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TITLE: The Role of Adenosine A2BR in Metastatic Melanoma

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14. ABSTRACT Adenosine signaling has been shown in many cancers including melanoma and has been associated with poor prognosis and increased risk of metastasis. Evidence indicates that adenosine receptor A2AR plays a role in inhibiting immune cells whereas A2BR is likely most critical on tumor cells and tumor endothelium. We propose that elimination of adenosine A2B receptor signaling in endothelial cells and tumor cells will result in a decrease of primary melanoma and metastasis. We were interested in the relevance of A2BR in the context of immunotherapy checkpoint inhibitors anti-PD1 or anti-CTLA4 or Braf inhibitors. We found that knocking down A2BR expression in melanoma cells SM1WT1 LWT1 did not change the growth of primary tumor nor experimental metastasis when compared to control tumor. Additionally, A2BR knocked out in the endothelium or the whole host did not affect primary tumor growth or metastasis of melanoma cell lines SM1WT1, B16F10 and HCMel12. Therefore, A2BR does not play a significant role in melanoma growth or metastasis.					
15. SUBJECT TERMS Adenosine, A2BR, melanoma, immune cells, vessel.					
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Table of Contents

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	18
5. Changes/Problems	18
6. Products	19
7. Participants & Other Collaborating Organizations	20
8. Appendices	20

1. INTRODUCTION:

The immunosuppressive metabolite adenosine plays an important role in tumor development, progression and responses to therapy. We would like to interrogate the role of adenosine receptor (A2BR) in regulating primary tumor growth and metastasis in experimental models of melanoma. Our hypothesis is that deletion of adenosine A2B receptor (A2BR) signaling in endothelial and tumor cells will result in a decrease of melanoma growth and metastasis. If A2BR plays a role in limiting tumor growth and metastasis, we will also investigate the role of A2BR in the context of immunotherapy using checkpoint inhibitors anti-PD1 or anti-CTLA4 or Braf inhibitors with A2BR knockout tumors. A2BR inhibitors and checkpoint inhibitors or BRAF inhibitors as a therapeutic for melanoma.

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

Adenosine, A2BR, melanoma, immune cells, vessel.

3. ACCOMPLISHMENTS:

▪ What were the major goals of the project?

Specific Aim1: 100% complete Determine the role of adenosine the A2B receptor (A2BR) in regulating tumor growth and metastasis in experimental models of melanoma.

Milestone # 1 ACURO approval obtained July 2016

Milestone # 2 Selection of the optimal A2BR knockout cell lines with similar proliferation characteristics to the control cell line to be used in in vivo experiments October 2016

Specific Aim2: 0% complete. Efficacy of combination therapy using A2B receptor (A2BR) inhibition and checkpoint inhibitors in regulating tumor growth and metastasis in experimental models of melanoma.

Milestone #3 Determination of the effect of anti-PD-1 and anti-CTLA4 on A2BR knockout melanoma metastasis.

Milestone #4 Identification of molecular signatures as a result of A2BR inhibition in the tumor microenvironment in the context of immunotherapies such as anti-PD-1 and anti-CTLA4

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

Specific Aim 1, Subtask 1: 100 % complete.

ACURO approval to perform animal studies was obtained July 2016.

Specific Aim 1, Subtask 2: 100 % complete. Use CRISPR/Cas9 technology to knockout A2BR expression in HcMel12, SM1WT1 LWT1 and B16F10 melanoma cell lines.

To dissect the role of A2BR in tumor-endothelial and tumor-immune cell interactions, the first objective was to use the CRISPR Cas9 system to knock out A2BR expression in melanoma cell lines. Melanoma cell lines were assessed for A2BR RNA expression (Figure 1A). SM1WT1 LWT1, which is a metastatic version of the Braf mutant SM1WT1 melanoma showed the highest expression of A2BR RNA and therefore was selected for genome editing. Small oligo guide RNA (sgRNAs) specific to the Adora2B locus were designed using the online tool provided by the Zhang lab (crispr.mit.edu) (Figure 1B). The workflow of this process is outlined including the approximate location of the sgRNA targets. The sgRNA was inserted into the px330 plasmid after the U6 and gRNA scaffold site and transformed. Clones were selected, run on PCR confirming a specific band at 100 bp (Figure 1C) and sequenced to confirm the successful insertion of the sgRNA into the px330 plasmid. SM1WT1 LWT1 cells were transfected to express Cas9, A2BRsgRNA and GFP then after 48 hrs were sorted based on GFP high expression. The workflow shows alternative ways to select the gene edited cells. This subtask was not conducted on the two additional cell lines as proposed because the initial A2BR RNA expression was so low.

Specific Aim 1, Subtask 3, 20 % complete: Assess the in vitro effects of knocking out A2BR expression.

qRTPCR was used to determine A2BR and A2AR expression in SM1WT1 LWT1 cells. A2AR binds adenosine with higher affinity than A2BR and has higher expression in the SM1WT1 LWT1 cells, thus it is interesting that sgRNA1 and sgRNA3 had a reduction in both A2BR and A2AR expression when compared to shcntl (Figure 1D). BLAST of the sgRNA1 and sgRNA3 sequence show they are specific for A2BR and not for A2AR. FACS analysis of A2BR surface expression using the Alomone Labs anti-mouse A2BR antibody showed that A2BR expression was not significantly different from the low expression level detected in the control cell line (Figure 1E). *In vitro* characterization of cell proliferation show that all the A2BRsgRNA cell lines had a defect in proliferation (Figure 1F). The desired phenotype of reduction of A2BR expression on the cell surface was not achieved; therefore, further *in vitro* characterization such as cytokine production or invasion assay was not performed.

