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11.0 APPENDICES

11.1 ECOG Performance Status

Grade	Description
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.
1	Symptoms, but ambulatory. Restricted in physically strenuous activity, but ambulatory and able to carry out work of a light or sedentary nature (e.g., light housework, office work).
2	In bed <50% of the time. Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	In bed >50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead.
* As published in Am. J. Clin. Oncol.: *Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982.* The Eastern Cooperative Oncology Group, Robert Comis M.D., Group Chair.	

11.2 Common Terminology Criteria for Adverse Events V4.0 (CTCAE)

The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for adverse event reporting.

Website: http://ctep.cancer.gov/reporting/ctc.html

11.3 Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 Criteria for Evaluating Response in Solid Tumors

RECIST version 1.1 will be used in this study for assessment of tumor response, with one modification. To account for tumor pseudo-progression that can sometimes occur with immunotherapy, it is required that RECIST PD be confirmed on a repeat scan before a patient is removed from study (see Section 7.1.3.5 for details).

For full details, refer to the manuscript as published in the European Journal of Cancer [43]:

E.A. Eisenhauer, P. Therasse, J. Bogaerts, L.H. Schwartz, D. Sargent, R. Ford, J. Dancey, S. Arbuck, S. Gwyther, M. Mooney, L. Rubinstein, L. Shankar, L. Dodd, R. Kaplan, D. Lacombe, J. Verweij. New response evaluation criteria in solid tumors: Revised RECIST guideline (version 1.1). Eur J Cancer. 2009 Jan;45(2):228-47.

[redacted]

11.3.1 Measurable Disease

Measurable lesions are defined as those that can be accurately measured in at least one dimension (longest diameter to be recorded) as ≥ 20 mm by chest x-ray, as ≥ 10 mm with CT scan, or ≥ 10 mm with calipers by clinical exam. All tumor measurements must be recorded in millimeters.

NOTE: Tumor lesions that are situated in a previously irradiated area are not considered measurable.

NOTE: Malignant Lymph Nodes. To be considered pathologically enlarged and measurable, a lymph node must be ≥ 15 mm in short axis when assessed by CT scan (CT scan slice thickness recommended to be no greater than 5 mm). At baseline and in follow-up, only the short axis will be measured and followed.

11.3.2 Non-measurable Disease

All other lesions (or sites of disease), including small lesions (longest diameter < 15 mm short axis), are considered non-measurable disease. Bone lesions, leptomeningeal disease, ascites, pleural/pericardial effusions, and lymphangitis cutis/pulmonitis, are considered as non-measurable. Non-measurable also includes lesions that are < 20 mm by chest x-ray.

NOTE: Cystic lesions that meet the criteria for radiographically defined simple cysts should not be considered as malignant lesions (neither measurable nor nonmeasurable) since they are, by definition, simple cysts.

'Cystic lesions' thought to represent cystic metastases can be considered as measurable lesions, if they meet the definition of measurability described above. However, if non-cystic lesions are present in the same patient, these are preferred for selection as target lesions.

11.3.3 Target Lesions

All measurable lesions up to a maximum of 2 lesions per organ and 5 lesions in total, representative of all involved organs, should be identified as **target lesions** and recorded and measured at baseline. Target lesions should be selected on the basis of their size (lesions with the longest diameter), be representative of all involved organs, but in addition should be those that lend themselves to reproducible repeated measurements. It may be the case that, on occasion, the largest lesion does not lend itself to reproducible measurement in which circumstance the next largest lesion which can be measured reproducibly should be selected.

A sum of the diameters (longest for non-nodal lesions, short axis for nodal lesions) for all target lesions will be calculated and reported as the baseline sum diameters. If lymph nodes are to be included in the sum, then only the short axis is added into the sum. The baseline sum of the diameters will be used as reference to further characterize any objective tumor regression in the measurable dimension of the disease.

11.3.4 Non-target Lesions

All other lesions (or sites of disease) including any measurable lesions over and above the 5 target lesions should be identified as **non-target lesions** and should also be recorded at baseline. Measurements of these lesions are not required, but the presence or absence of unequivocal progression of each should be noted throughout follow-up.

11.3.5 Evaluation of Target Lesions

Complete Response (CR)	Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to < 10 mm.
Partial Response (PR)	At least a 30% decrease in the sum of the diameters of target lesions, taking as reference the baseline sum diameters
Progressive Disease (PD)	At least a 20% increase in the sum of the diameters of target lesions, taking as reference the *smallest sum on study* (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. Note: the appearance of one or more unequivocal new lesions is also considered progression.
Stable Disease (SD)	Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study.

