Université de Montréal

The Role of Cardiotrophin-Like Cytokine Factor 1 on the Development of Atherosclerosis

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Mémoire présenté
en vue de l'obtention du grade de Maîtrise (M.Sc.)
En
Pharmacologie
Option Pharmacologie Moléculaire

Octobre, 2017

Université de Montréal

Faculté des études supérieures et postdoctorales

Ce mémoire intitulé

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Résumé

Le syndrome métabolique représente un problème majeur de la santé publique mondiale ; le taux augmente constamment en dépit de la technologie médicale innovante et des avancées thérapeutiques. Les maladies cardiovasculaires associées à la dyslipidémie demeurent la principale cause de décès et de morbidité à l'échelle mondiale en dépit d'une vaste recherche médicale et d'un large éventail de médicaments ciblant l'athérosclérose, l'obésité, le diabète, et autres. Des recherches récentes suggèrent l'existence et la persistance d'une inflammation de faible intensité dans la pathogenèse des pathologies comme l'athérosclérose et l'obésité. La « symbiose » entre le système métabolique et le système immunitaire est substantielle et toute perturbation contribue au développement des conditions métaboliques altérées qui aboutissent finalement à des troubles tels que l'obésité et l'athérosclérose.

L'objectif général de ma maîtrise était de caractériser l'effet de la *cardiotrophin-like cytokine factor 1* sur le développement de l'athérosclérose et de valider le dérivé de *cardiotrophin-like cytokine factor 1* couplé avec le fragment Fc de l'immunoglobuline G qui possède une longue durée de vie.

Cardiotrophin-like cytokine factor 1 est une cytokine du système immunitaire avec activité immunorégulatrice. *Cardiotrophin-like cytokine factor 1* appartient à la famille d'interleukine 6. *Cardiotrophin-like cytokine factor 1* est efficacement sécrétée en présence de *cytokine receptor like factor 1*, un récepteur soluble de la cytokine. *Cardiotrophin-like cytokine factor 1* possède des activités neurotrophiques médiées par le récepteur du *ciliary neurotrophic factor*. *Cardiotrophin-like cytokine factor 1* est également un ligand à haute affinité pour la sortiline. Des variantes du gène *Sort1* codant pour ce récepteur ont été associés à l'hyperlipidémie et au risque d'infarctus du myocarde dans plusieurs études d'associations génomiques. Il a été observé que la *cardiotrophin-like cytokine factor 1* lie et active les transfectants co-expriment de la sortiline et récepteur *du leukemia inhibitory factor*. Les deux récepteurs sont exprimés par les cellules myéloïdes et le récepteur du *leukemia inhibitory factor* est un puissant inducteur de la polarisation des macrophages de type M2 (anti-inflammatoire).

L'objectif général du projet était d'étudier si la *cardiotrophin-like cytokine factor 1* ou la *cardiotrophin-like cytokine factor 1* couplée avec le fragment Fc interagiraient avec des

macrophages des plaques d'athéroscléroses et réduirait la formation de cellules spumeuses et le développement de la plaque. Nos travaux montrent que l'expression de *cardiotrophin-like cytokine factor 1* dans le modèle murin d'athérosclérose $LDLR^{-/-}$ ne diminue pas le développement de la plaque. Cependant, certains résultats ont révélé une contribution significative de la *cardiotrophin-like cytokine factor 1* dans le gain de masse corporelle sans modification de l'apport calorique.

Mots-clés : système immunitaire, *cardiotrophin-like cytokine factor 1*, sortiline, syndrome métabolique, inflammation, macrophages, athérosclérose.

Abstract

Metabolic syndrome represents a major global health problem. Its rate is constantly increasing. Cardiovascular diseases emerging from dyslipidemia conditions are a worldwide leading cause of death and morbidity, despite extensive medical research and wide range of drugs targeting atherosclerosis, obesity, diabetes etc. Recent findings suggest the existence and persistence of low-grade inflammation in pathogenesis atherosclerosis and obesity. The "symbiosis" between metabolic and immune system is substantial and any perturbation contribute to the development of altered metabolic conditions that ultimately culminate in such disorders as obesity and atherosclerosis.

The overall goal of my Master internship was to characterise the effect of cardiotrophin-like cytokine factor 1 on development of atherosclerosis and validate a long half-life derivative of cardiotrophin-like cytokine factor 1 coupled with Fc fragment of immunolglobulin G.

Cardiotrophin-like cytokine factor 1 is a cytokine of the immune system with immunoregulatory activity. Cardiotrophin-like cytokine factor 1 belongs to the interleukin 6 family of monomeric cytokines. Cardiotrophin-like cytokine factor 1 is efficiently secreted in the presence of cytokine receptor like factor 1, a soluble cytokine receptor. Cardiotrophin-like cytokine 1 possesses neurotrophic activities mediated through the receptor of ciliary neurotrophic factor. Cardiotrophin-like cytokine factor 1 is a high affinity ligand for sortilin. Genome-wide association studies indicated that plasma low-density lipoprotein cholesterol levels and cardiovascular disease are associated with single nucleotide polymorphisms variants regulating sortilin expression. It was observed that cardiotrophin-like cytokine factor 1 binds and activates transfectants co-expressing sortilin and receptor of leukemia inhibitory factor. Both receptors are expressed by myeloid cells and leukemia inhibitory factor is a potent inducer of anti-inflammatory M2 macrophage differentiation.

The overall objective was to investigate if the interaction of cardiotrophin-like cytokine factor 1 or cardiotrophin-like cytokine factor 1 coupled with Fc fragment of immunoglobulin G with atherosclerotic plaque macrophages will reduce the foam cell formation and development of plaque. Our work shows that cardiotrophin-like cytokine 1 expression in mice on $LDLR^{-/-}$ atherosclerosis model does not decrease the development of plaque. However, some results

revealed a significant contribution of cardiotrophin-like cytokine factor 1 in gain of body mass without changes in food intake.
Keywords: immune system, cardiotrophin-like cytokine factor 1, sortilin, metabolic syndrome, inflammation, macrophages, atherosclerosis.

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***APC**: antigen-presenting cell; **ApoA**: apolipoprotein A; **ApoB**: apolipoprotein B; **ApoC**: apolipoprotein C; **ApoE**: apolipoprotein E; **ABCA(G)**: ATP-binding cassette(A), (G); **ATM**: adipose tissue macrophage; **BMDM**: bone marrow derived macrophage; **DC**: dendritic cells; **CNTF**: ciliary neurotrophic factor; **CNTFR**: receptor of CNTF; **CT-1**: cardiotrophin-1; **CLCF1**: cardiotrophin-like cytokine factor 1; **CRLF1**: cytokine receptor like factor 1; **CHD**: coronary heart disease; **CVD**: cardiovascular atherosclerotic diseases; **C/EBPδ**: CCAAT/enhancer-binding protein-δ; **CD 36**: cluster of differentiation 36; **CE**: cholesterol-ester; **CETP**: cholesterol ester transfer protein; **FAS**: fatty acid synthase; **FA**: fatty acid; **gp130**: glycoprotein 130; **AGE**: advanced glycation end product; **HFHC**: high-fat, high-cholesterol diet; **Th**: helper T lymphocyte; **HDL**: high-density lipoproteins; **HL**: hepatic lipase; **IFN**: interferon; **IL**: interleukin; **Ig**: immunoglobulin; **LCAT**: lethicin-cholesterol acyltransferase; **LIF**: leukemia inhibitory factor; **LIFR**: receptor of LIF; **LDL**: low-density lipoproteins; **LPL**: lipoprotein lipase; **LRP**: LDL receptor related protein; **MHC**: major histocompatibility complex; **MPO**: myeloperoxydase; **MMP**: metalloproteinase; **MTP**: microsomal triglyceride transfer protein; **MetS**: Metabolic syndrome; **M-CSF**: macrophage colony-stimulating; **NPY**: neuropeptide Y; **NK**: natural killer; **NP**: neuropoetin; **NNT-1/BSF-3**: novel neurotrophin-1/B cell-stimulating factor-3; **OSM**: oncostatin-M; **oxLDL**: oxidized LDL; **PRR**: pattern recognition receptors; **PAMP**: pathogen associated molecular pattern; **r**: recombinant; **sCNTFR**: soluble CNTFR; **SOCS 3**: suppressors of cytokine signaling-3; **SREBP1**: sterol regulatory element binding protein-1; **SMC**: smooth muscle cell; **SRAI/II**: scavenger receptor A I/II; **STAT**: signal transducer and activator of transcription; **TLR**: Toll-like receptors; **TG**: triglyceride; **TGF-β**: transforming growth factor-β; **TNF-α**: tumor necrosis factor α; **Vsp10p-D**: domain vacuolar protein-sorting 10 protein; **VLDL**: Very low-density lipoproteins*

This mémoire is dedicated to my parents,
my husband, my daughter and my son!

For their support, encouragement and immense patience!!!

Acknowledgements

First of all, I would like to express my sincere thanks to my scientific director Dr. Jean-François Gauchat for welcoming me in his laboratory. Thank you for your support, advice, patience and constant encouraging to not stop believing that at «the end of the tunnel will be the expected light»! I am grateful to the fate that I met notably you at the beginning on my scientific carrier. You have taught me to see the positive things instead of negative and never give up.

Equally, I would like to thank my co-director Dr. Gaetan Mayer. Without you my project could not be realized. Thank you for welcoming me in your laboratory, thank you for your kindness and professional support.

Special thanks to my lab mates: Sarah Pasquin, Salma Chehboun and Samaneh Samami. You were encouraging me all the time and due to all of you, you made my graduate studies unforgettable experience. You are amazing girls and I cannot imagine what I could do without you!!!

I would also like to mention and thank all my teachers from Pharmacology department for their professionalism, who largely have contributed to my academical progression.

I am grateful to all the members of the jury for taking their precious time to read and correct this memoire.

The most importantly, I have to thank my wonderful family: my husband Oleg Lapsin and my children Anastasia and Roman. Without your support and understanding, it would be impossible to path this difficult way and reach all goals.

Thank you to my parents, my parents-in-law and my brother for supporting me from abroad.

Chapter 1: Introduction

1.1 Immune system-generality

The immune system is an interactive network of lymphoid organs, cells, humoral factors and cytokines with specialized roles in defending against infection. Immunity is divided into two parts determined by the speed and specificity of the reaction. These are named the innate and the adaptive responses. Innate (natural) responses occur to the same extent, however, many times the infectious agent is encountered; whereas acquired (adaptive) responses improve on repeated exposure to a given infection.

The innate responses use phagocytic cells (neutrophils, monocytes, and macrophages), cells that release inflammatory mediators (basophils, mast cells, and eosinophils), and natural killer cells. The molecular components of innate responses include complement, acute-phase proteins, and cytokines such as the interferons (1, 2).

Acquired responses involve the proliferation of antigen-specific B and T cells, which occurs when the surface receptors of these cells bind to antigen. Specialized cells, called antigen-presenting cells (APC), display the antigen to lymphocytes and collaborate with them in response to the antigen. B cells secrete immunoglobulins, the antigen-specific antibodies responsible for the eliminating extracellular microorganisms. T cells help B cells to make antibodies and eradicate intracellular pathogens by activating macrophages and by killing virally infected cells. Innate and acquired responses usually work together to eliminate pathogens (1). All these cells develop from pluripotent stem cells in the fetal liver and in bone marrow and then circulate throughout the extracellular fluid. B cells reach maturity within the bone marrow, but T cells must travel to the thymus to complete their development (1).

1.1.1 The innate immune system

The innate immune system is comprised of a variety of cells, including antigen-presenting dendritic cells (DCs), phagocytic macrophages and granulocytes, cytotoxic natural killer (NK) cells, and gamma delta (γδ) T lymphocytes (3). Once an infectious agent penetrates physical and chemical barriers (e.g. epithelia), pattern recognition receptors (PRRs) of the innate immune

system sense their molecular structures so-called pathogen associated molecular patterns (PAMPs). Bacterial PAMPs often include cell wall components such as lipopolysaccharides (LPS), peptidoglycan and lipoteichoic acids. Additionally, viral proteins or unmethylated CpG motifs in bacterial and viral nucleic acids can be recognized (3). The best characterized PRRs are the Toll-like receptors (TLR), whose activation leads to pathogen uptake by phagocytic cells (including macrophages, DCs, neutrophils). After pathogen detection, APC migrate to the local lymph node, where they are involved in the activation and shaping of adaptive immunity (4).

1.1.2 The adaptive immune system

The two main cell populations of the adaptive immune system are T and B lymphocytes (T and B cells), which recognize a high diversity of antigens. The B cells originate from bone marrow and their maturation is accompanied by the expression of membrane bound immunoglobulins, called B cell receptors (BCRs). Activation of B cells by antigen leads to their differentiation into either memory or antibody-secreting plasma cells. Antibodies are composed of different classes (IgG, IgM, IgA, IgE, IgD) (5) and therefore mediate a variety of specific responses including antigen neutralization, opsonisation of pathogens or activation of the complement system (6).

The T cells also arise from bone marrow but migrate to the thymus to complete their maturation. Their receptors, T cell receptors (TCRs), recognize antigens only when displayed as peptides bound to self-major histocompatibility complex (MHC). Based on the class of MHC molecules they bind to, T cells are divided into CD4+ (bind to MHC class II) or CD8+ subsets (bind to MHC class I), TCD4+ and TCD8+, respectively. To fully activate T cells, additional costimulatory signals are necessary, which are displayed by antigen-presenting cells (APCs). The activation of naïve TCD4+ cells in the presence of an antigen induces their proliferation and differentiation into several subtypes of helper T lymphocytes (Th1, Th2, Th17, Treg and Tfh) (5). Each subtype of cells secretes specific cytokines involved in different biological function. Each subpopulation of Th has a negative influence on other populations. For example, interferon-γ (IFN-γ) produced by Th1 inhibits Th2 differentiation and Th17, whereas the interleukin 4 (IL-4) produced by Th2 inhibits the proliferation of Th1 and Th17 (7). The Treg

helps to prevent autoimmune T cell proliferation and decrease the intensity of the immune response (8).

By secreting cytokines, the T cells can act on the bone marrow to increase the production of monocytes (macrophages precursors that circulate in the blood) and neutrophils (9); they can activate endothelial cells lining blood vessels to express cell adhesion molecules that cause monocytes and neutrophils in the blood to adhere there. By secreting chemokines, they can direct the migration of adherent cells out the blood stream into the site of infection (10). The immune system is a very complex network that monitoring the balance between pro- and anti-inflammatory signals (cells, cytokines). Once this balance is disrupted, such disorders as autoimmune disease, chronic inflammation, cancer may appear.

1.1.3 Cytokines

Cytokines are small molecular weight glycoproteins (8 to 70 kDa), comparable to hormones and growth factors, secreted by specific cells of immune system to alter the behaviour of itself or another cell. Cytokines send intracellular signals by binding to specific cell-surface receptors. Cytokines are produced by virtually all cells and have a wide variety of functions. The biological effect depends on the cytokine and the cell involved, but typically these molecules will affect cell activation, division, apoptosis, or migration (11, 12). They are able to act locally, as autocrine, juxtacrine or paracrine response modifier (13). Cytokines produced by leukocytes and having effects mainly on other white cells are termed interleukins (14). Cytokines that have chemoattractant activity are called chemokines (15). Those that cause differentiation and proliferation of stem cells are called colony-stimulating factors (16). Those that interfere with viral replication are called interferons (17, 18).

1.2 Cardiotrophin-like cytokine factor 1-generality

1.2.1 Identification of cardiotrophin-like cytokine factor 1

Cardiotrophin-like cytokine factor 1 (CLCF1) is a member of the IL-6 family of cytokines which encompass interleukin 6 (IL-6), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor

(LIF), cardiotrophin-1 (CT-1), oncostatin-M (OSM), neuropoetin (NP) and interleukin 31 (IL-31) (19-21). All of them have the common signal transducing receptor molecule glycoprotein (gp130). Most of them are widely studied and their biological effects are well known. CNTF was tested in the treatment of neurodegenerative disease and obesity (22, 23). CT-1 is known to be a key regulator of energy homeostasis, as well as glucose and lipid metabolism (24). CT-1 enhances regeneration of cirrhotic liver remnants after hepatectomy through promotion of angiogenesis and cell proliferation (25). CT-1 has been already granted Orphan Drug Status by the European Medicine Agency for the prevention of the ischemia/reperfusion injury associated with solid organ transplantation and it is also obtained FDA Orphan Drug Status for protecting the liver from ischemia/reperfusion injury inherent to the procedure of transplantation (http://www.dignabiotech.com/newsdetail.asp?id=151).

Cardiotrophin-like cytokine factor 1 is one of the least defined cytokines that signal through the gp130 receptor subunit. Until 10 years ago, its main roles were thought to be restricted to the regulation of motor neuron development and regulation of immune system by targeting B-cells (26, 27). However, more recent work has identified potential activities in adult physiology, degenerative conditions, cancer, renal pathology (28-30). CLCF1 has several alternate names that are still commonly in use: CLC, CLCF1, NNT-1/BSF-3 (31, 32). In 1999, Senaldi et al. identified a novel member of the gp130 cytokine family using a sustraction cDNA library constructed from activated Jurkat human T-cell lymphoma cells (32). Due to neurotrophic and B cell-stimulating effects, it was termed novel neurotrophin-1/B cell-stimulating factor-3 (NNT-1/BSF-3). Shi et al. independently characterized the same protein using an algorithm incorporating neural network algorithms, applied to a large EST database. Due to its homology to CT-1, this group named it cardiotrophin-like cytokine (CLC) (31).

CLCF1 is a 225-aa protein: the primary structure of CLCF1 contains a putative conventional signal peptide spanning from amino acid 1 to amino acid 27 (31, 32), like IL-6, IL-11, LIF and OSM (33). There are four cysteine residues, two of which located in the signal peptide (32). CLCF1 contains one potential N-linked glycosylation site (amino acid 29) (32) and the mature CLCF1 is predicted to be a 198-aa peptide of 22 kDa (31, 32). The amino acid sequence of human CLCF1 has 27% homology with CT-1, 24% with IL-11, 23% with CNTF, 21% with LIF and 19% with IL-6 and OSM (32). Mouse CLCF1 displays a high amino acid homology of 96% to human CLCF1 and is also a 225-aa protein with a 27-aa signal peptide and

one potential N-linked glycosylation site (amino acid 29) (32), indicating a strong conservation throughout evolution.

1.2.2 Cytokine structure

CLCF1 is a Class-I helical cytokine, is folded in a four anti-parallel alpha-helices bundle. The four helices are arranged so that the helices A and B run in the same direction and C and D in the opposite direction. Linking the helices in this arrangement is made possible by a long loop joining the A and B helices, a short one between B and C and finally a second-long connection between C and the fourth main helix D (34). The tertiary structure of the cytokine (Figure 1) allows three binding sites (site 1, site 2, site 3) for its interaction with a heterotrimeric receptor composed of alpha (CNTFRα) and beta chains (gp130 and LIFRβ) (35).

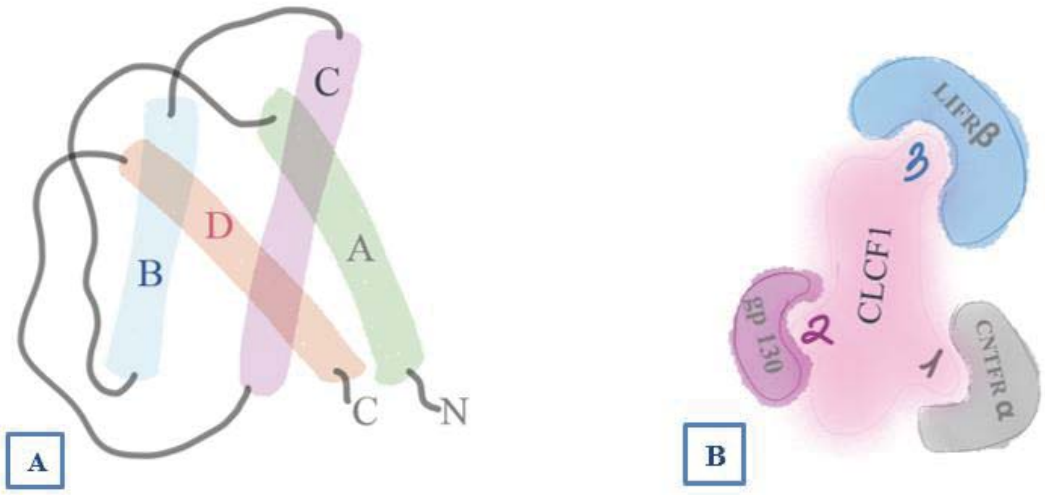


Figure 1. The secondary and tertiary structure of CLCF1.

