Title: A phase II trial of pembrolizumab with or without radiation in patients with recurrent or metastatic adenoid cystic carcinoma

Protocol Version Date: August 28th, 2019

Local Protocol #: 16-609

Coordinating Center: Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215

Principal Investigator (Overall): Jonathan Schoenfeld, MD, MPH

Co-Investigators: Roy Tishler, MD, PhD, Danielle Margalit, MD, MPH, Robert Haddad, MD, Jochen Lorch, MD, MS, Glenn Hanna, MD; Ravindra Uppaluri, MD, PhD; Harvey Mamon, MD, PhD, Tracy Balboni, MD, Ray Mak, MD, Scott Rodig, MD, Evisa Gijini, PhD, Mariano Severgnini, PhD, Jens Lohr, MD, PhD; Lori Wirth, MD; John Clark, MD; Jong Chul Park, MD; Paul Busse, MD; Annie Chan, MD

Statistician: Yu-Hui Chen, MS, MPH, Paul Catalano, ScD

Agent: Pembrolizumab (Supplied by Merck)

IND Holder: Jonathan Schoenfeld, MD

IND Number: 132576

SCHEMA

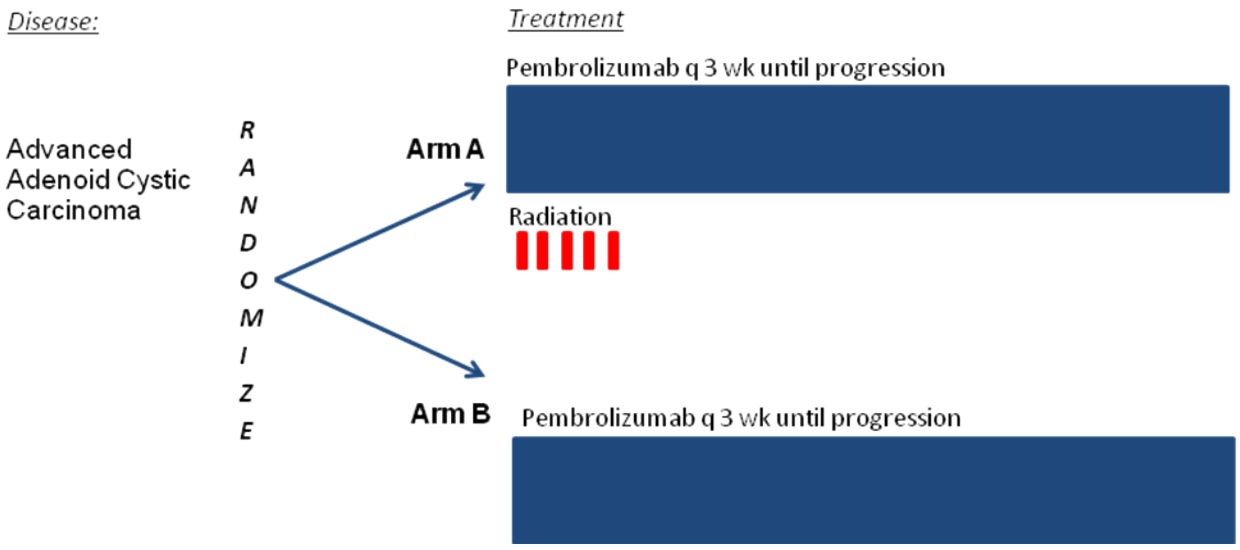

Disease:
Treatment
Pembrolizumab q 3 wk until progression
Advanced Adenoid Cystic Carcinoma
RANDOMIZE
Arm A
Radiation
Arm B
Pembrolizumab q 3 wk until progression

TABLE OF CONTENTS

1. OBJECTIVES

1.1 Study Design

This is an open label phase 2 study of pembrolizumab alone or with radiation therapy in subjects with recurrent or metastatic adenoid cystic carcinoma and at least one tumor or metastatic site that is amenable to palliative radiation. All subjects will have previously demonstrated disease progression, or lack of benefit from standard therapy. All subjects will have lesions amenable for palliative radiation therapy. In Arm A, up to 5 metastatic lesions will be targeted with radiation, in an attempt to provide palliative benefit and abscopal, out of field, systemic responses when given in combination with pembrolizumab. The lesions will be irradiated within 7 days following the first dose of pembrolizumab. Pembrolizumab will be administered intravenously every 21 days until disease progression or a maximum of 24 months from the first pembrolizumab dose. In Arm B, subjects will receive pembrolizumab intravenously every 21 days until disease progression or a maximum of 24 months from your first pembrolizumab dose. Correlative studies will include tumor biopsies performed before and on treatment if deemed safe and feasible.

1.2 Primary Objectives

- To evaluate the anti-tumor activity of pembrolizumab with or without radiation by determining the objective response rate using RECIST 1.1 (in non-irradiated sites) in subjects with recurrent and/or metastatic adenoid cystic carcinoma.

1.3 Secondary Objectives

- To estimate the progression-free survival and overall survival at 6 months and 12 months post treatment.

- To estimate the duration of response.

- To determine overall response rate using iRC in non-irradiated lesions.

- To confirm the safety and tolerability of pembrolizumab with radiation

- To investigate the mechanism of action of the combination of radiation and PD-1 inhibition and examine the relationship between candidate efficacy or resistance biomarkers and anti-tumor activity of pembrolizumab with or without radiation.

- To investigate mechanisms of resistance to PD-1 directed therapy in ACC by evaluating PD-L1 and PD-L2 expression, mutational burden and T cell infiltration in initial biopsies and at progression after pembrolizumab.

- To explore the effect of radiation in combination with pembrolizumab on circulating T-cell populations, and T-cell receptor diversity.

2. BACKGROUND

2.1 Study Disease

Adenoid cystic carcinoma (ACC) is one of the most common types of malignant salivary gland tumors. ACC typically occurs in young and middle-aged individuals with no history of tobacco or alcohol use, and 60% of cases involve women. Localized disease is managed with oncologic surgical resection with or without adjuvant radiotherapy or chemoradiotherapy. Despite upfront aggressive multimodality management, approximately 50% of patients develop distant metastases, and up to one third die within 2 years of diagnosis.[1, 2] ACC may have a slowly progressive natural history characterized by frequent recurrences and metastastic spread to lungs, liver and bones, and can also transform into rapidly progressive symptomatic disease.

No standard systemic chemotherapy regimen or FDA approved targeted therapy exists for recurrent or metastatic ACC. No drug therapy has demonstrated either survival or progression-free survival benefit.[3] In the past 20 years, multiple small phase II clinical trials have demonstrated generally low rates of objective response and lack of durable responses (Figure 1). Cytotoxic chemotherapy with combination regimens may result in short lived responses (ORR 0-44%) in a subset of patients but are associated with significant toxicities.[4-6] In addition, survival for patients to date has been the same whether patients underwent directed treatment for metastases or not.[7]

Figure 1. Phase II Trials in ACC (1996-2015)

Drug	Number of ACC patients evaluable	Objective Response Rate
Dovitinib[8]	32	3%
Sorafenib[9]	19	11%
Dasatinib[10]	40	3%
Everolimus[11]	31	0%
Vorinostat[12]	30	3%
Sunitinib[13]	13	0%
Imatinib[14]	15	0%
Cetuximab[15]	23	0%
Lapatinib[16]	19	0%
Vinorelbine, Cisplatin[4]	9	44%
Cisplatin, Doxorubicin, Cyclophosphamide[6]	12	25%
Cisplatin, 5-FU[5]	11	0%

Comprehensive molecular and genomic profiling studies in ACC have failed to identify a definitive genomic driver, and ACC tumors display a low rate of genomic instability compared to other cancer types.[17] Recently, somatic alterations in NOTCH1, FGF-IGF-PI3K pathway, CDKN2A/B, MDM2, PDGFRA, TRK have been reported,[18-20] and a gene fusion between the *MYB* and *NFIB* genes resulting from t(6;9)(q22-23;p24) regions has been identified.[21, 22] However the clinical significance and therapeutic targeting of these alterations remains elusive. ACC tumors frequently overexpress c-kit and EGFR by immunostaining, however the presence of activating mutations or amplifications are rare, and the use of targeted therapies against KIT and EGFR in ACC have yielded disappointingly low objective response rates in clinical trials to date (ORR 0-3%).[10, 13-16] A desperate need for novel drug therapies and treatment approaches in ACC remains.

Characterization of the immune infiltrates in ACC and the expression of checkpoint ligands has not been published to date. Data from our institution, obtained in collaboration with MD Anderson Cancer Center and the Adenoid Cystic Cancer Research Foundation, suggests that the majority of ACC tumors

demonstrate expression of the immunologic biomarker PD-L2. Expression of PD-L2 has been noted in more than 50% of patients and approximately 70% of metastatic deposits tested (Sridharan et al). Although we found that primary adenoid cystic carcinoma or metastatic ACC deposits generally do not express PD-L1, and there appeared to be low numbers of infiltrating immune cells, we did identify PD-L1 expression was present on infiltrating lymphocytes when they were present. Further interrogation of the immune microenvironment using mRNA profiling also identified candidate oncogenic pathways that may be linked to intratumoral immune infiltration. Distinct gene expression profiles suggested various immune populations present intratumorally. Lack of immune cell infiltrate appeared to be associated with expression of genes in the beta-catenin/Wnt and PI3K pathways. These results are consistent with a recent analysis showing activation of the WNT/ β-catenin signaling pathway correlating with the absence of a T-cell gene expression signature in melanoma.[23] When compared to tumors without immune infiltration on IHC, those tumor deposits with immune cells indicated significantly higher expression of B-cell, regulatory T-cell, CD8+ T-cell, and NK-cell associated mRNA transcripts. Conversely, when we stratified tumors by PD-L2 IHC status, those that stained positive for PD-L2 expression were associated with decreased mRNA expression indicating immune infiltration across all cell types, including NK cells, and CD4+ and CD8+ T-cells. Therefore, modulation of immune checkpoints in adenoid cystic carcinoma represents a promising therapeutic strategy that has yet to be tested.

2.2 Study Agents: Pembrolizumab

2.2.1 Pembrolizumab

2.2.1.1 Mechanism of action

Cancer immunotherapy is based on the premise that the body's immune system can recognize a tumor as foreign and mount an effective antitumor response capable of eliminating that tumor. This likely requires immune recognition of specific tumor antigens, but also effective functioning of activated T-cells capable of eliminating tumor cells as they arise and causing tumor shrinkage where existing tumor deposits are present. Conversely, tumor progression is likely intimately intertwined with mechanisms by which tumors evade immune recognition and attack.

One mechanism by which tumors may evade immune attack is by coopting inherent immune checkpoints that function under normal circumstances to maintain immune homeostasis and prevent harmful autoimmunity. Thus, one strategy that exists for cancer immunotherapy is to modulate these regulatory immune checkpoints that largely exist on the surface of T-cells. This can ideally overcome tumor mediated immune suppression, and potentiate nascent antitumor immune responses that might otherwise have been unable to lead to meaningful tumor regression.

Programmed death receptor-1 (PD-1, CD279), is a 55 kD type I transmembrane protein is a member of the CD28 family of T-cell costimulatory molecules that also includes CD28, CTLA-4, ICOS, and BTLA[24]. PD-1 contains an intracellular membrane proximal immunoreceptor tyrosine inhibitory motif (ITIM) and a membrane distal immunoreceptor tyrosine-based switch motif (ITSM). Two ligands specific for PD-1 have been identified: PD-L1 (B7-H1/CD274) and PD-L2 (B7-DC/CD273) [25]. PD-L1 and PD-L2 have been shown to down-regulate T-cell activation upon binding to PD-1 in both murine and human systems[25, 26]. PD-1 delivers a negative signal by the recruitment of SHP-2 to the phosphorylated

tyrosine residue in the ITSM in its cytoplasmic region[27, 28]. PD-1 is primarily expressed on activated T cells, B cells, and myeloid cells[28].

Further evidence for a negative regulatory role of PD-1 comes from studies of PD-1-deficient mice. PD-1-deficient mice develop various autoimmune phenotypes, including dilated cardiomyopathy, a lupus-like syndrome with arthritis and nephritis, and accelerated diabetes mellitus[29-31]. The emergence of these autoimmune phenotypes is dependent upon the genetic background of the mouse strain and many of these phenotypes emerge at different times and show variable penetrance. In addition to the phenotypes of null mutations, PD-1 inhibition by antibody-mediated blockade in several murine models has been found to play a role in the development of autoimmune diseases such as encephalomyelitis, graft-versus-host disease, and type I diabetes[32, 33]. Taken together, these results suggest that PD-1 blockade has the potential to activate anti-self T-cell responses, but these responses are variable and dependent upon various host genetic factors. Thus, PD-1 deficiency or inhibition is not accompanied by a universal loss of tolerance to self-antigens.

Preclinical animal models of tumors have shown that blockade by PD-1 by monoclonal antibodies (mAbs) can enhance the anti-tumor immune response and result in tumor rejection. Antitumor activity by PD-1 blockade functions in PD-L1-positive tumors as well as in tumors that are negative for the expression of PD-L1[34-39]. This suggests that host mechanisms (ie, expression of PD-L1 in antigen-presenting cells) limit the antitumor response. Consequently, both PD-L1 positive and negative tumors may be targeted using this approach.

2.2.1.2 Preclinical Safety

Please refer to the Investigator's Brochure for additional information on the preclinical testing of pembrolizumab.

2.2.1.3 Clinical pharmacology and safety

Pembrolizumab (MK-3475) (previously known as SCH 900475) is a potent and highly selective humanized monoclonal antibody (mAb) of the IgG4/kappa isotype designed to directly block the interaction between PD-1 and its ligands, PD-L1 and PD-L2. Keytruda™ (pembrolizumab) has recently been approved in the United Stated for the treatment of patients with unresectable or metastatic melanoma and disease progression following ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor.

As of the data cutoff dates for IB dated August 31, 2015, pembrolizumab monotherapy and combination therapy have been administered to 6,294 subjects, with hematologic malignancies and solid tumors, in a total of 18 Phase I, II, and III clinical trials sponsored by Merck.

Clinical data are derived from an ongoing, first-in-human phase I study (PN001, NCT01295827) to evaluate the safety and clinical activity of Pembrolizumab as a monotherapy, sponsored by Merck Sharp & Dohme. There are five parts to this study (Parts A-D and F).

Part A was a 3+3 dose escalation study in subjects with solid tumors to evaluate safety, tolerability, pharmacokinetics (PK), and pharmacodynamics, and to determine a maximum tolerated dose (MTD) or preliminary recommended phase 2 doses (RP2Ds). Doses were 1, 3, and 10 mg/kg every 2 weeks

(Q2W); doses of either 2 mg/kg or 10 mg/kg were also administered every 3 weeks (Q3W). All 3 dose levels were well tolerated and no dose-limiting toxicities (DLTs) were observed; therefore, the MTD was not determined. The RP2D was determined by the sponsor based on safety, PK, and pharmacodynamic measurements, along with the strength of antitumor activity signals observed.

In Parts B and D, subjects with metastatic melanoma were enrolled to assess the safety and antitumor activity of pembrolizumab. Additionally, Part B explored 3 different dose regimens in subjects with metastatic melanoma: 10 mg/kg Q2W, 10 mg/kg Q3W, and 2 mg/kg Q3W.

In Part C, subjects with NSCLC (with prior systemic therapy) were enrolled at 10 mg/kg Q3W to assess the tolerability, safety, and antitumor activity of pembrolizumab in NSCLC. In Part F, subjects with NSCLC in Cohort F-1 (without prior systemic therapy) and Cohort F-2 (with prior systemic therapy), whose tumors expressed PD-L1, were enrolled at 10 mg/kg Q2W and 10 mg/kg Q3W to characterize the tolerability, safety, and antitumor activity of pembrolizumab. A small cohort of previously treated subjects with NSCLC and at least 2 lines of systemic therapy, whose tumors did not express PD-L1, were enrolled and treated at a dose of 10 mg/kg Q2W in Cohort F-2. In Cohort F-3, previously treated subjects with NSCLC whose tumors express PD-L1 were enrolled at 2 mg/kg Q3W to better characterize the efficacy, safety, and antitumor activity of pembrolizumab. Each of the 2 disease specific cohorts (melanoma and NSCLC) were enrolled to confirm tolerability and evaluate tumor response to pembrolizumab.

Pharmacokinetics

The half-life ($t_{1/2}$) of pembrolizumab is approximately 4 weeks and there is no indication of dose dependency or half-life in the three dose groups (1, 3, and 10 mg/kg). The long $t_{1/2}$ supports a dosing interval of every 2 or 3 weeks.

There was a dose-related increase in exposure from 1 to 10 mg/kg. Serum concentrations of pembrolizumab were lower by a factor of approximately 5 in patients receiving 2 mg/kg Q3W than in those receiving 10 mg/kg Q3W. Steady-state trough concentrations were 20% greater in the patients receiving 10 mg/kg Q2W than in those receiving the same dose Q3W.

A population pharmacokinetic analysis has been performed using serum concentration time data from 476 patients. Within the resulting population PK model, clearance and volume parameters of pembrolizumab were found to be dependent on body weight. The relationship between clearance and body weight, with an allometric exponent of 0.59, is within the range observed for other antibodies and would support both body weight normalized dosing or a fixed dose across all body weights. Pembrolizumab has been found to have a wide therapeutic range based on the melanoma indication. The population PK evaluation revealed that there was no significant impact of tumor burden on exposure. In addition, exposure was similar between the NSCLC and melanoma indications. Therefore, there are no anticipated changes in exposure between different indication settings.

The rationale for further exploration of 2mg/kg and comparable doses of MK-3475 in solid tumors is based on: 1) similar efficacy and safety of MK-3475 when dosed at either 2 mg/kg or 10 mg/kg Q3W in melanoma patients, 2) the flat exposure-response relationships of MK-3475 for both efficacy and safety in the dose ranges of 2 mg/kg Q3W to 10 mg/kg Q3W, 3) the lack of effect of tumor burden or

indication on distribution behavior of MK -3475 (as assessed by the population PK model) and 4) the assumption that the dynamics of MK -3475 target engagement will not vary meaningfully with tumor type.

The choice of the 200 mg Q3W as an appropriate dose for the switch to fixed dosing is based on simulations performed using the population PK model of MK-3475 showing that the fixed dose of 200 mg every 3 weeks will provide exposures that 1) are optimally consistent with those obtained with the 2 mg/kg dose every 3 weeks, 2) will maintain individual patient exposures in the exposure range established in melanoma as associated with maximal efficacy response and 3) will maintain individual patients exposure in the exposure range established in melanoma that are well tolerated and safe. A fixed dose regimen will simplify the dosing regimen to be more convenient for physicians and to reduce potential for dosing errors. A fixed dosing scheme will also reduce complexity in the logistical chain at treatment facilities and reduce wastage.

Anti-Drug Antibodies (ADA) Data

The occurrence of ADA has been observed in less than 1% of the patients screened, indicating a low potential of pembrolizumab to elicit the formation of ADA. No impact of ADA on pembrolizumab exposure has been observed.

Safety

In general, the safety profile observed in P011 (monotherapy arm), P012, P013, and P028 was similar to that observed in P001/P002. In the pembrolizumab monotherapy trials (P001/P002, P012, P013, and P028, plus the P011 monotherapy arm), the overall incidence of AEs ranged from 83.0% (73 of 88 subjects in P012) to 100% (10 of 10 subjects in P011). The most commonly reported AEs included fatigue, diarrhea, decreased appetite, nausea, and anemia. The incidence of drug –related AEs (DRAEs) ranged from 39.8% (35 of 88 subjects in P013) to 80.0% (8 of 10 subjects in P011). The most commonly reported DRAEs across all studies were nausea, fatigue, and diarrhea. The incidence of Grade 3-5 DRAEs across studies ranged from 6.8% (6 of 88 in P013) to 12.0% (187 of 1562 subjects) in P001/P002. The most commonly reported Grade 3-5 DRAEs were anemia, alanine aminotransferase increased, and aspartate aminotransferase increased. Most subjects who experienced an AE continued in the study, with the incidence of AEs leading to discontinuation ranging from 1.9% (8 of 430 subjects in P028) to 12.3% (192 of 1562 subjects in P001/P002). The majority of AEs leading to discontinuation were not considered drug related. Discontinuations due to a DRAE were infrequent and ranged from 0% (no subjects in P011) to 4.5% (4 of 88 subjects in P013). The most commonly reported DRAEs leading to discontinuation were pneumonitis, alanine aminotransferase increased, and aspartate aminotransferase increased. The overall pattern of AEs observed in melanoma subjects enrolled in P002 demonstrates favorable safety profile when this immune therapy is compared to chemotherapy. Consistent with prior observations from randomized comparisons of the 2 mg/kg and 10 mg/kg dose levels when given every 3 weeks, there are no important differences in the safety profile of pembrolizumab at these 2 dose levels, and both doses appear to have a favorable safety profile compared to chemotherapy.

Pembrolizumab has also been well tolerated specifically in metastatic and recurrent head and neck cancer patients. KEYNOTE 012 is a Phase Ib study of pembrolizumab in patients with human papillomavirus (HPV)-negative and HPV-positive head and neck cancer. This trial enrolled an initial 60

patient cohort with recurrent and/or metastatic squamous cell carcinoma of the head and neck for treatment with single agent pembrolizumab. Preliminary results of this cohort were reported at Annual Meeting of the American Society of Clinical Oncology (ASCO) in 2014 [40]. In general, pembrolizumab was well tolerated with 58.3% reporting a DRAE and 16.7% reporting a Grade 3-5 DRAE. DRAEs with an incidence ≥ 5% were fatigue (10, 16.7%), pruritis (6, 10%), rash (5, 8.3%), nausea (4, 6.7%), decreased appetite (3, 5.0%), and myalgia (3, 5.0%). Of these DR AEs, Grade 3-5 was seen in rash (2, 3.3%). The reported pre- specified AEs were adrenal insufficiency (1, 1.7%); diarrhea (1, 1. 7%); pruritis (1, 1.7%); rash (2, 3.3%); rash, macular (1, 1.7%); pneumonitis (0); alanine aminotransferase (ALT) increase (2, 3.3%); and aspartate aminotransferase (AST) increase (2, 3.3%).

