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14. ABSTRACT
A recent breakthrough in treating melanoma patients is the use of immune checkpoint therapy (ICT). Unfortunately, the majority of patients do not respond to ICT. Complicating matters, is that as cancer develops, it can re-program immune cells to work in favor of tumor growth. One such population of cells are myeloid-derived suppressor cells (MDSCs). MDSCs dampen the immune system from recognizing cancer and decrease ICT treatment response. Preliminary data suggests that the enzyme myeloperoxidase is overexpressed in MDSCs. We hypothesize that by limiting the function of myeloperoxidase, we limit the effects of MDSCs to enable successful ICT response in melanoma.

15. SUBJECT TERMS
Immune checkpoint therapy, melanoma, myeloid-derived suppressor cells, myeloperoxidase, intravital imaging, immunosuppression

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1. INTRODUCTION:

A recent breakthrough in treating melanoma patients is the use of immune checkpoint therapy (ICT). ICT blocks these immune checkpoints allowing the immune system to recognize cancer as foreign and subsequently remove it from our body. A small set of melanoma patients after treatment with ICT demonstrated long-term survival. Unfortunately, the majority of patients do not respond to ICT; they either never respond to treatment or develop resistance to therapy. In order to increase the number of patients that respond to ICT, more knowledge is necessary in understanding the role of the immune system during melanoma progression and ICT treatment response. Complicating matters, as cancer develops, it can also re-program the immune system to work in favor of cancer growth. One such population of immune suppressing cells that result from melanoma is known as myeloid-derived suppressor cells (MDSCs). MDSCs are re-programmed immune cells that actively dampen the immune system from recognizing cancer while also helping cancer grow. These MDSCs have also been shown to decrease ICT treatment response in patients. This project aims to find strategies that limit the effects of MDSCs to enable successful ICT response in melanoma. Specifically, preliminary data suggests that myeloperoxidase (MPO), a major enzyme expressed in a subset of immune cells, is overexpressed in MDSCs. By hindering the function of myeloperoxidase, the hope is that this will limit the immune suppressing effects of MDSCs or even re-program MDSCs into proper immune cells that will fight against cancer. We expect that the combination of myeloperoxidase inhibitors with ICT will increase the number of melanoma patients that respond to treatment and possibly even overcome ICT resistance. This proposal aims to understand the role of myeloperoxidase in MDSCs and how myeloperoxidase is used to inhibit other immune cells from destroying cancer. An equally if not more important goal is to demonstrate that the combination of myeloperoxidase inhibitors with ICT enhances the effectiveness of ICT in treating melanoma and hopefully overcoming treatment resistance.

2. KEYWORDS:

Immune checkpoint therapy, melanoma, myeloid-derived suppressor cells, myeloperoxidase, intravital imaging, immunosuppression

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Confirm that MDSCs have increased expression and activity of MPO. Functionally identify the MDSC lineage during melanoma development. Image MDSC recruitment using a combination of the advanced intravital molecular imaging platform, functional imaging and surface marker expression during melanoma progression..

Major Task 1: Confirm that MDSCs have increased MPO activity and expression compared to healthy myeloid cells:

Subtask 1: Isolation and characterization (Luminol, L-012, Lucigenin, and DCFDA) of MDSCs from melanoma-bearing animals and myeloid cells from healthy animals. – 80% complete

Subtask 2: Real-time *in vivo* imaging of increased MPO activity in MDSCs during melanoma development in skinfold window chambers using our advanced molecular imaging platform. – 25% complete

Major Task 2: Develop a functional signature to identify the recruitment of MDSCs during melanoma progression:

Subtask 1: Real-time *in vivo* imaging and post processing of the tumor and microenvironment by immunohistochemistry of immune landscape during melanoma development in skinfold window chambers using our advanced molecular imaging platform – 25% complete

Subtask 2: Validation of immune landscape during subcutaneous model of melanoma growth using a combination of bioluminescent imaging, cytokine analysis of plasma samples, flow cytometry and immunohistochemical staining of tumors at different growth time points. – 90% complete (as suggested by reviewers, we have incorporated CyTOF in place of flow cytometry).

Specific Aim 3: Evaluate whether inhibition of MPO in MDSCs can increase melanoma ICT response and/or reverse melanoma ICT resistance. Develop a MDSC signature that predicts melanoma ICT responsiveness and/or ICT resistance.

Major Task 1: Confirm that there is an increase in melanoma ICT response in MPO-null and C57BL/6 animals when ICT is treated in combination with MPO inhibitors (4-ABAH, AZD5904, Verdiperstat (AZD3241)).

Subtask 1: Melanoma survival studies of ICT treatment in MPO-null animals. – 100% complete

Subtask 2: Melanoma survival studies of MPO inhibitors (4-ABAH, AZD5904, Verdiperstat (AZD3241)) in combination with ICT treatment in C57BL/6 animals. – 75% complete

What was accomplished under these goals?

1. The major achievement for this past year has been interviewing and accepting a Tenure-Track Assistant Professor position. I began applying for faculty positions in December 2020 uncertain of how COVID-19 had impacted job opportunities in academia. By June 2021, I had received 4 official offers where I accepted a tenure-track assistant professor position at West Virginia University (WVU) in the Department of Microbiology, Immunology & Cell Biology and the WVU Cancer Institute. I began my independent research career at WVU on August 31, 2021 and am in the midst of setting up my research program. I firmly believe that this DOD Career Development Award contributed to demonstrating my abilities to successfully obtain funding and allowed me to negotiate a sizeable start-up package.
2. I successfully published 3 first author papers related to this work in which this federal support was acknowledged.
    - **Liu TW**, Gammon ST, Yang P, Fuentes D, Piwnica-Worms D. HOCl is a Paracrine Effector Linking Myeloid Cells to NF-κB Signaling in melanoma by Trans-inhibition of IKK. Science Signaling. **2021**. 14 (677). DOI: 10.1126/scisignal.aax5971

- **Liu TW**, Gammon ST, Piwnica-Worms D. Multi-dimensional intravital microscopy of signaling dynamics in real-time during tumor-immune interactions. Cells. **2021**. 10(3). 499. DOI:10.3390/cells10030499
- **Liu TW**, Gammon ST, Fuentes D, Piwnica-Worms D. Multi-dimensional intravital macro-imaging of signaling dynamics in real-time during tumor-immune interactions. Cells. **2021**. 10 (3), 489. DOI: 10.3390/cells10030489

3. A major activity was the confirmation in the B16F10 melanoma model that MPO-deficiency enhanced ICT response (Figure 1). Identified an age-dependence and MPO-dependence in the response of B16F10 melanoma tumors in wild-type C57BL/6 ($MPO^{+/+}$) animals treated with immune checkpoint inhibitors (ICT, anti-PD-1 and anti-CTLA-4) without and with GVAX (Figure 1). ICT response in young (8 week old) $MPO^{+/+}$ was lost without combination GVAX treatment (light blue line). In contrast, adult (24 week old) $MPO^{+/+}$ demonstrated the reverse outcome, where no long term survivors were observed in combination ICT plus GVAX while, ~10% long term survivals was observed in adult $MPO^{+/+}$ when treated with ICT alone (dark blue line). Interestingly, no difference in ICT response was observed in syngeneic MPO-null ($MPO^{-/-}$), however, increase in ICT response was observed in adult animals $MPO^{-/-}$ (red line) compared to young $MPO^{-/-}$ animals (purple line). Therefore, in all further ICT studies, adult animals (24 weeks or older) were used and GVAX was not included to better mimic what occurs patients treated with ICT in the clinic.

