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Plasminogen: Binding and Activation on Mesothelial Cell Surface

by

Zachary Ditzig

A thesis

Submitted in partial fulfillment

of the requirements for the degree of

Master of Science in the Department of Biological Sciences

Idaho State University

Spring 2021
To the Graduate Faculty:

The members of the committee appointed to examine the thesis of Zachary Ditzig find it satisfactory and recommend that it be accepted.

____________________________________________________
Dr. Kinta Serve,  
Major Advisor

____________________________________________________
Dr. Peter Sheridan,  
Committee Member

____________________________________________________
Dr. Jared Barrott,  
Graduate Faculty Representative
September 20, 2018

Kinta Serve
Biological Sciences
MS 8007

RE: regarding study number IRB-FY2019-53: Analysis of Banked Sera from Libby, MT

Dear Dr. Serve:

I agree that this study qualifies as exempt from review under the following guideline: Category 4. Research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects.
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Sincerely,

Ralph Baergen, PhD, MPH, CIP
Human Subjects Chair
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<tr>
<td>ANAX2</td>
<td>Annexin A2</td>
</tr>
<tr>
<td>DTSSP</td>
<td>3,3’-Dithiobis (sulfosuccinimidyl propionate)</td>
</tr>
<tr>
<td>EACA</td>
<td>ε-aminocaproic acid</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>ENO-1</td>
<td>α-Enolase</td>
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<tr>
<td>LA</td>
<td>Libby Amphibole</td>
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<td>LPT</td>
<td>Lamellar Pleural Thickening</td>
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<tr>
<td>MCAA</td>
<td>Mesothelial Cell Autoantibody</td>
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<tr>
<td>PAD</td>
<td>Peptidyl Arginine Deiminase</td>
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<td>PAS</td>
<td>Plasminogen Activation System</td>
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<td>PLA</td>
<td>Plasmin</td>
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<td>Plasminogen</td>
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<td>PLG-R</td>
<td>Plasminogen Receptor</td>
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<td>PTM</td>
<td>Post-translational Modification</td>
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<td>RA</td>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
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Mesothelial cell autoantibodies (MCAA) are a form of tissue-specific autoantibody found in populations exposed to Libby Amphibole asbestos. We demonstrate that MCAA target plasminogen at the mesothelial cell surface. Mesothelial cells have not been previously recorded as binding plasminogen, meaning the receptors for plasminogen have also not been reported. Flow cytometry and plasmin activity assays were used to determine the plasminogen receptors on the surface of mesothelial cells. Mesothelial cells were found to express four plasminogen receptors on their surface including: α-Enolase, Annexin A2, Plg-RKT, and Histone H2B. Additionally, we assessed the effect of citrullination on plasmin activity, since this post-translational modification is common in systemic autoimmune diseases. Citrullination inhibited the activation of plasminogen on mesothelial cells but increased binding of MCAA. Together, these data help us understand the PLG-MCAA-mesothelial cell binding mechanism, and takes us one step closer to the development of a therapeutic for Libby Amphibole associated pathologies.

Keywords: Plasminogen, Plasmin, citrullination, Mesothelial cell autoantibodies
Asbestos exposure has been a health concern in the United States since the 1960s when a series of papers was published linking asbestos inhalation with mesothelioma development [1, 2]. An area with a high incidence of asbestos exposure is the mining town of Libby, located in northern Montana. In Libby, vermiculite was mined that was contaminated with a mixture of amphibole asbestos fibers, identified as 84% winchite, 11% richterite, and 6% tremolite [3]. This mixture is referred to as Libby amphibole (LA), and differs from other asbestos forms (i.e. chrysotile) due to its needle-like structure. The mining activities in Libby resulted in high rates of LA asbestos exposure among its residents [4, 5]. Notably, the amphibole asbestos exposure was not only occupational, but fibers were also present in the overall environment, increasing inhalation risk to the entire population of Libby and surrounding towns [6].

Amphibole asbestos has been uniquely linked to an increase in autoimmune disease and progressive pleural thickening in exposed populations [7, 8]. There is an increased risk of developing a rapidly-progressing, highly inflammatory, non-malignant pleural fibrosis, termed Lamellar Pleural Thickening (LPT) among the LA-exposed population [9, 10]. LPT is a predominant cause of mortality and morbidity among the LA-exposed population [5, 11]. Fibrotic diseases like LPT develop through remodeling of the extracellular matrix (ECM) in a manner that impairs organ function [12]. The ECM is a network of macromolecules that surround cells, contribute to cell morphology, and support organ structure. Remodeling of this network is utilized by the body to maintain organ homeostasis and for wound repair but can result in the generation of a scar if ECM homeostasis is disrupted [13]. This remodeling is facilitated by proteinases, like matrix metalloproteinases (MMPs), that degrade the ECM. MMPs are produced as zymogens or inactive forms of the protein and are secreted by cells. They
become activated by serine proteases, like plasmin, or by thiol group modifications via oxidation [14]. Dysregulation of ECM remodeling can result in excessive deposition of ECM components like collagen that can lead to impaired organ function. Pathogenic dysregulation of ECM remodeling is termed fibrosis and is observed in the pleural cavity of individuals with LPT [9, 10].

LA exposure not only increases the chance of developing LPT but may also increases the production of autoantibodies that drive inflammation and contribute to LPT progression [7, 15]. Among LA-exposed people, 61.3% are positive for anti-nuclear antibodies (ANA) [16, 17], which are associated with the development of systemic autoimmune diseases like rheumatoid arthritis (RA) and systemic lupus erythematosus [18]. In addition to these systemic autoantibodies, mesothelial cell autoantibodies (MCAA) were identified in 18.5% of an LA-exposed cohort [17]. MCAA are a form of tissue-specific autoantibody that may play a role in pulmonary disease [17]. In a study from 2013 by Serve et al., the binding of MCAA \textit{in vitro} led to an increase in collagen I deposition by mesothelial cells as well as to an increase in the expression of MMP 8 and 9. The formation of the collagen matrices was dependent on the MMP activity, suggesting a mechanism of MCAA-induced fibrosis by increasing collagen deposition via increasing MMP expression [19]. In a 2016 study by Gilmer \textit{et al.}, the binding of MCAA \textit{in vivo} led to increased collagen deposition in the peritoneal cavity of mice. Notably, the mice were treated with just the MCAA antibodies and were not exposed to any asbestos. This demonstrates that not only do MCAA induce collagen deposition \textit{in vivo} without asbestos exposure but also suggests a mechanism by which MCAA could play a role in the development of LPT [20]. MCAA have also been shown to positively correlate with the appearance of pleural abnormalities, like LPT, and may be a marker of pleural disease progression [17, 19, 20].
Because MCAA are likely involved in LA-associated plural disease development, we were interested in studying these MCAA to identify potential therapeutic targets that could slow or halt the development of LPT.

In order to target MCAA for the development of a therapeutic target, we must first understand the interactions between MCAA and the mesothelial cell surface. This involves determining all proteinaceous targets of MCAA and the responses they elicit upon binding. In a study from 2016 by Hanson et al., a cellular target of MCAA was identified as plasminogen (PLG). Additionally, it was found that not only do MCAA target PLG but that commercial antibodies against PLG were able to induce \textit{in vitro} collagen I deposition at levels comparable to those seen with MCAA binding [21]. Since the PLG cascade plays a role in the activation of MMPs (Figure 1), this study suggests a potential pathway for the excessive deposition of collagen. Specifically, PLG becomes activated into plasmin, which then activate MMPs for ECM remodeling. Disruption of this cascade may lead to excessive deposition of ECM macromolecules like collagen. However, in Hanson’s study (2016), when MCAA positive sera (MCAA (+ve)) were cleared of PLG-binding proteins, the level of collagen I deposition did not return to control levels [21]. This discrepancy in collagen deposition could be due to a variety of factors including the presence of another MCAA target on mesothelial cells or failure to completely clear all PLG-binding proteins from the sera. Since MCAA were identified by their binding to mesothelial cell surfaces and not to any one single protein, we hypothesized that these antibodies were polyclonal and targeted multiple proteins on the mesothelial cell surfaces [22]. Thus, one goal of the work presented here was to identify additional MCAA targets on mesothelial cells.
To identify additional MCAA targets, we took a different approach than that taken by Hanson et al. This group identified surface proteins through labeling all cell-surface proteins via biotinylation followed by shearing extracellular domains and isolating labeled proteins with a streptavidin column. The isolated proteins were then separated out via size-exclusion chromatography and run through a MCAA-binding ELISA [21]. This method of protein isolation, while isolating surface proteins, may leave out potential integral membrane proteins, proteins with low expression, and inducible proteins [23]. We hypothesized that using an alternative approach would allow for additional proteinaceous MCAA targets to be identified. This alternative approach determines MCAA targets through use of a different chemical crosslinker. 3,3’-Dithiobis (sulfosuccinimidyl propionate) (DTSSP) can be used to crosslink the MCAA with their targets on the cell surface, and then those targets can be isolated via immunoprecipitation with Protein A/G beads. We predicted that this method would better identify all MCAA targets by isolating all the proteins that MCAA bind without missing proteins with low expression or integral membrane proteins. Therefore, we used DTSSP to crosslink the MCAA to their protein target before lysing the cells and then performed immunoprecipitation and isolation of the MCAA-bound targets. Since DTSSP is cleaved by dithiothreitol, the proteins became separated in SDS-loading buffer, and, thus, the cellular proteins were isolated via SDS-PAGE. Proteins isolated from cells exposed to sera from LA-exposed individuals without MCAA (MCAA (-ve)) were used as a negative control in order to identify protein targets unique to MCAA.