2.2.1.4 Clinical Efficacy

Therapeutic studies in mouse models have shown that administration of antibodies blocking PD-1/PD-L1 interaction enhances infiltration of tumor-specific CD8+ T-cells and leads ultimately to tumor rejection, either as a mono-therapy or in combination with other treatment modalities. Anti-mouse PD-1 or anti-mouse PD-L1 antibodies have demonstrated anti-tumor responses as a mono-therapy in models of squamous cell carcinoma, pancreatic carcinoma, melanoma and colorectal carcinoma. Blockade of the PD-1 pathway effectively promoted CD8+ T-cell infiltration into the tumor and the presence of IFN-γ, granzyme B and perforin, indicating that the mechanism of action involved local infiltration and activation of effector T-cell function in vivo [41-45]. Experiments have confirmed the in vivo efficacy of PD-1 blockade as a mono-therapy (see the Investigator's Brochure [IB]).

When treated with pembrolizumab monotherapy, the overall response rate (ORR) for ipilimumab (IPI)-treated patients with melanoma was 25%/27% according to the Response Evaluation Criteria in Solid Tumors (RECIST)/investigator-assessed immune-related response criteria (irRC), respectively (Investigator's Brochure, 2014). The ORR for IPI-naïve patients with melanoma was 39%/43% by RECIST/investigator-assessed irRC, respectively. The majority of responses were seen in patients with melanoma by 16 weeks of therapy; however, some responses have been reported after 24 weeks or more of therapy with pembrolizumab. Responses can be delayed, and in some patients, a RECIST-defined progression followed by response has been observed. The preliminary ORR for 38 patients with non-small cell lung cancer was 21%/24% by RECIST/investigator-assessed irRC, respectively (Investigator's Brochure, 2014).

Ongoing clinical trials of pembrolizumab are being conducted in advanced melanoma, non-small cell lung cancer, a number of advanced solid tumor indications and hematologic malignancies. For study details please refer to the IB.

Trials evaluating pembrolizumab in head and neck cancer have demonstrated clinical activity in patients with recurrent and/or metastatic disease. KEYNOTE 012 is a Phase Ib study of pembrolizumab in patients with human papillomavirus (HPV)-negative and HPV-positive head and neck cancer. This trial enrolled an initial 60 patient cohort with recurrent and/or metastatic squamous cell carcinoma of the head and neck for treatment with single agent pembrolizumab. Preliminary results of this cohort were reported at Annual Meeting of the American Society of Clinical Oncology (ASCO) in 2014 [40], showing an overall response rate (confirmed and unconfirmed) of 19.6% (10 partial responses [PRs], 1 complete response [CR] out of 56 patients evaluable for response). An additional 16/56 patients (28.6%) experienced stable disease (SD), with 51% of patients experiencing some numerical decrease in tumor

burden from baseline. Seventeen total patients with CR, PR, or SD remain on therapy at the time of the reporting for > 6 months. There were no new or unexpected toxicity signals in this patient cohort, with infrequent grade 3-4 drug-related (DR) adverse events (AEs).

Preliminary PD-L1 biomarker data from KEYNOTE 012 suggests that the response rate may be enhanced for patients with high PD-L1 IHC expression. Using a Youden-Index derived PD-L1 IHC cutpoint, the response rate (RR) in patients with high PD-L1 expression was 45.5% (5/11), compared to 11.4% (5/44) in low PD-L1 expression patients.

2.3 Radiation therapy

Radiation therapy is the current standard of care for patients with unresectable adenoid cystic carcinoma.[46, 47] However, radiation therapy is typically not curative and patients frequently develop distant failure and ultimately die from progressive disease. Radiation is also used in the metastatic setting for palliation of symptomatic metastases, or when tumor progression may threaten critical structures.

Palliative radiation regimens used vary based on institutional practices and patient and disease factors, but are generally in the range of 8 to 37.5 Gy delivered in 1 to 15 fractions. There have been several attempts to increase the radiosensitivity of ACC, but none have been successful in the clinical setting to date. There is also some evidence that a higher bioequivalent dose will result in more durable disease control [48, 49] and preclinical evidence suggests that higher radiation doses may stimulate increased immunologic effects [50]. Stereotactic radiosurgical techniques are more commonly being employed for patients with oligometastatic disease and for salvage, with doses ranging from 12-30 Gy in 1-5 fractions.

2.4 Rationale

ACC is a common malignant salivary gland tumor for which no standard effective drug therapies exist. Data from recent immune profiling efforts in ACC suggest that multiple potential mechanisms of immune suppression may be relevant in ACC, including: 1) PD-L2 expression on tumor cells, 2) a majority of tumors with relatively sparse immune infiltrate, and 3) PD-L1 expression on infiltrating lymphocytes when they are present. While expression of PD-L1 has been demonstrated across multiple tumor types[51], comparatively less is known about the frequency and role of PD-L2 expression in modulating anti-tumor immune responses. Like PD-L1, PD-L2 also binds to the PD-1 receptor and decreases T-cell proliferation and cytokine production [25]. PD-L2 has also been found to bind to the RGMb receptor on the surface of macrophages and other immune cells to promote immune tolerance [52]. PD-L2 expression has been recently noted on esophageal adenocarcinoma, breast cancer, renal cell cancer and KRAS-mutant lung cancer [53-57]. Both PD-L1 as well as PD-L2, and infiltrating immune cells expressing the PD-1 receptor represent potential targets of treatment with PD-1 inhibitors in an attempt to stimulate an effective antitumor immune response.

Radiation therapy has known immunologic activity, and has demonstrated synergy with PD-1 blockade in multiple preclinical models [58, 59]. Preclinical studies and translational investigations in human patients have demonstrated that radiation may lead to increased antigen recognition and epitope spreading [59, 60]. Preclinical and human studies have also demonstrated that radiation may impact the tumor microenvironment, leading to increased infiltration of effector T-cells, and decreased levels of

inhibitory regulatory T-cells and myeloid derived suppressor cells [61]. There are now several case reports describing an "abscopal" response outside of the radiation field in patients treated with the combination of radiation therapy and checkpoint inhibition, including in some patients that appeared to be progressing on checkpoint therapy.[62-64]

In certain circumstances, these effects may lead to responses in patients who had previously progressed on checkpoint blockade, as has been demonstrated in case reports [60] and retrospective studies [65], including our experience that found improved systemic response following palliative radiotherapy in melanoma patients treated with ipilimumab [66]. Multiple radiation fractions / courses could potentially overcome resistance to PD-1 directed therapy and prime a systemic response in certain patients compared to a single fraction of radiation. Radiation therapy is commonly used to treat ACC patients, and our data also indicate that fractionated radiotherapy in ACC can result in increased numbers of CD8+ lymphocytes within the tumor microenvironment and increase in the ratio of CD8+ / FoxP3+ T-cells within the tumor microenvironment. Chemoradiation in ACC has been associated with an increase in CD8+ T-effector cells, decrease in T-regulatory cells, and promotion of a systemic humoral response. Indeed, traditional cytotoxic chemotherapy and radiation can also generate or potentiate immune responses under the proper circumstances, with some evidence for potential synergy with immune checkpoint blockade[64, 67].

Taken together, these data suggest a potential role for immune modulation with PD-1 blockade, with or without radiation, as a novel therapeutic strategy for patients with ACC. This study will examine the anti-tumor activity of PD-1 blockade in ACC and the role of targeted radiation to enhance the activity of PD-1 blockade. This study will explore potential mechanisms of response and resistance to PD-1 blockade in a tumor with predominant PD-L2 expression, and identify potential synergistic activity with radiation.

2.5 Correlative Studies Background

Correlatives studies in this trial are designed to explore mechanisms of sensitivity and resistance to PD-1 directed therapy and the subsequent effect of targeted radiation on this subset of tumors. Data suggests that radiation may increase PD-L1 expression and numbers of tumor infiltrating lymphocytes, and specifically may increase the ratio of CD8+ T-cells to FoxP3+ regulatory T-cells and decrease other inhibitory populations such as myeloid derived stem cells. The extent to which these changes in the tumor microenvironment are reflected in the peripheral circulation is currently unknown. Mutational burden has also been suggestive to correlate with response to checkpoint blockade and can be influenced by both high and low dose radiation. Finally, radiation may increase T-cell receptor diversity and lead to the development of dominant clones that may contribute to an effective anti-tumor immune response.

Tumor biopsies will be performed prior to the initiation of therapy. An optional biopsy will be performed during the study treatment between the 2nd and 3rd cycle of pembrolizumab if deemed safe and feasible. Another optional biopsy will be performed if deemed safe or feasible upon progression. Whole blood will also be collected prior to the initiation of therapy, at cycle 2 day 1, cycle 3 day 1, and then every 3 cycles while on treatment. We will correlate any signs of early tumor flare with immunologic markers and clinical response.

In addition to radiation dose, the location of the irradiated lesions may also impact the ability of targeted radiation to stimulate an abscopal response [68] due to the likelihood of T-cells trafficking from the irradiated lesions to distant sites of disease. The location may also impact the volume of tissue irradiated as well as safe administration of dosing, depending on surrounding organs of interest tolerance.

To evaluate PD-L1 expression, infiltrating lymphocyte subsets and other immunologic markers in the tumor microenvironment, tissue will be collected and fixed by 10% neutral buffered formalin overnight, dehydrated, and paraffin embedded. Four micrometer-thick sections will be cut. The paraffin blocks and unstained slides will be stored at room temperature. All immunohistochemical staining will be performed in the Center for Immuno-Oncology Pathology Core at Dana-Farber/Harvard Cancer Center (DF/HCC) Specialized Histopathology Core. Formalin fixed-paraffin embedded (FFPE) tumor slides will be prepared and H&E stained for assessment of TIL in pre- and post-treatment tumor samples. PD-L1 and PD-L2 expression will be assessed. To identify subsets of different immune populations (effector/memory CD8 cells, T-regulatory cells, etc.), IHC staining will be performed on FFPE tumor slices using antibodies including: PD-L1, CD8, PD-1, PD-L2, CD3, CD4, CD25, FoxP3. Investigators at our institution have also developed IHC staining protocols on FFPE tissue for additional surface markers such as PD-L2, TIM-3, and LAG-3 through our Center for Immuno-Oncology. Digital, quantitative scoring of stained tissue is performed using the Aperio slide scanning analysis platform. The scoring for markers (such as the PD-Ligands) that stain macrophages, dendritic cells, and other cells of heterogeneous morphology will be semi-quantitative and performed by a pathologist using a modified H-score to capture: 1) the percentage of neoplastic cells positive for biomarker expression, intensity of expression, and membrane or cytoplasmic expression, and 2) the percentage of non-neoplastic cells (macrophages, dendritic cells, endothelial cells) positive for biomarker expression, intensity of expression, and membrane or cytoplasmic expression. Scoring for PD-1 and other markers that stain lymphoid Tcells will primarily be performed by automated analysis using the Aperio system. Aperio scoring for PD-1+ (and other lymphoid markers) lymphocytes will be accomplished using a standard Aperio algorithm, developed for quantifying nuclear stains, but found to be applicable to quantifying membrane staining of cells with a very high N:C ratio, such as lymphocytes (Nuclear algorithm). For select patients, RNA profiling using nanostring platform will be performed to confirm immunohistochemical results.

Tumor sequencing will be performed in the Center for Advanced Molecular Diagnostics Laboratory of the Brigham and Women's Hospital using DNA isolated from FFPE slides containing at least 20% tumor nuclei using routine extraction methods and analysis by massively parallel sequencing using a solution-phase Agilent SureSelect hybrid capture kit and an Illumina HiSeq 2500 sequencer. Flow cytometry will be performed by the Dana-Farber Center for Immune Oncology to evaluate changes in circulating immune subsets over the course of therapy. Additionally, we will perform circulating tumor DNA studies as a surrogate marker for tumor burden and changes over the course of treatment. Whole blood samples will be drawn from patients included in this clinical trial for future evaluation of cell-free DNA and circulating tumor cell.

3. PARTICIPANT SELECTION

3.1 Eligibility Criteria

Participants must meet the following criteria on screening examination to be eligible to participate in the study:

1. Participants must have histologically confirmed adenoid cystic carcinoma with evidence of recurrent or metastatic disease not amenable to potentially curative surgery or radiotherapy.

2. Participants must have at least one RECIST v1.1 measurable non-CNS based lesions. Palliative radiation must be indicated for at least one measurable or non-measurable lesions (including bone lesion), and this lesion must be a candidate for radiation to a dose of 30 Gy of radiation over 5 fractions as deemed by a treating radiation oncologist. One measurable lesion must be in a location where it will not be incorporated into the radiation fields so systemic response can be assessed. However, inclusion of patients with more than 10 measurable lesions is strongly discouraged and all patients must have life expectancy > 6 months.

3. Participants must agree to undergo a research biopsy, if tumor is safely accessible, at baseline. Participants can be exempt if archival tumor tissue has been collected within 12 months of study enrollment that the Principal Investigator deems it appropriate/sufficient for analysis on this protocol. Biopsy of a lesion outside of the potential radiation treatment field is preferred to maintain consistency across cohorts.

4. Participants must have archival tissue from the primary tumor or metastases available for correlative studies. Either a paraffin block or twenty unstained slides are acceptable. If twenty slides are not available, a lesser amount may be acceptable after discussion with the Principal Investigator.

5. Prior systemic therapy: At least 2 weeks must have elapsed since the end of prior chemotherapy, biological agents (4 weeks for anti-cancer monoclonal antibody containing regimens) or any investigational drug product, with adequate recovery of treatment-related toxicity to NCI CTCAE Version 4.0 grade ≤1 (or tolerable grade 2) or back to baseline (except for alopecia or neuropathy). Any number of prior therapies for recurrent/metastatic ACC are allowed, with the exception of previous treatment with PD-1 pathway inhibitors.

6. Prior radiation therapy: At least 3 weeks must have elapsed from prior radiation therapy. The prior site of radiotherapy must be documented as reirradiation of the same site is not allowed in this protocol.

7. Be ≥ 18 years of age on day of signing informed consent.

8. Have a performance status of 0 or 1 on the ECOG Performance Scale (see Appendix A).

9. Participants must have documentation of a new or progressive lesion on a radiologic imaging study performed within 12 months prior to study enrollment (progression of disease over any interval is allowed) and/or new/worsening disease related symptoms within 12 months prior to study enrollment.

This assessment is performed by the treating investigator. Evidence of progression by RECIST criteria is not required.

10. Demonstrate adequate organ function as defined in **Table 1,** all screening labs should be performed within 14 days of treatment registration.

Table 1 Adequate Organ Function Laboratory Values

System	Laboratory Value
Hematological	
Absolute neutrophil count (ANC)	≥1,500 /mcL
Platelets	≥100,000 / mcL
Hemoglobin	≥9 g/dL or ≥5.6 mmol/L without transfusion or EPO dependency (within 7 days of assessment)
Renal	
Serum creatinine **OR** Measured or calculated[a] creatinine clearance (GFR can also be used in place of creatinine or CrCl)	≤1.5 X upper limit of normal (ULN) **OR** ≥60 mL/min for subject with creatinine levels > 1.5 X institutional ULN
Hepatic	
Serum total bilirubin	≤ 1.5 X ULN **OR**
	Direct bilirubin ≤ ULN for subjects with total bilirubin levels > 1.5 ULN
AST (SGOT) and ALT (SGPT)	≤ 2.5 X ULN **OR** ≤ 5 X ULN for subjects with liver metastases
Albumin	≥2.5 mg/dL
Coagulation	
International Normalized Ratio (INR) or Prothrombin Time (PT) Activated Partial Thromboplastin Time (aPTT)	≤1.5 X ULN unless subject is receiving anticoagulant therapy as long as PT or PTT is within therapeutic range of intended use of anticoagulants ≤1.5 X ULN unless subject is receiving anticoagulant therapy as long as PT or PTT is within therapeutic range of intended use of anticoagulants
[a]Creatinine clearance should be calculated per institutional standard.	

11. Baseline tumor measurements must be documented from tests within 28 days of study entry. Other non-laboratory tests must be performed within 28 days of study entry.

12. Female subjects of childbearing potential should have a negative urine or serum pregnancy test within 14 days of treatment registration. Female subjects of childbearing potential should have a negative urine or serum pregnancy test repeated within 72 hours prior to receiving the first dose of study medication. If the urine test is positive or cannot be confirmed as negative, a serum pregnancy test will be required..

13. Female and male subjects of childbearing potential must agree to use an adequate method of contraception, as outlined in section 5.6, prior to study entry, for the duration of study participation, and 4 months after completion of pembrolizumab administration. Contraception is required starting with the first dose of study medication through 120 days after the last dose of study medication.
    - Note: Abstinence is acceptable if this is the usual lifestyle and preferred contraception for the subject.

14. Be willing and able to provide written informed consent for the trial.

3.2 Exclusion Criteria

Participants who exhibit any of the following conditions at screening will not be eligible for admission into the study.

1. Metastatic disease impinging on the spinal cord or threatening spinal cord compression. Patients that have had previous treatment of disease with impinging on the cord with either surgery or radiotherapy with clinical or radiographic evidence of response or stability are eligible.

2. Surgical fixation of bone lesion to be irradiated is required and indicated to provide mechanical stability.

3. Participant has known active central nervous system (CNS) metastases and/or carcinomatous meningitis. Subjects with previously treated brain metastases may participate provided they are stable (without evidence of progression by imaging for at least four weeks prior to the first dose of trial treatment), have no evidence of new or enlarging brain metastases, and are not using steroids for the purposes of treating brain metastasis induced edema for at least 7 days prior to trial treatment. This exception does not include carcinomatous meningitis which is excluded regardless of clinical stability, because of their poor prognosis and because they often develop progressive neurologic dysfunction that would confound the evaluation of neurologic and other adverse events.

4. Prior treatment with PD-1 or PD-L1 inhibitor

5. Concurrent administration of other cancer specific therapy or investigational agents during the course of this study is not allowed.

6. Has a diagnosis of immunodeficiency or is receiving systemic steroid therapy or any other form of immunosuppressive therapy within 7 days prior to the first dose of trial treatment.

7. Has a known history of active TB (Bacillus Tuberculosis)

8. History of allergic reactions or hypersensitivity to pembrolizumab or any of its excipients.

9. Uncontrolled intercurrent illness including but not limited to ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements.

10. Has a known additional malignancy that is progressing or requires active treatment. Exceptions include basal cell carcinoma of the skin or squamous cell carcinoma of the skin that has undergone potentially curative therapy or in situ cervical cancer.

11. Has known history of, or any evidence of active, non-infectious pneumonitis.

12. Has an active infection requiring systemic therapy.

13. Has active autoimmune disease that has required systemic treatment in the past 2 years (i.e. with use of disease modifying agents, corticosteroids or immunosuppressive drugs). Replacement therapy (e.g., thyroxine, insulin, or physiologic corticosteroid replacement therapy for adrenal or pituitary insufficiency, etc.) is not considered a form of systemic treatment.

14. Has a history or current evidence of any condition, therapy, or laboratory abnormality that might confound the results of the trial, interfere with the subject's participation for the full duration of the trial, or is not in the best interest of the subject to participate, in the opinion of the treating investigator.

15. Subjects who are pregnant or breastfeeding, or expecting to conceive or father children within the projected duration of the trial, starting with the pre-screening or screening visit through 120 days after the last dose of trial treatment. Pregnant women are excluded from this study because immunotherapy has the potential for teratogenic or abortifacient effects. Because there is an unknown but potential risk of adverse events in nursing infants secondary to treatment of the mother with immunotherapy, breastfeeding should be discontinued if the mother is treated on this protocol. Women who could potentially become pregnant while undergoing treatment on this protocol must be willing to use 2 methods of contraception.

16. Has a known history of Human Immunodeficiency Virus (HIV) (HIV 1/2 antibodies).

17. Has known active Hepatitis B (e.g., HBsAg reactive) or Hepatitis C (e.g., HCV RNA [qualitative] is detected).

18. Has received a live vaccine within 30 days of planned start of study therapy.

3.3 Inclusion of Women, Minorities and Other Underrepresented Populations

Both men and women of all races and ethnic groups are eligible for this trial. Women, minorities and other underrepresented populations are all at risk to develop adenoid cystic carcinoma.

4. REGISTRATION PROCEDURES

4.1 General Guidelines for DF/HCC Institutions

ODQ will be responsible for randomization of participants to arms A and B. Institutions will register eligible participants in the Clinical Trials Management System (CTMS) OnCore. Registrations must

occur prior to the initiation of protocol therapy. Any participant not registered to the protocol before protocol therapy begins will be considered ineligible and registration will be denied.

An investigator will confirm eligibility criteria and a member of the study team will complete the protocol-specific eligibility checklist.

Following registration, participants may begin protocol therapy. Issues that would cause treatment delays should be discussed with the Overall Principal Investigator (PI). If a participant does not receive protocol therapy following registration, the participant's registration on the study must be canceled. Registration cancellations must be made in OnCore as soon as possible.

4.2 Registration Process for DF/HCC Institutions

DF/HCC Standard Operating Procedure for Human Subject Research Titled *Subject Protocol Registration* (SOP #: REGIST-101) must be followed.

4.3 General Guidelines for Other Investigative Sites

Not applicable

4.4 Registration Process for Other Investigative Sites

Not applicable

5. TREATMENT PLAN

5.1 Treatment regimen

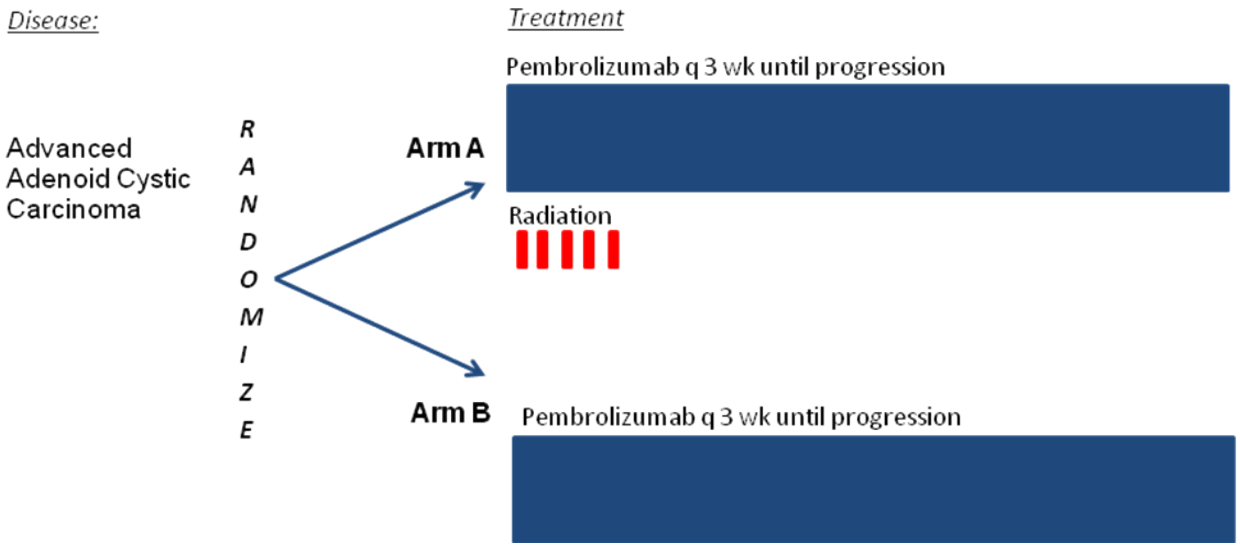
This is a randomized phase 2 study of pembrolizumab alone or with radiation therapy in subjects with recurrent or metastatic adenoid cystic carcinoma and at least one tumor or metastatic site that is amenable to palliative radiation. All subjects will have lesions amenable for palliative radiation therapy.