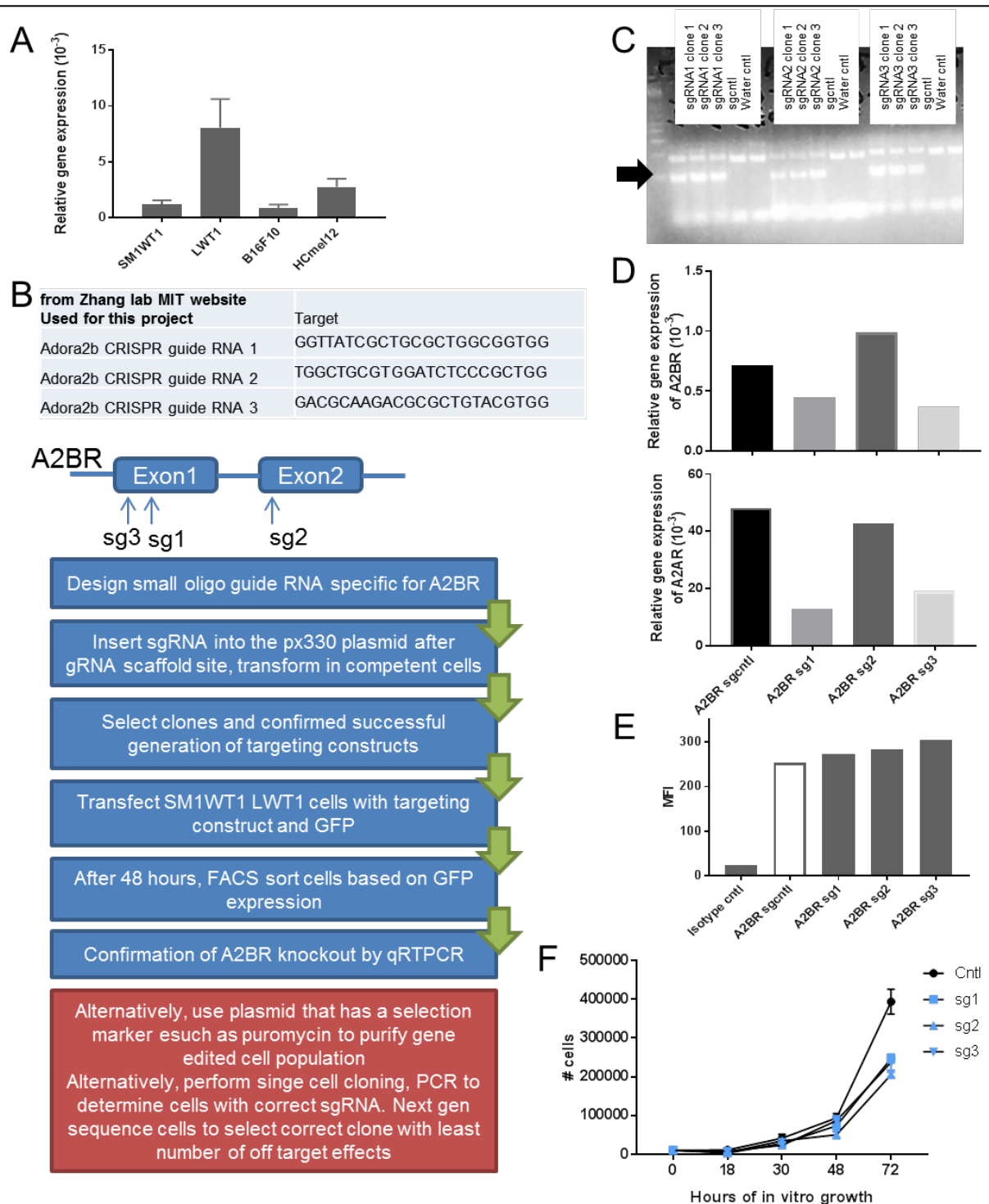


Figure 1: A2BR gene editing using CRISPR. Melanoma cell lines were assessed using qRTPCR for (A) A2BR expression. SM1WT1 LWT1 was found to have the highest expression. (B) Guide RNA sequence design, targeted region on A2BR and workflow, plus alternative in red box. (C) Clones were verified for correct sgRNA insertion via PCR, arrow indicates PCR products of sgRNA specific sites. (D) Tranfected SM1WT1 LWT1 cells were assessed for A2BR and A2AR expression via qRTPCR. (E) Tranfected SM1WT1 LWT1 cells were assessed for A2BR expression via FACS analysis. (F) *In vitro* cell proliferation was assessed in triplicate over 72 hours.

We now know that strong repression of a gene is observed when the sgRNA targets the DNA from -50 to +300bp relative to the transcriptional start site (Lo A, doi:10.12688/f1000research.11113.1). From this insight we would predict sgRNA1 and sgRNA3 to work but sgRNA2 would not be as efficient. We considered commercially available constructs to potentially improve the CRISPR knock down of A2BR, specifically the design provided by Genescript that was revised within 9 months. This indicated to us that other researchers have had similar difficulties with producing A2BR gene edited cells. We did not expect the expression of A2BR to be so low in these cells and thus the ability for us to detect surface A2BR for sorting was limited. We have recently purchased a plasmid which has a puromycin cassette to allow selection of the correctly targeted cells. In addition, the expression of A2AR could still be the dominant adenosine signaling receptor in this melanoma cell line and the interdependence of these two receptors is an interesting line of investigation.

Specific Aim 1, Subtask 4, 100 % complete: Primary tumor growth curves and analysis of distance metastases at necropsy.

