AWARD NUMBER: W81XWH-16-1-0128
OC150282

TITLE: Generation of a Suppressor tRNA-Mediated Antitumor Immune Response to Treat Ovarian Cancer

PRINCIPAL INVESTIGATOR: Thomas Weber

CONTRACTING ORGANIZATION: Icahn School of Medicine at Mount Sinai
One Gustave L. Levy Place
New Yor, NY, 10029

REPORT DATE: SEPTEMBER 2019

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
SEPTEMBER 2019	Final	15MAY2016 - 14MAY2019

4. TITLE AND SUBTITLE

Generation of a Suppressor tRNA-Mediated Antitumor Immune Response to Treat Ovarian Cancer

5a. CONTRACT NUMBER
W81XWH-16-1-0128

5b. GRANT NUMBER
OC150282

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Thomas Weber

E-Mail: Thomas.weber@mssm.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Icahn School of Medicine at
Mount Sinai
One Gustave L. Levy Place
New York, NY, 10028

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

14. ABSTRACT

In this project we were able to demonstrate that vaccination with lethally irradiated ovarian cancer cell lines that have been transfected with suppressor tRNAs protect against a subsequent challenge with live ovarian cancer cells. More importantly, vaccination with lethally irradiated ovarian cancer cell lines that have been transfected with suppressor tRNAs in animals that have been previously inoculated with live ovarian cancer cells reduced tumor growth ($p<0.0001$) and increased survival ($p<0.0001$) when compared to all the control groups. We were also able to develop a novel approach that will greatly facilitate the nature of neoantigen peptides on class I MHCs.

15. SUBJECT TERMS
Ovarian cancer, immunotherapy, suppressor tRNA, epitope spreading

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	12	USAMRMC

Table of Contents

Final PROGRESS REPORT

4. Introduction

This project was aimed at developing a novel immunotherapy approach to treat ovarian cancer. The basic premises of this approach are that 1) the introduction of suppressor tRNAs into tumor cells will result in translational readthrough through stop codons resulting in proteins with C-terminal extensions, 2) that proteins with these extensions are recognized as non-self and, hence, are *bona fide* neoantigens, 3) vaccination with lethally irradiated tumor cells transfected with suppressor tRNAs will trigger an immune response against these neoantigens and 4) that through intra- and intermolecular epitope spreading this will result in an immune response that leads to the rejection of the primary tumor and potential metastasis.

5. Keywords

Ovarian cancer, immunotherapy, suppressor tRNA, epitope spreading

6. Accomplishments

Task 1: Overexpression of Suppressor tRNAs in HM1 cells

Optimization of Transfection of ID8 Cells and Establish the Read through Efficiency upon Transfection with Suppressor tRNAs.

As described in our previous progress reports, the original cell line that we planned to use for our animal experiments, HM1, was not suitable and we had to change our ovarian cancer cell line to a subclone of ID8 cells.

We demonstrated in our previous report that we are able to transfect efficiently ID8 cells and that we can achieve read-through efficiencies of ~50%. To be able to complete successfully the experiments described in our original proposal,

Analysis of the Identity of C-Terminal Extension Peptides Presented on MHC Class I Molecules Upon Transfection of HM1 (ID8) Cells with Suppressor tRNAs

In this period, we created a plasmid that allows the metabolic biotinylation of MHC I alpha chain at their C-termini (Fig. 1A). To this end, we added a biotin acceptor peptide (BAP) to the C-terminal part of the cytosolic domain of H2-Kb (Fig. 1A). When these plasmids are co-transfected with a plasmid encoding the E. coli biotin ligase BirA, the H2 α-chains will be biotinylated at their C-termini (Fig. 1A). To test if we can capture MHCI complexes with peptides and then elute the peptides from the beads, we co-transfected ID8 cells with a MHCI(H2kb)-BAP, Bir A, GFP-OPAL-ovalbumin and opal suppressor tRNA. Doing so, will lead to the production of a GFP-ovalbumin fusion protein and biotinylated MHCI(H2kb). It is well known that in cells expressing MHCI(H2kb) and ovalbumin the dominant peptide SIINFEKL will be presented on the MHCI complexes. To demonstrate that we could capture MHCI complexes with bound SIINFEKL, we lysed the cells and then incubated the lysates with streptavidin-polystyrene beads. We then analyzed the beads by FACS with an antibody against MHCI(H2kb)-SIINFEKL. As can be seen from Fig. 1C we could not only capture these complexes but also elute them from the beads by acid treatment. SIINFEKL eluted from the beads could also be detected by mass spectrometry, although in low abundance (Fig. 1D). We are currently refining and scaling up our method in order to detect readthrough peptides by mass spec.