Subjects randomized to Arm A will have up to 5 metastatic lesions targeted with 30 Gy of radiation starting within 7 calendar days of the first dose of pembrolizumab and completing within 17 calendar days. The first dose of radiation can be given prior to the first infusion with the study drug. Pembrolizumab will continue on day 1 of each 21day cycle. Each cycle will have 21 days.

Subjects randomized to Arm B will receive pembrolizumab on day 1 of each 21day cycle.

Treatment will be administered on an outpatient basis. Reported adverse events and potential risks are described in section 7. Appropriate dose modifications are described in Section 6. No investigational or commercial agents or therapies other than those described below may be administered with the intent to treat the participant's malignancy during the course of treatment.

The treatment schema is shown below:



Pembrolizumab Regimen Description

Agent	Premedication ; Precautions	Dose	Route	Schedule	Cycle Length
Pembrolizumab	Not routinely necessary unless prior infusion reaction.	200 mg D1, *q3w, iv*	IV over approximately 30 minutes (range: 25-40 minutes). Please refer to Section 8 for compatible infusion set materials including in-line filter.	Day 1, q3w	21 days (3 week)

Arm A: Pembrolizumab and radiation

Radiation therapy to up to 5 metastatic lesions to a dose of 30 Gy of radiation will be administered within 7 calendar days of the first dose of pembrolizumab. Pembrolizumab will continue on day 1 of each 21day cycle. Each cycle will have 21 days. Radiation should in general be completed in the minimum amount of time possible, and shall in no instances extend over a period of more than 17 calendar days. Prescription details such as isodose prescription line, volume of treated tissue, and location of treated tissue will be collected. The first dose of pembrolizumab should be given within 2 days of the first day of radiation whenever possible, and the interval between the first day of radiation and first dose of pembrolizumab must not exceed 7 calendar days. Note: All sites do not need to be irradiated concurrently. Additionally, with the permission of the principal investigator, treatment to 30

Gy in 6 fractions (if more feasible than 5 fractions) or the additional radiation of 1-5 sites is allowed between future cycles of pembrolizumab in patients randomized to the radiation treatment arm, provided at least one measurable lesion remains unirradiated to monitor systemic response.

Pembrolizumab 200 mg will be administered intravenously on day 1 of a 21day cycle. Following this first cycle, pembrolizumab 200 mg will be continued intravenously every 3 weeks.

Arm B: Pembrolizumab alone

Pembrolizumab 200 mg will be administered intravenously on day 1 of a 21day cycle. Following this first cycle, pembrolizumab 200 mg will be continued intravenously every 3 weeks.

5.2 Pre-treatment Criteria

5.2.1 Screening for trial eligibility

Day -28 to Day 1: Screening Visit
Eligibility and exclusion criteria are provided in Section 3. These criteria will be assessed within 28 days of the first day of study treatment to establish eligibility and baseline values.

Informed consent will be obtained after the study has been fully explained to the subject and before the conduct of any screening procedures or assessments. If screening assessments occur within 3 days before start of study treatment, then they may serve as the baseline Cycle 1 Day 1 visit and cycle 1 day 1 labs do not need to be performed.

Demographic information and baseline characteristics will be collected at the Screening Visit. Standard demographic parameters include age, sex, and race/ethnicity (recorded in accordance with prevailing regulations). Baseline characteristics will include ECOG PS (Appendix A), disease status, medical histories, and prior and concomitant medications.

Additional testing required, as per Section 3, is: hematology panel (see Table 2), chemistry panel (see Table 2), coagulation panel, urine or serum HCG (in women of childbearing potential; see Section 3 for when serum HCG testing is required), TSH, and EKG.

Patients will also need to undergo a radiation planning appointment prior to the start of treatment.

Archival tumor sample should be collected (block or if not possible, 20 unstained slides). If a tumor outside the field of radiation is safely amenable to biopsy, a baseline tumor biopsy is also required and must be obtained within 28 days before starting protocol therapy. A second optional biopsy will be repeated (close to the end of cycle 2, within 14 days before the third cycle of pembrolizumab). A third optional biopsy will be performed if deemed safe or feasible at the time of confirmed disease progression.

Further details about collection and handling of tumor biopsy specimen can be found in Section 9 and appendices.

Table 2. Clinical Laboratory tests.

Category	Tests
Hematology panel	
	• Hematocrit, hemoglobin, platelet count, WBC with differential (bands, basophils, eosinophils, lymphocytes, monocytes, neutrophils), ANC
Chemistry Panel	
	• Chloride, potassium, sodium, BUN, serum creatinine, phosphorus, calcium, albumin, total protein, alkaline phosphatase, ALT, AST, total bilirubin (NOTE: the frequency of checking magnesium levels is at the discretion of the treating provider)

Abbreviations: ALT = alanine aminotransferase; ANC = absolute neutrophil count; AST = aspartate aminotransferase; β-hCG = beta-human chorionic gonadotropin; BUN = blood urea nitrogen; WBC = white blood cells, ANC=absolute neutrophil count

5.2.2 On treatment visits

Reasonable effort should be made to conduct study visits on the day scheduled (+/- 3 days).

Patients will also see the treating radiation oncologist at least once per week on the weeks they are receiving radiation as per standard clinical practice.

Any changes from screening clinical evaluation findings that meet the definition of an AE will be recorded on the AE page of the eCRF.

Cycle 1, Day 1
If screening assessments occur within 3 days before start of study treatment, then they may serve as the baseline Cycle 1 Day 1 visit and screening tests do not need to be repeated.
Draw blood/collect urine sample for:
- Hematology panel (Table 2)
- Chemistry panel (Table 2)
- Pregnancy test (for females of childbearing potential)

Record:
- ECOG PS
- Weight
- Vital signs
- Physical exam
- Concomitant medication use

Review all laboratory results before administering study treatment.

Criteria to treat at cycle 1 day 1:

- **Absolute neutrophil count ≥1000/** mcL
- **Platelets ≥75,000/** mcL
- **ALT and AST ≤3.0 x ULN in a patient with no documented liver metastases; ALT and AST ≤5.0 x ULN in a patient with documented liver metastases**
- **Total bilirubin ≤1.5 x ULN (2.0 x ULN in a patient with well documented Gilbert's syndrome)**

Every-3-week assessments, day 1 of every cycle:
The following assessments should be performed on the indicated weeks.

Draw blood/collect urine sample for:
- Hematology panel
- Chemistry panel
- Pregnancy test (for females of childbearing potential)
- TSH (every 2 cycles)

Record:
- ECOG PS
- Weight
- Vital signs
- Physical exam
- Concomitant medication use
- AEs or SAEs

Review all laboratory results before administering study treatment.

Criteria to treat at day 1:

- **Absolute neutrophil count ≥1000/** mcL
- **Platelets ≥75,000/** mcL
- **ALT and AST ≤3.0 x ULN in a patient with no documented liver metastases; ALT and AST ≤5.0 x ULN in a patient with documented liver metastases**
- **Total bilirubin ≤1.5 x ULN (2.0 x ULN in a patient with well documented Gilbert's syndrome)**

5.2.3 Additional On-Treatment Assessments

Tumor Assessments
Tumor assessments will be performed according to RECIST 1.1 and immune related response criteria (irRC) (see Section 11). Response evaluations will be performed every 9 weeks or as clinically indicated. In case of response, confirmatory scans will be performed 4 weeks after the scan that documented response. In the case of progressive disease, confirmatory scans are recommended between 4-8 weeks following the date of initial progression along with continued treatment, provided the patient is thought to be deriving clinical benefit and is counseled regarding the risks and benefits of continued treatment. If progression is confirmed, date of progression is dated at the time of the original scans. If progression is confirmed, continued treatment is only allowed if irRC demonstrates a response or stable disease, again provided the patient is thought to be clinically benefitting and is counseled regarding the risk and benefits of remaining on treatment.

Research Blood Sample Collection
Three research blood sample collections should be collected at the following time points:
1. At baseline, within 14 days prior to cycle 1 day 1 of protocol therapy (ideally as close to administration of cycle 1 day 1 therapy as possible)
At cycle 2 day 1, cycle 3 day 1 and then every 3 cycles
Specific instructions for research blood draw handling are described in Section 9 and appendices.

Tumor biopsy.
If a tumor is accessible, a tumor biopsy should be performed at the following time points:
1. At baseline, within 28 days prior to cycle 1 day 1 of protocol therapy (ideally as close to administration of cycle 1 day 1 therapy as possible)
2. After cycle 2, within 14 days prior to cycle 3 day 1 of protocol therapy

Further details about collection and handling of tumor biopsy specimen can be found in Section 9 and appendices.

5.2.4 End-of-Treatment Procedures

End-of-Treatment Visit
All subjects will be asked to return to the site for a final, End-of-Treatment visit, if possible. This visit must be performed within 30 days of final administration of study treatment. End-of-treatment assessments will not have to be repeated if the same assessments were performed within 7 days of this visit. The subject will be followed for 30 days after the last study intervention for adverse events.

Record:
- For patients who come off for reasons other than progressive disease: Tumor assessments to be performed every 6 weeks if patients come off within one year of therapy. If study treatment is discontinued due to PD, then tumor assessments are not required for that subject.
- ECOG PS
- Physical exam
- Vital signs and weight
- Concomitant medication use

- AEs or SAEs

Draw blood sample for:
- Hematology (Table 2)
- Chemistry panel (Table 2)

5.2.5 Follow-up Period Procedures

Subjects who do not have PD upon discontinuation of study treatment are required to have tumor assessments during the post-treatment follow-up period (using the same methodology and acquisition techniques as were used for previous assessments). Response evaluations will be performed every 9 weeks. Further details about collection and handling specimens can be found in Section 9 and appendices.

5.3 Agent Administration

5.3.1 Pembrolizumab

Pembrolizumab administration
Pembrolizumab will be administered by trained medical personnel at the investigational site. Treatment compliance will be monitored through documentation of study treatment administration in the subject's medical record.

Pembrolizumab will be administered in clinic on day 1 (+/- 3 days) of each cycle. It will be administered as a 30 minute IV infusion. Given the variability of infusion pumps from site to site, a window of -5 minutes and +10 minutes is permitted (i.e., infusion time is 30 minutes: -5 min/+10 min).

The Pharmacy Manual contains specific instructions for the preparation of the pembrolizumab infusion fluid and administration of infusion solution.

5.3.2 Radiation treatment

5.3.2.1 Prioritizing lesions for irradiation

In addition to providing palliative benefit, this study attempts to use radiation to engender a systemic immune response in the context of PD-1 inhibition. Therefore, the choice of site or sites irradiated will be based on a combination of safety, palliative benefit and, when feasible, correlative studies will evaluate whether there is an association between abscopal responses and site irradiated. To maintain homogeneity, lung lesions will be prioritized for radiation when this can be of palliative benefit.

5.3.2.2 Treatment simulation:

The treatment planning process will include CT based simulation with 1.25- 2.5-mm cuts to cover the area(s) of interest.

5.3.2.3 Contours: Several tumor contours will be defined by the physician:

CTV1-4 (CTV): Tumor volumes to be treated. CTV1-5 will be treated to 30 Gy in 5 fractions (6 fraction treatment is allowed as per 5.1 if deemed to be more safe and/or feasible). These volumes include gross disease; elective irradiation to other areas is not permitted. An optional 0-1 cm margin on gross disease (GTV) is allowed when determining the CTVs at the treating radiation oncologist's discretion to account for any uncertainty. The exact margins are patient- specific and larger margins can be considered with permission of the study PI, for example, in the case of bone lesions. A 2mm – 1cm planning tumor volume (PTV) margin added to the CTV will also be included to account for set up variation, as appropriate to each individual lesion. In the case of IMRT or SBRT planning, a smaller PTV margin of 2-5mm is strongly recommended.

5.3.2.4 Several contours of the organs at risk will be defined by the physician and/or treatment planner if visible within the axial slices covered by CTV1.

- Spinal cord
- Brainstem
- Brachial plexus
- Cauda equina
- Cochlea
- Esophagus
- Heart/pericardium
- Trachea and ipsilateral bronchus
- Skin
- Stomach
- Duodenum
- Jejunum/ilieum
- Liver
- Kidneys
- Lung
- Optic structures (Globes, right and left optic nerves and optic chiasm)
- Lacrimal gland
- Parotid gland

5.3.2.5 Prescriptions:

Target1 (CTV1+ individualized PTV margin) will be treated to a dose of 30 Gy.

Target2-5 (CTV2-5 + individualized PTV margin) will be treated to a dose of 30 Gy.

Photons or electron treatments are allowed, but graphic plans are mandated. Planning may be electron based, 3D conformal, IMRT or SBRT, as appropriate to each individual case / lesion to achieve coverage of the PTV target and maintain normal tissue tolerance. The percentage PTV covered to 100% of the prescription dose will be reported; reasonable effort should be made to keep this greater than 95% while maintaining normal tissue dose constraints as indicated below. Bolus may be used as appropriate. Maximum dose should not exceed 120% of prescription dose in any case, and must be documented.

5.3.2.6 Timing of treatment

Arm A: Radiation treatments will be delivered over a period that does not extend over more than 17 days. The first radiation fraction will start within 7 days following the first dose of pembrolizumab (ideally within 2 days of the first dose of pembrolizumab). Of note, radiation to all sites does not need to be administered concurrently.

5.3.2.7 Organs at risk (OAR) – Treating radiation oncologists will abide by accepted radiation dose constraints. Guidance for dose constraints are provided in the table below which are cumulative for all lesions treated.

Structure	**Volume (mL)**	**Volume Max (Gy)**	**Max Point Dose (Gy)**
Spinal cord	<0.25	22.5	D0.035<30
Brainstem	<0.5	23	30
Brachial plexus	<3	27.5	D0.035<35
Cauda equina	<5	30	35
Cochlea			25
Esophagus	<5	20	35
Heart/pericardium	<15	32	38
Trachea	<4	18	35
Skin	<10	30	D0.035<32
Stomach	<10	18	32
Duodenum	<5	18	32
Jejunum/ilieum	<5	20	32
Liver	700mL spared from volume max	21	
Kidneys	200mL spared from volume max	17.5	
Lung	1500mL spared from volume max	13	
Optic structures	<0.2	22	25

PTV coverage may be compromised to ensure an acceptable plan.

5.4 General Concomitant Medication and Supportive Care Guidelines

5.4.1 Concomitant Medication Guidelines

Medications or vaccinations specifically prohibited in the exclusion criteria are not allowed during the ongoing trial. If there is a clinical indication for one of these or other medications or vaccinations specifically prohibited during the trial, discontinuation from trial therapy or vaccination may be required. The investigator should discuss any questions regarding this with the Merck Clinical team. The final decision on any supportive therapy or vaccination rests with the investigator and/or the subject's primary physician.

Acceptable Concomitant Medications
All treatments that the investigator considers necessary for a subject's welfare may be administered at the discretion of the investigator in keeping with the community standards of medical care. Palliative radiation therapy can be prescribed in subsequent cycles at the discretion of the Overall PI if clinically indicated. All concomitant medication will be recorded on the case report form (CRF) including all prescription, over-the-counter (OTC), herbal supplements, and IV medications and fluids. If changes occur during the trial period, documentation of drug dosage, frequency, route, and date may also be included on the CRF.

All concomitant medications received within 28 days before the first dose of trial treatment and 30 days after the last dose of trial treatment should be recorded. Concomitant medications administered after 28 days after the last dose of trial treatment should be recorded for SAEs as specified in Section 7.2.

Prohibited Concomitant Medications
Subjects are prohibited from receiving the following therapies during the Screening and Treatment Phase of this trial:

- Antineoplastic systemic chemotherapy or biological therapy
- Immunotherapy not specified in this protocol
- Investigational agents other than pembrolizumab
- Any systemically active oral, injected, or implanted hormonal method of contraception except for progesterone coated intrauterine devices (IUDs) that had been previously implanted.
- Live vaccines within 30 days prior to the first dose of trial treatment and while participating in the trial. Examples of live vaccines include, but are not limited to, the following: measles, mumps, rubella, varicella/zoster, yellow fever, rabies, BCG, and typhoid vaccine.
- Systemic glucocorticoids for any purpose other than to modulate symptoms from radiation or an event of clinical interest of suspected immunologic etiology. The use of physiologic doses of corticosteroids may be approved after consultation with the Principal Investigator.

Subjects who, in the assessment by the investigator, require the use of any of the aforementioned treatments for clinical management should be removed from the trial. Subjects may receive other medications that the investigator deems to be medically necessary.

There are no prohibited therapies during the Post-Treatment Follow-up Phase.

5.4.2 Supportive Care Guidelines – general medications

The following treatments are permitted throughout the duration of the study treatment phase and during follow-up:

- Standard therapies for pre-existing medical conditions unless listed as prohibited therapy below. Any medication intended solely for supportive care (e.g., analgesics, anti-diarrheal, anti-depressants) may be used at the investigator's discretion. Antiemetics and anti-diarrheal medications should not be administered prophylactically before initial treatment with study drugs. At the discretion of the investigator, prophylactic antiemetic and anti-diarrheal medication(s) may be used as per standard clinical practice before subsequent doses of study drugs or before, during or after radiation treatment.
- Anticoagulants - Anticoagulation with heparin, heparin derivatives, and/or warfarin may be given at the discretion of the treating physician. Coagulation parameters should be checked at least once monthly, or more frequently at discretion of treating physician.
- Pain medications administered per standard clinical practice are acceptable while the patient is enrolled in the study.

Patients who experience toxicities should be treated symptomatically as clinically indicated. Medications that are considered necessary for the subject's welfare and that are not expected to interfere with the evaluation of study treatment or be restricted may be given at the discretion of the investigator. Ancillary treatments will be given as medically indicated.

5.5 Contraception, Pregnancy and Nursing considerations

5.5.1 Contraception

Pembrolizumab may have adverse effects on a fetus in utero. Furthermore, it is not known if pembrolizumab has transient adverse effects on the composition of sperm.
For this trial, male subjects will be considered to be of non-reproductive potential if they have azoospermia (whether due to having had a vasectomy or due to an underlying medical condition).
Female subjects will be considered of non-reproductive potential if they are either:
(1) postmenopausal (defined as at least 12 months with no menses without an alternative medical cause; in women < 45 years of age a high follicle stimulating hormone (FSH) level in the postmenopausal range may be used to confirm a post-menopausal state in women not using hormonal contraception or hormonal replacement therapy. In the absence of 12 months of amenorrhea, a single FSH measurement is insufficient.);
OR
(2) have had a hysterectomy and/or bilateral oophorectomy, bilateral salpingectomy or bilateral tubal ligation/occlusion, at least 6 weeks prior to screening;
OR
(3) has a congenital or acquired condition that prevents childbearing.
Female and male subjects of reproductive potential must agree to avoid becoming pregnant or impregnating a partner, respectively, while receiving study drug and for 120 days after the last dose of study drug by complying with one of the following:

(1) practice abstinence† from heterosexual activity;
OR
(2) use (or have their partner use) acceptable contraception during heterosexual activity.

Acceptable methods of contraception are‡:

Single method (one of the following is acceptable):
- intrauterine device (IUD)
- vasectomy of a female subject's male partner
- contraceptive rod implanted into the skin

Combination method (requires use of two of the following):
- diaphragm with spermicide (cannot be used in conjunction with cervical cap/spermicide)
- cervical cap with spermicide (nulliparous women only)
- contraceptive sponge (nulliparous women only)
- male condom or female condom (cannot be used together)
- hormonal contraceptive: oral contraceptive pill (estrogen/progestin pill or progestin-only pill), contraceptive skin patch, vaginal contraceptive ring, or subcutaneous contraceptive injection

> †Abstinence (relative to heterosexual activity) can be used as the sole method of contraception if it is consistently employed as the subject's preferred and usual lifestyle and if considered acceptable by local regulatory agencies and IRB. Periodic abstinence (e.g., calendar, ovulation, sympto-thermal, post-ovulation methods, etc.) and withdrawal are not acceptable methods of contraception.
>
> ‡If a contraceptive method listed above is restricted by local regulations/guidelines, then it does not qualify as an acceptable method of contraception for subjects participating at sites in this country/region.

Subjects should be informed that taking the study medication may involve unknown risks to the fetus (unborn baby) if pregnancy were to occur during the study. In order to participate in the study subjects of childbearing potential must adhere to the contraception requirement (described above) from the day of study medication initiation (or 14 days prior to the initiation of study medication for oral contraception) throughout the study period up to 120 days after the last dose of trial therapy. If there is any question that a subject of childbearing potential will not reliably comply with the requirements for contraception, that subject should not be entered into the study.

5.5.2 Use in Pregnancy

If a subject inadvertently becomes pregnant while on treatment with pembrolizumab, the subject will immediately be removed from the study. The site will contact the subject at least monthly and document the subject's status until the pregnancy has been completed or terminated. The outcome of the pregnancy will be reported to the Sponsor and to Merck without delay and within 24 hours to the Sponsor and within 2 working days to Merck if the outcome is a serious adverse experience (e.g., death, abortion, congenital anomaly, or other disabling or life-threatening complication to the mother or newborn).

The study investigator will make every effort to obtain permission to follow the outcome of the pregnancy and report the condition of the fetus or newborn to Merck. If a male subject impregnates his female partner the study personnel at the site must be informed immediately and the pregnancy reported to Merck and followed as described above.

5.5.3 Use in Nursing Women

It is unknown whether pembrolizumab is excreted in human milk. Since many drugs are excreted in human milk, and because of the potential for serious adverse reactions in the nursing infant, subjects who are breast-feeding are not eligible for enrollment.

5.6 Criteria for Taking a Participant Off Protocol Therapy

Duration of therapy will depend on individual response, evidence of disease progression and tolerance. In the absence of treatment delays due to adverse event(s), treatment may continue until one of the following criteria applies:

- Disease progression
- Intercurrent illness that prevents further administration of treatment
- Unacceptable adverse event(s)
- Participant demonstrates an inability or unwillingness to comply with the IV or oral medication regimen and/or documentation requirements
- Participant decides to withdraw from the protocol therapy
- General or specific changes in the participant's condition render the participant unacceptable for further treatment in the judgment of the treating investigator

Participants will be removed from the protocol therapy when any of these criteria apply. The reason for removal from protocol therapy, and the date the participant was removed, must be documented in the case report form (CRF). Alternative care options will be discussed with the participant.

