January 2021

Complement-Induced Post-Translational Regulation Of Tgf-SS Signaling On Endothelial Cells

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A Thesis Submitted to the

Yale University School of Medicine

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

By

Kevin Liu

2021

Acknowledgements

I would like to take this opportunity to thank my research mentor and PI Dr. Jane-wit for creating a stimulating and supportive research environment in which I was able to personally and professionally thrive, as well as providing guidance in navigating through some of my first steps into the field of medicine. I'd also like to thank Caodi Fang and Lufang Liu in the Jane-wit lab for their openness to collaboration and teaching while at the bench. Furthermore, I'd like to thank Dr. Pober and the other members of the Vascular Biology and Transplantation program for their suggestions and input to help me think more critically about aspects of my research. Finally, I'd like to thank my parents for their unwavering support and encouragement. I was in part supported by a T35 grant from the NIH (NHLB HL007649) and this work was also supported by generous grant support provided by the Jane-wit lab (NHLBI R00 HL125895, NHLBI R01 HL141137) as well as the Vasculitis Foundation and Yale Center for Clinical Investigation.

Complement-Induced Post-Translational Regulation of TGF-ß Signaling on Endothelial Cells

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Abstract

Cardiac allograft vasculopathy (CAV) is a complex vaso-occlusive complication of heart transplantation currently identified as a major limiting factor to long-term survival in those who receive a cardiac transplant. In CAV, neointimal lesions form in graft vessels leading to ischemic complications and ultimately allograft loss. CAV is medically untreatable and affects ~50% of heart transplant patients. Endothelial cells (ECs) are a critical site where CAV lesions form, and antibodies produced by the recipient that bind to donor HLA molecules on graft endothelium (donor specific antibodies) have been identified as a key mediator in this process. Recent studies have further identified a role for antibody-mediated complement fixation, specifically in determining pro-inflammatory signaling changes induced through this process. The laboratory of my mentor, Dr. Jane-wit, studies complement-mediated signaling in ECs and has previously identified a signaling pathway implicated in CAV. In an unbiased assay to identify new components of this pathway, I unexpectedly found that TGF-ß signaling molecules, canonically understood to be anti-inflammatory, were involved in complement-induced EC activation. During my thesis I defined a novel function for proteasomes as cellular chaperones to activate TGF-ß signaling in response to complement activation. My thesis studies identify a pathologic role for endothelial TGF-ß signaling in CAV.

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1. Introduction:

1.1 Heart Failure and Advanced Heart Failure: Prognosis and Treatment Modalities

Heart failure (HF) is a complex chronic and progressive syndrome resulting from structural or functional impairments in ventricular filling or ejection of blood.[1] Diagnosis is made clinically, and manifestations of HF include dyspnea and fatigue limiting exercise tolerance and fluid retention leading to pulmonary and peripheral congestion/edema.[1] In the US, for those over 40 years of age, the risk of developing HF is 20% with stable incidence.[1] Prevalence of HF is estimated to be greater than 5 million persons and increasing.[1,2] The increasing prevalence in context of stable incidence is thought to be due to evolving advancements in treatment options, including renin-angiotensin-aldosterone antagonists, beta-blockers, and implantable cardioverter-defibrillators.[2] A subset of those with HF are those with advanced or AHA Stage D HF (refractory HF) requiring specialized interventions in any NYHA functional class.[1] Etiologies vary from those with chronic HF and specific risk factors, such as diabetes mellitus, obesity, hypertension, and chronic kidney disease, to acute major insults (fulminant myocarditis, acute myocardial infarction with cardiogenic shock).[2-5] Advanced HF is defined as persistent symptoms of HF despite optimal medical therapy and is estimated to affect <1% to 25% of patients with heart failure with best estimates being around 5% from the Acute Decompensated Heart Failure national registry (ADHERE LM).[2-4] Treatment for those with advanced HF is often limited to advanced surgical HF therapies including left ventricular assist devices (LVADs) or cardiac transplantation as those on optimal medical therapy were found to have extremely poor prognoses as demonstrated in the medical arm (compared to LVAD) of the REMATCH

(Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure) trial.[2,4,6] Specifically, survival at 1 and 2 years in the medical treatment arm of advanced HF was found to be 25% and 8% respectively compared to 52% and 23% in the LVAD group.[2,6] While innovations in LVAD technologies have significantly improved since the REMATCH trial (especially centering around continuous flow technologies), those who receive a transplant still have the best long-term course.[2,7] Specifically, 1 and 2-year survival of those with LVADs is about 80% and 70% for continuous-flow pumps (with recent multi-center retrospective studies citing 45% survival to 4 years and mean survival of 4 year survivors to 7.1 years) while transplant survival rates are between 85-90% at one year with median survival between 11-13 years.[2,7-9] Thus again, while improvements in LVAD technologies as well as understanding of LVAD parameters has overall improved prognosis in patients with advanced HF, cardiac transplantation, where possible, remains the best option. However, given the complexity of host immunity in response to donor allograft, much is yet to be discovered/explained in the context of improving long-term sustainability of cardiac allografts.

1.2 Cardiac Allograft Vasculopathy: A Target

While many advances in cardiac transplantation have been made over the years in donor/recipient pairing, immunosuppression, and prevention and treatment of nosocomial infections, cardiac allograft vasculopathy (CAV) is among the top three causes of death in those who survive past the early post-transplant period (~6 months). Nonspecific graft failure, acute rejection, and infection are major causes.[10] CAV also remains as the most common reason for post-transplant candidates to require re-

transplant and is identified as a major limiting factor to longer survival in those who receive cardiac transplant.[10] Angiographic studies have indicated that CAV occurs in 42% of heart transplant patients at 3 years post-transplant while the more sensitive intravascular ultrasonography (IVUS) has pitted estimates of up to 75% at 3 years.[11] While CAV is the best studied in the realm of solid organ transplant and chronic allograft changes leading to dysfunction, it is conceptually very similar to other chronic post-transplant disorders in allograft vasculopathy (in renal transplantation), bronchiolitis obliterans (seen in lung transplantation and in rare situations secondary to inflammation), etc...[11,12] Thus, CAV has been identified as a target for improving outcomes in cardiac transplantation and may further have implications in improving transplant outcomes in other fields as well.[13] Moreover, understanding the underpinnings of the immune-mediated response to allografts could yield valuable information outside of the realm of transplant.

CAV is characterized by concentric fibrous intimal hyperplasia along the transplanted epicardial and intramyocardial vessels leading to diffuse stenosis, tissue malperfusion, ischemia, and ultimately graft loss.[11,14] Changes can be seen as early as 6 months post-transplant as a mild intimal thickening with potential for mild fibrosis and increase in extracellular matrix proteins.[11] Due to the nature of CAV being diffuse and non-focal as compared to focal atherosclerotic CAD, challenges have emerged in diagnosis and treatment. Specifically, traditional angiographic techniques have difficulties in picking up focal areas of stenosis (in attempting to compare proximal vs distal diameters/pressures) and angioplasty or stents are not able to be implemented as there is no focal plaque/atheroma or area to target.[11] Furthermore, no medical therapies have been identified or developed to ameliorate or halt the progression of CAV.[15,16]

Current therapies are all based on consensus expert opinion (level of evidence C) and typically involve modification of the immunosuppressive regimen to include mTOR inhibitors rather than anti-metabolites, but much work is ongoing in identifying targets and understanding underlying pathophysiology of CAV.[17] Thus, there is an urgent need to better understand CAV to develop more specific and effective therapies.

1.3 Pathophysiology of CAV

Early studies on CAV sought to delineate the roles that classical atherosclerotic risk factors (hyperlipidemia, diabetes, HTN, etc…) had to play in post-transplant coronary arterial disease.[18] These early studies found CAV to be clinically distinct from CAD due to atherosclerosis, and identified an immunological component to allograft vasculopathy/CAV.[11,18] Further analysis and studies of pathological samples of CAV tissue indicated some potential similarity between CAV and atherosclerotic disease in the presence of an immune reaction, but identified differences in the primary antigen driver.[13,18] In CAV, pathological areas of intimal hyperplasia were found to respect suture lines separating host from donor, indicating that the primary driver may be MHC incompatibility on luminal endothelial cells as opposed to oxidized LDL in the sub-endothelium as seen in classic atherosclerosis.[13] As such, CAV is often refractory to agents aimed at treating CAD, though superimposed effects of atherosclerosis on CAV may contribute in certain situations.[12,15] Studies performed in immunodeficient mice with implanted human coronary artery segments implicated interactions between transplanted donor endothelial cells (presenting MHC antigens) and host T cells (producing cytokines) as central to CAV.[13,19] Specifically, IFN- γ was identified as a key mediator of intimal expansion and CAV in this model.[13,19] IFN- γ was found to both

induce expression of MHC I and II on arterial endothelial cell surfaces (which they are minimally expressed to non-expressed in its absence) and lead to arteriosclerotic changes in causing intimal expansion.[19] Both innate and adaptive immunity were identified as contributing to CAV, however, the role of innate immune mediators such as natural killer (NK) cells appear to be dependent on the adaptive immune stimulus as, in their absence, NK cells alone do not produce CAV-type lesions in transplanted allografts in B and T-cell immunodeficient mice (scid mice).[14] However, other studies have posited a role for innate immune cells in responding to cellular injury in ischemia-reperfusion injury and cellular necrosis.[12] The role of the innate immune response thus is posited to be immunomodulatory in nature.[12-14]

Initial observational studies in CAV have indicated a higher risk of developing CAV in patients who develop donor specific antibodies (DSA) that are reactive with non-self MHC molecules expressed by donor endothelial cells.[13] Numerous studies have correlated higher DSA levels +/- the presence of complement deposition to worse outcomes in both survival and/or graft dysfunction due to CAV, potentially establishing a relationship between the two and identifying DSA as a potential lead as a therapeutic target.[17] Immunohistochemical analyses of endomyocardial biopsies and serological studies in post-transplant grafts reveal DSA correlates with C4d complement protein deposition and worsened outcomes in the presence of both C4d and C3d deposition (indicating progression down the complement cascade).[14,17,20] From these observations, C4d is used as a diagnostic marker for antibody mediated rejection (AMR) on endomyocardial biopsy due to the fact that it has a long half-life and is covalently bonded to the surface of arterial endothelial cells.[20] One accepted pathway by which DSA mediates damage to graft vasculature is by binding to foreign MHC antigens on

allograft endothelium (with evidence that DSA against class I and class I+II lead to worse outcomes)[20] and fixation of complement via classical pathway including components C1q, C4b, C4d, C3a, C3d, and C5 leading to endothelial cell injury and inflammation.[21] Studies have shown in vivo that antibodies targeting MHC I can provoke graft arteriosclerosis in immunodeficient scid/beige mice potentially indicating the primary target of DSA in causing CAV.[22]

