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

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Abstract

Autoimmune diseases, including Rheumatoid Arthritis and Systemic Lupus Erythematosus, are characterized by symptoms of chronic tissue swelling and inflammation. It remains largely unknown what effect this chronic inflammation has on lymph nodes, the major sites of immune cell activation. During inflammation, increased leakiness in blood vessels leads to the death of lymph node fibroblastic reticular cells (FRCs) in mice (unpublished data). The objective of the research described herein was to confirm this phenomenon and to determine the components of blood that cause the death of FRCs. It was hypothesized that a) thrombin is the molecule in plasma responsible for the observed cell death and b) cells exposed to a combination of plasma and an inhibitor of the cellular thrombin receptor (Protease-Activated Receptor 1, or PAR1) will not exhibit a significant decrease in cell viability. FRCs were exposed to varying plasma dilutions to determine how much plasma is needed to cause significant cell death. FRCs were then treated with varying dilutions of plasma + PAR1 inhibitor. Finally, FRCs were treated with plasma + hirudin, a direct thrombin inhibitor from the salivary glands of leeches. Results show a rescue in plasma-treated FRCs in the presence of PAR1 inhibitor or hirudin. The data suggest that thrombin is one of the blood components extravasated during chronic inflammation that significantly decrease the viability of FRCs. The observed cell death could potentially hinder the ability of FRCs to facilitate the activity of other immune cells, such as B and T cells.

Introduction

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, Sessa, 2015). 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 essential for responding to new infections. They foster the interactions between pathogen proteins and T cells, which then leads to the immune response against that pathogen (Milling, n.d.). A common sign of inflammation is swelling, especially of the lymph nodes. This sensation is prolonged in those who struggle with autoimmune disorders such as Rheumatoid Arthritis and Lupus. It is imperative to understand how the extravasation of blood contents into tissue such as the lymph nodes affects its cells, especially in the case of chronic inflammation, as these tissues are constantly being exposed to blood contents. There are many blood vessels in lymph nodes, thus increasing the amount of extravasated fluids a single lymph node may be exposed to (Figure 1).

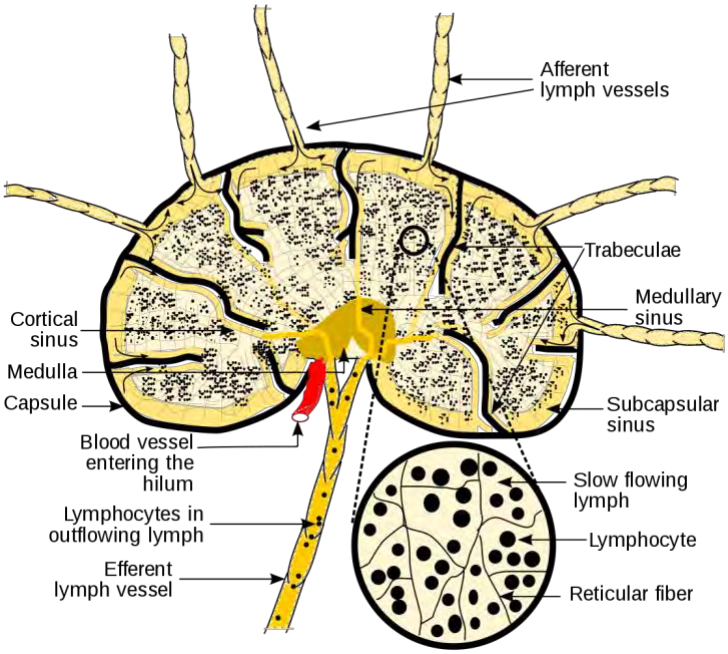

Figure 1: The physiology of a lymph node. Blood vessels run throughout the lymph node and into the medullary sinus, demonstrating the potential impacts of blood vessel leakage (Panchal, 2008).

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 2), were 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).