To determine the role of A2BR on immune cells in the tumor microenvironment, we used A2BR^{-/-} mice where exon II of the A2BR gene was deleted, resulting in an A2BR transcript lacking the poly(A) signal sequence which is unstable (doi: 10.4049/jimmunol.1001567). A2BR^{-/-} mice are genotyped by PCR to confirm that the genomic locus is missing (Figure 2A). The expression of A2BR is highest in the jejunum in WT mice, thus jejunum from WT mice were compared to A2BR^{-/-} mice by qRT-PCR and were shown to have no expression (Figure 2B). The positive control was a triple negative breast cancer cell line, E0771. To interrogate the role of A2BR in aiding tumor metastasis, we used VeCad Cre/WT mice where A2BR expression is knocked out only in the blood vessels. These mice are genotyped to confirm the deleted allele in whole tissue samples (Figure 2C). In addition, we have tested the VE-Cad-Cre x R26-LSL-eYFP mouse via confocal to confirm Cre expression in endothelial cells on vessels.

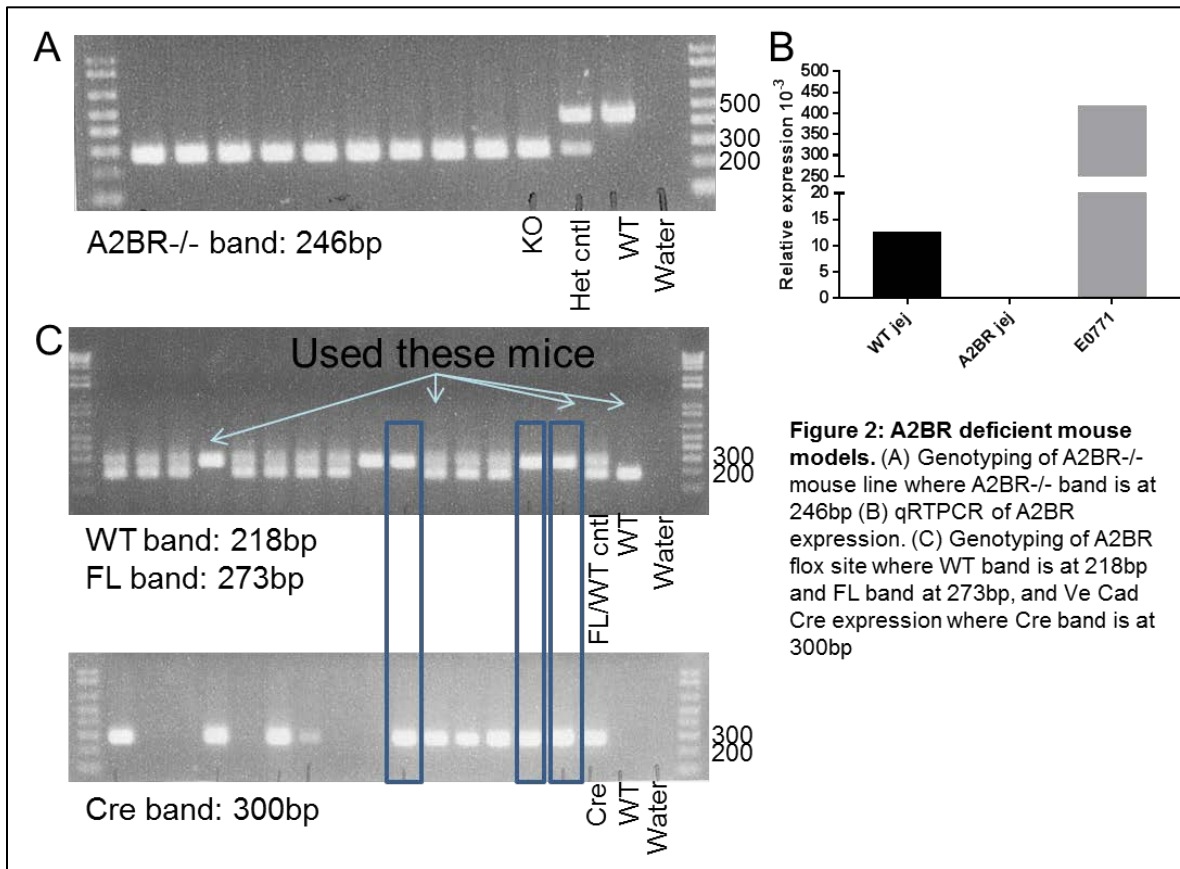
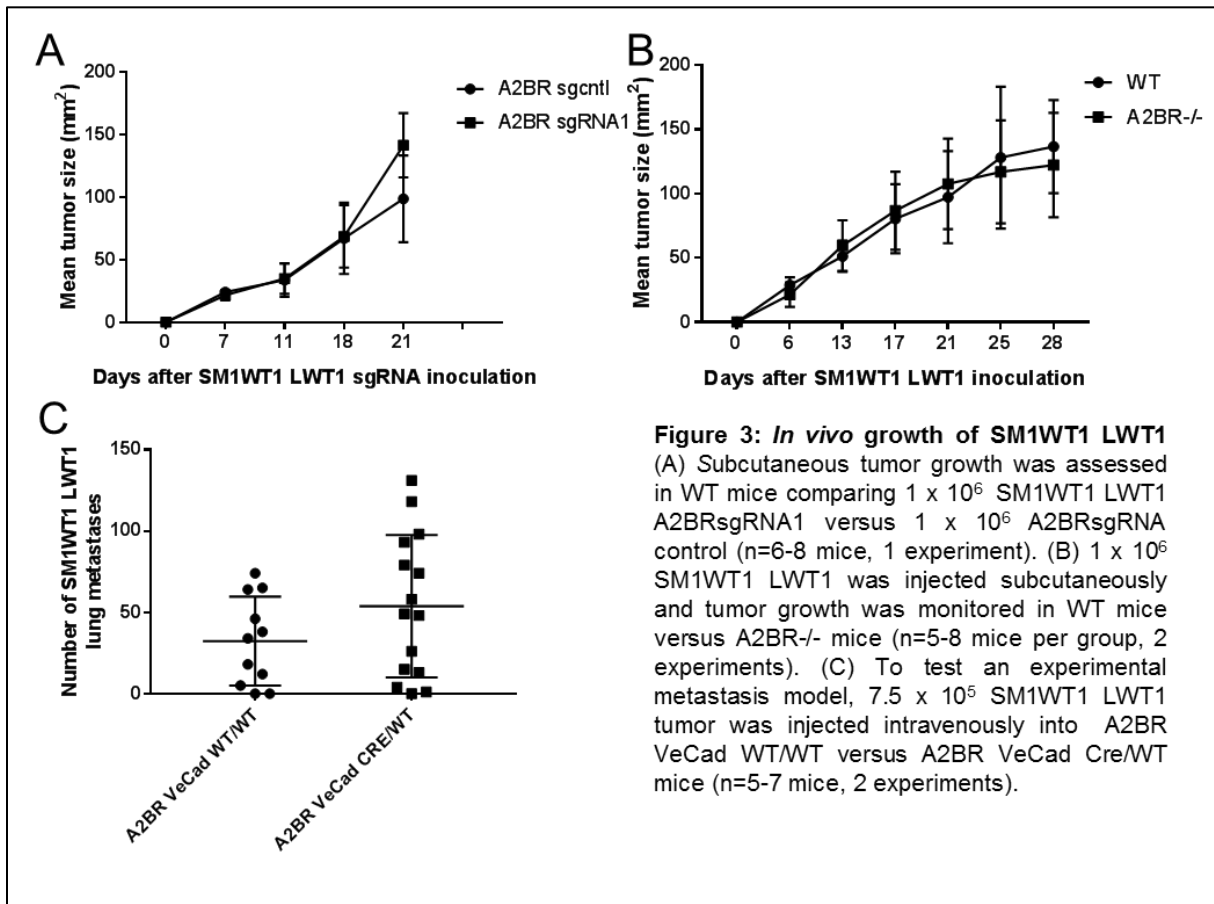