1.4 Current Management Recommendations for CAV

As above mentioned, no level I recommendations exist for treating AMR.[17] Current therapies for CAV center around the principles of modulation and suppression of immune-mediated injury as well as providing supportive therapy for allograft heart failure. Traditional immunosuppressive techniques including corticosteroids, calcineurin inhibitors (tacrolimus, cyclosporine), mTOR inhibitors (rapamycin, sirolimus), and anti-proliferative agents [azathioprine, mycophenolate mofetil (MMF)] targeting B and T cells are often included in managing AMR, though systematic studies have not been performed studying their efficacy.[17] As DSA has emerged as playing a central role in invoking chronic allograft rejection, targeting circulating alloantibodies via plasmapheresis in combination with other immunosuppression techniques (corticosteroids, MMF, cyclophosphamide, etc…) have been implemented in small studies indicating some potential efficacy.[17,23] Inhibition of circulating alloantibodies via IVIg therapy combined again with other immunosuppressive agents has also been utilized in small case series with reversal of rejection, but also with high incidence of recurrence.[17,24] In targeting B cell populations specifically, rituximab, an antibody engineered to target B cell surface marker CD20, has been used in small case series as

monotherapy as well as salvage therapy with good success and has been found to desensitize transplant candidates who have high reactivity against a standard panel of HLA subtypes (high panel reactive antibody activity).[17,25] As CD20 is not expressed on mature plasma cells, the primary alloantibody-producing cell, bortezomib and carfilzomib, proteasome inhibitors used in multiple myeloma to deplete plasma cells, have been trialed with successes leading to ongoing phase II clinical trials.[17,26,27] Specifically, a study in children receiving renal transplants showed stabilization of grafts in C4d positive antibody mediated rejection using bortezomib.[28] Larger studies centered around using bortezomib in adult renal transplant recipients are ongoing in a Phase II trial through the BORTEJECT Study.[26] Last, as C4d and complement are also implicated in the pathogenesis of CAV, it has been raised as a target as well. Eculizumab, a terminal complement inhibitor used in paroxysmal nocturnal hemoglobinuria, has been studied in preclinical trials in rats and murine models as successfully able to prevent antibody mediated rejection in renal and cardiac transplantation, and one report of utilization of eculizumab as salvage therapy was successful in a patient undergoing AMR of a kidney transplant, but a larger study in the renal transplant community showed no differences after 1 year with eculizumab treatment (though it was found to decrease acute clinical antibody-mediated rejection).[17,29-31] Again it is noted that current therapies for CAV are limited, with medical therapies for CAV limited to switching or increasing immunosuppression, and more invasive options including LVADs, PCI (if focal area to target), or re-transplantation.

1.5 Complement as an Active Area of Study

While the presence of C4d is used clinically as a diagnostic marker for AMR, the specific role that complement plays is an area of active study. Complement proteins (synthesized in the liver) function in a series of biochemical conversions in the realm of innate immunity and, in the setting of DSA fixation, initiate the complement cascade culminating in MAC formation via the classical pathway. In this pathway, immune complex deposits (IgG or IgM) on the cell surface expose complement binding sites on the constant portion (Fc) of the antibody leading to a conformational change of the C1 complex, in turn activating C4, then C2, to create the C3 convertase (C4b2a).[32,33] This, in turn, activates C3, leading to creation of the C5 convertase (C4b2a3b) ultimately leading to downstream creation of the membrane attack complex (MAC), composed of C5b, C6, C7, C8, and polymers of C9.[32,33] This terminal complex classically functions via osmotic and ion flux, creating pores on the surface of cells leading to rapid influx of water and ions.[34] However, in nucleated cells and in human cells, numerous protective elements exist in order to prevent rapid lytic destruction, a process called homologous restriction.[35] CD59, a cell surface glycoprotein, regulates terminal complement formation by inhibiting C9 (the final complement protein) from assembling the inner core of pores that are formed by the MAC complex. It is well studied as being defective in paroxysmal nocturnal hematuria, a genetic deficiency of CD55 or decay accelerating factor (DAF).[34] Presence of ion pumps, though they require energy, can also help to stabilize the cell in temporizing and maintaining ion gradients.[34] MAC complexes are also able to be endocytosed into the cell or ectocytosed via budding.[34] While these frank lytic effects of MAC are often avoided in nucleated human cells, studies have shown that assembly of terminal complement structures on different cell types can lead to various signaling changes leading to cell cycle alterations, protein synthesis modifications, and

inflammatory changes.[34] These studies have indicated that at least three potential pathways for signaling exist. First, while ion pumps may maintain membrane integrity, rapid calcium influx due to the large extracellular concentration may lead to modulation of calcium-binding proteins activating downstream effectors, though some of these changes have been observed in a low-extracellular calcium environment indicating possible calcium-independent pathways.[34] Studies have suggested that the MAC itself may interact with G-protein binding motifs as well as TLRs and other signaling receptors on the cell surface, though no formal structure or function has been elucidated.[34,36-38] Finally, the inhibitory CD59 GPI-anchored protein and other GPI anchored proteins could also initiate downstream signaling pathways via clustering by binding to MAC complexes, though this pathway of MAC-CD59 inducing signaling is still being elucidated.[34] Thus, while complement's role in CAV is established as being a downstream component of DSA fixation, the specific mechanism by which it acts in both CAV as well as more generally in cellular processes is an area of active study.

1.6 Developing a Model and System to Study DSA and Complement

A challenge associated with studying *in vitro* and *in vivo* DSA-mediated complement fixation on human endothelial cells is the specificity DSA have for a specific HLA molecule (namely those found on the allograft).[39] As a surrogate to DSA, polyclonal mixtures of antibodies with wide reactivities to a standard panel of HLA antigens, or highly reactive (generally >80%) panel reactive antibodies (PRA), have successfully been able to mimic the effects of DSA binding to a specific HLA surface molecule.[39,40] These polyclonal antibodies were found to successfully deposit on human endothelial cells with fixation of complement proteins potentiating EC-mediated activation of T-

cells.[39] Consistent with the variety of studies regarding terminal complement's effects on nucleated human cells as aforementioned, PRA deposition on endothelial cells led to downstream complement fixation and activation on cell surfaces which, instead of causing lysis, ultimately induced increased transcription of pro-inflammatory mediators.[39] These effects reproduced clinical observations where abundant complement deposition occurs in target vascular beds that show perivascular immune cell infiltrates in the absence of widespread vascular necrolysis.[39,41] Moreover, the above effects of PRA sera containing entirely human-derived components were not seen in other studies incorporating xenogeneic complement components, e.g. antisera, to experimentally induce complement activation.

In using PRA sera to induce terminal complement activation, i.e., MAC assembly, three principal mediators of EC activation were identified, namely DSA, anaphylatoxins, and MAC.[12,39,41] In prior studies involving fractionation and recombination of PRA sera and complement-deficient human reference sera, MAC was found to induce EC activation, characterized by upregulation of chemokines, cytokines, and adhesion molecules.[12,39,41] In interrogating the transcriptional and molecular changes associated with this response, it was found that the non-canonical NF-kB pathway was being activated by MAC.[39] Notable findings in these early studies were that the molecular changes associated with non-canonical NF-kB signaling were both rapidly inducible (changes seen within 30 minutes) and durable (changes lasting up to weeks).[39,41] These findings were inconsistent with the classically described non-canonical NF-kB signaling modality in which changes require ~8 hours in order to be detected.[41] Further work indicated that PRA-dependent MAC activation of non-canonical NF-kB functioned through a novel endosome signaling pathway, utilizing Rab5+ endosomes as opposed to

previously held beliefs that signaling occurred from the cell surface due to calcium flux as aforementioned being described as one of three potential pathways by which complement signaling may occur (calcium-dependent pathway).[41] The terminal complement MAC were found to be endocytosed via clathrin-mediated endocytosis into the cell while localizing to Rab5+ endosomes.[41] Rab5 itself is a small GTPase that mediates downstream functions through recruiting effector proteins and is a marker of early endosomes.[42] Ultimately, a Rab5 endosome effector protein ZFYVE21 was identified as an inducible effector that serves to modulate the endosome membrane lipid content in order to recruit members of the non-canonical NF-kB family in order to potentiate signal activation.[42] Specifically, ZFYVE21 was found to promote SMURF2-mediated polyubiquitinylation and proteasome degradation of endosome-associated PTEN in order to induce endosome-membrane enrichment of PI(3,4,5)P3 in order to recruit activated Akt and NF-kB-inducing kinase (NIK) leading to non-canonical NF-kB activation.[41,42] In follow-up studies performed subsequent to my thesis studies, the Jane-wit lab found that Rab5-associated NIK induced recruitment of inflammasome components including NLRP3 and caspase-1 from the ER and cytosol respectively, and that apposition of these molecules on Rab5 endosomes caused NLRP3 inflammasome activation, a process leading to EC release of IL-1b. [38,43] IL-1b was then found to activate canonical NF-kB.[38,43]

Thus, the pathway as described currently in the literature functions through the following steps: DSA (modeled by PRA) binds to MHC molecules on allograft luminal endothelial cell surfaces and affix complement proteins via the classical pathway.[41,42] Terminal complement forms and these MAC are endocytosed into the cell via clathrin-mediated endocytosis where they co-localize to Rab5+ endosomes.[41,42] This, in turn

leads to effector protein ZFYVE21 being recruited, activating the ubiquitin ligase SMURF2.[41,42] SMURF2 marks PTEN in the endosome for degradation, altering the endosome membrane content to include higher concentrations of PI(3,4,5)P3 to recruit activated Akt which activates NF-kB inducing kinase which leads to downstream NF-kB signaling.[41,42] ZFYVE21 was detected in a variety of patients with complement-mediated disease including transplant rejection, and a bioinformatics search identified a drug inhibitor of ZFYVE21, miltefosine, that blocked CAV in a humanized mouse model.[42]

Figure 1

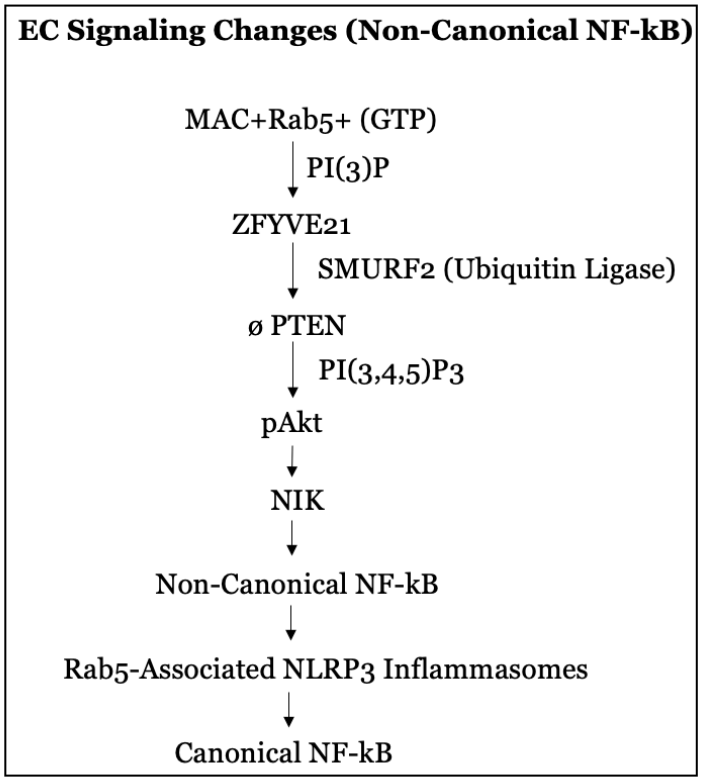

Figure 1: Flow-chart displaying previously described antibody-induced complement fixation pathway functioning through ZFYVE21 to activation of non-canonical NF-kB and downstream canonical NF-kB.

