Potential Roles of Necroptosis in the Development and Progression of Vascular Calcification, in Vitro

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Potential Roles of Necroptosis in the Development 
and Progression of Vascular Calcification, *in vitro*

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A Thesis in the Field of Biology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Vascular calcification (VC) is a major complication in individuals with chronic kidney disease (CKD). A constant inflammatory state remains a key characteristic in the development of VC. Necroptosis is a programmed form of cell death that results in an inflammatory phenotype. Early descriptions of necroptosis involve phosphorylation of mixed lineage kinase domain-like (MLKL) by receptor-interacting serine/threonine-protein kinase (RIPK) 1/3 signaling. Many studies have shown that necroptosis is a key contributor in various inflammatory diseases, but none have explored its place in VC. In this study, we aim to examine the roles of necroptosis in a model of VC in vitro.

We establish VC by utilizing Human Aortic Smooth Muscle Cells (HA-SMCs) treated with 5mM CaCl$_2$ and β-glycerolphosphate for 7, 14, 21 days. VC was confirmed by Arsenazo III and Alizarin Red Staining and by expression of Klotho and runt-related transcription factor 2 (RUNX2). Necroptosis is assessed through the expression of MLKL, phosphorylated-MLKL, RIPK 1 and 3, using Western Blot. Pan-caspase, apoptotic inhibitor ZVAD.fmK (20 μM), RIPK inhibitor necrostatin-1 (20 μM, 40 μM), and MLKL inhibitor necrosulfonamide (0.5 μM, 1 μM), were used to assess the effects of necroptosis inhibition.

VC was confirmed by down regulation of Klotho and up regulation of RUNX2. RIPK1 and RIPK3 expression were down regulated in a time-dependent manner in our VC model. The opposite was seen in MLKL and phosphorylated-MLKL, indicating the presence of necroptosis in VC. Furthermore, treatment with necrosulfonamide, alone
displayed a dose-dependent reduction of calcification. Neither ZVAD.fmк nor
necrostatin-1 resulted in significant changes in calcification. Calcification of HA-SMCs
highlight increased activity of MLKL, but not RIPK1/3. Our results indicate that RIPK-
independent activation of MLKL may play a significant role in the development of VC.
These findings suggest a novel pathway of necroptosis whose inhibition may be a target
in the treatment of VC.
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Chapter I.

Introduction

Chronic Kidney Disease

Chronic kidney disease (CKD) describes a heterogeneous group of disorders that affect kidney function and result in the progressive loss of renal function. Due to the decrease in renal function, CKD presents with increased blood pressure, accumulation of urea and potassium, metabolic acidosis, and anemia. The disease itself may be classified into 5 different stages, clinically differentiated by an individual’s glomerular filtration rate. Stage 1-3 describe slight to moderate reductions in kidney function. At Stage 4 and stage 5, the kidneys are described as having severely reduced function. Patients with end-stage renal disease (ESRD), require maintenance dialysis or kidney transplantations (Table 1) (Levey et al., 2005). Data from the Global Burden of Disease study suggest that the prevalence of CKD has increased by 19.6% worldwide in the past decade, affecting more than 497 million adults and a major cause of death among non-communicable diseases (Murray et al., 2015). In the United States, the Center for Disease Control found that CKD affected ~16.8% of the US adult population from 1999-2004 (Centers for Disease Control and Prevention, 2007) while data from the National Health and Nutrition Examination Surveys found an increase in cases of CKD across all age groups (Figure 1).

Unfortunately, CKD is often viewed in isolation, with clinical guidelines primarily focused on the consequences of impaired glomerular function and proteinuria; This approach neglects the reality that risk of CKD increases with age, therefore often
presents with multiple comorbid conditions (e.g. hypertension and diabetes) (Fraser et al., 2015). A 2015 retrospective study of 30,771 adults with CKD in Canada highlights that while the median number of comorbidities was 1, 25% and 7% of the population had 3 and more, or 5 and more comorbidities respectively (Tonelli et al., 2015). Similarly, during an analysis of 1,741 patients classified as CKD Stage 3, only 78/1741 (4%) had no comorbidities, with common comorbidities being hypertension, anemia, ischemic heart disease, diabetes and thyroid disorders (Fraser et al., 2015). Consequently, diabetes remains the major factor in CKD development, implicated in 44% of ESRD patients. Another 29% of ESRD cases may be attributed to hypertension (Figure 2).

