APPENDIX

METHODS

Eligibility Criteria

Inclusion criteria included patients who were at least 28 days from any treatment with chemotherapy, surgery, radiation, or immunotherapy, including high dose interferon, interleukin-2, ipilimumab, or PD-1 inhibitors, and recovered from any clinically significant treatment-related toxicities. Patients were required to have two or more measurable sites of disease ($\geq$ 1.5 cm), including the disease site requiring palliative radiation therapy, as well as at least one other disease site outside of the planned radiation field. We included patients with asymptomatic brain metastases, but excluded those with CNS metastases requiring palliative radiation therapy. We excluded patients not requiring palliative radiation therapy. Patients with ECOG of 3 or greater were excluded, as were patients with life expectancy of less than 16 weeks. We excluded patients who had experienced a serious adverse event related to previous anti-CTLA-4 treatment. We also excluded patients who had a history of inflammatory bowel disease or symptomatic connective tissue disorder, or patients with autoimmune motor neuropathy or conditions requiring high-dose immunosuppressants.

Patient and Treatment Characteristics

We enrolled 22 patients with stage IV melanoma, including 15 men and 7 women, median age 64 years (range 18-90 years) (Table 1). Twenty-one patients had cutaneous melanoma, and 1 patient had mucosal melanoma. Baseline tumor assessment included the sum of the products of the two largest diameters (SPD) of the target lesions (non-irradiated), including up to 5 lesions per organ, up to 10 visceral lesions, and up to 5 cutaneous lesions. Patients were treated with ipilimumab (3 mg/kg) every three weeks for a planned total of four treatments. All patients were treated with palliative radiation therapy to 1-2 sites of disease, with at least 1 measurable (>1.5 cm) non-irradiated site of disease for response assessment. The start of radiation therapy was initiated within 5 days after the first dose of ipilimumab. The total dose and fractionation regimen of radiation and treatment specifications including dose constraints were determined by the treating radiation oncologist, and were determined by factors including the size and location of the lesion to be treated, and the clinical status of the patient (Table 1).

Laboratory Assays Methods

Luminex

Human 63-plex kits were purchased from eBiosciences/Affymetrix and used according to the manufacturer's recommendations with modifications as described below. In brief, beads were added to a 96 well plate and washed in a Biotek ELx405 washer. Samples were added to the plate containing the mixed antibody-linked beads and incubated at room temperature for 1 hour, followed by

overnight incubation at 4°C with shaking. Cold and room temperature incubation steps were performed on an orbital shaker at 500-600 rpm. Following the overnight incubation plates were washed in a Biotek ELx405 washer and then biotinylated detection antibody added for 75 minutes at room temperature with shaking. Plates were washed as above and streptavidin-PE was added. After incubation for 30 minutes at room temperature, washing was performed as above and reading buffer was added to the wells. Each sample was measured in duplicate. Plates were read using a Luminex 200 instrument with a lower bound of 50 beads per sample per cytokine. Custom assay control beads by Radix Biosolutions were added to all wells.

Whole blood myeloid-derived suppressor cell staining

To assess for myeloid-derived suppressor cell (MDSC) populations, 200 microliters of whole blood were stained within two hours of the blood draw. The antibody panel shown in Table 2 was used. All antibodies were obtained from BD Biosciences, San Diego, CA). All reagents were used according to the manufacturer's recommended concentrations, with staining for 30 minutes at room temperature, followed by treatment with BD FACS Lysing Solution and freezing at -80C. Samples were later thawed, washed, and analyzed on a BD LSRII flow cytometer. Gating for MDSC populations was performed in accordance with the procedure described by Walter et al. (Walter, Weinschenk et al. 2012).