Interestingly, in interrogating changes associated with this pathway of induction of ZFYVE21, MG132, a pan-proteasome inhibitor, was found to induce a similar

repertoire of changes associated with PRA-induced MAC deposition on endothelial cell surfaces, upregulating ZFYVE21 and inducing NIK downstream in a similar fashion suggesting a potential role for proteasomes in initiating this pathogenic pathway.[42]

1.7 Rab5 and the Rab Protein Family

Rab proteins form the largest branch of the small GTP-binding proteins, commonly referred to as small GTPase proteins, that have an essential role in vesicular transport in eukaryotic cells.[44-46] Rab proteins structurally form one of at least 5 families of small GTP-binding proteins including Raw, Rho, Rab, Sar1/Arf, and Ran, in total constituting over 100 members with over 50 Rab proteins having been identified so far.[45] Identified Rab proteins have been shown to be ubiquitous in eukaryotic cells, though there are certain Rab proteins that are cell-type or tissue specific.[44] They, along with other small GTP-binding proteins, cycle between a GDP-bound off-state and GTP-bound on-state that is catalyzed by guanine exchange factor (GEF) proteins, however, it is noted that Rab proteins have been found to bind to effector proteins to some degree even in their "inactive" GDP-bound state.[44,46] Rab proteins have been found to cluster in membrane compartments on live-cell microscopy, establishing distinct micro-domains.[46] Furthermore, Rab proteins have been found to act through recruitment of effector proteins as well as other Rab proteins through specific GEF proteins in order to facilitate an organized cascade of downstream events.[44,46] Due to their distinct localization patterns and specificity for establishing micro-domains, Rab proteins initially were thought to function as markers for transport, ensuring vesicles leaving one compartment would arrive at a correct destination.[44] However, ongoing research has identified Rab proteins binding to effector proteins with very diverse functions,

implicating them in a larger scheme of intracellular signaling and transport.[44,46] Rab5 specifically has been identified as an early endosomal marker generally composed of smaller endosomes that, in a typical progression from early to late endosomes, undergo fusion events leading to fewer, larger endosomes with loss of Rab5 and acquisition of Rab7, a marker of late endosomes.[47] While their role as early endosome markers may be diverse, their apparent dynamic, but consistent expression on early endosomes prove to be useful as a consistent target in order to identify effector proteins associated with these early endosome populations following endocytosis of MAC on endothelial cell surfaces. Rab5 effectors including Rabaptin-5 and certain PI3 kinases initiate a myriad of downstream functions.[44-46,48] ZFYVE21, identified in the studies above, specifically and directly bound to Rab5-GTP complexes but not Rab5-GDP and initiated inflammatory signaling, thus operationally qualifying this protein as a Rab5 effector.[42]

1.8 Proteasome Structure and Function

The proteasome, with known functions in the MAC fixation pathway as targeting PTEN for degradation through the ubiquitin-proteasome pathway is regarded as the principle proteolytic machinery of the cell. Briefly, proteasomes have a core proteolytic element (20S core particle) in a cylindrical orientation that is sandwiched between one or two regulatory particles on either side (19S regulatory particle being the most well studied).[49,50] The regulatory particle is thought to confer a degree of specificity for the core proteolytic element, binding to poly-ubiquitylated substrates and regulating the opening of the core proteolytic element to degradation of proteins.[49,50] Different ubiquitin signals and conformations are thought to confer preference to the proteins that are allowed passage through the core proteolytic element of proteasomes and in

general, polyubiquitin chains have higher affinity compared to monoubiquitinlyated proteins.[49,50] Proteasomes also play a key role in classically described NF-kB activation in both canonical and non-canonical pathways.[49,50] Specifically, ubiquitin-mediated degradation is required for degradation of the NF-kB inhibitor IkB (generally classified in the canonical pathway) and plays a critical role in targeting NF-kB precursors p105 and p100 (p100 targeting classified in the non-canonical pathway) for proteasomal processing into mature forms p50 and p52.[51,52] Ubiquitination has also been found to play a role in NF-kB protein kinase function independent of proteasome function.[51,52] Thus, both canonical and non-canonical NF-kB activation require proteasome degradation of regulatory elements in order to allow for transcriptional activation.[49-52] There have been recent interests in the clinical use of proteasomes inhibitors thought previously to be too cytotoxic for systemic use. FDA-approved proteasome inhibitors including bortezemib and carfilzomib have been used in numerous clinical settings including CAV.[14,26,53]

1.9 NF-kB Signaling Pathway (Canonical, Non-Canonical, and Non-classical Non-Canonical)

Nuclear factor found near the kappa segment of B cells (NF-kB) was initially discovered by the Baltimore lab as a rapidly inducible transcription factor since found to have broad implications in a diverse set of cellular responses ranging from inflammation to oncogenesis. The NF-kB signaling pathway effector proteins consist of 5 family members p50, p52, Rel A (p65), Rel B, and c-Rel which all function through binding to promotor/enhancer sites of target genes regulating transcription by recruitment of activator and repressor proteins.[54-57] At baseline, these effector proteins reside in the

cytoplasm in inactive forms, bound to above-mentioned inhibitory IkB proteins.[54-57] Upon activation, the IkB protein is marked for degradation by proteasomes often after phosphorylation by a IkB kinase complex and the active NF-kB proteins heterodimerize and translocate to the nucleus to modulate expression.[54-57]

In studying activation of the NF-kB pathway, there classically has been a distinction between the "canonical" and "non-canonical" pathways based on the triggers as well as pathway mediators.[54-57] In the canonical NF-kB pathway, microbial products activating toll-like receptors (TLRs) or pro-inflammatory cytokines like TNF-α or IL-1 leads to activation of IkB kinase complexes by TRAF/RIP complexes inducing phosphorylation and degradation of inhibitory IkBα releasing NF-kB dimers which translocate to the nucleus.[54-57] In the non-canonical or alternative pathway (described mostly in lymph-organogenesis as well as B-cell activation), TNF-family cytokines (excluding TNF-α), CD40L, RANKL and B-cell activating factor leads to activation of IkB kinase complexes through TRAFs and NF-kB-inducing kinase (NIK) leading to proteasomal processing of p100 to p52.[54-57] Classically in non-canonical NF-kB signaling, NIK levels increase slowly over several hours and are correlated with degradation of TRAF3 (through ubiquitination).[39,41,54-57] What has been observed in DSA-mediated complement signaling on endothelial cells, however, is that there is a rapid induction of NIK that is not correlated with TRAF3 degradation and the levels are durably elevated for weeks after PRA treatment indicating a non-classical model of non-canonical NF-kB induction.[39,41] As previously described, phosphorylated Akt upstream of NIK plays a central role in this rapid induction and the ZFYVE21 pathway modifies the Rab5 endosomal lipid landscape (via PTEN) in order for phosphorylated Akt to be recruited.[39,41,42]

1.10 TGF-β Signaling Pathway and SMURF2

The TGF-ß family of proteins are nearly ubiquitously expressed in all cell types, including endothelial cells, and play key roles in growth, differentiation, and tissue morphogenesis.[58] TGF-ß signaling occurs primarily through surface complexes of Type I and Type II receptors and their downstream effector Smad proteins that exist in three subgroups encompassing R-Smads, Smad4 in vertebrates, and I-Smads.[58,59] Notably, TGF-ßRI is ubiquitously expressed on cells.[60] The signaling cascade is described as the following: ligand binding or a type III receptor presentation leads to TGF-ßRII (Type II receptor) that is constitutively active recruiting TGF-ßRI (Type I receptor) to form heterodimers.[58,59] Following complex formation between type I and II receptors, the active TGF-ßRI phosphorylates and activates downstream Smad2 and Smad3 which ultimately oligomerize with a common Smad4 to translocate to the nucleus to regulate expression of target genes.[58,59] Again, the roles of TGF-ß are complex and varied, and its role in novel signaling pathways are still being elucidated, though canonically, TGF-ß signaling has been shown to mediate anti-inflammatory effects in T cells, B cells, and macrophages, with the role of this signaling pathway in CAV unknown.[61]

As previously described above, proteomic analyses of FACS-sorted MAC+Rab5+ endosomes showed a reduction in spectral counts for PTEN, a PI(3,4,5)P3 phosphatase that functionally acts as an Akt inhibitor.[42] Prior studies showed that the MAC induced loss of PTEN from MAC+Rab5+ endosomes and that PTEN was inducibly ubiquitinylated and targeted by proteasomes for degradation.[42] To identify E3 ubiquitin ligases that could mediate this process, we looked at the literature for other proteins that could ubiquitinylate PTEN and found that 2 of these proteins were in the NEDD4 family, consisting of 9 proteins.[42,62-66] Of these, the Jane-wit lab identified SMURF2 as a

mediator of PTEN ubiquitinylation.[42] SMURF2, or Smad ubiquitin regulatory factor 2, is a type E3 ubiquitin ligase located primarily in the nucleus that has been best studied in its role as a regulator of the TGB-ß signaling pathway.[67-69] Classically, SMURF2, similar to its related isoform, SMURF1, has been characterized as a negative regulator of TGF-ß, translocating out of the nucleus in response to TGF-ß receptor activation, binding to I-Smads (inhibitory Smads) to ubiquitinate TGF-ß type I receptors leading to their proteasomal degradation to attenuate the TGF-ß signal.[67-69] However, SMURF2 has been found to play a role in numerous other capacities including tissue homeostasis, genomic stability, and even in tumorigenesis (as opposed to its classically studied and accepted role in tumor suppression).[67-69] In the DSA-induced complement signaling pathway, SMURF2 retains its ubiquitin ligase role in ubiquitinating PTEN on the Rab5 endosomal surface, leading to its degradation in order to influence the endosomal lipid content for downstream recruitment of effectors.[42]

2. Statement of Purpose:

As highlighted above, a potential link between antibody-mediated complement fixation has been established with CAV through the MAC-induced ZFYVE21 signaling pathway leading to downstream non-canonical NF-kB induction. To understand the nature by which this novel pathway of non-canonical NF-kB is activated, the focus turns to the means by which the downstream pro-inflammatory signaling proteins are induced on the Rab5+ endosomal membrane.