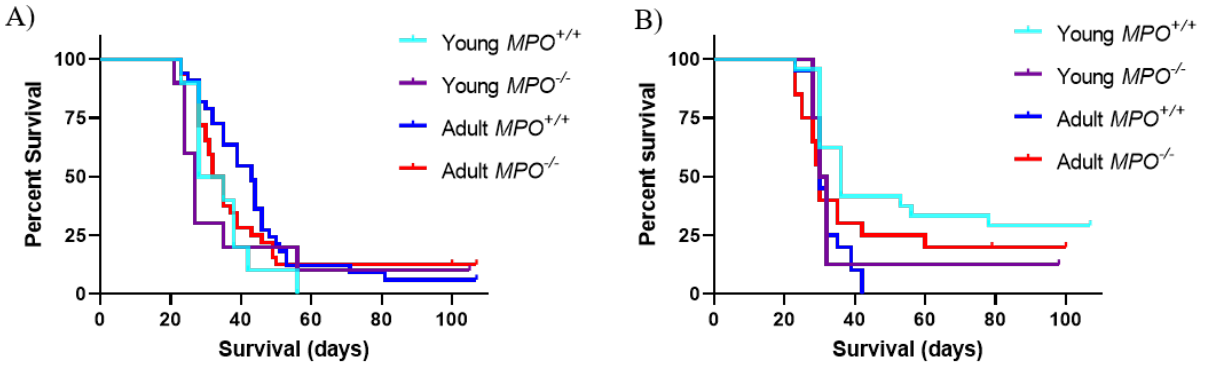


Figure 1. ICT treated survival curves of B16F10 subcutaneous tumors of (A) ICT alone and (B) ICT in combination with GVAX in young (8 week old) and adult (24 week old or older) wild type ($MPO^{+/+}$) and syngeneic MPO-null ($MPO^{-/-}$) animals. ICT treated animal numbers as follows: n = 10 young $MPO^{+/+}$ and $MPO^{-/-}$ animals, and n = 32 adult $MPO^{+/+}$ and $MPO^{-/-}$ animals. Combination ICT with GVAX animal numbers as follows: n = 24 young $MPO^{+/+}$, n = 10 young $MPO^{-/-}$ animals, and n = 20 adult $MPO^{+/+}$ and $MPO^{-/-}$ animals.

4. Confirmed that MPO-deficiency enhanced ICT response in two other melanoma models, YUMM3.3 and YUMM1.G1 (Figure 2).

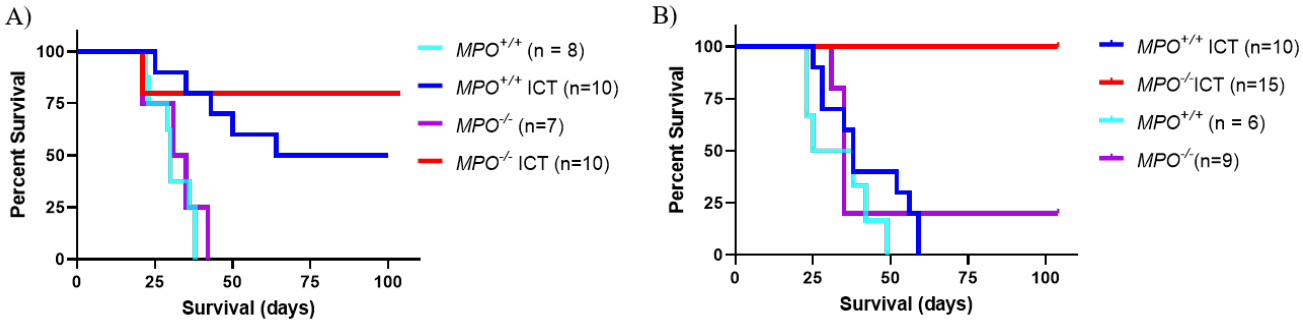


Figure 2. Survival curves of ICT-treated and tumor growth of (A) YUMM3.3 and (B) YUMM1.G1 subcutaneous tumors in adult (24 week old or older) $MPO^{+/+}$ and $MPO^{-/-}$ animals. Animal number in each group shown in legend.

5. Identified the effector cells which affected ICT response in B16F10 and YUMM3.3 subcutaneous melanomas using *in vivo* depletion studies with anti-CD8a, anti-CD4, anti-NK1.1 and IgG control antibodies in $MPO^{+/+}$ and $MPO^{-/-}$ animals (Figure 3). In both B16F10 and YUMM3.3 tumors regardless of mouse phenotype, CD8a *in vivo* depletion resulted in loss of ICT response as expected since these are considered the main effector cells of ICT. However, only in YUMM3.3 tumors did CD4 depletion result in loss of ICT, although on a slower time scale compared to CD8a depletion also suggesting that in YUMM3.3 tumors that CD4 contributes to ICT efficacy (Fig. 3C). MPO deficiency also demonstrated different responses in B16F10 compared to YUMM3.3 tumors. When MPO was deficient in B16F10 tumors, CD4 depletion resulted in lost of ICT response while NK1.1 depletion demonstrated enhanced ICT response suggesting that NK cells inhibited ICT response when MPO was deficient (Fig 3B). In contrast, when MPO deficiency in YUMM3.3 tumors demonstrated decreased ICT response when both CD4 and NK1.1 cells were depleted, suggesting these cells have pro-tumor activity during ICT when MPO is deficient (Fig 3D).

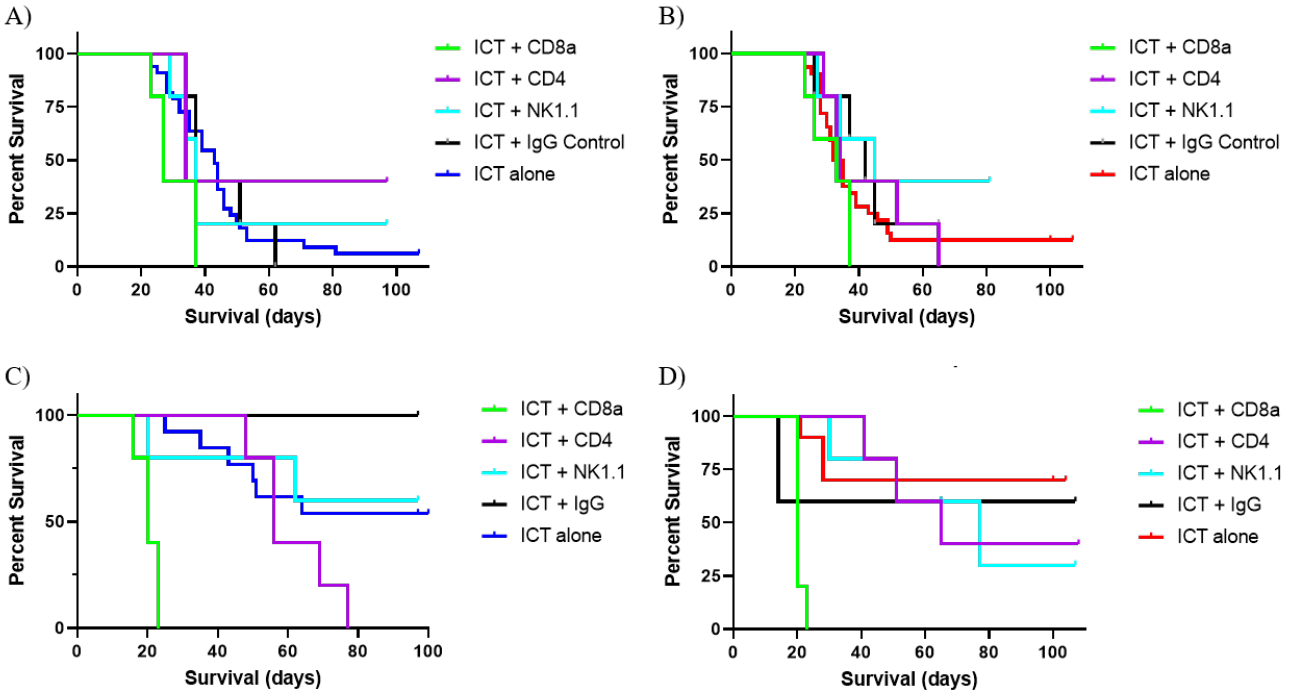


Figure 3. In vivo depletion studies using anti-CD8a, anti-CD4, anti-NK1.1 and IgG antibodies to evaluate effector cells involved in ICT response in B16F10 subcutaneous tumors-bearing (A) $MPO^{+/+}$ and (B) $MPO^{-/-}$ animals and YUMM3.3 subcutaneous tumor-bearing (C) $MPO^{+/+}$ and (D) $MPO^{-/-}$ animals; n = 5 animals in each group (ICT alone groups were reported in Fig. 1 & 2).