Figure 2: A2BR deficient mouse models. (A) Genotyping of A2BR^{-/-} mouse line where A2BR^{-/-} band is at 246bp (B) qRT-PCR of A2BR expression. (C) Genotyping of A2BR flox site where WT band is at 218bp and FL band at 273bp, and Ve Cad Cre expression where Cre band is at 300bp

SM1WT1 LWT1- A2BRsgRNA1 was chosen for *in vivo* testing because this cell line had the lowest A2BR surface expression via FACS and the least impediment to *in vitro* proliferation. 1×10^6 SM1WT1 LWT1- A2BRsgRNA1 and A2BRsgRNA control were injected subcutaneously into wild type mice and growth was monitored over 30 days. Tumors were measured twice a week with digital calipers, longest length x orthogonal length, shown as mean tumor size $\pm$ S.D. (Figure 3A). There was no statistical difference in growth between the A2BR deficient tumor cells and the control tumor (Mann-Whitney U test). No distant metastasis was detected.

Growth of 1×10^6 SM1WT1 LWT1 parental tumor in WT versus A2BR^{-/-} mice shows no statistical difference, shown as mean tumor size $\pm$ S.D., and no distant metastasis was detected (Figure 3B). The experimental metastasis model of SM1WT1 LWT1 parental tumor was tested in A2BR VeCad WT/WT versus A2BR VeCad Cre/WT mice. Lungs were harvested 14 days after intravenous injection of 7.5×10^5 of the cells, perfused with ink and surface tumors were counted, however there is no statistical difference in metastasis formation between the two groups (Figure 3C).

We conclude that A2BR deficiency in the tumor or the host does not affect tumor growth in the SM1WT1 LWT1 model. The results obtained with A2BRsgRNA1 may be due to a mixed population of cells. Cell sorting was performed but due to lack of adequate selection markers, the population could not be further purified upon subsequent passages.



We next tested if the parental melanoma would be controlled by A2BR-deficient mice, 1×10^6 SM1WT1 was injected subcutaneously into A2BR VeCad WT/WT versus A2BR VeCad Cre/WT (Figure 4A) and A2BR^{-/-} versus WT (Figure 4B) mice. Shown as mean $\pm$ S.D., there was no statistical difference in tumor growth in either strain of A2BR-deficient mice. We conclude that A2BR deficiency on the vessels or globally in the host does not affect tumor growth in the SM1WT1 model.

