AWARD NUMBER: W81XWH-21-1-0433

TITLE: Low-Dose Radiation Ex Vivo Reprogrammed/Activated CAR T Cells Targeting B7-H3 on Prostate Cancer

PRINCIPAL INVESTIGATOR: Xinhui Wang

CONTRACTING ORGANIZATION: Massachusetts General Hospital

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

Low-Dose Radiation Ex Vivo Reprogrammed/Activated CAR T Cells Targeting B7-H3 on Prostate Cancer

5a. CONTRACT NUMBER
W81XWH-21-1-0433

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts General Hospital

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:**

Metastatic prostate cancer (mPCa), which can be subdivided into hormone-sensitive (mHSPC) and castration-resistant PCa (mCRPC), is the lethal form of PCa , with a 5-year survival rate of 30%. Current therapies can prolong survival for mPCa patients; however, resistance invariably develops and eventually causes death. To address this unmet clinical need, we have recently developed a novel chimeric antigen receptor (CAR) T cell immunotherapy (CAR T therapy) by reprogramming/activation of CAR T cells that recognize B7- H3(CD276), an immune checkpoint which is almost uniformly expressed on differentiated (bulk) PCa cells and PCa stem cells (PCSPs), which can cause therapeutic resistance. B7-H3 expression increases in higher Gleason score prostate cancer and with progression to metastatic and castration-resistant disease, and is correlated with cancer-specific mortality. Conversely, B7-H3 expression on normal tissue is minimal. Low-dose radiation (IR) by upregulating NF-κB -stemness gene pathway empowers CAR T cells (IR CAR T) capable of producing a robust and long lasting anti-tumor activity. The IR B7-H3 CAR T compared to non-IR CAR T shows much increased potency in 1) in vitro killing of differentiated PCa and PCSCs in human PCa cell lines, and 2) in vivo inhibiting PCa or breast cancer xenograft growth, as measured by complete or substantial tumor regression and long-term survival in the absence of toxicity.

15. SUBJECT TERMS: NONE LISTED

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	19	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.*

The goal of this proposal is to test the hypothesis that Low-dose irradiation (IR) *ex vivo* reprogrammed/activated B7-H3 CAR T cells significantly prolong survival of mice bearing metastatic prostate cancer (mPCa), including metastatic hormone-sensitive prostate cancer (mHSPC) and metastatic castration-resistant prostate cancer (mCRPC), by eradicating differentiated bulk prostate cancer (PCa) cells and prostate cancer stem cells (PCSCs).

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

Radiation, reprogrammed/activated CAR T cells, B7-H3, metastatic prostate cancer

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.

1: Optimize the strategy of using low-dose IR to reprogram/activate B7-H3 CAR T cells and phenotypically and functionally characterize the reprogrammed CAR T cells.

2: Determine efficacy and safety of low-dose IR *ex vivo* reprogrammed/activated B7-H3 CAR T cells derived from mPCa patients to prolong survival of mice bearing human mHSPC or mCRPC cell-derived orthotopic xenografts.

3: Assess expression frequency and intensity of the B7-H3 epitope recognized by the B7-H3-specific mAb 376.96 used to make the B7-H3 CAR T cells, on PCa cells and PCSCs present in tissue samples from mPCa patients.

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.

Specific aim 1: Optimize the strategy of using low-dose IR to reprogram/activate B7-H3 CAR T cells and phenotypically and functionally characterize the reprogrammed CAR T cells.

Specific aim synopsis

In the initial year of the funding period, our focus was on titrating the doses and determining the post-irradiation (IR) duration for reprogramming and activating B7-H3 CAR T cells. We also conducted a thorough phenotypic and functional characterization of the reprogrammed CAR T cells using the optimized IR dose and duration. The progress report from our last progress report demonstrated significant success in completing these tasks.

However, due to various circumstances, such as Dr. Yufeng Wang's transition to graduation from his Ph.D. program, Dr. Ruochuan Sun's health issues, and David L. Drum's unexpected early admission to medical school, we encountered a gap between experienced and new, less-experienced lab members. We've taken proactive steps to train new team members, specifically Feng Chen and Dr. Cristina Martin, starting from the basics of CAR T cell production. I'm pleased to report that Ms. Chen has successfully overcome technical challenges and has mastered the techniques required to produce high-quality CAR T cells. She is now fully prepared to investigate the phenotypes of early stem T cells and memory CAR T cells reprogrammed through ex vivo IR.

Given the rapidly evolving nature of science, we strongly believe that conducting RNA-sequencing analysis on ex vivo IR-reprogrammed B7-H3 CAR T cells in comparison to the original non-IR-reprogrammed B7-H3 CAR T cells will yield valuable insights into the changes induced by low-dose IR on gene expression profiles within CAR T cells. This additional level of analysis promises to significantly enhance our understanding of the effects of IR on these crucial cellular reprogramming.

