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TITLE: Myeloid-Derived Suppressor Cells Expressing Myeloperoxidase Directly Inhibit Adaptive Immune Cells Limiting Immunotherapy in Melanoma

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CONTRACTING ORGANIZATION: The University of Texas MD Anderson Cancer Center

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

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

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

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.

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. – 50% 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. – 50% complete (as suggested by reviewers, we have incorporated CyTOF in place of flow cytometry).

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.

1. Isolated and functionally characterized myeloid cells and neutrophil cells comparing melanoma-bearing animals (B16F10, YUMM3.3 and YUMM1.G1 cell lines) and healthy animals and confirmed an increased in myeloperoxidase (MPO) activity. Using a neutrophil isolation kit and a CD3e depletion kit (results in myeloid cell population only), functional quantification of MPO and ROS activity was measured using three bioluminescent reporters, L-012, luminol, and lucigenin (Fig. 1).

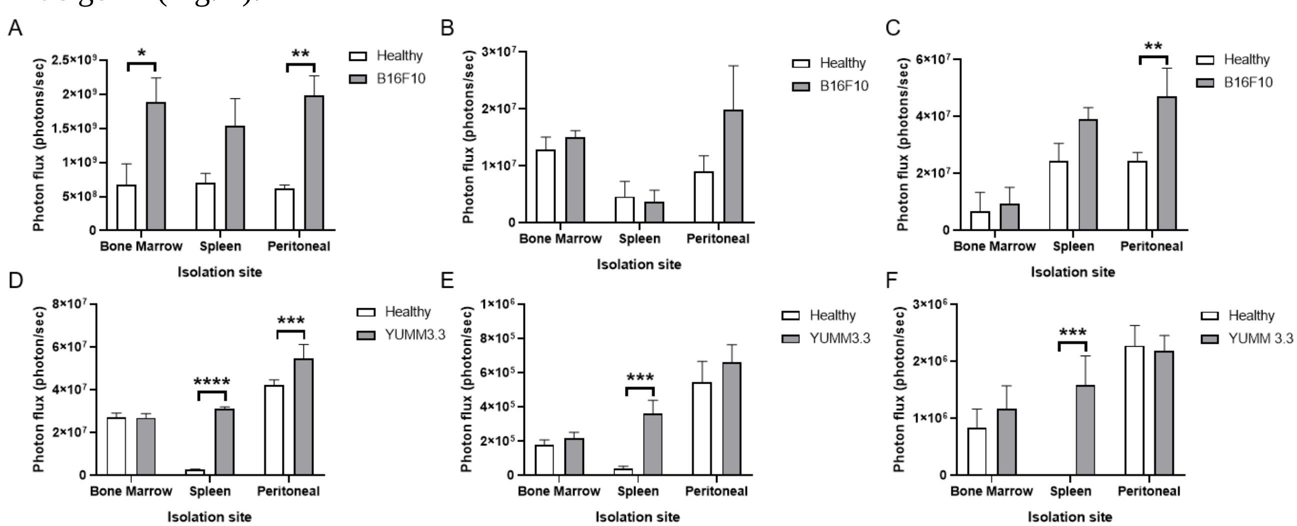


Figure 1. Functional assay of isolated myeloid cells from tumor bearing and age-matched healthy animals. (A) L-012, (B) luminol and (C) lucigenin bioluminescent quantification of MPO activity and ROS levels of isolated myeloid cells from B16F10 tumor-bearing and age-matched healthy animals. (D) L-012, (E) luminol and (F) lucigenin bioluminescent quantification of MPO activity and ROS levels of isolated neutrophils from YUMM3.3 tumor-bearing and age-matched healthy animals. Statistical significance calculated using a two-way ANOVA followed by Sidak's multiple comparison test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (n = 3 animals per group).

2. Real time *in vivo* imaging of B16F10 melanoma development in skinfold window chambers using our advanced molecular imaging platform. Utilizing intravital imaging with skinfold window chamber animal models, we evaluated in real-time *in vivo* the recruitment of MPO-active myeloid cells during melanoma tumor progression in live $MPO^{+/+}$ and $MPO^{-/-}$ animals.

