AWARD NUMBER: W81XWH-18-1-0418

TITLE: Altering the Tumor Microenvironment to Augment Neuroblastoma Immunotherapy

PRINCIPAL INVESTIGATOR: Shahab Asgharzadeh, MD

CONTRACTING ORGANIZATION: The Children's Hospital of Los Angeles
Los Angeles, CA 90027-6062

REPORT DATE: August 2019

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
August 2019	Annual	1 Aug 2018 - 31 Jul 2019

4. TITLE AND SUBTITLE
Altering the tumor microenvironment to augment neuroblastoma immunotherapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0418

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Shahab Azgharzadeh, MD

E-Mail: SAsgharzadeh@chla.usc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Children's Hospital of Los Angeles
4650 W Sunset Boulevard
Los Angeles, CA
90027-6062

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel 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

We are working to combine precision immunomodulatory therapy with chemoimmunotherapy to improve outcomes for high-risk neuroblastoma, and create a paradigm shift in our therapeutic approach to this highly lethal disease. Current treatment includes chemotherapy, surgery, myeloablative transplant, radiotherapy and then immunotherapy at the time of minimal residual disease. Our goals here are to leverage immunotherapy efficacy by tailoring specific immunomodulators to specific immunosuppressive TMEs, which are strongly impacted by the specific oncogenes activated in the cancer. We are studying two transgenic murine neuroblastoma models driven by distinct oncogenes (MYCN and T-Ag). We are using CyTOF and flow-cytometry, supplemented by transcriptional and cytokine profiles, to characterize the tumor TME. In year 1 we have shown that MYCN-driven neuroblastomas sculpt an immunosuppressive TME, but that the use of DFMO (an immunomodulator) leads to marked changes in the proportions and activity states of tumor-infiltrating leukocytes. We see markedly enhanced numbers of NK cells, and they express higher levels of activating-type surface receptors (in contrast to inhibitory-type receptors). Further, tumor cells in DFMO treated animals show upregulated expression of surface NK cell ligands. Whether this is a major contributor to the anti-tumor effect of DFMO is not yet known. It is possible these NK cells are poised for engagement and tumor cell killing but are inhibited, and a possible inhibitory axis to explore includes the TGFb axis. The reason this is important is because if that were the case, then combining DFMO with a TGFb inhibitor might significantly augment the anti-tumor activity of the regimen. Parallel work in the alternative model is proceeding but is delayed relative to the MYCN model.

15. SUBJECT TERMS
Neuroblastoma, tumor microenvironment, tumor infiltrating leukocytes (TILs), immunomodulation

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

Table of Contents

INTRODUCTION:

Our guiding hypothesis is that the solid tumor TME is a byproduct of the specific oncogenes driving the cancer, and neuroblastomas with different driver mutations will have distinct immunosuppressive TMEs. Indeed, it has been demonstrated in conditional tumor models that oncogene-specific immune remodeling is required for tumor progression. This heterogeneity complicates the integration of immunotherapy into the clinic but provides an opportunity to decode specific oncogene-TME phenotypes in solid tumors, enabling therapeutic intervention. Indeed, it is a natural extension of precision medicine from an isolated focus on cancer cell vulnerabilities to an expanded focus on the immune ecosystem of a cancer. We use complementary transgenic models of neuroblastoma, representing distinct common oncogenic pathways, to dissect the immune compartment remodeling accompanying neuroblastoma progression and the response to chemotherapy (Aim 1). We seek both shared and unique regulatory nodes that are amenable to immunomodulatory drug targeting, and these will be tested in our tumor models (Aim 2). In parallel, ongoing and recently completed neuroblastoma clinical trial samples will be studied for changes in these same immune compartment parameters (Aim 3) to inform subsequent trial design.

