AWARD NUMBER: W81XWH-22-1-0952

TITLE: Macrophage Regulation of the Tumor Microenvironment in Metastatic Melanoma

PRINCIPAL INVESTIGATOR: Ashley M. Holder

CONTRACTING ORGANIZATION: UT MD Anderson Cancer Center

REPORT DATE: OCTOBER 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2023	Annual	1SEPT2022 - 31AUG2023

4. TITLE AND SUBTITLE

Macrophage Regulation of the Tumor Microenvironment in Metastatic Melanoma

5a. CONTRACT NUMBER
W81XWH-22-1-0952

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Ashley M. Holder

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
UT MD Anderson Cancer Center

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT More than 7,000 patients will die from melanoma in the US this year. It is therefore critical that we design new therapies to treat and prevent the spread of melanoma from the skin. How melanoma spreads to lymph nodes (**LNs**) and other organs is not known. We suspect that immune cells, specifically macrophages, migrate from the primary tumor to help melanoma develop secondary tumors in LNs. Our hypothesis is that macrophages from the primary melanoma travel to LNs and alter cells there to suppress the immune response to cancer. The purpose of this research is to improve our understanding of lymphatic spread of melanoma and inform drug development to target tumor-promoting macrophages. Using a mouse model of melanoma, we are testing the hypothesis that macrophages from the primary tumor alter the expression of genes of immune cells in the LN. Thus far, we have validated our model for macrophage trafficking and are optimizing our read-out for assessing the impact of macrophage depletion on the melanoma LN metastases.

15. SUBJECT TERMS
Melanoma, lymph node, metastases, macrophage

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	21	19b. TELEPHONE NUMBER *(include area code)*

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

It is critical that we design new therapies to treat and prevent the spread of melanoma from the skin. How melanoma spreads to lymph nodes (LNs) and other organs is not known. We suspect that macrophages migrate from the primary tumor to help melanoma develop tumors in LNs. The purpose of this research is to improve our understanding of lymphatic spread of melanoma to inform drug development. Using a mouse model of melanoma, we are testing the hypothesis that macrophages from the primary tumor alter the gene expression of immune cells in the LN.

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

Melanoma, lymph node, metastases, macrophage

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: Determine the extent to which macrophages facilitate LN metastasis
- Months 0-14: 21.4%

Major Task 2: Determine the effect of adoptive transfer of macrophages from primary tumors on nodal metastasis
- Months 13-28: 7%

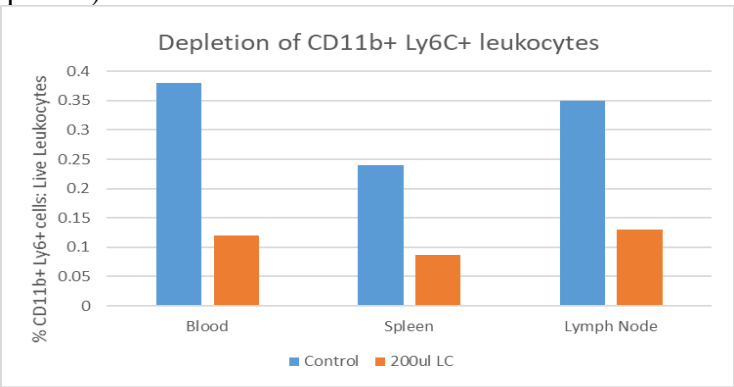
Major Task 3: Determine changes in the LN microenvironment using scRNAseq
- Months 27-42: 0%

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.

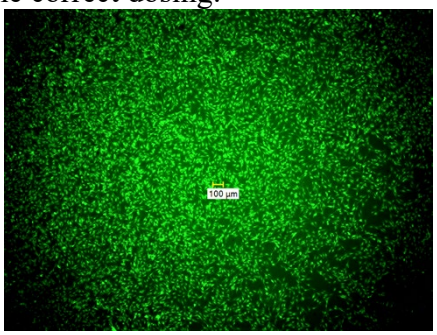
Portions of Major Task 1 have been completed and some remain in progress due to technical challenges that will be resolved. Firstly, ACURO Review was completed and the IACUC protocol has been approved.

For Subtask 1 and 2, there have been technical challenges associated with macrophage depletion and read-out/assessment of metastases. Firstly, Liposomal Clodronate depletion of macrophages required optimization. We spent 2 weeks refining technique using cull mice to ensure tail vein injection reproducibility. We then attempted macrophage depletion as per Subtask 1, only to have two failed experiments in which macrophage depletion was not observed over a period of approximately 1 month. After contacting the manufacturer, we were informed our lot of clodronate liposomes was problematic, and the manufacturer replaced the vial. With new liposomal clodronate, we repeated the attempt at macrophage depletion with my research assistant and a member of Dr.

Jim George's lab performing head-to-head tail vein injections with liposomal clodronate with the expected results obtained by both research assistants as measured by flow cytometry: 62-68% depletion (55-70% expected).



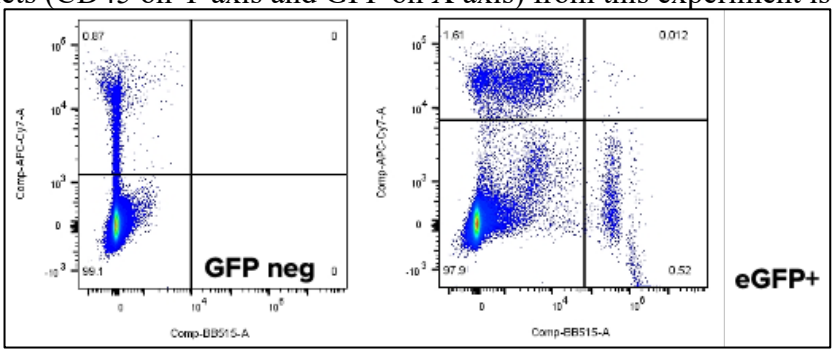
The next step for optimizing the approach for Subtask 1 was to obtain reproducible results with macrophage depletion using Diphtheria Toxin (DT) IP injection in CD11b-DTR mice. I selected the dosing based on published literature (25 ng/g). However, we repeated our experiments three times, and each time, the mice died within 72 hours of injection. These concerns were presented at Dr. Jim George's lab meeting with recommendations from other lab members to consider a dose as low as 6ng/g. My experimental plan is to attempt a spectrum of dosing and assess macrophage depletion to ensure mouse survival, while maintaining CD11b depletion of at least 70% compared to control. I was growing the colony to support this experiment when I left UAB. My plan upon resuming my DOD MASA project at MD Anderson is to test: 5ng/g, 10ng/g, 15ng/g, 20ng/g DT IP dosing along with control (5 mice/group). Once single dose has been evaluated for mouse survival and macrophage depletion (assessment by flow at 72 hrs), I will attempt repeated treatments (q3days x 3) to ensure we have identified the correct dosing.



