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TITLE: Optimizing Active Immunotherapy of Melanoma Through Metabolic Reprogramming of Melanoma Antigen-Specific CD8+ T Cells Combined with Checkpoint Blockade

PRINCIPAL INVESTIGATOR: Hildegund CJ Ertl

CONTRACTING ORGANIZATION: The Wistar Institute

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The Wistar Institute
3601 Spruce Street
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The grant focuses on testing the hypothesis is that the success rate of active immunotherapy of advanced melanoma based on vaccines or adoptive transfer of MAA-specific T cells can be optimized by metabolic reprogramming of T cells from glycolytic energy production towards the use of fatty acid oxidation. As we published, the interstitial fluids of melanomas have low glucose (Glc) contents while free fatty acid (FA) species increase during tumor progression. $CD8^+$ T cells upon activation in the periphery switch to glycolytic energy production. Once $CD8^+$ T cells enter the Glc-depleted environment of melanomas, starvation drives their differentiation towards functional exhaustion and apoptosis, unless they switch towards the use of alternative nutrients, such as FAs, for energy and biomass production. Metabolism can be modified by drugs, such as fenofibrate (FF), an agonist of PPAR-α. This in turn improves tumor infiltrating lymphocyte (TIL) functions, which results in more sustained tumor regression. $CD8^+$ TIL performance can be further enhanced by complementing metabolic reprogramming with a PD-1 checkpoint inhibitor, which in melanoma renders PD- $L1^+$ tumors cells more susceptible to cytolysis. These hypotheses are supported by our data.[22] Most of these studies were thus far conducted in mice using adoptive transfer models. Prior to clinical trials, the relevance of our findings for human tumors has to be confirmed using approaches that are suitable for use in melanoma patients.

15. SUBJECT TERMS

Cancer vaccine, mouse model, melanoma, $CD8^+$ T cells, metabolism, PPARa agonist, checkpoint blockade, human melanoma samples, iPDX model, NOD-SCID mice, human tumor transplantation, adoptive lymphocyte transfer.

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

   The grant focuses on testing the hypothesis is that the success rate of active immunotherapy of advanced melanoma based on vaccines or adoptive transfer of MAA-specific T cells can be optimized by metabolic reprogramming of T cells from glycolytic energy production towards the use of fatty acid oxidation. As we published, the interstitial fluids of melanomas have low glucose (Glc) contents while free fatty acid (FA) species increase during tumor progression. CD8+ T cells upon activation in the periphery switch to glycolytic energy production. Once CD8+ T cells enter the Glc-depleted environment of melanomas, starvation drives their differentiation towards functional exhaustion and apoptosis, unless they switch towards the use of alternative nutrients, such as FAs, for energy and biomass production. Metabolism can be modified by drugs, such as fenofibrate (FF), an agonist of PPAR-α. This in turn improves tumor infiltrating lymphocyte (TIL) functions, which results in more sustained tumor regression. CD8+ TIL performance can be further enhanced by complementing metabolic reprogramming with a PD-1 checkpoint inhibitor, which in melanoma renders PD- L1+ tumors cells more susceptible to cytolysis. These hypotheses are supported by our data. Year 1 focused on mouse studies, some of which were finished in year 2. In year 2 we started studies with human melanoma samples.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

   Cancer vaccine, mouse model, melanoma, CD8+ T cells, metabolism, PPARa agonist, checkpoint blockade, human melanoma samples, iPDX model, NOD-SCID mice, human tumor transplantation, adoptive lymphocyte transfer.