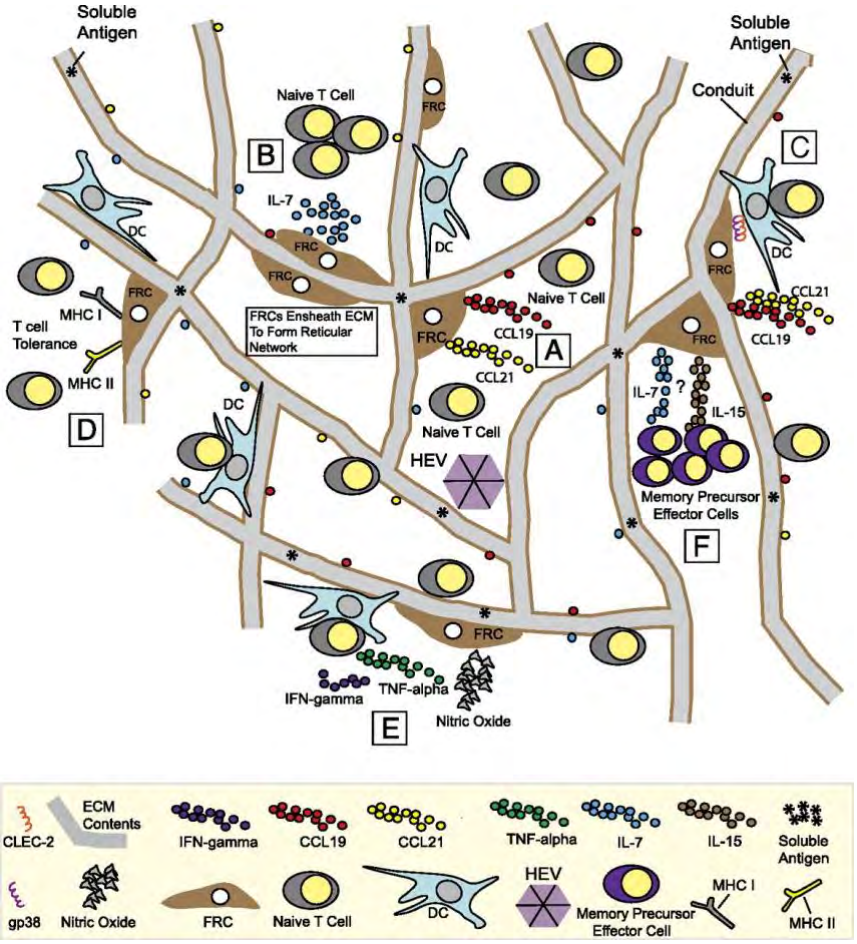

Figure 2: The cells involved in lymph node function (Chang, Turley, 2015). FRCs are woven throughout the stroma to foster interactions between immune cells.

Those with autoimmune diseases such as Rheumatoid Arthritis or Systemic Lupus Erythematosus experience periods of chronic inflammation, which is central to the focus of this research. While the inflammatory response is necessary for immune cells to positively act on an afflicted area, a continuous response can lead to prolonged pain and irritation. Understanding the effects of chronic inflammation

allows us to determine whether or not the constant exposure of tissue to extravasated blood contents is beneficial or detrimental to one's health.

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 3). For this study, plasma from mice was tested on murine FRC cultures to further understand the previously observed phenomenon of cell death. Both plasma and serum are derived from blood samples, but the collection and components of each differ. Plasma is harvested from the blood source and immediately collected in containers with anticoagulants (Yu et. al, 2011). Serum is collected from blood that has already coagulated following its collection. It is centrifuged in order to separate it from other blood components, which causes platelets to release cytokines and metabolites into the serum (Yu et. al, 2011). Unlike plasma, in serum, thrombin has been clotted out of the blood (Figure 3).

