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TITLE: Targeting the Microbiome to Enable Immunotherapeutic Efficacy in Pancreatic Carcinoma

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CONTRACTING ORGANIZATION: New York University, New York, NY

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The PRCRP Topic Area to be addressed is "Pancreatic cancer". The PRCRP Military Relevance Focus Area is "Gaps in cancer treatment". Pancreatic ductal adenocarcinoma (PDA) is characterized by immune-tolerance and resistance to immunotherapies. The microbiome has emerged as an important factor regulating health and disease. More specifically, we and other groups have shown that the microbiome has a pathogenic role in promoting the development of PDA and in mitigating response to therapy. Our recent published work indicates that the PDA-associated microbiome is markedly expanded by more than 1000-fold compared with the normal pancreas (Pushalkar et al, Cancer Discovery 2018). Further, we found that mouse and human PDA-bearing hosts exhibit bacterial dysbiosis in the gut. Moreover, we found that the microbiome corrupts tumor immunity in PDA. Ablation of the microbiome in PDA was tumor-protective, upregulated expression of checkpoint receptors on T cells, and enabled efficacy for immunotherapy in mouse models of PDA. Based on these data, the microbiome is an attractive target in the treatment of PDA.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

The PRCRP Topic Area to be addressed is "Pancreatic cancer". The PRCRP Military Relevance Focus Area is "Gaps in cancer treatment". Pancreatic ductal adenocarcinoma (PDA) is characterized by immune-tolerance and resistance to immunotherapies. The microbiome has emerged as an important factor regulating health and disease. More specifically, we and other groups have shown that the microbiome has a pathogenic role in promoting the development of PDA and in mitigating response to therapy. Our recent published work indicates that the PDA-associated microbiome is markedly expanded by more than 1000-fold compared with the normal pancreas (Pushalkar et al, *Cancer Discovery* 2018). Further, we found that mouse and human PDA-bearing hosts exhibit bacterial dysbiosis in the gut. Moreover, we found that the microbiome corrupts tumor immunity in PDA. Ablation of the microbiome in PDA was tumor-protective, upregulated expression of checkpoint receptors on T cells, and enabled efficacy for immunotherapy in mouse models of PDA. Based on these data, the microbiome is an attractive target in the treatment of PDA.

Hypothesis/Objective: Our overarching hypothesis is that targeting pathogenic bacteria will augment innate and adaptive immunity in human PDA and enable successful immunotherapy of this disease. Our objective is to identify specific bacterial species and cocktails associated with immunogenic activation of the PDA tumor microenvironment. We will then translate the knowledge gained from our experiments in mouse models and human pre-clinical models to a Phase I clinical trial testing the safety and efficacy of bacterial ablation in combination with αPD-1 treatment in PDA patients. We expect that this approach will broach an era of successful immunotherapy of PDA.

2. KEYWORDS:

Pancreatic Cancer, Microbiome, immunotherapy, immune suppression, bacteria, probiotics, cancer.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1. To determine effect of modulation of the microbiome on innate and adaptive immunity in PDA

a. To determine the optimal antibiotic and probiotic regimens to slow disease progression and enhance tumor immunity in mouse models of PDA
b. To determine the influence of select probiotic bacterial taxa on tumor immune response and tumor viability in a microfluidic-based organotypic tumor model derived from freshly resected human PDA

Aim 2. To determine whether targeting the microbiome enables efficacy for immunotherapy in PDA

a. To determine the optimal antibiotic and probiotic regimens to enable efficacy for checkpoint or costimulatory receptor-based immunotherapy in PDA in mouse models
b. To determine whether select bacterial taxa enable efficacy for combination immunotherapy in a microfluidic-based organotypic tumor model derived from freshly resected human PDA

Aim 3. To conduct a Phase I 'window of opportunity' clinical trial in resectable PDA patients treated with antibiotics plus checkpoint-receptor based immunotherapy

a. To determine the safety and efficacy of treatment with antibiotics plus checkpoint-receptor based immunotherapy in PDA patients
b. To determine the effect of antibiotics plus checkpoint-receptor based immunotherapy on systemic and intra-tumoral immunity in human PDA

What was accomplished under these goals?