**Plasminogen (PLG) Structure and Activity**

PLG is a plasmin (PLA) zymogen that, once activated, works as a serine protease in ECM degradation and fibrinolysis [24]. The activation of PLG into PLA is regulated by a
network of processes collectively known as the plasminogen activation system (PAS) [25]. The PAS is a highly regulated system that plays a role in a plethora of physiological processes, such as fibrinolysis, inflammation, thrombolysis, embryonic development, cancer progression, and wound healing [26-30]. Dysregulation of the PAS results in irregular wound healing, chronic inflammation, and infection [31]. Since PLG was identified as a MCAA target, we were interested in examining the role that PLG-mesothelial cell interactions play in the development and progression of LA-induced pathogenesis, like LPT. We hypothesized that MCAA binding to PLG would block its conversion to PLA, potentially preventing cleavage and activation.

At the N-terminus of native PLG there is a glutamate amino acid, denoting native PLG as Glu-PLG [32]. This form of PLG is compact and contains intermolecular forces amongst its 5 kringle domains and the N-terminus, thus protecting the bond that gets cleaved to form PLA (Arg561-Val562) [33]. When the N-terminus of Glu-PLG is truncated to either Lys62, Arg68, or Lys77, the conformation of PLG becomes altered to an open conformation and is referred to as Lys-PLG [34]. Formation of Lys-PLG from Glu-PLG is catalyzed by PLA concomitantly with the activation of PLG [32] (Figure 2). Because of the shift to an open conformation that removes the protection for the Arg561-Val562 bond, Lys-PLG is more easily converted to PLA than Glu-PLG [35]. Although Glu-PLG is the only form of PLG that is found natively in human sera, Lys-PLG is commonly used in studies because it binds to cells with higher affinity, and cells actively convert Glu-PLG to Lys-PLG before binding it [36]. Lysine-plasmin (Lys-PLA) is the final enzymatically active form of PLA and is generated one of two ways. First, Glu-PLG can be converted to glutamate-plasmin (Glu-PLA) through the activity of a plasmin activator, either urokinase-type plasminogen activator (uPA) or tissue plasminogen activator (tPA). Glu-PLA is then converted to Lys-PLA by another PLA via truncation to Lys62, Arg68, or Lys77 [37].
Alternatively, Lys-PLA is also generated from Lys-PLG through the activity of tPA or uPA (Figure 2). Since Lys-PLA is the final enzymatically active form of PLA, it is the form bound and utilized by cells, meaning all forms of PLG and PLA are converted to Lys-PLA before use [38]. For this reason, Lys-PLA is the form of PLA used and referenced in this thesis unless otherwise specified.

**Plasminogen Binding and Activation at the Cell Surface**

Due to the presence of the PAS in a wide-variety of physiological processes, plasminogen receptors (PLG-Rs) are distributed on a broad range of cells, including macrophages, monocytes, endothelial cells, fibroblasts, and carcinoma cells [39]. These PLG-Rs promote the conversion of PLG to PLA and also bind PLA to protect it from inhibitors [40]. Cell-bound PLA is important for fibrin proteolysis, ECM degradation, and cell migration, as well as for stimulating cell-signaling pathways that lead to the release of cytokines and other inflammatory signals [41]. Since PLG is a target of MCAA, we suspected that mesothelial cells express one or more PLG-Rs in order to bind and activate PLG into PLA at the cell surface.

Many PLG-Rs bind PLG through a C-terminal lysine, which is bound by kringle-associated lysine binding sites. Analogues of lysine like Ɛ-aminocaproic acid (EACA) and tranexamic acid block the lysine binding sites of PLG, inhibiting the ability of the PLG-Rs to bind it [29, 42, 43]. Over a dozen PLG-Rs have been identified, and at least half of them utilize this lysine-binding domain, including α-Enolase (ENO-1), Histone H2B protein, P11 (S100A10), Cytokeratin 8 (CK8), Plg-RKT, and TATA-binding protein-interacting protein [44]. Interestingly, many of these proteins have vital intracellular function in addition to acting as cell-surface PLG-Rs. For example, ENO-1 is a metalloenzyme that is an important glycolytic protein for the conversion of 2-phosphoglyceraldehyde to phosphoenolpyruvate [45, 46]. S100A10 forms a
tetramer complex with Annexin A2 (ANAX2) when it binds PLG, but it also plays a role in 
exocytosis [47], endocytosis [48], and membrane trafficking [49]. CK8 is a known internal 
structure protein in cells [50]. It is not known why these proteins move to the surface but, due to 
their C-terminal lysine residue, they are able to act as PLG-Rs on the cell surface. An exception 
to this is Plg-RKT, which is an integral membrane protein. Plg-RKT is relatively newer in its 
discovery but plays a role in the recruitment of macrophages in an inflammatory response 
through the activity of PLG/PLA, similar to other known PLG-Rs [41].

As of the writing of this thesis, there is no record outside of Hanson’s study (2016) of 
mesothelial cells directly binding PLG [21]. Thus, we sought to identify which PLG-Rs are 
responsible for the binding of PLG by mesothelial cells, and to determine if PLG is activated on 
the cell surface. Based on preliminary tests with EACA, we looked at the most probable PLG-Rs, 
i.e. PLG-Rs that contain a C-terminal lysine, including ENO-1, H2B, S100A10, ANAX2, CK8, 
and Plg-RKT [44]. We also measured the activity of PLG when bound to mesothelial cells, in the 
presence of MCAA, and in the presence of commercial antibodies to determine their effects on 
PLG binding and activation to PLA. Identifying PLG-Rs will allow us to determine the method 
of PLG interaction with mesothelial cells and will allow us to glean insight into the pathways and 
mechanics triggered by MCAA binding to PLG on the mesothelial cell surface.

Anti-Plasminogen Antibodies in Rheumatoid Arthritis (RA)

While anti-PLG autoantibodies (i.e. MCAA) were identified in the sera of LA-exposed 
people and mice [17, 20], and while evidence suggest they may contribute to progression of LA-
associated LPT [15], these antibodies are not unique to this population. Autoantibodies against 
PLG and PLG-Rs have been noted in a variety of autoimmune diseases. For example, 
autoantibodies against ENO-1 have been detected in RA patients [51] and autoantibodies against
ANAX2 have been found in SLE patients [52]. Since MCAA target PLG, and anti-PLG/anti-PLG-R antibodies are present in many different autoimmune diseases, we suspected that MCAA may also be present in patients with other autoimmune diseases. Therefore, we tested sera from patients with RA for antibody binding to mesothelial cells. We also predicted that antibody presence would be correlated with pleural disease, as noted in LA-exposed populations [15]. Pleural disease is a common complication of RA, but there are no effective treatment options for RA-associated pleural disease, likely due to the lack of identified mechanisms driving disease development [53]. Thus, if MCAA were found in RA sera and positively correlated with pleural disease, MCAA could prove to be a potential therapeutic target.

**Post-Translational Modifications of Plasminogen**

Although the exact reason for autoantibody production in autoimmune disease is complex, three main reasons are postulated: genetics, molecular mimicry, and post-translational modifications (PTMs) [54]. Genetic predisposition to autoantibody generation is correlated to a deficiency of IgA antibodies and is associated with HLA (human leukocyte antigen) and other autoimmunity risk alleles. Individuals deficient of IgA have an increased prevalence of autoantibodies that are correlated with systemic autoimmune diseases like RA and SLE [55]. Molecular mimicry is observed when antibodies are generated for proteins with similar structure to another native protein, allowing there to be dual reactivity against the native protein. For example, antibodies generated against Dengue virus [56] and antibodies against complementary PR3 protein in PR3-ANCA vasculitis [57] have been shown to react against PLG. In both of these studies, antibodies were produced for proteins with similar structure to PLG, allowing for dual reactivity against PLG. Lastly, PTMs have been linked to the generation of autoantibodies, most notable of these being anti-citrulline antibodies found in the sera of RA patients [58, 59].
However, citrullination is not the only form of PTM found to have autoantibodies generated against it. Methylation [60], acetylation [61], and carbamylation [62] are among the many PTMs found to have autoantibodies generated against them [63]. Understanding autoantibody generation is important for understanding the production of MCAA following LA-exposure or in RA patients. MCAA are not produced from any genetic predisposition or molecular mimicry that we know of, meaning PTMs are a probable cause of MCAA generation.

Citrullination is a PTM of arginine deimination, meaning that it alters a positively-charged arginine amino acid into an uncharged citrulline amino acid. This alteration of charge changes the hydrogen bonding and ionic interactions of the protein, commonly leading to a reduction in activity and binding affinity [64]. This modification is catalyzed by proteins known as peptidyl arginine deiminase (PAD) enzymes. Although humans produce 5 different PAD isozymes (1, 2, 3, 4, and 6), PAD2 and 4 are the enzymes most commonly associated with irregular citrullination [65]. PAD enzymes target many different proteins, but notably they target both ENO-1 and PLG [66, 67]. Also, irregular PAD activity has been observed in many different autoimmune disorders including RA [58, 67], multiple sclerosis [68], type-1 diabetes [69], SLE [70], and psoriatic hyperproliferative epidermis [71]. Since multiple autoimmune diseases are linked to the presence of autoantibodies against proteins with PTMs, and irregular PAD activity is also present in a plethora of autoimmune diseases, we sought to determine the effect of citrullination on the PLG-binding in mesothelial cells. We tested the ability of mesothelial cells to convert citrullinated PLG (citPLG) to PLA and the effect of citrullinated mesothelial cells on the activity of PLG/PLA. In addition, we tested the effect of MCAA and citrullination in tandem in both cases (Figure 3). We hypothesized that citrullination of either PLG or mesothelial cell
surface proteins would decrease the binding and activation of PLG, and MCAA would decrease the PLA activity even more when in the presence of citrullination.