In terms of protein induction, two general pathways can be considered:

A. "Slow pathway" involving transcription and translation leading to gradual protein accumulation in hours.
B. "Rapid pathway" involving protein activation (phosphorylation by kinases, cleavage, etc…) or rescue from basal degradation (basally transcribed, translated, and ubiquitinated for degradation by proteasomes under homeostatic conditions).

Figure 2

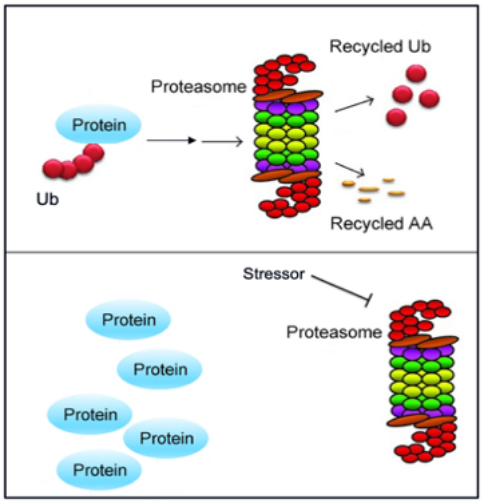

Figure 2: Graphic showing mechanism for proteasomes causing rapid protein induction.

A notable finding in the early studies of the DSA-mediated complement signaling pathway was the rapid nature (within 30 minutes) by which the NF-kB pro-inflammatory signaling proteins were induced. Furthermore, prior to induction, these proteins were not found in either their active or inactive states. Thus, it is hypothesized that these stress-related proteins are basally ubiquitinated and degraded by proteasomes while in homeostatic conditions, and in response to stressors like complement deposition, are rescued from proteasomes to rapidly accumulate to perform their effector function. As noted in the literature described above, proteasomes are known to have some degree of specificity as conferred through their lid element, and the pan-proteasome inhibitor MG132 was found to induce similar changes as DSA-mediated complement signaling.

After induction or rescue of these stress-related proteins, it is posited that the proteins are recruited or localized to a common signaling platform of Rab5+ endosomes where they are stabilized and positioned in proximity to each other to perform their downstream signaling function. Rab5+ endosomes were found to be a centralized location in which the initial components of DSA-mediated complement signaling were found.

The initial aim of my study was to identify other proteasome-mediated molecules like ZFYVE21 that were recruited to Rab5 endosomes to activate NF-kB. During the course of my studies, I unexpectedly uncovered a role for TGF-ß signaling in CAV, and I redirected my study to understand how this pathway became activated and how it mediated CAV.

3. Hypothesis:

Antibody-induced complement activation post-translationally sequesters pro-inflammatory signaling proteins on Rab5+ endosomes following a two-step model:

1. *Inhibition of a proteasome population leads to rapid up-regulation of inflammation-related proteins.*
2. *After rescue, inflammatory proteins are recruited to Rab5 endosomes in order to perform effector function.*

4. Methods:

*Student independently performed

**Student performed parts of experiment

***Performed by other members of lab

Endothelial Cell Culture and Treatment*: Human umbilical vein endothelial cells (HUVEC) obtained as de-identified tissue discarded from the Department of Obstetrics and Gynecology at Yale New Haven Hospital were plated in gelatin-coated flat bottom culture flasks, dishes, or multi-well plates (BD Biosciences, San Jose, CA) with Endothelial Cell Growth Medium-2 (EGM-2MV) containing 20% FBS, hydrocortisone, hFGF-B, VEGF, IGF-1, ascorbic acid, hEGF, and GA-1000 (Lonza Bioscience). Cells were serially cultured at 37°C with 5% CO2. All experiments in this manuscript were performed with cells at passage levels 2-6 at which point such cultures are free of contaminating leukocytes and uniformly express EC markers. To split the cells and/or plate for culture, trypsin-EDTA (0.25%) was used to dissociate the adherent cells from the vessel. Cells were trypsinized for no more than 60 seconds. Cells were plated at an ideal concentration of 10,000 cells in 200 µl. Discarded high-titer PRA sera were obtained as de-identified samples from Yale-New Haven Hospital's tissue typing laboratory and showed >80% reactivity to either HLA class I and/or II antigens. PRA sera underwent endotoxin testing according to manufacturer's specifications (Sigma) and human viral pathogen testing under h-IMPACT testing protocols (IDEXX RADIL, Columbia, MO). Prior to PRA treatment or in control groups for PRA treatment, HUVEC were pre-treated for 48-72 hours with IFN- γ (50ng/mL, Invitrogen, Grand Island, NY) in EGM-2 medium prior to addition of a 1:10 dilution of a PRA positive serum in gelatin veronal buffer (Sigma, St. Louis, MO) for the indicated times. For MG132 treatment,

MG132 (25 μM, Selleck Chemicals) was added to EGM2 media for the indicated times at the indicated concentration of 1:1000.

Western Blotting/Analysis*: Expression levels of intracellular proteins were quantified by western blotting. To do so, EC monolayers grown to 80-90% confluence from C6 or C12 wells (BD Biosciences, San Jose, CA) were washed in ice cold PBS three times and lysed in RIPA buffer (Sigma). Samples were then mixed with Laemmli's sample buffer, heated at 95°C for 10 minutes, and loaded at 30μg per lane. After electrophoretic resolution, samples were transferred onto a PVDF membrane (EMD Millipore) for two hours at room temperature. Membranes were blocked with 5% BSA and primary antibody was added at 1:1000 dilution overnight at 4°C while rocking gently. Primary antibodies were all used at 1:1000 dilution and included ZFYVE21 (Novus Biologicals), SMURF2 (Cell Signaling Technology), NIK (Cell Signaling), active Rab5 (NewEast Biosciences), Rab5 (Santa Cruz Biotechnology), TGF-ßRII (R&D), smad2 (R&D), smad3 (R&D), p-smad2/3 (R&D), TGF-ßRI (R&D), PSME4 (Abcam), PSMD3 (Abcam), KIAA0368 (Abcam), PSME1 (Bethyl), PSMF1 (Bethyl), PSMB8 (Bethyl), PSMA2 (Bethyl), PSMC2 (Bethyl), Rubicon (Cell Signal), and β-actin (Sigma). Anti-mouse and anti-rabbit horse radish peroxidase (HRP)-conjugated antibodies (ThermoScientific, Waltham, MA) were then added at room temperature for one hour at a dilution of 1:1000 and bound HRP was visualized using chemiluminescent developing of probed membranes on X-ray film (Denville Scientific, Metuchen, NJ) as per manufacturer's specifications (SuperSignal Pico West, Pierce, Junction City, OR). Densitometry was performed using NIH Image J software (Bethesda, MD).

Co-Immunoprecipitation****:** For co-IP experiments, 20 μL protein A/G beads (ThermoFisher) were incubated with 10 μL Rab5 antibody, PMSC2 antibody, or PSMA2 antibody at 4 °C overnight. Rab5 antibody (1 μg, abcam) and protein lysates (10ug) were incubated at 4 °C overnight (Pierce). Antibody-conjugated beads. The next day, antibody-bound lysates were mixed with agarose protein A/G beads. The beads were washed and resuspended at a volume of 32 μL using RIPA buffer (Cell Signaling). For western blotting, following the incubations, Laemli's buffer (12 μL) and 1 mM dithiothreitol (6 μL) were then added to samples, heated for 95 °C for 13 min, and subjected to western blotting. Antibodies used for western blotting were all used at 1:1000 dilution as described above. For proteomics analysis, after elution of protein, labeled samples were sent to Yale Proteomics lab (The Mass Spectrometry (MS) & Proteomics Resource of the W.M. Keck Foundation Biotechnology Resource Laboratory).

Transfection of Rab5 Constructs*****:** Rab5-GFP WT and DN constructs were gifts from Dr. Michael Simons (Yale University School of Medicine, New Haven, CT). To transfect plasmids, ECs at 60– 70% confluency in 24-well dishes were pretreated with IFN-γ as above for 48 h, followed by transfection of dynamin or Rab5 WT and DN constructs using lipofectomy. Then 500 ng of each construct was mixed with 0.4 μL of PLUS reagent (Invitrogen) in 200 μL/well of Opti-MEM for 5 min at room temperature, followed by the addition of 1.5 μL of Lipofectamine 2000 (In- vitrogen) for 30 min at room temperature. This mixture was added to cultured HUVECs at 37 °C for 8 h, followed by washing and buffer exchange with EGM2-MV medium. Using this protocol, ~30–50% of ECs remained viable for analysis. The transfection efficiency calculated

using flow cytometry measuring GFP fluorescence was typically >45%. GFP+ ECs were then sorted and replated to yield homogeneous cultures carrying each respective construct.

siRNA transfection of EC:** HUVECs were pretreated with IFN-γ for 48hrs prior to siRNA transfection. siRNA (Dharmacon, Waltham, MA) targeting PSMC2 or non-targeting siRNA (target sequence UAA CGA CGC GAC GAC GUA A) were purchased commercially (Dharmacon) and transfected into HUVECs at ~60–70% confluency in 24-well plates (BD Falcon). siRNAs were diluted at 40 nM concentration in Opti-Mem culture media (Gibco) and mixed at equal volume with RNAiMax transfection reagent (Invitrogen) diluted 1:50 in Opti-Mem for 45 min at room temperature. This mixture was then added to HUVEC cultures at 37 °C for 6 h prior to washing and buffer exchange with EGM2-MV. Cells were then analyzed by western blots 72 h after transfection.

Statistical methods*: Statistical analyses were performed using the computer software "Origin" (Origin, Northampton, MA). Absolute numbers and percentages of vesicles were analyzed by Student's t test and Chi-squared analyses, respectively. p values < 0.05 were considered statistically significant. Multiple comparison analyses were performed using analysis of variance. Standard deviations are reported throughout the text. References using the ImageJ image analysis software (Bethesda, MD) with the Just Another Colocalisation Plugin (JACoP).

5. Results:

5.1 Proteomics Screen to Identify Rab5+ Endosome-Sequestered Proteins

Given the central role that the Rab5+ early endosome population plays in the DSA-mediated complement signaling pathway, our focus initially centered on searching for and identifying pro-inflammatory signaling proteins that are post-translationally sequestered on Rab5+ endosomes in response to DSA. As Rab5 is ubiquitous on these endosomes, our strategy was to use Rab5 to perform co-immunoprecipitation to pull down endosomal populations of interest, elute peptides from said endosomes, and perform proteomic analysis to interrogate the effector proteins that are recruited to these endosomes (Figure 3). Specifically, we cultured five groups of human umbilical vein endothelial cells (HUVEC) with experimental Rab5 construct groups and treatment groups. The five groups were Rab5DN (constitutively inactive) control, Rab5Q79L (constitutively active) control, Rab5Q79L (constitutively active) with MG132 pan-proteasome inhibitor, Rab5Q79L (constitutively active) with PRA treatment for 4 hours, Rab5WT (wild type) with PRA treatment. Both Rab5DN and Rab5Q79L groups were used as controls to identify the spectrum of proteins normally recruited to Rab5 endosomes under homeostatic conditions. The Rab5Q79L group with MG132 was used in order to screen for proteins that are nonspecifically rescued from proteasomal degradation and

Figure 3

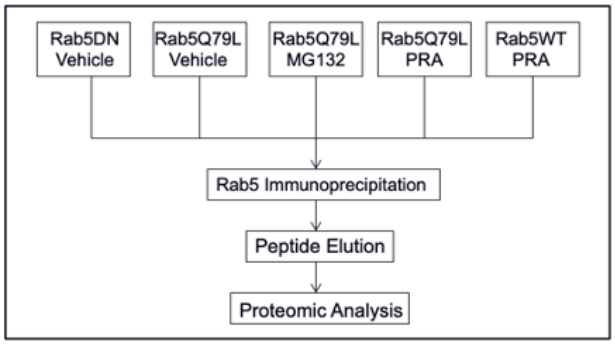

Figure 3: Proteomics screen workflow to identify differentially expressed and recruited proteins on Rab5+ endosomes.

recruited to active Rab5 endosomes. The Rab5Q79L group with PRA treatment served to comb through pro-inflammatory proteins rescued from degradation and recruited to active Rab5 endosomes. Last, the Rab5WT group with PRA treatment mimics the pathophysiological response to DSA binding onto allograft transplant vessels and to the DSA-mediated complement fixation pathway.

From this proteomics screen, 1956 unique proteins on Rab5 endosomes were identified and categorized based on their known associated pathways (Figure 4). Notably, 494 unique proteins were found to be in common between all 5 groups. The Rab5WT-PRA group was found to have 175 unique proteins and shared 67 proteins with the inactive Rab5 (Rab5-GDP) control group and 5 proteins with the active Rab5 (Rab5-GTP) control group (Figure 4a). In focusing on the proteins unique to the PRA-Rab5WT group that were not found in the control Rab5 groups, many of the proteins differentially unregulated and recruited on Rab5 endosomes fell into families of general inflammatory mediators such as in the Type 1 IFN response family, IL-12 signaling family, and IL-6 signaling family (Figure 4b). Other proteins were found to be related to vesicle trafficking pathways such as clathrin-mediated endocytosis signaling and secretory vesicle proteins, indicating a likely increase in vesicle transport in the PRA-Rab5WT group (Figure 4b). The top family that was differentially unregulated and recruited in the PRA-Rab5WT group compared to control groups, however, was the SMAD-Associated Signal Activation proteins, or proteins associated with the TGF-ß pathway (Figure 4b). Alongside this strong positive differential presence of TGF-ß family proteins on Rab5 endosomes in DSA-induced complement fixation states, it is again noted from prior literature (above-mentioned) that the DSA-mediated complement pathway utilizes SMURF2, a known TGF-ß signaling protein, suggesting a

link between these two seemingly divergent pathways. Preliminary analysis performed from the proteomics screen furthermore confirmed increased amounts of TGF-ß family proteins on Rab5 endosomes in the PRA-Rab5WT group compared to control groups such as TGF-ß1, TGF-ß2, TGF-ßR2, BMP-R2, and SMAD proteins (Figure 4c).

Figure 4

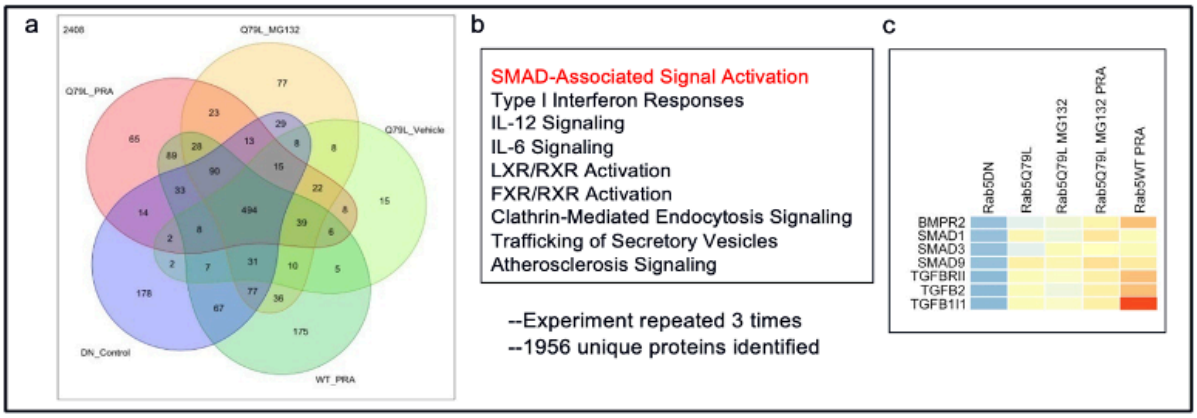

Figure 4: Proteomics screen results from Rab5 co-IP of experimental and control groups. a) Venn diagram indicating unique proteins on Rab5 endosomes in each experimental group and control group. b) Proteins identified on Rab5 endosomes unique to PRA-Rab5WT group compared to control groups classified into families with emphasis on the SMAD-Associated Signal Activation family or TGF-ß related proteins as being a top hit in proteomics screen. c) Preliminary comparative quantification of certain TGF-ß family proteins identified on Rab5 endosomes showing upregulation and recruitment of TGF-ß family proteins to Rab5 endosomes in PRA treated endothelial cells compared to control groups (baseline is Rab5DN or Rab5-GDP).

As the TGF-ß family of proteins has been identified as a potential target of interest that may serve effector functions in the DSA-mediated complement fixation pathway, our aim moving forward focuses on showing that TGF-ß proteins follow the two-step model described above of first being rescued from basal degradation by a proteasome population and second being recruited to Rab5 endosomes to perform their effector function.

5.2 TGF-β Signaling Components are Induced Following Antibody-Induced Complement Fixation

In order to confirm the initial results obtained by the proteomics screen that DSA-mediated complement fixation leads to induction of TGF-ß components, western blots of serial co-IPs of Rab5 were performed following PRA treatment of endothelial cells at 0, 5, 15, and 30 minutes of both previously described antibody-induced complement signaling pathway mediators such as ZFYVE21, SMURF2, and NIK, as well as proximal TGF-ß pathway mediators including TGF-ßR2, smad2, smad3, and the phosphorylated smad 2/3 complex (Figure 5).

Figure 5

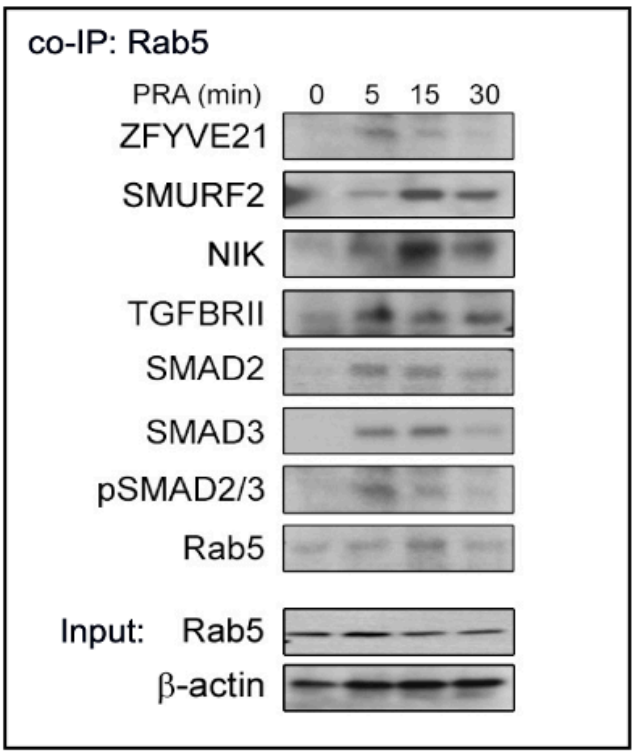

Figure 5: Western blots of serial co-IPs using Rab5 in PRA treated endothelial cells for previously described antibody-induced complement induction of non-canonical NF-kB (rows 1-3) as well as TGF-β signaling components (rows 4-6). Both antibody-induced complement effector proteins as well as TGF-β signaling components show rapid induction at 5 minutes and are sustained to at least 30 minutes. Experiment repeated two times.

In confirming prior studies describing DSA-induced complement signaling, ZFYVE21 was found to be up-regulated on Rab5 endosomes within 5 minutes, leading to subsequent increases in both SMURF2 as well as NIK, again displaying previously described rapid induction of non-canonical NF-kB (Figure 5). Furthermore, proximal

signaling effector proteins in the TGF-ß pathway including TGF-ßRII, smad2, smad3, and the activated heterodimerized phosphorylated smad2/3 complex were all found to be induced in a similar rapid timeframe as the antibody-induced complement signaling pathway with sustained increase up to 30 minutes (Figure 5). Thus, antibody-induced complement fixation is seen to lead to rapid induction of TGF-ß proximal effector proteins in a similar timeframe as induction of the ZFYVE21-mediated pathway activating non-canonical NF-kB through NIK.

In the case of antibody-induced complement fixation leading to up-regulation of ZFYVE21 and downstream non-canonical NF-kB induction, a pan-proteasome inhibitor MG132 was previously found to induce similar rescue of ZFYVE21 and associated proteins leading to the hypothesis that there is basal degradation of antibody-induced complement signaling pathway effectors by proteasomes. To test whether TGF-ß proximal effector proteins are similarly degraded at baseline by proteasomes, we performed similar experiments introducing MG132 at the same concentration to endothelial cells and subsequently performed serial western blots of proximal TGF-ß pathway effector proteins (Figure 6). Again, similar to previously described induction of ZFYVE21 and downstream proteins, TGF-ßRII and smad2 were found to be induced in endothelial cells within 30 minutes of treatment with MG132 and sustained to at least 4 hours (Figure 6). TGF-ßRI was found to be ubiquitously

Figure 6

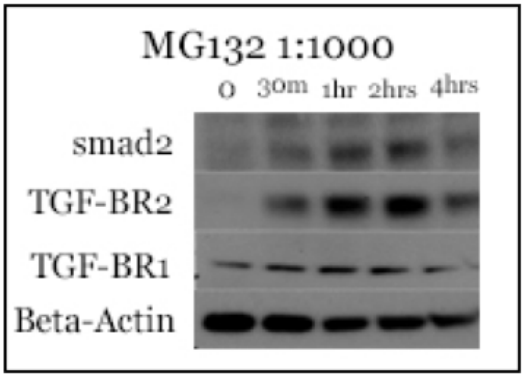

Figure 6: Serial western blots of endothelial cells treated with pan-proteasome inhibitor MG132 over time blotting for TGF-ß effector proteins including smad2, TGF-ßR2, and TGF-ßR1. TGF-ß effector proteins smad2 and TGF-ßRII were found to be induced within 30 minutes sustained to at least 4 hours, and TGF-ßRI expressed at baseline and unaffected by MG132. Experiment repeated 5 times.

expressed as previously described in the literature and minimally affected by MG132 treatment (Figure 6). Thus, it appears that similar to ZFYVE21 and other antibody-mediated complement signaling effectors described previously in the literature, TGF-ß effector proteins are similarly degraded at baseline by proteasomes and, in the presence of antibody-mediated complement fixation, are rescued from degradation.