B16F10 tumor growth, NF-κB signaling, and MPO-mediated activity arising from immune infiltrates were imaged simultaneously in MPO$^{+/+}$ animals and compared to MPO$^{-/-}$ mice using a multi-spectral, multi-modal imaging strategy (Fig. 2A & B). The dynamics of NF-κB signaling in B16F10 tumor *in vivo* were assessed using a transcriptionally-activated NF-κB-promoter-driven Firefly luciferase reporter (NF-κB-FLuc) enabling real-time bioluminescent imaging and quantitative monitoring of NF-κB transcriptional activation. B16F10 NF-κB-FLuc reporter cells also stably expressed a constitutively active CMV-driven dendra2 fluorophore to assess tumor mass by dendra2 fluorescence (λ_{ex} = 409 nm, λ_{em} = 507 nm). Concurrent, luminol and L-012 bioluminescence imaging assessed the level of enzymatic MPO activity and reactive oxygen species (ROS), respectively, from infiltrating myeloid cells. Interestingly, longitudinal live macroscopic intravital imaging of tumor NF-κB transcriptional activation, when quantified over time, showed an increase in NF-κB activation in MPO$^{-/-}$ mice compared to wild type MPO$^{+/+}$ mice during tumor progression. NF-κB transcriptional activation was normalized to tumor mass using dendra2 fluorescence imaging. MPO$^{-/-}$ animals demonstrated a 51.2% ± 3.6% normalized increase in NF-κB activation per unit tumor mass as a function of time compared to MPO$^{+/+}$ animals. Focusing on early melanoma development, here defined as between day 2 to day 5 post tumor inoculation, a 234.7% ± 71.9% increase in NF-κB transcriptional activation per unit tumor mass in MPO$^{-/-}$ animals compared to MPO$^{+/+}$ animals was observed (Fig 2C). Histology of B16F10 tumors confirmed the increased in NF-κB protein levels (Fig. 2D). Infiltrating MPO-active MDCs were imaged and peroxide activity quantified over time in individual animals using luminol and L-012 (Fig. 2A & B). Oxidation of luminol and L-012 results in photon emissions that primarily indicates the presence of enzymatically active MPO. Using quantitative image analysis, a spatially-encoded statistically significant inverse intensity correlation was observed between either luminol or L-012 signals, and B16F10 tumor NF-κB transcriptional activation only in MPO$^{+/+}$ animals (Pearson coefficients of r = -0.53 and r = -0.32, respectively) (Fig. 2E & F). This suggested that in the presence of MPO-active cells, tumor NF-κB activation was locally *dampened* in MPO$^{+/+}$ animals. As expected, L-012 or luminol did not demonstrate any global correlation with NF-κB transcriptional activation in MPO$^{-/-}$ animals (Fig. 2G & H).