A. The secondary structure of CLCF1 is formed by four anti-parallel alpha-helices bundles. B. Interaction sites 1,2,3 are the binding interface between cytokine and parts of complex receptor, comprising chains: CNTFRα, gp130 and LIFRβ, respectively.

1.2.3 CLCF1 tissue expression

The highest level of human CLCF1 mRNA expression is in lymph nodes and spleen, peripheral blood leukocytes/lymphocytes, bone marrow and fetal liver (31, 32). Moderate levels are expressed in ovary, placenta and kidney, whereas low but detectable CLCF1 mRNA levels were observed in the colon, heart, lung and pancreas (31). Various mouse tissues demonstrated strong CLCF1 mRNA expression in lymph nodes, spleen, liver, lung, uterus and ovary (32). Moderate to low levels were detected in thymus, heart, skin, adrenal and testis (32). CLCF1 has been detected in normal mouse pituitary tissue (20), fetal neuroepithelial cells (36) and in skeletal muscle fibers of embryonic mice (27). Thus, CLCF1 mRNA is expressed in a variety of tissues, suggesting an important biological role in numerous cellular functions.

1.2.4 CLCF1 secretory pathway

Despite the fact that the protein contains a putative signal peptide (32), its secretion is inefficient unless co-expressed with cytokine receptor like factor 1 (CRLF1), a protein that contains no transmembrane or cytoplasmic domain (37, 38). Alternatively, soluble CNTFR (sCNTFR) may act as a chaperone, in the same manner as CRLF1 (34). The site on CLCF1 that binds CRLF1 (site 3) is distinct from the tryptophan hotspot (site 1) through which it binds CNTFRα, but it is the same site 3 through which CLCF1 interacts with LIFRβ (35), suggesting the main role of CRLF1 in facilitating transport and secretion of CLCF1 (see the structure of receptor further in section 2.5.1).

Additional evidence that CRLF1 is not required for CLCF1 signaling comes from the finding that recombinant CLCF1 is biologically active (27, 32, 39). The clinical manifestations of CRLF1 deficiency have therefore been ascribed to the resulting decrease in CLCF1 secretion. However, a recent study (40) has provided compelling evidence to a more complex function. Thus, it appears that some CRLF1 mutations may cause disease without seriously affecting the cellular secretion of CLCF1, which implies that CRLF1 is more than just a facilitator of CLCF1 secretion.

Recent findings suggest that CRLF1 may have separate function and alternative partners (41-43). Thus, CRLF1 has been reported to complex with other cytokine components (44). Larsen and Petersen show that CRLF1 has additional and more important functions implicating both the signaling and turnover of CLCF1 and CNTFRα. Thus, it appears that CRLF1 contains three independent binding sites: one for its well-known binding to CLCF1; one that mediates direct binding to the CNTFRα; and a third site for interaction with VPS10P domain receptors, such as sorLA and sortilin (43, 45).

1.2.5 CLCF1 receptors

1.2.5.1 CNTFR

CLCF1 represents a developmentally important second secreted ligand for CNTF receptor (46). CNTFR is a tripartite receptor complex consisting of a non-signaling CNTFRα chain and two signal transducing beta chain receptors: gp130 and LIFRβ (Figure 2) (47). CLCF1 binds to its receptor in a stepwise manner by first recruiting the CNTFRα chain. The CLCF1/CNTFRα complex subsequently binds to gp130 and finally recruits the LIFRβ chains (37). The heterodimerization of signal-transducing chains gp130 and LIFRβ induce the activation of numerous signaling pathways (Figure 3) leading to the activation of gene transcription regulating cell proliferation, survival, migration etc (37, 38, 48).

1.2.5.2 Sortilin

Petersen and colleagues recently showed that both CNTF and CRLF1/CLCF1 strongly bind to the protein of the vacuolar protein-sorting 10 protein (Vsp10p) domain that contains sortilin (45), which recruits the gp130 and LIFRβ receptors to form a heterotrimeric signaling complex. This interaction provides rapid cellular uptake and endocytosis of the ligand, and second, it facilitates induction of gp130/LIFRβ signaling by CRLF1/CLCF1 in the presence of soluble CNTFRα (sCNTFRα) (45). Interestingly, the interaction of CRLF1/CLCF1 with sortilin in the presence of sCNTFRα resulting in increase of phospho-STAT3 levels, which was much higher than interaction of CRLF1/CLCF1 with sCNTFRα alone, without sortilin (45) (Figure 2). In genome wide association studies, the sortilin locus *Sort1* was associated with elevated LDL

levels and elevated risks of myocardial infarction (49). Sortilin deficiency protects against atherosclerosis by reducing uptake of native LDL by macrophages and foam cell formation (50). Targeting sortilin in immune cells attenuates inflammation by influencing IL-6 secretion from activated macrophages without changing lipoproteins metabolism, macrophages recruitment, or foam cell formation (51). Studies by several groups support an important role for sortilin in lipoprotein metabolism (50-55). However, the effect of sortilin on plasma cholesterol and its role in the secretion of hepatic lipoproteins remains controversial. Some groups support a role for sortilin in inhibiting lipoprotein export whereas other studies suggest that sortilin facilitates lipoprotein export (55, 56).

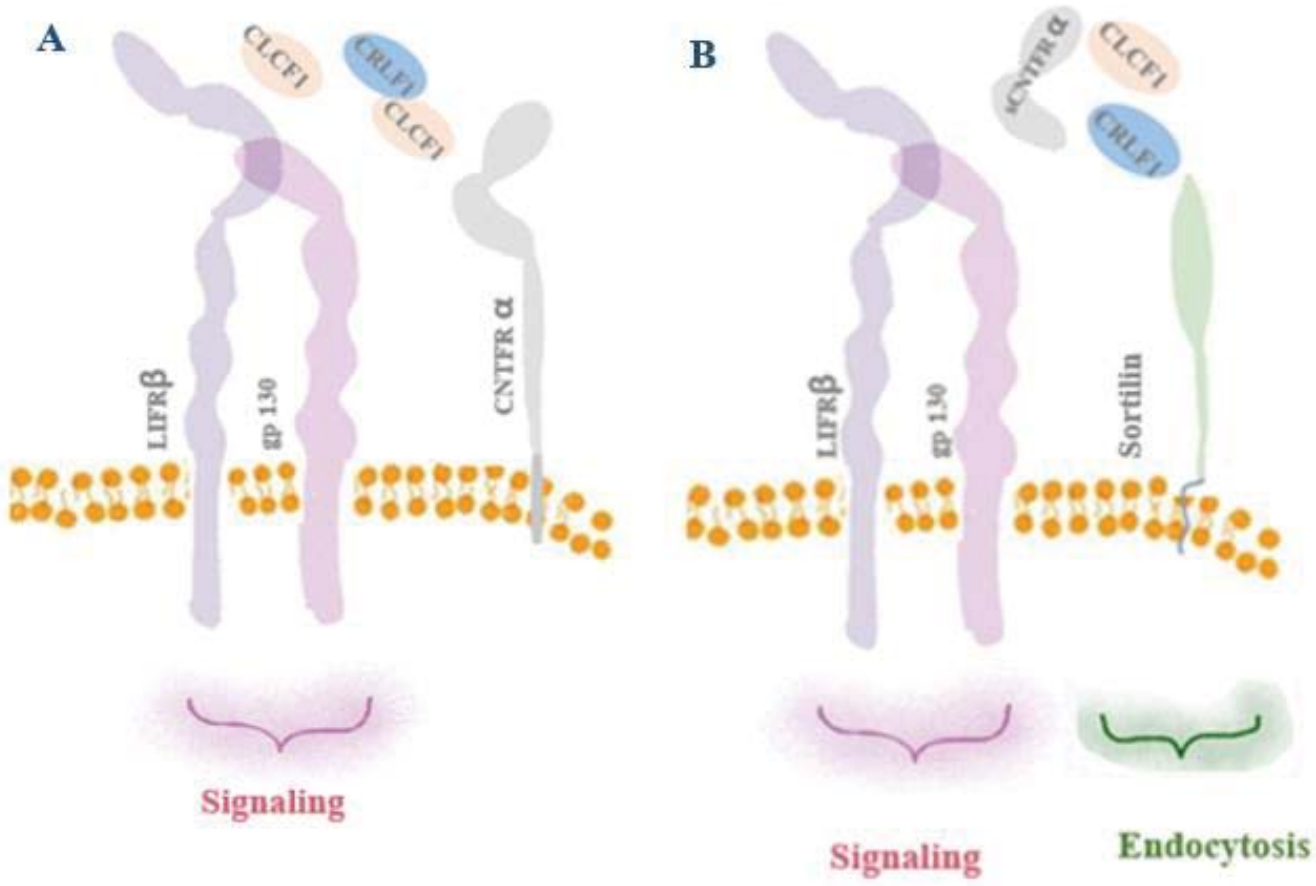


Figure 2. The structure of CNTFR and Sortilin receptor.

A, The trimeric receptor consists of non-signaling chains (CNTFRα) which binds the cytokine (CLCF1 or CRLF1/CLCF1), leading to the heteromerization of two signaling subunits (LIFRβ and gp130) with subsequent trans-phosphorylation and activation of tyrosine kinases.
B, CRLF1/CLCF1 can bind soluble CNTFRα, and via CRLF1 can bind Sortilin to facilitate signaling and endocytosis of ligands and receptor.

1.2.6 Signaling pathways

CLCF1, like many cytokines of the IL-6 family, mainly activates the JAK/STAT pathway (Figure 3). More specifically, CLCF1 activates JAK1, JAK2 and to a lesser extent TYK2 tyrosine kinases (38), leading to tyrosine phosphorylation of gp130 and LIFRβ subunits (37, 38). CRLF1/CLCF1 or CLCF1/sCNTFR induce downstream signaling events involving tyrosine phosphorylation of STAT3 and to a lesser extent STAT1 (34, 37, 38).

In addition to Jak/STAT signaling, other intracellular signal pathways which mediate functional responses to CLCF1 include SHP-2-mediated action of the PI3K/Akt pathway and the ERK1/2 MAPK pathway (57).

The earliest defined actions of CLCF1 included the induction of B cell hyperplasia, the mechanism by which this occurs remains obscure since B cells do not express the CNTFR (58). It has been suggested that a 3rd potential receptor is utilized by CLCF1 rather than CNTFR since binding of CLCF1 to B cells has been confirmed (59). Either CLCF1 can act on periphery by forming a complex with soluble CNTFRα (34, 45).

While gp130 and LIFRβ are broadly distributed, CNTFRα is mainly expressed in the brain, retina, skeletal muscles (60-62), and kidney cells (58). Recent proteomics analysis further demonstrated a substantial expression of CNTFRα in the bone, the gut and adipocytes (freely available at https://www.proteomicsdb.org/).

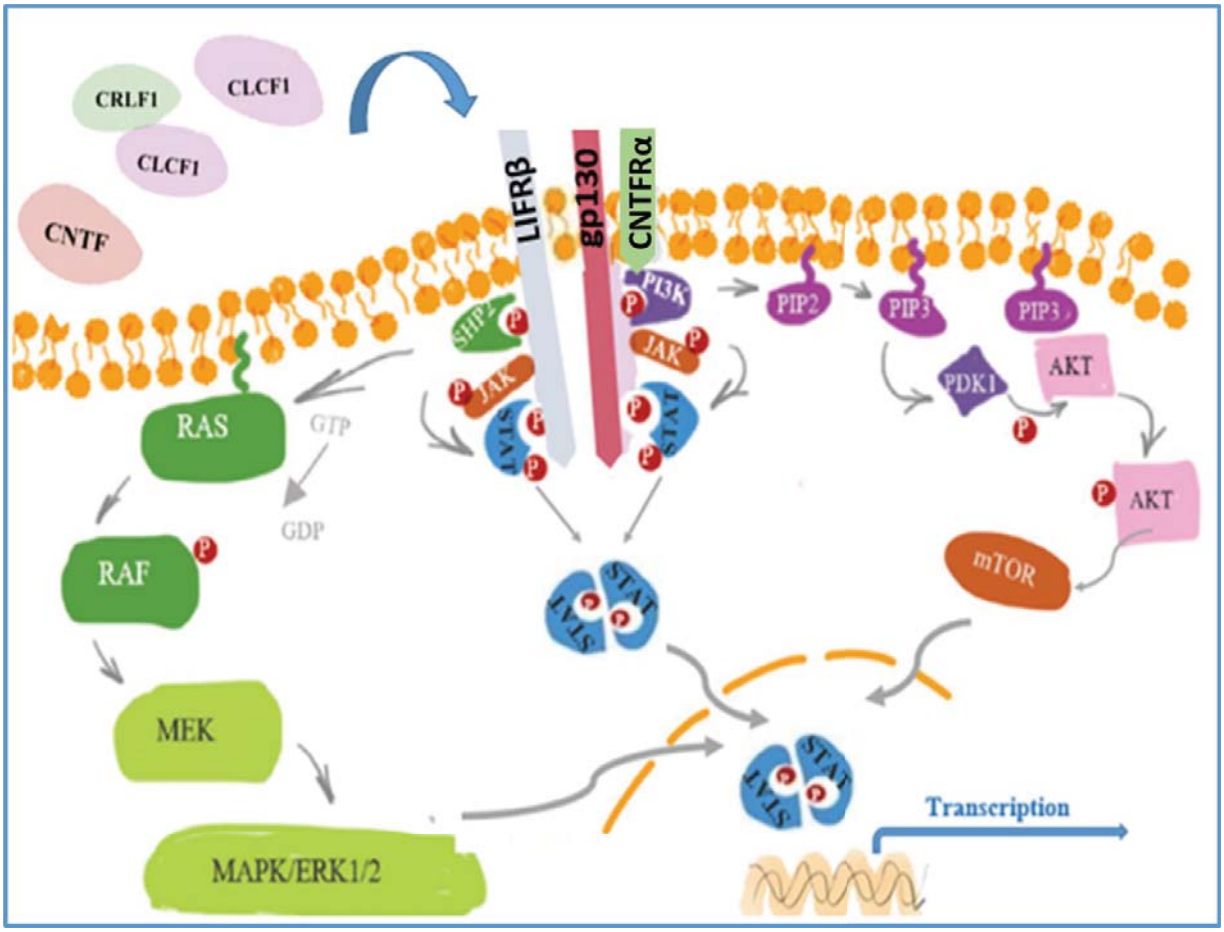


Figure 3. Main signaling pathways activated by CLCF1.

CLCF1, CRLF1/CLCF1 and CNTF bind CNTFRα, induce heterodimerization of gp130 and LIFRβ that trigger trans-phosphorylation and activation of tyrosine kinases JAK1, JAK2. Activated JAKs induces STAT1/3/5A phosphorylation, leading to STATs homo or heterodimerization, translocation to nucleus and activation of gene transcription. CLCF1-elicited activation of JAKs also induces PI3K phosphorylation leading to PDK1 activation and subsequently AKT phosphorylation. Phosphorylated AKT induces mTOR activation leading to upregulation of survival and proliferation. Alternatively, activated JAKs can phosphorylate SHP2 inducing a stepwise activation of Ras, Raf, MEK and ERK 1 or 2. ERK1/2 potentiates the signaling of transcription factors such as STATs.

1.2.7 CLCF1 knock out and mutation

Unlike CNTF, neither the CNTFRα nor CLCF1:CRLF1 is physiologically redundant and in contrast to CNTF-deficient mice which appear normal and healthy (63), CLCF1, CRLF1, or CNTFRα deficient mice suffer from a decreased number of neurons notably in the nucleus facialis and in motor neurons and die shortly after birth due to a failure to suckle (27, 64-66). Other factors may also contribute, such as atrophy of the facial muscles, and a defect in palate development, but these possibilities have not been explored, and it is not known whether they are a primary cause of the defect, or result from the loss of motor neurons.

Similar observations have been made in humans. CRLF1 (*CRLF*1) and CLCF1(*CLCF1)* gene mutations in humans both lead to a variety of syndromes that include "cold-induced sweating" (CISS) and Crisponi syndrome, with profuse sweating after exposure to cold, suckling problems during infancy and feeding difficulties in adult life manifesting with marked disinterest in food, as well as musculoskeletal abnormalities including spinal kyphoscoliosis, contracture of the muscles around the elbows, palatal and frontonasal malformations (67, 68). Patients with Crisponi syndrome tend to follow a more severe clinical manifestation and most die within the first year of life during high grade fever episodes (69).

The existing different grade of severity is explained by different degrees of mutated CLCF1 or CRLF1 secreted into media. Herholz and colleagues found that the patients with the most severe disease exhibited mutations that led to the least secretion of CLF1 into media (40). In contrast, the patients with the mildest manifestations of disease (originally characterized as CISS patients), showed relatively greater secretion of CLF1 (40). Since human mutations in CNTF are common, but are not associated with any notable health problems (70), this indicates that CLCF1 and CRLF1 are most biologically important ligands for CNTFR in human development.

1.2.8 Biological activity

1.2.8.1 Immunomodulatory function

CLCF1 is an integral molecule of the immune system. CLCF1 and CRLF1 are expressed in sites of hematopoiesis and immune cell maturation: bone marrow, adult spleen, thymus, and lymph node (31, 32, 71). CLCF1 has effects on adaptive immunity, directly stimulating B cells to proliferate and produce antibodies (Ab) with preference of Th2 over Th1 types (26).

The regulatory effects of CLCF1 was assessed using engineered transgenic mice with aberrantly expressing CLCF1 in the liver under control of the apolipoprotein E promoter. The cytokine was secreted into blood circulation (26). These mice show a phenotype consisting of B cell hyperplasia, hypergammaglobulinemia with anti-dsDNA Ab, and glomerulopathy with mesangial Ig deposition. Interestingly, B cell hyperplasia and hypergamma-globulinemia (IgG and IgM are increased) are also the main abnormalities seen in normal mice given a daily injection of recombinant CLCF1 for 7 days (32), indicating that the effects of CLCF1 supplementation, because of either transgenic expression or pharmacologic administration, are mainly to regulate immunity by stimulating B cell function and Ab production.

1.2.8.2 Neurotrophic function

CLCF1 has important functions within the nervous system, as indicated by its neurotrophic properties in vitro (32, 34, 37) and by the phenotype of mice lacking either CRLF1 or CNTFRα, two molecules assisting in CLCF1 secretion and signal transduction (64, 66). CLCF1 supports the survival of embryonic motor and sympathetic neurons (32, 37). Furthermore, CLCF1 may induce astrocyte differentiation at fetal neuroepithelial cells (36). In *vivo* injection of CLCF1 showed a regionally specific effect, increasing the number of lumbar spinal cord but not brachial or thoracic motoneurons (27).

1.2.8.3 Metabolic function

The non-redundant roles for the cytokines of the LIF family (LIF, CNTF, CT-1, OSM) was demonstrated in adipogenesis-regulation, insulin-signaling and metabolism (72-74). They exert significant yet diverse effects on lipogenesis in adipocytes and hepatic cells (72, 73, 75). Furthermore, through its central and peripheral action LIFRβ/gp130 signaling induces anorexic

effect and body weight regulation. CNTF administration was leading to a dose-dependent decrease in food intake and weight loss in leptin deficient *ob/ob* mice (76, 77), due to a hypothalamic inhibition of the orexigenic signal mediated by neuropeptide Y (NPY) (78). CNTF anorexic effect was also observed in patients, suffering of amyotrophic lateral sclerosis, receiving prolonged administration of the cytokine, triggering the interest of the potential of the cytokine in treatment of obesity (79). A recombinant (r) human CNTF derivative (Axokine) was tested in a phase II clinical study resulting in significant weight loss in obese patients (23, 74). Peripheral side effects and the development of neutralizing antibodies during the phase III trial led to the suspention of the development of this molecule (74). Chronic injection of rCLCF1 at high dose was also shown to induce 8% body weight loss in mice (32). Since CNTF and CLCF1 act through the same receptor complex, CLCF1 likely shares the mechanistic pathway with CNTF for body weight regulation.