Aim2: Determine efficacy and safety of low-dose IR *ex vivo* reprogrammed/activated B7-H3 CAR T cells derived from mPCa patients to prolong survival of mice bearing human mHSPC or mCRPC cell-derived orthotopic xenografts.

Specific aim synopsis

In the first year of this project, we achieved significant milestones. We successfully generated and reprogrammed CAR T cells using irradiation (IR) from both normal donors and PBMCs of patients. Subsequently, we conducted pivotal experiments with these CAR T cells. Firstly, we observed that IR-reprogrammed CAR T cells, whether derived from normal donors or patients with CRPC, exhibited enhanced efficacy in treating orthotopic PCa xenografts in mice. Additionally, we noted a higher infiltration of CAR T cells in tumors treated with IR-reprogrammed B7-H3 CAR T cells compared to those treated with non-IR-reprogrammed counterparts.

Over the second year, our primary focus was on the collection and isolation of peripheral blood mononuclear cells (PBMCs). We successfully banked PBMCs from 4 metastatic HSPC patients (a challenging task due to the low number of available patients and difficulty in obtaining blood samples) and 10 mCRPC patients. We are actively continuing these efforts to collect and isolate PBMCs from additional patients. This effort is of paramount importance for two reasons: firstly, our previous experience over the past two years revealed that PBMCs derived from 18-20 mL of fresh blood may not yield sufficient CAR T cells (it varies among patients' PBMCs) for our proposed mouse experiments. Secondly, evaluating the efficacy of CAR T cells sourced from a larger patient pool in metastatic HSPC and mCRPC mouse xenograft models holds the potential to offer invaluable clinically relevant insights into the potentially similar or diverse therapeutic responses associated with this approach.

Aim3: Assess expression frequency and intensity of the B7-H3 epitope recognized by the B7-H3-specific mAb 376.96 used to make the B7-H3 CAR T cells, on PCa cells and PCSCs present in tissue samples from mPCa patients.

Specific aim synopsis

Over the past year, our ongoing efforts have focused on the collection of both primary and metastatic prostate cancer tissues. Dr. Chin-Lee Wu, an esteemed PCa pathologist at MGH and a co-investigator on this grant, strongly recommended that we validate our homemade anti-B7-H3 monoclonal

antibody (mAb) 376.96 on formalin-fixed and paraffin-embedded (FFPE) PCa tissues before utilizing the precious and hard-to-obtain frozen PCa specimens.

In close collaboration with the Pathology Core at MGH, we conducted a comprehensive series of experiments involving various antigen retrieval methods, antibody titrations, and a variety of cancerous tissues. After approximately six months of dedicated work, it was determined that mAb 376.96 did not yield satisfactory staining results on FFPE PCa slides. Notably, we discovered that the commercial B7-H3 antibody (B7-H3 (D9M2L) XP® Rabbit mAb #14058) from Cell Signaling demonstrated staining on not only FFPE PCa tissues but also normal prostate tissues (**Figure 1**).