Flow cytometry immunophenotyping

Phenotyping was performed using standard panels as developed by the Human Immune Phenotyping Consortium (Maecker, McCoy et al. 2012). Peripheral blood mononuclear cells (PBMC) were thawed in warm media, washed twice and resuspended at $1x10^7$ viable cells/mL. 50 µL cells per well were stained for 45 min at room temperature with the antibodies shown in Table 2 (all reagents from BD Biosciences, San Jose, CA). Cells were washed three times with FACS buffer (PBS supplemented with 2% FBS and 0.1% sodium azide), and resuspended in 200 µL FACS buffer. 100,000 lymphocytes per sample were collected using DIVA 6.0 software on an LSRII flow cytometer (BD Biosciences). Data analysis was performed using FlowJo v9.3 by gating on live cells based on forward versus side scatter profiles, then on singlets using forward scatter area versus height, followed by cell subset-specific gating. Data were then graphed using GraphPad Prism 6.0d (GraphPad Software Inc., La Jolla, CA).

Intracellular cytokine staining

To assess potential melanoma-specific T cell responses, an intracellular cytokine assay was performed. PBMC were thawed in warm media, washed twice and resuspended at $1x10^7$ viable cells/ml. One freeze-thaw cycle was allowed before cytokine profiling. 200 µL of cells were plated per well in a 96-well U-bottom plate. After overnight incubation at 37°C, peptides at 1

µg/ml/peptide [gp100, MART-1, and NY-ESO-1 peptide mix, JPT, Berlin, Germany] + 10 µg/mL brefeldin A (Sigma, St. Louis, MO) were added and the cells were incubated for 6 hours at 37°C. EDTA was then added at 2 mM final concentration for 15 min, and the cells washed with FACS buffer (PBS supplemented with 2% FBS and 0.1% sodium azide). Cells were fixed in 200 µL of 2% PFA at 4^0C overnight. They were then washed and resuspended in 200 µL of 1x Permeabilizing buffer (eBioscience, San Diego, CA). After 30 minutes on ice, the cells were washed twice with FACS buffer, and resuspended in 200 µL FACS buffer. Staining with cell-surface and cytokine-specific antibodies [CD3 V500, CD8 V450, CD4 PerCP Cy5.5, IFNgamma FITC, IL17 PE, IL2 PE Cy7, TNF AlexaFluor 700, IL-4 APC, all from BD Biosciences] was then performed for 1 hour at room temperature, followed by two additional washes with FACS buffer. Data were collected using DIVA 6.0 software on an LSRII flow cytometer (BD Biosciences, San Diego, CA). Data analysis was performed using FlowJo vX.0.7 (FlowJo, LLC, Ashland, OR) and graphed with backgrounds (unstimulated) subtracted, using GraphPad Prism 6.0d (GraphPad Software Inc., La Jolla, CA).

CyTOF intracellular cytokine staining

To determine global immune competence, PBMC were also stimulated for 4 hours with PMA+ionomycin and used in a CyTOF assay. To determine global immune competence, PBMC were also used in a CyTOF intracellular cytokine staining (ICS) assay. The stimulation, intracellular cytokine staining and CyTOF assay were performed in accordance with the protocol published by Lin et al. (Lin

2015) (antibody panel shown in Table 3). Briefly, cryopreserved PBMC were thawed and incubated overnight in 96 well plates. They were then stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) in the presence of Brefeldin A (5 μg/ml) and Monensin (5 μg/ml) (Sigma-Aldrich, St. Louis, MO). After 4 hours of incubation, 2mM EDTA was added for 15 min. Cells were then washed with CyFACS buffer (0.1% BSA, 0.05% sodium azide in 1x PBS from Rockland Immunochemicals, Pottstown, PA). Surface antibody cocktail prepared in CyFACS was added, and cells were incubated for 45 min on ice. Cells were washed and incubated in 1:3000 diluted In115-DOTA maleimide (5 mg/ml) live/dead stain for 30 min on ice. Cells were then washed in CyFACS and fixed in 2% PFA at 4°C overnight. After washing twice in 1x permeabilization buffer (eBioscience, San Diego, CA), an intracellular staining cocktail prepared in permeabilization buffer was added for 45 minutes on ice. Cells were then washed and stained with Intercalator-Ir (Fluidigm, San Francisco, CA) diluted in 2% PFA for 20 minutes at room temperature, washed in CyFACS and MilliQ water. Data were collected on a CyTOF1 (Fluidigm, San Francisco, CA).