11.3.6 Evaluation of Non-Target Lesions

Complete Response (CR)	Disappearance of all non-target lesions. All lymph nodes must be non-pathological in size (<10 mm short axis)
Progressive Disease (PD)	Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions. Unequivocal progression should not normally trump target lesion status. It must be representative of overall disease status change, not a single lesion increase.
Non-CR/Non-PD	Persistence of one or more non-target lesion(s).

[redacted]

11.3.7 Evaluation of New Lesions

The appearance of new lesions constitutes Progressive Disease (PD).

A growing lymph node that did not meet the criteria for reporting as a measurable or non-measurable lymph node at baseline should only be reported as a new lesion (and therefore progressive disease) if it a) increases in size to ≥ 15 mm in the short axis, or b) there is new pathological confirmation that it is disease (regardless of size).

See RECIST 1.1 manuscript for further details on what is evidence of a new lesion.

11.3.8 Evaluation of Best Overall Response

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence or non-protocol therapy (taking as reference for progressive disease the smallest measurements recorded since the treatment started). The patient's best response assignment will depend on the achievement of both measurement and confirmation criteria.

Target lesions	Non-target lesions	New lesions	Overall response
CR	CR	No	CR
CR	Non-CR/non-PD	No	PR
CR	Not evaluated	No	PR
PR	Non-PD or not all evaluated	No	PR
SD	Non-PD or not all evaluated	No	SD
Not all evaluated	Non-PD	No	NE
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

11.4 Events of Clinical Interest Guidance Document

Please see companion document, "Pembrolizumab Program (MK-3475) Event of Clinical Interest Guidance Document, Version 5.0," for clinical guidance on the identification and management of immune-related adverse events and their reporting requirements, including which ECI must be reported within 24 hours.

[redacted]

11.5 Processing and Shipping of Tumor Tissue and Correlative Science Blood Samples

[redacted]

11.5.1 Tissue Collection

11.5.1.1 Collection of peripheral blood

a. Peripheral blood will be collected by venipuncture into six venous blood collection tubes (two Red Top tubes with no additive; BD vacutainers catalog# 366430 and four Green Top tubes with sodium heparin; BD vacutainers catalog# 366480).
b. The sample will be transported at room temperature (18°C to 25°C) in a double container from the collection site to the sample processing laboratory.
c. Red Top tubes for serum processing should be kept sitting upright at room temperature for at least 30 minutes and at most 60 minutes prior to processing to allow clotting. If the blood is not immediately processed after the clotting period, then tubes should be stored (after the 30-60 minutes of clotting time) at 4°C for no longer than 4 h.
d. Green Top tubes for peripheral blood mononuclear cell (PBMC) processing should be kept on a rocker at room temperature until processed (to avoid clotting) and processed as soon as possible (within 4 h maximum) after blood collection.
e. The collection tubes will be labeled with the patient ID, date, and time of venous blood draw.
f. Samples will be handled one patient at a time to avoid mix-ups.

11.5.1.2 Collection of tumor tissue and handling of archival tumor tissue

a. Fresh tumor tissue will be collected by biopsy and immersed (completely) in sterile cold (2-7°C) HBSS in a sterile specimen cup (for example, VWR catalog# 15704-088) on ice (care must be taken to not immerse the cup/tissue in ice to avoid freezing the tissue).
b. The specimen cup will be labeled with the patient ID, date, and time of tumor tissue collection.
c. The sample will be transported on ice from the collection site to the sample processing laboratory.
d. Tissue processing should be done as soon as possible after biopsy (and within 3 h maximum) after tissue collection.
e. Samples will be handled one patient at a time to avoid mix-ups.
f. Archival tumor tissue (for example, in paraffin blocks or pathology slides) should be kept at room temperature until shipping.