Please note: Covid-19 has impacted the work of this year. The grant was awarded in September 2019 and we started initial planning and recruitment of research staff. However, in early March 2020 there was major disruption in work due to COVID19. New York City was the most affected city in the country. New York University closed down all the research activities in the middle of March 2020. The labs were allowed to reopen in late June 2020 with limited capacity. Currently in 2021, we are working with restricted capacity in our institution to maintain social distancing. NYU has provided flexible hours to research staff to maintain social distancing.

Project 1 PI Dr. Xin Li:

Subtask 1 - Regulatory review and approval by the USAMRMC Animal Care and Use Review Office (ACURO) Completed and approved by ACURO

Subtask 3 – Determine the optimal antibiotic and probiotic regimens to slow disease progression in mouse models of PDA. An orthotopic PdxCre; LSL-KrasG12D; p53R172H (KPC) model will be used as tumor bearing mice to receive antibiotics or bacteria by oral gavage.

Experimental strategy. We postulate that immunogenic reprogramming of the microbiome will enhance tumor immunity in PDA. Our preliminary work that suggested an important role for the microbiome in corrupting anti-tumor immunity in PDA was performed in germ-free mice and in mice treated with broad spectrum antibiotics. However, while the use of germ-free mice and broad spectrum antibiotics are valid as proof of principal, there are critical questions that must be answered in more discriminating preclinical models to facilitate clinical translation. Specifically, we will need to determine whether a more selective antibiotic regimen which targets specific bacterial populations, rather than the entire microbiome, is protective. Further, we will test our hypothesis that re-population with probiotic immune-activating bacteria will enhance anti-tumor immunity in PDA.

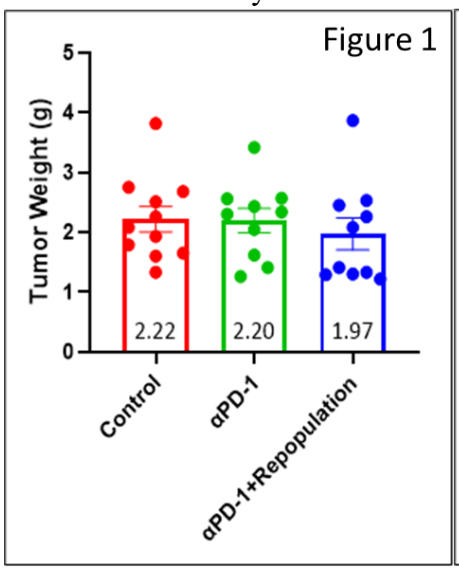
We selected Treatments regimens which included:

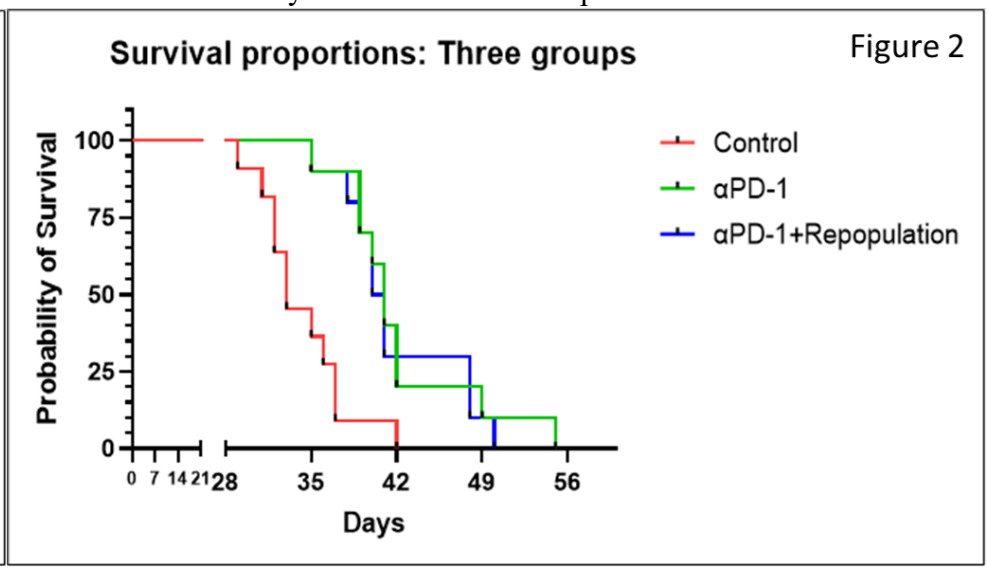
(i) broad spectrum antibiotics (vancomycin, neomycin, flagyl, ampicillin)
(ii) vancomycin alone
(iii) neomycin alone
(iv) flagyl alone
(v) ampicillin alone
(vi) ampicillin + flagyl