An important consideration in the study of CKD is the effect of ethnicity. For example, African Americans, Hispanics, and South Asians are at higher risks of developing CKD. Furthermore, 37% of ESRD cases in African Americans can be attributed to high blood pressure, compared to 19% among Caucasians (Appel et al., 2008). Another study reinforces the importance of ethnicity, highlighting significant decreases in hazard ratios for death in South Asian and black individuals compared to Caucasians, a finding independent of age, gender, socioeconomic status, kidney function and comorbidities (Jesky, Lambert, Burden, & Cockwell, 2013).

Other frequent characteristics of CKD include increased levels of oxidative stress and inflammation. The cyclical relationship between oxidative stress and inflammation promotes renal injury via damage to molecular components of the kidney. Unfortunately, oxidative stress and inflammation can become systemic, serving to injure tissues distal to the site of original insult. In addition to its renal effects, oxidative stress and
inflammation might also explain why patients with CKD are likely to experience cardiovascular diseases (CVDs) (Tucker, Scanlan, & Dalbo, 2015).

This is particularly critical as CVD is the leading cause of death in patients suffering from CKD, accounting for approximately 50% of all deaths in patients on dialysis and in recipients of renal transplants (Mizobuchi, Towler, & Slatopolsky, 2009; Weir et al., 1998). A study highlights an inverse association between renal function and cardiovascular mortality (Henry et al., 2002). Vascular calcification (VC) is the major cause of cardiovascular disease in patients with CKD. The increase of calcium minerals in the arteries results in vascular stiffening, increased pulse velocity and pressure that contribute to myocardial ischemia, impaired myocardial function, valvular insufficiency, arrhythmias and stroke (Blacher, Guerin, Pannier, Marchais, & London, 2001; Georg Schlieper, Schurgers, Brandenburg, Reutelingsperger, & Floege, 2016). In patients with ESRD, the risk of death increases with the number of vascular sites affected by calcification in the aorta and the carotid and femoral arteries (Blacher et al., 2001). The high prevalence of VC is frequently reported in early stages of CKD and in young dialysis patients (Goodman et al., 2000; Sigrist, Bungay, Taal, & McIntyre, 2006).

Vascular Calcification

There are two major types of VC, differentiated by location of development. The first is the more widely recognized atherosclerotic calcification, but the second type, medial artery calcification (arteriosclerosis), is more prevalent in diabetic and CKD patients (Georg Schlieper et al., 2016). In the past, VC was viewed as passive deposition
from elevated calcium-phosphate levels; however, recent findings recognize that the development of VC as active and multifactorial.

This idea is corroborated by the expression of many key regulators of osteogenesis during the calcification process. Transcription factors such as core-binding factor subunit alpha-1/Runt-related transcription factor 2 (Cbfa1/RUNX2) and homeobox protein MSX-2 (MSX-2), critical for normal bone development, have been identified in cells surrounding human arterial calcification in chronic kidney disease patients, in animal models, and in vitro (Moe, 2006). In culture, vascular smooth muscle cells (VSMC) can produce these same transcription factors and proteins, ultimately forming mineralized nodules (Lim et al., 2012). The increased expressions of these transcription factors are indicative of a phenotypic switch; one that allows VSMC to take on osteo/chondrocytic properties, lose contractility, and incorporate calcium and phosphorus into matrix vesicles for mineralization (Moe & Chen, 2008).

Given the complex mechanism of VC it should be no surprise that there are a multitude of inducers. The main constituents of VC are calcium and phosphate, primarily in the form of hydroxylapatite. Compared to the general population, CKD patients display altered Ca\(^{2+}\) and Pi\(^2-\) metabolism, leading to an influx of available phosphate and calcium for osteo/chondrocytic transformation and mineralization (G. Schlieper et al., 2010) (Figure 3). Furthermore, abnormal calcium and phosphate metabolism in uremia is often accompanied by dysregulation of PTH and FGF23. High PTH levels implicate rapid bone turnover with associated calcium and phosphorus release into the circulation while suppressed PTH can produce low-turnover bone disease, often associated with the presence of cardiovascular calcifications. While FGF23 itself does not seem to induce
VC, the deficiency of FGF23 co-receptor, Klotho, has been implicated in the development of arterial calcification (Lim et al., 2012; Scialla et al., 2013).