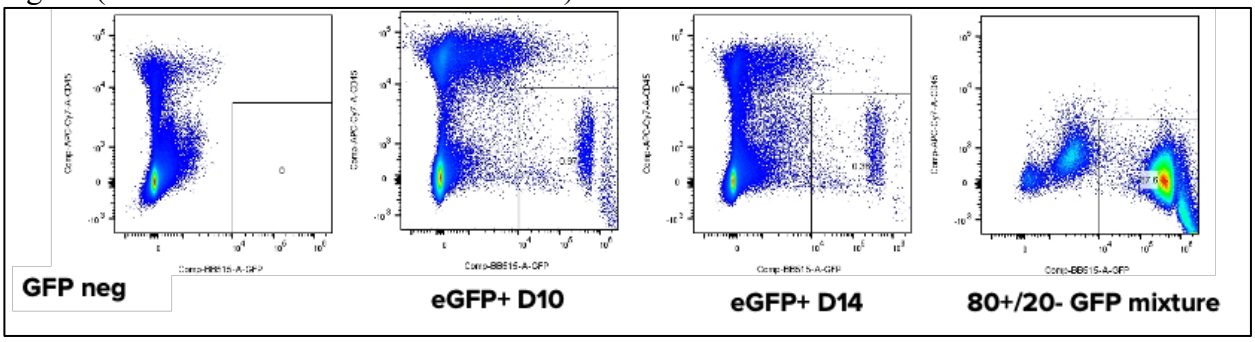
With regards to Subtask 2, we encountered challenges with using the UAB Animal Pathology Core for assessing lymph node metastasis using immunohistochemistry. Despite the small size of mouse lymph nodes, we were not consistently identifying metastases, as in our preliminary experiments. The pathologist anticipated we would need to increase our slides from 10 per mouse lymph node to at least 50 slides from each mouse. The cost associated with 50 slides for each mouse lymph node was prohibitive. I recognized the need to develop another methodology to quantify lymph node metastases. I determined that micrometastases may be best assessed by flow cytometry since this technique was already one with which I am familiar and is being utilized throughout Aim 1. I transfected the B16F10 cells with an eGFP reporter under control of a CMV promoter with puromycin resistance. We optimized our transfection within 3 weeks. We assessed the cells for mycoplasma and found them to be pathogen-free. We then spent approximately 2 months selecting cells and growing adequate cells and stock. We confirmed our fluorescent plasmid transfection with fluorescence microscopy (please see above image).

Using our newly created fluorescent B16F10 cells, my mentor's lab transitioned to a spectral cytometer from the instrument we had previously used in his lab. The GFP signal was so intense from the transfected melanoma cells, and we were unable to manually alter the voltages to accommodate for the intensity of that signal and the size of the melanoma cells compared to our immune cells of interest. In order to manually alter voltages, I transitioned our flow cytometry work to the UAB Flow Core's BD FACSymphony. We optimized our experiments there over

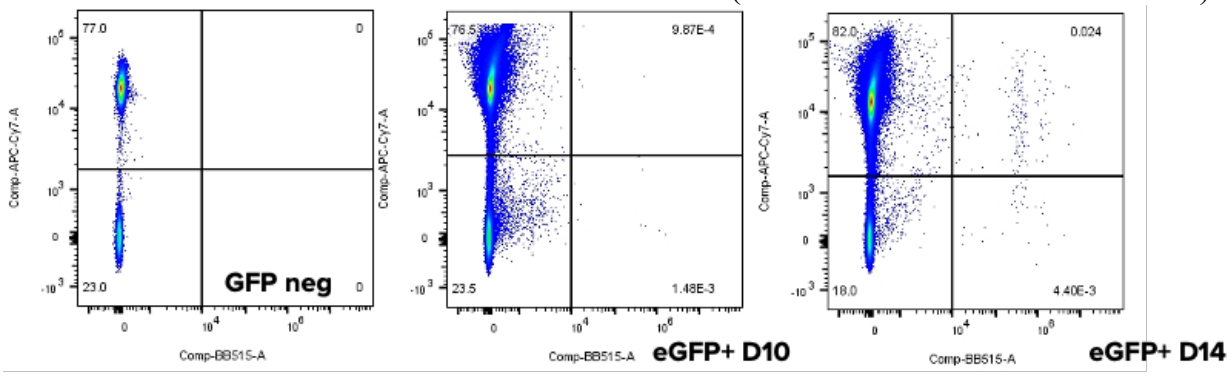
immune cells of interest. In order to manually alter voltages, I transitioned our flow cytometry work to the UAB Flow Core's BD FACSymphony. We optimized our experiments there over approximately 2 months, including antibody optimization and adjusting to using shared equipment that did have equipment failures/breakdown that we had not experienced with Dr. George's instrument. We confirmed that this methodology appears sensitive and specific for identifying melanoma B16F10-eGFP cells by comparing primary tumor and tumor-draining lymph node (TDLN) D10 and D14 along with PBS inoculation (in lieu of B16F10-eGFP) and B16F10 (without eGFP transfection) as controls. No GFP cells were counted in the PBS-treated TDLN or B16F10 cells (without GFP) establishing a background count of 0. All eGFP primary tumors had GFP cells counted but relatively low percentage compared to what I expected. Representative flow chart, eGFP as % of live singlets (CD45 on Y axis and GFP on X axis) from this experiment is above.



