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PRINCIPAL INVESTIGATOR: P'ng Loke

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P'ng Loke

E-Mail: png.loke@nih.gov

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New York University School of Medicine
550 First Avenue
New York, NY 10016-6402

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Macrophages play key roles in progression of atherosclerosis. Our goal is to understand the mechanisms by which atherosclerosis can be clinically regressed by altering the macrophage state in the plaques to resolve the inflammation, as well as to develop new therapeutic strategies to promote atherosclerosis regression by altering the macrophage activation state. We have found that successful atherosclerosis regression requires the alteration of macrophages in the plaques to a tissue repair "alternatively" activated state. This switch in activation state requires the action of TH2 cytokines interleukin (IL)-4 or IL-13. To accomplish our goals, we are testing if these molecules, or derivative of these molecules, will be able to accelerate atherosclerosis regression in mouse models. Additionally, we will develop nanomedicines that can favorably and rapidly affect the content and inflammatory state of macrophages in atherosclerotic plaques to promote regression. Concurrently, we will characterize the macrophages to understand the mechanisms that promote atherosclerosis regression.

15. SUBJECT TERMS

NONE LISTED

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1. INTRODUCTION

We currently have a limited capacity to **reverse** the high level of atherosclerotic plaques already present in the population. Our vision is to harness the immune system to reverse atherosclerosis. Inadequate resolution of inflammation is fundamental to all stages of atherosclerosis, with macrophages playing key roles in progression of the disease. The goal of our proposal is to understand the mechanisms by which atherosclerosis can be clinically regressed by altering the macrophage state in the plaques to resolve the inflammation, as well as to develop new therapeutic strategies to promote atherosclerosis regression by altering the macrophage activation state. By understanding and harnessing these mechanisms in mouse models of atherosclerosis, the final goal would be to develop new immunotherapeutic approaches that can complement existing lipid lowering treatments, thereby providing benefits to veterans, who experience a high rate of cardiovascular disease. We have recently found that successful atherosclerosis regression requires the alteration of macrophages in the plaques from an inflammatory "classically" activated state to a tissue repair "alternatively" activated state. This switch in activation state requires the action of TH2 cytokines interleukin (IL)-4 or IL13. To accomplish our goals, we are testing if these molecules, or derivative of these molecules, will be able to accelerate atherosclerosis regression in mouse models. Additionally, we will develop nanomedicines that can favorably and rapidly affect the content and inflammatory state of macrophages in atherosclerotic plaques to promote regression. Concurrently, we will characterize the macrophages to understand the mechanisms that promote atherosclerosis regression.

2. KEYWORDS: Atherosclerosis, cardiovascular disease, macrophages, interleukin 4, nanoparticles.

3. ACCOMPLISHMENTS:

The major goals and objectives of the project are:

Specific Aim 1: To determine the mechanism(s) regulating inflammation resolution in regressing plaques and to promote resolution by administration of TH2 cytokines (IL4 and IL13).

To accomplish this aim, the Major Tasks are:
(1) Determine the requirement for IL-4 in mediating M2 activation and regression and to generate conditional STAT6 deficient animals to independently determine the requirement for the IL-4 signaling pathway in specific cell types, including macrophages.
(2) Determine the cellular source of IL4 in mediating M2 activation and regression.
(3) Determine the optimal dosage of IL4 or IL13 required in vivo for M2 activation of peritoneal macrophages without adverse effects.
(4) Determine the optimal dosage and efficacy of IL4 or IL13 required in vivo to promote plaque regression.
(5) Generate ATAC-seq data from M2 macrophages from atherosclerosis plaques.
(6) Generate RNA-seq data from M2 macrophages from atherosclerosis plaques.
(7) Integrate ATAC-seq and RNA-seq data to build a transcriptional network for M2 activation in regressing plaques.

Specific Aim 2: To develop nanomedicines to favorably and rapidly affect the content and inflammatory state of macrophages in atherosclerotic plaques.

To accomplish this aim, the Major Tasks are:
(1) Determine the efficacy of nanoparticles containing LXR agonist to promote plaque regression.

(2) Characterize changes to plaque macrophages after treatment with LXR-agonist-NP.
(3) Determine the efficacy of nanoparticles containing Netrin1- and Unc5b-siRNA to promote plaque regression.
(4) Characterize changes to plaque macrophages after treatment with Netrin1-and Unc5b-siRNA – NP.

What was accomplished under these goals?

Major activities for this reporting period:

Aim 1 Major Tasks

Major Task 1 Determine the requirement and source of IL-4 required for atherosclerosis regression.

Major Task 1a: Fisher lab: Determine the requirement for IL-4 for atherosclerosis regression.