Other inducers of VC include high glucose, oxidized lipids, or cytokines (Mizobuchi et al., 2009). In a seminal study, Towler et al. established that male ldlr−/− mice on a Western diet developed type 2 diabetes along with metabolic syndrome characteristics, high circulating markers of inflammation, and medial calcification (Towler, Bidder, Latifi, Coleman, & Semenkovich, 1998). Results from in vitro studies of atherosclerosis also suggest that inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-6 (IL-6) promote VSMC differentiation and vascular intimal calcification (Shioi et al., 2002; Tintut, Patel, Parhami, & Demer, 2000). Further studies highlighted diabetes related drugs and diseases as highly associated with cardiovascular calcification. For example, warfarin is known to induce cardiovascular calcification through inhibition of the vitamin K cycle, thereby decreasing MGP activity, causing inflammation, and reducing fetuin-A levels (Buendía et al., 2015). Consequently, low fetuin-A levels in hemodialysis patients were shown to be associated with inflammation and connected VC to mortality in patients (Ketteler, Westenfeld, Schlieper, & Brandenburg, 2005).

It is widely accepted that aging is associated with a low-grade chronic inflammatory status, and that inflammation exacerbates age-related diseases such as CKD, osteoporosis, and diabetes. Moreover, VC is a complication that is often found in patients with CKD. As such, VC in CKD can be thought of as the consequence of a state of constant inflammation.
Programmed Cell Death

Excessive cell death is a prominent contributor to numerous inflammatory and degenerative diseases. Programmed cell death (PCD) describes the death of a cell that is mediated by intracellular process. It serves fundamental functions during both plant and animal tissue development. On the other hand, necrosis (non-programmed cell death) is a non-physiological process that occurs in response to external forces, such as infection or injury. PCD was first characterized by Lockshin in 1964, and of the types of PCD apoptosis was the earliest to be discovered, described by Karl Vogt in 1842 (Diamantis, Magiorkinis, Sakorafas, & Androutsos, 2008; Vandenabeele, Galluzzi, Vanden Berghe, & Kroemer, 2010). As such many times PCD has been incorrectly used to synonymize apoptosis; however, there are now numerous forms of PCD. Apoptosis and programmed necrosis are just two main forms, easily distinguished by their morphological differences (Figure 4).

Apoptosis

The initiation of apoptosis is a tightly regulated as its activation inevitably leads to the death of the cell. While there are several different activation mechanisms, the two best-understood are the intrinsic (e.g. mitochondrial pathway) and extrinsic pathway (Böhm & Schild, 2003). The intrinsic pathway is initiated through intracellular signals generated under cellular stress and depends on the release of proteins from the intermembrane space of mitochondria; in contrast, the extrinsic pathway is activated by extracellular ligands binding to cell-surface death receptors, which leads to the formation of the death-inducing signaling complex (DISC) (Alberts et al., 2002). In end-stage renal
disease, SMC apoptosis gives rise to apoptotic bodies that may serve as mineral nucleation points (Proudfoot et al., 2000).

**Necroptosis**

In contrast to apoptosis, necrosis was the accidental, passive subroutine of cellular death. This view of necrosis was not questioned until 1988 when treatment of cells with TNF manifested in deaths featuring either apoptotic or necrotic morphologies. Mounting evidence culminated in the introduction of necroptosis in 2005 to describe a regulated form of cell death that display the characteristics of necrosis: cytoplasmic and organelle swelling, loss of membrane integrity, and release of cellular contents into extracellular space. This release of cellular contents into the extracellular space is what ultimately gives rise to the inflammatory response seen in necroptosis (Jouan-Lanhouet et al., 2014; Vandenabeele et al., 2010).

Necroptosis may be induced through several different stimuli (e.g. TNF, FasL, TRAIL, TWEAK, oxidative stress). The canonical pathway of necroptosis begins with stimulation by TNF. As TNF also induces apoptosis, the pathway describes a balancing act between the two forms of cell death. The binding of TNF to TNF-receptor initiates the formation of the “necrosome” complex consisting of FADD, inactivated caspase-8, FLIP, RIPK1, and RIPK3. This complex in turn leads to RIPK1/3 phosphorylation, RIPK3 oligomerization, and the recruitment and phosphorylation of MLKL. It then translocates to the plasma membrane where it can induce plasma membrane rupture. Of course, given its multiple induction factors, recent discoveries have pointed to different RIPK1- or RIPK3-independent pathways (Figure 5). Due to this, MLKL and phosphorylated MLKL
(phospho-MLKL) remains the only reliable biomarker of necroptosis to date (Jouan-Lanhouet et al., 2014).