[redacted]

11.5.2 Tissue Processing

11.5.2.1 Processing of peripheral blood

a. Peripheral blood from Red Top tubes will be used for serum acquisition and peripheral blood from green top tubes will be used for PBMC acquisition. Please refer to the Post-processing and Shipping Table for post-processing handling details.
b. Red Top tubes will be processed as follows:
    i. Red Top tubes will be centrifuged for 20 minutes at 1100-1300 g at room temperature.
    ii. Using a pipette, serum from both Red Top tubes will be transferred into a 50 ml conical tube and mixed.
    iii. The serum will then be pipetted into the labeled cryovials at an aliquot volume of 250-500 µl per tube. The caps on the vials will be closed tightly.
    iv. This process should be completed within 1 hour of centrifugation.
    v. Care must be taken to not pick up red blood cells when aliquoting. This can be done by keeping the pipet above the red blood cell layer and leaving a small amount of serum in the tube.
    vi. All aliquots will be placed upright in a specimen box or rack in an -80°C or colder freezer. All specimens should remain at -80°C or colder prior to shipping. The samples should not be thawed prior to shipping.
c. Green Top tubes will be processed as follows:
    i. Green Top tubes will be mixed by inverting the tube gently 6 to 8 times.
    ii. Up to 10 ml of peripheral blood will be added to 10 ml of PBS in a 50 ml Falcon tube and mixed by inverting the tube gently 6 to 8 times.
    iii. The 20 ml peripheral blood/PBS mixture will be overlayed by slow careful pipetting onto a 20 ml layer of Ficoll in a 50 ml tube.
    iv. The peripheral blood/PBS/Ficoll tube will be centrifugated for 20 min at 1750 rpm at room temperature without the break.
    v. Using a transfer pipette the clear top plasma layer will be removed and discarded as biological waste.
    vi. Using a new transfer pipette, the cloudy PBMC layer will be transferred to a 50 ml conical tube.
    vii. Care must be taken not to disrupt the erythrocyte layer during the transfer by using gentle pipetting above the Ficoll layer and keeping the tube stationary.
    viii. The cells will be counted (using a standard hemocytometer) and the total cell count recorded.
    ix. PBS will be added to the PBMC tube up to the 50 ml mark and the tube will be centrifugated for 5 min at 1750 rpm at room temperature with the break.
    x. The supernatant will be discarded and the pellet dissolved in Cryopreserve solution (1 ml Cryopreserve solution per 1 ml of blood) and transferred to cryovials (1 ml per cryovial). The cell concentration and solution volume will be recorded.

[redacted]

xi. All aliquots will be placed upright in a in a Mr. Frosty (Thermo Scientific catalog #5100-0001) at 4°C for up to 24 hours and then in -80°C or colder freezer in a specimen box. All specimens should remain at -80°C or colder prior to shipping. The samples should not be thawed prior to shipping.

11.5.2.2 Processing of tumor tissue

a. Tumor tissue will be sectioned into three equal specimens. The RNA stabilization procedure should be completed first. Please refer to the Post-processing and Shipping Table for post-processing handling details.
b. After sectioning, the three samples will be preserved in HypoThermosol® FRS Hypothermic storage and shipping media (HTS-FRS) for flow cytometry analysis, formalin fixed for immunohistochemistry (IHC) analysis, and stored in an RNA stabilizing reagent for array analysis.
c. HTS-FRS preservation
    i. Sterile forceps will be used to gently place the specimen in a sterile labeled cryovial or specimen container (whichever is large enough to hold the tissue full submerged and surround by cold [2-8°C] HTS-FRS solution. Additonal HTS-FRS will be added with the volume required to fully submerge the tissue in the solution and fill the vial/container completely without air pockets.
    ii. Care should be taken to avoid crushing artifacts and a separate forceps must be used for each specimen to avoid cross contamination.
    iii. The cryovial/specimen container lid will be secured
    iv. The cryovial/specimen container must be kept at 2-8°C and shipped immediately the same day (maximum within 24 hours).
d. Formalin fixation for IHC
    i. Specimens intended for formalin fixation should be processed after the completion of other fresh tissue procedures, such as flash freezing and submersion in RNA stabilizing reagent.
    ii. The tissue sample should be trimmed such that it is maximally 0.5 cm in thickness. Specimens that exceed this dimension may not allow for adequate perfusion of the formalin.
    iii. After trimming, the specimen will be transferred to a container with formalin using a forceps. To avoid cross-contamination, a separate forceps should be used for each tissue sample. The volume of formalin should be a minimum of 15-20 times the volume of the tissue sample - e.g., 20 ml of formalin per 1 cm^3 of tissue. A 15 ml sterile centrifuge or conical tube or alternatively, a specimen cup can be used.
    iv. The specimen must be completely submerged in the formalin fixative and the container lid securely tightened to avoid any spillage.
    v. The tissue will be fixed at room temperature for a minimum of 24 hours, at which point it will be transferred to PBS in preparation for shipping.
    vi. Formalin should not be discarded down the drain. The chemical disposal plan at the institution will be followed for an appropriate method of disposal.