A Treatment Ended/Off Study Form will be filled out in OnCore when a participant is removed from protocol therapy.
In the event of unusual or life-threatening complications, treating investigators must immediately notify the Overall PI, Jonathan Schoenfeld at 617-632-5734 or paged at 58874.

5.7 Duration of Follow Up

Participants removed from protocol therapy will be followed for overall survival, every 6 months, until death (by phone).

Participants removed from protocol therapy for unacceptable adverse event(s) will be followed until resolution or stabilization of the adverse event. For these patients scans should be performed every 9 weeks for 1 year.

5.8 Criteria for Removal from Study

Participants will be removed from study when any of the criteria listed in Section 5.4 applies. The reason for study removal and the date the participant was removed must be documented in the study-specific case report form (CRF). Alternative care options will be discussed with the participant.

Participants will also be removed from study when any of the following criteria apply:

- Clear evidence of disease progression or lack of clinical benefit with investigational treatment
- Lost to follow-up
- Withdrawal of consent for data submission
- Death

The reason for taking a participant off study, and the date the participant was removed, must be documented in the case report form (CRF).

For Centralized Subject Registrations, the research team submits a completed Off Treatment/Off Study form to ODQ when a participant comes off study. This form can be found on the ODQ website or obtained from the ODQ registration staff.

For Decentralized Subject Registrations, the research team updates the relevant Off Treatment/Off Study information in OnCore.

In the event of unusual or life-threatening complications, participating investigators must immediately notify the Principal Investigator, Jonathan Schoenfeld, MD, at Partners pager 58874.

6. DOSING DELAYS/DOSE MODIFICATIONS

Dose delays and modifications will be made as indicated in the following table(s). The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for dose delays and dose modifications. A copy of the CTCAE version 4.0 can be downloaded from the CTEP website

http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm.

If possible, symptoms should be managed symptomatically. In the case of toxicity, appropriate medical treatment should be used (including anti-emetics, anti-diarrheals, etc.).

All adverse events experienced by participants will be collected from the time of the first dose of study treatment, through the study and within 30 days of the last study intervention. Participants continuing to experience toxicity at the last scheduled study visit may be kept on the study until the toxicity has resolved or is deemed irreversible.

6.1 Anticipated Toxicities

Dosing interruptions are permitted in the case of medical/surgical events or logistical reasons not related to study therapy (e.g., elective surgery, unrelated medical events, patient vacation, and/or holidays). Subjects should be placed back on study therapy within 3 weeks of the scheduled interruption, unless otherwise discussed with the Principal Investigator. The reason for interruption should be documented in the patient's study record.

No dose reductions are allowed for pembrolizumab. For toxicities that are attributable to pembrolizumab, this drug should be held as directed.

6.2 Management of toxicities attributable to pembrolizumab

Adverse events (both non-serious and serious) associated with pembrolizumab exposure may represent an immunologic etiology. These adverse events may occur shortly after the first dose or several months after the last dose of treatment. Pembrolizumab must be withheld for drug-related toxicities and severe or life-threatening AEs as per Table 4 below.

Table 4: Dose Modification and Toxicity Management Guidelines for Immune-related AEs Associated with Pembrolizumab

General instructions:

1. **Corticosteroid taper should be initiated upon AE improving to Grade 1 or less and continue to taper over at least 4 weeks.**
2. **For situations where pembrolizumab has been withheld, pembrolizumab can be resumed after AE has been reduced to Grade 1 or 0 and corticosteroid has been tapered. Pembrolizumab should be permanently discontinued if AE does not resolve within 12 weeks of last dose or corticosteroids cannot be reduced to ≤10 mg prednisone or equivalent per day within 12 weeks.**
3. **For severe and life-threatening irAEs, IV corticosteroid should be initiated first followed by oral steroid. Other immunosuppressive treatment should be initiated if irAEs cannot be controlled by corticosteroids.**

Immune-related AEs	Toxicity grade or conditions (CTCAEv4.0)	Action taken to pembrolizumab	irAE management with corticosteroid and/or other therapies	Monitor and follow-up
Pneumonitis	Grade 2	Withhold	• Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper	• Monitor participants for signs and symptoms of pneumonitis • Evaluate participants with suspected pneumonitis with radiographic imaging and initiate corticosteroid treatment • Add prophylactic antibiotics for opportunistic infections
	Grade 3 or 4, or recurrent Grade 2	Permanently discontinue		

Diarrhea / Colitis	Grade 2 or 3	Withhold	• Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper	• Monitor participants for signs and symptoms of enterocolitis (ie, diarrhea, abdominal pain, blood or mucus in stool with or without fever) and of bowel perforation (ie, peritoneal signs and ileus). • Participants with ≥ Grade 2 diarrhea suspecting colitis should consider GI consultation and performing endoscopy to rule out colitis. • Participants with diarrhea/colitis should be advised to drink liberal quantities of clear fluids. If sufficient oral fluid intake is not feasible, fluid and electrolytes should be substituted via IV infusion.
	Grade 4	Permanently discontinue		
AST / ALT elevation or Increased bilirubin	Grade 2	Withhold	• Administer corticosteroids (initial dose of 0.5- 1 mg/kg prednisone or equivalent) followed by taper	• Monitor with liver function tests (consider weekly or more frequently until liver enzyme value returned to baseline or is stable
	Grade 3 or 4	Permanently discontinue	• Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper	
Type 1 diabetes mellitus (T1DM) or Hyperglycemia	Newly onset T1DM or Grade 3 or 4 hyperglycemia associated with evidence of β-cell failure	Withhold	• Initiate insulin replacement therapy for participants with T1DM • Administer anti-hyperglycemic in participants with hyperglycemia	• Monitor participants for hyperglycemia or other signs and symptoms of diabetes.
Hypophysitis	Grade 2	Withhold	• Administer corticosteroids and initiate hormonal replacements as clinically indicated.	• Monitor for signs and symptoms of hypophysitis (including hypopituitarism and adrenal insufficiency)
	Grade 3 or 4	Withhold or permanently discontinue[1]		
Hyperthyroidism	Grade 2	Continue	• Treat with non-selective beta-blockers (eg, propranolol) or thionamides as appropriate	• Monitor for signs and symptoms of thyroid disorders.
	Grade 3 or 4	Withhold or permanently discontinue[1]		
Hypothyroidism	Grade 2-4	Continue	• Initiate thyroid replacement hormones (eg, levothyroxine or liothyroinine) per standard of care	• Monitor for signs and symptoms of thyroid disorders.

Nephritis and Renal dysfunction	Grade 2	Withhold	• Administer corticosteroids (prednisone 1-2 mg/kg or equivalent) followed by taper.	• Monitor changes of renal function
	Grade 3 or 4	Permanently discontinue		
Myocarditis	Grade 1 or 2	Withhold	• Based on severity of AE administer corticosteroids	• Ensure adequate evaluation to confirm etiology and/or exclude other causes
	Grade 3 or 4	Permanently discontinue		
All other immune-related AEs	Intolerable/ persistent Grade 2	Withhold	• Based on type and severity of AE administer corticosteroids	• Ensure adequate evaluation to confirm etiology and/or exclude other causes
	Grade 3	Withhold or discontinue based on the type of event. Events that require discontinuation include and not limited to: Gullain-Barre Syndrome, encephalitis		
	Grade 4 or recurrent Grade 3	Permanently discontinue		
1. Withhold or permanently discontinue pembrolizumab is at the discretion of the investigator or treating physician. **NOTE:** For participants with Grade 3 or 4 immune-related endocrinopathy where withhold of pembrolizumab is required, pembrolizumab may be resumed when AE resolves to ≤ Grade 2 and is controlled with hormonal replacement therapy or achieved metabolic control (in case of T1DM).				

Supportive care for pembrolizumab toxicity, particularly suspected immune-mediated toxicity

Subjects should receive appropriate supportive care measures as deemed necessary by the treating investigator. Suggested supportive care measures for the management of adverse events with potential immunologic etiology are outlined in Section 6 and in greater detail in the ECI guidance document, which is available from the study PI upon request. Where appropriate, these guidelines include the use of oral or intravenous treatment with corticosteroids as well as additional anti-inflammatory agents if symptoms do not improve with administration of corticosteroids.

Note that several courses of steroid tapering may be necessary as symptoms may worsen when the steroid dose is decreased. For each disorder, attempts should be made to rule out other causes such as metastatic disease or bacterial or viral infection, which might require additional supportive care. The treatment guidelines are intended to be applied when the investigator determines the events to be related to pembrolizumab.

Note: if after the evaluation the event is determined not to be related, the investigator is instructed to follow the ECI reporting guidance (see Section 7) but does not need to follow the treatment guidance (as outlined in the ECI guidance document).

It may be necessary to perform conditional procedures such as bronchoscopy, endoscopy, or skin photography as part of evaluation of the event. Suggested conditional procedures, as appropriate, can be found in the ECI guidance document.

- **Pneumonitis**:

o For **Grade 2 events**, treat with systemic corticosteroids. When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks.
o For **Grade 3-4 events**, immediately treat with intravenous steroids. Administer additional anti-inflammatory measures, as needed.
o Add prophylactic antibiotics for opportunistic infections in the case of prolonged steroid administration.

- **Diarrhea/Colitis**:

Subjects should be carefully monitored for signs and symptoms of enterocolitis (such as diarrhea, abdominal pain, blood or mucus in stool, with or without fever) and of bowel perforation (such as peritoneal signs and ileus).

o All subjects who experience diarrhea/colitis should be advised to drink liberal quantities of clear fluids. If sufficient oral fluid intake is not feasible, fluid and electrolytes should be substituted via IV infusion. For Grade 2 or higher diarrhea, consider GI consultation and endoscopy to confirm or rule out colitis.
o For **Grade 2 diarrhea/colitis** that persists greater than 3 days, administer oral corticosteroids.
o For **Grade 3 or 4 diarrhea/colitis** that persists > 1 week, treat with intravenous steroids followed by high dose oral steroids.
o When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks.

- **Type 1 diabetes mellitus (if new onset, including diabetic ketoacidosis [DKA]) or ≥ Grade 3 Hyperglycemia, if associated with ketosis (ketonuria) or metabolic acidosis (DKA)**

- **Hypophysitis**:

o For **Grade 2** events, treat with corticosteroids. When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks. Replacement of appropriate hormones may be required as the steroid dose is tapered.
o For **Grade 3-4** events, treat with an initial dose of IV corticosteroids followed by oral corticosteroids. When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks. Replacement of appropriate hormones may be required as the steroid dose is tapered.

- **Hyperthyroidism or Hypothyroidism**:

Thyroid disorders can occur at any time during treatment. Monitor patients for changes in thyroid function (at the start of treatment, periodically during treatment, and as indicated based on clinical evaluation) and for clinical signs and symptoms of thyroid disorders.
o **Grade 2** hyperthyroidism events (and **Grade 2-4** hypothyroidism):
▪ In hyperthyroidism, non-selective beta-blockers (e.g. propranolol) are suggested as initial therapy.
▪ In hypothyroidism, thyroid hormone replacement therapy, with levothyroxine or liothyroinine, is indicated per standard of care.

o **Grade 3-4** hyperthyroidism
▪ Treat with an initial dose of IV corticosteroid followed by oral corticosteroids. When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks. Replacement of appropriate hormones may be required as the steroid dose is tapered.

- **Hepatic**:

o For **Grade 2** events, monitor liver function tests more frequently until returned to baseline values (consider weekly).
▪ Treat with IV or oral corticosteroids
o For **Grade 3-4** events, treat with intravenous corticosteroids for 24 to 48 hours.
o When symptoms improve to Grade 1 or less, a steroid taper should be started and continued over no less than 4 weeks.

- **Renal Failure or Nephritis**:

o For **Grade 2** events, treat with corticosteroids.
o For **Grade 3-4** events, treat with systemic corticosteroids.
o When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks.

- **Management of Infusion Reactions**: Signs and symptoms usually develop during or shortly after drug infusion and generally resolve completely within 24 hours of completion of infusion. Table 3 below shows treatment guidelines for subjects who experience an infusion reaction associated with administration of pembrolizumab.

Table 5: Infusion Reaction Treatment Guidelines for pembrolizumab

NCI CTCAE Grade	Treatment	Premedication at subsequent dosing
Grade 1 Mild reaction; infusion interruption not indicated; intervention not indicated	Increase monitoring of vital signs as medically indicated until the subject is deemed medically stable in the opinion of the investigator.	None
Grade 2 Requires infusion interruption but responds promptly to symptomatic treatment (e.g., antihistamines, NSAIDS, narcotics, IV fluids); prophylactic medications indicated for < =24 hrs	**Stop Infusion and monitor symptoms.** Additional appropriate medical therapy may include but is not limited to: IV fluids Antihistamines NSAIDS Acetaminophen Narcotics Increase monitoring of vital signs as medically indicated until the subject is deemed medically stable in the opinion of the investigator. If symptoms resolve within one hour of stopping drug infusion, the infusion may be restarted at 50% of the original infusion rate (e.g., from 100 mL/hr to 50 mL/hr). Otherwise dosing will be held until symptoms resolve and the subject should be premedicated for the next scheduled dose. **Subjects who develop Grade 2 toxicity despite adequate premedication should be permanently discontinued from further trial treatment administration.**	Subject may be premedicated 1.5h (± 30 minutes) prior to infusion of pembrolizumab with: Diphenhydramine 50 mg po or IV (or equivalent dose of antihistamine). Acetaminophen 500-1000 mg po or IV (or equivalent dose of antipyretic).
Grades 3 or 4 Grade 3: Prolonged (i.e., not rapidly responsive to symptomatic medication and/or	**Stop Infusion.** Additional appropriate medical therapy may include but is not limited to: IV fluids	No subsequent dosing

NCI CTCAE Grade	Treatment	Premedication at subsequent dosing
brief interruption of infusion); recurrence of symptoms following initial improvement; hospitalization indicated for other clinical sequelae (e.g., renal impairment, pulmonary infiltrates) Grade 4: Life-threatening; pressor or ventilatory support indicated	Antihistamines NSAIDS Acetaminophen Narcotics Oxygen Pressors Corticosteroids Epinephrine Increase monitoring of vital signs as medically indicated until the subject is deemed medically stable in the opinion of the investigator. Hospitalization may be indicated. **Subject is permanently discontinued from further trial treatment administration.**	
Appropriate resuscitation equipment should be available in the room and a physician readily available during the period of drug administration.		

7. ADVERSE EVENTS: LIST AND REPORTING REQUIREMENTS

Adverse event (AE) monitoring and reporting is a routine part of every clinical trial. The following list of reported and/or potential AEs (Section 7.1) and the characteristics of an observed AE (Section 7.2) will determine whether the event requires expedited reporting **in addition** to routine reporting.

7.1 Adverse Events Lists

7.1.1 Adverse Event List(s) for pembrolizumab

Refer to the current version of Investigator's Brochure and/or FDA approved package insert (Appendix J) for detailed pembrolizumab safety/toxicity information.

7.2 Adverse Event Characteristics

- **CTCAE term (AE description) and grade:** The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 4.0. A copy of the CTCAE version 4.0 can be downloaded from the CTEP web site http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm.

- **Attribution** of the AE:
  - Definite – The AE *is clearly related* to the study treatment.
  - Probable – The AE *is likely related* to the study treatment.
  - Possible – The AE *may be related* to the study treatment.
  - Unlikely – The AE *is doubtfully related* to the study treatment.
  - Unrelated – The AE *is clearly NOT related* to the study treatment

7.3 Expedited Adverse Event Reporting

Investigators **must** report to the Overall PI any serious adverse event (SAE) that occurs after the initial dose of study treatment, during treatment, or within 30 days of the last dose of treatment on the local institutional SAE form. Investigative sites within DF/HCC will report AEs directly to the DFCI Office for Human Research Studies (OHRS) per the DFCI IRB reporting policy described below.

7.4 Submission to DFCI IRB

The DFCI IRB requires the following Adverse Events (AE) be reported for all subjects enrolled and actively participating in the trial or when the AE occurs within 30 days of the last study intervention (e.g. drug administration):

- **Grade 2 (moderate) and Grade 3 (severe) Events** – Only events that are Unexpected and Possibly, Probably or Definitely Related / Associated with the Intervention.

- **ALL Grade 4 (life threatening or disabling) Events** – Unless expected AND specifically listed in protocol as not requiring reporting.

- **ALL Grade 5 (fatal) Events**

Notes:

• **ALL unexpected Grade 5 events** must be communicated via email to the DFCI OHRS Event Reporting email box at the time of initial notification of the event. A full written report must then be submitted to OHRS as required below.
• If subject is in Long Term Follow Up, death is reported at continuing review.
• **Grade 2 and Grade 3 laboratory abnormalities** that are considered by the investigator to be clinically insignificant and do not require therapy, or adjustment in prior therapy, do not need to be reported to the DFCI IRB.

7.5 Expedited Reporting to the Food and Drug Administration (FDA)

The Overall PI, as study sponsor, will be responsible for all communications with the FDA. The Overall PI will report to the FDA, regardless of the site of occurrence, any serious adverse event that meets the FDA's criteria for expedited reporting following the reporting requirements and timelines set by the FDA.

7.6 Expedited Reporting to Hospital Risk Management

Participating investigators will report to their local Risk Management office any participant safety reports or sentinel events that require reporting according to institutional policy.

7.7 Routine Adverse Event Reporting

All Adverse Events **must** be reported in routine study data submissions to the Overall PI on the toxicity IRBcase report forms. **AEs reported through expedited processes (e.g., reported to the IRB, FDA, etc.) must <u>also</u> be reported in routine study data submissions.**

7.8 Immediate Reporting of Adverse Events and Events of Clinical Interest to Merck

7.8.1 Serious Adverse Events

A serious adverse event is any adverse event occurring at any dose or during any use of Merck's product that:

- Results in death;
- Is life threatening;
- Results in persistent or significant disability/incapacity;
- Results in or prolongs an existing inpatient hospitalization;
- Is a congenital anomaly/birth defect;
- Is a new cancer (that is not a condition of the study);
- Is associated with an overdose;
- Is another important medical event

Any serious adverse event, or follow up to a serious adverse event, including death due to any cause other than progression of the cancer under study that occurs to any subject from the time the consent is signed through 30 days following cessation of treatment, or the initiation of new anti-cancer therapy, whichever is earlier, whether or not related to Merck product, must be reported within 24 hours to the Principal Investigator and within 2 working days to Merck Global Safety.

Non-serious Events of Clinical Interest will be forwarded to Merck Global Safety and will be handled in the same manner as SAEs.

Additionally, any serious adverse event, considered by an investigator who is a qualified physician to be related to Merck product that is brought to the attention of the investigator at any time outside of the time period specified in the previous paragraph also must be reported immediately to Merck.

SAE reports and any other relevant safety information are to be forwarded to the Merck Global Safety facsimile number: +1-215-993-1220

A copy of all 15 Day Reports and Annual Progress Reports is submitted as required by FDA, European Union (EU), Pharmaceutical and Medical Devices agency (PMDA) or other local regulators. Investigators will cross reference this submission according to local regulations to the Merck Investigational Compound Number (IND, CSA, etc.) at the time of submission. Additionally investigators will submit a copy of these reports to Merck & Co., Inc. (Attn: Worldwide Product Safety; FAX 215 993-1220) at the time of submission to FDA.

All subjects with serious adverse events must be followed up for outcome.

7.8.2 Events of Clinical Interest (ECIs)

Selected non-serious and serious adverse events are also known as Events of Clinical Interest (ECI) and must be recorded as such on the Adverse Event case report forms/worksheets and reported within 24

hours to the Principal Investigator and within 2 working days to Merck Global Safety. (Attn: Worldwide Product Safety; FAX 215 993-1220). Events of clinical interest for this trial include:

1. An overdose of Merck product, as defined in Section 7.7.3 - Definition of an Overdose for This Protocol and Reporting of Overdose to Merck, that is not associated with clinical symptoms or abnormal laboratory results.

2. an elevated AST or ALT lab value that is greater than or equal to 3X the upper limit of normal and an elevated total bilirubin lab value that is greater than or equal to 2X the upper limit of normal and, at the same time, an alkaline phosphatase lab value that is less than 2X the upper limit of normal, as determined by way of protocol-specified laboratory testing or unscheduled laboratory testing.*

*Note: These criteria are based upon available regulatory guidance documents. The purpose of the criteria is to specify a threshold of abnormal hepatic tests that may require an additional evaluation for an underlying etiology. The trial site guidance for assessment and follow up of these criteria can be found in the Investigator Trial File Binder (or equivalent).

Additional adverse events:

A full document entitled "Event of Clinical Interest Guidance Document" (previously entitled, "Event of Clinical Interest and Immune-Related Adverse Event Guidance Document"), which provides guidance regarding identification, evaluation and management of ECIs and irAEs, is in the possession of the PI and can be accessed upon request.

ECIs (both non-serious and serious adverse events) identified from the date of first dose through 30 days following cessation of treatment, or 30 days after the initiation of a new anticancer therapy, whichever is earlier, need to be reported within 24 hours to the Principal Investigator and within 2 working days to Merck Global Safety. (Attn: Worldwide Product Safety; FAX 215 993-1220), regardless of attribution to study treatment, consistent with standard SAE reporting guidelines.

Subjects should be assessed for possible ECIs prior to each dose. Lab results should be evaluated and subjects should be asked for signs and symptoms suggestive of an immune-related event. Subjects who develop an ECI thought to be immune-related should have additional testing to rule out other etiologic causes. If lab results or symptoms indicate a possible immune-related ECI, then additional testing should be performed to rule out other etiologic causes. If no other cause is found, then it is assumed to be immune-related.

7.8.3 Definition of an Overdose of pembrolizumab for This Protocol and Reporting of Overdose to Merck

For purposes of this trial, an overdose of pembrolizumab will be defined as any dose of 1,000 mg or greater. No specific information is available on the treatment of overdose of pembrolizumab. Appropriate supportive treatment should be provided if clinically indicated. In the event of overdose, the subject should be observed closely for signs of toxicity. Appropriate supportive treatment should be provided if clinically indicated.

If an adverse event(s) is associated with ("results from") the overdose of a Merck product, the adverse event(s) is reported as a serious adverse event, even if no other seriousness criteria are met.

If a dose of Merck's product meeting the protocol definition of overdose is taken without any associated clinical symptoms or abnormal laboratory results, the overdose is reported as a non-serious Event of Clinical Interest (ECI), using the terminology "accidental or intentional overdose without adverse effect."