CyTOF data analysis

Data collected on the CyTOF were first analyzed on FlowJo vX.0.7 (FlowJo, LLC, Ashland, OR). Graphs were prepared using GraphPad Prism 6.0d (GraphPad Software Inc., La Jolla, CA). Spanning-tree Progression of Density-normalized Events or SPADE analyses were performed on Cytobank (https://cytobank.org). Data obtained from the mass cytometer were imported

into Cytobank and gated on live intact singlets, based on DNA content, cell length and live/dead staining. SPADE analysis was performed with the target number of nodes set to 200 and downsampled events target set to 10%. Major cell subsets were identified by lineage marker expression, and are illustrated as various ‘bubbles’. The node size represents the frequency of cells and the color represents the level of expression. All data were exported as PDF files from Cytobank and the appropriate scales are shown.

Statistics

Luminex assays were performed in duplicate for each sample time point. The average MFI of the duplicates was used in two-way ANOVA to look for significant differences between groups. CyTOF and flow cytometry, as is typical for these platforms, were done on a single aliquot for each sample time point. However, confidence in the results is increased by collecting a large number of events in each data file (typically 50,000-100,000 for flow cytometry and 200,000 for CyTOF). Percent of parent statistics for each gated cell subset, derived in FlowJo software, were then used in two-way ANOVA to look for significant differences between groups. All statistical analyses were performed using GraphPad Prism 6.0d (GraphPad Software Inc., La Jolla, CA). Luminex, flow cytometry and mass cytometry data were analyzed by two-way ANOVA with the Bonferroni post-test. Group-level differences and differences between PD and CR/PR at each visit are reported as significant if $p<0.05$.

*$p<0.05$, **$p<0.01$.

RESULTS

T cell responses following radiation plus immunotherapy

We used flow cytometry to study peptide-specific $CD8^+$ T cell responses (Supplementary Figure S1). We stimulated PBMC *ex vivo* with a mixture of MART-1, gp100 and NY-ESO and then used intracellular cytokine staining to detect peptide-specific responses. We found that CR/PR patients tended to show increases in IFNγ, IL-2 and TNFα after the 2^{nd} dose of ipilimumab, similar to the trend seen in some serum cytokines (Figure 3); but this trend did not reach statistical significance. There was a significant difference in TNFα+ CD8+ T cells responding to peptide stimulation at baseline ($p<0.05$). However, the frequency of antigen-specific $CD8^+$ T cells in the PBMC samples was very low overall, making the responses difficult to detect.

In addition, we used a high-dimensional single-cell protein detection technology similar to flow cytometry, termed mass cytometry (CyTOF). This method allows for the use of ~40 different antibodies, and uses metal-tagged antibodies instead of fluorochromes, so there is no spectral overlap, and no compensation is required (Table 4B). Using CyTOF, we investigated global immunocompetence by stimulating patient PBMC with PMA and ionomycin, followed by staining for surface as well as intracellular markers. We found that CR/PR patients had higher CD8+ IL-2 levels across visits as compared to PD

patients (Figure 4A, $p<0.01$). Central memory ($CCR7^{+}CD45RA^{-}$) CD8+ T (Tcm) cells were also higher in CR/PR patients across visits compared to patients with PD (Figure 4B, $p<0.05$). However, cytokine production by these Tcm cells was not different between responders and non-responders, suggesting that responders have a higher number of Tcm cells but they are not functionally different, at least in response to a general stimulator like PMA/Ionomycin. In addition, Tcm frequencies determined by conventional flow cytometric assays corroborated the CyTOF data, showing that CR/PR patients had a higher baseline percentage of Tcm $CD8^{+}$ T cells as compared to PD patients (Supplementary Figure S1A, $p<0.05$).

