AWARD NUMBER: W81XWH-21-1-0580

TITLE: Engineering a Biological Agent to Boost Brain Tumor Immunity

PRINCIPAL INVESTIGATOR: Hui Zong

CONTRACTING ORGANIZATION: Rector and Visitors of the University of Virginia
Charlottesville, VA

REPORT DATE: August 2022

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2022	Annual	01Aug2021-31Jul2022

4. TITLE AND SUBTITLE

Engineering a Biological Agent to Boost Brain Tumor Immunity

5a. CONTRACT NUMBER W81XWH-21-1-0580

5b. GRANT NUMBER GRANT13193408

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Hui Zong, Sarah Ewald, Tajie Harris

E-Mail: hz9s@virginia.edu; se2s@virginia.edu; thh9t@virginia.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Rector & Visitors of the University of Virginia
1001 N Emmett St.
Charlottesville, VA 22903-4833

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

Nearly 65% of brain cancer patients die within 5 years of diagnosis, a statistic that has not improved over the last 30 years. Even survivors suffer from debilitating defects in brain functions. While immunotherapy has been overwhelmingly successful in treating many types of tumors, clinical trials in brain tumors has not been successful, most likely due to two unique situations in brain tumors: first, the key anti-tumor immune cells called T cells cannot enter the brain, and second, even if they enter, the environmental factors in brain tumors are not supportive for T cell activities. In this grant application, we propose to harness a brain-tropic biological agent to solve both problems, by attracting T cells into brain tumors while creating a supportive brain environment for T cells. We also plan to engineer the biological agent to ensure its safety without compromising its effectiveness in boosting immunity. The knowledge gained here will inform us how the brain immune system can be activated to mount anti-tumor responses, which could lead to a paradigm shift in therapeutic strategies for brain cancer. Upon the completion of this project, we envision follow-up studies to test the combinatorial treatment between our agent and the current immunotherapy agents to determine if the combination will vastly increase the immune responses against brain tumors, which will take around two years. If successful, we should then move into clinical trials to assess the safety and efficacy of this treatment strategy in brain tumor patients, expecting not only to fundamentally improve survival but also to significantly enhance life quality.

15. SUBJECT TERMS

Brain tumor, tumor microenvironment, immunotherapy, *Toxoplasma gondii*

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRDC
a. REPORT	b. ABSTRACT	c. THIS PAGE	Unclassified	9	19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified			

TABLE OF CONTENTS

Page

1. **Introduction**……………………………………………………………………4
2. **Keywords** …………………………………………………………………...5
3. **Accomplishments**………………………………………………………….6
4. **Impact**…………………………………………………………………………..9
5. **Changes/Problems**…………………………………………………………9
6. **Products**…………………………………………………………………….....9
7. **Participants & Other Collaborating Organizations**……………………………..9
8. **Special Reporting Requirements**………………………………………………9
9. **Appendices**………………………………………………………………….9

1. INTRODUCTION

Background: Our proposed research addresses two FY20 PRCRP Topic Areas: brain cancer (primary), and pediatric brain tumors (secondary). Nearly 65% of brain cancer patients die within 5 years of diagnosis, a statistic that has not improved over the last 30 years. Even survivors suffer from debilitating defects in brain functions. While immunotherapy that uses checkpoint-blockade to sustain anti-tumor activities of T cells has been overwhelmingly successful in treating many types of solid tumors, the unique biology of the brain poses heightened challenges for its effectiveness in brain tumor treatment. On one hand, the 'immune-privileged' brain lacks T cells for immunotherapy to act on. Consistent with previous reports using patient samples, our work in mouse models rarely found T cells in both malignant glioma and pediatric brain tumor medulloblastoma [Yao et al, 2020 *Cell*], showing that one must first attract T cells into the tumor mass for checkpoint-blockade immunotherapy to be effective. On the other hand, we found that brain-resident cells (microglia and astrocytes) are both in an immune-suppressive state in brain tumors, which must be reverted to support T cells once they reach the brain. While cytokine administration has been shown to improve the immune environment in non-brain tumors, it has been nearly impossible to elicit sustained high level of cytokines in the brain through physical means. Therefore, we believe that it is imperative to adopt brain-immune modulating biological agents to fundamentally improve this situation.

