Investigating the role of coiled-coil domain containing protein 3 (CCDC3), a novel secreted protein, in endothelial inflammation

by

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Abstract

Endothelial inflammation is critical in the initiation and progression of atherosclerosis and other cardiovascular diseases (CVDs). Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine that induces endothelial inflammation via activation of nuclear factor κB (NF-κB) signaling. Coiled-coil domain containing 3 (CCDC3) is a newly identified secretory protein mainly expressed in endothelial cells (ECs) and in adipose tissues. However, the function of CCDC3 in ECs is unclear. A published paper showed that TNF-α downregulates CCDC3 expression in ECs. We therefore investigated the role of CCDC3 in TNF-α-induced inflammatory response in ECs.

In response to inflammation, ECs express adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1) that recruit leukocytes to the sites of infection or injury. In our study we found that stable overexpression of CCDC3 decreased, while stable knockdown of CCDC3 increased TNF-α-induced expression of VCAM-1 at the mRNA and protein levels in ECs. Mechanistically, stable overexpression of CCDC3 decreased TNF-α-induced p65 and p50 nuclear translocation and nuclear NF-κB activity, suggesting that CCDC3 attenuates TNF-α-induced gene expression by inhibiting NF-κB signaling in ECs. Importantly, we found that CCDC3 in the conditioned medium (CM) as well as the purified CCDC3 decreased TNF-α-induced expression of VCAM-1 in

receiving ECs, suggesting that CCDC3 has a paracrine/autocrine function. Interestingly, CCDC3 in CM can enter the receiving ECs. Taken together, our work demonstrates that CCDC3 represses TNF-α/NF-κB-induced pro-inflammatory response in ECs, suggesting a potential anti-inflammatory and atheroprotective role of CCDC3 in vascular ECs.

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List of Abbreviations

Abbreviations	Full Name
ACC	Acetyl-CoA carboxylase
ApoE	Apolipoprotein E
CAM	Cell adhesion molecules
CCDC3	Coiled-coil domain containing protein 3
CM	Conditioned medium
CRP	C-reactive protein
CVD	Cardiovascular disease
DMEM	Dulbecco's Modified Eagle Medium
EC	Endothelial cell
ECGS	Endothelial cell growth supplement
eNOS	Endothelial nitric oxide synthase
FAT	Fatty acid synthase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
HEK	Human embryonic kidney
HMEC-1	Human dermal microvascular endothelial cell-1
HUVEC	Human umbilical vein endothelial cell
IBD	Inflammatory bowel disease

Abbreviations	Full Name
ICAM-1	Intercellular adhesion molecule-1
IFR-1	Interferon regulatory factor-1
IKK	IκB kinase
IL	Interleukin
IP	Immunoprecipitation
IκB	Inhibitor of NF-κB
LDLR	Low-density lipoprotein receptor
LFA1	Lymphocyte function-associated antigen 1
LRC	Ligand based receptor capture
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony-stimulating factor
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor κB
NO	Nitric oxide
oxLDL	Oxidized low density lipoproteins
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RHD	Rel homology domain
RIPA	Radioimmune precipitation assay

Abbreviations	Full Name
ROS	Reactive oxygen species
SARA	Smad anchor for receptor activation
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin ribonucleic acid
SMAD 2	Mothers against decapentaplegic homolog 2
SMC	Smooth muscle cell
TGF-β	Transforming growth factor beta
TLR	Toll-like receptors
TNFR	Tumor necrosis factor receptor
VCAM-1	Vascular cell adhesion molecule-1
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VLA4	Very late antigen 4

Chapter One

Introduction

1.1 Endothelial cells (ECs) in regulating vascular homeostasis and integrity

The endothelium is a single layer of cells that line the entire blood vessels in the circulatory system and provide a semi-permeable barrier between blood or lymph within the vessels and surrounding tissues [1]. In the past, the thin layer of ECs was considered as an inert cellophane-like membrane without any specific functions, but to primarily maintain vessel wall permeability. However, it has become now clear that ECs participate in many dynamic cellular processes and have very important secretory, metabolic, and immunologic functions [2, 3]. ECs perform an array of functions critical to vascular health by maintaining vascular integrity and homeostasis, regulating vascular tone, leukocyte adhesion, angiogenesis and vasculogenesis by generating and releasing different bioactive molecules (***Fig. 1***) [3, 4].

Proper functioning of ECs is important in maintaining vascular homeostasis and integrity. ECs regulate vascular homeostasis through the balanced release of various vasodilators and vasoconstrictors, pro-coagulants and anti-coagulants, inflammatory and anti-inflammatory mediators [5, 6]. In normal physiological conditions, ECs maintain an active non-thrombogenic blood-tissue interface to help transport plasma and cellular constituents throughout the vasculature by regulating thrombosis and platelet adherence. For example, ECs prevent activation of different pro-coagulants such as thrombin

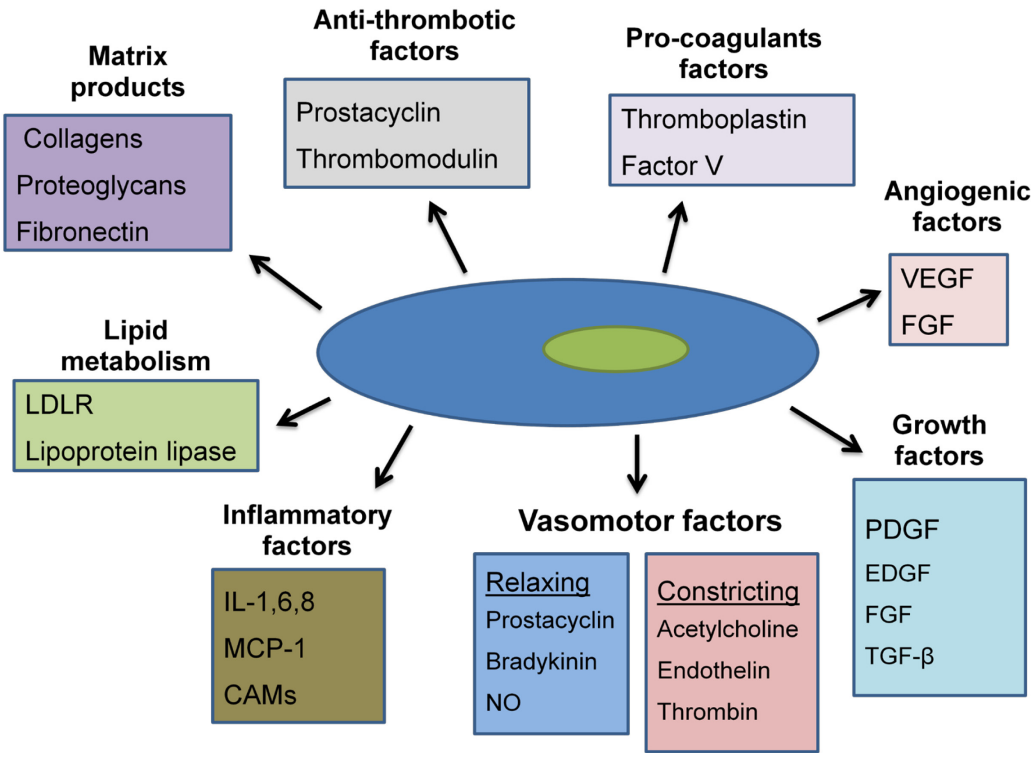

Figure 1: Endothelial cells derived bioactive molecules. Endothelial cells play a critical role in maintaining different cellular functions throughout the body by the secretion of a large variety of functional molecules. Figure adapted from [7].

and fibrin by producing thrombomodulin [8]. In addition, EC-derived functional molecules, such as nitric oxide (NO), endothelin and prostaglandins, are important in regulating vascular tone and blood pressure and thereby maintain the vascular homeostasis [9].

The integrity of blood vessels is an essential aspect in maintaining vascular homeostasis. The integrity of blood vessel is ensured by stabilization and maturation of newly formed vascular structure through a dynamic process, requiring a variety of active signaling events. For example, EC-derived vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), as well as vascular endothelial-cadherin (VE-cadherin) are important molecules that regulate vascular stabilization and vessels' integrity [10-12]. Any condition that poses a threat to vascular integrity either by disrupting VEGF and FGF signaling or VE-cadherin assembly in endothelial junctions leads to damage in endothelial monolayer, which ultimately gives rise to many pathological conditions [13]. Multiple studies showed that dysregulation of endothelial function results in impaired vascular reactivity that ultimately leads to various pathological conditions including cardiovascular diseases (CVDs), inflammatory disorders and even cancer [14-16].

1.2 Inflammation in endothelial dysfunction

Endothelial dysfunction is defined by the activation of ECs by various physical and chemical stimuli and an increase in the production of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule 1 (ICAM-1), E-selectin, interleukins and chemokines [17, 18]. Endothelial dysfunction could breach endothelial homeostasis and integrity, and contribute to the development CVDs (e.g., heart disease and stroke) and other inflammatory disorders [19]. Multiple studies have demonstrated that endothelial dysfunction is positively correlated with coronary artery disease development and is used as an early indicator for future cardiovascular disease progression [20, 21].

In terms of cardiovascular disease, endothelial dysfunction refers to impaired endothelium-dependent vasorelaxation, particularly due to reduced bioactivity of NO [22]. NO protects from various cardiovascular events by inducing the relaxation of smooth muscle cells (SMCs), and prevention of leukocytes-EC adhesion and the subsequent transmigration of leukocytes to arterial wall, thereby controlling the pro-inflammatory responses [22, 23]. However, endothelial dysfunction impairs NO bioactivity due to reduced production of endothelial NO synthase (eNOS), and increased level of reactive oxygen species (ROS) that inactivates NO, leading to initiation and progression of CVDs [24, 25].

Inflammation is considered as a host defense mechanism which allows the immune system to quickly responds to injury or infection. In response to inflammation, ECs express cell adhesion molecules (CAMs) and recruit circulating leukocytes from blood to the vessel wall, and the recruited leukocytes then pass through the endothelial monolayer into the injured tissues. Leukocytes then mediate the healing process by neutralizing the infectious agents and other pathogenic substances with an aim to restore normal tissue functions [26]. However, persistent inflammatory response will lead to endothelial dysfunction and development of different vascular diseases including atherosclerosis and rheumatoid arthritis [27, 28].

Acute inflammation could lead to endothelial dysfunction and pre-treatment with aspirin can inhibit inflammation-induced endothelial dysfunction. In their study, Rajesh *et al.* induced inflammation-induced endothelial dysfunction for 48h in healthy volunteers by administration of capsular polysaccharide typhoid vaccine, and showed that individuals pre-treated with aspirin was able to prevent endothelial dysfunction via modulation of the cytokine cascade, suggesting that acute inflammation is a transient risk factor for cardiovascular diseases [29]. Moreover, chronic inflammation mediated endothelial dysfunction has been shown to accelerate atherogenesis in patients with rheumatoid arthritis [30, 31]. The role of endothelial inflammation in the initiation, progression and development of atherosclerosis is well established, as it will be discussed later. In

fact, numerous studies have demonstrated the involvement of endothelial inflammation and dysfunction in different cardiovascular events including myocardial infarction, stroke and ischemic heart disease [32, 33].

1.3 Atherosclerosis as an inflammatory disease

Atherosclerosis is a systemic chronic disease characterized by thickening of artery wall due to accumulation of lipid and fibrous elements around the blood vessels. The atherosclerotic plaque narrows down the arteries and clogs the normal blood flow throughout the body, thereby becoming the most important contributor to CVDs including ischemic heart disease, peripheral arterial disease, and stroke [34]. According to a WHO report, an estimated 17.5 million people died from CVDs globally in 2012, representing 31% of all global death [35]. In Canada, 60,910 people died from heart disease and stroke in 2011, representing 25.2% of total national deaths [36]. The statistics here illustrate the severity of atherosclerosis on global health, which encourages seeking novel therapeutic intervention against atherosclerosis.

The development of atherosclerosis involves several risk factors including smoking, obesity, hypertension, hypercholesterolemia and inflammation [34, 37-39]. Traditionally, atherosclerosis was considered to be the result of lipid accumulation in arterial wall [40]. However, it is now widely acknowledged that

atherosclerosis is a disease of ongoing inflammatory response [41, 42]. Several lines of evidence demonstrated the involvement of inflammation in atherosclerosis including the presence of high levels of pro-inflammatory cytokines (e.g., IL-6 and TNF-α) [43, 44] and cell adhesion molecules (e.g., VCAM-1 and ICAM-1) [45]. In addition, clinical studies showed that high levels of inflammatory biomarkers such as c-reactive protein (CRP) predicts cardiovascular events in patients with or without previously known coronary heart disease [46, 47].

ECs in their normal state do not firmly interact with leukocytes; however, in response to inflammation ECs express different cell surface adhesion molecules including E-selectin, P-selectin, ICAM-1 and VCAM-1 that recruit different classes of leukocytes to the site of infection or injury [48, 49]. Among these adhesion molecules, VCAM-1 is crucial for recruiting monocytes to the arterial endothelium at lesion-prone areas [50, 51]. Upon binding to VCAM-1, monocytes transmigrate through the ECs basement membrane to the innermost layer of artery wall. This transmigration is mediated by different chemoattractant molecules such as interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) [52, 53]. Substantial studies have shown that recruitment of monocytes to the lesion site initiates the development of atherosclerosis [54, 55]. In addition, these inflammatory monocytes in turn augment the inflammatory response by the production of Toll-like receptors (TLR), TNF-α and IL-1 [41].

In arterial intima, inflammatory mediators such as macrophage colony-stimulating factor (M-CSF) and MCP-1 stimulate the differentiation of blood monocytes into tissue macrophages [56, 57]. Modified lipoproteins such as oxidized low density lipoproteins (oxLDL) are taken up through scavenger receptors present in macrophage, leading to the formation of foam cells [58]. These lipid-loaded foam cells are the early indication of atherosclerosis development, which can further amplify inflammatory responses by releasing ROS and other pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-β, and thereby augmenting the atherosclerosis progression (***Fig. 2***) [28, 59, 60].