[redacted]

e. RNA stabilizing for array analysis
   i. RNA preservation reagent (RNAlater or TRIZOL) will be transferred into a sterile specimen container. A minimum of 10 volumes of RNA reagent is required for each 1 mg of tissue - e.g., 10 l reagent per 1 mg of tissue (or for a specimen that is 0.5 cm^3, 5 ml of reagent will be used in a sterile container that is of sufficient size to allow for complete submersion of the specimen). A 15 ml sterile centrifuge or conical tube is sufficient. Prefilled tubes can also be purchased from the manufacturer.
   ii. Efforts must be made to expedite the transfer of harvested tissue to the sterile pre-filled containers of RNA reagent as quickly as possible after harvest.
   iii. It is imperative that height and width of the specimen not exceed 0.5 x 0.5 cm, as this tissue perfusion of the RNA stabilizing reagent is significantly impaired in larger specimens. However, specimens can exceed 0.5 cm in length.
   iv. Using sterile forceps, the tissue will be placed into the tube containing the RNA reagent and completely submerged. A separate forceps will be used for each specimen to avoid cross contamination.
   v. The tissue in RNA reagent will be incubated overnight at 2-8°C. Care must be taken to keep the tube in an upright position into order to keep specimens submerged. Even transient exposure of the specimens to air can result in DNA degradation.
   vi. After a minimum of 12 hours of refrigeration, the specimen will be transferred using a sterile forceps to a sterile labeled cryovial.
   vii. The cryovial will then be transferred to a liquid nitrogen freezer or a mechanical -80°C freezer for long-term storage.

Shipping Table

Tissue (processing endpoint)	Storage before shipping	Storage during shipping	Time allowed between processing and shipping
Blood (serum from Red Top tubes)	Room Temperature	Room Temperature	Shipped same day as obtained (max <24h)
Blood (PBMCs from Green Top tubes)	Room Temperature	Room Temperature	Shipped same day as obtained (max <24h)
Tumor (HTS-FRS preserved from fresh tissue)	2-8°C	Ice packs	Shipped same day as obtained (max <24h)
Tumor (formalin fixed from fresh tissue)	Room temperature	Room temperature	Shipped same day as obtained (max <24h)
Tumor (Archival tissue: blocks or slides)	Room temperature	Room temperature	Shipped same day as obtained (max <24h)

[redacted]

11.5.3 Shipping

a. [redacted] must be made so that one copy can be sent to [redacted] the samples and one can be maintained at the collection site for internal records.

g. Samples from each patient that are shipped under that same shipping condition should be consolidated in a single storage container (such as an 81-place freezer box). Samples from different patients should not be mixed in the same storage container but can be shipping in the same shipping container (as long as they are separated in different storage containers).

h. Please refer to the Post-processing and Shipping Table for post-processing and handling details.

i. Just prior to shipment, storage containers with samples from individual patients should be placed in the shipping container/s and the contents of the package should be matched to the shipping manifest. Both copies of the shipping manifest should be signed and dated and one copy of the shipping manifest and record should be placed in the box.

j. The box should be sealed and a shipping label attached onto the outside of the shipping container. The container should be labeled as containing biohazardous specimens.

k. [redacted]

11.5.4.1 Immunophenotypic analysis of immune cells and markers by multiparameter flow cytometry

Phenotypic analysis of immune cells will be conducted to determine cell population ratios, activation, differentiation, and suppressive and functional potential of T cells.