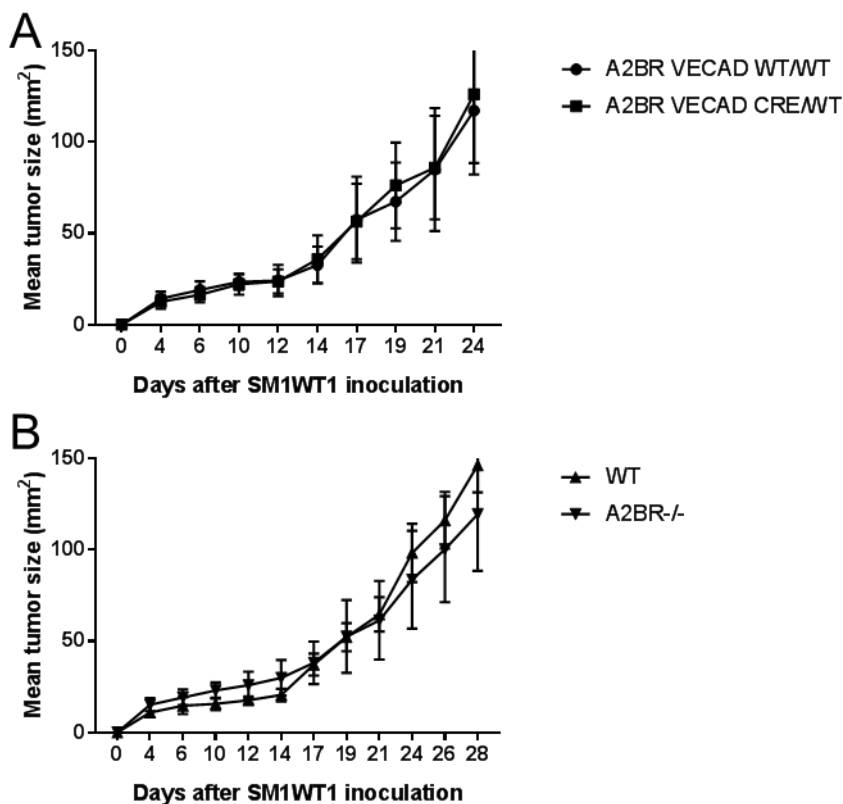


Figure 4: *In vivo* growth of SM1WT1 (A) 1×10^6 SM1WT1 was injected subcutaneously and tumor growth was monitored in A2BR VeCad WT/WT versus A2BR VeCad Cre/WT mice (n=6-8 mice, 2 experiments). (B) 1×10^6 SM1WT1 was injected subcutaneously and tumor growth was monitored in WT mice versus A2BR^{-/-} mice (n=5-8 mice per group, 2 experiments).

To obtain a further understanding if melanoma would be controlled by A2BR-deficient mice, 2×10^5 B16F10 melanoma was injected subcutaneously into A2BR VeCad WT/WT versus A2BR VeCad Cre/WT, shown as mean $\pm$ S.D. (Figure 5A). There was no difference in tumor growth in these mice and no distant metastasis was found. Finally, we tested HCMel12 a spontaneous metastatic melanoma where HGF is overexpressed and cell cycle is disrupted by CDK4(R24C). We injected 5×10^5 HCMel12 subcutaneously into A2BR VeCad WT/WT versus A2BR VeCad Cre/WT (Figure 5B) and A2BR^{-/-} versus WT, shown as mean $\pm$ S.D. (Figure 5C) mice and there was no statistical difference in tumor growth in either A2BR-deficient mice. We conclude that A2BR deficiency on the vessels or in the host does not affect tumor growth in the B16F10 or HCMel12 models.

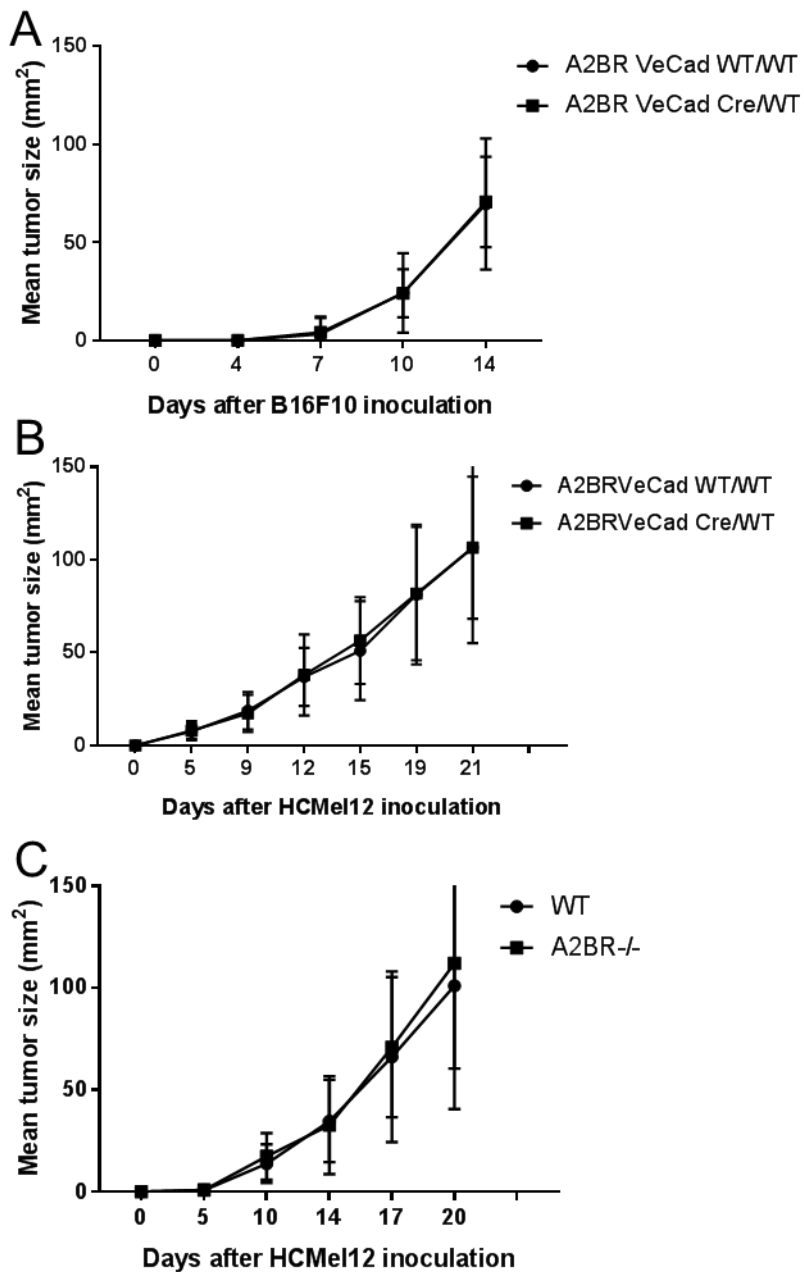


Figure 5: *In vivo* growth of B16F10 and HCMel12 (A) 2×10^5 B16F10 was injected subcutaneously and tumor growth was monitored in A2BR VeCad WT/WT versus A2BR VeCad Cre/WT mice (n=5-8 mice, 1 experiment). (B) 5×10^5 HCMel12 was injected subcutaneously and tumor growth was monitored in A2BR VeCad WT/WT versus A2BR VeCad Cre/WT mice (n=8 mice per group, 2 experiments). (C) 5×10^5 HCMel12 was injected subcutaneously and tumor growth was monitored in WT mice versus A2BR^{-/-} mice (n=6-8 mice per group, 2 experiments).

