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Functional association of a single nucleotide polymorphism in the human CD40 gene with the pathogenesis of atherosclerosis

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Abbreviations

ACS	Acute coronary syndrome
APC	Antigen presenting cells
ABCG-5	ATP-binding cassette sub-family G proteins
BCR	B cell receptors
Bp	Base pairs
CAD	Coronary artery disease
CARDIoGRAM	Coronary Artery Disease Genome Wide Replication and Meta-analysis
CHD	Coronary heart disease
cDNA	Complementary deoxyribonucleic acid
CDV	Cardiovascular diseases
D-PBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GWAS	Genome wide association studies
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIGM	Hyper-immunoglobulin M syndrome
HGP	Human genome project
HPRT	Hypoxanthine phosphoribosyltransferase
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intracellular cell adhesion molecule-1
IFN-γ	Interferon-γ
IgG	Immunoglobulin
IL	Interleukin
IRF1	Interferon regulatory factor 1
Jak-3	Janus family kinase-3

LD	Linkage disequilibrium
LDL	Low density lipoprotein
LIPA	Lipase A
MAF	Minor allele frequency
MAPK	Mitogen activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complexes
MI	Myocardial infarction
NF-κB	Nuclear factor-κB
PAMP	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
P13-K	Phosphoinositide-3-kinase
PE	Phycoerythrin
PsA	Psoriatic arthritis
PTK	Protein tyrosine kinases
PVDF	Polyvinylidene fluoride
RA	Rheumatoid arthritis
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse transcription polymerase chain reaction
SAPK	Stress activated protein kinase
sCD40	Soluble CD40
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
STAT1	Signal transducer and activator of transcription 1
sTNFR	Soluble tumour necrosis factor receptor
TACE	TNF-α-converting enzyme
TBS-T	Tris-buffered saline tween
TCR	T cell receptors

Abbreviations

TEMED   Tetramethylethylenediamine

Th17   T helper 17 cells

TNF-α   Tumour necrosis factor-α

TNFR   Tumour necrosis factor receptor

TRIB-1   Tribbles homologue-1 gene

UTR   Untranslated region

VDJ   Variable, diversity and joining genes

VCAM-1   Vascular cell adhesion molecule-1

1. ZUSAMMENFASSUNG

Für die Regulation einer vollständigen und adäquaten Immunantwort ist eine Kostimulation durch die Bindung des Oberflächenrezeptors CD40 an seinen ebenfalls auf der Zelloberfläche lokalisierten Liganden CD154 (CD40L) unabdingbar. Aus Untersuchungen an Mäusen und Menschen gibt es jedoch auch überzeugende Belege für eine Beteiligung dieses kostimulatorischen Rezeptor-Ligandenpaars an der Entwicklung und dem Fortschreiten chronischer Entzündungen. In ruhenden Endothelzellen fördert die durch CD40-CD40L-Interaktion ausgelöste proinflammatorische Reaktion einen Wechsel zu einer prothrombotischen und prokoagulierenden Zelloberfläche. Dieser Prozess geht der Atherosklerose voraus und ist an der Ätiologie von kardiovaskulären und rheumatischen Erkrankungen beteiligt.

Ein funktioneller Einzelnukleotidpolymorphismus (single nucleotide polymorphism, SNP) in der Kozak-Konsensussequenz des CD40-Gens (-1C>T, rs1883832) ist mit Autoimmun- und immun-entzündlichen Erkrankungen assoziiert, indem er die Effizienz der Proteintanslation beeinflusst. Basierend auf diesem Kontext haben wir die Hypothese aufgestellt, dass dieser SNP das Risiko einer koronaren Herzkrankheit (KHK) in der kaukasischen Bevölkerung Baden-Württembergs durch Beeinflussung der endothelialen CD40-Expression und -Signaltransduktion erhöhen könnte.

Um diese Hypothese zu testen, wurde die Verteilung der -1C/T CD40-Variante in einer krankenhausbasierten Fall-Kontroll-Studie von Patienten mit KHK untersucht. Die Ergebnisse zeigten ein mit dem C-Allel assoziiertes erhöhtes Risiko für KHK (Odds Ratio = 1,43; 95% Konfidenzintervall: 1,13-1,86). Die Genotyp-Phänotyp-Analyse ergab, dass klonal expandierte Endothelzellen von Trägern mit dem -1CC- gegenüber denen mit dem -1TT-Genotyp eine signifikant höhere CD40-Protein-Expression in Verbindung mit einem gesteigerten proinflammatorischen Phänotyp zeigen. Zusätzlich wurde bei -1CC- im Vergleich zu -1TT-Trägern ein erhöhtes Niveau an zirkulierendem löslichem CD40-Protein beobachtet. Diese Ergebnisse legen nahe, dass der -1C/T SNP des CD40-Gens durch verstärkte Endothelzellenaktivierung eine erhöhte Anfälligkeit für KHK induzieren kann. Schließlich war das C-Allel auch bei Patienten mit Psoriasis-Arthritis verglichen mit nicht erkrankten Kontrollpersonen signifikant überrepräsentiert, was auf eine

generelle Rolle dieses Einzelbasenaustauschs beim Fortschreiten chronischer Entzündungserkrankungen hinweist.

Insgesamt liefern diese Studie neue Belege, die nahelegen, dass das C-Allel des -1C/T SNP des CD40-Gens die Anfälligkeit für KHK erhöhen kann, indem es Endothelzellen durch eine gesteigerte CD40-Proteinexpression und -Signaltransduktion einen proinflammatorischen Phänotyp verleiht und somit eine überschießendezelluläre Immunantwort verstärken kann.

1. SUMMARY

The binding of the co-stimulatory receptor CD40 to its ligand CD154 (CD40L) is imperative for orchestrating a complete and adequate immune response. However, there is also compelling evidence from mice and human studies to indicate an involvement of this co-stimulatory receptor-ligand dyad in the development and progression of chronic inflammation. In endothelial cells, the proinflammatory response triggered by CD40-CD40L interactions facilitates a shift from a quiescent to a prothrombotic and procoagulant state; a process which precedes atherosclerosis and is implicated in the etiology of cardiovascular and rheumatic diseases.

A functional single nucleotide polymorphism (SNP) in the Kozak consensus sequence of the *CD40* gene (-1C>T, rs1883832) is associated with autoimmune and immune-inflammatory diseases by altering the efficiency of protein translation. Based on this context, we hypothesized that this SNP could enhance the risk of coronary heart disease (CHD) in the Caucasian population of Baden-Württemberg via influencing endothelial CD40 expression and signaling.

To test this hypothesis, distribution of the -1C/T SNP of the *CD40* gene was examined in a hospital-based case-control study of CHD. The findings showed an increased risk of CHD associated with the C allele (odds ratio = 1.43; 95% confidence interval: 1.13-1.86). Genotype-phenotype analysis revealed that endothelial cells from carriers with the -1CC versus -1TT genotype exhibited significantly higher CD40 protein expression, along with an enhanced proinflammatory phenotype. Additionally, higher circulating soluble CD40 protein was observed in -1CC compared with -1TT carriers. These results indicate that the -1C/T SNP of the *CD40* gene may induce susceptibility to CHD via enhanced endothelial cell activation. Lastly, the C allele was also significantly over-represented in patients with psoriatic arthritis compared to non-diseased controls, indicating a more general role for this SNP in driving chronic inflammation.

Collectively, this study provides novel evidence suggesting that the C allele of -1C/T SNP of the *CD40* gene may increase susceptibility to CHD by imparting a proinflammatory phenotype to endothelial cells through enhanced CD40 protein

expression and signaling and therefore augmenting an already exaggerated cellular immune response.

2 INTRODUCTION

2.1 Overview of the immune system

The vertebrate immune system has evolved into two distinct but complementary parts: an innate component which forms the first line of defense against invading pathogens, and a highly adaptive immune response, which is limited to higher animals. The innate system is evolutionarily conserved even in simple life forms and is composed of physical, chemical and biological barriers (Delves and Roitt, 2003). Its cellular components include leukocytes, phagocytes, bioactive molecules like the cytokines and the complement proteins. A central part of this system is the recognition of conserved motifs known as pathogen associated molecular patterns (PAMP) present on invading cells by the pattern recognition receptors encoded in the germline and displayed on antigen presenting cells (APCs).

The major cellular effectors of the adaptive immune response are the B and T lymphocytes which express B cell receptors (BCR) and T cell receptors (TCRs). By the process of VDJ (variable, diversity and joining genes) recombination the receptors undergo somatic re-arrangement and clonal expression, facilitating the recognition of a diverse range of antigens and altered non-self cells such as cancer cells (Schatz and Yanhong, 2011). T cells are activated following interaction with antigenic peptide fragments presented by major histocompatibility complexes (MHC) on cell surfaces. The CD8+ T cells recognize MHC class I presented on most nucleated cells, while CD4+ T cells bind to MHC class II complexes on APCs. Consequently, the cells differentiate into cytotoxic T cells (CD8+) and T helper cells (CD4+). B cells are stimulated upon recognition and interaction with antigens, which they internalize and present on MHC class II to type 2 T helper (Th2) cells. This process drives B cell proliferation into memory cells or antibody secreting plasma cells, and is known as T cell-dependent B cell activation.

One of the focal points of the immune-inflammatory responses is the CD40-CD40 ligand (CD40L) interaction. The CD40 receptor and its cognate ligand form a co-stimulatory pathway which is activated when CD40 expressed on B cells interacts with CD40L on the

Th2 cells. These interactions play a critical role in autoimmune diseases, signifying their importance in the immune system. They induce B cell proliferation, antibody secretion and immunoglobulin switching through T cell-dependent B cell activation (Noelle et al., 1992; Lane et al., 1992 and Kawabe et al., 1994). The CD40-CD40L pathway in conjunction with the CD28-CD80/86 pathway is also crucial for the activation of T cells and antigen presenting cells (APCs). Therefore, it is also implicated in the process of allograft rejection which is primarily a T-helper (Th) 1 response whereby Th1 cells interact with resident macrophages acting as APCs (reviewed by Harlan et al., 1999). Additionally, during inflammatory conditions CD40L primarily expressed on activated T helper cells, self-induced on endothelial cells, or shed from platelets activates endothelial cells. This results in the expression of adhesion molecules and release of chemokines thereby facilitating the recruitment and activation of circulating monocytes and contributing to the maintenance of a chronic immune response (Hollenbaugh et al., 1995; Karmann et al., 1995; Wagner et al., 2004).

2.2 CD40/CD40L system

2.2.1 Structure

CD40 is a type 1 transmembrane glycoprotein receptor of the tumour necrosis factor receptor superfamily (TNFR). The human CD40 gene is composed of 9 exons and it is located at chromosome 20 (q12-q13.2). It contains 277 amino acids (aa) with a 193 aa extracellular domain, a 22 aa transmembrane domain, and a 62 aa intracellular domain. In a manner similar to all TNFR family members, the extracellular domain contains several cysteine-rich regions which are critical for its function and are stabilized by disulfide bridges (Stamenkovic et al., 1989; Van Kooten and Banchereau, 2000).

CD40 signals through its natural ligand known as CD40L (CD154) which is a 5 exon, type II, 39 kDa, transmembrane protein mapped to chromosome X (q26.3-q27.1). Its extracellular structure consists of two β-sheets sandwiching an α-helix loop that allows receptor trimerization, which is typical for tumour necrosis factor (TNF) ligand family members (Karpusas et al., 1995). However, proteolytic cleavage can give rise to biologically active soluble forms known as sCD40L (Ludewig et al., 1996; Graf et al.,

1995).While the classical receptor for CD40L is CD40, it can also signal though other receptors, namely Mac-1 and α5β1 integrin (Zirlik et al., 2007).

2.2.2 Expression pattern and induction of the CD40-CD40L dyad

CD40 was initially characterized on B lymphocytes as a protein essential for all steps of B cell development, differentiation and maturation while CD40L was identified on CD4+ T cells (Armitage et al., 1992; Koho et al., 1984; Paulie et al., 1984). The importance of CD40-CD40L interactions in T cell-dependent-B cell responses was underscored by the discovery of point mutations in the CD40L gene in patients with the hyper-IgM syndrome (HIGM). This condition develops due to defective switching of the IgM antibodies to the IgG, IgE or IgA classes resulting in IgM accumulation. It is accompanied by reduced B cell proliferation, a restricted immune response, and increased susceptibility to opportunistic as well as viral infections. In experimental mouse models with inactivated CD40 or CD40L genes, a similarly defective immune response can be observed (Allen et al., 1993; Callard et al., 1993).

Further studies revealed that CD40-CD40L dyad expression is not restricted to the humoral immune system. CD40 is also expressed on other hematopoietic cells including monocytes, macrophages, platelets and dendritic cells, non-lymphoid cells like mast cells and eosinophils, as well as non-hematopoietic cells such as endothelial cells, epithelial cells and vascular smooth muscle cells. CD40L is mainly expressed by B and T cells and it is shed from activated platelets. However, under proinflammatory conditions its expression can also be induced on other cells including monocytes, mast cells and endothelial cells (reviewed by Banchereau et al., 1994 and Schonbeck and Libby, 2001).

CD40-CD40L interactions are integral in the Th1 immune response since they provide a co-stimulation signal which is imperative for T cell differentiation and proliferation, and its absence can result in T-cell anergy and depletion (Grewal and Flavell, 1998). The expression of CD40L on T cells enables their interaction with the CD40 receptor expressed by APCs like monocytes, macrophages and dendritic cells, leading to the expression of proinflammatory cytokines and adhesion molecules which facilitates atherogenesis. At the same time these bi-directional interactions prime the APCs for

activation of CD8+ T cells. In an atherosclerotic context, CD40-CD40L interactions on macrophages have particular pathophysiological relevance since they lead to macrophage activation which is followed by the release of matric metalloproteinases which induce plaque instability via digestion of extracellular collagen matrix (Lievens et al., 2009; Robertson and Hannson; Thim et al., 2008).

2.2.3 CD40 signal transduction

The CD40 signal cascade has been well-studied in B lymphocytes and dendritic cells since it plays a crucial role in B cell proliferation and dendritic cell activation. Due to a lack of intrinsic signaling activity in CD40, signal propagation occurs largely in conjunction with a family of structurally homologous adaptor proteins called tumour necrosis factor receptor associated factors (TRAF). Currently there are six known TRAFs while the CD40 cytoplasmic domain has simultaneous binding capacity for two (Schonbeck and Libby, 2000 and 2001).

The detectable downstream events of CD40 signal transduction include activation of protein tyrosine kinases (PTK), phosphoinositide-3-kinase (PI3-K) and the serine-threonine kinases, like the mitogen activated protein kinases (MAPK) and stress activated protein kinase (SAPK) (Bishop et al., 2007). In addition, the CD40 cytoplasmic tail can also associate with the Janus family kinase 3 (Jak 3) resulting in the activation of signal transducers and activators of transcription (STAT1). One of the best characterized pathways involved in CD40 activation is the prototypical proinflammatory nuclear factor-κB (NF-kB) pathway, which has a key role in inducing expression of proinflammatory cytokines, chemokines and adhesion molecules (Karmann et al., 1995; Saemann et al., 2002; Saemann et al., 2003). Overall the CD40-CD40L signal cascade is complex and can involve multiple pathways depending on the cell type where activation occurs, the adaptor molecules that are recruited, and the endocytic pathway involved after receptor internalization.

2.2.4 Post-transcriptional and post-translational regulation of CD40

CD40 expression is cytokine inducible and is markedly increased after exposure to a combination of tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) which

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synergistically induce *de novo* synthesis of CD40. This process is dependent on NF-kB, STAT1 and interferon regulatory factor 1 (Wagner et al., 2002). Additionally, CD40 is also regulated by gene splicing at the post-transcriptional level and at the post-translational level.

CD40 post-transcriptional regulation has not been widely studied. However, the presence of evolutionarily conserved isoforms has been detected in mice and humans. In addition to isoform I which encodes the fully functional CD40 receptor, the presence of three alternative CD40 mRNA isoforms has been confirmed. The major alternative isoform (type II) lacks exon 6 and encodes a soluble CD40 (sCD40) protein. Unlike other protein variants, type II which is also known as sCD40-6 retains three cysteine rich domains essential for binding to CD40L (**Figure 1**). Therefore, even though sCD40 generated from isoform II cannot signal, it can still bind to CD40L. Hence, it has the capacity to act as a decoy receptor which is a receptor that can structurally bind to a ligand but cannot activate it. Soluble CD40 can also be generated by the proteolytic cleavage of membrane CD40 via TNF-alpha-converting enzyme (TACE) and is likely to have clinical relevance (Eshel et al., 2008; Tone et al., 2001). For instance, elevated sCD40 levels have been reported in the serum of uremic patients which were decreased after normalization of kidney function (Contin et al., 2003). Increased sCD40 levels have also been associated with hematological malignancies (Hock et al., 2004) and Alzheimer's disease (Buchhave et al., 2009 and Ait-ghezala et al., 2008).

At the post-translational level CD40 is regulated by nitration of tyrosine residues. The tyrosine at position 82 has been shown to be particularly susceptible to nitration in resting
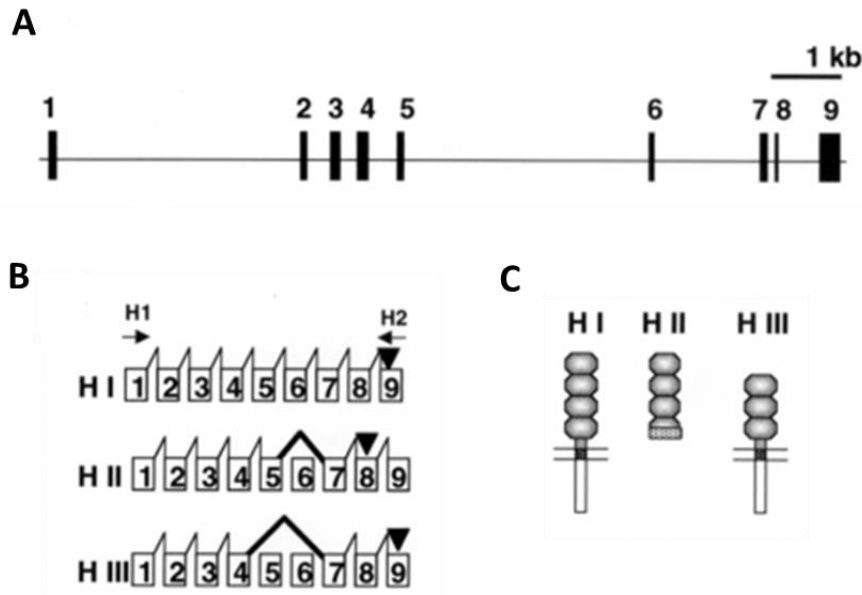
Figure 1: Human CD40 receptor isoforms. *(A) Gene structure of CD40 with 9 exons depicted as boxed structures. (B) CD40 isoform mRNA structures with lines and arrows indicating the splice sites and stop codons respectively. (C) Predicted translation product of the mRNA isoforms. H I, H II and H III denote human I, II and III isoforms respectively. Isoform I is the main CD40 receptor. Isoform II is the major alternative isoform with three cysteine-rich domains while in isoform III the intracellular regions are replaced by distinct 19 amino acids. (Image modified from Tone et al., 2001).*

and stretched endothelial cells. Consequences of tyrosine nitration include functional inactivation of CD40 followed by its targeting for proteasomal degradation (Wagner et al., 2011). There is also some evidence that CD40 can undergo S-nitrosylation, which is the binding of nitric oxide to thiol (SH) residues. It is possible that S-nitrosylation of CD40 augments the inflammatory response and its clinical relevance has been demonstrated in sepsis (Godoy et al., 2010).

2.3 Atherosclerosis

Atherosclerosis and its related complications such as coronary heart disease (CHD), myocardial infarction, heart failure and ischemic stroke are one of the leading causes of global morbidity, mortality and disability. Currently cardiovascular diseases (CVD) represent 31% of deaths worldwide but by 2020 this figure is expected to rise to 40%, enabling CVD-related mortality to exceed that of infectious diseases (World Health Organization, 2014). Atherosclerosis progression is asymptomatic and can be silent for

decades until it manifests itself in the form of angina or in more advanced cases as myocardial infarction or stroke (Biros et al., 2008). Therefore identification and early treatment of susceptible individuals is a feasible strategy for reducing the global burden of this chronic condition.

Atherosclerosis primarily affects large and medium sized arteries (Napoli et al., 1997). According to the response to injury hypothesis, the earliest event in atherosclerosis is an injury to the endothelium which precedes the development of atherosclerotic lesions. Endothelial injury can arise due to a number of factors such as physical stress, generation of reactive oxygen species and elevated levels of LDL, VLDL and glucose. A major contribution is made by altered hemodynamics, i.e. disturbed blood at arterial bifurcations and curves which weakens the anti-inflammatory defense of the endothelium, inducing endothelial dysfunction and thereby resulting in the early and maintained influx of proinflammatory leukocytes into the subendothelial spaces. Initially monocytes are recruited into the developing fatty streak which is composed of lipid-laden vascular smooth muscle cells, followed by Th1 cells (Bonetti et al., 2003; Ross, 1993; Widlandsky et al., 2003). Recently, the presence of polymorphonuclear neutrophils has also been confirmed in atherosclerotic lesions of human and murine origin (Drechsler et al., 2010; Ionita et al., 2010).

Collagen and matrix molecules produced mainly by activated vascular smooth muscle cells form the fibrotic cap underneath the endothelial cells which is eroded by the matrix metalloproteinases released following the interaction of CD40L expressing Th1 cells and CD40 expressing macrophages, leading to the formation of a rupture-prone atherosclerotic plaques. In addition to CD40L on Th1 cells, sCD40L is also shed from platelets (Elhage et al., 2003, Gerdes et al., 2016; reviewed by Tse et al 2013. In this environment CD40-CD40L interactions drive a plethora of proinflammatory responses which are reviewed below.

2.3.1 CD40-CD40L interactions in endothelial dysfunction and atherosclerosis
Inflammation is a protective cellular immune response to invasive pathogens, injury and irritation. However, continuous exposure to proinflammatory stimuli leads to chronic and

persistent inflammation which is a widely accepted hallmark of atherosclerosis and is implicated in all stages of its development and progression.

Endothelial dysfunction is an early marker for atherosclerosis. It is described as a shift of the endothelium from a quiescent towards a prothrombotic and proinflammatory phenotype (Lüscher and Barton, 1997). The endothelium is a selectively permeable barrier between the blood and the vessel wall. It fulfils essential functions including blood flow regulation and maintenance of vascular tone. Under physiological conditions it is an antithrombotic surface to which mononuclear cells cannot attach due to a lack of adhesion molecules. However, this condition is not maintained during disturbed blood flow which can be caused by inflammation, low shear stress and cardiovascular risk factors, and is aided by genetic predisposition (Asakura et al., 1990; Cataruzza et al., 2004; Celermayer, 1993; Libby et al., 2002).

CD40-CD40L interactions promote inadequate endothelial cell activation and ensuing dysfunction (**Figure 2**). During inflammation, cytokines such as TNF-α, IFN-γ, interleukin (IL)-1, -3 and -4 are released from activated monocytes and T helper cells. These cytokines induce CD40 expression on endothelial cells, which in an atherosclerotic context may be aggravated by oxidized LDL. The endothelial cells then interact with CD40L bound to T cells or released as a soluble form from activated platelets (reviewed by Antoniades et al., 2009).

