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PRINCIPAL INVESTIGATOR: Hildegund CJ Ertl

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Hildegund CJ Ertl

E-Mail: ertl@wistar.org

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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.[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.

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.

Tasks 1 and 2 focus on a mouse model of melanoma. In task 1, subtask 1 we were to gain IACUC and ACURO approval for the proposed animal experiments of tasks 1 and 2. This was achieved. In subtask 2 we were to determine tumor progression and characteristics of $CD8^+$ T cells induced by a melanoma vaccine comparing treatment with a PPARα agonist (fenofibrate) to diluent (DMSO) treatment. In task 2 fenofibrate treatment was to be combined with PD-1 checkpoint blockade again monitoring tumor progression and

characteristics of vaccine induced T cells. The goal of tasks 1 and 2 was to show if the PPARα agonist given directly to tumor-bearing mice slows tumor progression and if this is linked improved frequencies and functions of vaccine-induced TILs, changes in TIL metabolism and/or delayed TIL exhaustion.
Timeline: Year 1 of the award

Tasks 3 and 4 focus on human TILs from melanoma metastases tested in severely immunodeficient mice carrying tumor fragments from the same patients. In subtask 1 we are to gain IRB approval for subtasks 3 and 4. This has been obtained. We have submitted an animal protocol and are awaiting IACUC approval. Subtask 2 will determine if melanoma cells from human metastasis express PD-L1. In subtask 3 we will isolate and analyze lymphocytic infiltrates from the tumors. Subtask 4 will inject melanoma fragments into NOD/SCID mice, which will be treated with fenofibrate or diluent. Once tumors reach a certain size, lymphocytes will be isolated and characterized for expression of differentiation markers and transcripts involved in glucose and fatty acid metabolism. In subtask 5 we will take the same approach but combine fenofibrate treatment with PD-1 checkpoint blockade.

Task 4 will determine the effects of metabolic reprogramming and PD-1 treatment on adoptively transferred human $CD8^+$ T cells. Subtasks 1 and 2 will expand human melanomas in NOD/SCID mice and thereafter determine if the tumor cells express PD-L1. Subtasks 3- 5 will expand and then cryopreserve T cell from the same tumors or from matching PBMC samples. Once tumors have expanded in the NOD/SCID mice, lymphocytes will be thawed and cultured with diluent or fenofibrate. They will then in subtask 7 be infused into NOD/SCID mice bearing autologous tumors. Mice will be treated with fenofibrate or diluent. Tumor progression will be monitored and after euthanasia human TILs will be characterized. Subtask 8 will take the same approach but include a PD-1 checkpoint inhibitor into the treatment regimen.

The goal of the experiments of Task 3 is to determine if the PPARα agonist treatment with or without PD-1 checkpoint blockade affects human melanoma progression and characteristics of the tumors' lymphocytic infiltrate. The goal of aim 4 is similar but will used adaptively transferred lymphocytes that were expanded in vitro rather TILs that are present within the transplanted tumor fragments.
Timeline: Task 3 year 2 of the award; Task 4 year 3 of the award.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Research Accomplishments

Task 1 Effects of metabolic reprogramming on mouse TILs.
Subtask 1 Gain IACUC approval for the proposed animal experiments of tasks 1 and 2 by amending an existing protocol: We obtained IACUC and ACURO approval to conduct the mouse experiments of aims 1 and 2.

Subtask 2: Determine tumor progression in mice (5 mice/group) injected with B16BrafV600E cells and treated with the PPAR-α agonist.

We confirmed that the PPARα agonist fenofibrate does not affect tumor progression in unvaccinated animals (Figure 1). For this experiment groups of 5 C57Bl/6 mice were challenged with 5 X 10^4 B16Braf$_{V600E}$ cells/mouse. Six days later FF (100 mg/kg/day) in DMSO/PBS was given by oral gavage daily till tumors exceeded 1 cm in diameter. Control mice received diluent.

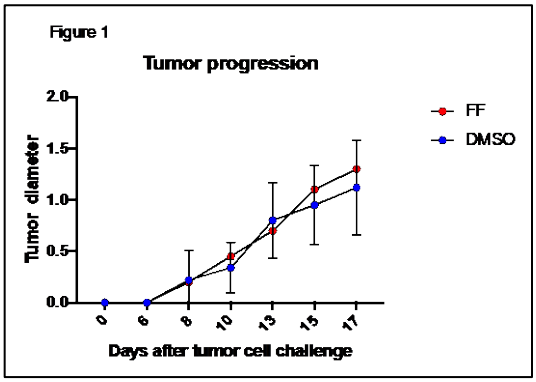


Fig 1 shows tumor progression in B16Braf$_{V600E}$ cell challenged mice treated with fenofibrate (FF) or DMSO per os.

Tumor progression was monitored by measuring the perpendicular diameters of tumors every other day. Comparing tumor diameters for each time point using multiple t-tests showed no significant differences between the groups. This is an important pre-experiment that we needed to conduct to ensure that fenofibrate without the vaccine does not affect tumor progression. We have since changed the protocol and increased the dose of fenofibrate to 200mg/kg/day. We therefore have to repeat the experiment with the new dose. This is ongoing.

Subtask 3: Vaccinate B16BrafV600E cell-challenged mice with the MAA-specific or the control vaccine and treat them with the PPAR-α agonist or the diluent using 10 mice per group (4 groups total). Determine tumor progression and characterize T cells from spleens and tumors for functions, phenotypes and metabolic characteristics by stains and flow cytometry.

The initial challenge experiment showed poor melanoma antigen-specific $CD8^+$ T cell responses to the AdC68gD-Melapoly vaccine and we therefore made a new batch, which was purified, titrated and quality controlled. Responses to this vaccine were excellent and we proceeded to use it for subsequent experiments (data were shown in the Q1 progress report).
We injected mice as described in the proposal with B16Braf$_{V600E}$ tumor cells. Three days later they were vaccinated with the new AdC68-gDMelapoly vaccine or the control vaccine, i.e., AdC68-gDE7. Three days later FF (100 mg/kg/day) in DMSO/PBS or DMSO/PBS was given by oral gavage daily for 3 weeks. Mice were euthanized once tumors were ~ 1 - 1.5 cm in diameter. Till then tumor progression was recorded. It should be noted that some of the mice did not develop tumors. Splenocytes and tumor cells were isolated and stained with a live cell stain, a tetramer to Trp-1 and antibodies to CD8, CD44, PPARα, HIF1α, T-BET, PD1, LAG3, KLRG1

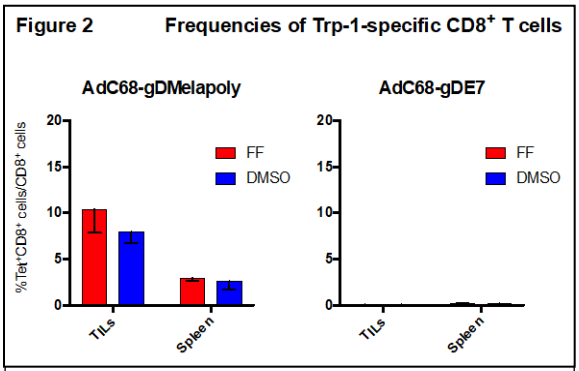


Figure 2. The graphs show for individual mice frequencies of Trp-1-tetramer$^+$CD8$^+$ T cells in tumors and spleens. Graph on the left shows results for AdC68-gDMelapoly-immunized mice, graph on the left shows those for AdC68-gDE7 immunized mice. Lines indicate mean frequencies.

and CD127. Cells were analyzed by flow cytometry and then after data acquisition by FlowJo. Cells were gated on lymphoid cells, and then live single cells. They were gated further on tetramer$^+$CD44$^+$CD8$^+$ cells. The frequencies of Trp-1$^+$ tetramer$^+$ CD8$^+$ T cells was determined (Figure 2).

Mice immunized with the AdC68-gDMelapoly vaccine developed a robust Trp-1-specific CD8$^+$ T cell response that was higher in tumors than spleens. It was only marginally higher in mice treated with fenofibrate (FF) rather than DMSO. Mice immunized with the control vaccine failed to develop Trp-1-specific CD8$^+$ T cells.

Trp-1-tetramer$^+$CD44$^+$CD8$^+$ T cells from AdC68-gDMelapoly immunized mice in comparison to CD44$^-$CD8$^+$ (naïve) T cells were tested for expression of the different markers by comparing mean fluorescent intensity (MFI) and % marker positive cells. These analyses were only conducted for AdC68-gDMelapoly-immune mice for which we isolated sufficient numbers of cells from the tumors.

Trp-1-tetramer$^+$ CD8$^+$ TILs from fenofibrate as compared to DMSO treated mice showed a trend towards higher PPARα, HIF-1α and T-bet expression, which failed to reach significance (type 1 error corrected 2-way Anova). Differences in PD1 (higher in the DMSO group) and KLRG1 (higher in the FF group) reached significance for TILs or TILs and splenocytes respectively (Figure 3a).

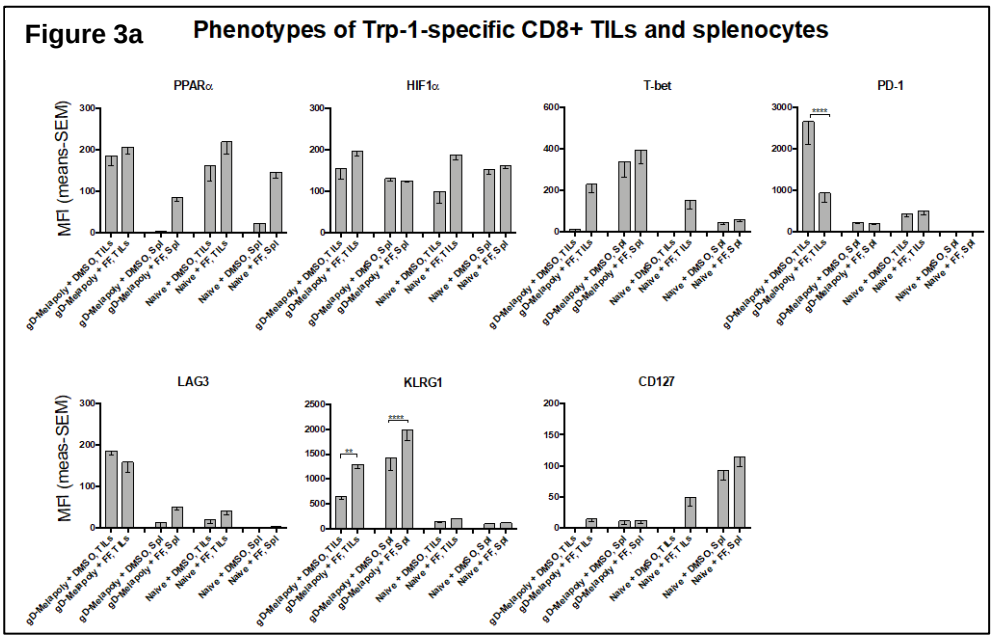


Figure 3a. The graphs show mean fluorescent intensity (MFI) for the indicated markers on/in Trp-1-tetramer^{+}CD8^{+} T cells from tumors and spleens as means - SEM. Lines with stars above indicate significant differences by type 1 corrected 2-way Anova (*) between cells from mice treated with FF or the diluent; p-value between 0.01-0.05, (**) p-value between 0.001-0.001, (***) p-value between 0.0001-0.001, (****) p-value < 0.0001.