a. Such phenotypic analyses will utilize 15-parameter flow cytometry with an LSR-II flow cytometer, whereby cells will be stained with fluorescent antibodies to the cell markers (purchased primarily from eBioscience).
b. Cell population ratios will include identifying cells as cytotoxic CD8+ T cells (Tcs), CD4+ helper T cell (Th) subsets (in conjunction with transcription factors defining such subsets), regulatory CD4+ T cells (Tregs), MDSCs, B cells, NK cells, and DCs.
c. Phenotypic analysis for cell subsets that can be distinguished most accurately by cytokine production and transcription factors (including, IL-2/IFN-γ with T-bet for Th1s; IL-4 with GATA-3 for Th2s; IL-17 with RORyt for Th17s, IL-22 for Th22s, IL-10 with FoxP3 for Tregs) will be performed using a six-hour stimulation assay with PMA/Ionomycin (as per manufacturer instructions).
d. Activation, differentiation, suppressive status/functional potential will include early and late markers of activation; markers of naïve, effector, memory (central, effector, and resident) cells. Antibodies to be used include: HLA class I (A,B,C) and II (DP, DQ, DR), CD3, CD4, CD8, CD11b, CD11c, CD14, CD16, CD19, CD25, CD27, CD34, CD45, CD45RA, CD45RO, CD56, CD57, CD62L, CD69, CD107a, CD127, perforin, Granzyme B, Foxp3, GITR, CCR7, HLA-DR, ICOS, ILT-2, ILT-3, ILT-4, ILT-5, PD-1, Tim3, Lag3, KIR2DL1, KIR2DL2, KIR3DL1.
e. To determine whether tissues express HLA-A2 and thus can be utilized in tetramer studies investigating anti-specific CD8+ T cell responses, staining and analysis of HLA-A2 on CD45+ PBMCs will be conducted.
f. PBMCs from Green Top tubes or extracted from flash frozen tumor tissue will be used to characterize immune responses. Specifically:
    i. Flash frozen tumor tissue will be quickly thawed by immersion of the bottom 90% of the cryovial in a 37oC water bath and processed after thawing as follows:

[redacted]

ii. The thawed tissue will be washed once in PBS and reconstituted in a solution of DNAse (40 µg/ml) and Collagenase (1 mg/ml) in 1 ml of HBSS in a Miltenyi C-tube.
iii. The tumor tissue will be mechanically dissociated using a GentleMACs OctoDissociator (Miltenyi) using the B_01 program, further dissociated in a shaking incubator for 30 minutes at 250 rpm, and further mechanically dissociated using the B_01 program of the OctoDissociator.
iv. The tumor tissue will then be passed through a 70 µm screen (BD Biosciences) to ensure a single cell suspension, and washed in PBS (with centrifugation for 5 min at 1500 rpm and 4°C).
v. Tumor tissue will be reconstituted in PBS ($2x10^6$ in 50 µl PBS, and the remainder frozen down, as described previously at $2x10^6$ in cryopreserve solution.

b. PBMCs from Green Top tubes and processed tissues will be thawed quickly by immersion of the bottom 90% of the cryovial in a 37°C water bath and washed once in PBS (with centrifugation and reconstitution of the cell pellet in 50 µl PBS per sample in a 96 well plate) prior to flow cytometric antibody staining.
c. For extracellular markers:
   i. Plates with cells to be stained will be centrifuged at standard conditions (4°C, 1500 rpm, 5 min) and decanted by inversion.
   ii. Extracellular marker antibodies will be added at titrated concentrations (for example, 0.5 µl antibody in 50 µl of PBS for antibodies used at 1:100).
   iii. The plate will be incubated in the dark for 30 minutes at room temperature and then centrifuged at standard conditions and washed once with PBS.
   iv. Cells not requiring intracellular staining (ICS) will be analyzed immediately by flow cytometry or fixed in 50/50 v/v 10% formalin in PBS, kept in the dark at 4°C, and analyzed within 24 hours. Prior to analysis, 5 µl of Countbright beads will be added to each well (and the bead concentration recorded from the Countbright bottle).
d. For intracellular markers:
   i. Cells requiring ICS, will be centrifugated at standard conditions and reconstituted with 100 µl of BD Perm/Fix per well, incubated at 4°C for 15 min, receive 100 µl of 10% PermWash to each well, and centrifuged immediately at standard conditions.
   ii. The plate will be then decanted and a 100 µl ICS stain mixture (with antibodies) added to each well in a 10% PermWash mixture.
   iii. The plate will be incubated at 4°C for 30 minutes, centrifuged at standard conditions, decanted, washed 2x with 200 µl of 10% PermWash per well, centrifuged at standard conditions, decanted, and analyzed by flow cytometry or fixed in 10% formalin for up to 24 hours.
   iv. Prior to analysis, 5 µl of Countbright beads will be added to each well (and the bead concentration recorded from the Countbright bottle).
e. Flow cytometry panels will be based on Optimized Multicolor Immunofluorescence Panel (OMIPs) published in the journal *Cytometry Part A.*