Objective: To harness the brain-tropic microbe *Toxoplasma gondii* to convert the "immune-cold" microenvironment in brain tumors into a tumoricidal immune environment.

Specific Aims and Study Design:
1) Characterize how *Toxoplasma* infection promotes the infiltration and activation of peripheral T cells in the tumor mass. Our preliminary data indicated that *Toxoplasma* infection is sufficient to induce T cell recruitment into the tumor mass, a promising lead that needs detailed functional studies. Here we will analyze the functions of infiltrated T cells in three complementary aspects: first, we will use flow cytometry to determine if they are effector (CD4, CD8) or regulatory (FoxP3) T cells, if they are active (CD44, CD62L) or exhausted (PD-1), and what is the effector capacity of the T cells based on cytokine production (IFN-γ and TNF-α) or lytic granules (granzyme B); second, we will use immunofluorescence followed by microscopic imaging to determine the spatial distribution and proliferation status of the T cell population within the tumor mass; and finally, we will evaluate the impact of *Toxoplasma*-induced immunity on tumor cell proliferation (Ki67) and cell death (cleaved caspase-3). Together, these studies will elucidate if infiltrated T cells are poised to eliminate tumor cells.
2) Determine whether *Toxoplasma* infection is sufficient to activate brain-resident immune cells in the tumor mass. The activation of brain-resident immune system is critical to support a productive immune response upon the infiltration of T cells. While literature report indicated that glioma and medulloblastoma establishes an immune-suppressive environment, our preliminary data shows that *Toxoplasma* infection is sufficient to induce high-level expression of multiple inflammatory cytokines (IL-12b, and IL-1b) in brain tumors. However, it is unclear whether the *Toxoplasma*-induced inflammatory response is sufficient to completely overcome tumor-derived immune-suppressive signals. To address this question, here we will assess pro- and anti-inflammatory phenotypes of brain-resident microglia and astrocytes, to determine if *Toxoplasma* infection could overcome the immune-suppressive TME to induce brain inflammation to recruit then sustain the activities of T cells.
3) Engineer safer *Toxoplasma* mutant strains to improve the translational potential of *Toxoplasma*. To eliminate a potential risk due to the persistent infection of *Toxoplasma*, we will engineer mutant *Toxoplasma* strains that cannot form latent cysts, and that can be induced to self-destruct on demand. These strains will be tested for their ability to induce immune activities in the tumor mass.

Innovation: The ineffective clinical trials of immunotherapy in brain tumors have been truly disappointing since conventional therapies only extend the lives of patients by a few months. Our proposed studies would be the first ever attempt to harness a non-viral biological agent to activate the immune system in brain tumors. Furthermore, engineering *Toxoplasma* to make it safer would also be the first line of investigation in this area.

Military Relevance: This proposal addresses the Military Health Focus area "mission readiness," specifically "gaps in cancer prevention, early detection/diagnosis, prognosis, and/or treatment that may impact mission readiness and the health and well-being of military members, Veterans, their beneficiaries, and the general public." By harnessing *Toxoplasma* to convert the "immune-cold" microenvironment into a tumoricidal immune environment, our study carries great promise to fundamentally improve the treatment of brain tumors.