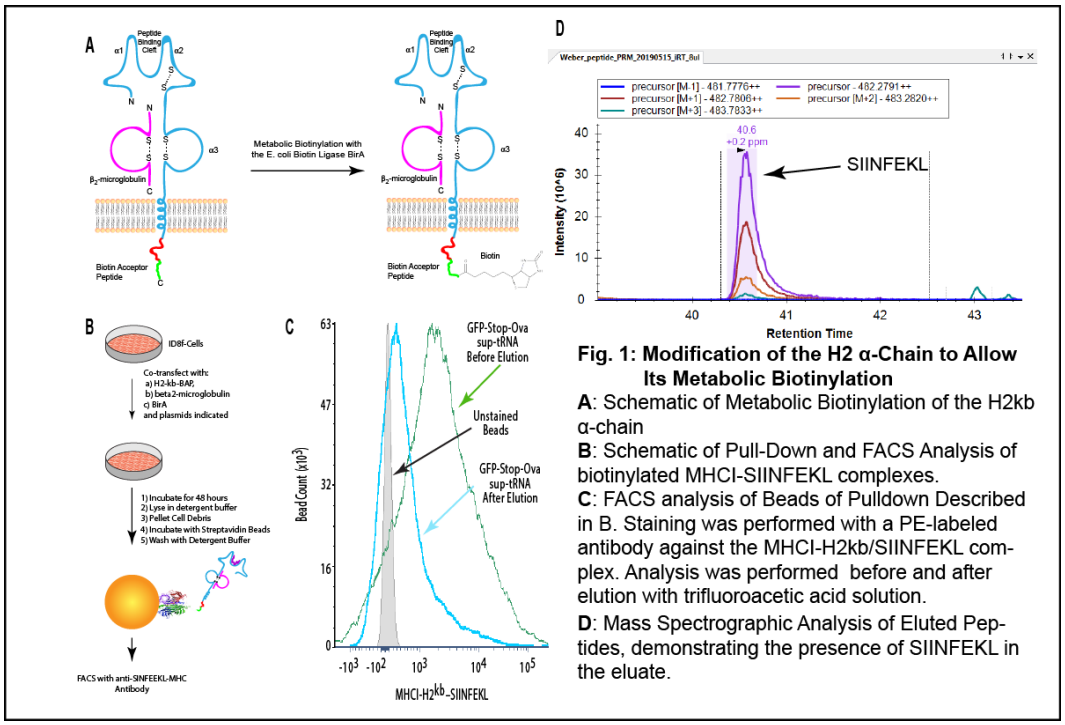

Fig. 1: Modification of the H2 α-Chain to Allow Its Metabolic Biotinylation

A: Schematic of Metabolic Biotinylation of the H2kb α-chain

B: Schematic of Pull-Down and FACS Analysis of biotinylated MHCI-SIINFEKL complexes.

C: FACS analysis of Beads of Pulldown Described in B. Staining was performed with a PE-labeled antibody against the MHCI-H2kb/SIINFEKL complex. Analysis was performed before and after elution with trifluoroacetic acid solution.

D: Mass Spectrographic Analysis of Eluted Peptides, demonstrating the presence of SIINFEKL in the eluate.

Milestone: 90%

Task 2: Obtain Regulatory Approval for Animal Experiments

We obtained regulatory approval by both the Icahn School of Medicine at Mount Sinai IACUC and the ACURO for the animal experiments described in our original proposal. We also obtained IACUC and ACURO approval to use ID8 cells instead of HM1 cells for the experiments described in our proposal.