Progression of atherosclerosis continues with the migration and proliferation of SMCs into the atherosclerotic lesion mediated by cytokines and growth factors released in the lesion site [61, 62]. SMCs synthesize extracellular matrix proteins such as collagen that forms a fibrous cap, leading to a more advanced atherosclerosis lesion [61]. Fibrous cap can be ruptured due to degradation of extracellular matrix proteins by matrix metalloproteinases (MMPs) released by macrophage foam cells, leading to exposure of the necrotic core to the blood stream, thus activating the coagulation cascade and leading to formation of thrombus [63-65].

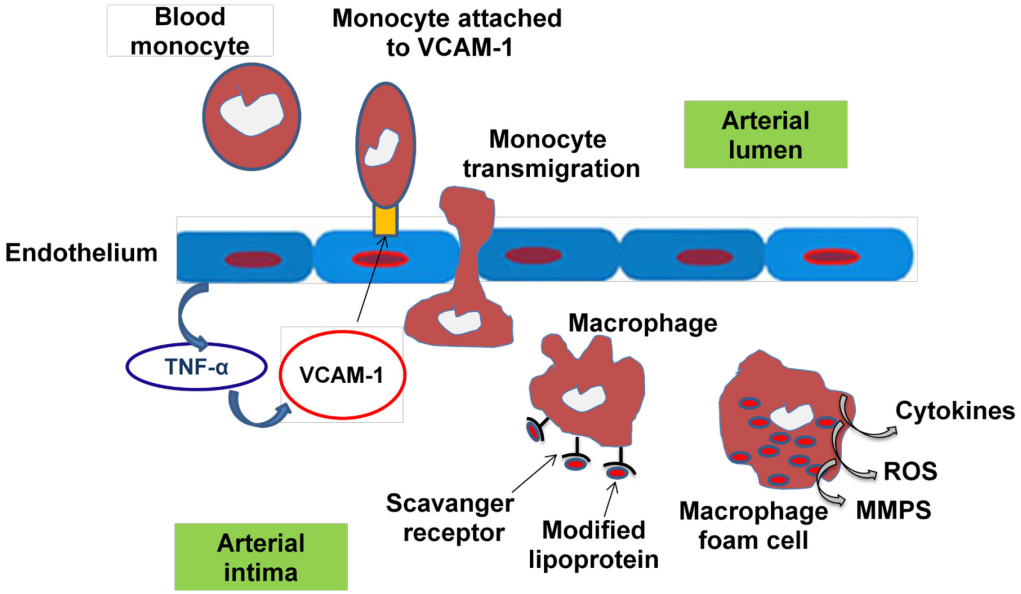

Figure 2: Inflammation and atherosclerosis. In response to inflammation, ECs secrete cytokines such as TNF-α that upregulates different CAMs. In the context of atherosclerosis, VCAM-1 is most critical in recruiting circulating monocytes. Monocytes transmigrate to the innermost layer of arterial wall, converting themselves into macrophages. Macrophages then uptake the modified lipoproteins and give rise to foam cells, which indicate the development of atherosclerosis. Figure and texts are adapted from [28].

1.4 Tumor necrosis factor-α (TNF-α) in endothelial inflammation

TNF-α is a critical pro-inflammatory cytokine that regulates ECs response to inflammation [66]. In response to inflammation, TNF-α can be secreted from a variety of cell types including macrophages, mast cells, lymphoid cells and ECs [67]. TNF-α then stimulates the expression of different CAMs such as E-selectin, VCAM-1 and ICAM-1 in ECs and thus helps to recruit leukocytes to the site of infection or injury [68].

TNF-α exerts its biological functions by interacting with two tumor necrosis factor receptors (TNFR): TNFR1 and TNFR2 [69]. Most of the tissues constitutively express TNFR1, whereas TNFR2 is typically found on the immune cells [70]. Upon contact with their receptors, TNF-α signals through three distinct signal transduction pathways: mitogen-activated protein kinase (MAPK), NF-κB and death signaling pathways [71]. Activation of these diverse signaling pathways stimulates different cellular responses including cell survival, proliferation, migration and apoptosis [72].

After binding to ligands, TNFR1 becomes activated and recruits TNFR1 associated death domain (TRADD), which in turn recruits TNFR-associated factor (TRAF) and receptor interacting kinase 1(RIP1). TRADD binds to fas-associated death domain (FADD), resulting in caspase 8 recruitment and activation and initiation of the extrinsic apoptosis pathway [71].

MAPK and NF-κB are the two most extensively studied signaling cascades that regulate TNF-α-induced pro-inflammatory genes expression in ECs [73, 74]. In addition, several transcription factors such as interferon regulatory factor-1 (IRF-1) and GATA have also been found to mediate TNF-α-induced pro-inflammatory genes expression [75, 76]. However, NF-κB is the main regulator of pro-inflammatory gene expression in ECs and has been proposed as a potential therapeutic target in inflammatory diseases such as atherosclerosis [77-79].

In mammals, five members of the NF-κB/Rel family including RelA/p65, RelB, c-Rel, NF-κB1/p50 (constitutively processed from its precursor p105 by proteolysis) and NF-κB2/p52 (which is inducibly processed from its precursor p100) share a highly conserved Rel homology domain (RHD) that mediates their dimerization, DNA binding, and nuclear translocation [80, 81]. In the unstimulated state, the nuclear location sequence of NF-κB is masked through interaction with members of the inhibitor of NF-κB (IκB) including IκBα, IκBβ, and IκBε, which results in retention of the NF-κB/IκB complex in the cytoplasm [82]. IκBα and IκBβ complex mainly with p50/p65 or p50/c-Rel heterodimers, whereas IκBε complexes mainly with p50/c-Rel or c-Rel homodimers [83, 84]. Extracellular stimuli, such as inflammatory cytokines, activate IκB kinase (IKK) that in turn phosphorylates IκB in the NF-κB/IκB complex [85-87]. Phosphorylation of IκB leads to its degradation by the 26S proteasome, resulting in release of NF-κB from the inhibitory complex and subsequent nuclear

translocation of the active NF-κB dimers [85-87]. In the nucleus, NF-κB dimers bind to specific sites on the promoter regions of target genes and regulate the expression of various proteins such as pro-inflammatory cytokines, chemokines, and adhesion molecules including VCAM-1 and ICAM-1 (***Fig. 3***) [85-87].

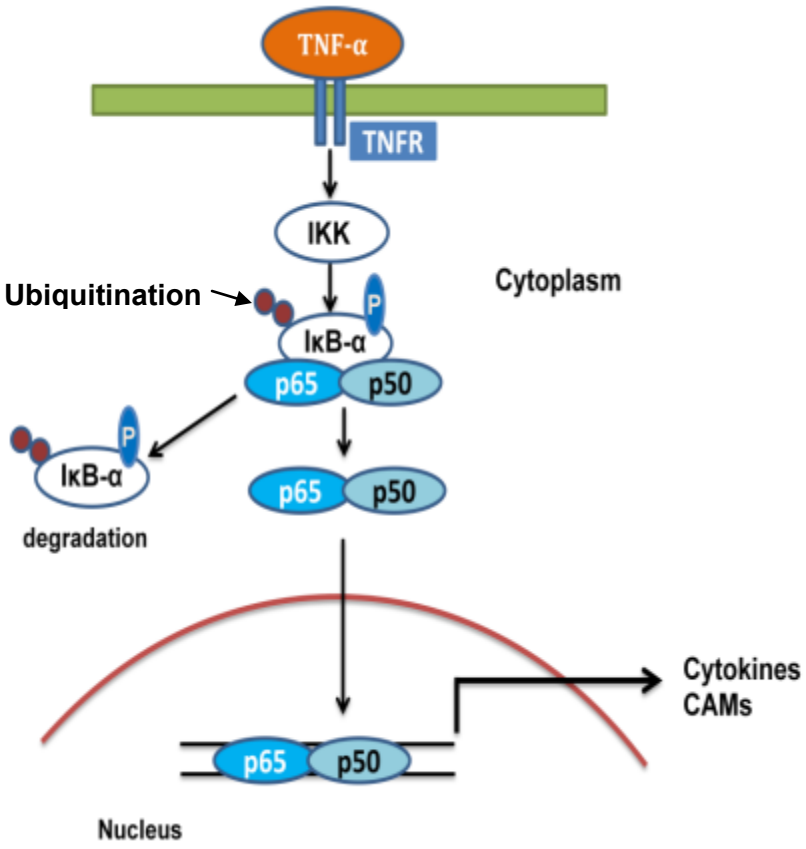

Figure 3: TNF-α signaling pathway. TNF-α binds to its receptor(s) and activates IKK that in turns phosphorylates IκB-α. Upon phosphorylation, IκB undergoes ubiquitination and proteasomal degradation, leading to release and subsequent nuclear translocation of NF-κB subunits (p65/p50) to the nucleus where NF-κB transcribes their target genes.

For its pro-inflammatory potential, TNF-α has been found to be involved in many inflammatory diseases including rheumatoid arthritis and inflammatory bowel disease (IBD), and anti-TNF-α therapies significantly improve clinical outcomes of the patients [88, 89]. TNF-α is also involved in endothelial dysfunction. It has been shown that intravenous administration of TNF-α impairs the endothelial-dependent vasodilation in rats [90]. TNF-α-induced endothelial dysfunction is mediated via reduced bioactivity of NO due to diminished expression of eNOS and increased scavenging by ROS [91, 92]. Reduced NO also increases inflammatory reaction, as NO has an anti-inflammatory effect by limiting the expression of VCAM-1 [23]. Interestingly, in Apolipoprotein E knockout mice ($ApoE^{-/-}$), a most widely used mouse atherosclerosis model, disruption of *Tnf* (the mouse TNF-α gene) diminishes atherosclerosis development compared to wild type mice [93]. The role TNF-α in atherosclerosis is summarized in ***Fig. 4*** [91].

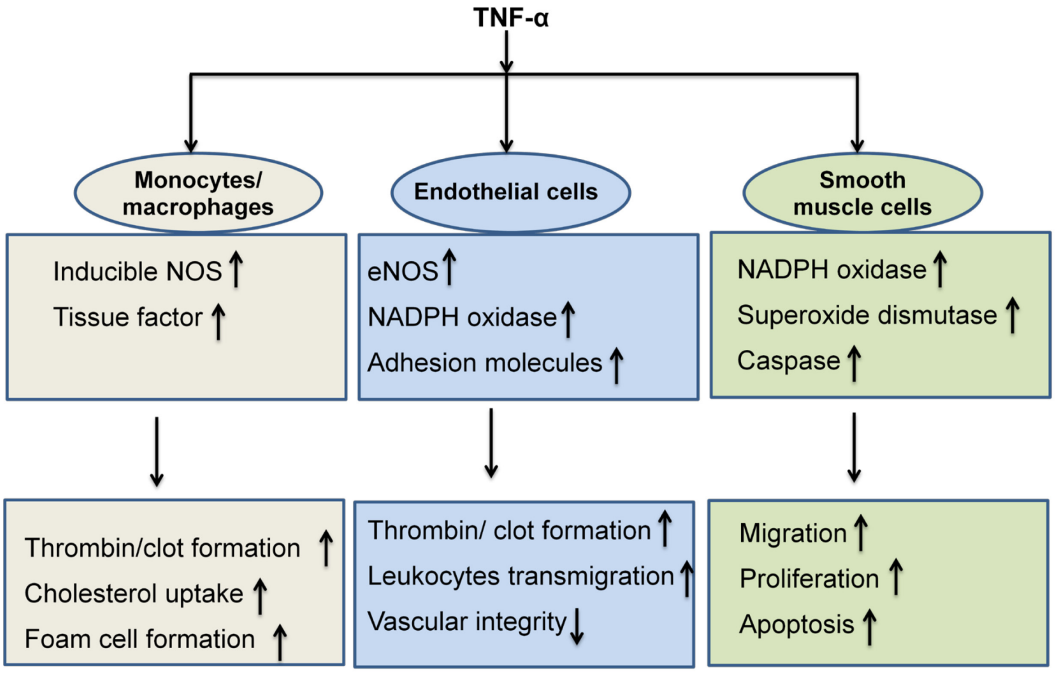

Figure 4: Involvement of TNF-α in atherosclerosis. TNF-α induces multiple cellular and molecular events that lead to development and progression of atherosclerosis. Figure and text are adapted from [91].

1.5 Cell adhesion molecules (CAMs) in inflammation and atherosclerosis

ECs play a fundamental role in inflammatory processes. Upon activation by various stimuli, ECs express different CAMs such as E-selectin, VCAM-1, and ICAM-1 that mediate the leukocytes recruitment and transmigration from the blood stream into the vascular wall [45, 68, 94]. Migration of leukocytes from the vascular lumen to the site of injury involves tightly regulated events: slow rolling of leukocytes, firm adhesion on vascular adhesion molecules and transmigration through the endothelial basement membrane (***Fig. 5***) [95, 96].

P-selectin and E-selectin capture the leukocytes from the bloodstream by interacting with glycosylated selectin ligands on leukocytes to initiate leukocytes rolling [97]. This initial interaction in turn promotes further activation of integrins in leukocytes via chemokine-driven intracellular signaling cascades, thereby initiating the later steps [98, 99]. The firm adhesion and subsequent trans-endothelial migration of leukocytes is mediated through interactions with VCAM-1 and ICAM-1 to the leukocyte integrins Very Late Antigen 4 (VLA4, also known as $\alpha_4\beta_1$) and lymphocyte function-associated antigen 1 (LFA1), respectively [95, 96].