2. KEYWORDS

Brain tumor

Tumor microenvironment

Immunotherapy

Toxoplasma gondii

3. ACCOMPLISHMENTS

A. Summary based on SOW

STATEMENT OF WORK
PROJECT START DATE: August 1, 2021

Specific Aim 1: Characterize how *Toxoplasma* infection promotes the infiltration and activation of peripheral T cells in the tumor	**Timeline (Months)**	**Site 1**	**Percent complete**
Major Task 1: Produce experimental mice			
Subtask 1: USAMRMC Animal Care and Use regulatory review	1-3	Dr. Zong	100%
Subtask 2: Expand two mouse stock lines for the project	4-6	Dr. Zong	100%
Subtask 3: Maintain 10 breeding pairs to produce medulloblastoma mice for experimentation	7-36	Dr. Zong	Ongoing
Milestone(s) Achieved: Obtain USAMRMC regulatory approval and maintain steady supply of medulloblastoma mice for analysis			Reached
Major Task 2: Flow analysis of T cell subtypes and functional states			
Subtask 1: Set up breeding pairs after mouse stock lines reach the sexual maturity, genotype, wean, and age them to 60 days of age	7-9	Dr. Zong	100%
Subtask 2: Infect a group of medulloblastoma mice with *Toxoplasma* and monitor them for 21 days, along with a group of control, uninfected medulloblastoma mice	10	Dr. Ewald	100%
Subtask 3: Freshly harvest tumors from brains, dissociate into single cells, and use flow cytometry to determine T cell number, composition, and functional states at 21-day-post-infection (dpi)	11-12	Drs. Zong, Harris, Ewald	100%
Subtask 4: Repeat the experiment at later dpi(s) to determine how T cell number, composition, and functional states shift along the time	13-18	Drs. Zong, Harris, Ewald	Not started
Subtask 5: Determine if the *Tgnst1*-null *Toxoplasma* strain elicits strong T cell recruitment and activation	13-18	Drs. Zong, Harris, Ewald	Not started
Milestone(s) Achieved: • Full characterization of T cells recruited into the tumor microenvironment by *Toxoplasma* and the sustainability of their activation status • Full understanding on the pros and cons of *Tgnst1*-null *Toxoplasma* strain over the WT strain, for both T cell biology and general mouse health			
Major Task 3: Use immunofluorescent staining to analyze T cell subtypes and functional states, and the impact on tumor cells			
Subtask 1: Infect a group of medulloblastoma mice with *Toxoplasma* and monitor them for 21 days, along with a group of control, uninfected medulloblastoma mice (after we gain information from the initial T cell analysis with flow cytometry)	13	Dr. Ewald	100%
Subtask 2: Harvest tumor brains at 21-dpi after perfusion with fixatives, OCT embedding, cryo-sectioning, and immunostaining and confocal imaging to determine the spatial distribution of T cells and their subtypes and functional states. Also determine the proliferative rate and death rate of GFP+ tumor cells.	14-15	Drs. Zong, Harris, Ewald	20%
Subtask 3: Repeat the experiment at later dpi(s) to determine how spatial distribution, composition, and functional states of T cells shift along the time, and the impact on tumor cells.	16-21	Drs. Zong, Harris, Ewald	Not started

Subtask 4: Determine if the *Tgnst1*-null *Toxoplasma* strain elicits stronger T cell recruitment and activation.	16-21	Drs. Zong, Harris, Ewald	Not started
Milestone(s) Achieved: • Full characterization of T cells recruited into the tumor microenvironment by *Toxoplasma*, with high spatial resolution along the time • Clear readouts of the impact on tumor cells, with spatial and temporal correlation with T cell infiltration			
Specific Aim 2: Determine whether *Toxoplasma* infection is sufficient to activate brain-resident immune cells in the tumor			
Major Task 1: Determine the responses of brain resident cells in the tumor microenvironment			
Subtask 1: Infect a group of medulloblastoma mice with *Toxoplasma* and monitor them for 10 days, along with a group of control mice (after we gain information from the initial T cell analysis)	13	Dr. Ewald	Not started
Subtask 2: Harvest tumor brains at 10-dpi: some fresh frozen for qRT-PCR and Luminex analysis to determine chemokine levels; others perfusion with fixatives, cryo-sectioning, and immunostaining and confocal imaging to determine the spatial distribution of astrocytes, microglia/macrophages and their functional states	14-15	Drs. Zong, Harris, Ewald	Not started
Subtask 3: Repeat the experiment at 21-dpi to determine the impact of infiltrated T cells on brain resident cells in the TME	16-18	Drs. Zong, Harris, Ewald	Not started
Subtask 4: Repeat the experiment at even later dpi, and with the *Tgnst1*-null *Toxoplasma* strain to determine if these changes could boost a stronger inflammatory state in brain resident cells	19-24	Drs. Zong, Harris, Ewald	Not started
Milestone(s) Achieved: full characterization of the inflammatory states of brain resident cells: how they recruit T cells, and how they can be further activated by infiltrated T cells			
Specific Aim 3: Engineer safer *Toxoplasma* mutant strains to improve the translational potential			
Major Task 1: Molecular cloning and characterization of mutant strains of *Toxoplasma*			
Subtask 1: Molecular cloning to engineer mutant strains of *Toxoplasma* that can self-destruct upon Tetracycline treatment, after we get well informed by data from experiments in Aims 1 and 2	17-18	Dr. Ewald	Not started
Subtask 2: Validate efficiency of Tetracycline-mediated clearance of engineered *Toxoplasma* strain in vivo	19-20	Dr. Ewald	Not started
Subtask 3: Characterize both the new strain and the BFD1 mutant strain in WT mice for their immune-modulating activities	21-24	Dr. Ewald, Harris	Not started
Major Task 2: Determine the impact on T cells, brain resident cells, and tumor cells by the mutant strains of *Toxoplasma*			
Subtask 1: Infect a group of medulloblastoma mice with mutant strains of *Toxoplasma* along with a group of control, uninfected medulloblastoma mice	25	Dr. Ewald	Not started
Subtask 2: Harvest the brains and determine the impact of mutant strain infection on T cells, brain resident cells, and tumor cells	26-30	Dr. Zong, Ewald, Harris	Not started
Major Task 3: Comprehensive data analysis, and manuscript writing and submission	31-36	Dr. Zong, Ewald, Harris	Not started