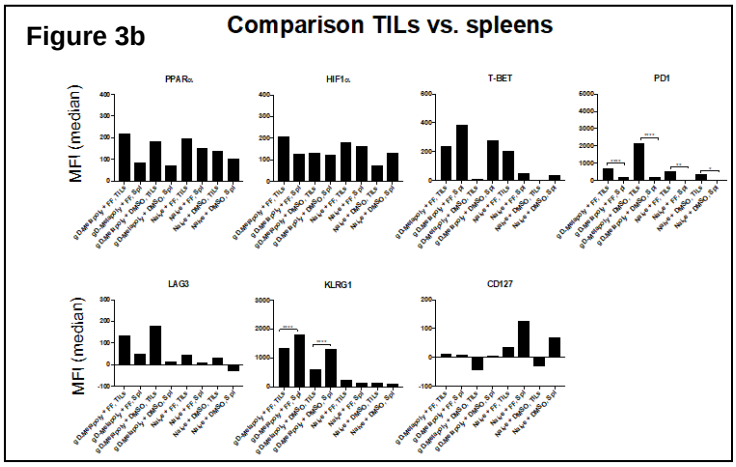


Figure 3b shows data from the same experiment as in Figure 3a comparing expression levels of the indicated markers in CD8^{+} TILs and splenocytes.

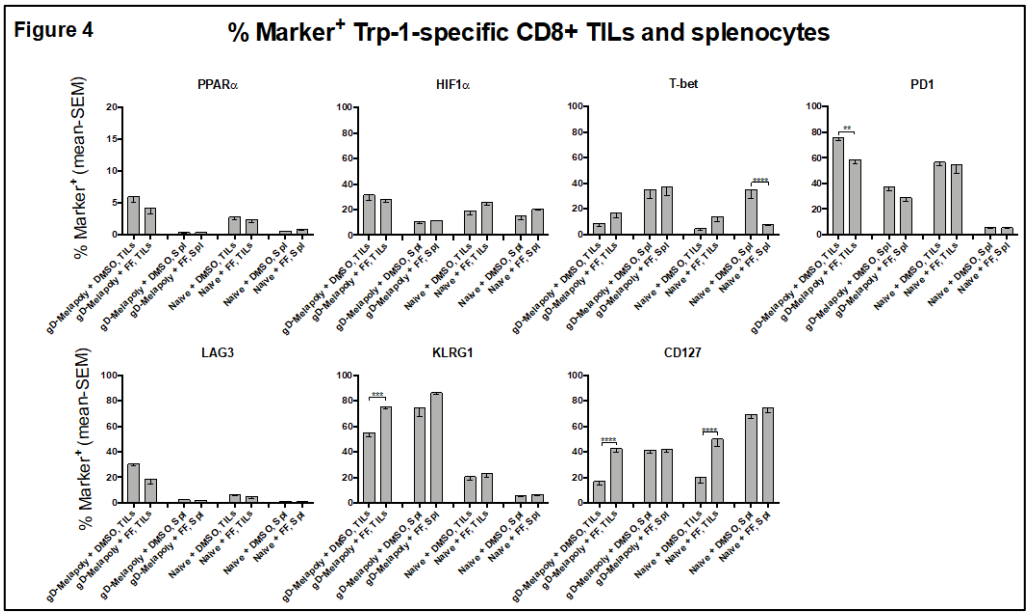


Figure 4. The graphs show % of Trp-1-tetramer$^+$CD8$^+$ T cells from tumors and spleens of mice expressing the indicated markers. Lines show mean results - SEM. (Lines with stars above indicate significant differences by type 1 corrected 2-way Anova (*) p-value between 0.01-0.05, (**) p-value between 0.001-0.001, (***) p-value between 0.0001-0.001, (****) p-value < 0.0001.

Comparing percentages of marker$^+$ Trp-1 tetramer$^+$ CD8$^+$ TILs and splenocytes from fenofibrate or DMSO treated mice (Figure 3b) showed significant differences for PD1 and KLRG1 for TILs and for CD127 for TILs and splenocytes. PD1 went down after fenofibrate treatment presumably reflecting that cells were less activated while KLRG1 and CD127 went up reflecting more pronounced differentiation towards terminal differentiation and memory formation (Figure 3b). We measured tumor progression during all of the experiments and the results are summarized later. In the experiment of Figure 3 we did not see a significant difference in tumor progression between DMSO and fenofibrate treated mice, so we increased the dose of fenofibrate to 200 mg/kg. In the next experiment for which mice were otherwise treated as described above, we analyzed CD8$^+$ T cells from spleens and tumors from AdC68-gDMelapoly-immunized mice that had been fed fenofibrate or DMSO for production of granzyme B, interferon (IFN)-γ, IL-2, perforin and tumor necrosis factor (TNF)-α by intracellular cytokine staining upon a short in vitro stimulation with a peptide carrying the immunodominant Tp-1 epitope. As shown in Figure 5 CD8$^+$ T cells produced mainly IFN-γ and TNF-α. All mice had low frequencies of perforin producing, Trp-1-specific CD8$^+$ T cells, only a few had cells producing granzyme B and stains for IL-2 were negative. The only significant difference was seen for CD8$^+$ T cells producing TNF-α which were more common in spleens than tumors (Figure 9).

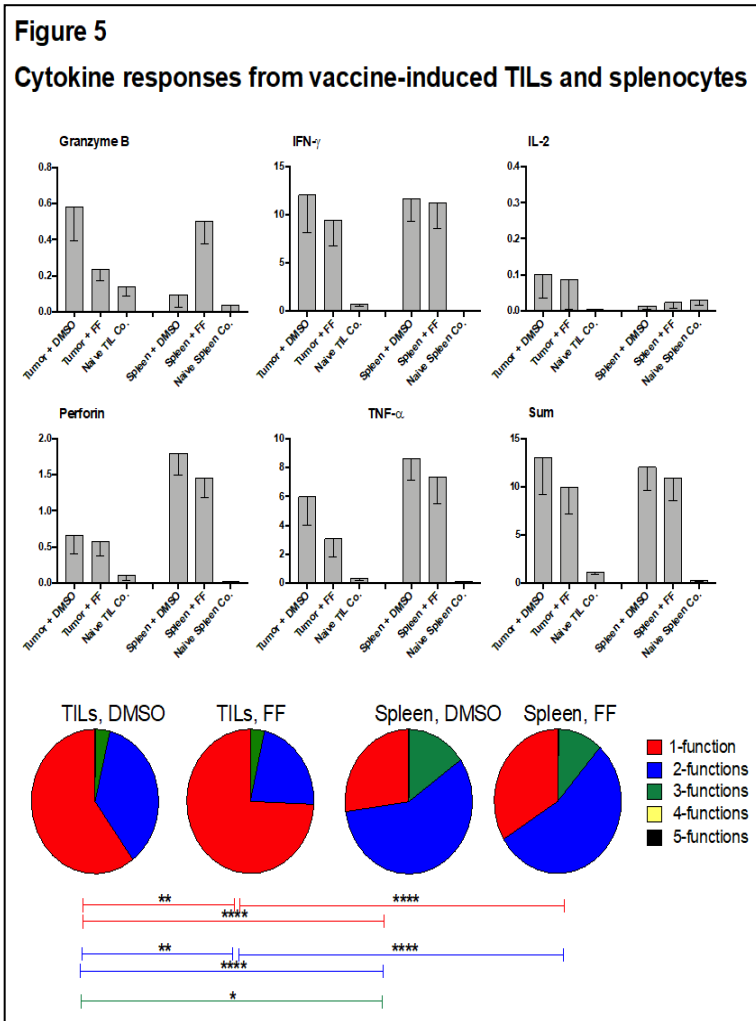


Figure 5. The graph shows frequencies of $CD8^+$ T cells producing the indicated cytokine combination after in vitro stimulation with the Trp-1 peptide. The sum reflects frequencies of all cytokine-producing $CD8^+$ T cells calculated upon Boolean gating. Background data (no peptides) were subtracted from spleens but not tumors. The circles at the bottom show the distribution of $CD8^+$ T cells producing 1-5 factors. Lines below indicate significant differences for frequencies of the different functions. Lines are colored according to the pie slices.

We used Boolean gating to determine cytokine profiles for the different Trp-1-specific CD8± T cell populations (Figure 5, bottom, Figure 6). Over 50% of Trp-1-specific TILs only produced 1 cytokine while multifunctional Trp-1-specific CD8± T cells were more common in spleens. Cells from fenofibrate-treated mice showed a significant reduction in polyfunctionality.

