Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Nghiem PT, Bhatia S, Lipson EJ, et al. PD-1 blockade with pembrolizumab in advanced Merkel-cell carcinoma. N Engl J Med. DOI: 10.1056/NEJMoa1603702

Supplementary Appendix for "PD-1 blockade with pembrolizumab in advanced Merkel cell carcinoma", by Nghiem et al.

Table of Contents to the Supplementary Appendix:

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II. SUPPLEMENTARY METHODS

Tumor Merkel Cell Polyomavirus (MCPyV) Status

Serology: Baseline serum samples from all patients were used to measure MCPyV small T-antigen oncoprotein antibody titers at Laboratory Medicine (University of Washington, Seattle, WA) as described.[1] Titers above 74 were considered positive.

Oncoprotein-specific T cells: All patients were low-resolution HLA genotyped to determine eligibility for CD8 T cell specific MCPyV peptide-MHC tetramer screening (Bloodworks Northwest, Seattle, WA). Pretreatment peripheral blood mononuclear cells (PBMCs) collected from patients with HLA-I types that corresponded to available MCPyV-specific tetramers (n=17) were tetramer stained to identify MCPyV-specific T cells, and analyzed by flow cytometry. Samples with >0.01% of CD8+ T cells co-staining with tetramers were considered positive. In addition, PBMCs from the first 12 patients with available pretreatment and week 12 post-treatment blood collections were stimulated with pools of MCPyV-specific peptides in a flow cytometry-based intracellular cytokine secretion assay (HIV Vaccine Trials Network, Seattle, WA). PBMCs that secreted interferon-gamma and/or IL-2 robustly (≥0.1% of CD8 T cells after background subtraction) were considered reactive to MCPyV.

Multispectral fluorescent immunohistochemistry

Position	Antibody	Clone (host)/Company	Dilution	Incubation	TSA dyes
1	PD-L1	SP142 (rabbit)/Spring Bio.	1:800	60 min	620
2	PD-1	EPR4877(2) (rabbit)/AbCam	1:1000	30 min	650
3	NSE	BBS/NC/VI-H14(mouse)/Dako	1:1000	60 min	570
4	CD68	PGM-1(mouse)/Dako	1:500	30 min	540
5	CD8	4B11(mouse)/AbD Ser	1:100	30 min	520
6	DAPI	Perkin Elmer Opal 7-color kit	2 drops/ml	5 min	

Formalin-fixed, paraffin-embedded tissues were cut in 4 um thick sections and placed on plus-charged slides. Slides were heated at 57°C overnight; then residual paraffin was removed using xylene, and tissue was rehydrated in a series of graded alcohols to distilled water. Antigen retrieval was performed using Tris-EDTA buffer and microwave treatment. Slides were washed and blocking was performed with 3% H2O2 blocking solution followed by Dako antibody diluent. The first primary antibody, i.e., Position 1 in the table above, was then applied and allowed to incubate. Opal polymer HRP Ms + Rb (Perkin Elmer, Hopkington, MA) was used as the secondary antibody. The slides were washed and the

tyramide signal amplification (TSA)-dye (Opal 7 color kit, Perkin Elmer, Hopkington, MA) for Position 1 was applied. Slides were then microwaved to strip the primary and secondary antibodies, washed, and blocked again using blocking solution. The second primary antibody, i.e., Position 2, was applied, and the process was repeated through Position 6, where DAPI was applied, rather than another primary antibody. After unbound DAPI was washed off, slides were coverslipped using Vectashield (Vector Laboratories, Burlingame, CA). In addition to the multiplex assay described above, a single color slide was generated for each antibody using an archival Merkel cell carcinoma case. Each single color control slide was compared to standard IHC methods for CD8, CD68, PD-L1, PD-1, and NSE (neuron-specific enolase).

Multiplexed and single color control slides were loaded onto the PerkinElmer Vectra automated multispectral microscope. Representative fields from the single color slides were imaged, and InForm Image Analysis software (Ver 2.1) was used to generate a spectral library for unmixing. Index cases stained using the multiplex method were then imaged. Channels were unmixed using the spectral library, and tissues and cells were segmented and scored.