B. Detailed report

In the first 3 months of this project, we first finished the task of getting IACUC protocol approval at local institution, then getting ACURO approval. Upon the approval for animal work, we expanded our mouse stocks and produced experimental mice in the follow 3 months. In the remaining of 6 months, we performed a few rounds of experiments to use flow cytometry to compare T cell number, composition, and functional states upon *Toxoplasma gondii* challenge.

First, we compare the number of T cells in WT mice vs. medulloblastoma-bearing mice in the presence or absence of *Toxoplasma gondii* challenge, and made two exciting findings (**Fig. 1**). While T cells were virtually absent in the brains of both WT and medulloblastoma-bearing mice in the control group, in the *Toxoplasma gondii* challenged group, the number of brain-infiltrating T cells increased significantly. Importantly, the number of T cells in the tumor mass was similar to that of either WT cerebellum or tumor-free regions in the cerebellum of medulloblastoma-bearing mice, suggesting that *Toxoplasma gondii* challenge was able to overcome the immune suppressive tumor microenvironment, as reported by our prior studies and other labs.

Second, we analyzed the composition of T cells: are they CD4+ helper T cells, or CD8+ cytotoxic T cells, or FoxP3+CD4+ regulatory T cells (Tregs) upon *Toxoplasma gondii* challenge? Our flow analysis revealed that most infiltrated T cells are CD4+ and CD8+ T cells, which are similar in number (**Fig. 2A,B**). In stark contrast, the number of brain-infiltrated Tregs was very small, consisting of less than 2% of total T cells (**Fig. 2C**).

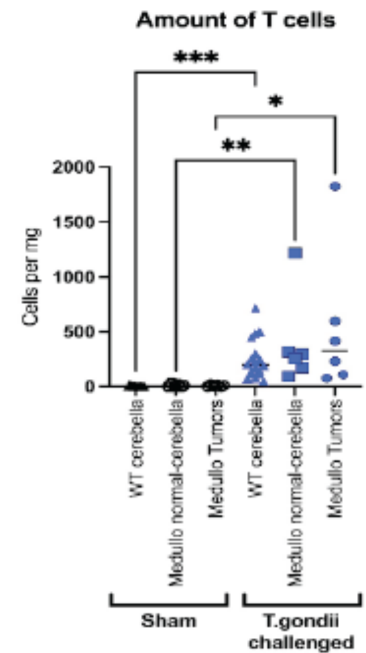

Figure 1. The number of T cells increased significantly in both WT and medolloblastoma-bearing brains upon *Toxoplasma* challenge.

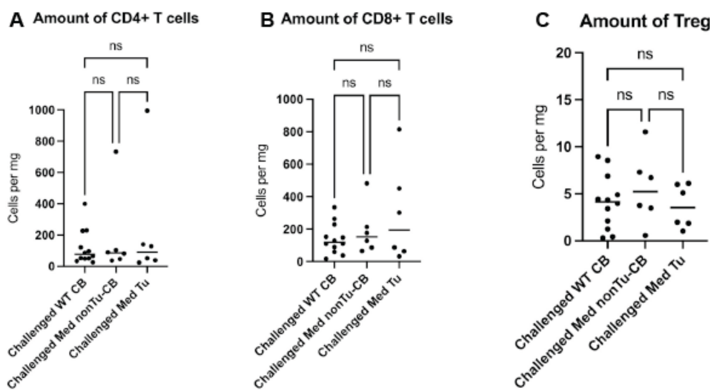

Figure 2. Infiltrated T cells are mainly CD4+ or CD8+ cells but rarely Tregs.