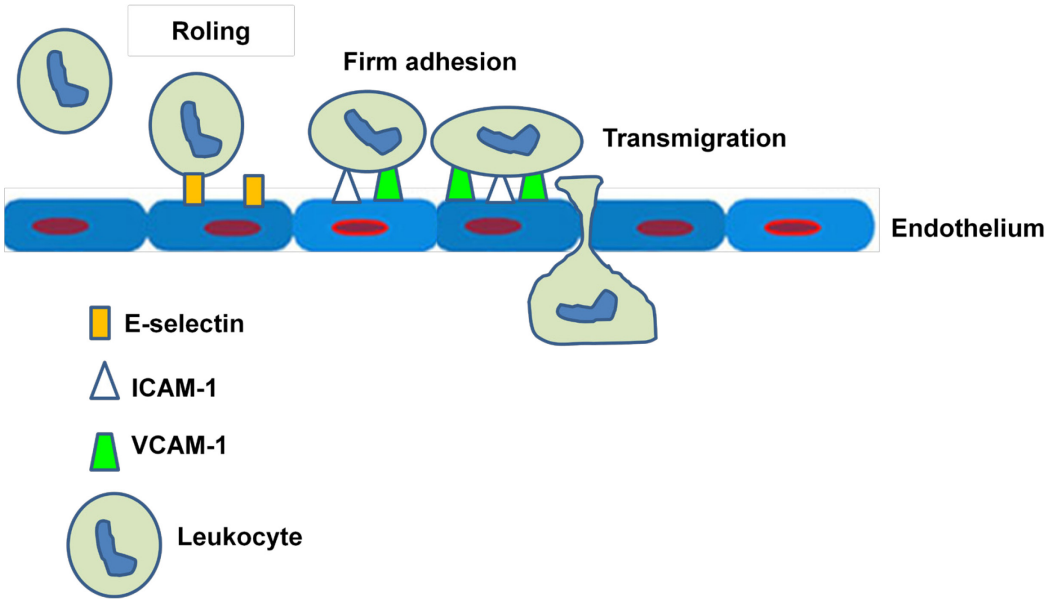

Figure 5: Multiple steps in the recruitment of leukocytes during the inflammatory response. Leukocytes are captured by E-selectin and start slow rolling. Firm adhesion of leukocytes to ECs is promoted by ICAM-1 and VCAM-1, followed by transmigration of leukocytes through endothelial basement membrane to the site of infection or injury. Figure is adapted from [100].

Among the adhesion molecules, VCAM-1 has been shown to be critical in atherosclerosis development [50, 101]. Increased expression of VCAM-1 has been found on endothelial cells overlying lesion-prone areas in rabbit models of atherosclerosis [50]. Moreover, VCAM-1 participates in recruiting the leukocytes that are found in early human and experimental atheroma, such as monocytes and T-lymphocytes, supporting the role of VCAM-1 in the development of atherosclerosis [28, 39].

Knockout of *Vcam1* (the mouse VCAM-1 gene) causes early embryonic lethality [102]. In order to circumvent embryonic lethality in *Vcam1* knockout mice, Cybulsky *et al*., disrupted the fourth Ig domain of VCAM-1 to generate $Vcam1^{D4D}$ mice [101]. $Vcam1^{D4D}$ mice partially overcome the embryonic lethality of VCAM-1 knockout mice, and the expression level of VCAM-1 is significantly decreased in $Vcam1^{D4D}$ mice compared to wild type mice [101]. Thus far, two most widely used atherosclerosis mice models are the low-density lipoprotein receptor (LDLR) knockout mice and ApoE knockout mice [103]. To investigate the importance of VCAM-1 in atherosclerosis, $Vcam\text{-}1^{D4D/D4D}$ mice were intercrossed with LDLR-null background and fed a cholesterol-enriched diet for 8 weeks [101]. They found that $Vcam\text{-}1^{D4D/D4D}LDLR^{-/-}$ mice showed a significant decrease in lesion formation compared to wild type mice [101]. Furthermore, $Vcam\text{-}1^{D4D/D4D}$ mice intercrossed with $apoE^{-/-}$ showed 84% reduction in lesion

formation, as well as marked decrease in monocyte adherence to endothelium, and fatty streak formation [104]. Taken together, these data show that VCAM-1 plays a central role in the development and progression of atherosclerosis.

1.6 Coiled-coil domain containing protein 3 (CCDC3)

CCDC3 gene is localized at human chromosome 10. Expression analysis in mouse tissues shows that *CCDC3* mRNA is abundant in the aorta and adipose tissues [105] and similar expression profiles have also been observed in various human tissues as well [106]. Database analysis showed a strong amino acid homology among species [105]. CCDC3 protein, comprised of 270 amino acid, contains a coiled-coil domain near the C-terminus and a signal peptide at the N-terminus [105]. Recent studies identified CCDC3 as a secretory protein and mainly expressed in adipose tissues and ECs [105]. CCDC3 is therefore also named as Favine (fat/vessel-derived secretory protein) by the authors. [105]. Transient expression experiments demonstrated that CCDC3 was secreted into culture medium and the expression of which was reduced by brefeldin A (inhibitor of Golgi-mediated secretory pathway) [105]. Overexpression in COS-7 cells showed that CCDC3 protein was post-transcriptionally modified with N-glycosylation [105].

Ugi *et al.* reported CCDC3 upregulation in visceral adipose tissues, but not in the subcutaneous adipose tissue in subjects with abdominal obesity, suggesting that CCDC3 may act as an adipokine (cytokines expressed from adipose tissue) and can be used a marker for visceral obesity [106]. They further showed that CCDC3 expression is upregulated during differentiation of murine 3T3-L1 cells into mature adipocytes [106]. Moreover, Eberlein *et al.* showed a significant positive correlation between intramuscular fat content and CCDC3 mRNA expression in bovine skeletal muscle, suggesting a role for CCDC3 in fat metabolism [107].

Our published studies demonstrate that Notch plays an important role in endothelial cells and heart development [108, 109]. To identify novel Notch target genes in ECs, we generated a database of the potential Notch-regulated genes in ECs using a microarray approach. From the microarray database, we found that Notch activation increases *CCDC3* mRNA expression by 51.5 fold in human umbilical vein endothelial cells (HUVECs) [108], which was further confirmed by quantitative RT-PCR (qRT-PCR) and Western blotting (unpublished data).

Kobyashi *et al.* reported that the expression of CCDC3 is upregulated during adipocyte differentiation and is regulated by different hormones and nutritional factors in adipocytes and ECs [105]. For example, insulin and pioglitazone (anti-diabetic drug) increases, while norepinephrine decreases

CCDC3 expression during adipocyte differentiation [105]. In the context of endothelial cells, Kobyashi *et al.* showed that TNF-α, which induces endothelial inflammation, downregulates CCDC3 mRNA expression [105]. However, the role of CCDC3 in vascular ECs is unclear. We therefore initiated this project to investigate the potential role of CCDC3 in TNF-α-induced endothelial inflammation.

1.7 Hypothesis

We hypothesize that EC-derived CCDC3 is involved in TNF-α-induced endothelial inflammation.

Chapter Two

Materials and Methods

2.1 Reagents

MCDB131 medium, Bay 11-7082 (IKK inhibitor), anti-FLAG M2 antibody and anti-FLAG M2 affinity gel were purchased from Sigma. M199 medium and fetal bovine serum (FBS) were obtained from Life Technologies. TNF-α was purchased from Calbiochem. NE-PER Nuclear and Cytoplasmic Extraction Reagents kit was obtained from Thermo Scientific. Rabbit polyclonal antibody against VCAM-1 and mouse monoclonal antibody against p50 were obtained from Santa Cruz. Rabbit polyclonal antibody against p65 and mouse monoclonal antibody against poly (ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technology. Rabbit polyclonal antibody against tubulin was purchased from Abcam. Endothelial cell growth supplement (ECGS) was obtained from VWR and Matrigel was purchased from BD Bioscience. The mouse monoclonal antibody against human CCDC3 was generated through Abmart.

2.2 Cell culture

Human dermal microvascular endothelial cell (HMEC-1) were cultured in MCDB 131 (Sigma) medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies) as previously described [109, 110]. Human embryonic kidney (HEK) 293T cells and the retroviral vector

packaging cell line Phoenix-Aphmo cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with the same supplements.

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as previously described [111]. The protocol was approved by the University of Alberta Ethics Committee. The investigation also conformed to the principles outlined in the Declaration of Helsinki and also Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. All subjects provided informed consent before inclusion into this study.

Briefly, the umbilical vein was flushed with phosphate buffered saline (PBS) and the HUVECs were isolated using type 1 collagenase dissolved in PBS. The cells were grown in M199 medium supplemented with 20% FBS as well as L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies) and 1% ECGS (BD Biosciences). Cells from both male and female infants were used without regard to sex differences. All the cells were maintained in a humidified incubator at 37^0C in 5% CO2.

2.3 Generation of overexpression cell lines

To make the CCDC3 overexpressing stable cell line, human CCDC3 cDNA was at first amplified from the CCDC3 cDNA clone MGC:59716

(IMAGE:6302570) by polymerase chain reaction (PCR) and cloned into a retroviral expression vector MSCVpac at the XhoI/EcoRI site to generate MSCVpac-CCDC3-FLAG plasmid. The primers used for the PCR reaction were:

- Forward primer:
  5’GGCTCGAGATGCTGCGCCAGCTGCTGCTCGCC3’
- Reverse primer:
  5’GGGAATTCTTACTTATCGTCGTCATCCTTGTAATCCCCCCGCA
  GGTAGGGGGGGCGCAC3’ (FLAG-tag sequence underlined).

CCDC3 cDNA was also PCR amplified and cloned into a mApple vector to generate a CCDC3-mApple expression vector that expresses a CCDC3-mApple red fusion protein. The primers used for the PCR reaction were:

- Forward primer:
  5'CCCGCTAGCGCCACCATGCTGCGCCAGCTGCTGCTCGCC3'
- Reverse primer:
  5'GGCGGAGCTCCCCCGCAGGTAGGGGGGGCGCAC3'

All newly generated expression plasmids were validated by sequencing.

HMEC-1 cells were stably transduced with empty MSCVpac vector or MSCVpac/CCDC3-FLAG to generate HMEC-1/Vector and HMEC-1/CCDC3-FLAG. Briefly, the retroviral vector packaging cell line Phoenix-Ampho cells

were transfected with 10 μg of expression plasmids and kept overnight in incubator at 37^0C. Next day the medium was replaced with fresh medium. The medium was collected after 48h and 72h post-transfection and used to infect HMEC-1 cells after being passed through the filters (Millex ® Syringe-driven Filter Unit 0.45 μm). Polybrene (8 μg/mL) was used to increase the transfection efficiency in target cells. Infected cells were then selected by puromycin (1 μg/ml) treatment. mApple alone or CCDC3-mApple vectors were transiently transfected into HEK 293T cells for generation of mApple and CCDC3-mApple CM.

2.4 Generation of stable knockdown cell lines

To knock down the expression of CCDC3 in HMEC-1 cells and HUVECs, we generated two lentiviral shRNA constructs (shCCDC3A and shCCDC3B) that express shRNAs targeting two distinct sequences of human *CCDC3* gene. A shRNA construct expressing a scrambled sequence, which does not target any known genes, was used as control [110]. All newly generated knockdown constructs were validated by sequencing. The shRNA target sequences were:

- shRandom : 5'GTTGCTTGCCACGTCCTAGAT3'
- shCCDC3A: 5'GGTCCAGGACTACTCCTATTT3'
- shCCDC3B: 5'GCAGGGCAGCAGGTAATTAAA3'

HEK 293T cells were transfected with pLentilox-GFP-shRandom, pLentilox-GFP-shCCDC3A or pLentilox-GFP-shCCDC3B together with lentiviral packaging plasmids (RRE, REV, VSVG) using the calcium phosphate transfection method. After 48h and 72h post-transfection, the virus-containing medium was collected and used to infect HMEC-1 cells and HUVECs. Generally, pLentiLox-GFP infected cells were purified using fluorescence-activated cell sorting (FACS) for GFP positive cells. However, in our case the infection efficiency was quite high, so we used the cells directly to avoid the side effects of FACS sorting on cells. The knockdown efficiency of CCDC3 in all prepared cells was confirmed by Western blotting and real time PCR.

2.5 Treatment

HMEC-1 cells or HUVECs cells were treated with 10 ng/ml TNF-α for various durations as indicated in each experiment. In the IKK inhibitor experiment, HMEC-1 cells were pre-treated with 5 μM Bay 11-7082 for 2h and then treated with 10 ng/ml TNF-α for 1, 4 or 24h.

2.6 Preparation of whole cell lysates and Western blotting

Cultured cells were washed once with 1X PBS and then whole cell lysates were prepared using modified radioimmune precipitation assay (RIPA) buffer as described previously [113]. Briefly, RIPA buffer (50 mM Tris pH 7.4, 150 mM

NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100 and 1X protease inhibitor cocktail) was added to the cells and then the cell lysates were collected, subjected to brief sonication, and cleared by centrifugation at 13000 rpm at for 15 min. Protein concentrations for all the cell lysates were quantified using the DC protein assay (Bio-Rad) and an equal amount of proteins were boiled at 95^0C in SDS sample buffer for 5min to perform Western blotting.

Proteins samples were separated by electrophoresis on 10% SDS polyacylamide gel (Stacking gel: 4X Upper Buffer, 30% Bis-Acrylamide, 10% APS (ammonium persulfate), TEMED (tetramethlethylenediamine), dH2O; Separating gel: 4X Lower Buffer, 30% Bis-Acrylamide, 10% APS, TEMED, dH2O). Proteins samples were then transferred from the gels to the nitrocellulose membranes at 100 V for 1.5h. Membranes were incubated overnight with primary antibodies at 4^0C. Bound proteins were detected by HRP-conjugated secondary antibodies and the images were analyzed and quantified by using an Odyssey® IR scanner and Odyssey® imaging software 3.0 (LiCor Biosciences).

