PROJECT TITLE **Effect of cytokine storm on vascular smooth muscle cell phenotype under healthy vs. diseased conditions**

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## ABSTRACT OF PROJECT

Recent Covid-19 pandemic has provided evidence for a potential link between Covid-19 infection and future risks of cardiovascular complications. Atherosclerosis is a major player in the development of several cardiovascular complications including myocardial infarction, heart failure and stroke. Elevated serum cytokine levels or systemic 'cytokine storm' (CS) is one of the most common clinical manifestations of severely ill hospitalized Covid-19 patients. Clinical studies have suggested that pre-existing risk factors for vascular disease, such as diabetes, obesity, and dyslipidemia, can exacerbate the inflammatory responses of the vasculature to CS prompting atherosclerotic complications. However, the molecular mechanism(s) by which CS may trigger atherosclerosis are unknown. De-differentiation of vascular smooth muscle cells (VSMC) from 'quiescent' contractile to 'synthetic' proliferative phenotype is a key event for development of atherosclerosis. This project aims to study whether CS conditions may induce VSMC phenotypic transition to a diseased proatherogenic phenotype, and further interrogate whether this effect is more pronounced under diabetic conditions. For these studies, we will utilize murine aortic SMC (MASMC) primary cultures isolated from healthy and diabetic mice. MASMCs will be exposed to different concentrations of CS cocktail in vitro for varying periods of time; VSMC signaling, and differentiation marker expression as a readout of VSMC phenotypic transition will be assessed using immunoblotting and immunocytochemistry.

## BACKGROUND AND RATIONALE

Cumulative evidence from basic sciences and clinical observations highlights a strong correlation between Covid-19 exposure and risks of accelerated vascular complications. 'Cytokine storm', a hallmark of host-immune responses and immune dysregulation induced by the deadly and pathogenic coronavirus, SARS-CoV-2, is a typical manifestation of critically ill Covid-19 patients. Systemic cytokine storm relevant to Covid-19 infection is clinically marked by elevated levels of serum inflammatory cytokines (e.g., TNF-$\alpha$, IFN-$\gamma$, CXCL9 and CXCL10, chemokines induced by IFN-$\gamma$), interleukins (IL)-1$\beta$, 6, 10, and soluble IL-2 receptor alpha. Clinical studies suggest that individuals with pre-existing risk factors for vascular disease, such as diabetes, obesity, and dyslipidemia, are more vulnerable to the onslaught of 'cytokine storm', characteristic of hospitalized Covid-19 patients, regardless of race, socioeconomic disparities, and ethnicity. However, the molecular mechanisms by which 'cytokine storm' exacerbates the inflammatory responses of the diseased vasculature, triggering accelerated vasculopathy in individuals with pre-existing cardiovascular risk factors remain poorly understood.

Atherosclerosis is a major player in the development of numerous cardiovascular complications and accounts for increased morbidity and mortality in individuals with pre-existing risk factors for vascular disease. Recent studies provide strong evidence for the direct involvement of vascular smooth muscle cells (VSMC), a major cell type in the blood vessel, in atherosclerotic lesion formation. Growing literature indicates that VSMC de-differentiation from 'quiescent' contractile to 'synthetic' proliferative phenotype is a critical mediator of augmented atherosclerotic lesion progression.

The proposed summer project is part of a larger research program that aims to interrogate whether pre-existing metabolic or vascular anomalies can stimulate VSMC proatherogenic phenotype prompting atherosclerotic complications following exposure to elevated concentrations of a cytokine cocktail, mimicking the systemic 'cytokine storm' manifested in hospitalized Covid-19 patients requiring intensive care. In the proposed project, we will determine the impact of 'cytokine storm' conditions *in vitro* on the cellular and molecular phenotypic properties of VSMCs isolated from healthy and diseased mice.

GOALS AND OBJECTIVES

Goal: We will investigate the effect of 'cytokine storm' conditions *in vitro* on the smooth muscle phenotypic characteristics of murine aortic smooth muscle cell (MASMC) primary cultures isolated from healthy and diabetic mice.

Objectives:

1. To compare expression of VSMC contractile and synthetic markers in healthy vs. diabetic MASMC primary cultures exposed to 'cytokine storm' cocktail *in vitro*.

2. To compare expression of signaling mediators of VSMC growth and migration in healthy vs. diabetic MASMC primary cultures exposed to 'cytokine storm' cocktail *in vitro*.

3. To compare expression of key transcriptional regulators of VSMC differentiation in healthy vs. diabetic MASMC primary cultures exposed to 'cytokine storm' cocktail *in vitro*.

Experimental Design. We will use MASMC primary cultures, isolated from healthy wild-type and diabetic agouti KKAy$^{+/-}$ mice, from our in-house cell culture banks for these studies. A subset of the wild-type MASMC cultures will be incubated with high glucose (30mM) to mimic the diabetic milieu *in vitro*. To induce systemic inflammation *in vitro* consistent with Covid-19-induced cytokine storm (CS), wild-type and diabetic MASMC cultures will be incubated with a cytokine cocktail mix containing the following components: IL2 (2ug), IL6 (1ug), IL10 (0.5ug), TNF-alpha (1ug), IFN-gamma (2ug), IL-4Ralpha (2ug), ACE2 (1ug), IL4 (1ug), IL13 (2ug). Our recipe for the CS cocktail is based on a recent publication where different CS concentrations were injected in murine models to study the cytokine storm-based mechanisms for extrapulmonary manifestations of SARS-CoV-2 infection (JCI Insight. 2023;8(10):e166012. https://doi.org/10.1172/jci.insight.166012). Cells will be exposed to different concentrations of CS cocktail (low, medium, high) for varying periods of time (short-term: 1d, 3d; long-term: 7d, 10d). This will be followed by cell harvests for the following in vitro assays.