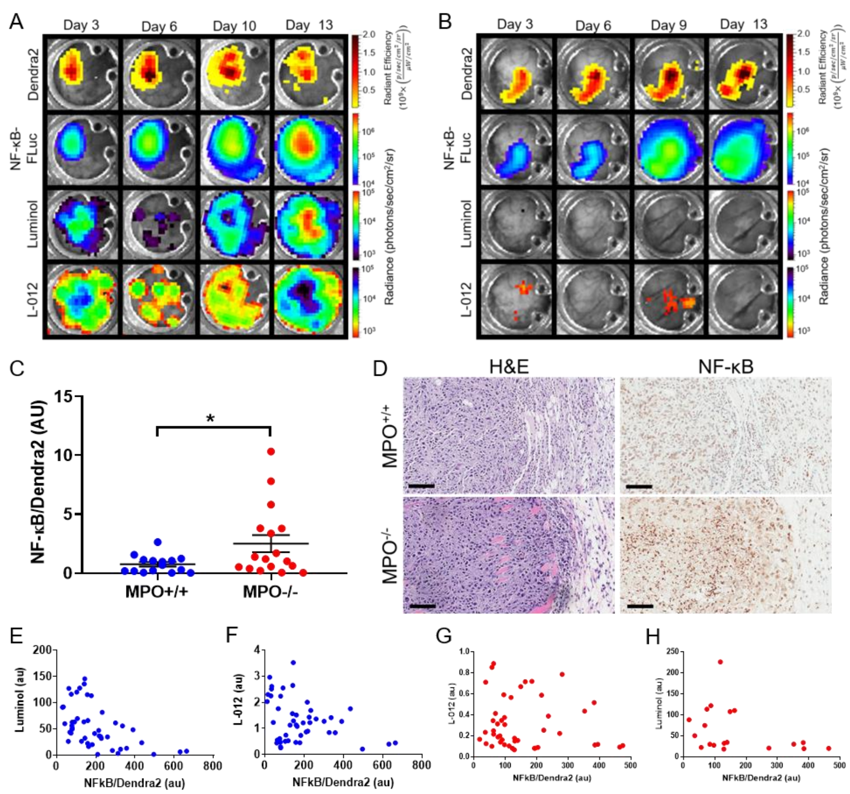


Figure 2. Longitudinal real-time imaging of tumor window chambers *in vivo*. Representative macroscopic bioluminescent image of the same skin window chamber over time in B16F10 melanoma tumor-bearing (A) MPO$^{+/+}$ (n = 17) and (B) MPO$^{-/-}$ (n = 16) animals. Window chamber bioluminescent quantification of macroscopic images (n = 135 images) of (C) NF-κB transcriptional activation normalized to tumor mass using dendra2 fluorescence in MPO$^{+/+}$ vs MPO$^{-/-}$ animals at early time points (measured between day 2 to day 5 post tumor inoculation); statistical significance calculated by identifying outliers, unpaired two-tail *t* test, * $P < 0.05$. (D) Representative immunohistology of MPO$^{+/+}$ and MPO$^{-/-}$ window chamber skin flaps showing H&E and NF-κB staining (20X; scale bar, 100 µm). Inverse correlation from window chamber macroscopic bioluminescent quantification between NF-κB tumor transcriptional activation and (E) luminol (MPO-active myeloid cells) or (F) L-012 (MPO-active myeloid cells) intensity *in vivo* in MPO$^{+/+}$ and NF-κB tumor transcriptional activation and (G) luminol (MPO-active myeloid cells) or (H) L-012 (MPO-active myeloid cells) intensity *in vivo* in MPO$^{-/-}$ following high throughput analysis (identified outliers; Pearson coefficients of r = -0.53 ($P < 0.0001$) and r = -0.32 ($P < 0.05$).

When MPO$^{+/+}$ window chamber tumor-bearing animals were imaged using intravital microscopy, heterogeneous activation levels of NF-κB between individual tumor cells within the same tumor was observed (Fig. 3A). To explore whether the spatial heterogeneity was a consequence of myeloid cell distribution within the microenvironment, myeloid cells were labeled *in vivo* using an intravenous injection of fluorophore-labeled dextran and fluorophore-labeled αGr-1 antibody. Remarkably, tumor cells *in close proximity* to myeloid cells *in vivo* demonstrated *decreased* NF-κB-FLuc bioluminescence (Fig. 3A, red arrow) compared to tumor cells that were distant from myeloid cells (Fig. 3A, white arrow). The bioluminescent signal from B16F10 NF-κB-FLuc tumors were quantified at 2X magnification between day 5 to day 7 post tumor inoculation (Fig. 3B). The inclusion of a constitutively active reporter, dendra2, provided a method to normalize signaling dynamics to tumor mass. Normalized NF-κB activation per unit tumor mass demonstrated an increase in NF-κB transcriptional activation within B16F10 tumors grown in MPO$^{-/-}$ animals compared to MPO$^{+/+}$ animals, similar to what was demonstrated macroscopically (Fig. 3C). Vasculature permeability was assessed following an intravenous injection of fluorophore-labeled dextran in real-time (Fig. 3D). The decrease in fluorescence corresponded to the leakage of dextran from the vasculature within B16F10 tumors. Interestingly, the leakage of dextran was significantly faster from B16F10 tumors in MPO$^{-/-}$ animals compared to MPO$^{+/+}$ animals suggesting that the vasculature was *more* permeable in melanoma tumors grown in MPO-deficient environments (Fig. 3E). These *in vivo* window chamber studies demonstrated that MPO-active myeloid cells suppressed NF-κB transcriptional activation within B16F10 melanoma tumors in a tight spatially-localized and proximity-dependent manner.

