AWARD NUMBER: W81XWH-16-1-0119

TITLE: Understanding the Immune Biology of Checkpoint Inhibitors to Develop New Strategies for Therapy

PRINCIPAL INVESTIGATOR: Meenhard Herlyn

CONTRACTING ORGANIZATION: The Wistar Institute of Anatomy & Biology
Philadelphia, PA

REPORT DATE: January 2021

TYPE OF REPORT: **Final Report**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
January 2021	Final	30Sep2016-29Sep2020

4. TITLE AND SUBTITLE

Understanding the Immune Biology of Checkpoint Inhibitors to Develop New Strategies for Therapy

5a. CONTRACT NUMBER
W81XWH-16-1-0119

5b. GRANT NUMBER
CA150619

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Meenhard Herlyn

E-Mail: **herlynm@wistar.org**

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Wistar Institute
3601 Spruce Street
Philadelphia, PA 19104-4265

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES N/A

14. ABSTRACT

This proposal deals with the development of novel treatment strategies for the deadliest of all skin cancers, melanoma. The disease is generally caused by excessive ultraviolet light (sun) exposure. Once the tumor cells have spread from the skin to other organs, there is only a ~15% chance of surviving more than five years. This may change because in the last five years seven new treatments have been approved by the Food and Drug Administration, each improving survival of patients and/or their well-being. The clinical and scientific evidence clearly points to the potential for further improvements in treatment outcome, mainly by combining different drugs. The core of the proposal is a clinical trial for the treatment of advanced melanoma, in which two newly-approved drugs are tested in combination when compared to the stronger drug alone. Now we need to extract as much information as possible from the trial by monitoring the blood and tumor before and during treatment and, in case the patients become resistant and progress with disease, after therapy. Ideally, we want to combine the stimulation of the immune system with new inhibitors that block MAPK signaling pathways in the cancer cells, but the initial studies done by others had to be stopped due to toxicities from the treatment. While we can progress in developing new strategies in patients, we need to advance faster. Therefore, we want to do all initial treatment work in experimental animals. To date, this has been difficult because the animal models do not faithfully reflect the human disease and, if they do, important components such as the immune (defense) system are missing. We have now developed two novel models of cancer, in which mice bear the human cancer cells but at the same time have a human immune system that is developed from blood stem cells. One model uses immune stem cells from newborns and the other uses stem cells from the same patients we have obtained tumors from. These models will allow us to investigate the mechanisms of tumor destruction (response) and tumor progression (resistance) by the drugs and also to develop novel rationale-guided combination therapies with a variety of drugs.

15. SUBJECT TERMS
BRAF, CTLA4, humanized mice, immune-checkpoint, immunodeficient, immune response, inhibitors, Ipilumumab, MAP kinase targeted-therapy, MEK, Nivolumab, program cell death (PD1), patient-derived xenograft (PDX).

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER**
Unclassified	Unclassified	Unclassified	Unclassified	13	***(include area code)***

TABLE OF CONTENTS

PAGE

1. INTRODUCTION:

Our long-term objective is to improve therapy outcome in melanoma. In the last few years, therapeutic responses in melanoma patients has considerably improved by using both targeted-therapies (signaling inhibitors) and immune-based therapies (anti-CTLA4 and anti-PD1 antibodies [immune checkpoints inhibitors]). BRAF inhibitor either alone or in combination with MEK inhibitor provided rapid clinical responses in patients; however, the therapy is applicable to ~50% of patients who carry the BRAFV600E mutation. Moreover, the responses are short-lived due to rapid development of acquired resistance. In contrast, clinical responses to immune-based (anti-CTLA4 or anti-PD1) therapies are slower but more durable targeting a broader cohort of melanoma patients. Recent reports, suggest that combined targeting of PD1 and CTLA4 provides synergy and more enhanced duration of clinical responses. This is the basis of our current ongoing clinical trial combining anti-PD-1 (Nivolumab [Nivo]), with anti-CTLA4 (Ipilumumab [Ipi]) at the MD Anderson Cancer Center. The trial, which is independently funded, provides the backbone of this collaborative grant between MD Anderson and The Wistar Institute with additional interactions with the University of Pennsylvania. Our overall objective is to combine targeted and immunotherapy to eradicate all malignant cells at the time of initial treatment because in each therapeutic strategy, a subpopulation of malignant cells can survive and subsequently develop acquired resistance. While in-depth molecular mechanisms of intrinsic and acquired resistance to BRAF and MEK signaling inhibitors in melanoma are known, very little information is available on why checkpoint inhibitors are active only in a subset (~25-30%) of patients, the cause of therapy non-responsiveness or resistance, or how to modify the immune response for achieving the highest therapeutic efficacy. In this collaboration, we are focusing on research efforts on mechanisms of action of checkpoint inhibitors *in vivo* to understand how we can optimally apply and translate them in patients and how we can use them in the future for designing combination studies that are rationale-based and personalized.