In our 2019 report, we summarized studies using the IL4/IL13 double knockout (DKO) mice to be the aortic transplant donors. Thus, these mice were first made hypercholesterolemic by an injection of an adenoviral vector expressing PCSK9. After 20 weeks of feeding the atherogenic "western diet", regression was initiated by injections of an anti-sense oligonucleotide (ASO) to apoB to reduce hepatic lipoprotein secretion (which reverses the hypercholesterolemia), and 3 weeks days after that, the aortic samples were collected and analysed. Regression (as assessed as we usually do by plaque macrophage content) was now impaired, indicating that these cytokines were indeed required, but because in a previous progress report we showed that if the cytokines were present in a donor plaque (i.e., prior to lipid lowering), regression was normal. Taken together, the plaque content of the cytokines prior to the regression stimulus was apparently sufficient for the stimulus to polarize macrophages to the inflammation resolving (M2) state. These results agree with our measurements that showed that neither cytokine changed significantly in plaques before and after regression and with data using mice with a reporter for IL4 (the "4get mouse") that showed the presence in plaques of IL4 expressing cells, but there was no change in their number associated with regression.

How do we explain, then, that unchanging concentrations of these cytokines apparently induced macrophages to become inflammation resolving? Upon examination of our prior transcriptomic data from macrophages selected from progressing and regressing plaques[1], we noticed an increase in Wnt signaling during regression. In recent experiments, we noted *in vitro* that the addition of a classical Wnt ligand (Wnt 3a) augments the response of macrophages to IL4 or IL13. Our working hypothesis became that there is induction of Wnt signaling in plaque macrophages when regression is initiated by lipid lowering, which amplifies their response to IL4/IL13. We undertook a long series of experiments to test this, and we were successful in establishing that the hypothesis was true *in vitro* and *in vivo*. We recently published the full story[2], so rather than reproducing figures and tables in the body of this report, I have appended the PDF of the article.

Major Task 1b: Fisher and Loke labs: Generate conditional STAT6 deficient animals to independently determine the requirement for the IL4 signaling pathway in specific cell types, including macrophages.

We received conditional STAT6 deficient animals from Ingenious Targeting Laboratories. After rederivation into our animal facility we have crossed these animals to macrophage specific Cre expressing mice. These include the CD169-Cre to delete STAT6 in tissue resident macrophages and CX3CR1-Cre and CSF1R-Cre mice to delete them in all other macrophages. Because of the long-term nature of many of the experiments we initially proposed and the dwindling time and funds, we

had to prioritize other studies and used STAT6-deficient mice only to show that the Wnt-signaling effects on macrophage responses to IL4 required STAT6. These results were reported in[2].

Major Task 2: Fisher Lab: Determine the cellular source of IL-4 in mediating M2 activation and regression.
As the research progressed, we became more focused on how the cells in the plaque become responsive to whatever IL4/IL13 is there already at the time we initiate regression. Also, we had initially thought from the IL-4 reporter experiment with the 4GET mice, that in plaques, it was mainly eosinophils were expressing IL-4. The emerging data, however, also implicated other leukocytes, making the determination of the relative contributions more complicated, in that IL4 expression would have to be inactivated on a cell type basis in any one experiment.

Major Task 3: Loke lab: Determine the optimal dosage of IL4 or IL13 required *in vivo* for M2 activation of peritoneal macrophages without adverse effects.

In a previous funding period, we had optimized the dosage of IL4 and IL13 for the treatment of plaques.

Major Task 4: Loke lab: Determine the optimal dosage and efficacy of IL4 or IL13 required *in vivo* to promote plaque regression.

In the previous Progress Report, we noted that IL4 treatment unexpectedly increased plaque size. Hence, instead of promoting plaque regression, it promoted plaque progression. Thus, we did not pursue IL4 or IL13 (expected to have the same effects) further.

Major Task 5: Loke lab: Generate ATAC-seq data from monocyte derived M2 macrophages from atherosclerotic plaques.

This was taken over by the Fisher lab, and they have generated one set of ATAC-seq data after improving the quality of the required libraries. These results are being analyzed by the bioinformatics core in cardiology.

Major Task 6: Fisher and Loke labs: Generate RNA-seq data from monocyte derived M2 macrophages from atherosclerosis plaques.
This work was published and the article[3] was included in the 2019 Progress Report.

Major Task 7: Integrate ATAC-seq and RNA-seq data to build a transcriptional network for M2 activation in regressing plaques.

This is still in progress, as the ATAC-seq data required new analytical skills to be first mastered by our bioinformatics core.

<u>Aim 2 Major Tasks:</u>

Major Task 1: Fisher lab: Determine the efficacy of nanoparticles containing LXR agonist to promote plaque regression in LDLR-/- mice.

All of these studies were successfully completed in the first funding period and published[4].

Major Task 2: Fisher lab: Characterize changes to plaque macrophages after treatment with LXR-agonist-NP.