Boolean gating only showed significant differences for both Trp-1-specific $CD8^+$ T cells from tumors and spleen which produced a combination of IFN-γ and TNF-α (Figure 6)

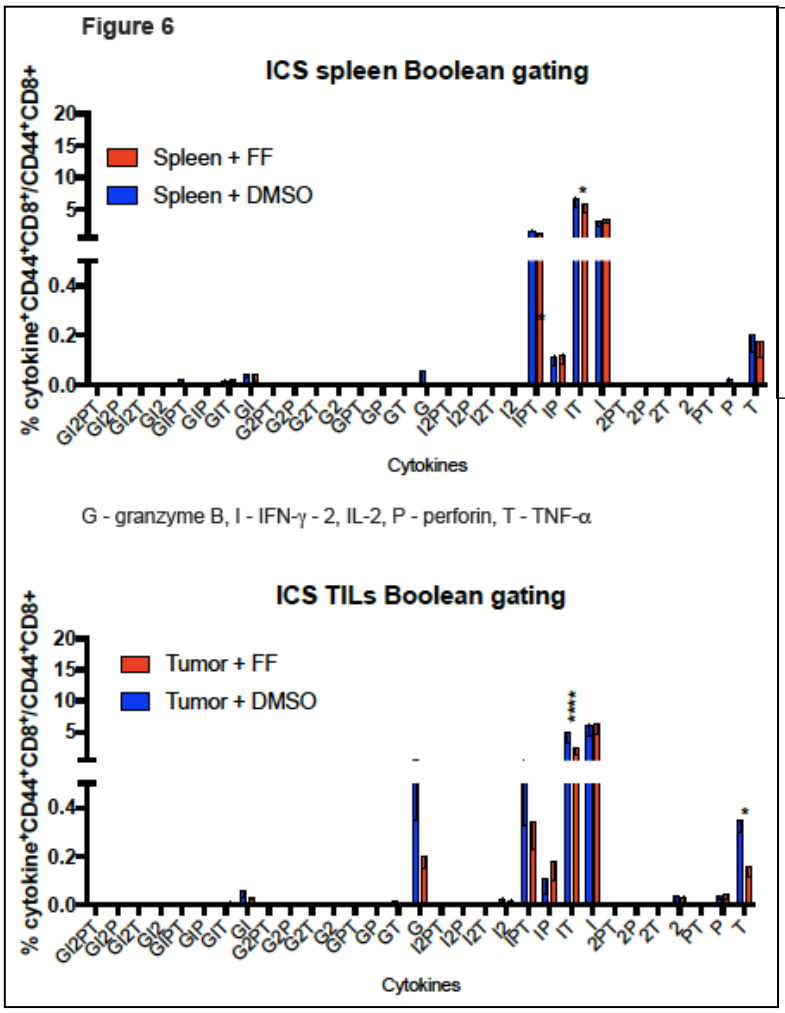


Figure 6. The graphs show frequencies of $CD8^+$ T cells producing the indicated cytokine combination after in vitro stimulation with the Trp-1 peptide. Background frequencies were subtracted from results obtains with lymphocytes from spleens but not tumors. Significant differences are indicated by stars above the bar graphs.

We compared distribution of Trp-1-specific $CD8^+$ T cells producing 1 to 5 cytokines. Multifunctional T cells producing 2 or 3 cytokines were more common the spleens that tumors. Only ~ 20 % of $CD8^+$ TILs from the DMSO group produced more than one cytokine while those from the fenofibrate group tended to be more polyfunctional, i.e., 34% produced 2 or 3 cytokines (Figure 6).

Subtask 4: Vaccinate B16Braf^{V600E} cell-challenged mice with the MAA-specific or the control vaccine and treat them with the PPAR-α agonist or the diluent using 10 mice per group (4 groups total). Determine tumor progression and measure levels of transcripts encoding factor involved in glucose or fatty acid metabolism by comparative PCR.

We analyzed transcripts of activate $CD44^+CD8^+$ T cells from spleens and tumors of DMSO or fenofibrate treated mice. Specifically, mice were challenged, immunized and treated with fenofibrate or DMSO. Tumors and splenocytes were isolated when tumors reached a diameter of ~ 1.2-1.5 cm. Lymphocytes were stained and sorted. Splenocytes and TILs were sorted into $CD44^+CD8^+$ cells and in the same run into $CD25^+CD4^+$ cells. The later population was not part of the application, but we felt that without undue cost we would potentially gain information on the effects of fenofibrate on regulatory T cells (Tregs). RNA was isolated from the sorted cells

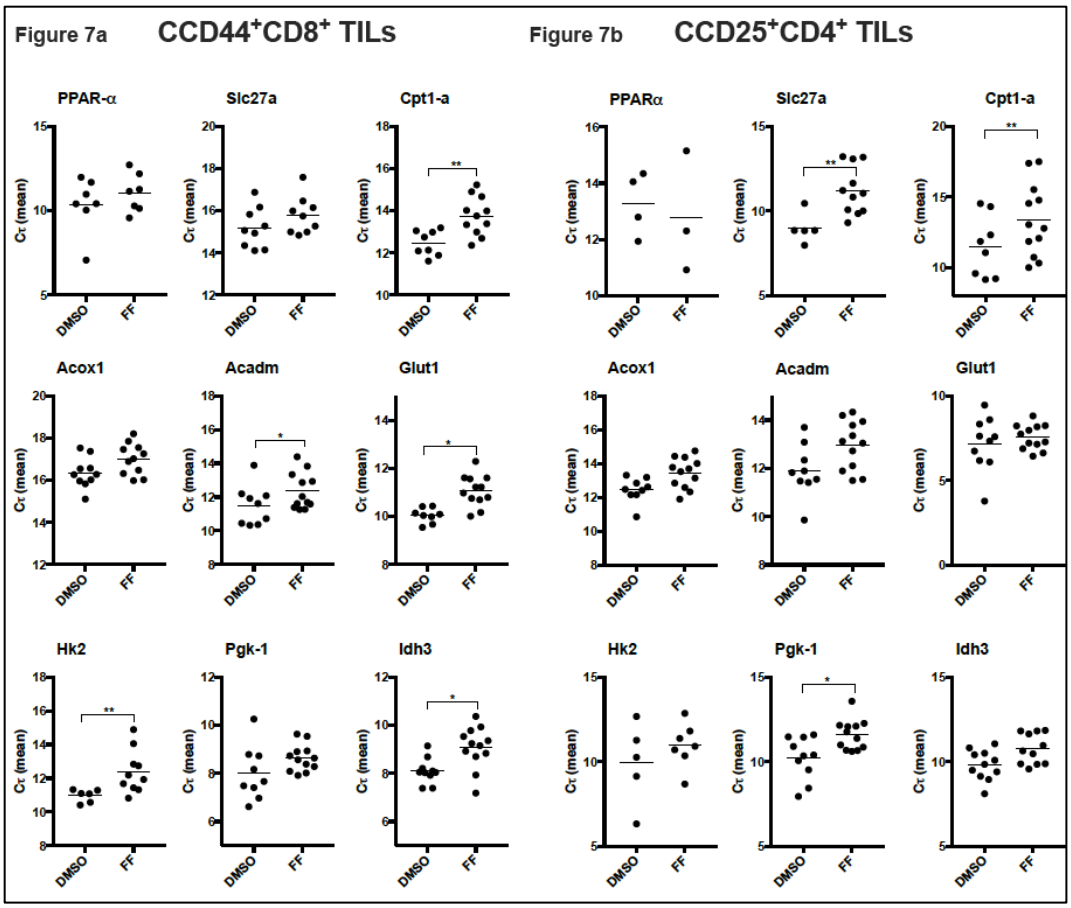


Figure 7a shows Ct values for individual $CD44^{+}CD8^{+}$ samples from tumors of fenofibrate or DMSO treated mice. Significant differences calculated by multiple t-test are indicated by lines with stars above, Figure 7b shows results for $CD25^{+}CD4^{+}$ cells from the same mice.

and reverse transcribed. cDNAs were probed for transcripts of factors involved in glucose and fatty acid catabolism, i.e., glucose transporter (Glut)1, hexokinase (Hk)-2, phosphoglycerate kinase (Pgk)1, isocitrate dehydrogenase (Idh)3, peroxisome proliferator-activated receptor (PPAR)α, solute carrier family (Slc)27a4, peroxisomal acyl-coenzyme A oxidase (Acox)1, carnitine palmitoyltransferase I (Cpt1)-a, acyl-CoA dehydrogenase medium chain (Acadm) and 18S ribosomal (r)RNA, the latter was used as the internal control. Data in Figure 7 are expressed as cycle threshold (Cτ) in which higher values reflect presence of lower levels of transcripts. In addition, we calculated fold differences in transcript levels between those isolated from cells of fenofibrate as compared to DMSO-treated mice. In latter analyses we used the average Cτ value obtained from DMSO samples for comparison to Cτ values from individual samples from fenofibrate-treated mice (Figure 8).

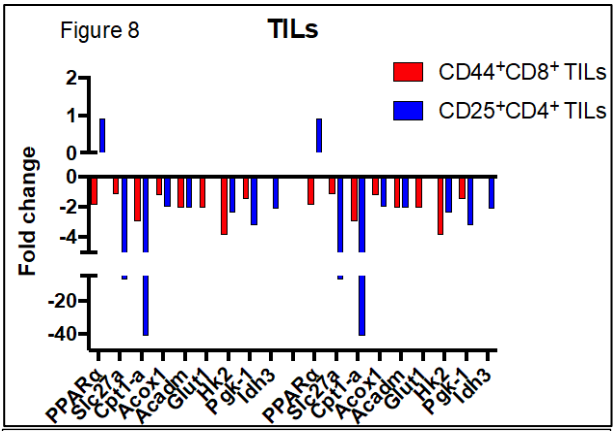


Figure 8 shows the same data as Figure 7 but as fold differences between the Ct values of transcripts from samples of DMSO or fenofibrate treated mice.

Figures 7 and 8 show results from TILs, Figure 9 shows results from $CD44^{+}CD8^{+}$ splenocytes,

there were a number of significant differences between transcripts levels in T cells from DMSO or fenofibrate treated mice. These reductions were not only seen for factors involved in glucose metabolism that would be expected to decline upon PPARα activation but also in factors involved in fatty acid metabolism. Overall these results suggest that fenofibrate interferes with T cell activation.