All reports of overdose with and without an adverse event must be reported within 24 hours to the Principal Investigator and within 2 working days hours to Merck Global Safety. (Attn: Worldwide Product Safety; FAX 215 993-1220)

7.8.4 Reporting of Pregnancy and Lactation to Merck

Although pregnancy and lactation are not considered adverse events, it is the responsibility of investigators or their designees to report any pregnancy or lactation in a subject (spontaneously reported to them), including the pregnancy of a male subject's female partner that occurs during the trial or within 120 days of completing the trial completing the trial, or 30 days following cessation of treatment if the subject initiates new anticancer therapy, whichever is earlier. All subjects and female partners of male subjects who become pregnant must be followed to the completion/termination of the pregnancy. Pregnancy outcomes of spontaneous abortion, missed abortion, benign hydatidiform mole, blighted ovum, fetal death, intrauterine death, miscarriage and stillbirth must be reported as serious events (Important Medical Events). If the pregnancy continues to term, the outcome (health of infant) must also be reported.

Such events must be reported 2 working days to Merck Global Safety. (Attn: Worldwide Product Safety; FAX 215 993-1220).

8. PHARMACEUTICAL INFORMATION

A list of the adverse events and potential risks associated with the investigational and other agents administered in this study can be found in Section 7.1.

8.1 Pembrolizumab

Please refer to the Investigator's Brochure for detailed agent information, and to the FDA label for additional information.

8.1.1 **Description**

Pembrolizumab is a humanized monoclonal antibody of the IgG4/kappa isotype. Other name: MK-3475, Keytruda. Pembrolizumab blocks negative immune regulatory signaling by binding to the PD-1 receptor, inhibiting the interaction between PD-1 and its ligands.

The molecular weight of Pembrolizumab is 148.9-149.5 KDa.

8.1.2 **Form**

Clinical supplies will be manufactured and provided by Merck as summarized in Table 7.

Table 7: Product Description

Product Name & Potency	**Dosage Form**
Pembrolizumab 100 mg/ 4mL	Solution for Injection

8.1.3 Storage and Stability

Store intact vials between 2°C-8°C (36°F-46°F). Do not freeze. Protect from light by storing in the original box.

Stability testing of the intact vials is ongoing.

Administer prepared solutions immediately after preparation. If not administered immediately, prepared solutions may be stored refrigerated for up to 20 hours. PEMBROLIZUMAB solutions may be stored at room temperature for a cumulative time of up to 4 hours. This includes room temperature storage of liquid drug product solution in vials, room temperature storage of infusion solution in the IV bag, and the duration of infusion.

8.1.4 Compatibility

Compatible IV bag materials: PVC plasticized with DEHP, non-PVC (polyolefin), EVA, or PE lined polyolefin.

8.1.5 Handling

Qualified personnel, familiar with procedures that minimize undue exposure to themselves and the environment, should undertake the preparation, handling, and safe disposal of the chemotherapeutic agent in a self-contained and protective environment.

8.1.6 Availability

Pembrolizumab is an investigational agent and will be supplied free of charge from Merck.

8.1.7 Preparation

Pembrolizumab solution for infusion must be diluted prior to administration. Allow the required number of vials to equilibrate to room temperature. Do not shake the vials. Do not use if opaque or extraneous particulate matter other than translucent to white proteinaceous particles is observed. Do not use if discolored. To prepare the infusion solution add the dose volume of Pembrolizumab to an infusion bag containing 0.9& Sodium Chloride Injection, USP of 5% Dextrose Injection, USP. Gently invert the bag 10-15 times to mix the solution. The final concentration must be between **1 mg/mL to 10 mg/mL.**

8.1.8 **Administration**

Route of administration: IV infusion only. Do not administer as an IV push or bolus injection.

Method of administration: Infuse over approximately 30 minutes (range: 25-40 minutes) using an infusion set containing a low-protein binding 0.2 to 5 m in-line filter make of polyethersulfone or polysulfone. Infusion rate should not exceed 6.7 mL/min. A central line is not required however if a subject has a central venous catheter in place, it is recommended that it be used for the infusion. Do not co-administer drugs through the same infusion line. Following the infusion, flush the IV line with normal saline.

8.1.9 **Ordering**

Pembrolizumab will be obtained directly from Merck.

8.1.10 **Accountability**

The investigator, or a responsible party designated by the investigator, should maintain a careful record of the inventory and disposition of the agent using the NCI Drug Accountability Record Form (DARF) or another comparable drug accountability form. (See the NCI Investigator's Handbook for Procedures for Drug Accountability and Storage.)

8.1.11 **Destruction and Return**

At the end of the study, unused supplies of pembrolizumab should be destroyed according to institutional policies. Destruction will be documented in the Drug Accountability Record Form.

9. BIOMARKER, CORRELATIVE, AND SPECIAL STUDIES

Background information on the pre-clinical and clinical rationale for these investigations is discussed in Section 2.

In all patients in whom a tumor deposit is safely accessible, a baseline tumor biopsy is required. If biopsy was performed prior to enrollment on study and extra specimen is available from this biopsy, then this tissue may fulfill this requirement. We plan to use baseline biopsy tissue to perform a number of immune profiling assays, detailed below. On baseline tumor biopsies, we will perform characterization based on histology (TILs), protein expression, and mRNA expression. Additionally, we will bank specimens for possible future DNA analysis, and other further testing. Optional sequential biopsy will also be performed after cycle 2, within 14 days prior to cycle 3 of therapy to determine the impact of combined radiation PD-1 blockade on immunologic parameters. A third optional biopsy will be performed if deemed safe or feasible at the time of confirmed disease progression to study potential markers of resistance.

Serial blood draws for correlative science are required on this trial; blood draws will be obtained at baseline, cycle 2 day 1, cycle 3 day 1 and then every 3 cycles. On each blood draw, we will perform flow cytometry to characterize protein expression of immune mediators, detailed below, and additional

blood will be banked for future testing. We will also perform circulating tumor DNA studies to evaluate potential correlation with tumor burden.

Sequential imaging is included in this trial for staging purposes and to monitor subsequent response to treatment. Deidentified images will also be used for correlative imaging studies evaluating the potential of irradiated lesions to stimulate distant abscopal responses based on hypothetical T-cell trafficking as described above and by Poleszczuk et al. [68].

9.1 Characterizing the immunologic microenvironment of ACC

9.1.1 Hypotheses

This study will evaluate the efficacy of pembrolizumab with or without radiation in ACC. We will attempt to evaluate whether targeted radiation can change the tumor microenvironment and confer greater sensitivity to PD-1 inhibitors. Specifically, we hypothesize that localized radiation may have effects on unirradiation lesions, specifically:

- Radiation increases PD-L1 expression on tumor cells
- Radiation increases T-cell infiltration into tumor deposits
- Tumors sensitive to PD-1 inhibition will express higher levels of PD-L1 and PD-L2 compared to tumors resistant to PD-1 inhibition.

9.1.2 Collection, handling and transportation of biopsy specimens

Research biopsy kits will be provided by the study.

Biopsies should not be performed on Friday afternoons, as there may not be time for processing of the fresh tissue. If a biopsy must be performed on Friday morning, the lab of Mariano Severgnini must be notified ahead of time to ensure that there will be adequate time for processing fresh tissue, since fresh tissue cannot be stored over the weekend. The specimens in formalin may be stored over the weekend and shipped on Monday. Specimens in formalin should be stored at room temperature until processing

Ideally three core biopsies will be obtained:
- One core should be placed in 10% neutral buffered formalin tube supplied by the study.
- Two cores should be placed in sterile DMEM

The order of specimen collection should be:
- First core: 10% neutral buffered formalin
- Second core: Sterile DMEM
- Third core: Sterile DMEM

If additional cores are obtained, they should be processed as follows:
- Fourth core: 10% neutral buffered formalin
- Fifth core: Sterile DMEM
- Sixth core: 10% neutral buffered formalin
- Seventh core: Sterile DMEM

After being obtained, processing of the cores is as follows:

- All samples should be de-identified and labeled with the Participant initials, Participant Study ID number and date of procedure.
- Two cores in sterile DMEM should be brought as fresh tissue immediately to the lab of Mariano Severgnini at:

Center for Immuno-Oncology
Dana-Farber Cancer Institute
1 Jimmy Fund Way, JF0406
Boston, MA 02215
Phone: (617) 632-2421
Pager: 42093

This core must arrive to the lab to be processed for TILs (as described below) within 1.5 hours of its collection. In addition, a small piece of this core will be immediately frozen in liquid nitrogen upon arrival to Mariano Severgnini, for later use for RNA sequencing.

- One core in formalin should be brought to the Brigham and Women's SPL lab on the 6th floor of the Thorn building, where a block will be made. An email will be sent to the CRC within 2-3 days to confirm that the block has been made. The block should then be picked up from the SPL lab and provided to the CRC, Matthew Sanborn.

- Two cores in formalin should be brought to the Brigham and Women's SPL lab on the 6th floor of the Thorn building, where a block will be made. An email will be sent to the CRC within 2-3 days to confirm that the block has been made. The block should then be picked up from the SPL lab and brought to Dr. Scott Rodig on the 6th floor of the Thorn building.

9.1.3 Assay 1: Tumor infiltrating lymphocyte (TIL) percentage

Paraffinized, hematoxylin and eosin-stained slides taken from two tissue planes will be derived from each biopsy and will be reviewed by certified pathologists. In the research setting, all cases are reviewed by two pathologists and any discordant results resolved by consensus review. The extent of lymphocytic infiltrate in tumor tissue will be assessed, and stromal TIL percentage will be determined.

9.1.4 Assay 2: Immunohistochemistry

Tissue will be collected and fixed by 10% neutral buffered formalin overnight, dehydrated, and paraffin embedded. Four micrometer-thick sections will be cut. The paraffin blocks and unstained slides will be stored at room temperature. All immunohistochemical staining will be performed in the Center for Immuno-Oncology Pathology Core at Dana-Farber/Harvard Cancer Center (DF/HCC) Specialized Histopathology Core. PD-L1 IHC testing will also be performed at QualTek Molecular Laboratories as per Merck.

Formalin fixed-paraffin embedded (FFPE) tumor slides will be prepared and H&E stained for assessment of TIL in pre- and post-treatment tumor samples. To identify subsets of different immune populations (effector/memory CD8 cells, T regulatory cells, dendritic cells, tumor associated

macrophages, and Tie-2 expressing monocytes (TEM)), immunohistochemical (IHC) staining will be performed on FFPE tumor slices using some or all of the following antibodies:
Core set: CD3, CD8, PD-1, PD-L1, PD-L2, CD68, p16, Ki67
Other potential antibodies to be used: CD4, CD25, FoxP3, Indoleamine 2,3 deoxygenase-1 (IDO1), CD11c, CD83, CD86, CD56, CD14, CD16, Tie2 (See also Appendix E)

Investigators at our institution have developed IHC staining on paraffin embedded tissues for PD-L1, PD-L2, TIM-3, OX40, GITR and LAG-3 through our center for Immuno-Oncology Pathology Core (Scott Rodig MD, PhD Core Director, is a co-investigator on this protocol). PD-L1 IHC has recently been established in a CLIA approved laboratory and the remaining assays for CLIA laboratory conduct are being finalized.

As part of the validation assays in a CLIA-certified laboratory, identical cases were stained multiple times and under a variety of staining conditions and the results reviewed by two certified pathologists. A positive control sample (classical Hodgkin Lymphoma for PD-L1 expression; primary mediastinal large B-cell lymphoma for PD-L2 expression) and negative control sample (benign lymph node) is stained with each experimental tissue biopsy sample. The controls are reviewed by a certified pathologist at the time of review of the experimental sample.

An IHC assay for PD-1 (CD279, clone NAT105, Cell Marque Inc.) expression has been in standard surgical pathology diagnostic practice for several years, used to confirm the diagnosis of antioimmunoblastic T-cell lymphoma (AITL).

PD-1 IHC is performed routinely in the CLIA-certified laboratory and interpreted by a certified pathologist with an appropriate control (reactive lymph node, intra-follicular T cells are positive for PD-1) as described above.

Briefly, staining is scored according to intensity (0=no staining, 1=weak staining, 2=moderate staining, 3=strong staining), staining patter (M=predominantly cell membrane; C=predominantly cell cytoplasm), and the percentage of cells showing positive staining (0-100%). The semi-quantitative scoring is performed for: 1) the neoplastic tumor cells and 2) the non-neoplastic infiltrating immune cells. In the research setting, all cases are reviewed by two pathologists and any discordant results resolved by consensus review.

Digital, quantitative scoring of stained tissue is performed using the Perkin-Elmer Mantra slide scanning analysis platform. Quantitative assessment of positive staining uses the commercially provided algorithm for cell identification and positive pixels counted within a predefined DAB (brown, chromogenic) channel. It has been shown that this method of analysis showed good correlation with pathologists' scoring[69]. This method has been used to score PD-L1 expression in tumor cells[70].

The scoring for markers (such as the PD-Ligands) that stain macrophages, dendritic cells, and other cells of heterogeneous morphology will be semi-quantitative and performed by a pathologist using a modified H-score to capture: 1) the percentage of neoplastic cells positive for biomarker expression, intensity of expression, and membrane or cytoplasmic expression, and 2) the percentage of non-neoplastic cells (macrophages, dendritic cells, endothelial cells) positive for biomarker expression, intensity of expression, and membrane or cytoplasmic expression.

Scoring for PD-1 and other markers that stain lymphoid cells (CD3, CE4, CD8, CD25, FOXP3, IDO, CD16, CD56, LAG-3, TIM-3) will primarily be performed by automated analysis using the Perkin-Elmer system.

Perkin-Elmer scoring for PD-1+ (and other lymphoid markers) lymphocytes will be accomplished using a standard algorithm, developed for quantifying nuclear stains, but found to be applicable to quantifying membrane staining of cells with a very high N:C ratio, such as lymphocytes (Nuclear algorithm). The output is number of positive-staining cells per unit area (micron^2).
Below is a schematic of the workflow for the tissue-based biomarker analysis.

For image analysis:
1. IHC stained slides will be digitally scanned using the Perkin-Elmer Mantra system. The instrumentation is housed in the Tissue Microarray and Imaging Core (TMI) facility at the Dana-Farber/Harvard Cancer Center (DF/HCC). This facility is located adjacent to the office of Dr. Scott Rodig in the Department of Pathology. All digital images are stored on servers owned by the TMI core facility and accessed via the internet using a password-protected login.
2. Digital images are viewed using ImageScope Software (version 10.0.35.1800; Lecia) on standard PCs. Slides are digitally annotated by the pathologist to identify the region of interest and analysis.
3. Quantitative analysis is performed using analytical software associated with ImageScope, specifically Aperio Color Deconvolution V.9 (for PD-Ligands) and nuclear algorithm (for PD-1+ lymphocytes) and the results given as the percentage of positive pixels per unit area (for PD-Ligands) or number of positive cells per unit area (for PD-1+ lymphocytes). Intensity of staining is also captured automatically using the above algorithms and assigned a score (0, 1, 2, or 3) based upon the average optical density of the region or cells. All results are exported into an Excel spreadsheet.
4. Individual scoring data will be compared to clinical parameters to determine if there is an association with outcome. Scores using a combination of biomarker data will also be considered.

Tumor will be considered positive if >5% (PD-L1) or >10% (PD-L2) of the tumor cell population demonstrates unequivocal staining. PD-1 positivity will be defined as >3% positive cells/high power field. All IHC stained slides will be evaluated and scored by a pathologist. A subset of slides will be reviewed by a second pathologist to ensure concordance of interpretation.

The semi-quantitative scoring for this study is in accordance with those published previously and, as described above, will include scores for both the neoplastic and non-neoplastic cells within the tumor microenvironment. Data derived from pathologist visual scoring (semi-quantitative, but with increased specificity for delineating neoplastic and non-neoplastic cells) and pathologist-assisted, automated scoring (quantitative, but without accurately delineating neoplastic and non-neoplastic cells) for each marker of interest will be assessed for its clinical value. As necessary, the data from combinations of markers will also be considered (i.e. combined scores from PD-L1 and PD-L2 expression). All data will be analyzed in conjunction with the biostatistics group.

Further details of the immunohistochemical assay and assessment are described in Appendix G

9.1.5 Assay 3: Flow cytometry

TILs will be isolated from the biopsy specimen as described in Appendix H.

Surface staining followed by flow cytometry on the resultant TILs will then be performed as described in Appendix I. The following antibodies will be used on all specimens: (core set)
CD8
PD-1
PD-L1
PD-L2

A selection of the following antibodies may also be used, and additional antibodies may be used as well, as deemed appropriate and informative based on the state of the immune profiling literature at the time of correlative science performance:
CD4
FOXP3
(Other antibodies as listed in Appendix F)

9.1.6 Tissue banking

All leftover tissue will be banked in the Center for ImmunoOncology or the lab of Ravindra Uppaluri, MD, PhD as per standard lab protocol, such that it can be used for additional or optional future analyses as needed.

These analyses may include RNA analyses using the most current and informative methodologies at the time that correlative science is performed on all specimens. NanoString signatures and comprehensive RNA sequencing may be used.

Additionally, available tissue, may be used for whole exome sequencing to determine mutational burden and neoantigen detection, T-cell receptor sequencing to determine intratumoral TCR diversity and flow cytometry to quantify immune populations.

9.2 Characterizing immune markers in serum and peripheral blood mononuclear cells (PBMCs), and circulating tumor DNA studies prior to and after therapy with pembrolizumab plus radiotherapy

9.2.1 Hypotheses

- We hypothesize that the immune marker profile in the peripheral blood will change over the course of pembrolizumab plus radiation.
- We hypothesize that a larger increase in markers of immune activity in the peripheral blood will correlate with a better disease response as assessed on concurrent restaging scans, and in terms of best radiographic response at any time on trial.
- We hypothesize that an immune marker or composite of markers in the peripheral blood at baseline will correspond to TIL percentage in baseline tumor biopsy
- We hypothesize that changes in circulating tumor DNA will correlate with disease burden

9.2.2 Collection, handling, and shipping of blood specimens

Research blood collection is mandatory for all patients for cytokine analyses, flow cytometry and potential DNA isolation. The samples will be banked in the DFCI Center for ImmunoOncology laboratories and/or lab of Ravindra Uppaluri MD, PhD or Jens Lohr MD PhD for these and future research purposes. These specimens will become the property of the DF/HCC.

Blood draws should not be performed on Friday afternoons, as there may not be time for processing of the blood. If a blood draw must be performed on Friday morning, the lab of Mariano Severgnini and Jens Lohr must be notified ahead of time to ensure that there will be adequate time for processing the blood, since it cannot be stored over the weekend.

The following research blood samples are included as per the appendix:

-
- Two 6 mL purple-top (EDTA) tubes for circulating tumor DNA studies
- Three 10 mL green-top (heparin) tubes for PBMC analyses

All samples should be de-identified and labeled with the Participant initials, Participant Study ID number, protocol number, and date of collection (mm/dd/yyy) and time point (24 hour clock).

ALL samples from MGH should be shipped to the Lohr Lab.

1. Inform the CRC, Matthew Sanborn with the sample and tracking information the day before shipping a specimen.

   Email: matthewt_sanborn@dfci.harvard.edu
   Phone: (617) 632-5260

2. Ship specimen to the address below:

Laboratory of Jens G. Lohr, M.D., Ph.D.
Dana-Farber Cancer Institute
Dana-Building 542
450 Brookline Avenue
Boston, MA 02215

<u>All samples for serum and PBMC analyses</u> will then be hand carried at ambient temperature to Mariano Severgnini at:
Center for Immuno-Oncology
Dana-Farber Cancer Institute
1 Jimmy Fund Way, JF0406
Boston, MA 02215
Phone: (617) 632-2421
Pager: 42093

Blood must be processed within 3-4 hours of its being drawn.

All samples for circulating tumor DNA studies and cell-free DNA studies must be collected on Mondays, Tuesdays, Wednesdays or Thursdays for same-day shipment. Once drawn, samples may be shipped via overnight air, on wet ice (NOT dry ice) at +4 - 8*C (for purple top and green top tubes) Monday- Thursday to:

Jens G. Lohr, M.D., Ph.D.
Dana-Farber Cancer Institute
Dana-Building 542
450 Brookline Avenue
Boston, MA 02215
Phone: (617) 632-2069
Pager: 42255

9.2.3 Assays on serum and isolated PBMCs

Studies of immune function performed on blood will include:
- Analysis of antibody response (both quantitative antibody titer and qualitative antibody binding and functionality)
- Study of T-cell composition and functioning
- Analysis of cytokine response
- Identification of circulating antigen
- Comparison of immune responsiveness in serum
- Analysis of blood cell composition using flow cytometry
- Effect of immune status on circulating tumor cells
- Analysis of genomic or protein polymorphisms that may affect immune functioning and inflammation
- Analysis of inflammatory status in correlation to toxicity response
- Analysis of intracellular signaling pathways

9.3 Additional analysis

The above-mentioned analyses may be altered based on novel developments in the field of cancer immune profiling at the time of correlative science. Additional markers or alternative technologies (based on scientific developments and/or novel technologies) may also be used, to explore potential prognostic or predictive candidate markers/panels or markers related to treatment benefit and/or safety, to improve diagnostic tests, or to understand ACC biology.

9.4 Site Performing Correlative Studies

The IHC staining will be conducted in a research laboratory by Dr. Evisa Gjini in conjunction with Dr. Scott Rodig and / or Qualtek centralized laboratories. Dr. Rodig is a hematopathologist at the Brigham and Women's Hospital with prior expertise evaluating PD-1, PD-L1, and other immunologic markers in paraffin embedded tumor samples, and he has many prior publications in this area. Flow cytometry assessment of both tissue and blood will be performed by Mariano Severgnini, who has performed similar assays on many specimens collected on clinical trials of melanoma. Other analyses on tumor tissue and collected samples will be performed in the laboratory of Dr. Ravindra Uppaluri, MD, PhD, a

surgical oncologist at the Dana Farber Cancer Institute, who has performed similar analyses on other human tissues.

10. STUDY CALENDAR

Baseline evaluations are to be conducted within **28 days** prior to start of protocol therapy (except for pregnancy test and baseline tumor biopsy, as detailed). If these screening assessments occur within 3 days before start of study treatment, then they may serve as the baseline Cycle 1 Day 1 values. Scans must be done within **28 days** prior to the start of therapy.

As detailed in the Study Calendar, a negative pregnancy test in women of child-bearing potential must be documented within 72 hours before the first dose of study medication.