Furthermore, CD40-CD40L interactions facilitate leukocyte adhesion to the endothelial cell surface via upregulation of adhesion molecules such as vascular and intracellular cell adhesion molecule (VCAM-1, ICAM-1) and E-selectin as well as P-selectin (Karmann et al., 1995; Hollenbaugh et al., 1995). The penetration of the adhered leukocytes is facilitated by monocyte chemoattractant protein-1 (MCP-1), which is a key chemokine primarily released from stretched vascular smooth muscle cells (Demicheva et al., 2008) and also upregulated by endothelial CD40-CD40L interactions. It provides a signal to recruit circulating monocytes to the vascular endothelium where they transmigrate and differentiate into macrophages and foam cells (Wagner et al., 2004).

CD40 signal transduction also leads to increased expression of interleukins, matrix metalloproteinases and tissue factors. These changes are accompanied by a decrease in the expression, activity and stability of the cardio-protective endothelial nitric oxide synthase. All together these changes confer an activated proatherogenic phenotype to the endothelium, which is strongly implicated in chronic inflammatory diseases particularly atherosclerosis (Pluvinet et al., 2008).
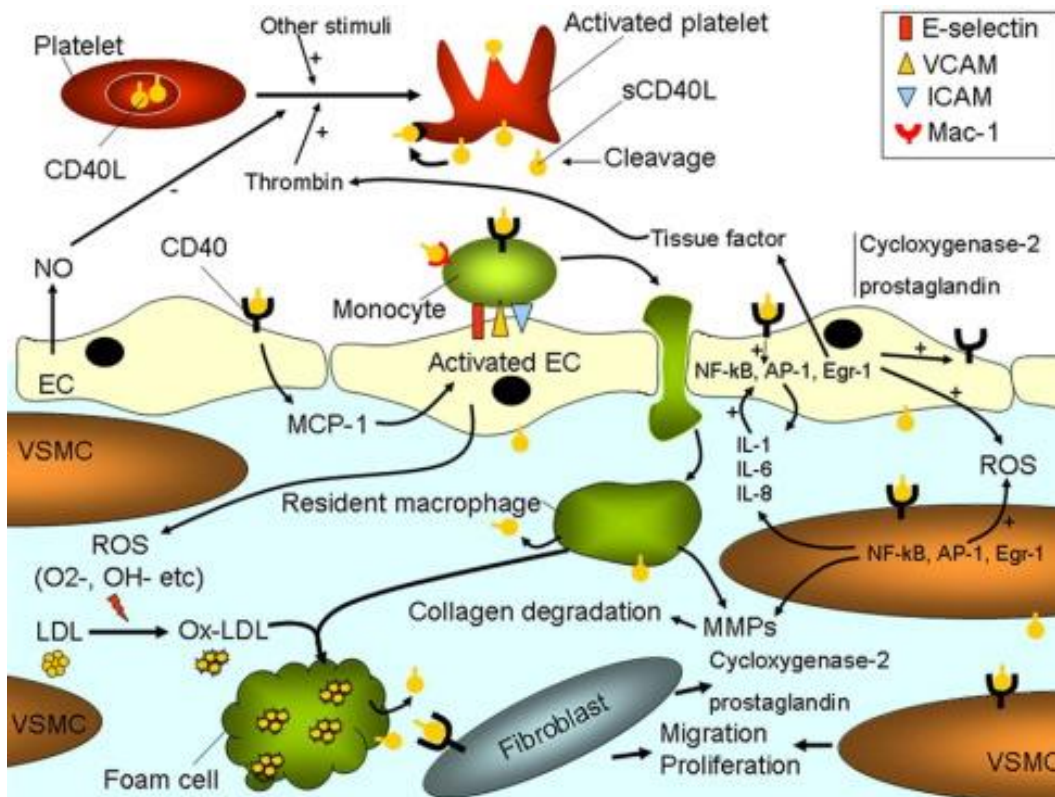
Figure 2: CD40-CD40L interactions in atherothrombosis: *A simplified illustration of the impact of CD40-CD40L interactions in the context of atherosclerosis; CD40L cleaved and released from activated platelets binds to CD40 expressed on endothelial cells. The resulting expression of transcription factors such as NFkB and activator protein-1 (AP-1) leads to endothelial activation through upregulation of chemokines (MCP-1) and adhesion molecules (VCAM-1, E-selectin and I-CAM). Monocytes are attracted to the endothelial surface, adhere and transmigrate into the subendothelial region. In activated vascular smooth muscle cells and macrophages, especially upon interaction with infiltrating Th1 cells, there is upregulation of matrix metalloproteinase (MMP) expression which results in plaque instability through collagen degradation. Additionally, foam cell formation is promoted by uptake of oxidized-low density lipoprotein by macrophages and VSMCs. Altogether the consequences of CD40-CD40L interactions create a pro-thrombotic environment favoring plaque formation and disruption which can lead to vessel occlusion (Figure is taken from Antoinades et al., 2009).*

2.3.2 Atherosclerosis and rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory exaggerated immune response likely mediated via Th1 cells which primarily affects the small joints in the hands and feet. The correlation between RA and CVD is indisputable. Cardiovascular mortality is 50% higher

in RA patients as compared to non-RA groups (Avina-Zubeita et al., 2009). Additionally, the risk for myocardial infarction (MI) is two-fold higher in RA patients, which is comparable to the risk imparted by type II diabetes (Solomon et al., 2003). There is a strong possibility that the underlying reason is accelerated inflammation since RA is characterized by inflammation which promotes and exacerbates atherosclerosis (reviewed by Yang et al., 2016). CVD incidence is also enhanced in other systemic inflammatory diseases such as systemic lupus erythematosus (SLE) (Nikpour et al., 2003) and inflammatory bowel disease (Singh et al., 2014). There are high similarities in the proinflammatory processes in an atherosclerotic plaque and the hypertrophied synovium (pannus) in RA. C-reactive protein is a marker for both RA (Ridker et al., 1998) and CVD (Del Ricon et al., 2003). In both conditions, cytokines primarily TNF-α and IL-1 are elevated, accompanied by increased expression of adhesion molecules which promote leukocyte extravasation. TNF-α reduces nitric oxide (NO) bioavailability in the vessel wall by decreasing the half-life of endothelial nitric oxide synthase mRNA and thereby impairs NO-mediated endothelium-dependent anti-inflammatory responses, setting the stage for endothelial dysfunction (reviewed by Zhang et al., 2009).

Endothelial activation and ensuing dysfunction is a well-known driver of inflammation in atherosclerosis. Similarly, its presence has been reported at various stages of RA (reviewed by Steyers and Miller, 2014). Animal models of RA also exhibit early endothelial dysfunction. For instance, in collagen-induced arthritis in mice as well as in a sheep model, there is considerably greater impairment of endothelium-dependent NO release as compared to non-diseased controls (He et al., 2013; Dooley et al., 2014). Also, aortic endothelium isolated from the same mouse model exhibits elevated VCAM-1 expression (Denys et al., 2014). Hence it is not farfetched that the underlying factor integrating RA and CDV is an inadequately activated endothelium that precedes atherosclerosis.

2.3.3 Genetic risk of atherosclerosis

Atherosclerosis has a complex phenotype arising from the interplay between genetic and environmental factors. Epidemiological investigations including the Framingham study have identified family history as an independent risk factor of fatal cardiac events

(Yarnell et al., 2003). The Swedish Longitudinal Heart study has shown a high relative risk of death by coronary artery disease (CAD) in monozygotic and dizygotic male and female twins when one of the pair has suffered from a cardiac event prior to age 65 (Zdravkovik et al., 2002). In addition, primary risk factors such as diabetes, dyslipidemia or hypertension also have a significant individual genetic component. These observations place the heritability factor of CAD between 50-60% (Peden and Farrall, 2011). However, due to the polygenic and multifactorial nature of CAD, precise identification of the molecular genetic architecture has remained elusive.

The most common type of genetic variation in the human genome is represented by single nucleotide polymorphisms (SNP), i.e. single DNA base changes that occur rather frequently and can be a marker of disease development and progression. Recent advances in technology and data analysis made possible by the Human Genome Project have led to the development of genome wide association studies (GWAS). In GWAS millions of SNPs are simultaneously examined in thousands of case-control populations (https://www.ebi.ac.uk/gwas/).

While GWAS is a bias-free method, another way of harnessing SNPs to understand genetic associations is the candidate gene approach in which pre-specified genomic regions are analyzed to determine association with disease states. A high throughput candidate gene approach in humans analyzing data obtained from 15,596 CAD cases and 34,992 controls using the cardiovascular gene-centric 50K SNP array customized for CAD has implicated several novel genes including lipase A (LIPA), interleukin 5 (IL-5), Tribbles homologue-1 gene (TRIB-1) and ATP-binding cassette sub-family G proteins (ABCG-5 and 8) with increased CAD risk. Combined with data from the CARDIoGRAM (Coronary Artery Disease Genome Wide Replication and Meta-analysis), the association of 33 loci with common variants significantly influencing the risk of CAD was confirmed (Brendan et al., 2008, Butterworth et al., 2009). However, these studies account for only approximately 10% of the total genetic risk. This is likely because of the stringent threshold for GWAS studies ($P<5x10^{-8}$).

2.4 Single nucleotide polymorphism and disease susceptibility

SNPs are the most common form of genetic variation and occur at a frequency of once per every 300 nucleotides. There are approximately one million known SNPs in the human genome. A vast majority of them have no pathophysiological relevance. However, some occur within, or in linkage disequilibrium with other SNPs, in the regulatory regions of genes. As such they can directly affect disease development by influencing gene expression and function (Van der Broeck et al., 2014). For instance, a G to A transition at position 75 of the apolipoprotein A1 gene promotor is associated with early onset Alzheimer's disease potentially through influencing cholesterol metabolism (Helbecque 2008). Another promoter polymorphism present in the gene for NOS3, the endothelial isoform of NO synthase (-786 C/T), is an independent predictor of vascular inflammatory diseases including CHD and RA through inducing endothelial dysfunction (Cataruzza et al., 2004; Melchers et al., 2006). Following sequencing of the human genome, a database characterizing more than a million genetic variants in world populations has been organized by the human genome project (HGV) in collaboration with the HapMap project (International HapMap Consortium, 2005).

2.4.1 The -1C/T CD40 single nucleotide polymorphism

In 2002, linkage analysis identified chromosome 20q11 as a susceptibility locus for the autoimmune condition known as Graves' disease. Sequencing of the entire CD40 gene, which maps to the specified locus, identified a cytosine to thymine transition one base pair upstream of the initiating AUG codon which was classified as a -1C/T CD40 SNP **(Figure 3)**. This SNP is located in the Kozak consensus sequence, which is a region of 6-8 nucleotides surrounding the initiating codon. A conserved Kozak sequence is necessary for optimal protein translation (Kozak 1984 and 1986). In Graves' disease, patients present a preferential transmission of the C allele (Tomer et al., 2002). A follow up study investigating a possible mechanism for this phenotype showed an association of the C allele with higher CD40 protein expression in B cells as well as increased translation in an *in vitro* system, indicating that the C allele predisposes towards Graves' disease by increasing CD40 levels (Jacobson et al, 2005). This SNP is also implicated with other auto-immune conditions including the prototypic autoimmune disease systemic lupus

erythematosus (Chen et al., 2015; Wu et al., 2016). In light of the importance of CD40-CD40L interactions in inflammation, the -1C/T CD40 SNP has also been studied in relation with diseases associated with increased inflammatory responses such as CHD, and the C allele has been consistently implicated as a risk factor (Tian et al., 2010; Yan et al., 2010; Yun et al., 2014).
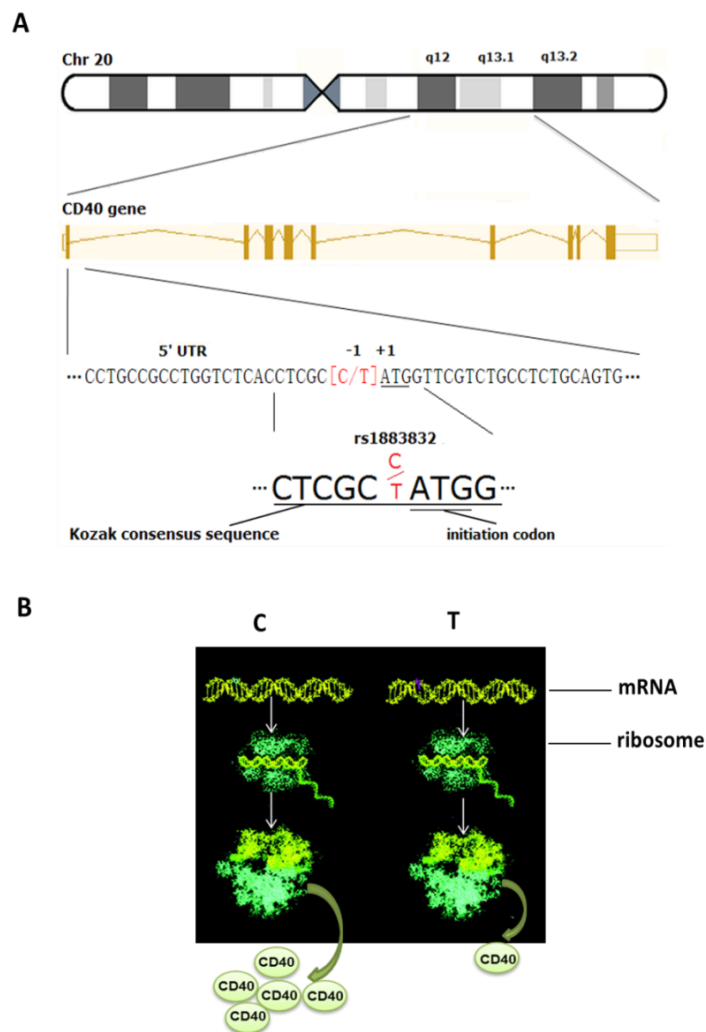
Figure 3: Location and impact of the -1C/T SNP of the *CD40* gene. *(A) The chromosomal location of the -1C/T CD40 is illustrated. The CD40 gene is located on chromosome 20 (q12-q13.2). One base pair upstream of the initiation codon a cytosine or a thymine can be present. This is referred to as the -1C/T CD40 SNP or Rs1883832. 5'UTR refers to the untranslated region (**B**) Mechanism of action. The -1C/T SNP affects the initiation of translation therefore both alleles are transcribed equally by RNA polymerase II. The initiation of translation is more effective in the C allele resulting in a greater quantity of translated product (adapted from Jacobson et al., 2005 and Yun et al., 2014).*

2.5 Aim of the study

The proinflammatory response triggered by CD40-CD40L interactions makes a significant contribution to endothelial dysfunction, which is the underlying basis of chronic inflammatory conditions, like cardiovascular and rheumatic diseases. Given the importance of these interactions, even slight imbalances in expression would further enhance immune dysregulation and prolong inflammation. A common genetic polymorphism in the *CD40* gene which is a C to T variation at position -1 of the 5' UTR is known to influence CD40 gene expression in various cell types and is a risk factor for autoimmune and immune-inflammatory diseases. This study investigated if the -1C/T SNP of the *CD40* gene is a genetic risk factor for susceptibility towards endothelial dysfunction by influencing endothelial CD40 gene expression and function. Considering that endothelial dysfunction is an early predictor for atherosclerosis, we further hypothesized that the -1C/T SNP may influence susceptibility to pathologies in which atherosclerosis is dominant, namely CHD, and related inflammatory diseases such as rheumatoid arthritis. To test this hypothesis, allele frequencies for the -1C/T SNP of the *CD40* gene were compared in non-diseased controls and patients with CHD, psoriatic and rheumatoid arthritis. Since activation of the CD40 receptor and downstream signaling maybe regulated by its soluble form, we further investigated if sCD40 levels were influenced by the –1C/T SNP and its implications in a CHD context. The main aims of this thesis are summarized below:

1. To investigate the distribution of the -1C/T SNP in the local population of Baden-Württemberg and compare the allele and genotype frequencies with those reported by the 1000 Genome project.

2. To analyze whether the -1C/T SNP can be functionally considered as a risk factor for inducing endothelial dysfunction via influencing CD40 expression and signaling in endothelial cells under baseline and proinflammatory conditions.

3. To determine if the -1C/T SNP is a true risk factor for CHD and related inflammatory arthritic conditions in a hospital based case-control study.

4. To evaluate the clinical relevance of sCD40 in a CHD context and analyze its possible association with the -1C/T SNP, namely the CC genotype.

3. MATERIALS

3.1 Buffers and Solutions

Table 1: Composition of buffers and solutions

Buffer/Solution	Composition
Agarose gel electrophoresis	
Loading buffer	10 mM Tris HCl, pH 7.5
	10 mM EDTA, pH 8.0
	30% Glycerol
	0.01% Bromophenol blue
	0.01% Xylene green
Running buffer	450 mM Tris
	450 mM Boric acid
	20 mM EDTA, pH 8.0
FACS buffer	Ice-cold DPBS and 10% FCS
Protein extraction	
Lysis buffer (whole cell extract)	10 mM Hepes, pH 7.9
	10 mM KCl
	0.1 mM EDTA
	0.1 mM EGTA
	0.1 M DTT
	50 µM Pefabloc
	25 µM Protease inhibitors
Pefabloc	15 mM HEPES buffer, pH 7
	4 4 % Pefabloc®-SC
Protease inhibitor mixture	1 % Pepstatin A in 20 DMSO
	80 % 15 mM Hepes, pH 7.4

Protease inhibitor mixture	1 % Leupeptin in 20 % DMSO 80 % 15 mM Hepes, pH 7.4
Phosphate-buffered Saline (PBS)	8.0 g NaCl 0.2 g KCl 1.44 g Na2HPO4 0.2 g KH2PO4
SDS-PAGE Blocking buffer	5% milk powder in TBST
Running buffer	248 mM Tris-HCl, pH 8.7 192 mM Glycine 35 mM SDS in 2 L water
Stripping buffer	0.2 M NaOH
Transfer buffer	25 mM Tris 192 mM glycine, pH 8.3
Tris-buffered saline (TBS)	248 mM Tris-HCl pH 8.3 192 mM glycine dissolved in 2 litre H2O
TBS-T	TBS+0.05% Tween®20

3.2 Chemicals and reagents

Table 2: Chemical and reagents used in this work

Chemicals and reagents	**Supplier**
Agar-Agar	Carl Roth
Ampicillin	Sigma-Aldrich
Ammonium persulfate	Carl Roth

Chemicals and reagents	Supplier
Bovine Serum Albumin	Sigma-Aldrich
Calceim AM	Molecular Probes Life Technologies
Dispase	Gibco, Life Technologies
Dithiothreitol (DTT)	Carl Roth
DNA ladders	Thermo Scientific
D-Phosphate-buffered saline solution	Gibco, Life Technologies
Endothelial cell growth medium	PromoCell
Endothelial cell growth supplements	PromoCell
EDTA (Ethylenediaminetetraacetic acid)	PanReac AppliChem
Ethidium bromide	Carl Roth
Fungizone	Gibco, Life Technologies
Gelatin	Merck Millipore
Hank's balanced salt solution	Gibco, Life Technologies
Heat inactivated fetal bovine serum	Gibco, Life Technologies
HEPES	Sigma-Aldrich
Luminata^{TM} and Luminata^{TM} Forte	Merck Millipore
Methanol	Sigma-Aldrich
Oligo dT	Qiagen
Penicillin	Gibco, Life Technologies
Pefabloc	Sigma-Aldrich
Pepstatin A	Sigma-Aldrich

Chemicals and reagents	Supplier
Polyacrylamide	Carl Roth
Precision Plus Protein Standard^{TM}	Bio-Rad Laboratories
Restriction enzyme (NcoI)	New England Biolabs
RPMI 1640 medium	Invitrogen
Sodium dodecyl sulfate (SDS)	Serva Electrophoresis
Streptomycin	Gibco, Life Technologies
TEMED (Tetramethylethylenediamine)	Carl Roth
Tween 20	Carl Roth
Tris	Carl Roth
Triton-X 100	Sigma-Aldrich
Taq Polymerase	Bioron

3.3 Consumables

Table 3: Materials utilized in the study

Materials	Company
Cell culture dishes	Techno Plastic Products (TPP)
Cell culture flasks	Sarstedt
Cell culture plates	
Cell scraper	
PCR tubes	
Pipette tips	
Serologic pipettes	
PVDF Transfer membranes	Merck Millipore
Round bottom glass FACS tubes	Falcon-Corning

3.4 Kits:

Table 4: Kits used in this work

Kit	Catalog Number	Supplier
Omniscript RT Kit	205113	Qiagen
QuantiTect SYBR Green R PCR kit	204143	
RNA Isolation (RNeasy Mini Kit)	74104	
Quantikine ELISA CD40 CCL2/MCP-1 sE-selectin/CD62E	 DCCD40 DCP100 DSLE00	R&D

3.5 Miscellaneous Equipment

Table 5: Miscellaneous equipment utilized

Equipment	Model	Supplier
Agarose gel electrophoresis power supply	PowerPack 25	Biometra
CO2 incubator	Innova 4230 and CO-170	Heraeus Instruments
Centrifuge	Universal 32	Hettich Zentrifugen
Digital Imaging System	ImageQuant LAS 4000 mini	GE Healthcare
Dry block heater	HBT-1131	Haep Labor Consult
FACS machine	BD FACSCanto II	BD Biosciences

Equipment	Model	Supplier
Fluorescence microscope	ScanR Inverted Microscope	Olympus
Image analyzer	Gel Doc® XR Imager	Bio-Rad
Lab pH meter	pH 720	InoLab®
Laminar flow	HS18 Hera safe	Heraeus Instruments
Light cycler®	480 software, version 1.5	Roche
Light microscope	Axiovert 25	Carl Zeiss
Microplate spectrophotometer	PowerWave XS	Bio Tek Instruments
Mini transfer chamber	Mini Trans-Blot®	Bio-Rad
NanoDrop spectrophotometer	ND-1000	Thermo Fisher Scientific
PCR cycler	Thermocycler	Biometra
Refrigerated centrifuge	Mikro 22	Hettich Zentrifugen
SDS-PAGE system	Mini-Protean® Tetra Cell	BioRad
Ultrasonic homogenizer	UP50H	Hielscher Ultrasonics
Water bath	Ecotherm E11	Dinkleberg Analytics

3.6 Primer sequences (qRT-PCR and conventional PCR)

Table 6: Primers and their sequences

Gene product	Primer sequence (Direction 5' to 3')	Annealing Temperature
CD40	For GCATGCAGAGAAAAACAGTACCT Rev GTGCAGTCACTCACCAGTTTCT	60° C
CD40 Δ6	For GGGGTCAAGCAGATTGGTCCCCA Rev AGACCAGCACCAAGAGGATGGCA	60° C
E-selectin	For TTCGCCTGTCCTGAAGGATG Rev TCAGTTGAAGGCCGTCCTTG	60° C
GAPDH	For CCACTCCTCCACCTTTGAC Rev ACCCTGTTGCTGTAGCCA	60° C
HPRT	For CAAGCTTGCTGGTGAAAAGGAC Rev GTCAAGGGCATATCCTACAACAAA	59° C
MCP-1	For CAAACTGAAGCTCGCACTCTCGCC Rev GCAAAGACCCTCAAAACATCCCAGG	63° C
VCAM-1	For CATGGAATTCGAACCCAAACA Rev GACCAAGACGGTTGTATCTCTGG	53° C
Genotyping primer		
CD40 (-1C/T SNP)	For CCTC TTCCCCGAAGTCTTCC Rev GAAACTCCTG CGCGGTGAAT	61° C

3.7 Recombinant proteins

Table 7: Concentrations of recombinant proteins used

Recombinant protein	Concentration	Company
IFN-γ	100 U/ml	R&D systems
MCP-1/CCL2	30 ng/ml	R&D systems
Soluble trimeric CD40Ligand	200 ng/ml	Enzo Life Sciences
TNF-α	1000 U/ml	Biomol GmbH
Antibodies	**Dilution**	**Company**
Immunoblotting Rabbit polyclonal anti-CD40(C-20)	1:500	Santa Cruz Biotechnology
Mouse monoclonal anti-beta-actin	1:2000	Abcam
Flow cytometry FITC- mouse anti-human CD40	2μl/test	BD Pharmingen
PE mouse anti-human CD106		
FITC mouse IgG1 (κ Isotype control)		

4. METHODS

4.1 Cell culture and isolation

4.1.1 Human umbilical vein endothelial cells (HUVEC)

Human umbilical vein endothelial cells were isolated from fresh umbilical cords obtained from the University Clinic Heidelberg with the permission of the local Ethics Committee (S-383/2013). For isolation a cannula was inserted into the ends of the umbilical vein and blood was cleared with 20 ml of D-PBS buffer. For endothelial cell isolation, the umbilical veins were filled with dispase solution (3.1 g/l) through the cannula whose ends were tied. After 30 min incubation at 37°C, the contents were centrifuged at 1000 rpm for 5 min. The cell pellet was re-suspended in endothelial cell growth medium containing supplements, antibiotics and antimycotic agents. The isolated cells were cultured in a CO_2 incubator at 37°C on 60 mm polystyrene dishes coated with 2% (w/v) gelatin. Cells were passaged once (P1) on gelatin coated 6 well dishes.