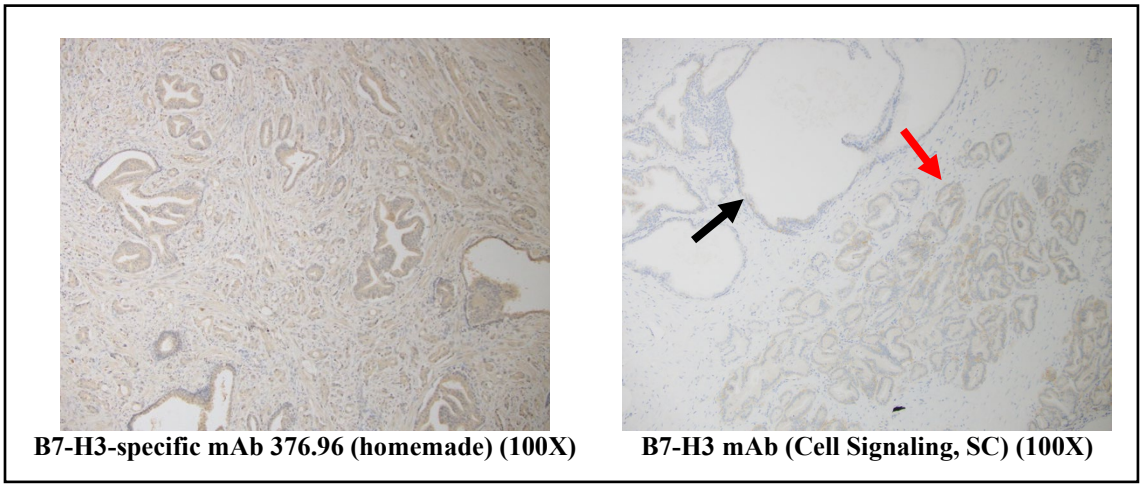

Fig1. Lack of immunohistochemical staining of formalin-fixed and paraffin-embedded (FFPE) primary PCa tissues with B7-H3-specific mAb 376.96. Various antigen retrieval methods and antibody titrations were used to establish IHC staining of B7-H3 expression by mAb 376.96 on primary PCa or kidney cancer FFPE tissues obtained from different patients. No IHC staining was detected (left). The representative staining with PCa is shown. The rabbit anti-B7-H3 mAb was used as a positive control. However, it stained both cancer and normal prostate glands (right). The **red arrow** indicates cancer cells and the **black arrow** indicates normal glandular cells.

In light of these findings, we collectively agreed to advance the project by establishing an immunohistochemical staining (IHC) protocol tailored for frozen tissue slides within my lab. We initiated our practice runs using slides derived from human PCa cell line pellets with mAb 376.96. As the IHC method proved successful, we progressed to staining frozen PCa tissue slides obtained from two patients using mAb 376.96. Through meticulous antibody titrations and optimization of the

staining process, this week, we achieved successful and specific staining of PCa tissues from 3 patients, as demonstrated by the lack of staining observed in adjacent normal prostate tissues (**Figure 2**).