Like CNTF, CT-1 was recently shown to have an important effect on body weight. The lack of CT-1 in mice leads to adult onset obesity (80). Moreover, chronic rCT-1 administration reduces body weight and fat accumulation in diet-induced or genetically obese rodents (73). These observations can be explained by the potential activation of the hypothalamic anorexigenic pathway by CT-1, as previously observed with CNTF (78). Similarly, daily intraperitoneal injections of OSM in diet-induced and *ob/ob* obese mice induced beneficial effects in these models of obesity and metabolic syndrome (81). LIF administration led also to a significant reduction of food intake and body weight gain (75). In *vitro* and in *vivo* studies have demonstrated that LIF can significantly modulate the release of the orexigenic neuropeptide NPY (82).

Moreover, cytokines of LIF family act directly on periphery to modulate adipogenesis. For example, CT-1 can regulate lipolysis in murine adipocyte cell lines and in the mouse white adipose tissue (WAT) by increasing adipose triglyceride lipase activity (83). OSM upregulates STAT 5 phosphorylation and ERK signaling pathway in murine preadipocytes attenuating C/EBPβ-induced adipogenesis (75).

CNTF directly regulates adipocyte metabolism. CNTF induces STAT3 phosphorylation in cultured 3T3-L1 adipocytes and rodent primary adipocytes (72). CNTF was shown to trigger the AKT pathway in preadipocytes, while activation of MAPK-Erk1/2 was observed in mature adipocytes (82).

LIF activates STATs 1 and 3 in preadipocytes and mature adipocytes (83). LIF inhibits triacylglyceride (TAG) accumulation during adipogenesis. Acute treatment with LIF resulted in increased expression of suppressors of cytokine signaling-3 (SOCS3) and CCAAT/enhancer-binding protein-δ (C/EBPδ) mRNA in 3T3T-L1 adipocytes. Chronic treatment resulted in decreased protein levels of sterol regulatory element binding protein-1 (SREBP1) and fatty acid synthase (FAS) (83).

1.3 Metabolic syndrome-generality

Metabolic syndrome (MetS) is a complex disorder with high socioeconomic cost that is considered one of the most alarming public health issues facing the world today (84). MetS is defined by a cluster of interconnected factors that directly increase the risk of coronary heart disease (CHD), other forms of cardiovascular atherosclerotic diseases (CVD), and diabetes mellitus type 2 (DMT2). Its main components are dyslipidemia (elevated triglycerides and apolipoprotein B (apoB)-containing lipoproteins, and low high-density lipoproteins) (85), elevation of arterial blood pressure and dysregulated glucose homeostasis (86).

Besides the multiple components and clinical implications of MetS, there is still no universally accepted pathogenic mechanism, but it seems to be largely attributable to insulin resistance with excessive flux of fatty acid (86).

Recently, it has become evident that systemic low-grade inflammation in the liver, muscle, and adipose tissue is a major contributor to the development of obesity and insulin resistance (87-91). The term “para-inflammation” was proposed by Ruslan Medzhitov (92) to characterize immune responses in which persistent tissue stress by a variety of stimuli, such as advanced glycation end products (AGEs) (glucose forms covalent adducts with the plasma proteins, lipids and nucleic acids which play an important role in the pathogenesis of diabetic complications like retinopathy, nephropathy, neuropathy, cardiomyopathy), free fatty acid and oxidized lipoproteins, triggers maladaptive chronic non-resolving immune activation, which is capable of resetting homeostatic set-points and thereby inhibiting insulin signaling pathways and prompting the development of insulin resistance, ultimately driving all components of the metabolic syndrome (93, 94). Hence, insulin resistance could serve as a transient adaptive mechanism, diverting blood glucose to leukocytes and other cell types required for the

preservation of homeostasis and tissue repair upon acute infection. However, the long-term consequences of such adaptive mechanism may be metabolically detrimental, particularly in cases of chronic non-resolving inflammation. Multiple lines of evidence link chronic activation of pro-inflammatory pathways to obesity-related insulin resistance (91, 95, 96). Cytokines and chemokines such as IL-6, IL-1β, MCP-1 and TNF-α are released by both adipocytes and immune cells, thereby contributing to the development of obesity (97). Thus, inflammation may be one of the links between obesity and insulin resistance, and may also promote endothelial dysfunction and early atherogenesis.

1.3.1 Link between immunity and metabolic syndrome

The cross-talk between the immune and metabolic systems is pivotal in promoting "metabolic health" throughout life. Perturbation of such "symbiosis" contributes to the tendency to develop altered metabolic states that may culminate in such well-known disorders as obesity and atherosclerosis (98).

The pathogen-associated molecular patterns (PRRs) are components of the innate immune system well known for their ability to sense foreign molecules and initiate a defense response with consequent release of pro-inflammatory cytokines (99). However, the PRRs are capable also to sense endogenous ligands formed in pathologic state which could be a trigger of inflammation in metabolic disorders (100, 101). Of these PRRs, TLR4 has received the most attention, as this receptor can be activated by free fatty acids (FAs) to generate pro-inflammatory signals and activate NF-κB (81). *TLR4*-deficient mice are protected from the inflammatory activation induced by obesity and demonstrate protection from insulin resistance induced by lipid infusion (102). The modified low-density lipoproteins (LDL), such as oxLDL can also activate TLR2, TLR4. The intracellular cholesterol crystals in foam cells activate the inflammasome NLRP3 (NLR family, pyrin domain containing 3) and trigger the secretion of IL-18 and IL-1β (pro-inflammatory cytokines) (103, 104).

Moreover, all metabolic tissues contain resident populations of leukocytes, presented even in lean healthy animals, indicating that the immune system is ready to respond to nutrient-derived signals (105, 106). For example, the extent of adipose tissue macrophage (ATM) infiltration is dynamically altered with lipid flux in adipocytes in lean and obese states, and may

serve to suppress lipolytic signals (107). ATMs are recruited to adipose tissue when chemokine or lipid release (lipolysis) are triggered and may function to promote lipid storage by suppressing lipolysis. These events could be classified as an inflammatory response, as it involves the acute recruitment of leukocytes to fat, but it lacks many of the cardinal signs of classic inflammation (*dolor, rubor, calor, and tumor*) (90).

1.4 Atherosclerosis

1.4.1 Generality

Atherosclerosis gives rise to cerebrovascular disease and coronary artery disease (CAD) through a slowly progressing lesion formation and luminal narrowing of arteries. Upon plaque rupture and thrombosis, these most common forms of cardiovascular disease manifest as acute coronary syndrome (ACS), myocardial infarction or stroke (108, 109). Coronary artery disease arising from atherosclerosis is still the leading cause of death and morbidity worldwide despite the availability to a wide range of drugs, targeting multiple and diverse pathological pathways (110). There is still an enormous need for additional therapies and to discover the new risk factors of cardiovascular disease (CVD).

Atherosclerosis is considered not only a disorder of lipid accumulation in the arterial wall as it was traditionally thought, but also a chronic inflammatory disease that contains components of both innate and adaptive immune systems (108, 111-113), implicated in all disease stages (Figure 4).

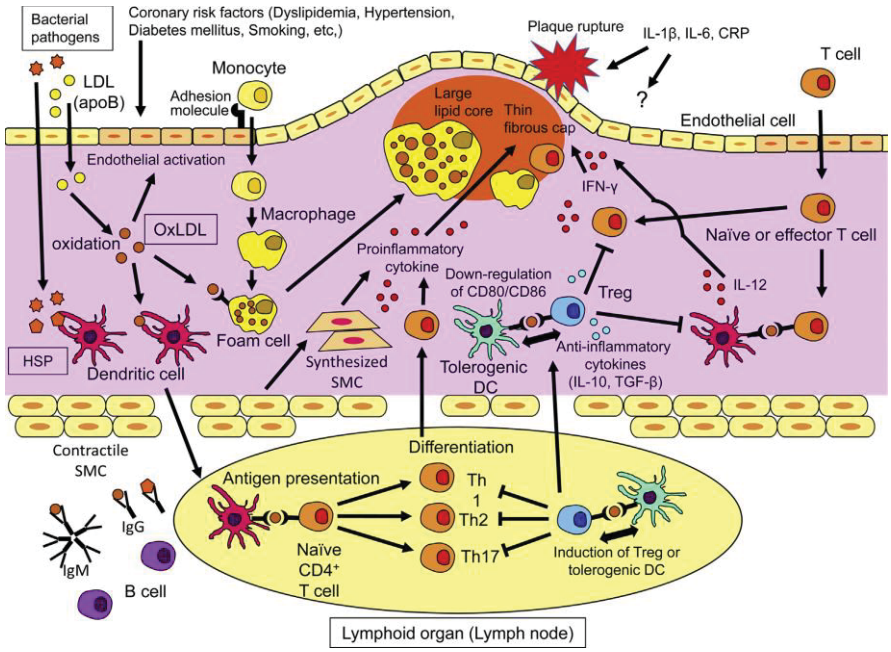


Figure 4. Role of inflammatory cells and immune responses in atherogenesis.

LDL is deposited in the subendothelial space, and the accumulated LDL is oxidized to oxLDL that activates the endothelium. Coronary risk factors also activate the endothelium and induce the adhesion molecules. Monocytes migrate into the subendothelial space using the adhesion molecules, differentiate into macrophages, take up oxLDL, and change to foam cells. The protein components of the oxLDL particle are processed and presented as antigens to T cells by macrophages and dendritic cells (DCs). Other self and foreign antigens may also trigger similar immune reactions. T cells differentiate into effector T cells (Th1, Th2, and Th17) and release cytokines and chemokines, and stimulate the migration of smooth muscle cell (SMC) and other inflammatory reactions. Migrated SMCs change their phenotype from contractile SMCs to synthesized ones that produce cytokines. Synthesized SMCs and foam cells contribute to form the atherosclerotic plaques including the lipid core and fibrous cap formation. Proatherogenic cytokines including IFN-γ secreted by Th1, and IL-12 secreted by DCs and macrophages deteriorate the lesion formation, might be associated with destabilizing the plaque, and induce the plaque rupture. Regulatory T cells (Tregs) suppress effector T cell activation, the differentiation of naïve T cell into effector T cells, and downregulate antigen presentation of DCs via secretion of anti-inflammatory cytokines including IL-10 and transforming growth factor (TGF)-β. Tolerogenic DCs, characterized by downregulated expressions of CD80/CD86, maintain the tolerance to self-antigens by inducing Tregs or by inhibiting effector T cells. Immunoglobulins produced by B cells are also thought to play a role in atherogenesis. apo B, apolipoprotein B; CRP, C-reactive protein; HSP, heat shock protein; IFN, interferon; Ig, immunoglobulin (114).

The first step preceding the atherosclerotic lesion formation is endothelial activation or dysfunction and structural alterations, including the absence of a confluent luminal elastin layer and exposure of proteoglycans (115), which permit subendothelial accumulation of low-density lipoprotein (LDL), which are mediated by coronary risk factors such as dyslipidemia, hypertension (116), diabetes mellitus (117), and smoking (118). Secondly, elevated levels of circulating cholesterol transported by apolipoprotein B100 (apoB100)-containing LDL binds to negatively charged extracellular matrix proteoglycans which leads to retention of LDL particles in the intima, where they are susceptible to oxidative modification by reactive oxygen species or enzymes such as myeloperoxidase or lipoxygenases released from inflammatory cells (119-121). The resultant formation of oxidized LDL (oxLDL) has been suggested to be the critical event in inducing inflammation in vascular wall (122). Thirdly, oxidized lipids retained in the subendothelial space promotes activation of endothelial cells. Activated endothelial cells have increased expression of monocyte interaction/adhesion molecules (VCAM-1, ICAM-1 and P-selectin), proinflammation receptors (Toll-like receptor 2, TLR 2) and chemoattractants (MCP-1 and IL-8) leading to attachment and transmigration of monocytes into intimal space (123-125). In addition, other cells contribute to development of lesions including dendritic cells (126, 127), mast cells (128), T cells (129) and B cells (130). Subsequently, monocyte-derived macrophages take up oxLDL via scavenger receptor leading to the formation of lipid-laden foam cells (131, 132). Following such steps, the initial fatty streaks contain lipids and numerous immune cells such as macrophages, dendritic cells (DCs), T lymphocytes etc. Advanced atherosclerotic lesions involve the migrating smooth muscle cells (SMCs), debris, apoptotic cells, and extracellular matrix such as collagen and proteoglycans (113).

Fatty streaks do not result in clinical complications and can even undergo regression. However, once smooth muscle cells infiltrate, and the lesions become more advanced, and such vulnerable plaque regression is less likely to occur (133, 134). Small populations of vascular smooth muscle cells (VSMCs) already present in the intima proliferate in response to growth factors produced by inflammatory macrophages (135). In addition, macrophage-derived chemoattractants cause tunica media smooth muscle cells to migrate into the intima and proliferate. Critical smooth muscle cell chemoattractants and growth factors include platelet-derived growth factors (136), matrix metalloproteinases (137), fibroblast growth factors (138), and heparin-binding epidermal growth factor (139). The accumulating VSMCs produce a

complex extracellular matrix composed of collagen, proteoglycans, and elastin to form a fibrous cap over a core comprised of foam cells (140). As lesions advance, substantial extracellular lipid accumulates in the core, in part due to large cholesterol-ester (CE)-rich particles arising from dead macrophage foam cells (141, 142). As the intimal volume enlarges due to accumulating cells, the fibrous cap becomes weak. The advanced plaque rupture leads to acute exposure of procoagulant and prothrombotic factors from the necrotic core of the lesion to platelets and procoagulant factors in the lumen, thereby causing thrombus formation (143). Thrombus formation at sites of plaque rupture accounts for the majority of clinical events with acute occlusive lumenal thrombosis causing myocardial infarction, unstable angina, sudden cardiac death, and stroke (144, 145). Calcifications are also common in advanced atherosclerotic lesions and increase with age (146). The necrotic core can completely calcify with time and calcifications can constitute most of plaque volume (147). Osseous metaplasia is sometimes seen in human lesions but these are rare and only occur in arteries that are already heavily calcified (148).

1.4.2 Lipoprotein metabolism

Whole body lipid homeostasis is maintained through a balance between exogenous uptake and endogenous synthesis of fatty acids and cholesterol and the pathway of reverse cholesterol transport. The transport of these lipid in the blood occurs in macromolecular complexes is called lipoproteins.

1.4.2.1 Lipoproteins

All lipoproteins are spherical, soluble lipid carriers comprised of a hydrophobic triglyceride (TG) and cholesterol-ester (CE) rich core encapsulated by a hydrophilic monolayer of phospholipids, free cholesterol and apolipoproteins (149). The classification of lipoproteins is based on their density, lipid composition and apolipoprotein association (150) (Figure 5). In addition to provide structural support to lipoprotein complexes, apolipoproteins also determine the interaction of lipoproteins with cell surface receptors as well as their rate of catabolism (151, 152). The largest lipoproteins are chylomicrons which are formed in the intestine and transport dietary TGs on an apoB48 backbone, with trace amounts of dietary cholesterol (153). Very low-

density lipoproteins (VLDL), secreted by hepatocytes, carry endogenous TG with modest amounts of endogenous as well as exogenous CE on an apoB100- backbone, and are associated with apoEs and apoCs. The catabolism of VLDL results in VLDL-remnant lipoproteins called intermediate density lipoproteins (IDL), which transport roughly equal partitions of TG and CE. Further catabolism of IDL followed by a series of modifications and lipid exchanges with various lipoproteins and peripheral tissue, results in the formation of CE-rich low-density lipoproteins (LDL) particles (149).

LDL carries about 60-70% of serum cholesterol (154). It transports cholesterol from the liver to the peripheral tissues. High levels of LDL cholesterol are harmful because it can build up on the arterial walls to initiate the formation of atherosclerotic plaques (85). Cholesterol is also transported by high-density lipoproteins (HDL), the smallest and densest lipoprotein particle. HDL is the key lipoprotein involved in reverse cholesterol transport and the transfer of cholesteryl esters between lipoproteins (85, 155). Recent studies discovered a number of pleotropic atheroprotective effects of HDL which include antioxidative (156, 157), anti-inflammatory (158-160), antiapoptotic (161), antithrombotic properties (162-164). HDL cholesterol is commonly known as the "good" cholesterol (85), as high levels of HDL cholesterol are associated with reduced levels of CVD. This concept was first demonstrated in the Framingham study in the 1970s and 1980s (165). However, recent human studies have cast some doubt on the "good cholesterol" HDL hypothesis (110).

HDL cholesterol concentration was identified several years ago, as an ideal target for lowering the incidence of atherosclerosis (166-168). However, the atheroprotective properties of HDL may not be directly related to its concentration in plasma (110, 169-171). It was suggested that the cholesterol efflux (reverse cholesterol transport) capacity of an individual's plasma is a better predictor of CVD status than HDL cholesterol concentration alone (172). Moreover, sometimes HDL does not appear protective and it might be classified as dysfunctional, a term associated with HDL particles that have been modified and thus, are no longer protective (173, 174). Accumulating data suggest that HDL can easily be modified and lose its antiatherogenic activities through multiple mechanisms. Based on the nature of modification, the alterations were classified into three types: spontaneous oxidative modification (175-177), due to the presence of free metal ions and free radicals in the atherosclerotic plaques, similar to the oxidation of LDL; enzyme-induced modification,

including myeloperoxidase (MPO), chymase-tryptase, matrix metalloproteinases (MMPs), PMN-associated enzyme, endothelial lipase, and so on (178-183). These enzymes can degrade or oxidize apolipoproteins without significant changes in lipid moiety. Metabolic modification, such as glycation (184-186) that occurs under hyperglycemic conditions, and acute phase reactants-induced modification during inflammation (169, 187, 188).

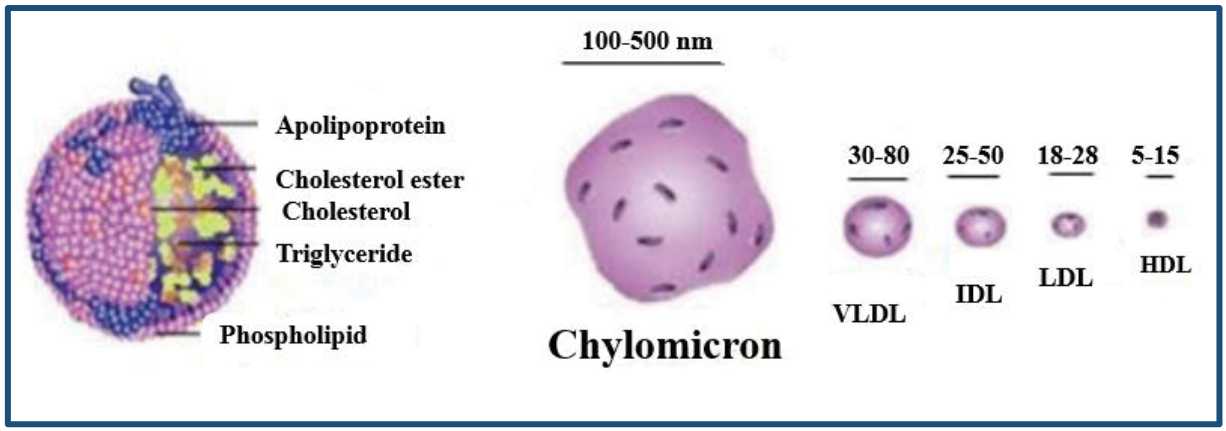


Figure 5. The composition and size of different lipoproteins.

On the left, the lipoproteins form a sphere consisting of cholesterol-esters and triglycerides enveloped by a membrane formed of a monolayer of phospholipids and free non-esterified cholesterol and apolipoproteins. On the right, a drawing showing the size of different lipoproteins ranging from 100 to 500 nm for chylomicron down to 5 to 15 nm for HDL (150).