**Goals for this Thesis**

This three main goals of the work presented in this thesis are:

1. To determine novel proteinaceous targets of MCAA on the surface of mesothelial cells. We hypothesized that MCAA bind another target on mesothelial cells besides PLG.

2. To determine which PLG-Rs are responsible for the binding of PLG by mesothelial cells, and to determine if PLG is active on the cell surface. We hypothesized that mesothelial cells bind PLG through the expression of multiple PLG-Rs including: ENO-1, ANAX2, H2B, and Plg-RKT.

3. To determine if citrullination affects the PLG-binding system of mesothelial cells. We hypothesized that citrullination would inhibit PLG conversion, activity, and binding by mesothelial cells.

We anticipate that the data derived from this research will be important because MCAA-targeted protein(s) can be evaluated as a potential therapeutic targets of pleural fibrosis. This would allow for alleviation or treatment of asbestos-induced plural disease, or more specifically, LPT in LA-exposed populations or in RA patients. By determining the PLG-Rs utilized by mesothelial cells, we can glean insight into the mechanisms that trigger excessive collagen deposition following MCAA-mesothelial cell binding, noted both *in vitro* and *in vivo* [19, 20]. Further, analysis of the effect of citrullination on PLG activity will help determine the effect of PTMs on the PLG-binding system of mesothelial cells and give insight into the role they may play in overall asbestos-induced pathogenesis. Together, these data will provide a more complete
understanding of LA-induced LPT and autoimmunity, thus enabling the development of better therapeutics.
Figure 1. Role of the Plasminogen Cascade in MMP Activation and Fibrinolysis
Figure 2. The Formation of Lys-PLA. Lys-PLA is generated in two ways: 1) Glu-PLG is truncated to Lys-PLG via PLA, then convert to Lys-PLA via uPA/tPA, and 2) Glu-PLG is activated to Glu-PLA via uPA/tPA, then truncated via PLA to Lys-PLA.
Figure 3. Schematic of Experimental Design for Testing the Effect of Citrullination on PLA Activity at MeT5A Cell Surface. This diagram depicts the surface of a MeT5A cell with PLG represented by the blue circle, citrullination represented by the red highlight, and MCAA represented by the upside-down letter Y. From left to right, we will be testing PLA activity of mesothelial cells and the effects of: added PLG, added citrullinated PLG, citrullinating PLG-Rs on MeT5A surface and adding PLG, added MCAA (+ve) sera, added citrullinated PLG and MCAA (+ve) sera, and added MCAA (+ve) sera after citrullinating PLG-Rs on MeT5A cell surface.
Chapter 2: Materials and Methods

Human Serum Samples

Human sera samples from an LA-exposed population were obtained from the Center for Asbestos Related Disease (CARD) in Libby Montana and identified as MCAA positive (+ve) or negative (-ve) as previously described [17]. All samples were stored at -80⁰C and used in accordance with Idaho State University IRB project approval #FY2019-53.

Sera from RA patients was obtained from the University of Washington in accordance with IRB project approval #STUDY00006196. All samples were stored at -80⁰C and used in accordance with Idaho State University IRB project approval #FY2019-53.

Cell Cultures

Non-malignant, transformed human pleural mesothelial cells, MeT-5A (ATCC, Manassas, VA) were grown and maintained in RPMI medium supplemented with FBS (5%), penicillin/streptomycin (1%), L-glutamine (1%), and other essential amino acids (1%). The cells were incubated at 37⁰C with 5% CO₂. Unless otherwise stated, cells were incubated in 0% FBS media 48hrs before any tests were ran.

Human monocytes, THP-1 cells (ATCC, Manassas, VA) were grown and maintained in RPMI medium supplemented with FBS (10%), penicillin/streptomycin (1%), L-Glutamine (1%), 2-mercaptorothanol (20mM), and other essential amino acids (1%). The cells were incubated at 37⁰C with 5% CO₂. THP-1 cells were differentiated into macrophages in medium supplemented with 14ng/mL of PMA (phorbol 12-myristate 13-acetate) overnight.
Primary human peritoneal mesothelial cells (Coriell Institute for Medical Research, Camden, NJ) were grown and maintained in Medium 199 media supplemented with 15% FBS, 1% L-Glutamine, and 0.4µg/ml hydrocortisone. The cells were incubated at 37°C with 5% CO₂.

**Cell-based MCAA ELISA**

MeT5A cells were seeded in a 96-well plate at 1x10⁶ cells per well and incubated overnight to allow for adherence. The cells were then fixed with 2% paraformaldehyde for 20min, rinsed with 0.05% PBS-T, and then blocked for 1hr at RT with 5% milk (w/v). The cells were then incubated for 1hr with either RA of LA MCAA (+ve/-ve) human sera diluted 1:100 in 3% BSA-PBS. All samples were run in triplicate. Cells were then rinsed with PBS-T, then blocked again for 30 min at RT with 5% milk. The cells were then incubated with 100µl of HRP-conjugated rabbit anti-human IgG secondary antibody, diluted 1:2500 in 1% BSA-PBS for 1hr at RT. The plate was rinsed with 0.05% PBS-T, and ULTRA-TMB ELISA (ThermoFisher) was added to the plate. Once the plate had developed, the reaction was quenched with an addition of 2N H₂SO₄. The plate was read at 450nm on a Synergy HTX Multi-Mode Microplate Reader (BioTek).

**SDS-PAGE and Western Blot**

MeT5A cells were trypsinized for 7min, then scraped and pulled from culture, then centrifuged down at 5000 x g for 5min. Cells were washed 2x with ice-cold PBS, then resuspended in NP40 Cell Lysis Buffer (ThermoFisher) supplemented with 1mM protease inhibitor cocktail/PMSF and added at a concentration of 1mL per 10⁸ cells. The cells were incubated in the buffer on ice and vortexed every 10min. The samples were then centrifuged at 12,000 rpm for 10min, and the supernatant was collected. BCA assay (Pierce) was used to determine protein concentration of lysates. Samples were diluted to 20 µL with a maximum
15µg of protein for cell lysates, then 5µl of 5x SDS loading dye was added, and finally the samples were incubated at 100°C for 5min. Proteins were separated on a 10% or 15% SDS-PAGE gel at 100V for 90mins. Gels were stained in Coomassie blue, unless a western blot was being run instead (refer to the next paragraph).

Prior to use, a PVDF (polyvinylidene fluoride) membrane was activated in methanol for 5min, then incubated in transfer buffer for 10min. Proteins were transferred to the activated PVDF membrane at 125V for 90mins. After running, the membrane was removed and allowed to dry out completely. The membrane was then placed in methanol for 30secs, rinsed 3x with PBS, and then blocked for 1hr with 5% milk (w/v). The membrane was then incubated in primary antibody (diluted 1:1000-1:2000 in 3% BSA-PBS) overnight at RT with constant mixing. The next day, the membrane was washed 3x with 0.05% PBS-T and then blocked with 5% milk for 30min. The membrane was then incubated in either a goat anti-rabbit IgG HRP-conjugated secondary antibody or a mouse anti-human IgG HRP-conjugated secondary antibody at a 1:1500 dilution in 3% BSA-PBS for 1hr at RT. Afterwards, the membrane was rinsed 3x with 0.05% PBS-T and then developed using ULTRA-TMB BLOTTING (ThermoFisher). After the membrane developed, the reaction was quenched with PBS and the membrane was imaged on the Bio-Rad ChemiDoc Gel Imager if applicable.

**Silver Staining of SDS-PAGE Gels**

SDS-PAGE gels were run as noted above but were not Coomassie Blue stained. Instead, the gel was incubated in formaldehyde fixing solution (40% methanol, 0.037% formaldehyde) for 10mins. Gel was washed 2x for 5min with water, then soaked for 1min in 12mM Na₂S₂O₃ diluted in water. Gel was washed 2x for 20sec with water, then soaked in 0.1% silver nitrate for 10min. Gel was washed with thiosulfate developing solution (0.2M Na₂CO₃, 2.5nM Na₂S₂O₃,
0.02% formaldehyde) for 20 seconds, then fresh thiosulfate developing solution was added until bands developed. Reaction was quenched with 2.3M citric acid and incubated for 10min. Gel was washed 2x in water for 10min each.

MCAA-Binding Protein Isolation

MeT5A cells were trypsinized for 7mins, then scraped and pulled from culture, then centrifuged down at 5000 x g for 5min. Cells were resuspended in PBS and human sera (previously determined MCAA (+)ve or MCAA (-ve)) was added to each sample. The cells were incubated with the sera for 1hr with agitation at RT. DTSSP was added to each sample for a final concentration of 20mM and then incubated for 1hr at RT. The samples were subjected to three rounds of freeze-thaw lysis. The samples were then run through immunoprecipitation via a Protein A/G magnetic bead kit (ThermoFisher) as previously described [20]. In brief, the beads were washed with lysis buffer, and then added to the cell lysate samples. The beads were incubated with the samples for 1hr at RT. The beads were collected and the supernatant was removed. The protein was eluted off of the beads and concentrated via speed vacuum concentrator. The samples were reconstituted in PBS with protease cocktail inhibitor (Promega). The protein samples were separated by SDS-PAGE for visualization of protein bands unique to MCAA (+ve) exposed cells compared to MCAA (-ve) exposed cells.