Specific Aim 1, Subtask 5: 100 % complete. Immune FACs analysis of tumor and metastatic sites.

To obtain a deeper understanding of the role of A2BR in the tumor microenvironment we performed cellular analysis of the local recruitment of immune cells using flow cytometry. Primary tumors were assessed but not metastatic sites since metastases were not detected.

We examined the numbers of infiltrating immune cells into the primary tumor site. We inspected TCR β ⁺CD4⁺ T cells, TCR β ⁺CD8⁺ T cells, TCR β ⁻NK1.1⁺ NK cells, CD11b⁺ total myeloid cells, CD11b⁺F4/80⁺ macrophages (Figure 6). 1 x 10⁶ SM1WT1 tumors were injected s.c. into WT, A2BR^{-/-}, A2BR VeCad WT/WT and A2BR VeCad Cre/WT mice and on day 24, tumors were harvested, digested in collagenase I with DNase 1 and stained with antibodies for immune cells markers and analyzed on a BD LSR Fortessa 4. At time of harvest, there was no statistical difference in the tumor weights from the different genotypes. There was an increase in the number of CD4⁺ T cells per gram of tumor grown in A2BR VeCad Cre/WT compared to A2BR VeCad WT/WT mice (ANOVA with Mann-Whitney U test), however, the percentage of CD4⁺ T cells of the total immune cell infiltrate was not different from the control group. There were no significant differences in the number or percentages of CD8⁺ T cells, NK cells, or myeloid cells, which is to be expected as the tumor sizes were not significantly different.

Flow cytometry was performed on A2BR^{-/-} mice versus wildtype and A2BR VeCad WT/WT versus A2BR VeCad Cre/WT, where A2BR expression is only knocked out only in the vasculature, to determine if there are any baseline differences in immune cell populations in these mice. We inspected TCR β ⁺CD4⁺, TCR β ⁺CD8⁺, TCR β ⁻NK1.1⁺, TCR β ⁻CD19⁺, CD11b⁺CD11c⁺, CD11b⁺GR-1^{lo}, CD11b⁺GR-1^{int}, CD11b⁺GR-1^{hi} and found there were no differences in the percentage of cell populations in the blood, bone marrow, spleen or lung of these mice (data not shown).