1.4.2.2 Exogenous lipoprotein metabolism

Absorption of dietary lipids occurs in the jejunal portion of the small intestine, and upon internalization into intestinal enterocytes, TG-rich chylomicron particles are formed (189). Chylomicrons, upon entry into the lymphatics and into plasma, become associated with apoEs and apoCs, both of which are required for the metabolism of chylomicron particles (153). Following TG hydrolysis by lipoprotein lipase (LPL) anchored on capillary endothelial cells, the chylomicron-derived FAs and glycerol are taken up predominantly by adipose tissue and re-esterified into TGs for storage. The resultant chylomicron remnant particles are efficiently taken up by the liver through LDLR- and LDL receptor related protein (LRP)- mediated uptake by recognition of apoE (190), concluding the exogenous lipid metabolism pathway.

1.4.2.3 Endogenous lipoprotein metabolism

The endogenous lipoprotein pathway begins with hepatic *de novo* synthesis of lipoproteins. Approximately 70% of circulating cholesterol is produced in hepatocytes, making the liver the primary regulator of whole body cholesterol and lipid homeostasis (191). VLDL are particles that transport TG and cholesterol from the liver for redistribution to various tissues. Assembly of VLDL particles occurs within the secretory pathway of liver cells. This is a two steps process (192). The first series of events in VLDL assembly process occurs in the rough endoplasmic reticulum (193, 194). This process gives rise to a partially lipidated form of apoB (pre-VLDL) (195). The pre-VLDL formation is highly dependent on MTP (196). Microsomal triglyceride transfer protein (MTP) is essential for VLDL formation, and lack of it leads to a total loss of apoB-containing lipoproteins from plasma (197, 198). MTP transfers lipids: mainly triglycerides, but also cholesterol-esters and phospholipids. The second step, which occurs outside the rough ER in a smooth membrane compartment, pre-VLDL associates with the major proportion of lipids, forming a *bona fide* VLDL (real VLDL) (192). This step depends on ADP-ribosylation factor 1 (199) and its activation of phospholipase D (200). So, once VLDL particles are formed and secreted by hepatocytes, in capillary beds of target tissues such as adipose and muscle, endothelial cell lipoprotein lipase (LPL) hydrolyzes the VLDL liberating FA and glycerol which are taken up via soluble passive diffusion or CD36-mediated uptake in order to be re-esterified into TGs in adipose tissue or used for fatty acid oxidation in muscle (201). Following LPL-mediated hydrolysis of VLDL particles, VLDL-remnants or IDLs are formed, which can be taken up by recognition of apoE by the LDLR or the LRP following hepatic lipase (HL) modification (202). Alternatively, IDL can be hydrolyzed by HL resulting in the formation of LDL particles (203). The principal mechanism of LDL uptake is receptor-mediated endocytosis via LDL receptor (204). The LDLR is expressed ubiquitously. It accounts for approximately one third of LDL uptake by extrahepatic tissues. However, the liver is the primary site of LDLR expression and therefore regulates most of the clearance of circulating LDL particles (204).

Cholesterol is also transported by HDL, the smallest and densest lipoprotein particle. HDL is a heterogeneous collection of lipoprotein particles. Two-dimensional electrophoresis of

plasma lipoproteins separates the HDL based on their size and charge. Five major HDL particles have been identified: pre-β-1 HDL, α-4 HDL, α-3 HDL, α-2 HDL, and α-1 HDL (205, 206). The overwhelming majority of HDL particles contain apoAI, it comprises roughly 70% of the HDL mass (207, 208). Many HDL particles also contain apoAII, second most abundant protein in HDL. The metabolism of HDL initiates with apoAI synthesis in the liver and intestine, but in order to form HDL, lipid-free apoAI must interact with cells expressing ATP-binding cassette transporter A1 (ABCA1) that moves cellular lipids across the bilayer in a process requiring hydrolysis of ATP (209, 210). Nascent HDL released by ABCA1 expressing cells contains cellular phospholipids (PL) and free cholesterol (FC), and this particle is the substrate for lethicin-cholesterol acyltransferase (LCAT) which esterifies FC into cholesteryl ester (CE), building up the hydrophobic core necessary to generate spherical alpha HDL particles (211, 212). Additional lipidation of HDL can take place with ABCG1 which has been reported to lipidate mature, spherical HDL (213). Another enzyme-cholesterol ester transfer protein (CETP) moves the cholesteryl ester to apoB-containing lipoproteins in exchange to triglycerides. The concerted action of CETP-mediated cholesteryl ester transfer and hepatic lipase-mediated hydrolysis of triglycerides and phospholipids leads to formation of smaller HDL particles that are the preferred binding partners for scavenger receptor B type I (SR-BI), the major HDL receptor in hepatic cells. Thus, in humans, HDL-cholesterol can be returned to the liver via two pathways: direct hepatic uptake by scavenger receptor B1 (SR-B1) (214, 215); or through CETP exchange of HDL-CE for TG in apoB-containing lipoproteins, followed by hepatic uptake of these apoB-containing particles by the LDL receptor (216). In the liver the cholesterol is converted to bile acids for subsequent excretion (217).

1.4.2.4 LDLR $^{-/-}$ mice model

Deletion of the *Ldlr* in C57Bl/6J mice disrupts normal murine lipoprotein metabolism, resulting in elevated plasma cholesterol, particularly in the LDL fraction as a result of defective LDL clearance (218). Furthermore, these animals have increased cholesterol in both VLDL and IDL when fed a high-fat diet (219). LDLR$^{-/-}$ mice fed in a high-fat high-cholesterol (HFHC) diet for 12 weeks recapitulate many features of the metabolic syndrome such as dyslipidemia, hyperinsulinemia, insulin resistance and hepatic steatosis (220). Furthermore, these animals

develop atherosclerotic lesions that are relatively advanced, exhibiting significant lipid accumulation, increased macrophage and smooth muscle cell infiltration and enhanced collagen deposition (220).

1.4.3 Monocyte fate in atherosclerosis

Monocytes are essential for the development and exacerbation of atherosclerosis. They arise from proliferating and differentiating hematopoietic stem and progenitor cells in the bone marrow. Increased production of bone marrow monocytes in experimental models of atherogenesis has been reported in hypercholesterolemic swine, rabbits, and rodents (221, 222). It was also shown that hypercholesterolemia induces monopoiesis in extramedullary organs, including the spleen (223). There are several subtypes of monocytes based on their cell surface expression of the glycoprotein Ly6C in mice. $Ly6C^{high}$ monocytes are short-lived, transport tissue antigens to lymph nodes (224), and accumulate at sites of inflammation (225) where they differentiate to macrophages and dendritic cells. $Ly6C^{low}$ monocytes are longer lived, patrol the vasculature, respond early to infection (226), and survey endothelial integrity (227). The activation of endothelial cells by components of oxLDL, and possibly also by the turbulent blood flow at the arterial branching points, lead to the expression of adhesion molecules such as E-selectin and VCAM-1 on the endothelial surface of atherosclerotic artery and promotes the recruitment of circulating monocytes. These act in synergy with chemokines which attract monocytes, dendritic cells and T cells into the intima (228). Monocytes in the intima are stimulated by macrophage colony-stimulating (M-CSF) factor produced by activated endothelial cells to differentiate into macrophages; this process is necessary for the development of atherosclerosis (229).

1.4.4 Macrophage and foam cell formation

A central hallmark of atherosclerosis is the cholesterol-loaded macrophage or foam cell. This process is initiated by the ingestion and processing of LDL in both its native and modified forms (230). Native LDL uptake occurs via the LDL receptor (LDLR), which undergoes negative feedback regulation under high intracellular sterol concentrations (231). Although this pathway

certainly contributes to foam cell development (231), the predominant LDL uptake pathway by macrophages in lesions is that of modified LDL via the scavenger receptors cluster of differentiation (CD) 36 and scavenger receptor A I/II (SRAI/II). Upon entry into the intima, LDL particles undergo oxidative modification rendering them high-affinity scavenger receptor ligands (131). Unlike the native LDLR, scavenger receptors do not undergo negative feedback regulation in response to intracellular sterol accumulation (131). Consequently, macrophage uptake of modified LDL particles in lesions can persist indefinitely, and is only limited by substrate availability and cell viability. There is also an alternative mechanism for macrophages foam cell formation; receptor-independent uptake of unmodified native LDL called macropinocytosis, by which cells ingest fluids within vacuoles (232).

Hence, the macrophages uptake of modified LDL is mediated by scavenger receptors such as SR A I/II and CD36. However, deletion of scavenger receptors does not reduce macrophage oxLDL uptake. Even in $CD36^{-/-}$, $SR\ A^{-/-}$ double knock out mice there are abundant lipid laden macrophages in the vessel wall and these mice can develop atherosclerosis (233), although lesions complexity is reduced (234). Moreover, targeting macropinocytosis by PI3K inhibitors and Cytochalasin D (inhibitor of actin polymerization) reduced uptake, but residual LDL uptake still take place (233), suggesting existence of another additional pathway leading to foam cell formation.

Genome-wide association studies for coronary artery disease reported noncoding genetic variants at chromosome 1p13 to be significantly associated with CVD (235) and plasma level of LDL cholesterol (56, 236, 237). The *SORT1* gene, encoding the protein sortilin, seems to be the causal gene at the locus regulating LDL cholesterol levels (53, 55, 238). Sortilin is involved in macrophage LDL uptake. Increased concentrations of extracellular LDL cause an upregulation of macrophage *Sort1* mRNA and protein (50), promoting continuous uptake of LDL by Sortilin. Sortilin deficiency led to reduced atherosclerosis in $ApoE^{-/-}$, $LDLR^{-/-}$ mice models (50, 51).

1.4.5 Macrophage polarization and atherosclerosis

Macrophages are heterogeneous cell population. Several classes of macrophages have been described based on their expression of markers, the production of specific factors, and their

biological functions (239, 240). Classically activated (M1) macrophages are typically induced by cytokines, such as IFN-γ and tumour necrosis factor (TNF), or by lipopolysaccharide recognition. These macrophages are characterized by production of high levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and by ability to promote Th1 and Th17 immune response via production of IL-12 and IL-23 (239, 240). From a functional point of view, M1 macrophages participate in the removal of pathogens during infection through activation of the NADPH oxidase system, and the subsequent generation of reactive oxygen species (ROS). Chronic M1 macrophage activation can, therefore, mediate ROS-induced tissue damage and impair wound healing (241). To protect against such tissue damage, the inflammatory response is spatially and temporally counterbalanced by regulatory mechanisms driven by alternatively activated (M2) macrophages (242). Three different subclasses of M2 macrophages have been identified: M2a macrophages are induced by the IL-4 and IL-13 cytokines; M2b macrophage subtypes are induced by immune complexes in combination with IL-1β or lipopolysaccharide; and M2c macrophages are induced by IL-10, transforming growth factor-β (TGF-β), or glucocorticoids (243). All M2 macrophages have an anti-inflammatory cytokine profile characterized by low production of IL-12 and high production of both IL-10 and TGF-β (240). Functionally, M2 macrophages can scavenge debris and apoptotic cells (244) and promote tissue repair and healing. Furthermore, they possess proangiogenic (245) and profibrotic (246) properties.

The distribution of various macrophages phenotypes within atherosclerotic plaque is different and stage-dependent (247). The total number of macrophages gradually increases with plaque progression and severity, and is higher in symptomatic than asymptomatic plaques (248-250). Macrophages located in the lesion shoulder, which is considered one of the most unstable areas within the plaque (251), mainly express M1 polarization markers, whereas macrophages present in the fibrous cap surrounding the necrotic core express both M1 and M2 markers. In the adventitia, M2 macrophages are twofold to threefold more abundant than M1 macrophages (248).

1.5 Hypothesis and objectives

CLCF1 is a type I cytokine expressed in the immune system. It is a part of IL-6 family which has the common signal transducing receptor molecule glycoprotein (gp130). Most of the CLCF1 partners (IL-6, CNTF, CT-1, OSM, NP, IL-31) are widely studied and their biological effects are well known (22-25). CNTF was tested in the treatment of neurodegenerative disease and obesity (22, 23). Until 10 years ago CLCF1 main roles were thought to be restricted to the regulation of motor neuron development and regulation of the immune system by targeting B cells (26, 27). However, more recent work has identified potential activities in adult physiology, degenerative conditions, cancer and renal pathology (28-30). CLCF1 represents a developmentally important second secreted ligand for CNTF receptor (46). The CNTFRα chain of CNTFR is not expressed by the immune system. Therefore, the CLCF1 receptor implicated in the immune function of CLCF1 has not been yet identified. Early studies indicated that it signals through the leukemia inhibitory factor receptor (LIFR) and supports the growth of a mouse myeloid leukemia cell line (32). CLCF1 was later shown to be a high affinity ligand for sortilin (45) and we observed that it binds and activates Ba/F3 transfectants co-expressing sortilin and LIFR. Both sortilin and LIFR are expressed by myeloid cells (252, 253) and LIF (ligand for LIFR) is a potent inducer of anti-inflammatory M2 macrophage differentiation (252).

We hypothesised that CLCF1 administered in circulation will have therapeutic effects on atherosclerosis by targeting macrophages and promoting the anti-inflammatory M2 macrophages polarization through LIFR and inhibiting foam cell formation and atherosclerosis plaque formation.

For that purpose, we first investigated the effect of acLDL with or without CLCF1 on differentiation of macrophages into foam cells *in vitro.* Secondly, the effect of CLCF1 was assessed *in vivo* in a mice model of atherosclerosis; mice $LDLR^{-/-}$ were on an atherogenic diet for 12 weeks. To avoid multiple CLCF1 injections, it was generated the recombinant adeno-associated virus 8 (AAV 8, liver tropisme) encoding CRLF1/CLCF1. We decided to incorporate into our construct CRLF1 as it is not only a facilitator of CLCF1 secretion, but CRLF1 can have separate function and alternative partners (41, 42).

At the same time, the FcCLC1, stable derivative of CLCF1 molecule, was tested *in vivo* on the same mice model. Fusion with IgG Fc derivative can be used to generate long-lived

therapeutic derivatives of cytokines (254). We have generated a construct coding for a mouse CLCF1-IgG2bFc using a Fc mutant sequence that does not induce antibody-dependent cell-mediated cytotoxicity **(**ADCC). FcCLCF1 can be considered as functional form of CLCF1 (it proliferates Ba/F3 cells expressing CNTFR). It can be used *in vivo* assays in order to be validated as a more stable form of CLCF1 and could be used in further *in vivo* tests.

CLCF1 is involved in body mass control as well, as it was shown in literature: after 7 days treatment with recombinant CLCF1, WT mice reduced the body mass by 8% (32). Thus, as the *in vivo* test was performed, the body weight of atherosclerotic mice had been followed up as well.

Chapter 2: Materials and Methods

2.1 *In vitro* assay

2.1.1 Cell culture of 3T3-L1 preadipocytes and standard differentiation into mature adipocytes

Murine preadipocyte (3T3-L1) were cultured using DMEM-F12 medium supplemented with 10% fetal calf serum (FBS) and kept at 37˚C in a 5% CO_2 incubator. Experiments were performed when the cells were at low passage number (maximum 12 passages) and were routinely subdivided at <70% confluence. For standard 3T3-L1 adipocyte differentiation, preadipocytes were seeded in 6-well plates (3.0×10^5 cells per well) and grown to confluence. A second-day postconfluence differentiation was initiated with induction medium containing 167 nM insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (all from Sigma-Aldrich) in DMEM-F12 with 10% FBS. Two days later, medium was replaced with DMEM-F12 containing 10% FBS and 167 nM insulin (insulin medium) and was refed each second day with fresh medium with insulin till the end of differentiation period (9 days total). Differentiated cells (>80% with lipid droplet accumulation) were used for functional assays on day 9 after differentiation was initiated (255).

For assessment of triglyceride accumulation AdipoRed™ Assay Reagent was used for intracellular lipid droplets quantification. A Wallac 1420 VICTOR2^{TM} system was used to measure fluorescence in 6-well plates at wavelengths of 485 nm excitation and 572 nm emission.

The following antibodies were used for Western Blot to identify the changing of surface expression of scavenger receptor CD36: goat anti-CD36 (cat# AF 2519, R&D system) and a rabbit anti-β Actin (cat#A2066, Sigma Aldrich) as a control intern.

2.1.2 Foam cell formation and flow cytometry analysis

For *in vitro* tests, murine macrophage cell line RAW 264.7 and primary macrophages derived from bone marrow (described below) were loaded with the mix, previously incubated at 37ºC for 30 minutes: 100 µg/ml of acLDL+ 5 µM of Bodipy-cholesterol (Avanti Polar lipids, cat#810255) +/- 100 ng/ml CLCF1. Macrophages were incubated with the mix acLDL+ Bodipy-cholesterol+/-CLCF1 at 37ºC for 24 h in the medium (DMEM or RPMI for RAW264.7 and BMDM, respectively) without serum in order to load the cells and induce foam cells formation. Before analysing cells by flow cytometry (FACS), the cells were maintained in corresponding medium without serum and 0.2% BSA for 4 hours in order to equilibrate the level of intra- and extracellular cholesterol. To perform FACS analysis and measure the level of fluorescent lipids loading the FITC channel or Alexa 488 can be used to detect Bodipy.

2.1.3 Biological activity of recombinant FcCLCF1

To validate that mouse recombinant FcCLCF1 protein can be used in an *in vivo* model, the biological activity was assessed using Ba/F3 cells transfected with cDNA coding for mouse CNTFR by proliferation assay (44). Recombinant FcCLCF1 was produced using Flp-In-293 cells (Thermo Fisher Scientific) stably transfected with expression vector pcDNA^{TM}/FRT with inserted cDNA coding for FcCLCF1. Transfectants were expanded and recombinant FcCLCF1 was isolated from the culture medium, concentrated, purified by immobilized metal-affinity chromatography (IMAC) using Ni-NTA agarose beads (Qiagen Inc). Purified protein was dialysed against PBS, sterile-filtered and analysed by Western Blot. To assist the biological activity of FcCLCF1, the transfected Ba/F3 CNTFR 1.0×10^5 cells/96-well plate were incubated for 72 h in triplicate with increased protein concentrations (from 0 to 1 µg/ml) in RPMI-1644 medium supplemented with 5% FBS. Before fluorescence measurement, incubation with 50 µl/well Alamar Blue was performed (Alamar Blue, AbD Serotec, Cedarlane). The Alamar Blue is an indicator dye, that incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes colour in response to the chemical reduction of growth medium, resulting from cell growth. The Alamar Blue cell proliferation assay reagent is designed to quantitatively measure the proliferation of various cell lines. A Wallac 1420 VICTOR2^{TM}

system was used to measure fluorescence in 96-well plates at wavelengths of 530 nm excitation and 590 nm emission (44).

2.2 *In vivo* assay

2.2.1 AAV vector construction and virus generation as performed in the lab of Dr. Gaetan Mayer

The codon optimized mouse *CRLF1/CLCF1* and *FcCLCF1* synthetic cDNA were obtained from GeneArt™ (Thermo Fisher Scientific Inc). The *CRLF1/CLCF1* and *FcCLCF1* cDNA were inserted into the AAV serotype 8 vector downstream from the constitutive apolipoprotein E promoter to generate AAV8*CRLF1CLCF1* and AAV8*FcCLCF1*. The empty plasmid for AAV was used as a vector control (AAV8*CTR*). The AAV8*CRLF1/CLCF1*, AAV8*FcCLCF1* and AAV8*CTR* plasmids and adenovirus helper plasmid (pDG8) were co-transfected into 293 T-cells using Polyethylenimine (PEI) for large-scale recombinant AAV (rAAV) production (Figure 6). The transfected cells were harvested, the cell extracts were prepared, and the AAV8 were purified by discontinuous iodixanol density gradient centrifugation. After the freeze-thaw cycles and enzyme treatment, the viral preparations were concentrated and desalted by centrifugation through the Amicon ultra-15 centrifugal filter devices (100 kDa nominal molecular weight limit, Millipore, Billerica, MA). The viral genome titers were determined by qPCR. Viral preparations used for animal liver transduction had titers $\approx 1\times10^{12}$ viral genome particles per mouse.