Plasmin Activity Assay

MeT5A cells were seeded at 1x10^5 cells per well on a 96-well plate and incubated overnight at 37ºC to allow for cell adherence. Media was changed to 0% FBS media and incubated for 48hrs. Either Glu-PLG or Lys-PLG were diluted to 1mM in 0% FBS media and incubated on the cells for 12hrs. Afterwards, assay was run according to manufacturer’s specification (Abcam, Plasmin Activity Assay Kit). In short, after a 12hr incubation with a PLG
solution, 50µl of plasmin substrate was added to the wells. Fluorescence of plate was read at Ex/Em 360/450 in kinetic mode (read every 2.5min for 20min) on a Synergy HTX Multi-Mode Microplate Reader.

**Fluorescence Microscopy**

MeT5A cells were seeded on a positively charged microscopy slides (Leica) at 1x10^4 cells per ml. The slide was incubated overnight at 37°C to allow for cell adherence. The cells were then incubated in 0% FBS media for 48hrs. Afterwards, the cells were fixed for 20min in 2% paraformaldehyde. The slide was then washed 3x with PBS-T and then PLG (1µM) with mouse anti-human PLG (1:1000) in 3%BSA/PBS was added and incubated for 1hr at RT. The slide was then washed 3x with PBS-T and FITC-conjugated rabbit anti-mouse IgG secondary antibody diluted 1:1000 in 3% BSA/PBS was added and incubated for 1hr at RT. The slide was then washed 3x with PBS-T and incubated in 300nM DAPI solution for 2.5min, then washed 3x with PBS. The slides were sealed with VECTASHIELD (Vectorlabs) and imaged on Olympus FV1000 Confocal Microscope.

**MCAA with Plasmin Activity Assay**

Plasmin activity assay protocol followed as noted above with changes to the added PLG solution. Instead, either RA MCAA (+ve) or LA MCAA (+ve/-ve) human sera was diluted 1:100 in 0% FBS media and 50µl was added to cells and incubated for 12hrs. PLA activity assay was run as noted above.

**Flow Cytometry**

MeT5A cells were collected and aliquoted into groups of 1x10^6 cells. Cells were washed 2x with FACS buffer (500mL PBS, 0.5g NaN_3, 0.1% BSA, pH 7.7) and pelleted down at 7,000 RPM for 5min. The cells were incubated with the primary antibody at the dilution noted below in
FACS buffer for 15mins. The cells were then washed twice with FACS buffer and incubated with the secondary antibody (either anti-mouse IgG FITC [1:1000] or anti-rabbit IgG FITC [1:1000]) for 15min. The cells were then washed twice with FACS buffer and resuspended in FACS Analysis buffer (250mL PBS, 5g NaN₃, 1%BSA, pH 7.8). Cells were analyzed on a FACSCalibur Flow Cytometer. The following primary antibodies were used (all from BD Biosciences):

- Rabbit anti-H2B diluted 1:1000
- Rabbit anti-ENO-1 diluted 1:1000
- Rabbit anti-Plg-RKT diluted 1:3000
- Rabbit anti-ANAX2 diluted 1:1000
- Rabbit anti-S100A10 diluted 1:1000
- Mouse anti-CK8 diluted 1:100
- Mouse anti-PLG/PLA diluted 1:2500

**Biotinylation and Isolation of MeT5A Surface Proteins**

MeT5A cells were trypsinized for 7min, scrapped, collected, and then centrifuged at 5,000 x g for 5min. Cells were washed 3x with ice-cold PBS and then incubated for 30min at RT in 100µl of 20mM solution of NHS-Biotin (ThermoFisher) per 25 x 10⁶ cells/ml. The cells were washed 3x with PBS supplemented with 100mM glycine to quench the reaction. Cells were suspended in 500µL of NP40 Cell Lysis Buffer (ThermoFisher) supplemented with 1mM protease cocktail inhibitors/PMSF and incubated on ice for 30min, with sonication every 5min for 1sec at 40kHz. Samples were centrifuged at 12,000rpm for 10 min and supernatant was placed into a new tube.
A NeutrAvidin agarose affinity column was prepped by added 500µl of NeutrAvidin agarose resin (ThermoFisher) to a spin column and washed 3x with PBS. Samples were then added to the affinity column and incubated for 60min at RT with constant rocking. The column was centrifuged for 1min at 1000 x g and then washed 3x with PBS supplemented with 1mM protease inhibitor cocktail. Protein was eluted from the column by incubating the affinity column in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 50mM DTT) for 60min at RT with constant rocking. The protein was collected by centrifuging the column at 100 x g for 2min. Collected protein was stored at -20℃ until analyzed by SDS-PAGE, as noted above.

Citrullination Assay

Human Plasminogen (hPLG) was diluted to 0.25mg/ml in citrullination buffer (100mM Tris-HCl, 1mM DTT, 5mM CaCl₂, pH 7.5). PAD2 and PAD4 were added to a final concentration of 2µg PAD per mg protein, unless otherwise stated. The solution was allowed to incubate at RT for 3hrs. The citrullination protein solution was diluted into coating buffer (30mM Na2CO3, 70mM NaHCO3, pH 9.6) to a final concentration of protein at 10ug/ml. The protein coating solution was plated on a 96-well plate and incubated overnight at 4⁰C. The plate was washed with PBS-T and blocked with 5% milk (w/v). A rhodamine-conjugated citrulline specific antibody was added to the wells diluted 1:100 in acidified citrullination buffer (pH 2.5). Fluorescence was read at Ex/Em 532/590 on a BioTek plate reader.

Citrullination ELISA

Citrullination of hPLG was set up as noted in Citrullination Assay section, except after the plate was washed and blocked, human sera (either RA or LA MCAA (+ve)) was added to the wells at a 1:100 dilution in 3%BSA/PBS and incubated at RT for 2hrs. The plate was then
washed with PBS-T and then HRP-conjugated rabbit anti-human IgG secondary antibody diluted 1:2000 in 3% BSA/PBS was added and incubated for 1hr at RT. The plate was then washed with PBS-T, then treated with TMB-ULTRA ELISA. After the plate had developed, 2N H₂SO₄ was added to quench the reaction. The plate was read at 450nm on a BioTek plate reader.

**Cell-based Citrullination ELISA**

MeT5A cells were seeded in a 96-well plate at 1x10⁵ cells per well. The plate was incubated overnight at 37°C to allow for cell adherence. PAD2 and PAD4 were diluted to 0.20µg/ml in citrullination buffer. The protein citrullination solution was added to the wells and the plate was incubated for 3hrs at RT. The plate was then washed with PBS-T and blocked with 5% milk (w/v). Sera was added to the wells at a 1:100 dilution in 3% BSA/PBS and incubated for 1hr at RT. The plate was washed with PBS-T and then HRP-conjugated rabbit anti-human IgG secondary antibody diluted 1:2000 in 3%BSA/PBS was added and incubated for 1hr at RT. The plate was then washed with PBS-T, and then treated with TMB-ULTRA ELISA. After the plate had developed, 2N H₂SO₄ was added to quench the reaction. The plate was read at 450nm on a BioTek plate reader.

**Citrullination on PLA activity assay**

For citPLG tests, protocol followed as noted in Plasmin Activity Assay section above with alterations to the added PLG solution. hPLG was diluted to 0.25mg/ml in citrullination buffer, and PAD4 was added to a final concentration of 2µg PAD per mg protein. The solution was allowed to incubate at RT for 3hrs. The citrullinated PLG solution was added to the wells and incubated for 12hrs. The PLA activity assay was then run as noted above.
For citrullinated MeT5A surface proteins, MeT5A cells were seeded at $1 \times 10^5$ cells per well on a 96-well plate and incubated overnight at 37°C to allow for cell adherence. PAD4 was diluted to 0.20ug/ml in citrullination buffer. The protein citrullination solution was added to the wells and the plate was incubated for 3hrs at RT. Either a PLG solution or sera solution (noted above in PLA activity assay protocol) was added to the wells. Afterwards, assay was run according to manufacturer’s specification as noted above.

**Statistical Analysis**

One-way ANOVA and Tukey’s HSD post-hoc tests were performed using R ver.4.0.3 [72]. Statistical significance was defined as $P<0.05$. 
Chapter 3: Results

MCAA Targets

The identity of additional MCAA targets on mesothelial cells was assessed utilizing DTSSP crosslinking and SDS-PAGE. SDS-PAGE analyses demonstrated an absence of any unique targets between MCAA (+ve) and MCAA (-ve) sera (Figure 4). No unique bands were detected when DTSSP was added either at 20mM (Figure 4A) or at a lower concentration of 2mM (Figure 4B). Silver stain analyses of the SDS-PAGE gel was also run and, again, no unique bands were detected between MCAA (+ve) and MCAA (-ve) sera (data not shown).

Binding of PLG to MeT5A (Glu-PLG vs Lys-PLG)

The ability of mesothelial cells and macrophages (used as a positive control) to bind PLG was assessed. Flow cytometry analyses demonstrated that MeT5A cells bind PLG significantly higher than the isotype control, but THP-1 differentiated macrophages have higher PLG binding overall (Figure 5A). Macrophages had a higher geographic mean fluorescent intensity (gMFI) of 320.17 and PLG bound on 96% of all gated cells, whereas MeT5A had a gMFI of 69.7 and PLG appeared on 80% of the gated cells. The binding of PLG by MeT5A cells was also demonstrated by confocal fluorescent microscopy. The FITC-labeled anti-PLG antibodies bound to the MeT5A cells, allowing for visualization of PLG and MeT5A interaction (Figure 5B). Notably, PLG was found to bind one side of the MeT5A cells and did not bind around the entirety of the cell surface evenly.