III. SUPPLEMENTARY FIGURES

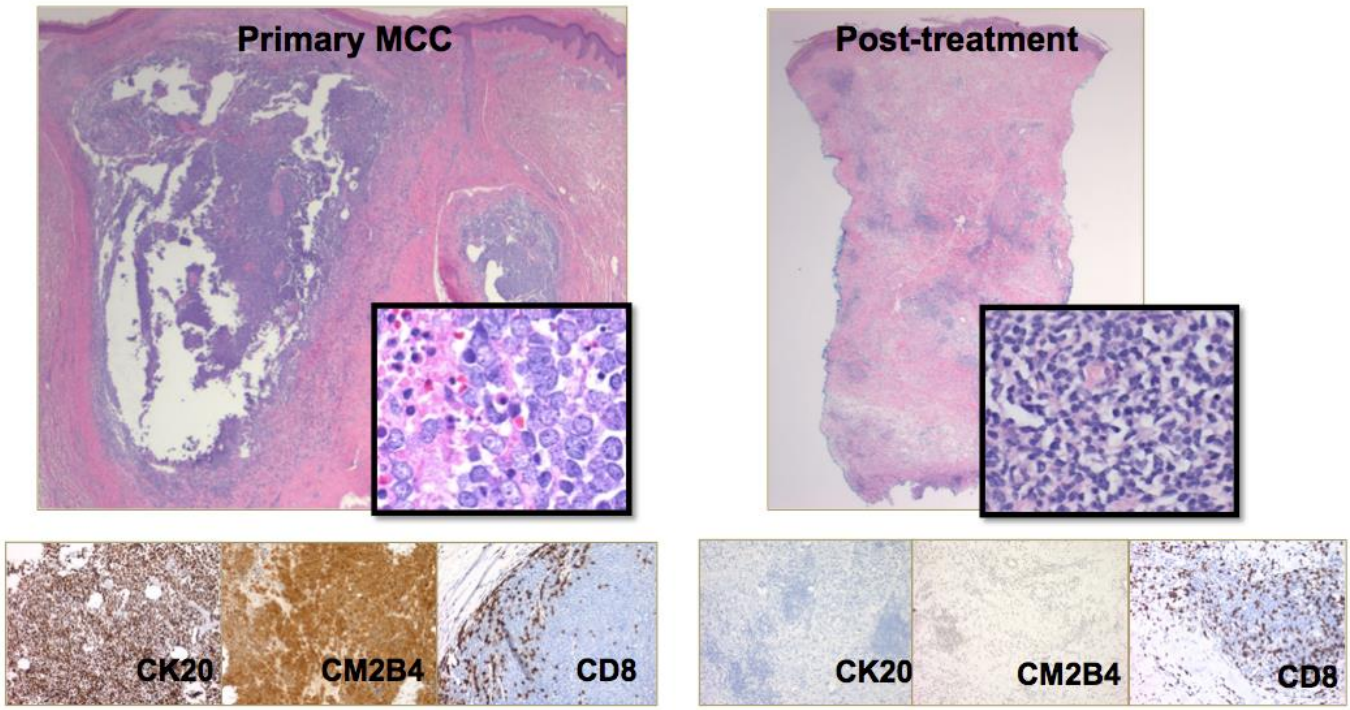

Figure S1: Pre- and on-treatment biopsy analysis in a patient responding to anti-PD-1

Biopsies of the primary Merkel cell carcinoma (MCC) lesion before anti-PD-1 therapy (left panels), and an adjacent subcutaneous metastasis regressing 3 weeks after initiating therapy (right panels) from the responding patient represented in **Figure 3**. Top panels, H&E stain (showing typical "salt & pepper" chromatin of MCC in primary lesion, and lymphoid infiltrates in post-treatment sample). Immunohistochemistry in lower panels shows no evidence of residual tumor cells and a robust CD8 infiltrate in post-treatment biopsy. CK20, cytokeratin marker for MCC; CM2B4, marker for MCPyV large T-antigen [2,3]; CD8, cytolytic T cell subset.