Milestone: Completed June 2016.

Task 3: Test Effect of Immunization with Suppressor tRNA Transfected HM1 (ID8) Cells on Tumor Growth and Survival and Analysis of Immune Response in Preventive Model.

Establish number of HM1 (ID8) cells for tumor inoculation.

As mentioned above, we needed to switch our cell line to ID8 cells. As described in our previous report we determined that a dose of 5e6 ID8 cells results in a survival of ~6 weeks, which was suitable to proceed to determine the maximum number of lethally irradiated, untreated ID8 cells that would *not* prevent tumor growth in our prophylactic model.

Establish number of irradiated, non-transfected HM1 (ID8) cells that can be injected i.p. ***without*** *affecting tumor growth.*

In a next step, we established the cell number for inoculation in our preventive model. To this end, we first plated ID8 cells in regular medium and irradiated them in a X-ray irradiator for increasing amounts of time. The optimal dose to arrest cell growth, but not result in excessive cell lysis, was determined to be 150 Gy.

We then vaccinated mice with these lethally irradiated cells at days -14 and -7 followed by s.c. inoculation with 5e6 life ID8 cells (Fig.1). From these experiments we determined that the maximum number of cells per vaccination that did **not** have any effect on tumor growth following inoculation with 5e6 ID8 cells was 1e6 of lethally irradiated ID 8 cells.

Analysis of vaccination with suppressor tRNA-transfected, lethally irradiated ID8 cells on tumor growth and survival upon challenge with live ID8 cells.

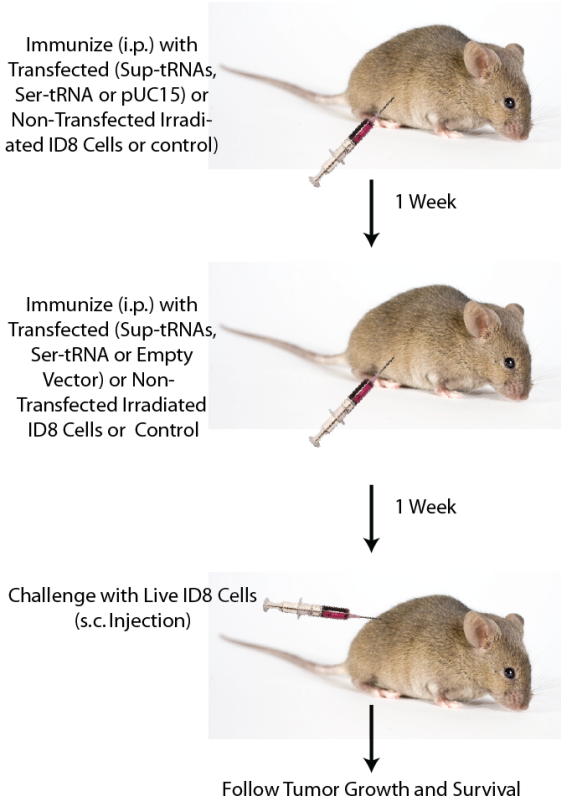

Figure 2: Vaccination Protocol in Preventive Model

As described in our proposal, we then proceeded to test our immunotherapy approach in a prophylactic model. In these experiments, we immunized C57/b6 mice at day -14 and day -7 with lethally irradiated ID8 cells that were either non-transfected, transfected with plasmid (pUC15), or pUC15 carrying the Ser-tRNA, ochre-tRNA, amber-tRNA or opal-tRNA gene. At day 0 the immunized animals, or non-immunized control animals, were injected s.c. with 5e6 live ID8 cells. We then followed tumor growth (twice a week) and survival. As can be seen from Fig. 3 tumor growth was significantly delayed in animals treated with suppressor tRNA transfected, lethally irradiated ID8 cells. Moreover, all groups of animals that have been vaccinated with suppressor tRNA transfected, lethally irradiated ID8 cells showed dramatically increased survival Fig. 4 and Table 1.