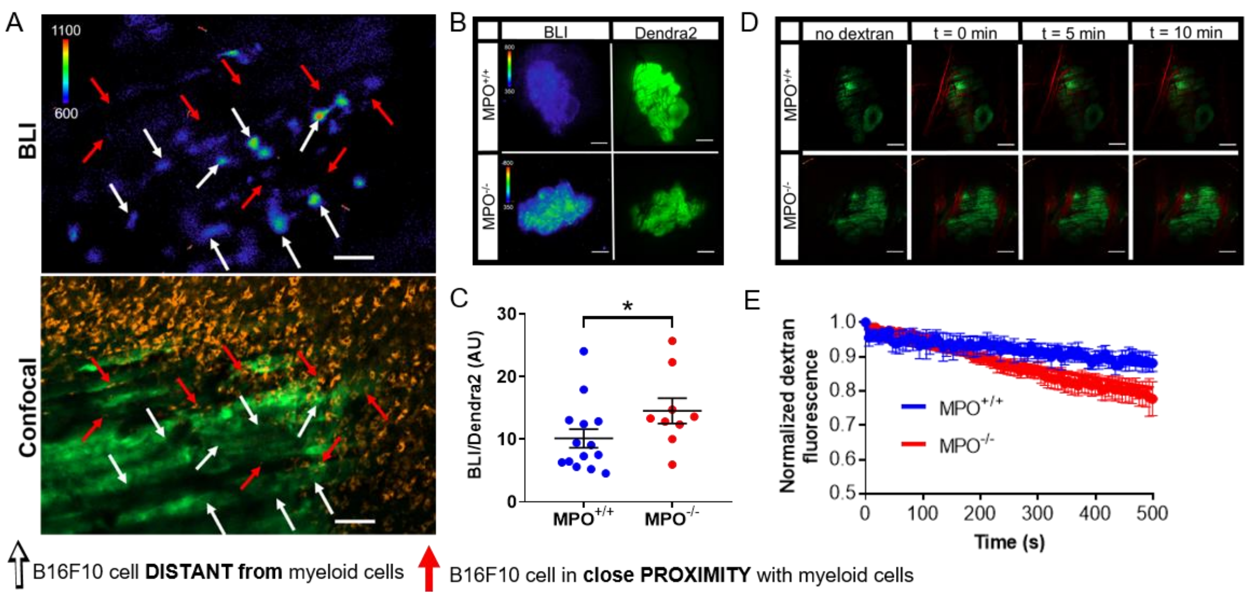


Figure 3. Intravital microscopy of wild type (MPO$^{+/+}$) and MPO-null (MPO$^{-/-}$) window chamber-bearing animals with B16F10 NF-κB-FLuc dendra2 melanoma tumors. (A) Representative intravital image of bioluminescence (BLI) and corresponding confocal image of B16F10 NF-κB-FLuc tumor cells (dendra2; green), myeloid cells (αGr-1+; red, and dextran uptake; orange), red arrows indicate B16F10 cells in contact with myeloid cells, white arrow indicate B16F10 cells not in contact with myeloid cells (10X objective; scale bar, 100 μm; n = 4 MPO$^{+/+}$ animals). (B) Representative intravital image of NF-κB-FLuc bioluminescence (BLI, 20 min acquisition, open filter) and dendra2 epifluorescence (GFP cube, 1 s exposure) at 2X in MPO$^{+/+}$ and MPO$^{-/-}$ animals at day 7 post window chamber implantation and tumor inoculation; scale bar represents 1000 μm. (C) Quantification of NF-κB-FLuc bioluminescence normalized to tumor dendra2 fluorescence between day 5 and day 7 post tumor inoculation (data are represented as mean ± SEM, statistical significance calculated by unpaired two-tail Mann Whitney t test, * $P < 0.05$, , n = 14 MPO$^{+/+}$ animals, n = 9 MPO$^{-/-}$ animals). (D) Quantitative decrease in Texas Red fluorescence corresponding to leakage of dextran from the vasculature of B16F10 tumors over time (data are represented as mean ± SEM, n = 4 animals per group). Dextran leaks out of MPO$^{-/-}$ B16F10 tumor vasculature significantly faster than from MPO$^{+/+}$ B16F10 tumor vasculature (least squares fit, slope significantly different, $P < 0.0001$). (E) Representative confocal images in MPO$^{+/+}$ and MPO$^{-/-}$ animals of tumor vasculature post i.v. injection of dextran-Texas Red (70,000 MW, 4 mg/mL) at different time points (green, B16F10 dendra2 fluorescence; red, dextran-Texas Red); 2X objective, scale bar indicates 1000 μm.