Next, the ability of mesothelial cells to bind the different PLG forms (Glu-PLG and Lys-PLG) was determined. A PLA activity assay demonstrated a significantly high amount of PLA activity from both Lys-PLG and Glu-PLG treated cells (Figure 6). However, Glu-PLG had
lower PLA activity than Lys-PLG, but the activity was still significantly higher than the negative control (p=6). Based on this data, Lys-PLG was used in all subsequent experiments.

**Effect of FBS on PLG Binding**

PLG binding to MeT5A cells was assessed in the presence of different FBS concentrations. Flow cytometry analyses demonstrated that when cells were incubated in media without FBS (0%), PLG showed significantly higher binding compared to isotype control. However, addition of FBS (5% or 10%) to the cell culture media reduced PLG binding compared to 0% FBS, though binding was still slightly higher than isotype control (Figure 7). 0% FBS had the highest gMFI of 100.72 and PLG bound to 20% of the gated cells. Cell culture media with added FBS (either 5% or 10%) had similar gMFIs at 24.28 but PLG bound a higher percentage of the gated cells (51%).

Next, PLG binding to MeT5A cells was assessed in the presence of 0% FBS for different amounts of time (Figure 8). Flow cytometry analyses demonstrated that cells incubated with 0% FBS for 48hrs had the highest PLG binding compared to isotype control with a gMFI of 791.68 and PLG appeared on 89% of the gated cells. Cells incubated for 72 or 96hrs had lower binding with a gMFI of 403 and 462 respectively and PLG appeared, on average, on 90% of the gated cells. The 24hr incubation had a gMFI of 876.18 on 96% of the gated cells, but had a lower read on the secondary peak of higher intensity compared to the 48hr incubation. Therefore, all subsequent experiments were performed using cells incubated for 48hrs in FBS-free media.

**Effect of MCAA on the Activity of PLA**

To assess PLG conversion to PLA on the mesothelial cell surface, a PLA activity assay was used. PLG was readily converted to PLA, noted by a significant increase of PLA activity, as indicated by the cleavage of a PLA-specific substrate (Figure 9). Upon addition of MCAA, PLA
activity significantly decreased, suggesting a decrease in PLG conversion to PLA. Cells treated with PLG and MCAA (-ve) sera demonstrated higher amounts of PLA activity compared to MCAA (+ve) treated cells, but the activity was not as high as seen with PLG added alone (control).

**Effect of EACA on the Activity of PLA**

In order to confirm the mode by which PLG binds to mesothelial cells, PLG binding and activation was tested in the presence or absence of a known lysine-binding domain inhibitor, EACA (Figure 10). The effect of EACA was first tested with PLG incubated in differing concentrations of EACA. This assay showed that both 0.5M and 0.3M of EACA significantly dropped PLA activity below levels seen with untreated mesothelial cells (Figure 10A). MeT5A cells treated with PLG only had significantly higher binding than any of the EACA test groups. Furthermore, flow cytometry analyses demonstrated that cells treated with 0.5M EACA had lower PLG binding than observed in the control (Figure 10B). MeT5A cells treated with PLG had a significantly higher binding with a gMFI of 118 on 68% of the gated cells than either EACA-PLG treated cells or the isotype control. EACA-PLG treated cells had lower PLG binding with a gMFI of 22.46 as assessed for 92% of the gated cells.

**Receptor Identification**

Since experiments with EACA indicate that the lysine-binding domain is necessary for PLG binding to mesothelial cells, the presence of PLG-Rs with a lysine-binding domain were examined. Since macrophage cells are known to bind PLG via the lysine-binding domain, and known to express many different PLG-Rs, these cells were used as a positive control. As noted above, PLG binding was higher for macrophages than MeT5A cells (Figure 6); this trend was also observed for all tested PLG-Rs as well (data not shown). Figure 11 demonstrates the flow
cytometry analyses for all of the PLG-Rs tested. ENO-1 (Figure 11A) had the highest gMFI of 51.82 and appeared on 75% of all gated cells. ANAX2/S100A10 (Figure 11B) had the next highest expression on the surface of MeT5A cells with a gMFI of 38.25 and appeared on 85% of all gated cells. H2B (Figure 11C) and Plg-RKT (Figure 11D) had lower expression than either ENO-1 or ANAX2/S100A10, each with a gMFI of 10, but had higher expression than the isotype control. Plg-RKT had higher expression than H2B, appearing on 73% of the gated cells, whereas H2B had the lowest expression of the receptors tested, only showing up on 24% of the gated cells. Western blotting of surface proteins isolated following biotinylation was also run to confirm PLG-R presence. ENO-1, ANAX2, and Plg-RKT were detected in the isolated surface proteins from MeT5A cells (Figure 11E), while H2B did not show up in the western blot. Unexpectedly, ANAX2 was not detected in proteins isolated from macrophages.

Presence of MCAA in RA Sera

RA sera (n=24) was investigated for binding to MeT5A cells. A cell-based ELISA demonstrated that out of the 24 samples tested, 4 were statistically positive for binding. This lead to a 16.7% seropositivity in the RA samples, whereas in populations exposed to LA, the frequency is around 18.5% [17].

PAD2 vs PAD4 Citrullination of PLG

The ability of PLG to be citrullinated by PADs was assessed using PAD2, PAD4, and PAD2/4 in tandem. A citrullination assay demonstrated a dose-response curve following PLG exposure to PAD2/4 (Figure 12A), with increased citrullination observed with increasing PLG concentration. Furthermore, the citrullination of PLG from either PAD2 or PAD4 was assessed by a citrullination assay (Figure 12B). Analyses demonstrated that PLG is citrullinated more by
PAD4 and PAD2/4 in tandem compared to PAD2 alone. PAD2 had higher citrullination than the control but had lower citrullination than that of PAD4.

**PAD 2 vs PAD 4 Citrullination of Met5A Surface**

Citrullination of proteins on MeT5A cell surface was assessed using a cell-based citrullination ELISA with PAD 2/4 (Figure 13). The citrullination ELISA demonstrated the ability of PAD4 to citrullinate MeT5A surface proteins significantly better than PAD2 or PAD2/4 in tandem.

**Effect of Citrullination on the Binding of RA MCAA**

Binding of RA sera to citrullinated proteins was tested. A citrullination ELISA showed that RA bound to citPLG significantly higher than native PLG (Figure 14A). Furthermore, a cell-based citrullination ELISA demonstrated the ability of RA sera to bind to citrullinated MeT5A cells significantly higher than unaltered MeT5A cells (Figure 14B).

**Effect of Citrullination on the Binding of LA MCAA**

Binding of LA MCAA to MeT5A cells was assessed in the presence of citrullination. A citrullination ELISA showed the ability of both MCAA (+ve) and MCAA (-ve) sera to bind citPLG significantly higher than native PLG. However, a cell-based citrullination ELISA demonstrated that MCAA (+ve) sera bound significantly higher to citrullinated MeT5A cells than to unaltered MeT5A cells. This was not seen with MCAA (-ve) sera, instead the binding was significantly decreased in the presence of citrullination (Figure 15).

**Effect of Citrullination and MCAA on PLG Conversion**

The effect of citrullination on PLA activity was assessed with citPLG and citrullinated MeT5A cells. A PLA activity assay showed a significant decrease in PLA activity upon
citrullination of PLG (**Figure 16**). Notably, the activity of citPLG was brought down to control levels. Furthermore, citrullination of MeT5A cell surface significantly decreased PLA activity, but did not reduce activity to control levels.

Next, the effects of MCAA and citrullination on the activity of PLG was determined by utilizing MCAA and altering the citrullination target (either MeT5A surface proteins or PLG). The presence of citrullination, either citPLG or citrullinated MeT5A cells, decreased the relative PLA activity in the presence of MCAA. CitPLG plus MCAA decreased the PLA activity levels to control levels, whereas the citrullination of MeT5A cells followed by added PLG and MCAA in tandem decreased the activity, but not to control levels (**Figure 17**).

Finally, the effect of citrullinated MeT5A cells in tandem with MCAA (+ve) sera (either LA or RA) was assessed. LA MCAA (+ve) sera had overall lower relative PLA activity in comparison to RA MCAA (+ve) sera (**Figure 18**). Addition of RA MCAA (+ve) sera to MeT5A cells resulted in significantly higher PLA activity compared to the control. When MeT5A cells were citrullinated prior to RA MCAA (+ve) addition, PLA activity decreased. However, PLA activity was still significantly higher than with cells treated with LA MCAA (+ve) sera. The addition of LA MCAA (+ve) sera to citrullinated MeT5A cells significantly increased PLA activity compared to LA MCAA (+ve) sera added to unaltered MeT5A cells.
Figure 4. SDS-PAGE Gel Analyses of MCAA Targets. DTSSP was used to crosslink MCAA to their proteinaceous targets and then the MCAA were immunoprecipitated and separated out on a SDS-PAGE gel. DTSSP was added at a final concentration of A) 20mM or B) 2mM. Gels were stained using Coomassie blue. Order of gel is Molecular weight marker, MCAA (+ve), MCAA (-ve). No unique bands appeared between the immunoprecipitated MCAA (+ve) or MCAA (-ve) sera.
Figure 5. The Presence of PLG on MeT5A and Macrophage Cells. A) PLG binding of macrophages (—) and MeT5A cells (■) was compared to the isotype control (····). M-gates were used to calculate gMFI of individual peaks. The highest PLG binding was seen on the macrophage cells (M3 gate), but PLG binding to MeT5A cells (M2 gate) was higher than the isotype control (M1 gate). B) Binding of PLG to MeT5A cells assessed by confocal fluorescence microscopy. DAPI was used to identify nuclei and FITC-antiPLG used to visualize PLG. PLG binding tended to localize on one side of the cells.
Figure 6. Relative PLA activity of Glu-PLG and Lys-PLG on MeT5A cells. After 12hr incubation of MeT5A cells with either Glu-PLG or Lys-PLG, PLA activity assay was run. Lys-PLG (●···) and Glu-PLG (- -●- -) showed significantly higher PLA activity on MeT5A cells compared to cell-only control (―●―). Error bars represent standard error, with significance being **p<0.05 compared to the control. Each sample was run in triplicate.
Figure 7. Effect of FBS on PLG binding. Flow cytometric analysis of PLG binding to MeT5A cells incubated in 0%, 5%, or 10% FBS. 0% FBS ( ), 5% FBS (- - - -), and 10% FBS (—) were compared to the secondary antibody as isotype control (·····). Cells incubated in 0% FBS showed the highest binding of PLG as indicated by the shift in the peak to the right, with no detectable difference between 5% and 10% FBS.
Figure 8. Time-course of PLG Binding. Flow cytometric analysis of PLG binding to MeT5A cells incubated in 0% FBS for 24hr ( ), 48hr (—), 72hr (•••), or 96hr (—). The highest PLG binding was seen when cells were incubated in FBS-free media for 48hrs prior to addition of PLG, as indicted by the shift in the peak to the right.
Figure 9. Relative Plasmin Activity of MeT5A treated with MCAA. A plasmin activity assay was run with MeT5A cells treated with Lys-PLG (1µM), MCAA (+ve) sera, or MCAA (-ve) sera. MCAA (+ve) treatment (··· ●···), MCAA (-ve) treatment (---●---), and Lys-PLG treatment (·−·●−·−·), were compared to untreated cells (—–●–—), used as a negative control. MCAA (-ve) treatment led to higher PLA activity than MCAA (+ve) treatment. Error bars represent standard error with **p<0.01. Experiment was run in triplicate and repeated twice.
Figure 10. The effect of EACA on PLG Binding and Activation on MeT5A Cells. A) The effect of EACA on the activity of PLA. MeT5A cells treated with PLG (---·---), PLG plus 0.3M EACA (--●--), and PLG plus 0.5M EACA (··●··) were compared against untreated cells (––●––). MeT5A cells treated to PLG plus EACA had significantly lower levels of PLA activity than the control. Error bars represent standard error and significance is *p<0.05 and **p<0.01, as compared to control, n=6. B) The effect of EACA on PLG binding. MeT5A cells were treated with PLG ( ) or PLG incubated in 0.5M EACA (——) and binding was compared to the isotype control (----). MeT5A cells treated with PLG had a clear shift in PLG binding, whereas MeT5A cells treated with PLG plus EACA peak did not shift past the isotype control peak.
Figure 11. The Presence of PLG-Rs on MeT5A Cells. Flow cytometric analyses of A) ENO-1, B) ANAX2/S100A10, C) H2B, and D) Plg-RKT expressed on MeT5A cell surface. For all histograms, PLG-R of interest ( ) was compared to the isotype control (—). M-gates were used to calculate gMFI: M2 gate was set based on background staining with isotype control, indicated by M1 gate. E) Western blots run testing the PLG-Rs presence on MeT5A cell surface. Surface proteins ENO-1 showed up at 48kDa, ANAX2 showed up at 37kDa, and Plg-RKT showed up at 60kDa. Protein presence was compared for MeT5A and macrophage cells. All 4 of the PLG-Rs appeared on the surface of MeT5A cells following flow cytometry analyses, but only ENO-1, ANAX2, and Plg-RKT showed up on the western blots.
Figure 12. Citrullination of PLG. A) Citrullination of PLG with increased PLG concentration. Citrullination was measured by ELISA utilizing amounts of PLG with a constant ratio of 2µg of PAD2/4 per 1mg of PLG. B) Citrullination of PLG by PAD enzymes. Citrullination was determined by an ELISA utilizing 0.4µg/ml PLG with 2µg of PAD2, PAD4, or both PAD2 and PAD4 for every 1mg of PLG. Thus, we demonstrated that PLG can be citrullinated and was more likely to become citrullinated by PAD4 or PAD2/4. Error bars show standard deviation with *p<0.01 and **p<0.005, n=6.
Figure 13. Citrullination of Met5A Cells and its effect on MCAA Binding. The determination of citrullination was carried out by a cell-based citrullination ELISA utilizing MCAA. MCAA had highest binding to MeT5A cells citrullinated by PAD4. Error bars show standard deviation with *P<0.01, n=6.
Figure 14. Effect of Citrullination on the Binding of RA Sera. ELISA was used to assess the presence of antibodies in RA sera against citrullinated or unaltered PLG (A) and MeT5A cells (B). RA sera bound both PLG and MeT5A cells better when citrullination was present. Citrullination was carried out using PAD4 with data showing mean relative absorbance ±S.D., n=6, and *p<0.01
Figure 15. The effect of citrullination on the MCAA binding. ELISA was used to assess the binding of MCAA to citrullinated or unaltered PLG (A) and MeT5A cells (B). Citrullination was carried out by PAD4. MCAA bound better to citPLG over PLG and this trend was also observed with MCAA (-ve) sera. MCAA bound better to citrullinated MeT5A cells over unaltered MeT5A cells, but this trend was not observed with MCAA (-ve) sera. Data shows mean relative absorbance ±S.D., n=6, and *p<0.05, **p<0.01.
Figure 16. The effect of citrullination on PLG activity. A plasmin activity assay was run to assess the activity of native PLG, citrullinated (cit) PLG, and citrullinated MeT5A with added Lys-PLG. Normal Human Serum was used as a control. MeT5A with added PLG (⋯●⋯) and citrullinated MeT5A with added PLG (−−●−−) showed significantly higher activity compared to cell-only control (––●––). MeT5A cells with added citPLG (⋯●⋯) showed activity below that of the control. Error bars show standard error with **p<0.01, n=6.
Figure 17. The effect of citrullination and MCAA in tandem. A PLA activity assay was run with citrullinated targets (either MeT5A cell surface proteins or PLG) and LA MCAA (+ve) sera at the same time. MeT5A cells treated with PLG and MCAA (●●●), citrullinated MeT5A cells treated with PLG and MCAA (−−●−−), and MeT5A cells treated with citPLG and MCAA (−−●−−) all had significantly higher activity of PLA compared to the cell-only control (−−−−−). Error bars show standard error with **p<0.01, n=6.
Figure 18. Comparison of the effect of citrullination on MeT5A cells in the presence of MCAA. A plasmin activity assay was run in the presence of MeT5A cells or citrullinated MeT5A cells. Citrullinated MeT5A cells treated with RA MCAA (+ve) sera (− • − −) had significantly lower activity than control MeT5A cells treated with RA MCAA (+ve) sera (···). Citrullinated MeT5A cells treated with LA MCAA (+ve) sera (···) had significantly higher binding than the control, MeT5A cells treated with LA MCAA (+ve) sera (—−). Error bars demonstrate standard error and *p<0.05, **p<0.01, n=6.
Chapter 4: Discussion

MCAA (Mesothelial Cell Auto-Antibodies) have been found in 18.5% of individuals exposed to LA and correlate with the appearance of LPT, a unique form of pleural fibrosis [17, 20]. Although the mechanism linking MCAA to asbestos-induced fibrosis is not fully understood, a possible mechanism is through inducing excessive collagen deposition [19, 20]. Following MCAA binding to mesothelial cell, increased collagen 1 deposition was detected in vitro [19] and in vivo [20]. Hanson et al. identified PLG as a MCAA target and demonstrated that the removal of PLG lead to the excessive deposition of collagen by mesothelial cells, similar to that seen with MCAA binding [21]. In this thesis, we sought to further examine the PLG-MCAA-mesothelial cell interaction in order to better understand the role MCAA play in LA-associated pathologies.

MCAA Targets

Although Hanson et al. previously identified PLG as a MCAA target, they also postulated that additional MCAA targets were present on MeT5A cells [21]. We sought to isolate other proteins bound by MCAA through the use of the crosslinker DTSSP, thus allowing us to isolate just the protein targets of MCAA and not all surface proteins, as previously done by Hanson et al. Preliminary data showed the appearance of a unique band between MCAA (+ve) and MCAA (-ve) that was later identified as fibromodulin (data not shown). However, follow-up studies were unable to replicate these results and all subsequent SDS-PAGE gels showed no unique bands (Figure 4). In addition to Coomassie staining, SDS-PAGE gels were silver stained in an attempt to reveal unique bands as silver staining is more sensitive than Coomassie staining. However, this approach also revealed the same bands between the two groups. Therefore, our experiments did not reveal any novel MCAA targets.
We suspect that the lack of unique bands in the MCAA (+ve) treated cells compared to MCAA (-ve) treatment was due to DTSSP’s propensity to crosslink all the proteins together, MCAA included. Upon lysis of the cells, there is no way to keep DTSSP from entering the cells, resulting in intracellular proteins being immunoprecipitated out with MCAA and their cell surface targets. To combat this, we attempted to use concentrations lower than the recommended concentration. However, when using lower concentrations of DTSSP, either all of the background proteins appeared in both MCAA (+ve) and MCAA (-ve) samples, or no protein was immunoprecipitated other than IgG itself (Figure 4B). Together, these data confirm that MCAA likely do not bind other proteins on MeT5A cells. Another approach to test the targets of MCAA on mesothelial cells would be to isolate the surface proteins first, then run the DTSSP crosslinking protocol. This would alleviate any concerns of intracellular proteins being present and would allow for better determination of surface proteins that MCAA bind.