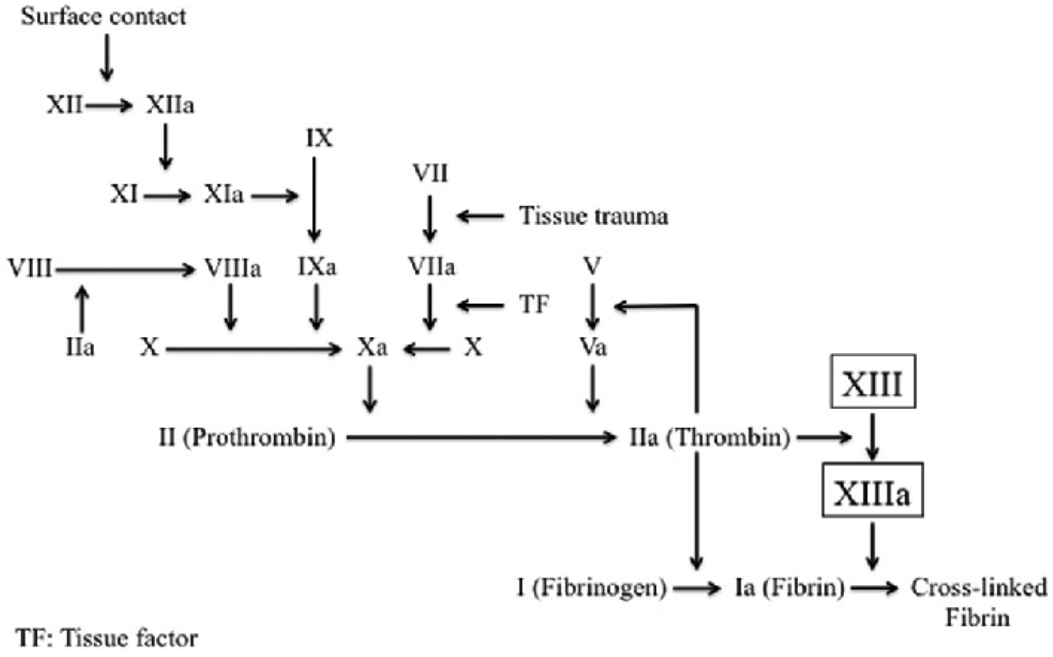

Figure 3: The coagulation cascade. When prothrombin, factor II, is cleaved to form thrombin, factor IIa, fibrinogen, factor I, is converted into fibrin, factor Ia, and a blood clot forms (Shimogawa et. al, 2017).

One of the most important molecules in the coagulation cascade is thrombin. 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.

Methods

Harvesting Tissue and Blood

For this study, murine models were used. In order to obtain the blood components and FRCs needed for experimentation, blood and lymph nodes were harvested. Blood was drawn from freshly euthanized mice using a 1 mL syringe and collected in 10 mL EDTA tubes for plasma. Then, inguinal, axillary, brachial, and mesenteric lymph nodes were 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 were placed into the digestion solution, they were kept on ice until next needed.

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

Preparing Plasma

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

FRC Cell Culture

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

Tissue was 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 was then centrifuged at 1200 rpm for six minutes at 4ºC. The supernatant was discarded. The pellet of cells was then resuspended under the hood in 1 mL 10% FCS + RPMI media. 20 µL of resuspended cells were used for counting in a Coulter Counter. 1 x 10_7 cells were plated per well in a 12-well tissue culture plate and incubated for 24 hours at 37ºC. The cells were then washed twice with 1 mL of warm PBS to remove non-adherent cells and particles. Remaining FRCs were incubated with 2 mL 10% FCS + RPMI media. Cells were maintained by replacing media every three days until confluence was reached at approximately six days.

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 were replated into 96-well tissue culture plates.

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

The cells were then 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 were treated with plasma, plasma + PAR1 inhibitor, plasma + DMSO, and plasma + hirudin from Sigma Aldrich. The main dilutions used during experimentation were 1:10, 1:20, 1:50, and 1:60 dilutions in 10% FCS + RPMI culture media.

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. These dilutions were used 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.

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

Microscopy

In order to assess morphological changes caused by treatments, micrographs of cells were taken using a microscope with a Canon lens. In order to avoid condensation on the tissue culture plate lid affecting the quality of the micrographs, each well was photographed through a coverslip instead. PBS was added into each well until a dome of liquid formed. Glass coverslips were then placed over the wells slowly as to not form bubbles.

The micrographs were taken under 400x magnification. Micrographs between the control groups and experimental groups were compared in order to assess the qualitative effects of various treatments on FRCs.