Detailed protocol:

AAV vectors

Two vectors were used in our experiment:

- An AAV vector containing GOI (gene of interest) (*CRLF1/CLCF1* and *FcCLCF1*) flanked by the inverted terminal repeats (ITRs) which allow synthesis of the complementary DNA strand (cDNA).
- A packaging vector pDG8 containing the AAV serotype REP2 and CAP8 genes-translated to produce multiple proteins required for an AAV life cycle and capsid

proteins for viral encapsulation. The E4, E2A, and VA genes required for virus replication.

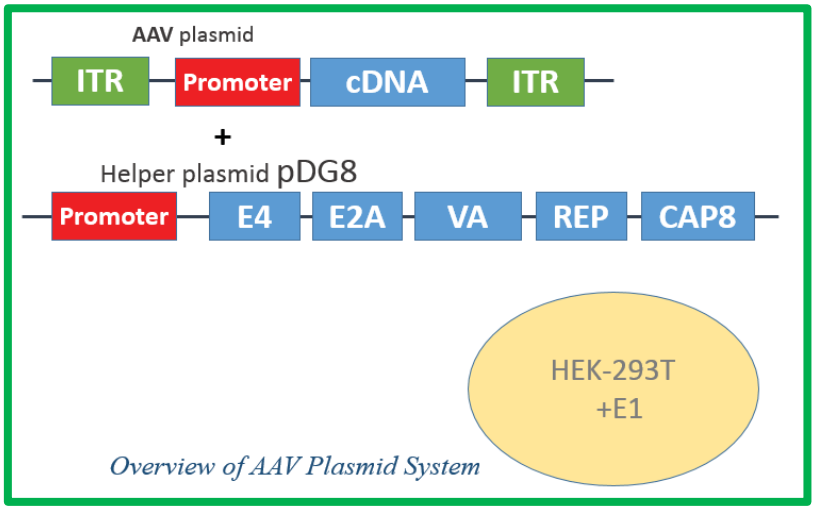


Figure 6. Overview of AAV Plasmid System.

The AAV plasmid is flanked by two 145 base inverted terminal repeats (ITRs) and contains promoter with cDNA coding for gene of interest. The helper plasmid pDG8 contains genes from adenovirus: REP and CAP8 required for the AAV life cycle and viral encapsulation. The E4, E2A and VA genes mediate AAV replication. The AAV plasmid and pDG8 plasmid are transfected into HEK 293T cells, which contain the adenovirus gene E1+, to produce infectious AAV particles.

Preparation of HEK-293T cells for transfection

HEK 293T cells (available from ATTC) were maintained in complete medium (4.5 g/L Glucose and 2 mM L- Glutamine containing DMEM supplemented with 10% FBS) and incubated at 37°C, 5% CO_2. The HEK 293T cells were seeded into sixteen 100 mm plates in 9 ml of complete medium for each AAV construction. The cells were approximately 70-80% confluent on the day of transfection.

Co-transfection of two plasmids

The transfection mixture included 8.6 µg of pDG8, 2.9 µg of AAVGOI, 46 µl of PEI and 580 µl of DMEM serum free, was vortexed 15 times for 1 second, and incubated at room temperature for 10 min. The mixture (625 µl in total per plate) was then added to each 100 mm plate in a dropwise manner. The plates were returned to the incubator for 72 hours at 37°C, 5% CO_2.

Virus Harvesting

The transfected cells were harvested 72 hours post-transfection. The medium was discarded and the cells with 2-3 ml of added new media without serum were gently detached with cell scrapper to prevent cells lysis. The cells were harvested by centrifugation at 1140 g for 10 min at 4°C, the medium was discarded. The cells were than lysed in 4.5 ml of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.5) by three freeze-thaw cycles in dry ice-ethanol and 37°C baths. The mixture was treated with 1 M $MgCl_2$ and Benzonase nuclease 250 units (Sigma) for each ml of lysate followed by 45 min of incubation at 37°C. The lysate was clarified by centrifugation at 6000 g for 20 min at 4°C to pellet cell debris. The virus-containing supernatant was considered as crude lysate.

Centrifugation and virus retrieval

To retrieve the AAV particules the iodixanol gradients was used (Optiprep Density Gradient Medium, Sigma). The iodixanol gradient was prepared in a 5 ml Beckman ultracentrfuge tube as shown in schema (Table 1). The 40% and 25% iodixanol layer are used to remove contaminants with lower densities including empty capsids and a 60% layer serves as a cushion for genome-containing recombinant AAV virions. Tubes were sealed and centrifuged in a Type 70 Ti rotor (Beckman) at 33,000 rpm for 3 hours at 4°C. To retrieve the virus, the tube was then secured in a clamp stand set to eye-level, after which an 18 G 11/2 needle was inserted just below the interface of the 40% and 60% iodixanol layers with the bevel of the needle up. Approximately ¾ of the 40% layer containing the AAV particules was aspirated.

Iodixanol gradient solutions

	25%	*40%**	*60%*
Optiprep 60 (mL)	50	68	100
5X PBS-MK (mL)	24	20	-
Phenol Red 0.5%	300 uL	-	250 uL
H_2O (mL)	46	12	-
Total (mL)	120	100	100

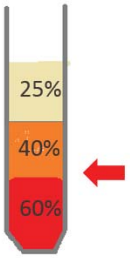


Table 1. Preparation of iodixanol gradient solutions.

Dialysis and virus concentration

The extracted virus was prediluted with vehicle (PBS 0.001% Tween) and added to a clean concentrator tube Amicon Ultra-15 100 kDa (Fisher) followed by a 1-5 min centrifugation at 1500 g. When most of the solution had sufficiently passed through the filter, the concentrator was reloaded with 10 ml of vehicle and spun down to 1 ml. Washing and spinning was repeated 3 more times. After the final wash, the solution was aliquoted and stored at −80°C.

Determination of virus titer

The vector genome copies number (VG) was determined by qPCR using iTaq Universal SYBR Green Supermix (BioRad). qPCR was run in an Mx3000P real-time thermal cycler (Agilent) with primers for the ITRs common to AAV transfer vector plasmids: forward primer 5′-GGA ACC CCT AGT GAT GGA GTT-3′ and reverse primer 5′-CGG CCT CAG TGA GCG A-3′; set with a program: 95°C 10 secs, then cycled 40 times at 95°C for 5 secs, 60°C for 30 secs. To generate a standard curve, a pAAV*CRLF1/CLCF1* plasmid was used in serial dilutions from 1×10^7 to 1×10^3 genome copies, performed in triplicate. A no-template negative control was also performed in triplicate.

2.2.2 Animal treatment

LDLR$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Three groups of male mice, composed of four animals each, at 6 weeks old were injected with AAV8*CRLF1/CLCF1*, AAV8*FcCLCF1* or AAV8*CTR* virus at a titer of 1×10^{12} viral genome particles / per mouse via tail vein. The injection (100-300 µl) was done once at the beginning of the experience (day "0"). The mice were fed with high-fat, high cholesterol containing diet (HFHC) (Harland Teklad, TD88137). HFHC diet was provided from the first day of injection and maintained for the entire study period (12 weeks). Such diet, commonly used to induce metabolic abnormalities and atherosclerosis is composed by 42% calories as fat (21.2 by weight) (256, 257). Of the total fat, saturated fatty acids comprise >60% by weight. The carbohydrate component is 34% (by weight) is derived from sucrose. This diet contains 0.2% cholesterol (0.15% added, 0.05% from fat source). All mice were housed in an animal facility with a 12:12-h light-dark cycle and constant temperature (22-24˚C). The mice had free access to water and

diet. All procedures were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the Montreal Heart Institute Animal Care Committee.

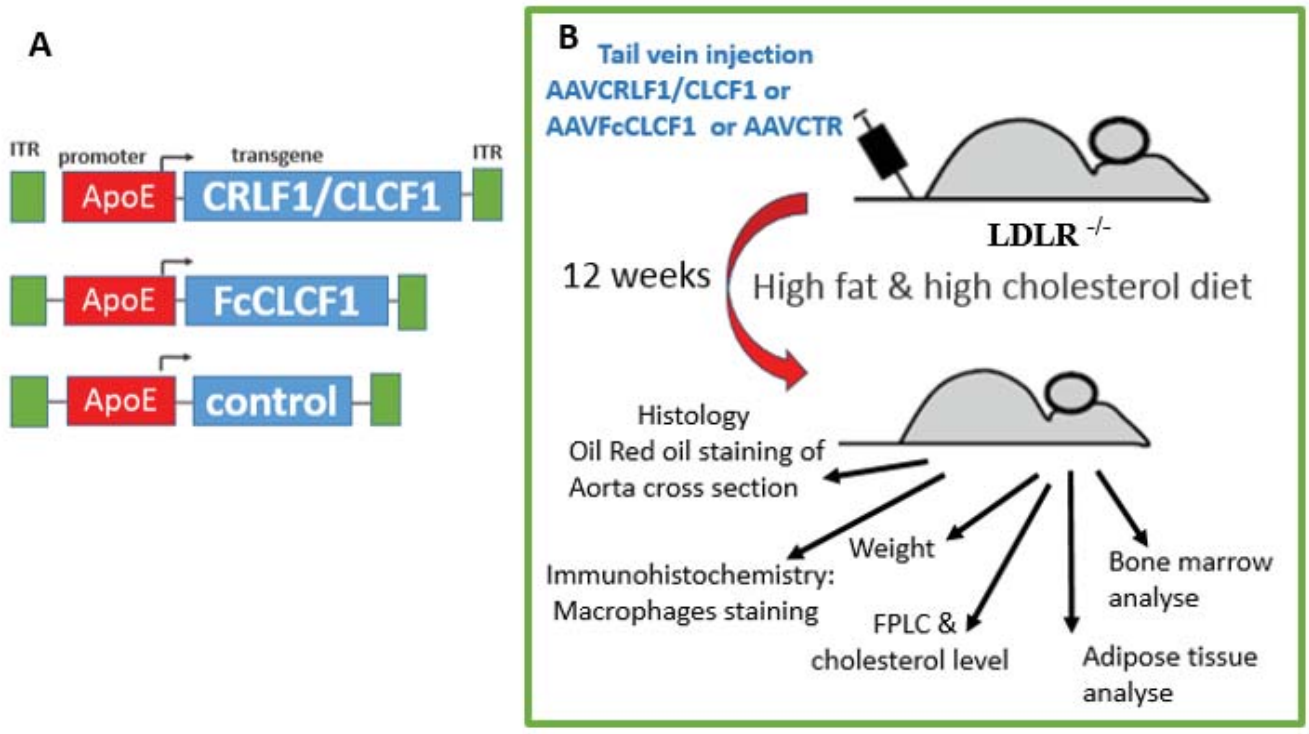


Figure 7. Animal experimental overview.

A, The structure of the AAV8 virus vectors. **B,** The overall structure of the study. The experimental details are provided in the «Materials and Methods» section.

2.2.3 Experimental setup

Mice legs (for bone marrow extraction), liver, spleen, aortas, hearts and adipose tissue (visceral and subcutaneous) were harvested and either immediately processed for flow cytometry analysis, or fixed in 10% formalin, or frozen in liquid nitrogen for further analysis, as described below.

2.2.3.1 Bone marrow derived macrophages and spleen cells isolation

To generate bone marrow derived macrophages (BMDM), bone marrow cells from femurs and tibias were obtained using aseptic techniques. Marrow cores were flushed into sterile plates using syringes fit with 26G needles and filled with RPMI 1640/10% FBS. Cells were filtered through a 70 μm Nylon cell strainer to remove solid fragments, washed several times with RPMI 1640/10% FBS, centrifuged and the collected pellet was dissolved and cultured in RPMI 1640 supplemented with 1% penicillin/streptomycin, 1% HEPES, 0.001% β-mercaptoethanol, 10% FBS and M-CSF 100 ng/ml (PeproTech). The M-CSF is needed to promote differentiation of bone marrow cells into macrophages (7–10 days). The cells were cultured in 6-well plate at a density of 0.4×10^6 cells/ml. On day 4, the floating, non-adherent cells were discarded, but remaining attached cells were refed with medium containing M-CSF again for 3 extra days.

Spleens were dissociated and passed through 70 μm Nylon cell strainer to obtain single cell suspensions. Following red cell lysis by RBC buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.4), the cells were suspended in FACS buffer (PBS with 0.2% BSA).

2.2.3.2 Flow cytometry cell staining

For spleen cells and bone marrow cells staining, all were first incubated with anti-mouse FcR antibody (CD16/CD32, BD Bioscience) for 15 min at 4°C in FACS buffer, then were surface stained using the following fluorescently labeled antibodies: CD11b (clone M1/70, PE, eBioscience), CD11c (clone N418, FITC, eBioscience), Ly6C (clone HK 1.41, PerCP-Cyanine 5.5), F4/80 (clone BM 8, Alexa Fluor 488, eBioscience), CD19 (clone 1D3, APC, BD bioscience), CD3 (clone 17A2, PE, BD Bioscience). Cells were gated based on their forward and side scatter properties. For analysis of myeloid cell population, they were gating on $Ly6C^+$ cells and F4/80 cells were measured from CD11b positive cell population. For absolute counting cells 123 count ebeads (eBioscience) were used. All data collection and sorting were performed using BD FACS Diva (BD Bioscience). Data analyses were done using FlowJo Software (Tree Star, Ashland, OR, USA).

2.2.3.3 Analysis of plasma

After 8 h fasting period blood was collected into heparin-containing tubes by cardiac puncture and plasma was obtained following centrifugation at 2400 g for 10 minutes. The mice $LDLR^{-/-}$ plasma (100 µl per each mouse) was fractionated by fast protein liquid chromatography (FPLC) using on Superose 6^{TM} HR 10/30 column at the Lipid and Lipid Metabolite Analysis Core Facility (University of Alberta, Edmonton, AB). Total plasma cholesterol and cholesterol in lipoprotein fractions were measured using a Wako kit Cholesterol E (Wako), according to manufacturer's instructions.

2.2.3.4 Atherosclerotic lesion analysis

After 12 weeks HFHC diet, animals were euthanized and the hearts were harvested, fixed, and embedded in paraffin. Serial 6-µm cross-sections of the entire aortic valve area were prepared and stained with hematoxylin-phloxine-saffron (HPS) for histological analysis; ten sections unstained were kept for immunohistofluorescence (immunofluorescent macrophage staining). Four sections of each specimen, containing 3 valve segments each, were analysed at 50-µm intervals to determine the average lesion surface. In addition, aortic arch and descending aorta (thoracic part) were isolated and fixed in 10% formalin. The vessels were cleaned of adherent fat, and then stained for lipids using Oil Red O for "en face" morphometry of the atherosclerotic lesion area. The "en face" aorta is a longitudinally opened from iliac artery and proceed along the curvature of the aortic arch. This procedure allows you to explore the endothelium along the entire length of the aorta and visualize intact atherosclerotic lesions.

Immunofluorescence staining of macrophages within atherosclerotic plaques was performed using monoclonal antibody rat MAC-2 (clone M3/38, 1:1000, CEDARLANE), secondary antibody anti-Rat Alexa Fluor 555 (1:500, Thermo Fisher). Sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Antigen retrieval was performed by steam heating slides in citrate buffer (pH 6.0) in a steamer for 20 minutes at 98˚C. Pictures were taken with an Olympus B45 microscope and visualized for quantification using ImagePro Plus 7.0 software.

2.2.3.5 Adipose tissue morphometry

Perigonadal adipose tissue and back skin of the animal for analysing the subcutaneous adipose tissue was collected in 10% formalin, fixed overnight, and embedded in paraffin. Hematoxylin and eosin staining was used for adipocyte morphometry. The 10X objective images were taken by Olympus B45 microscope and visualized for quantification adipocyte area using ImagePro Plus 7.0 software.

2.2.3.6 Validation of efficient hepatic cells transduction by AAV8

The integrity of total RNA samples, isolated from frozen liver tissue using Ribozol (Amresco), was verified by agarose gel electrophoresis or using an Agilent 2100 BioAnalyser. cDNA was prepared using iScript cDNA Synthesis Kit according the manufacturer's instructions (BioRad). Quantitative RT-PCR was performed in an Mx3000P real-time thermal cycler (Agilent) using iTaq Universal SYBR Green Supermix (BioRad). For each gene of interest, dissociation curves were performed to ensure unique PCR product. Arbitrary units were determined from PCR duplicates for each sample using the hypoxanthine guanine phosphoribosyl transferase (HPRT) as a normalizer.

The used oligonucleotides sequences were:

mFcCLCF1-
(5'CTCAACAGAACCGGCGATCCTGG,5'CCAGGCTTCTCAGCTGGTGTTCC);
mCRLF1/CLCF1-
(5'GCAGAAGAGGCGCTGCTAGAGGA;5'CAAGAATCGCCGGCTCTCAGGTC);
HPRT- (5'-TCATTATGCCGAGGATTTGG; 5'-ACAGAGGGCCACAATGTGAT).

2.2.3.7 Statistical Analysis

The data are repoted as mean ± S.D. Statistical analyses were performed using GraphPad Prism (version 7). Data were compared with Student T-test for unpaired comparison or a one-way ANOVA followed by *post hoc* comparisons Bonferroni or Dunnett's test for mice weight gain. The P value < 0.05 was considered significant.

Chapter 3: Results

3.1 *In vitro* assay

3.1.1 Biological activity of recombinant FcCLCF1 protein

Recombinant FcCLCF1 (rFcCLCF1) protein was produced in eukaryotic cells in order to be validated as a more stable form of CLCF1 protein for *in vivo* tests. The rFcCLCF1 induced proliferation of Ba/F3 CNTFR cells at the same extent as rCLCF1. Proliferation was not observed with CLCF1 mutated for site 1 (Figure 8). Thus, the FcCLCF1 can be considered as a functional form of CLCF1, biologically active and can be used in *in vivo* assays. However, the stability (the extended half-long live of cytokine *in vivo*) was not assessed. We were basing on the work of Kin-Ming Lo, who engineered a pharmacologically superior form of leptin for treatment of obesity by ligating the cytokine with constant heavy chain of IgG and extended the circulating half-life of leptin from a few minutes to many hours for FcLeptin with enhanced pharmacological properties (258).

3.1.2 CLCF1 and foam cells formation

The *in vitro* study was focused on foam cell formation, which are strongly associated with atherosclerosis. For this reason, RAW 264.7 macrophages cell line and primary bone marrow derived macrophages were used. To induce foam cell formation, RAW 264.7 murine macrophages or macrophages derived from bone marrow (BMDM), which were stimulated for 7 to 10 days with M-CSF (macrophages colony stimulating factor) were exposed to acLDL and cholesterol for 24 hours. Acetylated LDL is a modified form of LDL which binds to scavenger receptors (CD36 and SRAI/II) and induces foam cell formation. Cells engulf excessive lipids becoming foam cells, which have abundant lipid droplets in their cytoplasm. We found that foam cells (macrophages loaded with acLDL+cholesterol) treated with CLCF1 displayed a significant decrease in area of intracellular lipid droplets compared to the cells exposed to acLDL+ cholesterol alone, as detected and quantified by Bodipy on confocal images (Figure 9A and C). Quantification of lipid droplets of unstimulated and CLCF1 stimulated macrophages

which were not pre-treated with acLDL+cholesterol showed no difference (Figure 9B). Then, the process of uptake of modified lipoprotein by macrophages was investigated. For this purpose, macrophages were loaded with acLDL labeled with fluorescent cholesterol (Bodipy cholesterol). The uptake or influx of labeled acLDL with Bodipy cholesterol was assessed by flow cytometry. The quantification uptake of fluorescent acLDL-cholesterol by primary macrophages derived from bone marrow revealed that co-treatment with CLCF1 significantly decreased uptake of lipids indicating that CLCF1 may impair acLDL-induced foam cell formation (Figure 10A). As the negative control for the uptake of acLDL, bone marrow derived macrophages of mice double knock out $Pcsk9^{-/-}CD36^{-/-}$ were used, which exhibit less uptake of acLDL-cholesterol; as CD36 scavenger receptor is required for the uptake of modified lipoproteins, such as acLDL. However, the same experiment on RAW 264.7 macrophages demonstrated inverse results (Figure 10B). Moreover, acLDL-cholesterol uptake by RAW 264.7 macrophages was performed several times, each time the results were variable. It can be explained by variable subtype of macrophages with opposed action, as macrophages type M1 and M2 (see description at chapter I). Macrophages are cells with great plasticity and versatility depending on the microenvironment signals. BMDM or RAW 264.7 macrophages are considered as M0 macrophages and different signals can change their polarisation either toward M1 or M2 macrophages. For example, they can change their phenotype depending on the surface roughness of plate, cells passage number or plate density (259, 260).