6. Currently completing the melanoma survival studies of MPO inhibitors (4-ABAH, AZD5904, Verdiperstat (AZD3241)) in combination with ICT treatment in $MPO^{+/+}$ animals. Survival studies with 4-ABAH are completed but animals treated with AZD5904 and Verdiperstat will continue to be monitored for another 45 days. All MPO inhibitor treatments began on day 2 post tumor inoculaton, one day prior to the initiation of ICT treatment. However, the data thus far demonstrates that pharmacological inhibition of MPO combined with ICT can enhance response in both B16F10 and YUMM3.3 preclinical animal models (Figure 4). Generally, all MPO inhibitor treatments demonstrated enhanced ICT response in B16F10 tumors compared to ICT treated only $MPO^{+/+}$ and $MPO^{-/-}$ animals (Figure 4A – C). In the YUMM3.3 treated tumors, combination 4-ABAH with ICT did not improve response compared to $MPO^{+/+}$ animals (Fig. 4D), AZD5904 in combination with ICT demonstrated similar efficacy as when MPO was

deficient (Figure 4E), while ICT in combination with Verdiperstat demonstrated significant enhanced survival over ICT treated only $MPO^{+/+}$ and $MPO^{-/-}$ animals (Figure 4F).

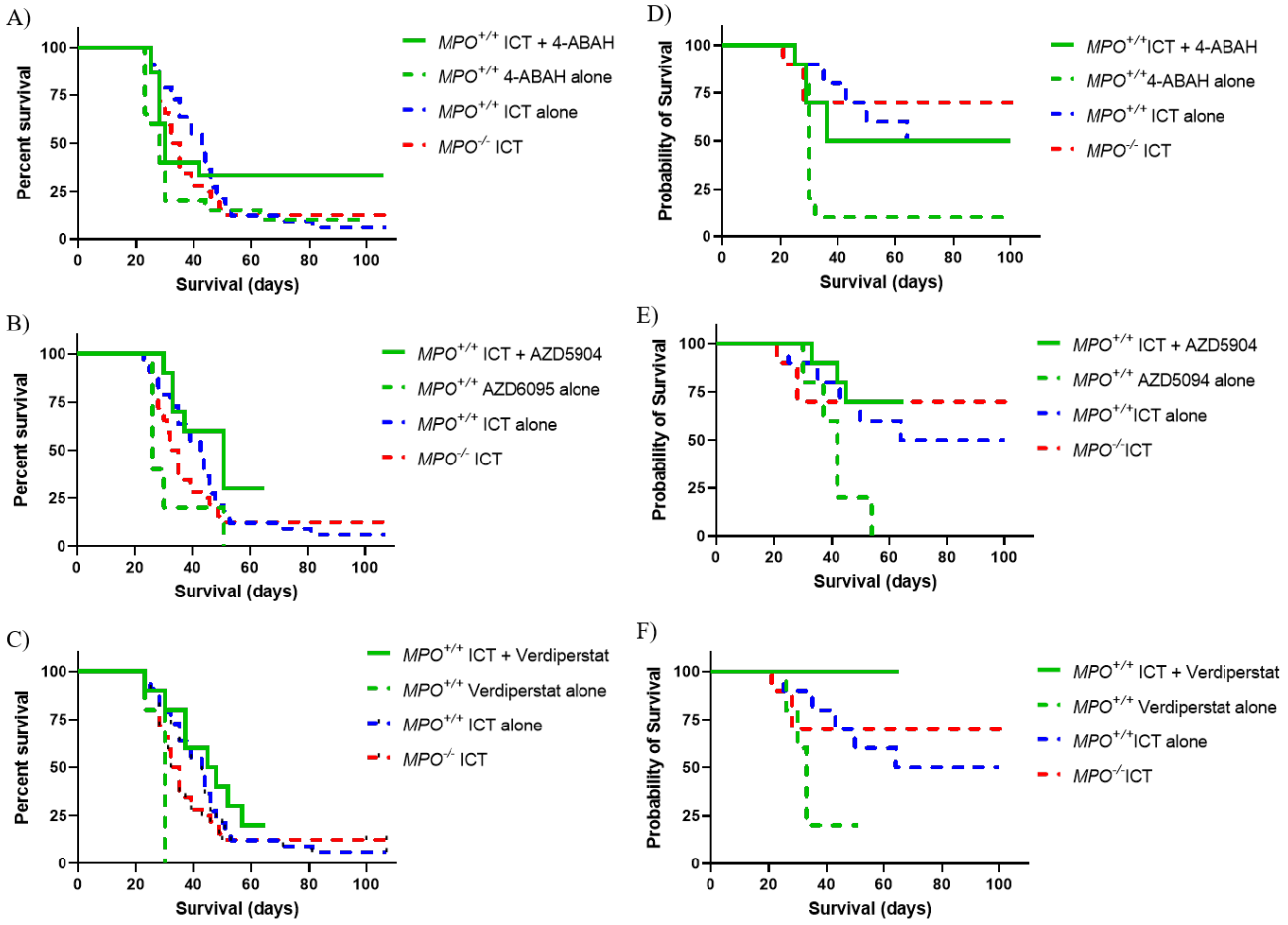


Figure 4. Survival studies of melanoma tumors treated with combination ICT and MPO inhibtors. Survival studies of B16F10 subcutaneous tumors treated with ICT and (A) 4-ABAH, (B) AZD5904, and (C) Verdiperstat.; n = 15 animals treated with ICT + 4-ABAH animals, n = 25 animals treated with 4-ABAH alone, n = 10 animals treated with ICT + AZD5904 or Verdiperstat, n = 5 animals treated with AZD5904 or Verdiperstat alone. Survival studies of YUMM3.3 subcutaneous tumors treated with ICT and (D) 4-ABAH, (E) AZD5904, and (F) Verdiperstat.; n = 10 animals treated with ICT + MPO inhibitors, n = 10 animals treated wtih 4-ABAH alone, n = 5 animals treated with AZD5904 or Verdiperstat alone. (ICT alone groups were reported in Fig. 1 & 2).

7. Confirmed an increase in MPO activity by isolating and functionally characterizing myeloid cells and neutrophil cells comparing melanoma-bearing animals and healthy animals. Using a neutrophil isolation kit and a magnetic rack for separation, myeloid cell isolation from the bone marrow, spleen and peritoneal cavity occurred comparing healthy and B16F10 tumor-bearing animals, we observed an increase in the MPO activity and ROS levels in the presence of B16F10 tumors (Fig. 5A – C). In contrast, cells isolated from YUMM3.3 tumor bearing animals did not demonstrate a similar increase in MPO activity compared to healthy animals (Fig. 5D – F). Since YUMM3.3 are ICT partial responders, perhaps this broad myeloid cell subset is not all immunosuppressive as is the case in the B16F10 model. We then used a magnetic column for specific isolation of neutrophils in healthy compared to YUMM3.3 tumor-bearing animals. Here we observed an increase in MPO activity and ROS levels (Fig. 5 G – I). Myeloid cells isolated from young B16F10 tumor-bearing animals had significantly higher ROS levels compared to mature B16F10 tumor-bearing and healthy animals (Fig. 5J – L). No difference in the Western blots which evaluated the MPO protein levels were observed in healthy vs to B16F10 tumor-bearing animals or YUMM3.3 tumor-bearing animals was observed (Fig. 5M & N). Lastly, the

number of CD11b+Ly6G+ cells by flow cytometry demonstrated no difference in the isolated myeloid cells when comparing young and mature healthy and B16F10 tumor-bearing animals (Fig. 5O). Still need to evaluate MPO activity in isolated neutrophils from B16F10 animals.