Cell Viability Assay

In order to collect quantitative data, a crystal violet assay for cell viability was conducted. Cells were washed with PBS and then incubated for 10 minutes with 50 µL of crystal violet solution (0.2% crystal violet, 2% ethanol (95%), and 97.8% dH_2O) per well at 25ºC. After 10 minutes, the plate was washed twice in a beaker of warm water. The plate was then drained upside down on paper towels. 1% SDS solution was added to each well thereafter to solubilize the stain. The plate was then agitated in an orbital shaker at 25 rpm and 25ºC until the purple color inside each well was uniform. The absorbance of each well was read at 570 nm using a Tecan Plate Reader.

Results

Incubation of FRCs with Plasma Causes Cell Death

The effects of varying dilutions of plasma on the viability of FRCs were assessed qualitatively through microscopy. Morphologically, healthy FRCs appear elongated, exhibiting contact inhibition as they adhere to the bottom of the tissue culture plates. When compared with the micrographs of the control group (Figure 4A), micrographs of FRCs treated with high plasma dilutions suggest that high concentrations of plasma cause cell death (Figure 4B). Micrographs of FRCs treated with lower dilutions of plasma (Figure 4C) show cells that are morphologically similar to FRCs under control conditions (Figure 4A). This suggests that low concentrations of plasma are not toxic to FRCs.

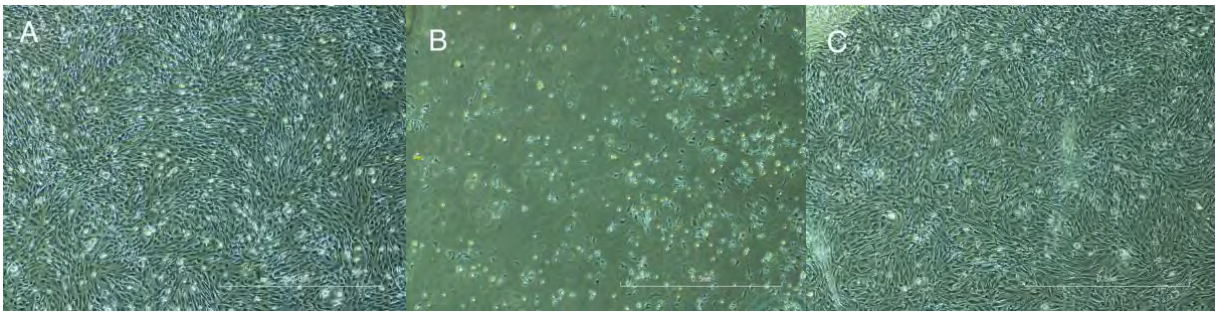

Figure 4: Micrographs of FRCs (A) exposed to only tissue culture media (control conditions), (B) treated with a 1:10 plasma dilution, (C) treated with a 1:100 plasma dilution. The photo scales are each 0.5 mm.

Based off of the qualitative data, a high concentration of plasma causes most FRCs to die and a low concentration of plasma does not affect FRC viability. In quantifying this data, there was a statistically significant difference in cell viability between the groups ($p < .05$) (Figure 5).