3. Plasma cytokine analysis from B16F10 and YUMM3.3 subcutaneous model of melanoma growth comparing $MPO^{+/+}$ and $MPO^{-/-}$ animals demonstrated differential expression of circulating cytokines but surprisingly, only at early tumor progression time points on day 3 post tumor inoculation (Fig. 4). Interestingly, CCL11 and CXCL5 were significantly different between $MPO^{+/+}$ and $MPO^{-/-}$ animals on day 3 post tumor inoculation in both tumor models.

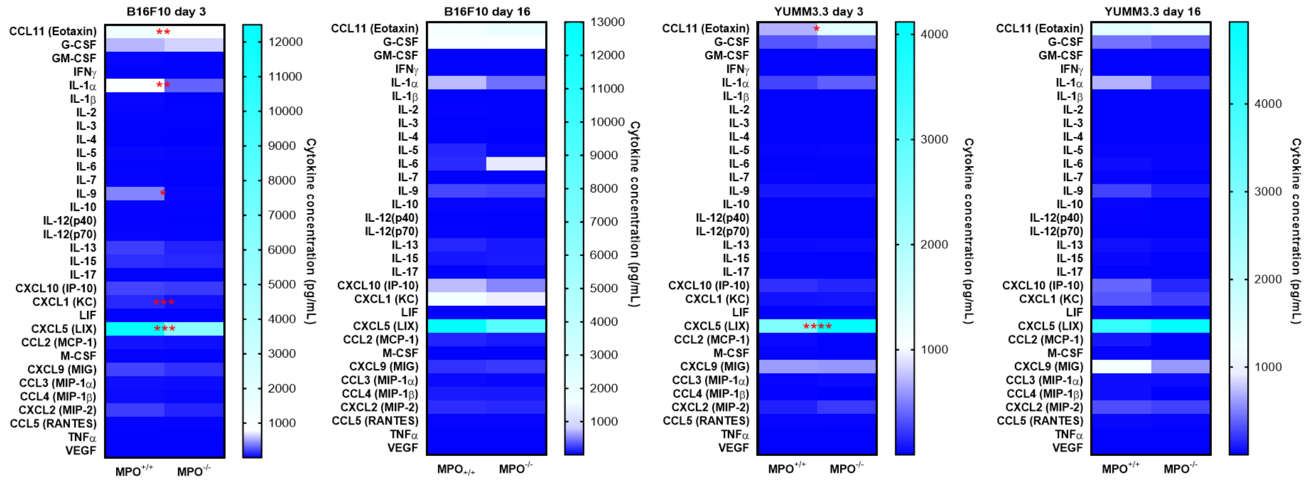
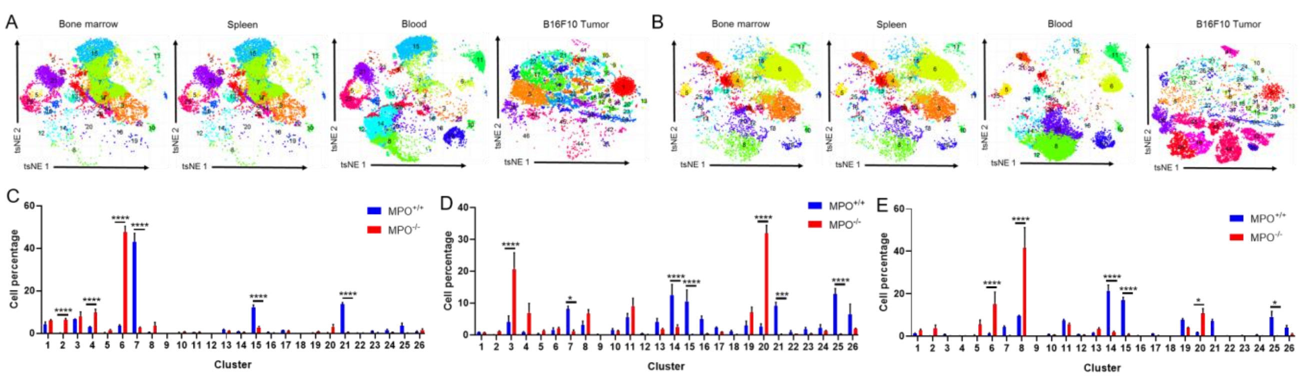
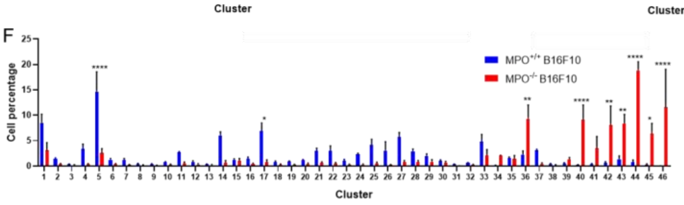