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

   What were the major goals of the project?
   List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

   The overall goal of the application is to test first in mice and then with human samples if metabolic reprogramming of tumor antigen specific CD8+ T cells improves their functions and delays their progression towards exhaustion and thereby delays tumor progression. Year 1 focused on mouse studies and addressed the following major 2 aims divided into subtasks. Aim 1 was to identify effects of metabolic reprogramming on mouse TILs using the B16BrafV600E model. The initial subtask 1 was to gain regulatory approval for animal studies. This task was completed. In the next subtask 2 we determined if metabolic reprogramming of mice affected tumor progression. This experiment was completed. Subtask 3 was to vaccinate B16BrafV600E cell-challenged mice with the melanoma-associated antigen (MAA)-specific or the control vaccine and treat them with the PPAR-α agonist or the diluent. We were to determine tumor progression and characterize T cells from spleens and tumors

for functions, phenotypes and metabolic characteristics by stains and flow cytometry. This task was completed. Subtask 4 was similar, but we used levels of transcripts encoding factor involved in glucose or fatty acid metabolism by comparative PCR. This task was completed. Aim 2 tested the effect of metabolic reprogramming combined with PD-1 blockage on the performance of vaccine-induced T cells. The tasks of this aim were completed. The end points of both aims were to determine if the treatments (1) delay or prevent tumor progression, (2) improve frequencies and functions of vaccine-induced TILs, (3) change TIL metabolism and/or (4) delay TIL exhaustion. These endpoints were met.
We have obtained institutional approvals to conduct the animal experiments with human materials for aims 3 and 4, which are described below. We have obtained DoD approval of our ACURA document.
Aim 3 addresses the effects of metabolic reprogramming on human TILs. We are using the iPDX model to test if treatments with FF with or without PD-1 blockade affect human TIL frequencies, phenotypes, and metabolism with the expectation that the PPAR agonist will prolong TIL persistence within human melanoma fragments by shifting their metabolism towards FA catabolism and thereby slow down tumor growth. PD-1 blockade may have a synergistic or additive effect.
Aim 4 will test the effects of metabolic reprogramming and PD-1 treatment on adoptively transferred human CD8+ T cells. Human melanoma fragments will be engrafted into NOD/SCID mice, expanded, and maintained through serial passages in NOD/SCID mice. PBMCs or TILs from the same patients will be expanded in vitro using established techniques. Some of the cultures will be treated with modified FFA or its diluent. Upon expansion, PPAR-α agonist treatment, CD4+ T cell depletion and characterization, lymphocytes will be injected into NOD/SCID mice bearing autologous melanoma fragments. Mice will be treated with FF or diluent. Subgroups will further receive either an anti-human PD-1 Ab or an isotype control Ab. Tumor progression will be monitored. Frequencies of human CD8+ T cells will be tested over time. At the end of the experiment mice will be euthanized and human CD8+ T cells from blood and tumors will be characterized for frequencies, functions, phenotypes, and metabolic features.

What was accomplished under these goals?

Aims 1 and 2: Effects of metabolic reprogramming without or combined with PD-1 blockade on mouse TILs. The original aim was to combine vaccination and metabolic reprogramming with PD-1 checkpoint blockade.
These aims have been completed and resulted in one publication.

Aim 3: Effects of metabolic reprogramming on human TILs. We focused on task 3. Data that were shown in the 3 monthly program reports are described more recent data are shown as figures.

Table 1 shows the available human samples. Table 2 shows samples we are currently working with.