2. KEYWORDS:

BRAF, CTLA4, humanized mice, immune-checkpoint, immunodeficient, immune response, inhibitors, Ipilumumab, MAP kinase targeted-therapy, MEK, Nivolumab, program cell death (PD1), patient-derived xenograft (PDX).

3. ACCOMPLISHMENTS:

- **Major goals of the project.**

We incorporated a clinical trial into the study and compare the data from the trial with those in humanized mice. Thus, in Aim 1 we collect specimens from tumor and blood to monitor patients and provide material for experimental studies including the production of patient-derived xenografts (PDX).

In Aim 2 we proposed to develop new experimental models to investigate human immune responses to tumors. We proposed to develop two models, an HLA-matched model, in which the mice receive hematopoietic stem cells from either cord blood or fetal liver and then monitor the immune response to human melanoma; in the second model, we are taking blood from

patients to create induced pluripotent stem cells (iPS cells) that we can in turn differentiate to blood cells. This model was not complete yet and needed further fine tuning.

In Aim 3 we investigate the immune mechanisms and compare the data in patients with those in mice.

For Aim 1,

Major task 1 and all subtasks (1, 2 and 3) are completed at Wistar. Local IRB/IACUC approvals from Wistar has been completed.

Major task 2: Collection and analysis of patient tumor and blood samples.

(**Please refer to Annual report submitted independently by MD Anderson**).

For Aim 2,

Major task 2: Obtain PDX use approvals and select PDX to be used for model development.

1. Wistar IRB approval for use of patient tumor and blood samples for PDX usage were obtained (Protocol #2802240 [use of human melanoma tissues] approved and renewed on 13th July 2020 and Protocol # 21602279 was approved and renewed on April 1st, 2020.
2. IACUC approval for PDX inoculation continuation was approved on May 30, 2020.
3. HRPO and ACUORO approval July 2020.

- **What was accomplished under these goals?**

Specific Aim 1. Bio specimens and monitoring. MD Anderson will provide updated information in their Annual report. Original trial was closed due to high grade toxicities for combination therapy arm (Ipi + Nivo) and the low response to single agent Nivo arm. We have received 60 tumor specimens from 22 patients have been delivered to Wistar since June 1, 2016 for PDX generation. Amongst the 22 patients delivered to Wistar 12 of them are treated with PD1 (Nivo) therapy (1 patient received treatment off study) and 10 were treated with Nivo + CTLA4 (Ipi) therapies. Twenty PDX are established, 37 specimens failed, and 6 specimens are in the process of establishment and too soon to determine their status of establishment. For iPS production, 11 blood samples were received.

Specific Aim 2 (humanization of mice), we have injected 60 batches of 40 to 60 mice (each