Figure 4. Cytokine concentration in plasma samples from B16F10 and YUMM3.3 subcutaneous tumor bearing $MPO^{+/+}$ and $MPO^{-/-}$ animals taken on day 3 and day 16 post tumor inoculation (B16F10 – n = 9 animals per group, YUMM3.3 – n = 3 animals per group). Statistically significant difference found between $MPO^{+/+}$ and $MPO^{-/-}$ animals of each cytokine using Student's *t*-test at each time point; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

4. CyTOF analysis of endpoint B16F10 tumors and immune compartments (bone marrow, spleen and blood) in melanoma-bearing $MPO^{+/+}$ vs $MPO^{-/-}$ animals demonstrated differences in myeloid cells, dendritic cells and B cell populations in the immune and tumor compartments. In addition, an increase in CD8 and CD4 T cell populations in the B16F10 tumor compartment in $MPO^{-/-}$ compared to WT animals was observed (Fig. 5).





G

Clusters	Markers
2	TIM-3, CTLA-4, Arg-1, TIGIT, MPO, FoxP3, IFNg, CD11c, CD115, CD68, Ly6G, CD11b, Ly6C, iNOS, LAG-3, p-NFkB, CD3e, IFNa, F4/80
3	
4	CD11c, Ly6G, CD11b, Ly6C, iNOS
6	IFNg, CD11c, CD115, CD68, Ly6G, CD11b, Ly6C, iNOS, LAG-3, p-NFkB, CD3e, CD80
7	Ly6G, CD11b
8	MHC II, PD-L1, CD19
14	MHC II, CD19
15	Ly6G, CD11b
20	MHC II, PD-L1, CD19
21	MPO, CD68, CD11b, Ly6C, iNOS, p-NFkB, IFNa
25	PD-L1, CD68, CD11b, IFNa

H

Cluster	Marker
5	TIM-3, CTLA-4, PD-L1, FoxP3, CD68, iNOS, LAG-3, NFkB, IFNa, GranzymeB
17	CTLA-4, PD-L1, FoxP3, CD68, NFkB, GranzymeB
36	MHCII, TIM-3, CTLA-4, PD-L1, FoxP3, CD11c, CD115, CD68, CD11b, iNOA, LAG-3, NFkB, IFNa, F4/80, CD206, CD80, GranzymeB, ICOS
40	MHCII, CD19
42	TIM-3, CTLA-4, CD8a, LAG-3, CD3e, ICOS
43	TIM-3, CTLA-4, PD-L1, FoxP3, CD68, CD11b, Ly-6C, LAG-3, GranzymeB, ICOS
44	TIM-3, CTLA-4, LAG-3, CD3e, CD4, GranzymeB, ICOS
45	PD-L1, GranzymeB
46	MHCII, PD-L1, CD19, LAG-3

Figure 5. CyTOF tsNE plot of (A) $MPO^{+/+}$ and (B) $MPO^{-/-}$ animals of immune compartments, bone marrow, spleen and peripheral blood, and endpoint B16F10 tumor (n = 3 animals per group). CyTOF analysis using Phenograph of quantified cluster cell percentage in (C) bone marrow, (D) spleen, (E) blood and (F) B16F10 tumor. Statistically significant differences found between $MPO^{+/+}$ and $MPO^{-/-}$ animals of clusters found within each isolation site using a two-way ANOVA followed by Tukey's multiple comparisons test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Marker expression of (G) Immune compartment and (H) B16F10 tumor compartment of significantly different clusters.