KEYWORDS:

Pediatric cancer, neuroblastoma, tumor microenvironment (TME), tumor-infiltrating leukocytes (TILs), immunomodulation, immunotherapy, *MYCN* oncogene, immunogenic chemotherapy

ACCOMPLISHMENTS:

The following tasks of our research program are presented as outlined in our SOW. Items targeted for work effort in YR1 (months 1-12) are included with an update on their status:

Major Task 1 (Aim 1). Identify the circulating and tumor-infiltrating immune cells (cellular) and regulatory cytokines (soluble) accompanying oncogene-initiated tumor progression, and the impact of chemotherapy on these processes.

Subtask 1.1 Characterize the TME in *TH-MYCN* model tumors during *MYCN*-driven progression.

1.1a. We obtained regulatory approval for these animal studies. The *TH-MYCN* mouse model work was approved by the CHOP IACUC on 2/8/2019, and by ACURO on 2/27/2019. From that point we initiated our mouse work at CHOP (Hogarty/CHOP).

1.1b-1.1e. We bred *TH-MYCN* mice from +/- x +/- crosses: 20% are *TH-MYCN* +/+ suitable for studies with 10% attrition; we obtained ~50 +/+ mice for experiments: 10 for CyTOF; 40 for flow-cytometry profiling.

1.1b. Profile functional states and abundance of TILs using murine CyTOF mass cytometry at two time-points, n~5 tumors each. We worked with the CHLA group to optimize a CyTOF panel for this work, using discoveries from our flow-based assay to refine the cell types to target.

The approach to the use of CyTOF and Flow Cytometry analyses was modeled after two-tier testing, with the first tier (CyTOF) encompassing sensitive but broad immunophenotypic testing, followed by the second tier (Flow Cytometry) to probe specific subsets in greater detail in greater numbers of tumors. We held monthly conference calls to identify antigens to target in the CyTOF panels. We constructed this to detect changes in **effector lymphocytes** (T cells, T_{REG} cells, NK cells, NKT cells, and B cells (assessed by markers such as CD45, CD3, TCRb, CD4 vs CD8, NK1.1, NKp46, CD25 and FoxP3) and their functions (cytokine production [IFNg, TNFa], activation and skewing [CD69, phospho-ERK, Tbet, Eomes], cytolytic activity [perforin, granzyme B], naïve vs memory markers [CD62L, CD44], proliferation [Ki-67], and checkpoint receptors [CTLA4, PD1, LAG3, Tim3]), **myeloid cells** (dendritic cells, tumor-associated macrophages, and myeloid derived suppressor cells [as subsetted by staining for CD11c, CD16/32, F4/80, CD11b, Ly6C and Ly6G] and their functions [PDL1, iNOS, I-A/I-E, CD206, CD170]), and **tumor cells** (hematopoietic lineage marker negative, GD2, LAP, PHOX2B, FAP, CD140a/b). These panels were generated using antibodies that would work in diverse mouse backgrounds (including those of the *TH-MYCN*$^{+/+}$ and the NB-Tag transgenics).

Given that the CyToF workflow is time-consuming and resource intensive, following the initial identification of altered cell populations, we confirm and extend findings via second tier flow cytometry panels. As an illustration, we preliminarily identified changes in intra-tumoral NK cell frequencies

associated with the administration of DFMO to *TH-MYCN*$^{+/+}$ transgenic mice. To better understand these changes, we subsequently designed multiparameter flow cytometric panels to examine the activating (NKG2D, Ly49H, Ly49D) and inhibitory receptors (CD94-NKG2A, Ly49A) of NK cells and the corresponding ligands for these receptors expressed by tumor cells (MHC class I, Rae1g, Rae1d). These flow cytometry panels allowed us to easily test dozens of mice in a cost-effective manner.See **Table 1**.

We shipped our first batch of samples to CHLA for CyTOF testing (August 2019) and we will compare results with shared antibodies included in our flow-based panel. Hogarty/CHOP- mouse work; Hogarty/ Asgharzadeh/Bassiri- CyTOF panel development; CHLA-CyTOF assays; months 5-9 estimated, revised estimate months 10-14 due to delay in animal approvals.