Table 1

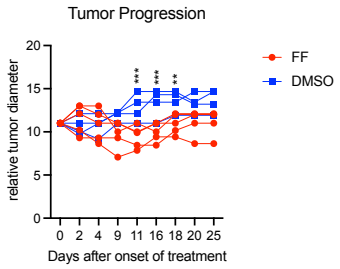
Mice (ID)	Group	Treatment	Tumor growth	Tumor type	Treatment (FF/DMSO)	Analysis
1	Group A	FF	no growth	Primary melanoma	3 mo after transplant for 21 days	Phenotyping blood (3,5 mo)
2		FF	no growth			
3		FF	no growth			
4		FF	no growth			
5		FF	mouse died			
6	Group B	DMSO	growth (slow)	Merkel cells carcinoma	2 mo after transplant for 21 days	Phenotyping blood (2, 4 mo)
7		DMSO	growth (slow)			
8		DMSO	no growth			
9		DMSO	no growth			
11	GroupC	FF	rowth, euthanize	Recurrent melanoma	1 week after transplant for 21 days	2 mice (sick) phenotyping TILs
12		FF	rowth, euthanize			
13		FF	growth (slow)			Phenotyping blood (1, 3 mo)
14		DMSO	no growth			
15	Group D		no growth	Primary melanoma		Phenotyping blood (2mo)
16			no growth			
17			no growth			
18			no growth			
19			mouse died			
20	Group E	DMSO	growth (fast)	Recurrent melanoma under nivolumab	2 mo after transplant for 21 days	Phenotyping blood (2, 3 mo), mice euthanized, tumor progression
21		DMSO	growth (fast)			
22		FF	growth (fast)			
23		FF	growth (fast)			
24	Group F		no growth	Primary melanoma		
25			growth (slow)			
26			no growth			
27			no growth			
28			no growth			
29	Group G	FF	growth	Metastatic melanoma	2 mo after transplant for 21 days	Transcriptome analysis
30		FF	growth			
31		FF	mouse died			
34	Group H	DMSO	growth			
35		DMSO	growth			
36		DMSO	growth			
37	Group I	FF	growth	Metastatic melanoma	2 mo after transplant for 21 days	Transcriptome analysis
38		FF	growth			
39		FF	growth			
40	Group J	DMSO	growth			
41		DMSO	growth			
42		DMSO	growth			
43	Group K	None	growth	Metastatic melanoma	Used for expansion of T and tumor cells	Testing the effect of DMSO and FF on tumor cells cultured in vitro or in vivo
44			growth			
45			growth			
46			growth			
47			no growth			

Table 2

Mouse group	Tumor type	T cells expansion in vitro	Tumor cell expansion in vitro	Sequential tumor fragment transplants in mice
Groups G/H	Metastatic melanoma	cells died	Yes	Yes
Groups I/J	Metastatic melanoma	cells are growing but poorly	Yes	Yes
Group K	Metastatic melanoma	cells are growing well	yes	yes

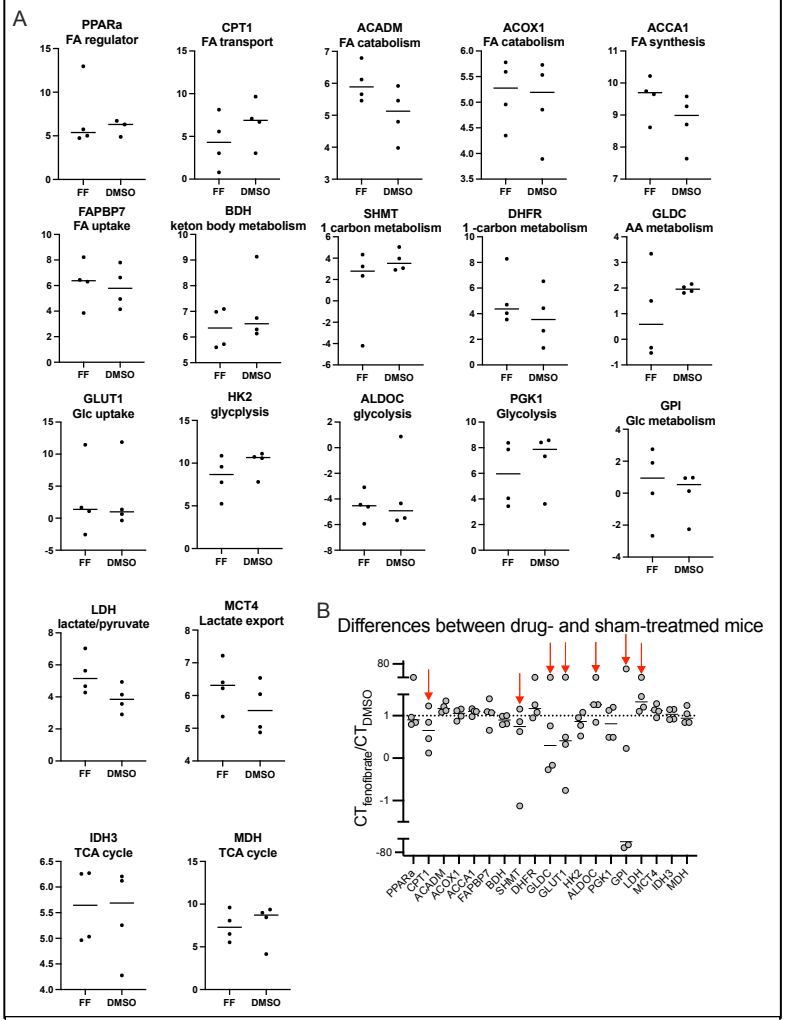
NSG mice that carried human tumor transplants had readily detectable human T cells in their blood which presumably originated from the tumor fragments. Treatment of mice with fenofibrate once tumor reached a certain size delayed tumor progression. It is possible that the delay of tumor progression was caused by the human T cells which upon fenofibrate treatment showed increased expression of PD-1 and fatty acid uptake by flow cytometry which is an indication that T cells were switching from glycolysis to fatty acid metabolism. This was confirmed by an analysis of the T cells transcriptome. Alternatively it could reflect that the drugs affected tumor cells directly. This was tested by transferring tumor cells that that we had cultured from one of the melanoma patients (Patient K, see Table 1) into NSG mice. Once tumors became visible, we treated mice with DMSO or fenofibrate and measured tumor progression (Figure 1). Treatment initially delayed tumor progression and differences between the two groups were significant between days 11-18 of treatment but then differences declined, suggesting that fenofibrate delayed tumor progression although the effect might be transient. At euthanasia we isolated RNA from the tumors and upon reverse transcription analyzed samples for expression of transcripts for metabolic markers by qPCR. The normalized CT values are shown in Figure 2A while the differences between the CT values are shown in Figure 2B, with lower CT values indicating higher levels of transcripts. Due to the small number of samples the data did not reach significance. Nevertheless, there were some trends (Figure 2A). Increases were seen upon

Figure 1



Legend Figure 1: Graph shows tumor diameter over time as of the day of onset in drug treatment. Data were normalized to the overall average tumor size at the 1st day of treatment. Significant differences by repeated corrected student t-test are highlighted by stars (** - p value below 0.01, *** p value below 0.001).

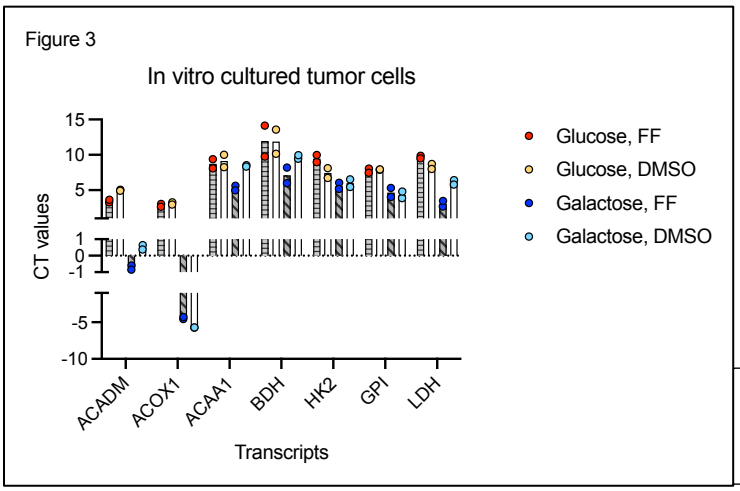
Figure 2



Legend Figure 2: A shows CT values of the two groups for the different transcripts, data were normalized to CT values of 18sRNA. B shows the differences in CT values between the t wo groups (CT value of fenofibrate samples divided by average CT value of matching DMSO samples). Samples which showed differences are highlighted by red arrows.

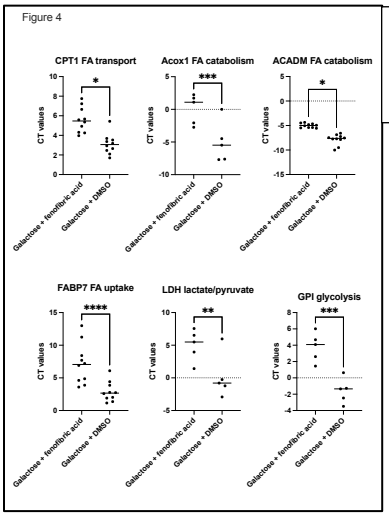
fenofibrate treatment in transcripts for CPT-1, a fatty acid transporter, SHMT, which plays a role in one carbon metabolism and GDLC involved in amino acid metabolism and GPI, an enzyme of the glycolytic pathway, which is also considered a tumor cell cytokine and has angiogenic functions while LDH, which converts pyruvate to lactate, decreased.

To further assess how fenofibrate affects metabolism of human melanoma cells we cultured tumor cells of patient K in medium containing glucose or galactose. Twenty-four hours of changing medium fenofibric acid (a metabolite of fenofibrate that is more suitable than fenofibrate for in vitro studies) or DMSO were added to the cells. Cells were harvested 7 days later, and transcripts were analyzed.



Legend Figure 3: CT values are shown for melanoma cells of patient K cultured under the different conditions.

In cell cultured in presence of glucose changes in presence of fenofibric acid were marginal presumably reflecting that availability of this nutrient overcame the effects of the PPARα agonist on fatty acid metabolism. In contrast in cells cultured in galactose without glucose increased enzymes of fatty acid metabolism. We repeated the galactose part of this experiment with larger numbers of replicates. In this experiment we shortened the time of drug-treatment to 3 days. The results were unexpected – they showed that fenofibric acid decreased transcripts for all tested markers (Figure 4). The likely explanation for the discrepancy in results was that we shortened the duration of drug treatment ad that cells need a period of adjustment before they show metabolic changes.



Legend Figure 4: CT values are shown for melanoma cells of patient K cultured under the indicated conditions.

We are currently expanding tumor cells and T cells from Patient J. Both grow well and will be. Used to repeat the above-described experiments with samples from a different individual and to start the adoptive transfer experiments of aim 4.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report

How were the results disseminated to communities of interest?

***Manuscript**:* Chekaoui A, Ertl HCJ. PPARα Agonist Fenofibrate Enhances Cancer Vaccine Efficacy. Cancer Res. 2021 Sep 1;81(17):4431-4440. doi: 10.1158/0008-5472.CAN-21-0052. Epub 2021 Jul 8. PMID: 34244236.
***Invited talk**:* Hildegund Ertl, **In Pre-clinical Models Metabolic Manipulations Improve the Efficacy of T cell Immunotherapy of Melanoma.** Immuno-Resistance and Sequencing Symposium, SITC 2021, Washington, DC