Mouse Model: We postulate that modulation of the microbiome with the selective antibiotics and/or probiotic repopulation will enhance anti-tumor immunity. Because of many experimental conditions are proposed to test, we require a high throughput in vivo system. Therefore, we initially employed an orthotopic PdxCre;LSL-KrasG12D;p53R172H (KPC) model where KPC cells are implanted in the pancreas of WT hosts. This high throughput system will allow for testing of all our antibiotic and probiotic conditions. In our initial experiment we tested one antibiotic combination and one formulation. Mice were sacrificed at experimental endpoints to measure tumor progression and intra-tumoral myeloid cell and T cell programming. We also tested the survival rate in another cohort.

(i) Tumor size and survival rate:
We used 10 -11 mice/group and implanted 40,000 cells in all of them. After depletion with a combined antibiotic, PD-1 antibody (αPD-1) was injected to two groups with one of them received probiotic formulation by oral gavage using published protocol. We used broad spectrum antibiotic in this experiment. Survival in the control and treated groups was measured by Kaplan-Meier analyses.

Results: In our initial experiment we established the feasibility of experiments and dosage. The analysis indicated there were no major differences in the tumor weight (Figure 1) however the animals with αPD1 and αPD1-repopulated with probiotic survived much longer as compared to the control group (Figure 2). We noticed the cocktail of probiotics has low solubility in the vehicle solution which could compromise the efficacy of the probiotics. We will have to modify our protocol for formulation. We have tried a few combination and noticed that some probiotics have better solubility and we will consider to use them only in our next in vivo experiment.





Project 2 PI Dr. Deepak Saxena:

Subtask 2 – Culture probiotics and generate cocktails to share with Project 1.

In addition, based on our 16S bacterial DNA sequencing experiments we have identified a number of bacterial strains that may be protective against PDA. We have chosen these bacterial taxa as putative 'probiotics' based on their greater abundance in healthy control compared to PDA patients in our 16S analysis and an established literature designating these strains as either having immune activating effects or protecting against pathogenic bacterial colonization. Therefore, we will also treat mice with either single strains or select combinations of:

(i) Lactobacillus - Enhances innate immunity.
(ii) Akkermansia muciniphila – Improves gut barrier integrity limiting translocation.
(iii) Bifidobacterium bifidum – Helps colonization of other probiotics, enhances immunity.
(iv) Escherichia coli Nissle 1917 or Streptococcus thermophilus – Improves survival capacities of 'immunogenic' bacteria.
(v) Bifidobacterium lactis - Increases the intestinal barrier against pathogens.

As negative controls, we will also repopulate with tumor-promoting strains B. pseudolongum and F. nucleatum which we have shown promote accelerated oncogenesis.

We initiated developing probiotics using various combination of Bacteria. As proposed in Aim1 we selected 8 bacterial strains. The bacterial cultures were obtained from ATCC.

Bacterial Growth & Preparation

Bacterial strain	Liquid Growth Medium	Aerobic/Anaerobic	Growth time	Cfu/mL @ **<time>**
Lactobacillus acidophilus ATCC#4356	MRS Broth (BD 288130)	Anaerobic	14h	~3.00×10^{8} cfu/mL
Lactobacillus casei ATCC#393	MRS Broth (BD 288130)	Anaerobic	14h	~2.0×10^{9} cfu/mL