Next, I repeated the experiment and included an 80/20 mixture of GFP+ and GFP negative B16F10 cells as a validation of the quantification technique. Representative flow chart, eGFP as % of live singlets (CD45 on Y axis and GFP on X axis):

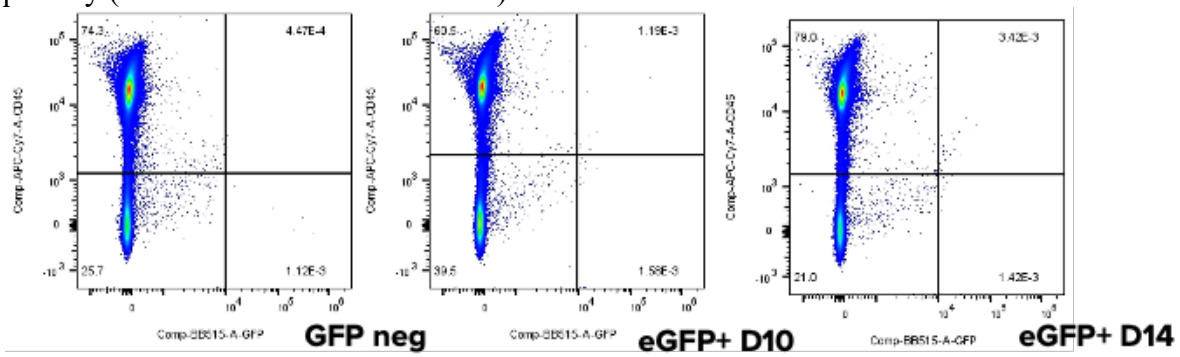


From evaluating the TDLNs in both experiments, the percentage and discrete number of B16F10-eGFP cells detected was very low. I identified that I would need to capture more events (10,000,000 as opposed to 1,000,000) to obtain results in a reproducible range. For experiment 1, 4/5 D10 animals had GFP detected and 2/4 D14 animals had GFP detected (CD45 on Y axis and GFP on X axis):

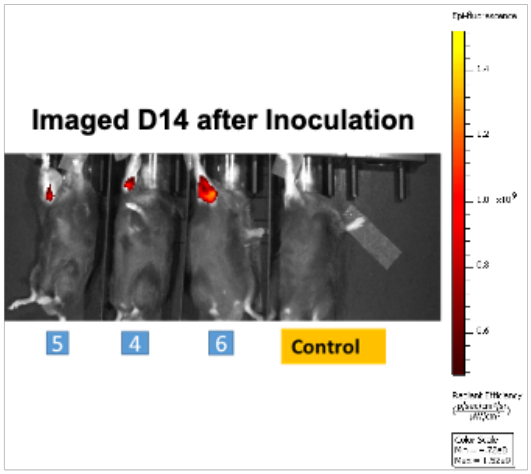


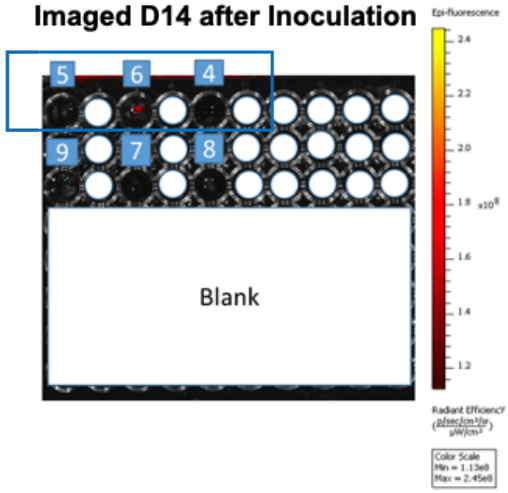
For experiment 2, GFP cells were identified in the TDLNs of mice inoculated with B16F10 (no eGFP) but not in PBS TDLNs. I suspect there may have been contamination in the cytometer and have adjusted the SOP to run GFP negative samples first. In this experiment, 3/5 D10 animals had GFP detected and 3/4 D14 animals had GFP detected. Once again, very few GFP+ cells were detected in each TDLN (CD45 on Y axis and GFP on X axis, see flow plots below please). I will first attempt to capture more events before being concerned that the micrometastases to the TDLNs are too small

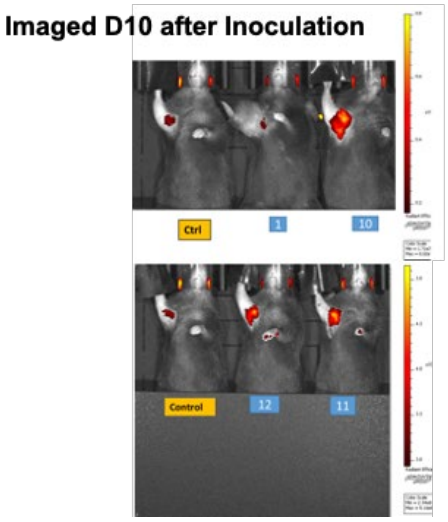
to quantify (below the detection threshold).

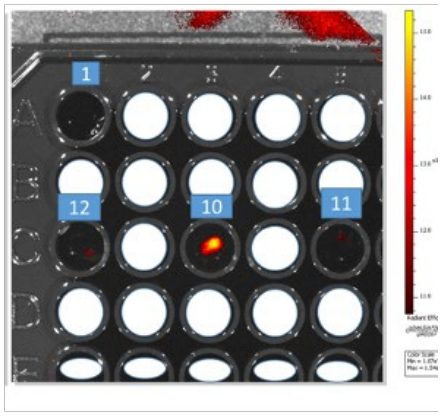


As an alternative to flow cytometry, we simultaneously attempted to develop In Vivo Imaging as a readout of melanoma lymph node metastases. We found that IVIS was too time sensitive and labor intensive without high reproducibility despite two separate attempts. Our results are demonstrated in the below figures:









As I have now had my animal protocol approved at MD Anderson, my cells have transferred from UAB with an MTA, and my mice from UAB are in quarantine, I am ready to continue experiments but am still awaiting transfer of funds from DoD as they have been returned by UAB. I have also established lab space and hired a research scientist. I have also identified another KikGR mouse strain available through Jax that I have purchased in case my mice from UAB are too old to be successful breeders. And I am starting to build my colony of CD11b-DTR mice as well.

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.

1) My Year 1 Melanoma Academy Activities have included that I have begun attending Dr. Davies' lab meetings which has been beneficial to me by offering collaborative opportunities and a future forum at which to present my research and receive feedback. I was also invited to present at the Melanoma Research Seminar Series, where I connected with multiple potential collaborators and advisors in Melanoma Research at MD Anderson. With the support of my mentors, I submitted a multi-PI R01 with Dr. Jen Wargo for the June deadline, published an editorial on the impact of SWOG S1801 with Drs. Wargo and Ross in the *Annals of Surgical Oncology*, and submitted a review article on biomarkers of response to immunotherapy for *Nature Medicine* with Dr. Boland (sustained collaboration). The above work on melanoma model development for tracking immune cell migration from the primary melanoma to the draining nodal basin was accepted as a poster presentation at the upcoming annual meeting of the Society for Melanoma Research.
2) The patient advocates who present to the Melanoma Academy have allowed me to engage with patient communities in a different way than as a surgical oncologist in clinic. I have appreciated the opportunity to hear and see them through their stories of courage.
3) The Melanoma Academy has impacted my career goals by allowing me to join a section with clinical expertise in a department where I am well-supported by my chair, Dr. Matt Katz, and my section chief, Dr. Merrick Ross, with protected time for melanoma research. I benefit from the ongoing meetings with Drs. Fisher, Davies, and Boland to help me navigate my grant transfer and continue my productivity while awaiting those funds. I have also appreciated the mentorship of Dr. Jeff Gershenwald, with whom I am actively developing a DoD Team Science Application. Dr. Gershenwald and I meet weekly not only to review cases but also to discuss clinical research opportunities including my serving as a Co-Investigator on MELMART and working with him on the AJCC Neoadjuvant Landscape Ad Hoc Working Group that led to my selection to the AJCC Education and Promotions Committee. I am also developing a potential collaborative project with Dr. Fisher about squaric acid and macrophage recruitment/trafficking to the draining nodal basin.
4) I look forward to continuing to learn more about the impact of cancer on Military Health and mission readiness throughout my time in the Melanoma Academy. Thus far, I have been honored to share about my Melanoma Academy project and ask my veteran and active-duty service member patients about their own experiences with melanoma and military health.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report at this time.

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.

Since starting my new faculty position at MD Anderson, I have met with five other researchers to discuss some of the experimental challenges that I have faced. The director of the flow cytometry and cell imaging core has proven to be an excellent resource; he has recommended that I modify my methodology to use cyclic immunofluorescence (COMET) as a read-out and create a tissue microarray (TMA) with 2-3 punches of each mouse lymph node and primary tumor. This technique would allow for assessment of metastatic burden along with tumor microenvironment with the option for spatial transcriptomic analysis to be performed on the same TMA. We are actively working to create our first TMA for COMET analysis. Lastly, there is not an obvious difference between D10 and D14 TDLN metastases; thus, will select D14 as timepoint going forward to maximize capture of GFP+ cells in TDLN

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).

Nothing to report

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.

Nothing to report

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

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

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:*

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

Please see above for methodologic adjustment from flow cytometry to cyclic immunofluorescence/COMET.

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 primary delay that has been encountered has been associated with my move to MD Anderson and the transfer of the DoD MASA. It has been a long process to transfer the grant, and I have been unable to work in earnest from January 27, 2023, when I left UAB until the last month or so when I had laboratory supplies, equipment, and cells. I have had to utilize start-up funds, that I had to apply and be awarded before being funded in June 2023 to have funding to support continuing the work on this grant since the funds have not transferred for the DoD grant yet.

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

Nothing to report aside from above. I have been unable to spend funds on this grant since it has not transferred yet.

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

Significant changes in use or care of human subjects

No changes to report

Significant changes in use or care of vertebrate animals

No changes to report

Significant changes in use of biohazards and/or select agents

No changes 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).*

1) Holder AM, Wargo JA, Ross MI. Timing is Everything: Neoadjuvant Versus Adjuvant Immunotherapy in Patients with Resectable Metastatic Melanoma. Ann Surg Oncol. 2023 Aug 19. doi: 10.1245/s10434-023-14133-8. Epub ahead of print. 2023 Sep 3;: PMID: 37597079. No acknowledgement of federal support
2) Montgomery KB, Holder AM, Burgan CM, Galgano SJ, Broman KK. Is it Time for Synoptic Reporting in Melanoma Nodal Surveillance Ultrasonography? Ann Surg Oncol. 2023 Sep;30(9):5327-5328. doi: 10.1245/s10434-023-13749-0. Epub 2023 Jun 16. PMID: 37326810. Acknowledgement of federal support.
3) Holder AM, Cohen S, Liu D, Parikh A, Boland GM. Defining clinically useful biomarkers of immune checkpoint inhibitors in solid tumors. *Nature Medicine*. Submitted. Will have acknowledgement of federal support

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

None

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

None yet

- **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.

Not applicable

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

None

- **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.

None

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

None yet

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:	Ashley Holder
Project Role:	PI
Researcher Identifier:	0000-0002-6506-5871
Nearest person month worked:	7
Contribution to Project:	Performed work to date
Funding Support:	MD Anderson Start-up Funds American College of Surgeons Clowes Award

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.

Primary change has been awaiting DoD funds in transfer since leaving UAB January 27, 2023

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.

Ann Surg Oncol
https://doi.org/10.1245/s10434-023-14133-8

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ASO PERSPECTIVES

Timing is Everything: Neoadjuvant Versus Adjuvant Immunotherapy in Patients with Resectable Metastatic Melanoma

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CURRENT ADJUVANT THERAPY LANDSCAPE FOR RESECTED STAGE III AND IV MELANOMA

Immune checkpoint blockade (ICB)-based immunotherapy (anti-CTLA-4 and anti-PD-1 antibodies) and tyrosine kinase inhibitor-based targeted therapy (BRAF/MEK) have revolutionized the treatment of patients with unresectable stage III or IV melanoma. The resultant improved survival outcomes have provided the rationale to investigate the role of these novel agents in the post-surgical adjuvant setting in patients with resectable high-risk stage III nodal and oligometastatic stage IV disease. The results of three randomized trials demonstrating disease-free survival advantages with the post-resection use of BRAF/MEK combination targeted therapy in stage III patients with BRAF-mutated tumors (Combi-AD[1]) and single-agent anti-PD-1 immunotherapy in stage III (CheckMate 238[2] and Keynote-054[3]) and stage IV (CheckMate 238[4]) patients regardless of BRAF mutational status provide the basis for the current US FDA-approved standards of care. A fourth adjuvant trial, ImmuNED[5], accrued patients exclusively with resected stage IV disease and used a three-arm randomization schema of placebo, single-agent anti-PD-1, or combination checkpoint blockade (anti-PD-1 plus anti-CTLA-4). A recent updated report showed improved survival outcomes with either of the two immunotherapy regimens compared with placebo and best outcome with the standard high-dose combination ICB regimen.

First Received: 27 July 2023
Accepted: 30 July 2023

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Published online: 19 August 2023

While these results have been transformational, survival outcomes are still unfavorable, particularly for patients with resected palpable regional lymph node metastases and stage IV disease. Furthermore, it is important to recognize that the realized survival outcomes are likely somewhat inflated as they are not reflective of the true recurrence rate of the entire at-risk population undergoing resection with curative intent. Since the randomizations occurred after resection, some patients, thought to be eligible for adjuvant therapy, were ineligible for trial entry because of early relapse;[6] these patients missed the opportunity to potentially benefit from adjuvant therapy. These patients harbor the most biologically aggressive disease and are thought to represent 10–15% of patients with resectable metastases. Improving survival for the entire high-risk patient population, beyond what has been achieved with the current standards, represents an important unmet need. CheckMate 915,[7] attempting to build on the success of the CheckMate 238 trial, randomized a similar patient population (resected stage III and IV) to receive single-agent anti-PD-1 or a modified, less toxic, combination ICB regimen. An updated 2022 report demonstrated no outcome benefit with combination ICB over single agent. Another strategy was to explore the role of various regimens or combination therapies, with proven efficacy, in the neoadjuvant setting in patients with measurable, resectable disease, acknowledging there are advantages and disadvantages to both neoadjuvant and adjuvant therapy approaches (Fig. 1).

THE RATIONALE FOR AND EARLY RESULTS FROM NEOADJUVANT STRATEGIES

After the achievement of high clinical response rates and improved survival outcomes with both targeted and ICB therapy in patients with disseminated stage IV and unresectable stage III disease, interest developed in investigating

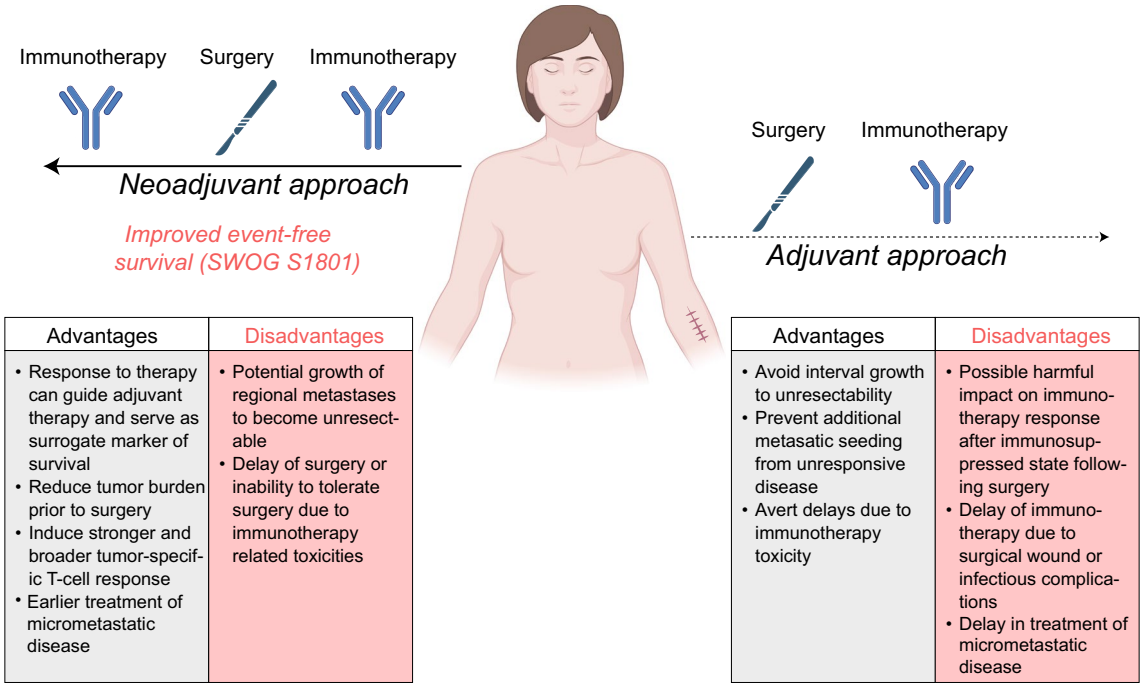

FIG. 1 Neoadjuvant versus adjuvant approaches for immunotherapy in melanoma. Advantages and disadvantages exist for both approaches; however, SWOG S1801 demonstrated improved event-free survival for neoadjuvant-adjuvant immunotherapy over adjuvant immunotherapy

