and low expression of β2M, respectively (blue boxes). A subset of cases showed higher amounts of intact/positive membrane staining for MHC class I compared with amounts of intact/positive membrane staining for β2M (red box), consistent with greater sensitivity of the MHC class I IHC assay for expression of the antigen presentation complex. (**B**) Box plot showing relative expression of the *HLA* genes. *HLA-A*, *HLA-B*, and *HLA-C* were summarized into one score by gene set variance analysis. The samples were grouped into two categories: MHC class I high (>50% tumor cells with positive membrane staining by IHC) and MHC class I low. A two-sided Wilcoxon test showed a significant difference between the two groups ($p = 0.007$). β2M, beta-2-microglobulin.

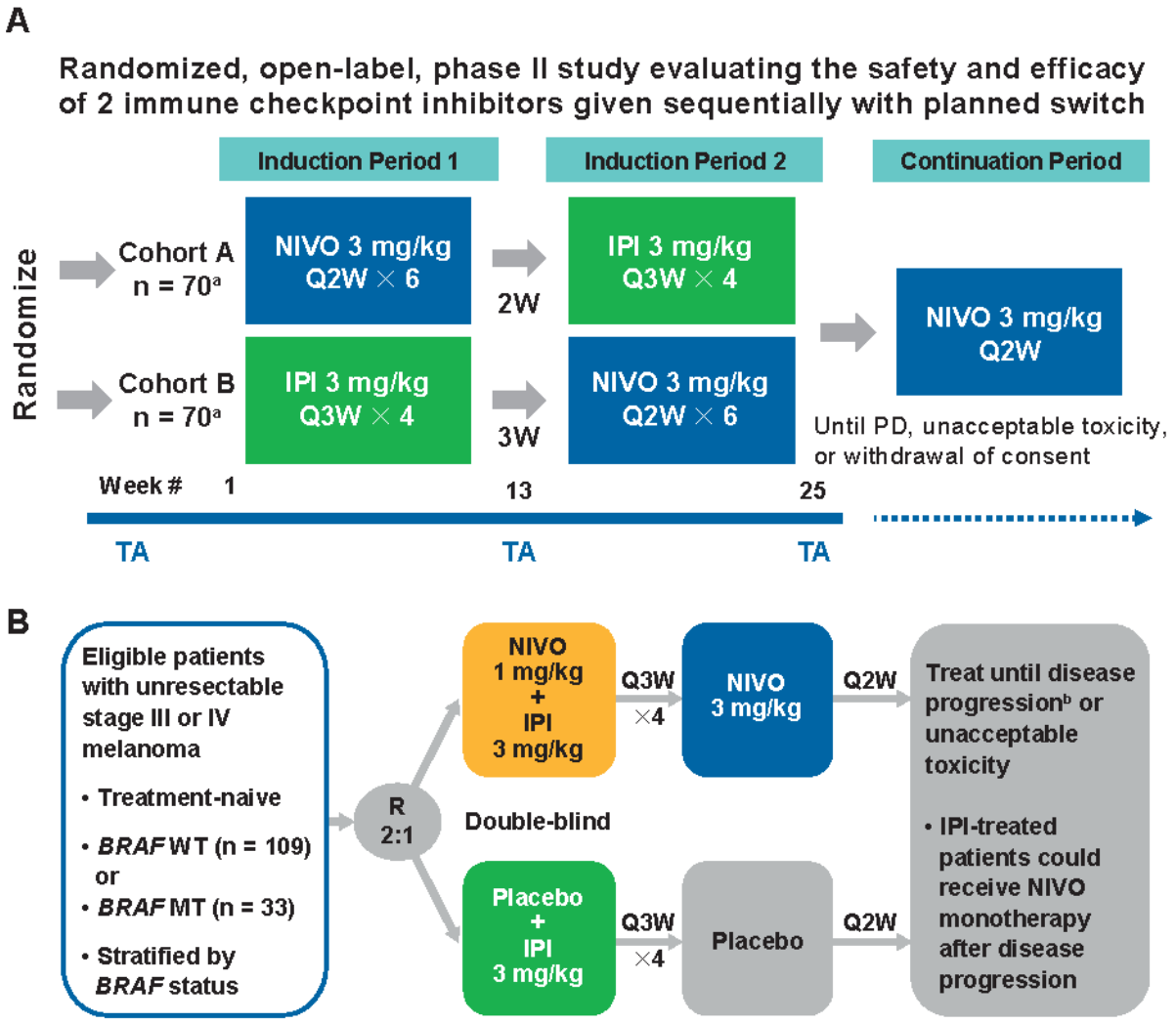
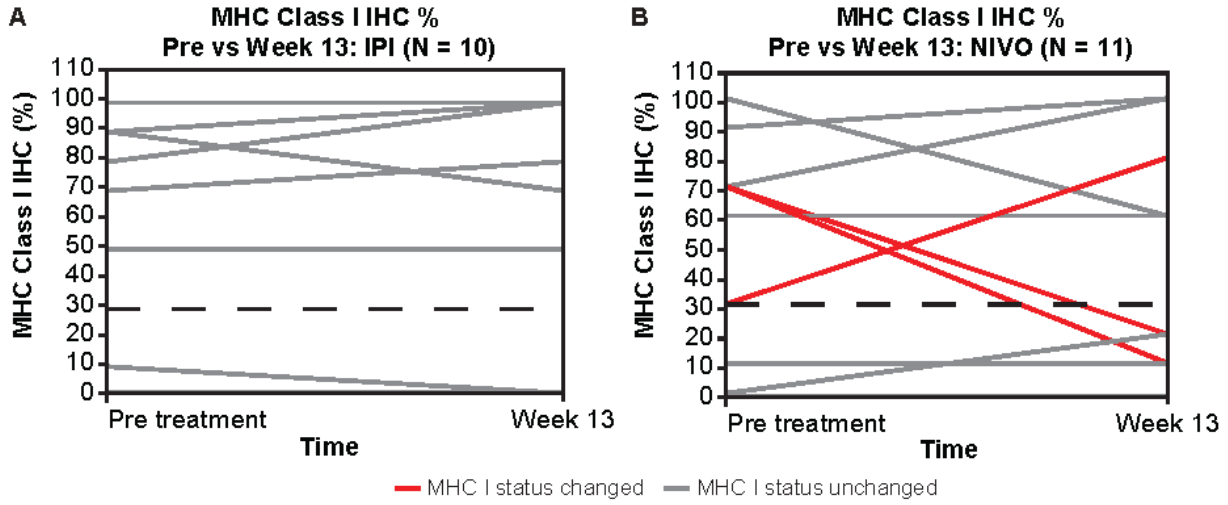