5.3 Downstream TGF-β Signaling Components are Post-Translationally Rescued from Proteasomal Degradation and Proximal TGF-βR2 is Post-Translationally Stabilized on Activated Rab5 Endosomes

To further interrogate the dynamics of TGF-ß superfamily proteins after post-translational rescue from proteasomal degradation and their relationship to Rab5 endosomes, we transduced HUVEC cells with two different Rab5 constructs: Rab5 S43N (dominant negative) and Rab5 Q79L (constitutively active) (Figure 7). After establishing these groups, we treated each with pan-proteasome inhibitor MG132 which binds to the 20S catalytic core, and performed serial western blots over time (0, 1, 2, and 4 hours) for ZFYVE21 (as an internal control) and members of the TGF-ß superfamily of proteins including TGF-ßRII, smad2 and smad3 (Figure 7).

Figure 7

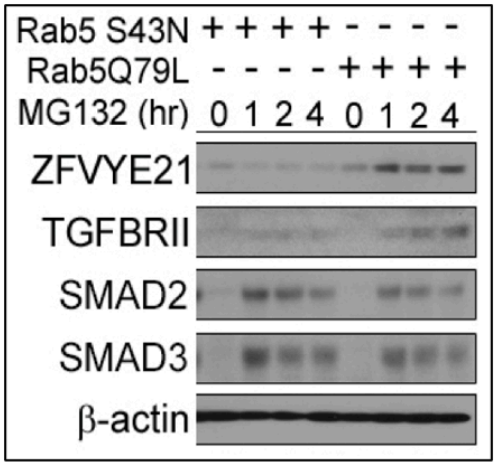

Figure 7: HUVEC cells in two groups: with transduction of Rab5S43N construct (dominant negative Rab5-GDP) and with transduction of Rab5Q79L construct (constitutively active Rab5-GTP) treated with pan-proteasome inhibitor MG132 with serial western blots performed at 0, 1, 2, and 4 hours for ZFYVE21, proximal TGF-βR2, and distal signaling SMAD2 and SMAD3 proteins. Upregulation of SMAD2 and SMAD3 can be seen in both groups treated with MG132 at 1 hour sustained to 4 hours. ZFYVE21 and TGF-βR2 are seen to be upregulated in only the Rab5 constitutively active group treated with MG132 at 1 hour sustained to 4 hours. Experiment repeated 2 times.

As can be seen in both of the groups, the pan-proteasome inhibitor MG132 induced post-translational rescue of the downstream TGF-ß signaling components SMAD2 and SMAD3 beginning at 1 hour after treatment sustained to 4 hours after treatment (Figure 7). However, levels of both ZFYVE21 and TGF-ßRII were not seen to be increased in the Rab5 dominant negative group treated with MG132 alone (Figure 7). In comparison, in the constitutively active Rab579L group, both ZFVYE21 and TGF-ßRII were found to be increased (Figure 7). From this, it can be deduced that while TGF-ßR2 and ZFYVE21 are both post-translationally rescued from proteasome degradation by a pan-proteasome inhibitor, there may be a secondary requirement of being translocated to Rab5-GTP or active Rab5 endosomes in order for them to be stabilized.

This combined with Figure 6 shows again the integral role that proteasomes appear to play in the signaling modality that is induced by antibody-mediated complement fixation.

5.4 The PA200 Proteasome Population is Selectively Inactivated by Antibody-Induced Complement Activation

As discussed above in literature review, proteasomes are posited to have some degree of specificity in the proteins that they target. Furthermore, it is noted in the proteomics analysis performed at the outset that the proteins discovered in the MG132-Rab5-GTP group (pan-proteasome inhibitor and constitutively active Rab5) did not entirely overlap with the PRA-Rab5 group (Figure 4a, 4c) suggesting that a specific proteasome population is selectively inactivated in the presence of antibody-induced

complement fixation. In order to interrogate whether there is selective inactivation of proteasome populations by antibody-induced complement activation, cells treated with PRA underwent serial co-IP over time (0, 5, 15, 30, and 60 minutes) using a common core proteolytic element PSMA2, and western blots were performed to identify specific proteasome populations of interest (Figure 8). Co-IP was performed using the common core component PSMA2 in order to ensure internal validity by normalizing the levels of proteasomes sampled over time.

Figure 8

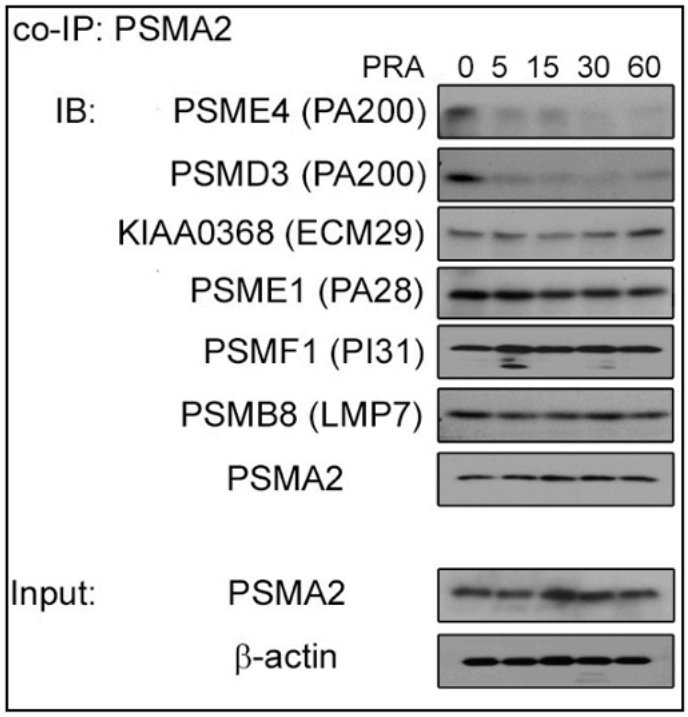

Figure 8: Western blots of serial co-IPs using PSMA2 (a common core proteolytic element) in PRA treated endothelial cells for specific proteasome populations showing two specific PA200 lid populations (PSME4 and PSMD3) being degraded within 5 minutes in rows 1 and 2 and stable levels of other proteasome specific elements up to one hour in rows 3 to 7. Experiment repeated two times.

From this, it is noted that two components that confer specificity to the PA200 proteasome population, PSME4 in row 1 (encoding the specific PA200 molecule that binds to and affects proteasome function) and PSMD3 in row 2 (a regulatory 26S subunit on the cap of proteasomes), are selectively inactivated within 5 minutes of PRA treatment, while levels of other proteasome population lid elements in rows 3-7 are stable up to one hour following PRA treatment (Figure 8). This finding serves to suggest that antibody-induced complement activation specifically targets the PA200 population.

5.5 The PA200 Proteasome Basally Degrades ZFYVE21 Effector Proteins and TGF-β Signaling Molecules

While antibody-induced complement signaling appears to specifically target the PA200 proteasome population for degradation, attention then was turned to whether the PA200 proteasome population indeed targets and degrades antibody-mediated complement signaling pathway mediators such as previously described ZFYVE21 mediators as well as our newly found TGF-ß superfamily proteins.

In order to confirm that the PA200 proteasome targets components induced by antibody-mediated complement fixation, we knocked down a PA200 proteasome lid element via siRNA of the PSMC2 gene in the presence and absence of PRA and performed western blots of ZFYVE21 mediators and proximal TGF-ß pathway mediators (Figure 9). In order to confirm knock-down of PSMC2 with the siRNA, PSMC2 was blotted in Row 8 indicating partial, but not complete

Figure 9

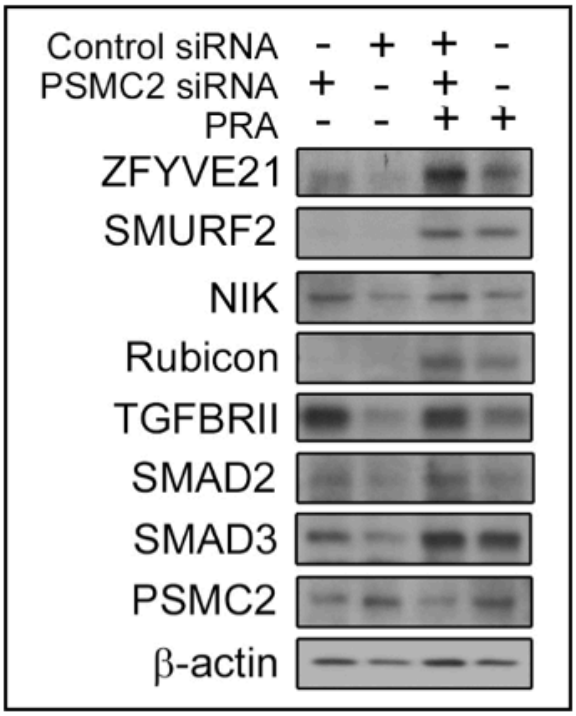

Figure 9: PSMC2 siRNA (PA200 proteasome lid component) was introduced into endothelial cells in the presence and absence of PRA treatment and western blots were performed for ZFYVE21 signaling proteins as well as TGF-βRII signaling proteins. PSMC2 blotted in row 8 was used to determine effectiveness of siRNA knockdown. In comparing lanes 1 and 2, it is seen that there is rescue of ZFYVE21 signaling proteins as well as TGF-β signaling proteins in the PSMC2 knockdown group, and similarly in lanes 3 and 4 rescue of both families of proteins in the knockdown group. In comparing lanes 2 and 4 (control siRNA in the absence and presence of PRA treatment), it is once again seen that both families of signaling proteins are induced following PRA treatment.

knock-down in the PSMC2 siRNA groups as compared to the control siRNA groups (Figure 9). When comparing lanes 1 and 2 in the non-PRA-treated group, it is observed that ZFYVE21 and its downstream effectors as well as TGF-ß pathway effectors all increase in the presence of the proteasome lid knockdown group compared to the control siRNA group (Figure 9). A similar increase can be seen when one compares lanes 3 and 4 in the PRA-treated groups (Figure 9). Specifically, ZFYVE21, SMURF2, NIK, and Rubicon in the ZFYVE21 pathway induced by antibody-mediated complement fixation and TGF-ßRII, smad2, and smad3 in the TGF-ß superfamily of proteins were found to increase in the proteasome knockdown group compared to controls in both physiological and PRA-treated settings (Figure 9). It is also noted that when one compares the control groups to the PRA groups, there were increases in proteins from both the ZFYVE21 pathway as well as TGF-ß pathway (specifically by comparing lanes 1 and 3 and 2 and 4) (Figure 9).