Lactobacillus paracasei ATCC#25302	MRS Broth (BD 288130)	Anaerobic	14h	~2.8×10^9 cfu/mL
Lactobacillus reuteri ATCC#23272	MRS Broth (BD 288130)	Aerobic	TBD	~1.9×10^9 cfu/mL
Akkermansia muciniphila ATCC#BAA-835	BHI Broth (BD 237500)	Anaerobic	TBD	TBD
Streptococcus thermophilus ATCC#19258	BHI Broth (BD 237500)	Aerobic	TBD	TBD
Bifidobacterium breve ATCC #15700	Modified Reinforced Clostridial (custom)	Anaerobic	TBD	TBD
Ruminococcus bromii ATCC#27255	PYG Broth (AS-822)	Anaerobic	TBD	TBD

Bacterial cells were grown to log-phase in 100mL of the appropriate medium (see table above).
- Cells btwn 10^{8-10} cfu/mL, depending on species.
- 100mL culture volume was produce approx. 10^{10-12} cfu total

Cells were harvested by centrifugation at 4000×g/5min/4°C, and washed 3x with sterile distilled water[1].
Resuspend cells in 5mL cryoprotectant solution (see below).

Cryoprotectant Solution

Gelatin-glycerol medium:
1.5% (w/v) gelatin
1.0% (v/v) glycerol
Mix in MilliQ water, sterilize by autoclaving on liquid cycle at 121°C/30min.
Allow to cool before using.

Microencapsulation Procedure
Polymer mix:
1.0% (w/v) xanthan gum
0.75% (w/v) gellan gum
Mix in MilliQ water, sterilize by autoclaving on liquid cycle at 121°C/15min.
Allow to cool before using.

Hardening solution:
0.2M sterile $CaCl_2$

Mix cryoprotectant-cell suspension with polymer solution (1:1) (5mL)
- 10mL should give ~10^{9-11} cfu/mL
- Collect 100µL for cfu-determination (pre-freeze drying)

Load cell suspension into syringe fitted with 21G (or similar) needle.[2]
Drop-wise, add cell suspension to $CaCl_2$ solution.

[1] To completely remove any media residue

- Allow beads to harden for 30min by sitting in $CaCl_2$ solution.

Collect beads on sterile gauze, rinse with MilliQ water.
Aliquot beads to 5g/50mL conical tube, resuspend in 5mL cryoprotectant media.
Freeze overnight at -80°C.
Lyophilize next day: may have to work out conditions, safe point to start seems -40°C/48h.

Viability Testing

Initial bacterial titer

Perform several serial-fold dilutions on 100µL aliquot from above. (to at least 10^{-8})
Plate duplicate plates from 10^{-6} and upwards.

- 100µL per plate, per dilution
- $total\ cfu = (plate\ count) \times 10 \times 10^{dilution\ factor}$

Viability of cells after lyophilization

Weigh empty 50mL conical tubes beforehand.
Weigh after lyophilization: $dry\ pellet\ weight = (pre)weight - (post)weight$

Resuspend lyophilized pellet in equal volume appropriate medium for bacterial strain.
Perform several serial-fold dilutions. (to at least 10^{-8})
Plate duplicate plates from 10^{-6} and upwards.

- 100µL per plate, per dilution
- $total\ cfu = (plate\ count) \times 10 \times 10^{dilution\ factor}$
- $\frac{cfu}{g} = total\ \frac{total\ cfu}{dry\ pellet\ weight}$

Perform viability test immediately after lyophilization, and at (time period[s]) afterwards.

Results:

We have developed 3 different formulation:

1. POC18 and 19: Using 6 strains combination.
2. POC20 (LL): Using two lactobacillus strains which has better solubility.

These formulations are being test in Project 1 (Dr Li) using KPC orthotopic models.

Project 3 PI Dr. Deirdre Cohen:

Specific Aim 3: To conduct window of opportunity clinical trial in resectable PDA patients with antibiotics plus checkpoint receptor based immunotherapy.

Subtask 1 To determine the microbiome composition in tumor tissue prior to and following treatment with antibiotics, chemotherapy and PD1 inhibition.