Analysis of immune response caused by immunization with irradiated, suppressor or Ser-tRNA transfected and non-transfected ID8 cells

We then started to analyze the immune response in the preventive model. Our initial experiments analyzing a cytolytic T-cell (CTL) response indicate that, as expected, splenocytes from animals that have been vaccinated with opal suppressor tRNA are able to kill $CD45^{-}$ (ID8) cells (Fig. 4). This demonstrates that epitope expression has indeed occurred and suggests

strongly that the reduced tumor growth and increased survival in the animals transfected with suppressor tRNAs is due to a CD8 T-cell response against **naïve** tumor cells.

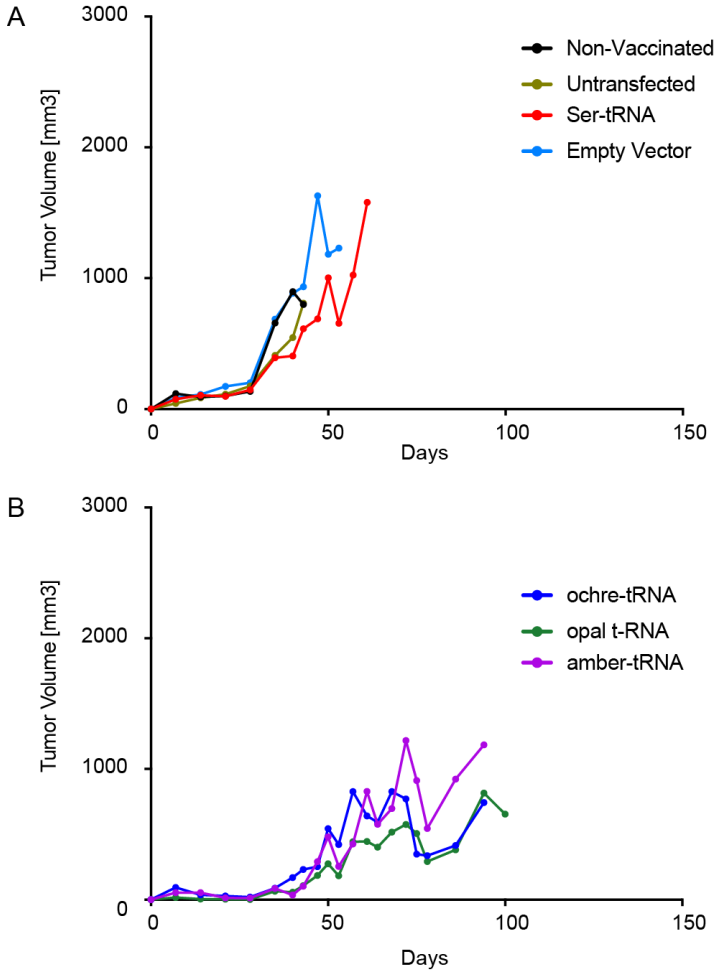

Figure 3: Tumor Growth in Animals in Control or Treatment Groups.

A) Animals were either not vaccinated (Non-Vaccinated), vaccinated with untransfected, irradidated ID8 cells or irradiated cells transfected with the empty vector or a vector containing the Ser-tRNA gene.

B) Animals were vaccinated with a vector containing the suppressor-tRNA gene.