We initially hypothesized that MCAA targeted other proteins in addition to PLG because of previous work. Hanson et al. demonstrated that removal of anti-PLG proteins in MCAA (+ve) sera did not return collagen I deposition to control levels [21]. While we initially thought that this discrepancy was due to the presence of additional MCAA targets, other factors need to be considered. It is possible that in the previous study, the MCAA (+ve) sera was not completely cleared of anti-PLG antibodies prior to the addition to the cells; if anti-PLG remained, the results would demonstrate mesothelial cell binding. We think this is a possibility because in Hanson’s study (2016) when testing sera that had been cleared for either 24 or 48hrs, there were differences in cell binding levels. The sera cleared for 24hrs had higher levels of binding compared to the sera cleared for 48hrs [21]. This demonstrates that clearance might not have removed all of the anti-PLG antibodies in the sera. Determining the efficacy of the anti-PLG
clearance would allow for a stronger evaluation of MCAA targets, i.e. allow for determination if MCAA do indeed bind another protein besides PLG.

**Determination of PLG-Rs**

PLG plays an important role in the human body. While PLG-Rs are widely distributed in the body [39, 73], there are no reports of mesothelial cells binding PLG directly. To demonstrate that mesothelial cells will bind PLG, flow cytometry and fluorescence confocal microscopy assays were run (Figure 5). We tested PLG binding to macrophages as a known positive control, then we tested binding to MeT5A cells. We found that MeT5A cells bind PLG, but at a significantly lower level than macrophages; however, binding was significantly higher than the isotype control. Notably, upon fluorescence microscopy analyses of MeT5A cells binding PLG, the binding of PLG appeared strongly on one side of the cell, and not equally on the cell surface (Figure 5B). However, more research needs to be done to further evaluate this phenomenon. In addition, to demonstrate that PLG binding was not unique to the MeT5A pleural cell line, primary mesothelial cells from human peritoneum were also tested for PLG binding. The peritoneal mesothelial cells also demonstrated positive PLG binding (data not shown).

In order to determine the ideal culture conditions for PLG binding, we tested several factors. First, we demonstrated that Lys-PLG was converted more efficiently than Glu-PLG following binding to MeT5A cells (Figure 6). This is likely due to cells utilizing Lys-PLG and Lys-PLA instead of their glutamate counterparts. In addition, Glu-PLG conformation protects it from PLG activators [33], making activation into PLA slower and less efficient. Second, we suspected that FBS may interfere with detection of PLG because FBS contains bovine PLG as part of its slew of protein [74]. Therefore, we tested the binding of PLG to MeT5A cells grown in 0%, 5%, and 10% FBS media (Figure 7). PLG bound best to cells grown in media with 0%
The presence of FBS in the growth media lowered the binding of PLG but did not reduce the binding to control levels. Since bovine PLG is a human PLG homolog, it is likely that PLG binding was decreased in the presence of FBS because the bovine PLG inhibited the binding of the added human PLG, which was detectable by the human specific anti-PLG antibody. Finally, we performed a time-course assay of PLG binding in the absence of FBS. We assessed PLG binding at 24, 48, 72, and 96hrs after removal of FBS and detected the highest PLG binding at 48hrs (Figure 8). PLG binding at 72 and 96hrs was decreased down to levels seen below 24hrs with gMFIs of 403 and 462, respectively, but was higher than that observed with cells grown in culture media supplemented with FBS. The 24hr incubation had the highest gMFI of 876 and PLG appeared on 96% of the gated cells. However, although the 48hr incubation had a lower gMFI of 791 and only appeared on 89% of the gated cells, the 48hr incubation had a larger secondary peak which was detected at a higher fluorescent intensity. Since most of the incubations had similar primary peaks, we determined 48hrs was the best incubation time due to the larger, higher intensity fluorescence peak. Together, these experiments established the best binding of PLG for MeT5A cells as: Lys-PLG with mesothelial cells grown in 0% FBS for 48hrs prior to testing. Therefore, these were the conditions used in the subsequent flow cytometry and PLA activity assays. These results could also explain why others have not detected PLG binding on mesothelial cells before, as mesothelial cells are commonly grown in media with FBS.

In addition to demonstrating PLG binding to mesothelial cells, we also established that PLG is converted to PLA on the mesothelial cell surface. We tested the ability of MeT5A cells to convert PLG to PLA with a PLA activity assay and demonstrated that PLA was activated on the surface of MeT5A cells (Figure 9). MeT5A cells alone had low levels of PLA activity, but upon the addition of PLG, PLA activity significantly increased. Next, we suspected that when MCAA
bound PLG, they would block conversion and activation into PLA. The PLA activity assay showed that MeT5A cells treated with MCAA (-ve) sera had higher activity of PLA than MCAA (+ve) treated cells. This demonstrates that MCAA are actively reducing the activity of PLA by inhibiting its conversion and activation from PLG (Figure 9). This observed inhibition of PLA activity is a form of PAS dysregulation. Interestingly, symptoms of PAS dysregulation are also found in individuals exposed to LA including dysregulated ECM remodeling (fibrosis) and chronic inflammation [31]. Additionally, PLG may contribute to ECM remodeling by activating MMPs, which then degrade and recycle ECM proteins. Since MCAA binding blocks PLG activity, it may also reduce MMP activation, thus reducing ECM degradation and increasing collagen deposition. This model fits with previous data showing that MCAA binding increases collagen detection both in vitro and in vivo [19, 20]. This also suggests a possible mechanism for the development of chronic physiological problems like fibrosis seen in LA-exposed populations [17].

A known inhibitor of PLA activity, EACA, binds the lysine-binding domain of common PLG-Rs, thus preventing PLG binding [43, 75]. We used EACA to confirm that the lysine-binding domain was important for PLG binding to MeT5A cells. In the presence of EACA, the binding of PLG dropped below control levels, demonstrating that EACA blocked not only PLG binding, but also nonspecific binding of the commercial anti-PLG antibodies (Figure 10). This trend was also seen with PLA activation in the presence of EACA. EACA actively dropped the activity of PLA on MeT5A cells to control levels, demonstrating again that addition of EACA blocked all PLG binding (Figure 10). From this, we postulated that the dominate PLG-R(s) of MeT5A cells bind and activate PLG through the use of a C-terminal lysine. There are a broad range of PLG-Rs that bind via a lysine domain, including ENO-1, ANAX2/S100A10 complex,
H2B, PlgRKT, and CK8 [44]. We determined that all of these receptors were present on MeT5A cells with the exception of CK8. In order to quantify presence of PLG-Rs on MeT5As, cells were analyzed by flow cytometry and resultant histogram peaks were gated and geographic mean fluorescent intensity (gMFI) was calculated (Figure 11). Overall, ENO-1 and ANAX2/S100A10 seem to be the primary PLG-Rs on MeT5A cells with the highest gMFIs of the PLG-Rs and appearing on 75% and 85% of the gated cells, respectively. Plg-RKT also appeared on MeT5A cell surfaces, but appeared at a lower rate with a gMFI of 10 on 73% of the cells. H2B showed up the least amount with a gMFI of 10 on 24% of the cells. Notably, H2B was only sometimes present on MeT5A cells and did not show up every test. Interestingly, after isolating the proteins from the MeT5A cell surface, western blot analyses showed that ENO-1 and ANAX2 were the only receptors present where we expected them (Figure 11E). H2B failed to show up on any of the western blots ran. Plg-RKT showed up in the blots but appears at a higher weight than expected. Plg-RKT is a 17kDa protein but was detected at 60kDa on the western blots. We suspect this is due either to Plg-RKT complexing with other proteins or the antibody not being specific to Plg-RKT. Further research would need to be done to determine the identity of the band. Altogether, this data confirms that ENO-1 and ANAX2 are the primary PLG-Rs on mesothelial cells, with H2B and Plg-RKT appearing on the cell surface but at a lower frequency. Follow-up studies are needed to further assess the contribution of each PLG-R to overall PLG binding and activation on the mesothelial cell surface.

Together, our data demonstrate for the first time that mesothelial cells not only bind, but also activate, PLG. The lack of previous reports of direct mesothelial-PLG interaction is probably due to the common use of FBS in cell culture growth media. We showed that FBS in culture media reduces the exogenous PLG binding, which may have allowed the binding to be
overlooked previously. We further demonstrated that PLG is bound by mesothelial cells via C-terminal lysine PLG-Rs, namely ENO-1, and ANAX2. Although we did detect H2B and Plg-RKT on the mesothelial cell surface, their appearance was not consistent, and they never appeared as strongly as ENO-1 or ANAX2, suggesting that they may be inducible receptors while ENO-1 and ANAX2 are constitutive. However, follow-up studies are needed to confirm these expressions.

When looking at the activity of PLG on mesothelial cells, PLG becomes readily converted to PLA. Interestingly, MCAA act as an inhibitor of PLA activity. This works into the current proposed model for MCAA induced fibrosis, i.e. MCAA binding PLG on mesothelial cell surfaces, inhibiting PLA production and MMP activation, dysregulating ECM remodeling and leading to overall excessive collagen 1 deposition, or fibrosis.