1.1c. TIL flow-cytometry profiling. Up to 3-4 time-points, n~10-15 tumors each. We optimized a series of flow cytometry panels for TIL detection in our tumors and this initial characterization is complete. Samples for subsequent gene expression, IHC, and PB studies have been collected on this same cohort. Hogarty- mouse work; Bassiri- cytometry; months 6-12. TIL profiles for untreated tumors are provided below in **Figure 1**, relative to TIL profiles for DFMO treated mice.

1.1d. Gene expression profiling (nanoString, nCounter) of tumors. Hogarty- mouse work; Bassiri- nanoString. At initiation (t=1) and terminal progression (t=2), n~5 tumors each; months 6-12. Samples for gene expression have been collected on this same cohort from 1.1c.

1.1e. Confirm cytokines and signaling molecules in PB using Luminex bead-array detection. Hogarty. At initiation (t=1) and terminal progression (t=2), n~6 samples each; months 6-12. Samples for subsequent PB studies have been collected on this same cohort from 1.1c.

1.1d and 1.1e studies will be completed in YR2, having had samples collected in YR1. We are batching these to run contemporaneously and minimize batch effects and maximize work efficiencies.

Table 1. CyTOF markers used in this work-flow.

Cell types (General)	Antibody	Specific Information (Cell Type, etc.)	Metal
Immune Cells	CD45	Pan leukocyte marker	89
B cells	CD19	B cells	144
	MHC II(IA/IE)	Antigen Presenting Cells (B cells, monocytes, macrophages, dendritic cells), and activated T lymphocytes	176
T cells	CD 25	Activated T cells, T reg, B-cells, pre B-cells	141
	CD8a	Cytotoxic T-cells	143
	CD69	Activated T cells, B cells, NK cells, neutrophils and eosinophils	145
	T-bet (HS/Ms)	Th1 CD4+ T-cells	147
	CD4	Helper T-cells	149
	CD62L	B and naïve T cells, a subset of memory T cells, monocytes, granulocytes, most thymocytes, and a subset of NK cells	154
	CD223 LAG3	Activated CD4+ and CD8a αβ Tcells, γδ T-cells, NK subset	156
	eomes	Effector and memory CD8+ T cells, NK cells	164
	FoxP3	Regulatory T-cells (CD4+ CD25+)	165
	LAP (TGFbeta)	Activated regulatory T-cells. Important in regulating development of Treg, Th17, and Th9 cells	167
	CD3e	T-cells (TCR component)	172
	CD44	Memory T-cells, Effector T cells, Hematopoietic and non-hematopoietic cells, bone marrow myeloid cells, peripheral B and T cells can upregulate its expression	174
T-Cells Exhaustion Marker	CD366 (TIM3)	Th1 CD4+ and CD8+ T cells	150
NK cells	CD161 NK1.1	NK cells, rare subsets of T-cells and monocytes	168
	Granzyme B	Cytotoxic T-cells and NK cells	175
Immune Checkpoint	CD279 (PD-1)	Activated T- and B- lymphocytes	152
	CD274 (PD-L1)	Majority of Leukocytes	159
Macrophage / Monocyte	CD11c	Dendritic cells, NK cell subset, intestinal Intraepithelial Lymphocytes (IEL) and some activated T cells	209
	CD11b	Expressed on NK, stem cell precursor, macrophage / monocyte (hi in monocytes), eosinophils, granulocytes	115
	CD206	M2a tissue macrophages, dentritic cells, subpopulation of endothelial cells	151
	Ly-6C	Monocyte/macrophage cells, endothelial cells, Grandulocytes/neutrophils, and some T cell subsets	153
	F4/80	Macrophage, DC subset, eosinophils	173
Neutrophils/Granulocytes	CD170 siglecF	Eosinophils, alveolar macrophage	163
	Ly6G	Granulocytes, peripheral Neutrophils, transient on monocytes	142
Stroma / Vascular Cells	ALPHA SMA	Smooth muscle vessel walls, gut wall, myometrium, myoepithelial cells in breast and salivary glands	146
	CD140a (PDGFRa)	Embryonic tissues and mesenchymal-derived cells	148
	FSP1	Fibroblasts, epithelial cells undergoing epithelial–mesenchymal transition (EMT),	155
	CD31	Endothelial cells, low in Leukocytes	158
	CD317 BST2	Stroma,	160
	FAP	Stromal fibroblasts	161
	CD326/epCAM	Epithelial cells (Pan-carcinoma marker)	166
	CD102 (CCR2)	Endothelial cells, leukocytes	169
	CD140b (PDGFRb)	Embryonic tissues and mesenchymal-derived cells	170
Tumor	C-MYC	Tumor - Neuroblastoma	162
	MYCN	Tumor - Neuroblastoma	162
	Phox2b	Tumor - Neuroblastoma	171
	GD2	Tumor - Neuroblastoma	139