5. First small cohort of real-time *in vivo* imaging in skinfold melanoma-bearing window chambers using our advanced molecular imaging platform. B16F10 tumors stably expressed a constitutively active CMV-driven fluorescent protein, dendra2 (λ_{ex} = 409nm, λ_{em} = 507nm), to assess tumor mass. B16F10 tumors were imaged at multiple time points prior to, during and post ICT treatment. ICT response was only observed in MPO$^{-/-}$ animals where tumor fluorescence was non-detectable as early as 5 days post ICT treatment (Fig. 6A & B). L-012 bioluminescence imaging assessed the levels of enzymatic MPO activity and ROS in the tumor microenvironment (Fig 6C). In MPO$^{-/-}$ animals, oxidation of L-012 is primarily from ROS as MPO is deficient in these animals. Although preliminary, ICT responders appeared to have localized L-012 signal at the tumor site and the fold change in L-012 bioluminescence was greater in WT and MPO$^{-/-}$ ICT resistant animals compared to MPO$^{-/-}$ ICT responders (Fig. 6D & E).

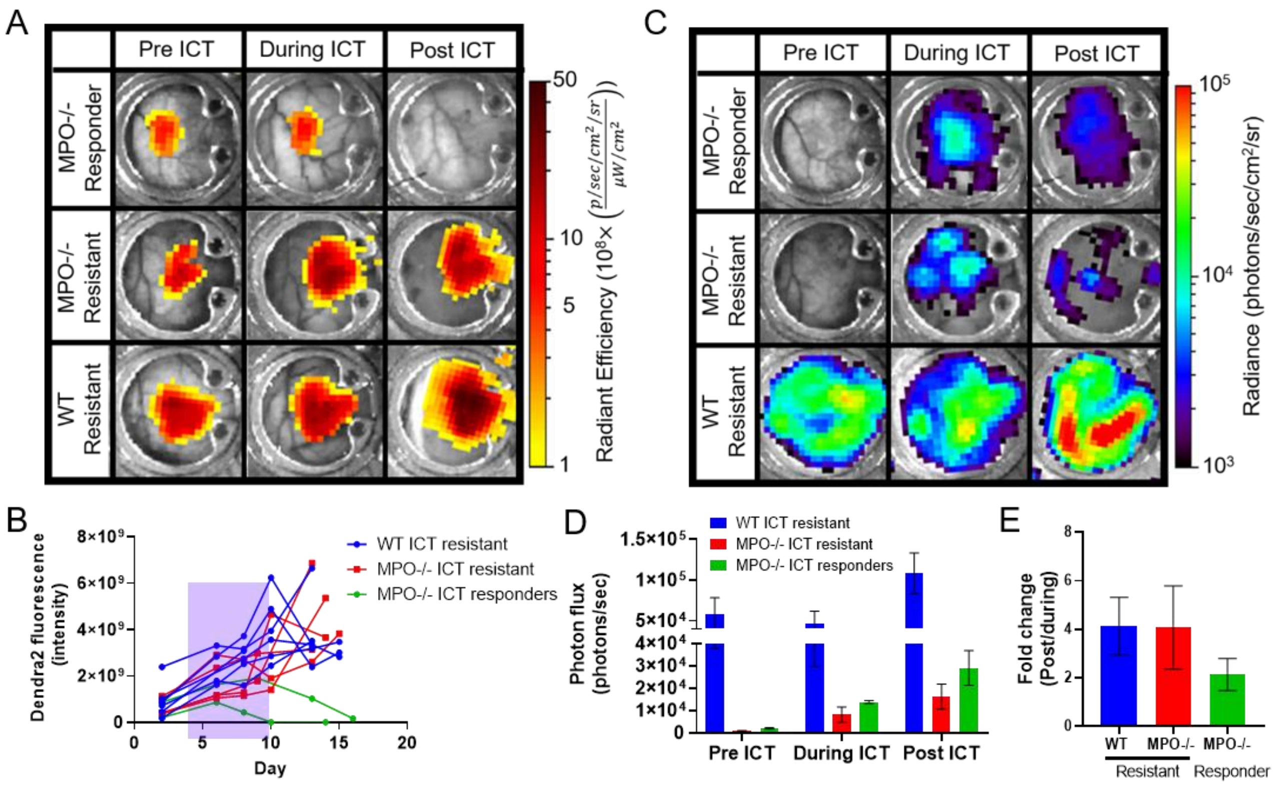


Figure 6. Dynamic imaging and quantification of ICT response in skin window chamber B16F10-tumor bearing in WT and MPO-/- animals using the advanced molecular imaging platform (n = 6 WT ICT resistant animals, n = 5 MPO-/- ICT resistant animals, n = 2 MPO -/- ICT responders). (A) Representative fluorescent images and (B) quantitative graphical representation of tumor growth prior (pre), during and post ICT treatment using dendra2 fluorescence; purple box indicates ICT treatment time. (C) Representative L-012 bioluminescent images and (D) quantitative graphical representation of MPO activity and ROS levels within tumor microenvironment prior (pre), during and post ICT treatment. (E) Quantitative fold change comparing L-012 bioluminescence post and during ICT.