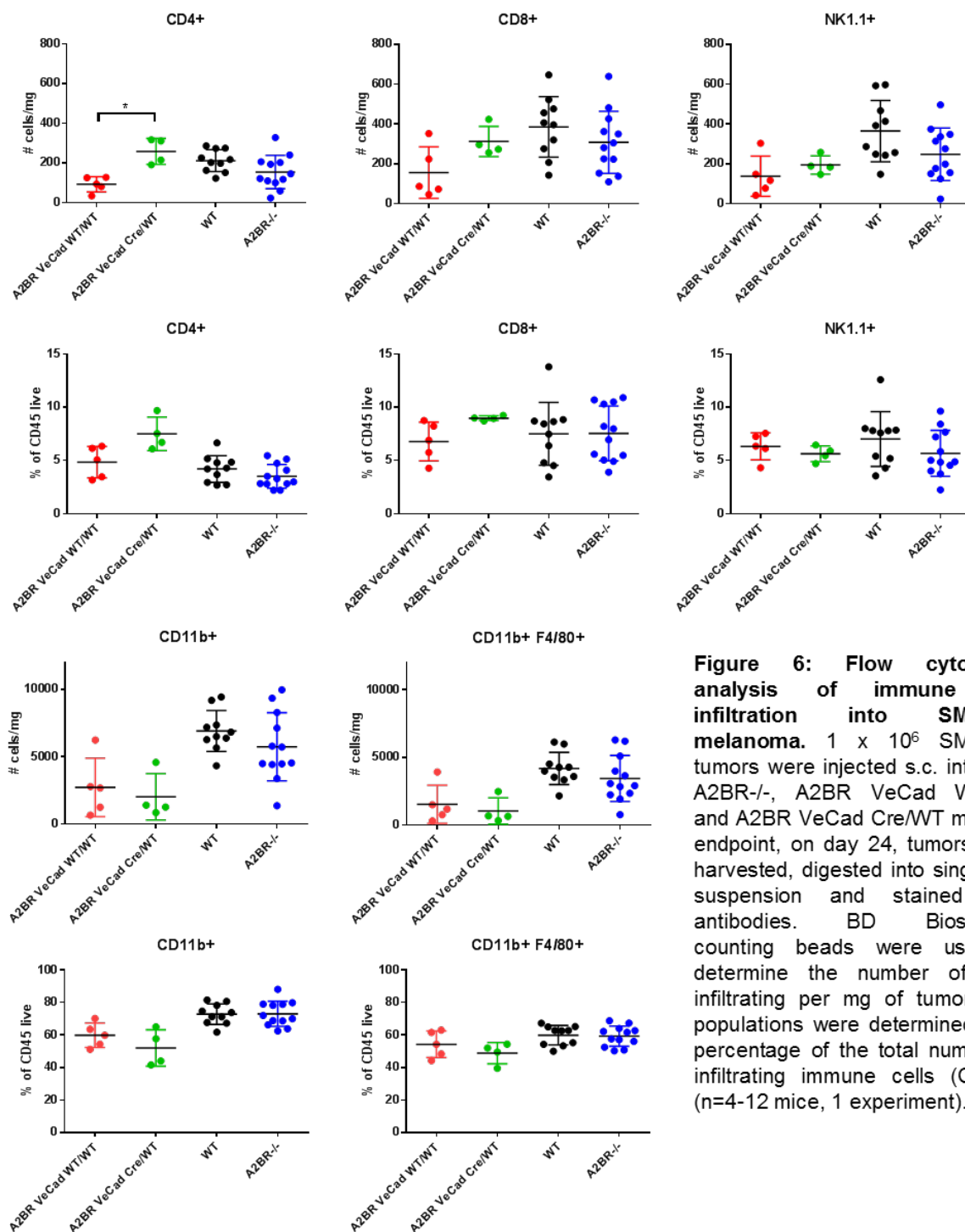
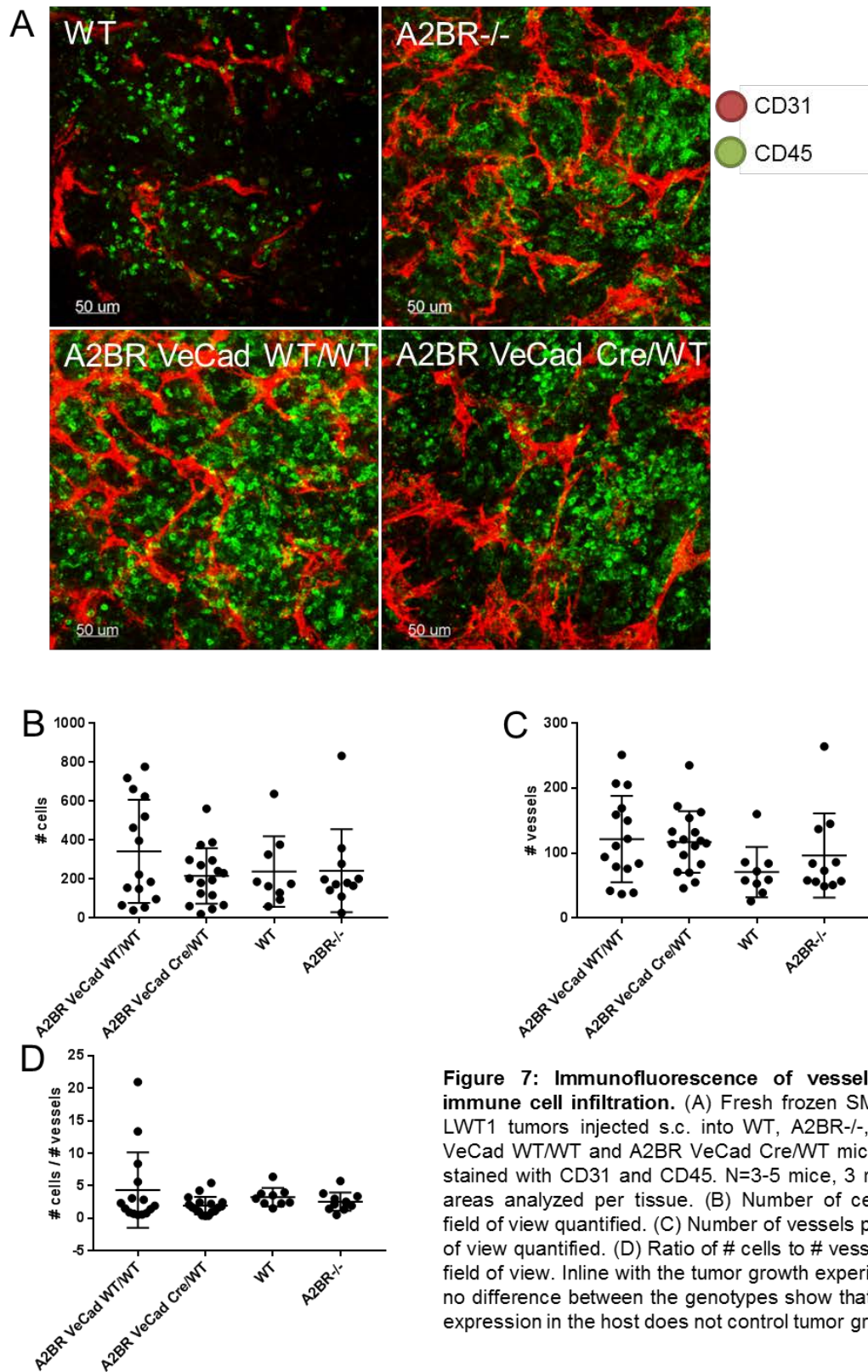


Figure 6: Flow cytometry analysis of immune cell infiltration into SM1WT1 melanoma. 1×10^6 SM1WT1 tumors were injected s.c. into WT, A2BR^{-/-}, A2BR VeCad WT/WT and A2BR VeCad Cre/WT mice. At endpoint, on day 24, tumors were harvested, digested into single cell suspension and stained with antibodies. BD Bioscience counting beads were used to determine the number of cells infiltrating per mg of tumor. Cell populations were determined as a percentage of the total number of infiltrating immune cells (CD45⁺) (n=4-12 mice, 1 experiment).

Specific Aim 1, Subtask 6: 100% complete. Immunohistochemistry of primary tumor and metastatic site with particular interest in immune cell subsets around CD31 stained vessel endothelium.

Fluorescent immunohistochemistry was employed to further understand the spatial relationship between the immune cells and endothelium. Primary tumor was assessed but metastasis was not detected. Tumor was taken from mice on day 24 and fresh frozen in OCT. A cross-section of the tissue was cut. The tissue was blocked with Background sniper (Biocare) and stained total immune cell infiltrate with CD45 and assessed the vessel density by staining CD31, both in Tris-buffered solution. Three random areas were imaged per tissue, for melanoma injected into WT, A2BR^{-/-}, A2BR VeCad WT/WT, and A2BR VeCad Cre/WT using a Zeiss 780 confocal microscope (Figure 7a).

There was no statistical difference in the number of cells counted per field of view (Figure 7b), nor was there a statistical difference in the number of vessels counted per field of view (Figure 7c) nor the ratio of the number of cells to the number of vessels (Figure 7d).