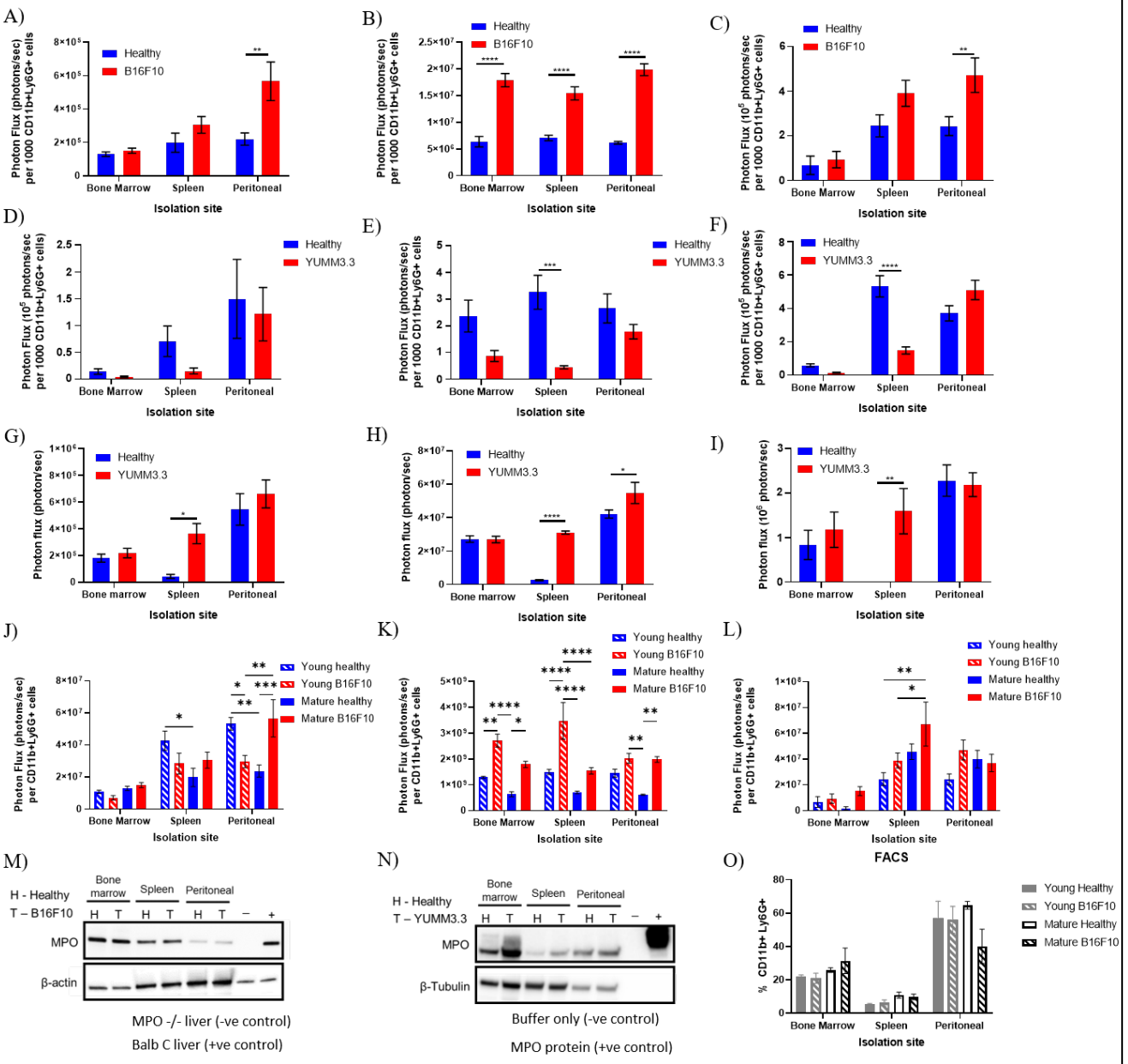


Figure 5. Functional assay of isolated myeloid cells from tumor-bearing and age-matched healthy animals. Bioluminescent quantification of MPO and ROS activity from myeloid cells isolated from healthy compared to B16F10-tumor bearing animals using (A) luminol, (B) L-012 and (C) lucigenin. Bioluminescent quantification of MPO and ROS activity from myeloid cells isolated from healthy compared to YUMM3.3 tumor-bearing animals using (D) luminol, (E) L-012 and (F) lucigenin. Bioluminescent quantification of MPO and ROS activity from neutrophils isolated from healthy compared to YUMM3.3 tumor-bearing animals by (G) luminol, (H) L-012, and (I) lucigenin. Bioluminescent quantification of myeloid cells isolated from young (8 week old) compared to mature (same dataset as in A – C) from healthy compared to B16F10 tumor-bearing animals using (J) luminol, (K) L-012, and (L) lucigenin. Western blots evaluated the MPO protein levels in the myeloid cells isolated from healthy compared to (M) B16F10 tumor-bearing animals and neutrophils isolated from health compared to (N) YUMM3.3 tumor-bearing animals. (O) Flow cytometry of the number of CD11b+Ly6G+ cells isolated from young compared to mature healthy versus B16F10 tumor-bearing animals. n = 3 animals per group used in each experiment. Statistical significance calculated using a two-way ANOVA followed by Sidak's multiple comparison test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.00001).

8. Using a CD3e depletion kit (resulting in myeloid cell population only) followed by magnetic column separation, myeloid cells were isolated from the spleen and the bone marrow of healthy and age matched B16F10 tumor-bearing and YUMM3.3 tumor-bearing. Secreted cytokine analysis of myeloid cell media demonstrated significant differences between isolated myeloid cells from healthy and tumor-bearing animals (Fig. 6).