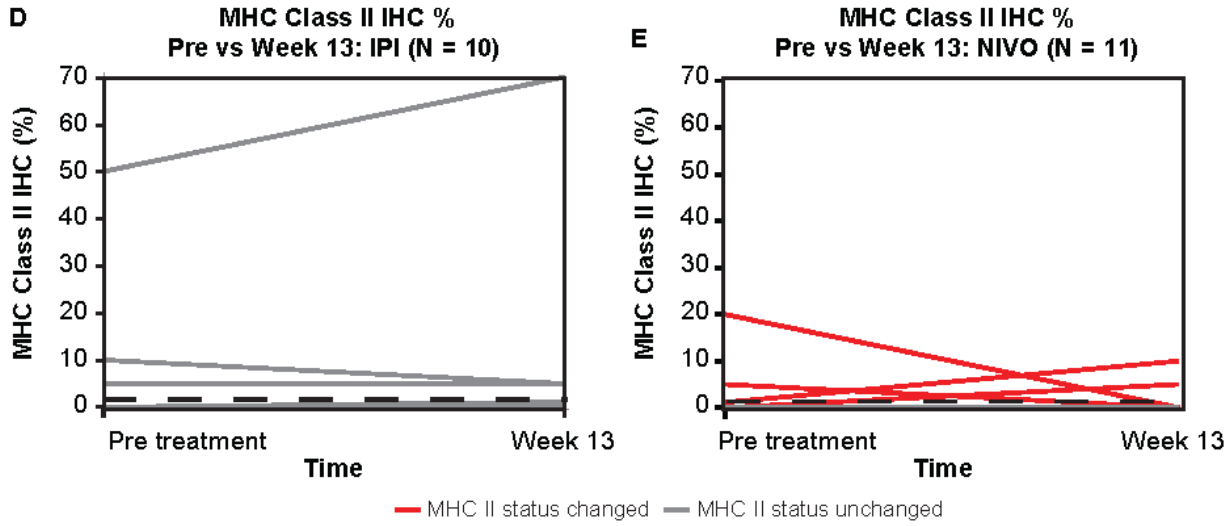
Fig. S3. Study schemas for the CheckMate 064 and CheckMate 069 trials. (**A**) Trial schematic for CheckMate 064. [a]68 patients in cohort A and 70 patients in cohort B received at least one dose of study drug. (**B**) Trial schematic for CheckMate 069. [b]Treatment beyond initial investigator-assessed progression as assessed by Response Evaluation Criteria In Solid Tumors version 1.1 was permitted in patients experiencing clinical benefit and tolerating study therapy. IPI, ipilimumab; MT, mutant; NIVO,

nivolumab; PD, progressive disease; Q2W, every 2 weeks; Q3W, every 3 weeks; R, randomize; TA, tumor assessment; WT, wild type; MT, mutated.