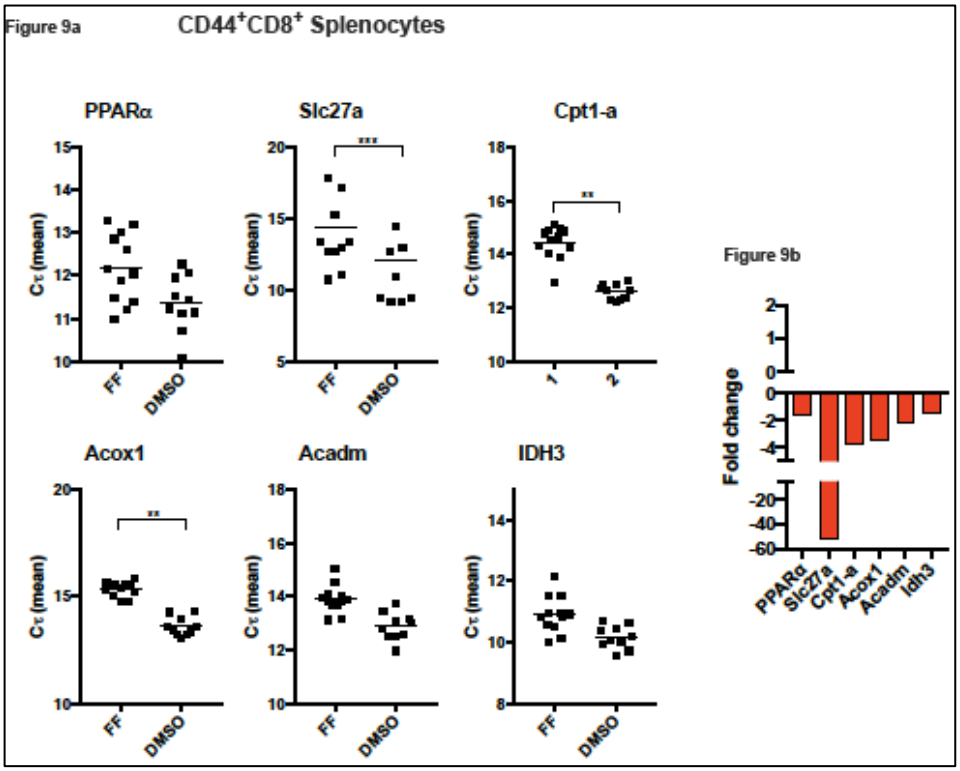


Figure 9 shows levels of transcripts in $CD44^{+}CD8^{+}$ splenocytes.

We monitored all mice that had been challenged for aim 1 for tumor progression. As shown in Figure 10 fenofibrate failed to slow tumor progression but instead in 2 out of 3 experiments tended to have a detrimental effect.

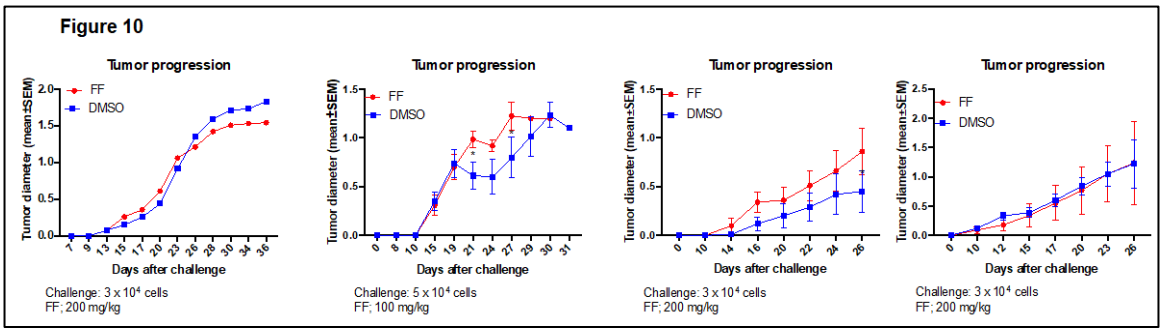


Figure 10 shows tumor progression in diluent (DMSO, blue) and fenofibrate (red) treated mice in different aim 1 experiments.

Our previous experiments, which showed that fenofibrate improves the efficacy of a cancer vaccine, were based on adoptive transfer models where T cells were either stimulated in vitro or

in vivo in presence of fenofibrate. They were then transferred into tumor-bearing vaccinated recipient mice. These mice were not treated with fenofibrate. T cell activation in vitro fundamentally differs from their stimulation in vivo. In vitro cultures have higher levels of key nutrients than plasma or lymphatic tissues. T cells are in general cultured under atmospheric levels of oxygen unlike T cells that encounter their antigen within lymph nodes, which are relatively hypoxic. Furthermore, activation of T cells in vitro occurs rapidly, while in vivo T cell activation takes place over a period of days or even weeks. Adoptive transfer from donor to tumor-bearing recipient mice may also affect T cell metabolism during in vitro manipulations as well as upon their injection into the blood stream of another animal. In previous experiments we treated cells with fenofibrate immediately upon injection of a vaccine or upon in vitro activation, but we did not treat them once they had been transferred into tumor-bearing mice. Under the assumption that timing will influence the effectiveness of metabolic manipulations we next explored different times for onset of fenofibrate treatment relative to vaccination. This was not part of the original application but was necessitated by our disappointing results. We explored different schedules for the fenofibrate treatment starting on day 3, 5 or 7 following vaccination with 10^{10}vp of the AdC68-gDMelapoly vaccine.

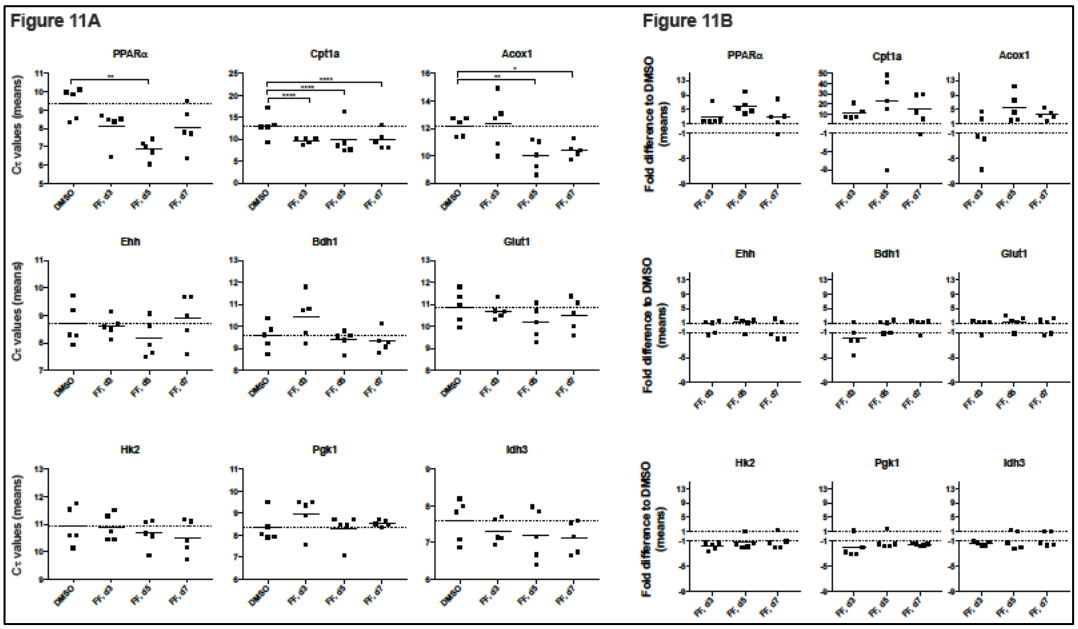


Figure 11A shows levels of transcripts in splenocytes treated with DMSO or fenofibrate starting at different days after vaccination. Figure 11B shows the same data as fold differences between DMSO and fenofibrate treated mice.

Mice were challenged with tumor cells. Fenofibrate was given at 200 mg/kg dose for up to 15 days orally, control mice received DMSO. Mice were euthanized 18 days after vaccination and splenocytes were stained for T cell markers and sorted onto $CD44^{+}CD8^{+}$ T cells. Transcripts were isolated and upon reverse transcription probed by a comparative quantitative polymerase chain reaction (qPCR) for those encoding factors and enzymes involved in glucose and fatty acid metabolism. Figure 11A, which illustrates the cycle thresholds (Cτ) of the PCR reaction, with higher thresholds representing lower levels of transcripts, and 2B, which reports on fold differences between drug and diluent treated mice, show significant increases in some of the transcripts involved in fatty acid metabolism. Transcript levels for PPARα, and acyl-CoA

oxidase 1 (Acox-1), the 1st enzyme of the fatty acid beta-oxidation pathway did not change when fenofibrate was given 3 days after vaccination but then showed increases if drug treatment was delayed till day 5. Transcripts for Cpt1a, a key factor that is essential to transport long chain fatty acids into mitochondria where they are converted to acetyl-CoA, which can feed the citric acid cycle, increased after fenofibrate treatment regardless of the time frame of drug treatment. We observed some other trends which failed to reach significance such as decreased transcript levels for 3-hydroxybuttyrate dehydrogenase 1 (Bdh1) an enzyme that is involved in the conversion of acetyl-CoA into ketone bodies and Pgk1, an enzyme of the glycolysis pathway, upon early drug treatment, confirming that timing of drug-induced PPARα activation affects the metabolism of activated $CD8^+$ T cells.

In the next set of experiments mice were injected with $B16Braf_{V600E}$ cells three days prior to AdC68-gDMelapoly vaccination. Mice were then fed DMSO 3 days later or fenofibrate starting 3, 5 or 7 days after vaccination. We tracked tumor progression, analyzed peripheral blood mononuclear cells (PBMCs) for frequencies and functions of melanoma antigen-specific $CD8^+$ T cells 18 days later and tested $CD44^+CD8^+$ tumor infiltrating lymphocytes for transcripts indicative for metabolic changes once tumors exceeded 1 cm in diameter.
As shown in Figure 12 fenofibrate treatment delayed onset of tumor development and this reached significance if the drug treatment was started on days 5 or 7 after vaccination. Fenofibrate treatment also delayed tumor progression and by day 23 after challenge tumor diameters of all groups were significantly lower than those of control mice that had received DMSO instead. The delay was most pronounced if fenofibrate was given 5 days after vaccination and was only marginally significant for the last time point if fenofibrate was given as of day 3 after vaccination.