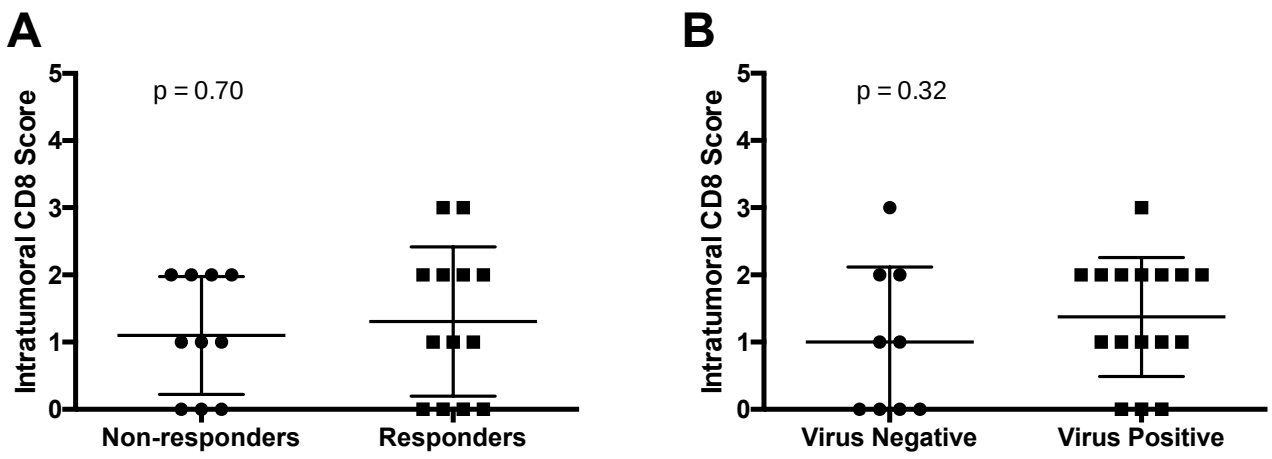

Figure S2: Pretreatment intratumoral CD8+ lymphocyte infiltration does not correlate with response to pembrolizumab or tumor viral status

Pretreatment tumor biopsy samples were obtained and analyzed. The period between the pretreatment tumor biopsy and treatment initiation ranged from 7 days to 8.4 years (median 5.2 months). Intratumoral (IT) CD8+ cells were defined as those surrounded completely by tumor cells without direct contact with stroma, as described previously. IT CD8+ infiltration was scored semi-quantitatively on 25 tumors with evaluable staining, on a 0-5 scale: 0 = no IT CD8+ cells; 1= 1-179 IT CD8+ cells/mm^2; 2=180-433 IT CD8+ cells/mm^2; 3= 434-582 IT CD8+ cells/mm^2; 4= 583-731 IT CD8+ cells/mm^2; 5 = ≥732 IT CD8+ cells/mm.[2,4] (**A**) IT CD8+ infiltration did not correlate with response (complete and partial responses) to pembrolizumab (n = 23; three patients were excluded because one patient did not yet have a response evaluation, one patient had an unconfirmed response, and one patient did not have sufficient tumor for CD8 interpretation). The mean score (on the 0-5 point scale) among responders was 1.3 versus 1.10 for non-responders; p = 0.70 by Mann-Whitney U test. (**B**) IT CD8+ infiltration did not correlate with viral status (n = 25 from all tumors with interpretable CD8 IHC; mean score of 1.38 in virus-positive vs 1.00 in virus-negative tumors; p = 0.32 by Mann-Whitney U test).