Subtask 1.2 Characterize the TME in tumors arising in the *TH-MYCN* model following chemotherapy. Mice are treated with irinotecan and temozolomide at non-curative doses that induce transient tumor regression.
We are currently in the process of breeding 165 *TH-MYCN* mice from +/- x +/- crosses: 20% are *TH-MYCN* +/+ suitable for studies with 10% attrition; results in ~30 +/+ mice for experiments: 10 used for CyTOF; 20 used for extended TIL flow-cytometry profiling.
1.2a. Profile functional states and abundance of TILs using murine CyTOF mass cytometry (Helios, Fluidigm). This is a YR2 task as per our SOW.
1.2b. Extended TIL flow-cytometry profiling. Two time-points, n~10 tumors each. Hogarty- mouse work; Bassiri- cytometry; months 12-18. This work has initiated and is ongoing, having started earlier than estimated.
1.2c, 1.2d and 1.2e are tasks for gene expression profiling of tumors, cytokine profiling in PB, and IHC assessment. They are to be pursued in YR2 as per our SOW.

Subtask 1.3. Characterize the TME *T-Ag* model tumors during *cell cycle-deregulated* tumor progression.
1.1a. We obtained regulatory approval for these animal studies. The *T-Ag* mouse model work was approved by the CHLA IACUC on 4/10/2019, and ACURO on 8/15/2019. From that point we initiated our mouse work at CHLA (Asgharzadeh/CHLA).
1.3a-1.3f. Task 1.3 workflow mirrors Task 1.1 but in a complementary tumor model; all mouse work is done in parallel at CHLA; assays done in labs/sites as per Task 1.1 and time-lines as per Task 1.1. We are currently breeding 115 TAg mice from +/- x wt crosses: 50% are TAg +/- suitable for studies with 10% attrition; this results in ~50 +/- mice for experiments: 10 used for CyTOF; 40 used for extended TIL flow-cytometry profiling. Gene expression, IHC, PB studies will be done on this same cohort (Asgharzadeh/CHLA).

Subtask 1.4 Characterize the TME in tumors arising in the *T-Ag* model following chemotherapy. Mice are treated with irinotecan and temozolomide at non-curative doses that induce transient tumor regression.
We will breed 70 TAg mice from +/- x wt crosses: 50% are TAg +/- suitable for studies with 10% attrition; this results in ~30 +/- mice for experiments: 10 used for CyTOF; 20 used for extended TIL flow-cytometry profiling. Gene expression, IHC, PB studies will be done on this same cohort. 1.4a-1.4e. Task 1.4 workflow mirrors Task 1.2 but in a complementary tumor model; all mouse work is done in parallel at CHLA; assays done in labs/sites as per Task 1.2 and time-lines as per Task 1.2 (Asgharzadeh/CHLA).

Subtask 1.5 Bioinformatic analyses of datasets obtained in Subtasks 1. Hogarty/CHOP BDMC and Asgharzadeh/CHLA Bioinformatics Core; months 6-30. This work will be initiated in Year 2 (frame-shifted due to delay in animal work approvals).

