Student Checklist (1A)

This form is required for ALL projects.

1. a. Student/Team Leader: Mary Sotiryadis Grade: 12

   Email: marysotiryadis@gmail.com Phone: 516-857-7980

   b. Team Member: _____ c. Team Member: _____

2. Title of Project:

   Bloodborne thrombin promotes the death of murine lymph node fibroblastic reticular cells

3. School: North Shore High School School Phone: 516-277-7000

   School Address: 450 Glen Cove Avenue, Glen Head, NY 11545

4. Adult Sponsor: Dr. Molly Mordechai Phone/Email: mordechaim@northshoreschools.org

5. Does this project need SRC/IRB/IACUC or other pre-approval? ☐ Yes ☑ No Tentative start date: 06/28/19

6. Is this a continuation/progression from a previous year? ☐ Yes ☑ No

   If Yes:

   a. Attach the previous year's ☐ Abstract **and** ☐ Research Plan/Project Summary

   b. Explain how this project is new and different from previous years on

   ☐☐ Continuation/Research Progression Form (7)

7. This year's laboratory experiment/data collection:

   06/28/19 (Actual Start Date: (mm/dd/yy)) 08/23/19 (End Date: (mm/dd/yy))

8. Where will you conduct your experimentation? (check all that apply)

   ☑ Research Institution ☐ School ☐ Field ☐ Home ☐ Other: _____

9. List name and address of all non-home and non-school work site(s):

   Name: Hospital for Special Surgery

   Address: 535 East 70th Street, New York, NY 1002

   Phone/email: 212-774-2532

10. **Complete a Research Plan/Project Summary following the Research Plan/Project Summary instructions and attach to this form.**

11. **An abstract is required for all projects after experimentation.**

3) Research Plan and Project Summary

A) RATIONALE

Inflammation is the body's response to irritation from a number of sources, including trauma, overuse, infection, and exposure to pathogens and chemicals (Stone, 2019). When an inflammatory response is underway, blood vessels dilate to increase blood flow to the affected area (Pober, 2014). The purpose of this is to catalyze interactions between immune cells and the affected area, inducing a natural relief to irritation. As a consequence, however, the contents of blood not necessarily related to the suppression of irritation can also leak out into the surrounding tissue. The extravasation of this fluid allows for molecules such as plasma proteins to interact with the cells in the surrounding tissue. This is especially pertinent to organs heavily involved in immune responses, such as lymph nodes.

Lymph nodes are comprised of various types of cells that interact systematically to foster an immune response. This study focuses on lymph node stromal cells due to their ability to maintain lymph node homeostasis and function in the movement of immune cells during an immune response (Chang, Turley, 2015). Specifically, fibroblastic reticular cells (FRCs), which create matrices throughout the stroma to support the survival of and interactions between immune cells (Figure 1), will be used. Without FRCs in the lymph nodes, B and T cell compartments would not be segregated, T cell numbers would be abnormal, B and T cells would not be recruited for active immunity, and the flow of lymph and smaller molecules would not be as effective (Chang, Turley, 2015).

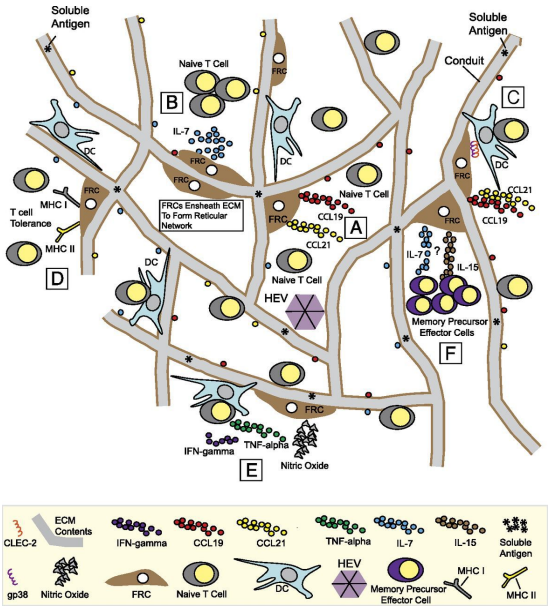

Figure 1: The cells involved in lymph node function (Chang, Turley, 2014). FRCs are woven throughout the stroma to foster interactions between immune cells.

Unpublished data in the lab have shown that plasma causes cell death in murine models *in vivo* while serum does not (personal communication, D. Dasoveanu). This may relate to the coagulation cascade, which occurs when blood clots (Figure 2). For this study, plasma from mice was tested on murine FRC cultures to further understand the previously observed phenomenon of cell death. Unlike plasma, in serum, thrombin, one of the most important molecules in the coagulation cascade, has been clotted out of the blood (Figure 2). Thrombin is responsible for the conversion of fibrinogen into fibrin, allowing for the formation of a blood clot. Because of its pivotal role, it was hypothesized that thrombin is the molecule in plasma responsible for causing FRC death. Therefore, it was predicted that cells exposed to plasma will die, cells exposed to plasma treated with a thrombin receptor inhibitor will exhibit less death, and cells exposed to plasma treated with a direct thrombin inhibitor (DTI) will not exhibit death.