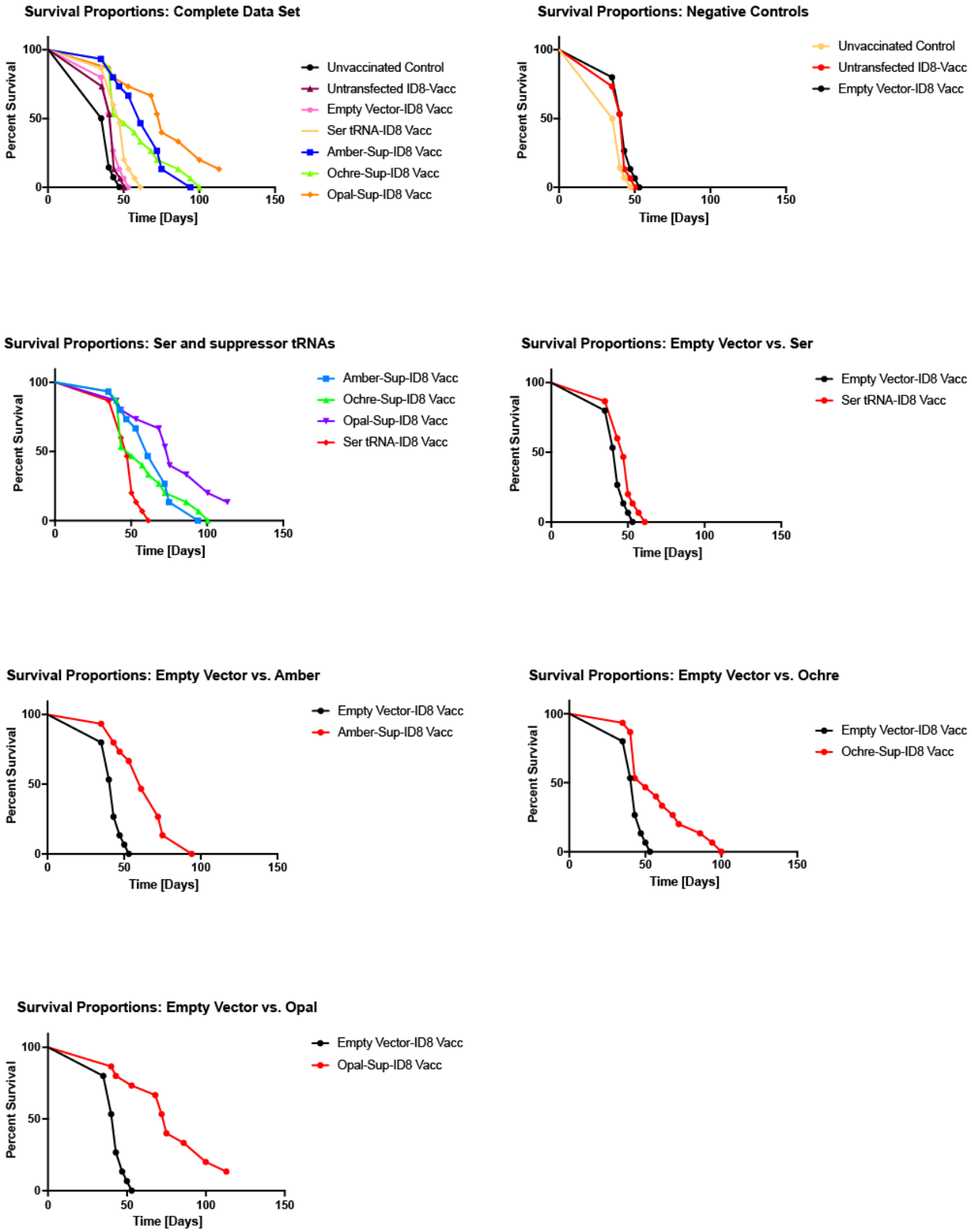

Figure 4: Survival of Animals in Control or Treatment Groups

A) Animals were either not vaccinated (Non-Vaccinated), vaccinated with untransfected, irradidated ID8 cells or irradiated cells transfected with the empty vector or a vector containing the Ser-tRNA gene.

B) Animals were vaccinated with an empty vector or a vector containing the suppressor-tRNA gene.

Survival vs. Empty Vector	p-Value (Log-Rank)
Unvaccinated	0.024
Non-Transfected	0.43
Empty Vector	N/A
Ser-tRNA Transfected	0.027
Ochre-tRNA Transfected	0.002
Amber-tRNA Transfected	<0.0001
Opal-tRNA Transfected	<0.0001

Table 1: Survival Statistics

Survival of each group was compared (Log-rank test) to animals vaccinated with ID8f cells that were transfected with empty vector.

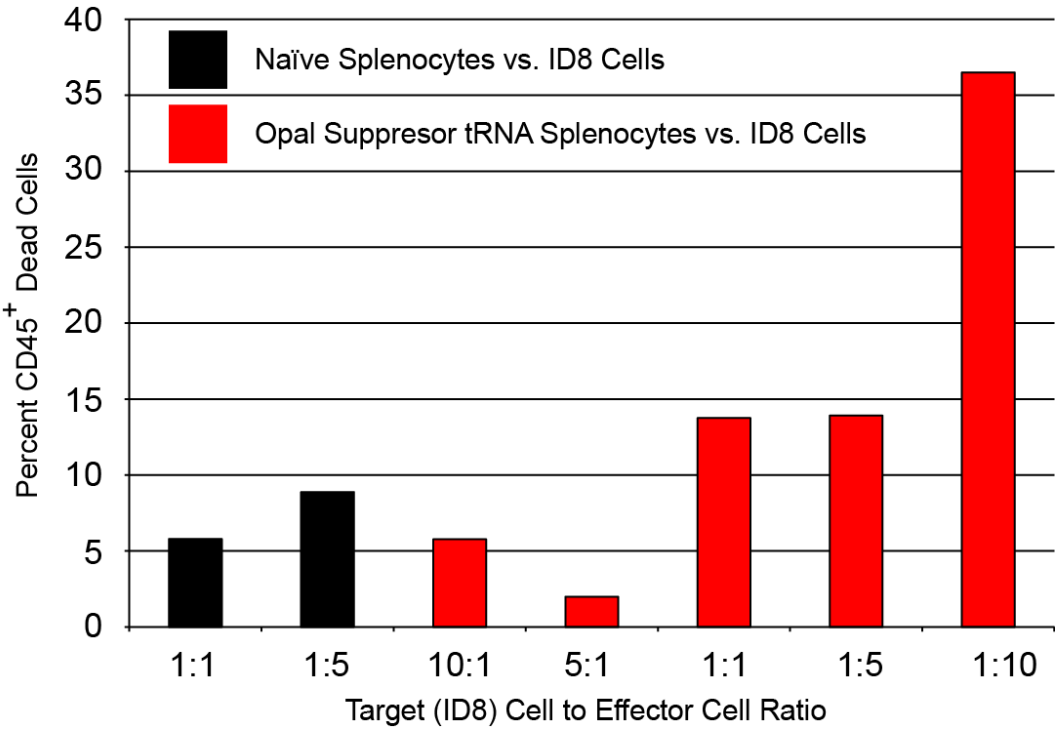

Figure 5: CTL Response Against Untransfected ID8 Cells

Milestone: 80% completed.

Task 4: Test Effect of Immunization with Suppressor tRNA Transfected HM1 (ID8) Cells on Tumor Growth and Survival and Analysis of Immune Response in Therapeutic Model.

Based on these very promising results, we proceeded to test our approach in a therapeutic model. In this model, at day 0, we injected mice s.c. with life tumor cells in their left flank. The animals were then vaccinated at day 3, day 6 and day 10 with lethally irradiated cells that were either not transfected or lethally irradiated cells that had been transfected with empty vector or a combination of all sup-tRNAs. As can bee seen from Fig. 4, tumor growth was dramatically decreased in animals that were vaccinated with cells that had been transfected with a combination of the three sup-tRNAs. Changes in tumor growth were analyzed over time via a mixed modeling procedure for repeated measures using PROC MIXED (SAS). This statistical analysis demonstrates that tumor growth in the animal group vaccinated with lethally irradiated ID8f cells that have been transfected with a combination of all sup-tRNAs is lower than tumor growth in all other groups ($p < 0.0001$).

Importantly, the animals vaccinated with lethally irradiated ID8f cells that have been transfected with a combination of all sup-tRNAs showed also increased survival compared to all other groups (Fig. 5, Wilcoxon rank-sum test: $p < 0.0001$).