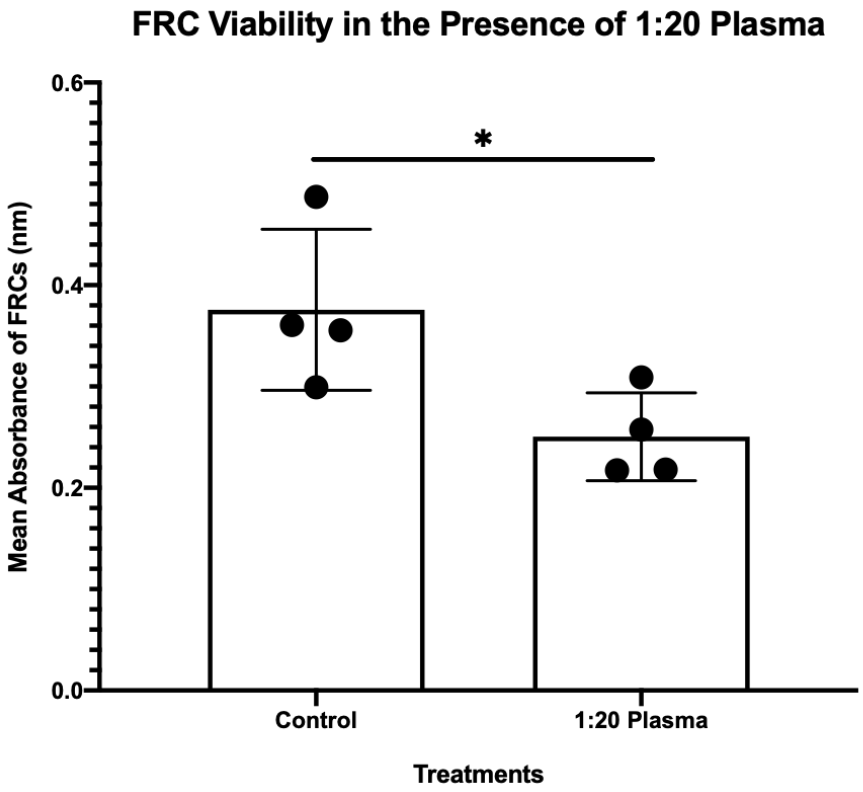

*Figure 5: Viability of FRCs under control conditions vs. viability of those exposed to a 1:20 plasma dilution. Error bars represent +/-1 SD (*p < .05, unpaired Welch's t-test).*

Inhibition of PAR1 Results in Rescue of FRCs Exposed to Plasma

Cells were treated with dilutions of plasma + PAR1 inhibitor in order to block the major site of thrombin binding, thus blocking the signal for cell death from being transduced. A one-way ANOVA test shows statistical significance among these data ($p < .01$). A Tukey post-hoc test shows that when compared to cells treated with 1:20 plasma only, significantly more cells survive under 1:20 plasma + 1:20 PAR1 inhibitor conditions ($p < .01$) (Figure 6). A Tukey post-hoc test also shows that when compared to cells treated with 1:20 plasma + 1:20 DMSO, significantly more cells survive under 1:20 plasma + 1:20 PAR1 inhibitor conditions ($p < .05$) (Figure 6). The PAR1 inhibitor used is dissolved in DMSO, which is why a DMSO dilution was used as a control. Finally, a Tukey post-hoc test shows that when compared to cells treated with 1:20 plasma + 1:60 PAR1 inhibitor, significantly more cells survive under 1:20 plasma + 1:20 PAR1 inhibitor conditions ($p < .01$) (Figure 6).