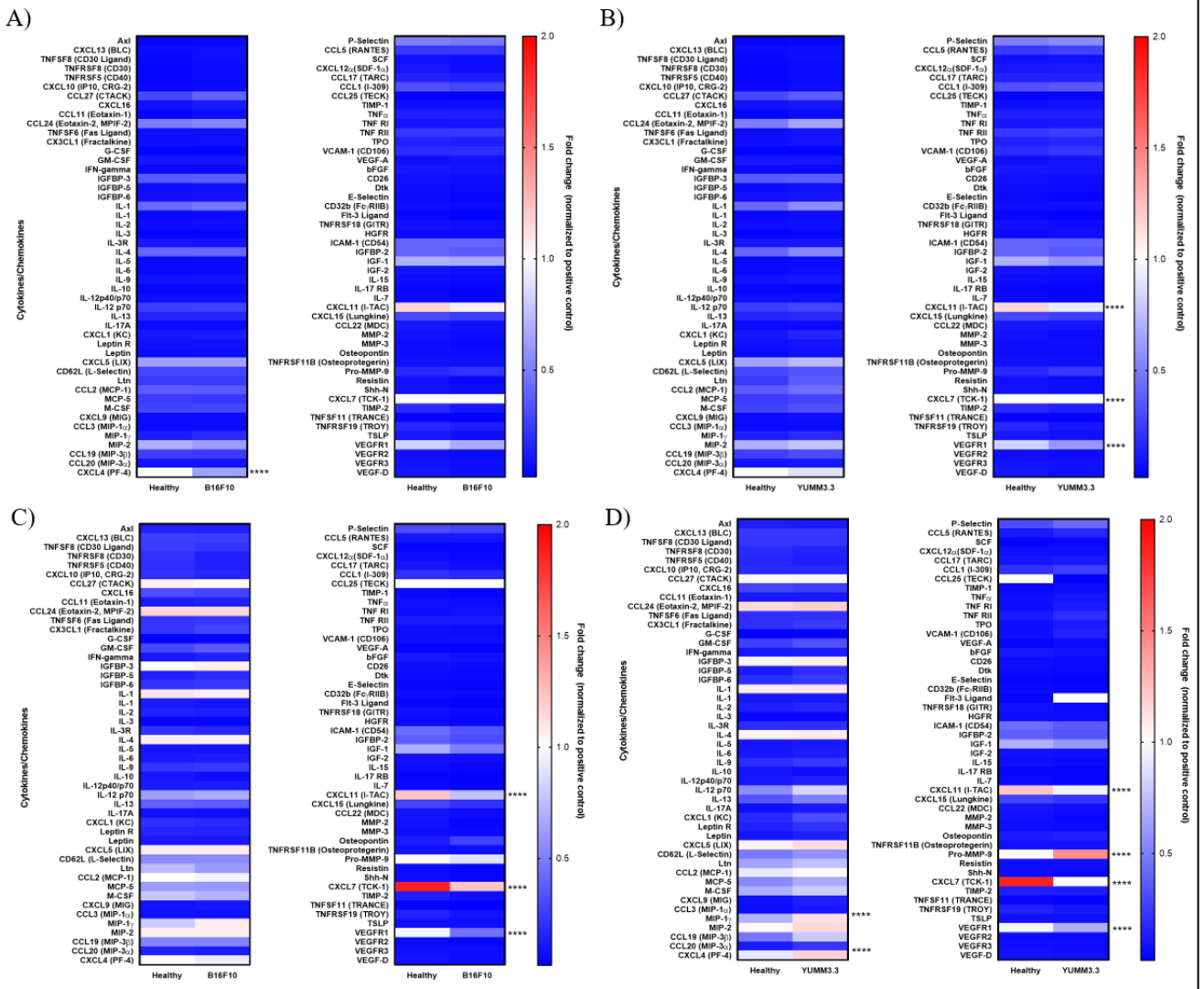


Figure 6. Isolated myeloid cell analysis comparing healthy versus age-matched tumor-bearing animals. Secreted cytokine analysis of spleen isolated myeloid cells from healthy and (A) B16F10 tumor-bearing animals or (B) YUMM3.3 tumor-bearing animals, and bone marrow isolated myeloid cells from healthy and (C) B16F10 tumor-bearing animals or (D) YUMM3.3 tumor-bearing animals (n = 3 animals per group). Statistical significant evaluated using a multiple comparison Student *t* test, **** $P < 0.0001$.

RNASeq analysis of these isolated myeloid cells also demonstrated differences between healthy and tumor-bearing myeloid cells (Fig. 7). We are currently collaborating with bioinformaticians, Dr. Jing Wang and Dr. Wencai Ma, to evaluate whether there are pathway changes or signatures that correlate with the differences in the immune checkpoint response observed *in vivo*.

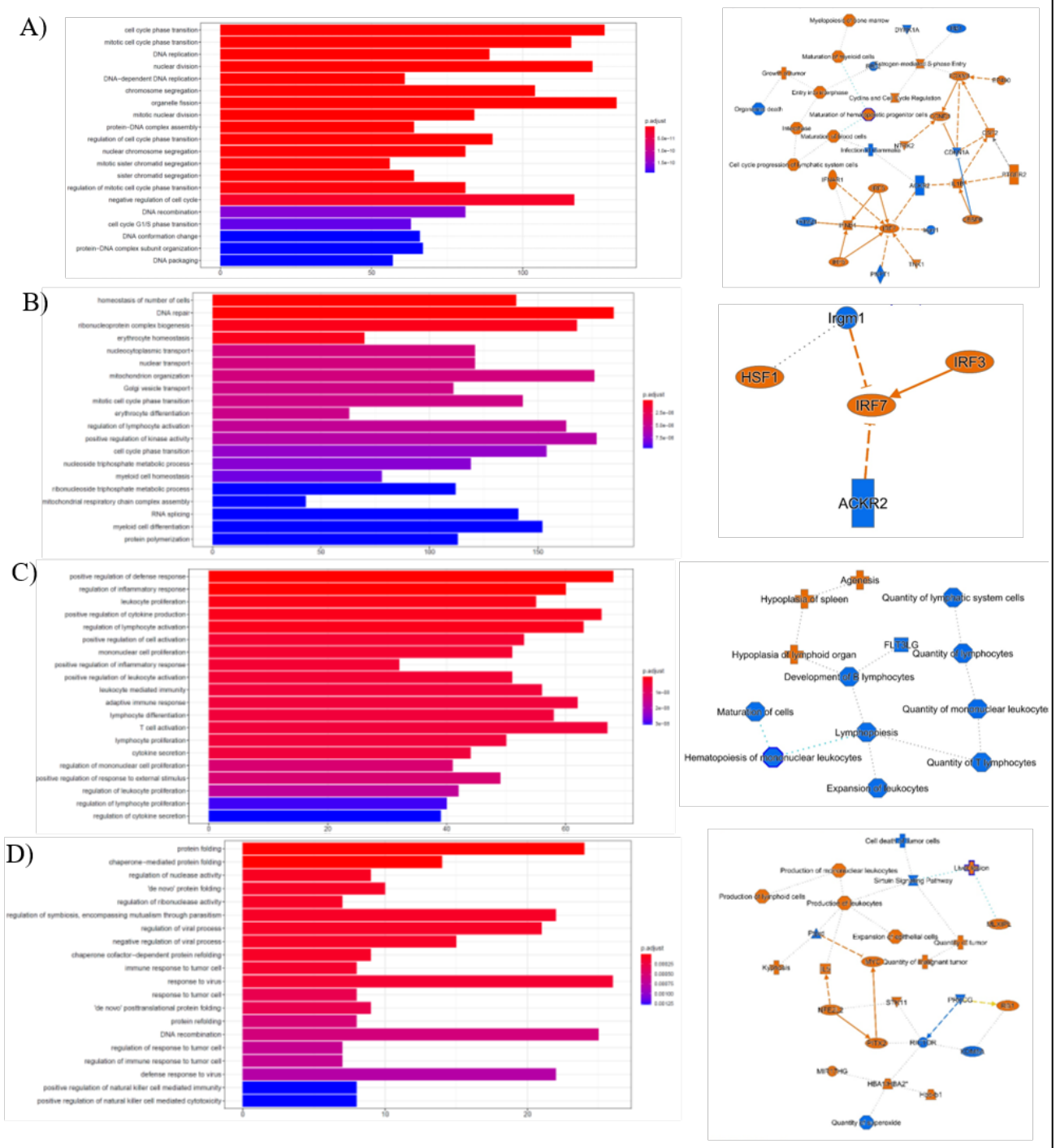
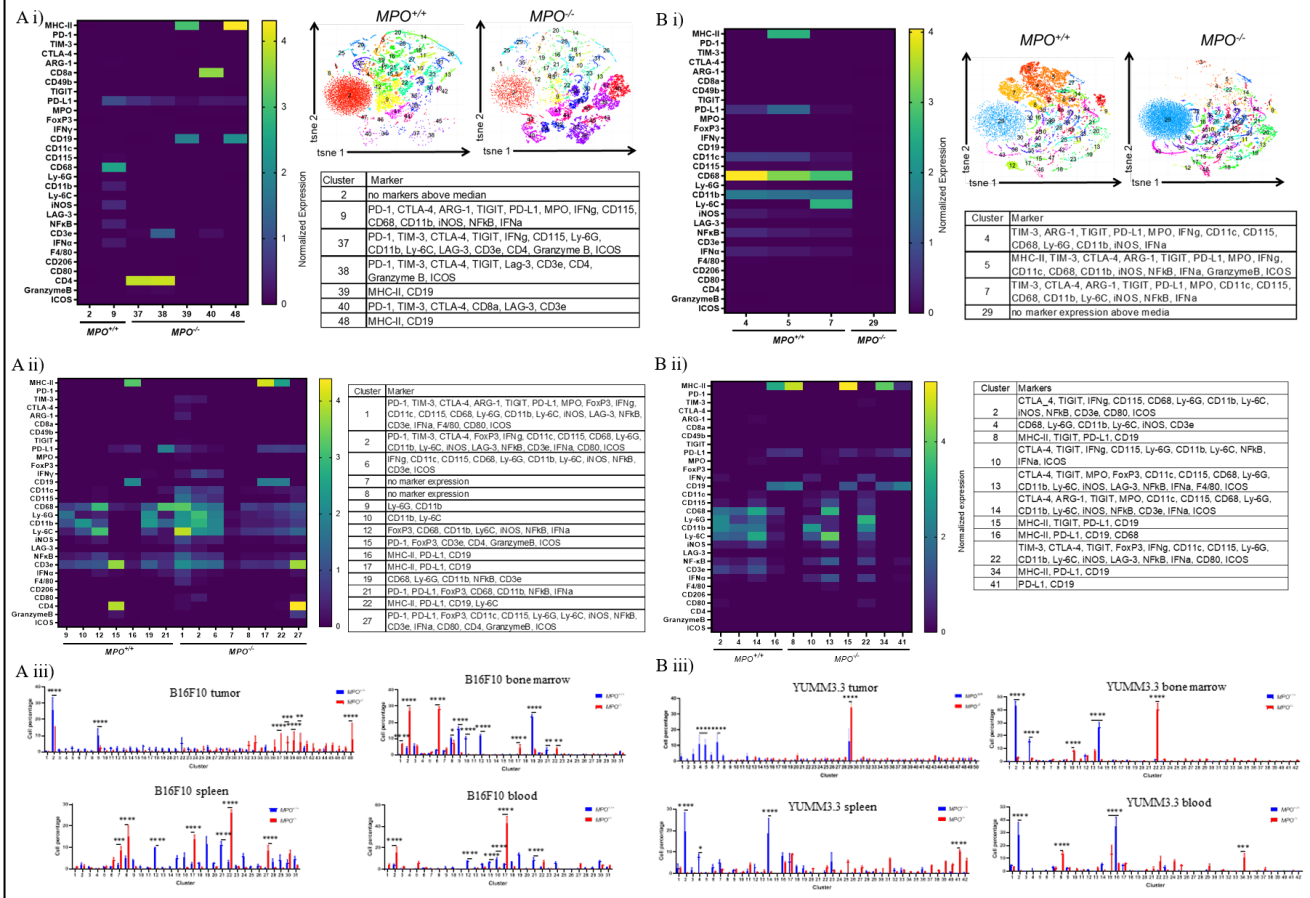