What do you plan to do during the next reporting period to accomplish the goals?
We are planning to use tumor and TIL samples from patient J to confirm that the effect of fenofibrate to slow tumor progression in the PDX mouse model was cause primarily by an effect of the drugs on TILs rather than tumor cells. Otherwise we will conduct the adoptive transfer experiments of aim 4 starting with material of patient J and additional melanoma samples we expect to obtain from the University of PA Hospital.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
In the experiments of aims 1 and 2 we confirmed that fenofibrate given to melanoma bearing mice that were vaccinated with a melanoma vaccine reduced tumor progression. Reduced tumor progression was caused by an effect of the drug on tumor cell metabolism. In presence of fenofibrate melanoma cell switched from the use of glycolysis to the use of fatty acids for energy production. This in turn made more glucose available for TILs improving their fitness. In the PDX model where NSG mice were transplanted with tumor fragment from human melanoma patients, which in additional to melanoma and stroma cells contain TILs treatment of mice with fenofibrate also slowed tumor progression. This was likely cause by an effect of the drug on TILs as mice injected with in vitro cultured melanoma cells from one of the same patients only transiently showed reduced tumor growth with only marginal metabolic changes within cells isolated from the tumors indicating that in humans the effect of the metabolic drug requires the presence of TILs

What was the impact on other disciplines?
Our data show that treatment of melanoma patients with a drug that alters the metabolism of cells within the tumors may have benefits. Results are likely to other forms of solid tumor where TIL functions are impaired to te metabolic constrains.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

CHANGES/PROBLEMS:
Nothing to Report.

Changes in approach and reasons for change
We had planned in conduct in the mouse melanoma. Model studies which combined vaccination, fenofibrate treatment and checkpoint blockade by PD-1. Our. Results showed that the benefits of fenofibrate reflected that the drugs reduced glucose uptake by tumor cells making more of this key nutrient available to TILs, which in turn increased their fitness. We therefore only conducted a limited number of experiments with anti-PD-1 treatment. Results were not promising as would be expected considering the underlaying mechanism by which fenofibrate affected tumor progression in the mouse model.

Actual or anticipated problems or delays and actions or plans to resolve them
We do not anticipate any problems.

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

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.

5. PRODUCTS:
Nothing to Report.

- **Publications, conference papers, and <u>presentations</u>**

<u>Invited talk</u>: Hildegund Ertl, **In Pre-clinical Models Metabolic Manipulations Improve the Efficacy of T cell Immunotherapy of Melanoma.** Immuno-Resistance and Sequencing Symposium, SITC 2021, Washington, DC

Journal publications.

Chekaoui A, Ertl HCJ. PPARα Agonist Fenofibrate Enhances Cancer Vaccine Efficacy. Cancer Res. 2021 Sep 1;81(17):4431-4440. doi: 10.1158/0008-5472.CAN-21-0052. Epub 2021 Jul 8. PMID: 34244236.

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers and presentations.
Nothing to Report.

- **Website(s) or other Internet site(s)**
  Nothing to Report..

- **Technologies or techniques**
  Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Nothing to Report.

- **Other Products**
  Nothing to Report.

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	*Hildegund CJ Ertl*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*3.6 cal.mo*
Contribution to Project:	*Dr. Ertl wrote the animal protocol, designed experiments, analyzed data, interpreted results, and wrote the manuscript and progress reports*

Name:	*Zhiquan Xiang*
Project Role:	*Investigators*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	4.8 cal. mo.
Contribution to Project:	*Dr. Xiang trained and helped Dr. Hasanpourghadi*

Name:	*Wynetta Giles-Davis*
Project Role:	*Research Technician*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	6 cal. mo.
Contribution to Project:	*Ms. Giles-Davis maintained tumor cell lines and ordered supplies.*

Name:	*C. Cole*
Project Role:	*Lab Manager*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*1.2 cal.mo*
Contribution to Project:	*C. Cole submitted the animal protocol and ran the T cell experiments on the LSRII*

Name:	*Mohadeseh Hasanpourghadi*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*0.7 cal. mo.*
Contribution to Project:	*She expanded human T cells and conducted mouse experiments*

Name:	*Arezki Chekaoui*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*7.2 cal. mo.*
Contribution to Project:	*He expanded human T cells and conducted mouse experiments*

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.

7. SPECIAL REPORTING REQUIREMENTS
Nothing to report.

8. APPENDICES:
Nothing to report.