IV. SUPPLEMENTARY TABLES

Table S1: Adverse events

System organ class/ preferred term	All grades (1-4)** (no. patients, %)	Grades 3-4** (no. patients, %)
Total subjects with drug-related* adverse events	**20 (77%)**	**4 (15%)**
Blood and lymphatic system disorders	**3 (12%)**	
Anaemia	1 (3.8%)	
Leukocytosis	1 (3.8%)	
Microcytic anaemia	1 (3.8%)	
Thrombocytopenia	1 (3.8%)	
Cardiac disorders	**1 (3.8%)**	**1 (3.8%)**
Acute myocardial infarction	1 (3.8%)	1 (3.8%)
Myocarditis	1 (3.8%)	1 (3.8%)
Ventricular arrhythmia	1 (3.8%)	1 (3.8%)
Ventricular tachycardia	1 (3.8%)	1 (3.8%)
Atrial fibrillation	1 (3.8%)	
Bundle branch block left	1 (3.8%)	
Eye disorders	**2 (7.7%)**	
Eyelid ptosis	1 (3.8%)	
Ophthalmoplegia	1 (3.8%)	
Vision blurred	1 (3.8%)	
Gastrointestinal disorders	**4 (15%)**	**1 (3.8%)**
Small intestinal haemorrhage	1 (3.8%)	1 (3.8%)
Diarrhoea	1 (3.8%)	
Dry mouth	1 (3.8%)	
Nausea	1 (3.8%)	
General disorders and administration site conditions	**13 (50%)**	
Fatigue	12 (46%)	
Chills	2 (7.7%)	
Asthenia	1 (3.8%)	
Feeling hot	1 (3.8%)	
Local swelling	1 (3.8%)	
Nodule	1 (3.8%)	
Infections and infestations	**1 (3.8%)**	
Oral candidiasis	1 (3.8%)	
Injury, poisoning and procedural complications	**1 (3.8%)**	
Fall	1 (3.8%)	
Investigations	**6 (23%)**	**3 (12%)**
Aspartate aminotransferase increased	4 (15%)	3 (12%)
Alanine aminotransferase increased	2 (7.7%)	2 (7.7%)
Blood creatine phosphokinase increas	1 (3.8%)	1 (3.8%)
Blood alkaline phosphatase increased	1 (3.8%)	
Blood bilirubin increased	1 (3.8%)	
Blood corticotrophin decreased	1 (3.8%)	
Blood creatinine increased	1 (3.8%)	
Blood urine present	1 (3.8%)	
Weight decreased	1 (3.8%)	

Metabolism and nutrition disorders	**3 (12%)**	**2 (7.7%)**
Hyponatraemia	2 (7.7%)	2 (7.7%)
Hyperglycaemia	1 (3.8%)	1 (3.8%)
Decreased appetite	1 (3.8%)	
Malnutrition	1 (3.8%)	
Musculoskeletal and connective tissue disorders	**3 (12%)**	
Myalgia	2 (7.7%)	
Arthralgia	1 (3.8%)	
Neoplasms benign, malignant and unspecified (including cysts and polyps)	**2 (7.7%)**	**1 (3.8%)**
Tumour pain	2 (7.7%)	1 (3.8%)
Nervous system disorders	**4 (15%)**	**1 (3.8%)**
Encephalopathy	1 (3.8%)	1 (3.8%)
Cerebral ischaemia	1 (3.8%)	
Dizziness	1 (3.8%)	
Headache	1 (3.8%)	
Hypoaesthesia	1 (3.8%)	
Nystagmus	1 (3.8%)	
Paraesthesia	1 (3.8%)	
Sciatica	1 (3.8%)	
Psychiatric disorders	**1 (3.8%)**	
Delirium	1 (3.8%)	
Disorientation	1 (3.8%)	
Renal and urinary disorders	**3 (12%)**	**1 (3.8%)**
Renal failure acute	1 (3.8%)	1 (3.8%)
Proteinuria	1 (3.8%)	
Renal failure	1 (3.8%)	
Urinary retention	1 (3.8%)	
Reproductive system and breast disorders	**2 (7.7%)**	
Breast pain	1 (3.8%)	
Pruritus genital	1 (3.8%)	
Respiratory, thoracic and mediastinal disorders	**3 (12%)**	
Cough	2 (7.7%)	
Pneumonitis	1 (3.8%)	
Skin and subcutaneous tissue disorders	**4 (15%)**	
Alopecia	2 (7.7%)	
Itching scar	1 (3.8%)	
Pruritus	1 (3.8%)	
Rash	1 (3.8%)	
Uncoded	**1 (3.8%)**	**1 (3.8%)**
Acute Combined Systolic And Diastolic Heart Failure	1 (3.8%)	1 (3.8%)
Vascular disorders	**5 (19%)**	
Hypotension	2 (7.7%)	
Hot flush	1 (3.8%)	
Hypertension	1 (3.8%)	
Phlebitis	1 (3.8%)	

*Adverse events recorded as having a Definite, Probable or Possible association with the study drug were considered drug-related.
**A subject that experienced multiple occurrences of an adverse event was counted once at the maximum grade recorded.

V. REFERENCES

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