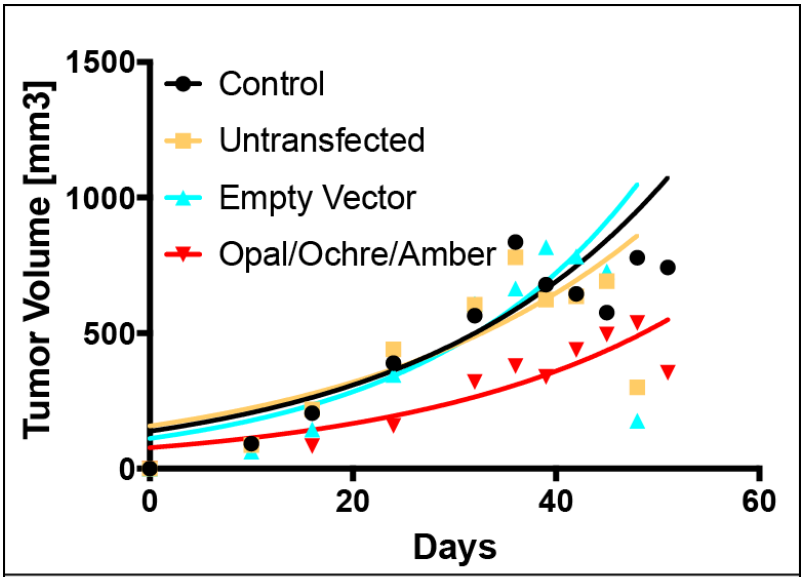

Fig. 6: Tumor Growth in a Therapeutic Model.
Tumor growth over time

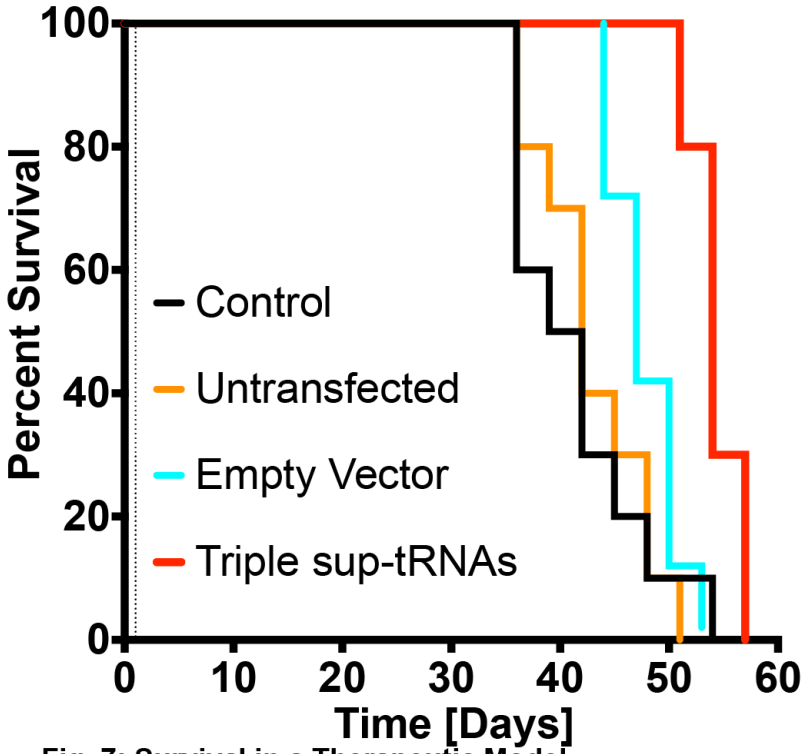

Fig. 7: Survival in a Therapeutic Model
Animals were inocculated with live ID8F cells by s.c. injection into the left flank. One group of animals was not vaccinated (Control). The animals in the other groups were vaccinated at days 3, 6 and 10 with lethally irradiated untransfected ID8f cells, or vaccinated with lethally irradiated ID8F cells that had been transfected with either empty vector or a combination of all thre suppressor tRNAs.

Milestone 75% complete

7. Impact

Our results described describe a completely novel approach for the immunotherapeutic treatment of (ovarian) cancer. Furthermore, we developed a novel approach to identify peptides of neoantigens presented by class I MHCs.

8. Changes/Problems

Not Applicable

9. Products

Nothing to report.

10. Participants

Dr. Thomas Weber, Dr. Nina Bhardwaj, Dr. Miriam Merad, Dr. Ananda Mookerjee

11. Special Reporting Requirements

Not Applicable.

12. Appendices

Not applicable