these therapies in the neoadjuvant setting in patients with resectable, measurable nodal metastases so as to (1) determine the extent of clinical and pathological responses; (2) correlate clinical outcomes with extent of response or resistance; (3) prevent early progression of disease by initiating effective systemic therapies earlier to eradicate micrometastatic disease; and (4) establish a translational research platform for biomarker discovery to elucidate mechanisms of response and resistance, since access to tumor is more straightforward in these patients. Single-arm and randomized phase II trials were undertaken and demonstrated high clinical and pathological response rates with combination targeted and ICB regimens (single agent and combination). Significantly improved survival outcomes correlated with complete and major (< 10% viable tumor) pathological responses compared with patients with less responsive or non-responding tumors. The encouraging results of these early neoadjuvant trials and the need to improve upon the outcomes of the phase III adjuvant trials motivated investigators to formalize a multidisciplinary collaborative effort that led to the establishment of the International Neoadjuvant Melanoma Consortium (INMC).[8] One of the major contributions of the INMC was a pooled analysis of 184 patients participating collectively in six prospective clinical trials utilizing a variety of targeted or ICB neoadjuvant regimens. This analysis demonstrated that pathological complete response (pCR) occurred in 41% of patients overall and 33% of patients treated with immunotherapy, with an increased pCR rate observed in those receiving combination ICB therapy.[9] While pCR was achieved with a higher frequency with targeted therapy and correlated with very favorable survival outcomes, any pathological response less than pCR was associated with a rapid decline in recurrence-free survival (RFS). Remarkably, pCR generated by ICB was associated with nearly 100% RFS, and, in contrast to targeted therapy, even a partial response correlated with high and durable RFS outcomes. In an attempt to compare the impact of neoadjuvant immunotherapy with adjuvant immunotherapy, data from the pooled analysis demonstrated that neoadjuvant immunotherapy was associated with an RFS of 75% at 2 years, which compared favorably with an RFS of 60% at 2 years obtained from adjuvant immunotherapy trials, without accounting for the patients who were not captured in adjuvant trials due to early recurrence. Additional evidence suggesting that neoadjuvant immunotherapy might lead to superior outcomes compared with adjuvant immunotherapy was reported from the OpaCIN trial,[10] which studied the use of neoadjuvant versus adjuvant combination ICB, albeit with a relatively small number of patients. Beyond the survival curve analysis demonstrating trends of improved outcomes with neoadjuvant immunotherapy, translational analysis demonstrated a larger number of tumor-resident T cells in the peripheral blood of patients in the neoadjuvant versus adjuvant treatment arm.[10] These results are consistent with findings from preclinical models showing improved outcomes with neoadjuvant therapy.[9,11] While

the aforementioned reports of improved survival outcomes with neoadjuvant immunotherapy in high-risk patients were interesting and even provocative, the relatively few patients in the study and the limitations inherent in these types of analyses categorized the findings as hypothesis-generating. A well-designed, narrowly focused, prospective randomized trial was needed to support substantive changes to the current standard clinical practice of adjuvant single-agent ICB.

SWOG S1801: DESIGN, RESULTS, IMPACT, AND QUESTIONS

The SWOG S1801 trial was crafted to evaluate whether pembrolizumab administered both before and after surgery (3 doses neoadjuvantly and 15 doses adjuvantly) compared with pembrolizumab administered after surgery only (adjuvantly), with both treatment arms receiving a total of 18 doses, would increase event-free survival (EFS) in patients with resectable stage IIIB-IVm1C melanoma.[12] Importantly, randomization occurred prior to the initiation of either therapy (surgery or pembrolizumab), to ensure that all patients at risk for relapse were included and evenly distributed between the two arms. Power calculations were based on an anticipated EFS of 64% in the adjuvant arm (RFS from Checkmate-238) and 74% in the neoadjuvant-adjuvant arm (from an INMC neoadjuvant systemic therapy pooled analysis). Ultimately, 154 patients were randomized to the neoadjuvant-adjuvant arm and 159 to the adjuvant arm. At 2 years, the EFS was 72% for the neoadjuvant-adjuvant group and 49% in the adjuvant-only group (hazard ratio 0.58, 95% confidence interval 0.39–0.87; $p = 0.004$). A deeper dive into the results reveals that the reduction in events observed in the neoadjuvant arm (improved EFS) occurred almost completely during the adjuvant phases of the trial (Fig. 2). Given the simplicity in trial design, with only a single perturbation between the two arms (sequence of modalities), one can conclude that administration of pembrolizumab in the context of intact tumor burden primed the immune response to generate more efficacious and tumor-specific T cells, especially the adjuvant phase. In terms of the potential for preventing early relapse, a smaller absolute number of patients experienced an event during the neoadjuvant phase prior to surgery compared with those who relapsed in the surgery arm prior to initiating adjuvant therapy. However, the number of these events overall was surprisingly small. A significant reduction of these early events would not necessarily be expected given the current understanding of the time required for a PD-1-induced anti-tumor response that prevents or thwarts relapse to take place. In contrast, extrapolating from the Combi-AD BRAF/MEK adjuvant trial,[1] which demonstrated a significant reduction in early events compared with a placebo control, along with the recognition that targeted therapy has a direct tumoricidal effect, a neoadjuvant targeted therapy trial would more likely be expected to prevent early relapses during neoadjuvant therapy.

The results of the S1801 trial may be readily applied: treatment with neoadjuvant ICB should be offered to patients with high-risk resectable stage III or oligometastatic stage IV melanoma. Decisively, the results from S1801 eliminated concerns about delaying surgery for patients receiving neoadjuvant therapy due to progression that would prevent an attempt at a surgical cure. Additionally, these results will most certainly intensify efforts in the neoadjuvant space to address critically important questions.

(1) *What is the most appropriate type and duration of neoadjuvant treatment for patients with resectable metastatic melanoma?* The INMC provides recommendations to address this question in their white paper.[4] Specifically, they recommend a duration of neoadjuvant therapy of 6–8 weeks, given the risk of systemic and/or regional disease progression becoming unresectable.