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.*

This project has provided me one-on-one work with my career mentor, Dr. David Piwnica-Worms. I gave an invited talk for the World Molecular Imaging Society's Immune Cell Imaging Webinar Series 2020 titled "Optical Imaging of the Immune System". I presented posters at two virtual meetings, AACR annual meeting 2020 and Keystone Symposia 2020 Advances in Cancer Immunology. I've also increased my skill set by learning CyTOF experimental approaches and analysis.

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.

I gave an invited talk for the World Molecular Imaging Society's Immune Cell Imaging Webinar Series 2020 titled "Optical Imaging of the Immune System". I presented posters at two virtual meetings, AACR annual meeting 2020 and Keystone Symposia 2020 Advances in Cancer Immunology.

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.

Complete Specific Aim 1 and begin work on Specific Aim 2 and Aim 3.

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).*

This work will impact melanoma research and the field of immunology. My early findings have demonstrated that the enzyme myeloperoxidase is increased in innate immune cells in the presence of melanoma when compared to healthy subjects. Furthermore, we developed and demonstrated the applicability of our advanced molecular imaging platform in this project which can be easily be applied to many other studies in the field of molecular biology and immunology. 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?

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.

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?

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.*

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.*

Nothing to Report.

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:*

One of the melanoma cell lines, YUMM4.1, does not grow consistently in mature (23 weeks and older) wild type C57BL/6 $MPO^{+/+}$ animals or syngeneic MPO-null $MPO^{-/-}$ animals which has hindered 100% completion of Major Task 1. Another problem we have encountered is that the YUMM1.G1 cell line which have a 100% take rate in wild type C57BL/6 $MPO^{+/+}$ animals, do not consistently grow in syngeneic MPO-null $MPO^{-/-}$ animals. To address these problems, I hope to change from using mature (23 weeks and older) animals to using young 6-8 week old animals to see if that increases the take rate of YUMM4.1 and YUMM1.G1.

Another change as suggested by reviewers previously in the Peer and Programmatic Review Considerations document, is to use CyTOF instead of flow cytometry to study the immune cell landscape of subcutaneous melanoma models.

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.

The COVID-19 pandemic shut down our laboratories beginning March 18, 2020. We were able to return back to the laboratory but in short shifts June 8, 2020. Laboratory shifts were just lifted beginning on Sept 7, 2020 but we are currently still not running at full capacity. Because of the pandemic, we were unable to complete all of Specific Aim 1 and begin Specific Aim 2. One major delay in this project has been breeding our $MPO^{-/-}$ colony. Unfortunately, during the pandemic, we were required to stop all breeding colonies and were only able to begin breeding again in June. Due to the use of mature animals in this project, we are still a few months away before $MPO^{-/-}$ animals will be of age to begin studies again which are needed to complete the Specific Aims.

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.

Nothing to report.

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 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:** *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."*

- **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).*

  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; 2020; Under Review; 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; Molecular Cell; 2020; Submitted; 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; Molecular Cell; 2020; Submitted; acknowledgement of federal support yes.

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).*

Nothing to report.

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.*

AACR Annual Meeting 2020 – virtual poster presentation
Keystone Symposia 2020 Advances in Immunology – virtual poster presentation

- **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.

Nothing to report.

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

Nothing to report.

- **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.

Nothing to report.

- **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.*

An ImageJ macro was developed used to align and crop intravital macro-images which allows for downstream quantitative analysis of imaging.

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".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

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 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 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?
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.

(Liu) 06/01/2020 – 05/31/2021
0.12 CM
The University of Texas MD Anderson Cancer Center / Melanoma SPORE CEP
PET Imaging of the Innate Immune System Predicts Immune Checkpoint Therapy Response
Goal: To test the hypothesis that real-time, non-invasive [18F]4FN PET imaging of MPO activity and ROS levels will emerge as predictive markers for ICT response.
Role: Principal Investigator

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.*

Nothing to report

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.*