[redacted]

http://qap2.onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4930/homepage/information_on_omips.htm

11.5.4.2 Analysis of PD ligand expression (Qualtek analysis)

a. Formalin-fixed tissue will be utilized for analysis of PD-L1 and PD-L2 using commercially available reagents from the Dako EnVision FLEX+ HRP-Polymer kit (DAKO K8012; Dako, Carpinteria, CA) and the 22C3 antibody.
b. Briefly, slides will be baked at 60°C, deparaffinized in xylenes, and rehydrated with graded ethanols to distilled water.
c. Antigen retrieval will be performed using the Dako EnVision FLEX Target Retrieval Solution, low pH (supplied as a citrate-based buffered solution, pH 6.1) in a steamer.
d. Cooled slides will be stained using the automated TechMate IHC staining platform including an additional Proteinase-K antigen retrieval step (Dako #S3020
e. The 22C3 antibody will be diluted at 2 µg/mL in Dako Primary Antibody Diluent for off-platform overnight incubation in a dark humidified chamber.
f. The EnVision FLEX+ Polymer reagents will be utilized for primary antibody detection including the mouse linker, HRP-polymer, DAB chromogen and DAB enhancer.
g. EnVision FLEX+ wash buffer will be used between incubation steps, with slides counterstained with hematoxylin.
h. Slides will then be rinsed in distilled water and subjected to an ethanol dehydration series and xylene changes before coverslipping.

11.5.4.3 Measurement of antigen-specific T cells and evaluation of cellular functionality by intracellular cytokine and effector molecule staining

PBMCs derived from Green Top tubes and flash frozen tumor tissue will be utilized for evaluation of cellular functionality by cytokine detection after stimulation. Briefly,

a. Antigen presenting cells (APCs; monocytes, DCs, etc., $1x10^5$ cells) will be obtained from PBMCs isolated from Green Top tubes by HLA-DR positive bead selection (Miltenyi Biotec), washed in Aim V media and pulsed at 37°C in a 5% CO_2, humidifying incubator in Aim V media for 1 hour with tumor antigen peptides of interest (gp100, Mart-1, tyrosinase) and washed 3x Aim V media.
b. Non-APCs from the PBMCs (80-90% T cells) will then be cultured with these loaded cells APCs for 24 hours at 37°C in a 5% CO_2, humidifying incubator (or anti-CD3 stimulation antibody at 1mg/ml [positive control] or unloaded APCs or no APCs [negative controls]. During the last 6 hours in incubation, Golgi Plug [Pharmingen] will be added.
c. After stimulation cells will be washed 3x in PBS and stained for extracellular antibodies identifying CD8+ cells subsets and intracellular antibodies for cytokines and effector molecules of interest (IFNγ, TNFα, IL-2, granzyme B, perforin, and CD107), according to protocols described in the "Immunophenotypic analysis of immune cells and markers by multiparameter flow cytometry" [redacted]

d. Cells will be analyzed by flow cytometry. Antigen-specific T cells will be defined as cells producing any one of the cytokines or effector molecules of interest, while functionality will be defined by the ability to co-produce combinations of the cytokines or effector molecules of interest.

11.5.4.4 Measurement of antigen-specific T cells by MHC dextramer staining

To measure tumor antigen-specific responses PBMCs from Green Top tubes or extracted from flash frozen tumor tissue of patients found to be HLA-A2-positive by protocols in the "Immunophenotypic analysis of immune cells and markers by multiparameter flow cytometry" section" will be stained with MHC dextramers (including those identifying cells specific against melanoma-specific antigens gp100, MART-1, and tyrosinase, or positive and negative control antigens CMV, EBV, Influenza, HIV, and empty). Specifically:

a. PBMCs from Green Top tubes and processed tissues will be thawed quickly by immersion of the bottom 90% of the cryovial in a 37°C water bath and washed once in PBS (with centrifugation and reconstitution of the cell pellet in 50 µl PBS per sample) prior to dextramer staining.
b. As per manufacturer instructions, 10-20 µl of tetramer will be added to the cells and incubated for 30 min in the dark at room temperature.
c. Thereafter, the ECS protocol described above will be followed to determine CD8+T cells (and exclude B, monocyte/macrophage, and dendritic cells from dextramer analysis).