We also performed an unsupervised analysis of the high-dimensional CyTOF data using SPADE, a clustering algorithm. Representative SPADE plots (Figure 4C and D) from one PD patient and one CR patient show IL-2 expression in different immune cell subsets. The node size indicates the frequency of cells and the color represents the expression level of IL-2.

Lastly, the frequency of different types of myeloid-derived suppressor cells (MDSC) was assessed by flow cytometry on whole blood (Supplementary Figure S2). Although clear patterns were not discernable from these data, there was a tendency for responders to have a higher percentage of MDSC1, MDSC2 and MDSC3 at the second sampling, which corresponded to three weeks after administration of the first cycle of ipilimumab.

Table 3. Grade 2-4 toxicity

	Grade 2	Grade 3	Grade 4
Colitis*	2 (9%)	1 (5%)	1 (5%)
Hypophysitis*	2 (9%)	1 (5%)	NR
Rash*	3 (14%)	NR	NR
Anemia*	2 (9%)	NR	NR
Nausea**	2 (9%)	NR	NR
Radiation Dermatitis**	4 (19%)	NR	NR

*: primarily immunotherapy-related
**: primarily radiation-related

Table 4. Myeloid-derived suppressor cells (MDSC) panel

Label	Specificity
FITC	CD15
PE	CD124
PE-Texas Red	Live/dead
PerCP-Cy5.5	CD33
PE-Cy7	CD56
APC	CD11b
Alexa 700	CD45
APC-H7	CD3+19+20
V450	CD14
V500	HLA-DR

Table 5A. Flow phenotyping panel

Label	T cell	Treg	B cell	DC/mono/NK	Th1/2/17
FITC	Live/dead	Live/dead	Live/dead	Live/dead	Live/dead
PE	CCR7	CD25	CD24	CD56	CXCR3
PerCP-Cy5.5	CD4	CD4	CD19	CD123	CD4
PE-Cy7	CD45RA	CCR4	CD27	CD11c	CCR6

APC	CD38	CD127	CD38	CD16	CD38
APC-H7	CD8	CD45RO	CD20	CD3+19+20	CD45RO
V450	CD3	CD3	CD3	CD14	CD3
BV510	HLA-DR	HLA-DR	IgD	HLA-DR	HLA-DR

Table 5B. Staining panel for phenotypic and intracellular cytokine analysis by mass cytometry (CyTOF)

CyTOF ICS			
Metal label	**Specificity**	**Clone**	**Source**
141Pr	CD49d	9F10	DVS
142Nd	CD19	HIB19	DVS
145Nd	CD4	RPA-T4	DVS
146Nd	CD8	RPA-T8	DVS
147Sm	CD20	2H7	DVS
148Nd	CD57	HCD57	in-house
149Sm	CD85j	292319	in-house
150Nd	MIP1b	D21-1351	DVS
151Eu	CD107a	H4A3	DVS
152Sm	TNFa	Mab11	DVS
153Eu	CD45RA	HI100	DVS

154Sm	CD3	UCHT1	DVS
155Gd	CD28	L283, BD	in-house
156Gd	CD38	HB-7, BD	in-house
157Gd	HLA-DR	G46-6, BD	in-house
158Gd	CD33	WM53	DVS
159Tb	GMCSF	BVD2-21C11	DVS
160Gd	CD14	M5E2	DVS
161Dy	IFNg	4S.B3	in-house
162Dy	CD69	MCA 1442	DVS
163Dy	TCRgd	B1	in-house
164Dy	IL-17	N49-853	DVS
165Ho	CD127	A019D5	DVS
166Er	IL-2	MQ1-17h12	DVS
167Er	CD27	L128	DVS
168Er	CD154	24-31	DVS

	(CD40L)		
169Tm	CCR7	150503	in-house
170Er	PD1	EH12.1, BD	in-house
171Yb	Granzyme B	GB11	DVS
172Yb	NKG2C	134591	in-house
173Yb	CD25	M-A251, BD	in-house
174Yb	CD16	3G8	in-house
175Lu	Perforin	B-D48	DVS
176Yb	CD56	NCAM16.2	DVS