Third, we assessed the activation status of CD4+ and CD8+ cells with flow cytometry, and found that they were $CD44^{hi}$, $CD62L^{lo}$ (**Fig. 3**), suggesting that they are activated T cells.

Finally, we carefully analyzed the proportion of T cells that entered brain parenchyma rather than remained in blood vessels, using immuno-fluorescent staining. Our result demonstrated that, while control brains were free of T cells, in *Toxoplasma gondii* challenged brains, both WT and medulloblastoma-bearing, ~30% T cells reside in the brain parenchyma (**Fig. 4**).

Taken together, the promising data from our first year's studies indicated that *Toxoplasma gondii* challenge overcame the immune suppressive nature of tumor microenvironment to not only successfully shuttled T cells into the brain parenchyma but also maintained their activation status.

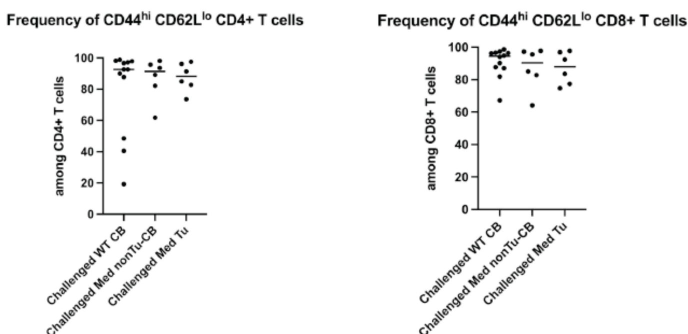

Figure 3. CD4+ and CD8+T cells both express activation markers.

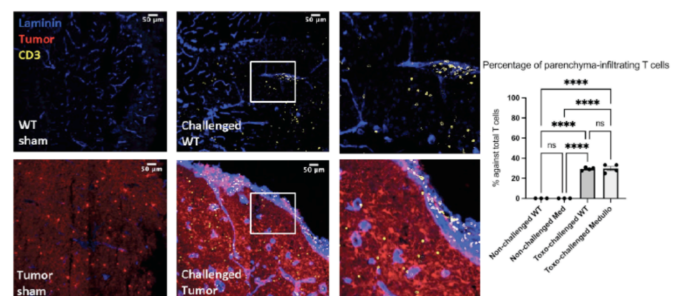

Figure 4. T cells entered the brain parachyma of medulloblastoma.

4. IMPACT
The results from our first year's studies firmly demonstrated the capability of *Toxoplasma gondii* challenge to enable T cell infiltration to previously impenetrable brain tumor parachyma. Even more importantly, these T cells are effector T cells rather than Tregs, and express activation markers, suggesting that they could carry anti-tumor activities. Therefore, we are very excited to continue our studies in the coming year to understanding how *Toxoplasma gondii* challenge alters the tumor microenvironment from immune-suppressive to immune-supportive, providing critical information for formulating future immunotherapy strategies for brain tumors.

5. CHANGES/PROBLEMS
N/A

6. PRODUCTS
N/A

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
Hui Zong (PI, 1.8 cal mo) obtained IACUC/ACURO approval, oversaw all aspects of the research, coordinated efforts with Drs. Ewald and Harris and their labs, critically evaluated data, and wrote the annual report.

Sarah Ewald (co-PI, 1.2 cal mo) contributed her expertise in *Toxoplasma* biology to guide two graduate students for all the experiments along with Drs. Zong and Harris.

Tajie Harris (co-PI, 1.2 cal mo) contributed her expertise in brain immunology to guide Yen Nguyen for all the experiments along with Drs. Zong.

Yen Nguyen (graduate student, Zong lab, 12 cal mo) managed mouse colony to produce tumor-bearing mice, then coordinated with Xiaoyu Zhao in Ewald lab for *Toxoplasma* injection and tissue harvest for flow and histological analysis.

Xiaoyu Zhao (graduate student, Ewald lab, 6 cal mo) produced *Toxoplasma* strains for mouse injection in this project, measured parasite burden and localization in the brain.

8. SPECIAL REPORTING REQUIREMENTS
N/A

9. APPENDICES
N/A