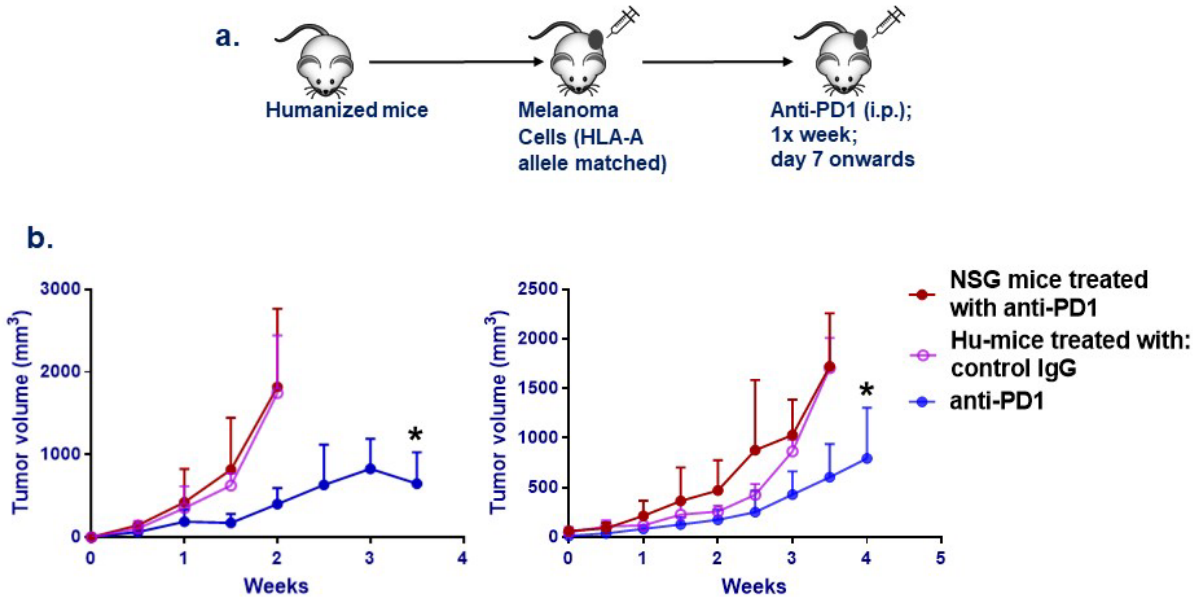


Fig. 1. a. Schema for anti-PD-1 therapy in Hu-mice. b. Mixed anti-PD-1 therapy response in established tumor model of Hu-mice. Two different batches of Hu-mice were challenged with HLA-A2 or HLA-A3 melanoma cells and followed with anti-PD-1 injections every 5 to 6 days for a total of 6 injections. Mixed therapy responses to anti-PD-1 was observed.

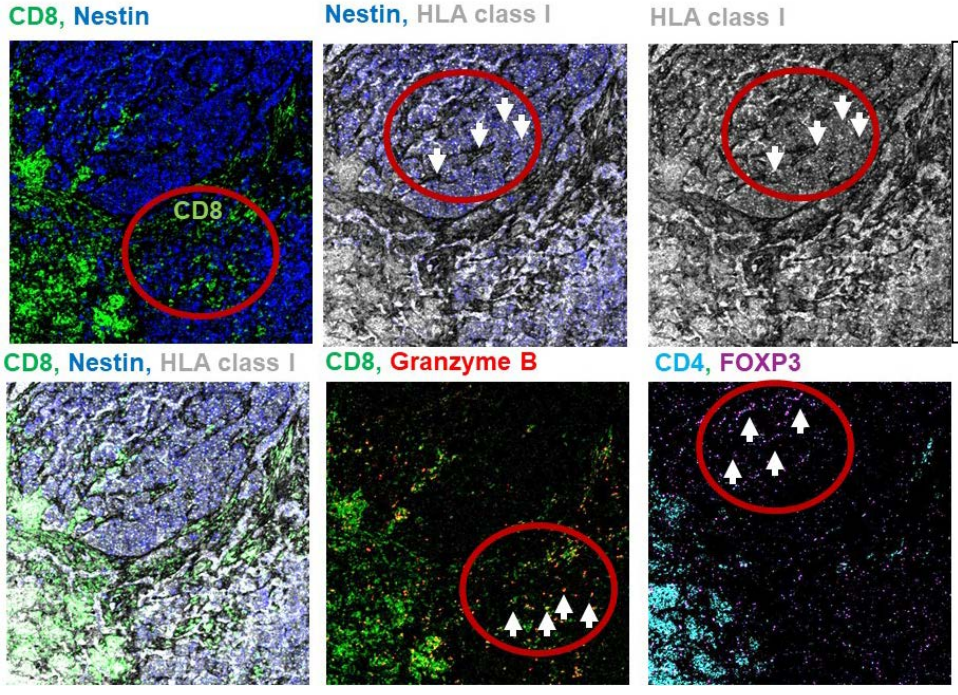


Fig. 2. Heterogeneous distribution of CD8+ Granzyme B+ cells, downmodulation of HLA-class I and increase in FOXP3+ cells in tumors after anti-PD-1 therapy.