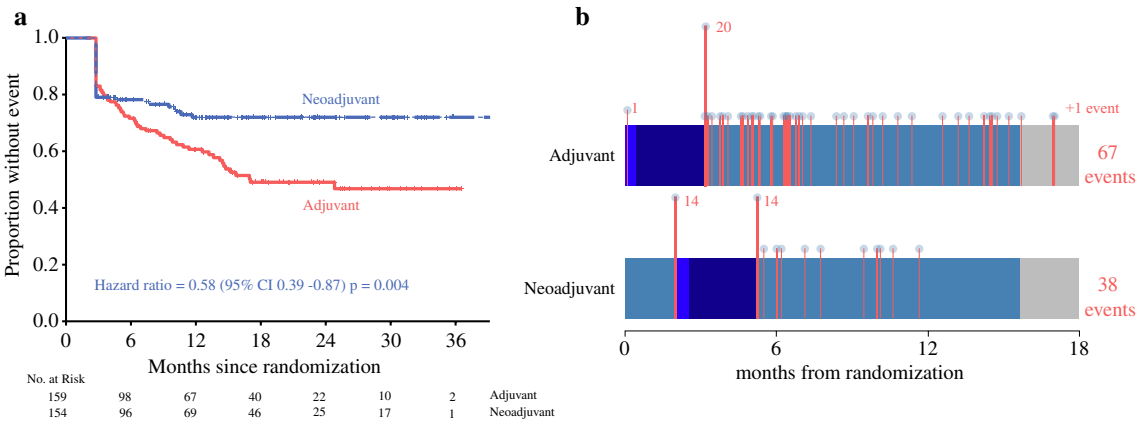

FIG. 2 Results of the SWOG S1801 trial. **a** Event-free survival curves for the neoadjuvant versus adjuvant treatment arms. **b** Timeline of events in each treatment arm. *CI* confidence interval

They similarly advise that trials include a post-surgical, adjuvant component until the field has determined how best to employ the individualized pathological response assessment to determine the need for and type of additional post-surgical therapy. Regarding the composition of neoadjuvant therapy, multiple options exist, from targeted therapy (BRAF/MEKi) to anti-PD-1 plus anti-CTLA-4 ICB to intralesional therapy. Notably, neoadjuvant immunotherapy has been shown to induce superior RFS with pCR compared with BRAF/MEK inhibition,[9] suggesting that BRAF/MEKi may be a strategy best suited to the adjuvant setting. In patients with unresectable stage III or IV melanoma even with *BRAFV600E/K* mutation, the DREAMseq trial was stopped early due to the 2-year overall survival (OS) benefit with nivolumab/ipilimumab followed by dabrafenib/trametinib, calling into question whether BRAF/MEKi should be considered in lieu of immunotherapy in the neoadjuvant setting.[13] Talimogene laherparepvec (T-VEC), an intralesional oncolytic immunotherapy approved for the treatment of unresectable melanoma, demonstrated improved RFS and OS in stage IIIB-IVM1a patients who received T-VEC followed by surgery versus surgery alone, which notably persisted at the 3-year analyses.[14] LAG-3 inhibition has been approved in combination with anti-PD-1 in the unresectable or metastatic setting,[15] and has also demonstrated promise in the neoadjuvant setting, with a high pCR rate and excellent safety profile.[16]

(2) *How good of a surrogate is pathologic response for long-term outcomes after treatment with neoadjuvant immunotherapy (and targeted therapy)?* Preclinical data and small neoadjuvant immunotherapy studies[9] suggest that pCR and likely pathologic major response will portend favorable RFS and OS.[9,11] Preliminary data from the SWOG S1801 trial reveal a 21% pCR with neoadjuvant pembrolizumab, but we need to wait for final response assessment data to better correlate pCR, partial response, and non-response to outcomes. The above-described combination ICB regimens will likely be further evaluated in an attempt to increase the rates of pCR.

(3) *Can we limit the extent of surgical resection (and subsequent adjuvant therapy) in patients who have an effective response to neoadjuvant systemic therapy?* The PRADO trial attempted to address this question. Ninety-nine patients with stage IIIB-D melanoma were treated with 6 weeks of neoadjuvant combination ICB (flipped dosing schedule of nivolumab/ipilimumab). Subsequent surgery and adjuvant therapy were determined by the pathological response assessment of the resected index lymph node (ILN, largest metastatic lymph node at baseline): major pathologic response (mPR, $\leq$ 10% viable tumor) had both therapeutic lymph node dissection (TLND) and adjuvant therapy omitted; pathologic partial response (pPR, > 10 to $\leq$ 50% viable tumor) underwent TLND only; and pathologic non-response (pNR, > 50% viable tumor) underwent TLND, adjuvant systemic therapy (targeted if BRAF mutated or nivolumab if wild-type) $\pm$ synchronous radiation. Relapse-free survival and distant metastasis-free survival were as follows: mPR 93 and 98%, pPR 64 and 64%, and pNR 71 and 76%, respectively. Not only did this study suggest that it may be safe to de-escalate treatment in patients with mPR but also that escalation in a non-responding patient could improve RFS.

(4) *If neoadjuvant immunotherapy is the 'new normal', what considerations must surgical oncologists have related to immune checkpoint blockade toxicity?* Surgical oncologists must be attuned to subtle symptoms of immune-related adverse events (irAEs) and refer patients for treatment. As Helmink and others outlined, fatigue and poor energy symptoms that may be attributed to metastatic disease may be presenting symptoms of hypophysitis and adrenal insufficiency.[17] They propose a thorough history, including bowel habit changes to uncover possible autoimmune colitis, complete skin examination for dermatologic irAEs, and complete blood work to uncover endocrinopathies preoperatively. There are no studies to date that suggest increased wound-healing complications or anastomotic leaks linked to ICB. However, Helmink et al. warn about the surgical implications of high-dose steroids used for the treatment of irAEs preoperatively as a risk factor for postoperative wound complications and stress-induced adrenal insufficiency.

BOTTOM LINE?

The implications of SWOG S1801 on the treatment of resectable stage III and IV melanoma are paradigm-shifting. The translational studies from SWOG S1801 and future neoadjuvant trials will provide critical insight into predicting recurrence, determining prognosis, and personalizing treatment according to the pathologic response.

DISCLOSURE Jennifer A. Wargo reports compensation for speaker's bureau and honoraria from Imedex, Dava Oncology, Omniprex, Illumina, Gilead, PeerView, Physician Education Resource, MedImmune and Bristol Myers Squibb; serves as a consultant and/or advisory board member for Roche/Genentech, Novartis, AstraZeneca, GlaxoSmithKline, Bristol Myers Squibb, Merck, Biothera Pharmaceuticals, Microbiome DX and Micronoma; and receives research support from GlaxoSmithKline, Roche/Genentech, Bristol Myers Squibb and Novartis. Merrick I. Ross reports consultant compensation from Merck and travel expense support from Merck for Advisory Board participation. Ashley M. Holder has no conflicts of interest to declare.