Figure 12

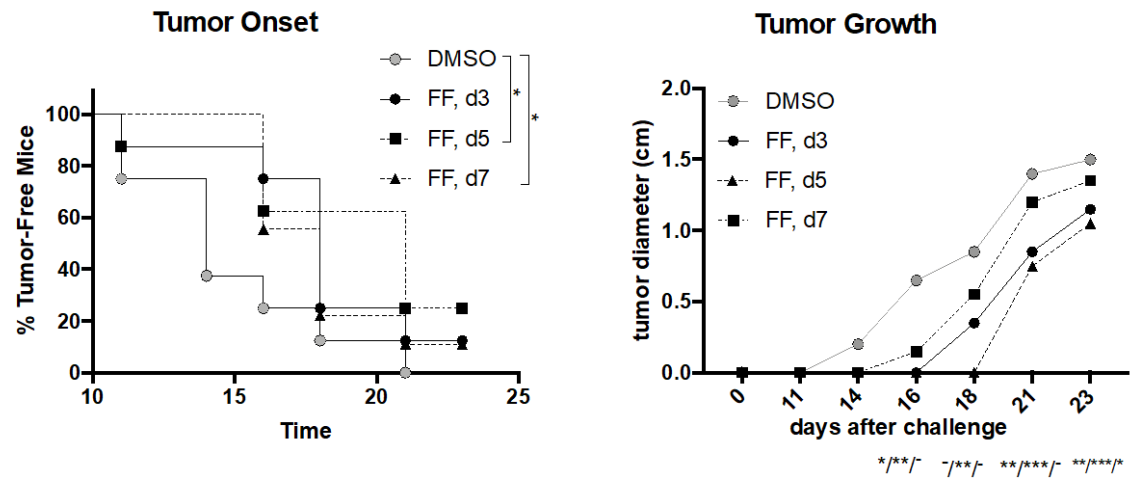


Figure 12 shows on the left onset of tumors in the 4 groups. Statistically significant differences between groups are shown in the legends. The right graph shows tumor sizes over days after challenge. Significant differences between the drug treated as compared to the control group (DMSO, isotype control) are shown below the x-Axis for the different time points the following order: FF, d3/FF, d5/FF, d9.

Delay of tumor progression was accompanied by improved functions of circulating vaccine-induced melanoma antigen-specific $CD8^+$ T cells (Figure 13). Fenofibrate-induced increases in frequencies of melanoma antigen-specific $CD44^+CD8^+$ T cells were highly significant for those that produced IFN-γ in response to a short in vitro stimulation. Increases in frequencies in comparison to the DMSO group were more pronounced if fenofibrate treatment was started on day 3 than on day 5. Frequencies of $CD44^+CD8^+$ T cells producing TNF-α for all time points or IL-2 for day 5 were also increased in the fenofibrate treated groups while those of T cells producing perforin decreased. The latter differences failed to reach significance. Boolean gating showed that in all four groups the majority of responding $CD8^+$ T cells produced IFN-γ and about 25-30% also produced TNF-α. Fenofibrate did increase polyfunctionality even if given on day 3 after vaccination. This is in contrast to our findings shown in Figure 5 but as difference in both experiments were subtle, they may not be biologically meaningful but rather represent assay variability.

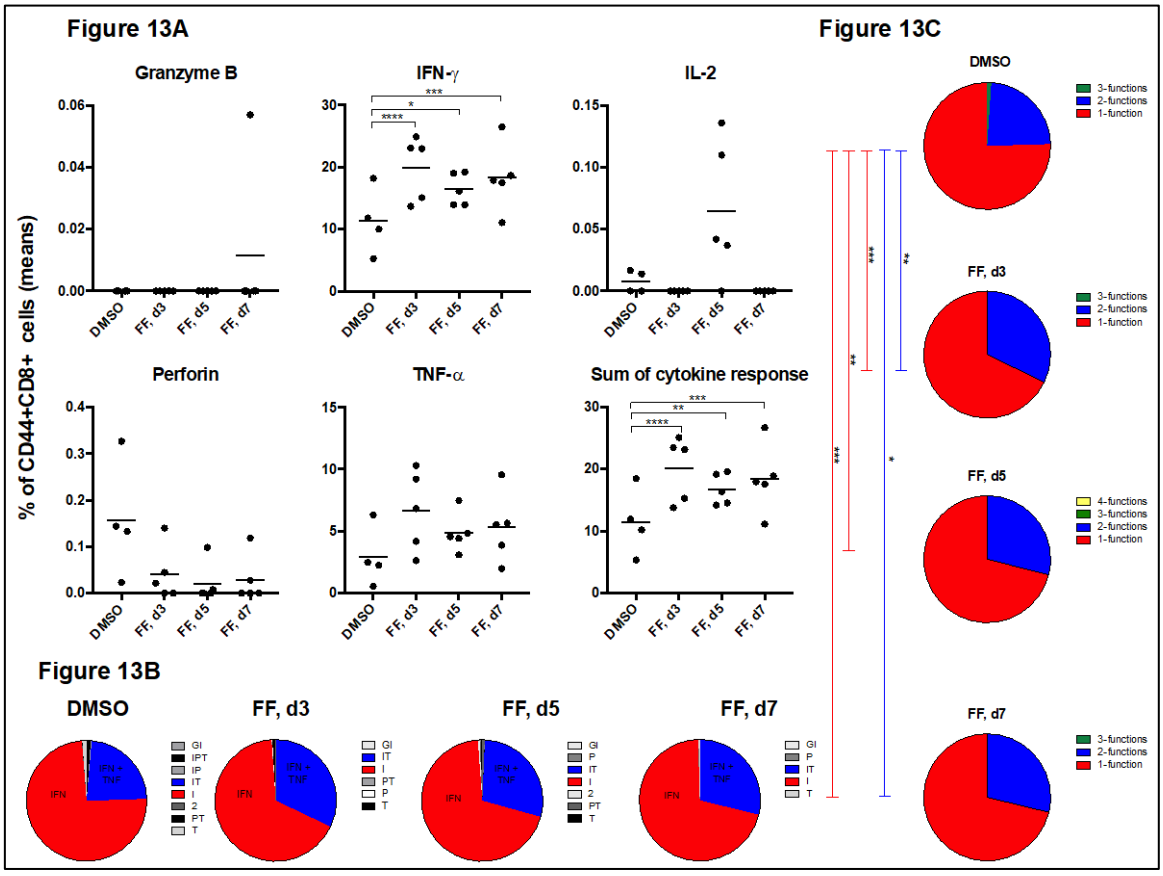


Figure 13A shows frequencies of $CD44^+CD8^+$ T cells from blood producing the indicated factors in response to a short in vitro stimulation with a peptide expressing the immunodominant epitope of Trp-1. Sum of cytokine responses was calculated based on Boolean gating. Figure 3B shows the distribution of T cells producing different mixtures of cytokines. Figure 13C shows the distribution of T cells producing 1-5 factors. Significant differences are indicated by vertical lines to the left of the circles. Lines are color coded according to the pie-slices.

Mice were euthanized when tumors exceeded 1 cm in diameter and TILs were isolated and sorted into $CD44^+CD8^+$ cells. RNA was isolated and upon reverse transcription levels of transcripts for factors involved in fatty acid and glucose metabolism were determined by qPCR.

As shown in Figure 5A and B fenofibrate given early on day 3 after vaccination did not have a significant effect on T cell transcripts while treatment as of day 5 after vaccination increased levels of transcripts for PPARα and Cpt-1a. A further delay of drug treatment till day 7 after vaccination resulted in increased expression of transcripts for Bdh, an enzyme involve in ketone body formation, Glut1 the receptor involved in glucose uptake, Pgk1, an enzyme of glycolysis and Idh3 an enzyme that converts isocitrate to 2-oxoglutarate in the tricarboxylic acid (TCA) cycle. Fenofibrate-induced changes in transcripts within $CD44^{+}CD8^{+}$ TILs were similar but not identical to those in the corresponding cells from spleens of mice which did not carry y tumors (Figures 2A,B). This most likely reflects that the effects of PPARα activation on down-stream transcripts are influenced by the local availability of nutrients and oxygen which differ between spleens and tumor.

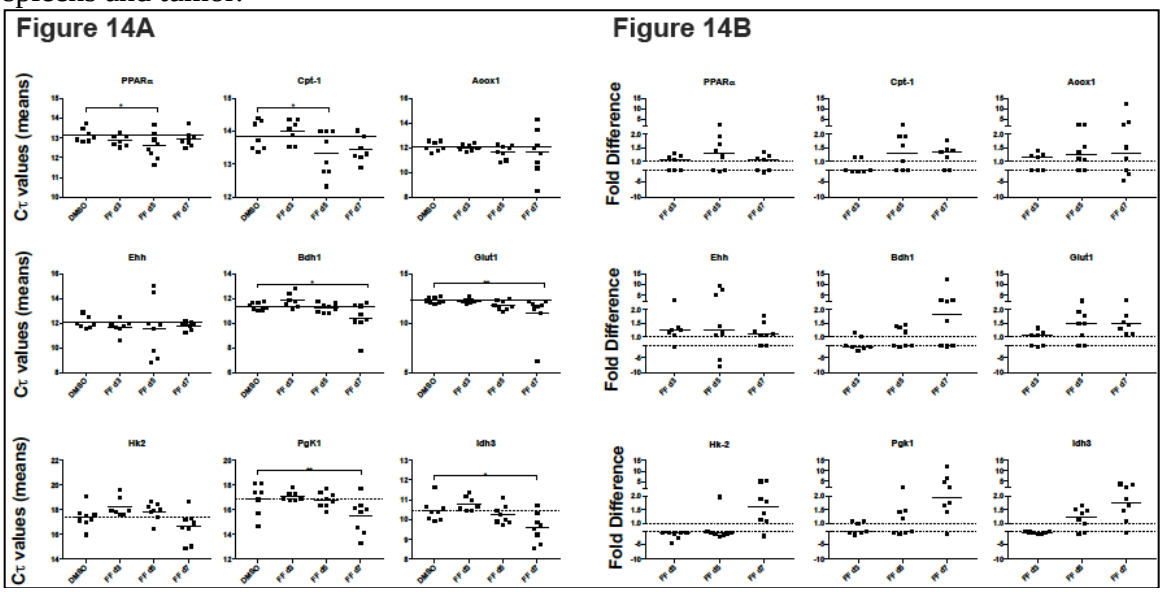


Figure 14A show levels of transcripts in $CD44^{+}CD8^{+}$ TILs from vaccinated mice treated with DMSO or fenofibrate (FF) starting 3-7 days later. Figure 14B shows fold differences between DMSO and fenofibrate treated mice.