INVESTIGATIVE METHODS TO BE USED

**Cell Culture:** We have already isolated MASMC primary cultures from wild-type and diabetic KKAy$^{+/-}$ mice in our lab and have multiple frozen vials stored at different passages. MASMC primary cultures will be grown in DMEM/F12 media supplemented with 10% FBS and appropriate antibiotic and antimycotic solutions. About 80% confluent cells will be allowed to grow overnight in serum-starved media. This will be followed by incubation of cells with different concentrations of CS cocktail for short-term (1-3 days) and long-term (7-10 days) duration. In a parallel study, following overnight serum-starvation, 80% confluent wild-type cells will be incubated with 30 mM glucose to mimic the diabetic milieu *in vitro* ± CS cocktail to induce systemic inflammation *in vitro*. Following these treatments, cells will be harvested and utilized for immunoblotting and immunocytochemistry experiments as outlined below.

**Immunoblotting**: Whole cell lysates will be prepared in 1X RIPA lysis buffer containing protease and phosphatase inhibitors. Protein content will be measured using the BCA protein assay. Equal concentrations of protein lysates (10-20 µg) will be resolved on 8-12% SDS-PAGE followed by wet transfer to PVDF membranes. Immunoblotting will be performed using the following antibodies: ACTA2, CNN1, LMOD1, SM22 (SM contractile markers); VIM, OPN (SM synthetic markers); p-ERK1/2, t-ERK1/2, p-p38, t-p38, p-AKT, t-AKT (VSMC signaling mediators); SRF, YY1, ELK1, KLF4 (transcriptional regulators of SM differentiation). Equal protein loading of samples will be confirmed by staining the membranes with Ponceau S and probing with tubulin or β-actin antibodies (loading controls).

**Immunocytochemistry**: Cells will be grown on coverslips in 6-well cell culture clusters and treated as described above under "Cell Culture". At endpoint, cells will be fixed and permeabilized in a solution containing 4% PFA and 0.2% TTX. Following this, cells will be blocked in 5% donkey serum followed by incubation with primary antibodies against SM contractile markers (ACTA2, CNN1, SM22). After a brief PBS wash, cells will be incubated with appropriate Alexa Fluor 594 or 647 secondary antibodies. Coverslips will then be mounted on DAPI-containing mounting media for cell nucleus visualization. To

control for non-specific staining, identical set of cells will be incubated with species-specific IgG control antibody in the absence of the corresponding primary antibodies or no primary antibody. Coverslips will be observed using the Olympus fluorescence IX71 microscope (10× magnification) and images will be digitally captured using an identical set of parameters across all samples, specific for each antibody.

PROPOSED METHOD OF DATA ANALYSIS

Each experiment will be repeated at least three times with two to three replicates for each treatment within an independent experiment. For immunofluorescence experiments, six to eight images will be collected for each individual treatment within an independent experiment. Densitometric quantification of immunoblots and immunofluorescence quantification will be performed using ImageJ software. All data will be presented as Mean ± SEM; statistical significance will be analyzed by one-way ANOVA (using GraphPad Prism Software) followed by post-hoc Tukey HSD test or unpaired Student's t-test (two-tailed), as appropriate. Any data that does not meet the assumptions of ANOVA will be analyzed using non-parametric statistics; $p \leq 0.05$ considered statistically significant.

SIGNIFICANCE OF ANTICIPATED FINDINGS

Expected Outcome. We predict that exposure to CS cocktail will induce VSMC phenotypic transition to a disease proatherogenic phenotype and this effect will be more pronounced in diabetic MASMC cultures compared to healthy MASMC.

Impact. The proposed studies will provide key pilot data that will lead to future studies aimed to interrogate the role of Covid-related cytokine storm in VSMC phenotypic transformation and development and progression of atherosclerotic complications under conditions of diabetes, obesity and hyperlipidemia, and further elucidate the underlying molecular mechanisms.

SUMMER RESEARCH FELLOW TRAINING/MENTORING PLAN

Plan for Training/Mentoring: The summer research fellow will be supervised and mentored by Dr. Priya Raman. The student will receive hands-on training from Dr. Raman and her laboratory personnel during the first 2-3 wks of the program. Upon demonstration of adequate independence, the summer fellow will be expected to run independent experiments under close supervision by Dr. Raman and her team. Dr. Raman will be responsible for student training on all aspects of this project, including data analysis and graphical presentations, interpretation of data and poster preparation and presentation. After the initial period involving casual interactions, Dr. Raman will meet with the student one-on-one at least once per week to discuss progress/data and will be trained in scientific reading relevant to the field of study. The student is also expected to participate in departmental seminars and weekly group meetings. These meetings will develop the student's research horizons and enhance his/her scientific presentation and perception skills. At the end of the training period, the student will be expected to submit a brief report summarizing the project and results as well as present work during NEOMED's Annual Poster Day.

Description of Resources available: The student will have access to Dr. Raman's laboratory, including all necessary supplies and reagents in her lab as well as other departmental core facilities, as needed for completion of the proposed studies. The summer fellow will also have access to graphing and imaging software, as needed.

Site where the research will be conducted: This project will be conducted in Dr. Raman's laboratory within the department of Integrative Medical Sciences at NEOMED.