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Role of Vav2 in Podocyte Inflammasome Activation and Glomerular Injury During Hyperhomocysteinemia

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Role of Vav2 in Podocyte Inflammasome Activation and Glomerular Injury during Hyperhomocysteinemia

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Deciding to pursue graduate studies, has been an eye-opening experience filled with opportunities to grow and deal with obstacles. My favorite piece of poetry is a poem entitled "Don't Quit". In this composition the author details of inevitable circumstances that occur in life which causes us as individuals to want to give up. Many times when we look at the reality of our situations, it seems unclear how the story will unfold and we become fearful of the outcome. Sticking to the fight when you are hardest hit has never proven to be an easy feat. This poem was a regular reminder to me as I inched closer to the finish line that there will be times where I felt defeated and moments when I have achieved success. But I had to maintain in my mind if I continue to put one foot in front of the other I remain closer to reaching my aspirations.

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LIST OF ABBREVIATIONS

ACEi	Angiotensin Converting Enzyme Inhibitors
ARB	Angiotensin Receptor Blockers
ANOVA	Analysis of Variance
ASC	Apoptosis-Associated Speck Like Protein with a Caspase Recruitment Domain
BSA	Bovine Serum Albumin
CAPS	Cyropyrin-Associated Periodic Syndromes
CBS	Cystathione β-Synthase
cDNA	Complementary DNA
CKD	Chronic Kidney Disease
CMH	1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrro-lidine
COX	Cyclo-oxygenases
DAMP	Danger Associated Molecular Patterns
DN	Diabetic Nephropathy
ELISA	Enzyme-Linked Immunosorbent Assay
ESR	Electron Spin Resonance
ESRD	End-Stage Renal Disease
ER	Endoplasmic Reticulum
FCU	Familial Cold Urticaria
FF	Folate-Free
FGF2	Fibroblast Growth Factor 2
GFP	Green Fluorescent Protein
GFR	Glomerular Filtration Rate
GLISA	GTPase Linked Immunosorbent Assay
GN	Glomerulonephritis
GNEF	Guanine Nucleotide Exchange Factor
GAP	GTPase Activating Protein
hHcys	Hyperhomocysteinemia
Hcys	Homocysteine
HFD	High Fat Diet
HG	High Glucose
HMBG1	High Mobility Group Box 1
H_2O_2	Hydrogen Peroxide
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
IL	Interleukin
IL-1R	Interleukin-1 Receptor 1
IP	Intraperitoneal
K^+	Potassium
LN	Lupus Nephritis
MeOH	Methanol
mRNA	Messenger RNA
MS	Methionine Synthase
MSU	Monosodium Urate
MTHFR	Methylenetetrahydrofolate Reductase

MWS	Muckle-Wells Syndrome
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ND	Normal Diet
NLR	Nod-Like Receptor
NLRC	Nod-Like Receptor Containing Caspase Domain
NLRP	Nod-Like Receptor Containing Pyrin Domain
NOX	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NTN	Nephrotoxic Serum Nephritis
$O_2^{\cdot -}$	Superoxide
PAMP	Pathogen Associated Molecular Patterns
PAS	Periodic-Acid Schiff
PBS	Phosphate Buffered Saline
PCC	Pearson Correlation Coefficient
PFA	Paraformaldehyde
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene Fluoride
RAGE	Receptor for Advanced Glycation End-Products
ROS	Reactive Oxygen Species
RT-PCR	Real Time Polymerase Chain Reaction
SE	Standard Error
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
SOD	Superoxide Dismutase
sRNA	Scrambled RNA
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
TBP	Tri-n-butylphosphine
TBS-T	Tris Buffered Saline with 0.2% Tween
TCA	Trichloroacetic Acid
tHcys	Total Hcys
TNF	Tumor Necrosis Factor
TXNIP	Thioredoxin-Interacting Protein
TRPC6	Transient Receptor Potential Cation Channel-6
UTP	Uridine Triphosphate
VEGF	Vascular Endothelial Growth Factor

ABSTRACT

ROLE OF VAV2 IN PODOCYTE INFLAMMASOME ACTIVATION AND GLOMERULAR INJURY DURING HYPERHOMOCYSTEINEMIA

By Sabena M. Conley

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Major Director: Pin-Lan Li, MD, PhD, Professor, Pharmacology and Toxicology

Hyperhomocysteinemia (hHcys) is a widely known pathogenic factor in the progression of end-stage renal disease (ESRD) and it is also associated with an increased risk for injurious cardiovascular pathologies during ESRD. HHcys is linked to the formation and activation of the NOD-like receptor protein 3 (NLRP3) inflammasome, characterized as a critical early mechanism initiating the inflammatory response. NADPH oxidase (NOX)-derived reactive oxygen species (ROS) mediate the activation of the NLRP3 inflammasome in podocytes in response to elevated levels of homocysteine (Hcys) *in vitro* and *in vivo*. However, it remains unknown how NLRP3 inflammasome activation is triggered by NOX. The aim of the present study sought to determine the signaling cascade that triggers glomerular injury and sclerosis during hHcys mediated by Vav2,

a guanine nucleotide exchange factor (GNEF). Using both genetic and pharmacological interventions of Vav2, we first tested whether this GNEF is involved in hHcys-induced NLRP3 inflammasome activation in podocytes by its role in activation of the Rac-1-NOX complex. Further, we explored whether pharmacological targeting of Vav2 activation may regulate NLRP3 inflammasome signaling pathway during hHcys-induced glomerular injury. We found that mice with hHcys (on the FF diet) or oncoVav2 (a constitutively active form of Vav2) transfection in the kidney exhibited increased colocalization of NLRP3 with apoptosis-associated speck-like protein (ASC) or caspase-1 and elevated IL-1β levels in glomeruli, indicating the formation and activation of the NLRP3 inflammasome. This glomerular NLRP3 inflammasome activation was accompanied by podocyte dysfunction and glomerular injury, even sclerosis. Local transfection of Vav2 shRNA plasmids significantly attenuated hHcys-induced NLRP3 inflammasome activation, podocyte injury, and glomerular sclerosis. In cultured podocytes, Hcys treatment and oncoVav2 transfection increased NLRP3 inflammasome formation and activation. This NLRP3 activation was inhibited by Vav2 shRNA, associated with reduction of Rac-1 activity and ROS production. Administration of NSC23766, a Rac-1 inhibitor substantially attenuated inflammasome formation, desmin expression and decreased podocin expression in glomeruli of hHcys mice. These results suggest that elevated Hcys levels activate Vav2 and thereby increase NOX activity, leading to ROS production. ROS trigger NLRP3 inflammasome activation, podocyte dysfunction and glomerular injury. Therefore, the present study defines a novel mechanism underlying hHcys-induced NLRP3 inflammasome activation and its progression to ESRD.

CHAPTER ONE

INTRODUCTION

Hyperhomocysteinemia (hHcys) is recognized as a significant contributor to the development of glomerular dysfunction; if left untreated eventually, hHcys-associated glomerular injury can progress into consequent end-stage renal disease (ESRD). It has been reported that the molecular mechanisms mediating the pathogenic action of hHcys is dependent on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), which generates the primary enzymatic product, superoxide ($O_2^{\cdot-}$). NOX-derived oxidative stress has been associated with the initiation and progression of glomerular diseases including diabetic nephropathy, hHcys-induced nephropathy, hypertension and obesity-related kidney diseases [1]. It is imperative to understand how this cascade of glomerular injury is instigated and amplified during redox signaling. In this regards, the regulation of NOX activation is dependent on Rac-1 GTPase activity, in which activated Rac stimulates the aggregation of the NOX complex as a functional enzyme to produce $O_2^{\cdot-}$. Vav2, a guanine nucleotide exchange factor may be key in Rac-1 activation.

The nucleotide-binding oligomerization domain Nod-like receptor containing pyrin domain 3 (NLRP3)-centered inflammasome has been well characterized as a redox sensor that turns on the inflammatory response and instigates damage in the glomeruli, ultimately leading to glomerular sclerosis. Activation of the NLRP3 inflammasome is considered ubiquitous in different organs and cells and is a potent intracellular inflammatory machinery that proteolytically cleaves interleukin-1β (IL-1 β) or IL-18 into their active forms [2–4]. Some investigators believed that this inflammasome activation may be the root of many chronic degenerative diseases. In the kidney, the formation and activation of the NLRP3 inflammasome plays a critical role in a broad spectrum of glomerular and tubulointerstital diseases as well as tubular injury and repair. Inhibition of this complex may serve

as an innovative therapeutic strategy to possibly prevent or treat glomerular diseases associated with inflammation.

1.1 Hyperhomocysteinemia

HHcys has been identified as one of the leading pathogenic factors in the progression of ESRD and it is also associated with an increased risk for cardiovascular complications. Amongst the growing prevalence of chronic kidney disease (CKD), hHcys is a prevalent comorbidity occurring in 85% of diagnosed patients [5]. At present there are no diagnostic biomarkers for accurately predicting these group of patients and with the prolonged clinical silence, it is a challenge to completely reverse due to the functional abnormalities already present that develops into fibrosis. The early mechanisms responsible for increased homocysteine (Hcys) levels during ESRD or other associated kidney diseases still remain unclear. Prior studies have demonstrated that a reduction in glomerular filtration rate (GFR) contributes to the increases in both serum creatinine and Hcys [6–8]. Elevated concentrations of Hcys leads to endothelial damage and dysfunction that progress into a buildup of extracellular matrix proteins, ultimately inducing renal glomerular sclerosis and atherosclerosis [9–11]. It has become increasingly evident that hHcys may result in glomerular degenerative diseases mainly through podocyte dysfunction and injury similar to other metabolic disorders such as diabetes mellitus, where glomerular pathological modifications will develop even in the absence of initiating factors.

More than 40 years ago, Dr. Kilmer S. McCully reported that elevated plasma Hcys level is a key factor in the pathogenesis of cornary artery disease [9]. Through extensive epidemiological studies, high plasma Hcys has been implicated in numerous other conditions throughout the body such as cardiovascular diseases [12–14] , stroke [15], neurodengerative diseases [16] and ESRD [7,17,18].

Patients with ESRD often develop hHcys with total blood Hcys (tHcys) levels of three times higher than the normal range of < 10 μmol/L [19].

1.1.1 Homocysteine Metabolism

The kidney is a major participant in the homeostatic balance of amino acids and protein metabolism; however, alterations in glomerular function have been closely linked with defective synthesis, degradation or clearance, diet deficiencies and uremic cardiovascular complications [19,20].

A basic illustration of the Hcys metabolic pathway is presented in **Figure 1**. Hcys is a methionine-containing essential amino acid synthesized from food or the metabolism of endogenous proteins. Following two separate enzymatic reactions methionine is converted into S-adenosyl methionine (SAM), a universal methyl donor for neurotransmitters, nucleic acids, hormones and phospholipids. Demethylation of SAM yields S-adenosyl homocysteine (SAH) which is hydrolyzed to form Hcys and adenosine as byproducts. At this phase in the metabolic pathway, Hcys serves as a key regulatory molecule that can undergo remethylation or transsulfuration to maintain a balance in Hcys concentrations [19,21]. If Hcys is remethylated back to methionine by methionine synthase (MS), folate and cobalamin (vitamin B_{12}) are required cofactors. Alternatively, Hcys can undergo transsulfuration to cystathionine by cystathionine β-synthase (CBS), in an irreversible pyridoxal-5'-phosphate (vitamin B_6) dependent reaction. Cystathionine is then degraded into cysteine and converted into sulfates which are released in the urine.

Normally 75% of total Hcys is covalently bound to a protein molecule, whereas the remaining fraction is freely filtered through the glomeruli [21]. Disturbances in Hcys metabolism have been linked to the progressive loss of kidney function, endothelial dysfunction and

atherogenesis [22]. HHcys-associated nephropathy orginates from defective clearance of Hcys in the plasma. There is an increasing body of evidence indicating that age, smoking, coffee consumption and folate antagonist may also affect Hcys concentrations [21]. In addition, alterations in enzymatic activity and cofactors necessary for proper synthesis and catabolism have been associated with overwhelmingly elevated plasma Hcys levels.

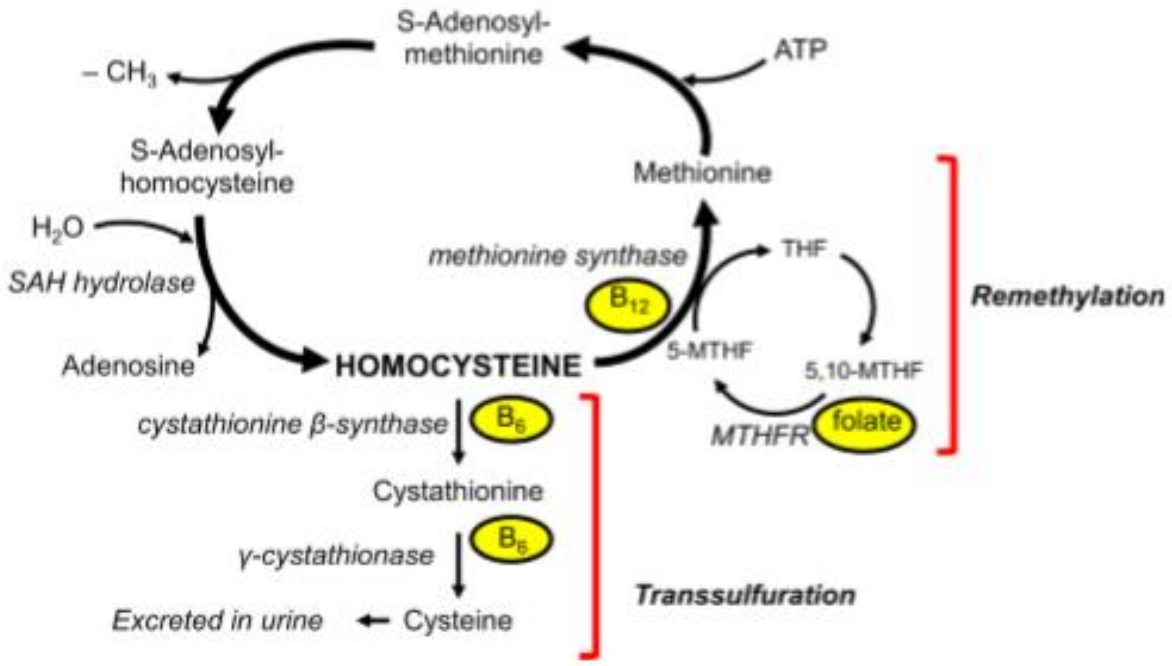

Figure 1. Homocysteine Metabolism.

Initially, dietary methionine is converted to S-adenosyl methionine (SAM); releasing of a methyl group converts SAM to S-adenosylhomocysteine (SAH) by the enzyme SAH hydrolase. Following hydrolase, SAH forms adenosine and Hcys. Once formed, Hcys then can be remethylated into methionine by methylenetetrahydrofolate reductase (MTHFR) or degraded by cystathione β-synthase (CBS) and γ-cystathionase.

1.1.2 Models of Hyperhomocysteinemia in Animals

Initial studies led by Dr. Kilmer S. McCully revealed children with mental retardation, accelerated growth during childhood, dislocated ocular lenses and thrombosis were found to excrete Hcys in the urine as well as advanced arteriosclerosis [9,23]. Through careful review of these clinical cases, Dr McCully discovered inherited abnormalities of the enzymes controlling homocysteine levels in these patients. Several animal models of hHcys have been developed to examine the effects of altered Hcys metabolism in the development of organ dysfunction and progression of disease. Elevated Hcys concentrations can be induced by diet, gene knockouts or a combination of both to investigate the causal role of Hcys *in vivo*. Dietary approaches include addition of methionine to the diet; reducing the dietary content of vitamin B_6 which prevents the conversion of Hcys to cystathionine; limiting the remethylation of Hcys by implementing a diet deficient in folate and/or vitamin B_{12} or adding Hcys directly to the drinking water [24]. Modulations of these factors *in vivo* have direct relevance to human disease progression, since low levels of these vitamins are commonly seen in patients with hHcys. Additionally, hHcys can be induced by deletion of CBS, MTHFR or MS, targeting of these enzymes in the Hcys metabolism pathway mimic the inborn errors seen in patients with severe hHcys (Hcys > 100 μmol/L).

1.2 NAPDH Oxidase in the Kidney

An ever growing body of work has characterized the numerous intrarenal enzymatic systems that contribute to the generation of reactive oxygen species (ROS). These sources include xanthine oxidases, lipoxygenases, cyclo-oxygenases (COXs), P450 mono-oxygenases, mitochondrial respiratory chain and NOX [25]. However, the latter is considered the predominant source of $O_2^{\cdot -}$ in the kidney [26]. Assembly of both membrane-associated ($gp91^{phox}$ and $p22^{phox}$) and cytosolic ($p47^{phox}$, $p40^{phox}$, and $p67^{phox}$) subunits activates this complex, resulting in the shift of electrons to

molecular oxygen, leading to the generation of $O_2^{\cdot -}$ [25,27,28]. Initial studies using a rodent model of spontaneous hypertension reported upregulation of all NOX subunits localized in the renal cortex [29]. In addition, all components of the NOX complex have been documented to be prominently expressed throughout the kidney including areas such as the renal vessels, tubules, interstitium and glomeruli [30].

Under normal circumstances, ROS are short-lived species that are rapidly metabolized by scavenging antioxidant enzymes. Low levels of ROS generated by NOX participate in normal physiologic processes such as intracellular signaling as second messengers [31], mediating hormonal effects [32,33], regulating ion channel activity, oxygen sensing [34], adipocyte differentiation [35], gene expression [31,36], reproduction [37], as well as cell growth, senescence, and apoptosis [38,39]. More specifically, NOX-derived ROS are implicated in various physiological regulation in the kidney. For example, the kidney maintains glucose homeostasis by participating in gluconeogenesis (production of glucose to glutamine) in proximal tubular cells and reabsorbing filtered glucose into the blood [28]. Type II diabetes is a chronic systematic disorder metabolically characterized by elevated levels of glucose. Winiarska *et al.* demonstrated that pretreatment of apocynin, a known NOX inhibitor reduced tubular cell gluconeogenesis in a diabetic rabbit model, indicating the importance of NOX regulation during diabetic conditions in order to maintain glucose balance [40]. In addition, tubuloglomerular feedback is a crucial mechanism which regulates the hemodynamic response upon alterations in tubular salt overload in the kidney [28]. Production of $O_2^{\cdot -}$ leads to constriction of the afferent arteriole [41] and scavenging of nitric oxide in the macula densa [42]. However, silencing of NOX and apocynin treatment inhibited high salt-induced $O_2^{\cdot -}$ generation [42].

1.2.1 Pathophysiological Role of NADPH Oxidase in the Kidney

During pathological conditions, renal NOX has been connected to oxidative stress and disease progression. Metabolic disruptions in the kidney leads to increased renal NOX expression, resulting in overproduction of ROS which disrupts the homeostatic balance and causes injurious consequences by altering the structure and function of various lipids, proteins and DNA. Generation of ROS exceeds the normal catabolism processes affecting the concentration in the kidney.

We have documented that NOX-induced oxidative stress is crucial in the pathophysiological role of renal injury associated with hHcys [43,44]. Podocytes, known as the structural framework of the glomerular filtration barrier are the most susceptible targets of injury in a number of renal diseases. Following injury, podocytes become effaced, detached from the basement membrane and undergo hypertrophy and apoptosis. We observed protection of podocyte structure and function in $gp91^{phox}$ gene KO mice when they were exposed to hHcys [44]. These mice exhibited significantly attenuated proteinuria, foot process effacement and podocyte loss compared to their wild-type counterparts. Additionally, inhibition of the $gp91^{phox}$ gene in cultured podocytes prevented Hcys-induced $O_2^{\cdot -}$ generation. We have also shown that NOX redox signaling mediates hHcys-induced inflammasome activation inducing glomerular inflammatory injury and consequently leading to glomerular sclerosis [43]. Altogether, we have determined that podocyte ROS generation is an early mechanism mediating hHcys-induced glomerulosclerosis. NOX-derived ROS generation is a common observation related to other renal-associated pathologies including diabetes and hypertension [30]. During focal and segmental glomerulosclerosis, the transient receptor potential cation channel-6 (TRPC6) co-localizes with NOX to form a lipid raft signaling complex with the

podocyte specific protein, podocin, producing a localized burst of ROS that may modulate the podocyte morphology and function [28].