4.1.2 THP1 cells

THP1 is a human acute monocytic leukemia cell line (ATCC® TIB-202™) commonly used to investigate the function and regulation of monocytes and macrophages in the cardiovascular system. THP1 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1% of penicillin/streptomycin at 37 °C and 5% CO_2. Cells were maintained at a density between $1x10^5$ and $5x10^5$ cells/ml. Cells were sub-cultured every two-three days (density $7-8x10^5$ cells/ml).

4.2 Cell treatments

HUVECs were stimulated with sCD40L (200ng/ml) for 8 or 24 hours for measuring changes in mRNA and protein respectively. For cytokine priming, the cells were incubated with a mixture of TNF-α (1000U/ml) and IFN-γ (100 U/ml) for 16 hours with 8 (mRNA) or 24 hours (protein) further culture with sCD40L. To analyze the effect of cytokine priming without sCD40L, the cells were cultured with TNF-α and IFN-γ for 24 hours (mRNA) or 40 hours (protein).

4.3 Monocyte adhesion assay

HUVECs cultured for 48 hours on gelatin coated 12 well plates were stimulated for 14 hours with 200 ng/ml sCD40L. THP1 cells (2×10^5) were labelled with Calcein-AM (2µM) by incubation at 37°C for 30 mins with rotations every 10 minutes. The labelled cells were centrifuged at 1000 rpm for 5 minutes and resuspended in 1 ml EC medium. HUVECs were washed to remove the stimuli and overlaid with the THP-1 cells for 2 hours at 37°C. The non-adherent cells were removed by careful washing (5 times) with pre-warmed D-PBS. The numbers of adherent cells were imaged immediately **(Figure 4)** using a fluorescence microscope (Olympus ScanR) and cells were counted in multiple planes using the CellSens software (Version 1.12).
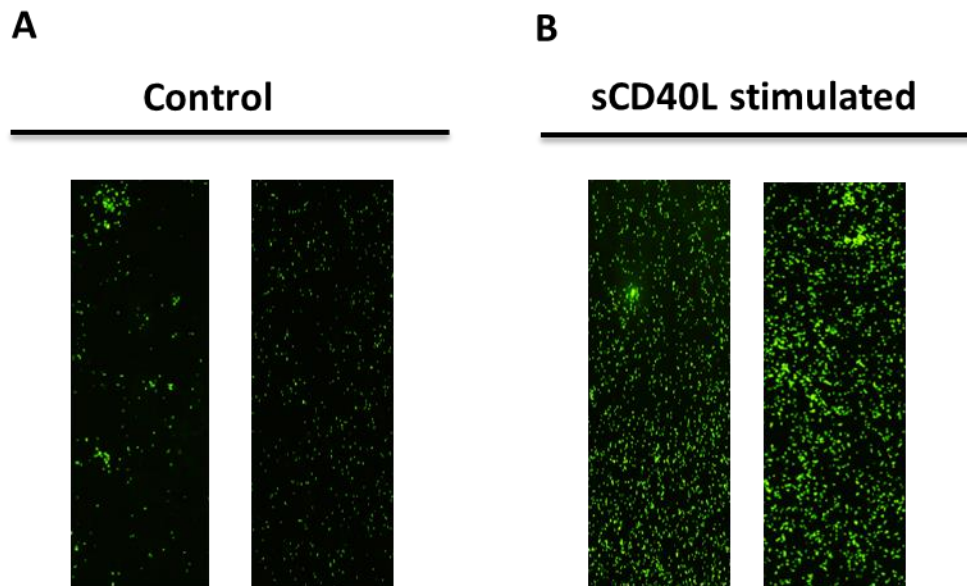
Figure 4: Monocyte adhesion to the endothelial monolayer. *THP-1 adhesion to the endothelial monolayer before (**A**) and after sCD40L stimulation (**B**). THP-1 adhesion to the different genotypes of the -1C/T SNP of the CD40 gene was analyzed using this set-up.*

4.4 Genomic DNA extraction from human blood

DNA was extracted from whole blood using Qiagen DNA Mini Kit as per the manufacturer's protocol. This kit uses a silica membrane spin column that selectively adsorbs DNA in the sample while impurities and contaminants are eluted using low salt buffers. Pure DNA was eluted in 30 µl elution buffer and its purity was assessed by the

ratio of A_{260}/A_{280} which was determined spectrophotometrically using the NanoDrop ND-1000 (PeQlab Biotechnology, GmbH). Samples with a ratio between 1.7 and 1.9 were genotyped for the -1C/T CD40 SNP.

4.5 RNA extraction from cells

To isolate RNA from adherent cells, they were rinsed with cold D-PBS or Hank's balanced salt solution (HBSS) and lysed with 350 µl RNA lysis buffer supplemented with 1% β-mercaptoethanol. Suspension cells (THP1 cells) were centrifuged at 1000 rpm for 5 mins and lysed with the same method as for the adherent cells. Total RNA was extracted using Qiagen RNEasy extraction kit following the manufacturer's protocol briefly outlined below. Column silica membranes form the basis of this method. The lysed samples were mixed with an equal volume of 70% ethanol to facilitate binding of RNA to the silica membranes. Washing steps with buffers supplied ensure the elution of pure RNA after DNA digestion and elimination of other cellular components. Purity and concentration of the RNA eluted in 30 µl of RNase free water was analyzed spectrophotometrically. For assessment of purity the ratio of absorbance readings at 260 and 280 nm (A_{260}/A_{280}) was analyzed. Samples within a ratio of 1.9 to 2.1 were analyzed further.

4.6 Reverse transcription and cDNA synthesis

The Qiagen Omniscript® Reverse Transcription Kit was used for the transcription of the RNA template to complementary DNA (cDNA), according to the manufacturer's instruction. Briefly, 100 ng of the template (12 µl) was incubated with a master mix composed of reverse transcriptase enzyme, RNase inhibitor which inhibits RNases from the human placenta as well as RNase A,B and C, and a primer mix containing dNTPs and random primers to facilitate transcription from all regions including the 5′ untranslated region. The volume was made to 20 µl with RNase free water and the mixture was heated at 56°C for 1 hour. After cooling the mixture on ice, the resulting cDNA was diluted 1:10 and used as a template for conventional or RT-qPCR.

4.7 Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RT-qPCR reactions were carried out using the QuantiTect SYBR Green® Kit (Qiagen, Germany) with the LightCycler 1.5 instrument (Roche Diagnostics, Germany). A master mix of 20 µl was prepared, the constituents of which are listed in **table 8**. The annealing temperatures varied according to the primer pairs being used for amplification and are tabulated in **table 6.** Experiments were carried out in duplicates and the Ct values of the technical duplicates were typically within 0.3 cycles. To adjust for inter-experimental variability changes in gene expression were normalized to housekeeping genes: GAPDH or HPRT-1 (Primer sequence from Raychaudhuri et al., 2008). Ct values of these genes did not vary considerably between the control and stimulated conditions. Changes in gene expression were analyzed by relative quantification which is based on the comparative Ct method, where Ct denotes the cycle number required for exceeding the fluorescence threshold (Livak et al., 2001). Fold changes in gene expression were calculated by computing the ratio of the Ct values of target and housekeeping genes under untreated vs treated conditions.

Gene expression was quantified by a mathematical model which enables relative quantification of the target gene by comparison to a reference gene (Pfaffl, 2001). The gene transcript of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. GAPDH is widely considered to be a housekeeping gene since it is essential in glycolysis and has a stable expression across various cells and experimental conditions. Consequently, it is commonly used as a reference gene for mRNA and protein normalization (De Kok et al., 2005; Sikand et al., 2012). Additionally, selected experiments were also compared to mRNA expression of hypoxanthine guanine phosphoribosyl transferase (HPRT), an enzyme essential for purine generation, and also a standard housekeeping gene for use as endogenous standard (Lee et al., 2002). The ratio was calculated as follows:

$$ratio = \frac{(E_{target})^{\Delta CP_{target(control-sample)}}}{(E_{ref})^{\Delta CP_{ref(control-sample)}}}$$

E_{target}, E_{ref} = amplification efficiencies of the target and reference gene

CP_{target}, CP_{ref} = fluorescence crossing points

The PCR amplification efficiency was assessed by the methods published by Pfaffl in 2001 (E_{target} and $E_{ref} \approx 2$). CP values for each transcript were determined by setting a threshold in the segment of the highest fluorescence intensity increase using the Light cycler software (Version 1.5, Roche).

Table 8: Composition of the reaction mixture for RT-qPCR

Component	Volume (µl)
cDNA (20 ng)	5
Primers (10-20 µM) (Forward and Reverse)	2
SYBR green	10
Water	3

4.8 Conventional PCR

Semi-quantitative PCR was used for genotyping the -1C/T CD40 SNP and isolating soluble CD40 from HUVEC cDNA. The composition of the PCR reaction mixture is outlined in **table 9** and it was amplified in an automated thermocycler (Biometra), for which the cycling parameters are presented in **table 10.**

Table 9: Composition of the reaction mixture for PCR

Component	Volume (µl)
PCR buffer (10X)	5
Primers (10µM) (Forward and reverse)	1
dNTPs (10mM)	4

DNA	3
Taq polymerase	0.2
Water	36.8
Total	50

Table 10: PCR program for the genotyping of the -1C/T SNP of the *CD40* gene

Step	Temperature (°C)	Time
Pre-denaturation	94	5 min
Denaturation	94	30 s
Annealing	61	45 s
Synthesis	72	45 s
Extension	72	10 min

4.9 Restriction fragment length polymorphism analysis

For genotypic analysis of the -1C/T CD40 SNP, PCR-based restriction fragment length polymorphism (PCR-RFLP) analysis was performed. Genomic DNA extracted from umbilical arteries, human patients and healthy control subject's blood was genotyped. A 320 bp fragment of the 5' untranslated region surrounding the SNP was amplified by PCR and digested for 1 hour at 37°C with the restriction enzyme *NcoI*. The resulting fragments were visualized on an agarose gel and the genotype determined according to the band pattern obtained which was indicative of the genotype **(Figure 5).**
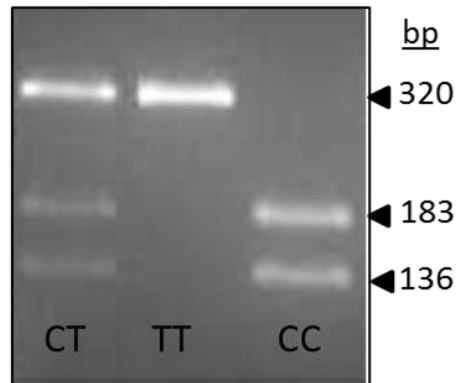
Figure 5: Representative 3 genotypes of the -1C/T SNP of the *CD40* gene. *Genotyping was performed by polymerase chain reaction-restriction fragment length analysis of genomic DNA.*

4.10 Agarose gel electrophoresis

Agarose gel electrophoresis is a molecular biology technique for the size based separation of DNA fragments, which migrate towards the positive electrode depending upon the size of the agarose gel. This technique was utilized for restriction fragment length polymorphism (RFLP) and to confirm the presence of the correct PCR product after amplification. Gels with a density of 2% were prepared by dissolving 3 g agarose in 150 ml TBE buffer supplemented with 4 µl ethidium bromide for visualization of the DNA fragments. Samples consisting of either PCR products or restriction digests were mixed with 6µl loading dye and electrophoresed at 120 V for 60 mins. For verification of the DNA size fragments standard DNA ladders were used. The resolved samples were visualized using the Gel Doc XR+ system and the Quantity One software (Version 4.6.9, Bio-Rad Germany) was used for data analysis.

4.11 Protein extraction

For total cellular protein isolation, cells were rinsed twice with ice cold HEPES buffer and scraped in 1.5 ml HEPES, followed by centrifugation at 3000 rpm for 5 mins at 4°C. The pellet was lysed in 150 µl lysis buffer containing protease inhibitors and Pefabloc to protect the samples from proteolysis, and dithiothreitol to prevent protein oxidation. Cellular contents were disrupted by exposure to sound waves of 30 Hz in short bursts of 10 sec. Samples were kept on ice in between sonication to regulate the heat generated. A

second centrifugation step ensured the removal of nonhomogenized cells and cellular debris.

Light absorbance of the samples at 595 nm wavelength was measured using the Bradford dye reagent in a colorimetric assay for estimation of the protein concentration. Increase in absorbance is directly proportional to the protein concentration. Standard curves were generated with known bovine serum sample standards and the samples were immediately frozen in liquid nitrogen and stored at -80°C for further analysis.

4.12 Immunoblotting

Separation of protein samples was achieved using SDS-PAGE. 10% denaturing gels overlaid with 4% stacking gel **(table 11)** were freshly prepared for polymerization. Total protein (20µg) was denatured by heating at 95°C for 5 mins with 4X sample loading buffer (Roth) and loaded onto 1.5mm thick gels. A vertical system (Mini-Protean Tetra Cell, Bio-Rad) was utilized for electrophoresis with dual colour protein standards for determining the molecular mass and for monitoring the transfer. Proteins were stacked at 80V for 30 mins and separated at 120 V. Electrotransfer at 350 A for 60-90 mins of a combination of gel, membrane and electrodes immersed in a transfer buffer enabled migration of proteins from the gel onto a pre-activated PVDF membrane (0.45 µM, Mini Trans-Blot Bio-Rad). The membrane were blocked with 5% non-fat milk (w/v) in TBS-T to reduce non-specific binding and incubated with the appropriate primary antibody over-night at 4°C. Subsequently, they were washed thrice for five minutes with TBS-T to wash away weakly or unbound antibodies and incubated with the species specific secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature. Finally, the membranes were washed thoroughly with TBS-T and TBS and chemiluminescence (Luminata Forte) was used for band visualization in combination with ImageQuant LAS 4000 system. Band intensity was quantified using ImageJ and β-actin was used for normalization.

Table 11: SDS gel components

	Separating gel (10%)	Stacking gel (4%)
1.5M Tris-HCl (pH 8.8)	2.5 ml	-
1.5M Tris-HCl (pH6.8)	-	0.5 ml
SDS (10%)	100 µl	40 µl
APS (10%)	100 µl	40 µl
Acrylamide (30%)	3.3 ml	0.67 ml
TEMED	4 µl	4 µl
Water	4 ml	2.7 ml
Total	10 ml	4ml

4.13 Flow Cytometry

HUVECs ($1x10^6$) were washed twice with FACS buffer and detached with 300 µl Accutase® cell detachment solutions. Cells were centrifuged at 1000 rpm for 5 minutes and the pellet was washed twice with FACS buffer followed by incubation with the appropriate antibodies (CD40 and VCAM-1) and their respective IgG controls for 30 mins at 4°C. Unstained and untreated cells were used as controls. Finally cells were washed with 1 ml FACS buffer, re-suspended in 300 µl FACS buffer and analyzed immediately. 10,000 cells were measured in every experiment which was performed on a BD FACSDiva® flow cytometer using BD FACSDiva version 6 software. Analyses were carried out in collaboration with Dr. Michael Weitnauer, Department of Medical Microbiology and Hygiene, Heidelberg University.

4.14 Coronary heart disease patient sample collection

Blood samples were provided by Dr. Florian Leuschner (University Hospital Heidelberg, Antragsnummer: S-390/2011) and Dr. Thorsten Kessler (German Heart Centre, Munich, Antragsnummer: S-689/2015). Patients were diagnosed with CHD when blockage of at least one coronary artery by 25% was confirmed by coronary angiography. Additional

requirements for diagnosis were a lack of myocardial infarction, unstable angina and confounding chronic inflammatory conditions like rheumatoid arthritis and inflammatory bowel disease. The average age of the patients was 71.3±12.7. Age-matched control samples which had a mean age of 64±3, were provided by Dr. Maik Brune (University Hospital Heidelberg).

4.15 Inflammatory arthritis patient sample collection

Blood samples of RA and PsA patients were kindly provided by Prof. Dr. Hanns Marti-Lorenz (University Hospital Heidelberg Antragsnummer: S-689/2015). Patients were diagnosed as per the 2010 Rheumatoid Arthritis Classification Criteria (Aletaha et al., 2010). According to these criteria, definite RA is diagnosed when there is the presence of synovitis in at least one joint coupled with an inadequate explanation for its presence. Additionally, six out of ten possible points in a scoring system measuring 4 domains must be obtained. The four domains are: number and site of joints affected, serologic abnormality, elevated acute-phase response and symptom duration. The CASPAR classification criterion was used for PsA diagnosis (Taylor et al., 2006). To be classified as PsA positive, a patient must have inflammatory articular disease and score a minimum of 3 out of 5 points in the following domains: 1) evidence of psoriasis 2) psoriatic nail dystrophy 3) negative test result for rheumatoid factor 4) current of a history of dactylitis and 5) radiological evidence of juxta-articular new bone formation.

4.16 Statistical analysis

All data are represented as mean ± SEM with 'n' denoting biological replicates unless stated otherwise. To determine if the data followed Gaussian distribution the D'Agostino-Pearson omnibus test was used. Differences between two groups (control vs stimulated) were determined using the unpaired Students t-test or Mann-Whitney U test (parametric vs non-parametric respectively). Variations between three or more groups (e.g., CC, CT and TT genotype endothelial cells) were computed by one-way ANOVA with Tukey's post-hoc test. Data which was not normally distributed was analyzed by Kruskal-Wallis ANOVA test with Dunn's multiple comparison. Mean differences between two independent variables (genotype and stimulation) were analyzed by two-way ANOVA

with Tukey's post-hoc test. Hardy-Weinberg equilibrium for the -1C/T CD40 SNP was determined by Chi-square goodness of fit test with 1 degree of freedom and odds ratio by Fischer's exact test. Additionally, the Grubb's test was used to determine outliers. All data was analyzed by GraphPad® Prism 6 (Version 6.01) software.

5 RESULTS

5.1 Distribution of the -1C/T *CD40* SNP in Baden-Württemberg

To determine the distribution of the –1C/T SNP in the *CD40* gene in the local population, 470 genomic DNA samples isolated from collected umbilical cords from Heidelberg hospitals were subjected to polymerase chain reaction-restriction fragment length polymorphism analysis. Since the presence and distribution of single nucleotide polymorphisms varies depending on the ethnicity and race of the population being studied, DNA samples from the local region were analyzed to estimate allele frequency and genotype distribution. Genotyping efficiency was <95% and three distinct genotypes in Hardy-Weinberg equilibirum were present. The results showed a clear dominance of the C allele (**Figure 6A**) and of the homozygous C genotype (**Figure 6B**).
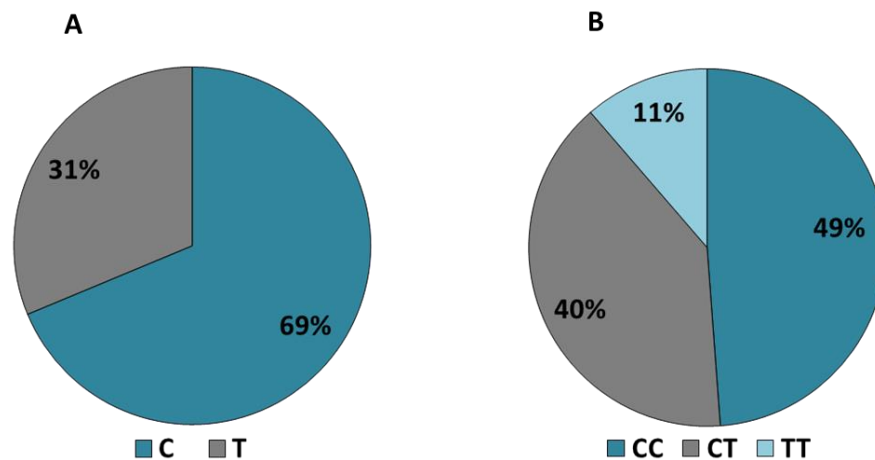
Figure 6: The C allele is dominant in the local population. *(A) Allele frequency and (B) genotype distribution of -1C/T SNP. Genotyping was performed by PCR-based RFLP analysis of genomic DNA isolated from umbilical cords of newborns; n=470. HWE (p>0.1)*