Last, of note is that the level of PSMC2 appears to have been unaffected by PRA treatment, potentially indicating the lid component of the PA200 proteasome remains intact, but unable to associate with the PA200 proteasome or perform effector function (Figure 9). The idea that these lid components dissociate from the core proteasome element is supported from data shown in Figure 8 as, following PRA treatment, the lid elements PSME4 and PSMD3 are no longer found to be pulled down with core element PSMA2 in co-IP studies.

5.6 PA200 Proteasome Components are Recruited to Rab5+ Endosomes

In further exploring the PA200 proteasome dynamics in relation to the Rab5 endosomes where antibody-mediated complement signaling proteins appear to localize,

attention was turned to whether or not proteasome components are recruited to Rab5 endosomes at baseline or whether they translocate following antibody-mediated complement fixation. From the proteomics screen, when comparing constitutively active Rab5 endosomes (Rab5-GTP), constitutively active Rab5 endosomes with MG132 pan-proteasome inhibitor treatment, and wild-type Rab5 treated with PRA, numerous proteasome components are seen to be upregulated on Rab5 endosomes in endothelial cells treated with PRA compared to cells with constitutively active Rab5 or with constitutively active Rab5 with proteasome inhibitor MG132 (Figure 10a). This suggests that antibody-induced complement fixation differentially recruits proteasome elements onto Rab5 endosomes. However, as noted again in Figure 8, specific components of the PA200 proteasome are not found to be associated with functional core PSMA2 proteasome components following PRA treatment, indicating that apparent recruitment of lid components does not equate to proteasome function.

Furthermore, when performing serial co-IP of the proteasome lid component PSMC2 and blotting for signaling proteins induced by antibody-induced complement fixation, it is seen that over time there are increases in Rab5, TGF-ßRII, and ZFYVE21 after PRA treatment (Figure 10b). Increase in Rab5 co-localization with PSMC2 after PRA treatment shown in row 1 once again points towards recruitment of the PSMC2 lid component to these Rab5 endosomes and increase in TGF-ßRII and ZFYVE21 reaffirms previously described co-localization on the Rab5 endosome in rows 2 and 3 (Figure 10b). Presence to some degree of TGF-ßRII and ZFYVE21 at time 0 in rows 2 and 3 with PSMC2 could further be indicative of these proteins bound to PSMC2 destined for degradation, but prior to entering the proteasome core element to be fully degraded (Figure 10b).

Figure 10

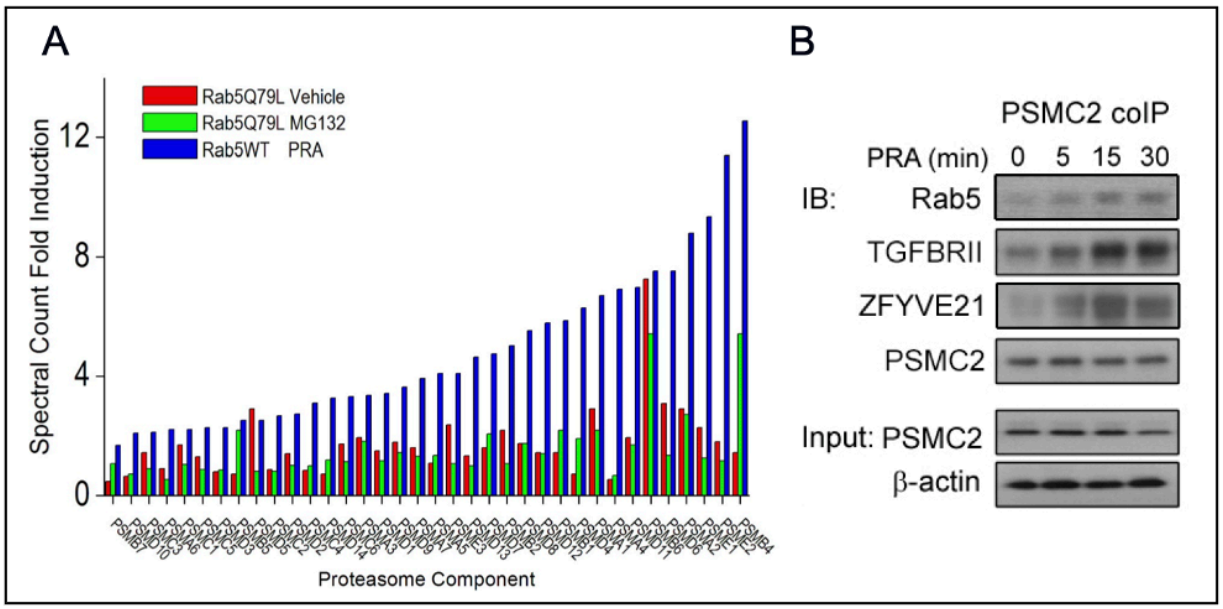

Figure 10: Recruitment of proteasome components to Rab5+ endosomes. a) Proteomic analysis of proteasome component relative amounts comparing constitutively active Rab5-GTP transduced endothelial cells, constitutively active Rab5-GTP transduced endothelial cells with MG132 pan-proteasome treatment, and wild-type Rab5 treated with PRA. Relative counts are compared to constitutively inactive Rab5-GDP. Numerous proteasome components are found to be recruited with highest relative induction in endothelial cells treated with PRA as compared to other two groups. b) Western blot of serial co-IPs over time of proteasome lid component PSMC2 in endothelial cells after PRA treatment showing increased pulldown of Rab5 endosomes with TGF-βRII and ZFYVE21 proteins.

6. Discussion:

The present work aims to establish the TGF-ß superfamily of proteins as a potential component in the pathogenesis of cardiac allograft vasculopathy induced by DSA-mediated complement fixation as well as study more generally the mechanism by which antibody-mediated complement fixation leads to rapid signaling changes, such as leading to activation of non-canonical NF-kB (and subsequently canonical) as previously described in the ZFYVE21 pathway.[42]

As ZFYVE21 and its downstream mediators were found to localize to Rab5 endosomes following antibody-mediated complement fixation, we hypothesized initially that the previously described landscape of protein makeup on Rab5 endosomes in

homeostatic conditions differed from the proteins recruited to Rab5 endosomes following a stressor such as antibody-mediated complement fixation. Thus, in order to elucidate or search for those differences, we grouped HUVEC cells into five different conditions/treatments reflecting homeostatic vs non-homeostatic conditions and performed co-IPs of Rab5 in order to capture early Rab5 endosomes and sent the eluted peptides of the endosomes for proteomic analysis. The five groups we chose included HUVEC cells with constitutively inactive Rab5 under homeostatic conditions, constitutively active Rab5 under homeostatic conditions, constitutively active Rab5 with pan-proteasome inhibitor MG132, constitutive active Rab5 with PRA treatment, and a wild-type Rab5 with PRA treatment. Both the constitutively inactive and active Rab5 groups under homeostatic conditions served as controls in order to sample all the proteins that would be recruited to early Rab5 endosomes. The MG132 group with constitutively active Rab5 was meant to sample all the proteins that are rescued from proteasomal degradation that have peptide motifs that would allow them to be nonspecifically recruited to active Rab5 endosomes. The constitutively active Rab5 endosomes treated with PRA were meant to understand the specific sub-population of proteins that are induced by antibody-mediated complement fixation and recruited to active Rab5 endosomes and, through comparison with the Rab5 wild-type group treated with PRA, one could deduce the proteins that are recruited to inactive Rab5 endosomes treated with PRA. Finally, the wild-type Rab5 group with PRA treatment served to closely mimic the proteins and signaling events occurring in antibody-mediated complement fixation seen as one component causing CAV.

Classically, rigorous experimental design would necessitate the presence of a wild-type Rab5 group under homeostatic conditions instead of a constitutively active

and inactive Rab5 under homeostatic conditions in order to prevent the possibility of confounding variables, however, the spectrum of proteins recruited to constitutively active and inactive Rab5 endosomes under homeostatic conditions was seen as more informative as a dual-control variable given the ability to sample all proteins in homeostatic conditions that would be recruited to Rab5 endosomes, and would give more insight down the road in terms of proteins that are differentially recruited in GTP and GDP states. One must recognize, however, the potential confounding variable the process of transfection of the HUVEC cells with gene constructs may cause in inadvertently altering the landscape of signaling events. While there is no specific literature discussing the role of transfection affecting the landscape of Rab5 endosomes, transfection, whether it be viral (biological) or chemical has been theoretically proposed to affect the cell.[70,71] However, in the case of this study, all further analysis after the proteomics screen was done with native HUVEC cells with or without PRA treatment. Thus, there is greatest risk of a beta error, or missed signaling pathway, from this proteomics screen as there may be proteins recruited to Rab5 endosomes in the control groups that otherwise would not be without transfection.

Through this proteomics screen, we identified the TGF-ß superfamily of proteins as being differentially upregulated on Rab5 endosomes as compared to homeostatic conditions, pointing towards its role as an antibody-mediated complement pathway mediator. As previously stated, the TGF-ß superfamily protein SMURF2 was previously identified as playing a role in the ZFYVE21 pathway, hinting at the role that the TGF-ß pathway may play in the greater signaling changes induced by antibody-mediated complement.[42] Furthermore, it is well described in the literature that proximal TGF-ß receptors localize to and signal from early endosomes following clathrin-mediated

endocytosis and a less clearly described lipid/caveolae-mediated endocytosis.[72-75] Thus, we decided to move forward with studying the TGF-ß superfamily of proteins in its relation to antibody-mediated complement signaling.

In order to confirm the upregulation of TGF-ß component proteins on Rab5 endosomes following antibody-mediated complement fixation, western blots of co-IP of Rab5 showed rapid induction of TGF-ß component proteins on Rab5 endosomes similar to both previously described ZFYVE21 pathway mediators as well as repeat ZFYVE21 blots performed for the sake of internal validity.[42] It was observed that within 5 minutes, TGF-ß signaling components were upregulated on Rab5 endosomes. Furthermore, in returning to our original hypothesis, these TGF-ß signaling components seem to follow a similar "rapid induction" that would not be expected in the case of differential gene expression leading to transcription and translation. Thus, in order to test the hypothesis that TGF-ß signaling components follow closely with ZFYVE21 pathway mediators as being rescued from a baseline proteasomal degradation, we treated HUVEC cells with MG132, a pan-proteasome inhibitor in order to study whether or not these TGF-ß mediators were basally transcribed, recruited to proteasomes, and degraded. Serial western blots over time after MG132 treatment was notable for upregulation of TGF-ß signaling components more firmly implying a role for proteasomes in the antibody-mediated complement signaling as well as partially satisfying the first posit of our initial hypothesis that inhibition of proteasomes leads to rapid upregulation of inflammation-related proteins.