Milestone(s) Achieved: We showed upon modification of the protocol that the PPAR-α agonist given directly to tumor- bearing mice slows tumor progression and this is linked to improved T cell functions and changes in T cell metabolism within the tumors.

Aim 2: Effects of metabolic reprogramming combined with PD-1 blockade on mouse TILs. The original aim was to combine vaccination and metabolic reprogramming with PD-1 checkpoint blockade.

The initial experiment was conducted with the original protocol. We injected C57Bl/6 mice as described in the proposal with $B16Braf_{V600E}$ tumor cells. Three days later they were vaccinated with the AdC68-gDMelapoly vaccine. Three days later fenofibrate (200 mg/kg/day) in DMSO/PBS or DMSO/PBS was given by oral gavage daily for 3 weeks. Starting 10 days after vaccination with AdC68-gDMelapoly, 10 mice of each group were injected intraperitoneally (ip) with an anti-PD-1 Ab (clone 29F.1A12) or the isotype control Ab (Iso, Clone: 2A3, Bio X Cell) every 3rd day at a dose of 200 μg/mouse. A small group of naïve mice that were challenged with tumors served as controls. Mice were euthanized once tumors exceeded 1 cm in diameter and cells were stained with T cell markers and an MHC class I tetramer specific for the

immunodominant Trp-1 epitope of the vaccine insert. Cells were analyzed by multi-color flow cytometry. Post-acquisition analysis was conducted by FlowJo. We determined frequencies of $tet^{+}CD8^{+}$ T cells over all $CD8^{+}$T cells in tumors and spleens (Figure 15). As expected, frequencies of Trp-1-specific CD8+ T cells were higher in vaccinated than naïve mice and they were higher in tumors than spleens. In tumors anti-PD1 treatment significantly increased frequencies of Trp-1-specific $CD8^{+}$ TILs in DMSO but not fenofibrate treated mice.

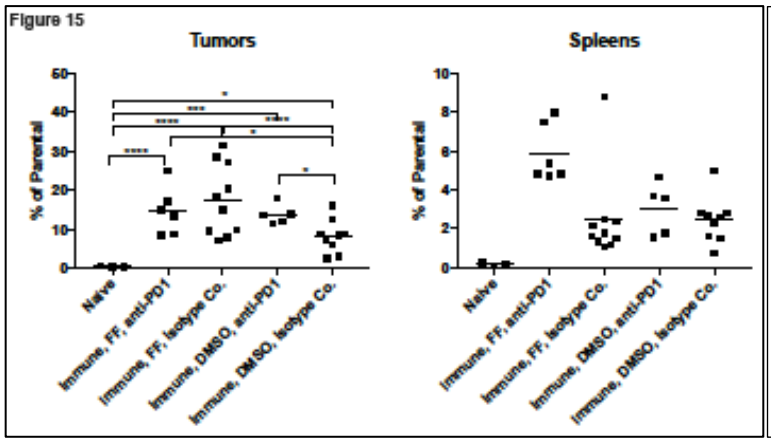
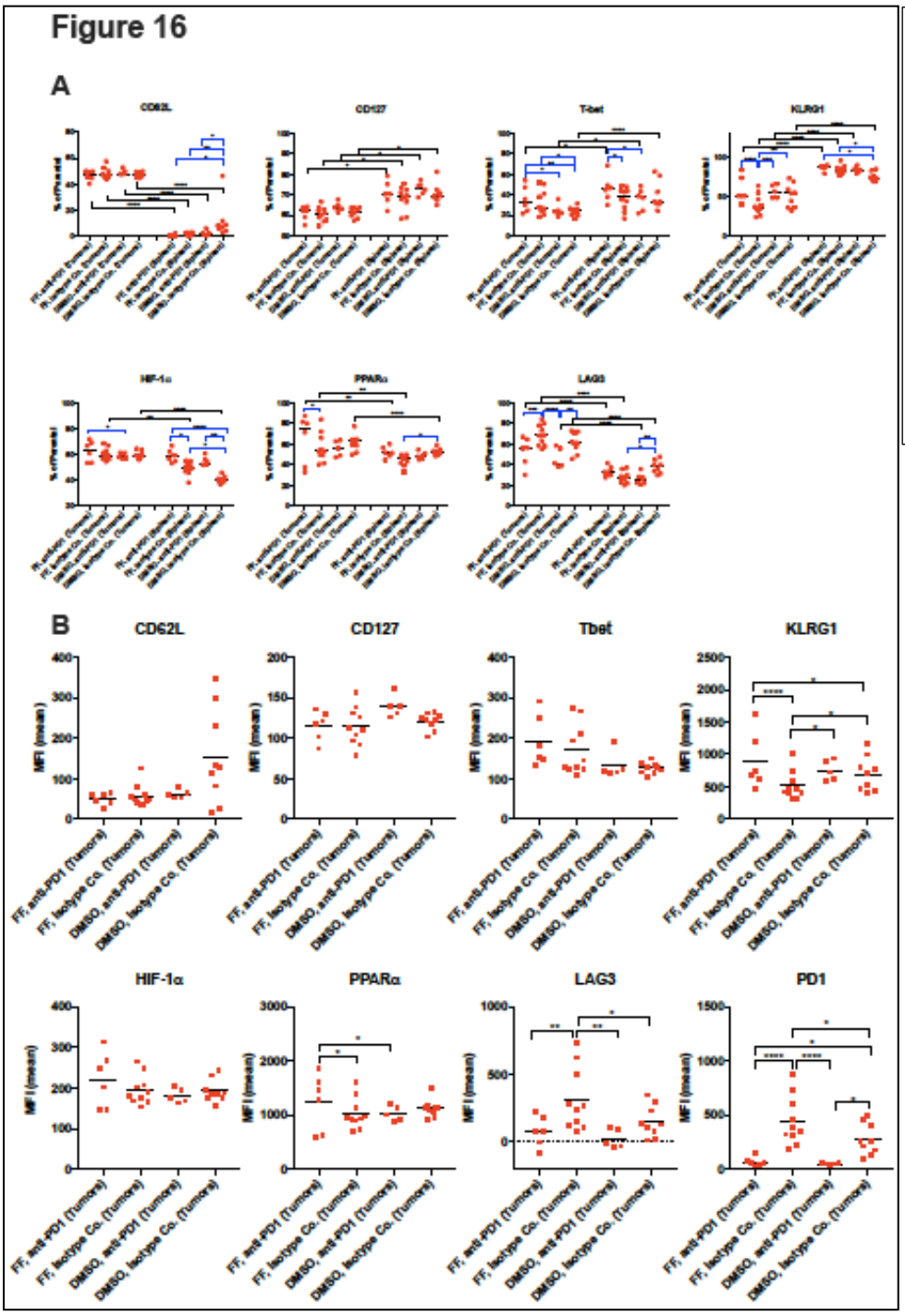


Figure 15. The graphs show for individual mice frequencies of Trp-1-specific $CD8^{+}$ T cells tumors (left) and spleens (right) Lines indicate mean counts. Lines with stars above indicated significant differences by Fisher LSD test (uncorrected 2-way Anova). (*) p-value between 0.01-0.05, (**) p-value between 0.001-0.001, (***) p-value between 0.0001-0.001, (****) p-value < 0.0001.

We analyzed phenotypes of vaccine-induced Trp-1-specific $CD8^{+}$ T cells by staining with antibodies indicative of T cell receptor density (MHC class I tetramer), T cell differentiation (CD62L, CD127), functions (T-bet), exhaustion (PD-1, LAG-3), terminal differentiation (KLRG1), glucose metabolism (HIF-1α) and fatty acid metabolism (PPAR-α). We included lymphocytes from spleens and tumors of vaccinated mice undergoing various treatments. We determined the percentages of cells that had high expression levels of a given marker (Figure 16A) and we analyzed the mean fluorescent intensity (MFI) of each marker on TILs (Figure 16B). As shown in Figure 16A, percentages of $CD62L^{hi}$ cells were regardless of treatment consistently higher on Trp-1-specific $CD8^{+}$T cells from tumors than spleens. In spleens treatment with fenofibrate or anti-PD-1 and fenofibrate reduced CD62L expression. Percentages of CD127 were lower in tumors than spleens regardless of treatment. Percentages of Trp-1 specific $CD8^{+}$ T cells expressing T-bet, a transcription factor that controls T cell functions, were in most of the treatment groups higher in spleens than tumors suggestive of loss of T cell functions potentially due to exhaustion within the tumor microenvironment. The Fenofibrate increased percentages of $T\text{-}bet^{hi}$ Trp-1 specific $CD8^{+}$ T cells and in tumors this was more pronounced in presence of the anti-PD-1 antibody. Percentages of Trp-1-specific $CD8^{+}$ T cells expressing high levels of KLRG1, a terminal differentiation marker, were higher in spleens than tumors. Within spleens treatment with fenofibrate slightly elevated percentages of $KLRG1^{hi}$ Trp-1-specific $CD8^{+}$ T cells. The opposite was observed for Trp-1-specific $CD8^{+}$ T cells from tumors which upon fenofibrate treatment given without the anti-PD-1 antibody showed reduced percentages of $KLRG1^{hi}$ Trp-1-specific $CD8^{+}$ T cells. Percentages of Trp-1 specific $CD8^{+}$ T cells expressing high levels of HIF-1α, a marker for hypoxia, which drives cells towards glycolytic energy production were as expected for some groups higher of cells isolated from oxygen deprived tumors than from spleens. Fenofibrate with anti-PD-1 increased percentages of $HIF\text{-}1\alpha^{hi}$ cells in tumors compared to DMSO + anti-PD-1. Within spleens $HIF\text{-}1\alpha^{hi}$ cells were more common in groups that had been treated with the anti-PD-1 antibody. Increases upon anti-PD-1 treatment were expected as PD-1 signally reduced signaling through the PI3K/mTOR/Akt pathways, which promote