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Ann Surg Oncol (2023) 30:5327–5328
https://doi.org/10.1245/s10434-023-13749-0

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ASO RESEARCH LETTER

Is it Time for Synoptic Reporting in Melanoma Nodal Surveillance Ultrasonography?

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With the increased use of nodal surveillance in sentinel lymph node positive (SLN+) melanoma following the Second Multicenter Selective Lymphadenectomy Trial (MSLT-II),[1,2] the availability of high-quality, clinically actionable nodal surveillance ultrasonography (U/S) has become critical. Based on MSLT-II, U/S criteria regarding nodal recurrence include length-to-depth ratio >2, hypoechoic lymph node (LN) hilum, and changes in LN vascularity, with biopsy recommend if two or more features are present.[1] While prior qualitative work has identified a potential disconnect between surgeons' and radiologists' awareness of MSLT-II criteria,[3] little is known about how these criteria have been adopted and reported outside of clinical trial settings or used by surgical teams when interpreting ultrasound results.

METHODS

Patients with SLN+ melanoma undergoing nodal surveillance at a single tertiary cancer center from July 2017 to September 2022 who received at least one nodal ultrasound were identified retrospectively. Reporting language from each ultrasound was analyzed for number of MSLT-II nodal ultrasound criteria reported. Additionally, we abstracted whether a clinically actionable recommendation was made (e.g. continued surveillance or biopsy). Descriptive statistics and Chi-square tests were performed using Stata 17 (StataCorp LLC, College Station, TX, USA). This study was deemed exempt by the University of Alabama at Birmingham Institutional Review Board.

RESULTS

Overall, 269 nodal ultrasounds were performed in 78 patients (median three U/S per patient; interquartile range [IQR] 1–5). The majority of ultrasounds (81.0%) reported normal findings versus abnormal findings (19.0%). As detailed in Table 1, only a small proportion of normal ultrasounds had one or more MSLT-II criteria reported (33/215, 15.3%) versus the majority of abnormal ultrasounds (48/54, 88.9%; $p < 0.0001$). While most abnormal ultrasounds had only one MSLT-II criterion reported (20/54, 37.0%), fewer had two (15/54, 27.8%) or three criteria (13/54, 24.1%). Of the eight abnormal ultrasounds with biopsy recommendation, six (75%) had two or more MSLT-II criteria reported. Clinically actionable recommendations were provided in 94.9% of normal ultrasounds compared with 64.8% of abnormal ultrasounds ($p < 0.0001$).

DISCUSSION

In this single-institution retrospective study at a tertiary cancer center, clinically actionable recommendations were provided in the majority of nodal U/S reports, but few documented specific criteria associated with nodal recurrence as

This work was accepted as an ePoster at the Society of Surgical Oncology International Conference on Cancer Care, Boston, MA, USA, 23 March 2023.

First Received: 9 May 2023
Accepted: 4 June 2023
Published online: 16 June 2023

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TABLE 1 Reporting of MSLT-II criteria and clinically actionable recommendations in melanoma nodal surveillance ultrasonography

Variable	Normal ultrasound [$n = 215$]	Abnormal ultrasound [$n = 54$]	Total [$N = 269$]	p-Value
MSLT-II criteria reported				
0	182 (84.7)	6 (11.1)	188 (69.9)	< 0.0001
1	28 (13.0)	20 (37.0)	48 (17.8)	
2	2 (1.0)	15 (27.8)	17 (6.3)	
3	3 (1.4)	13 (24.1)	16 (6.0)	
Clinically actionable recommendation made				
Continue surveillance	204 (94.9)	27 (50.0)	231 (85.9)	< 0.0001
Biopsy	0 (0.0)	8 (14.8)	8 (2.9)	
Combined	204 (94.9)	35 (64.8)	239 (88.8)	

Variables are expressed as frequency (%)

MSLT-II Second Multicenter Selective Lymphadenectomy Trial

defined in MSLT-II.[1] More importantly, when ultrasound findings were abnormal, they were much less likely to be accompanied with a clinically actionable recommendation (e.g. biopsy or continued surveillance). We suspect that this discrepancy is due at least in part to the lack of a shared mental model between surgeons and radiologists for the intention behind nodal surveillance ultrasounds and the evidence supporting the use of U/S to identify specific findings of nodal recurrence that would prompt biopsy.[3] In their current format, nodal ultrasound reports may be difficult for surgical team members, including both surgeons and advanced practice providers, to interpret results and plan the next steps.

The rapid adoption of nodal surveillance as the predominant management strategy for SLN+ melanoma in the surgical oncology community[2,4] presents an opportunity for collaboration between surgeons and radiologists to ensure high-quality, clinically actionable nodal U/S. A synoptic reporting system for melanoma nodal ultrasound may standardize reporting and improve surgeon interpretation of the results, particularly when abnormal findings are present. Additionally, a synoptic reporting template could potentially be disseminated to non-specialized centers to increase access for rural or underserved patients who may face financial, transportation, or other barriers in returning to their treating center for frequent examinations.[3,5,6] Following a multidisciplinary collaborative effort to develop and implement a synoptic reporting template for melanoma nodal U/S at our institution (electronic supplementary material), we plan to study its adoption and perceived utility in a multidisciplinary cohort of surgeons and radiologists in future work.

SUPPLEMENTARY INFORMATION The online version contains supplementary material available at https://doi.org/10.1245/s10434-023-13749-0.

ACKNOWLEDGMENT Grant support was provided to KBM by the Agency for Healthcare Research and Quality under grant T32 HS013852; to AMH by the American College of Surgeons Clowes Award and the Department of Defense through the Melanoma Academy Scholar Program; and to KKB from the American College of Surgeons, the American Society of Clinical Oncology, and the National Institutes of Health on grant KL2 TR003097.

DISCLOSURE Kelsey B. Montgomery, Ashley M. Holder, Constantine M. Burgan, Samuel J. Galgano, and Kristy K. Broman have no conflicts of interest to declare.

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