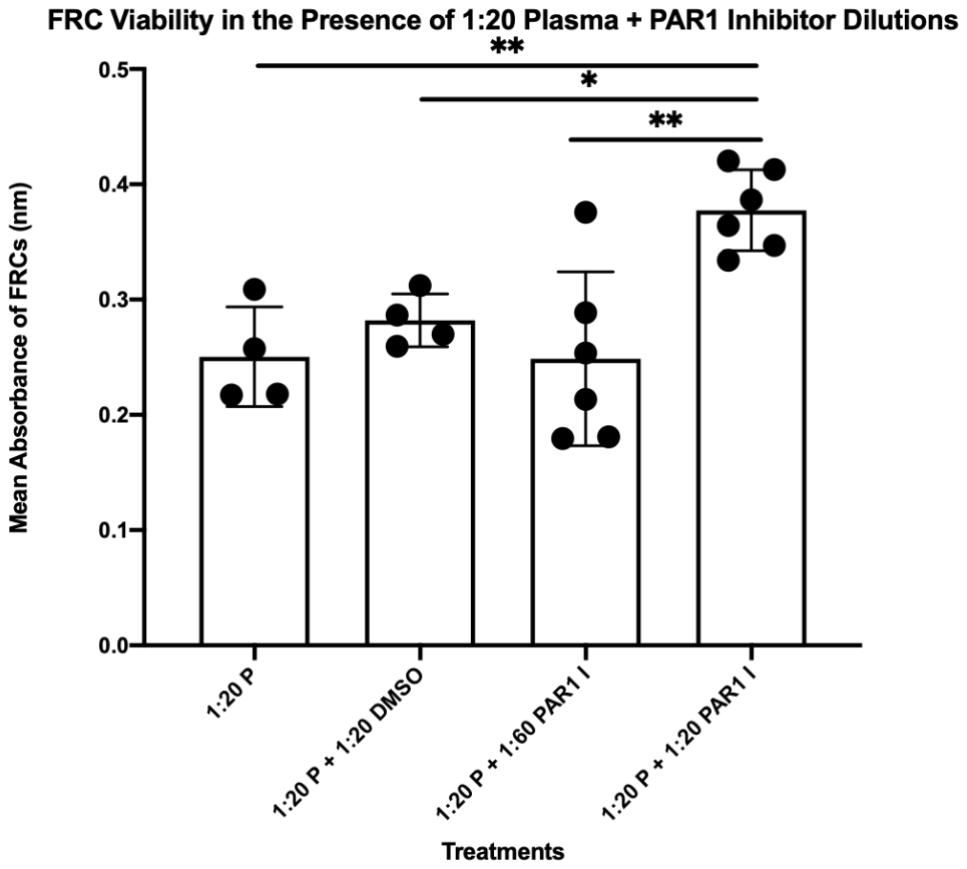

*Figure 6: Cell viability of FRCs exposed to 1:20 plasma dilutions with and without PAR1 inhibitor. Error bars represent +/- 1 SD (*p < .05, **p < .01, by a Tukey post-hoc test).*

Blocking Thrombin Activity Rescues FRCs Exposed to Plasma

In order to determine if thrombin is necessary for cell death, hirudin was added to cells incubated with plasma. This was needed to deduce if thrombin is the only molecule that acts on PAR1 to transduce the signal for cell death. FRCs exposed to plasma were treated with hirudin, a direct thrombin inhibitor. When a 1:60 dilution of hirudin was added to FRCs exposed to plasma, average cell viability increased marginally (Figure 7). This effect was somewhat elevated at a higher concentration of hirudin (1:20) (Figure 7) but these data were not statistically significant by a one-way ANOVA test.