An interesting component of the canonical pathway is the presence of inactivated caspase-8. Activated caspase-8 interacts is necessary to interact with caspase-3 in initiating the apoptotic pathway; however, the activation of caspase-8 also inhibits necrosome formation, and necroptosis. This has promoted the idea that necroptosis and apoptosis is a balancing act, raising the question of why there is a need for a highly immunogenic immune response (Linkermann, Stockwell, Krautwald, & Anders, 2014)? In mice, infections by vaccinia virus were found to be lethal in RIPK3−/− mice but not in wild type (Cho et al., 2009). In essence, necroptosis acts as an evolutionary defense mechanism should apoptosis fail.

Necroptosis in Human Diseases

The past decade has implicated the role of necroptosis in numerous pathologies, encompassing the heart, brain, eye, pancreas, intestine, liver, skin, and kidneys. In ischemia-reperfusion injuries, the restoration of blood flow results in a burst of reactive oxygen species (ROS) leading to inflammation-related cell death (Jouan-Lanhouet et al., 2014). Similarly, chronic liver diseases are characterized by hepatic inflammation and cell death, an effect that becomes attenuated with the suppression of RIPK1 (Silke, Rickard, & Gerlic, 2015). Examination of RIP3−/−; Ldlr−/− mice were no different from RIP3+/+; Ldlr−/− mice in early atherosclerosis, but displayed significant reduction in advanced atherosclerotic lesions (Lin et al., 2013).
In kidneys, the presence of necroptosis in acute kidney injury (AKI) was first determined in a murine model of renal ischemia–reperfusion injury (Linkermann et al., 2014). This study highlighted the protective effect of necrostatin-1 (Nec-1), a chemical inhibitor of RIP1. In contrast, treatment with the pan-caspase inhibitor ZVAD.fmik (apoptotic blocker) did not provide any detectable protection. Further studies showed that necroptosis also contributes to AKI induced by cisplatin, a widely used chemotherapy agent with nephrotoxic effect. Tristao et al. found that combined use of ZVAD.fmik and Nec-1 can provide protection (Tristão et al., 2016). Another recent work examined the cytotoxic effects of calcium oxalate, monosodium urate, calcium pyrophosphate dihydrate, and cystine crystals on kidney epithelial cells in vitro. Consistent with other studies, deficiencies of RIPK3 or MLKL prevents oxalate crystal-induced acute kidney injury (Mulay et al., 2016). In short, much of necroptosis-implicated pathologies involve a combination of cell death and inflammation.

As stated, VC may be thought of as a state of constant inflammatory response. Given the strong link between necroptosis and inflammatory diseases, the aim of this study is to examine the role of necroptosis in the development of arteriosclerotic VC.

Research Aims, Goals, and Hypothesis

The primary research goal of this thesis is to examine the role of necroptosis in the development of VC, in vitro. Based on previous literature, it is hypothesized that the inflammatory nature of VC is in large part due to increases in necroptotic activity of cells under stress. Thereby, the inhibition of key players in the necroptotic pathway would
result in decreased calcification. To assess this hypothesis, three specific aims work
together to answer the primary objective.

Primary objective: Establish a model of VC, *in vitro*, to elucidate the role of
necroptosis in disease development and progression. Furthermore, show a reduction in
calcification severity through the inhibition of necroptotic modulators.

Specific Aim 1: Establish a model of long-term calcification in Human Aortic
Smooth Muscle Cells (HA-SMCs).

Methods: Western Blot, Alizarin Red Staining, Arsenazo III.

Expected results: Calcified samples will exhibit a reduction in normal smooth
muscle cell markers (e.g. calponin, myocardin) as well as Klotho. Similarly, diseased
samples that successfully undergo osteo/chondrocytic transformation will have increased
expressions of RUNX2 (transcription factor associated with osteoblasts). Alizarin Red
Staining and arsenazo III will be used as qualitative and quantitative means to assess the
VC, respectively.

Specific Aim 2: Examine the role of necroptosis in VC, *in vitro*.

Methods: Western Blot

Expected Results: There will be an increase in necroptosis markers (e.g. MLKL,
RIPK1, RIPK3).

Specific Aim 3: Explore effects of necroptosis inhibition on the development of
VC.

Methods: Western Blot, Alizarin Red Staining, Arsenazo III
Expected results: There will be a reduction in disease represented by reduction of necroptotic markers and calcification (as measured by alizarin red staining and arsenazo III).
Chapter II.