3.1.3 CLCF1 and adipocyte differentiation

As mentioned in Chapter I, adipocytes express CNTFR (82) and mice injected with recombinant CLCF1 showed reduction in body weight (32). Therefore, we tested whether CLCF1 alters adipocyte differentiation *in vitro*. Thus, 3T3-L1 preadipocyte differentiation was analysed in the presence of CLCF1. Among the first assays on preadipocytes differentiation, when 100 ng/ml of CLCF1 was added during whole period of differentiation, it was very clear that CLCF1 was slowing down the lipids accumulation and adipocyte formation, but none of the tests was possible to perform since after inducing the differentiation, the medium in the dishes becomes very viscous and cell layers become very unstable. The fact that CLCF1 was added at the beginning of differentiation, cells continued to proliferate which caused their detachment

making impossible to analyse cells at the end of the differentiation protocol. After multiple attempts, such as using more adherent plastic for cells plating, and adjusting the washing procedure, we could not prevent cell detachment. In this case, to assess lipid accumulation in adipocytes, different protocols were used such as CLCF1 stimulation at different time points with shorter period of stimulation. As shown in Figure 11A-B, the fact of adding 100 ng/ml of CLCF1 once with induction medium at the beginning of differentiation protocol or with medium containing insulin, adipocytes accumulate less triglyceride as determined by AdipoRed staining; once this reagent is added in a hydrophobic environment, it becomes fluorescent. To investigate the mechanism of decreased lipid accumulation, the expression of CD36 was estimated. The CD36-scavenger receptor contributes to adipose TG storage, by binding long-chain fatty acids and facilitate their transport into cells (261). As shown by Western Blotting in Figure 11C, the CD36 expression decreased after the treatment of fully differentiated adipocytes with CLCF1 in a concentration-dependent manner.

3.2 *In vivo* assay

3.2.1 Validation of efficient liver transduction by adeno-associated virus serotype 8 (AAV8)

To determine the liver expression and plasma levels of CRLF1/CLCF1 or FcCLCF1 after adenovirus-mediated gene transfer into 6 weeks old $LDLR^{-/-}$ mice following tail vein injection at day "0", plasma was taken after 14 days and 1-month post-injection from mandibular vein (~20 µl) to confirm efficient transduction and protein secretion in blood circulation. However, it was impossible to detect the proteins in plasma by Western Blot, or by immunoprecipitation due to the lack of sensitivity of the Abs (data not shown). Then we decided to go with more sensitive ELISA test as it has a detection range around 15-1000 pg/ml. Unfortunately, no CLCF1 was detected in mice plasma by ELISA (Figure 12B). Most likely the ELISA test was not sufficiently sensitive to permit measurement of CLCF1 in mice plasma. The group of VJ Savin (Renal Research Laboratory, Kansas City, USA) faced the same problem while working with focal segmental glomerulosclerosis (FSGS) patients. CLCF1 was identified as a potential plasma permeability factor in those patients (262). They could determine the plasma

concentration of CLCF1 only using immunocapillary electrophoresis (ICE) (263). This technique is linear across concentrations from 200 to 10 pg/ml and permits measurement of concentrations as low as 10 pg/ml (263, 264). ICE technology is recognized as a powerful tool for quantification of ultra-low abundance analytes in biological fluids, cells and tissues (264).

Despite the fact, that efficient transduction and protein secretion could not been validated, starting from the 7th week post-transfection with AAV8, the difference in mice weight started to be significant. The qPCR was conducted at the end of treatment to confirm the mRNA expression in the liver. As shown in Figure 12A, injection with AAV8 led to persistence of the viral genome until the time when mice were sacrificed. The level of CRLF1/CLCF1 or FcCLCF1 mRNA expression was high but variable between mice: from 1500 to 5000-fold changes in gene expression in comparison with control mice.

3.2.2 Body and organ weight

The mice were followed up during 12 weeks of treatment. At the day "0" of injection, they had different weight (14-21 gr) (Figure 13A). After 1-month post-injection, the difference in body weight became distinguishable and at week "7" significant. The mice group expressing CRLF1/CLCF1 or FcCLCF1 had increased body weight by 10-13% than control group. Such tendency persisted until the end of the treatment and there was no difference between CRLF1/CLCF1 group and FcCLCF1 group (Figure 13A). As shown in Figure 13B, this difference was not due to increased caloric intake. At the end of the treatment period, once the mice were sacrificed, the visceral organs were examined for the presence of hyperplasia which could cause increased body mass. To assess subsequent reasons which might be one of the existent cause of such difference, the adiposity was taken into consideration also. As shown in Figure 13C, there was no significant difference in liver weight. However, the spleen of CRLF1/CLCF1 group was much bigger than control mice group (the medium difference approximately 2 mg), confirming the well-known effect of CLCF1 as B-cell stimulating cytokine causing spleen hyperplasia (26) (Figure 13D). Unexpectedly, the FcCLCF1 had no such action (Figure 13D). This suggests that the Fc fragment made the cytokine inaccessible to the receptor through which CLCF1 stimulates spleenocytes.

The visceral (perigonadal) and subcutaneous adipose tissue were investigated for histological appearance and adipocytes size. It can be clearly noticed on converted binary images (Figure 14B-F) that the adipocytes from visceral tissue are significantly bigger in mice group expressing CRLF1/CLCF1 than control mice which can explain the difference in mice weight. However, there was no difference in subcutaneous adipose tissue (Figure 15). The FcCLCF1 adipose tissue was not tested.

3.2.3 Quantification of atherosclerosis lesions

To compare the degree of atherosclerotic lesion formation in the three groups of CRLF1/CLCF1, FcCLCF1 and control (CTR) mice, the standard atherogenic diet was fed to the $LDLR^{-/-}$ mice for 12 weeks to induce the atherosclerotic lesions. The mice were euthanized to perform cardiac puncture and blood collection for plasma analyses. The aortas, from ascending part to the thoracic aorta were cut, cleaned from periaortic fat, and vessels were longitudinally sectioned and stained with Oil Red O, in order to detect lipid deposition. As shown in Figure 16A, the "en face" aorta staining show that all three mice groups developed atherosclerotic lesions throughout all sectioned aorta. The measurement of plaque area did not confirm our hypothesis, that mice expressing CLCF1 would have less atherosclerosis plaque.

Furthermore, despite the nonsignificant difference between control and treated groups as it is shown in Figure 16A, the observed tendency is that expression of either CRLF1/CLCF1 or FcCLCF1 increases the lesions area. The histological analysis of aortic sinus plaque area did not give us more clear information, either (Figure 16C-D). All three layers of aortic valve should be visualised on histological cuts to have good and reliable results of plaque area. Unfortunately, due to a small number of mice per group and a limited quality of histological aortic valve cuts, it was impossible to obtain clear results and to make any conclusion. The quantification of percentage of macrophages in plaque area gave us big variation within all three groups, making the results nonsignificant (Figure 16E-F).

3.2.4 Effects of CLCF1 expression on plasma lipids

To address whether CLCF1 could influence lipoprotein metabolism, the mice plasma was fractionated by fast protein liquid chromatography (FPLC) in order to verify if CRLF1/CLCF1 or FcCLCF1 expression can change the lipoprotein cholesterol distribution in atherosclerotic mice model. Taking into consideration small mice group (4 per group), instead of using the pool, we individually subjected plasma of each mouse to FPLC. Proteins content of eluted fractions was determined at 280 nm and graphical presentation of FPLC peaks, which represents a separated compound, as VLDL, LDL and HDL, is presented at Figure 17A. The protein level can be visualised also by separating them by apoB et non-apoB fractions, since the major protein constituent of VLDL and LDL is apoB and major protein component of HDL is apoA. The analysis of each mouse from each group showed that there was no difference between groups (Figure 17B). The cholesterol level was measured in each fraction, so three peaks can be observed on the graph (Figure 17C). Those peaks correspond to VLDL, LDL, HDL cholesterol profile. To obtain clearer results, the cholesterol level was divided into apoB et non apoB fractions, in other words: “bad” (apoB) and “good” (nonapoB or HDL) cholesterol. There was a significant difference in cholesterol level of apoB fraction (VLDL+LDL) (Fig 17D) between control and treated groups, suggesting contribution of CRLF1/CLCF1 and FcCLCF1 on atherosclerosis development. The measurement of total cholesterol level in plasma was also performed. Despite nonsignificant difference in total plasma cholesterol, revealed while examine each mouse individually, there was some variation within each group, but still it was possible to observe the same tendency: the total cholesterol of mice expressing CRLF1/CLCF1 and FcCLCF1 was higher than in control group (fig 17E). Those findings correlate with the same pattern of protein distribution in apoB fractions (fig 17B) between groups (higher apoB protein level in treated groups), but still do not reach statistical significance because of a big variation within each group of mice.

3.2.5 Analysis of bone marrow and spleen cells population by flow cytometry

Fresh bone marrow and spleen cells were analysed for total cell number in tissue and absolute or percentage of each stained cell population. After the exclusion of doublets, dead cells and debris, different immune cell population were identified using the marker for B-cells (CD19+), T-cells (CD3+) and several myeloid lineage markers sorting them on dendritic cells (CD11c+) and macrophages (CD11b+), by gating them on Ly6C positive cell population.

We determined the percentage and absolute number of B cells (CD19+), T cells (CD3+). As expected, mice expressing CRLF1/CLCF1 had bigger total cell counts, B-cells increased in absolute and percentage number in spleen, confirming B-cell stimulating effect of CLCF1 in periphery (Figure 18A-B). Surprisingly, FcCLCF1 had only a slight increase of B-cell percentage, but not in absolute number (Figure 18A-B).

Afterwards, the detailed analysis of myeloid lineage was performed by gating them on Ly6C positive, antigen expressing on macrophages, dendritic cells, granulocytes; we also identified activated macrophages in inflammatory tissues. The analyses revealed that CRLF1/CLCF1 treatment decreased the population of Ly6C positive cells in spleen (Figure 18D), but not in bone marrow (Figure 19C). By using CD11b and CD11c markers, we were able to separate macrophages and dendritic cells. CRLF1/CLCF1 increased absolute number of CD11b positive cells in spleen (Figure 18F). No significant differences were determined in bone marrow cell population (Figure 19).

Chapter 4: Discussion

CLCF1 is a neurotrophic factor, a body weight regulator and an immunomodulator (26, 27, 32). CLCF1 activates the tripartite ciliary neurotrophic factor receptor (CNTFR), comprising CNTFRα, gp130 and LIFRβ (46). CLCF1 and CNTF share signaling pathways, suggesting that the extensive pre-clinical and clinical investigations conducted on CNTF might also be indicative of the therapeutic potential of CLCF1. CNTF derivative was tested in clinical trials in which significant weight loss was observed, indicating that activation of CNTFR can regulate food intake and metabolism (23). The manifestations of genes mutations in *CRLF1* and *CLCF1,* resulting in Crisponi and CISS syndromes, suggest that CLCF1 can also regulate food intake. Crisponi syndrome patients tend to suffer from high grade fever episodes and most die during the first year of life, which support the role of CLCF1 in the regulation of metabolism.

Our work was aimed on revealing whether CLCF1 could have effect on atherosclerosis development since the sortilin and LIFR are expressed by myeloid cells. Sortilin is implicated in uptake of LDL and foam cell formation; LIF, a ligand for LIFR, is a potent inducer of anti-inflammatory M2 macrophages differentiation (50, 252, 253).

For this purpose, two different constructs for CLCF1 *in vivo* expression were designed: first construct code for CLCF1 with its soluble receptor CRLF1; second construct code for CLCF1 fused with Fc fragment of IgG. This approach allows to engineer the FcCLCF1 protein with significant therapeutic potential and a number of additional beneficial biological and pharmacological properties. Perhaps most important, the presence of the Fc domain markedly increases their plasma half-life, which prolongs therapeutic activity, owing to its interaction with the neonatal Fc-receptor (FcRn) (265), as well as to the slower renal clearance for larger sized molecules (266).

The latest data regarding the new CRLF1 separate functions and alternative partners led us to incorporate soluble receptor CRLF1 in the construct with CLCF1. Since, CRLF1 could have its distinct function in the periphery and FcCLCF1 has not been tested for enhanced stability *in vivo* yet*,* it was decided to test and to compare the *in vivo* effects of both constructs.

To address all our objectives, mice $LDLR^{-/-}$, were on HFHC diet, were transduced with AAV8 for CRLF1/CLCF1 or FcCLCF1 expression for 12 weeks. To confirm efficient liver

transfection and construct expression, mice plasma was used for ELISA test. Unfortunately, neither CRLF1/CLCF1 nor FcCLCF1 was detected in plasma. However, the gene expression by liver was confirmed by qPCR. The level of CRLF1/CLCF1 and FcCLCF1 expression was high and variable within treatment groups: from 1500 to 5000-fold changes in gene expression.

Most likely, ELISA test was not sufficiently sensitive to permit measurement of CLCF1 in mice plasma, since mice started to show difference in weight after 7 weeks post-transfection and spleen hyperplasia was observed at the end of the treatment. The group of VJ Savin (Renal Research Laboratory, Kansas City, USA) faced the same problem while working with focal segmental glomerulosclerosis (FSGS) patients. CLCF1 was identified as a potential plasma permeability factor in those patients (262). They could determine the plasma concentration of CLCF1 only using immunocapillary electrophoresis (263). This technique is more sensitive than ELISA test and it is recognized as a powerful tool for quantification of ultra-low abundance analytes in biological fluids, cells and tissues (264).

CLCF1 targets immune peripheral cells. However, they do not express chain α of CNTFR, suggesting existence of the second receptor (58) or CLCF1 can form a complex with soluble CNTFR (CNTFRs) (45). The multiple dose treatment revealed that CLCF1 reduced mice body weight by 8% and stimulated B cells, resulting in hyperplasia of B cell areas of secondary lymphoid organs and augmented B cell count in mice (32).

In mice $LDLR^{-/-}$, 12 weeks expression of CRLF1/CLCF1 confirmed B cell stimulating capability. Moreover, the expression of FcCLC unexpectedly did not show considerable B cell stimulating effect. The absolute total number of spleen cells and B cell number in mice expressing CRLF1/CLCF1 was 30% higher than in control mice. Mice expressing FcCLCF1 showed slight, but statistically significant increase in percentage of spleen B cells. Mice expressing CRLF1/CLCF1 expressed both CLCF1 and CRLF1. Probably, the considerable B cell stimulating effect was obtained due to CRLF1; as it was shown in recent publication that complex CRLF1/CLCF1 represent more potent signal transducer as CLCF1 alone. Larsen showed that via complex formation with CRLF1, CLCF1 gains a higher affinity for CNTFRα which enhances its accumulation on the cell membrane and formation of the tripartite signaling complex (CLCF1/CRLF1/CNTFRα) and, hence, its ability to promote engagement of gp130/LIFRβ for signaling. The magnitude of induced signaling was measured by determining

the resulting level of phosphorylated STAT3 (pSTAT3). It was shown that CLCF1/CRLF1 induced a three times stronger signal than CLCF1 alone (43).

Besides B cells, CRLF1/CLCF1expression in mice LDLR$^{-/-}$ increased the absolute number of CD11b positive cells in spleen, suggesting influence on myeloid lineage cells in spleen, including macrophages, neutrophils, DC.

Regarding our observations on atherosclerosis development, the results showed no impact of CLCF1 expression on disease development. However, i would not consider the results of plaque quantification reliable, as there was a lot of nonspecific staining of surrounding fat. Moreover, some parts of aortic arch, where usually the biggest accumulation of plaque lesion is observed, were lost. Unfortunately, due to a small number of mice per group and a limited quality of histological aortic valve cuts, it was impossible to obtain cumulative results. Nevertheless, we could observe some distinct effects of CLCF1 on the level of plasma lipoproteins, notably on apoB-containing lipoproteins (VLDL and LDL). Interestingly, sortilin is expressed in the liver and plays role in the lipoprotein metabolism. However, the role of sortilin in VLDL secretion is controversial (267), and inconsistencies have been summarized (238, 268). Some studies suggest, that sortilin serves as a chaperone where sortilin is hypothesized to facilitate secretion of VLDL (55). Other studies demonstrate, that sortilin inhibits VLDL secretion by trafficking towards degradation (53). A number of reviews were published (269-272) proposing a variety of explanations for the discrepant findings: total body *Sort1* deficiency versus liver-specific manipulations, the genetic background used in each study, adenovirus versus adeno-associated virus, western-type diet versus chow diet, and the nature of the knockout mouse model itself, though no conclusions were reached.

CRLF1/CLCF1 is secreted via the classical secretory pathway, and it can so far not be excluded that sortilin, once its propeptide has been cleaved, can target and transport this cytokine in Golgi compartments as well as at the cell surface (273, 274). In such case, it could be insufficient sortilin available for another ligand, as VLDL or/and LDL, since our constructs were expressed in the liver. Thus, if we presume, that apoB-containing lipoproteins are less subjected to the trafficking towards degradation, as CRLF1/CLCF1 is expressed in the liver and binds sortilin in the secretory pathways, the liver will secrete more VLDL in the blood circulation or degrade less VLDL. In such case, the dyslipidemia in mice expressing CLCF1 could be more pronounced than in control mice. VLDL are lipoprotein rich in TG, deliver FA

to the heart, skeletal muscle and adipose tissue for energy expenditure and storage. Adipose tissue is taken up the liberated FA and re-synthesized into TG within the cytoplasm. Thus, the gain in body weight and increased adipocyte size volume in our *in vivo* experiment can be assumed from higher level of apoB-containing lipoprotein rich in TG and increased fat storage.

CLCF1 is a member of large family which share gp130 as a common signal transducer in their receptor complex and typically activate STAT3. Various laboratories have conducted studies to demonstrate that gp130 cytokines can modulate adipocyte development and function (275). However, the difference in gp130 cytokine activity can be partially attributed to the use of additional receptor components that are utilized by each family member, including the LIF receptor and the cytokine's specific α receptor (72). Both CNTF and CLCF1 utilize a signaling complex, including CNTFRα, gp130, and LIFR for signal transduction. CNTFR is expressed in the region of the brain controlling energy balance, and in adipose tissues (82). A CNTFR derivative was tested in clinical trials in which significant weight loss was observed (23), indicating that activation of the CNTFR can regulate food intake and metabolism. Treatment of wild type mice with recombinant CLCF1 resulted in body weight loss, as well (32). *In vitro* experiments with 3T3-L1 cell line showed significant decrease in TG accumulation and inhibition of adipocytes differentiation during CLCF1 stimulation. Though, the CLCF1 expression during 12 weeks in $LDLR^{-/-}$ mice model demonstrated completely inverse effect on body adiposity and weight. Mice expressing CRLF1/CLCF1 and FcCLCF1 showed increased body weight by ~14 % and increased adiposity in visceral adipose tissue (the histological test was not performed for mice expressing FcCLCF1). Such discordance can be explained by the fact that CLCF1 interacts with lipoprotein, and such interaction abrogate the biological activity of CLCF1. Same deduction was made in the lab of Dr Gauchat; it was revealed that CLCF1 co-purifies with lipoproteins in plasma and VLDL-CLCF1 and LDL-CLCF1 binding was confirmed using proximity and ligand blots assays. The physiological relevance of that finding was demonstrated in a mouse model of oxygen-induced retinopathy, were the beneficial anti-angiogenic properties of CLCF1 were abrogated when co-administrated with VLDL (Article submitted for publication). Thus, it can be presumed that in the current *in vivo* test, CLCF1 and FcCLCF1 complexed with lipoproteins in the blood circulation of dyslipidemic mice, behaved as "lipocytokine" and modulated their biological activity by abrogating its role in the regulation

of body weight. However, the B cell stimulating effect in mice expressing CRLF1/CLCF1 was very significant, but this effect was excluded in mice expressing FcCLCF1. This fact can be explained by the expression of CRLF1 in mice CRLF1/CLCF1. As it was shown, CRLF1 could have its own distinct function or, as it was mentioned before, CRLF1 facilitate CLCF1 signalling and induce three times stronger phosphorylation of STAT3 than CLCF1 alone (43).