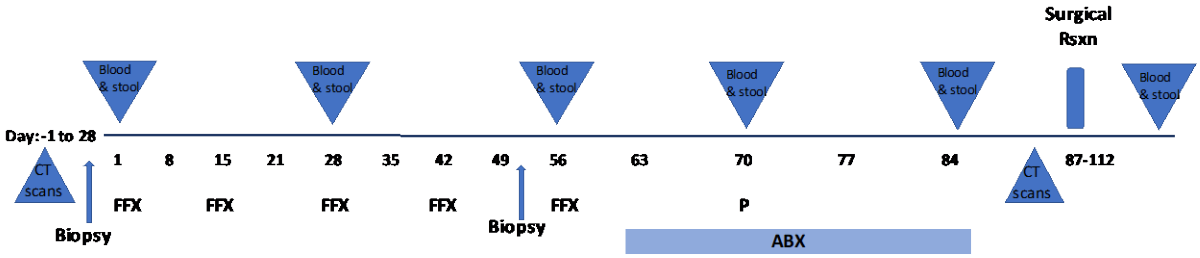
Subtask 2 To determine the microbial signature in the intestines of enrolled patients with PDAC using 16S sequencing

The clinical trial was redesigned and the protocol rewritten to include pre-operative multi-agent chemotherapy followed by a window of opportunity to test the combination of microbiome depletion with antibiotics in combination with immune checkpoint inhibition. Following protocol revision, it was reviewed and approved by the Mount Sinai Biostatistics Design Workshop. Subsequently, the study was presented at the GI Disease Focus Group and approved to move forward for review by the Mount Sinai scientific review committee (PRMC). Most recently, the study was deferred by the PRMC in order to clarify and address various concerns regarding

feasibility and design. The protocol has been resubmitted and we are now awaiting approval. In parallel, ongoing negotiation between clinical study sponsor and Mount Sinai is underway. Following PRMC approval, we will submit an IND application to the FDA. In parallel, the protocol will be submitted to the Mount Sinai IRB for review and approval.

Next funding cycle we will open the redesigned clinical trial (see attached schema, Appendix 1) which will enable us to collect the biological samples (tumor tissue and stool) in order to perform subtasks 1 and 2 of Aim 3.. We will plan to report interim accrual numbers and possibly early results from the paired biopsies.

Clinical protocol schema



Intervention:
FFX: 5FU 2400mg/m^2, oxaliplatin 85mg/m^2, Leucovorin 400mg/^{2}, irinotecan 150m/m^2
P: Pembrolizumab 200mg IV
Abx:
Ciprofloxacin 500mg PO BID & Metronidazole 500mg PO TID

Project 1, 2 and 3 (PI Li, Saxena, Cohen)

We tried addressing Aim 1 whether there is any functional changes in the microbiome which can help in developing optimal probiotics which can be used in combination with immunotherapy in PDA. The manuscript on this finding is under revision. The Major findings:

1. To investigate the association between the dysbiotic gut microbiome and the altered host metabolic or signaling pathways, we performed PICRUSt analysis. The electron transfer carriers (p=0.0001) and secretion system (p=0.003) were differentially enriched in the gut of PDA patients than of non-PDA controls. The pathways of amino acid, ascorbate, and aldarate (p=0.01), nucleotide (p=0.0004), glycan biosynthesis and metabolism (p=0.05), cofactors and vitamins (p=0.03), signal transduction mechanisms (p=0.03) and bladder cancer (p=0.002) were significantly expanded in PDA gut. In addition, bacterial motility proteins (p=0.04), replication, recombination and repair proteins (p=0.02) and pathways involving bacterial invasion of epithelial cells (p=0.001) were differentially downregulated in non-PDA control guts. On contrary, pathways involved in oxidative phosphorylation (p=0.004), peroxisome proliferator-activated receptors (PPAR) signaling (p=0.005) and adipocytokine signaling (p=0.02) were upregulated in the non-PDA gut. Moreover, alanine, aspartate and glutamate (p=0.01) and histidine metabolism (p=0.04) as well as fatty acid biosynthesis were significantly decreased in the PDA gut.
2. We assessed the host metabolic changes in response to the dysbiotic pancreatic microbiota in PDA and healthy (non-PDA) patient population. We found that the pathways of PPAR signaling (p=0.05) and ether lipid metabolism (p=0.02) were significantly overrepresented while, protein machinery for replication, recombination and repair (p=0.05) were significantly underrepresented in cancer cohorts. The metabolic pathways for fatty acid, tryptophan, lipid metabolism and biosynthesis, valine, leucine and isoleucine biodegradation, oxidative phosphorylation and bacterial toxins were enriched in PDA pancreata. In contrast, flavone and flavonol biosynthesis, alanine, aspartate and glutamate metabolism,

amino acid metabolism, glycan biosynthesis and metabolism, signal transduction mechanisms and electron transfer carriers were upregulated in non-PDA pancreata.