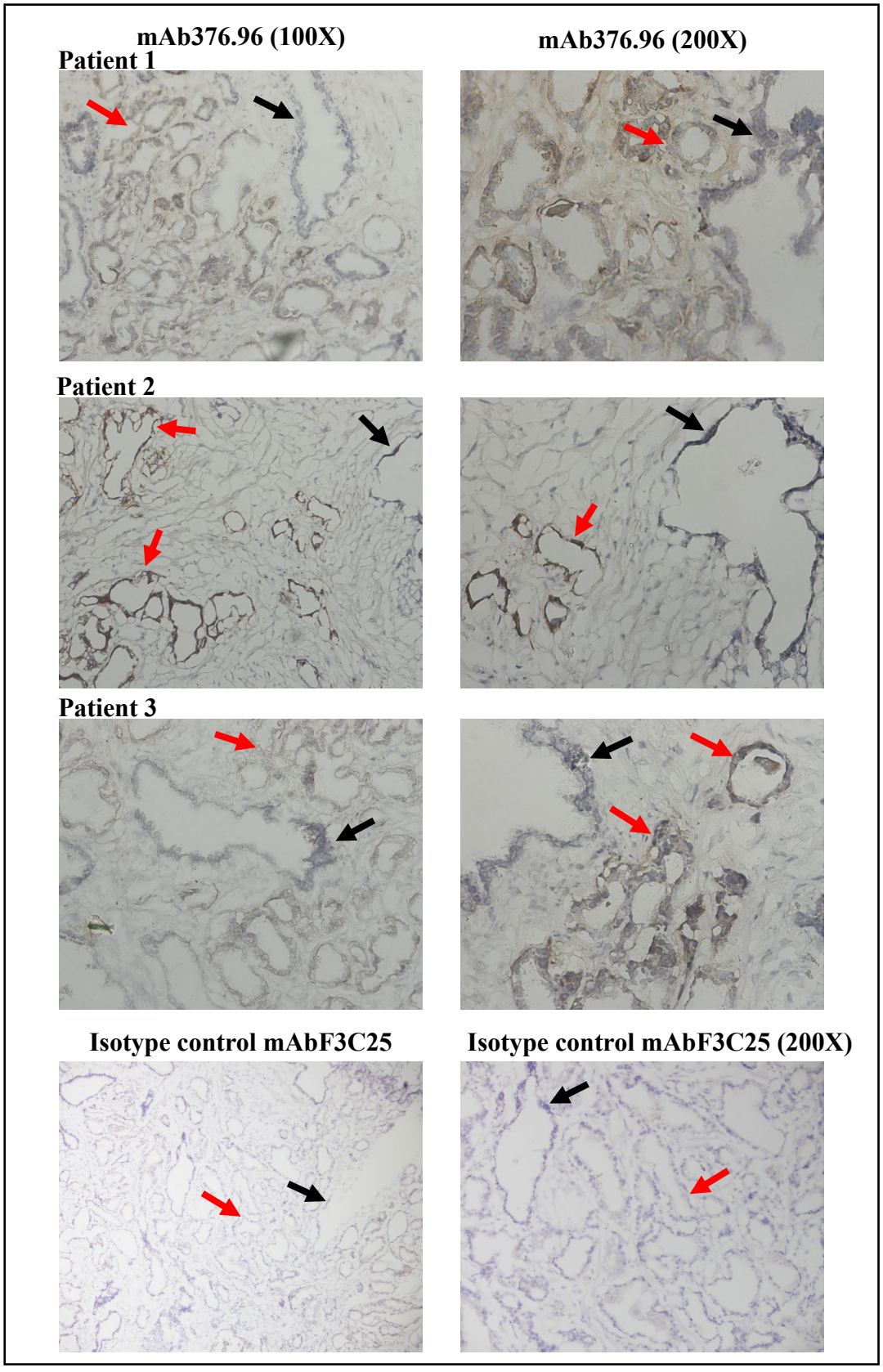

Fig2. Immunohistochemical staining of frozen primary PCa tissue with B7-H3-specific mAb 376.96. B7-H3 expression was detected by mAb 376.96 (0.75µg/ml) and avidin-biotin complex kits (Vector laboratories) on 3/3 primary PCa tissues obtained from different patients while adjacent cancer free tissue was negative for B7-H3 expression. The staining was specific since the same PCa tissue was stained simultaneously with an isotype matched control mAb F3C25 as the primary antibody was negative. The **red arrow** indicates cancer cells, and the **black arrow** indicates adjacent normal glandular cells.

With this milestone achieved, we are now prepared to transition to the next phase of the project, which entails staining the valuable and hard-to-obtain primary and metastatic PCa tissues with mAb 376.96—the same antibody utilized in generating the CAR T cells.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr. X. Wang and her group at MGH: have trained two graduate students; one was recently awarded Ph.D., and one pre-medical school student was accepted by a medical school in CA, USA. Now with the support of this grant, one Ph.D student and a post-doctoral fellow have been trained in the lab.

Dr. Xin Gao at MGH

Dr. Chin-Lee Wu at MGH

Dr. Joseph Schwab at Cedars Sinai Medical Center (We continue to collaborate)

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

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

We plan to continue the project as proposed based on our current progress.

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).

More results are to be developed and published. The results obtained thus far look promising to impact upcoming clinical trial design.

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.

More results are to be developed and published. The results obtained thus far look promising to impact upcoming clinical trial design, such as for other cancer types including leukemia.

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

No significant changes were made.

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.

There is a slight delay in progressing aims 1 and 2, due to personal transition and required training. This kind of challenge is present from time to time in almost every laboratory. While we have people who could do the proposed work, we will do it in a timely manner.

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

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

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:** *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. Ventin M, Cattaneo G, Maggs L, Jia J, Arya S, Ferrone S, **Wang X** and Ferrone CR . B7-H3-targeted CAR T cell activity is enhanced by radiotherapy in solid cancers. Front Oncol 2023. July 7;13:1193963. PMID: 37483496.

2.Liu Z, Liu W, Wang W, Ma Y, Wang Y, Drum D, Cai J, Blevins H, Lee E, Shah S, Fisher PB, **Wang X**, Fang X*, Guo C*, Wang X* (*corresponding authors). CPT1A-mediated fatty acid oxidation confers cancer cell resistance to immune-mediated cytolytic killing. PNAS. 2023. Sep 26;120(39). PMID: 37722058.

3. Wang Y, Drum DL, Sun R, Zhang Y, Chen F, Sun F, Dal E, Yu L, Jia J, Arya S, Jia L, Fan S, Isakoff SJ, Kehlmann AM, Dotti G, Liu F, Zheng H, Ferrone CR, Taghian AG, DeLeo AB, Ventin M, Cattaneo G, Li Y, Jounaidi Y, Huang P, Maccalli C, Zhang H, Wang C ,Yang J, Boland GM, Sadreyev RI, Wong L, Ferrone S, **Wang X**. Stressed target cancer cells drive nongenetic reprogramming of CAR T cells and solid tumor microenvironment. Nat. Commun. 2023 Sep 14(1): 1-17. PMID: 36865255.

4. Ventin M, Cattaneo G, Maggs L, Arya S, **Wang X**, Ferrone CR. Implications of High Tumor Burden on CAR T Cell Immunotherapy. A Review. JAMA Onc. Dec 2023. In press.

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.*

Nothing to report

- **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.*

Nothing to report

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: Feng Chen M. S.

Project Role Graduate student

Nearest person month worked: *12*

Contribution to Project: Ms. Chen has collected all PMBCs and learned how to make CAR T cells.

Name: Fengfei Sun MD, PhD

Project Role Post-doctoral fellow

Nearest person month worked: *8*

Contribution to Project: Dr. Sun performed the IHC staining work.

Name: Cristina Martin, PhD

Project Role Post-doctoral fellow

Nearest person month worked: *4*

Contribution to Project: Dr. Martin was under training to produce CAR T cells.

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.

Dr Schwab has moved recently from MGH to Cedars Sinai Medical Center. Our collaboration cotunnites.

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.*

None

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: *N/A*

9. APPENDICES: *N/A*