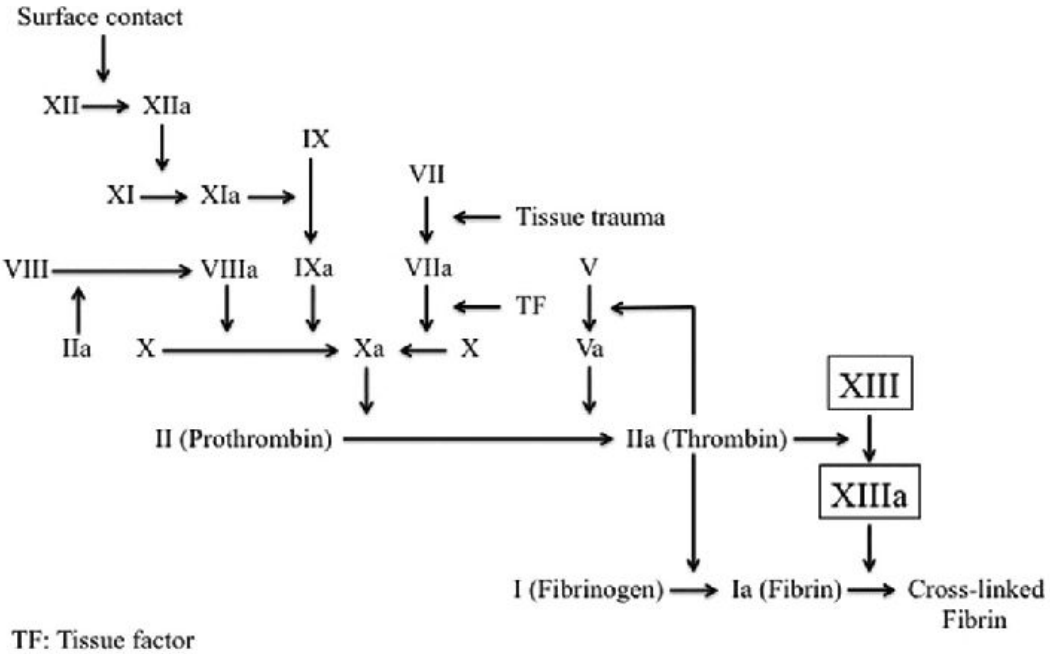

Figure 2: The coagulation cascade. When prothrombin, factor II, is cleaved to form thrombin, factor IIa, fibrinogen, factor I, is converted into fibrin, factor Ia, and a blood clot forms (Shimogawa et. al, 2017).

B) RESEARCH QUESTIONS, HYPOTHESES

Research Questions

1) How do varying concentrations of plasma affect the viability and morphology of FRCs?
2) How does a thrombin inhibitor added to plasma affect the viability of FRCs to which it is exposed?

Hypotheses

1) If plasma is toxic to FRCs, then FRCs will exhibit a change in morphology and decreased viability in the presence of plasma.
2) If thrombin is toxic to FRCs, then cells exposed to plasma plus a thrombin inhibitor will exhibit rescue compared to those treated with plasma-only controls.

C) Procedures, Risks and Safety, Data Analysis

Procedures

Harvesting Tissue and Blood

For this study, murine models will be used. In order to obtain the blood components and FRCs needed for experimentation, blood and lymph nodes will be harvested from freshly euthanized mice. Blood will be drawn using a 1 mL syringe and collected in 10 mL EDTA tubes for the collection of plasma.

EDTA tubes containing blood for plasma will be centrifuged at 2500 rpm for 10 minutes at 10ºC. Plasma supernatant will then be transferred to 100 µL Eppendorf tubes under the hood and labeled and stored at -20ºC until needed.

Then, inguinal, axillary, brachial, and mesenteric lymph nodes will be harvested and placed into one 15 mL Falcon tube with 2 mL of 5% BSA + RPMI + Collagenase solution to be digested. The RPMI solution is made from RPMI + 1% L-Glutamine + 1% PennStrep + 1% HEPES Buffer. After the lymph nodes are placed into the digestion solution, they will be kept on ice until needed.

All mice used for this study will be donated by fellow lab members who have no use for their blood and lymph nodes. No mice will be euthanized for the purpose of this study alone.

FRC Cell Culture

Using a glass pipette, media will be suctioned out of the lymph nodes, which will be chopped using a sterile surgical blade in a petri dish under the hood. Chopped tissue will then be incubated in 10 mL of a 1:1 solution of RPMI/BSA : Collagenase in fresh 15 mL Falcon tubes. These tubes will be placed in an orbital shaker at 50 rpm for 40 minutes at 37ºC in order to further digest the chopped tissue.