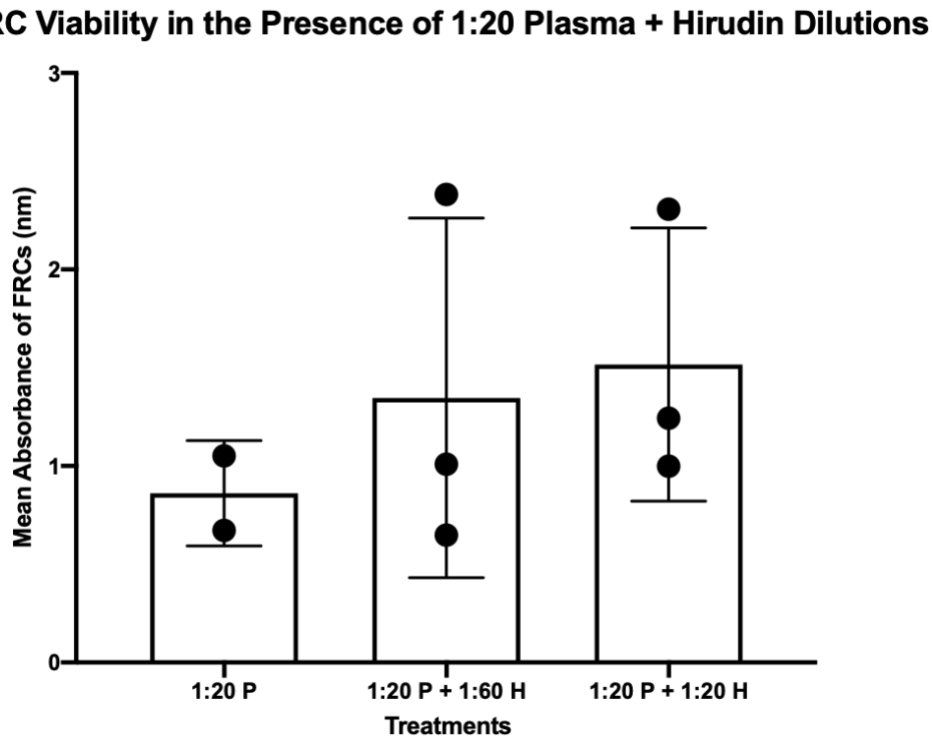

Figure 7: Viability of FRCs exposed to 1:20 plasma and 0, 1:20 or 1:60 dilutions of hirudin. Error bars represent +/- 1 SD.

Discussion

Plasma makes up 55% of blood (Mathew, Varacallo, 2019). Therefore, extravasated fluid from blood vessels contains mostly plasma. During inflammation, blood vessels dilate in order for immune cells to reach the area of the body affected by an invader (Pober, Sessa, 2015). As blood vessels expand, the likelihood of plasma leaking out increases. While the effects of the toxicity of plasma may not drastically compromise the health of someone unaffected by an autoimmune disease, these effects can be extremely damaging to the tissues of one who is chronically undergoing an inflammatory response. Conditions such as Rheumatoid Arthritis and Systemic Lupus Erythematosus are characterized by symptoms of chronic inflammation. Therefore, the tissues of these patients are constantly exposed to the fluids that have leaked out of blood vessels. Micrographs of FRCs incubated with high concentrations of plasma show severe morphological changes and cell death (Figure 4B). Cell viability assays reflect this trend quantitatively (Figure 5). Based on these results, it is clear that plasma extravasated during an inflammatory response can drastically affect surrounding tissue.

The purpose of this research was to uncover the plasma component responsible for the toxic effects it has on FRCs. One potential candidate was thrombin due to its central role in the coagulation cascade (Figure 3). Thrombin is responsible for cleaving fibrinogen into fibrin and thus allowing for a

blood clot to form. Moreover, because thrombin is the single protease that activates PAR1 (Coughlin, 1999), various concentrations of a known PAR1 inhibitor were added to plasma in order to determine the effects of thrombin on FRC viability. PAR1 is a G-protein coupled receptor. When it comes in contact with thrombin, the N-terminus is proteolytically cleaved. This newly formed N-terminus acts as a tethered ligand which activates the receptor and tranduces multiple signaling pathways (Neiman, Schmaier, 2007). Data from research involving Vorapaxar, a PAR1 antagonist, show that PAR1 inhibition slows the coagulation cascade and the inflammatory response (Schoergenhofer et al., 2018). Data from experiments involving astrocyte and hippocampal neuron exposure to thrombin suggest that thrombin signals for death in these cell lines (Donovan et al., 1997). The results from experiments with neurons are likely similar to the results from this study because both neurons and fibroblasts are mesenchymal cells (Wislet-Gendebien et al., 2005).

The results of experiments involving dilutions of plasma and PAR1 inhibitor suggest that thrombin is in fact the molecule in plasma that causes FRC death, supporting my hypothesis that thrombin is the component of plasma that causes cell death (Figure 6). To determine thrombin's role in cell death seen during inflammation, FRCs were incubated with hirudin at the time of exposure to plasma. Hirudin blocks thrombin's proteolytic activity in plasma when it binds to thrombin because it is a direct thrombin inhibitor (DTI) (Lee, Ansell, 2011). Hirudin and its derivatives, such as lepirudin and desirudin, bind thrombin at its active site and exosite I rather than blocking its cellular receptor (Figure 8). The results of these experiments do not support the hypothesis that thrombin is necessary to cause cell death. Quantitative data show that hirudin-treated plasma does not result in a statistically significant rescue when compared to cells that are exposed to unaltered plasma, although a trend may be emerging (Figure 7). Future experimentation is needed to determine if thrombin is necessary for FRC death. Specifically, higher concentrations of hirudin should be explored because a higher amount of the inhibitor would be better able to counteract equal or lesser volumes of plasma, as shown when comparing 1:20 plasma + 1:20 hirudin against 1:20 plasma + 1:60 hirudin (Figure 7). However, it is possible that increasing concentrations of hirudin could yield similar results. In this case, thrombin alone would not be necessary for cell death and other molecules in plasma that may transduce the signal for cell death would be explored.