Next, we sought to test the conditions in which antibody-mediated pathway proteins are recruited to Rab5 endosomes. We introduced pan-proteasome inhibitor MG132 to cells with constitutively inactive Rab5 and constitutively active Rab5. From

this, we found that the level of downstream smad2/3 in the TGF-ß signaling pathway were all rescued from basal degradation in both groups irrespective of Rab5 status. However, ZFYVE21 and TGF-ßRII were both only found to be upregulated in the constitutively active Rab5 group (Rab5-GTP), implying a prerequisite Rab5-GTP state for these proteins to be stabilized. The levels of smad2/3 being elevated irrespective of Rab5 endosome status is not surprising given they are not classically described as signaling from endosomes, whereas TGF-ßRII, being a membrane protein receptor, is described as functioning from an endosomal base, similar to ZFYVE21.[42,72-75]

As non-specific inhibition of proteasomes leading to up-regulation of TGF-ß signaling components suggests a post-translational mechanism of antibody-mediated complement signaling, we sought to identify the specific proteasome population that was targeted in antibody-mediated complement signaling as it is both unlikely that antibody-mediated complement signaling shuts down the proteasome function entirely in a cell and the proteomics screen we initially performed found differential protein up-regulation in the MG132-treated constitutively active Rab5 group and the PRA-treated constitutively active Rab5 group, as well as compared to the PRA-treated wild-type Rab5 group. In order to interrogate the specific lid components that are targeted by antibody-mediated complement signaling, we pulled down a common core proteolytic component and western blotted for specific proteasome components that were representative of different proteasome populations, ultimately finding the PA200 proteasome population as being selectively inactivated by PRA treatment. This finding suggested that antibody-mediated complement fixation leads to selective degradation or targeting of the PA200 proteasome population, making it a proteasome population of interest.

However, to show that the PA200 proteasome population selectively targets antibody-mediated complement signaling proteins including ZFYVE21 and TGF-ß superfamily proteins, we needed to show that the PA200 proteasome indeed targets these pathway mediators. To show this, we transfected HUVEC with siRNA against a PA200 proteasome lid component to knock-down PA200 proteasome function in order to establish its role. One limitation of this strategy, seen in our western blot confirmation of knock-down, was the limited efficiency of knock-down as compared to other methods such as knock-out models. Namely, the PSMC2 lanes that were blotted showed partial knock-down, but not complete. However, differences in levels of ZFYVE21 mediators and TGF-ß mediators were still appreciated in the control siRNA groups compared to the PSMC2 siRNA groups in both homeostatic as well as PRA-treated groups. In the future, different strategies can be employed in order to have better knock-down efficiency with the PSMC2 siRNA in order to more clearly delineate these differences. Thus, our siRNA studies established that the PA200 proteasome population is implicated in targeting these antibody-induced complement signaling mediators including ZFYVE21 pathway proteins as well as TGF-ß proteins. Combined with the earlier co-IP studies of the proteasome core protein western blots, a pathway can be deduced whereby antibody-mediated complement fixation leads to selective inactivation of the PA200 proteasome population, further leading to inhibited degradation of ZFYVE21 signaling proteins and TGF-ß proteins.

An interesting phenomenon noted through the siRNA knock-down studies performed was the fact that PRA treatment, or antibody-mediated complement fixation, did not lead to breakdown of the PSMC2 lid component, instead, led to disruption of its proteasome function as evidenced by post-translational rescue of ZFYVE21 and TGF-ß

pathway mediators. This finding implies that the lid components themselves are not targeted for destruction, but rather are either unable to associate with the core proteolytic proteasome elements or are in some way disrupted from their binding capacity. Again, when looking at the core PSMA2 co-IP experiments, it would appear that the core proteolytic element is dissociated from the PA200 lid components following antibody-mediated complement fixation, leaving proteasome lid components bound to ZFYVE21 and TGF-ß mediators.

In order to determine the spatial relationship that these proteasome components have to the Rab5 endosomes that ultimately serve as the platform for previously described antibody-mediated complement signaling via ZFYVE21, we turned back to our initial proteasomal analysis that were performed on the Rab5 endosomes, specifically comparing the untreated Rab5 constitutively active group, the pan-proteasome MG132-treated Rab5 constitutively active group, and the PRA-treated Rab5 wild-type group and noted numerous proteasome components were differentially recruited to Rab5 endosomes in the PRA-treated group. Furthermore, when pulling down for a proteasomal lid component PSMC2 and blotting for Rab5 and other antibody-mediated complement signaling mediators after PRA treatment, we found increased associations of the proteasomal lid component PSMC2 with all of the above, suggesting that PRA treatment leads to recruitment of this PA200 lid component to Rab5 endosomes, likely while carrying ZFYVE21 and TGF-ß pathway proteins originally destined for degradation. It was also noted that at time 0 during the co-IP, the PSMC2 lid component was already found to be bound to some amount of ZFYVE21 and TGF-ß pathway proteins, likely indicating active degradation.

Taken all together, these experiments serve to suggest that proteasomes do not simply function to basally degrade antibody-mediated complement signaling proteins such as ZFYVE21 and TGF-ß proteins while under homeostatic conditions, but also potentially serve a second role as a chaperone in bringing these pathway mediators to the correct platform of Rab5 endosomes in order to perform their effector function. In other words, at baseline, the PA200 lid components bind to ZFYVE21 and TGF-ß signaling proteins and are associated with proteasomal core components that lead to degradation. However, upon introduction of antibody-mediated complement fixation, the PA200 lid components are dissociated from their core proteolytic element and are instead translocated to Rab5 endosomes, bringing their cargo (ZFYE21 and TGF-ß signaling proteins) along with them to ensure proper co-localization for further downstream signaling changes.

Figure 11

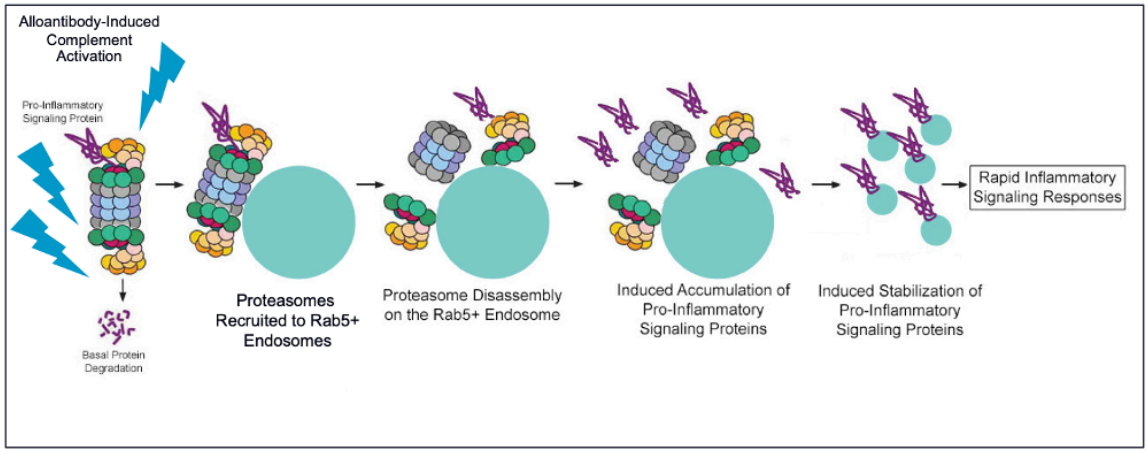

Figure 11: Proposed mechanism for proteasomes serving dual function as basal inhibitor of effector protein function, but also chaperone to ensure proper localization of effector proteins to Rab5+ endosomes.

To summarize briefly and recap at this stage, we have shown that for antibody-mediated complement fixation, the TGF-ß superfamily of proteins follows in our

proposed two-step model. Namely, we have shown that inhibition of proteasomes leads to rapid upregulation of TGF-ß components and have more specifically shown a role for the PA200 proteasome population as mediating this effect. Furthermore, we have shown that after rescue from basal degradation by proteasomes, these proteins are recruited via PA200 proteasome lid components to Rab5 endosomes which serve as a platform for further downstream signaling. This would suggest that the same proteasome components that specifically target these pathway mediators for degradation under homeostatic conditions may dually serve a novel chaperone function in aiding recruitment to the proper signaling platform. In this updated model, proteasomes may serve as a stress sensor and chaperone. In basal homeostatic states, they degrade pro-inflammatory proteins. However, with introduction of a stressor such as antibody-mediated fixation of complement, proteasomes bound to pro-inflammatory proteins are recruited to Rab5 endosomes and disassembled, bringing their pro-inflammatory mediators along in the process in order for them to co-localize and perform downstream signaling function. Through this, cells are able to rapidly adapt and respond to stressors without undergoing the typical process of transcription and translation.

Through identification of the proteasome as being potentially central in the pathogenesis of antibody-mediated complement signaling and one arm of allograft vasculopathy, one may turn towards the clinical correlate in potential drugs targeting proteasomes. Interestingly, as there is a well-described B-cell component to allograft vasculopathy, efforts are already underway in studying the effect modulation of this response has. Specifically, as described above in the literature review, the renal transplant community has initiated the BORTEJECT Study, a Phase II study using

bortezumib, a proteasome inhibitor, in preventing late allograft loss. Given the novel findings that proteasomes may play in this pathway as described here, there may be a dual function of preventing antibody-mediated complement signaling as well.

While we have been working up the more global mechanism by which we believe antibody-mediated complement fixation leads to rapid signaling changes in endothelial cells, we have also performed preliminary experiments in order to better characterize the TGF-ß response that is induced through this pathway. Specifically, we have found that antibody-induced complement activation generates TGF-ß1 and TGF-ß2 in endothelial cells via ELISA in preliminary studies. We have also seen preliminary data that TGF-ß signaling elicits endothelial cell activation via both RT-qPCR as well as T-Cell adhesion studies. Last, to better understand dynamics of co-localization, IHC would be most informative in being able to observe the interactions between proteasome components, ZFYVE21 and TGF-ß pathway mediators, and Rab5 endosomes pre- and post-PRA treatment and those experiments are tentatively planned.

7. COVID-19 Statement:

Much of the research presented was performed in the first year I was engaged in the lab and my original plans were to resume research in the summer/winter 2020. As much of the research involves physically being at the lab, these were disrupted as labs were closed for much of the early summer. Moreover, my board exams, sub-internship, and electives were all cancelled or pushed back during this time. In order to make the most of my time then when my in-person activities were limited, I chose to engage in research through reading in order to frame the work that is presented here as well as focus more on thesis writing. When clinical electives and sub-internships were opened again, I opted to complete those before returning to reading and thesis writing in order to ensure I would be able to apply to residency this cycle.

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