11.5.4.5 Serological assays to evaluate antibody titers to tumor-associated antigens

Serum from Red Top tubes will be thawed for analysis of antibody titers to tumor associated antigens. Briefly:

a. Tumor-associated antigens (gp100, MART-1, and tyrosinase) will be prepared within PBS for plate well coating 200 µl per well of 96 well plate) and incubated at 37 °C for 30 min., or incubated (covered) overnight at 4 °C.
b. The coating solution will be removed and the plate washed 3x with PBS prior to the additional blocking step (30 min. 5% BSA-PBS) Dilute the monoclonal primary antibody in PBS. The optimal dilution should be determined using a titration assay.
c. Then 0.2 ml of the diluted monoclonal antibody will be added to each well. The negative control will be a species- and isotype-matched, non-specific immunoglobulin diluted in PBS.
d. The plate will be incubated at room temperature for 2 hours, washed 3x in PBS, followed by addition of the secondary antigen (0.2 ml per well).
e. The plate will be incubated at room temperature for 2 hours, washed 3x in PBS, and the freshly prepared enzyme substrate will be added (0.2 ml of the freshly prepared substrate to each well).

[redacted]

f. Color development will be observed (expected after 30 min of incubation at room temp) and absorbance will be read (at 405 nm) immediately in a microplate reader or the reaction will be stopped with 50 µl per well of the appropriate stopping reagent and absorbance read within 2 hours.

11.5.4.6 Multiplex cytokine analysis

Serum from Red Top tubes will be thawed for analysis of cytokines using the Bio-Plex Cytokine Assay (BioRad). Briefly:

a. Thawed serum samples will be prepared for by diluting 1 volume of the serum sample with 3 volumes of the Bio-Plex human serum sample diluent. Extremely lipemic samples should be filtered through a 0.22 µm filter to prevent clogging.
b. The cytokine standard dilutions will be prepared as per manufacturer instructions from the manufacturer's master standard stock, for the cytokines of interest, including IFNα, IFNβ, IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, and TNF-alpha.
c. A multiplex bead working solution will be prepared from the anti-cytokine conjugated 25x beads and the multiplex plate will be prepared using Bio-Plex assay and wash buffers.
d. Diluted standard or serum-derived samples will be pipetted (50 µl each) per well, sealed within the plate, and incubated on a microplate shaker for 30 min at room temperature.
e. The wells will be washed with Bio-Plex wash buffer (100 µl; 3x), and detection antibody solution (0.5 µl/well) will be added to the wells, sealed within the plate, and incubated on a microplate shaker for 30 min (1,100 rpm for 30 sec and
f. 300 rpm at room temperature for the remainder of the incubation).
g. The wells will be washed with Bio-Plex wash buffer (100 µl; 3x), and pre-diluted streptavidin-PE (1x; 50 µl/well) will be added to each well, sealed within the plate, and incubated on a microplate shaker for 10 min (1,100 rpm for 30 sec and 300 rpm at room temperature for the remainder of the incubation) at room temperature.
h. The wells will be washed with Bio-Plex wash buffer (100 µl; 3x), and Bio-Plex assay buffer will be added (125 µl/well) to the wells, sealed within the plate, and incubated on a microplate shaker for 30 seconds (1,100 rpm) at room temperature.
i. The plate will be analyzed on a Bio-Plex array reader, as per the manufacturer instructions within 1 hour of last incubation.

11.5.4.7 Nanostring Gene Expression Analysis

RNA from preservation media will be utilized for gene expression analysis using the Nanostring nCounter System. Briefly:

a. Extracted total RNA will be further purified using RNeasy mini spin columns (Qiagen) according to the manufacturer's protocol. The [redacted]

[redacted]

of the total RNA preparation will be determined by measuring absorbance at 260 and 280 nm using the Nanodrop system (Thermo Scientific).