Major Task 2 (Aim 2). Characterize changes induced by DFMO and lenalidomide on the cellular and soluble immune-TME during tumor progression, and identify synergistic and/or antagonistic activities when combined with chemotherapy. Aim 2 involves both tumor models and was defined as a YR2-YR3 task as per our SOW. However, we have in fact started DFMO treatment trials in the TH-MYCN model and performed TIL profiling; see **Figure 1**. Our data show that 1% DFMO in drinking water alters TIL profiles in tumors, with increased proportions of NK cells and dendritic cells, and no significant alterations in other immune cell types (Tregs, MDSCs). Further, our data show that not only are NK cells increased, but the expression of activating-type NK receptors like NKG2D and IL15Ra is also increased (but not inhibitory-type receptors) and the tumor cells themselves express elevated levels of activating NK cell ligands like Rae1g. It is not clear if this NK receptor-ligand engagement is participating in providing the anti-tumor activity seen with DFMO, or if this simply identifies that NK cells in the TME are poised for tumor cell killing but are not fully engaged. It is possible that inhibitory TGFb signaling may be relevant, and we have shown increased LAP expression on tumor cells from DFMO treated mice supporting this.

Major Task 3 (Aim 3). Identify changes in circulating immune cells and cytokines in children with neuroblastoma treated with chemoimmunotherapy, with or without DFMO or lenalidomide, in Phase 1 and 2 clinical trials. These are YR2-4 tasks as outlined in our SOW. However, as an update, the ANBL1821 trial has opened as of May 2019, patients are being enrolled and biospecimens collected.

3.1a. Regulatory approval for human anatomical substances research; Asgharzadeh has submitted this work to the IRB at CHLA and received approval on 5/22/2019. A HRPO application for approval for this work was submitted on 6/27/2019. Follow up email was sent on 9/20/2019 and we received a response that initial administrative review was completed and the application is with the Approval Authority for additional comments or approval.

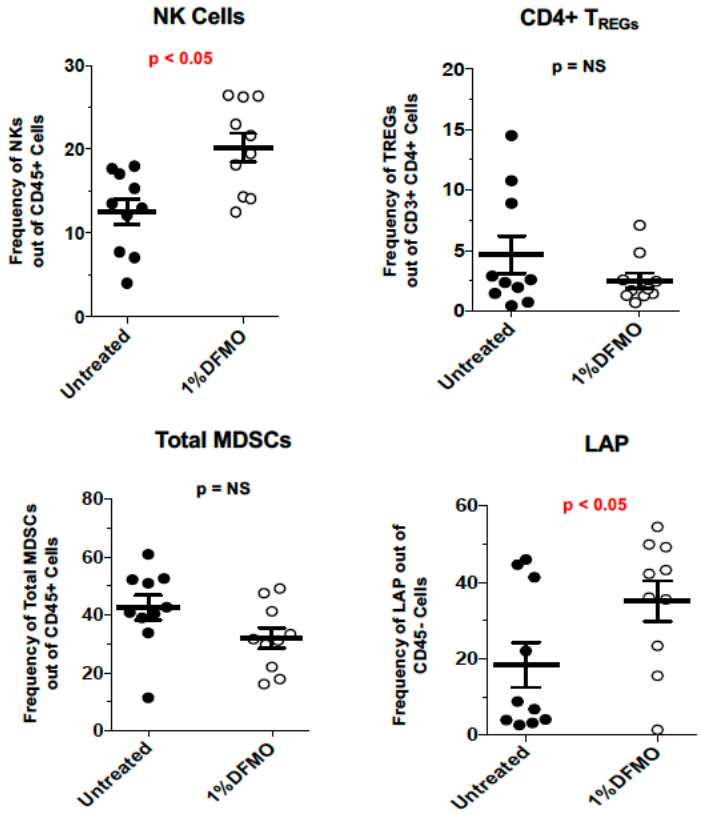

Figure 1. Selected flow-cytometry data from TH-MYCN tumors harvested at lethality from mice (genotype TH-MYCN +/+), enzymatically and mechanically dissociated. Mice were treated with 1% DFMO in drinking water ad libitum or regular water. Median intake of DFMO allometrically scales to a human equivalent dose of ~7gm/m2/day. The frequency of NK cells (shown) and DC (not shown) are increased by DFMO treatment, whereas inhibitory cell types such as Tregs and MDSCs, both total (shown) and G-MDSCs and M-MDSCs (not shown) are not altered. Further, LAP expression on tumor cells (from the CD45- compartment) is elevated). Shown are dot-plots of individual tumors plus mean and SEM as whisker-plot overlays. P value tested by two-tailed unpaired T-test.