Figure 7. Isolated myeloid cell analysis comparing healthy versus age-matched tumor-bearing animals. RNASeq analysis demonstrating differences in biological processes and preliminary IPA pathway summary of spleen isolated myeloid cells from healthy and (A) B16F10 tumor-bearing animals or (B) YUMM3.3 tumor-bearing animals, and bone marrow isolated myeloid cells from healthy and (C) B16F10 tumor-bearing animals or (D) YUMM3.3 tumor-bearing animals (n = 3 animals per group).

9. CyTOF analysis of B16F10 and YUMM3.3 endpoint tumors and immune compartments (bone marrow, spleen and blood) in melanoma-bearing *MPO*$^{+/+}$ and *MPO*$^{-/-}$ animals (Fig. 8). Using phonograph to analyze the different immune cells that were recruited to the tumors; B16F10 tumors demonstrated an increase in clusters associated with myeloid cells with MPO (cluster 9) in *MPO*$^{+/+}$ animals and in B cells (clusters 38 & 48), CD4 (cluster 37 & 38) and CD8a T cells (cluster 40) in *MPO*$^{-/-}$ animals (Fig. 8A), while YUMM3.3 tumors demonstrated an increase in clusters associated with dendritic/myeloid cell markers with MPO in *MPO*$^{+/+}$ animals (Fig. 8B). While the immune compartment demonstrated a mix of clusters associated with B cell and myeloid cell markers. The CyTOF data was also analyzed by astrolabe using the immune hierarchy in figure 9A, B16F10 tumor-bearing animals demonstrated an increase in polymorphonuclear myeloid derived suppressor cells (PMN MDSCs) and neutrophils in *MPO*$^{+/+}$ animals while B cells and dendritic cells were increased in *MPO*$^{-/-}$ animals (Fig. 9B – E). YUMM3.3 tumor-bearing animals demonstrated an increase in monocytic MDSCs, CD8 T cells and Ly6C^{-} monocytes in *MPO*$^{+/+}$ animals while B cells and neutrophils were increased in *MPO*$^{-/-}$ animals (Fig. 9F – J). Differences demonstrated by astrolabe were only observed in the immune compartments however. We are continuing to understand how best to analyze this CyTOF data, however, our concern with using astrolabe is that there is no room for ambiguity which could potentially mean we are missing cells that don't fall into our hierarchy (Fig. 9A). With the known plasticity of immune cells, especially in the presence of tumors, conventional identification may not apply as so an unambiguous analysis without any preconceived characterization using phenograph may be better suited for this data.



Cluster	Marker
2	no markers above median
9	PD-1, CTLA-4, ARG-1, TIGIT, PD-L1, MPO, IFNg, CD115, CD68, CD11b, iNOS, NFkB, IFNa
37	PD-1, TIM-3, CTLA-4, TIGIT, IFNg, CD115, Ly-6G, CD11b, Ly-6C, LAG-3, CD3e, CD4, Granzyme B, ICOS
38	PD-1, TIM-3, CTLA-4, TIGIT, Lag-3, CD3e, CD4, Granzyme B, ICOS
39	MHC-II, CD19
40	PD-1, TIM-3, CTLA-4, CD8a, LAG-3, CD3e
48	MHC-II, CD19

Cluster	Marker
4	TIM-3, ARG-1, TIGIT, PD-L1, MPO, IFNg, CD11c, CD115, CD68, Ly-6G, CD11b, iNOS, IFNa
5	MHC-II, TIM-3, CTLA-4, ARG-1, TIGIT, PD-L1, MPO, IFNg, CD11c, CD68, CD11b, iNOS, NFkB, IFNa, GranzymeB, ICOS
7	TIM-3, CTLA-4, ARG-1, TIGIT, PD-L1, MPO, CD11c, CD115, CD68, CD11b, Ly-6C, iNOS, NFkB, IFNa
29	no marker expression above media

Cluster	Marker
1	PD-1, TIM-3, CTLA-4, ARG-1, TIGIT, PD-L1, MPO, FoxP3, IFNg, CD11c, CD115, CD68, Ly-6G, CD11b, Ly-6C, iNOS, LAG-3, NFkB, CD3e, IFNa, F4/80, CD80, ICOS
2	PD-1, TIM-3, CTLA-4, FoxP3, IFNg, CD11c, CD115, CD68, Ly-6G, CD11b, Ly-6C, iNOS, LAG-3, NFkB, CD3e, IFNa, CD80, ICOS
6	IFNg, CD11c, CD115, CD68, Ly-6G, CD1b, Ly-6C, iNOS, NFkB, CD3e, ICOS
7	no marker expression
8	no marker expression
9	Ly-6G, CD11b
10	CD11b, Ly-6C
12	FoxP3, CD68, CD11b, Ly6C, iNOS, NFkB, IFNa
15	PD-1, FoxP3, CD3e, CD4, GranzymeB, ICOS
16	MHC-II, PD-L1, CD19
17	MHC-II, PD-L1, CD19
19	CD68, Ly-6G, CD11b, NFkB, CD3e
21	PD-1, PD-L1, FoxP3, CD68, CD11b, NFkB, IFNa
22	MHC-II, PD-L1, CD19, Ly-6C
27	PD-1, PD-L1, FoxP3, CD11c, CD115, Ly-6G, Ly-6C, iNOS, NFkB, CD3e, IFNa, CD80, CD4, GranzymeB, ICOS

Cluster	Markers
2	CTLA_4, TIGIT, IFNg, CD115, CD68, Ly-6G, CD11b, Ly-6C, iNOS, NFkB, CD3e, CD80, ICOS
4	CD68, Ly-6G, CD11b, Ly-6C, iNOS, CD3e
8	MHC-II, TIGIT, PD-L1, CD19
10	CTLA-4, TIGIT, IFNg, CD115, Ly-6G, CD11b, Ly-6C, NFkB, IFNa, ICOS
13	CTLA-4, TIGIT, MPO, FoxP3, CD11c, CD115, CD68, Ly-6G, CD11b, Ly-6C, iNOS, LAG-3, NFkB, IFNa, F4/80, ICOS
14	CTLA-4, ARG-1, TIGIT, MPO, CD11c, CD115, CD68, Ly-6G, CD11b, Ly-6C, iNOS, NFkB, CD3e, IFNa, ICOS
15	MHC-II, TIGIT, PD-L1, CD19
16	MHC-II, PD-L1, CD19, CD68
22	TIM-3, CTLA-4, TIGIT, FoxP3, IFNg, CD11c, CD115, Ly-6G, CD11b, Ly-6C, iNOS, LAG-3, NFkB, IFNa, CD80, ICOS
34	MHC-II, PD-L1, CD19
41	PD-L1, CD19

Figure 8. Phenograph analysis of CyTOF data comparing *MPO*$^{+/+}$ and *MPO*$^{-/-}$ animals with (A) B16F10 and (B) YUMM3.3 subcutaneous tumors. Marker expression of clusters significantly increased in *MPO*$^{+/+}$ or *MPO*$^{-/-}$ animals from (i) tumors with TSNE plots and (ii) immune compartment (bone marrow, spleen and blood) and (iii) bar plots of statistically significantly different clusters evaluated using multiple comparison Student *t* test, *P <0.05, ** P < 0.01, *** P < 0.0001, **** P < 0.00001. The markers expressed above median expression of each statistically significant cluster is identified in the corresponding chart. n = 3 animals per group.