Materials and Methods

Model of Vascular Calcification

Commercially available HA-SMCs were obtained from different sources (ScienCell Lots: 0295, 0573, 3523, 9865). Cells were cultured in 5% CO₂/37°C incubator with the SMC medium (2% FBS; Cat No. 1101; ScienCell Research Lab). All experiments were repeated using at least two different cell sources. HA-SMCs were grown to 80% confluence and then treated every other day with calcification media containing 5 mM calcium chloride and 5 mM β-glycerolphosphate disodium. Equal volume of distilled water was added to controls.

Inhibitor Treatments

Pan-caspase inhibitor ZVAD.fmkk (20 µM), RIPK inhibitor necrostatin-1 (20 µM, 40 µM), and MLKL inhibitor necrosulfonamide (0.5 µM, 1 µM), were used to assess the effects of necroptosis inhibition.

Western Blot

Sample media was mixed with 4X loading buffer containing 5% mercaptoethanol (Sigma, MO, USA) and Radio-Immuno Precipitation Assay (RIPA) buffer, pH 7.4 (Catalog No. BP-115, Boston BioProducts, MA, USA) for equal protein concentrations. The samples were then heated for 5 minutes at 95°C. 10-30 g of sample was loaded onto SDS-PAGE, NuPAGE Bis-Tris pre-cast polyacrylamide gels using the mini-cell system (Invitrogen, CA, USA). NuPAGE MOPS SDS running buffer
(Invitrogen, CA, USA) was used. 500 μl of antioxidant was added to the running buffer. Electrophoresis was performed at 140V- 200V until adequate spread of the protein molecular marker was achieved. Following SDS-PAGE gel electrophoresis, proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). Transfer was achieved using a wet-blot (Bio-Rad) transfer system. Standard Towbin transfer buffer was used containing 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol. Proteins were then visualized with an enhanced chemiluminescence detection system.

**Alizarin Red Staining**

HA-SMCs were grown in 24-well plates. The cultures were then washed in distilled water and exposed to alizarin red (2% aqueous, Sigma) for 5 minutes and then washed again with distilled water. The results were observed under light-microscopy (Olympus IX70) and photographed by digital camera (Olympus America).

**Arsenazo III**

Calcification was also quantified with Arsenazo III, calcium sensitive dye (Fisher Scientific, Pittsburgh, PA). Briefly, cells were decalcified in 0.1 M HCl, and the supernatant removed for colorimetric assay with Arsenao III at 650 nm. Cells were harvested for Lowry protein quantification (BioRad, USA). Calcium concentration was normalized to the cellular protein content.
Chapter III.

Results

Validation of *in vitro* Calcification Model

A previous study by Hsiao’s lab established that Klotho deficiency potentiates VC and decreased Klotho expression may be a marker of VC (Lim et al., 2012). Likewise, increased RUNX2 expression is also a driver of calcification as it promotes the phenotypic transformation of VSMCs to more osteo/chondrocytic stage. Myocardin and calponin were also utilized as additional markers. Studies have shown that RUNX2 expression represses the transcriptional activity of VSMCs differentiation-associated genes through the inhibition myocardin-SRF binding. Meanwhile the expression of calponin plays a role in contractility and is specific to differentiated mature, normal VSMCs. To establish a long-term model of *in vitro* calcification we utilized primary HA-SMCs treated with a calcification media (5 mM of calcium chloride and 5 mM of β-glycerolphosphate disodium) every other day for 7, 14, and 21 days. Significant, time-dependent decreases in Klotho and calponin were evident as early as day 7 while decreases in myocardin and increases in RUNX2 expression could be seen at day 14, confirming successful calcification for the *in vitro* model (Figure 6).

Necroptotic Markers Increased in Vascular Calcification

We then examined markers of apoptosis and necroptosis in our long-term calcification model to elucidate the roles of programmed cell death in VC. As previously described, both Bcl-2-associated X protein (Bax) and caspase-3 are crucial mediators of apoptosis and exhibit no significant increases over 21 days when treated with our
calcification media (Figure 7). This suggests that apoptosis may not be a significant contributor in the development of VC, *in vitro*. In contrast, both MLKL and phospho-MLKL display clear increases at 21 days, confirming the presence and activity of necroptosis. Interestingly, RIPK1 and RIPK3, which were described as critical in the canonical necroptosis pathway, displayed a time-dependent down-regulation in our VC model (Figure 8).