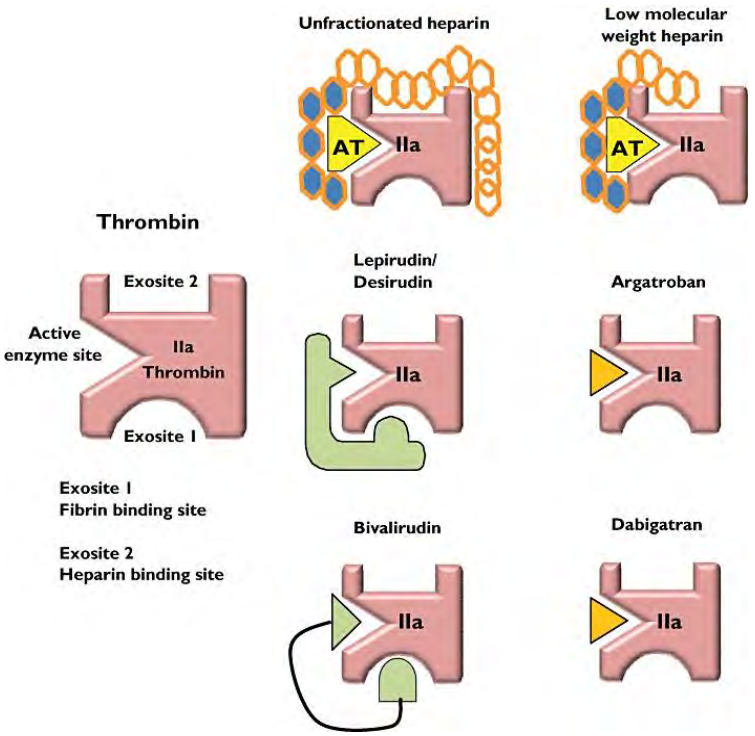

Figure 8: A diagram of how thrombin is inhibited using DTIs (Lee, Ansell, 2011). Lepirudin and desirudin are derivatives of hirudin from leeches. This diagram shows that these DTIs bind to thrombin's active site as well as its exosite I, where fibrin binds, in order to inhibit thrombin directly.

Based on unpublished data, FRCs treated with serum do not undergo cell death (personal communication, D. Dasoveanu). One of the fundamental differences between plasma and serum is that plasma is uncoagulated blood whereas serum is blood that has clotted already (Yu et al., 2011). During the coagulation cascade, a myriad of molecules are activated through chemical processes to ultimately lead to the conversion of fibrinogen to fibrin, thus allowing for a blood clot to form (Overbey et al., 2014). Before this reaction occurs, thrombin is proteolytically cleaved from prothrombin and therefore clotted out of the blood. Similar changes have been shown in over 100 other metabolites in serum (Yu et al., 2011). It is likely that plasma causes cell death but serum does not because plasma is composed of molecules that have not been clotted out of the blood during the coagulation cascade (Yu et al., 2011), allowing thrombin to signal for cell death.

The anatomy of a human lymph node shows that there is a complex network of blood vessels woven throughout the body of the node (Figure 1). FRCs in the lymph nodes of patients who are afflicted by chronic inflammation are therefore constantly being killed by plasma. Other immune cells found in lymph nodes have an interdependent relationship with FRCs (Chang, Turley, 2015). If FRCs die, they cannot support the interdependent network of immune cells that exist within the lymph nodes (Figure 2).

Therefore, during an immune response, the immune cells from lymph nodes would not be able to efficiently do their jobs. Clearly, the symptoms of chronic inflammation in a patient with an autoimmune disease have detrimental impacts on important tissues such as lymph nodes. The current research elucidates one pathway by which the inflammatory response can be modulated. By understanding the potential harms that the symptoms of inflammatory diseases can cause, doctors and researchers can develop improved methods of controlling and potentially treating these conditions.

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