5.2 Effect of the -1C/T CD40 SNP on endothelial CD40 expression

The -1C/T SNP is located in a strategic position surrounding the AUG initiation codon in the Kozak consensus sequence. Since the sequence neighboring the AUG initiation codon in an mRNA is a determinant of the efficiency of the protein translation machinery, we analyzed the impact of the -1C to T transition on CD40 expression in genotype stratified

primary HUVECs. Predictaby, the CD40 mRNA levels were comparable in all genotypes (**Figure 7A**). However, a genotype-dependent effect was clearly seen in intracellular and surface CD40 protein levels, measured by immunoblotting and flow cytometry, respectively (**Figure 7B and C**). Intracellularly, homozygous C genotype (CC) cells expressed twice as much protein than endothelial cells isolated from heterozygous (CT) or homozygous T genotype (TT) carriers (**Figure 7B**). This phenotypic difference was clearly more pronounced with respect to CD40 protein detected on the surface of the CC cells as compared to the CT or TT cells (**Figure 7C**). Collectively, cell surface expression levels on CC cells were 1.3- and 5.7-fold higher as compared to the CT and TT genotype cells, respectively. These measurements indicate an additive effect of the T allele with CD40 protein levels decreasing significantly in the presence of one T allele and further in the presence of two T alleles.
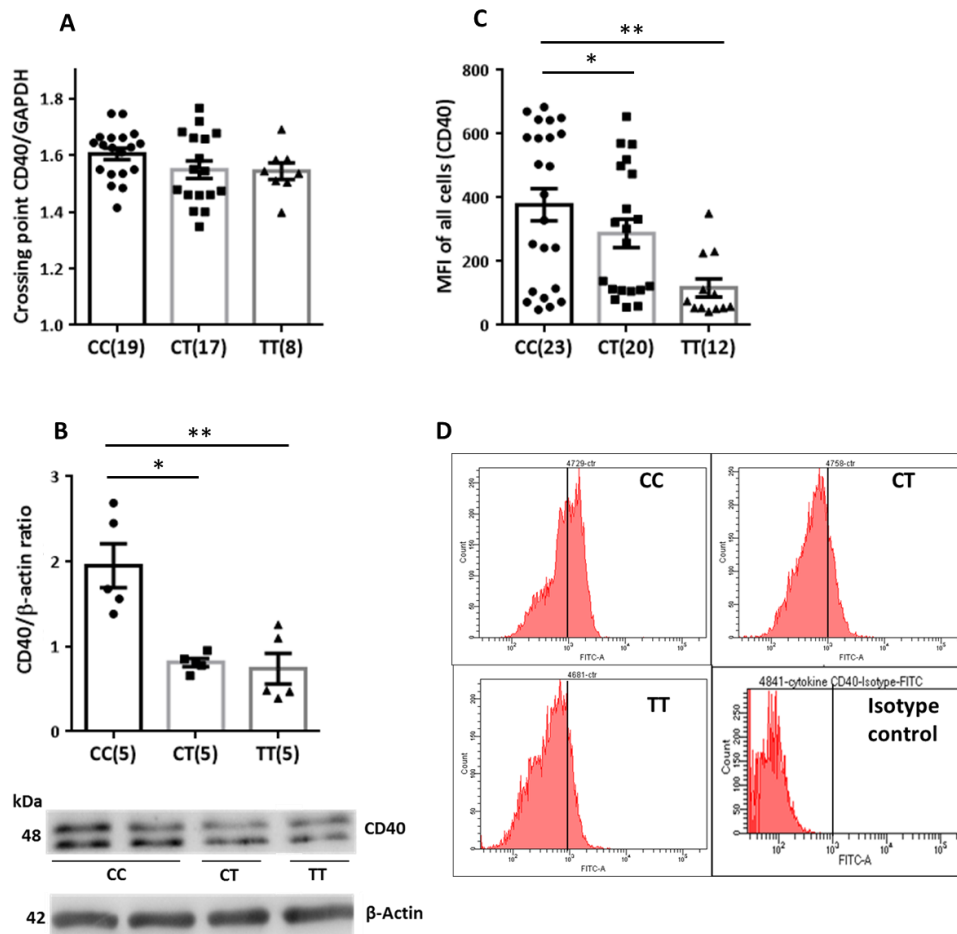
Figure 7: The -1C/T SNP affects endothelial CD40 protein expression. *(A) Quantitative RT-PCR analysis of CD40 mRNA levels in CC, CT and TT genotypes. Crossing points were normalized to the housekeeping gene GAPDH. (B) Western blot analyses of CD40 protein normalized to β-actin as a loading control in whole cell lysates from HUVECs with different genotypes.**p<0.01 (C) Flow cytometry analyses of CD40 cell surface protein abundance in HUVECs with different genotypes (10,000 cells were used per FACS measurement). Isotype control and unstained cells were analyzed to determine sensitivity of the staining protocol (D) Representative FACS histograms are depicted; *p<0.05; **p<0.01, n-numbers are denoted in brackets on the graph adjacent to the genotype.*

5.3 Effect of the -1C/T *CD40* SNP on sCD40L-induced proinflammatory gene expression

CD40 protein has a critical role in immunity and inflammation. Associated signaling is known to increase the expression of proinflammatory genes and adhesion molecules to facilitate the binding of monocytes and lymphocytes to the vascular endothelium. We

were interested to determine if the effect of the -1C/T SNP of the CD40 gene is limited to protein abundance or if there are also downstream consequences which take place by influencing sCD40L-induced signaling. To test if variation in CD40 protein abundance has an impact on proinflammatory gene expression in endothelial cells, expression of three proinflammatory mediators, VCAM-1, MCP-1, and the leuckocyte adhesion molecule E-selectin, were analyzed under three different proinflammatory conditions that are described in the following three sections.

The following section presents data comparing gene expression in non-stimulated and sCD40L-stimulated HUVECs with and without genotype stratification. HUVECs were stimulated with sCD40L (200ng/ml) for 8 hours and 24 hours to measure changes in mRNA and protein expression respectively.

5.3.1 Impact on CD40 cell surface abundance

CD40 belongs to the TNFR superfamily in which signal transduction varies depending on whether the receptor remains on the cell surface or is internalized. Following ligation the CD40 receptor is rapidly internalized into early stage endosomes in a cholesterol-dependent manner. The internalized protein is sequestered into vesicles with the capacity to phosphorylate Akt and lead to NF-kB activation (Chen et al., 2007). Therefore, it was not surprising to note an obvious and significant decrease in surface CD40 levels 24 hours post-ligation with sCD40L **(Figure 8A)**. Moreover, TT cells internalized CD40 to a greater extent (0.44-fold) than CT or TT (0.33-fold) cells **(Figure 8B)**.
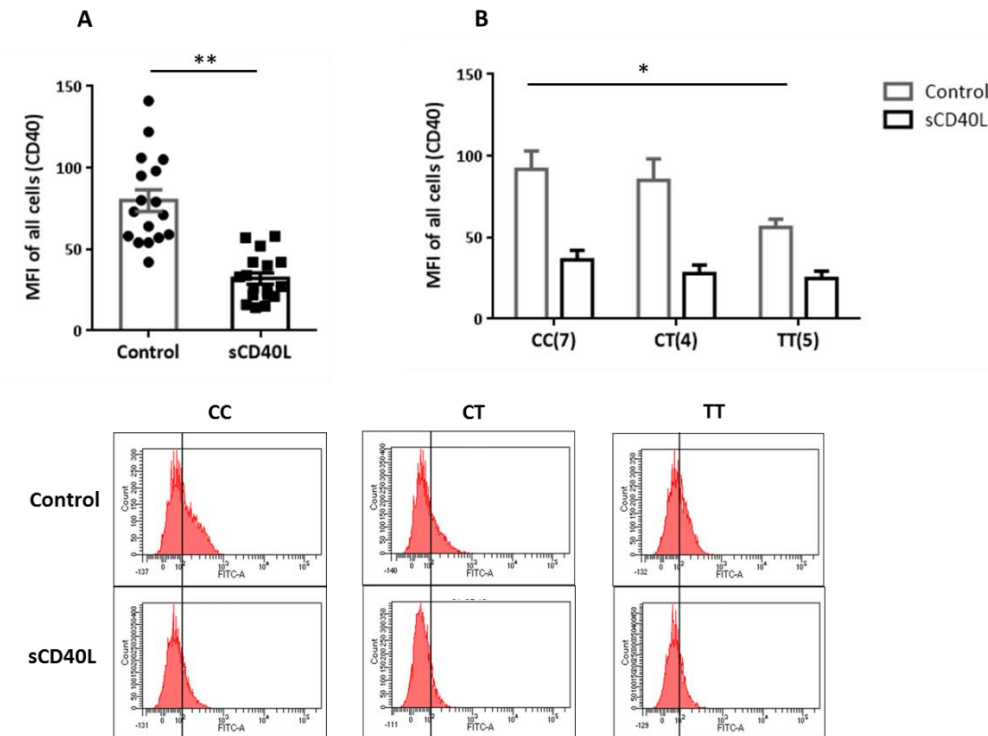
Figure 8: sCD40L induces genotype-dependent internalization of surface CD40.
*FACS analyses of CD40 cell surface protein appearance. MFI of 10,000 cells was analyzed. (**A**) sCD40L stimulation induces a significant decrease in surface CD40; n=17;**p<0.01 vs control. (**B**) The decrease was significant in CC and CT but not TT genotype cells; *p<0.05, n-numbers are denoted in brackets on the graph adjacent to the genotype.*

5.3.2 Impact on sCD40L-induced VCAM-1 expression

The first read-out for measuring the effect of CD40-sCD40L-interaction on proinflammatory gene expression was VCAM-1. Exposure of HUVECs to sCD40L induced a 44-fold increase in VCAM-1 mRNA expression independent of the genotype **(Figure 9A)**. Genotype-dependent effects were clearly observed with CC cells having the highest capacity to upregulate VCAM-1 (70-fold) compared to 15- and 6-fold upregulation in CT and TT genotype cells, respectively **(Figure 9B)**. Exposure of HUVECs to sCD40L elicited a total 13-fold increase in surface VCAM-1 protein **(Figure 10A)**. The CC cells exhibited the highest upregulation (30-fold) compared to CT and TT genotype cells (6- and 8-fold, respectively). Similar to mRNA levels, the presence of one T allele was sufficient to diminish sCD40L-induced VCAM-1 protein upregulation significantly **(Figure 10B)**.
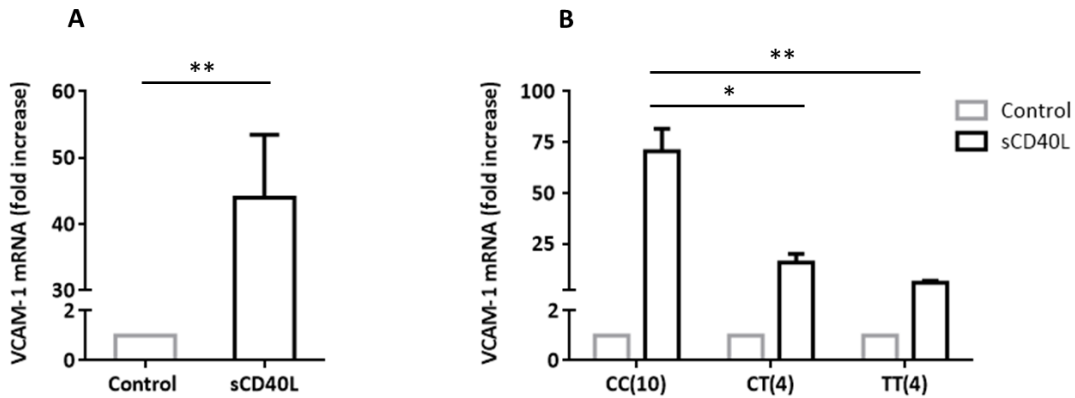
Figure 9: sCD40L-induced VCAM-1 mRNA expression is affected by the -1C/T SNP of the *CD40* gene. *(**A**) Soluble CD40L induces a marked upregulation of VCAM-1 mRNA levels in all cells; n=16; **p<0.01 vs control. (**B**) The -1C/T genotype differentially affects this upregulation; *p<0.05, **p<0.01, n-numbers are denoted in brackets on the graph adjacent to the genotype.*
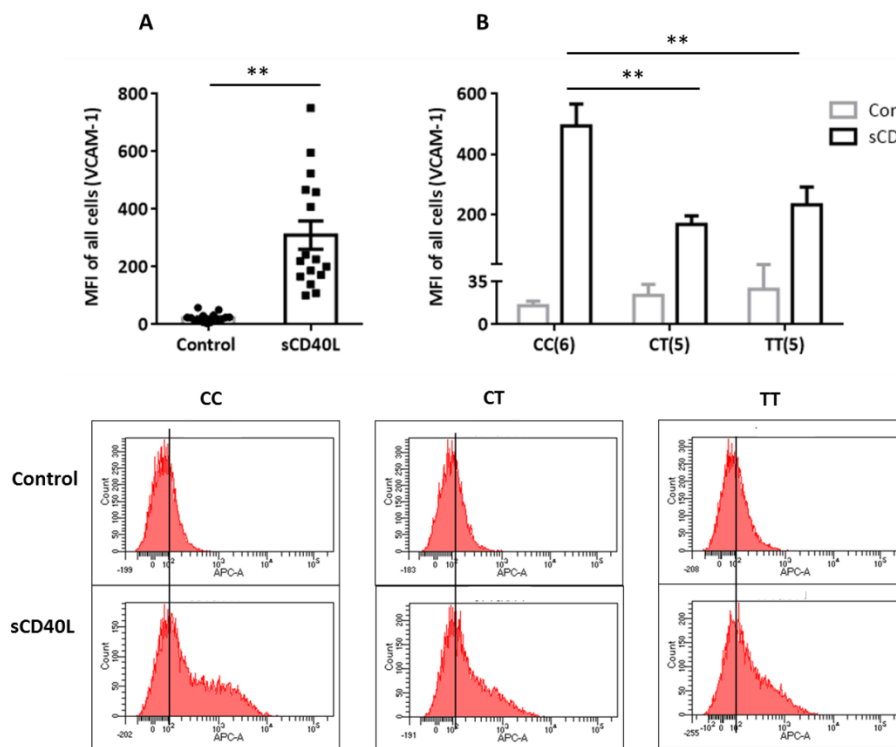
Figure 10: sCD40L-induced VCAM-1 protein expression is affected by -1C/T SNP. *Flow cytometry analyses of VCAM-1 cell surface protein levels. MFI of 10,000 cells was analyzed. (**A**) Genotype-independent upregulation of surface VCAM-1 protein; n=16;**p<0.01 vs control. (**B**) Genotype-dependent upregulation of surface VCAM-1 protein; **p<0.01, n-numbers are denoted in brackets on the graph adjacent to the genotype.*

5.3.3 Impact on sCD40L-induced MCP-1 expression

The second gene analyzed as a read-out was MCP-1. Monocyte transmigration and differentiation into macrophages occurs along a MCP-1 gradient, underlying the key role of this chemotactic agent in immunity and inflammation (Randolph and Furie, 1995). Soluble CD40L induced a genotype-independent 13-fold increase in MCP-1 mRNA expression in HUVECs **(Figure 11A).** This increase was variable depending on the genotype with 18-fold upregulation of MCP-1 mRNA in CC genotype cells, 10-fold in CT and 8-fold in TT genotype cells (**Figure 11B**). Upregulation thus peaked in cells homozygous for the C allele and progressively decreased with addition of the T allele. Upregulation of MCP-1 protein followed a similar pattern. Exposure to sCD40L for 24 hours elicited a genotype-independent increase in protein by 1.8-fold (**Figure 11C**). No significant differences were observed within the genotypes but the recorded increase was maximum in the CC genotype cells (2.3-fold) as compared to the CT (1.7-fold) and TT (1.1-fold) genotype cells (**Figure 11D**).
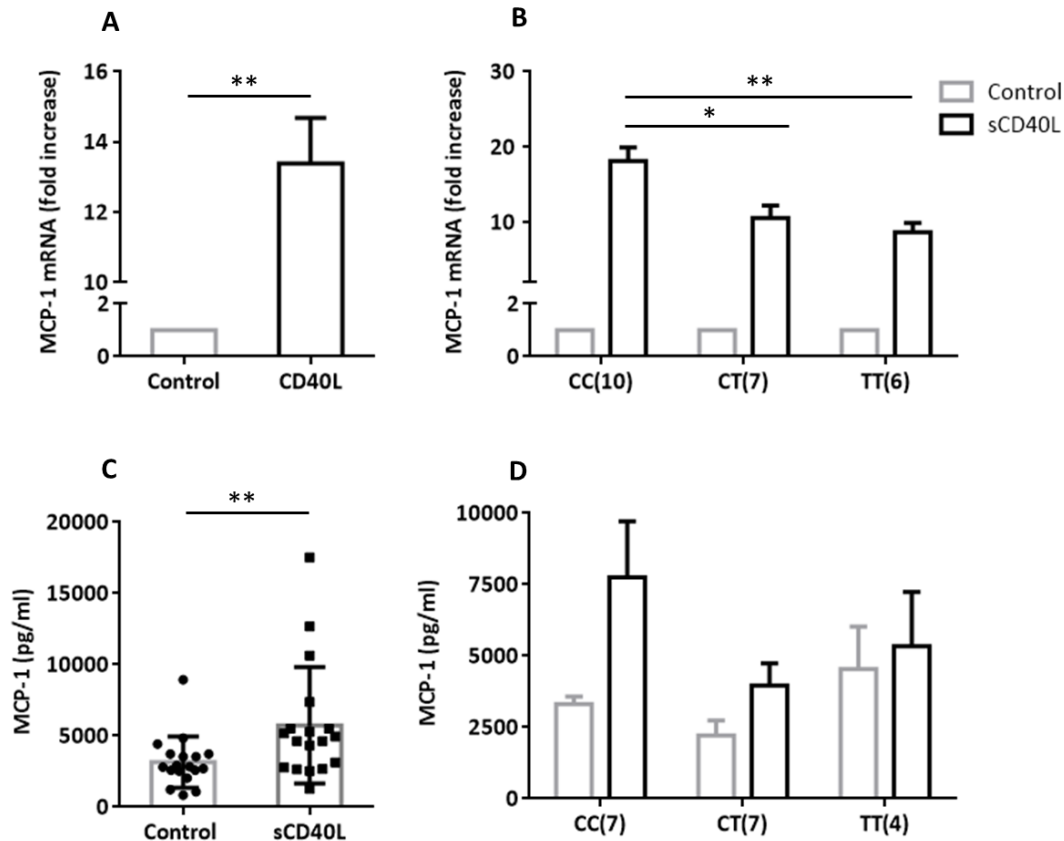
Figure 11: sCD40L-induced MCP-1 mRNA expression is affected by the -1C/T CD40 genotype. *(A) Genotype-independent upregulation of MCP-1 mRNA; n=23, **p<0.01 vs control. (B) Genotype-dependent upregulation of MCP-1 mRNA; *p<0.05, **p<0.01. ELISA based quantitation of MCP-1 in HUVEC supernatants. (C) Genotype-independent upregulation of MCP-1 protein; n=18; **p<0.01 vs control. (D) Genotype-dependent upregulation of MCP-1 protein, n-numbers are denoted in brackets on the graph adjacent to the genotype.*

5.3.4 Impact on sCD40L-induced E-selectin expression

E-selectin facilitates leukocyte rolling to enable leukocyte recruitment and extravasation. Enhanced leukocyte adhesion to HUVECs is dependent upon CD40L-induced E-selectin expression (Urban et al., 2011). Soluble CD40L induced a striking, genotype-independent 230-fold upregulation in E-selectin mRNA (**Figure 12A**) which was even higher (335-fold) in CC genotype cells but lower in CT (120-fold) or TT (180-fold) genotype cells (**Figure 12B**). Soluble CD40L induced a genotype-independent 1.6-fold upregulation in E-selectin protein released into the cell culture supernatant (**Figure 12C**) which was comparable in all genotypes (**Figure 12D**).
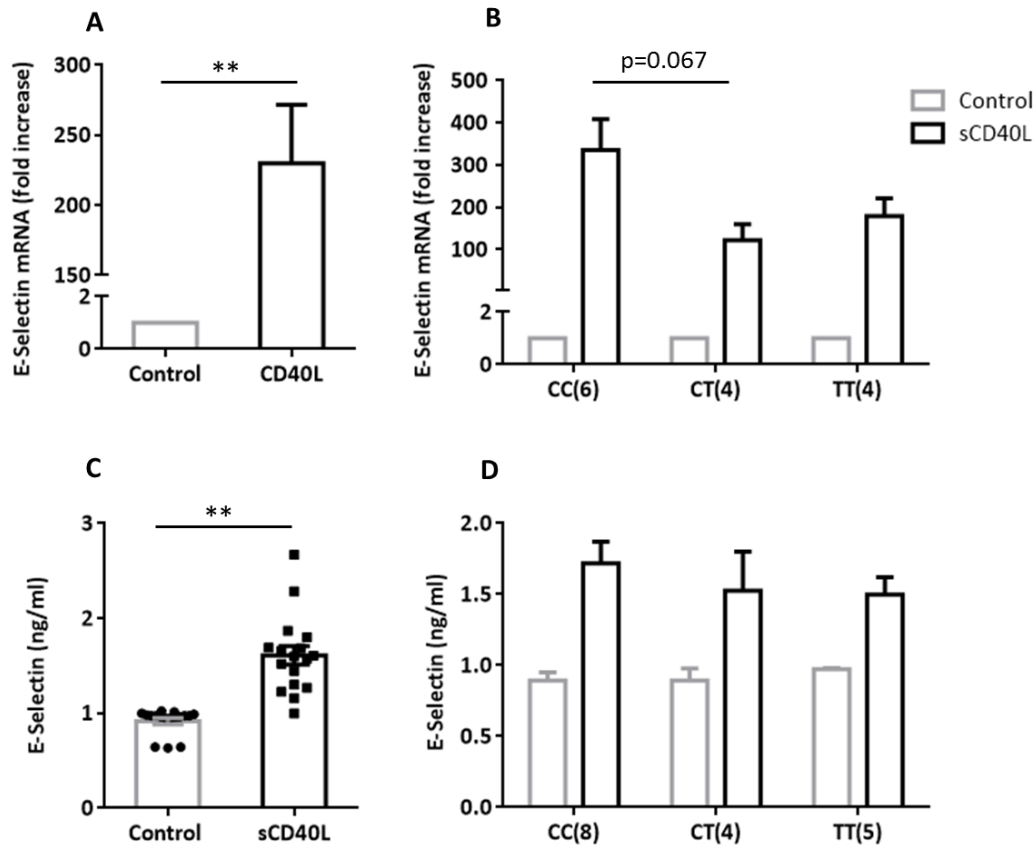
Figure 12: Impact of the -1C/T CD40 genotype on E-selectin expression. (*A*) *Genotype-independent upregulation of E-selectin mRNAs; n=14; **p<0.01 vs control.* (*B*) *Genotype-dependent upregulation of E-selectin mRNA.* (*C*) *Genotype–independent upregulation of E-selectin protein; n=17; **p<0.01 vs control.* (*D*) *Genotype-dependent upregulation of E-selectin protein, n-numbers are denoted in brackets on the graph adjacent to the genotype.*

5.4 Functional impact of the -1C/T CD40 SNP on monocyte adhesion to endothelial cells

Taken together, the aforementioned results indicate that the degree of sCD40L-induced proinflammatory gene expression in endothelial cells is dependent on the genotype for the -1C/T SNP of the *CD40* gene. The functional consequences of this effect were analyzed by employing a monocyte adhesion assay. Leukocyte adhesion to the vascular endothelium is an early event in atherosclerosis. It is initiated by leukocyte rolling, mediated by E-selectin while stable adhesion and leukocyte extravasation through the endothelium is facilitated by VCAM-1 and ICAM-1 (Hogg and Landis, 1993; Luscinskas

et al. 1996). Since the C allele of the -1C/T SNP was found to be associated with higher VCAM-1 expression post-sCD40L stimulation, its functional consequence was analyzed by quantifying the number of monocytes that adhered to sCD40L pre-stimulated endothelial cells with different -1C/T genotypes. Independent of the genotype of the endothelial cells, there was a 6-fold increase in monocyte adhesion post-stimulation with sCD40L (**Figure 13A**). Compared with non-stimulated cells the maximum adhesion occurred in CC genotype cells (12-fold) but was reduced to 3-fold by the presence of one T allele (CT and TT genotype cells) (**Figure 13B**).
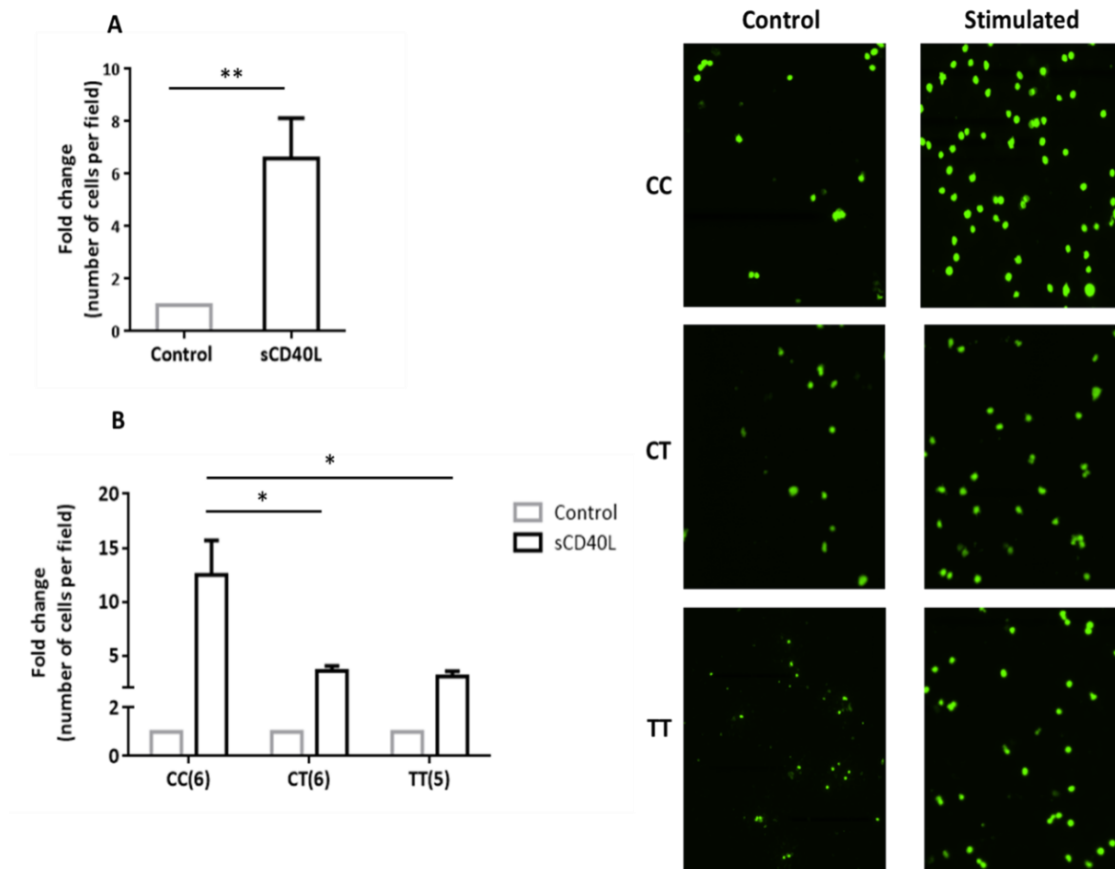
Figure 13: **SolubleCD40L-induced monocyte attachment to the endothelial monolayer is highest in CC genotype cells.** *Quantification of fluorescently labelled (green) THP-1 cells adhering to HUVECs stimulated for 2 hours with sCD40L.* **(A)** *Genotype independent adhesion of THP-1 cells; n=17, **p<0.01 vs control.* **(B)** *Genotype-dependent adhesion of THP-1 cells;*p<0.05, n-numbers are denoted in brackets on the graph adjacent to the genotype.*

5.5 Impact of the -1C/T *CD40* SNP on cytokine-activated and sCD40L-stimulated endothelial cells

During inflammatory conditions sCD40L is released from platelets while at the same time cytokines are released from Th1 cells and macrophages. Consequently, in an inflammatory context endothelial cells are simultaneously acted upon by CD40L and cytokines like TNF-α and IFN-γ which act synergistically to upregulate CD40 (Henn et al., 1998 and 200; Wagner et al., 2002; Yellin et al., 1995). Therefore, we investigated the impact of the -1C/T CD40 SNP on sCD40L-induced signaling in a proinflammatory context which would include cytokines. Such conditions were simulated *in vitro* by exposing cytokine-primed HUVECs to sCD40L. Their combined effect on CD40 protein expression was examined. Additionally, as read-outs for CD40-dependent intracellular signaling, the same panel of genes as in section 5.2 through 5.4 was analyzed and the results are presented in the following section. Stimulation details are described in the Materials and Methods section.