The decreased expressions of RIPK1 and RIPK3 point to an alternative pathway of necroptosis in the role of VC development and progression. One such described RIPK-independent pathway is mediated by TIR-domain-containing adapter-inducing interferon-β (TRIF) and poly (ADP-ribose) polymerase (PARP). We therefore examined the expressions of both at 21-days of our VC model and found PARP, cleaved-PARP (active form), and TRIF to be down-regulated when treated with the calcification media (Figure 9).

**Inhibition of Necroptosis, Not Apoptosis, Decreases Total Calcification**

Having established that necroptosis is increased in VC, we then wanted to examine if its suppression might attenuate the development of VC. HA-SMCs were treated with 20 μM pan-caspase/apoptotic inhibitor (ZVAD.fm), 20 μM and 40 μM RIPK inhibitor (necrostatin-1), or 0.5 μM and 1 μM MLKL inhibitor (necrosulfonamide) for 21 days to further elucidate the roles of programmed cell death in VC. Neither treatment with ZVAD.fm nor necrostatin-1 displayed any significant changes in calcification; however, treatment with necrosulfonamide highlighted dose-dependent reductions in calcification as assessed through alizarin red staining and arsenazo III
quantification (Figure 10). In fact, both doses of necrosulfonamide reduced calcification levels to that of the control.

These results further serve to reinforce that necroptosis, not apoptosis, is the critical form of programmed cell death in the development of VC. In addition, the lack of response to necrostatin-1 treatment highlights that VC progression occurs via a RIPK-independent pathway of necroptosis. In contrast, treatment with necrosulfonamide displayed almost complete attenuation of calcification, suggesting that suppression of MLKL alone may be a viable therapeutic approach in future treatments of VC.
Chapter IV.

Discussion

Significance of Results

The burden of non-communicable diseases has increased significantly during this century and account for much of the health-related morbidity and mortality in the United States and worldwide. This is evidently the case with CKD as its prevalence has been increasing over the years. For individuals with CKD, CVD remains the number one cause of death, accounting for near 50% of all deaths in ESRD patients (Georg Schlieper et al., 2016). The occurrence of CVD in these patients often display with accelerated calcification of the intima, media, heart valves and likely the myocardium. Medial VC contributes to cardiovascular mortality in CKD patients as it leads to loss of vessel elasticity, subsequent increases in systolic blood pressure, and left ventricle hypertrophy. These may ultimately result in arrhythmias and heart failure (Blacher et al., 2001).

Recent studies provide compelling evidence that VC is associated with inflammatory status and is enhanced by inflammatory cytokines (Shioi et al., 2002; Tintut et al., 2000). Despite numerous studies having highlighted the roles of necroptosis in inflammatory diseases, this is the first to examine its significance in the development and progression of arteriosclerotic VC. Furthermore, unlike previously reported, the results of this study found that apoptosis was not a significant contributor to the development of VC. This difference may be due to the longer time needed for apoptotic bodies (28 days in vitro) to become nucleation points for calcification, suggesting that necroptosis is the
dominant form of programmed cell death in the development of VC (Proudfoot et al., 2000).

Since its discovery, several pathways for necroptosis has been described. These include the canonical caspase-8 guided mechanism, which proceeds through RIPK1/3 phosphorylation of MLKL (Feltham, Vince, & Lawlor, 2017). In addition, however, there are mediators such as TRIF and PARP that guide RIPK1 or RIPK3 independent pathways of necroptosis (Kaiser et al., 2013; Sosna et al., 2014). Despite these findings, there is currently no description of a RIPK1 and RIPK3 independent path of necroptosis. This study highlights, for the first time, a RIPK-independent phosphorylation of MLKL in VC. Furthermore, inhibition of MLKL is sufficient to reduce calcification levels to normal. The results reinforce the need for a better understanding of the mechanisms behind necroptosis and provide a novel target for the treatment of vascular calcification.

Study Limitations

While this study provides promising preliminary results, more work is needed to further confirm the extent to which necroptosis drives the development of VC. One critical factor that needs to be taken into consideration is the use of single-type, primary cell cultures for this study. Its simplicity allowed for development of a VC model and exploration of necroptosis in pathogenesis; however, there are inherent limitations to this design. The first is the lack of understanding on physiological, mechanical stress. This is particularly important as mechanical stress is an important component in the development and progression of VC (Chatzizisis et al., 2007; Hoshino et al., 2009). Secondly, cross-talk between cell types is an important consideration for the study of pathogenesis. In the
case of vascular calcification, macrophages and endothelial cells are known to have profound effects on the regulation of VSMCs (Chang et al., 2014; Liu, Yuan, Xu, & Wang, 2007).