1.2.2 Role of Rac-1 GTPase in NADPH Oxidase Regulation

During the NOX activating process, the major regulatory step necessary for activation of NOX involves heterodimerization of $gp91^{phox}$ and $p67^{phox}$ mediated by the Rac protein. Rac-1 GTPase has been reported to rotate between two conformational states either a GDP-bound "resting" state or GTP-bound "active" state. The transition between these two confirmations is regulated by either GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activation causing the switch to the resting condition or guanine nucleotide exchange factors (GNEFs) that facilitate the exchange of GDP for GTP. Conversion to activated Rac-1 initiates the translocation of the cytosolic subunits to the membrane, resulting in the formation of a functional NOX complex [45,46]. It has been reported that the Vav subfamily represents distinct GNEFs with high specificity to Rac-mediated NOX activation [47–49]. It is possible that the activation of Vav2 through elevated Hcys accelerates the switch from GDP to GTP, thereby enhancing NOX activity and $O_2^{\cdot -}$ production.

1.3 NLRP3 Inflammasome in the Glomeruli

Both microbial and non-microbial inflammation has been reported to participate in glomerular inflammatory responses, which may represent a critical pathogenic mechanism responsible for glomerular injury and subsequent ESRD. In particular, the non-microbial inflammation is widely recognized as a key pathological mechanism that mediates or promotes the development of glomerular sclerotic pathology during ESRD associated with several common systemic diseases such as hypertension, hHcys, diabetes mellitus, and erythematosus lupus [50,51] . In this regard, activation of inflammatory responses in the residential renal cells may be critical in

initiating glomerular injury and renal dysfunction. There is evidence that glomerular IL-1β is mainly generated from podocytes, but not other cell types. Increased IL-1β production from podocytes are critically involved in the progression of many non-proliferative forms of glomerulonephritis and other glomerular diseases induced by local inflammatory processes in humans and rodents [52–54]. Using different approaches, our recent studies have shown that the 3 major NLRP3 components were indeed enriched in murine podocytes and that their assembling into a multiprotein complex in podocytes occurs during stimulations by various danger factors such as L-Hcys, uric acid crystals, and adipokine-visfatin [51,55]. It has been proposed that NLRP3 inflammasome activation may importantly contribute to the onset or gradual progression of glomerular injury under different pathological conditions [51,56,57].

1.3.1 NLRP3 inflammasome

Originally, the NLRP3 inflammasome was identified as a crucial player necessary for activation of caspase-1, and the primary function is to activate cysteine proteases that subsequently induce inflammatory responses in tissues or organs by generation of pro-inflammatory interleukins (ILs) [23]. It is known that there are 23 Nod-like receptor (NLR) genes in the human genome including the NLRP family that contains a pyrin domain and the NLRC family that contains a caspase recruitment domain. Among all NLRs, only a few can be assembled into the inflammasome to activate caspases [58], which are NLRP1, NLRC4, and NLRP3 inflammasomes. NLRP1 was the first identified inflammasome, and it was found to provide immunity against bacterial cell wall components, which directly activates caspase-5 without ASC (apoptosis-associated speck like protein with a caspase recruitment domain) [59,60]. The presence of ASC, however, greatly accelerates NLRP1 inflammasome activity [60]. Another NLR-associated

inflammasome is NLRC4, and its activation is only triggered by bacterial flagellin and endogenous cell cytoplasmic components [61–63].

The NLRP3 inflammasome has been well characterized and its activation strongly links to sterile inflammation in a variety of chronic degenerative diseases [64]. The NLRP3 gene was first found to have mutations in patients with familial cold urticaria (FCU) and Muckle-Wells syndrome (MWS) [65]. Consequently, the over exuberant inflammatory response seen in these patients was attributed to an NLRP3-dependent mechanism that results in defective apoptosis and transcriptional regulation. Further studies in these MWS patients demonstrated that they spontaneously secrete active IL-1β, which is due to the interaction of NLRP3 with the ASC protein. This interaction was later recognized as the formation of the NLRP3 inflammasome [66]. Over the last 5 years, many bacterial and viral pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) released from damaged tissues or cells have been identified as activators of the NLRP3 inflammasome. Tremendous efforts have been made worldwide to address the possible role of this inflammasome in the initiation or development of different diseases and to find inducers or activators of the NLRP3 inflammasome under different pathological conditions, which may help identify therapeutic targets for treatment of related diseases.

1.3.2 NLRP3 inflammasome activators

There are a very diverse range of danger factors that initiate activation of the NLRP3 inflammasome through different mechanisms. It has been reported that microbes such as the influenza virus, adenoviruses, *Staphylococcus aureus*, *E. coli*, *Neisseria gonorrhoeae*, and *Candida albicans* may stimulate NLRP3 inflammasome activation [67–72]. One of the mechanisms activating the NLRP3 inflammasome during infections of these microorganisms may be associated

with the formation of pannexin-1 channel that permits the entry of these microbial toxins into the cytoplasm. In addition, monosodium urate (MSU) crystals, aluminum salts, silica or asbestos are also reported to stimulate the NLRP3 inflammasome [2,73,74] and as non-microbial materials they are phagocytosed into the cell leading to lysosomal damage, triggering NLRP3 inflammasome activation to produce IL-1β and other cytokines. Additionally, extracellular ATP and potassium were shown to induce NLRP3 assembly and activation, which may be mediated by the purinergic P2X7 receptor and pannexin-1 [75].

NLRP3 formation and activation have been originally attributed to auto-inflammatory diseases and more than 20 such auto-inflammatory diseases can be treated by inhibition of NLRP3 activation and by antagonizing the action of IL-1β. Recently, many studies found that the activation of the NLRP3 inflammasome also contributes to the development of various chronic metabolic diseases such as diabetes mellitus, gout, silicosis, and obesity. In addition, in acute myocardial infarction, atherosclerosis, glomerular sclerosis, Alzheimer's disease and liver cirrhosis the NLRP3 inflammasome is also shown to be activated [51,76–78]. In this regard, accumulation of the amyloid-β protein in the brain is considered as an activator of the NLRP3 inflammasome to promote local inflammation or cell injury leading to the development of Alzheimer's disease [79]. In addition, the high levels of IL-1β have been detected in the brains of patients suffering from Alzheimer's disease. During atherosclerosis, cholesterol crystals or even oxidative products of cholesterol may serve as a danger factor to cause phagolysosomal damage, activating the NLRP3 inflammasome and thereby promoting a pro-atherosclerotic phenotype in arteries [80]. In addition, trauma and excessive exercises may produce some chemokines that may activate the NLRP3 inflammasome. This process may be associated with hyaluronan-mediated cellular responses [81].

In a murine model of diabetes, hyperglycemia stimulated NLRP3 inflammasome activation, subsequently causing injury to pancreatic islet cells, glucose intolerance and insulin resistance [82]. Recent findings in our lab demonstrated that Hcys can stimulate NLRP3 inflammasome formation and activation in different types of cells, if it is increased in blood or extracellular space. In endothelial cells, this NLRP3 inflammasome activation will lead to endothelial dysfunction and induce atherogenic effects. In podocytes, increased activation of the NLRP3 inflammasome results in local glomerular inflammation and podocyte injury or transformation, ultimately leading to glomerular sclerosis [51]. We have shown that inhibition of the NLRP3 inflammasome via genetic or pharmacological interventions prevented Hcys-induced podocyte injury and glomerular sclerosis [51]. We also demonstrated that the adipokine, visfatin instigates vascular inflammation and injury by activation of the NLRP3 inflammasome, which ultimately leads to atherosclerosis [81]. To our knowledge, increasing numbers of danger factors including PAMPs and DAMPs are reported as activators of the NLRP3 inflammasome under different pathological conditions. Therefore, the NLRP3 inflammasome is evolving as a new common pathogenic mechanism for different diseases, in particular, those chronic degenerative diseases associated with inflammatory pathology.

1.3.3 Mechanisms Mediating NLRP3 Inflammasome Activation

Detection of pathogenic microorganisms and sterile stressors, recruitment of ASC, caspase-1 cleavage and production of inflammatory molecules are the main steps directing the pathological path to NLRP3 inflammasome activation. Given that more than 120 substrates were predicted for caspase-1, it is predictable that its maturation or activation will produce a variety of biological responses in addition to production of cytokines. However, IL-1β and IL-18 are well-studied products from the NLRP3 inflammasome. It is now known that the production of various cytokines

via activation of caspase-1 triggers the inflammatory response and other cellular activities [83]. In phagocytes, the NLRP3 inflammasome activation follows a tightly regulated intracellular signaling and regulatory process, which include two important steps. Initially, NF-κB initiates the increased expression of genes encoding for inflammasome formation such as NLRP3, pro-caspase-1, pro-IL-1β and pro-IL-18 [4]. This initial step is referred to as "priming", which is influenced by a cellular disturbance that activates pattern recognition receptors (PRRs). Signal II involves the detection of PAMPS or DAMPs by NLRP3, which promotes the recruitment of the inflammasome components to form the molecular complex [4]. Aggregation of these molecules together leads to the cleavage of pro-caspase-1 into two subunits, p10 (10 kDa) and p20 (20 kDa), and active caspase-1 is the heterodimer [23]. Considerable evidence has indicated that the regulation of inflammasome activity can be controlled by transcription of NLRP3 via signaling cytokine receptors [84]. Additionally, tight transcriptional regulation through microRNA, miR-223, influences inflammasome activation by manipulating NLRP3 mRNA levels [85,86]. In some other cells including endothelial cells, podocytes, neurons, epithelial cells and hepatic stellate cells, however, a sustained and small scale inflammasome activation may not require the priming step. Several signaling pathways have been reported to account for activation of the NLRP3 inflammasome. Among them, potassium (K^+) efflux, ROS generation, and cathepsin B leakage into the cytosol due to phagolysosomal rupture are often studied [87]. For example, an intracellular surge of K^+ ions into the cellular milieu is capable of triggering NLRP3 inflammasome activation. This model of activation occurs when extracellular ATP interacts with gated cation channels like P2X7R or bacterial toxins to produce pore formation [70,88]. Researchers have shown experimentally that reduction of high K^+ concentrations in cells diminishes the activation of the inflammasome, although this molecular mechanism has not been characterized in all K^+ efflux inflammasome

inducers [89–91]. It has been reported that many inflammasome activators stimulate production of ROS and these activators could be exogenous stimuli such as microbes or endogenously produced or secreted molecules including DAMPs, Hcys and uric acids [92]. Uptake of particulate and crystalline matter is reported to cause disruption of the lysosomal compartment causing cathepsin B release that may lead to NLRP3 inflammasome activation [74,79,93].

In recent studies, we have demonstrated that during hHcys podocyte injury and glomerular sclerosis are consequences of NLRP3 inflammasome activation [43,51,56]. We found that this activation is associated with NOX-derived $O_2^{-\cdot}$ and related oxidants [43,94]. Chemical scavengers and pharmacological inhibitors of ROS were used to confirm that elevated plasma Hcys may serve as a DAMP that activates the NLRP3 inflammasome in podocytes, which leads to podocyte dysfunction, glomerular injury, inflammation and ultimate glomerular sclerosis [56]. It has been suggested that DAMPs like Hcys, cholesterol, or visfatin may increase NOX activity via membrane raft clustering to form lipid raft redox signaling platforms in podocytes or glomerular endothelial cells, thereby producing $O_2^{\cdot-}$. NLRP3 can sense and monitor intracellular redox changes. If intracellular $O_2^{\cdot-}$ increases, it dissociates from thioredoxin-interacting protein (TXNIP) and then binds to ASC, forming the NLRP3 inflammasome [56] where IL-1β, IL-18 and high mobility group box 1 (HMGB1) are produced. These factors, in particular, IL-1β act to recruit inflammatory cells to the glomeruli, in which $O_2^{\cdot-}$ and additional cytokines are generated, creating a chronic sterile inflammatory cascade that contributes to glomerular injury and sclerosis. Excessive amounts of active caspase-1 and IL-1β or other inflammasome products may reduce the level of podocyte-specific proteins, nephrin and podocin, which will result in slit diaphragm derangement and proteinuria. These inflammasome products may also directly induce podocyte pyroptosis, consequently reducing podocyte numbers and causing foot process effacement [43,51,57].

1.3.4 Effector Response of Inflammasome Activation

There is substantial evidence that activation of the NLRP3 inflammasome turns on inflammatory responses, which may influence the progression to many chronic degenerative disease including CKDs by eliciting damaging insults independent of inflammation [95]. This NLRP3 inflammasome activation can cause both inflammatory and non-inflammatory effects [96]. In this regard, there is evidence that activated caspase-1 may act on more than 120 substrates and that pyroptosis, enhanced glycolysis and lipid metabolism, altered cell survival and many other direct effects during NLRP3 inflammasome activation may influence cell function and associated metabolism. It is believed that the physiological or pathological role of activated NLRP3 inflammasome extends far beyond inflammation [97].

With respect to the classical pathway, various cellular proteins such as cytokines or chemokines may be produced and secreted during NLRP3 inflammasome activation, which is associated with an internal signal or amino-terminal that direct their translocation from the endoplasmic reticulum (ER) lumen. These factors produced during inflammasome activation are then actively transported to the Golgi complex, packaged into vesicles, fusing with the plasma membrane and excreted out of the cells [98,99]. Fibroblast growth factor 2 (FGF2), galectin 1 and 3 as well as mature IL-1β and the DAMPs including HMBG1 have been documented to be released during NLRP3 inflammasome activation. These secreted factors induce inflammatory, cell survival and repair reactions by activating cell surface receptors including FGF receptor-1, the IL receptors, and the receptor for advanced glycation end-products (RAGE) [97]. Among these cytokines or chemokines, IL-1β and IL-18 are often studied over the current years. Both have extensive biological activities that result in the onset and development of inflammation as well as in the disturbance of cell functions. Moreover, IL-1α, may also contribute to the inflammatory

process. IL-1α, is released from necrotic cells and further processing of this interleukin is not necessary because it descends from an active precursor [100]. Interestingly, some recent studies have indicated that almost all inflammasome activators are able to induce co-secretion of IL-1α with other interleukin molecules [101]. However, IL-α release is not directly from the NLRP3 inflammasome activation, but may be dependent upon intermediate reactions after inflammasome activation [4].

Pyroptosis, a specialized form of cell death serves as a non-inflammatory or non-canonical effector mechanism during NLRP3 inflammasome activation. It is caspase-1 mediated and proceeds independently of pro-inflammatory cytokine production. This form of cell death enhances the immune responses by exposing foreign agents to the immune system's surveillance [102]. Cytoplasmic swelling, osmotic lysis and release of intracellular molecules into the outside of the cell are all characteristics of pyroptosis [97]. Although attempts have been made to fill the gaps in knowledge of pyroptosis, characterization of its molecular features still remains under debate. A distinctive characteristic that may contribute to the pathogenesis of organ failure include the abnormal overexpression of collagen leading to fibrosis [103]. With the initial insult often unknown in fibrosis, this form of wound healing can cause high mortality rates and proceed independent of inflammation [103]. In addition to pyroptosis, many other non-canonical effects induced by activation of the NLRP3 inflammasomes or consequently enhanced caspase-1 activity include enhanced glycolysis, abnormal cellular lipid metabolism, disturbance of cell survival programs, or altered cell membrane permeability. These non-inflammatory effects may also contribute to the functional and structural pathology of NLRP3 inflammasome activation in a variety of chronic degenerative diseases.

1.4 NLRP3 Inflammasomes in Glomerular Diseases

Growing evidence reveal that pathological progression of many kidney diseases originates from inflammation as a result of renal injury. Routes to resolving renal damage involves activation of transmembrane and intracellularly expressed PRRs as well as induction of transcriptional inflammatory mediators that are crucial in the innate immune response and cellular homeostasis [104,105], which may be reflected by the activation of the NLRP3 inflammasome. In the subsequent section, we highlighted some evidence showing the implications of this inflammasome in the onset or development of several different glomerular diseases.

1.4.1 Chronic glomerulonephritis (GN)

Chronic GN is referred to as inflammation of the glomeruli, which develops over several years with no or very few symptoms, but causes irreversible kidney damage, ultimately leading to ESRD. In GN, infiltrating mononuclear phagocytes generate canonical NLRP3 inflammasome signaling, and the activation of NLRP3 inflammasomes also occurred in some non-immune glomerular cells such as podocytes and glomerular capillary endothelial cells [52–54]. There is evidence that in a rodent model of nephrotoxic serum nephritis (NTN) IL-1 and tumor necrosis factor (TNF) are critical in promoting glomerular injury by interaction with the IL-1 receptor (IL-1R) [106]. Inflammasome signaling components were upregulated during NTN, in particular, in renal dendritic cells, which led to increased production of mature IL-1β. In *Nlrp3* and *Asc* gene knockout mice having NTN, glomerular injury and related inflammatory responses such as leukocyte infiltration or T-cell activation were significantly attenuated compared to their wild type littermates. Interestingly, reduced secretion of active IL-1β was only observed in *Asc* knockout mice, but not in *Nlrp3*-deficient mice, suggesting another potential molecular mechanism to be involved, which is independent of the NLRP3 inflammasome. Additionally, NLRP3 may have

non-canonical early inflammatory effects in NTN, which may be associated with glomerular release of the inflammatory protein, HMGB1 in a NLRP3-mediated manner. This HMGB1 is currently considered as one of the important mediators to promote the non-canonical functions of NLRP3/ASC inflammasome activation [106].

Acute and chronic inflammatory responses have also been considered as an important pathogenic mechanism of lupus nephritis (LN). Participation of the NLRP3 inflammasome may promote the progression of LN [107]. Recent studies have demonstrated that a selective inhibitor of NLRP3 inflammasome activity, Bay11-7082, prevented assembling and activation of the inflammasome and thereby decreased proteinuria, blood urea nitrogen, and glomerular damage during LN. Accompanied with these beneficial effects, Bay11-7082 treatment also decreased renal immune complex deposition and lowered the level of glomerular IL-1β, TNF-α and chemokine (C-C Motif) ligand 2 (CCL2). The infiltration of macrophages was also found to be significantly reduced. However, other studies have shown that active caspase-1 and renal secretion of pro-inflammatory cytokines such as IL-1β were not significant during acute heterologous NTN and other glomerulonephritis [108]. It is clear that more studies are needed to further establish the contribution of this inflammasome and other related molecular mechanisms in GN.

1.4.2 Hyperhomocysteinemic nephropathy

HHcys is characterized as a Hcys level that exceeds 15 μmol/L in the plasma of patients. Elevated plasma Hcys levels have been linked to the progression of many chronic systemic diseases including hypertension, peripheral vascular disease, Alzheimer's disease, diabetes and atherosclerosis [109]. In regard to renal disease, hHcys is considered one of the important pathogenic factors leading to the progression of ESRD. Additionally, the development of cardiovascular complications related to ESRD has also been attributed to hHcys [48]. Increasing evidence has

indicated that glomerular injury and ultimate sclerosis during chronic hHcys proceeds in a manner independent of hypertension. Accumulation of Hcys in plasma or hHcys was shown to result in the glomerular pathology including extracellular matrix accumulation and podocyte injury. Some studies demonstrated that the inability of Hcys to be properly cleaned or degraded from blood eventually leads to compromised renal function and glomerulosclerosis [110,111]. Although the mechanism remains unclear on how Hcys causes cellular injury and sclerotic changes in many organs and tissues, inflammatory cytokines were found increased in blood and many tissues during hHcys. It has been indicated that the inflammation and related molecular machinery, inflammasomes, may play a crucial role in these processes [112,113]. Under some pathological conditions, Hcys-induced glomerular injury was indeed found to activate local inflammatory responses by enhancing the production of monocyte chemoattractant protein 1 in glomerular mesangial cells and tubular epithelial cells [114]. Inhibition of the inflammatory process could significantly protect the kidney against hHcys-associated damage [114].

However, it remains poorly understood how the inflammation in glomeruli during hHcys is activated and whether this is associated with NLRP3 inflammasome formation and activation. Our recent studies revealed that treatment of glomerular epithelial cells (podocytes) with Hcys induced the NLRP3 inflammasome, forming molecular complex and producing IL-1β and other cytokines. This NLRP3 inflammasome activation critically contributes to the development of glomerular sclerosis during hHcys. Moreover, there is evidence that knocking out of the *Nlrp3* gene protected podocytes and glomeruli against hHcys-induced injury as observed by decreased urinary protein excretion, reduced glomerular damage and diminished expression of the podocyte-specific damage marker, desmin in a mouse model of hHcys. The *Nlrp3* gene has been well documented as an essential component of NLRP3 inflammasomes, which serves as a sensor for monitoring redox

changes. This sensing function of NLRP3 is confirmed to be mediated by its association and dissociation with TXNIP [56].