Specific Aim2: 0 % completed. Efficacy of combination therapy using A2B receptor (A2BR) inhibition and checkpoint inhibitors in regulating tumor growth and metastasis in experimental models of melanoma.

The premise of this aim was to determine if checkpoint inhibitors such as anti-PD1 and anti-CTLA4 or BRAF inhibitors could be used to enhance the effect of A2BR deficiency in the tumor microenvironment. It has become clear from the experiments completed in Aim 1 that A2BR deficiency in the tumor, on the blood vessels or throughout the host does not affect tumor growth at all. Therefore, it was decided to not proceed with this aim because it would likely simply be a study of the effects of anti-PD1, anti-CTLA4 or Braf inhibitors on melanoma tumor growth.

However, a component of this aim (subtask 4) was to collect tumor injected into A2BR-deficient mice in the presence or absence of anti-PD1, anti-CTLA4 and BRAF inhibitors, prepare lysates and analyse the microenvironment by using the Nanostring nCounter PanCancer Immune Profiling Panel. This method would allow the assessment of 500 genes by direct RNA detection, related to the immune response towards melanoma. This analysis was intended to not only provide insight into the molecular function of adenosine in the microenvironment, but to provide leads to improve combination therapies. When enquiring about the cost of the Nanostring nCounter PanCancer Immune Profiling Panel, it was decided that a custom set of targets would allow us to assess the adenosine related status of the immune cells. Figure 8 lists the targets selected for analysis which includes the adenosine receptors and adenosine generating ectonucleases, transcription factors, checkpoint inhibitors, chemokines, cytokines, and activation markers.

A	Adenosine related	Cell activation	Cell function	Cytokine/chemokine
	A1	Eef1g	Akt3	CCL3
	Adora2a	Prf1	Bcl2	CCL4
	Adora2b	Gzma	Bcl6	CCL5
	A3	Gzmb	GM-CSF	CCR1
	Hif1a	Hpse1	Mapk3	CCR5
	CD39	Icosl	PI3K	CCR7
	CD73	Ifnar1	PTPN2	CXCR3
		Ifnar2	Socs3	CXCR4
	Checkpoint inhibitors	Ifng	Stat1	CXCR6
	PD1	Ikzf2	Stat3	IL10
	CTLA4	CRTAM	Tgfb1	IL12a
	LAG3	CD160	TNF	IL15
	TIM3	CD244	Tnfrsf9	IL17a
	BTLA	CD28		IL18
		CD44		IL1a
	Transcription factors	Itga1		IL1b
	Tbx21	Itga2		IL2
	Eomes	Klrg1		IL21
	Foxp3	Klrk1		IL22
	Gata3	Nfatc1		IL23a
	Rorc	Zap70		IL2rb
	Tbp			IL4
				IL5
				IL6
				IL7
				IL7r
				IL9

Figure 8: Nanostring panel. A custom set of targets were designed to determine the adenosine related status, activation status and checkpoint inhibitors of T cells and/or NK cells within the tumor microenvironment.

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

I attended the Immunotherapy@Brisbane 2017 Conference, 10-12 May. This international conference allowed me to learn about the newest clinical outcomes and related metrics with checkpoint inhibitor therapy. In addition, I was able to engage with researchers that have the potential to turn into future collaborators.

The development of a Nanostring panel provided an opportunity to learn about a new technology and employ a technique that might be utilized not only to determine the status of the tumor microenvironment but to develop biomarkers that could be used for direct clinical applications.

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

▪ *Nothing to Report.*

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

- *Nothing to Report.*

4. **IMPACT:**

We have shown that in melanoma, that RNA expression of A2BR is relatively low and that gene editing to generate loss of receptor expression in melanoma does not have a profound effect on tumor growth in vivo. We have also shown that deficiency of A2BR expression on the blood vessels and throughout the host also does not impact the growth of melanoma in vivo. However, this gene editing approach might be beneficial in other cancer types that have higher A2BR expression such as triple negative breast cancer.

- **What was the impact on the development of the principal discipline(s) of the project?**
 - These experiments suggest cancers other than melanoma, such as triple negative breast cancer, might be better studied in the context of A2BR.
- **on other disciplines?**
 - *Nothing to Report.*
- **What was the impact on technology transfer?**
 - *Nothing to Report.*
- **What was the impact on society beyond science and technology?**
 - *Nothing to Report.*

5. **CHANGES / PROBLEMS:**

The loss of A2BR expression on the tumor, the endothelium or throughout the host entirely did not change the growth of the tumor. The goal of Aim 2 was to enhance the anti-tumor effect of inhibiting A2BR expression by adding checkpoint inhibitors or BRAF inhibitors. However, combining these therapies with an approach that clearly does not provide benefit over the control tumor setting is not likely to yield useful information.

- **Changes in approach and reasons for change**
 - Performing experiments outlined in Aim 2 will not be performed because the grant term is completed and it will not likely add to our knowledge about combination therapies.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
 - *Nothing to Report*
- **Changes that had a significant impact on expenditures**
 - *Nothing to Report*
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

- *Nothing to Report.*
- **Significant changes in use or care of human subjects.**
 - *Nothing to Report.*
- **Significant changes in use or care of vertebrate animals.**
 - *Nothing to Report.*
- **Significant changes in use of biohazards and / or select agents**
 - *Nothing to Report.*

6. **PRODUCTS:** *Nothing to Report.*

- **Publications, conference papers, and presentations.** *Nothing to Report.*
- **Journal publications.** *Nothing to Report.*
- **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.*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

Name:	<i>Deborah Barkauskas</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	0000-0002-1872-4109
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Ms. Barkauskas has performed all the work in this project</i>
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
 - *Nothing to Report.*
- **What other organizations were involved as partners?**
 - *Nothing to Report.*

8. APPENDICES: Nothing to Report.