**Future Research Directions**

Future work will need to build upon these results by elucidating the extent and mechanisms of necroptosis in VC. Transient knockdown of necroptosis via siRNA or full-knockout via Crispr should be utilized to reinforce findings given the potential off-target effects of inhibitors. Currently it is unknown what other players outside of RIPK1/3 may drive the phosphorylation of MLKL. One possibility is that of nitric oxide. Studies have shown that oxidative stress can induce necrotic cell death (Choi, Kim, Kim, & Choi, 2009). Furthermore, it is known that nitric oxide and oxidative stress are important components in the development of VC (Kanno, Into, Lowenstein, & Matsushita, 2008). Shindo, *et al.* notes that nitric oxide is involved in the necroptotic pathway downstream of the RIPK1/3 complex while Tamura, *et al.* highlighted the RIPK1-independent nature of nitric oxide in the activation of necroptosis (Shindo, Kakehashi, Okumura, Kumagai, & Nakano, 2013; Tamura et al., 2011). The importance of nitric oxide and oxidative stress in vascular calcification and necrotic cell death provides an intriguing target for examining this RIPK-independent form of necroptosis.

Another component for further research is cross-talk between endothelial and VSMCs. While medial VC primarily involves SMCs, intracellular interactions remain a critical consideration (Bardeesi et al., 2017; Milliat et al., 2006; Sprague & Khalil, 2009). Endothelial cells are a major source of nitric oxide and endothelial dysfunction is a major
source of reactive oxygen species. It would be interesting to examine whether endothelial dysfunction further increases the presence of necroptosis and calcification of SMCs. A co-culture of endothelial and SMCs can be utilized to examine this relationship.

Conclusions

I hypothesized that necroptosis plays critical roles in the development and progression of VC; consequently, the inhibition of necroptosis would attenuate VC. Data is presented to demonstrate a viable long-term, in vitro model of VC through treatment of HA-SMCs with a combination of CaCl$_2$ and β-glycerolphosphate. This model displays protein expression patterns characteristically linked to the development of VC (Figure 6). Furthermore, this model highlighted association in the expression of necroptosis with the progression of VC, as seen through the time-dependent increases in MLKL and phospho-MLKL. This is further confirmed when HA-SMCs treated with necrosulfonamide displayed significant reductions in calcification back to control levels. Significantly, this effect was only seen when MLKL was inhibited, and not for RIPK1/3 or apoptosis. Together these results highlight a novel pathway of necroptosis that drives the development of vascular calcification, in vitro and may be a viable target for further therapeutics research.
Appendix

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<th>Potential complications of reduced GFR (in alphabetical order)</th>
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<td>Kidney damage with normal or ↑ GFR</td>
<td>≧90</td>
<td>• Anemia, including functional iron deficiency</td>
</tr>
<tr>
<td>2</td>
<td>Kidney damage with mild ↓ GFR</td>
<td>60–89</td>
<td>• Blood pressure increases</td>
</tr>
<tr>
<td>3</td>
<td>Moderate ↓ GFR</td>
<td>30–59</td>
<td>• Calcium absorption decreases</td>
</tr>
<tr>
<td>4</td>
<td>Severe ↓ GFR</td>
<td>15–29</td>
<td>• Dyslipidemia /heart failure/volume overload</td>
</tr>
<tr>
<td>5</td>
<td>Kidney failure</td>
<td>&lt;15 or dialysis</td>
<td>• Hyperkalemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Hyperparathyroidism</td>
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<td></td>
<td></td>
<td></td>
<td>• Hyperphosphatemia</td>
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<td></td>
<td></td>
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<td>• Left ventricular hypertrophy</td>
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<td></td>
<td></td>
<td></td>
<td>• Metabolic acidosis</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• Malnutrition potential (late)</td>
</tr>
</tbody>
</table>