Chapter 5: Conclusion

Though, the role and mechanisms underlying the impact of CLCF1 on atherosclerosis development by targeting macrophages have not been elucidated. However, the expression of CLCF1 in $LDLR^{-/-}$ mice model under hypercholesterolemic condition, revealed potential function on lipoprotein metabolism and body adiposity state. Additional study from the laboratory of Dr. Gauchat provides evidence of changing biological activity of CLCF1 under hypercholesterolemic conditions. It can be assumed that opposite effect on $LDLR^{-/-}$ mice body weight was due to formation a complex of CLCF1 with plasma lipoproteins. Further studies are needed to examine and confirm the hypothesis of "lipocytokine". In such case, it would be valuable to repeat the same experiment *in vivo* on $LDLR^{-/-}$ mice model, but using the construct of CRLF1/CLCF1 for gene expression since expression of both CRLF1 and CLCF1 had better B cell stimulating effect and probably would have effect on myeloid cells, as we observed increased CD11b positive cells in spleen; but we did not remark any difference in atherosclerotic plaque, very likely because of small experimental group. It would be relevant to investigate the role of CLCF1 on progression of atherosclerosis by using the same mice model, but with some changes of protocol: AAV8 injections for CRLF1/CLCF1 expression should be performed after atherosclerotic plaque formation. In addition, mice could be transferred from HFHC diet to a normal low-fat diet to prevent complex formation of CLCF1 with elevated level of lipoproteins and avoid the lost of biological action on body weight.

It is important to confirm the modulation of CLCF1 activity by formation a "lipocytokine", by *in vitro* experiments on the differentiation of adipocytes by stimulating with VLDL+/- CLCF1.

For a greater understanding, the role of CLCF1 on lipoprotein metabolism by targeting sortilin, a series experiments *in vitro*, could be performed. The HEK293 cell line is well-suited for that purpose, as they have only a minor endogenous expression of CNTFRα and sorLA (sortilin partner), are easy to transfect (expression of both construct of sortilin and CLCF1 can be performed), express gp130/LIFRβ, and have a large cell body, which is suited for immunocytochemistry (276). Either, any hepatic cell line, as Huh7, Hep G2, can be used, but in that case, it is not necessary to transfect with sortilin, as there is endogenous expression in hepatic cells. Finally, to validate FcCLCF1 construct (as a more stable form of CLCF1) with enhanced biological and pharmacokinetical properties, it would be better to use WT mice model.

Taken together, it is important to understand the action of CLCF 1 on adipose tissue, lipoprotein metabolism and define the mechanism; the new role of CRLF1 on CLCF1 signalisation and biological impact on immune peripheral cells. It may provide valuable information in the potential development of novel therapeutic strategies of metabolic disorders.

Chapter 6: Figures

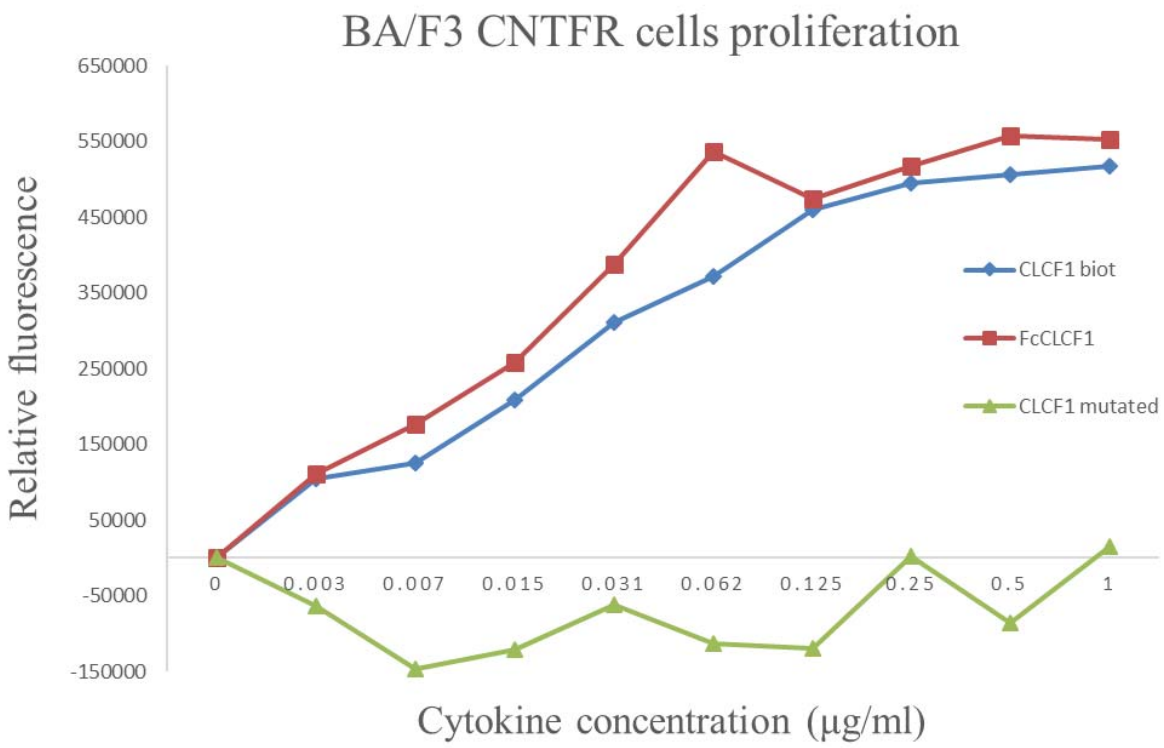


Figure 8. Proliferation assay of Ba/F3 CNTFR cells with increasing concentrations of cytokines.

The transfected Ba/F3 CNTFR cells were incubated for 72 h in triplicate with increased proteins concentrations (from 0 to 1 µg/ml). Proliferation was measured using a fluorometric assay (Alamar Blue, AbD Serotec, Cedarlane). The CLCF1 mutated for site 1 was used as negative control. The CLCF1 biotinylated as positive control. Mean of unstimulated cells fluorescence background was sustracted from the values obtained with stimulated cells. Wallac 1420 VICTOR2^{TM} system was used to measure fluorescence at wavelengths of 530/590 nm.

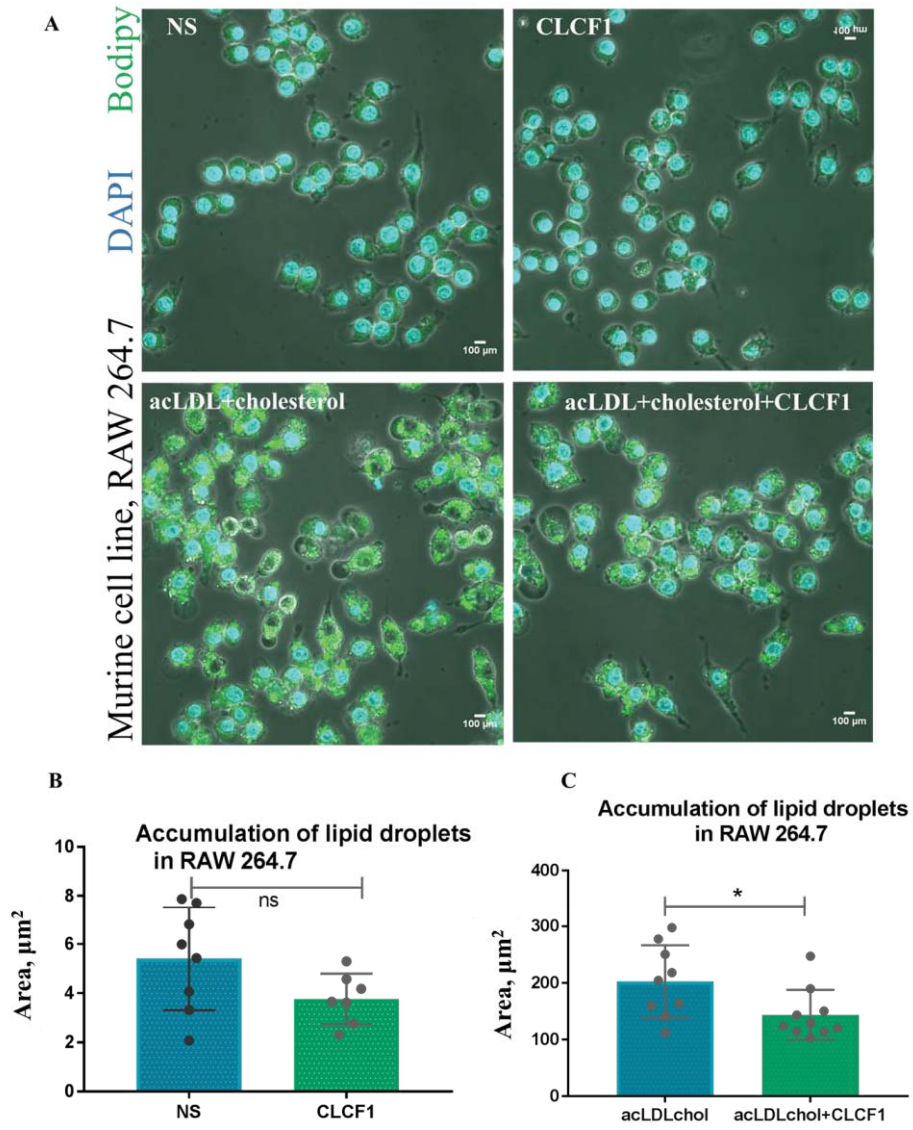


Figure 9. CLCF1 and foam cell formation.

A, Confocal images of fixed RAW264.7 macrophages and stained with Bodipy 493/503 nm (lipid droplets, green) and DAPI (nucleus, blue). The macrophages were loaded with 100 µg/ml acLDL+ 5 µM cholesterol +/- 100 ng/ml CLCF1 in DMEM 0% FBS, equilibrated for 4 h in DMEM 0.2% BSA; NS (non-stimulated cells). **B**, Quantification of unloaded macrophages; NS (non-stimulated cells). **C**, Quantification of loaded macrophages. Fluorescent area of confocal images was quantified by Image J and represent 7-10 selected regions. Scale bar represents 100µm. The Student T-test was used; * P value is < 0.05.

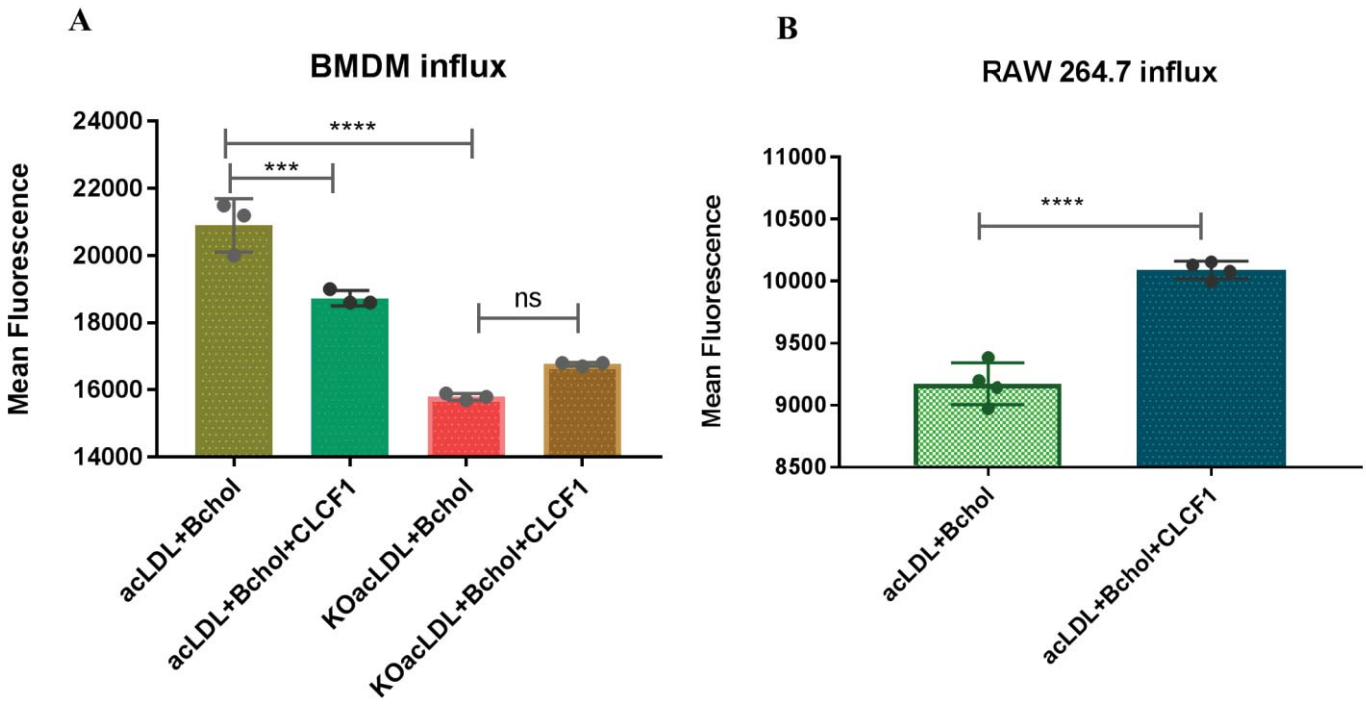


Figure 10. The assessment of cholesterol influx in cultured macrophages.

A, The bone marrow derived macrophages (BMDM) or RAW264.7 macrophages (**B**) were incubated for 24 h with 100 µg/ml acLDL labelled with 5 µM Bodipy-cholesterol -/+ 100 ng/ml CLCF1 in 0% FBS RPMI medium, equilibrated for 4 h (RPMI+0.2% of BSA). The mean fluorescence was measured by flow cytometry. As negative control for influx, BMDM from mice double knock out ($PCSK9^{-/-}CD36^{-/-}$) was used. The one-way ANOVA or Student T-test was used; *** P value < 0.001; **** P value < 0.0001.

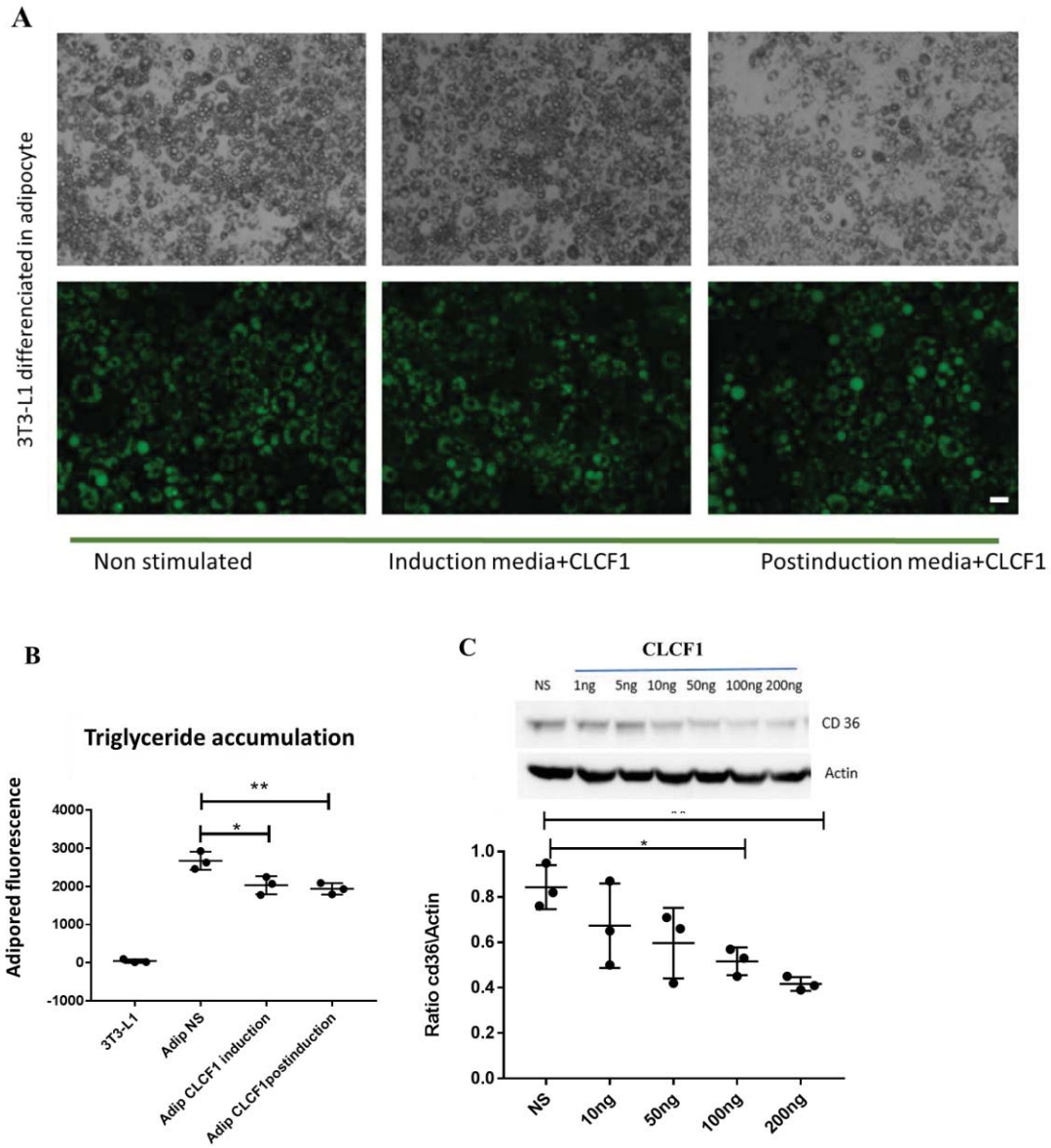


Figure 11. CLCF1 decrease adipocyte differentiation *in vitro.*

A, Visualisation of differentiated adipocyte in white and black images, and with green-open channel. The triglyceride accumulation was assessed by AdipoRed Assay Reagent (Lonza) and (**B**) fluorescence was measured at wavelengths 485/ 572 nm. 100 ng/ml CLCF1 was added with induction medium or post-induction (with insulin medium). **C**, Quantification of Western Blot. Completely differentiated adipocytes were treated with increasing concentrations of CLCF1 for 48 h and the ratio CD36/Actin was quantified. These experiments were repeated 3 times. Scale bar represents 150 µm; The one-way ANOVA test was performed; * P value < 0.05; ** P value < 0.01.