1.4.3 Diabetic nephropathy

Diabetic nephropathy (DN) is the leading cause of ESRD and remains a great clinical concern due to high mortality and morbidity rates. Poor management of diabetes often results in the loss of renal function, which is due to gradual changes in the glomerular structure and associated functions such as thickening of the basement membrane, mesangial expansion, proteinuria/albuminuria and glomerular fibrosis. DN is different from other types of glomerular diseases that result in ESRD, and it is generally categorized as a non-inflammatory glomerular disease. However, recent genome-wide transcriptome analysis revealed that several inflammatory signaling pathways are present or activated during DN. Although some reports have shown hyperglycemia-induced cell death and immune cells accumulation in glomeruli, an inflammatory response, the precise mechanisms by which inflammation is activated during DN remains to be elucidated [115].

More recent studies have now suggested that infiltration of inflammatory cells is crucial in the pathogenesis of DN [116]. IL-1β and IL-18 secreted from immune cells and glomerular resident cells such as podocytes, endothelial cells or mesangial cells may possibly promote DN [51,54,117]. Initial studies from Shahzad et al [117] revealed that diabetic mice have upregulated expression of inflammasome molecules and pro-inflammatory cytokines in circulation when compared to their non-diabetic counterparts. Morphological and progressive functional changes were also observed in the kidney of these diabetic mice. In the db/db mouse model of diabetes, however, transplantation of *Nlrp3* and *caspase-1* deficient mice bone marrow did not attenuate the kidney damages compared to control db/db mice, suggesting that blocking of myeloid-lineage immune cells is insufficient in preventing the progression to DN. In other studies, the production of

mitochondrial ROS was shown to initiate NLRP3 inflammasome activation in diabetic conditions, further establishing the causative link between NLRP3 inflammasome activation and DN. In addition, inhibition of NLRP3 or caspase-1 in the kidney led to protective outcomes seen by inactivation of the inflammasome. This provides a strong foundation in how the NLRP3 inflammasome serves as an important mediator for pro-inflammatory cytokine production by caspase-1 activation and how inhibition of this complex formation may serve as a promising approach to treat DN. With respect to the mechanism responsible for NLRP3 inflammasome activation, it has been reported that high glucose (HG) treatment in mice induced NOX activity triggering NLRP3 inflammasome activation in glomerular podocytes and leading to podocyte injury during DN. However, inhibition of TXNIP by shRNA and its inhibitors abolished this DN-induced inflammasome activation [118]. Since deletion of mouse *Nlrp3* gene, antagonism of IL-1R and inhibition of mitochondrial ROS production all were shown to protect or even reverse DN in mice, targeting the NLRP3 inflammasome has been indicated to serve as a beneficial strategy for treatment of DN [117].

1.4.4 Glomerular injury and sclerosis in obesity

CKD is now considered as one of the strongest risk factors for the morbidity and mortality in obese patients, with the prevalence of obesity increasing worldwide [119,120]. Previous studies have identified visceral fat as a generator of bioactive substances that contribute to the pathophysiologic and structural changes in glomeruli [121]. Mechanistically, obesity-induced glomerular sclerosis and ultimate ESRD is involved in chronic inflammation, abnormal glomerular vascular remodeling, rise in renal plasma flow, hyperfiltration and renal lipotoxicity [122].

Most recently, we have indeed shown that the NLRP3 inflammasome is implicated in the development of obesity and associated chronic glomerular injury. It serves as an important

initiating mechanism to activate local glomerular inflammation leading to ultimate glomerular sclerosis in obese mice [57]. Inhibition of the *Asc* gene significantly protected mice from high fat diet (HFD)-induced obesity, podocyte damage and glomerular injury. Although the molecular mechanism of HFD-induced inflammasome activation remains unknown, it is possible that increased production of fatty acid metabolites, ceramide and palmitate is a key trigger. There is evidence that the abundance of fatty acid metabolites in adipose tissue positively correlates with the development of obesity and type II diabetes in mice. In this regard, Vandanmagsar et al [123] showed that supplement of ceramide to adipose tissue explants led to NLRP3-dependent caspase-1 activation and IL-1β production, suggesting that ceramide acts a danger signal to stimulate the NLRP3 inflammasome. We have also shown that a HFD increased the glomerular ceramide production due to the activation of acid sphingomyelinase, a ceramide producing enzyme. The increased ceramide production induced the formation and activation of NLRP3 inflammasome in glomeruli, which contributes to the obesity-induced glomerular injury. All these results have indicated that the NLRP3 inflammasome may also be a therapeutic target for obesity and related glomerular injury and sclerosis.

1.5 The NLRP3 Inflammasome as a Therapeutic Target in Chronic Glomerular Diseases

The prolonged period of clinical silence in chronic glomerular disease or CKD leads to irreversible pathological damage leading to glomerular fibrosis. It has been well known that early detection of these alterations and abnormalities in the impaired kidneys is necessary to facilitate therapeutics that can improve clinical outcomes. Management of risk factors such as hypertension, increase blood glucose level and albuminuria has been vital in slowing the progression to ESRD. Although current treatments including angiotensin converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARB), mineralocorticoid receptor antagonists and statins [124] have

been used clinically for many years, the morbidity and mortality of CKD patients still remain high. It is obvious that identification of new therapeutic targets and development of new strategies for treatment of ESRD are imperative.

There is growing interest in administering anti-inflammatory therapies to halt renal and cardiovascular functional loss, for example, targeting oxidative stress and inflammatory responses. However, these therapies are met with a bit of skepticism and may only elicit partial improvements in the pathogenic process of CKD characterized by glomerular sclerosis. The root of glomerular dysfunction and other renal diseases previously associated with inflammation may potentially be eliminated by inhibiting NLRP3 inflammasome activation. Convincing evidence has shown that by blocking this complex there is a reduction in tissue and cellular inflammation. Because this strategy targets both the non-inflammatory and non-canonical damaging effects on cell function and metabolism, it may be more efficient during glomerular diseases. Indeed, treatments with IL-1β antibodies have proven very effective in patients with cyropyrin-associated periodic syndromes (CAPS) [125].

Activation of the NLRP3 inflammasome following MSU and other crystal-induced pathologies translate into the disease manifestation of gout or pseudogout [2]. There is also evidence that antagonism of the IL-1 signaling pathway immediately improved the clinical outcome of patients with these arthropathic diseases [126,127]. However, whether crystal-associated nephropathies have the similar mechanisms has yet to be established. To our knowledge, approved inhibitors of IL-1β are commercially available for treatment of various inflammasome-mediated autoinflammatory diseases; however, they produced adverse effects at the injection site [128,129]. Anakinra, a recombinant IL-1R antagonist competitively inhibits IL-1 signaling [130], and administration of Anakinra has been reported to improve the inflammatory response in

hemodialysis patients [131]. In addition, Glyburide, commonly used to treat type II diabetes, was found to block chloride and potassium channels in pancreatic β cells to regulate insulin release [132]. This compound is able to inhibit IL-1β release upon stimulation with LPS in human monocytes [133], but it does not block capsase-1 activation unless *Nlrp3, NF-κB* or *P2X7* genes are knocked out in mice [134–137]. It is expected that more therapeutic strategies will be forthcoming. For example, the use of NLRP3 inhibitors and the P2X7 receptor blockers may block NLRP3 inflammasome activation. IL-1 and IL-18 receptor blockers, caspase-1 inhibitors can antagonize the effects of NLRP3 inflammasome products, and H_2S donor Na_2S, lysosome stabilizer, cathepsin-B inhibitors, and milk fat globule EGF-8 may serve as an endogenous inhibitor of inflammasome-induced IL-1β production. These potential therapeutic strategies target different stages of NLRP3 inflammasome formation and activation, which may be selected for the use in prevention or treatment of ESRD and associated glomerular diseases.

1.6 Aims of Study

The hypothesis to be tested in the present study states that: Vav2 associated redox signaling associated with NOX contributes to Hcys-induced NLRP3 inflammasome formation and activation, thereby causing glomerular inflammatory responses and associated pathologies.

In order to test this hypothesis the following specific aims were proposed:

1. To determine whether GNEF, Vav2 contributes to NLRP3 inflammasome activation and associated podocyte injury and glomerular sclerosis *in vivo* in hyperhomocysteinemic mice.
2. To determine whether Vav2 exerts its pathogenic role by activation of the Rac-1-NOX complex, inducing NLRP3 inflammasome formation and activation in murine podocytes.

3. To investigate pharmacological targeting of Vav2 as a potential therapeutic to prevent CKD progression associated with hHcys-associated nephropathy.

The overall hypothesis and three specific aims of this project are schematically presented in **Figure 2.**

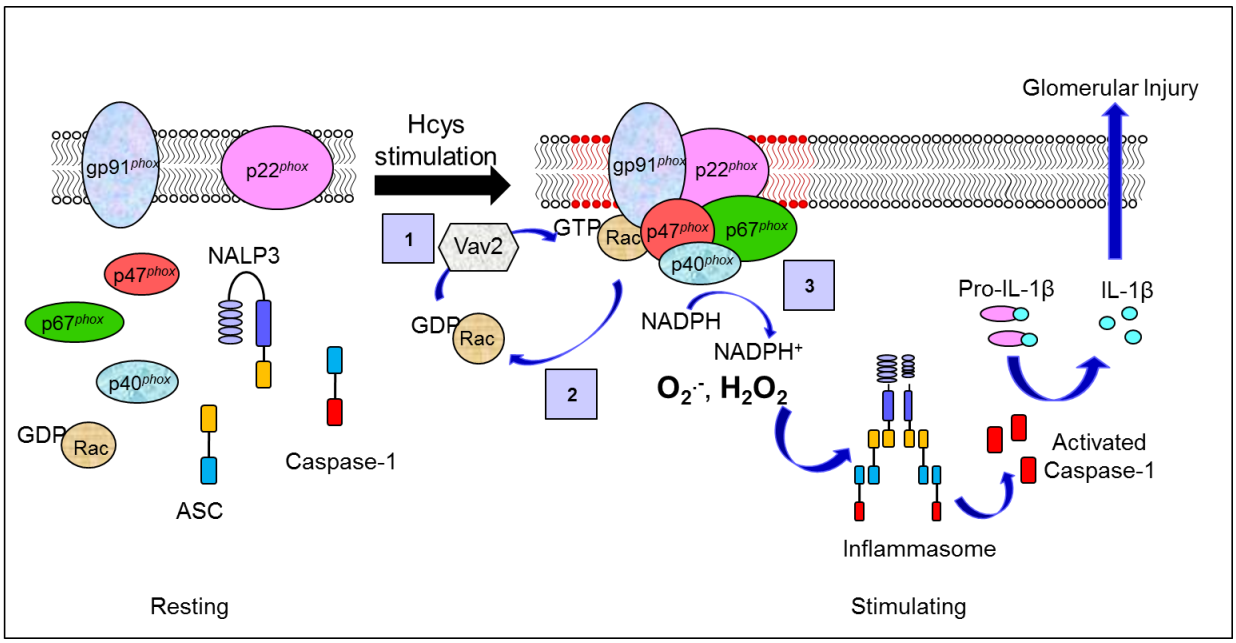

Figure 2. The Overall Hypothesis.

By inhibition and overexpression of the Vav2 gene, Aim 1 and 2 will demonstrate the role of Vav2 in NOX-mediated redox signaling leading to NLRP3 inflammasome formation and activation in podocytes. More specifically, these aims will reveal the involvement of Rac-1-NOX signaling in the Hcys-induced sclerotic process. Aim 3 will further elucidate if Vav2 may serve as a therapeutic target for termination of early events in hyperhomocysteinemic nephropathy and ESRD.

CHAPTER TWO

GENERAL METHODS

2.1 Hyperhomocysteinemic Mouse Model

Eight week old mice on a C57BL/6J background were purchased from Jackson Laboratories (Bar Harbor, ME). To produce a rapid and advanced model of hHcys, mice were uninephrectomized and afterward allowed 1-week to recover. Uninephrectomized mice were fed either a normal diet (ND) or a folate-free (FF) diet to induce hHcys (Dyets Inc, Bethlehem, PA) for 4 weeks. Before sacrifice, 24-hour urine samples were collected using mouse metabolic cages and afterward blood samples were collected. The mice were then sacrificed and renal tissues were harvested for biochemical and molecular analysis. All protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.1.1 *In vivo* Treatments

For *in vivo* genetic studies, Vav2 short hairpin RNA (shRNA) or a dominant-active Vav2 variant (oncoVav2) plasmid with a luciferase expression vector was co-transfected into the kidneys via a femoral artery injection using the ultrasound-microbubble system. Plasmid containing scrambled small RNA (sRNA) was used as a control. After introduction of plasmid into the kidney, these mice were maintained on either a ND or FF diet for 4 weeks.

For *in vivo* pharmacological studies, Nlrp3 knockout ($Nlrp3^{-/-}$) mice were purchased from the Mutant Mouse Research and Resource Center. Groups of mice received intraperitoneal (IP) injections of the Vav2 activator, Uridine Triphosphate (UTP) or Rac-1 inhibitor, NSC-23766 (1 mg/kg/day) throughout the ND or FF diet feeding period, doses were chosen according to a recent report [138].

2.1.2 Ultrasound-microbubble Assisted Plasmid Transfection

After allowing one week for recovery from uninephrectomy surgery, a preparation of plasmid encoding either Vav2 shRNA, oncoVav2 or the reporter gene luciferase was mixed with cationically charged Optison microbubbles (GE Healthcare, Piscataway, NJ) then injected into the femoral artery and locally transfected to the kidney by sonoporation with a continuous wave output of 1 MHz at 10% power output at 30 sec intervals for a total of 6 minutes. To daily monitor the efficiency of gene expression, mice were anesthetized with ketamine (100 mg/kg IP) and xylazine (10 mg/kg IP), and an aqueous solution of luciferin (150 mg/kg IP) was injected 5 minutes before imaging as described previously [48]. The anesthetized mice were imaged using the Xenogen IVIS200 *in vivo* imaging system (Perkin Elmer, Waltham, MA). Photons emitted from luciferase-expressing cells and transmitted through tissue layers were quantified over a defined period of time ranging up to 5 minutes using the software program Living Image (Xenogen) as an overlay on an Igor program (Wavemetrics). If transfection was not detected by *in vivo* imaging, the mice were sacrificed and experiments terminated due to unsuccessful transgene expression.

2.2 Culture of Murine Podocytes

A conditionally immortalized mouse podocyte cell line gifted by Dr. Paul E. Klotman (Division of Nephrology, Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA) was cultured undifferentiated with 10 U/mL recombinant mouse interferon-γ at 33°C on collagen I-coated flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Prior to experiments, podocytes were thermoshifted at 37°C for 10-14 days to differentiate in the absence of interferon-γ and afterward used for experiments.

2.2.1 *In vitro* Treatments

Our previous studies have demonstrated a 40 µM treatment of L-Hcys for 24 hours to be the optimal concentration and time to produce a significant activation of the NLRP3 inflammasome [43,56,107]. Pharmacological interventions of Vav2 UTP (100 µM) and NSC-23766 (50 µM) were added to the cells 1 hour prior to Hcys treatment. Their doses for effective activation or inhibition were confirmed in previous studies [139,140].

2.2.2 RNA interference

Podocytes were transfected with a scrambled sRNA, Vav2 shRNA or constitutively active form of Vav2 (oncoVav2) plasmid directly to the nucleus via 4D- Nucleofector Technology (Lonza, Basel, Switerland). The dominant active oncoVav2 plasmid containing an N-terminal truncation was a generous gift from Dr. Keith Burridge from the University of North Carolina at Chapel Hill [141]. Small pores in the cell membrane develop temporarily through electrical impulses and cell-specific solutions. Substrates are then delivered through the cytoplasm and into the nuclear membrane. 2 x 10^6 podocyte cells were resuspended in SF Cell Line nucleofector solution containing 2 µg plasmid shRNA or DNA, transferred into a certified cuvette and placed into the nucleofector system. Podocytes were subjected to cell-type specific program CM-137, and then resuspended in pre-warmed medium in cultured plates. An additional group of podocytes was used to transfect Vav2 siRNA medium (Life Technologies, Carlsbad, CA) using the siLentFect Lipid Reagent (Bio Rad, Berkeley, CA), which was incubated in serum-free for 25 minutes according to the manufacturer's instructions. After 4 hours incubation at 37ºC in both nucleo- and lipid-transfected cells, medium was replaced with fresh serum containing medium and podocytes were allowed an overnight recovery prior to treatment with Hcys.

2.3 High Performance Liquid Chromatography (HPLC) Analysis of Plasma Hcys

Plasma Hcys levels were measured as previously described [142] by using 100 µL plasma or standard solution mixed with 10 µL of internal standard, thionglycolic acid (2.0 mmol/L) then treated with 10 µL of 10% tri-n-butylphosphine (TBP) solution in dimethylformamide at 4°C for 30 minutes. Then, 80 µL of ice-cold 10% trichloroacetic acid (TCA) in 1 mmol/L EDTA was added and centrifuged to remove proteins in the sample. 100 µL of the supernatant was transferred into the mixture of 20 µL of 1.55 M sodium hydroxide, 250 µL of 0.125 M borate buffer (pH 9.5), and 100 µL of 1.0 mg/mL ABD-F solution. The resulting mixture was incubated at 60°C for 30 minutes to accomplish derivatization of thiols. HPLC was performed with a HP 1100 series equipped with a binary pump, a vacuum degasser, a thermo stated column compartment, and an auto sampler (Agilent Technologies, Waldbronn, Germany). Separation was carried out at an ambient temperature on an analytical column, Supelco LC-18-DB (1504.6 mm ID, 5 m) with a Supercoil LC-18 guard column (204.6 mm ID, 5 m). Fluorescence intensities were measured with an excitation wavelength of 385 nm and emission wavelength of 515 nm by a Hewlett-Packard Model 1046A fluorescence spectrophotometer. The peak area of the chromatographs was quantified with a Hewlett-Packard 3392 integrator. The analytical column was eluted with 0.1 M potassium dihydrogen phosphate buffer (pH 2.1) containing 6% acetonitrile (v/v) as the mobile phase with a flow rate of 2.0 mL/min.

2.4 Confocal Microscopy and Immunofluorescence of Frozen Tissue and Cells

To observe colocalization of inflammasome and podocyte marker proteins, indirect immunofluorescent staining was used in frozen tissue sections and podocytes. Podocytes seeded in 8-well chambers were fixed in 4% paraformaldehyde (PFA), washed with phosphate-buffered saline (PBS), and blocked with 1% bovine serum albumin (BSA) in PBS before being incubated

in primary antibodies (1:100) overnight at 4°C. The primary goat anti-NLRP3 (Abcam, Cambridge, MA) antibody was used in combination with the following: rabbit anti-ASC (Santa Cruz, Santa Cruz, CA) or rabbit anti-caspase-1 (Santa Cruz). Frozen slides with mouse kidney tissue were fixed in acetone, blocked with 3% donkey serum, then incubated with the same aforementioned primary antibodies (1:50) overnight at 4°C. Additional groups of podocytes and frozen kidney sections were only stained for antibodies against podocyte markers podocin (1:50; Sigma, St. Louis, MO), desmin (1:50; BD Biosciences, San Jose, CA) or Vav2 (Santa Cruz, Santa Cruz, CA). Double immunofluorescent staining was performed by Alexa-488 or Alexa-555-labeled secondary antibodies (1:200 podocytes, 1:50 frozen kidney slides; Life Technologies, Grand Island, NY) with an incubation of 1 hour at room temperature. Slides were then washed, mounted and observed using a confocal laser scanning microscope (Fluoview FV1000, Olympus, Japan) and tools in Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD) were used to analyze colocalization, or the degree of overlap between the two wavelengths, which was expressed as the Pearson Correlation Coefficient (PCC).