TRAINING AND PROFESSIONAL DEVELOPMENT OPPORTUNITIES:
Nothing to report.

DISSEMINATION OF RESULTS:
Nothing to report. We anticipate presenting preliminary data obtained through this award as abstracts submitted to the AACR and ANR Meetings in Spring 2020.

IMPACT:
Our collective work to create a CyTOF antibody panel to profile the tumor immunoenvironment of neuroblastomas in murine models is being used within the CHLA CyTOF Core Laboratory as a standard approach to immunophenotyping solid tumor TMEs across additional transgenic mouse cancer models. Over time this will enable the comparison of tumor infiltrating leukocyte populations among diverse pediatric and/or adult murine tumor models.

Impact on technology transfer: Nothing to report.

Impact on Society beyond Science and Technology: Nothing to report.

CHANGES/PROBLEMS:
We have not made changes to our work plans so far, beyond being slightly delayed with the time to obtain animal work approvals. We have worked efficiently to obtain institutional and ACURO approvals, which are now in place. Nothing to report.

PRODUCTS:

Nothing to report. We anticipate presenting preliminary data obtained through this award as abstracts submitted to the AACR and ANR Meetings in Spring 2020.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS:

CHOP Participants:

Name: Michael Hogarty
Project Role: PI (initiating PI, CHOP)
ORCID ID: https://orcid.org/0000-0002-9221-4852
Nearest person months worked: 3
Contribution to project: Oversee CHOP portion of research program, coordinate with partnering PI; processed IACUC and ACURO approvals; TTSA monthly conference calls; input into CyTOF panel antibody inclusion.
Funding support (if other than this award): N/A

Name: Hamid Bassiri
Project Role: Co-investigator
ORCID ID: https://orcid.org/0000-0001-6532-8478
Nearest person months worked: 1
Contribution to project: Develop and oversee flow-based TIL identification within tumors; coordinate with CyTOF center; TTSA monthly conference calls; planning for CyTOF panel antibody inclusion.
Funding support (if other than this award): N/A

Name: Annette Vu
Project Role: Research Technician
ORCID ID: N/A
Nearest person months worked: 5
Contribution to project: Oversees all aspects of *TH-MYCN* neuroblastoma-prone mouse program (breeding, treatment, monitoring, tumor harvest and processing at endpoint)
Funding support (if other than this award): N/A

CHLA Participants:

Shahab Asgharzadeh
Role: PI (partnering PI, CHLA)
ORCID ID: https://orcid.org/0000-0002-0510-3848
Nearest person months worked: 2
Contribution to project: Overseeing all CHLA investigations; processed IACUC and ACURO approvals; TTSA monthly conference calls; CyTOF panel design and validation.
Funding support (if other than this award): N/A

Name: Hiroyuki Shimada
Project Role: Co-investigator
ORCID ID: https://orcid.org/0000-0002-5168-3222
Nearest person months worked: <1
Contribution to project: CyTOF panel antibody design and validation.
Funding support (if other than this award): N/A

Name: Sakunthala Muthougounder
Project Role: Research Technician
ORCID ID: N/A
Nearest person months worked: 6
Contribution to project: CyTOF panel antibody conjugation, titration, testing, and validation; CyTOF assay development and sample processing SOP.
Funding support (if other than this award): N/A

There are no changes in the active other support of the key personnel (nothing to report).

There are no changes or additional organizations as partners in this work (nothing to report).

SPECIAL REPORTING REQUIREMENTS:

The Translational Team Science Award is a collaborative award with an initiating PI (M. Hogarty, CHOP) and a partnering PI (S. Asgharzadeh, CHLA). Both PIs will submit duplicate (identical) Annual Progress Reports defining the contributions of each, under the unique award number for each PI and institution.

APPENDICES:

No appendices are included in this Year 1 Progress Report.