b. Each hybridization reaction will require 100-150 ng of total RNA. The integrity of the total RNA preparation will be verified on a Bioanalyzer (model 2100, Agilent Technologies) before proceeding with the hybridization reaction. RNA should be aliquoted (to avoid freeze/thaw cycles) and stored for up to several years at −80◦C.
c. Hybridization of the target mRNA to the gene-specific probe pairs will be carried out in triplicate with each sample containing 5 µl RNA (150 ng), 10 µl reporter probe (final 40 pM), 5 µl capture probe (final 200 pM), and 10 µl hybridization buffer (5× SPPE, pH 7.5, with 0.1% Tween-20).
d. Using a thermocycler, the hybridization reaction will be conducted will be at 65°C for optimally 16 h (at least 12 h but not more than 30 h).
e. After the hybridization reactions are completed, post-hybridization processing will immediately continue using the nCounter Prep Station, as per the manufacturer instructions on using the instrument.
f. After post-hybridization processing, the nCounter Digital Analyzer will be used to acquire images of the immobilized fluorescently labeled target mRNA molecules in the sample cartridge using a CCD camera and a microscope objective lens and the expression level of a gene will be measured and tabulated in a CSV format by the system.
g. Using Excel, the individual data files will be imported into a collector file template provided by the analyzer manufacturer. The abundance of target mRNA molecules will be compared across multiple different samples and thus normalized for all target genes in all samples based on the positive spike-in controls to account for differences in hybridization efficiency and post-hybridization processing, including purification and immobilization of complexes.
h. To determine if the normalized, background-subtracted counts are statistically above background, a Student's t test will be performed against eight human negative controls. A gene will be considered to be above background if the average count for the target gene is greater than the average counts for the eight negative control genes and if the P value of the t test is less than 0.05.
i. The relative changes in the abundance of target mRNA molecules will be calculated using the normalized, background-subtracted counts for one or more reference genes included manufacturer set.

11.5.4.8 Other Analyses

If samples are available, some or all of the following analyses may also be performed: Genetic studies, proteomics, and grafting of fresh tissue into experimental animals using blood and tumor tissue. The experimental protocol will follow laboratory SOPs and published procedures.

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11.5.5 Safety Precautions

Universal precautions (*i.e.*, a method of infection control in which all human blood and body fluids are treated as if they are infectious for Hepatitis Viruses, Human Immunodeficiency virus, and other known and unknown infectious agents) will be utilized when handling all unfixed cells and tissues.

a. Hepatitis B and Hepatitis C viruses may be transmitted through blood and other body fluids, and are associated with acute hepatitis, chronic liver disease, and hepatocellular carcinoma in humans. The probability of seroconversion after needlestick exposure is estimated at 7%. Untreated virus can persist for up to one week at room temperature. All staff who work with human tissue must provide evidence of Hepatitis B vaccination.
b. Human Immunodeficiency virus (HIV) is a retrovirus that causes severe immunodeficiency. Infection increases the risk of developing malignancies, infection by opportunistic organisms, and death. The probability of seroconversion after needlestick exposure is estimated at 0.5%. Infectivity of untreated virus persists for up to one week at room temperature.
c. Other potentially infectious agents, both known and unknown, pose hazards to those working with human tissue. Included are tuberculosis, HTLV1, Coccidiomycosis, Creutzfeldt-Jacob disease, amongst others.
d. Individual institutional and OSHA guidelines must be followed when handling human cells and tissues, and referred to for additional information on bloodborne pathogens, laboratory safety, chemical safety, and biohazardous waste disposal. Briefly:
    i. Personal protective equipment (PPE) must be used at all times while working with human tissue. These include disposable latex or nitrile gloves, face shield, protective splash-resistant laboratory coat (disposable preferred), and covered protective shoes.
    ii. Gloves should be immediately removed and replaced in the event that they become torn or perforated. Gloves must be removed prior to leaving the work area, and disposed of in an appropriate waste disposal container. Hands must be washed in a "clean" sink after removal of gloves.
    iii. Face shields, goggles and masks should be worn whenever a potential for exposure to splashes, spray, splatter, droplets, aerosols of blood or tissue fluid, or other potentially infectious materials may be generated, and if there is a potential for eye, nose or mouth contamination. They should be worn at all times while handling tissue in the for processing.
    iv. Protective lab coats, preferably disposable types, must be donned while working with tissue. Contaminated clothing must be removed prior to leaving the work area, and appropriately laundered or discarded, as per individual institutional guidelines.
    v. All waste must be disposed of prior to leaving the work area. Biohazardous sharps must be properly disposed of in an approved "sharps" container. All other non-sharp waste must be disposed of in an approved orange or red biohazardous waste disposal bag.

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vi. After completion of work with human tissue, all work surfaces must be disinfected with a product that has been demonstrated to be effective against bacteria, viruses, pseudomonas, tuberculosis and fungi. Product literature should be referred to for appropriate use.

e. Any injuries or exposure to human tissue or potentially infectious biologic agents must be reported promptly as specified in individual institutional safety guidelines.

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