2.5 Immunohistochemistry

Kidneys were perfused in 4% PFA, removed and fixed in 10% neutral buffered formalin. 5-µm tissue sections embedded in paraffin were cut and mounted onto microscope slides. Deparaffination was induced through heat, tissue cleaning agent, ethanol and water. To unmask antigen binding sites, slides were boiled in 0.01 M citrate buffer pH 6.0 for 20 minutes. Endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide (H2O2) in 100% Methanol (MeOH) for 30 minutes. The sections were then incubated at room temperature for 30 minutes in 10% goat serum to block nonspecific binding and incubated overnight at 4°C in a humidified chamber with an antibody against IL-1β (Abcam, Cambridge,

MA) diluted 1:50 in Tris Buffered Saline with 0.2% Tween (TBS-T) containing 4% goat serum. Then, the slides were incubated for 30 minutes at room temperature in a humidified chamber with a biotinylated goat anti-rabbit IgG-B antibody diluted 1:200 in TBS-T. These slides were subsequently placed in streptavidin-horseradish peroxidase for 30 minutes at room temperature in a humidified chamber, then incubated with 50 µL of diaminobenzadine (BioGenex, San Ramon, CA) as a substrate, counterstained with hematoxylin (Sigma-Aldrich, Saint Louis, MO), dehydrated, and fixed with Permount histological mounting medium (Fisher Scientific, Hampton, NH).

2.6 Urinary Protein and Albumin Measurements

Total urinary protein excretion was determined spectrophotometrically using a Bradford assay. Determination of urinary albumin was analyzed by a commercially available albumin Enzyme-Linked Immunosorbent Assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX). The procedure for processing of albumin followed the instructions from the manufacturer. Briefly, using an anti-rat albumin coated 96 well plate. The plate was washed 4 times in prepared wash solution provided in kit, then incubated with an anti-albumin detection antibody urine samples were diluted 1:200 in 1X dilution buffer and incubated for 1 hour at room temperature and exposed to horseradish peroxidase (HRP) color substrate. Absorbance was analyzed by a microplate reader using Gen5 software (BioTek, Winooski, VT).

2.7 Glomerular Morphological Examinations

Glomerular structure was examined using fixed paraffin-embedded kidneys, stained with a Periodic-Acid Schiff (PAS) stain. Glomeruli were scored on a scale of 0-4 depending on the extent of sclerotic changes. In general, 50 glomeruli were counted under the microscope, 0 represents no lesion, 1 represents sclerosis <25% of the glomerulus while 2, 3 and 4 represent sclerosis of 25%

to 50%, >50% to 75%, and >75% of the glomerulus respectively. A whole kidney average sclerosis index was obtained by averaging scores from counted glomeruli. This observation was examined by 2 investigators and averaged under blind conditions.

2.8 Cellular Protein Homogenates

Cultured podocytes were washed three times with ice-cold PBS and scraped in ice-cold sucrose buffer (20 mM Tris-HCl, 250 mM sucrose, pH 7.2). Cellular extract was transferred into a pre-chilled microcentrifuge tube and sonicated 3 times using a handheld sonicator for 10 pulses on ice. Samples were incubated on ice for 30 minutes prior to centrifugation at 7,000 x g for 10 minutes at 4ºC to remove cellular debris. The supernatant was transferred to a new, pre-chilled microcentrifuge tube and stored at -80ºC until used for biochemical analysis. Protein concentrations were determined by the Braford Reagent Protein Assay method.

2.9 Western blot analysis

Expression levels of Vav2 were quantified in cultured mouse podocytes. Equal amounts of protein samples were loaded onto a prepared 8% polyacrylamide gel and allowed to separate electrophoretically at 120 Volts for 1 hour. Proteins from the gel were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, Richmond, CA). Non-specific binding sites on the membranes were blocked in 5% fat-free milk made in TBS-T for 1 hour at room temperature. The PVDF membrane was then probed using a specific antibody against Vav2 and incubated at 4ºC overnight. The primary antibody used was mouse monoclonal anti-Vav2 (Santa-Cruz, Santa Cruz, CA) diluted 1:1000. Membranes were washed three times for 10 minutes in TBS-T and incubated with anti-mouse IgG-HRP (Santa Cruz, Santa Cruz, CA) for 1 hour, diluted 1:10,000 in TBS-T. The membranes were then washed in TBS-T for 15 minutes three times, exposed to Super Signal

West Pico Substrate Solution (Thermo Scientific, Rockford, IL) and protein bands were developed by exposure to X-Ray film. The intensity of the protein bands were quantified by densitometry using ImageJ Software.

2.10 RNA Isolation and Real Time RT-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from podocytes using TRIzol reagent (Thermo Fisher, Waltham, MA) according to the protocol described by the manufacturer. The resultant RNA was resuspended in RNase-free water and messenger RNA (mRNA) levels for Vav2 were analyzed by quantitative reverse transcription using a Bio-Rad iCycler system (Bio Rad, Hercules, CA). The mRNA was reverse transcribed to complementary DNA (cDNA) using an iScript cDNA synthesis kit (Bio Rad). Real-time polymerase chain reaction (RT-PCR) was performed using a SYBR supermix kit (Bio Rad) for 40 cycles at 95 ºC for 15 seconds and 53°C for 45 seconds. PCR efficiency was examined by serially diluting the template cDNA and the melting curve data were collected to check the PCR specificity. The mRNA levels of each sample for each gene were normalized to that of β-Actin. The specific primers for Vav2 based on the core sequence of rat Vav2 cDNA (Accession number XM_216030) were 5'- GTT CAA ACT CGG AAG TCA GG -3' (Forward) and 5' CCA CGG GTA TGC AGT GTA AT -3' (Reverse).

2.11 Caspase-1 Activity, IL-1β Production and Vascular Endothelial Growth Factor (VEGF) Measurements

Caspase-1 activity (Biovision, Mountain View, CA) was measured by a commercially available colorimetric assay. IL-1β production (R&D Systems, Minneapolis, MN) and VEGF (Bender Medsystems, San Diego, CA) were quantified through an ELISA using cellular supernatant, according to manufacturer's instructions.

2.12 Rac-1 GTPase Activation Assay

A GTPase Linked Immunosorbent Assay (GLISA) was performed to determine Rac-1 activation *in vitro* (Cytoskeleton, Denver, CO). Podocytes were lysed in provided lysis buffer and clarified to remove cellular debris by centrifugation at 10,000 x g for 1 minute at 4°C. Equalized amounts of protein (1 mg/mL) was loaded onto a Rac-1 GTP affinity plate for 30 minutes. Following incubation, the plate was washed in wash buffer then incubated with primary and secondary antibodies against Rac-1. The activated Rac-1 was determined after exposure to HRP detection reagents by measuring absorbance at 490 nm using a microplate spectrophotometer.

2.13 Electron Spin Resonance (ESR) Spectrophotometry of $O_2^{\cdot -}$ Production

Cellular protein samples were prepared by using modified Kreb's-Hepes buffer containing deferoximine (100µM) and diethyldithio-carbamate (5 µM). NOX-dependent $O_2^{\cdot -}$ production was examined by addition of 1 mM NADPH as a substrate in 30 µg protein in the presence or absence of superoxide dismutase (SOD) (800 U/ml), and then supplied with 10 mM $O_2^{\cdot -}$ specific spin trapping compound, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrro-lidine (CMH). The mixture was loaded in glass capillaries and immediately analyzed for $O_2^{\cdot -}$ production kinetically for 10 min in an ESR spectrometer. Results were obtained by subtracting the total CMH signal without SOD, representing the SOD-specific signal. All values were expressed as the fold change from control.

2.14 Statistical Analysis

All data are represented as mean $\pm$ standard error (SE); n represents the number of independent experiments unless otherwise stated. Differences in mean values within and between multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by

Dunnett's post hoc test. The statistically significant differences were also assessed by applying the unpaired Student's t test and the significance level was $p<0.05$.

CHAPTER THREE

Contribution of guanine nucleotide exchange factor Vav2 to homocysteine-induced NLRP3 inflammasome activation in mouse podocytes during hyperhomocysteinemia

3.1 Rationale and Hypothesis

One of critical steps for NOX to induce glomerular injury may be due to activation of the NLRP3 inflammasome, which senses the overall integrity and health of the cell. As illustrated in **Figure 3,** the present study hypothesized that activation of Vav2 through elevated Hcys accelerates the switch from GDP to GTP, thereby enhancing NOX-mediated activity and $O_2^{\cdot -}$ production, which may instigate the cascade to glomerular sclerosis by activation of the NLRP3 inflammasome in podocytes. To test this hypothesis, we first used 8 week old uninephrectomized mice on a C57BL/6 background to examine the molecular participation of Vav2 in the actions of NLRP3 inflammasome formation and activation which results in glomerular inflammatory responses and functional alterations in the glomerulus of hyperhomocysteinemic mice. Additionally, we explored the molecular mechanisms by which Hcys results in an active Rac-1-NOX complex using cultured murine podocytes.

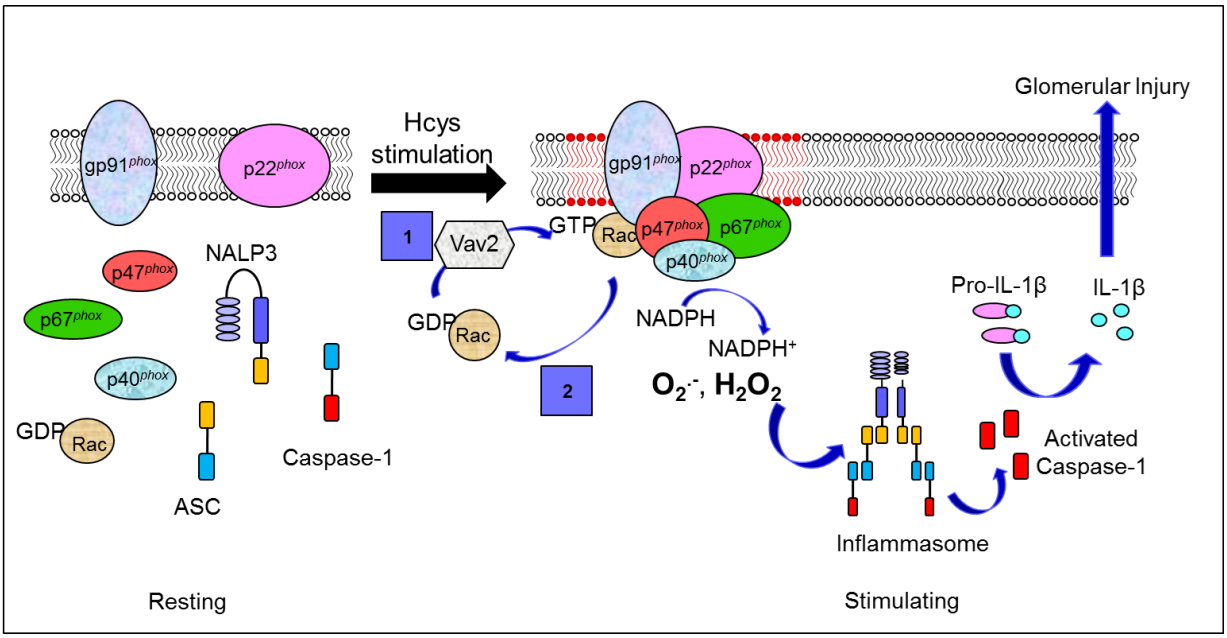

Figure 3. Representative schematic illustrating the goals of Aim 1 and 2.

Through genetic inhibition of Vav2 both *in vivo* and *in vitro*, Aims 1 and 2 will explore the effects of Vav2 during hHcys.

3.2 Results

3.2.1 Confirmation of hHcys-induced Model

Using HPLC analysis, we found that plasma Hcys concentrations was similar in all that consumed the ND. However, we observed increased plasma Hcys levels in FF-fed Scram-, Vav2 shRNA- and oncoVav2-transfected mice, indicating a successful establishment of hHcys and that Vav2 is not involved in Hcys metabolism (**Figure 4**).

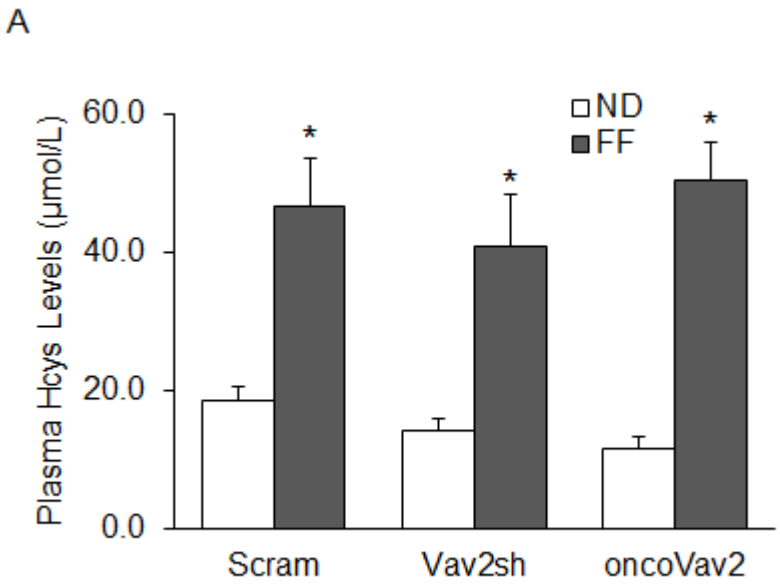

Figure 4. Effect of normal and FF diets on plasma Hcys concentrations.

Plasma Hcys levels measured by HPLC in 6 groups of mice (n=4). Scram: Scramble, Vav2sh: Vav2 shRNA, ND: Normal Diet and FF: Folate-Free Diet. * $p<0.05$ vs. Scram on ND.

3.2.2 *In vivo* inhibition of Vav2 prevented glomerular NLRP3 inflammasome formation and activation

Using a hyperhomocysteinemic mouse model, we tested whether Vav2 facilitates hHcys-induced inflammasome formation and glomerular injury. By confocal microscopy, we found that hHcys and oncoVav2 transfected mice fed a normal or FF diet exhibited increased colocalization of NLRP3 with ASC and NLRP3 with caspase-1 (**Figure 5**). However, this colocalization was inhibited in mice transfected with Vav2 shRNA (**Figure 5**). The summarized data is shown in **Figures 5C** and **5D**. Since activation of the NLRP3 inflammasome is associated with the maturation of the pro-inflammatory cytokine, IL-1β, we measured the IL-1β levels in mouse glomeruli using immunohistochemistry. We detected a significant increase in IL-1β levels in hHcys mice and mice receiving the oncoVav2 plasmids on both normal and FF diets (**Figure 6A**). However, blockade of Vav2 abolished further increases in hHcys-induced glomerular IL-1β (**Figure 6A**). This data indicates that Vav2 is involved in both assembling and activation of the NLRP3 inflammasome in glomeruli of mice with hHcys, which could be mimicked by overexpression of Vav2.

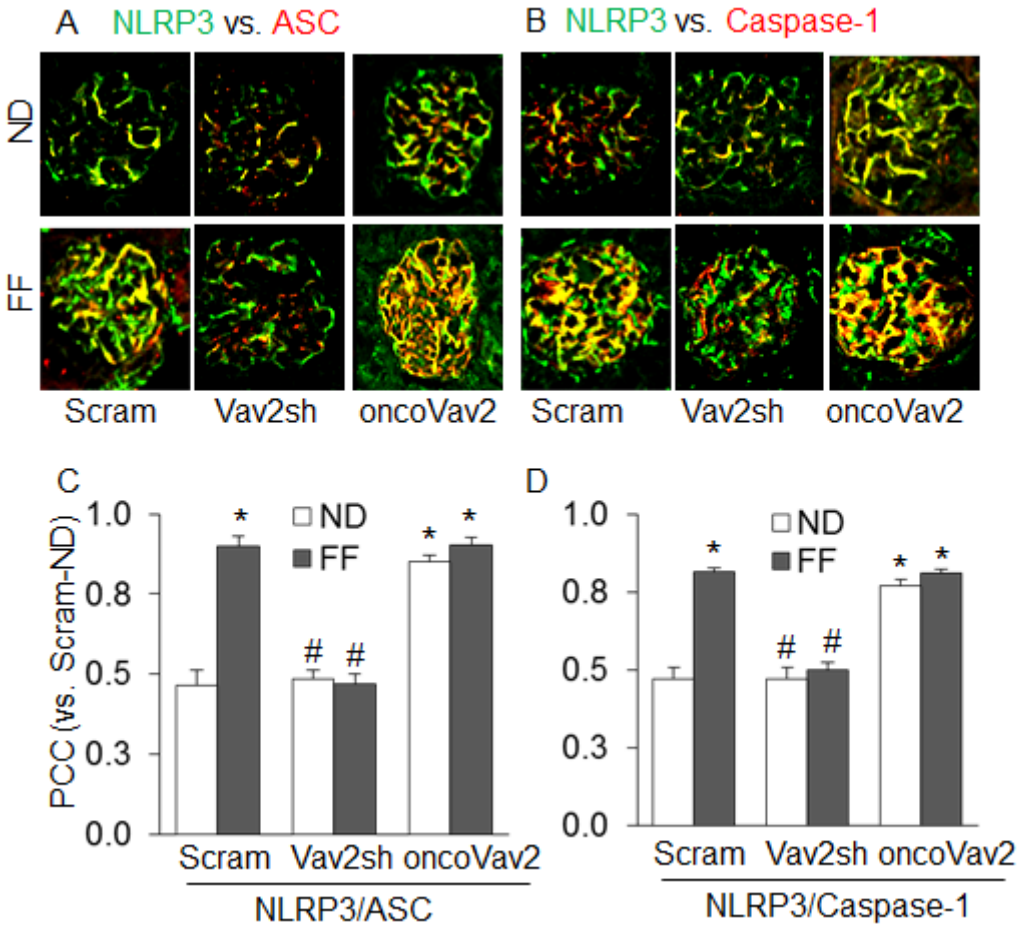

Figure 5. Inhibition of Vav2 abolished glomerular NLRP3 inflammasome formation in mouse kidney.

A-B. Colocalization of NLRP3 (green) with ASC (red) or caspase-1 (red) in mouse glomeruli fed a normal or FF diet. C-D. Summarized data showing the fold changes in PCC for the colocalization of NLRP3 with ASC or with caspase-1 (n=6). Scram: Scramble, Vav2sh: Vav2 shRNA, ND: Normal Diet and FF: Folate-Free Diet. * $p<0.05$ vs. Scram on ND; # $p<0.05$ vs. Scram on FF Diet.

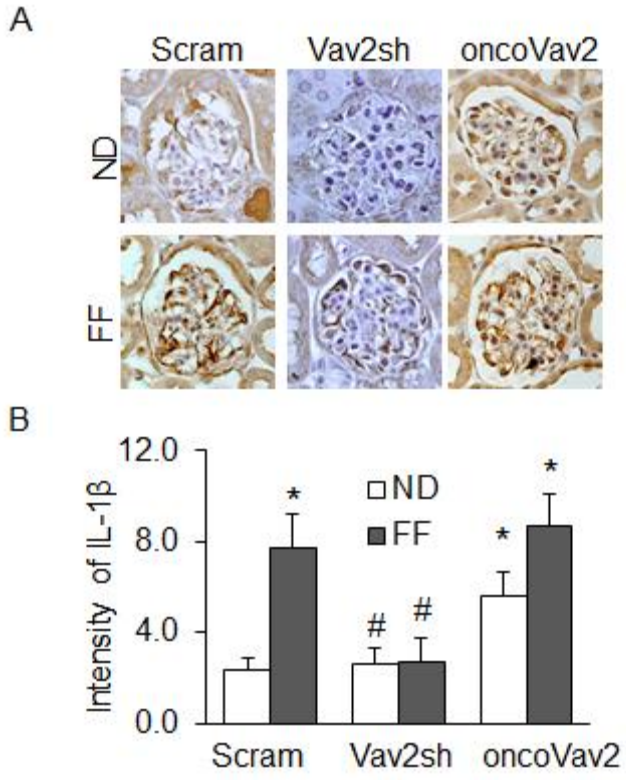

Figure 6. Vav2 overexpression induced NLRP3 inflammasome activation in podocytes of the mouse kidney.

A. Representative images of immunohistochemical staining of IL-1β in glomerular podocytes in mice on the normal or FF diet. B. Summarized data depicting relative intensity of IL-1β brown staining (n=5). Scram: Scramble, Vav2sh: Vav2 shRNA, ND: Normal Diet and FF: Folate-Free Diet. * $p<0.05$ vs. Scram on ND; # $p<0.05$ vs. Scram on FF Diet.

3.2.3 Glomerular protection by Vav2 inhibition

It is well recognized that protein in the urine is a hallmark of many glomerular diseases. After a 24-hour urine collection, protein and albumin levels were evaluated in collected mouse urine to serve as an indicator of glomerular injury. As illustrated in **Figures 7A** and **7B**, the FF diet produced both proteinuria and albuminuria in scramble sRNA-transfected mice, and the increased urinary excretion of protein and albumin was also observed in oncoVav2 transfected mice on a ND. However, this glomerular damage was attenuated in mice receiving intrarenal Vav2 shRNA transfection (**Figures 7A** and **7B**). Furthermore, this glomerular dysfunction was correlated to modifications in the glomerular architecture as shown by morphological examinations. It was found that scramble sRNA and oncoVav2 plasmid-transfected mice fed a FF diet developed increased sclerotic damage to their glomeruli, as shown by mesangial expansion, fibrosis and hypercellularity with an increased glomerular damage index (GDI) (**Figures 7C** and **7D**). Inhibition of Vav2 by shRNA protected mice from this glomerular sclerotic pathology. Immunofluorescence analysis demonstrated that the expression of podocin decreased in oncoVav2 transfected mice on either the ND or the FF diet, but this decrease in podocin levels was not seen in Vav2 shRNA transfected mice on the same diet (**Figure 8A**). In contrast, the levels of desmin, a marker of podocyte injury, increased in oncoVav2 transfected mice, but not in Vav2 shRNA transfected mice (**Figure 8B**). Furthermore, as shown in **Figure 8C**, Vav2 protein levels were significantly lower in scramble sRNA and Vav2 shRNA transfected mice, as depicted by green staining. **Figure 8D** showed the relative intensity of Vav2 staining in oncoVav2 transfected mice, which exhibited a two-fold increase in comparison to that seen in scramble sRNA transfected mice.

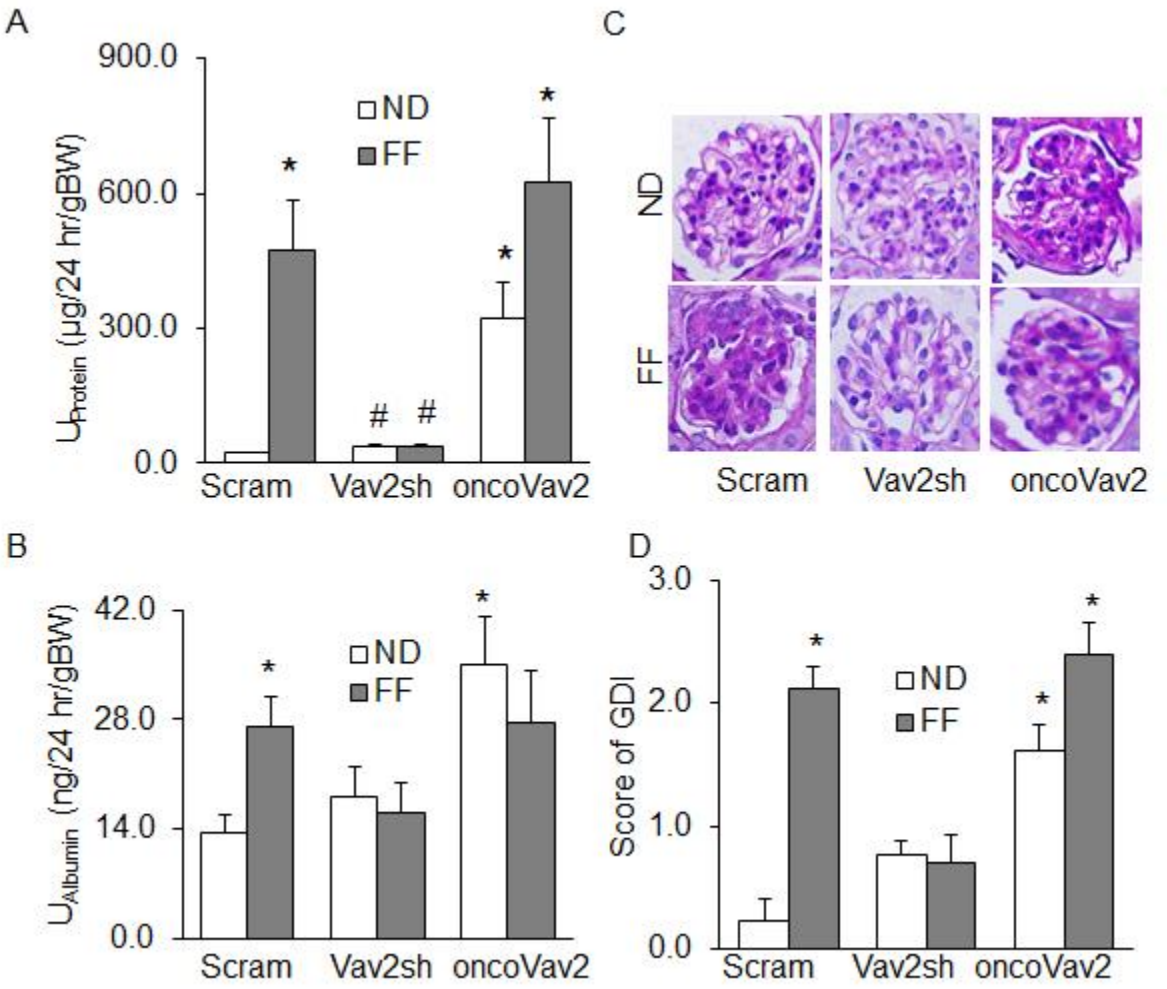

Figure 7. *In vivo* inhibition of Vav2 attenuated hHcys-induced glomerular damage.

Urinary protein (A) (n=4) and albumin (B) (n=5) measurements in 24 hr urine samples of hHcys mice receiving shRNA- and onco-Vav2 transfection. C. Microscopic microphotograph of glomerular structure in PAS stained kidney sections. D. Semiquantitative assessment of glomerular damage index (GDI) from PAS images (n=4). Scram: Scramble, Vav2sh: Vav2 shRNA, ND: Normal Diet, FF: Folate-Free Diet, U: Urinary, and GDI: Glomerular Damage Index. * $p<0.05$ vs. Scram on ND; # $p<0.05$ vs. Scram on the FF Diet.

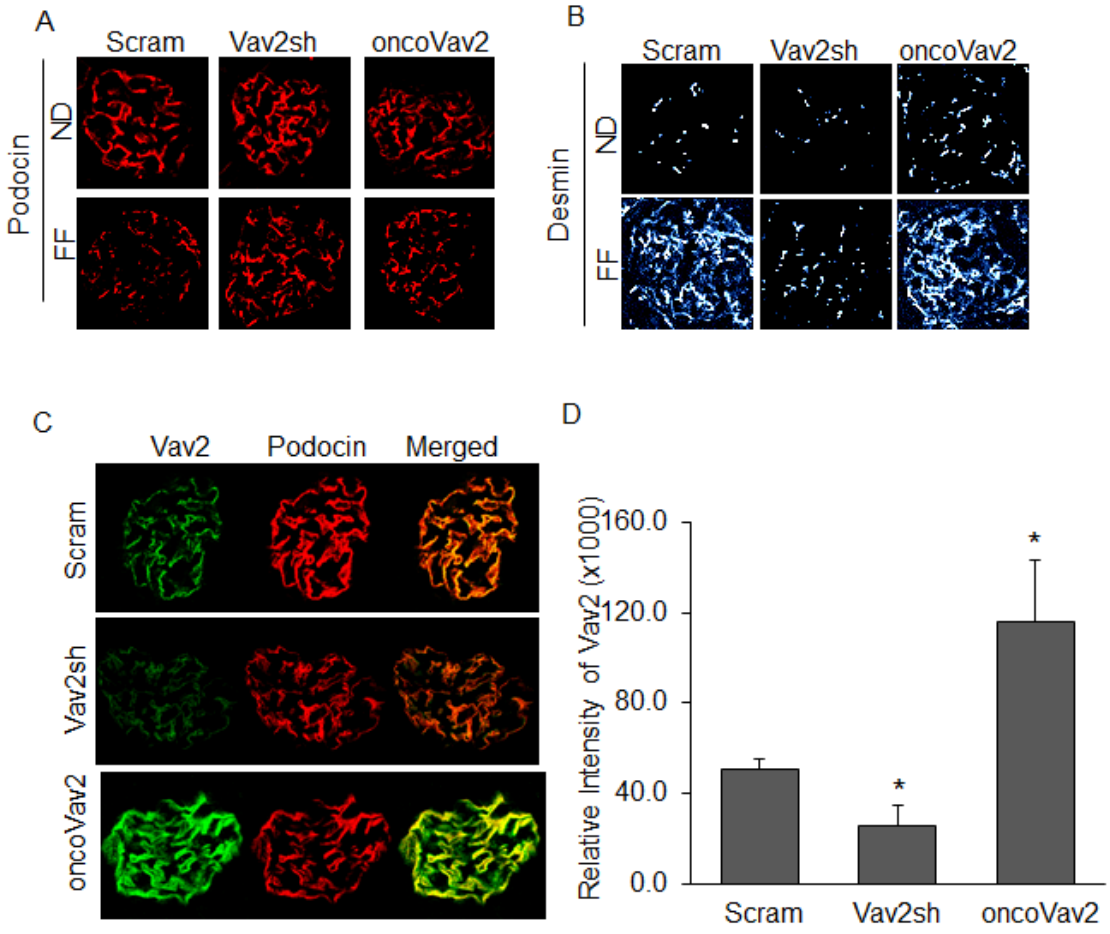

Figure 8. Glomerular dysfunction associated with overexpressed Vav2 in mouse glomeruli.

A. Florescent staining of podocyte markers, podocin (A) and desmin (B) in mouse glomeruli (n = 5). C. Confirmation of Vav2 (green) expression in glomerular podocytes. D. Quantification of relative intensity of Vav2 in glomeruli (n=4). Scram: Scramble, Vav2sh: Vav2 shRNA, ND: Normal Diet and FF: Folate-Free Diet. * $p<0.05$ vs. Scram on ND; # $p<0.05$ vs. Scram on the FF Diet.

3.2.4 Analysis of Vav2 expression in mouse podocytes

To verify efficiency of the Vav2 gene podocytes were co-transfected with the appropriate plasmid encoding for either Vav2 shRNA or oncoVav2 with an amplified green fluorescent protein (GFP) vector to visualize the transfected podocyte density. In **Figure 9**, representative images 48 hours-post transfection depict an intense stable gene expression in each group of transfected cells. Using genetic manipulations, we observed Vav2 cellular protein levels (**Figure 10B**) and mRNA expression (**Figure 10C**) were significantly increased 48 hours-post transfection of oncoVav2.

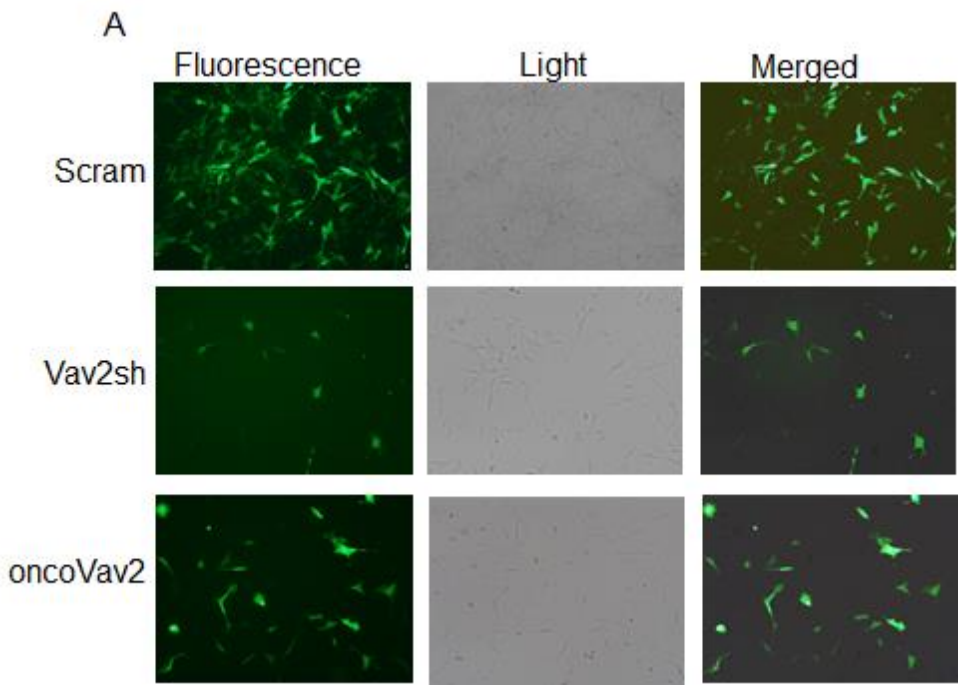

Figure 9. Determination of podocyte density 48 hours post-transfection

A. Representative images of podocytes 48 hours post-transfection, displaying fluorescence, light and merged overlay (n=4). Scram: Scramble and Vav2sh: Vav2 shRNA.

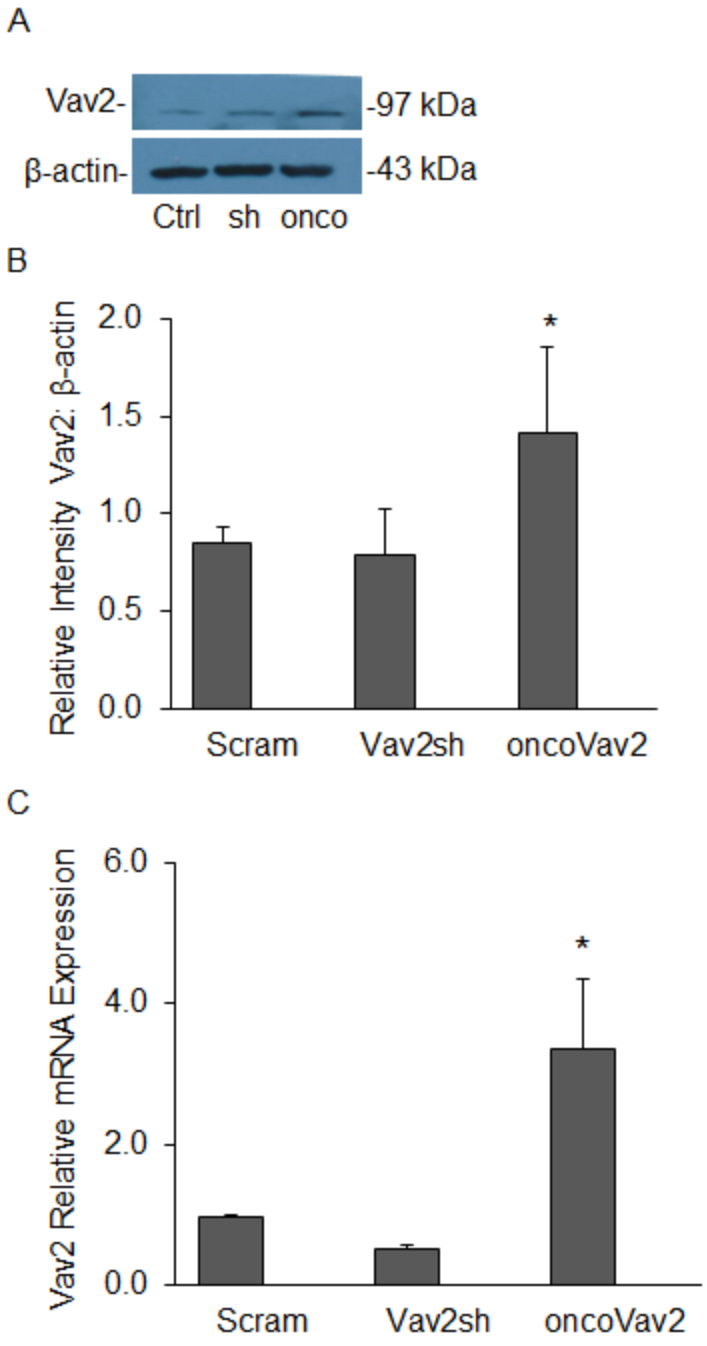

Figure 10. Vav2 gene efficiency.

A. Western blot gel document and summarized data (B). C. RT-PCR detection of Vav2 mRNA levels after shRNA and oncoVav2 transfection. (n=4) Scram: Scramble, Vav2sh: Vav2 shRNA, sh: Vav2 shRNA, onco: oncoVav2 and kDA: kiloDalton. * $p<0.05$ vs. Scram.

3.2.5 Role of Vav2 in Hcys-induced NLRP3 inflammasome formation in podocytes

The glomerulus is comprised of specialized cells that operate in a partnership to preserve the integrity of the kidney. Evidence strongly links podocyte injury to the pathogenesis of many glomerular diseases [143,144]. To better understand the impact of glomerular dysfunction during hHcys, we chose to further investigate the molecular mechanisms initiating injury in podocytes by employing cultured podocytes. Our initial studies determined if Vav2 is involved in Hcys-induced inflammasome formation in these cells. By confocal microscopic analysis, we detected increased colocalization of NLRP3 with ASC or caspase-1 in Hcys-treated podocytes when compared with untreated cells (**Figure 11**). However, this Hcys-induced increase in colocalization was inhibited in podocytes transfected with Vav2 shRNA (**Figure 11**). Additionally, oncoVav2 overexpression increased colocalization of inflammasome molecules in both control and Hcys-treated groups of podocytes. The quantitative colocalization of NLRP3 with ASC or caspase-1 is summarized in **Figures 11C** and **11D**.

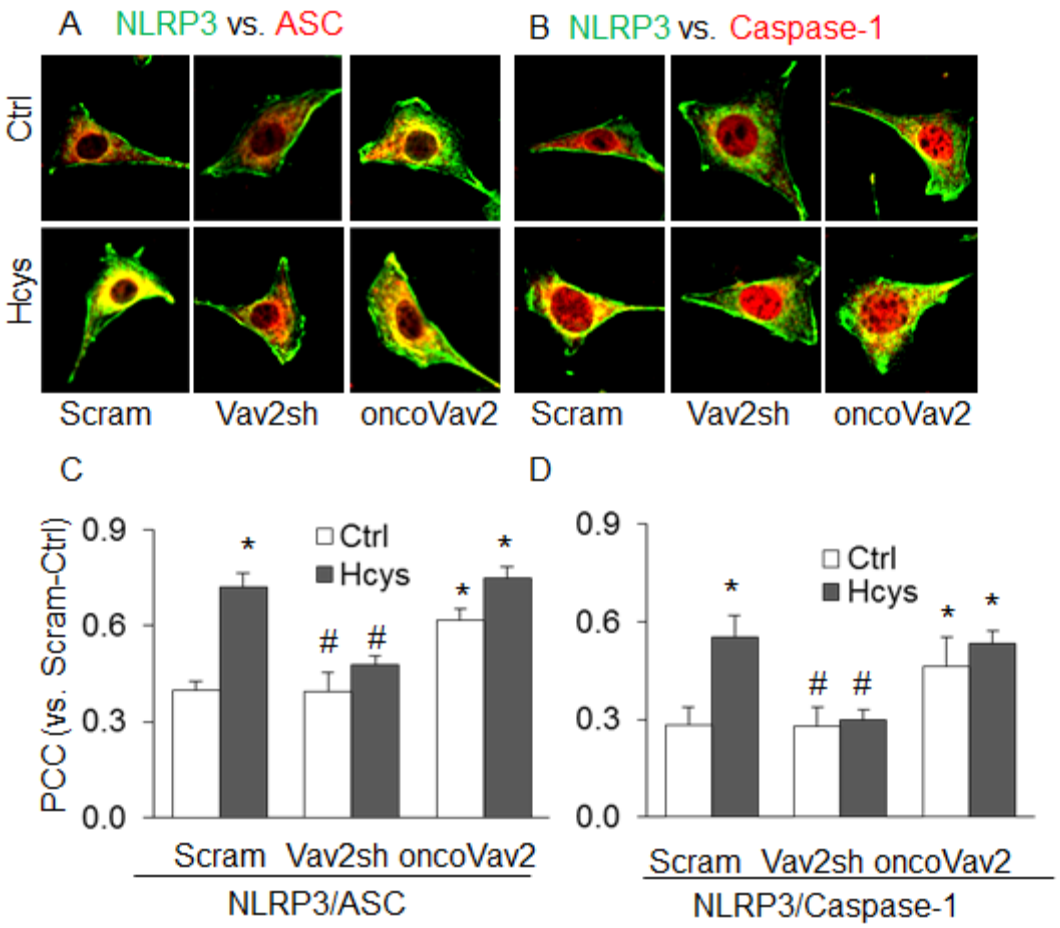

Figure 11. Hcys treatment and oncoVav2 transfection increased NLRP3 inflammasome formation in podocytes.

A-B. Confocal images signifying the colocalization of NLRP3 (green) with ASC (red) and NLRP3 (green) with caspase-1 (red) in cultured podocytes transfected with Vav2sh or oncoVav2. C-D. Summarized data showing the fold change in PCC colocalization (n=6). Scram: Scramble, Vav2sh: Vav2 shRNA and Ctrl: Control. * $p<0.05$ vs. Ctrl; # $p<0.05$ vs. Hcys.

3.2.6 Activation of the NLRP3 inflammasome *in vitro*

We confirmed the role of Vav2 in Hcys-induced inflammasome activation by measuring the levels of active or cleaved caspase-1 and the level of secreted IL-1β. As shown in **Figure 12**, Hcys treatment increased caspase-1 activity and IL-1β production in comparison to control podocytes. Hcys-induced increase in caspase-1 activity and elevation in IL-1β levels were significantly attenuated in podocytes transfected with Vav2 shRNA (**Figure 12**). Transfection with oncoVav2 mimicked the effects of Hcys which increased caspase-1 activity and elevated IL-1β levels in cultured podocytes (**Figure 12**).

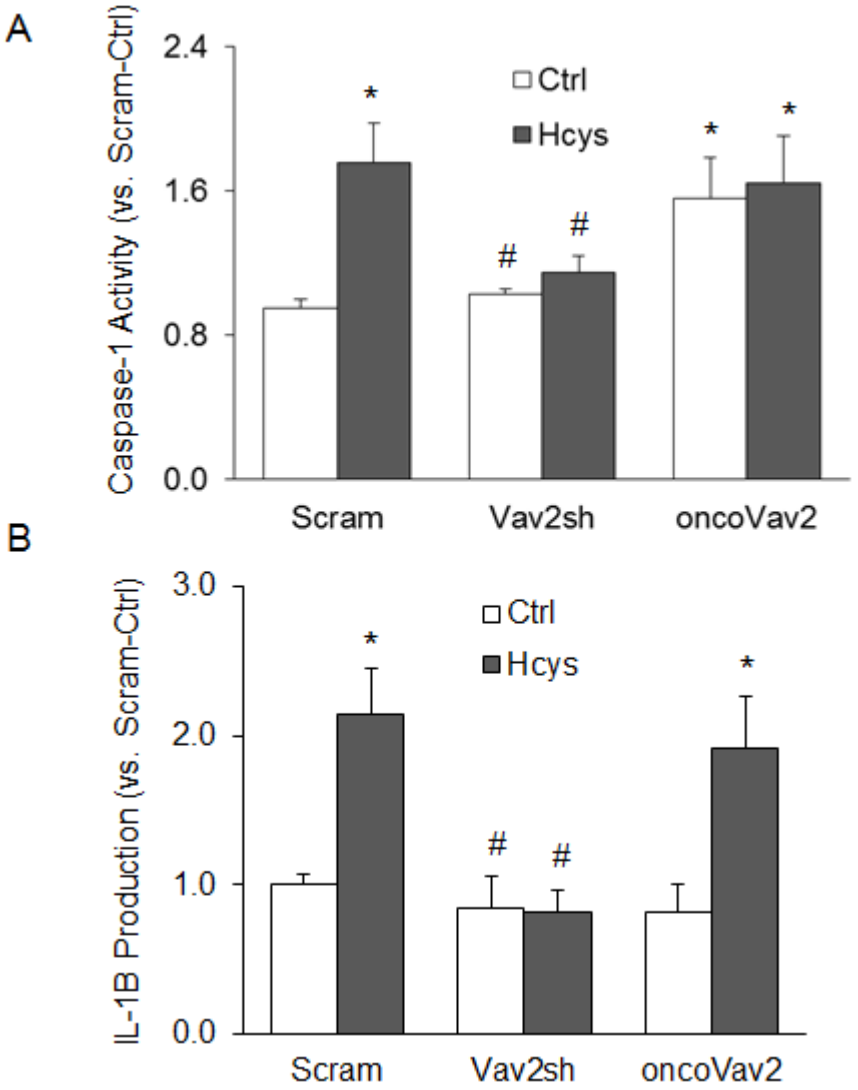

Figure 12. In vitro induction of NLRP3 inflammasome activation in podocytes, independent of Hcys treatment.

A. Caspase-1 activity, shown as fold vs. Ctrl, measured in transfected podocytes treated with Hcys (n=5). B. IL-1β production measured in the supernatant of transfected podocytes treated with Hcys (n=6). Scram: Scramble; Vav2sh: Vav2 shRNA. * $p<0.05$ vs. Ctrl; # $p<0.05$ vs. Hcys.

3.2.7 Inhibition of Vav2 improves podocytes damage

We further assessed the participation of Vav2 in podocyte dysfunction induced by Hcys. It was found that upon stimulation with Hcys, there was decreased staining of the podocyte marker podocin in Hcys-treated and oncoVav2-transfected podocytes, whereas desmin levels increased in these groups, suggesting damage to podocytes (**Figure 13A**). However, Vav2 shRNA reversed Hcys-induced podocyte damage (**Figure 13A**). Additionally, we observed that Hcys treatment and oncoVav2 transfection resulted in significant impairment of podocytes to secrete vascular endothelial growth factor (VEGF), a major product of healthy and mature podocytes (**Figure 13B**). Inhibition of Vav2 protected podocytes from such injury or dysfunction, which was seen by restored levels of VEGF secretion (**Figure 13B**).

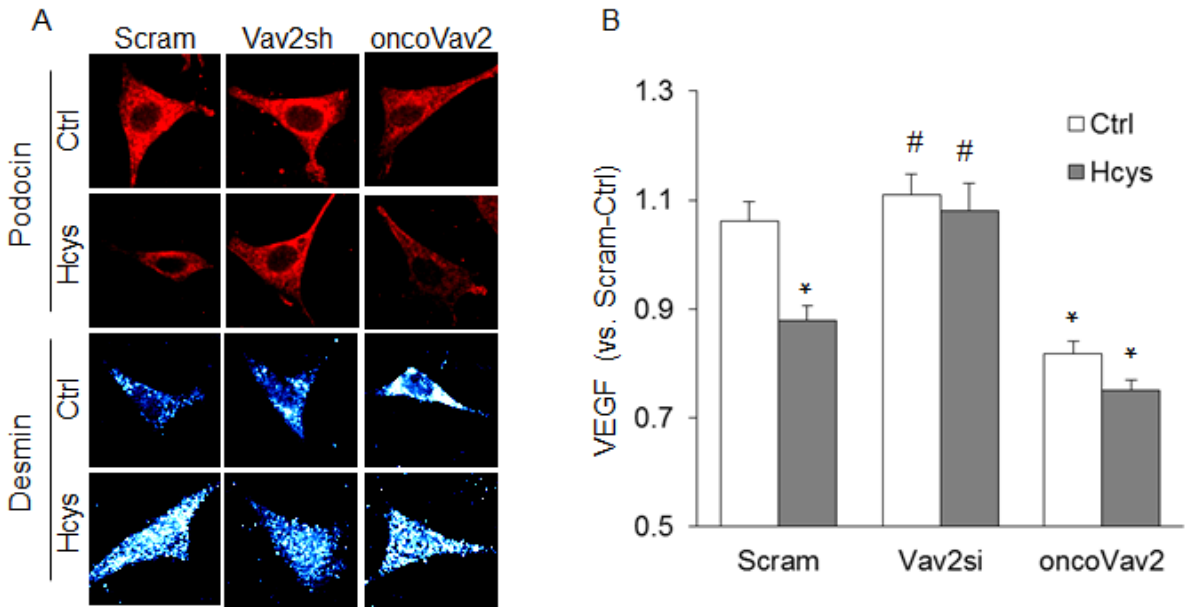

Figure 13. NOX activation by Vav2 overexpression promoted podocyte dysfunction.

A. Podocin (red) and desmin (blue) staining following Hcys treatment (n = 5). B. Measurement of secreted VEGF in cellular supernatant (n=7). Scram: Scramble; Vav2sh: Vav2 shRNA; Vav2si: Vav2 siRNA, * $p<0.05$ vs. Ctrl; # $p<0.05$ vs. Hcys.

3.2.8 Effect of Rac-1 activation on Hcys-induced NOX activity

It is well-defined that activated Rac serves as an anchor protein to regulate NOX activity in many cell types [145–147]. We have shown that Hcys treatment and oncoVav2 transfection resulted in increased Rac-1 activation in cultured podocytes (**Figure 14A**). A significant decrease in Rac-1 activity was observed in podocytes with silenced Vav2 gene, suggesting that more Rac-1 remains in the GDP-bound or inactive state (**Figure 14A**). Coupled with increased GTP-bound Rac-1, we also found that oncoVav2 transfection produced a great increase in $O_2^{\cdot-}$, which was inhibited in the Vav2 shRNA transfected podocytes (**Figure 14B**).

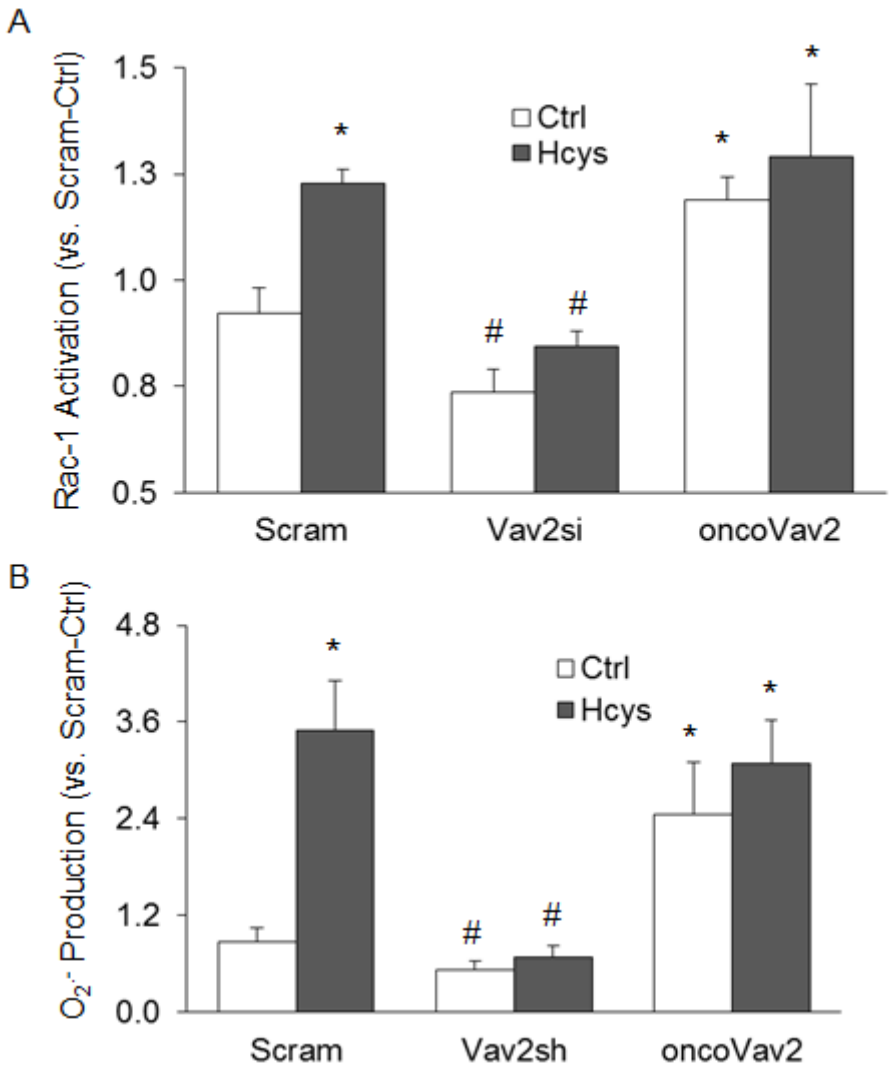

Figure 14. Vav2 blockade prevented in vitro inhibition of NOX-derived $O_2^{\cdot-}$ production.

A. Rac-1 activity (n=6) and $O_2^{\cdot-}$ production (n=5) (B) in Vav2- and oncoVav2-transfected podocytes in the presence or absence of Hcys. * $p<0.05$ vs. Ctrl; # $p<0.05$ vs. Hcys.

3.3 Summary

In summary, the results from these experiments signify that Vav2 is critically involved in Hcys-induced NLRP3 inflammasome formation and activation, which may critically contribute to podocyte dysfunction and glomerular injury. These results demonstrate that Rac-1-NOX activation and subsequent production of $O_2^{\cdot -}$ are main triggering mechanisms of the NLRP3 inflammasome in podocytes, which is consistent with previous reports [56,94]. Vav2 may be a therapeutic target for termination of early events in hyperhomocysteinemic nephropathy and ESRD.

CHAPTER FOUR

Pharmacological targeting of Vav2 to inhibit the NLRP3 inflammasome and glomerular injury during hHcys

4.1 Rationale and Hypothesis

We have established that Vav2 signaling initiates a pathogenic mechanism facilitating hHcys-induced glomerular injury by activation of the NLRP3 inflammasome. This multi-protein complex has been shown to elicit direct damaging insults both inflammatory and non-inflammatory, suggesting that its activation may turn on glomerular inflammation and other cellular damages, contributing to the onset of glomerular injury and ESRD. We have shown from the chapter above that Vav2 is functionally required to initiate the progression of these downstream signaling events, indicating that targeting of Vav2 signaling could potentially serve as an innovative therapeutic to halt glomerular damage associated with hHcys (**Figure 15**). To test this hypothesis, we administered pharmacological interventions of Vav2 signaling to glomerular epithelial cells and $Nlrp3^{-/-}$ mice.

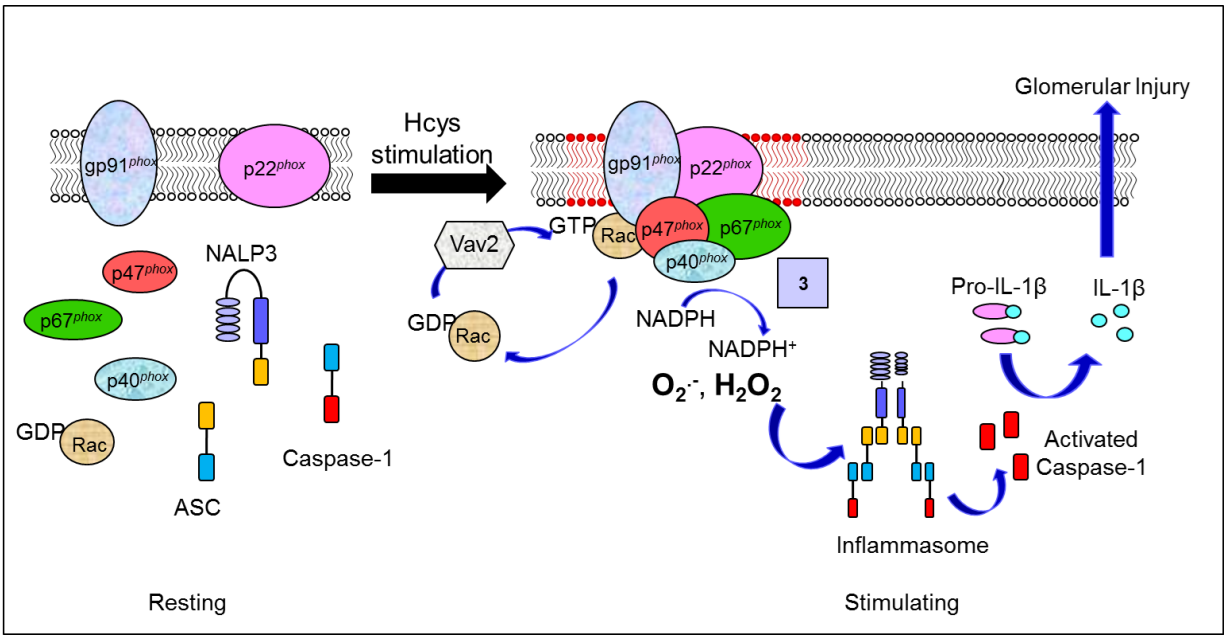

Figure 15. Representative schematic illustrating the goal of Aim 3.

Aim 3 will investigate the targeting of Vav2, pharmacologically to prevent or treat hHcys-induced inflammasome activation and glomerular injury.

4.1 Results

4.2.1 Rac-1 inhibition by NSC23766 ameliorated Hcys-induced inflammasome activation and podocyte injury *in vitro*

To determine whether targeting of Vav2 can prevent excessive NLRP3 inflammasome signaling during Hcys-induced injury, podocytes were treated with either uridine triphosphate (UTP), a Vav2 activator or NSC23766, a selective inhibitor of Rac-1 in the presence or absence of Hcys. As depicted in **Figure 16**, pretreatment of podocytes with UTP and Hcys resulted in a significant elevation of GTP-bound Rac in comparison to untreated cells. However, NSC23766 treatment blocked Hcys-induced Rac GTPase activity. By confocal microscopy, we demonstrated that following Hcys treatment, podocytes increased colocalization of NLRP3 with ASC or caspase-1 in comparison to control cells (**Figures 17A** and **Figure 17B**). Stimulation of podocytes with UTP, mimicked this Hcys-induced inflammasome formation and had no further enhancing effects on Hcys response (**Figures 17A** and **Figure 17B**). Pretreatment with NSC23766, blocked Hcys-induced increases in inflammasome formation as shown by reduced NLRP3 colocalization with ASC or caspase-1 (**Figure 17A** and **Figure 17B**). Using caspase-1 activity and IL-1β production as indicators of NLRP3 inflammasome activation, we found increased activation of caspase-1 in Hcys-treated podocytes, and prior treatment of podocytes with NSC23766 led to attenuated caspase-1 levels (**Figure 18A**). Similar to Hcys, UTP treatment increased the level of cleaved caspase-1 (**Figure 18A**). Elevated production of IL-1β displayed in Hcys- and UTP treated podocytes, signifying inflammasome activation (**Figure 18B**). Inhibition of Rac-1, by pre-incubation of NSC23766 reduced IL-1β production in Hcys-induced podocytes (**Figure 18B**). During glomerular podocyte maturation, VEGF is secreted from the cell and is an essential molecule necessary for podocyte survival and function. We observed in both Hcys- and UTP-

injured podocytes VEGF secretion was significantly reduced in comparison to control cells (**Figure 19**). Additionally, prior treatment with NSC23766 resulted in increased secretion of VEGF, indicating preserved podocyte function (**Figure 19**).

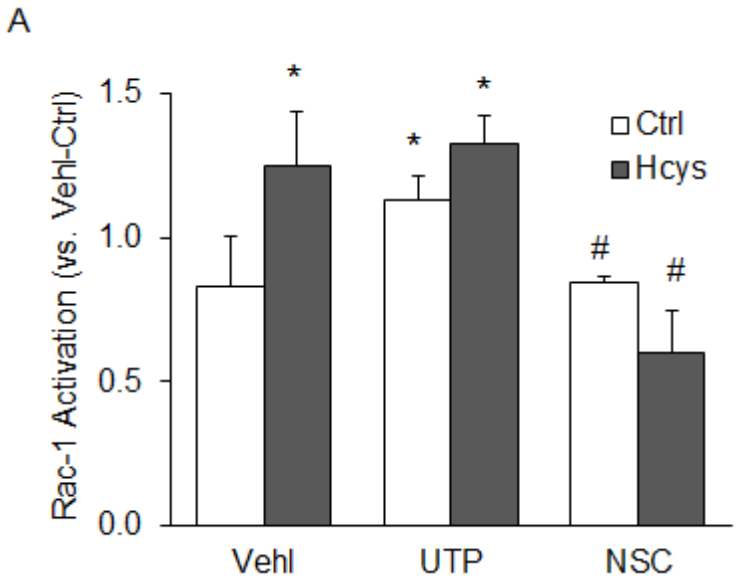

Figure 16. NSC23766 attenuated Hcys-induced Rac-1 activation.

A. Rac-1 activity in UTP-and NSC23766-treated podocytes (n=3).Vehl: Vehicle and NSC: NSC23766, * $p<0.05$ vs. Ctrl; # $p<0.05$ vs. Hcys.

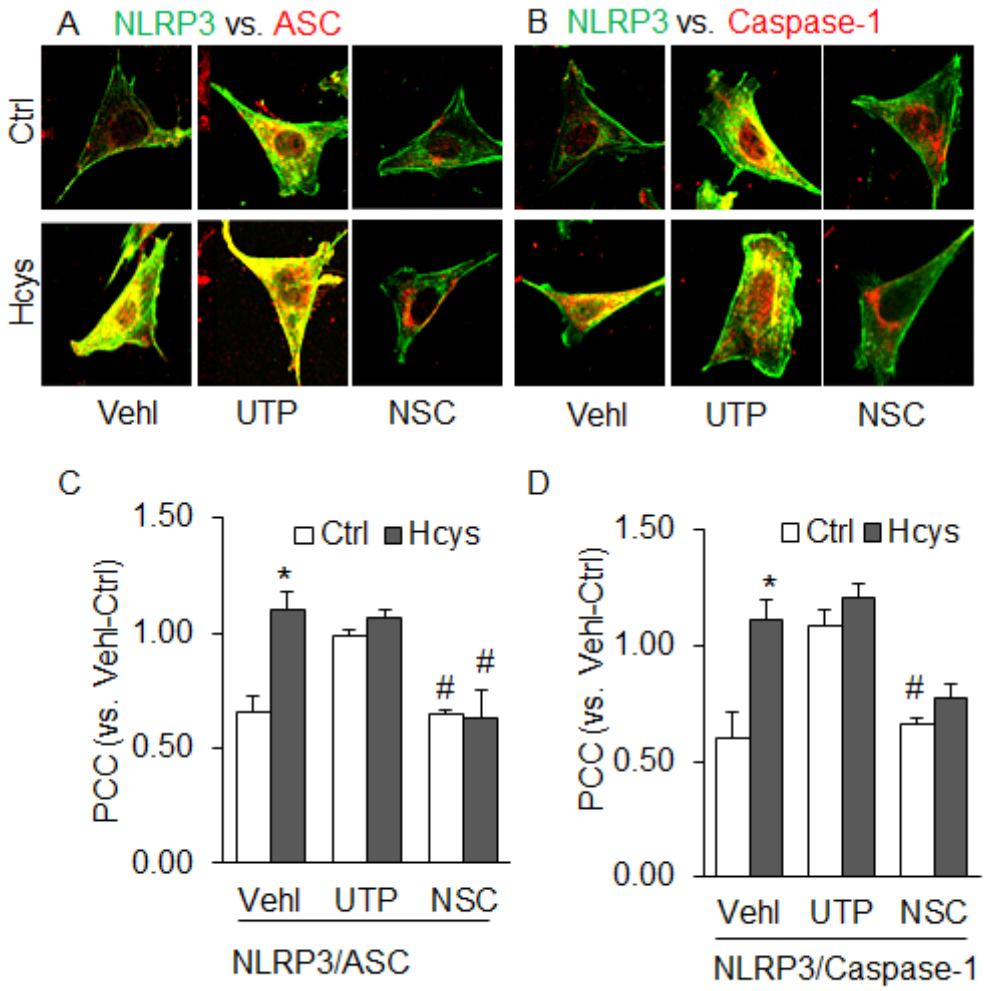

Figure 17. Rac-1 Inhibition prevented Hcys-induced NLRP3 inflammasome formation.

Confocal microscopy analysis of NLRP3 (green) with ASC (red) (A) and NLRP3 (green) with caspase-1 (red) (B) in treated podocytes. C-D. Summarized data showing the quantification of colocalization of inflammasome components (n=3). Vehl: Vehicle and NSC: NSC23766, * $p<0.05$ vs. Ctrl; # $p<0.05$ vs. Hcys.

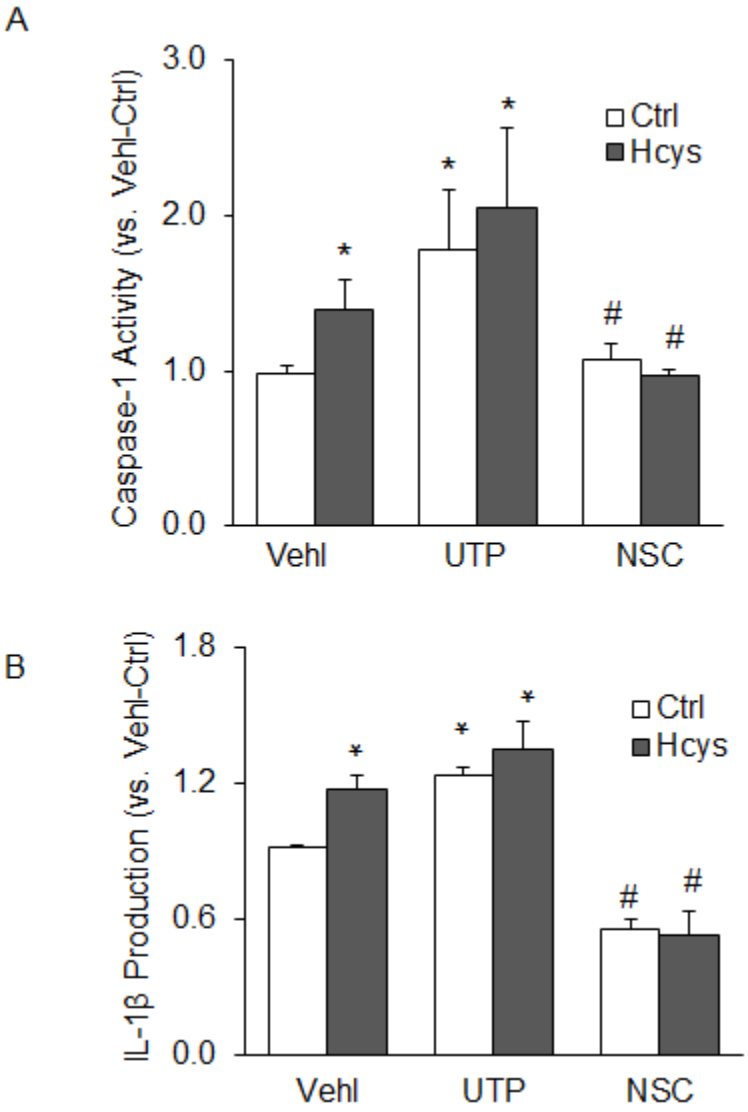

Figure 18. Inhibition of Vav2 attenuated Hcys-induced NLRP3 inflammasome activation.

Effect of inhibition of Vav2 on caspase-1 activity (A) and IL-1β production (B) induced by Hcys in podocytes treated with UTP or NSC23766 (n=6). Vehl: Vehicle and NSC: NSC23766, * $p<0.05$ vs. Ctrl; # $p<0.05$ vs. Hcys.

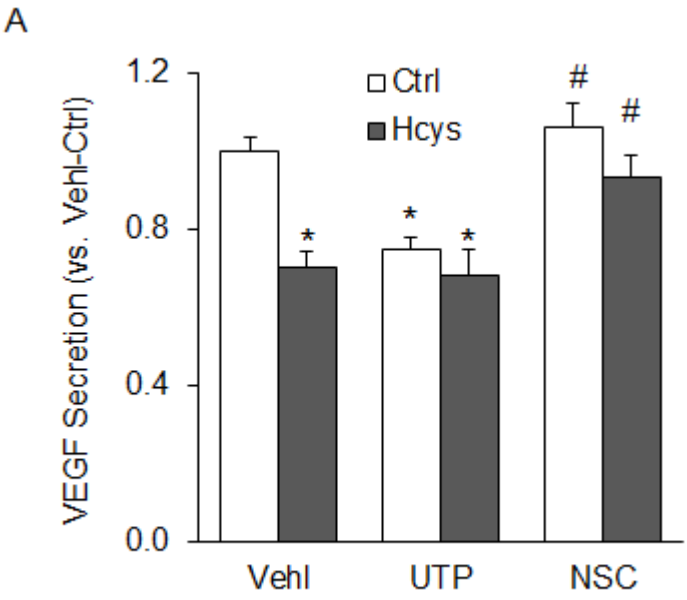

Figure 19. Blockade of Vav2 signaling maintained podocyte function.

A. Measurement of VEGF secretion as a podocyte function parameter was detected in the supernatant of treated podocytes (n=6). Vehl: Vehicle and NSC: NSC23766, * $p<0.05$ vs. Ctrl; # $p<0.05$ vs. Hcys.

4.2.2 Mouse *Nlrp3* gene deletion and Vav2 inhibition prevented hHcys-induced NLRP3 inflammasome formation and activation in glomeruli

To further determine whether targeting of Vav2 signaling attenuates hHcys-induced NLRP3 inflammasome formation and activation *in vivo*, *Nlrp3*$^{-/-}$ (KO) and *Nlrp3*$^{+/+}$ (WT) mice were administered UTP or NSC23766, intraperitoneally and fed either a ND or FF diet for 4 weeks. Approximation of plasma Hcys concentration was evaluated by HPLC. In both KO and WT mice consuming the FF diet significantly increased total Hcys levels in comparison to their ND-fed littermates (**Figure 20**). Neither gene deletion nor treatment with UTP or NSC23766 altered Hcys levels. As shown in **Figures 21**, hHcys *Nlrp3*$^{+/+}$ mice as well as those mice administered UTP displayed increased inflammasome formation seen by colocalization of inflammasome components, NLRP3 with ASC and NLRP3 with caspase-1 as well as increased IL-1β production in the glomeruli, indicating inflammasome activation (**Figure 22**). However, this formation and activation was inhibited in hHcys Nlrp3$^{-/-}$ mice and mice receiving NSC injections (**Figure 21** and **Figure 22**). The summarized data of quantitative colocalization of NLRP3 with ASC or with caspase-1 in glomeruli of mice were shown in **Figures 21C** and **21D**.

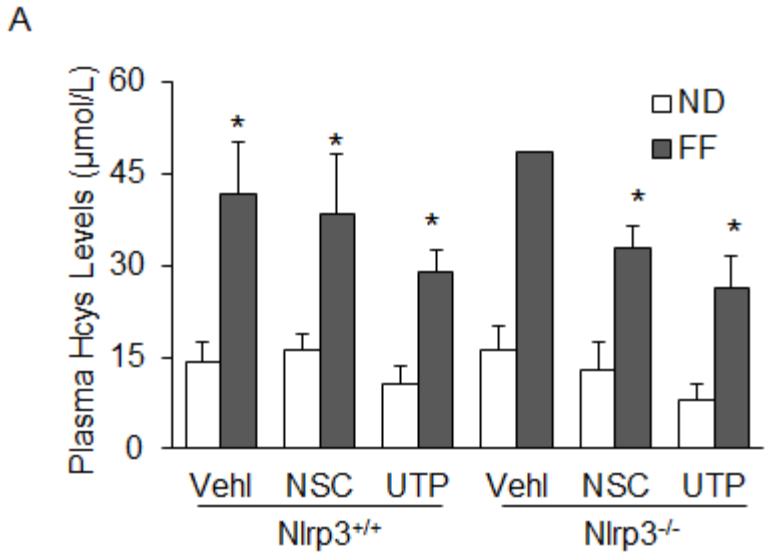

Figure 20. Effects of gene knockout or *in vivo* UTP and NSC23766 administration on FF diet-induced Hcys concentration.

A. Plasma Hcys levels measured by HPLC in *Nlrp3*$^{-/-}$ and *Nlrp3*$^{+/+}$ mice (n=1-7). Vehl: Vehicle, NSC: NSC23766, ND: Normal Diet and FF: Folate-Free Diet. * $p<0.05$ vs. Vehl on ND.

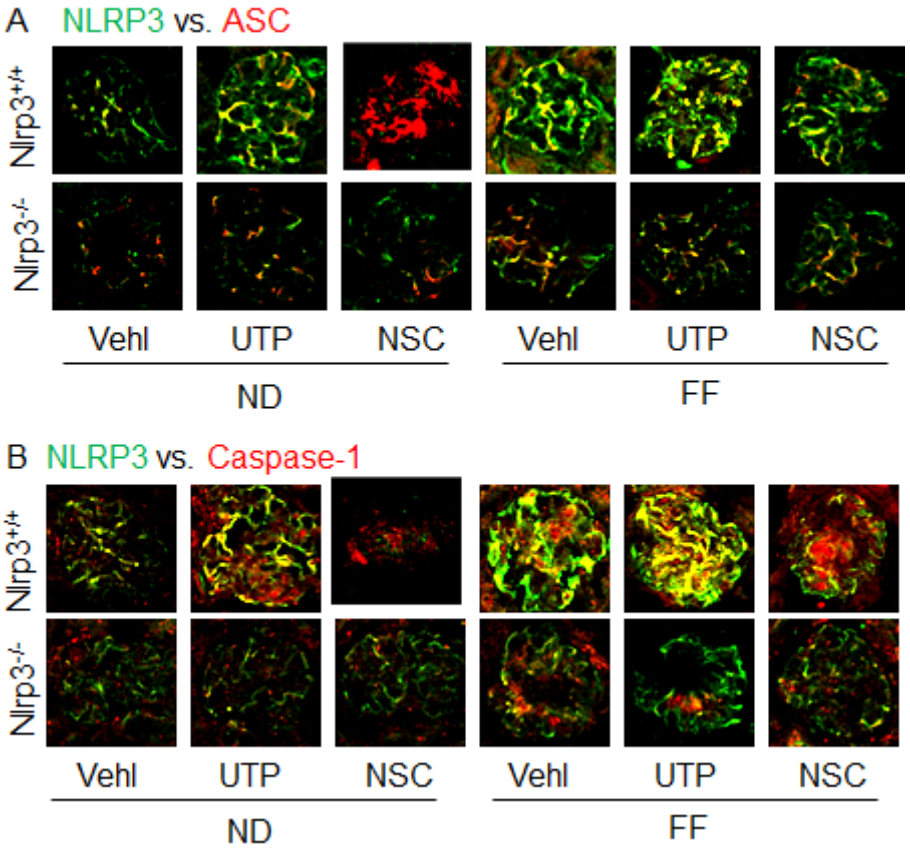

Figure 21. HHcys and UTP administration induces NLRP3 inflammasome formation.

Colocalization of NLRP3 (green) with ASC and NLRP3 with caspase-1 (red) in mouse glomeruli following UTP or NSC23766 administration in FF- or ND-fed mice (n=3). Vehl: Vehicle, NSC: NSC23766, ND: Normal Diet and FF: Folate-Free Diet. * $p<0.05$ vs. Scram on ND. * $p<0.05$ vs. Vehl on ND; # $p<0.05$ vs. Vehl on the FF diet.

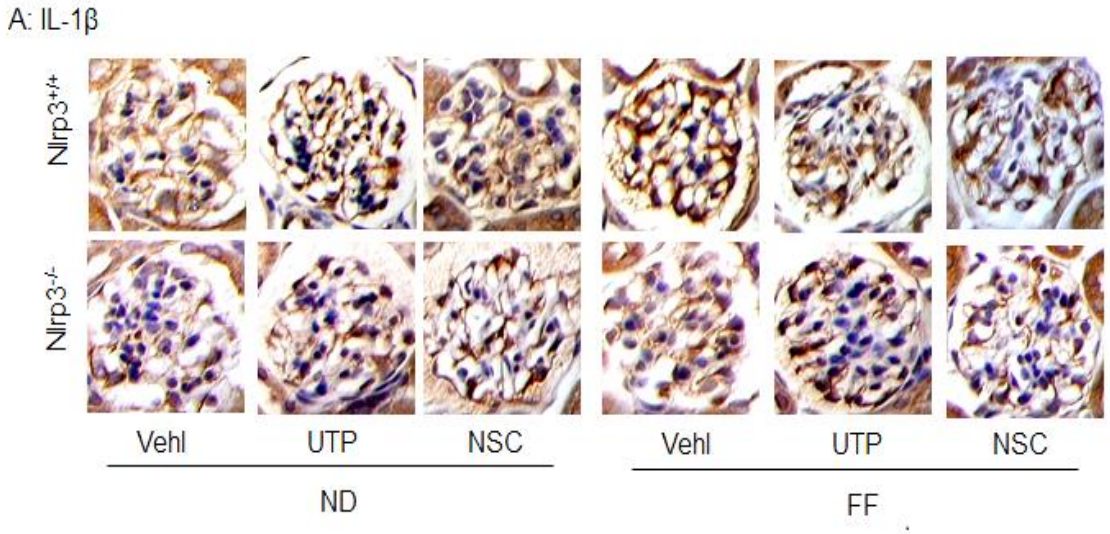

Figure 22. *In vivo* administration of NSC23766 attenuated NLRP3 inflammasome activation.

A. Immunohistochemical staining of IL-1β in glomerular podocytes (n=2). Vehl: Vehicle, NSC: NSC23766, ND: Normal Diet and FF: Folate-Free Diet. * $p<0.05$ vs. Scram on ND. * $p<0.05$ vs. Vehl on ND; # $p<0.05$ vs. Vehl on the FF diet.

4.2.3 Administration of NSC23766 protected glomerular podocytes from hHcys-induced dysfunction and injury

To assess whether inflammasome-mediated glomerular damage is a consequence associated with podocyte dysfunction we determined the expression levels of podocyte specific markers, podocin and desmin by immunofluorescence analysis. Our results displayed that in both hHcys Nlrp3$^{+/+}$ mice and UTP-treated mice there was dramatic decreases in podocin staining (**Figure 23A**). Rac-1 inhibition by NSC23766 administration resulted in ameliorated hHcys-induced glomerular damage shown by restoration of podocin expression levels (**Figure 23A**). Moreover, Nlrp3$^{+/+}$ UTP treated mice exhibited a high abundance of desmin expression in glomeruli of both ND and FF-fed groups, whereas NSC23766 administration protected against hHcys-induced increases in glomerular desmin in Nlrp3$^{+/+}$ mice, with similar expression levels as the Nlrp3$^{+/+}$ mice on a ND (**Figure 23B**).

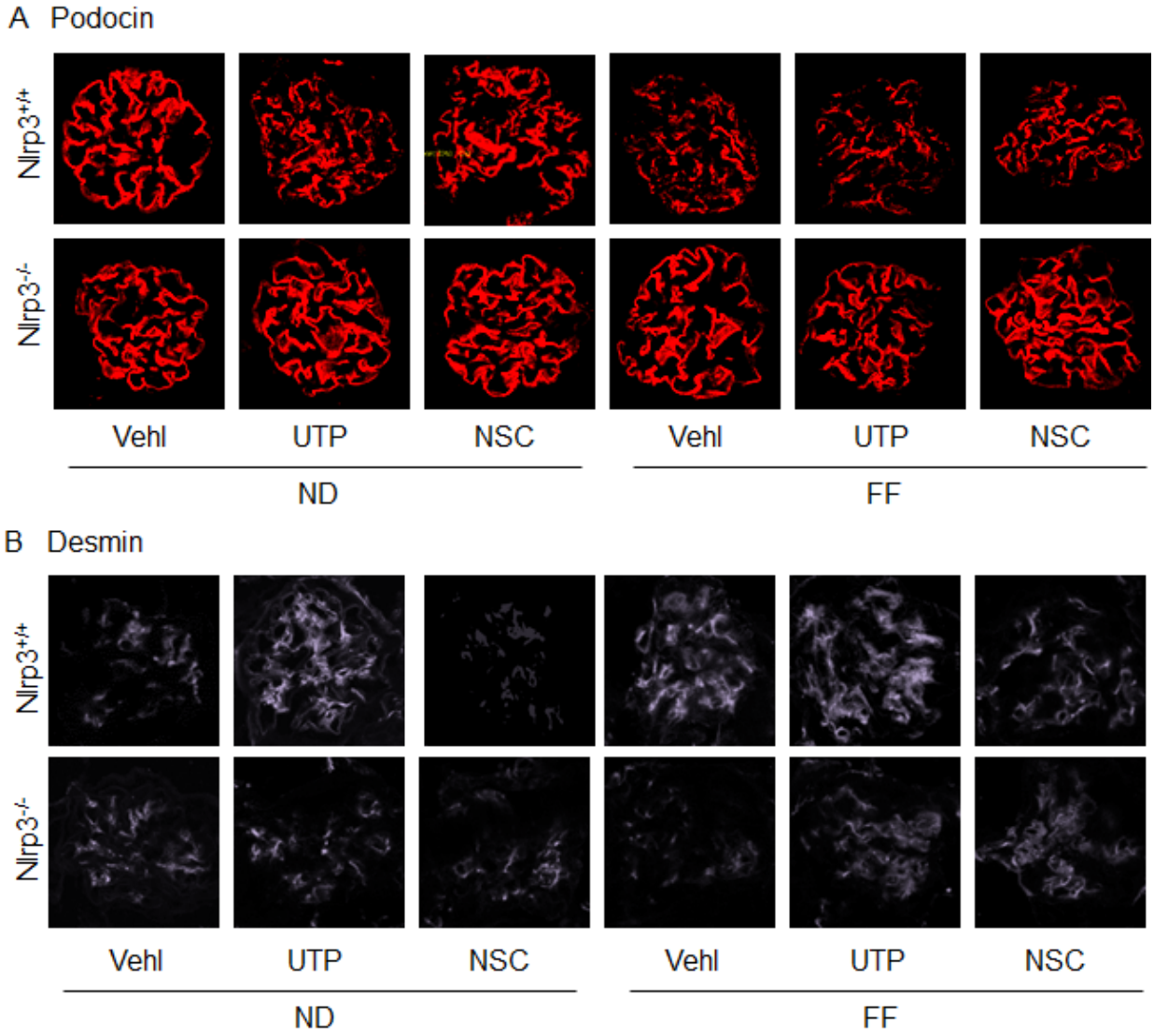

Figure 23. NSC23766 protected glomerular podocytes from dysfunction.

Immunofluorescent staining of podocin (A) and desmin (B), podocyte-specific markers (n=3). Vehl: Vehicle, NSC: NSC23766, ND: Normal Diet and FF: Folate-Free Diet. * $p<0.05$ vs. Scram on ND. * $p<0.05$ vs. Vehl on ND; # $p<0.05$ vs. Vehl on the FF diet.

4.3 Summary

Taken together, our results demonstrate that blockade of Vav2 exchange activity diminished NLRP3 inflammasome formation and activation *in vitro* and *in vivo*. Additionally, inhibition of the NLRP3 inflammasome protected podocytes from the early adverse effects of increased Hcys, demonstrating that tight regulation of the NLRP3 inflammasome by intervention of Vav2 activation could prevent unwarranted damage and excessive inflammation seen in hHcys-associated glomerular injury.

CHAPTER FIVE

DISCUSSION

5.1 Activation of NADPH oxidase by Vav2 overexpression is sufficient to activate the NLRP3 inflammasome in glomerular podocytes, independent of hHcys

The primary goal of this study was to reveal the involvement of the GNEF, Vav2 in activation of the NLRP3 inflammasome during hHcys. Our results showed that Vav2 inhibition by RNA interference attenuated hHcys-induced NLRP3 inflammasome formation and activation in podocytes. Conversely, overexpression of Vav2 resulted in enhanced inflammasome formation and activation in these cells independent of Hcys treatment. Results from the present study also demonstrated that Vav2 inhibition protected podocytes against Hcys-induced glomerular injury. This Vav2-mediated action was associated with NOX activation and increased ROS production in glomeruli.

Distinctive GNEFs facilitate the conversion of GDP for GTP, inciting Rho protein activation [148]. Among more than 100 GNEFs, the Vav subfamily is highly selective in coordinating intracellular signal transduction pathways associated with NOX activation [141,149]. Our earlier studies have demonstrated that of 3 Vav isoforms, both Vav2 and Vav3 are localized in the glomeruli, whereas it is already established in other reports that Vav1 is solely present in hematopoietic cells [48]. We have shown that by sensing intracellular ROS during hHcys, thioredoxin-interacting protein (TXNIP) associates with NLRP3 to trigger the cascade of downstream events leading to inflammasome activation [56]. Recently, evidence links Vav2 as a binding partner of TXNIP [150]. Therefore, we sought to identify whether the detrimental actions of Vav2 are involved in NLRP3 inflammasome assembling, activation and subsequent podocyte

dysfunction. It has been reported that internal or external danger signals are capable of initiating the recruitment of ASC and caspase-1 forming the NLRP3 inflammasome complex [2,50]. This inflammasome complex triggers the production of IL-1β and IL-18, inducing a heightened inflammatory response. Processing of not only the interleukins, but also caspase-1 is considered crucial in the inflammatory process that leads to tissue injury, organ or systemic diseases [96]. In particular, NLRP3 inflammasome activation has been implicated in the pathogenesis of various renal diseases including acute kidney injury, chronic kidney disease, diabetic nephropathy, crystal related nephropathy and hyperhomocysteinemic nephropathy [3,151,152]. Renal cells such as podocytes have been documented as the main enriched sites for maturation of glomerular IL-1β production [43,107].

To our knowledge, we are the first to report that silencing the Vav2 gene and reduction of its activity using genetic manipulations inhibited the NLRP3 inflammasome by preventing the activation of the small GTPase, Rac-1. Consistent in both our *in vivo* and *in vitro* models, our results show that Vav2 shRNA interventions decreased the interactions of inflammasome components NLRP3, ASC and caspase-1 (Figures 5 and 11). Consequently, the absence of inflammasome machinery resulted in decreased IL-1β production in mouse glomeruli (Figure 6) which was associated with diminished caspase-1 activity in podocytes (Figure 12). The most remarkable finding in the present study was that Vav2 overexpression triggered NLRP3 inflammasome formation irrespective of Hcys exposure (Figure 5 and 11), suggesting that Vav2 is crucial in bridging the redox signals derived from NOX activity and NLRP3 inflammasome activation, which may be a critical mechanism responsible for glomerular inflammatory response during hHcys.

There is evidence that abundance of protein leaked in the urine and excessive reabsorption of protein in renal proximal tubular cells are linked to the pathophysiological states of renal disease development [153,154]. In this regard, Connell et al. demonstrated that in response to albumin overloads, proximal tubule cells encounter direct toxic effects associated with Rac GTPase and NOX activation as well as receptor-mediated endocytosis [155]. Our results add to this by showing that mice with overexpressed Vav2 had typical features of glomerular damage as evidenced by proteinuria, albuminuria and increased GDI (Figure 7). Downregulation of the Vav2 gene effectively ameliorated these detrimental effects in mouse glomeruli. With respect to the mechanism of glomerular injury during different pathological conditions such as hHcys, podocyte injury is considered to play an important role. It has been reported that disruption of the slit diaphragm, actin rearrangement and foot process effacement are unique indicators of podocyte injury [143,156]. Our data revealed that Hcys treatment and Vav2 overexpression decreased a podocyte-specific molecule, podocin (Figure 8 and 13), which may result in derangement of the slit diaphragm structure and related cell signaling pathways [156]. Furthermore, resultant injury to podocytes may lead to the upregulation of the intermediate filament, desmin, which was indeed confirmed in our studies as shown by increased desmin levels in podocytes during hHcys or exposure to high levels of extracellular Hcys. Interestingly, this desmin increase in podocytes during hHcys could be blocked by shRNA of Vav2, but mimicked by oncoVav2 transfection. The results suggest that Vav2 critically contributes to hHcys-induced podocyte injury and glomerular sclerosis.

To explore the mechanisms by which Vav2 activates the NLRP3 inflammasome and thereby lead to podocyte injury and glomerular sclerosis, we examined the effects of Vav2 interventions on Rac activity and consequent NOX activation. It is well known that instigation of NOX as a

functional signaling complex is dependent on the translocation of its cytosolic subunits ($p47^{phox}$ and $p67^{phox}$) to the membrane in which this platform initiates the generation of superoxide ($O_2^{\cdot -}$) [25,157]. The Rac protein is crucial in these migratory events, serving as a binary switch that cycles between active GTP-bound and inactive GDP-bound states. However, the molecular cues directly responsible for Rac protein activation and subsequent NOX activity following prolonged exposure to Hcys remained unclear. From our findings, we confirm that expression of constitutively active Vav2, can itself drive the activation of the Rac-1 protein (Figure 14A) and the aggregation of a functional NOX complex, which produces $O_2^{\cdot -}$ even without Hcys stimulus (Figure 14B). Moreover, blockade of Vav2 attenuated enhancement of Rac activity and $O_2^{\cdot -}$ generation, even in the presence of Hcys, a known activator of ROS. Results from these experiments illustrate that Vav2 can promote injurious consequences in podocytes and that inhibition of Vav2 function may terminate the development and progression of Hcys-induced podocyte injury and consequent glomerular sclerosis.

5.2 Inhibition of Vav2 signaling by NSC23766 ameliorated hhHcys-induced glomerular dysfunction

In the present study, we clearly demonstrated that inhibition of Vav2 exchange activity by administration of NSC23766 prevented upregulation of Rac-1 GTPase production in Hcys-treated podocytes, resulting in attenuated NLRP3 inflammasome formation, inhibition of caspase-1 activation and a subsequent reduction in IL-1β processing and release. Additionally, we showed that administration of NSC23766 *in vivo* attenuated excessive hHcys-induced inflammasome activation and glomerular dysfunction, thus highlighting the significance of pharmacologically targeting Vav2 activity.

ESRD is regarded as one of the world's most prevalent chronic disorders [7,154]. Although many therapeutic approaches focus on slowing the progression of renal failure, alleviating the severity of symptoms, management of risk factors and prolonging life remain a large challenge clinically given that many patients in the early stages of CKD are asymptomatic. Therefore, early diagnosis and effective interventions are crucial in management and amelioration of kidney injury. We have found that the underlying mechanism associated with Hcys-induced podocyte dysfunction and injury is dependent on the activation of Vav2. Expression of Vav2 in podocytes promotes the activation of Rac-1 which leads to the aggregation of the multi-protein NOX complex, promoting $O_2^{\cdot -}$ production, inflammasome activation and initiation of glomerular injury. Progression of these downstream signaling events are primary contributing factors associated with hHcys-induced nephropathy. It is clear that novel therapies to target Vav2 and the inflammasome may be an important therapeutic strategy to lessen the extent of glomerular injury.

In our previous studies, we revealed that the Nlrp3 gene is a critical component necessary for assembling and corresponding injury during hHcys [158]. Furthermore, we reported that chronic elevations of Hcys contribute to the development of glomerular disease independent of hypertension [159]. In previous studies, *Nlrp3* deficient mice have been reported to have cardio-protective effects during myocardial ischemia [160] as well as protection against pancreatic damage associated with diet-induced obesity [161]. Utilization of these mice in our study demonstrated remarkable protection against hHcys-induced inflammasome activation and glomerular injury. Additionally, using a Rac-1 selective inhibitor in $Nlrp3^{+/+}$ mice proved to exhibit reno-protective effects against hHcys-induced alterations in the glomeruli. We found that these mice had attenuated inflammasome formation (Figure 21), enhanced podocin expression (Figure 23) and a downregulation of the injury marker, desmin (Figure 23). *In vitro*, following pretreatment of

glomerular podocytes with Rac-1 inhibitor, NSC23766 abolished Hcys-induced inflammasome formation (Figure 17) and activation (Figure 18). Through biochemical analysis we also observed protective effects associated with increased VEGF secretion in this group of podocytes (Figure 19). Taken together, these results confirm that the Nlrp3 gene mediates hHcys-induced inflammasome formation and activation, but most significantly these findings demonstrate that pharmacological modification of Vav2 exchange activity abolished these pathological events associated with increased Hcys.

5.3 Significance and perspectives

There is indeed increasing evidence for an association between glomerular diseases and NLRP3 inflammasome activation, which leads to caspase-1-mediated IL-1β/IL-18 production. More knowledge is needed in this area to further assess the underlying relationship between the NLRP3 inflammasome activation and decline of glomerular function in various chronic kidney diseases, in particular, to define the temperospatial contribution of the activated inflammasomes to the onset or development of glomerular disease and ultimate ESRD. Moreover, recognition and clarification of the non-canonical effects of NLRP3 inflammasome activation in glomeruli as well as the alternative pathways to activate this inflammasome may be particularly interesting and important, because this non-canonical action and alternative inflammasome activating pathways may result in a combination of injurious actions independent of typical inflammation. Possible direct damage to glomerular cells, interference with synthesis of cell-specific proteins, enhanced cell membrane permeability and cell pyroptosis may importantly contribute to glomerular injury during NLRP3 inflammasome activation in response to pathological stimuli. These direct effects of NLRP3 inflammasome activation may suggest a new therapeutic strategy for treatment and

prevention of glomerular sclerosis and ESRD, namely, targeting the NLRP3 inflammasome-mediated uncanonical mechanism responsible for glomerular injury. This therapeutic strategy may be more efficient for treatment or prevention of progressive chronic glomerular injury or sclerosis compared with the approaches that just target the inflammatory pathways.

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VITA

Sabena Michelle Conley is a native of Fayetteville, NC. She was born September 13, 1989, the youngest of two daughters to her parents, Michael and Juanita Conley. Sabena is a loving and proud aunt of Darius and Amyiah Smith. Sabena attended schools in the Cumberland County School District. During her middle and high school years Sabena expressed a love of music. She played the flute and performed in concerts and marched with the band in competitions and during seasonal holidays. In her junior and senior years of high school, she participated in the Cape Fear Valley Medical Center Junior Volunteer Auxiliary Program. Her exposure to the hospital environment peaked her interest in the biomedical field. She has received many honors and monetary awards toward her academic endeavors.

In 2007, she began her college career entering Fayetteville State University in Fayetteville, NC. While working on her undergraduate studies, in 2010 she was accepted into the Ronald E. McNair Postbaccalaureate Achievement Program. This program aims to increase the number of underrepresented minorities entering into graduate school. During the program she conducted research, attended scientific conferences and met with prominent scientists. In 2011, she graduated with a Bachelor's degree in Biology, with honors. In the fall of 2011, Sabena enrolled in the MS Biology program at North Carolina Agricultural & Technical State University. She worked as a laboratory instructor for an undergraduate-level course (BIOL 100), where she provided support for the course lecturer. Sabena's thesis research was supported by a Basic Immune Mechanisms Training Grant from the National Institutes of Health.

After successful completion of her Master's degree, Sabena enrolled in Fall of 2013 at Virginia Commonwealth University in the Biomedical Sciences Doctoral Portal Program.

Sabena Michelle Conley

Education

Virginia Commonwealth University, Richmond, VA 2013-Present
Doctorate of Philosophy in *Pharmacology* and *Toxicology*
• One of the top 10 Pre-Doctoral finalists in APS Renal Section Excellence in Renal Research
North Carolina Agricultural and Technical State University, Greensboro, NC 2011-2013
Master of Science in *Biology*
• Basic Immune Mechanisms Training Grant Pre-Doctoral Recipient
Fayetteville State University, Fayetteville, NC 2007-2011
Bachelor of Science in *Biology*, with honors
• 2010 Bronco McNair Scholar
• First place in FSU Innovative Curriculum Approach for Mathematics and Science (FICAMS) poster presentation competition
• First place in FSU McNair Program and OpTIMUM Program Summer Research Internship Closing Ceremony oral presentation competition

Research Experience

Doctoral Dissertation, Virginia Commonwealth University, Richmond, VA 2014-Present
Adviser: Dr. Pin-Lan Li
Role of Vav2 in Podocyte Inflammasome Activation and Glomerular Injury during Hyperhomocysteinemia
• Determining the early molecular mechanisms leading to the development of end-stage renal disease associated with hyperhomocysteinemia.
Master's Thesis, North Carolina Agricultural and Technical State University, Greensboro, NC 2011-2013
(Research Adviser: Dr. Elimelda M. Ongeri
Role of Meprins in the Pathogenesis of Diabetic Nephropathy
• Studied diabetic nephropathy in meprin deficient mice.
• Investigated the role of hypoxia inducible factors in acute kidney injury.
Undergraduate Ronald E. McNair Scholar, Fayetteville State University, Fayetteville, NC Summer 2010
Adviser: Dr. Shirley Chao
Impact of *Pseudomonas aerguinosa* on Reducing Toxicity of Diazinon in the Earthworm (*Lumbricus terrestris*) and Mealworm (*Tenebrio molitor*)
• Investigated the toxicity of Diazinon in the environment of soil-dwelling organisms. Observed how bioremediation reduces the effects of Diazinon on acetylcholinesterase in the nervous system.
FICAMS Undergraduate Researcher, Fayetteville State University, Fayetteville, NC 2008- 2009
Adviser: Dr. Stephen Salek
A Comparison of the Effectiveness of Traditional and Computer Simulated Biology Laboratory Exercises
• Used two methods of instruction to teach undergraduate non-biology majors about the anatomy and physiology of the squid (*Loligo pealei*)
• Formatted a short lab practical using a dissected specimen.

Teaching Experience

Teaching Assistant, Biological Science Laboratory North Carolina Agricultural and Technical State University, Fall 2011
• Taught laboratory section and provided support for faculty member in charge.

Leadership Experience

Secretary, Virginia Commonwealth University Pharmacology and Toxicology Student Officer 2015-2016
• Responsibilities included maintaining detailed records of meetings and upcoming club events.
Graduate Student Mentor, Initiative for Maximizing Student Diversity Virginia Commonwealth University 2014-Present
• Advised incoming undergraduate and graduate students about graduate school and VCU campus.
Community Outreach, Sunshine Committee Sandy Lane Church of Christ 2015-Present
• Served Richmond and surrounding area residents to provide personal care to the sick and support for bereaved families.
Shift Leader, Quiznos Sub 2006-2011
• Directed team of employees in daily operations during night shift and interacted with customers to ensure a satisfactory experience.
• Assisted in food prepping and marketed new promotions.
• Maintained a clean work environment.
•Trained newly hired personnel.

Technical Skills

• Maintenance of cell lines
• Gene manipulation by RNAinteference or nucleofection
• Protein purification by size exclusion chromatography
• Immunoprecipitation of complexes for identification by Western blot analysis
• Detection of proteins by Immunohistochemistry and biochemical assays such as ELISA
• Handling of mice
• Fluorescence and Confocal Microscopy imaging

Research Publications

Conley SM, Abais JM, Boini KM and Li PL. Inflammasome Activation in Chronic Glomerular Diseases. Current Drug Targets. Accepted May 2016

Martin BL, **Conley SM**, Harris RS, Stanley CD, Niyitegeka JM and Ongeri EM. Hypoxia Associated Proteolytic Processing of OS-9 by the Metalloproteinase Meprin β. Int J Nephrol. 2016: 2851803, 2016. PMCID: PMC4961814

Zhu Q, Li XX, Wang W, Hu J, Li PL, **Conley SM** and Li N. Mesenchymal stem cell transplantation inhibited high salt-induced activation of the NLRP3 inflammasome in the renal medulla in Dahl S rats. Am J Physiol Renal Physiol. 310: F621-F627, 2016. PMCID: PMC4824142

Xia M, **Conley SM**, Li G, Li PL and Boini KM. Inhibition of hyperhomocysteinemia-induced Inflammasome activation and glomerular sclerosis by NLRP3 gene deletion. Cell Physiol and Biochem 34: 829-841, 2014. PMCID: PMC4864609

Abais JM, Xia M, Li G, Chen Y, **Conley SM**, Gehr TW, Boini KM and Li PL. Nod-like receptor protein 3 (NLRP3) Inflammasome activation and podocytes injury via thioredoxin-interacting protein (TXNIP) during hyperhomocysteinemia. J Bio Chem. 289: 27159-27168, 2014. PMCID: PMC4175351

Professional Presentations

Conley S and Li N, Possible Role of the Renal Medullary Inflammasome in Salt Sensitivity Hypertension. Presented November 18, 2013 at the First Laboratory Rotation Research Colloquium, Virginia Commonwealth University, Richmond, VA.

Selected Conference Presentations

Conley SM, Chen Z, Li G, Xia M, Boini KM, Gehr TW and Li PL, Role of Vav2 in Podocyte Inflammasome Activation and Glomerular Injury during Hyperhomocysteinemia. Experimental Biology 2016, San Diego, CA.

Chen Z, **Conley SM**, Li G, Xia M, Gehr TW, Boini KM and Li PL, NLRP3 Inflammasome as a Novel Target for Docosahexaenoic Acid and Its Metabolites to Abrogate Glomerular Injury during Hyperhomocysteinemia. Experimental Biology 2016, San Diego, CA

Conley S, Han J, Hurley S and Ongeri EM, Meprin Deficient Mice Have a More Severe Form of Diabetic Nephropathy. Experimental Biology 2013, Boston, MA.

Conley S, Martin B and Ongeri EM, Meprins Cleave OS-9 Present in Mouse Kidneys Subjected to Ischemia Reperfusion Acute Kidney Injury. Experimental Biology 2013, Boston, MA.

Conley S and Chao S, Impact of *Pseudomonas aerguinosa* on Reducing Toxicity of Diazinon in the Mealworm (*Tenebrio molitor*). Presented November 20, 2010 at the State of North Carolina Undergraduate and Creativity Symposium (SNCURCS), Raleigh, NC.

Conley S and Chao S, Impact of *Pseudomonas aerguinosa* on Reducing Toxicity of Diazinon in the Mealworm (*Tenebrio molitor*). Presented November 11, 2010 at ABRCMS (Annual Biomedical Research Conference for Minority Students), Charlotte, NC.