5.5.1 Impact on CD40 gene expression

Stimulation induced a significant increase in CD40 at the mRNA (2.9-fold) and protein (2-fold) level (**Figure 14A and B**). In genotype stratified cells, upregulation of CD40 mRNA was significantly higher in TT genotype cells (**Figure 15A**). This effect was not translated to the protein level where all genotypes showed a similar upregulation of total CD40 protein (**Figure 15C**). However, basal levels of CD40 protein on the cell surface were significantly different between the three genotypes (**Figure 15B**), and interestingly this difference remained the same following cytokine-priming and sCD40L stimulation with surface CD40 protein being highest in CC genotype cells followed by the CT and the TT genotype (**Figure 15B**).
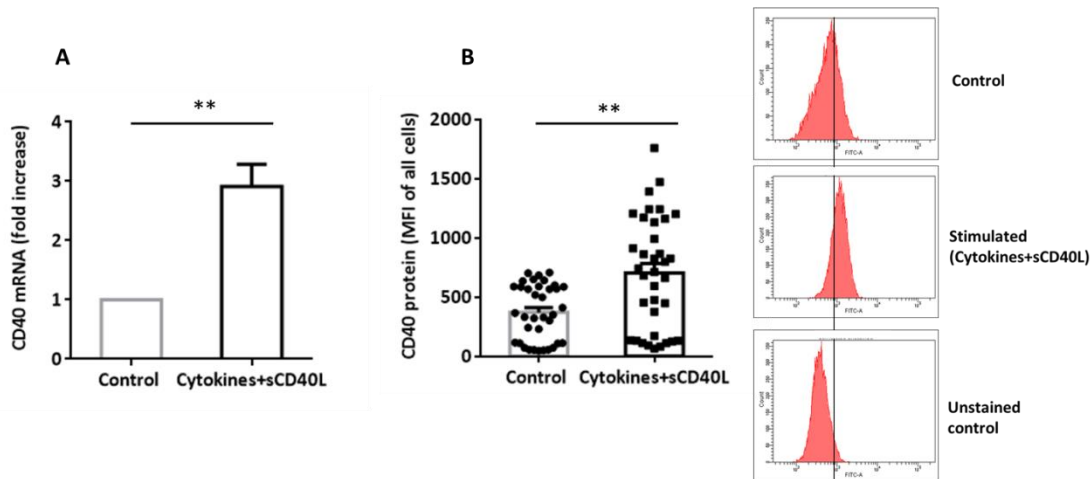
Figure 14: CD40 gene expression is upregulated under proinflammatory conditions.
*(**A**) CD40 mRNA and (**B**) protein expression is induced by sCD40L stimulation of primed cells of all -1C/T genotypes of the CD40 gene. (**A**) qRT-PCR of CD40 mRNA expression; n=23, **p<0.01 vs control. (**B**) FACS analysis of CD40 stained cells. MFI of ten thousand cells was analyzed. Representative histograms with unstained controls are depicted; n=40, **p<0.01 vs control.*
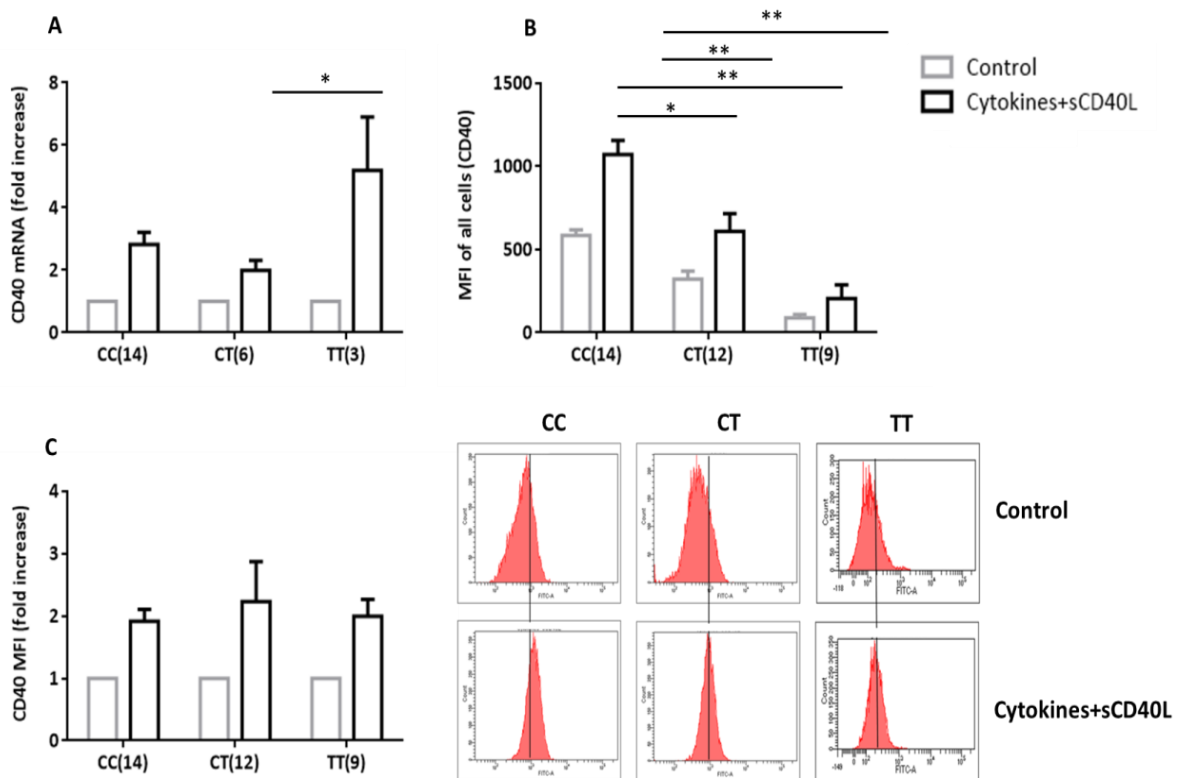
Figure 15: Impact of the -1C/T SNP on CD40 surface protein under proinflammatory conditions. *(A) Genotype-dependent upregulation of CD40 mRNA; *p<0.05. (**B&C**) Genotype-dependent upregulation of CD40 protein expressed as MFI of all cells (**B**) and fold increase relative to control (**C**)*p<0.05, **p<0.01, n-numbers are denoted in brackets adjacent to the genotype.*

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5.5.2 Impact on VCAM-1 gene expression

Under these simulated proinflammatory conditions VCAM-1 mRNA expression increased up to 75-fold independent of the genotype (**Figure 16A**). The presence of one T allele was sufficient to halve this effect which not reduced further in TT genotype cells (**Figure 16B**). Independent of the genotype there was also a marked 48-fold increase in VCAM-1 protein (**Figure 17A**) that rose to 140-fold in CC genotype cells, 120-fold in CT genotype cells and was non-significantly less in TT genotype cells (40-fold) (**Figure 17B**). Total VCAM-1 protein abundance post-stimulation was 3-fold higher in CC genotype cells as compared to the TT genotype (**Figure 17C**).
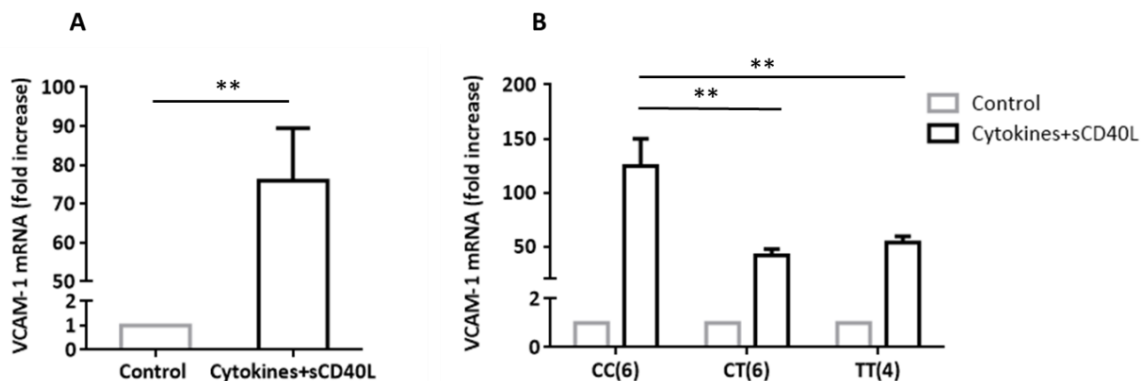
Figure 16: CC genotype is associated with higher VCAM-1 mRNA expression. (*A*) *Genotype independent up-regulation of VCAM-1 mRNA after cytokine priming and sCD40L stimulation; n=16; **p<0.01 vs control. (**B**) Genotype-dependent up-regulation of VCAM-1;**p<0.01, n-numbers are denoted in brackets adjacent to the genotype.*
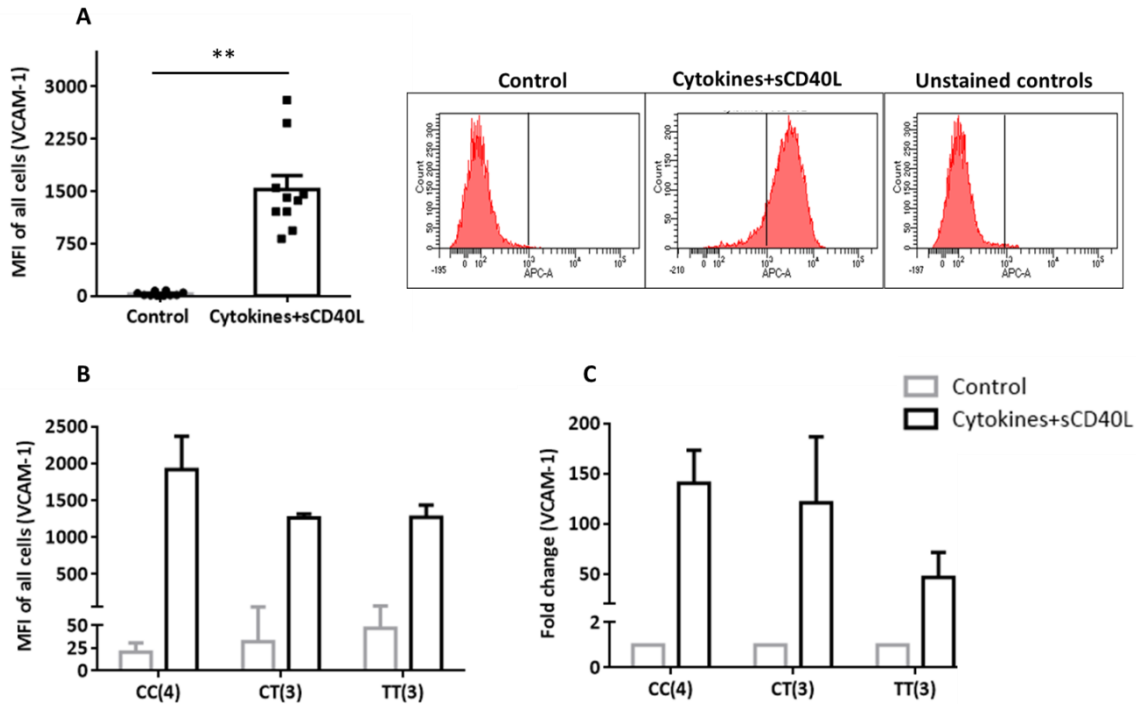
Figure 17: s. *Flow cytometry analyses of VCAM-1 cell surface protein upregulated after cytokine priming of sCD40L stimulation.(**A**) Genotype-independent upregulation of VCAM-1 protein; n=10, **p<0.01 vs control. (**B & C**) Genotype-dependent upregulation of VCAM-1 protein expressed as MFI of all cells (**B**) and fold increase relative to control (**C**), n-numbers are denoted in brackets adjacent to the genotype. MFI of 10,000 cells analyzed per FACS measurement.*

5.5.3 Impact on MCP-1 gene expression

Following stimulation with cytokines and sCD40L, the MCP-1 mRNA expression increased up to 38-fold independent of the genotype (**Figure 18A**). The presence of the T allele halved this effect which was further reduced when the T allele was homozygous (**Figure 18B**). MCP-1 protein quantified in the cell supernatants increased up to 1.4-fold independent of the genotype (**Figure 18C**) with the maximum increase (2-fold) in the CC genotype cells (**Figure 18D**).
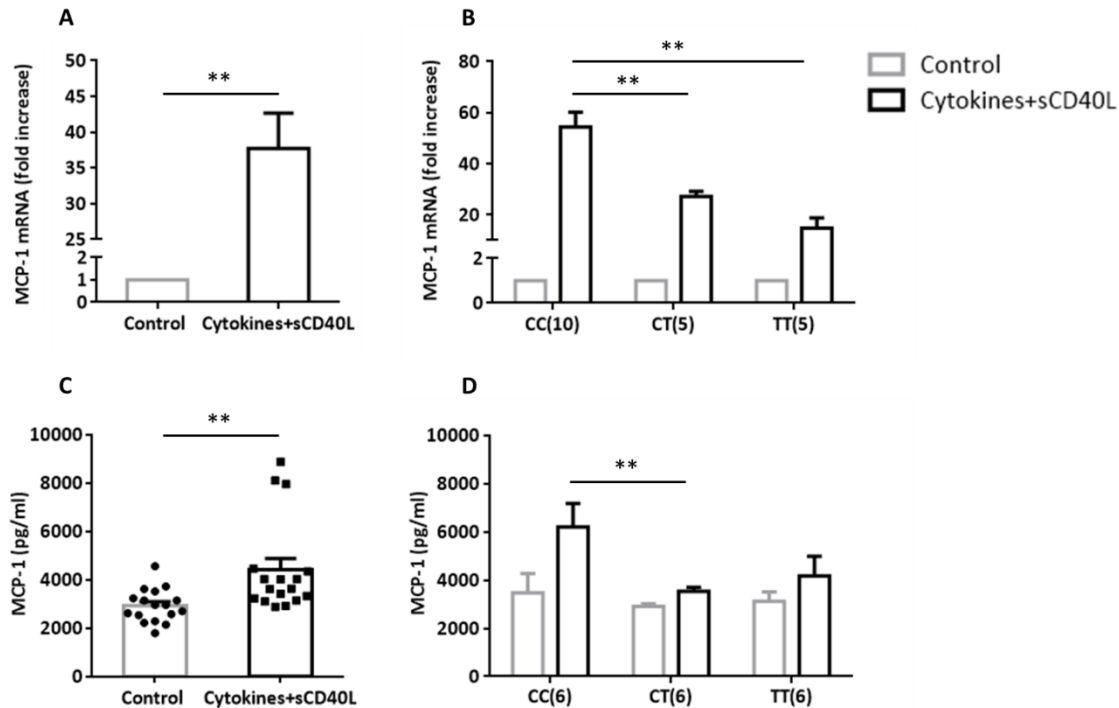
Figure 18: sCD40L-induced MCP-1 mRNA expression is affected by the -1C/T CD40 genotype. *(A) Genotype-independent upregulation of MCP-1 mRNA; n=20, **p<0.01 vs control. (B) Genotype-dependent upregulation of MCP-1 mRNA; **p<0.01. (C&D) ELISA based quantitation of MCP-1 in HUVEC supernatants. (C) Genotype-independent upregulation of MCP-1 protein; n=12; **p<0.01 vs control. (D) Genotype-dependent upregulation of MCP-1 protein, n-numbers are denoted in brackets on the graph adjacent to the genotype.*

5.5.4 Impact on E-selectin gene expression

Finally, soluble CD40L-induced E-selectin expression was compared in cytokine-primed HUVECs derived from homozygous or heterozygous carriers of the -1C/T SNP of the *CD40* gene. E-selectin mRNA expression exhibited a strong upregulation (354-fold) independent of the genotype (**Figure 19A**) which was comparable in the CC (594-fold) and CT (414-fold) genotype cells but was diminished to 54-fold in the TT genotype cells (**Figure 19B**). Independent of the genotype there was an 11.5-fold increase in the E-selectin protein released into the cell culture supernatants (**Figure 19C**) which was even higher (15-fold) in the CC genotype cells but lowed in the CT (11-fold) and TT (8-fold) genotype cells (**Figure 19D**).
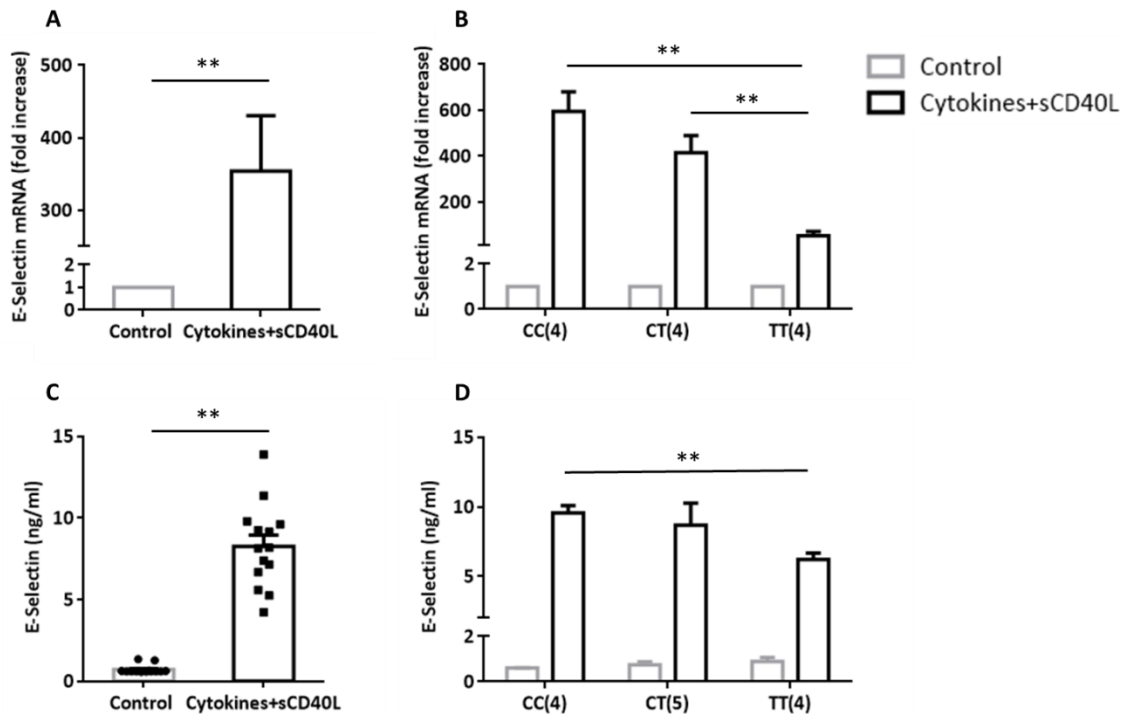
Figure 19: Impact of the -1C/T CD40 genotype on E-selectin expression. (*A*) *Genotype-independent upregulation of E-selectin mRNA; n=12; **p<0.01 vs control.* (*B*) *Genotype-dependent upregulation of E-selectin mRNA;**p<0.01.* (*C*) *Genotype–independent upregulation of E-selectin protein; n=14; **p<0.01 vs control.* (*D*) *Genotype dependent upregulation of E-selectin protein; **p<0.01, n-numbers are denoted in brackets on the graph adjacent to the genotype.*

The -1C/T SNP of the *CD40* gene affects the baseline CD40 abundance on the endothelial cell surface. This phenotype was maintained following cytokine upregulation of CD40 and sCD40L stimulation. Against this background, a genotype-dependent difference in CD40-mediated signaling measured via monitoring changes in the expression of proinflammatory endothelial gene products was also observed. Altogether, the aforementioned results indicate that the endothelial cell response to proinflammatory stimuli following sensitization to sCD40L is dependent on the genotype of the -1C/T SNP of the *CD40* gene and is highest in cells which are homozygous for the C allele.