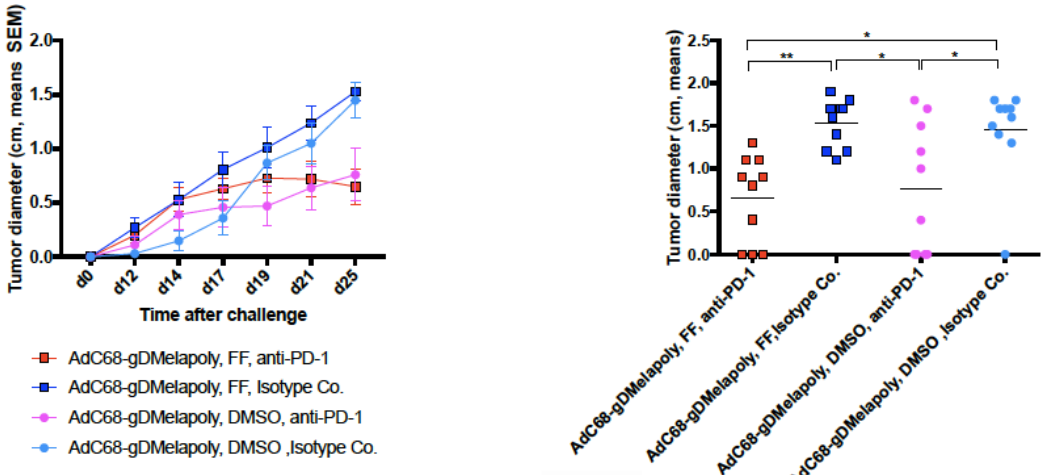


Legend Figure 16A. The graphs show the percentages of Trp-1-specific $CD8^+$ T cells that were high for the indicated markers. Lines with stars above indicate significant differences. Blue lines show difference between groups for a given tissue, black lines show differences between spleens and tumors for the same groups Figure 16B show the mean fluorescent intensity for the same markers on Trp-1-specific $CD8^+$ TILs.

glycolysis and instead drives a switch towards fatty acid oxidation. Percentages of Trp-1-specific $CD8^+$ T cells that expressed high levels of PPARα, the master regulator of fatty acid metabolism were in most groups higher in tumors than spleens, which is an expected result as according to our previous results glucose is scarce in tumors which contain high levels of fatty acid; this in turn forces T cells to adjust their metabolism to the available nutrient. Within tumors fenofibrate given with a control antibody did not increase the percentages of Trp-1-specific $PPAR\alpha^{hi}$ $CD8^+$ T cells while this was achieved upon combination of fenofibrate with the anti-PD-1 antibody. In spleen both fenofibrate and the anti-PD-1 antibody increased percentages of $PPAR\alpha^{hi}$ $CD8^+$ T cells. Percentages of Trp-1-specific $CD8^+$ T cells expressing elevated levels of LAG-3, an exhaustion marker that increases upon hypoxia were as expected higher in tumors than spleens. Within tumors anti-PD-1 treatment reduced LAG-3 expression. Within spleens, fenofibrate or the anti-PD-1 antibody had

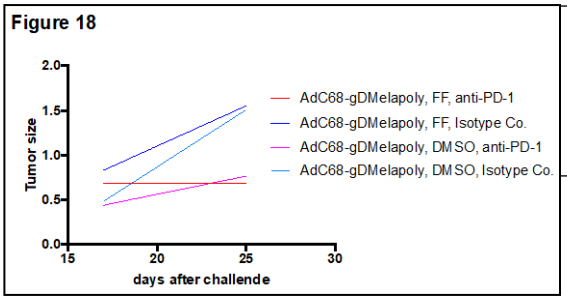
slightly reduced levels of Trp-1-specific LAG-3^{hi} $CD8^+$ T cells compared to the sham-treated control group. We did not include PD-1 into this analysis as the same antibody clone was used for treatment and staining.
For the analyses of mean fluorescent intensity (MFI) of the different markers we focused on cells isolated from tumors. As shown in Figure 16B treatment with fenofibrate or anti-PD-1 or both increased this parameter. Treatment did not change the MFI for CD62L, CD127, T-bet or HIF-1α or Trp-2 $tetramer^+$ $CD8^+$ T cells. KLRG1 expression increased upon treatment with the anti-PD-1 antibody. . PPARα increased in Trp-1-specific $CD8^+$ T cells from mice that received the combination treatment. LAG-3 levels were lower in mice that had been treated with the anti-PD-1 antibody and increased in mice that received only fenofibrate. As expected, mice that had been treated with the anti-PD-1 antibody stained poorly for PD-1 which most likely does not reflect a reduction in expression but rather competition between the antibody used for treatment and for staining. Nevertheless, it is of interest to note that treatment with fenofibrate increased expression levels of PD-1 as we had reported earlier.
We monitored tumor progression. It should be noted mice were euthanized early when tumors were still comparatively small. We can therefore not conclude if any of the treatments would have prolonged survival. As shown in Figure 11, the anti-PD-1 treatment effectively reduced tumor progression in both the fenofibrate and DMSO treated groups. Fenofibrate had no significant effect although it should be noted that the tumor progression curve in the group that received fenofibrate and the anti-PD-1 antibody started to stabilize as of day 17 after tumor cell challenge while the curves for the other groups continued to increase. Plotting linear regression curves for these data as of day 17 of the measurements further highlights the potential differences in tumor progression (Figure 17).

Figure 17



	d17	d19	d21	d25
AdC68-gDMelapoly, FF, anti-PD-1 vs. AdC68-gDMelapoly, FF,Isotype Co.			**	****
AdC68-gDMelapoly, FF, anti-PD-1 vs. AdC68-gDMelapoly, DMSO, anti-PD-1				
AdC68-gDMelapoly, FF, anti-PD-1 vs. AdC68-gDMelapoly, DMSO ,Isotype Co.				****
AdC68-gDMelapoly, FF, Isotype Co. vs. AdC68-gDMelapoly, DMSO, anti-PD-1		**	**	***
AdC68-gDMelapoly, FF, Isotype Co. vs. AdC68-gDMelapoly, DMSO ,Isotype Co.	*			
AdC68-gDMelapoly, DMSO, anti-PD-1 vs. AdC68-gDMelapoly, DMSO ,Isotype Co.		*	*	***

Legend Figure 17. The graphs show the MFI for the indicated markers on or in Trp-1-specific $CD8^+$ T cells from tumors. Lines with stars above indicate significant differences (see legend Fig. 7).



Legend Figure 18. The graph shows linear regression curves plotted as of day 7 for the tumor progression curves shown in Figure 17.

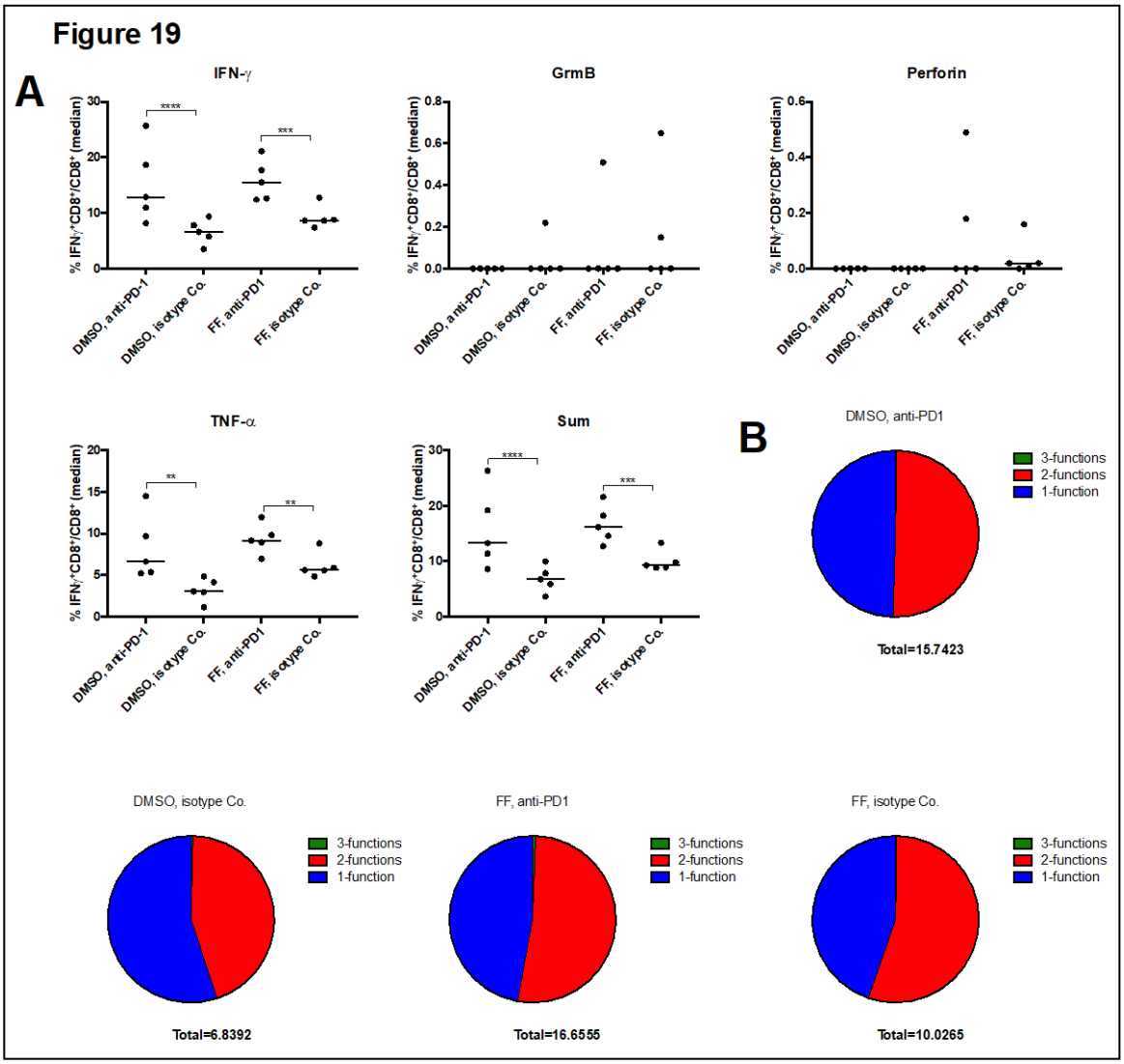
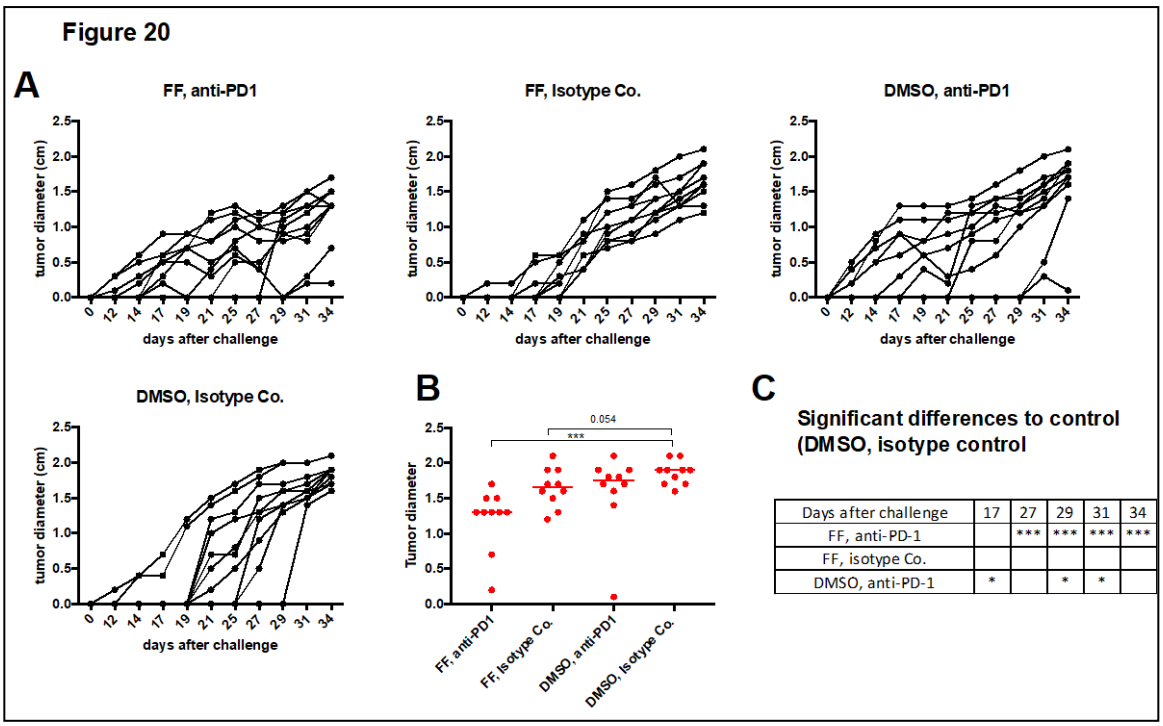