C **MHC I +/- Status Change From Week 0 to Week 13**

Patient ID	Pre	Week 13	Response Week 13
1	70	20	Non-PD
2	70	10	Non-PD
3	30	80	PD



F **MHC II +/- Status Change From Week 0 to Week 13**

Patient ID	Pre	Week 13	Response Week 13
2	5	0	Non-PD
3	0	5	PD
4	1	10	Non-PD
5	20	0	PD

Fig. S4. Comparison of MHC class I and MHC class II expression in paired baseline and week 13 biopsy samples from CheckMate 064. (**A**) The percentage of tumor cells with positive membrane expression for MHC class I before treatment and after 13 weeks of IPI. (**B**) The percentage of tumor cells with positive membrane expression for MHC class I before treatment and after 13 weeks' treatment with NIVO. (**C)** Progressive disease (PD) versus non-PD status at week 13 for patients with paired biopsies that cross the threshold for MHC class I expression (high versus low). (**D**) The percentage of tumor cells with positive membrane expression for MHC class II before treatment and after 13 weeks' treatment with IPI. (**E**) The percentage of tumor cells with positive membrane expression for MHC class II before treatment and after 13 weeks' treatment with NIVO. (**F**) PD versus non-PD status at week 13 for patients with paired biopsies that cross the threshold for MHC class II expression (high versus low). The dashed lines show the 30% threshold for MHC class I (as determined for week 13 PD [Table 1]) and 1% threshold for MHC class II expression, respectively. Patients with MHC class I or MHC class II expression that cross the threshold between baseline and week 13 are depicted in red; otherwise they are depicted in gray.

Table S1. CheckMate 064 MHC class I and MHC class II IHC. Provided as an Excel file.

Table S2. Best overall response of PD and non-PD according to optimally defined biomarker thresholds.

Biomarker	Threshold#	Number of patients			Proportion With Low Biomarker Expression and PD	Proportion With High Biomarker Expression and PD	Fisher's Exact p-value
		In Analysis	Low Expression	High Expression			
IPI→NIVO							
MHC class I	30	42	14	28	0.93	0.57	**0.03**
MHC class II	50	42	41	1	0.71	0.00	0.31
NIVO→IPI							
MHC class I	30	50	16	34	0.44	0.32	0.53
MHC class II	1	50	35	15	0.49	0.07	**0.005**

IPI, ipilimumab; MHC; major histocompatibility complex; NIVO, nivolumab; PD, progressive disease
[#]Optimal thresholds for week 13 PD data.

Table S3. CheckMate 069 MHC class I and MHC class II IHC. Provided as an Excel file.

Table S4. Genes used in the published IFN-γ signature.

Gene	Description
HLA-DRA	Major histocompatibility complex, class II, DR alpha
CXCL9	Chemokine (C-X-C motif) ligand 9
GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)
PRF1	Perforin 1 (pore-forming protein)
CCR5	Chemokine (C-C motif) receptor 5 (gene/pseudogene)
IFNG	Interferon-gamma
CXCL10	Chemokine (C-X-C motif) ligand 10
IDO1	Indoleamine 2,3-dioxygenase 1
STAT1	Signal transducer and activator of transcription 1
CXCL11	Chemokine (C-X-C motif) ligand 11

[#]From Ayers et al. (*23*).

Table S5. Genes used in the NK cell and γδ T cell gene sets.

NK Cell-associated Gene Set	
KLRG1	*KLRC4-KLRK1*
SIGLEC7	*KLRA1P*
B3GAT1	*KLRF2*
FCGR3A	*KIR3DX1*
KLRB1	*KIR2DL1*
KLRF1	*KIR3DL1*
KLRC4	*KIR2DL4*
KLRG2	*KIR2DS4*
KLRC3	*KIR3DL2*
KLRK1	*KIR2DL3*

γδ **T cell-Associated Gene Set**
TRDC
TRGC1
TRGC2