5.6 Role of the -1C/T *CD40* SNP in predisposing HUVECs towards an inflammatory phenotype in the absence of recombinant sCD40L

As shown before, the proinflammatory gene products MCP-1, VCAM-1 and E-selectin were expressed in endothelial cells after cytokine (TNF-α, IFN-γ) priming followed by exposure to sCD40L. As summarized in table 12, this combined stimulation induced an approximately 2-fold greater increase in VCAM-1, MCP-1 and E-selectin mRNA expression as compared to sCD40L stimulation alone **(for detailed analysis please refer to sections 5.3-5.5),** presumably through enhanced cytokine-induced CD40 receptor expression which augmented CD40 receptor-mediated downstream signaling. It is also possible that the -1C/T SNP influences the proinflammatory response of endothelial cells to cytokines in the absence of sCD40L. To test this idea, genotype-stratified HUVECs were stimulated *in vitro* only by using the combination of TNF-α and IFN-γ.

Table 12: *Effects of cytokine priming in combination with sCD40L stimulation as compared to sCD40L stimulation alone on proinflammatory gene expression (mRNA) in the cultured HUVECs independent of the -1C/T SNP of the CD40 gene. qRT-PCR measurement of mRNA levels of VCAM-1, MCP-1 and E-selectin. *p<0.05.*

	VCAM-1 (n=16-18)	MCP-1 (n=20-23)	E-selectin (n=12-14)
sCD40L	44.0 ± 9.4	13.4 ±1.3	229.8±41.9
TNF-α+IFN-γ+sCD40L	72.6 ± 14.0	37.7±5.0	450.3±98.0
Fold increase	1.7	2.8**	2.0*

5.6.1 Impact on CD40 gene expression

Stimulation with cytokines (TNF-α, IFN-γ) in the absence of sCD40L induced the highest CD40 protein increase independent of the -1C/T SNP of the *CD40* gene, as compared to stimulation with sCD40L alone or in combination with cytokines (**Figure 22A**). As mentioned before, exposure to sCD40L induces internalization of the CD40 receptor leading to a decrease in the CD40 protein detectable on the cell surface, while proinflammatory cytokines such as TNF-α and IFN-γ induce its release (for greater detail, please refer to 5.3.1 and 5.5.1). Consequently, cytokine stimulation in the absence of sCD40L induced a higher (3.5-fold) increase in CD40 protein expression, as compared to

the increase post-stimulation (2-fold) with a combination of cytokines and sCD40L (**Figure 22A**). Following the same pattern as depicted in 5.5.1, the highest CD40 surface protein expression after cytokine priming was observed in the CC genotype, followed by the CT and the TT genotype (**Figure 22B**).
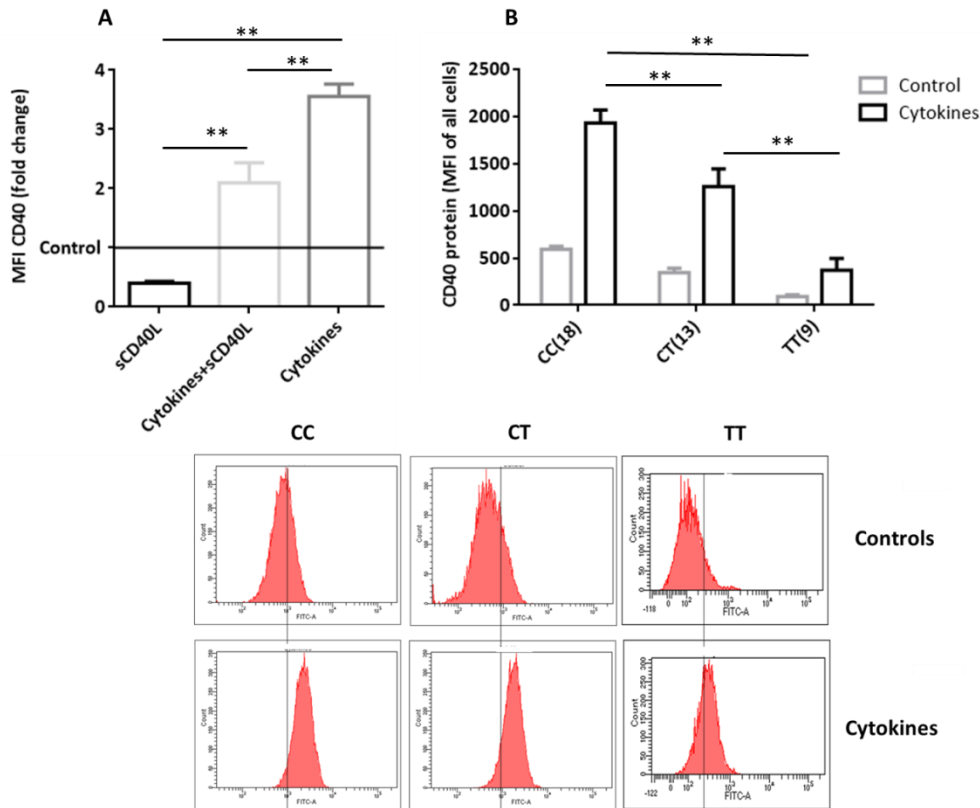
Figure 20: Cytokine treatment induces CD40 expression in a manner dependent on the genotype of the -1C/T SNP of the *CD40* gene. *(A) CD40 protein expression independent of the -1C/T SNP of the CD40 gene following stimulation with sCD40L, a combination of sCD40L and cytokines (TNF-α and IFN-γ) and cytokines without sCD40L, n=17, 23, 23 for sCD40L, cytokines and combination of cytokines and sCD40L respectively;**p<0.01. (**B**) Genotype-dependent upregulation of CD40 protein following exposure to cytokines;**p<0.01, n-numbers denoted in brackets adjacent to the genotype.*

5.6.2 Impact on VCAM-1 expression

Exposure of HUVEC to TNF-α and IFN-γ induced a highly significant, genotype-independent 34-fold increase in VCAM-1 mRNA (**Figure 21A**) which was even higher (65-fold) in the CC genotype cells but reduced to 20-fold in the CT genotype cells and 9-fold in the TT genotype cells (**Figure 21B**). Independent of the genotype there was a significant (26-fold) increase in VCAM-1 protein (**Figure 22A**) that was highest in the

CC genotype cells (69-fold) compared to 42-fold in the CT genotype cells and 33-fold in the TT genotype cells (**Figure 22 B**).
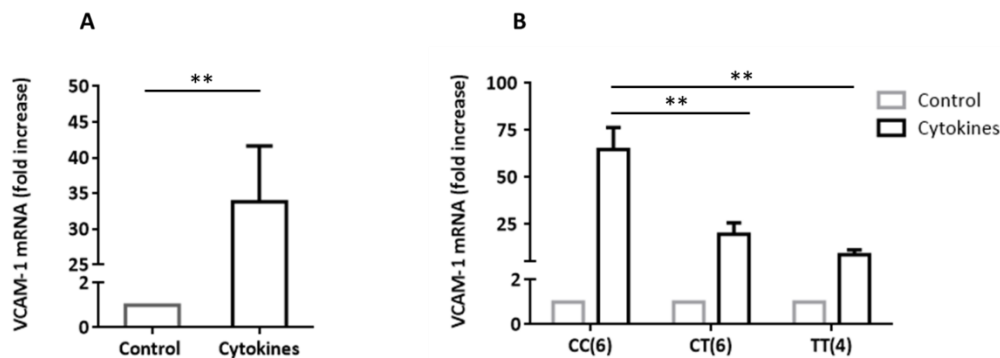
Figure 21: Cytokine-induced VCAM-1 mRNA expression is affected by the -1C/T CD40 genotype *(A) Genotype-independent upregulation of VCAM-1 mRNA; n=16, **p<0.01 vs control. (B) Genotype-dependent upregulation of VCAM-1 mRNA; **p<0.01, n-numbers are denoted in brackets on the graph adjacent to the genotype.*
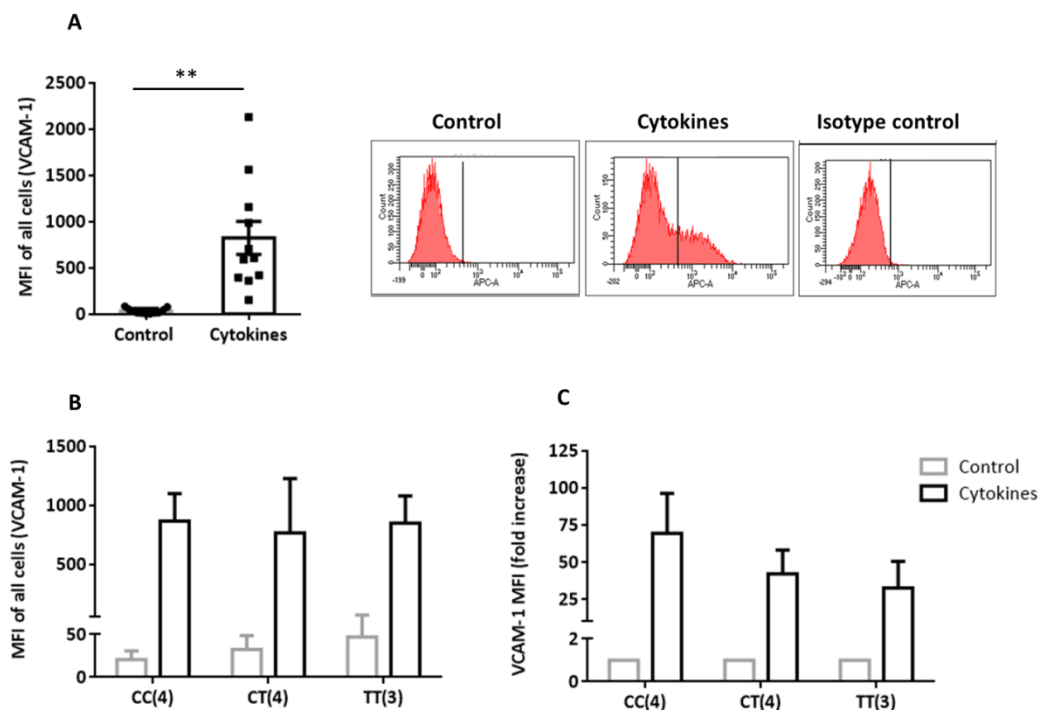
Figure 22: Impact on cytokine-induced VCAM-1 protein expression. *Flow cytometry analyses of VCAM-1 cell surface protein levels. MFI of 10,000 cells was analyzed. (A) Genotype-independent upregulation of surface VCAM-1 protein; n=11; **p<0.01 vs control. (B &C) Genotype-independent upregulation of surface VCAM-1 protein expressed as total MFI (B) and folds relative to controls (C); n-numbers are denoted in brackets on the graph adjacent to the genotype.*

5.6.3 Impact on MCP-1 expression

Cytokine exposure induced a genotype-independent 24-fold increase in MCP-1 mRNA (**Figure 23A**). The increase in the homozygous C genotype cells was 36-fold, which was significantly greater than the 17- and 5-fold increases observed in the heterozygous and homozygous T genotype cells (**Figure 23B**). MCP-1 protein quantified in the cell supernatant showed a genotype-independent 1.5-fold increase (**Figure 23C**). No significant differences were observed within the genotypes however an increase could be seen in only the CC genotype cells while the CT and TT genotype cells did not respond to the stimulation (**Figure 23D**).
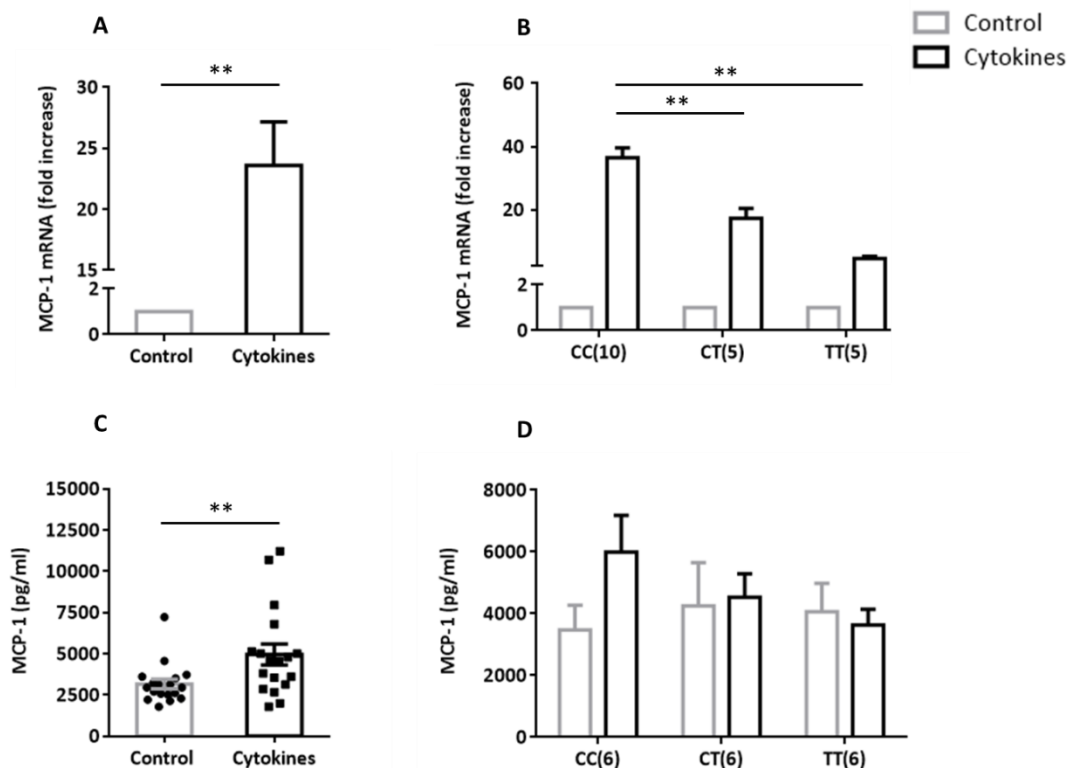
Figure 23: Impact of -1C/T genotype on cytokine induced MCP-1 gene expression.
(A) Genotype-independent upregulation of MC-1 mRNA post-cytokine stimulation; n=20; **p<0.01 vs control. (B) Genotype-dependent upregulation of MCP-1 mRNA; **p<0.01, n-numbers are denoted in brackets adjacent to the genotype. (C) Genotype-independent upregulation of MCP-1 protein quantified by ELISA; n=20, **p<0.01 vs control. (D) Genotype-dependent upregulation of MCP-1 protein, n-numbers are denoted in brackets on the graph adjacent to the genotype.

5.6.4 Impact on E-selectin expression

Exposure to cytokines induced a genotype-independent 94-fold increase in E-selectin mRNA **(Figure 24A).** The presence of two T alleles dramatically reduced this effect from a 154 and 125-fold induction in the CC and CT genotype cells to only 1.5-fold in the TT genotype cells **(Figure 24B)**. Independent of the genotype, there was a 10-fold increase in E-selectin protein post-cytokine stimulation **(Figure 24C)** which was highest in the CC genotype cells (16-fold) but was markedly reduced in the TT genotype (4-fold) cells **(Figure 29D)**.
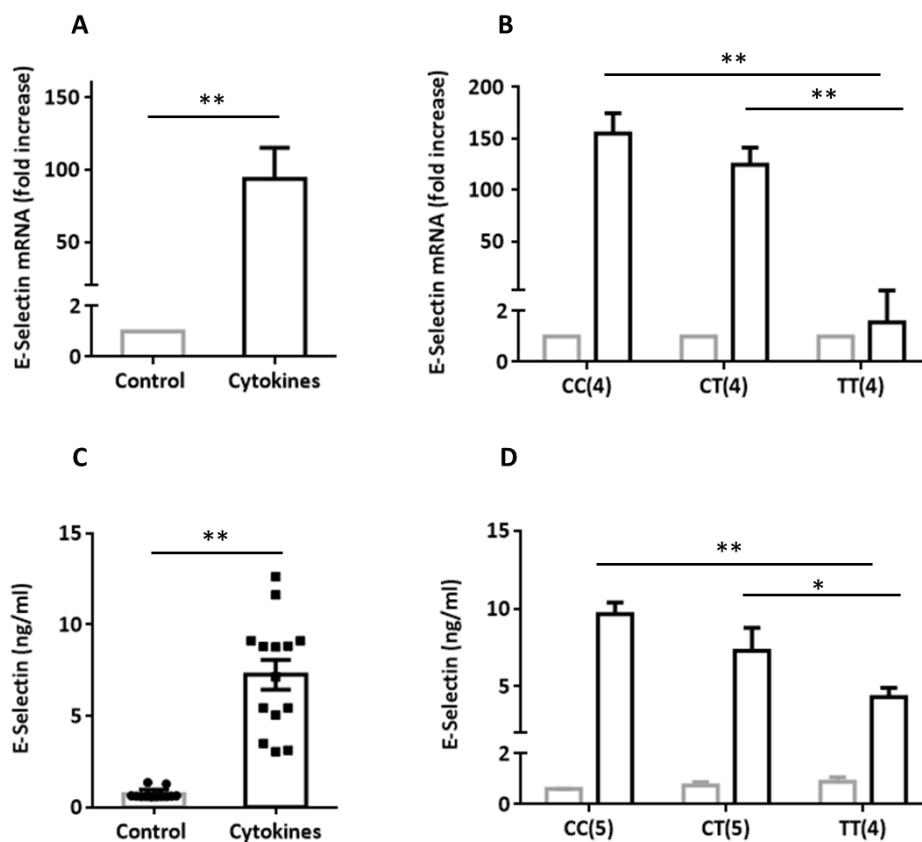
Figure 24: Impact of -1C/T genotype of the *CD40* gene on cytokine induced E-selectin gene expression. *(A) Genotype-independent upregulation of E-selectin mRNA post-cytokine stimulation; n=12; **p<0.01 vs control. (B) Genotype-dependent upregulation of E-selectin mRNA; **p<0.01, n-numbers are denoted in brackets adjacent to the genotype. (C) Genotype-independent upregulation of E-selectin protein quantified by ELISA; n=14, **p<0.01 vs control. (D) Genotype-dependent upregulation of E-selectin protein, *p<0.05; **p<0.01, n-numbers are denoted in brackets on the graph adjacent to the genotype.*

5.7 CD40 isoforms in endothelial cells

Regulation of mouse and human CD40 expression through the generation of alternative splice variants has been shown previously (Tone et al., 2000; Eshel et al., 2008). The major alternative isoform in which exon 6 is spliced out is known as isoform 2 or sCD40-6. This soluble receptor isoform has been reported to reduce expression of the full length signaling competent CD40 in a human B lymphocyte cell line by acting as a decoy receptor (Eshel et al., 2008). However, there is no information regarding the presence or function of CD40 isoforms in human endothelial cells. Therefore we were interested to determine if sCD40-6 is conserved in human endothelial cells, if it is affected by proinflammatory stimuli, and if the -1C/T SNP of the *CD40* gene is associated with changes not only in full-length CD40 but also sCD40-6.

5.7.1 Expression of inducible sCD40-6 in human endothelial cells

The presence of three additional isoforms could be detected by running the products of a semi-quantitative PCR on an agarose gel (**Figure 25A**). sCD40-6 lacks exon 6, sCD40-5 is missing exon 5 and sCD40-5+6 is missing both of them. Of these isoforms only sCD40-6 was studied further since this isoform is known to retain three cysteine rich domains and the capacity to bind CD40L, therefore it can act as a soluble decoy receptor for CD40L. Cytokine priming with and without additional sCD40L stimulation significantly increased mRNA expression of sCD40-6 (**Figure 25B**). To investigate if there is an association between the -1C/T SNP of the *CD40* gene and sCD40-6 levels under proinflammatory conditions genotype-stratified endothelial cells were analyzed, however all genotypes upregulated sCD40-6 in a comparable manner (**Figure 25C**).
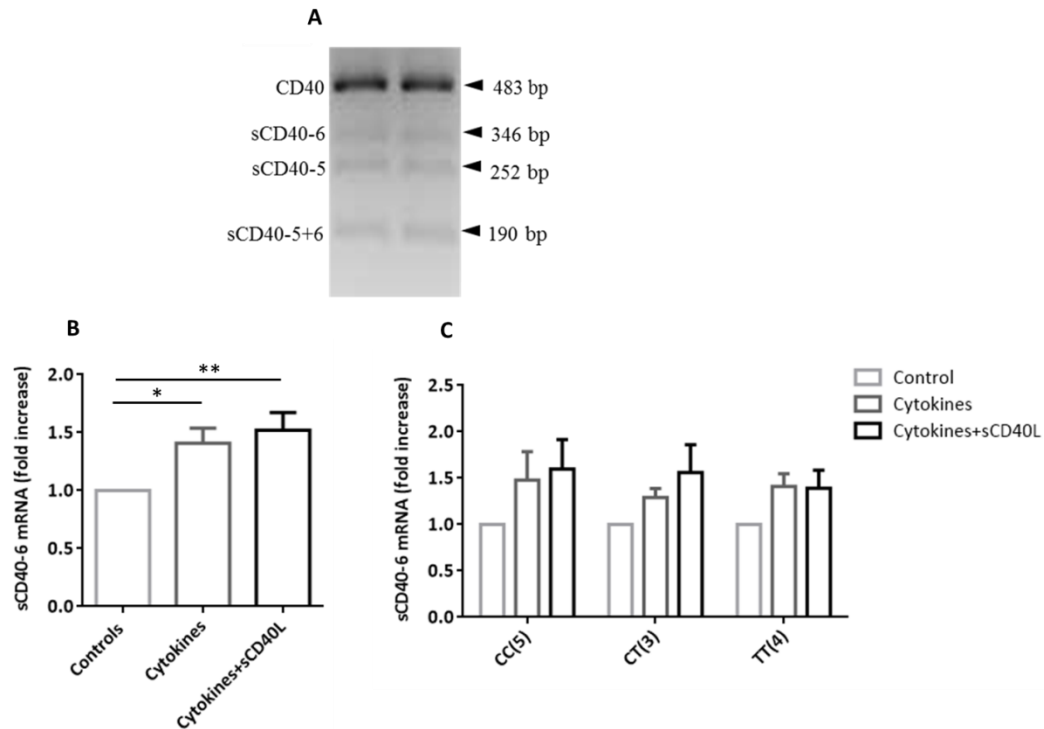
Figure 25: Endothelial cells express cytokine-inducible CD40 isoforms at mRNA level. *(A) Representative image of PCR products separated on a 2% agarose gel. (B) qRT-PCR of mRNA isolated from HUVECs stimulated with cytokines ± sCD40L; *p<0.05; **p<0.01 vs control, n=12. (C) No effect of -1C/T CD40 genotype on cytokine inducible sCD40-6 mRNA expression, n-numbers are indicated on the graph adjacent to the genotype.*

5.7.2 Association of soluble CD40 protein with the -1C/T SNP of the *CD40* gene

Soluble CD40 proteins can be generated by alternative splicing or TNF-α converting enzyme (TACE)-mediated cleavage of transmembrance CD40. Soluble CD40 levels have been shown to be elevated in the plasma of patients in a number of pathological conditions (refer to section 2.2.4). To elucidate if the -1C/T SNP of the *CD40* gene correlates with altered sCD40 protein levels, gentoype-stratified HUVECs were analyzed post-sCD40L stimulation (**Figure 26**). A trend indicating an increase in sCD40 protein expression (p=0.087) following 24-hour stimulation with sCD40L was observed (**Figure 26A**) with no significant effect of the -1C/T SNP of the *CD40* gene obtained (**Figure 26B**).
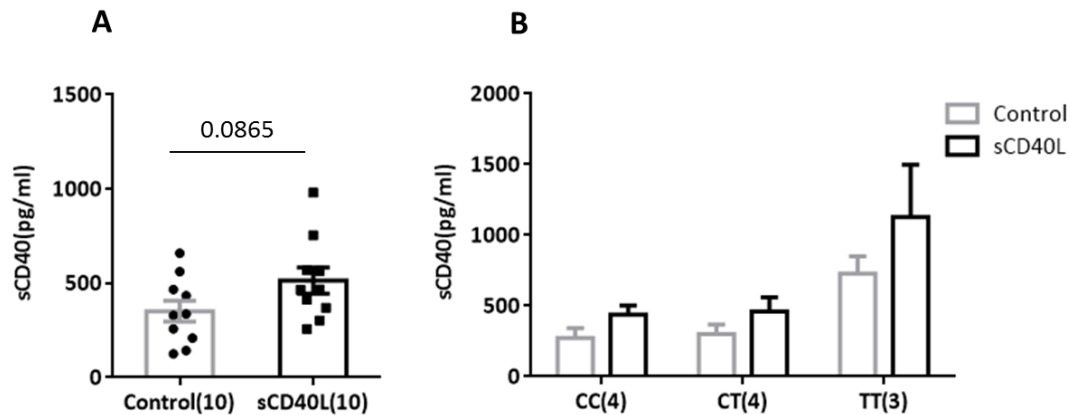
Figure 26: Effect of sCD40L-induction on endothelial sCD40 protein expression. (A) *ELISA based quantitation of soluble CD40 protein released into HUEVC supernatants 24 hour post-sCD40L stimulation independent of -1C/T CD40 genotype, n=10. (**B**) Genotype-dependent effect of sCD40L on sCD40 release, n-numbers are denoted in brackets on the graph adjacent to the genotype.*

5.8 Association of the -1C/T SNP of the *CD40* gene with coronary heart disease

Endothelial dysfunction is accepted as an independent risk factor for vascular disease and its contribution to atherosclerotic disease development and progression is undisputed. Data presented in sections 5.2 to 5.6 indicate that the C allele of -1C/T SNP of the *CD40* gene acts as a gain of function allele (in comparison to the T allele) and confers an activated phenotype to the CC genotype endothelial cells.