A baseline tumor biopsy, obtained within **28 days** before starting protocol therapy, is also required if tumor tissue is safely accessible. A second optional tumor biopsy sample will be obtained at the end of cycle 2, within 14 days of cycle 3, day 1. A third optional biopsy will be performed if deemed safe or feasible at the time of confirmed disease progression.

Baseline Laboratory evaluations must be completed within 14 days prior to registration. In the event that the participant's condition is deteriorating, laboratory evaluations should be repeated within 48 hours prior to initiation of the next cycle of therapy.

Assessments must be performed prior to administration of any study agent. Study assessments and agents should be administered within $\pm$ 3 days of the protocol-specified date, unless otherwise noted.

	Screening[a]	C1 D1	C1[b] D1-D8	C2 D1	C2 D15-D21	Cycle 3 and subsequent cycles D1	Off-Study[m]	Follow-Up
Pembrolizumab		X		X		X		
Radiotherapy[b]			X					
Informed consent	X							
Demographics	X							
Medical history	X							
Concurrent medications[c]	X	X----------X						
Physical exam	X	X		X		X	X	
Vital signs	X	X		X		X	X	
Weight	X	X		X		X	X	
Performance status[d]	X	X		X		X	X	
Hematology panel (CBC with platelets)	X	X		X		X	X	
Chemistry panel[e]	X	X		X		X	X	
Coagulation panel (PT/PTT)	X							
Pregnancy test[f]	X	X		X		X		
EKG	X							
Tumor archival	X[g]							
Tumor biopsy	X[h]				X[i]		X[p]	
Adverse event evaluation	X	X----------X					X[o]	
Tumor measurements	X	Tumor measurements are repeated every 9 weeks. Documentation (radiologic) must be provided for participants removed from study for progressive disease. Confirmatory scans 4 weeks after documented response should be obtained						X[j]
Blood collection for correlative science[k]	X			X		X		
TSH[l]	X	X				X		
MD/NP/PA evaluation required		X		X		X	X	
Survival								X[n]

[a] Baseline evaluations are to be conducted within 28 days prior to start of protocol therapy. If these screening assessments occur within 3 days before start of study treatment, then they may serve as the baseline Cycle 1 Day 1 values. Baseline Laboratory tests must be completed within 14 days prior to registration.
[b] Radiation will be initiated as specified in Arm A
[c] See Section 5 for concomitant medications guidelines
[d] See Appendix A
[e] Chloride, potassium, sodium, BUN, serum creatinine, phosphorus, calcium, albumin, total protein, alkaline phosphatase, ALT, AST, total bilirubin (NOTE: the frequency of checking magnesium levels is left up to the treating provider)
[f] Female subjects of childbearing potential should have a negative urine or serum pregnancy test within 14 days of treatment registration. Female subjects of childbearing potential should have a negative urine or serum pregnancy test repeated within 72 hours prior to receiving the first dose of study medication..
[g] Archival tumor sample should be collected (block or if not possible, 20 unstained slides could be sent as an alternative). This is required, unless not available.
[h] Baseline tumor biopsy obtained within 28 days before starting protocol therapy is required for those with accessible tumor tissue. See Section 9.1.2 for biopsy handling and processing instructions. This biopsy is required, unless not possible.

[i] A second tumor biopsy should be performed in patients after cycle 2, within 14 days prior to cycle 3 day 1 of protocol therapy (ideally as close to administration of cycle 3 day 1 therapy as possible). This biopsy is optional.
[j] For those taken off the study for toxicity tumor measurements should be repeated every 9 weeks for 1 year.
[k] Research blood samples are required at baseline, cycle 2 day 1, cycle 3 day 1 and then every 3 cycles, as specified in Section 5.2.3. See Section 9 for blood handling and processing instructions.
[l] TSH test must be performed every other cycle.
[m] Off-Study visit is to occur within 30 days of final administration of study treatment. End of treatment assessments do not have to be repeated if the same assessments were performed within 7 days prior to the visit.
[n] Patients with documented PD will be followed for overall survival every 6 months, until death (by phone).
[o] Adverse events are to be recorded until 30 days after the last dose of trial treatment
[p] A third optional biopsy will be performed if deemed safe or feasible at the time of confirmed disease progression.

11. MEASUREMENT OF EFFECT

11.1 Antitumor Effect– Solid Tumors

Response and progression will be evaluated in this study using the new international criteria proposed by the Response Evaluation Criteria in Solid Tumors (RECIST) guideline (version 1.1) [*Eur J Ca* 45:228-247, 2009]. Changes in the largest diameter (unidimensional measurement) of the tumor lesions and the shortest diameter in the case of malignant lymph nodes are used in the RECIST criteria.

11.1.1 Definitions

Evaluable for Target Disease response. Only those participants who have measurable disease present at baseline, have received at least one cycle of therapy, and have had their disease re-evaluated will be considered evaluable for target disease response. These participants will have their response classified according to the definitions stated below. (Note: Participants who exhibit objective disease progression prior to the end of cycle 1 will also be considered evaluable.)

Evaluable Non-Target Disease Response. Participants who have lesions present at baseline that are evaluable but do not meet the definitions of measurable disease, have received at least one cycle of therapy, and have had their disease re-evaluated will be considered evaluable for non-target disease. The response assessment is based on the presence, absence, or unequivocal progression of the lesions.

11.1.2 Disease Parameters

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 or ≥10 mm with CT scan, MRI, or calipers by clinical exam. All tumor measurements must be recorded in millimeters (or decimal fractions of centimeters).

Note: Tumor lesions that are situated in an irradiated area are not considered measurable.

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.

Non-measurable disease. All other lesions (or sites of disease), including small lesions (longest diameter <10 mm or pathological lymph nodes with ≥10 to <15 mm short axis), are considered non-

measurable disease. Bone lesions, leptomeningeal disease, ascites, pleural/pericardial effusions, lymphangitis cutis/pulmonitis, inflammatory breast disease, abdominal masses (not followed by CT or MRI), and cystic lesions are all considered non-measurable.

Note: Cystic lesions that meet the criteria for radiographically defined simple cysts should not be considered as malignant lesions (neither measurable nor non-measurable) 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 participant, these are preferred for selection as target lesions.

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 diameters will be used as reference to further characterize any objective tumor regression in the measurable dimension of the disease.

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, absence, or in rare cases unequivocal progression of each should be noted throughout follow up.

11.1.3 Methods for Evaluation of Measurable Disease

All measurements should be taken and recorded in metric notation using a ruler, calipers, or a digital measurement tool. All baseline evaluations should be performed as closely as possible to the beginning of treatment and never more than 4 weeks before the beginning of the treatment.

The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up. Imaging-based evaluation is preferred to evaluation by clinical examination unless the lesion(s) being followed cannot be imaged but are assessable by clinical exam.

Clinical lesions. Clinical lesions will only be considered measurable when they are superficial (*e.g.*, skin nodules and palpable lymph nodes) and ≥10 mm in diameter as assessed using calipers (*e.g.*, skin nodules). In the case of skin lesions, documentation by color photography, including a ruler to estimate the size of the lesion, is recommended.

Chest x-ray. Lesions on chest x-ray are acceptable as measurable lesions when they are clearly defined and surrounded by aerated lung; however, CT is preferable.

Conventional CT and MRI. This guideline has defined measurability of lesions on CT scan based on the assumption that CT thickness is 5mm or less. If CT scans have slice thickness greater than 5 mm, the minimum size of a measurable lesion should be twice the slice thickness. MRI is also acceptable in certain situations (*e.g.* for body scans).

Use of MRI remains a complex issue. MRI has excellent contrast, spatial, and temporal resolution; however, there are many image acquisition variables involved in MRI, which greatly impact image quality, lesion conspicuity, and measurement. Furthermore, the availability of MRI is variable globally. As with CT, if an MRI is performed, the technical specifications of the scanning sequences used should be optimized for the evaluation of the type and site of disease. Furthermore, as with CT, the modality used at follow-up should be the same as was used at baseline and the lesions should be measured/assessed on the same pulse sequence. It is beyond the scope of the RECIST guidelines to prescribe specific MRI pulse sequence parameters for all scanners, body parts, and diseases. Ideally, the same type of scanner should be used and the image acquisition protocol should be followed as closely as possible to prior scans. Body scans should be performed with breath-hold scanning techniques, if possible.

FDG-PET. While FDG-PET response assessments need additional study, it is sometimes reasonable to incorporate the use of FDG-PET scanning to complement CT scanning in assessment of progression (particularly possible 'new' disease). New lesions on the basis of FDG-PET imaging can be identified according to the following algorithm:

(a) Negative FDG-PET at baseline, with a positive FDG-PET at follow-up is a sign of PD based on a new lesion.
(b) No FDG-PET at baseline and a positive FDG-PET at follow-up: If the positive FDG-PET at follow-up corresponds to a new site of disease confirmed by CT, this is PD. If the positive FDG-PET at follow-up is not confirmed as a new site of disease on CT, additional follow-up CT scans are needed to determine if there is truly progression occurring at that site (if so, the date of PD will be the date of the initial abnormal FDG-PET scan). If the positive FDG-PET at follow-up corresponds to a pre-existing site of disease on CT that is not progressing on the basis of the anatomic images, this is not PD.
(c) FDG-PET may be used to upgrade a response to a CR in a manner similar to a biopsy in cases where a residual radiographic abnormality is thought to represent fibrosis or scarring. The use of FDG-PET in this circumstance should be prospectively described in the protocol and supported by disease-specific medical literature for the indication. However, it must be acknowledged that both approaches may lead to false positive CR due to limitations of FDG-PET and biopsy resolution/sensitivity.

Note: A ‘positive’ FDG-PET scan lesion means one which is FDG avid with an uptake greater than twice that of the surrounding tissue on the attenuation corrected image.

PET-CT. At present, the low dose or attenuation correction CT portion of a combined PET-CT is not always of optimal diagnostic CT quality for use with RECIST measurements. However, if the site can document that the CT performed as part of a PET-CT is of identical diagnostic quality to a diagnostic CT (with IV and oral contrast), then the CT portion of the PET-CT can be used for RECIST

measurements and can be used interchangeably with conventional CT in accurately measuring cancer lesions over time. Note, however, that the PET portion of the CT introduces additional data which may bias an investigator if it is not routinely or serially performed.

Ultrasound. Ultrasound is not useful in assessment of lesion size and should not be used as a method of measurement. Ultrasound examinations cannot be reproduced in their entirety for independent review at a later data and, because they are operator dependent, it cannot be guaranteed that the same technique and measurements will be taken from one assessment to the next. If new lesions are identified by ultrasound in the course of the study, confirmation by CT or MRI is advised. If there is concern about radiation exposure from CT, MRI may be used instead of CT in selected instances.

Endoscopy, Laparoscopy. The utilization of these techniques for objective tumor evaluation is not advised. However, such techniques may be useful to confirm complete pathological response when biopsies are obtained or to determine relapse in trials where recurrence following complete response (CR) or surgical resection is an endpoint.

Tumor markers. Tumor markers alone cannot be used to assess response. If markers are initially above the upper normal limit, they must normalize for a participant to be considered in complete clinical response. Specific guidelines for both CA-125 response (in recurrent ovarian cancer) and PSA response (in recurrent prostate cancer) have been published [*JNCI* 96:487-488, 2004; *J Clin Oncol* 17, 3461-3467, 1999; *J Clin Oncol* 26:1148-1159, 2008]. In addition, the Gynecologic Cancer Intergroup has developed CA-125 progression criteria which are to be integrated with objective tumor assessment for use in first-line trials in ovarian cancer [*JNCI* 92:1534-1535, 2000].

Cytology, Histology. These techniques can be used to differentiate between partial responses (PR) and complete responses (CR) in rare cases (*e.g.*, residual lesions in tumor types, such as germ cell tumors, where known residual benign tumors can remain).

The cytological confirmation of the neoplastic origin of any effusion that appears or worsens during treatment when the measurable tumor has met criteria for response or stable disease is mandatory to differentiate between response or stable disease (an effusion may be a side effect of the treatment) and progressive disease.

11.1.4 Response Criteria

11.1.4.1 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 new lesions is also considered progressions).

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.

11.1.4.2 Evaluation of Non-Target Lesions

Complete Response (CR): Disappearance of all non-target lesions and normalization of tumor marker level. All lymph nodes must be non-pathological in size (<10 mm short axis).

Note: If tumor markers are initially above the upper normal limit, they must normalize for a patient to be considered in complete clinical response.

Non-CR/Non-PD: Persistence of one or more non-target lesion(s) and/or maintenance of tumor marker level above the normal limits.

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.

Although a clear progression of "non-target" lesions only is exceptional, the opinion of the treating physician should prevail in such circumstances, and the progression status should be confirmed at a later time by the review panel (or Principal Investigator).

The finding of a new lesion should be unequivocal (i.e. not due to difference in scanning technique, imaging modality, or findings thought to represent something other than tumor (for example, some 'new' bone lesions may be simply healing or flare of pre-existing lesions). However, a lesion identified on a follow-up scan in an anatomical location that was not scanned at baseline is considered new and will indicate PD. If a new lesion is equivocal (because of small size etc.), follow-up evaluation will clarify if it truly represents new disease and if PD is confirmed, progression should be declared using the date of the initial scan on which the lesion was discovered.

11.1.4.3 Evaluation of Best Overall Response

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

For Patients with Measurable Disease (i.e., Target Disease)

Target Lesions	**Non-Target Lesions**	**New Lesions**	**Overall Response**	**Best Overall Response for when Confirmation is Required:**
CR	CR	No	CR	≥4 wks confirmation
CR	Non-CR/Non-PD	No	PR	≥4 wks confirmation
CR	Not evaluated	No	PR	
PR	Non-CR/Non-PD/Not evaluated	No	PR	
SD	Non-CR/Non-PD/Not evaluated	No	SD	Documented at least once ≥4 wks from baseline
PD	Any	Yes or No	PD	No prior SD, PR or CR
Any	PD*	Yes or No	PD	
Any	Any	Yes	PD	

* In exceptional circumstances, unequivocal progression in non-target lesions may be accepted as disease progression.

Note: Participants with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be reported as *"symptomatic deterioration"*. Every effort should be made to document the objective progression even after discontinuation of treatment.

For Participants with Non-Measurable Disease (*i.e.*, Non-Target Disease)

Non-Target Lesions	**New Lesions**	**Overall Response**
CR	No	CR
Non-CR/non-PD	No	Non-CR/non-PD*
Not all evaluated	No	not evaluated
Unequivocal PD	Yes or No	PD
Any	Yes	PD

* 'Non-CR/non-PD' is preferred over 'stable disease' for non-target disease since SD is increasingly used as an endpoint for assessment of efficacy in some trials so to assign this category when no lesions can be measured is not advised

11.1.5 Duration of Response

Duration of overall response: The duration of overall response is measured from the time measurement criteria are met for CR or PR (whichever is first recorded) until the first date that recurrence or PD is objectively documented, taking as reference for PD the smallest measurements recorded since the treatment started.

Duration of overall complete response: The duration of overall CR is measured from the time measurement criteria are first met for CR until the first date that recurrent disease is objectively documented.

Duration of stable disease: Stable disease is measured from the start of the treatment until the criteria for progression are met, taking as reference the smallest measurements recorded since the treatment started.

11.1.6 Clinical Benefit rate

Clinical benefit rate: defined as CR, PR and stable disease (SD) ≥ 24 weeks.

11.2 Other response parameters

11.2.1 Definition of Tumor Response Using Immune-Related Response Criteria (irRC)

The sum of the longest diameter of lesions (SPD) at tumor assessment using the immune-related response criteria (irRC) for progressive disease incorporate the contribution of new measurable lesions. Each net Percentage Change in Tumor Burden per assessment using irRC criteria accounts for the size and growth kinetics of both old and new lesions as they appear.

11.2.2 Impact of New Lesions on irRC

New lesions in and of themselves do not qualify as progressive disease. However, their contribution to total tumor burden is included in the SPD which in turn feeds into the irRC criteria for tumor response. Therefore, new non-measurable lesions will not discontinue any subject from the study.

11.2.3 Definition of Target Lesions Response Using irRC

- **irComplete Response (irCR):** Complete disappearance of all target lesions. This category encompasses exactly the same subjects as "CR" by the mWHO criteria.
- **irPartial Response (irPR):** Decrease, relative to baseline, or 50% or greater in the sum of the products of the two largest perpendicular diameters of all target and all new measurable target lesions (i.e., Percentage Change in Tumor Burden). Note: the appearance of new measurable lesions is factored into the overall tumor burden, but does not automatically qualify as progressive disease until the SBD increases by ≥25% when compared to SPD at nadir.
- **irStable Disease (irSD):** Does not meet criteria for irRC or irPR, in the absence of progressive disease.
- **irProgressive Disease (irPD):** At least 25% increase Percentage Change in Tumor Burden (i.e. taking SPD of all target lesions and any new lesions) when compared to SPD at nadir.

11.2.4 Definition of Non-Target Lesions Response Using irRC

- **irComplete Response (irCR):** Complete disappearance of all non-target lesions. This category encompasses exactly the same subjects as "CR" by the mWHO criteria.
- **irPartial Response (irPR) or irStable Disease (irSD):** Non-target lesion(s) are not considered in the

definition of PR; these terms do not apply.

- **irProgressive Disease (irPD):** Increases in number or size of non-target lesion(s) does not constitute progressive disease unless/until the Percentage Change in Tumor Burden increases by 25% (i.e. the SPD at nadir of the target lesions increases by the required amount).

11.2.5 Definition of Overall Response Using irRC

Overall response using irRC will be based on these criteria:

- **Immune-Related Complete Response (irCR):** Complete disappearance of all tumor lesions (target and non-target) together with no new measurable/unmeasurable lesions for at least 4 weeks from the date of documentation of complete response.
- **Immune-Related Partial Response (irPR):** The sum of the products of the two largest perpendicular diameters of all target lesions is measured and captured as the SPD baseline. At each subsequent tumor assessment, the SPD of the two largest perpendicular diameters of all target lesions and of new measurable lesions are added together to provide the Immune Response Sum of Product Diameters (irSPD). A decrease, relative to baseline, of the irSPD compared to the previously SPD baseline of 50% or greater is considered an irPR.
- **Immune-Related Stable Disease (irSD):** irSD is defined as the failure to meet criteria for immune complete response or immune partial response, in the absence of progressive disease
- **Immune-Related Progressive Disease (irPD):** It is recommended in difficult cases to confirm PD by serial imaging. Any of the following will constitute PD:
  - At least 25% increase in the SPD of all target lesions over baseline SPD calculated for the target lesions.
  - At least 25% increase in the SPD of all target lesions and new measurable lesions (irSPD) over the baseline SPD calculated for the target lesions.

Criteria for determining overall response by irRC are summarized as follows:

Immune-Related Response Criteria Definitions

Target Lesion Definition	Non-Target Lesion Definition	New Measurable Lesions	New Unmeasurable Lesions	Percent change in tumor burden (including measurable new lesions when present)	Overall irRC Response
Complete Response	Complete Response	No	No	–100%	irCR
Partial Response	Any	Any	Any	≥ –50%	irPR
				<–50% to <+25%	irSD
				>+25%	irPD
Stable Disease	Any	Any	Any	<–50% to <+25%	irSD
				>+25%	irPD
Progressive Disease	Any	Any	Any	≥+25%	irPD

11.2.6 Immune-Related Best Overall Response Using irRC (irBOR)

irBOR is the best confirmed overall response over the study as a whole, recorded between the date of first dose until the last tumor assessment before subsequent therapy (except for local palliative radiotherapy for painful bone lesions) for the individual subject in the study. For the assessment of irBOR, all available assessments per subject are considered.

irCR or irPR determinations included in the irBOR assessment must be confirmed by a second (confirmatory) evaluation meeting the criteria for response and performed no less than 4 weeks after the criteria for response are first met.

11.2.7 Local response is defined as irRC applied to the irradiated lesions. This is not applicable in the case of an irradiated bone lesion.

11.2.8 Abscopal response rate

Abscopal response is defined as described previously [71] to be a decrease in the longest diameter of at least 30% in any measurable (>1cm) non-irradiated lesion from baseline. A complete abscopal response is defined as the complete disappearance of a measurable non-irradiated lesion and a partial abscopal response was defined as at least a 3-% decrease in the longest diameter. Progressive disease in this context is defined as at least a 20% increase in the longest diameter of the best measurable non-irradiated lesion, whereas stable disease was defined as insufficient shrinkage or growth to qualify for a partial abscopal response or complete abscopal response or progressive disease.

12. DATA REPORTING / REGULATORY REQUIREMENTS

Adverse event lists, guidelines, and instructions for AE reporting can be found in Section 7.0 (Adverse Events: List and Reporting Requirements).

12.1 Data Reporting

12.1.1 Method

The ODQ will collect, manage, and perform quality checks on the data for this study.

12.1.2 Responsibility for Data Submission

Investigative sites within DF/HCC or DF/PCC are responsible for submitting data and/or data forms to the ODQ according to the schedule set by the ODQ.

12.2 Data Safety Monitoring

The DF/HCC Data and Safety Monitoring Committee (DSMC) will review and monitor toxicity and accrual data from this study. The committee is composed of clinical specialists with experience in oncology and who have no direct relationship with the study. Information that raises any questions about participant safety will be addressed with the Overall PI and study team.

The DSMC will review each protocol up to four times a year or more often if required to review toxicity and accrual data. Information to be provided to the committee may include: up-to-date participant accrual; current dose level information; all grade 2 or higher unexpected adverse events that have been reported; summary of all deaths occurring with 30 days of intervention for Phase I or II protocols; for gene therapy protocols, summary of all deaths while being treated and during active follow-up; any response information; audit results, and a summary provided by the study team. Other information (e.g. scans, laboratory values) will be provided upon request.

12.3 Multicenter Guidelines

N/A

13. STATISTICAL CONSIDERATIONS

13.1 Study Design/Endpoints

The primary endpoint of this study is objective response in non-irradiated lesions per RECIST 1.1. This phase 2 study will enroll subjects with advanced ACC that is not amenable to curative treatment. This is a population for whom no effective or FDA approved therapies exist and where cytotoxic chemotherapy has minimal activity. Therefore, the treatment in either arm of the study with pembrolizumab alone (Arm B), or pembrolizumab in combination with radiation (Arm A), will be considered promising and

worthy of further evaluation if the objective response rate (outside of the radiation field) is 25%. On the other hand, a given arm will not be of interest if the objective response rate is 5%.

Eligible patients will be randomized to either pembrolizumab alone or pembrolizumab in combination with radiation therapy and a two-stage design will be used in this study. The first stage will accrue 10 evaluable patients in each arm. If at least 1 response is observed in a given arm, an additional 10 patients will be entered to that arm. If 3 or more responses are observed among the 20 patients in a given arm, that treatment will be considered promising and worthy of further study. With this design, the probability of declaring a treatment arm effective is 0.88 if the true objective response rate is 25% and 0.07 if the true objective response rate is 5%. Under the null hypothesis (true objective response rate of 5%), the probability of stopping at the first stage accrual is 60% for each arm.

13.2 Sample Size, Accrual Rate and Study Duration

In order to enroll 40 eligible, treated and evaluable patients, a maximum of 44 patients (including 10% increase for ineligible patients or patients who never start treatment) will be accrued. Between 22 and 44 patients are estimated to be enrolled in this study.

The anticipated accrual rate is 1-2 patients per month, and the accrual is expected to complete in 30 months.

13.3 Stratification Factors

None.

13.4 Interim Monitoring Plan

N/A

13.5 Analysis of Primary Endpoint

Objective response will be assessed in non-irradiated lesions among all eligible and treated patients pursuing RECIST 1.1. Objective response rate along with 90% two-stage confidence intervals will be reported in each arm.

13.6 Analysis of Secondary Endpoints

Efficacy Endpoints
Secondary endpoints include progression-free survival, overall survival, and duration of response. All efficacy endpoints will be evaluated using both RECIST 1.1 and immune-related response criteria (irRC), and both sets of results will be reported in each arm. Progression-free survival is defined as the time from randomization to disease progression or death, whichever occurs first. Patients who are alive without disease progression will be censored at the date of last disease assessment. Overall survival is defined as the time from randomization to death or date last known alive. Duration of response will be analyzed among patients with objective response and is defined as the time from onset of objective response (complete response or partial response) to documentation of disease progression. Kaplan-Meier

method will be used to analyze all time-to-event endpoints. Exact binomial confidence intervals will be used to describe the proportion of patients with immune-related response (irCR or irPR) by treatment arm.

Safety and tolerability
All patients who receive treatment, regardless of eligibility, will be evaluable for toxicity. Toxicity will be graded according to NCI CTCAE, Version 4.0. The proportions of patients with various toxicities will be reported by treatment arm. For each arm, with 22 patients, the binomial exact 90% confidence interval for toxicity rate would be no wider than 38%.

Correlative endpoints
Analyses of correlative endpoints are largely exploratory and hypothesis-generating. Any promising findings will be tested in future studies. All correlative analyses will be descriptive in nature.

Tumor biopsies are required at baseline and cycle 2 for analyses of tumor infiltrating lymphocytes (expressed as a percentage) as well as PD-L1 and PD-L2 expression (expressed as H-scores and also as percent positive). Wilcoxon signed rank test will be used to evaluate the changes on these measurements from baseline to cycle 2. Assuming all patients have both tumor biopsies available (20 eligible and treated patients per arm), the study has 90% power to detect a 0.6 SD change using a one-sided test with 10% type I error. There is 84.5% power to detect the above change with 80% of patients (16 eligible and treated patients per arm) submitting paired biopsy samples.

13.7 Reporting and Exclusions

Evaluation of Efficacy
For this Phase II trial, the efficacy evaluable population consists of all eligible and treated patients.

Evaluation of Safety

The safety population will be used in the safety data summaries. The safety population consists of all patients who receive any protocol treatment and who have at least one post-baseline safety assessment. Note that a patient who had no adverse events constitutes a safety assessment. Patients who have received at least one dose of study drug but have no post-treatment safety data of any kind would be excluded.

14. PUBLICATION PLAN

The results should be made public within 24 months of reaching the end of the study. The end of the study is the time point at which the last data items are to be reported, or after the outcome data are sufficiently mature for analysis, as defined in the section on Sample Size, Accrual Rate and Study Duration. If a report is planned to be published in a peer-reviewed journal, then that initial release may be an abstract that meets the requirements of the International Committee of Medical Journal Editors. A full report of the outcomes should be made public no later than three (3) years after the end of the study.

15. REFERENCES

1. Spiro, R.H., *Distant metastasis in adenoid cystic carcinoma of salivary origin.* Am J Surg, 1997. **174**(5): p. 495-8.
2. Bobbio, A., et al., *Lung metastasis resection of adenoid cystic carcinoma of salivary glands.* Eur J Cardiothorac Surg, 2008. **33**(5): p. 790-3.
3. Laurie, S.A., et al., *Systemic therapy in the management of metastatic or locally recurrent adenoid cystic carcinoma of the salivary glands: a systematic review.* Lancet Oncol, 2011. **12**(8): p. 815-24.
4. Airoldi, M., et al., *Phase II randomized trial comparing vinorelbine versus vinorelbine plus cisplatin in patients with recurrent salivary gland malignancies.* Cancer, 2001. **91**(3): p. 541-7.
5. Hill, M.E., et al., *Cisplatin and 5-fluorouracil for symptom control in advanced salivary adenoid cystic carcinoma.* Oral Oncol, 1997. **33**(4): p. 275-8.
6. Licitra, L., et al., *Cisplatin, doxorubicin and cyclophosphamide in advanced salivary gland carcinoma. A phase II trial of 22 patients.* Ann Oncol, 1996. **7**(6): p. 640-2.
7. Gao, M., et al., *Clinicopathological study of distant metastases of salivary adenoid cystic carcinoma.* Int J Oral Maxillofac Surg, 2013. **42**(8): p. 923-8.
8. Keam, B., et al., *Phase 2 study of dovitinib in patients with metastatic or unresectable adenoid cystic carcinoma.* Cancer, 2015. **121**(15): p. 2612-7.
9. Thomson, D.J., et al., *Phase II trial of sorafenib in advanced salivary adenoid cystic carcinoma of the head and neck.* Head Neck, 2015. **37**(2): p. 182-7.
10. Wong, S.J., et al., *Phase II trial of dasatinib for recurrent or metastatic c-KIT expressing adenoid cystic carcinoma and for nonadenoid cystic malignant salivary tumors.* Ann Oncol, 2016. **27**(2): p. 318-23.
11. Kim, D.W., et al., *A multicenter phase II study of everolimus in patients with progressive unresectable adenoid cystic carcinoma.* BMC Cancer, 2014. **14**: p. 795.
12. Goncalves, P., et al., *A phase II study of suberoylanilide hydroxamic acid (SAHA) in subjects with locally advanced, recurrent, or metastatic adenoid cystic carcinoma (ACC).* J Clin Oncol, 2013. **31**: p. Suppl; abstr 6045.
13. Chau, N.G., et al., *A phase II study of sunitinib in recurrent and/or metastatic adenoid cystic carcinoma (ACC) of the salivary glands: current progress and challenges in evaluating molecularly targeted agents in ACC.* Ann Oncol, 2012. **23**(6): p. 1562-70.
14. Hotte, S.J., et al., *Imatinib mesylate in patients with adenoid cystic cancers of the salivary glands expressing c-kit: a Princess Margaret Hospital phase II consortium study.* J Clin Oncol, 2005. **23**(3): p. 585-90.
15. Locati, L.D., et al., *Cetuximab in recurrent and/or metastatic salivary gland carcinomas: A phase II study.* Oral Oncol, 2009. **45**(7): p. 574-8.
16. Agulnik, M., et al., *Phase II study of lapatinib in recurrent or metastatic epidermal growth factor receptor and/or erbB2 expressing adenoid cystic carcinoma and non adenoid cystic carcinoma malignant tumors of the salivary glands.* J Clin Oncol, 2007. **25**(25): p. 3978-84.
17. Yu, Y., et al., *Concurrent loss of heterozygosity and copy number analysis in adenoid cystic carcinoma by SNP genotyping arrays.* Lab Invest, 2007. **87**(5): p. 430-9.
18. Ho, A.S., et al., *The mutational landscape of adenoid cystic carcinoma.* Nat Genet, 2013. **45**(7): p. 791-8.

19. Ross, J.S., et al., *Comprehensive genomic profiling of relapsed and metastatic adenoid cystic carcinomas by next-generation sequencing reveals potential new routes to targeted therapies.* Am J Surg Pathol, 2014. **38**(2): p. 235-8.
20. Stephens, P.J., et al., *Whole exome sequencing of adenoid cystic carcinoma.* J Clin Invest, 2013. **123**(7): p. 2965-8.
21. Mitani, Y., et al., *Comprehensive analysis of the MYB-NFIB gene fusion in salivary adenoid cystic carcinoma: Incidence, variability, and clinicopathologic significance.* Clin Cancer Res, 2010. **16**(19): p. 4722-31.
22. Persson, M., et al., *Clinically significant copy number alterations and complex rearrangements of MYB and NFIB in head and neck adenoid cystic carcinoma.* Genes Chromosomes Cancer, 2012. **51**(8): p. 805-17.
23. Spranger, S., R. Bao, and T.F. Gajewski, *Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity.* Nature, 2015. **523**(7559): p. 231-5.
24. Freeman, G.J., et al., *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation.* J Exp Med, 2000. **192**(7): p. 1027-34.
25. Latchman, Y., et al., *PD-L2 is a second ligand for PD-1 and inhibits T cell activation.* Nat Immunol, 2001. **2**(3): p. 261-8.
26. Carter, L., et al., *PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2.* Eur J Immunol, 2002. **32**(3): p. 634-43.
27. Sheppard, K.A., et al., *PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta.* FEBS Lett, 2004. **574**(1-3): p. 37-41.
28. Nishimura, H. and T. Honjo, *PD-1: an inhibitory immunoreceptor involved in peripheral tolerance.* Trends Immunol, 2001. **22**(5): p. 265-8.
29. Nishimura, H., et al., *Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor.* Immunity, 1999. **11**(2): p. 141-51.
30. Nishimura, H., et al., *Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice.* Science, 2001. **291**(5502): p. 319-22.
31. Okazaki, T., et al., *Autoantibodies against cardiac troponin I are responsible for dilated cardiomyopathy in PD-1-deficient mice.* Nat Med, 2003. **9**(12): p. 1477-83.
32. Salama, A.D., et al., *Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis.* J Exp Med, 2003. **198**(1): p. 71-8.
33. Blazar, B.R., et al., *Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism.* J Immunol, 2003. **171**(3): p. 1272-7.
34. Iwai, Y., S. Terawaki, and T. Honjo, *PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells.* Int Immunol, 2005. **17**(2): p. 133-44.
35. Iwai, Y., et al., *Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade.* Proc Natl Acad Sci U S A, 2002. **99**(19): p. 12293-7.
36. Strome, S.E., et al., *B7-H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma.* Cancer Res, 2003. **63**(19): p. 6501-5.
37. Dong, H., et al., *Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion.* Nat Med, 2002. **8**(8): p. 793-800.
38. Hirano, F., et al., *Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity.* Cancer Res, 2005. **65**(3): p. 1089-96.

39. Blank, C., et al., *PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells.* Cancer Res, 2004. **64**(3): p. 1140-5.
40. Seiwert, T., et al., *A phase Ib study of MK-3475 in patients with human papillomavirus (HPV)-associated and non-HPV–associated head and neck (H/N) cancer.* J Clin Oncol, 2014. **32**(5s): p. 1-2.
41. Ropponen, K.M., et al., *Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer.* J Pathol, 1997. **182**(3): p. 318-24.
42. Dudley, M.E., et al., *Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma.* J Clin Oncol, 2005. **23**(10): p. 2346-57.
43. Hunder, N.N., et al., *Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1.* N Engl J Med, 2008. **358**(25): p. 2698-703.
44. Okazaki, T., et al., *PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13866-71.
45. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe, *The B7 family revisited.* Annu Rev Immunol, 2005. **23**: p. 515-48.
46. Schoenfeld, J.D., et al., *Salivary gland tumors treated with adjuvant intensity-modulated radiotherapy with or without concurrent chemotherapy.* Int J Radiat Oncol Biol Phys, 2012. **82**(1): p. 308-14.
47. Chen, A.M., et al., *Long-term outcome of patients treated by radiation therapy alone for salivary gland carcinomas.* Int J Radiat Oncol Biol Phys, 2006. **66**(4): p. 1044-50.
48. Hartsell, W.F., et al., *Randomized trial of short- versus long-course radiotherapy for palliation of painful bone metastases.* J Natl Cancer Inst, 2005. **97**(11): p. 798-804.
49. Wu, J.S., et al., *Meta-analysis of dose-fractionation radiotherapy trials for the palliation of painful bone metastases.* Int J Radiat Oncol Biol Phys, 2003. **55**(3): p. 594-605.
50. Golden, E.B., et al., *Radiation fosters dose-dependent and chemotherapy-induced immunogenic cell death.* Oncoimmunology, 2014. **3**: p. e28518.
51. Teng, M.W., et al., *Classifying Cancers Based on T-cell Infiltration and PD-L1.* Cancer Res, 2015. **75**(11): p. 2139-45.
52. Xiao, Y., et al., *RGMb is a novel binding partner for PD-L2 and its engagement with PD-L2 promotes respiratory tolerance.* J Exp Med, 2014. **211**(5): p. 943-59.
53. Calles, A., et al., *Expression of PD-1 and its ligands, PD-L1 and PD-L2, in smokers and never smokers with KRAS mutant lung cancer.* J Thorac Oncol, 2015.
54. Derks, S., et al., *Epithelial PD-L2 Expression Marks Barrett's Esophagus and Esophageal Adenocarcinoma.* Cancer Immunol Res, 2015. **3**(10): p. 1123-9.
55. Baptista, M.Z., et al., *Prognostic significance of PD-L1 and PD-L2 in breast cancer.* Hum Pathol, 2016. **47**(1): p. 78-84.
56. Shin, S.J., et al., *Clinicopathologic Analysis of PD-L1 and PD-L2 Expression in Renal Cell Carcinoma: Association with Oncogenic Proteins Status.* Ann Surg Oncol, 2015.
57. Barrett, M.T., et al., *Genomic amplification of 9p24.1 targeting JAK2, PD-L1, and PD-L2 is enriched in high-risk triple negative breast cancer.* Oncotarget, 2015. **6**(28): p. 26483-93.
58. Sharabi, A.B., et al., *Stereotactic Radiation Therapy Augments Antigen-Specific PD-1 Mediated Anti-Tumor Immune Responses via Cross-Presentation of Tumor Antigen.* Cancer Immunology Research, 2014.

59. Victor, C.T.-S., et al., *Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer*, in *Nature*. 2015.
60. Postow, M.A., et al., *Immunologic correlates of the abscopal effect in a patient with melanoma*, in *N Engl J Med*. 2012. p. 925-31.
61. Sridharan, V. and J.D. Schoenfeld, *Immune effects of targeted radiation therapy for cancer.* Discovery medicine, 2015. **19**(104): p. 219-28.
62. Golden, E.B., et al., *An abscopal response to radiation and ipilimumab in a patient with metastatic non-small cell lung cancer.* Cancer Immunol Res, 2013. **1**(6): p. 365-72.
63. Hiniker, S.M., et al., *A systemic complete response of metastatic melanoma to local radiation and immunotherapy.* Transl Oncol, 2012. **5**(6): p. 404-7.
64. Postow, M.A., et al., *Immunologic correlates of the abscopal effect in a patient with melanoma.* N Engl J Med, 2012. **366**(10): p. 925-31.
65. Grimaldi, A.M., et al., *Abscopal effects of radiotherapy on advanced melanoma patients who progressed after ipilimumab immunotherapy.* Oncoimmunology, 2014. **3**: p. e28780.
66. Chandra, R., et al., *A systematic evaluation of abscopal responses following radiotherapy in patients with metastatic melanoma treated with ipilimumab.* Oncoimmunology, 2015. **in press**.
67. Chandra, R.A., et al., *A systematic evaluation of abscopal responses following radiotherapy in patients with metastatic melanoma treated with ipilimumab.* Oncoimmunology, 2015. **4**(11): p. e1046028.
68. Poleszczuk, J., et al., *A Systems Biology Approach to Predict Immunotherapy Augmented Radiation-Induced Abscopal Effects*, in *AACR Tumor Immunology and Immunotherapy*. 2014: Orlando, FL. .
69. Mino-Kenudson, M., et al., *A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry*, in *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2010. p. 1561-71.
70. Green, M.R., et al., *Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma*, in *Blood*. 2010. p. 3268-77.
71. Golden, E.B., et al., *Local radiotherapy and granulocyte-macrophage colony-stimulating factor to generate abscopal responses in patients with metastatic solid tumours: a proof-of-principle trial*, in *Lancet Oncol*. 2015. p. 795-803.
72. Quine, M.A., et al., *Prospective audit of upper gastrointestinal endoscopy in two regions of England: safety, staffing, and sedation methods.* Gut, 1995. **36**(3): p. 462-7.
73. Topalian, S.L., et al., *Safety, activity, and immune correlates of anti-PD-1 antibody in cancer.* N Engl J Med, 2012. **366**(26): p. 2443-54.
74. Bachireddy, P., et al., *Reversal of in situ T-cell exhaustion during effective human antileukemia responses to donor lymphocyte infusion.* Blood, 2014. **123**(9): p. 1412-21.

16. APPENDICES

APPENDIX A: Performance Status Criteria

ECOG Performance Status Scale		**Karnofsky Performance Scale**	
Grade	Description	Percent	Description
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.	100	Normal, no complaints, no evidence of disease.
		90	Able to carry on normal activity; minor signs or symptoms of disease.
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).	80	Normal activity with effort; some signs or symptoms of disease.
		70	Cares for self, unable to carry on normal activity or to do active 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.	60	Requires occasional assistance, but is able to care for most of his/her needs.
		50	Requires considerable assistance and frequent medical care.
3	In bed >50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.	40	Disabled, requires special care and assistance.
		30	Severely disabled, hospitalization indicated. Death not imminent.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.	20	Very sick, hospitalization indicated. Death not imminent.
		10	Moribund, fatal processes progressing rapidly.
5	Dead.	0	Dead.

APPENDIX B: Pembrolizumab Events of Clinical Interest

Pneumonitis (reported as ECI if ≥ Grade 2)		
Acute interstitial pneumonitis	Interstitial lung disease	Pneumonitis
Colitis (reported as ECI if ≥ Grade 2 or any grade resulting in dose modification or use of systemic steroids to treat the AE)		
Intestinal Obstruction	Colitis	Colitis microscopic
Enterocolitis	Enterocolitis hemorrhagic	Gastrointestinal perforation
Necrotizing colitis	Diarrhea	
Endocrine (reported as ECI if ≥ Grade 3 or ≥ Grade 2 and resulting in dose modification or use of systemic steroids to treat the AE)		
Adrenal Insufficiency	Hyperthyroidism	Hypophysitis
Hypopituitarism	Hypothyroidism	Thyroid disorder
Thyroiditis	Hyperglycemia, if ≥Grade 3 and associated with ketosis or metabolic acidosis (DKA)	
Endocrine (reported as ECI)		
Type 1 diabetes mellitus (if new onset)		
Hematologic (reported as ECI if ≥ Grade 3 or any grade resulting in dose modification or use of systemic steroids to treat the AE)		
Autoimmune hemolytic anemia	Aplastic anemia	Thrombotic Thrombocytopenic Purpura (TTP)
Idiopathic (or immune) Thrombocytopenia Purpura (ITP)	Disseminated Intravascular Coagulation (DIC)	Haemolytic Uraemic Syndrome (HUS)
Any Grade 4 anemia regardless of underlying mechanism		
Hepatic (reported as ECI if ≥ Grade 2, or any grade resulting in dose modification or use of systemic steroids to treat the AE)		
Hepatitis	Autoimmune hepatitis	Transaminase elevations (ALT and/or AST)
Infusion Reactions (reported as ECI for any grade)		
Allergic reaction	Anaphylaxis	Cytokine release syndrome
Serum sickness	Infusion reactions	Infusion-like reactions
Neurologic (reported as ECI for any grade)		
Autoimmune neuropathy	Guillain-Barre syndrome	Demyelinating polyneuropathy
Myasthenic syndrome		
Ocular (report as ECI if ≥ Grade 2 or any grade resulting in dose modification or use of systemic steroids to treat the AE)		
Uveitis	Iritis	
Renal (reported as ECI if ≥ Grade 2)		
Nephritis	Nephritis autoimmune	Renal Failure
Renal failure acute	Creatinine elevations (report as ECI if ≥Grade 3 or any grade resulting in dose modification or use of systemic steroids to treat the AE)	
Skin (reported as ECI for any grade)		
Dermatitis exfoliative	Erythema multiforme	Stevens-Johnson syndrome
Toxic epidermal necrolysis		
Skin (reported as ECI if ≥ Grade 3)		
Pruritus	Rash	Rash generalized
Rash maculo-papular		
Any rash considered clinically significant in the physician's judgment		
Other (reported as ECI for any grade)		
Myocarditis	Pancreatitis	Pericarditis
Any other Grade 3 event which is considered immune-related by the physician		

APPENDIX C: Guidelines for collecting research biopsy tissue

Tissue specimens will be collected from metastatic lesions using standard institutional procedures. The amount of tissue collected may follow the guidelines listed below:

Skin/chest wall: A goal of 2 5-mm punch biopsies will be obtained.

Lymph node: A goal of 3-6 core biopsy specimens will be obtained using an 18-gauge needle.

Liver: A goal of 3-6 core biopsy specimens will be obtained using an 18-gauge needle.

Lung: Because of the risk of pneumothorax associated with core needle biopsies of lung nodules, no core biopsies of lung nodules are mandated on this protocol, unless they are clinically indicated.

Bone: Because the yield of malignant tissue from bone biopsies tends to be relatively low, if a patient has another accessible site of disease (i.e. skin, lymph node, liver), that site should be biopsied preferentially. If bone is the only biopsy-accessible site, then a goal of 3-6 core biopsy specimens will be obtained using an 11-13 gauge needle.

Please note that the above are guidelines for the amount of tissue to be obtained, and are not meant to replace clinical judgment at the time the procedure is performed. Less than the goal quantity of tissue is accepted for each type of biopsy, and will be left to the clinical judgment of the physician performing the procedure.

If a patient is undergoing resection of a lesion for clinical reasons (i.e. wedge resection of a new lung lesion for confirmation of diagnosis or re-testing of hormone receptor or HER2 status; or, resection of a chest wall lesion; or, resection of a lymph node), then the patient may opt to have a portion of that tissue (roughly equivalent to the goal amount of tissue listed in the guidelines above, i.e. the equivalent of two 5-mm punch biopsies of the skin, or 3-6 18-gauge core biopsies) stored for research at the time of the procedure (provided that the tissue is processed as specified), in which case, the patient would not be required to undergo a separate research biopsy at baseline on this protocol.

Coded laboratory specimens will be stored in the Tumor Bank of the DFCI. These specimens will become the property of DFCI. Patients will be informed that their specimens may be used for research by investigators at DF/HCC and other approved collaborators. Shared specimens will be identified with a sample ID number; all patient identifying material will be removed.

Risks of Research Biopsy and Procedures for Minimizing Risk

Potential risks according to site are:
Skin/chest wall (punch biopsy):
- Likely: local discomfort and minor bleeding
- Less likely: moderate or major bleeding, or infection

Lymph node, liver, or bone (core needle biopsy):
- Likely: local discomfort and minor bleeding
- Less likely: moderate or major bleeding, need for blood transfusion, hospitalization due to bleeding or other complications, infection, damage to adjacent organs. Additional risks may be present if intravenous conscious sedation is required

Breast (core biopsy):
- Likely: local discomfort and minor bleeding.
- Less likely: moderate or major bleeding, need for blood transfusion, hospitalization due to bleeding or other complications, infection, pneumothorax, damage to adjacent organs.

Pleural fluid (thoracentesis):
- Likely: local discomfort and minor bleeding
- Less likely: moderate or major bleeding, need for blood transfusion, hospitalization due to bleeding or other complications, infection, pneumothorax, damage to adjacent organs

Ascites fluid (paracentesis):
- Likely: local discomfort and minor bleeding
- Less likely: moderate or major bleeding, need for blood transfusion, hospitalization due to bleeding or other complications, infection, bowel perforation or damage to adjacent organs. In order to minimize the risk of a biopsy, only qualified personnel will perform these procedures.

Prior to the procedure, the physician performing the procedure will discuss the risks with each study participant, answer any questions, and obtain separate procedure consent. Patients will be evaluated for comorbidities or concomitant medications that may increase the risk of potential complications. For biopsies of lesions that are not superficial and clearly palpable, imaging studies such as CT or ultrasound will be used to guide the biopsy in order to minimize the risk of damage to adjacent structures. After lymph node biopsies, patients will be observed a minimum of 2 hours (range 2-4 hours) after the procedure, or according to standard institutional guidelines. After liver biopsies, patients will be observed a minimum of 4 hours (range 4-6 hours) after the procedure, or according to standard institutional guidelines. Less than the goal quantity of tissue is accepted for each type of biopsy, and will be left to the clinical judgment of the physician performing the procedure.

Risks of Anesthesia

Local Anesthesia
All biopsy procedures require local anesthesia using lidocaine, xylocaine, or related compounds. There is a small risk of an allergic reaction associated with these drugs. In order to minimize the risk of local anesthesia, only qualified personnel will perform the biopsy procedure. Patients will be queried if they have had previous allergic reactions to local anesthetics.

Intravenous Conscious Sedation
Certain biopsy procedures, such as lymph node, liver, or bone biopsies, may require

intravenous conscious sedation (IVCS). IVCS is a minimally depressed level of consciousness that retains the patient's ability to maintain a patent airway independently and continuously and respond appropriately to physical stimulation and verbal commands.

The risks of intravenous conscious sedation include: inhibition of the gag reflex and concomitant risk of aspiration, cardiopulmonary complications (myocardial infarction, cardiac arrhythmias, hypoxemia), and allergic reactions to the sedative or analgesic medications. These risks are small but real; for example, in a prospective study of 14,149 patients undergoing IVCS during upper gastrointestinal endoscopies, the rate of immediate cardiopulmonary events was 2 in 1000.[72] The 30-day mortality was 1 per 2,000 cases. In this study, there was a strong association between lack of monitoring and use of high-dose benzodiazepines with adverse outcomes. There was also an association between the use of local anesthetic sprays to the oropharynx and the development of pneumonia. In order to minimize the risk of intravenous conscious sedation, only qualified personnel will be responsible for conscious sedation. A minimum of two individuals will be involved in the care of patients undergoing conscious sedation—the physician performing the biopsy procedure, and the individual (M.D. or R.N.) who monitors the patients and his/her response to both the sedation and the procedure, and who is capable of assisting with any supportive or resuscitative measures. The room where the procedure utilizing IVCS takes place will have adequate equipment to provide supplemental oxygen, monitor vital signs, and maintain an airway should this be necessary. An emergency cart will also be immediately accessible to the room where the procedure is to take place, and emergency support services will be available on page. Patients will be screened and evaluated for their fitness to undergo conscious sedation by a trained physician. Patients with active cardiac disease are excluded from this study. No local anesthetic spray to the oropharynx will be necessary, given that endoscopy is not a planned procedure. Following the procedure, patients will be observed closely in the recovery room for a minimum of 2 hours.

General Anesthesia
Because of the higher risk of general anesthesia compared with local anesthesia or intravenous conscious sedation, biopsies that would require general anesthesia in order to be performed *are not permitted* on this protocol, unless they are being done for clinical reasons, and excess tissue that otherwise would have been discarded is then banked for the purpose of this protocol.

For Biopsies of Soft Tissue, Liver, Bone, Breast, Etc:
1. After biopsy is performed, the tissue mass is placed on a sterile gauze
2. Using forceps, separate the tumor tissue
3. Place 2 pieces (cores) of tumor tissue in each cassette (typically end up with 3 cassettes per biopsy); the last cassette will contain many small pieces of tumor tissue
4. Fill cassettes with OCT
a. Completely cover tissue
b. Limit the amount of bubbles
5. Place cassettes on dry ice and prepare for transport by limiting OCT leakage
6. Return samples to the lab and complete freezing of samples in OCT with dry ice (about 10 minutes freezing time)
7. Once samples are frozen, place in plastic bag; label bag with date, protocol number, patient number, and number of initials included
8. Store in –80C freezer

For Effusions and Ascites
1. Fluid sample should be split into two equal aliquots
2. One aliquot should be spun down into a pellet and snap frozen in an ETOH/dry ice bath or in liquid N2
3. One aliquot should be fixed and processed as a standard cell block.
Note: if the sample preparation is done by a clinical cytopathology laboratory, it is important to explain that the sample is for research purposes only and that no thin prep should be performed as this uses up a significant portion of the sample.

For Fine Needle Aspiration Samples
A goal of 3 passes:
1. One pass should be evacuated and rinsed directly into 2mL of room temperature Trizol for RNA analysis.
2. One pass should be evacuated and rinsed directly into 2mL of room temperature Trizol for DNA analysis.
3. One pass should be evacuated and rinsed directly into 10-20mL of RPMI to prepare a cell block.

Fresh Tissue Shipping Procedures
Please ship frozen specimens over-night on dry ice to the CRC.

APPENDIX D: Antibodies that can be used for immunohistochemistry in correlative studies

IHC Biomarkers	Priority	Clone/ Cat #	Source	Host species	Dilution	Optimized?
PD-L1	1	9A11	G. Freeman	Mouse	1/125	Yes
PD-L2	1	9E 6	G. Freeman	Mouse	1/10000	Yes
PD-1	1	EH33	G. Freeman	Mouse	1/600	Yes
CD3	1	IS503	Dako	Rabbit	1/250	Yes
CD4	1	4B12	Vector Labs	Mouse	1/200	Yes
CD8	1	144B	Dako	Mouse	1/100	Yes
FOXP3	1	206D	BioLegend	Mouse	1:50	Yes
TIM3	1	AF2365	R&D Systems	Goat	1:50	Yes
LAG3	1	17B4	LifeSpan BioSc	Mouse	1/200	Yes
Tie2	2	AF313	R&D Systems	Goat	1/500	Yes
ANGPT2	2	sc-74403	Santa Cruz Bio	Mouse	1/200	Yes
IDO1	2	ab55305	Abcam	Mouse	1/100	No
CD38	3	SPC32	Abcam	Mouse	1/300	Yes
CD56	3	123C3	Dako	Mouse	1/100	Yes
CD14	3	ab49755	Abcam	Mouse	1/100	Yes
CD16	3	ab183354	Abcam	Rabbit	1/100	No
CD11c	3	EP1347Y	Abcam	Rabbit	1/500	Yes

APPENDIX E: Antibodies that may be used for flow cytometry in correlative studies

Cell Type	Antibody	Color	Clone
T effector	CD4	FITC	SK3
		PC7	SK3
	CD62L	APC	DREG-56
	CD69	PE	FN50
T regs	CD4	FITC	SK3
		PC7	SK3
	CD25	PE	Bc96
		PC5	B1.49.9
	FOXP3	PE	PCH101
		FITC	PCH101
	CD127	APC	eBioRDR5
NK	CD3	FITC	UCHT1
		PC7	UCHT1
	CD56	PE	NCAM16.2
	CD57	PE	TB01
NKT	CD3	FITC	UCHT1
		PC7	UCHT1
	CD56	PE	NCAM16.2
	TCR a/b	APC	BW242/412
	CD314 (NKG2D)	PE	ON72
MDSC	HLA-DR	PC7	L243
		FITC	L243
	CD11b	FITC	Bear1
	CD14	APC	61D3
	CD33	PE	WM53
Cytotoxic	CD8	APC	BW135/80
		PE	BW135/80
	CD3	FITC	UCHT1
		PC7	UCHT1
Memory T	CD197 (CCR7)	PE	3D12
	CD45RO	FITC	UCHL1
	CD45RA	PC7	HI100
	CD4	FITC	SK3
		PC7	SK3
	CD8	APC	BW135/80
		PE	BW135/80
B cells	CD5	BV421	UCHT2
		FITC	UCHT2
	CD19	PC7	SJ25C1
		PE	SJ25C1

		APC	SJ25C1
	CD20	FITC	2H7
Classic Monocytes	CD14	APC	61D3
	CD16	FITC	eBioCB16
Dendritic	CD123	APC	6H6
	CD303a	FITC	201A
	CD11c	FITC	11-0116
	CD141	APC	M80
	CD1c	PE	L161
Macrophages	CD40	APC	5C3
Progenitors	CD34	PE	4H11
Intracellular Cytokines	IL-10	PE	JES3-9D7
	IL-17a	PercP Cy5	eBio64DEC17
	INFg	APC	B27
	TNFa	FITC	Mab11
Co-stimulatory and inhibitory markers	CD134 (OX40)	APC	ACT-35
	CD137 (4-1BB)	FITC	4B4
	CD154 (CD40L)	PercP 710	24-31
	CD223 (LAG3)	PercP 710	3DS223H
	CD252 (OX40L)	PE	11C3.1
	CD278 (ICOS)	FITC	ISA-3
	Tim-3	BV421	F38-2E2
	CD274 (PD-L1)	PE	MIH1
	CD279 (PD-1)	FITC	MIH4
		PE	MIH4
		APC	MIH4
	CD357 (GITR)	APC	621
	CD152 (CTLA-4)	efluor 660	14D3
Proliferation	Ki-67	FITC	20Raj1

APPENDIX F: Immunohistochemical staining assays

Design of immunohistochemical assay

The immunohistochemical assay for PD-L1 and PD-L2 is semi-quantitative while PD-1 stained slides will be scanned by an automated scanning microscope and quantitatively analyzed by Aperio image analysis system (Lecia Biosystems) after they are evaluated and positive cells are manually counted by a pathologist.

Standard EnVision two-step (indirect) staining method will be utilized. Four micrometer-thick sections will be cut, deparaffinized, rehydrated, and subjected to heat modified antigen retrieval in citrate buffer (pH 6) (Invitrogen) by steaming for 30 minutes. After cooling, tissue sections will be incubated with peroxidase block (DAKO, Carpinteria, CA) for five minutes, then serum free protein block (DAKO) for 20 minutes. Slides will be incubated at room temperature for one hour with a primary antibody. Antibodies will be diluted in Da Vinci Green Diluent (Biocare Medical, Concord, CA). EnVision™ anti-mouse HRP-labeled polymer (DAKO) will be applied to the sections for 30 minutes, followed by visualization using the chromogen 3,3-diaminobenzidine (DAKO). All the sections will then be counterstained with hematoxylin, dehydrated, mounted, and coverslipped. Positive and negative controls shall be included in each staining. Known positive stained Hodgkin Lymphoma (PD-L1), tonsil (PD-1), and melanoma (PD-L2) slides will be used as external control (separate slides). Stained slides will be stored at room temperature.

In a pilot study performed by our correlative scientists, immunoreactivity for PD-L1 was detected in the cytoplasm and membrane while PD-L2 and PD-1 expression was observed in the cytoplasm. Scoring for PD-L1 and PD-L2 will be semi-quantitative/ordered categorical. The percentage of the tumor cells staining positive for PD-L1 or PD-L2 and the intensity of the staining will be recorded (using the scale 0=no staining, 1=weak staining, 2=moderate staining, 3=intense staining). Absolute PD-1 positive cells will be counted under microscope lens x20 power field. Five representative areas will be chosen to count. The average number from 5 areas will be recorded and compared with data from image analysis.

For PD-1 staining, slides will be scanned by an automated scanning microscope and analyzed by Aperio image analysis system (Lecia Biosystems). Tumor areas will be marked by a pathologist to exclude non-neoplastic areas, such as stroma, normal epithelial, and necrotic regions. The software will be used to count the number of positive cells in each tissue. The percentage of PD-1 positive cells will be calculated. Data will be compared with that of manual counting by a pathologist to exclude tissue artifacts that cannot be recognized by computer image software.

Assay performance

Protocols of these three antibodies have been optimized and standardized to minimize staining variance. Positive control and negative controls were used and stained separately with each batch of slides. The IHC staining of three markers (PD-1, PD-L1, PD-L2) has been performed in two different labs by three different technicians on whole tissue sections of Hodgkin lymphomas, melanomas, lung cancers, and renal cell carcinomas. Three readers were involved, confirming the good reproducibility of the assay.

Thresholds of positivity

Tumor will be considered positive if >5% (PD-L1)[73] or >10% (PD-L2) of the tumor cell population demonstrates unequivocal staining. PD-1 positivity will be defined as >3% positive cells/high power field.[74] All IHC stained slides will be evaluated and scored by a pathologist. A subset of slides will be reviewed by a second pathologist to ensure concordance of interpretation.

APPENDIX G: TIL isolation from solid tumors

1. Prepare an enzyme solution of collagenase, hyaluronidase and deoxyribonuclease in advance:
a. Dissolve collagenase (collagenase type I, cat#17100-017, Invitrogen) in DMEM at a concentration of 1 mg/ml
b. Add hyaluronidase (hyaluronidase type V, cat#H6254, Sigma-Aldrich) to a final concentration of 1mg/ml (1,500 units/ml) and deoxyribonuclease (deoxyribonuclease I, type IV, cat#5025, Sigma-Aldrich) to a final concentration of 50 micrograms/ml (100 units/ml)
c. Filter the solution with a 10 ml sterile syringe, a sterile 23G needle, and a sterile 0.2 μm filter.
2. Record the date and time of the start of TIL isolation.*
3. Dissect patient tumor sample into pieces as small as possible with sterile scissor or scalpel. **Note**: Mincing of tumor may be facilitated by lining up two scalpels in parallel.
4. Submerge the pieces of tumor in 5 - 10 ml prepared enzyme solution in a 50 ml conical tube.
5. Enzymatically digest tumor pieces in 37°C waterbath for one to two hours; every 15 min., vigorously shake the tube.
6. Put a sterile cell strainer (100 μm, 352360, BD Falcon) on a 50 ml conical tube, and pass the digested tumor solution through the strainer; the flowthrough will be collected in the 50 ml conical tube. Rinse the strainer and undigested tumor once with PBS.
7. Add 2-5 ml complete DMEM medium (with 10% FBS + 50 μg/ml gentamycin) into flowthrough to stop digestion.
8. Spin the tube at 1500 rpm for 5 min in a centrifuge at room temperature.
9. In the meantime, put undigested tumor tissue into a sterile 50 ml conical tube, and add 5-10 ml enzyme solution and continue with digestion from step 4.
10. Repeat step 3 to step 8, based on tissue digestion

NOTE: For samples with lot of red blood cells and/or undigested debris that has passed through the cell strainer, the following is recommended before proceeding to step 11:

A. Resuspend the cell pellet in 10 ml complete DMEM medium

B. Add 10 ml Ficoll Paque Plus (Cat# 17-1440-03; GE Healthcare) in a 50 ml conical tube.

C. Slowly and gently layer the digested tumor suspension onto the Ficoll Paque Plus.

D. Centrifuge the tube at room temperature at 1500 - 2000 rpm (1000 g) with slow acceleration and deceleration for 20 - 30 min.

E. Pipette off the interface between complete DMEM and the Ficoll Paque Plus (lower part), and transfer the layer into a 50 ml conical tube. The bottom pellet will be composed of red blood cells and undigested debris.

F. Add 2 - 3X bed volume of PBS to dilute Ficoll.

G. Spin the tube at 1500 rpm for 5 min in a centrifuge at room temperature.

H. Aspirate supernatant and proceed to step 11.

11. Resuspend cell pellets in complete DMEM medium plus gentamycin 50ug/ml and combine cells-TIL into one sterile 50 ml conical tube.
12. Centrifuge at 1500 rpm for 5 min in a centrifuge at room temperature.

13. Aspirate off the supernatant and remove a small aliquot to record the cell count and viability, then place the tube on ice.
14. For each timepoint, collect the following parameters:

a. Cell viability (%) before freezing*
b. Total yield of TIL (x 10^6 cells/mL/vial) isolated prior to freezing*
15. Resuspend the cell suspension in pre-chilled PBMC freezing media (CTL-cryoABS kit, CTL cellular Technology)
a. Transfer 1 ml aliquots of the cell suspension to a cryovial labeled with the **supplied Quintiles labels**. A minimum of one (1) cryovial should be obtained with a minimum concentration of cells at $1x10^6$ cells/mL/vial.
b. For each cryovial prepared, please record the total # of cells in the cryovial*. If there are more than $2x10^6$ cells, then aliquot cells into as many cryovials as possible at a concentration of $1x10^6$ cells/ml/vial.
16. Store in 1 ml aliquots in cryovials at -80^0C in a slow freeze container. Leave undisturbed overnight or for a **minimum of 12 hrs and a maximum of 24 hrs.**
17. Transfer into liquid nitrogen for long-term storage. Record the time, date, and location that the samples* were placed in liquid nitrogen storage.
18. At the Principal Investigator's request, samples should be batch shipped at the end of study. Please, follow the shipping instructions provided. Samples will be shipped on liquid nitrogen.

APPENDIX H: Flow cytometry procedures

Prep without Permeabilization

KEEP EVERYTHING ON ICE

1. Thaw cell vial in 37 degree water bath until completely thawed.
2. Resuspend cells in 50 ml of RPMI medium (Gibco, 11A75-093) + 10% FBS + 1X final Anti-Anti (Gibco, 15240-062) in a 50 ml conical tube (Corning, 430290).
3. Culture cells in 2 T-150 culture flasks (Corning, 431465) overnight (25 ml per flask)

- In one flask, activate cells by adding 0.4 ml (whole vial) of Dynabeads Human T- Activator (Gibco, 1161D). Before adding beads to flask, wash beads according to manufacturer's protocol.

4. Incubate cells for 24 hours at 37 degrees with 5% CO_2
5. Remove cells and media from flask and filter through 70 micron cell filter (Biologix, 15-1070) into 50 ml conical tube.
6. Spin conical tubes for 5 min at 1800 rpm in a Sorvall Legend XTR centrifuge.
7. Make wash/blocking media: PBS +2.5% FBS (Gibco, 14040).
8. Vacuum media off pellet, resuspend pellet in calculated volume of wash/blocking media according to calculations from cell density + number of wells and tubes for 700,000 cells/tube in 100 µl.
9. Pipet 100 µl/ well of cells + wash/blocking media containing FcR Blocking Reagent (Milteny Biotec, 130-059-901) into v-bottomed plate (Costar, 3894) according to well map (let sit for 20-30 minutes on ice).
10. Spin plate at 1800 rpm for 5 minutes at 4C in Sorvall Legend XTR centrifuge
11. Mix antibody cocktails in flat bottomed plate (amount according to manufacturer specifications or from previously developed assays)
12. After plate with cells is finished spinning, aspirate liquid off pellet by carefully tilting the plate. Add appropriate antibody cocktails from flat bottomed plate according to well map after pipetting up and down to mix at least three times.
13. Let plate with cells + antibodies sit for 45 minutes on ice in the dark.
14. Spin plate as previously described in step 11
15. Aspirate off liquid by tilting plate and wash with 150 µl/well of wash/blocking media, pipetting up at down to mix at least 3 times (described in step 6)
16. Resuspend cells in 150 µl/well in wash/blocking media
17. Keep plate and single tubes (single color controls) on ice in the dark or covered with aluminum foil until read by Fortessa LTS II (Beckton- Dickinson).

APPENDIX I: Generation of PBMCs

1. Pour blood from green-cap tubes (heparin treated tubes) into two 50 ml conical tubes (Corning, 430290).
2. Spin tubes at 1500 rpm for 10 min (Sorvall Legend XTR centrifuge).
3. Aspirate 2 ml plasma/tube and aliquot into 4 tubes mircocentrifuge tubes (Fisherbrand, 05-408-138)
4. Spin plasma at 3000 RPM for 5 minutes (Sorvall Legend Micro 21R centrifuge)
5. Aspirate plasma into Cryogenic tubes 2 ml plasma/ tube (Corning, 430488).
6. Dilute blood 1:1 with PBS. (Blood amount should not exceed 25 ml per tube.)
7. Take 2 new 50 ml conical tubes and add 12 ml ficoll-paque (Cat# 17144003; GE Healthcare) per tube.
8. Slowly and gently layer the diluted blood on top of the ficoll-paque of the tube with a maximum volume of 35 ml.
9. Centrifuge the tube at 1900 rpm for 20 min at room temperature with slow acceleration (#7) and deceleration (#7) (Sorvall Legend XTR centrifuge).
10. Remove the PBMC layer from between the upper layer (diluted plasma) and middle layer (ficoll-paque) and transfer into a 50 ml conical tube. The lower layer is composed of red blood cells.
11. Completely fill conical tube containing isolated PBMC with PBS, mixing well.
12. Count viable cells by mixing 10 µl Trypan Blue with 10µl PBMC/PBS dilution in a microcentrifuge tube. Load 10 µl of mixture onto Countess Cell Counting Chamber Slide (Invitrogen, C10283) and read with Countess Automated Cell Counter (Invitrogen).
13. Centrifuge the tubes containing PBMC/PBS mixture at 1500 rpm for 5 min at room temperature (Sorvall Legend XTR centrifuge).
14. Remove PBS, and resuspend PBMC pellet in appropriate amount of freezing solution so that there are approx 5×10^6 cells/cryo vial in 300-500 µl of Fetal Bovine Serum (heat inactivated) plus 15% DMSO.
15. Put vials in CoolCell container (Biocision Inc.) and transfer to -80C freezer overnight.
16. Transfer cells to liquid nitrogen tank