2.7 RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Total cellular RNA was extracted by using TRIzol® (Invitrogen) according to the manufacturer's instructions and treated with DNase to remove

DNA contamination. Briefly, cells were washed once with 1X PBS and then lysed by pipetting with 500 µl of TRIzol for each 6 cm culture dish. 100 µl of chloroform was added, mixed and centrifuged at 12000 rpm at 4^0C for 10 min. Aqueous upper phase solution was collected and precipitated by adding 250 µl isopropanol. Samples were then incubated at room temperature for 10 min and subjected to centrifugation at 12000 rpm for 10 min at 4^0C. The gel-like pellet was washed with 75% ethanol, air dried for 5 min, the RNA was resuspended in 50 µl RNase-free water.

cDNA was synthesized by using the superscript II reverse transcriptase in the presence of random primers and RNaseOUT™ recombinant ribonuclease inhibitor (Invitrogen) according to the manufacturer's instructions. qRT-PCR was carried out using the Mastercycler®ep realplex real-time PCR system (Eppendorf). The reaction mixture consisted of 1 µl of cDNA, 1 µl of 10 µM primers, and 10 µl of SYBR® Select Master Mix (Applied Biosystems) in a total volume of 20 µl. Experimental samples were first normalized to GAPDH and then to the control samples, and then the fold changes were calculated based on ΔΔCT method. The PCR primers used for VCAM-1 were:

- Forward primer sequence: 5'CATTTGACGGGCTGGAGATA3'
- Reverse primer sequence: 5'GAACAGGTCATGGTCACAGA3'

And the PCR primers used for CCDC3 were:

- Forward primer sequence: 5'CCCAGACACTCAAGAGAACAGAAGG3'
- Reverse primer sequence: 5'TGGTCCTCCTCCTCAAACAAGG3'

2.8 Subcellular fractionation

HMEC-1/Vector and HMEC-1/CCDC3-FLAG at approximately 80% confluency were treated with 10 ng/ml TNF-α for 1h. After washing with PBS, cells were trypsinized and centrifuged to obtain cell pellets. The pellets were lysed and nuclear and cytosolic components were prepared by using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit according to the manufacturer's instructions (Thermo Scientific). Briefly, cells were washed with 1X PBS, harvested by trypsinization, and then washed with chilled 1X PBS. Cytosolic proteins were extracted by disrupting cell membranes with cytosolic extraction buffer, followed by centrifugation at 13000 rpm. The intact nuclei were washed with chilled 1X PBS to remove cytosolic proteins, and then lysed with nuclear extraction buffer to collect nuclear proteins. Expression of p65 and p50 in cytosolic and nuclear fractions was determined by Western blotting.

2.9 Determination of NF-κB transcriptional activity

To determine the effect of CCDC3 on TNF-α-induced NF-κB transcriptional activity, luciferase reporter gene assay was performed by using the

Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. HMEC-1/Vector and HMEC-1/CCDC3-FLAG were seeded onto a 24-well plate. Next day, the cells were transfected with a NF-κB firefly luciferase reporter driven by a promoter containing three NF-κB binding sites (provided by Dr. Shairaz Baksh, University of Alberta) and a Renilla luciferase reporter driven by the CMV promoter (transfection control). 24h post-transfection, cells were treated with 10 ng/ml TNF-α for 24h. Subsequently, cells were lysed in a Passive Lysis Buffer (Promega) and luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega). The NF-κB reporter activity was determined by normalizing the firefly luciferase activity to the co-transfected Renilla luciferase activity. The relative NF-κB activity in HMEC-1/Vector treated with TNF-α was set as 100.

2.10 Conditioned medium (CM) experiments

CM was collected from HMEC-1/Vector, HMEC-1/CCDC3-FLAG, or HEK 293T cells that were transiently transfected with the empty MSCVpac vector, MSCVpac-CCDC3-FLAG, mApple alone or CCDC3-mApple vector. The expression of CCDC3 in CM was at first determined by Western blotting. The CM was applied to receiving HMEC-1 cells at approximately 80% confluency and cells were incubated overnight. Next day the receiving HMEC-1 cells were

treated with 10 ng/ml TNF-α for 20h. Whole cell lysates were collected for Western blotting.

2.11 Purification of CCDC3 from CM

CM was collected from HEK 293T cells transiently transfected with MSCVpac or MSCVpac-CCDC3-FLAG. CCDC3-FLAG was purified from the CM using the anti-FLAG M2 affinity gel according to the manufacturer's instruction manual. Briefly, 150 µl of anti-FLAG M2 affinity gel was washed three times with 1X PBS and added to 8 ml CM. After overnight incubation at 4^0C with shaking, the CM and the anti-FLAG M2 affinity gel mixture was centrifuged at 850*g* for 5 min. The supernatant was collected as the CCDC3-depleted CM, and the pelleted anti-FLAG M2 affinity gel was washed three times with the buffer containing 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% TRITON X-100 and 1X proteinase inhibitor cocktail. CCDC3-FLAG bound to the anti-FLAG M2 affinity gel was then eluted with 300 µg/ml FLAG peptide in TBS buffer (50 mM Tris HCl, with 150 mM NaCl, pH 7.4). The eluate was collected as the purified CCDC3-FLAG. Presence of CCDC3 in the CM before purification, depletion of CCDC3 in the CM after purification and the presence of the purified CCDC3 in eluate were confirmed by Western blotting using the mouse monoclonal CCDC3 antibody.

2.12 CCDC3 uptake assay

Uptake of CCDC3 in CM by the receiving cells was examined using fluorescence microscopy and Western blotting approaches. For fluorescence microscopy, HMEC-1 cells were incubated with CM collected from HEK 293T cells that were transiently transfected with mApple alone or CCDC3-mApple vector overnight. The medium was then aspirated and the HMEC-1 cells were washed once with 1X PBS, fixed with 4% paraformaldehyde for 10 min, and permeabilized with 1X PBS containing 4% FBS and 0.15% TRITON X-100 for 10 min at room temperature. Cells were then incubated with 4',6-diamidino-2-phenylindole (DAPI) (1 μg/mL) for 5min to stain the nucleus. After one wash with 1X PBS, the uptake of CCDC3-mApple was examined via fluorescence microscopy and images were captured.

For Western blotting, HMEC-1 cells were incubated overnight with CM from HEK 293T cells that were transiently transfected with empty vector or MSCVpac-CCDC3-FLAG. Receiving HMEC-1 cells were then washed once with 1X PBS, twice with 150 mM NaCl and 0.1% acetic acid for 2 min at 4^0C to remove the extracellular CCDC3-FLAG [114]. Cells were then washed once more with 1X PBS and cell lysates were collected using RIPA buffer for Western blotting analysis to determine the presence of CCDC3-FLAG in the receiving HMEC-1 cells.

2.13 Statistical analysis

Data are shown as mean ± SEM of three independent experiments. Statistical analysis was performed using GraphPad Prism5. Statistical significance between two groups was determined by two-tailed t-test and defined as $P < 0.05$.

Chapter Three

Results

3.1 Overexpression of CCDC3 represses TNF-α-induced VCAM-1 expression in ECs

Our previous microarray data showed that activation of Notch signaling upregulates CCDC3 in ECs [108]. We further validated the upregulation of CCDC3 upon Notch activation by by qRT-PCR and Western blotting (unpublished data). A recent published paper demonstrated that CCDC3 expression is downregulated by TNF-α in ECs [105]. Together, these results suggest that CCDC3 may play functional role in ECs. We initiated this project to determine whether CCDC3 is involved in TNF-α signaling in ECs. To this end, we stably transduced HMEC-1 cells with an empty retroviral vector MSCVpac or MSCVpac-CCDC3-FLAG to generate HMEC-1/Vector and HMEC-1/CCDC3-FLAG. Overexpression of CCDC3 was confirmed by Western blotting using an anti-FLAG antibody ***(Fig. 6A).*** After making the stable CCDC3 overexpressing cell lines, we treated HMEC-1/Vector and HMEC-1/CCDC3-FLAG with 10 ng/ml TNF-α for 4h or 24h and measured the expression of VCAM-1 by qRT-PCR and Western blotting. As shown in ***Fig 6B***, TNF-α-induced VCAM-1 mRNA expression was more pronounced at 4h than 24h, and it was decreased by CCDC3 overexpression by 67.2% and 54.5%, respectively.

Western blotting results showed that TNF-α-induced VCAM-1 protein expression was higher at 24h than 4h and it was inhibited by CCDC3

overexpression by 37.9% and 47.5%, respectively ***(Fig. 7A and 7B)***. These results demonstrate that overexpression of CCDC3 represses TNF-α-induced VCAM-1 expression in ECs.

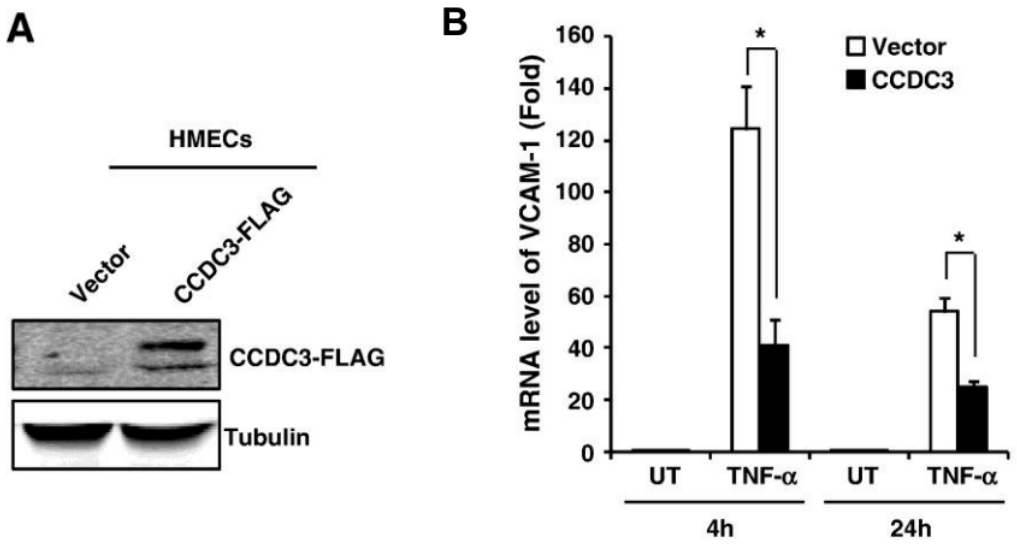

Figure 6: Overexpression of CCDC3 inhibits TNF-α-induced VCAM-1 mRNA expression in ECs. (A) HMEC-1 cells were stably transduced with an empty vector or CCDC3-FLAG to generate HMEC-1/Vector and HMEC-1/CCDC3. Expression of CCDC3-FLAG was determined by Western blotting using an anti-FLAG antibody. Tubulin was used as the loading control. **(B)** HMEC-1/Vector and HMEC-1/CCDC3 were left untreated or treated with 10 ng/ml TNF-α for 4h or 24h. The mRNA levels of VCAM-1 were examined by qRT-PCR, normalized to that of GAPDH, expressed as fold change relative to untreated HMEC/Vector cells at 4h and shown as mean ± SEM of three independent experiments. *Significantly different ($P < 0.05$).

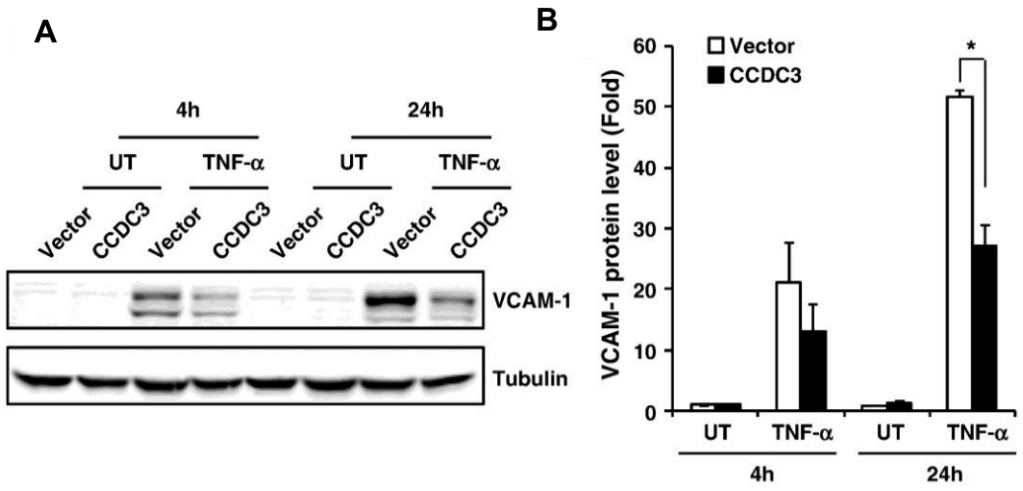

Figure 7: Overexpression of CCDC3 inhibits TNF-α-induced VCAM-1 protein expression in ECs. (A) HMEC/Vector and HMEC/CCDC3 were left untreated or treated with 10 ng/ml TNF-α for 4h or 24h. The protein levels of VCAM-1 were examined by Western blotting. Tubulin was used as the loading control. **(B)** Quantification of Western blotting data by densitometry analysis and expressed as the fold change. The VCAM-1 expression level in untreated HMEC-1/Vector at 4h was designated as 1 and shown as mean ± SEM of three independent experiments. *Significantly different ($P < 0.05$).

3.2 Knockdown of CCDC3 expression enhances TNF-α-induced VCAM-1 expression in ECs

To further ascertain the inhibitory effect of CCDC3 on TNF-α-induced VCAM-1 expression in ECs, we stably knocked down the expression of the endogenous CCDC3 in HMEC-1 cells using a lentivirus-delivered shRNA approach as previously described [110]. HMEC-1 cells infected with lentivirus expressing shRandom, shCCDC3A or shCCDC3B were referred to as HMEC-1/shRandom, HMEC-1/shCCDC3A and HMEC-1/shCCDC3B, respectively. Knockdown of CCDC3 expression with shCCDC3A and shCCDC3B was confirmed by real time PCR and by Western blotting using a mouse monoclonal antibody against human CCDC3 ***(Fig. 8A and 8B).***

HMEC-1/shRandom, HMEC-1/shCCDC3A and HMEC-1/shCCDC3B cells were left untreated or treated with 10 ng/ml TNF-α for 24h and VCAM-1 protein expression was measured by Western blotting. As shown in ***Fig. 9A and 9B***, TNF-α-induced VCAM-1 expression was more pronounced in CCDC3 knockdown cells compared to the shRandom control cells. Specifically, TNF-α-induced VCAM-1 protein expression was 2.3 and 3.8 fold higher in HMEC-1/shCCDC3A and HMEC-1/shCCDC3B, respectively, compared to HMEC-1/shRandom (***Fig. 9B***).

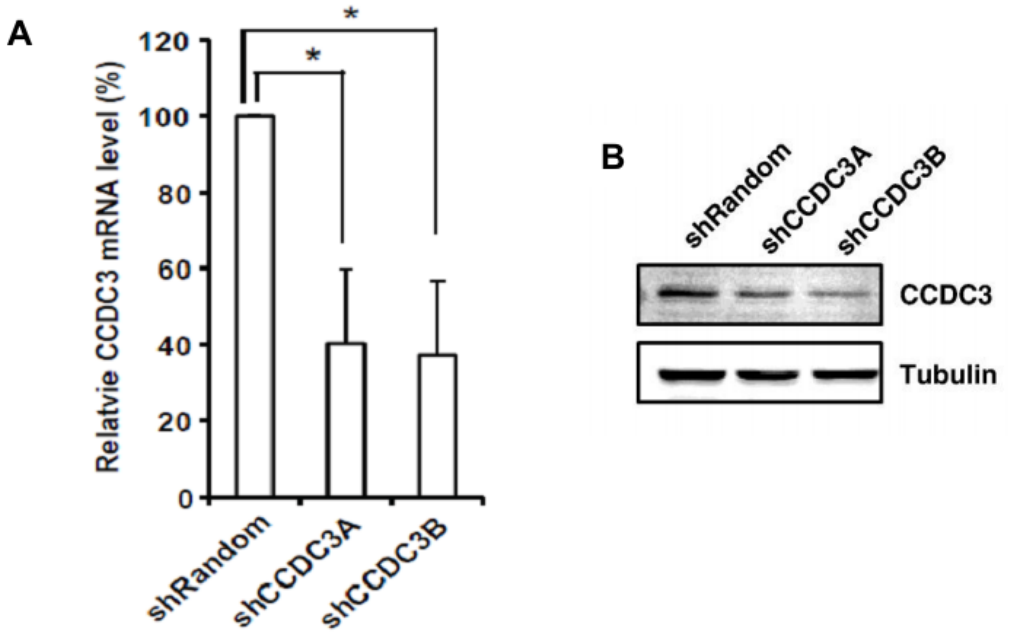

Figure 8: Knockdown of CCDC3 in HMEC-1 cells. Expression of CCDC3 in HMEC-1 cells was stably knocked down by two shRNAs (shCCDC3A and shCCDC3B) targeting two distinct sequences of CCDC3 genes. shRandom that does not target any known gene was used a control. Knockdown of CCDC3 was confirmed by real time PCR **(A)** and by Western blotting **(B)** using a mouse monoclonal antibody against human CCDC3. Tubulin was used as the loading control.

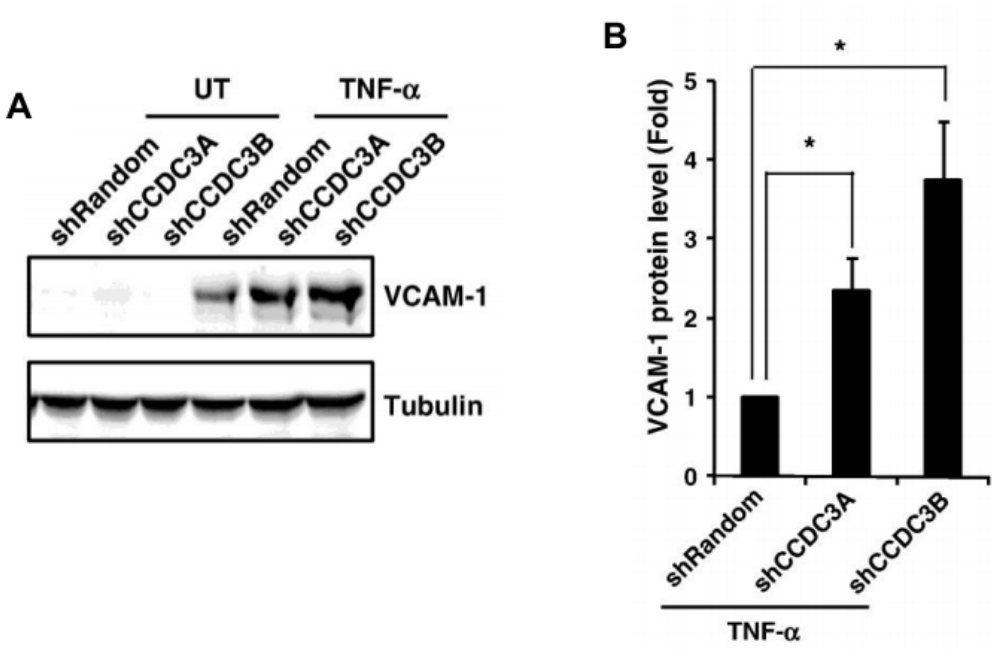

Figure 9: CCDC3 knockdown enhances TNF-α-induced VCAM-1 expression in ECs. (A) The shRandom and shCCDC3 HMEC-1 cells were left untreated or treated with 10 ng/ml TNF-α for 24h. Protein levels of VCAM-1 were examined by Western blotting. Tubulin was used as the loading control. **(B)** Quantification of Western blot data by densitometry analysis. The expression of VCAM-1 in TNF-α-treated shRandom cells was designated as 1 and shown as mean ± SEM of three independent experiments. *Significantly different ($P < 0.05$).

3.3 TNF-α-induced VCAM-1 expression in ECs is NF-κB-dependent

Multiple signaling pathways are activated by TNF-α stimulation. For example, MAPK and NF-κB pathways have been shown to be activated by TNF-α and to be involved in TNF-α-induced expression of cell adhesion molecules including E-selectin and VCAM-1 [73, 74]. To determine if NF-κB signaling is required for TNF-α-induced VCAM-1 expression in ECs, we used Bay 11-7082 (an inhibitor of IKK) compound to block NF-κB signaling.

We pre-treated HMEC-1 cells with 5 μM Bay 11-7082 for 2h to inhibit cytokine-induced IκB phosphorylation and hence NF-κB activation prior to TNF-α treatment (10 ng/ml) for 1h, 4h or 24h. TNF-α-induced expression of VCAM-1 was measured by Western blotting. As shown in ***Fig. 10***, Bay 11-7082 completely abolished TNF-α-induced VCAM-1 expression in HMEC-1 cells, confirming that TNF-α-induced VCAM-1 expression in HMEC-1 cells depends on NF-κB activation.

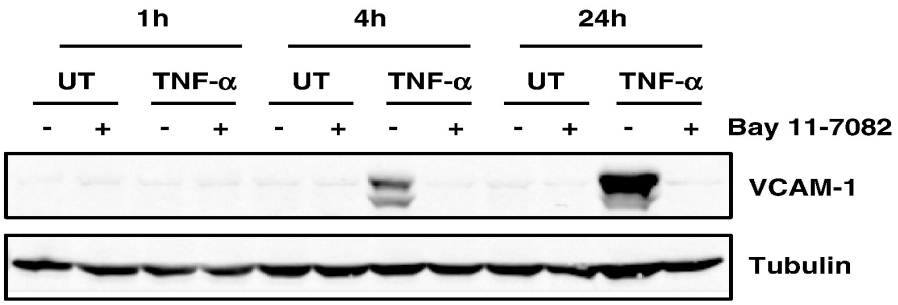

Figure 10: TNF-α-induced VCAM-1 expression in ECs is NF-κB-dependent. HMEC-1 cells were pretreated with 5 μM Bay 11-7082 for 2h, and then left untreated or treated with 10 ng/ml TNF-α for 1h, 4h or 24h. TNF-α-induced VCAM-1 expression was examined by Western blotting. Tubulin was used as the loading control.

3.4 CCDC3 overexpression inhibits TNF-α-induced NF-κB activation in ECs

Since TNF-α-induced expression of VCAM-1 in ECs is NF-κB-dependent, we then investigated whether the inhibitory effect of CCDC3 is mediated by repressing TNF-α-induced NF-κB activation. Upon activation, NF-κB dimers, such as the p50 and p65 heterodimer, are released from cytosolic inhibitory protein IκB and translocate to the nucleus where NF-κB dimers bind to the promoters of the target genes and regulate their expression [85-87]. We therefore examined whether CCDC3 overexpression affected TNF-α-induced nuclear translocation of p50 and p65 in HMEC-1 cells. We treated HMEC-1/Vector and HMEC-1/CCDC3-FLAG with 10 ng/ml TNF-α for 1h and collected cytosolic and nuclear fractions for Western blotting analysis. As shown in ***Fig. 11***, CCDC3 overexpression decreased TNF-α-induced p50 and p65 nuclear translocation, suggesting that CCDC3 overexpression represses TNF-α-induced NF-κB activation in ECs.

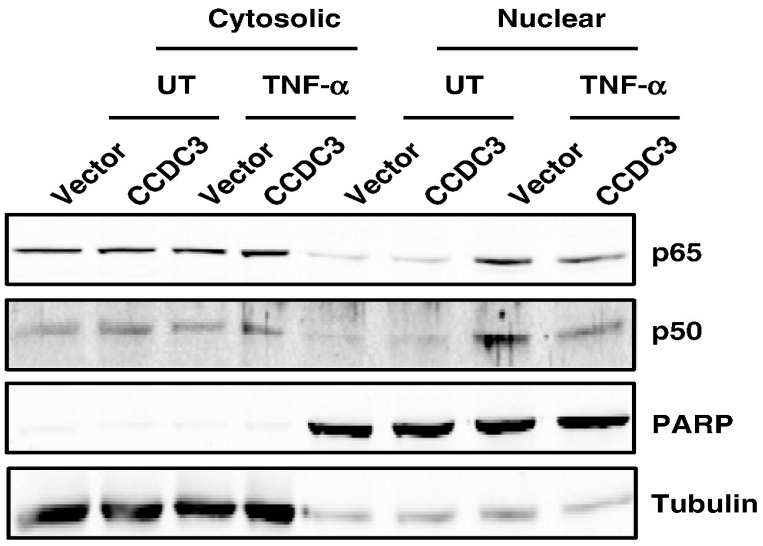

Figure 11: CCDC3 inhibits nuclear translocation of p50 and p65. Cytosolic and nuclear fractions were prepared from HMEC-1/Vector and HMEC-1/CCDC3 that were left untreated or treated with 10 ng/ml TNF-α for 1h. Protein levels of p65 and p50 in the cytosolic and nuclear fractions were examined by Western blotting. PARP was used as nuclear loading control and tubulin was used as cytosolic loading control. Two independent experiments were performed that showed similar results.

To further confirm that CCDC3 represses TNF-α-induced NF-κB activity, we performed a luciferase based assay. We determined the effect of CCDC3 overexpression on nuclear NF-κB transcriptional activity by using a firefly luciferase reporter driven by a promoter containing three NF-κB binding sites. In the presence of high levels of active NF-κB, we would expect to see high levels of luciferease activity, and a low level of luciferase activity would suggest inhibition NF-κB activity.

HMEC-1 cells were transiently transfected with the NF-κB firefly luciferase reporter and a Renilla luciferase reporter driven by a constitutively active CMV promoter (a transfection control). 24h post-transfection, the cells were left untreated or treated with 10 ng/ml TNF-α for 24h. Nuclear NF-κB activity was then determined by dual luciferase assay. As shown in ***Fig. 12***, CCDC3 overexpression decreased TNF-α-induced NF-κB activity by 36.1%. Together, our results indicate that CCDC3 inhibits TNF-α-induced NF-κB activation in ECs.

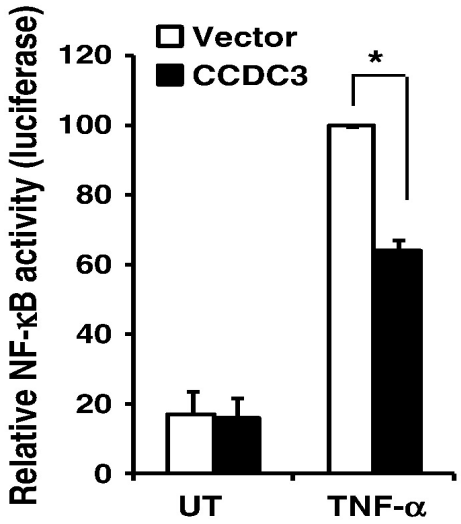

Figure 12: CCDC3 overexpression inhibits TNF-α-induced NF-κB activation in ECs. HMEC-1/Vector and HMEC-1/CCDC3 were transiently transfected with a NF-κB firefly luciferase reporter (NF-κB reporter) driven by a promoter containing three NF-κB binding sites and a Renilla luciferase reporter as a transfection control. 24h after transfection, cells were treated with 10 ng/ml TNF-α for 24h. Cell lysates were then collected and luciferase activity in the cell lysates was measured. The NF-κB reporter activity was determined by normalizing the firefly luciferase activity to the Renilla luciferase activity. The relative NF-κB activity in HMEC-1/Vector treated with TNF-α was set as 100 and shown as mean ± SEM of three independent experiments. *Significantly different ($P < 0.05$).

3.5. CCDC3 represses TNF-α-induced VCAM-1 expression in a paracrine/autocrine manner

CCDC3 has an N-terminal signal peptide for secretion and the published papers also demonstrated that CCDC3 is a secreted protein [105, 115]. Therefore, we wanted to investigate whether CCDC3 also affects TNF-α signaling in a paracrine/autocrine manner. In order to determine if the secreted CCDC3 represses TNF-α-induced gene expression in ECs, we collected CM from HMEC-1/vector and HMEC-1/CCDC3-FLAG and applied the CM to HMEC-1 cells. The presence of CCDC3 in the CM was confirmed by Western blotting using the mouse monoclonal antibody against CCDC3 (***Fig. 13A***).

CM with and without CCDC3 was applied to HMEC-1 cells. After overnight incubation in the CM, the receiving HMEC-1 cells were left untreated or treated with 10 ng/ml TNF-α for 20h. Western blotting showed that the CCDC3 CM decreased TNF-α-induced expression of VCAM-1 in the receiving HMEC-1 cells by 57.3% compared to vector CM (***Fig. 13B and 13C***), suggesting that CCDC3 also represses TNF-α-induced expression via a paracrine/autocrine mechanism.

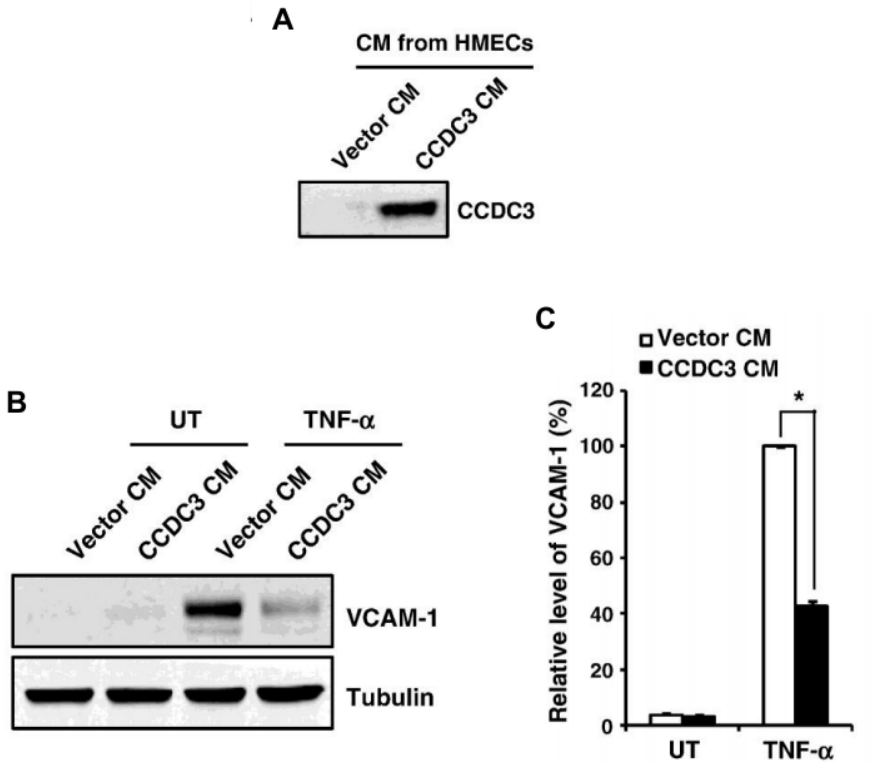

Figure 13: CCDC3 CM collected from HMEC-1 cells inhibits TNF-α-induced VCAM-1 expression in ECs. (A) CCDC3 in CM collected from HMEC-1/Vector and HMEC-1/CCDC3 was confirmed by Western blotting. **(B)** HMEC-1 cells were cultured in the CM collected from HMEC-1/Vector and HMEC-1/CCDC3 overnight and then were left untreated or treated with 10 ng/ml TNF-α for 20h. Protein levels of VCAM-1 in the receiving HMEC-1 cells were examined by Western blotting. **(C)** Quantification of Western blotting data by densitometry analysis. Tubulin was used as the loading control. The expression of VCAM-1 in TNF-α-treated Vector CM cells was designated as 100 and shown as mean ± SEM of three independent experiments. *Significantly different ($P < 0.05$).

CM collected from CCDC3 overexpressed in HMEC-1 cells may contain many different bioactive molecules secreted by ECs. We therefore wanted to test whether CM collected from non-endothelial cells inhibits TNF-α-induced expression of VCAM-1 in HMEC-1 cells. To test this, CM was collected from HEK 293T cells that were transiently transfected with MSCVpac or MSCVpac-CCDC3-FLAG. Secretion of CCDC3 into the CM was confirmed by Western blotting using the mouse monoclonal anti-CCDC3 antibody (***Fig. 14A***).

CCDC3 CM collected from HEK 293T cells was applied to HMEC-1 cells. After overnight incubation, cells were left untreated or treated with 10 ng/ml TNF-α for 20h. Similar to CM collected from HMEC-1 cells, the CCDC3 CM collected from HEK 293T cells inhibited TNF-α-induced expression of VCAM-1 in receiving HMEC-1 cells by 51.7% (***Fig. 14B and 14C***). These results demonstrate that CCDC3 CM collected from CCDC3-overexpressing HMEC-1 cells and HEK 293 cells inhibits TNF-α-induced gene expression.

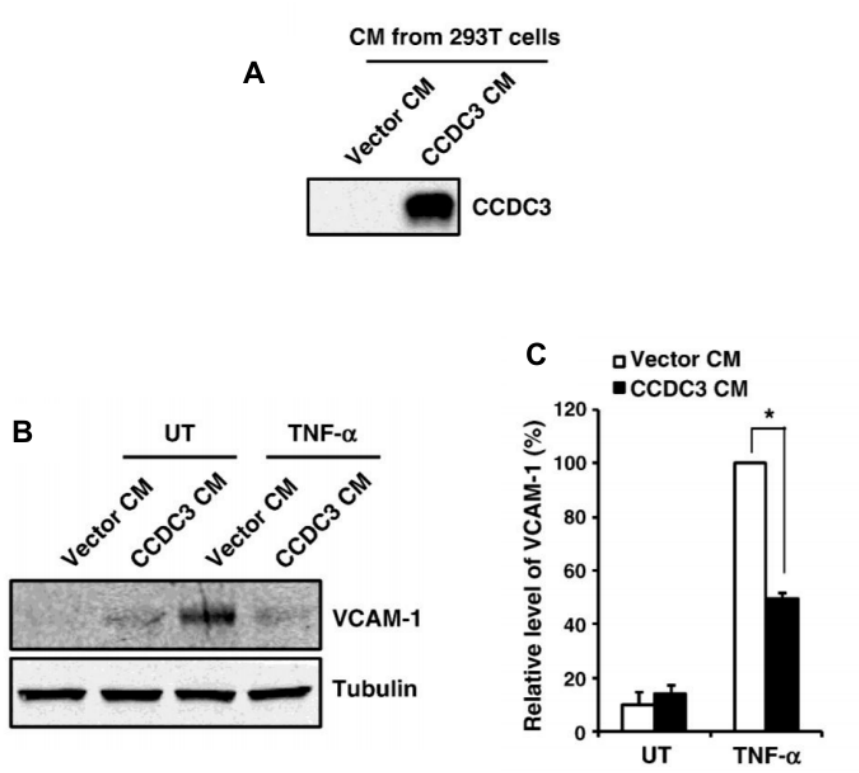

Figure 14: CCDC3 CM collected from HEK 293T inhibits TNF-α-induced VCAM-1 expression in ECs. (A) CCDC3 in CM collected from HEK 293T cells transiently transfected with empty vector MSCVpac or MSCVpac-CCDC3-FLAG was confirmed by Western blotting. **(B)** HMEC-1 cells were cultured in the CM collected from HEK 293T cells overnight and then were left untreated or treated with 10 ng/ml TNF-α for 20h. Protein levels of VCAM-1 in the receiving HMEC-1 were examined by Western blotting. **(C)** Quantification of Western blotting data by densitometry analysis. Tubulin was used as the loading control. The expression of VCAM-1 in TNF-α-treated Vector CM cells was designated as 100 and shown as mean ± SEM of three independent experiments. *Significantly different ($P < 0.05$).

3.6 Purified CCDC3 protein inhibits TNF-α-induced VCAM-1 expression in ECs

To further confirm that the effect of CCDC3 containing CM on TNF-α-induced VCAM-1 expression is a direct consequence of CCDC3, we transiently transfected HEK 293T cells with MSCVpac or MSCVpac-CCDC3-FLAG and purified CCDC3-FLAG from the CM using anti-FLAG M2 affinity gel. The presence of CCDC3 in the CM before purification, depletion of CCDC3 from the CM after purification and presence of the purified CCDC3 in the eluate were confirmed by Western blotting (***Fig. 15A***).

We incubated HMEC-1 cells with the purified CCDC3 overnight and then left the cells untreated or treated with 10 ng/ml TNF-α for 20h. Western blotting showed that, similar to CCDC3 CM, purified CCDC3 inhibited TNF-α-induced expression of VCAM-1 in HMEC-1 cells by 51.0% (***Fig. 15B and 15C***).

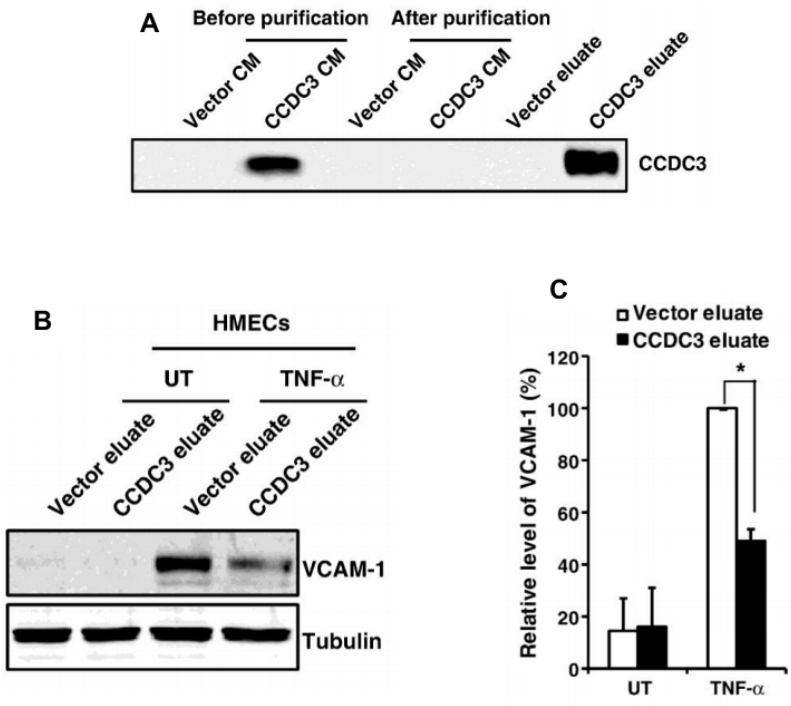

Figure 15: Purified CCDC3 represses TNF-α-induced VCAM-1 expression in HMEC-1 cells. CM was collected from HEK 293T cells transiently transfected with empty vector MSCVpac or MSCVpac-CCDC3-FLAG. CCDC3 in the CM was purified using the anti-FLAG M2 affinity gel. **(A)** CCDC3 in the CM before purification, depletion of CCDC3 after purification and purified CCDC3-FLAG in the eluate were confirmed by Western blotting. (**B)** HMEC-1 cells were cultured in the medium with Vector eluate (no CCDC3) or with CCDC3 eluate (purified CCDC3) overnight and then left untreated or treated with TNF-α for 20h. Protein levels of VCAM-1 in the receiving HMEC-1 cells were examined by Western blotting. **(C)** Quantification of Western blot data by densitometry analysis. Tubulin was used as the loading control. The expression of VCAM-1 in TNF-α-treated Vector eluate cells was designated as 100 and shown as mean ± SEM of three independent experiments. *Significantly different ($P < 0.05$).

Since HMEC-1 is an immortalized cell line, we wanted to determine the inhibitory effect of CCDC3 in primary cells. To this end, we treated primary cultures of HUVECs of three individual isolations with purified CCDC3 and examined the TNF-α-induced expression of VCAM-1 expression. Western blotting confirmed that purified CCDC3 inhibited TNF-α-induced expression of VCAM-1 in HUVECs by 42.5% (***Fig. 16A and 16B***). Taken together, these results demonstrate that the effect of CCDC3 CM on TNF-α-induced gene expression is a direct consequence of CCDC3 in the CM and CCDC3 acts as a paracrine/autocrine factor to negatively regulate TNF-α-induced gene expression in ECs.

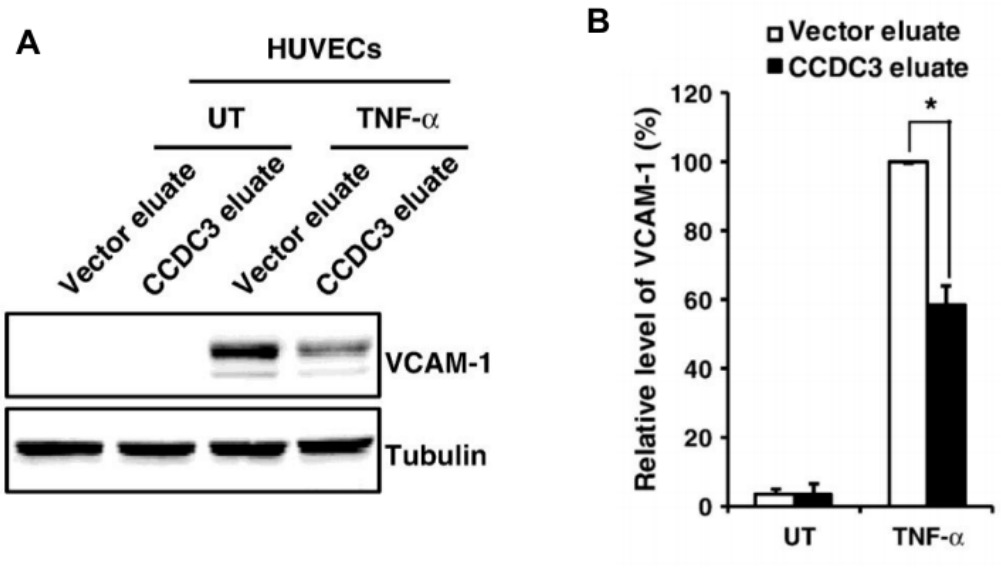

Figure 16: Purified CCDC3 represses TNF-α-induced VCAM-1 expression in HUVECs. **(A)** HUVECs were cultured in the medium with Vector eluate (no CCDC3) or with CCDC3 eluate (purified CCDC3) overnight and then left untreated or treated with TNF-α for 20h. Protein levels of VCAM-1 in the receiving HUVECs were examined by Western blotting. **(B)** Quantification of Western blotting data by densitometry analysis. Tubulin was used as the loading control. The expression of VCAM-1 in TNF-α-treated Vector eluate cells was desigatned as 100 and shown as mean ± SEM of three independent experiments. *Significantly different ($P < 0.05$).

3.7 CCDC3 in CM is taken up by the receiving ECs

To determine whether CCDC3 in CM can enter the receiving HMEC-1 cells, we generated a vector expressing human CCDC3 fused with the red fluorescent protein mApple at the carboxyl terminus of CCDC3. HEK 293T cells were transiently transfected with mApple alone or CCDC3-mApple vector. The presence of CCDC3-mApple in CM was confirmed by Western blotting (***Fig 17A***).

Similar to CCDC3-FLAG CM shown in ***Fig. 13B and 14B***, CCDC3-mApple CM inhibited TNF-α-induced VCAM-1 expression in the receiving HMEC-1 cells (***Fig. 17B***), confirming that the mApple fused to the carboxyl terminus of CCDC3 does not affect the CCDC3 inhibitory effect on VCAM-1 expression.

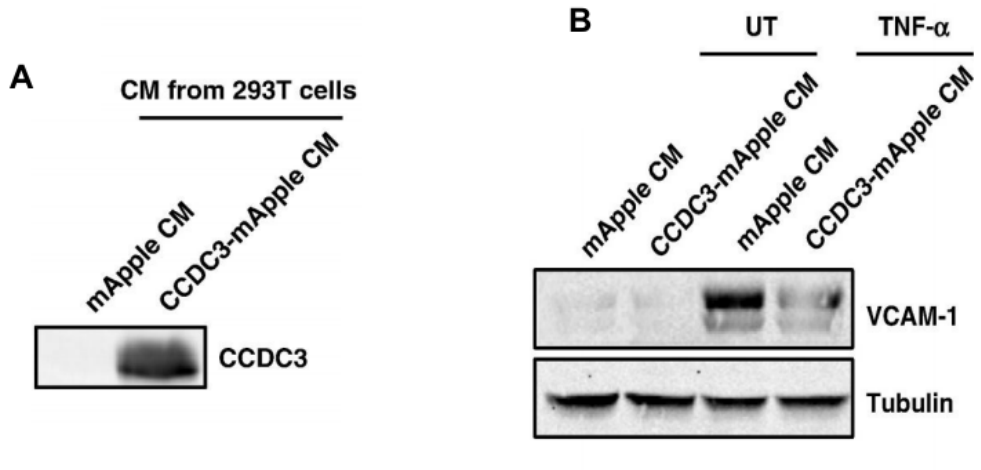

Figure 17: CCDC3-mApple fusion protein retains the inhibitory effect of CCDC3 in TNF-α-induced VCAM-1 expression in ECs. (A) HEK 293T cells were transiently transfected with mApple alone or CCDC3-mApple vector. The presence of CCDC3-mApple in the CM collected from 293T/CCDC3-mApple cells was confirmed by Western blotting. **(B)** HMEC-1 cells were cultured in the mApple alone or CCDC3-mApple CM overnight and then left untreated or treated with TNF-α for 20h. Protein levels of VCAM-1 in the receiving HMEC-1 cells were examined by Western blotting. Tubulin was used as the loading control.

To determine whether CCDC3-mApple could enter the receiving HMEC-1 cells, we incubated HMEC-1 cells with mApple alone or CCDC3-mApple CM for 20h. The receiving HMEC-1 cells were then fixed and stained with DAPI for the nucleus. Fluorescence microscopy showed that CCDC3-mApple was taken up by the receiving HMEC-1 cells (***Fig. 18***) and accumulated around the nucleus.

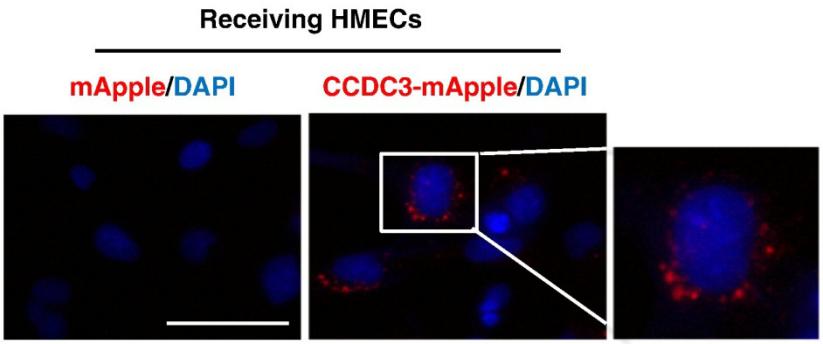

Figure 18: CCDC3 in CM is taken up by the receiving ECs. HMEC-1 cells were cultured in mApple alone or CCDC3-mApple CM overnight. Uptake of CCDC3-mApple by the receiving HMEC-1 cells was determined by fluorescence microscopy. DAPI was used to stain the nucleus. Scale bar, 50 μm.

To further confirm the uptake of CCDC3 by the receiving HMEC-1 cells, we incubated HMEC-1 cells with vector or CCDC3-FLAG CM overnight. Cell lysates were prepared from the receiving HMEC-1 cells after thorough washing to remove the extracellular CCDC3-FLAG. Western blotting results showed that CCDC3-FLAG was present in the cell lysates (***Fig. 19***), indicating that CCDC-FLAG in CM was taken up by the receiving HMEC-1 cells. Together, we demonstrate that CCDC3 in CM can enter the receiving cells, suggesting that CCDC3 may regulate intracellular signaling events in receiving cells.

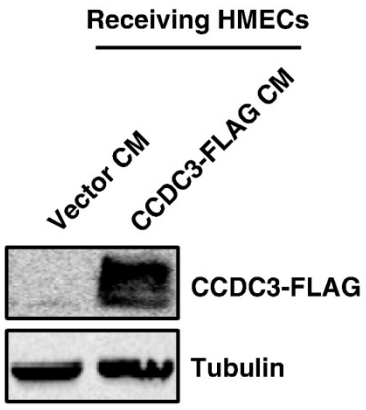

Figure 19: CCDC3 in CM is taken up by the receiving ECs. HMEC-1 cells were cultured overnight in Vector or CCDC3-FLAG CM collected from HEK 293T cells. Uptake of CCDC3-FLAG in the CM by the receiving HMEC-1 cells was examined by Western blotting. Tubulin was used as the loading control.

Chapter Four

Discussion and Future Directions

4.1 CCDC3 inhibits TNF-α-induced VCAM-1 expression in ECs

Atherosclerosis is a disease of ongoing inflammatory response on vascular ECs and characterized by thickening and hardening of artery walls due to accumulation of lipids and fibrous elements inside the blood vessels [39]. Since endothelial inflammation plays an important role in initiation and progression of atherosclerosis, there is a strong interest in developing novel therapies targeting the endothelial inflammatory response.

TNF-α is a central mediator of inflammation and is involved in initiation and progression of atherosclerosis [61]. TNF-α participates in inflammatory reaction by rapidly inducing the expression of CAMs in ECs through activation of TNFR1 [116]. TNF-α-induced activation of TNFR1 also limits the production of NO, which acts as a known inhibitor of CAMs expression in ECs [117, 118]. For this reason inhibition of TNFR1 is an attractive target for the development of anti-atherosclerosis therapy. Interestingly, Xanthoulea *et al.* showed that TNFR1 deficient mice reduce the expression of CAMs and develop significantly smaller size of atherosclerotic lesions compared to the wild type mice [119]. Moreover, $ApoE^{-/-}$ mice deficient in TNF-α exhibits 50% reduced lesion size compared to control mice [120]. These studies clearly indicate a role of TNF-α in the development of atherosclerosis.

In response to inflammation, the recruitment of monocytes to the site of infection and their subsequent transmigration through endothelial basement membrane to the subintimal inflammatory foci is the initial step of atherosclerosis development [59, 121]. The recruitment of circulating monocytes to inflammatory sites is a multi-step process that is predominantly mediated by different classes of CAMs expressed on the vascular ECs. The initial tethering and rolling of monocytes to the activated ECs is mediated by different selectin molecules such as L-, P-, and E-selectins [122, 123]. In the next step, monocytes firmly attach to the ECs primarily through interaction with VCAM-1 and ICAM-1 [49]. In the context of atherosclerosis, high level of VCAM-1, ICAM-1 and L-selectin expression has been observed on ECs overlaying atherosclerotic lesions and fatty streak [124, 125]. However, multiple studies have showed that VCAM-1 is most critical in mediating monocytes recruitment to the early atherosclerotic lesion site [51, 101, 126].

It has been shown that in a dietary-induced atherosclerosis rabbit model, the expression of VCAM-1 is rapidly increased by ECs in areas prone to lesion formation [51]. VCAM-1 interacts with its integrin counter receptor VLA4 ($\alpha_4\beta_1$) present on the activated leukocytes to promote their recruitment to the site of inflammation. Patel *et al.* showed that blocking antibody against the α-subunit of the $\alpha_4\beta_1$ integrin reduces macrophages migration to the atherosclerotic sites in $ApoE^{-/-}$ mice [127]. Interestingly, blocking antibody against VCAM-1 itself

significantly decreases the development of atherosclerosis in ApoE$^{-/-}$ mice [128]. Moreover, Cybulsky *et. al.* showed that ApoE$^{-/-}$ mice with VCAM-1 molecule lacking the fourth IgG domain significantly reduces atherosclerotic lesion formation [101]. Therefore, these data proved that VCAM-1 is strongly involved in the development of atherosclerosis.

As CCDC3 expression is downregulated by TNF-α in ECs [105], we investigated whether CCDC3 could modulate TNF-α-induced endothelial inflammation. We demonstrated that overexpression of CCDC3 inhibits TNF-α-induced VCAM-1 expression in ECs (***Fig. 6B and 7***), and knockdown of endogenous CCDC3 increases TNF-α-induced VCAM-1 expression in ECs (***Fig. 9***). Our findings indicate that CCDC3 negatively regulates TNF-α-induced VCAM-1expression in ECs. However, our findings are made in cultured endothelial cells and whether CCDC3 plays a role in atherosclerosis needs to be investigated using *in vivo* models.

4.2 CCDC3 inhibits TNF-α-induced activation of NF-kB in ECs

In response to inflammation, TNF-α prominently exerts its biological effects through activation of transcription factor NF-κB [77, 79]. The activation of NF-κB leads to upregulation of a wide range on inflammatory molecules such as IL-6, IL-8, TNF-α, MCP-1, MMPs and CAMs, which are all implicated in the

development and progression of atherosclerosis [77, 129]. The involvement of NF-κB in atherosclerosis has been elucidated by the detection of nuclear translocation of NF-κB subunit p65 in the intima of atherosclerotic lesion, in SMCs and in macrophages within the lesion site [130, 131]. In a seminal study, Gareus *et al.* showed that inhibition of EC-specific NF-κB protects mice from atherosclerosis due to diminished expression of CAMs in ECs and the subsequent impairment in recruiting macrophages to atherosclerotic plaques [132]. Therefore, it is evident that inhibition of NF-κB activation could prevent atherosclerosis development.

Several studies showed that TNF-α stimulates the expression of CAMs in ECs through the activation NF-κB and p38/MAPK [73, 74]. However, multiple studies have shown that NF-κB alone is sufficient to induce the expression of CAMs in ECs [73, 133]. In our current study we also showed that TNF-α-induced VCAM-1 expression is NF-κB-dependent (***Fig. 10***). Furthermore, we showed that CCDC3 inhibits TNF-α-induced nuclear translocation of p65 and p50 (***Fig. 11***) and NF-κB transcriptional activity as measured by a luciferase reporter assay (***Fig. 12***), indicating that CCDC3 inhibits TNF-α-induced VCAM-1 expression by inhibiting the nuclear translocation of p65 and p50 and thereby blocking NF-κB transcriptional activity. These results suggest that CCDC3 represses some molecular event(s) upstream of NF-κB activation induced by TNF-α in ECs.

However, the molecular mechanism underlying the inhibitory effect of CCDC3 on these events is not clear. Further mechanistic studies are needed to determine at which steps CCDC3 inhibits NF-κB activation in order to better understand the molecular function of CCDC3. Since CCDC3 has a coiled-coil interacting domain, it will be interesting to test if CCDC3 interacts with the TNF-α receptor complex, thereby inhibiting downstream signaling pathway. CCDC3 can also potentially interact with the components of NF-κB signaling pathway downstream of TNFR activation to repress NF-κB activation.

4.3 CCDC3 is a EC-derived secretory factor and has a paracrine/autocrine function

ECs play a critical role in the cardiovascular system during embryonic development and in adults by generating and releasing many bioactive molecules including small molecules (e.g., NO), cytokines (e.g., IL-6 and TNF-α), and growth factors (e.g., VEGF). These factors exert significant autocrine, paracrine and endocrine actions and thus influence vascular development, SMC proliferation and coagulation [9, 134]. For example, NO is a key molecule secreted from ECs that maintains vascular integrity by inhibiting platelet aggregation, ECs and leukocyte adhesion and SMC proliferation [135-137]. NO also has anti-inflammatory effects that limit the expression of VCAM-1,

suggesting a potential anti-atherosclerosis role of NO [23, 138]. Interestingly, Kuhlencordt *et al.* demonstrated that knockout of eNOS (generates NO) in $ApoE^{-/-}$ (eNOS/ApoE double knockout) mice significantly increases atherosclerosis development compared to $ApoE^{-/-}$ control mice [139].

In this current study we showed that CCDC3 is an EC-derived secreted protein which functions in a paracrine/autocrine manner. At first, we confirmed that CCDC3 is a secreted protein by detecting the presence of CCDC3 in culture medium (***Fig. 13A***). Then we showed that, similar to CCDC3 overexpression, CCDC3 in the medium also inhibits CCDC3 TNF-α-induced VCAM-1 expression (***Fig. 13B***). Our results thus suggest that CCDC3 negatively regulates TNF-α signaling not only via a cell autonomous manner but also via a paracrine /autocrine mechanism.

We further confirmed that the CCDC3 CM effect is a direct consequence of CCDC3. Firstly, we used CM collected from non-endothelial (HEK 293T) cells overexpressed with CCDC3 to exclude the effect of other bioactive molecules that might be present in the CCDC3 CM collected from ECs. In our study we showed that similar to HMEC-1 cell CM, the CCDC3 CM collected from HEK 293T also inhibits TNF-α-induced VCAM-1 expression in ECs (**Fig. *14B***). Secondly, we purified CCDC3 from CM and found that purified CCDC3 also exhibits a similar inhibitory effect as CCDC3 CM (**Fig. *15B and 16A***), suggesting that it is CCDC3,

and not other endothelial factors secreted by ECs, that inhibit TNF-α-induced VCAM-1 expression in the receiving ECs. Together, our results clearly demonstrate that CCDC3 is a paracrine/autocrine factor and may play a potential anti-inflammatory and thus athero-protective role in the cardiovascular system.

4.4 Entry of CCDC3 into the receiving cells

We demonstrated that CCDC3 as a secreted protein enters the receiving HMEC-1 cells (***Fig. 18 and 19***) and inhibits TNF-α-induced VCAM-1 expression (***Fig. 17B***). One of the most common mechanisms involved in extracellular protein internalization is receptor-mediated endocytosis. In endocytosis, the ligand-receptor complex is internalized through lipid vesicles called endosomes. Although endocytosis has been viewed as a signal-terminating process via degradation of ligand-receptor complexes, accumulating evidence suggests that endocytosis actively participates in many signal transduction pathways via signaling from endosomes [140, 141]. For example, upon binding to its receptor, transforming growth factor beta (TGF-β) undergoes internalization where the endosomal scaffolds SARA (smad anchor for receptor activation) enhances TGF-β signaling by bringing its phosphorylation targets such as mothers against decapentaplegic homolog 2 (SMAD2) and SMAD3 in close proximity [142]. Here, we showed that upon entering the receiving HMEC-1 cells, CCDC3 is

potentially localized around the nuclear region. It is our future interest to investigate the molecular mechanisms underlying the entry of CCDC3 into the receiving cells and to identify the intracellular proteins that interact with CCDC3 in the receiving cells.

4.5 *In vivo* function of CCDC3

After the publication of our manuscript [143], an *in vivo* study of CCDC3 was been reported [144]. Kobyashi *et al*. generated CCDC3 knockout mice and showed that CCDC3 knockout mice are leaner and have decreased fat mass and smaller adipocyte size compared to wild type mice [144]. They demonstrated that knockout of CCDC3 decreases the expression of lipogenic genes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) in adipose tissue, indicating that CCDC3 is involved in lipid accumulation [144]. In their previous study, Kobyashi *et al*. showed that CCDC3 is expressed in murine aorta [105], however, in this study the authors did not find any morphological changes in aorta of CCDC3 knockout mice [144]. Our study with a cell culture model showed that CCDC3 attenuates TNF-α-induced inflammation in ECs, suggesting that CCDC3 might have significant biological effects in pathological settings in the vascular system. However, the vascular function of CCDC3 needs to be further

investigated using *in vivo* models (more details are indicated in future direction section ***4.6.3***).

4.6 Future directions

4.6.1 To identify the receptor(s) that mediate CCDC3 entry

To identify the receptors that interact with CCDC3, we will use a ligand-based receptor-capture (LRC) technology as described by Frei *et al.* [145]. In this method a specifically designed chemoproteomic reagent (TRICEPS) will be used to couple to CCDC3 for the subsequent ligand-based capture of corresponding receptors on living ECs. TRICEPS contains three functional moieties: one that enables conjugation to an amino group containing ligands, a second for the ligand-based capture of glycosylated receptors on gently oxidized living cells and a biotin tag for purification of receptor peptides for analysis by quantitative mass spectrometry. This LRC technology has been shown to be an unbiased and sensitive approach to identify specific receptors for a given ligand under near physiological conditions [145].

4.6.2 To identify the intracellular proteins that interact with CCDC3

To identify the proteins that interact with CCDC3 in HMEC-1 cells, we will collect cell lysates from HMEC-1/Vector and HMEC-1/CCDC3-FLAG and

perform an immunoprecipiation (IP) using the anti-FLAG M2 affinity gel. CCDC3-FLAG and its interacting partners in the HMEC-1/CCDC3-FLAG will be pulled down by the antibody. Any proteins that are pulled down by the antibody in HMEC-1/Vector will be non-specific proteins due to the nature of the antibody. Proteins that are eluted from the anti-FLAG M2 gel will be separated by SDS-PAGE and stained using Coomassie Brilliant Blue. Protein bands that are present only in HMEC-1/CCDC3-FLAG but not in HMEC-1/Vector will be cut out from the gel. The gel slices will be applied to an ingel trypsin digestion. The extracted peptides will be analyzed by mass spectrometry to identify the proteins that interact with CCDC3. This experiment will help us to elucidate the molecular mechanisms underlying the inhibitory effect of CCDC3 in TNF-α-induced VCAM-1 expression.

4.6.3 To investigate the role of CCDC3 in atherosclerosis in vivo

We will also test the effect of CCDC3 in an atherosclerosis *in vivo* mice model. So far, ApoE and LDLR knockout mice are widely used to investigate molecular mechanisms underlying atherosclerosis [103]. These mice readily develop atherosclerotic lesions when fed with a high fat diet [103, 146]. Since CCDC3 negatively regulates TNF-α-induced VCAM-1 expression, we will introduce CCDC3 protein intravenously in $ApoE^{-/-}$ and $LDLR^{-/-}$ mice and test if

CCDC3 has an atheroprotective role by reducing the expression of VCAM-1 and a subsequent decrease in monocyte recruitment in the atherosclerotic lesion site.

Recently, Kobayashi *et al.* used CCDC3 knockout mice to investigate the involvement of CCDC3 in lipid metabolism in adipocytes [144]. Given that CCDC3 knockout mice are viable, we will use this mice model in the background of $ApoE^{-/-}$ and $LDLR^{-/-}$ to investigate the role of CCDC3 in atherosclerosis. If CCDC3 indeed has an atheroprotective role, we would expect to see accelerated rate of atherosclerosis development in $CCDC3^{-/-}$ $ApoE^{-/-}$ and $CCDC3^{-/-}$ $LDLR^{-/-}$ mice compared to the respective control mice after feeding a high fat diet.

4.6.4 To further characterize the role of CCDC3 in angiogenesis

Angiogenesis is a process by which new blood vessels are formed from pre-existing ones and is involved in normal growth and development, as well as in wound healing [147, 148]. It is also an essential process for tumor growth and metastasis whereby continuous supply of oxygen and nutrients are ensured [149].

A large variety of pro- and anti-angiogenic factors regulate the angiogenesis process. *In vitro* tube formation assay is most widely used to investigate the reorganization stage of angiogenesis [150]. To determine the role of CCDC3 on tube formation in ECs, we stably knockeddown the expression of

CCDC3 in HUVECs using the same lentivirus-delivered shRNA approach as used for making CCDC3 knockdown in HMEC-1 cells. HUVECs/shRandom, HUVECs/shCCDC3A and HUVECs/shCCDC3B cells were seeded in 96-well plates containing matrigel containing M199 medium supplemented with 20% FBS and 1% ECGS, then the images were captured under microscope after 6h incubation at 37^0C. Our preliminary results showed that stable knockdown of CCDC3 reduced tube formation of HUVECs (***Fig. 20***).

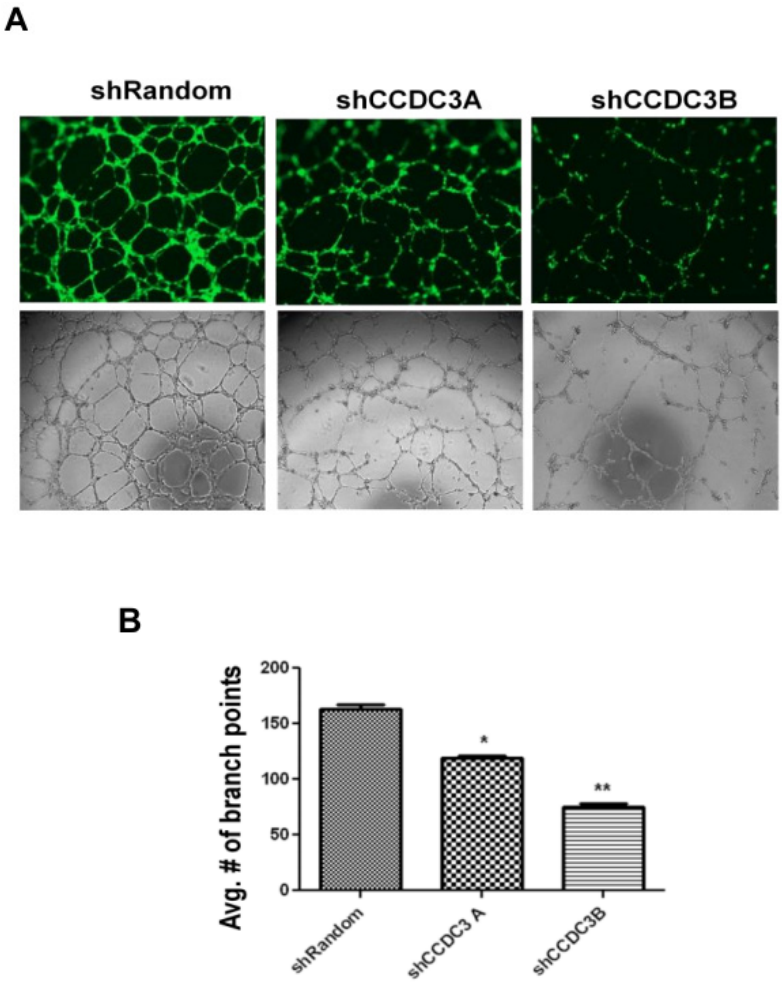

Figure 20: Knockdown of CCDC3 decreases tube formation in HUVECs. (A) Expression of CCDC3 was stably knocked down in HUVECs by using two shRNAs (shCCDC3A and shCCDC3B). shRandom is the negative control. Cells were seeded in 96-well plates containing matrigel. Images of the tubular network were captured at 6 h under a fluorescence microscope (shRNA constructs express green fluorescent protein). **(B)** Tube formation was quantified by counting the branch points of the tubular network on three random fields. *Significantly different ($P < 0.05$) from the shRandom cells.

In the future, we would like to validate these results *in vivo* by using the onplant chick chorioallantoic membrane (CAM) angiogenesis assay. Briefly, a collagen onplant that has a large bottom mesh (4 x 4 mm) and a smaller top mesh (2 x 2 mm) overlaid on the bottom mesh at a slight angle is applied to the CAM of an ex ovo chicken embryo to provide an avascular environment adjacent to the richly vascularized capillary bed [151]. The inclusion of defined pro-angiogenic factors (e.g., VEGF) reproducibly induces angiogenesis that can be quantified by counting the blood vessels that grow into the onplant using the mesh grid as a scoring array. To determine the role of CCDC3 in angiogenesis using this model, we will use collagen onplants that contain ECs with stable knockdown of CCDC3 vs. shRandom control and with or without purified CCDC3. These onplants will be applied to ex ovo chicken embryos and blood vessels that grow into the onplants will be quantified to determine whether purified CCDC3 promotes, while CCDC3 knockdown decreases, angiogenesis *in vivo*.

4.7 Conclusions

In this study we, for the first time, showed that overexpression of CCDC3 inhibits while knockdown of CCDC3 increases the TNF-α-induced VCAM-1 expression in ECs. As a secreted protein, CCDC3 blocks TNF-α-induced inflammatory gene expression via a paracrine mechanism. We further showed that

CCDC3 inhibits TNF-α-induced inflammatory pathway by attenuating the activation of NF-κB. Taken together our findings suggest that CCDC3 is a functional molecule in ECs and may have a potential anti-inflammatory and atheroprotective role of CCDC3 in the vascular system. Our results also suggest that CCDC3 may be involved in angiogenesis. However, the physiological function of CCDC3 needs to be further investigated using in vivo models.

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