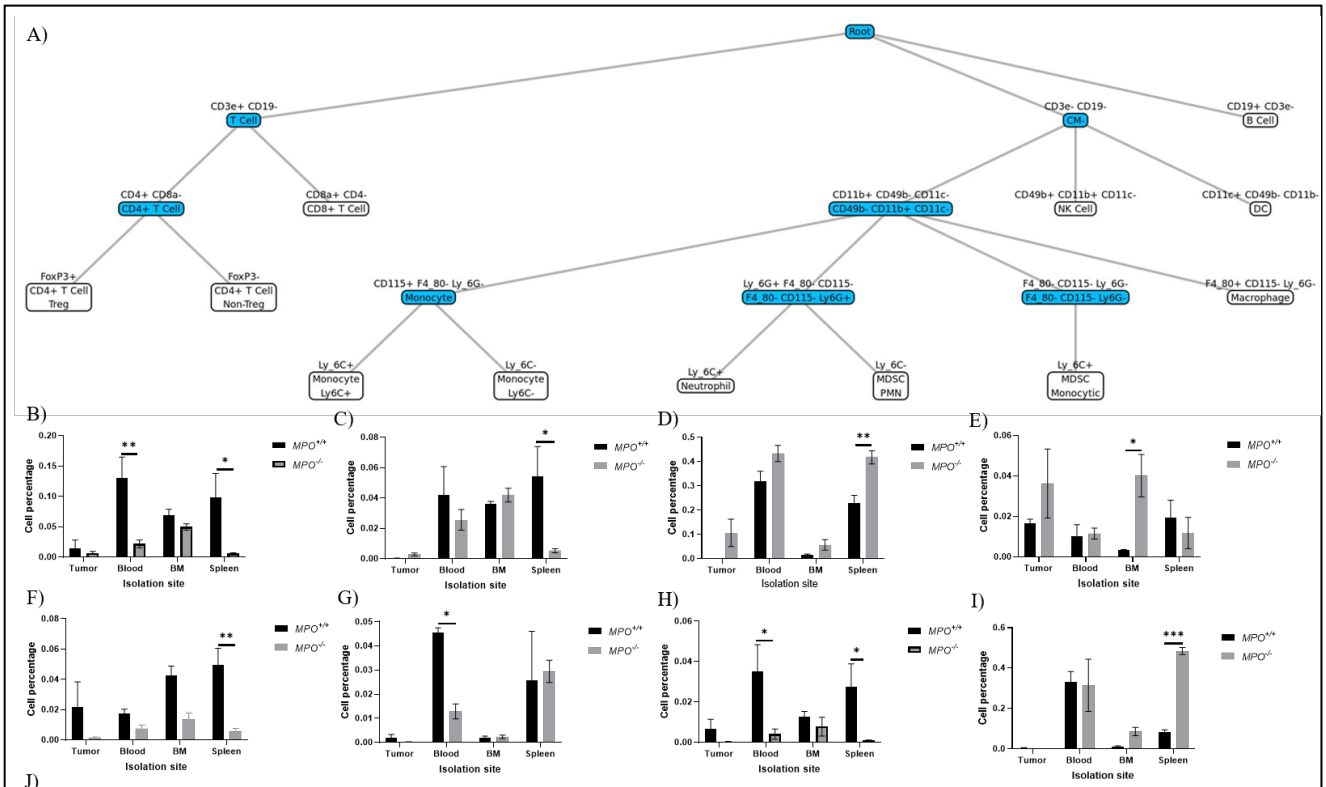


Figure 9. Astrolabe analysis of CyTOF data comparing *MPO*$^{+/+}$ and *MPO*$^{-/-}$ animals with B16F10 and YUMM3.3 subcutaneous tumors. (A) Astrolabe hierarchy for identification of immune cells. Significant differences observed from B16F10 tumor-bearing animals in (B) PMN MDSCs, (C) neutrophils, (D) B cells, and (E) dendritic cells of the isolated immune compartments. Significant differences observed from YUMM3.3 tumor-bearing animals in (F) Mono MDSCs, (G) CD8 T cells, (H) Ly6C^{-} monocytes, (I) B cells, and (J) neutrophils. Statistical significant calculated using multiple comparison Student *t* test, *P <0.05, ** P < 0.01, *** P < 0.0001. n = 3 animals per group.

10. CyTOF analysis of YUMM3.3 ICT responders compared to ICT non-responders of the immune compartments in *MPO*$^{+/+}$ animals (Figure 10); tumor compartments could not be evaluated since ICT responders do not develop tumors. Phenograph analysis demonstrated that a single cluster with markers associated with immunosuppressive myeloid cells was increased in ICT non responders (Fig. 10A – D). Similarly, astrolabe analysis using the same immune hierarchy above (Fig. 9A), demonstrated a statistically significant increase in immunosuppressive PMN MDSCs suggesting that these immunosuppressive myeloid cells significantly contribute to ICT efficacy.