**PTM of Citrullination**

Citrullination, or arginine deimination, is the most notable of PTMs found to have autoantibodies generated against it [59]. This PTM is catalyzed by PAD enzymes, which have been known to citrullinate PLG and have irregular activity in a multitude of autoimmune disorders, including RA and SLE [67, 70]. Since we determined that MCAA consist mostly of anti-PLG antibodies, and anti-PLG antibodies have been shown in a number of autoimmune diseases, we postulated that their presence may not be unique to LA-exposed people. Therefore, we examined sera of RA patients for antibody binding to mesothelial cells. After screening 24 sera samples from RA patients, we found that 4 were positive for MeT5A binding, or 16.7%. This percentage is close to the percentage found in LA-exposed individuals, which is 18.5% [17]. Furthermore, we evaluated the two PAD isozymes 2 and 4 for their efficiency in citrullinating PLG and MeT5A cell surface. PAD4 seemed to be the most able to citrullinate of
the two as it catalyzed higher amounts of citrullination both on PLG and on MeT5A cell surfaces (Figure 12 & 13). That is not, however, to discredit PAD2, as it was also able to citrullinate both PLG and MeT5A, just not as highly as was seen with PAD4. Therefore, PAD4 was used in all following citrullination experiments unless otherwise noted.

The effect of citrullination was evaluated on the binding of both LA and RA MCAA. Since citrullination is a known target of RA autoantibodies, the binding increased significantly for RA sera with both citrullinated PLG and citrullinated MeT5A cells compared to their unaltered counterparts (Figure 14). However, this trend was not observed for LA MCAA (Figure 15). When comparing PLG to citrullinated PLG, binding was increased in both LA MCAA (+ve) and MCAA (-ve) sera, demonstrating an increase in nonspecific binding. When comparing MeT5A to citrullinated MeT5A, only LA MCAA (+ve) sera demonstrated an increase in antibody binding. MCAA (-ve) sera actually decreased in antibody binding compared to the non-citrullinated control. Although citrullination of PLG increased overall antibody binding, the citrullinated MeT5A did not, demonstrating that citrullinated MeT5A cells decreased nonspecific antibody binding while increasing MCAA-specific binding. The citrullination of MeT5A cells may have altered any endogenous, nonspecific antibody receptors by altering the charge of the protein. Since citrullination of MeT5A cells increased MCAA binding but decreased nonspecific binding, MCAA may either target citrullinated PLG-Rs or citrullinated PLG-Rs bound PLG better, thus increasing MCAA binding.

Since citrullination affected the binding of MCAA, we evaluated the effects of citrullination and MCAA on PLA activity (Figure 16). All tests run with citPLG had levels of PLA activity at levels seen with MeT5A cells without added PLG (negative control). This is probably due to the fact that the cleaved bond of PLG (Arg561-Val562) contains an arginine
If that arginine becomes citrullinated, the overall charge of the cleaved site is altered, which could lead to significantly reduced or even absent PLG activation. Citrullination of MeT5A surface proteins also dropped PLA activity, but did not drop activity to control levels, signaling that not all PLG activation was lost. Activation of PLG may have decreased due to citrullination of the PLG-Rs on the mesothelial cells, leading to inhibition of PLG binding or the inhibition of activation by binding PLG differently. As has been observed with citrullination of ENO-1 in RA conditions, PLG binding and activation is diminished but not fully inhibited [51]. This trend was also observed when MCAA and citrullination of either PLG or MeT5A surface proteins were run at the same time (Figure 17).

MeT5A cells treated with citPLG had PLA activity dropped to control levels both in the presence and absence of MCAA (Figure 17 & 18). PLA activity also decreased with citrullination of MeT5A cells and MCAA, but this decrease was not as strong as the decrease observed with citPLG. As was observed earlier, citrullination of MeT5A surface proteins, including any PLG-Rs, hindered but did not fully block the activation of PLG. Since this was observed without MCAA, we determined that citrullination plays a bigger role in the decrease of PLG activation compared to MCAA in this scenario. Notably, when looking at both RA and LA MCAA with MeT5A citrullination, this trend was not observed (Figure 18). Citrullination of MeT5A cells with LA MCAA (+ve) sera increased the activity compared to non-citrullinated MeT5A cells treated with LA MCAA (+ve). However, this trend was not observed with RA sera. Citrullination of MeT5A cells with RA MCAA (+ve) sera decreased the PLA activity compared to non-citrullinated MeT5A cells treated with RA MCAA (+ve) sera. Interestingly, whether citrullination was present or not, RA sera on MeT5A cells led to overall higher levels of PLA
activity compared to MCAA (+ve) sera. We hypothesized that this is due to a higher titer of antiPLG antibodies in LA MCAA (+ve) sera than there is in RA MCAA (+ve) sera.

Overall, citrullination significantly affected the binding and activation of PLG on mesothelial cells. CitPLG always dropped the PLA activity levels to control or below control levels, while citrullinated MeT5A cells decreased PLA activity by themselves and when tested with RA sera in tandem. Interestingly, LA MCAA (+ve) sera in tandem with MeT5A surface protein citrullination was variable in either dropping or increasing PLA activity and needs to be investigated further.
Chapter 5: Conclusion

In this work, we addressed and examined three major ideas: 1) The binding of MCAA to other proteinaceous targets on the surface of MeT5A cells; 2) The activity of PLA on MeT5A surface as well as the PLG-Rs utilized by mesothelial cells to bind and activate PLG; 3) The effect of citrullination on the binding and activity of PLG as well as its effect on the binding of MCAA.

We hypothesized that MCAA bind another target besides PLG on mesothelial cells. However, we found no other target for MCAA besides PLG. This does not eliminate the possibility that there are other MCAA targets.

Next, we hypothesized that mesothelial cells bind PLG-Rs through the use of multiple receptors: including ENO-1, ANAX2, H2B, and Plg-RKT. We demonstrated PLA activity on mesothelial cells and showed with flow cytometry analysis that ENO-1 and ANAX2 were present on the surface of mesothelial cells. Although H2B was detected, its appearance was never constant, sometimes it showed up strongly and other times not at all. Plg-RKT showed up on the surface of mesothelial cells as well, but through western blotting, we cannot be sure that the antibody is specific for just Plg-RKT. Since the commercial anti-Plg-RKT antibody demonstrated binding to a protein with a higher mass than Plg-RKT, we cannot definitively say Plg-RKT exists on the cell surface until the unknown protein is identified. Also, we did not test every single PLG-R that can exist on a cell surface. Since EACA dropped all binding and activity of PLG on mesothelial cell surfaces, we only tested the lysine-binding PLG-Rs.

However, there are still unknowns about the presence of PLG-Rs on mesothelial cells. First, although we demonstrated that ENO-1 and ANAX2/S100A10 were the most prominent PLG-Rs on the surface of mesothelial cells, Plg-RKT and H2B did show up. Most notably,
western blots run with anti-Plg-RKT antibodies showed a band appear around 60kDa. Plg-RKT is a 17kDa protein, and the band appears too strongly to be background. We suspect that this band is Plg-RKT complexed with another protein or that the antibody is binding a different protein and is not specific to Plg-RKT. However, to confirm the protein identity, the 60kDa band should be excised out of a gel and analyzed via LCMS. This would allow for the determination of the specificity of the Plg-RKT antibody or if Plg-RKT complexes with other proteins and shows up as a higher band.

Second, testing the expression of the receptors themselves would allow for insight into the binding and mechanism of PLG activation on mesothelial cell surfaces. This could be tested by knocking down the receptors via siRNA or shRNA and testing the binding of PLG after knockdown. Third, would be the determination of the actual mechanism behind why mesothelial cells bind PLG. Since many PLG-Rs are translocated to the surface via L-type calcium channels [44], testing differing calcium concentrations on mesothelial-PLG binding would allow for some insight to be gleaned into this mechanism.

The final hypothesis addressed in this work was that citrullination would decrease binding and activity of PLG on mesothelial cells. We showed through the use of citrullination assays and PLA activity assays that mesothelial-PLG binding is vulnerable to citrullination. Citrullination of PLG dropped all activity and citrullination of the mesothelial cell surface significantly dropped the binding and activity of PLG. Therefore, citrullination of mesothelial cells did alter the PLG-binding mechanism. However, further testing is required to determine the importance of citrullination. In addition, citrullination was the only PTM tested. More tests should be run, to examine other PTMs and their effects on this binding mechanism.
Phosphorylation and glycosylation are two other PTMs commonly seen on PLG, with both PTMs existing at the same time on PLG found in human plasma [76, 77].

In addition, the ability of MCAA to bind different cells should be assessed. Since MCAA are mainly comprised of LA-associated, anti-PLG antibodies, MCAA may also potentially bind to other cell types that bind PLG, like macrophages. The ability of these antibodies to bind as well as their effect on cell activity is unknown and should be addressed moving forward as these interactions may contribute to the overall development of LA-associated fibrosis.

Overall, the work presented here shows for the first time that mesothelial cells directly bind to PLG and that PLG is converted to active PLA on the cell surface. It additionally demonstrates binding by a suite of PLG-Rs including: ENO-1, ANAX2, S100A10, H2B, and Plg-RKT and the PLA activity may be reduced by the binding of MCAA or the presence of citrullination on either PLG of mesothelial cell surface proteins. These findings are important because they allow for better understanding of the mechanism behind the development of LPT in LA-exposed populations and the role PLG plays in this mechanism. This takes us one step closer to the development of a therapeutic for the alleviation or inhibition of the exacerbation of LPT.
References


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