All of these studies were successfully completed in the first funding period and published[4].

Major Task 3: Moore lab: Determine the efficacy of nanoparticles containing Netrin1- and Unc5b-siRNA to promote plaque regression in LDLR-/- mice.

As mentioned last year, the "empty" nanoparticles (control) were also anti-atherogenic, most likely because they effluxed cholesterol from plaque macrophages. After reviewing all of the data, we decided to terminate this study.

Major Task 4: Moore lab: Characterize changes to plaque macrophages after treatment with Netrin1- and Unc5b-siRNA -NP.

As noted just above, the study was terminated, so this task was not undertaken.

What opportunities for training and professional development did the project provide?

Postdoctoral Fellows (Loke Lab):

Dr. Jian-da Lin came to the Loke lab with a background in parasitical disease. He had some experience in immunology as part of that, and was trained to adapt and expand this training to the study of atherosclerosis. He was the first author of the previously reported paper on single-cell sequencing of plaque macrophages[3], and in the studies summarized in this report, he contributed to characterizing the roles of IL-4 and IL-13 in atherosclerosis regression.

Graduate Students: None trained under this grant.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
We were the first to publish a single-cell RNA seq analysis of macrophages in progressing and regressing plaques[3]. This work has been widely cited, as it uncovered much more diversity of the phenotypes of macrophages in atherosclerotic plaques than had been appreciated. We also established the requirement of IL4 for the regression of atherosclerosis and discovered a novel pathway by which Wnt signaling augments the responses of macrophages to IL4 to resolve inflammation[2]. Overall, our studies emphasize that lipid lowering alone will not be sufficient to maximally reduce the risk of cardiovascular disease- inflammation resolution must also occur. The studies from our model system are consistent with recent clinical studies, in particular, the CANTOS trial.

What was the impact on other disciplines?
The results are relevant to immunologists and rheumatologists because of the basic findings in macrophage biology and inflammatory diseases.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. CHANGES/PROBLEMS:

None to note.

6. PRODUCTS:

Weinstock A, Rahman K, Yaacov O, Nishi H, Menon P, Nikain CA, Garabedian ML, Pena S, Akbar N, Sansbury BE, Heffron SP, Liu J, Marecki G, Fernandez D, Brown EJ, Ruggles KV, Ramsey SA, Giannarelli C, Spite M, Choudhury RP, Loke P, Fisher EA. Wnt signaling enhances macrophage responses to IL-4 and promotes resolution of atherosclerosis. Elife. 2021;10. Epub 2021/03/16. doi: 10.7554/eLife.67932. PubMed PMID: 33720008; PMCID: PMC7994001.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Name: Loke Png
Project Role: PI
Nearest person month worked: 1
Contribution to Project: Dr. Loke was the PI and supervised the research staff to complete the tasks as described in this report

Name: Jian-Da Lin
Project Role: Post-doctoral fellow
Nearest person month worked: 12
Contribution to Project: Dr. Lin played the main role in characterizing the phenotype of the macrophages and the role of IL-4 and IL-13 during atherosclerosis.

Name: Caroline McCauley
Project Role: Research Associate
Nearest person month worked: 9
Contribution to Project: Ms. McCauley helped Dr. Lin on various aspects of the project, including sectioning tissue samples as well as some other assays

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS:
Dr. Loke was the partnering PI, and he relinquished the grant as of February 3, 2020 because he accepted a position at the NIH. Dr. Edward Fisher, the initiating PI, has also submitted a Final Progress Report.

9. APPENDICES: NONE

References:

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3. Lin JD, Nishi H, Poles J, Niu X, McCauley C, Rahman K, Brown EJ, Yeung ST, Vozhilla N, Weinstock A, Ramsey SA, Fisher EA, Loke P. Single-cell analysis of fate-mapped macrophages reveals heterogeneity, including stem-like properties, during atherosclerosis progression and regression. JCI Insight. 2019;4(4). Epub 2019/03/05. doi: 10.1172/jci.insight.124574. PubMed PMID: 30830865; PMCID: PMC6478411.

4. Yu M, Amengual J, Menon A, Kamaly N, Zhou F, Xu X, Saw PE, Lee SJ, Si K, Ortega CA, Choi WI, Lee IH, Bdour Y, Shi J, Mahmoudi M, Jon S, Fisher EA, Farokhzad OC. Targeted Nanotherapeutics Encapsulating Liver X Receptor Agonist GW3965 Enhance Antiatherogenic Effects without Adverse Effects on Hepatic Lipid Metabolism in Ldlr(-/-) Mice. Adv Healthc Mater. 2017;6(20). Epub 2017/07/22. doi: 10.1002/adhm.201700313. PubMed PMID: 28730752; PMCID: PMC5656530.