*Table 1. Clinical cut-offs of eGFR for Various Stages of Chronic Kidney Disease.* (Weber, Beaulieu, Karr, & Levin, 2008)
Figure 1. Prevalence of Chronic Kidney Disease Has Increased across all Age Groups and Stages in 1999-2004 as compared to 1988-1994. There has been an increase in reported cases of chronic kidney disease across all age groups except in >70 individuals with CKD Stage 1,2. There were no cases recorded in 1988-1994. Data from the National Health and Nutrition Survey (NHANES). (Centers for Disease Control and Prevention (CDC), 2007)
Figure 2. Reported Causes of New Cases of End-Stage Renal Disease in the United States in 2014. Data from US Renal Data System. The majority of End-Stage Renal Disease cases in the United States are due to diabetes and high-blood pressure. Consequently, cardiovascular diseases are a major cause of death in these patients. n = 118,014. (Centers for Disease Control and Prevention (CDC), 2017)

*Includes glomerulonephritis and cystic kidney disease
Figure 3. Mechanism of Vascular Calcification in Chronic Kidney Disease Patients. Normally, mesenchymal stem cells differentiate to adipocytes, osteoblasts, chondrocytes, and vascular smooth muscle cells. In the setting of chronic kidney disease, VSMC transform into osteo/chondrocytic-like cells through upregulation of RUNX-2 and MSX2. Transformed VSMCs then incorporate calcium and phosphorus into matrix vesicles to initiate mineralization. The overall positive calcium and phosphorus balance of most dialysis patients feeds the cellular transformation and the generation of matrix vesicles. Whether an artery calcifies or not depends on the strength of inhibitors (I) (e.g. fetuin-A, PPI = pyrophosphate, MGP = matrix Gla protein, and OP = osteopontin). (Moe & Chen, 2008)
Figure 4. Regulated Cell Death Plays Critical Roles in Inflammation. Cytokines, such as TNF, expressed by cells may trigger the death of additional cells, generating a vicious cycle that results in chronic non-resolving inflammation. In this setting, DAMP release may drive the inflammatory response. Apoptosis and Necroptosis have varying degrees of DAMP release and subsequent inflammatory response. In apoptotic cell death the orderly disassembly of dying cells allows limited release of DAMPs. In contrast, disintegrating necroptotic cells is believed to be a strong trigger for inflammation.
Figure 5. There are a number of Necroptotic Pathways that have been described. The binding of TNF induces the formation of the cytoplasmic complex, resulting in caspase-8-dependent apoptosis. FLIPL keeps caspase-8 in a heteromeric complex that controls RIPK1 and RIPK3. When caspase-8 is inhibited, the necrosome (RIPK1, RIPK3, MLKL) is formed, inducing RIPK1/3-dependent necroptosis (a). Stimulation of Fas or TRAIL can similarly induce formation of the necrosome via a caspase-8 independent mechanism (b). TLR4 or TLR3 stimulation triggers formation of the necrosome through the RHIM-containing adapter TRIF, resulting in RIPK3-dependent necroptosis (c). DNA-dependent activator of IFN regulatory factors (DAI) recognizes viral double-stranded DNA and through its RHIM domain recruits RIPK3 and results in RIPK1-independent necroptosis (d). IFNα/β induce necroptosis through IFNARI, leading to activation of JAK1. These cause the formation of the ISGF3 complex (STAT1–STAT2–IRF9), which induces the necrosome (e).
Figure 6. Decreased Klotho and Increased RUNX2 expressions validate in vitro model of long-term Vascular Calcification. Klotho and RUNX2 showed significant changes in expression by day 14. Decrease in calponin at day 7 is indicative of initially phenotypic shift.
Figure 7. Apoptosis is not critical for the development of vascular calcification, in vitro. Bax and Caspase-3 (apoptotic markers) display no significant changes in 21 days of Calcification Media treatment.
Figure 8. MLKL and phospho-MLKL both display significant increases in expression in calcified HA-SMCs, highlighting the role of necroptosis in VC. RIPK1 and RIPK3 expressions were decreased, suggesting a novel pathway of necroptosis at play.
Figure 9. Neither the PARP nor TRIF mediated pathways of necroptosis were involved in VC. PARP, cleaved-PARP, and TRIF all showed decreased expressions in calcified HA-SMCs.
Figure 10. Treatment with MLKL inhibitor (necrosulfonamide) significantly reduced presence of calcification in vitro. VC was attenuated with treatment by necrosulfonamide (NSA), but not by apoptotic inhibitor (ZVAD.fmk) nor RIPK1 inhibitor, necrostatin-1 (Nec), highlighting a RIP1/3-independent pathway of necroptosis in the development of VC. Calcification was assessed via Alizarin Red Staining (top) and Arsenazo III (bottom).