batch) with CD34+ cells derived from fetal liver. We have completed therapy experiments with anti-PD-1 in 10 batches. We observed mixed responses to anti-PD-1 and none of the treated-tumor bearing Hu-mice showed complete regression (**see Fig. 1**). To understand the mechanism of resistance, tumor sections were examined by multiplex imaging using MassCyTOF and RNA seq studies. We found

heterogeneous distribution of CD8+ T cell infiltration, down-modulation of HLA class I molecules, and increased presence of FOXP3+ T cells and mast cells in areas where tumor cells survived (**see Figs. 2 and 3**). We have analyzed RNASeq dataset from Dr. Jen Wargo's clinical trials and the results showed that there is an increased number of mast cells in non-

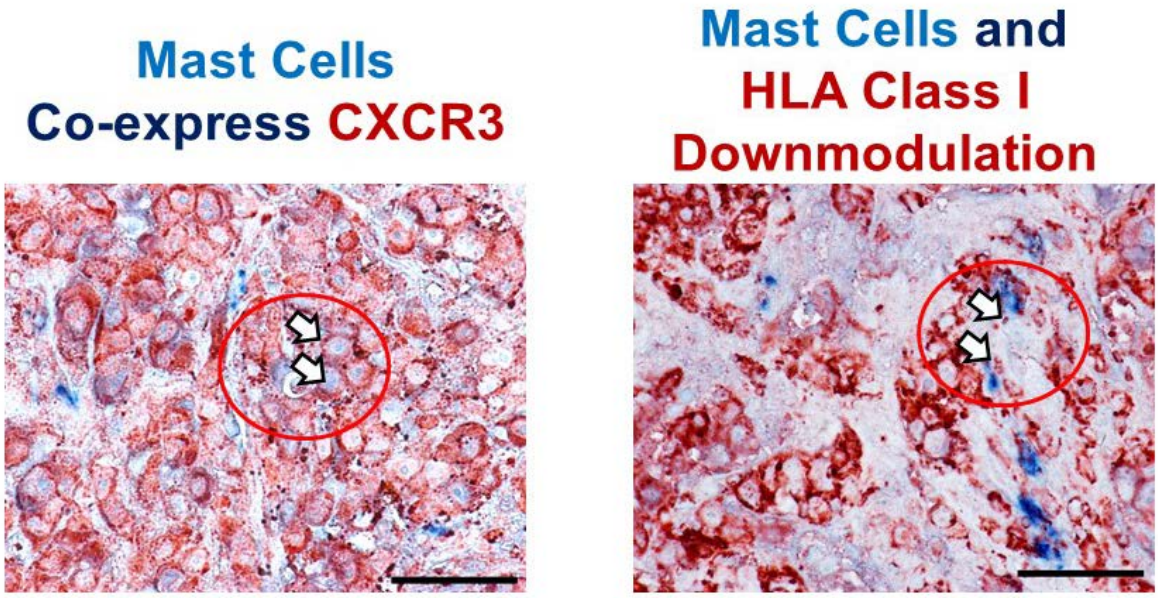


Fig 3. Mast cells co-express CXCR3 (left panel) and tumor cells secrete CXCL10 a ligand for CXCR3 and this results in attraction of mast cells to the tumor area. Mast cell infiltration causes HLA class I downmodulation (right panel).

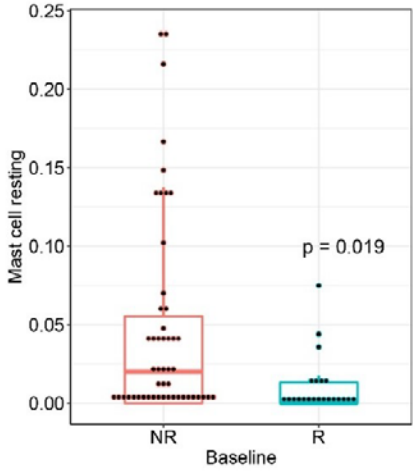


Fig. 4 Increased number of mast cells in immune-checkpoint therapy non-responders.

responders to immune-checkpoint therapy antibodies (**see Fig. 4**). Mast cells are generally c-kit+ and we have targeted them by using Sunitinib. Treatment of tumor-bearing Hu-mice by Sunitinib alone did not show restriction of tumor growth. However, a combination of Sunitinib and anti-PD-1 showed complete regression of tumors (**see Fig. 5**). Cured Hu-mice were given a drug holiday for 4 weeks and then re-challenged with fresh tumors. None of the tumors grew for 4 weeks to indicate there is a memory T-cell response that rejects freshly implanted tumors. We have repeated the experiment using a combination of Imatininb (targeting c-kit+ cells) and anti-PD-1. As in Sunitinib study, we observe complete regression of tumors. One another drug, Cediranib not so specific for c-kit but targeting VEGFR and PDGFR was included in the study and the tumors continued to grow. We have sequenced the T-cell receptors (TCR) from tumor infiltrating cells and found that sequences matched to TCR reactive to melanoma differentiation antigen (MART; **see Fig. 6**). We have identified new mechanism of

therapy resistance that involves mast cells and FOXP3+ cells (**see schema Fig. 7**). This work is accepted for publication in **Nature Communications**. Combination therapy with signaling

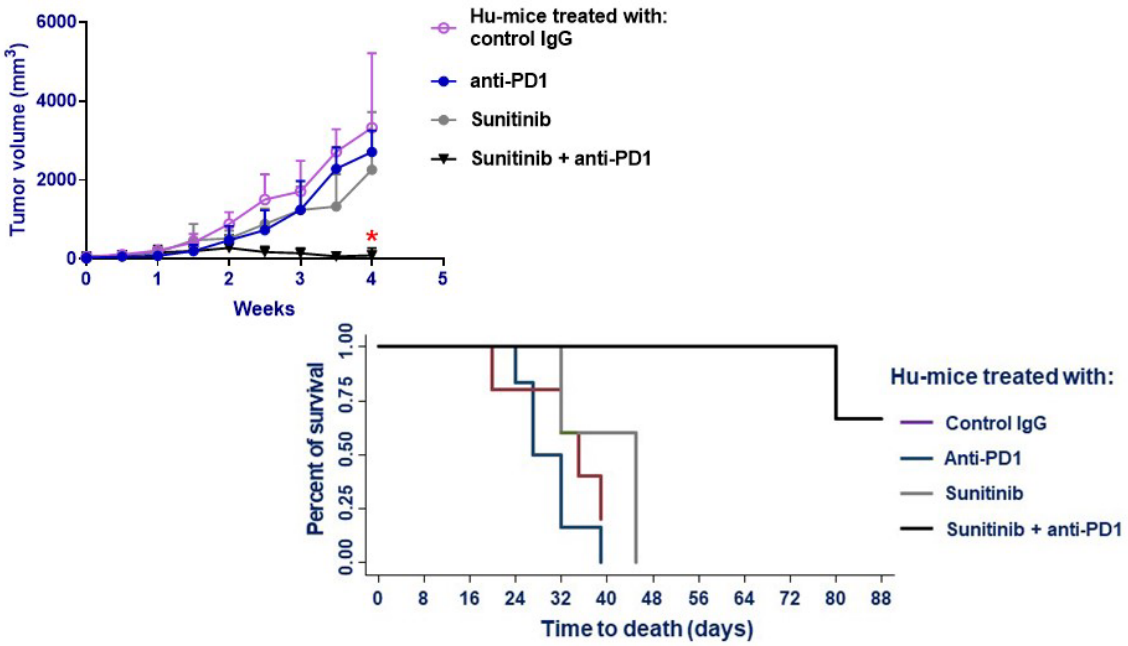


Fig. 5. Complete regression of tumors after combination of sunitinib and anti-PD therapy. Established tumors in Hu-mice were treated with sunitinib (20mg/kg) daily by oral gavage and after 72 h, anti-PD-1 therapy (10 mg/kg) was given weekly for a total of 6 injections. Complete tumor regression was observed in presence of combination therapy (**black inverted triangle; p<0.0001; top panel**), while sunitinib alone (**grey circles**), anti-PD-1 alone (**blue line, closed circles**) or control IgG (**magenta; open circles)** did not have any effect of tumor growth. **Bottom panel.** Survival curve from the above treated mice indicates significant (*p<0.0001) survival advantage of tumor bearing Hu-mice that received combination therapy of sunitinib and anti-PD-1 group when compared to sunitinib alone **(grey),** or anti-PD-1 alone **(dark blue)** or control IgG **(magenta)** groups**.**

inhibitors (BRAF) and anti-PD-1 is underway. We have made enormous progress with the iPS model (subtasks 5 and 6). Combination therapy with signaling inhibitors (BRAF) and anti-PD1 are underway (subtask 7). We made progress with the iPS model (subtasks 5 and 6). Several batches of CD34+ cells derived from iPS has been injected into mice into the tail vein for lymphocyte reconstitution. We consistently observe increased presence of CD45+ cells in in the mouse peripheral blood. However, percentage of CD45+ cells in circulating blood is still low (~10%). We have transduced CD34+, mCherry promoter+ with GATA3 or FOS transcription factors to aid in accelerated lymphoid cell differentiation. Initial observations suggest that the transduction of CD34+ cells with above transcription factors promote CD5+ lymphoid cell differentiation. Several batches of liver or iPS derived-CD34+ were transduced with CD45, mCherry promoter construct for live tracing of these cells in NSG mice. Thymic progenitor cells transduced with FOXN1 are implanted in the renal capsule to aid in the lymphoid cell differentiation of iPS-derived CD34+ cells. We have developed thymic progenitor organoids to

propagate thymus cells that is suitable for grafting. George Xu continues to offer histology staining support for iPS derived CD34s and thymic progenitor cells.

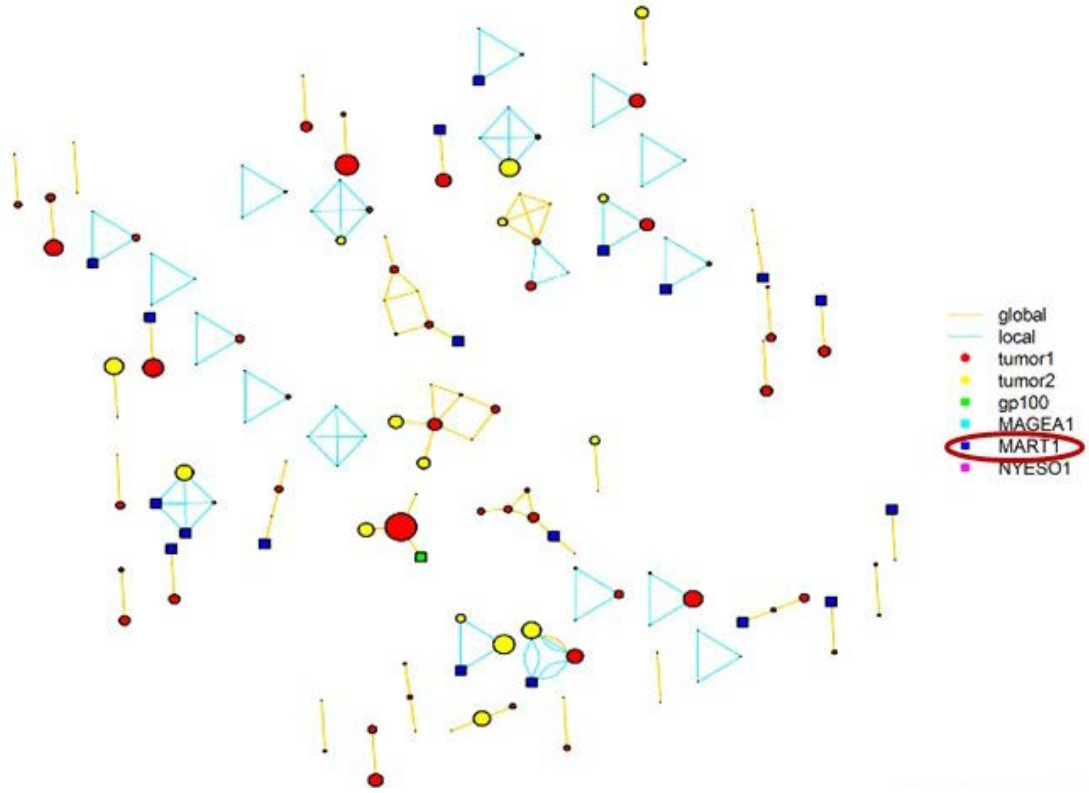
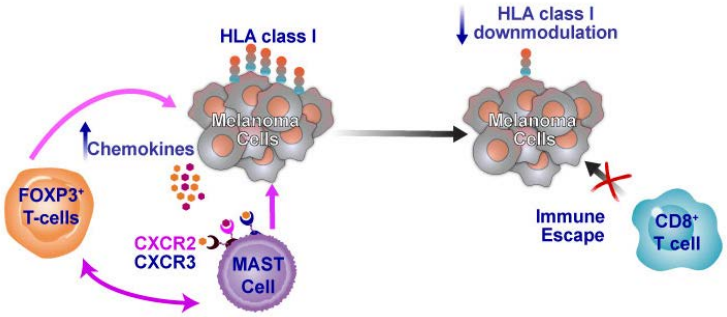


Fig. 6. MART-1 reactive T cells were found in TILs of tumor challenged mice. TcR sequences analysis revealed the presence of anti-MART-1 reactive T cells in TILs of tumor cells obtained from Hu-Mice.

Fig. 7. Proposed schema of new therapy resistance mechanism to anti-PD-1 therapy. Co-localization of mast cells and Foxp3+ causes downmodulation of HLA-class I molecules that results in lack of CD8+ T-cell mediated lysis of tumor cells. This results in immune escape of tumor cells.



- **What opportunities for training and professional development has the project provided?**

Nothing to Report.

- **How were the results disseminated to communities of interest?**

Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

This is a Final report.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**

Nothing to Report.

- **What was the impact on other disciplines?**

Nothing to Report.

- **What was the impact on technology transfer?**

Nothing to Report.

- **What was the impact on society beyond science and technology?**

Nothing to Report.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report.

- **Changes that had a significant impact on expenditures**

Nothing to report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

- **Significant changes in use or care of human subjects**

Nothing to report.

- **Significant changes in use or care of vertebrate animals.**

Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Somasundaram R, Connelly T, Choi R, Choi H, Samarkina A, Li L, Gregorio E, Chen Y, Thakur R, Abdel-Moshen M, Beqiri M, Kiernan M, Perego M, Wang F, Xiao M, Brafford P, Yang X, Xu X, Secreto A, Danet-Desnoyers G, Traum D, Kaestner KH, Huang A, Hristova D, Wang J, Fukunaga-Kalabis M, Krepler C, Ping-Chen F, Zhou X, Gutierrez A, Rebecca VW, Vonteddu P, Dotiwala F, Bala S, Majumdar S, Dweep H, Wickramasinghe J, Kossenkov AV, Reyes J, Santiago K, Nguyen T, Griss J, Keeney F, Hayden J, Gavin BJ, Weiner D, Montaner LJ, Liu Q, Peiffer L, Becker J, Burton EM, Davies MA, Tetzlaff MT, Muthumani K, Wargo JA, Gabrilovich D and Herlyn M.

Tumor-infiltrating mast cells are associated with resistance to anti PD 1 therapy. ***Nature Communications, in press, December 2020.***

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Yes, Hu-mice model.

- **Inventions, patent applications, and/or licenses**
- Humanized Mouse Model, PCT/US2019/026552, was filed on 4/9/2019 (18-23)
- Engineered Optimized Cytokine Compositions", PCT/US2019/026562, was filed on 4/9/2019 (18-32)

- **Other Products**

No

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Meenhard Herlyn
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.2 cal month
Contribution to Project:	Project planning and coordination.
Funding Support:	This award

Name:	Rajasekharan Somasundaram
Project Role:	Research Assistant Professor
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.6 cal month
Contribution to Project:	Humanized model #1. Supervision of technicians.
Funding Support:	This award
Name:	Min Xiao
Project Role:	Research Assistant IV

Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.6 cal month
Contribution to Project:	Growth of PDX.
Funding Support:	This award

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report.

- **What other organizations were involved as partners?**

Nothing to report.

8. APPENDICES:

None