Figure 19 shows functions of vaccine-induced melanoma-specific $CD44^+CD8^+$ T cells in blood. A shows individual functions as well as the sum of functions determined by Boolean gating. Significant differences are indicated by lines between groups with stars above. B shows the proportion of melanoma antigen-specific $CD8^+$ T cells exhibiting 1 or 2 functions (cells exhibiting 3 or 4 functions were to rare to be seen in the pie charts).

For the next experiment we changed the protocol and delayed onset of fenofibrate treatment till day 5 after vaccination. Mice were treated with DMSO or fenofibrate and then with anti-PD-1 antibody or the isotype control antibody. Anti-PD-1 treatment was started 10 days after vaccination. Five mice in each of the four groups were bled 30 days after challenge and function of vaccine-induced melanoma antigen-specific CD8+ T cells were determined by ICS. As shown in Figure 18, anti-PD-1 treatment significantly increased production of IFN-γ and TNF-α and overall functions of circulating $CD8^+$ T cells. There was a trend of further increases if the anti-PD-1 treatment was combined with fenofibrate but this failed to reach significance. We tracked tumor progression and as shown in Figure 19.



Days after challenge	17	27	29	31	34
FF, anti-PD-1		***	***	***	***
FF, isotype Co.					
DMSO, anti-PD-1	*		*	*	

Figure 20A shows tumor diameters in cm over time for individual mice of the 4 groups. B shows tumor diameters on day 34, the day of euthanasia. Significant differences to the control group by t-tests (DMSO and isotype control) are indicated by lines with stars above. C shows significant differences of drug-treated mice to the control group for the different days after challenge. Numbers of stars indicate level of significance as shown in legend to Figure 3.

We tracked tumor progression till tumors in the control groups reached sizes that required euthanasia of the mice. As shown in Figure 20 the combination of fenofibrate and anti-PD-1 antibody achieved a highly significant delay in tumor progression.

We harvested the tumors and isolated $CD8^+$ T cells. We are in the process to analyze their transciptome for the factors shown in Figure 14.

Aim 3
We have obtained human subject approval and submitted an animal protocol. We revised the protocol and are awaiting approval prior to its submission for DoD approval.

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."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars,

study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr. A. Chekaoui was recruited as a postdoctoral fellow to this project. He has learned a number of techniques and is now in the process to write a manuscript. He participated in training related activities at The Wistar Institute, including training in 'Ethical Research Standards".

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report yet. We are planning to submit a manuscript within the next 2 months.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Once we obtain animal approval for the next set of experiments, we will proceed with aim 3. The experiments are designed to test how PPAR-α agonist treatment with or without PD-1 blockade affects TILs within human melanomas. Fragments of freshly resected human melanoma metastases (n = 8-10) will be obtained from the Hospital of the University of Pennsylvania. Tumors fragments will be transplanted under the flank skin of NOD/SCID mice. For metastases that are large enough, lymphocytes will be isolated from remaining tumor fragments and stained to identify different leukocyte subsets, the T cells' differentiation and their metabolic status. A small section of tumor will be embedded in paraffin and stained with an antibody to human PD-L1. Starting 2 days after tumor transplantation, mice will be fed FF or diluent for 3 weeks as in aim 1. Tumor progression will be monitored as in aim 1. At 28 days after transplantation, mice will be euthanized. TILs will be isolated, counted and tested for the markers described above. If numbers do not suffice for stains $CD8^+CD95^+$ TILs will be purified by sorting and probed for the metabolically relevant transcripts. In a second set of experiments, fenofibrate treatment will be combined with PD-1 blockade. PD-1 antibody treatment will be started 2 days after FF treatment. These experiments will preferentially be conducted with larger tumor samples so that a minimum of 8 mice can be transplanted, which will be divided into following 4 groups: (1) diluent and isotype control Ab, (2) diluent and the anti-human PD-1 Ab, (3) FF and isotype control Ab, (4) FF and anti-human PD-1 Ab.

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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We show that combining the melanoma vaccine with fenofibrate treatment reduces tumor progression in mice.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The concept of metabolic manipulation of T cells may increase treatment efficacy in other diseases such as some chronic viral infections where T cells migrate to a microenvironment that is poor in nutrients

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Wistar's business development team believes that the findings disclosed during the performance of this grant are novel, non-obvious and useful and will be used to support the filing of a new provisional patent application. Wistar is actively seeking a co-development partner to assist with the translation of these findings.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*

- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

This would require confirmation of the mouse studies by clinical trials, which may inform if and what type of metabolic manipulations which can in theory not only be achieved by drugs but also by changes in diets or by changes in the microbiome would benefit patients with solid tumors.

5. CHANGES/PROBLEMS: *The PD/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. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

We made some minor technical adjustments but no significant changes.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Due to the COVID-19 pandemic the facility at the Hospital of the University of Pennsylvania was temporarily closed which caused a slight delay in our ability to obtain approval for the aim 3 and 4 animal protocols (which required information that we needed to obtain from the facility. Starting this aim thus has been delayed by about 2-3 months.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

None

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

Nothing to report

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Review article: Zhang Y, Ertl HCJ. T and B cell Metabolism in Older Adults. Immunometabolism. 2020;2(3):e200001. https://doi.org/10.20900/immunometab20200001 Published

  Chekaoui A and Ertl HCJ. Fenofibrate, a PPARa agonist increases the effectiveness of a cancer vaccine to reduce tumor progression. In preparation

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

None

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

None

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  None

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  Timing and dosing of fenofibrate in relation to vaccination. These data will be shared in the publication that is in preparation.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  We are currently assessing if a PPARa antagonist given directly to a tumor bearing host in combination with immunomodulatory agents such as vaccines or checkpoint inhibitors is patentable.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*

- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

None

7. 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: *Areski Cherkaoui, PhD, Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *12*

Contribution to Project: *Dr. Areski performed the T cell assays, cell sorting and PCR studies*
Funding Support: *Fully supported on this award.*

Name: *Christina Cole, Lab Manager*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1.2*

Contribution to Project: *Christina Cole writes, submits and amends animal and human subject protocols and assists Dr. Ertl in assembling and submitting progress reports and manuscripts and in interactions with outside facilities such as the Core Facilities at the University of PA*
Funding Support: *Current award 1.2 cal; Virion 6.0 cal.; Mathers Foundation 4.8 cal.*

Name: *Hildegund Ertl, MD, PI*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *3*

Contribution to Project: *H. Ertl designs experiments, analyses data obtained by flow cytometry and qPCR, writes progress reports and manuscripts and oversees the efforts of the team*

Funding Support: *Current award 3 cal; Spark Therapeutics 0.6 cal; Virion 4.08 cal; Mathers Foundation 3.6 cal.*

Name: *Wynetta Giles-Davis, Research Technician*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*

Contribution to Project: *Wynetta Giles-Davis takes care of cell cultures, prepares media and orders supplies and animals*

Funding Support: *Current award 6 cal.; Spark Therapeutics 1.2 cal; Virion 1.8 cal; Mathers Foundation 3 cal*

Name: *Zhiquan Xiang, MD, Senior Staff Scientist*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4.8*

Contribution to Project: *Dr. Xiang initially trained Dr. Areski, he is in charge of production and quality of vaccines and he assists Dr. Areski in handling of mice*

Funding Support: *Current proposal 4.8 cal; Spark Therapeutics 1.8 cal; Virion 1.56 cal; Mathers Foundation 3.6 cal*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

No other organizations were involved in the 1st year funding period. In the upcoming years, a Core Facility of the University of Pennsylvania will provide melanoma samples.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.