To determine if the -1C/T SNP of the *CD40* gene could be a risk factor for coronary heart disease (CHD) via increasing CD40 abundance on endothelial cells and therefore imparting the risk of enhanced adhesion molecule and chemokine expression, an association study was carried out. DNA isolated from the blood of 243 patients with coronary heart disease, diagnosed by coronary angiography, was genotyped. Age-matched controls (76) were also genotyped. The sample size of the umbilical vein cohort was higher than the age-matched control group and the genotype distribution as well as the allele frequency of these two groups was highly comparable **(Figure 27)**, hence the umbilical vein group was used as a reference control cohort for computing odds ratios **(table 13)**.
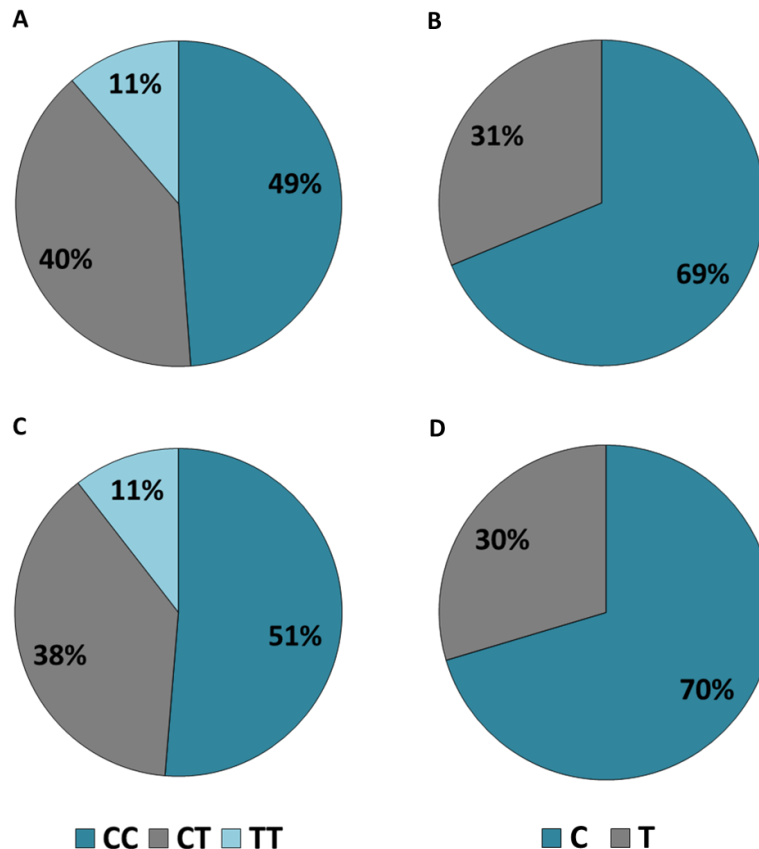
Figure 27: The -1C/T SNP of the *CD40* gene has comparable genotype distribution and allele frequency in non-diseased and age-matched control groups. *(A) Genotype distribution and (B) allele frequency of the non-diseased control group, n=470. (C) Genotype distribution and (D) allele frequency of age-matched controls, n=76. Values expressed as percentages.*

Both control populations were in Hardy-Weinberg equilibrium therefore an allelic association test was used for comparison. A significant difference in the C and T allele frequency between control and CHD cohorts was observed **(table 13)**. Carriers of the enhanced function C allele of the -1C/T SNP of the *CD40* gene exhibited an elevated odds ratio for CHD (75.9% vs 68.5% cases vs controls, OR=1.43; 95% CI=1.13-1.86, p=0.0038). Therefore the C allele was found to be associated with a 1.4 times increased risk of CHD compared with the T allele. To analyze the association of CHD with the genotypes of this SNP, genotypic odds ratio were computed. Genotypic analysis (CC vs TT, cases vs controls) showed a significant association of the CC genotype with CHD (OR=2.38; 95% CI= 1.28-4.42, p=0.0056). Taking the CT genotype into account, the

recessive model of penetrance was analyzed. According to this model, two copies of the C allele would be required to increase the risk of disease (Clarke et al., 2011). Comparison of the TT genotype vs combined CC and CT genotypes in cases and control groups revealed an association of the TT genotype with decreased risk of CHD (OR=0.48; 95% CI=0.26-0.89, p=0.02). Altogether, these results confirm an association of the C allele and the CC genotype with susceptibility to CHD **(table 13)**.

Table 13: Association of the -1C/T SNP of the CD40 *gene* with susceptibility to CHD
The distribution in total n numbers and percentages of the CC, CT and TT genotypes in coronary heart disease patients, controls (DNA from umbilical veins) and age-matched controls. N indicates number of subjects; ref: reference; CI, confidence interval; ns, non-significant. Odds ratio computed by Fischer's exact test.

Allele	Controls n=470	CHD patients n=243	Odds ratio (95% CI)	P value
C	644 (*68.5%*)	369 (*75.9%*)	Reference	
T	296 (*31.5%*)	117 (*24.1%*)	1.43 (1.13-1.86)	0.038
Genotype				
CC	228 (*48.5%*)	140 (*57.6%*)	_	
CT	188 (*40.0%*)	89 (*36.6%*)	_	
TT	54 (*11.4%*)	14 (*5.7%*)	CC vs TT 2.38 (1.28-4.42)	0.02
Recessive model				
TT	54 (*11.4%*)	14 (*5.7%*)	0.48 (0.26-0.89)	0.02
CC+CT	416 (*88.5%*)	229 (*94.2%*)		

5.8.1 Association between sCD40 levels and -1C/T CD40 SNP in coronary heart disease

Soluble CD40 can act as a negative regulator of CD40L-induced signaling. However there is no information regarding sCD40 levels in a CHD context and if it is affected by the -1C/T SNP genotype. To investigate this, the sCD40 levels in CHD patients' plasma was quantified after genotype stratification. Analysis showed that the CC genotype was

associated with 1.5- and 2-fold higher sCD40 protein levels compared to the CT and TT genotype, respectively (**Figure 28**).
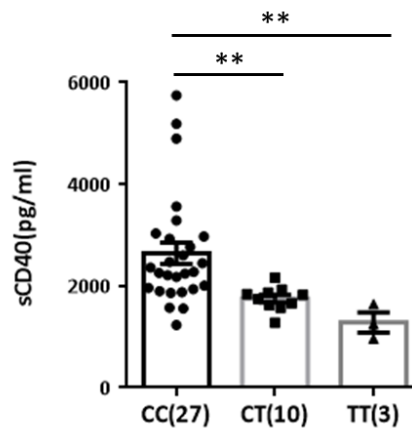
Figure 28: Association between sCD40 serum levels and the -1C/T SNP of the *CD40* gene in patients with CHD. *Genotype-dependent release of sCD40 in CHD patients; **p<0.01, n-numbers are denoted adjacent to the genotypes .*

5.9 Association analysis of the -1C/T SNP of the *CD40* gene with inflammatory arthritic diseases

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are chronic inflammatory diseases associated with increased risk of cardiovascular morbidity and mortality. The CD40 locus has been shown by genome wide association studies to be implicated in RA pathogenesis (Raychaudhri et al., 2008). Therefore, the -1C/T SNP of the *CD40* gene was also analyzed in patients with RA and PsA to determine its distribution and to confirm whether the C allele is a risk factor not only for CHD but also other chonic inflammatory conditions. Comparison of the allele frequencies of RA and Controls(umbilical vein DNA) did not show a significant difference however the C allele was significantly associated with PsA (OR=1.93% CI: 1.20-3.11, p=0.005,**table 14).**

Table 14: The -1C/T SNP of the *CD40* gene and Inflammatory arthritis risk.
The Distribution in total n numbers and percentages of the CC, CT and TT genotypes in rheumatoid arthritis patients, controls (DNA from umbilical veins) and psoriatic arthritis patients. N indicates number of subjects; ref: reference; CI; confidence interval. Odds ratio (OR) computed by Fischer's exact test

Allele	RA patients n=122	Controls n=470	PsA patients n=60
C	179 (*73.3%*)	644 (*68.5%*)	97 (*80.8%*)
T	65 (*26.63%*)	296 (*31.5%*)	23 (*19.1*)
OR (95% CI)	ns	Reference	1.93 (1.20-3.11)
P value	-	-	0.0058
CC	70 (*57.3%*)	228 (*48.51%*)	38 (*63.3%*)
CT	39 (*31.9%*)	188 (*40%*)	21 (*35.0%*)
TT	13 (*10.7%*)	54 (*11.48%*)	1 (*1.7%*)

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6. Discussion

CD40 and its ligand represent an important co-stimulatory pathway with a wide expression profile. Since CD40-CD40L co-stimulation regulates both adaptive and innate immunity, changes in expression and/or function of these co-stimulatory molecules would have pathophysiological relevance. The C allele of a commonly occurring bi-allelic SNP in the 5' UTR of the CD40 gene has been described as a risk factor for a variety of auto-immune and inflammatory diseases (Jacobson et al., 2005; Tanizawa et al., 2011; Tomer et al., 2002). While this SNP has been well studied, particularly in East Asian and Turkish populations (Inal et al., 2015; Yun et al., 2014), there are no reports which focus specifically on a CHD risk in a German subset. Therefore, in our study we investigated the effect of this common SNP in the local population, to acquire representative data on the importance of this SNP in the primarily Caucasian population of Northern Baden-Württemberg.

6.1 The C allele is dominant in the local population

SNPs are the most common type of genetic variation and they form the basis of human diversity. The most extensive and up-to-date information regarding human genetic variation is catalogued by the 1000 Genomes Project, which characterized 84.7 million SNPs in 26 populations (The 1000 Genomes Project Consortium, 2015). In this project, the European population is represented by the British, Finnish, Iberian Spanish, and Tuscan Italians, while Northern and Western Europe is represented by Utah residents with corresponding ancestry. The database reports a combined minor allele frequency (MAF) for the -1C/T SNP of the *CD40* gene as 23% (T allele).

However, ethnic differences play an important role in SNP presence and distribution. It is estimated that 85% of all SNPs are common in every human population while 15% are limited to certain geographical regions (Barbujani et al., 1997). For example, a SNP in the gene encoding the blood clotting cofactor protein S (Rs138925964) which is almost missing in the European population is significantly present in an African-descent group (Huang et al., 2015).

Analysis of the global distribution of the allele and genotype frequency of the -1C/T SNP of the CD40 gene also exhibits a clear geographical gradient (**Figure 29**). While the lowest MAF is present in the African (3%) and the highest in the East Asian populations (43%), it is comparable in the European and South Asian populations (26 and 27% respectively). In addition to population specificity, ethnic variability in allele and genotype frequency is not rare. One example is a C to T transition in the methylenetetrahydrofolate reductase (MTHFR) gene (Frosst et al., 1995). The distribution of the TT genotype ranges from 10-33% in the European, Asian and American populations, but is only 0-3% in the African populations (Wilcken et al., 2003).
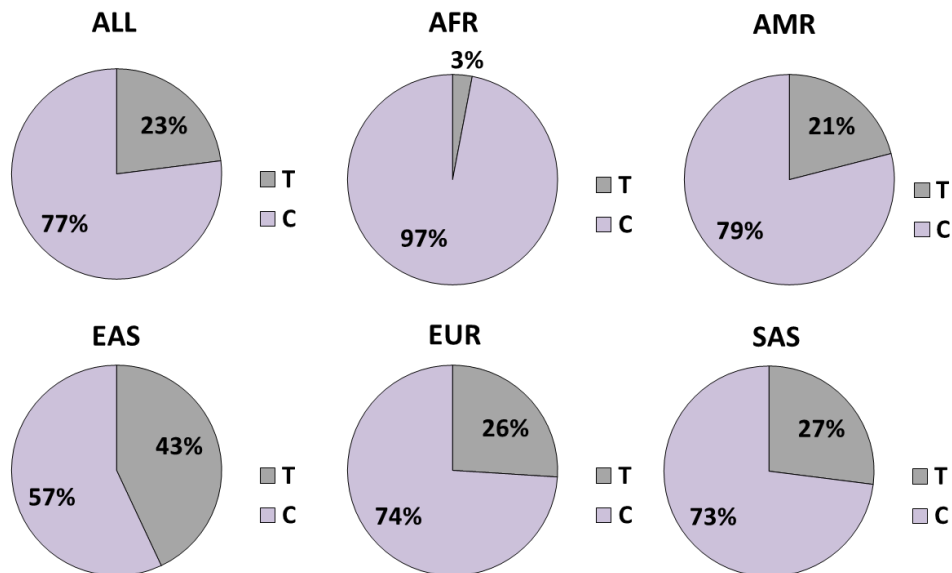
Figure 29: Global allele frequencies of the -1C/T SNP of the CD40 gene from the 1000 Genomes Project. *AFR, AMR, EAS, EUR and SAS refer to African, American, East-Asian, European and South-Asian populations, respectively*

In view of the importance of ethnic groups in SNP studies and the geographical gradient reported regarding the -1C/T SNP of the CD40 gene, we investigated its distribution in the local region of Northern Baden-Württemberg, Germany. DNA extracted from 470 umbilical cords showed a MAF of 31% with genotype distribution of 49, 40 and 11% for the CC, CT, and TT genotypes, respectively (*cf.* **Figure 6**). This distribution follows the Hardy-Weinberg equilibrium, and the CC and CT percentages are comparable to the reported European distribution of 52 and 44%, respectively (1000 Genomes Project,

2015). However, the observed MAF was somewhat higher as the MAF (26%) reported by the 1000 Genomes Project consortium. There are certain possibilities for the discrepancy. Firstly, since 14% of the population in Heidelberg is from ancestry other than German and we do not have access to the ethnic heritage of our samples, it is not possible to rule out some population mixing. Additionally, as mentioned above, the 1000 Genomes Project does not include German samples but refers to a general Northern and Western European population which migrated to Utah, USA. It is possible that the Utah population does not properly reflect the European population and in particular the primarily Caucasian population of Northern Baden-Württemberg. Therefore it is possible that in Germany, the MAF of the -1C/T SNP of the CD40 gene is higher than in a combined Northern and Western European population.

6.2 The -1C/T SNP of the CD40 gene is a regulator of endothelial cell CD40 protein

Gene expression is a heritable, quantifiable and complex trait that is controlled by genetic, epigenetic and environmental factors (Cheung et al., 2005; Petronis, 2006). Gene transcription and translation are intricate and sophisticated mechanisms which require stringent control at multiple levels, including the initiation of translation. In eukaryotes, up to 12 different factors are known to influence this process and enable interaction of the methionyl-initiator tRNA with the AUG initiating codon in the P subunit of a ribosome (Heryshey and Merrick, 2000). The context in which the AUG codon is placed is known as the Kozak consensus and it is a strong determinant of the rate of translation initiation. The strongest sequence is one that contains a guanine at position +4 and an adenine at position -3. Presence of a cytosine at positions -1 and -2 also contributes to the overall strength of the Kozak sequence (Kozak 1984 and 1986).

The -1C/T SNP of the *CD40* gene is a cytosine to thymine transition in the Kozak sequence. As mentioned above, the presence of cytosine at position -1 enables the Kozak sequence to resemble the ideal sequence. Since the presence of the thymine nucleotide results in a disruption in this location, we hypothesized that there would be an effect on CD40 protein translation. As discussed in the introduction, CD40-CD40L interactions confer a pro-inflammatory phenotype to endothelial cells hence changes in gene expression in these cells could have significant pathophysiological consequences. Hence

the impact of this SNP on endothelial cell expression of pro-inflammatory gene products was evaluated. In resting HUVECs intracellular CD40 protein content and surface receptor density were both significantly higher in CC genotype cells than in CT or TT genotype cells. Interestingly, we observed a seemingly additive effect of the T allele regarding surface CD40 abundance, with an even greater decline in TT as compared to CT genotype cells. As expected, there were no differences in CD40 mRNA levels suggesting little influence of this SNP on transcription and stability of the CD40-encoding mRNA. Our results correspond to other studies regarding the -1C/T SNP of the *CD40* gene which consistently correlate the disrupted Kozak sequence with lower CD40 protein levels. An *in vitro* study with transfected fibroblasts showed about 30% more CD40 protein in the C allele as compared to the T allele transfectants (Jacobson et al., 2005). Functional studies using B lymphocytes (Park et al., 2007), dendritic cells (Skibola et al., 2008) and platelets (Yan et al., 2010) have also demonstrated increased CD40 protein in the presence of the C allele. Similarly, we detected up to 5.6-fold higher protein expression on the cell surface of endothelial cells with the CC genotype compared to the TT genotype.

Kozak sequence SNP' are also relevant in other genes. For instance, a G to C transition at the -6 position in the β-globin gene is associated with increased risk of thalassemia intermedia by decreased efficiency of translation of the β-globin chain (Angioletti et al., 2004). Since the beta-globin protein is a subunit of hemoglobin, alteration in its protein levels would have clinical significance in blood-related disorders such as thalassemia. A -5C/T SNP in the glycoprotein Ib-α gene (-5C/T) is an independent risk factor for ischemic stroke (Baker et al., 2001) via influencing plasma membrane levels of the platelet GP Ib-clotting factor IX-clotting factor V complex on platelets and transfected cells (Afshar-Kharghan et al., 1999).

6.3 Correlation of increased CD40 surface content with stronger signal transduction

CD40 ligation in endothelial cells has a similar effect as the pro-inflammatory cytokine TNF-α, which is characterized by increased expression of chemokines, cytokines and adhesion molecules. Consequently, there is increased leukocyte recruitment, adhesion and rolling, which are essential steps in leukocyte extravasation (Hollebaugh et al., 1995;

Yellin et al., 1995). We could demonstrate a clear impact of the -1C/T SNP of the *CD40* gene on CD40 protein abundance, namely on the endothelial cell surface which expresses low levels of CD40 protein under basal conditions (Wagner et al., 2011). It is interesting to note that even under this condition the difference in the protein expression of the genotypes of this SNP was detectable. Thus, we next analyzed functional activation of the receptor by using its recombinant soluble ligand. As read-outs for CD40 downstream signaling, the expression of MCP-1, VCAM-1 and E-selectin was compared in CC, CT and TT genotype endothelial cells. These gene products were selected since they are well known to be upregulated by CD40 ligation in endothelial cells and all considered to be pivotal for the onset and development of atherosclerosis (Pluvinet et al., 2008).

Compared to non-stimulated cells, exposure to sCD40L elicited a marked and highly significant increase in expression of all three gene products. The most pronounced genotype-dependent effect was noted for VCAM-1 followed by MCP-1 and E-selectin which were consistently upregulated to a much greater extent in the CC genotype as compared to the CT or TT genotype endothelial cells. These pro-inflammatory molecules are markers of endothelial dysfunction and their role in macrophage activation and leukocyte adhesion in human blood vessels is indisputable (reviewed by Smitzko et al., 2003; Zhang, 2008). MCP-1 deficient mice on an atherosclerotic background have lower levels of aortic lipid deposition despite long-term exposure to a high-fat diet (Brown et al., 2001) while in VCAM-1 deficient mice the development of atherosclerotic lesions is impaired (Cybulsky et al., 2001). Therefore, a CD40-mediated increased expression of these gene products would in particular incline the CC genotype cells towards endothelial dysfunction.

The results indicate that increased CD40 abundance on the cell surface corresponds to enhanced CD40-mediated downstream signaling to the nucleus, which would induce a pro-inflammatory phenotype in the CC genotype endothelial cells (Iezzi et al., 2009). Such an amplification of the strength of CD40-CD40L interactions due to constitutive or stimulus-dependent differences in CD40 expression has previously been reported by Iezzi et al., 2009. Moreover, CD40 belongs to the TNFR superfamily which mediates the biological activity of TNF-α (Vandanabeelee et al., 1995). Genetic variations in these

receptors are common with a number of SNPs present in the promotor region of these genes that significantly affect gene expression (Kim et al., 2005), and which are linked to immune-regulatory diseases such as SLE or RA.

Furthermore, our results also depict a preferential binding of monocytes to endothelial cells with the CC genotype. Monocyte adhesion to the endothelial cell surface is facilitated by adhesion molecules such as VCAM-1 (Luscinskas et al., 1996). VCAM-1 expression at mRNA and protein levels was upregulated by sCD40L to a greater extent in CC as compared to CT or TT genotype endothelial cells; therefore the functional implication of these differences was investigated in an adhesion assay. Monocyte adhesion was halved in CT and TT genotype endothelial cells indicating that homozygosity of the C allele is required for an enhanced response of these cells to sCD40L stimulation. Collectively, CC genotype cells presented a more pro-inflammatory phenotype sCD40L stimulation than the CT or TT genotype cells, which both responded in a comparable manner. Moreover, these results provide first functional data explaining the association of the C allele with inflammatory diseases such as atherosclerosis(Ma et al., 2013; Tian et al., 2010; Yun et al., 2014).