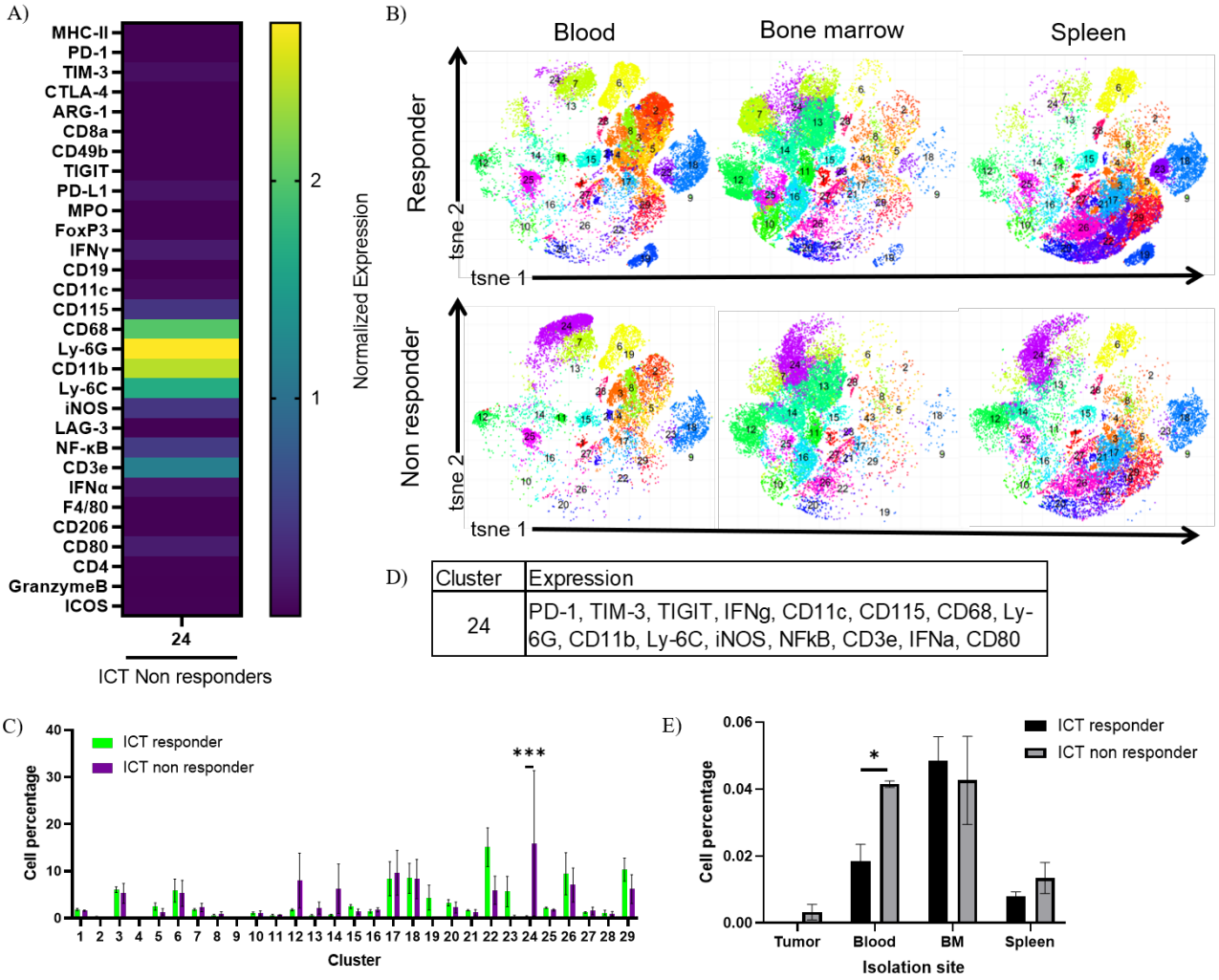


Figure 10. CyTOF analysis of YUMM3.3 ICT responders compared to ICT non responders in *MPO*$^{+/+}$ animals. Phenograph analysis demonstrates (A) marker expression of cluster 24 that significantly increased ICT non responders with corresponding (B) TSNE plots of immune compartment (blood, bone marrow and spleen) and (C) representative bar plots of the spleen. (D) The markers expressed above median expression of each statistically significant cluster is identified in the corresponding chart.(E) Astrolabe analysis of CyTOF data demonstrated significant increase in PMN MDSCs in ICT non responders. Statistical significant calculated using multiple comparison Student *t* test, *P <0.05, *** P < 0.0001; n = 3 animals per group.

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

This work provided me the framework to successfully attain a Tenure-track Assistant Professor position. I began my independent research position at West Virginia University in the Department of Microbiology, Immunology, and Cell Biology. In addition, the intravital imaging work from this grant (reported in the 2019 – 2020 annual report, Fig. 6) provided the preliminary data for a Melanoma SPORE grant from the University of Texas MD Anderson Cancer Center titled "PET Imaging of the Innate Immune System Predicts Immune Checkpoint Therapy Response". This grant provided me the opportunity to work with PET imaging scientists and radiochemists, Dr. Seth Gammon and Dr. Federica Pisaneschi. More importantly, this grant assigned myself a melanoma expert as my mentor, Dr. Cassian Yi, in which we had bi-monthly one-on-one meetings. This grant also provided me the opportunity to participate in monthly SPORE melanoma meetings that included researchers from University of Pittsburgh, New York University, Yale University and Wistar Institute/University of Pennsylvania.

How were the results disseminated to communities of interest?

I presented at the melanoma research working group at the University of Texas MD Anderson Cancer Center in April 2021.

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

Complete all Specific Aims this year at West Virginia University.

4. IMPACT:

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

This work will impact melanoma research and the field of immunology. My preclinical results suggest that blocking an innate immune cell enzyme, myeloperoxidase, enhances immune checkpoint therapy response. The completion of this project would could impact melanoma patient care with immune checkpoint therapy, as two myeloperoxidase inhibitors that we evaluated preclinically have previously used in clinical trials for neurodegenerative disease. Thus, with our promising results, we anticipate that we can easily repurpose these myeloperoxidase inhibitors for use in cancer as an adjuvant to enhance immune checkpoint therapy. Completion of this project will not only increase the knowledge base on melanoma progression and immunotherapy response, but also enhance our understanding of the contributions of the innate immune system in melanoma development. The ultimate goal of this project is to find treatment strategies that enhance melanoma response to immunotherapy treatments.

What was the impact on other disciplines?

First, our results suggest that MPO inhibitors previously used for neurodegenerative diseases could be repurposed for use in cancer, specifically as an adjuvant to enhance immune checkpoint therapy. Second, our advanced molecular imaging platform provides molecular biologists and immunologists an imaging tool that provides real-time monitoring of intact biological systems. We expand the utility of intravital imaging by modifying an off-the-shelf commercial system with the addition of bioluminescent imaging easily achieved by the addition of a CCD camera and demonstrate high quality imaging within reach of any biology laboratory. Our advanced molecular imaging platform is an advance of technology; this molecular imaging window chamber platform uniquely combines both bioluminescent and fluorescent genetically-encoded and exogenous reporters, providing a powerful multi-plex strategy to study molecular and cellular processes in real-time in intact living systems at single cell resolution.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

I have changed roles during the duration of this grant from an instructor at the University of Texas MD Anderson to a Tenure-Track Assistant Professor at West Virginia University.

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

The COVID-19 pandemic prevented the laboratory from running at 100% capacity for the past year. Because of the laboratory shut downs, as mentioned in the 2019 – 2020 annual report, we were required to stop all breeding colonies, and were unable to start them back up until June of 2020. Due to the use of mature animals in this project, *MPO*$^{-/-}$ animals were not of age until January 2021. Due to limited animal numbers, the tasks from Specific Aim 3 were prioritized over Specific Aim 2. Additionally, starting my independent career at West Virginia University (WVU) has delayed further work on the project as it will take several months for my lab here to be fully functional. Equipment has been ordered and protocols are all under review at WVU and I am confident that by Jan 2021, I will have my research program fully operational.

Changes that had a significant impact on expenditures

With the COVID-19 pandemic and my search for a Tenure-Track Assistant Professor position limiting my time, I have not spent as much on this grant this academic year as anticipated.

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

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

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

Journal publications.

Liu TW, Gammon ST, Yang P, Fuentes D, Piwnica-Worms D. HOCl is a Paracrine Effector Linking Myeloid Cells to NF-κB Signaling in melanoma by Trans-inhibition of IKK. Science Signaling. **2021**. 14 (677). DOI: 10.1126/scisignal.aax5971. Published; acknowledgement of federal support yes.

Liu TW, Gammon ST, Piwnica-Worms D. Multi-dimensional intravital microscopy of signaling dynamics in real-time during tumor-immune interactions. Cells. **2021**. 10(3). 499. DOI:10.3390/cells10030499. Published; acknowledgement of federal support yes.

Liu TW, Gammon ST, Fuentes D, Piwnica-Worms D. Multi-dimensional intravital macro-imaging of signaling dynamics in real-time during tumor-immune interactions. Cells. **2021**. 10 (3), 489. DOI: 10.3390/cells10030489. Published; acknowledgement of federal support yes.

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:	Tracy Liu, PhD
Project Role:	Principal Investigator
Researcher Identifier:	0000-0003-0671-8390
Nearest person month worked:	11
Contribution to project:	Dr. Liu has performed work in the area of animal studies, myeloid and neutrophil isolation studies, window chamber molecular imaging, plasma cytokine studies, and CyTOF studies.
Funding support:	Non-sponsored research projects
Name:	Ping Yang
Project Role:	Senior Research Assistant
Researcher Identifier:	N/A
Nearest person month worked:	1
Contribution to project:	Mrs. Yang has performed work by assisting with animal studies, blood draws for plasma cytokine studies and assisted with a few window chamber molecular imaging sessions.
Funding support:	National Institutes of Health/National Cancer Institute

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

Employee of West Virginia University

What other organizations were involved as partners?

- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: