Table of contents

Draft questions

Draft question #	Contributor	Topic area
D14	Grossniklaus	Uveal melanoma metastasis
D15	Henry	Aging and immunotherapy
D31	Rafiq	chimeric antigen receptor (CAR)
D39	Waller	Immunotherapy
D43	Yun	Autophagy

Question D14
(Grossniklaus)

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults. Treatment, either by enucleation (removing the eye) or brachytherapy (irradiating the tumor in the eye) almost always leads to successful local control. However, there is a 40% to 50% mortality rate from liver metastases of this tumor. These metastases are diagnosed by imaging (MRI, CT) at about 3 years after the primary tumor is treated.

a. (3 pts) What are possible mechanisms regarding why UM metastasizes virtually exclusively to the liver? How can you test these possible mechanisms?

b. (4 pts) One early hypothesis was that enucleation spread the tumor to the liver. However, the Collaborative Ocular Melanoma Study (COMS) found that the rate of metastasis and death was identical in a large cohort of patients who were randomized to either enucleation or brachytherapy. What do these results tell us about the original hypothesis? What alternative hypotheses can you develop to explain the findings? How can you test your hypothesis?

c. (3 pts). Thus far there are no systemic medications that prolong life significantly for patients with metastatic uveal melanoma to the liver. Neither conventional chemotherapy nor immune checkpoint inhibitors have been effective. What are possible reasons why these therapies haven't worked? What are some strategies that may be tested to overcome these problems? What experiments would you design to test these strategies?

Answer A: Uveal melanoma that metastasizes to the liver expresses high levels of the surface receptors cMet and CXCR4. The ligands for these receptors, HGF SF and CXCL12 (SDF) are found in relatively high levels in the liver. This receptor/ligand gradient appears to play a role for UM metastases localization in the liver. One could utilize melanoma cell lines in a mouse model of metastatic UM, including those with high versus low levels of cMet and/or CXCR4. One could create these cell lines using transfection vectors, shRNA and/or CRISPR Cas9. One would express more liver metastases in cell lines that express high levels of cMet and/or CXCR4 versus low levels of cMet and/or CXCR4.

Answer B: Many chemotherapeutic agents work when cells are dividing, and UM has a very long tumor doubling time, therefore most UM cells are not cycling through the cell cycle at a give time. These agents might not effective reach the tumor in the liver as when they are clinically detectable, there is fibrosis including collagen present around the metastases, thus limiting drug penetration. There is little angiogenesis in metastases in the liver parenchyma, again limiting drug penetration to the metastasis. UM expresses low levels of PDL-1. However, UM may express higher levels of other checkpoints, including TIGIT and IDO. Additionally, recent work has shown that CTLs in the liver associated with metastatic UM, express the checkpoint LAG3 rather than CTLA4. A strategy would be to test checkpoint inhibitors targeting TIGIT, IDO and/or LAG3 in animal models or metastatic UM. One potential strategy would be to obtain biopsies of the primary or metastatic UM, evaluate for TIGIT, IDO and/or PDL-1 and choose an appropriate checkpoint inhibitor based on the tumor's checkpoint expression. Other strategies would be to use agents that may potentiate checkpoint expression by the UM cells. LSD1 is an epigenetic modulator of gene expression. LSD1 inhibition has been shown to increase response to checkpoint inhibitors

in cutaneous melanoma. Experiments may be designed to test for increased checkpoint expression and inhibition after siRNA suppression of LSD1 in UM cell lines and after treatment with compounds that block LSD1 expression, both *in vitro* and in metastatic UM animal models. There are additional strategies under investigation, including tebentafusp, a molecule that re-directs T cells against UM cells by binding to receptors both on UM cells and T lymphocytes.

Answer C: The main hypothesis is that there are dormant, clinically undetectable micrometastases present in the liver at the time; the primary UM is diagnosed in the cases in which the metastases become clinically detectable at 3 years. These "dormant" micrometastases have the capacity to grow in the liver. In this case, the treatment choice for the primary tumor does not matter as these micrometastases are present or not present at the time of diagnosis of the primary tumor. One could test this by calculating tumor doubling times in the primary and metastatic tumor by analyzing images and extrapolate backwards to find when the metastasis occurred. A possible mechanism is that the primary tumor produces a substance, such as angiostatin, which suppresses growth of any metastases that are already present. Removing the eye or irradiating the tumor removes the angiostatin and the micrometastases grow. This has essentially been disproven and other mechanisms have been proposed. The main hypothesis is that there are host immune and non-immune mechanisms that are involved with emergence from dormancy of the micrometastases. These immune mechanisms may include NK cell tumor suppression, which may be blocked by CD11b+ myeloid derived suppressor cells (MDSCs) and possibly IL10+ marrow derived cells and CTL tumor suppression which is overcome by checkpoints (such as PD1, PDL-1 and CTLA4). The non-immune mechanisms include proteins locally produced in the liver, such as PEDF, which limits VEGF mediated angiogenesis in the metastases, which is overcome by TGFβ and PDGFbb production by the metastatic melanoma cells. One could test these mechanisms i*n vitro* with co cultures of UM cells and liver cells and by treating mouse models with NK boosting agents, anti-checkpoint inhibitors, agents that block TGFβ and that block PDGFbb. Another possible mechanism is that any treatment of the primary tumor (enucleation, irradiation) causes substances to be released that cause pre-existing micrometastases, which may be present in virtually all UM patients, to grow. This may be potentiation of inflammatory mediators that potentiate metastasis growth, such as COX1/Thromboxane A2. One might block these mediators using Ketorolac in an animal model of metastatic UM and evaluate for efficacy.

Question D15
(Curtis J. Henry)

Non-Small Cell Lung Cancer (NSCLC) has a median age at diagnosis of 70 years. Unfortunately, chemotherapy treatment is largely ineffective in older patients with NSCLC.
The emergence of immune checkpoint inhibitors (ICI), particularly αPD-1 antibody, has shown promise in patients with NSCLC. However, age-related disparities persist, as shown in the Kaplan-Meier plot below (from Lichtenstein et al., J Thorac Oncol. 2019 Mar;14(3):547-552).

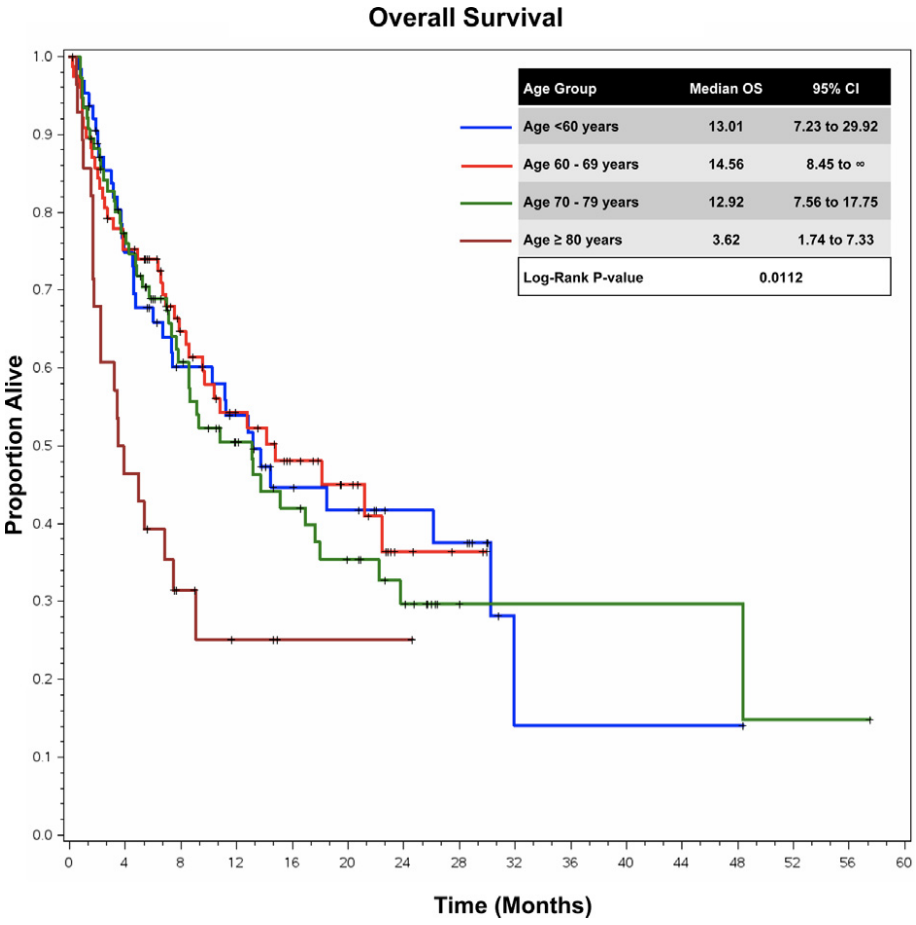
BRIEF REPORT

IASLC

Check for updates

Impact of Age on Outcomes with Immunotherapy in Patients with Non-Small Cell Lung Cancer

Morgan R. L. Lichtenstein, MD, Ryan D. Nipp, MD, MPH, Alona Muzikansky, MA, Kelly Goodwin, CNP, Danyon Anderson, BA, Richard A. Newcomb, MD, Justin F. Gainor, MD*



a. (**2 pts**) What factors might account for the observation that *chemotherapy* is largely ineffective in older patients?

b. (**3 pts**) State a mechanism-based, immunological hypothesis why *immunotherapy* is less effective, specifically why the therapeutic outcomes shown in the figure above are significantly worse in the age ≥ 80 years cohort. Before contemplating experiments to test this hypothesis,

what confounding factors might you wish to rule out.

c. (**5 pts**). Assume that you have been granted IRB approval to use blood and tumor samples from these patients. Propose a set of experiments using these sample to test your hypothesis. This should include one *in vitro* and one *in vivo* approach. Provide an experimental design, specifying the experimental groups including control groups. Discuss potential outcomes and whether they support or refute your hypothesis.

Answer A:

- Changes in drug pharmacokinetics due to increased adiposity with age.
- Decreased proliferation of target cells. Chemotherapies preferentially target rapidly proliferating cells. If the aged microenvironment induces a senescent-like phenotype in malignant cells, the efficacy of chemotherapies will decline.

Answer B:

- Acceptable Hypotheses: 1) Aging-associated immunosenescence [compromised adaptive immunity and augmented immunosuppressive mechanisms], and 2) Aging-associated increases in chronic inflammation which create a more permissive microenvironment for the initiation and progression of malignant cells

- Confounders: Differences in gender, race, underlying conditions [diabetes, cardiovascular disease, etc.], weight, chronic infections, *etc.*

Answer C:

In vitro:

- [**Immune Functional Assays**]
  - Option 1: Isolate T-cells (MACs selection or FACs-sorted) from NSCLC tissue homogenates from the groups studied above. Stimulate with PMA/Ionomycin or αCD3/αCD28 and assess T-cell proliferation, cytokine production, and the induction of cytolytic mediators using flow cytometric analysis (controls=unstimulated T-cells).
  - Option 2: Immunophenotyping Experiments. Perform targeted analysis of activating and inhibitory proteins expressed on tumor-resident immune cells in NSCLC tissue homogenates. Given the focus of this study, you should conduct surface staining experiments for PD-1 expression (inhibitory protein) on $CD4^+$ and $CD8^+$ T-cells using flow cytometry. T-cells could also be stained for CD44, CD69, and CD62L as proxies for more functional T-cells (via flow cytometry). Antigen presenting cells (**APCs**) could be surface stained to detect levels of T-cell stimulatory (CD80, CD86, CD40, and MHC molecules) and inhibitory (PD-L1, PD-L2, etc.) proteins (controls= unstained and isotype control stained samples).
  - Option 3: Single-cell RNA-sequencing or Nanostring experiments using RNA isolated from NSCLC tissue from patients in your study. These global approaches will allow you to assess multiple immune cell populations in

your samples as well as their activation state (using the gene expression profiles for the markers described above).

- [**Cytokine Profiling Assays**]
  - Option 4: Perform Luminex (for global cytokine/chemokine profiling) or ELISA (targeted cytokine studies) analysis on serum collected from patients. You would determine the levels of cytokines known to promote T-cell mediated immunity (IL-12, IL-2, IFN-γ, etc.) and those that suppress T-cell activation (IL-10, TGF-β, high levels of TNF-α, etc.). You would focus on cytokines that regulate T-cell function in your experiments due to the usage of αPD-1 antibody treatment in these patients.

In vivo:
Patient-derived xenograft (PDX) experiments would be performed using samples from this study. Once cancer cells are transplanted, and signs of morbidity are observed, mice would receive weekly treatments with αPD-1 antibody or control immunoglobin. After treatment initiation, mice should be bled weekly to determine the activation state of T-cells and APCs using the methods described above. Mouse survival would also be assessed.
In parallel experiments, mice would be left untreated to determine disease progression. In these experiments, immunophenotyping of circulating blood cells would begin 2 days post-transplantation and occur weekly thereafter. Mouse survival would also be assessed.

D31
(Rafiq)

Your lab designed a novel chimeric antigen receptor (CAR) against a tumor-associated antigen found on solid tumor X. In preclinical testing, these CAR T cells were effective in killing tumor cells *in vitro* cytotoxicity assays as well as eliminating established tumors engrafted in immunodeficient (NSG) mice. Phase I trials conducted in patients with relapsed/refractory solid tumor X treated with the CAR T cells demonstrated that the CAR T cells were well tolerated and there are no dose-related toxicities. However, no objective clinical response was seen.

a. (1 pt) Propose two reasons why the preclinical data did not accurately predict or correspond to the patient data.

b. (3 pts) Correlative analysis performed on tumor biopsies from the trial show that CAR T cells are trafficking to the tumor but are only found on its periphery. You hypothesize that solid tumor X creates a microenvironment that is functionally suppressive to the CAR T cells. Design an experiment with proper controls to identify a factor by which the tumor cells are suppressing the CAR T cells. Include anticipated results and how you would interpret them.

c. (3 pts) After identifying a factor by which the tumor could be creating a hostile microenvironment in (b), you next want to validate this factor's effect on CAR T cell function and determine the mechanism of suppression in T cells. Design an experiment with proper controls to test this and include anticipated results and how you would interpret them.

d. (3 pts) Given what you have learned in questions (b) and (c), hypothesize a therapeutic strategy to overcome CAR T cell suppression in the solid tumor X microenvironment and design an experiment with proper controls to test your hypothesis.

Answer A: Important CAR T cell functions such as cytokine production, expansion and persistence were not tested in vitro and the xenograft models may not be informative enough for these. Also, these experiments did not take into account the tumor microenvironment.

Answer B: This question is asking what tumor-specific factors are suppressing the T cells. This could include suppressive ligands, cytokines, and/or suppressive cell types. There are a number of experiments that can be proposed here such as mass cytometry, immunofluorescence, or RNA-Seq of the tumor and microenvironment. This could be performed on human tumor samples or syngeneic mouse models of the cancer.

Answer C: This question is asking how you would validate the factor identified in b is indeed suppressive and determine the mechanism by which it suppresses T cell function. For instance, if a ligand expressed on tumor cells is suppressive, this would be validated by expressing this ligand on cells and co-culturing T cells with it to verify suppression of functions such as cytokine release and proliferation. The mechanism of suppression could be elucidated by identifying the ligand's binding receptor on T cells or the signaling pathway involved. This applies similarly to cytokines or suppressive cells and there are many different approaches that could be proposed to study this.

Answer D: Depending on what is proposed in b and c, this answer can be to include combination therapies such as with checkpoint blockade or cytokines to enhance CAR T cells function or oncolytic viruses, radiation, etc. to convert the suppressive environment to an

inflammatory one. This answer can also be an armored CAR T cells that is engineered to express cytokines/blocking scFv or with genetic knockout of receptors. These experiments would ideally be in immunocompetent mice.

Question D39
(Ned Waller)

Describe an experimental approach to test a new cancer immunotherapy.

a. (2 pts) What is the mechanism of action for the new immunotherapy that you will be testing?

b. (4 pts) Provide a set of preclinical experiments to test the new immunotherapy. What considerations inform your choice of a model system? What would be the primary end-point to evaluate this new therapy? What secondary end-points would you use to evaluate efficacy? How would you evaluate toxicity? Provide an experimental design, showing experimental groups including control groups. Discuss the anticipated results and their interpretation, including how results from the control groups would inform your analysis of the results with the other experimental groups.

c. (4 pts) Based upon promising results in the model system, you wish to conduct a clinical trial. What would be your concerns regarding about translating these results into a human clinical trial?

Answer A Options:
Adoptive therapy with chimeric antigen receptor (CAR) T cell or NK cell therapy- Cytotoxic attack of cancer cells based upon expression of antibody on the surface of effector cells that recognize a cancer-associated antigen on the surface of the cancer cell. Direct binding of effector cell to cancer cell with activation of cytolytic program. Effector cells can be autologous or allogeneic.

Or

Adoptive therapy with recombinant TCR receptor T cell or NK cell therapy- Cytotoxic attack of cancer cells based upon expression of expression of recombinant TCR on effector cells that recognize a cancer-associated peptide expressed on the surface of the cancer cell in the context of restricting MHC element. Direct binding of effector cell to cancer cell with activation of cytolytic program. Effector cells can be autologous or allogeneic.

Or

Adoptive therapy with T cell or NK cell therapy derived from tumor-infiltrating lymphocytes. Endogenous anti-cancer T cells (or NK cells) with a diverse repertoire of surface receptors that recognize antigens/peptides presented on the surface of cancer cells are collected form the patient's blood (NK cells) or tumor (T cells), expanded ex vivo, then reinfused into the same patients from which they were harvested. Direct binding of effector cell to cancer cell with activation of cytolytic program. Effector cells are autologous (T cells) or can be allogeneic (NK cells).

Or

Activated macrophages that have down-regulated SIRPalpha. Monocytes are harvested form patient, expanded ex vivo into SIRPalpha low macrophages and reinfused back into

patient- directly iv or intra-tumoral. Macrophgaes phagocytoses tumor, present peptides derived from tumor -associated antigens to endogenous T cells leading to their activation and indirect cytolytic attack on the tumor.

Or

Infusion of an antibody or small molecule that blocks immune check-point signaling. T cells that are normally constrained by signaling through an immune check-point receptor are activated and lead to cytolytic attack on tumor.

Or

Infusion of a monoclonal antibody to a tumor-associated antigen. Antibody binding to the tumor cell may lead to direct complement-mediated cytolysis, recruitment of NK cells with cytolytic activity via ADCC, inhibition of key signaling molecule on the surface of the cancer cell that leads to tumor cell death. Variation is infusion of a monoclonal antibody coupled to a toxin or radio-pharmaceutical.

Or

Infusion of a bi-specific antibody with specificity to a surface molecule on the cancer cell and a surface receptor on a T cell or NK cells. Binding of the bi-specific antibody activates effector cell in close proximity to the tumor cell which is killed via cytolysis.

Or

Infusion of an agonists monoclonal antibody or drug that activates T cells or NK cells (i.e., agonistic antiCD40L antibody). Activated effector cells mediate anti-tumor cytolysis.

Or

Infusion of cytokines that lead to expansion of T cells or NK cells which then have cytolytic activity against the tumor cells (i.e., IL15)

Answer B: Scoring would include the student touching upon the following:

1. Animal model system with intact immune system- mouse, dog, etc... Or humanized NSG mice. In vitro systems would not fully recapitulated interactions of immune cells that are responsible for cancer immunotherapy efficacy. Treatment of animals with established tumor is more relevant to clinical practice that treating simultaneously with tumor challenge. Treatment of animals that develop spontaneous or natural tumors (dogs) is more relevant than transplantation of a cancer cell line.
2. Primary end-point would be tumor regression and/or tumor-free survival.
3. Secondary end-points would be frequency of activated, tumor-specific effectors, infiltration of effectors into tumor microenvironment, cytokine levels, and elimination of metastases.
4. Control groups would include: no effectors, irrelevant effector infused, animals with no tumor, animals lacking specific effector populations (in case of immune check-point antibodies, BITES, etc..)

Answer C:

1. Transplantable murine cell lines may not be isogenic with host (i.e., presence of xeno-protein (i.e., GFP, luciferase, etc..) or genetic drift from original derivation.
2. Implanting tumor may cause an initial inflammatory response that is not seen in human when tumors develop spontaneously.
3. Results with inbred mice using clonal transgenic T cells may not transplant with fidelity to out-bred human population.
4. Relevance of signaling pathways may not be uniform across species- relevant to cytokines and antibodies that affect signaling pathways (i.e., anti-PD1)
5. Immune activation pathways may be distinct between mice/primates and humans, and humans may experience unexpected toxicity.
6. Scale of expansion of cells for mice very different than humans; high expense of cell therapy in humans.
7. Human tumors do not express the same molecules present on mouse tumors (i.e., murine CD20 not the same as human CD20).

Question D43
(Yun)

Activation of the transcription factor TRIM1 has been documented in a wide range of tumors, including colorectal cancer (CRC). Your goal is investigate the role of TRIM1 in tumorigenesis using a loss-of-function approach in intestinal epithelial cells (IECs) in mice. For this purpose, you have crossed $Trim1^{f/f}$ mice with villin-cre to generate $Trim1^{\Delta IEC}$ mice, which lack Trim1 expression in IECs. $Trim1^{\Delta IEC}$ mice were then treated with the somatic mutagen, azoxymethane, (AOM) ,and tumor development was examined 20 weeks later. AOM induced tumors in wild type (wt) mice, but not $Trim1^{\Delta IEC}$ mice. Consistent with this, AOM-treated $Trim1^{\Delta IEC}$ mice survived much longer than AOM-treated wt mice.

a. (2 pts) Histological examination of AOM-challenged $Trim1^{\Delta IEC}$ mice revealed a pronounced accumulation of lymphocytes in mouse intestinal mucosa. You suspect these are T-lymphocytes, but based on the morphological features under a light microscopy you are not sure if these are CD4+ or CD8+ cells. Describe an experiment to determine if these are CD4+ or CD8+ T cells.
b. (4 pts) Based on your experiment, you found out that these are CD8+ T cells. However, you are not sure whether the survival advantage of $Trim1^{\Delta IEC}$ mice is dependent on the adaptive immune response rather than on Trim-dependent IEC autonomous effects such as proliferation. Describe an experiment how you will test this. Provide an experimental design showing experimental groups and control groups. Discuss anticipated results and their interpretation.
c. (4 pts) In order to find the underlying mechanism, you consulted a pathologist friend to look at the histochemical feature of tumors of wt and $Trim1^{\Delta IEC}$ mice treated with AOM. Your friend found double membrane-bounded cytosolic vesicles in IECs of $Trim1^{\Delta IEC}$ mice but not in those of wt mice. Based on your knowledge, the presence of double-membrane vesicles is a feature of autophagosomes, which is formed by autophagy. Propose a hypothesis how Trim1 loss prevents tumor formation. Describe one experimental approach that you could use to test your hypothesis. Provide an experimental design showing experimental groups and control groups. Discuss anticipated results and their interpretation.

Answer A: (IHC or FACS analysis)

Answer B: (use neutralizing anti-CD8 antibody or CD8 knockout mice.)

Answer C:
(Hypothesis- Trim1 loss activates autophagy which induces adaptive immune response to activate CD8 T cells.

1) Experiments – Test whether autophagy is activated by Trim1 loss. Determine autophagy flux and determine whether expression of genes regulating autophagy is altered. To test the role of autophagy in CD8 cell infiltration, inhibit autophagy using an inhibitor like chloroquine or a genetic approach such as breeding ATG5 KO mice with Trim1 KO mice to test tumor formation by AOM and determine mice survival)