The aforementioned results provide evidence of a -1C/T SNP-dependent effect on sCD40L-induced endothelial cell activation. However, under resting conditions CD40 abundance on the cell surface is maintained at low levels for preservation of a quiescent endothelial cell phenotype. Constitutive CD40 expression, which is largely restricted to venous and capillary endothelial cells, is affected by micro-environmental factors like the presence of inadequate cyclic stretch or pro-inflammatory cell traffic in and out of the vessel wall (Korff et al., 2007; Wagner et al., 2011). Moreover, atherosclerotic plaques are abundant in pro-inflammatory cytokines like TNF-α and IFN-γ, which are powerful stimulators of CD40 expression *in vivo* (Schoenbeck and Libby, 2001) and *in vitro* (Kresz et al., 1999; Nguyen et al., 2009; Wagner et al., 2002). Furthermore, in inflammation endothelial cells are acted upon by sCD40L released from activated platelets, which are estimated to contribute to 95% of the sCD40L released into the circulation, in the presence of cytokines (Henn et al., 1998 and 2001). Consequently, a combination of pro-inflammatory mediators which act synergistically is a more suitable mimic of

inflammation as opposed to single cytokines, which are commonly used to simulate this condition (Franscini et al., 2004). Hence, we next investigated if endothelial cell reactivity to cytokine-priming followed by exposure to sCD40L was affected by the -1C/T SNP of the *CD40* gene. Independent of the genotype, endothelial cells responded to cytokine priming plus sCD40L stimulation by significantly upregulating both CD40 mRNA and protein levels, namely on the cell surface. While the relative increase in CD40 abundance was similar in all genotypes, absolute CD40 levels were highest in CC genotype endothelial cells both at rest and following cytokine plus sCD40L exposure. We were curious if these cells would protect themselves from adopting a pro-inflammatory phenotype by reduced reactivity to stimulation, however this was not the case as evidenced by the comparable relative rise in CD40 protein in all genotypes following stimulation.

Expression of the selected pro-inflammatory marker genes was also significantly upregulated under these conditions, namely that of MCP-1 and E-selectin genes in the CC genotype endothelial cells. Regarding VCAM-1, significant differences were not observed among the different genotypes; however, as observed in endothelial cells exposed to sCD40L alone, maximum upregulation of all marker genes occurred in the CC genotype cells.

These observations suggest that all three genotype have a comparable capacity to upregulate CD40 protein under strong pro-inflammatory conditions and the relatively greater downstream signaling to the nucleus in CC genotype cells is due to a more pronounced cytokine-priming of CD40 expression hence leading to greater abundance of CD40.

It was interesting to note that the presence of one T allele (CT genotype) is sufficient to decrease CD40 protein abundance and consequently downstream signaling to the nucleus. Presence of the second T allele (TT genotype) mostly had no additional effect on downstream signaling despite causing a further decrease in CD40 abundance, suggesting that beyond a certain threshold a further decline in the amount of CD40 on the endothelial cell surface does not have a measurable impact. Hence sensitivity of the CT and TT genotype endothelial cells to stimulation by sCD40L is comparable. This may be

due to differential adaptor molecule recruitment by the different genotypes of the -1C/T SNP of the *CD40* gene. As mentioned before, (section 2.2.3) CD40 mainly signals through TRAFs. Whether the TRAFs activate or inhibit downstream signaling depends on the cell type and TRAF molecule involved. For instance, in B cells TRAF-3 appears to negatively regulate NF-kB, while an opposite effect has been observed in epithelial cells using a TRAF3 overexpression system (Propst et al., 2002; Urbich et al., 2001). TRAF-6 is particularly important in CD40-mediated signaling and is required for the CD40-mediated activation of important signaling pathways like Akt and p38 in epithelial cells (Davies et al., 2005). Therefore, it is plausible that a different TRAF is recruited post-ligation in CT and TT genotype cells On the whole, these findings suggest that homozygosity for the C allele of the -1C/T SNP is required for genetic inclination towards a pro-inflammatory endothelial cell phenotype. However, this conclusion may not necessarily be applicable to other CD40 expressing cell types.

Endothelial cell activation promoted by increased adhesion molecule expression is not only induced by CD40L but also by the cytokines employed to upregulate CD40 expression (Landsberger et al., 2007). To investigate if the -1C/T SNP has a general influence on endothelial cells under pro-inflammatory conditions; genotype-stratified cells were analyzed for differences in pro-inflammatory marker gene expression in the absence of CD40L.

Cytokine exposure induced a similar shift in phenotype as described in the previous two sections, with maximum upregulation of CD40 abundance and expression of pro-inflammatory marker genes in the CC genotype endothelial cells. Changes in gene expression were significantly more pronounced than those in non-stimulated cells but less than in CC genotype endothelial cells exposed to cytokine priming plus sCD40L stimulation (*cf.* **table 12**) which could be expected because exposure to the ligand is known to amplify the response of endothelial cells to pro-inflammatory cytokines (Kotowicz et al., 2010). Since studies regarding the impact of the -1C/T SNP have mostly focused on CD40 expression in other cell types and not on CD40-signaling mediated susceptibility to atherosclerosis, there is essentially no literature on this subject. However,

our results match findings of increased CD40 protein abundance on B lymphocytes from CC genotype individuals' pre and post stimulation with IFN-γ (Tian et al., 2010).

The impact of the -1C/T SNP on the endothelial cell shift from a quiescent to a pro-inflammatory phenotype even in the absence of CD40L is noteworthy also because it suggests a critical role for this SNP (and of CD40) in a more general pro-inflammatory context. This is the first study to functionally investigate the consequences of the -1C/T SNP of the *CD40* gene on endothelial cell signaling so far. Therefore, it is difficult to draw a conclusion as to why the SNP would facilitate such a more general pro-inflammatory phenotype. One possibility is that the SNP exists in linkage disequilibrium (LD) with other SNPs that affect pro-inflammatory endothelial cell responses and thus contribute to the observed phenotype. However, this statement is purely speculative and a LD of the -1C/T SNP of the *CD40* gene with genes in pathways affecting responses to pro-inflammatory cytokine is not known except the T/G SNP in intron 2 of the CD40 gene (Rs4810485) which is in near perfect LD with the -1C/T SNP and associated with, e.g. rheumatoid arthritis (Raychaudhri et al., 2009). But since this SNP also exists in the CD40 gene, it does not provide a suitable biological explanation for our observation.

6.4 The C allele of the -1C/T SNP of the *CD40* gene is associated with increased susceptibility to CHD

CD40-CD40L interactions are consistently implicated in the pathogenesis of atherosclerosis in animal experimental studies, namely in mice (Bruemmer et al., 2001; Hakkinen et al., 2000), and downstream signaling in particular in macrophages and possibly also in vascular smooth muscle cells (Stojakovic et al., 2007) promotes plaque instability (Shoenbeck and Libby, 2001). These interactions play a particularly significant role in endothelial dysfunction in inducing pro-inflammatory cytokines, chemokine and adhesion molecule expression in endothelial cells (Henn et al., 1998; Pluvinet et al., 2008). Since downstream CD40 signaling in these cells in the arterial vessel wall promotes a pro-atherogenic phenotype, it is highly likely that genetic variations in this co-stimulatory receptor-ligand dyad would have an impact on the individual susceptibility to atherosclerosis and consequently CHD. Indeed, the CD40 locus is a confirmed risk factor for RA; a condition which itself is a risk factor for atherosclerosis

and hence CHD (Raychaudhuri et al., 2008). Moreover, CHD and RA seem to have an overlapping risk gene profile and there are striking similarities in the pathogenesis, namely in the nature of the chronic inflammatory response in the joint or blood vessel, of both diseases.

However, the CD40 gene locus is not included in the 33 loci that have been identified by GWAS as confirmed CHD risk factors (Peden and Farrall, 2011). Apart from these 33 loci, many others have been identified in multiple studies to be nominally significant. It is also possible that some genuine disease-related loci are not detected because their contribution is too small to cross the stringent threshold of GWAS (must undercut a probability value of $5x10^{-8}$) so that large meta-analyses are required for their detection (DiPetrillo et al., 2005). LD, the non-random allelic association of different loci, adds another layer of complexity to genetic inheritance. Due to LD, it is possible that the loci detected in GWAS are the not the disease-causing ones, but are present because of their linked inheritance with the true causative variant resulting in a false positive association (Platt, 2010).

In the light of the importance of CD40-CD40L interactions for inflammation, the -1C/T SNP of the *CD40* gene has been studied in relation with diseases associated with increased inflammation. Thus, the C allele has been linked to acute coronary syndrome (ACS) via enhanced CD40 protein expression on platelets and mononuclear cells. No association has been reported with stable angina, indicating a direct link of the C allele with instability of atheromatous plaques instead of long-term atherosclerosis development (Yan et al., 2010; Tian et al., 2010).

Our experimental results show increased CD40 expression and signaling in CC genotype endothelial cells. This allele is also implicated in autoimmune conditions like Graves' disease (Jacobson et al., 2005; Tomer et al., 2002) and Crohn's disease (Blanco-Kelly, 2010). Therefore, we compared the distribution of this SNP in a case-control population composed of CHD patients and non-diseased controls. The frequency of the C allele was significantly higher in the CHD cohort compared to non-diseased controls, and its presence conferred a 1.4-fold increased odds of developing CHD compared to the T allele which was protective. Subgroup analysis using a recessive model also showed that the TT

genotype is associated with reduced risk for CHD (*cf.* **table 13**). This observation is in line with a meta-analysis pooling results from seven case-control studies investigating this SNP with susceptibility to atherosclerosis in a Chinese population, which found a significant effect of the C allele under a dominant model. Further sub-group analysis showed the C allele to be a risk allele for CHD and ACS under all genetic models (Yun et al., 2014).

Interestingly, the T allele which must be considered protective factor in terms of CHD risk, is associated with increased risk of non-Hodgkin's lymphoma (Skibola et al., 2008; Nieters et al., 2011), breast cancer (Shuang et al., 2011), multiple sclerosis (Field et al., 2011) and chronic hepatitis B viral infection (Zhou et al., 2015). The opposite genetic association of the -1C/T SNP of the *CD40* gene where the C allele is a risk for autoimmune and inflammatory diseases, but provides protection against cancerous and infective conditions is intriguing. These findings can be interpreted in view of the association of the C allele with increased CD40 protein expression in all cell types reported so far (for details please refer to section 5.2). Since CD40 downstream signaling rather augments exaggerated immune-regulatory processes, enhanced expression and activity would be harmful in a context where inflammation already exists, for instance in CHD or RA. However, a reduction may impede B and Th2 cell interactions and hamper the adaptive immune system in launching a powerful offensive attack against infectious microbes or cancerous cells. In this way, the C allele could be protective for cancer and infectious diseases but a risk for autoimmune and inflammatory diseases. In fact, considering that this allele is ancestral and dominant in every human population analyzed, it would make evolutionary sense for its presence to also confer some advantage to its carriers.

6.5 The -1C/T SNP of the *CD40* gene and its effect on circulating soluble CD40

The association of the -1C/T SNP with autoimmune and inflammatory diseases is widely considered to be a consequence of altered CD40 protein expression in various cell types due to disruption of the Kozak sequence. However, it is possible that this SNP also affects other processes such as control of CD40 downstream signaling through soluble CD40 (sCD40) release which may act as a decoy receptor for soluble CD40L.

Receptors belonging to the TNF superfamily can exist in membrane-bound or soluble forms. The soluble forms (sTNFR) are generated by proteolytic cleavage via the TNF-α converting enzyme (TACE) and retain ligand binding capacity, thereby enabling natural inhibition of TNF-α signaling through receptor sequestration (Glossop et al., 2005; Kohno et al., 1990). They have clinical relevance and are elevated in inflammatory conditions like RA (Cope et al., 1992; Steiner et al., 1995). Furthermore, sTNFRII is known to be influenced by at least one SNP in RA, indicating genetic regulation (Glossop et al., 2005).

CD40 is a TNFRII and even though sCD40 is known to exist, yet it has not been characterized to the same extent as other such molecules. In addition to cleavage of plasma membrane-bound CD40 through TACE, sCD40 can also be the product of alternative splicing (Tone et al., 2001; Eshel et al., 2008). However, independent of its source, current literature suggests an inhibitory role for sCD40 in the immune response which is evidenced by its ability to reduce immunoglobulin production by B-lymphocytes (Van Kooten et al., 1994; reviewed by Chatzigeorgiou et al., 2009).

We show here for the first time that human endothelial cells express a transcript that can encode sCD40, which indicates their ability to generate sCD40. This transcript, referred to as CD40-Δ6, was significantly upregulated after exposure to TNFα and IFNγ or a combination of these cytokines plus soluble CD40L. However, its upregulation was independent of the -1C/T SNP genotype. Furthermore, sCD40 protein was detected in endothelial cell supernatants, and this was increased after exposure to sCD40L. These results correspond to previous publications reporting the presence of sCD40 in cell culture supernatants of human epithelial cells (Eshel et al., 2008) and B lymphocytes (Tone et al., 2001).

In patients with CHD, we observed sCD40 levels exceeding 2300 pg/ml. Interestingly, in this cohort the serum level of sCD40 seemed to correlate with the genotype of the -1C/T SNP of the *CD40* gene. This was highest in the CC genotype and lowest in the TT genotype individuals. Thus, our results are at variance with a recent report by Chen et al. (2015) who observed increased sCD40 serum levels in TT genotype individuals in a SLE patient cohort. Certainly, it is possible that the serum level of sCD40 is variable and

depends on the type of disease. Since sCD40 can be generated by cleavage of the membrane-bound receptor, it is possible that it is not functionally relevant but exists in higher amounts in the serum from CC genotype individuals simply because of enhanced shedding. However, putatively sCD40 acts as a natural inhibitor of CD40L-induced signaling by providing an alternative receptor which can bind to CD40L but cannot propagate the signal. Therefore, it is also possible that the elevated sCD40 serum levels in the CC genotype individuals with CHD is a form of compensation to protect the endothelial cells from the deleterious effects of an increased sensitivity towards CD40L because of an increased abundance of the receptor on the cell surface. It would be intriguing to determine if the effect of the -1C/T SNP on sCD40 levels is specific for CHD patients or if it also affects age-matched healthy controls. To determine this, sample analysis of sCD40 in control serum is currently underway.

6.6 The -1C/T SNP of the *CD40* gene and its association with rheumatic diseases

The -1C/T SNP of the *CD40* gene is associated with enhancement of inflammation, therefore we have also analyzed whether it is associated also with rheumatic diseases such as rheumatoid arthritis (RA) and psoriatic arthritis (PsA).

In comparison to PsA, RA has been studied in greater detail. CD40-CD40L interactions are implicated in RA as evidenced by the association of CD40L-expressing Th1 cells with a more active form of the condition (Liu et al., 2001). Moreover, according to our data it is strongly associated with increased cardiovascular mortality due to atherosclerosis hence CHD.

There is a significant role for genetic inheritance in the susceptibility to RA, made evident by twin and family-based epidemiological studies. The etiology of RA has not been completely elucidated but gene-gene and gene-environment hypotheses are widely accepted to be critical factors (Kallberg et al., 2007). Family history is a reliable predictor for RA incidence (Smolik et al., 2013) while disease probability in monozygotic twins is 15% compared to 3.5% in dizygotic twins (Silman et al., 1993). Taken together, the genetic component of RA risk is estimated to be as high as 60% with a significant contribution from the locus of the human leukocyte antigen gene (Bax et al., 2011;

Macgregor et al., 2000). Recently, a meta-analysis of three GWAS with a total of 3,393 cases and 12,462 controls confirmed CD40 as a risk locus for RA in a European population (Raychaudhri et al., 2009).

Considering that both RA and CHD are inflammatory, it was expected that the C allele frequency would be similar. As predicted, the MAF in both disease cohorts was comparable. However, in the case of RA, the C allele was not significantly associated with increased odds of disease development, which is likely because of the comparatively small sample size of the RA cohort. Similarly, a nominal but significant association of the C allele with RA has been reported in a Spanish cohort (Garcia-Bermudez et al., 2012).

Furthermore, we analyzed distribution of the -1C/T SNP of the *CD40* gene in patients with PsA which also is a chronic episodic disease of the joints. While inflammation is the hallmark of both types of arthritis analyzed, there are still considerable differences in their pathologies and damage pattern. For instance, RA is limited primarily to the phalangeal joints of the hands and feet while PsA is more heterogeneous and also involves skin lesions and enthesis sites (reviewed by Feale and Veeron, 2015).

Since this study and the sample collection are ongoing, the sample numbers are not high enough to make finite statements. Nevertheless, it is interesting to note the stark differences in the distribution of the -1C/T SNP in the RA and PsA cohorts (*cf.* **table 14**). The PsA cohort has the highest distribution of the C allele (80.8%) and CC genotype (63.3%) among all sample sets analyzed thus far. Using umbilical cords as a control population, we found a significant association of the C allele with PsA but not RA. These results may be interpreted in view of the differential inflammation in RA and PsA. While RA is inflammatory, there is greater representation of Th17 cells in PsA (Yeremenko et al., 2001). Consequently, the systemic inflammatory response in PsA is considerably more enhanced than in RA, with PsA synovial tissue biopsies exhibit greater vascular tissue infiltration (Fraser et al., 2001). Furthermore, PsA is thought to be triggered in genetically susceptible individuals, in whom the immune system is disrupted, leading to joint and skin inflammation (Fitzgerald and Winchester, 2009). Therefore, it is tempting to speculate that the C allele significantly contributes to the inflammatory response and is thus over-represented particularly in the PsA group. These preliminary results indicate

that the -1C/T SNP of the *CD40* gene, either directly or as a tag SNP, could be a strong predictor or genetic risk factor particularly for PsA development. Since this is the first study to associate the -1C/T SNP of the *CD40* gene with two different types of rheumatic diseases, we cannot compare our results with those of previous reports.

6.7 Critical analysis of this thesis

Our study has certain limitations which should be kept in mind. One main critical point is that we considered umbilical vein DNA as a non-diseased control sample set and the MAF and genotype frequencies of our patients (CHD, RA and PsA) have been compared to that cohort instead of an age-matched control. The reason is the difficulty to obtain high numbers of age-matched samples which are free from other confounding pathologies, particularly in the case of CHD. Nevertheless, we have currently obtained 76 age-matched non-diseased samples for CHD patients and the MAF and genotype distribution was highly comparable with the umbilical cord set. Further sample collection is currently underway.

Another critical point is that we did not stratify the patient cohorts by traditional risk factors such as smoking or dyslipidemia. Therefore, odds ratios have been computed without such stratification. However, our results are comparable to other studies regarding the -1C/T SNP of the *CD40* gene in an inflammatory context and are supported by *in vitro* data from endothelial cells; therefore we make the assumption that the association of the CC genotype that we detected for both CHD and PsA is in fact clinically relevant.

Finally, we have focused on the -1C/T SNP of the *CD40* gene in the context of CHD and RA. This SNP is in strong LD with Rs4810485, which is a confirmed risk factor for RA. Therefore, in the context of RA and PsA we cannot be certain that our observation is not being confounded by the presence of Rs4810485. However, Rs4810485 has been studied in RA but not PsA, therefore our study indicates that the -1C/T SNP is a risk factor for PsA as a causative or a tag SNP.

6.8 Summary and Perspective

CD40-CD40L interactions are critical for the development and maintenance of a fully functional immune system. They have a profound impact on endothelial cells and significantly contribute towards their shift from a quiescent to an activated phenotype. In humans, a Kozak consensus sequence polymorphism at the -1 position is associated with variations in protein translation through altered binding of the mRNA to the ribosome. Presence of the cytosine enables the sequence to be ideal and consequently the C-variant is associated with increased CD40 expression.

The present study provides novel evidence demonstrating an association of the homozygous C allele with enhanced endothelial CD40 protein expression and subsequently stronger CD40-CD40L signaling. A detailed analysis of the pro-inflammatory phenotype of the CC genotype endothelial cells indicates that the C-variant acts as a gain-of-function allele which could genetically predispose individuals towards endothelial dysfunction. Since endothelial dysfunction is an early event in chronic inflammatory conditions, alterations in endothelial CD40 abundance, namely on the cell surface, would have pathophysiological consequences. Accordingly, a case-control study of CHD and two types of inflammatory arthritis was carried out which demonstrated an association of the homozygous C allele with significantly increased odds of developing CHD and PsA; a particularly inflammatory type of arthritis. Furthermore, the homozygous C allele was also significantly associated with high sCD40 levels in patients with CHD, which is encoded by a transcript that increased under pro-inflammatory conditions in endothelial cells.

While this study adds to the current knowledge of a genetic predisposition to endothelial dysfunction with particular regard to the role played by the -1C/T SNP of the CD40 gene, yet it also raises further questions which need to be addressed for a more complete understanding. For instance, analysis of CD40 expression in genotype-stratified adult endothelial cells from CHD patients would provide information regarding the extent of the difference that the C allele makes in a CHD context. Furthermore, comparison of sCD40 levels in serum of healthy controls with the data from CHD patients would help in understanding if the association that we have observed occurs specifically under

inflammatory conditions. For this purpose, sample collection of serum from age-matched healthy controls is underway.

This work also provides intriguing preliminary data exhibiting a strong over-representation of the C allele and CC genotype in patients with PsA. Currently, we are expanding this study to include more samples so that a statistically stronger analysis can be made. Further studies performing a comparison of endothelial cell proliferation in biopsies from PsA and RA patients with the different genotypes would yield interesting functional data regarding the inflammatory and proliferative role of this genetic variation in an arthritic context. Additionally, sCD40 measurements in arthritic patients would provide further information regarding its role as potential (prognostic) biomarker of inflammation.
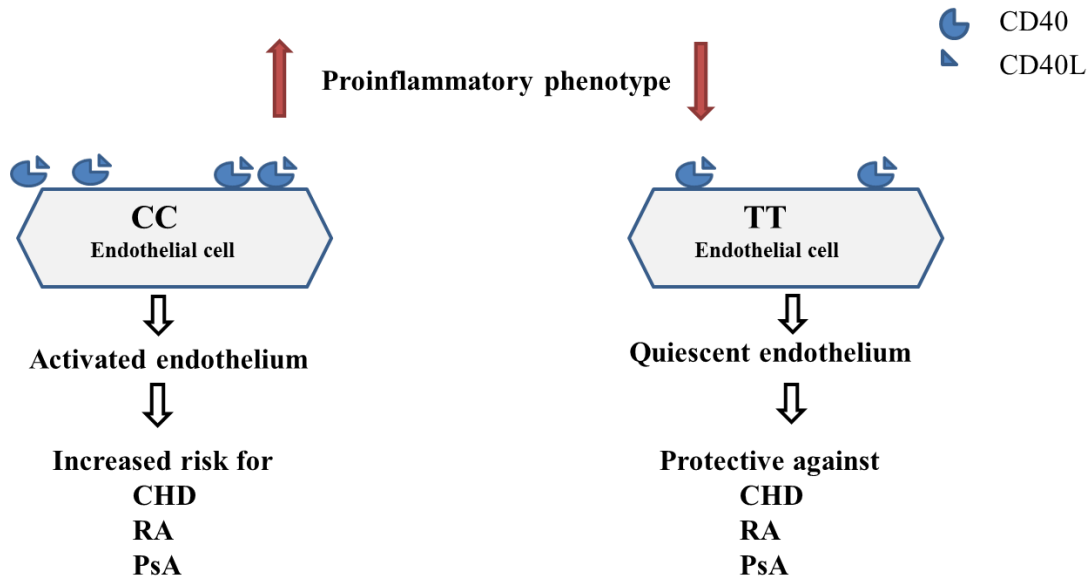
Figure 30: Endothelial cells with the CC genotype of the -1C/T SNP are genetically inclined towards endothelial dysfunction through enhanced CD40 protein expression. *Endothelial cells with the C allele and the homozygous C genotype express higher CD40 levels due to an optimal Kozak consensus sequence. Consequently, the strength of CD40-CD40L signaling is also higher in cells with the CC genotype. Therefore, the CC genotype carriers present a more proinflammatory phenotype compared to the T allele carriers, which protects the former from susceptibility towards endothelial dysfunction. In a case-control study, the C allele was associated with increased odds of developing chronic inflammatory diseases represented by CHD and RA. Hence the C allele and particularly the CC genotype of -1C/T CD40 is potentially a risk factor for immune dysregulation.*

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