To conclude, these host-microbiota interactions are chiefly driven by accessible metabolites produced by bacteria and those that they utilize as substrates. Thus, amalgamating metabolome and microbiome as a unique approach to functionally distinguish the microbiota in terms of their metabolic activity in relation to cancer will increase our understanding of this complex interactions. Next funding cycle we will expand our finding and larger sample size. We will also include longitudinal investigation of the microbiome and the metabolites production in the gut and the pancreata at different stages of pancreatic cancer to explicate the functions of metabolically upregulated pathways in oncogenic progression.

Training and Professional Development

What opportunities for training and professional development has the project provided?

Nothing to Report

Dissemination of Results

How were the results disseminated to communities of interest?

Present invited seminar at NCI meeting: Glycans, Microbes & Cancer Workshop
Session I: Role of Glycans in Host-Microbe Interactions August 20-21, 2020

Plans for Next Reporting Period

What do you plan to do during the next reporting period to accomplish the goals?

1. Continue working on developing optimal antibiotic and probiotic regimens to slow disease progression and enhance tumor immunity in mouse models of PDA
2. Continue working on optimal antibiotic and probiotic regimens to enable efficacy for checkpoint or costimulatory receptor-based immunotherapy in PDA in mouse models.
3. Obtain human subject approval for new recruitment.
4. Submit manuscript on functional analysis of microbiome in PDA.

4. IMPACT:

Impact on principal discipline.

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

Impact on other disciplines.
What was the impact on other disciplines?

Nothing to Report

Impact on technology transfer.
What was the impact on technology transfer?

Nothing to Report

Impact on society.
What was the impact on society beyond science and technology?

- **Nothing to Report**

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

We proposed to use either Patient-derived oganotypic tumor spheroids (PDOTS) system or human 3D organotypic cancer model which are recently validated as a platform for evaluating immunotherapies. If it is possible we will plan to use commercial available animal or human 3D organotypic pancreatic cancer models.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

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:

- **Publications, conference papers, and presentations**

Journal publications. Smruti Pushalkar, Fangxi Xu, Scott Thomas, Constantinos P. Zambirinis, Donnele Daley, Qianhao Li, Mautin Hundeyin, Mykhaylo Usyk, Cohen, Deirdre, George Miller, Xin Li and Deepak Saxena. 2021. Microbial Dysbiosis and Altered Functional Metabolic Pathways in Pancreatic Ductal Adenocarcinoma. Cancers (under review)

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Nothing to Report

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on project

Name:	Deepak Saxena
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-5506-5827
Nearest person month worked:	1
Contribution to Project:	Design experiment and analysis
Funding Support:	

Name:	Xin Li
Project Role:	PI Project 2
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Design experiment and analysis
Funding Support:	
Name:	Deirdre Cohen
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-6178-9266
Nearest person month worked:	1
Contribution to Project:	Redesigned and re-written clinical Protocol
Funding Support:	

Name:	Fangxi Xu
Project Role:	Jr Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	11
Contribution to Project:	Bench research (Project 2)
Funding Support:	

Name:	Yuqi Guo
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Bench research	Bench research (Project 1)
Funding Support:	

Name:	
Project Role:	
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	
Funding Support:	

Change in support since last reporting period

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The PI of Project 1 has been changed to Dr Xin Li, Professor, NYU College of Dentistry. It has been approved by Science Officer (SO)/Grants Officer Representative (GOR).

NIH/NIA R01 AG068857 Li (Contact PI) 08/15/2020-04/30/2025
Title: Succinate triggers gut dysbiosis and activates SUCNR1 to enhance inflammaging

Role: MPI
There is no overlap with current proposal.

Other organizations.

- **Organization Name:**
- **Location of Organization:** *(if foreign location list country)*
- **Partner's contribution to the projec**t *(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:

QUAD CHARTS:

9. APPENDICES.