Tissue will be physically disrupted using a glass pipette under the hood and then strained for sterility into a 50 mL Falcon tube using a 70 µm cell strainer under the hood. The tissue will be centrifuged at 1200 rpm for six minutes at 4ºC, and the supernatant discarded. The pellet of cells will be resuspended under the hood in 1 mL 10% FCS + RPMI media. 20 µL of resuspended cells will be used for counting in a Coulter Counter. 1 x 10^7 cells will be plated per well in a 12-well tissue culture plate and incubated for 24 hours at 37ºC. The cells will be then washed twice with 1 mL of warm PBS to remove non-adherent cells and particles. Remaining FRCs will be incubated with 2 mL 10% FCS + RPMI media. Cells will be maintained by replacing media every three days until confluence.

Replating Cells

In order to work with FRCs from cell culture, they must be partitioned into smaller quantities. Cells in the 12-well tissue culture plate will be replated into 96-well tissue culture plates.

At 100% confluency, FRCs will be washed twice with PBS and then trypsinized using 500 µL of warm 0.25% trypsin under the hood. Cells will be centrifuged at 1200 rpm for six minutes at 4ºC. The pellet will be resuspended under the hood in 1 mL 10% FCS + RPMI media.

The cells will then be counted in a hemocytometer and plated at 15,000 cells per well in 96-well plates in 200 μL of 10% FCS + RPMI media under the hood.

Treatment of Cells

For this study, cells will be treated with plasma, plasma + PAR1 inhibitor, plasma + DMSO, and plasma + hirudin from Sigma Aldrich. Proper working dilutions will be determined in the initial steps of experimentation.

Treated cells will be incubated for 24 hours at 37ºC. After this period, qualitative and quantitative data will be collected.

Qualitative data will be collected by assessing changes in FRC morphology via micrographs. Images of each well of cells will be taken 24 hours after treatment in order to assess the effects of plasma on FRC morphology and viability.

Quantitative data will be collected through cell viability assays using crystal violet. 24 hours after being treated, the cells will be stained with Crystal Violet and the mean absorbance of each well will be recorded using a Tecan Plate Reader. Comparing the differences in mean absorbance readings across different groups will reveal the effect of plasma on cell viability.

Risk and Safety

Working with euthanized mice and certain reagents will present some considerable safety risks. To mitigate harm caused by coming into contact with mice or their cells, gloves and a full length lab coat will be worn at all times. Gloves will be disposed of in a toxic waste bin after handling mice and their cells. Goggles will be worn when working with hazardous reagents, like Crystal Violet. A mask was worn when measuring out SDS, which was needed to solubilize cell stains. Pants and closed toe shoes will be worn.

After conducting cell viability assays using Crystal Violet solution, both the crystal violet solution and the cells will be disposed of properly. Crystal Violet solution will be rinsed off of the cells in a large beaker of water. The contents of this beaker will then be poured down the drain of a sink with the water running. The sink will be clear before disposing of the diluted Crystal Violet Solution.

In order to dispose of the cells, a 10% dilution bleach will be poured into each well of the 96-well plate used for cell viability assays. The mixture of bleach and cells will be left to sit for 10-15 minutes before being disposed of in the hazardous waste bin.

Data Analysis

Qualitative data will be collected via cell viability assays. These assays will use the mean absorbance of Crystal Violet solution as a metric for showing cell viability. With these data, an ANOVA test will be conducted. If statistical significance is found, a Tukey post-hoc test will be performed to locate specific areas of significance among the data.

D) Bibliography

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3) Potentially hazardous biological agents research

When working with euthanized mice, gloves and a lab coat will be worn at all times. Tissue will be digested and cultured underneath the hood to ensure sterility. Blood plasma will be stored in sterile eppendorf tubes. Mouse tissue and plasma will be discarded of in BioHazard containers when experimentation is finished. When experimentation with cultured tissue is finished, cells will be bleached and discarded in a BioHazard container.

4) Hazardous chemicals, activities, and devices

I will work with Crystal Violet when conducting cell viability assays. When working with Crystal Violet solution, gloves and a lab coat will be worn at all times to mitigate the risk of skin contact. When I rinse the Crystal Violet stain off of the cells, it will be done in a laboratory sink with a constant flow of water. When I am finished working with the stained cells, I will bleach the cells and discard them in a BioHazard container.

Post Research Summary

Initial experimentation showed that 1:10 dilutions of plasma kill most FRCs, while plasma dilutions of or above 1:100 do not have a visible effect on the viability of FRCs. Therefore, subsequent experiments used primarily 1:20 and 1:50 plasma dilutions. These dilutions were tested at least four times.

In terms of PAR1 inhibitor and hirudin concentrations, 1:20 and 1:60 dilutions were used in order to show the effects of thrombin on cell death when the concentration of plasma was equal to or greater than the concentration of its inhibitors. These dilutions were tested at least twice.