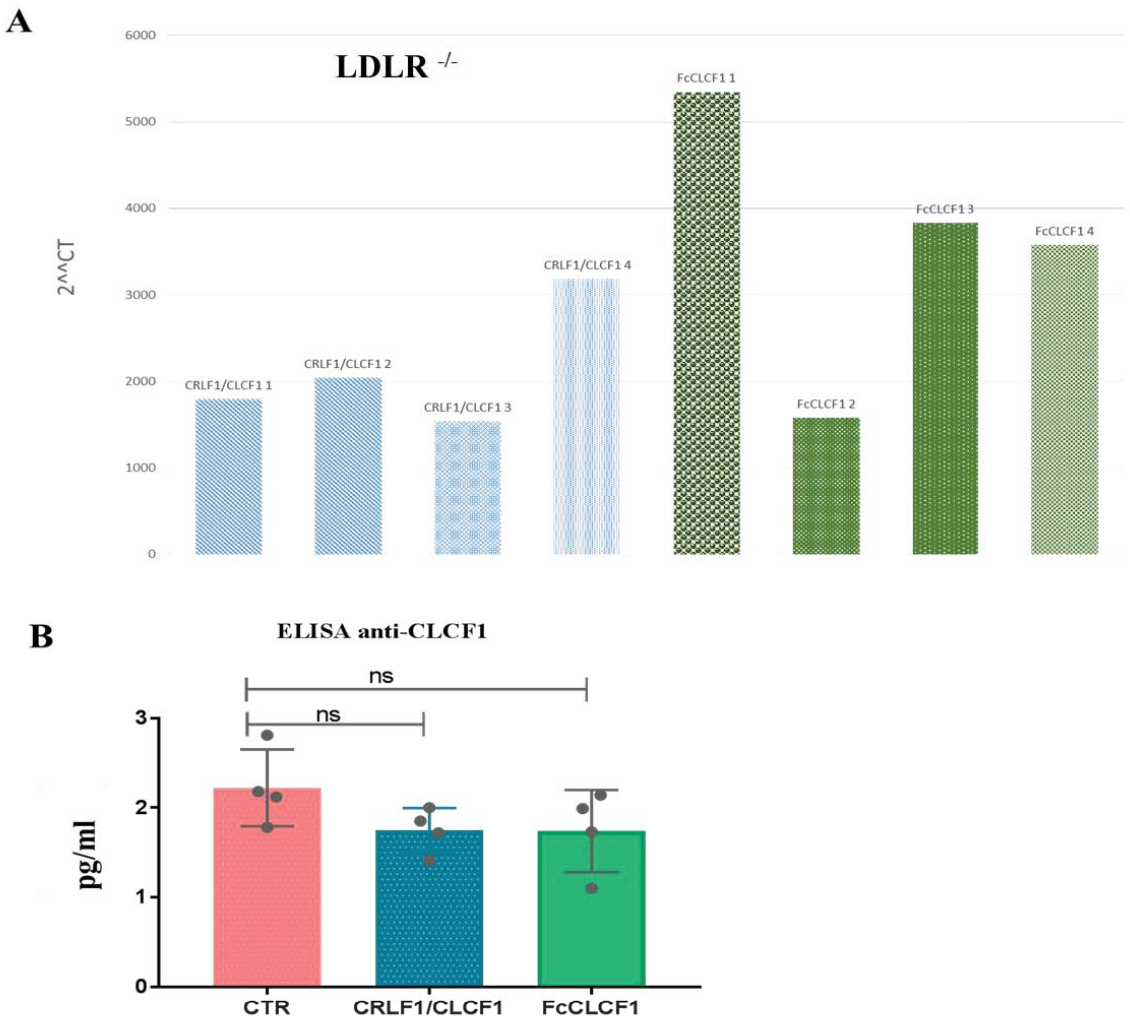


Figure 12. Validation of construct expression.

Adeno-associated virus 8 (AAV8) vectors either containing no gene, CRLFCLCF1 cDNA or FcCLCF1 cDNA were administered via intracaudal vein injection at day "0" to target liver transduction at a titer of $1*10^{12}$ viral genome particles/per mouse. **A,** The CRLFCLCF1 and FcCLCF1 expression in the liver of each mouse was quantified using RT-qPCR. Data were normalised relative to HPRT internal gene expression. The $2^{\wedge\wedge CT}$ value represents the fold changes in gene expression, it was calculated using following equation: 2^- (CT GOI-CT HPRT) - (CT Control-CT HPRT). **B,** Protein detection in mice plasma by antimouse CLCF1 antibody using sandwich ELISA assay (LSBio, Inc). P value was calculated using one-way ANOVA. P value-ns (non-significant).

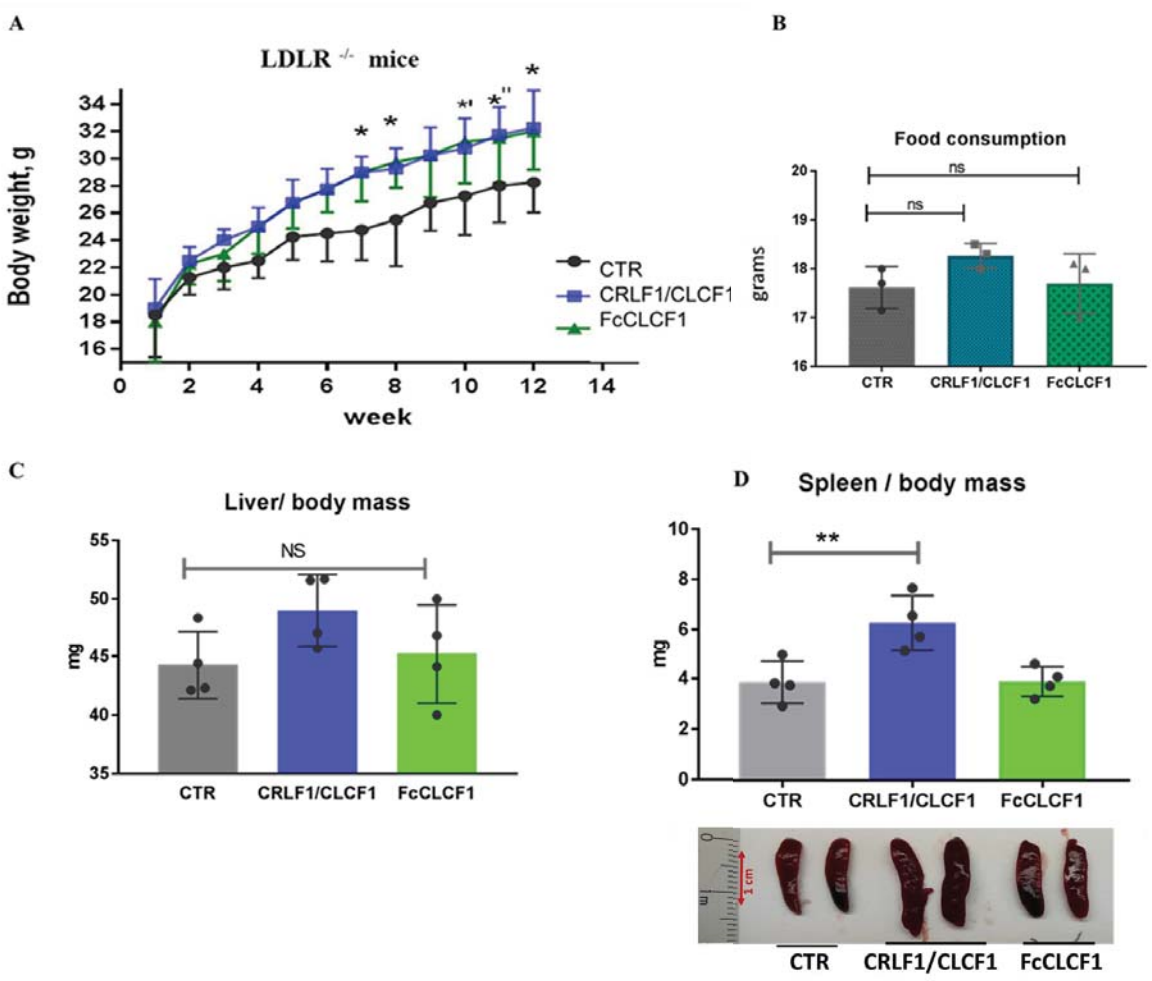


Figure 13. Effect of CLCF1 on diet-induced weight gain and body organs weight.

Six weeks old *Ldlr*$^{-/-}$ mice transfected with AAV8CRLFCLCF1, AAV8FcCLCF1 or AAV8CTR (n=4 per group) were placed on a HFHC diet for 12 weeks. **A,** Body weight gain was measured weekly during 12 weeks of HFHC diet. **B**, The measurement of food intake starting from week 7 of HFHC diet. Represent the mean of food intake for 4 weeks. **C**, Liver/body mass ratio. **D**, Spleen/body mass ratio. Visualised spleen dimension. Scale bar represents 1 cm. P value was calculated using one-way ANOVA, P value-ns (non-significant); ** P value < 0.01. Error bar shows S.D. For body weight gain was used two-way ANOVA with Dunnett's multiple comparisons test within each week; *' and *'' for CRLFCLCF1 or FcCLCF1 group, respectively; * P value <0.05.

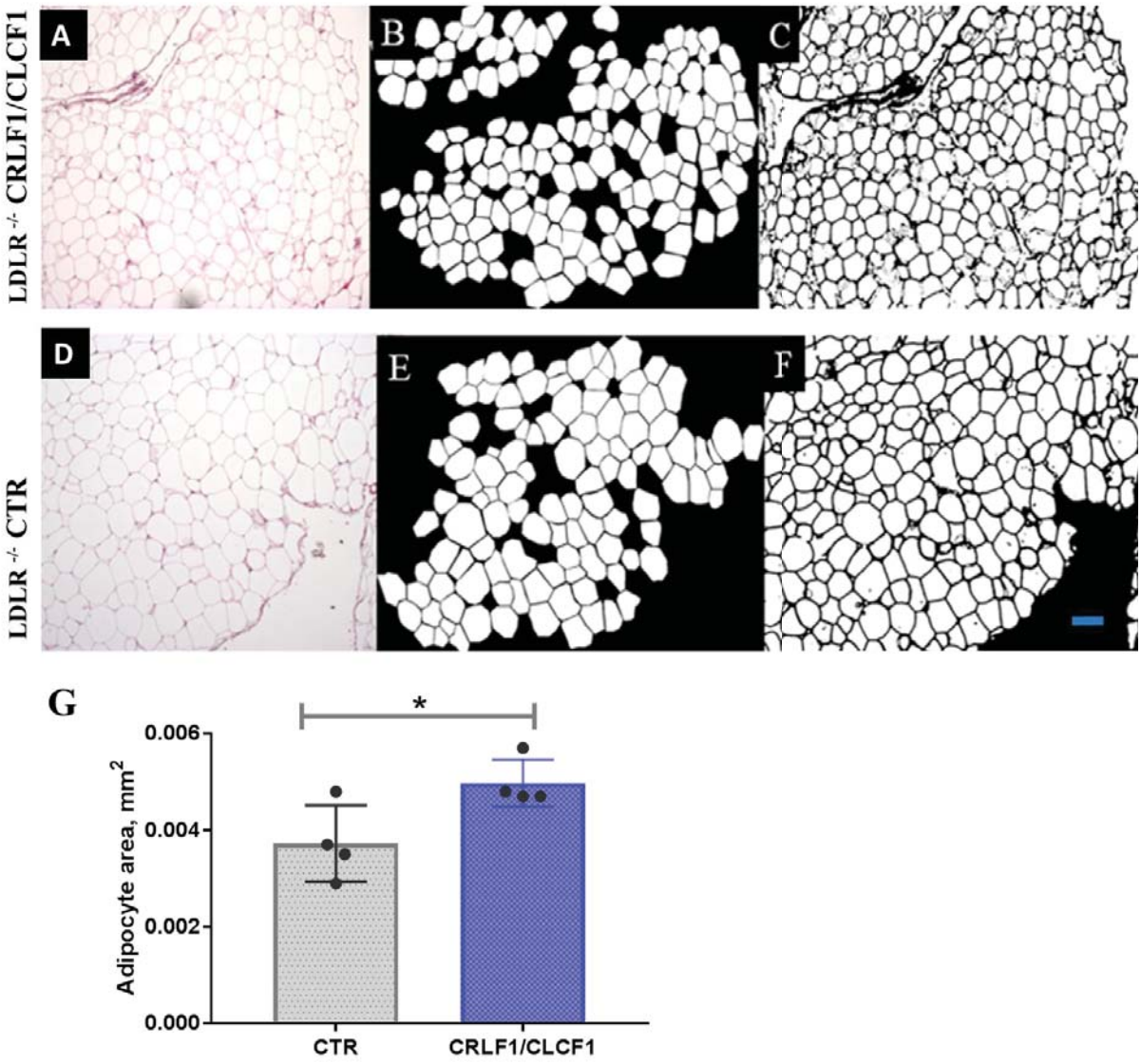


Figure 14. Effect of CLCF1 on visceral adiposity.

Manuel adipocyte size measurement. Conversion of histological images into binary representation. **A** and **D**, H&E stained sections of adipose visceral tissue. **C** and **F**, Computer-generated binary images of A and D. **B** and **E**, Binary images with visualised and quantified cells with exclusion of unclear or/and broken cells. Images captured at 10X magnification. Pictures were taken with an Olympus B45 microscope and analysed by ImagePro Plus 7.0 software. Bar represent 100 µm. **G**, Mean surface area of adipocytes. n = four mouse per group. > 250 cells were measured for each mouse. P value was calculated using Student T-test, * P value <0.05.

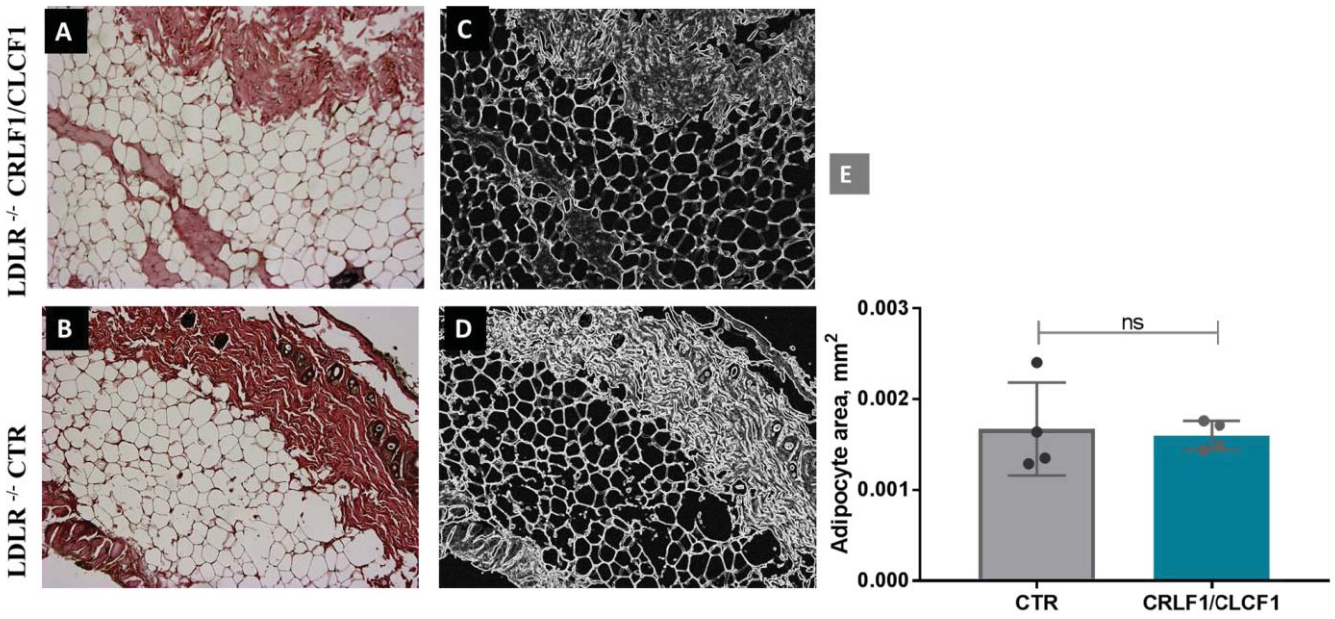


Figure 15. Effect of CLCF1 on subcutaneous adiposity.

Manuel adipocyte size measurement. Conversion of histological images into binary representation. **A** and **B**, H&E stained sections of adipose subcutaneous tissue. **C** and **D**, Computer-generated binary images of A and D. Images captured at 10X magnification. Pictures were taken with an Olympus B45 microscope and analysed by ImagePro Plus 7.0 software. Bar represent 100 μm. **G**, Mean surface area of adipocytes. n = four mice per group. 100-150 cells were measured for each mouse. P value was calculated using Student T-test. P value-ns (non-significant).

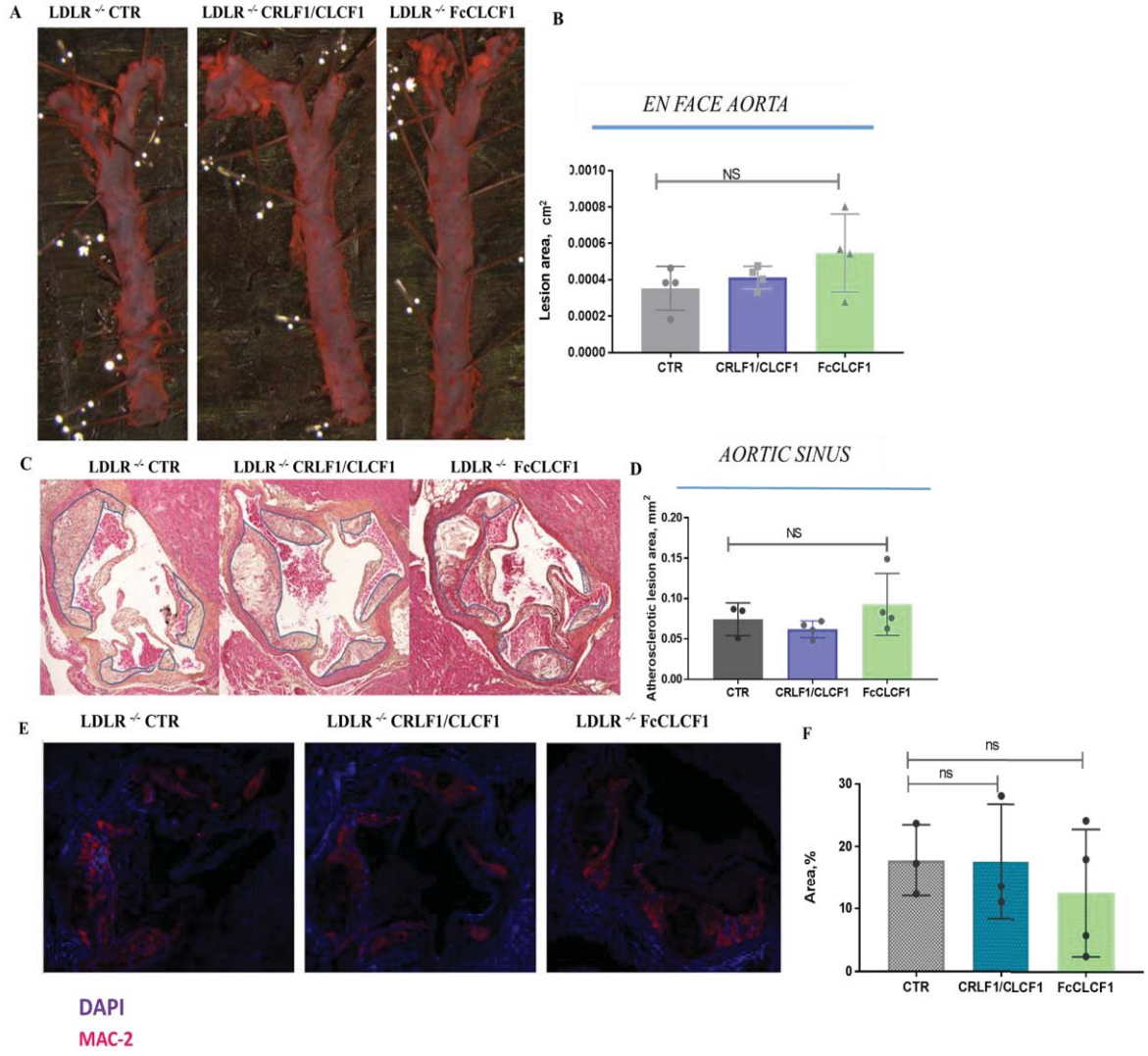


Figure 16. Effect of CLCF1 on atherosclerotic lesion development.

Six-weeks old LDLR$^{-/-}$ mice transfected with AAV8CRLFCLCF1, AAV8FcCLCF1 or AAV8CTR (n=4 per group) were placed on a HFHC diet for 12 weeks. **A**, Arch and thoracic aortas were dissected and tissues harvested. Aortas were stained with Oil Red O. **B**, Quantification of atherosclerotic lesions on aorta. **C**, Sections from cardiac-aortic junctions were sectioned and stained with H&E to quantify atherosclerotic lesion area, **D** (defined by blue lines). **E,** Cardiac-aortic sections were stained with anti MAC-2 (pink, CEDARLANE) antibody to detect macrophages in lesion and DAPI (blue). **F**, Quantification of percentage of MAC-2 positive cells per total lesion area. Pictures were taken with an Olympus B45 microscope. For quantification cardiac-aortic junctions, four sections per mice was analysed by ImagePro Plus 7.0 software. P value was calculated with ordinary one-way ANOVA test. P value-ns (non-significant). Error bar shows S.D.

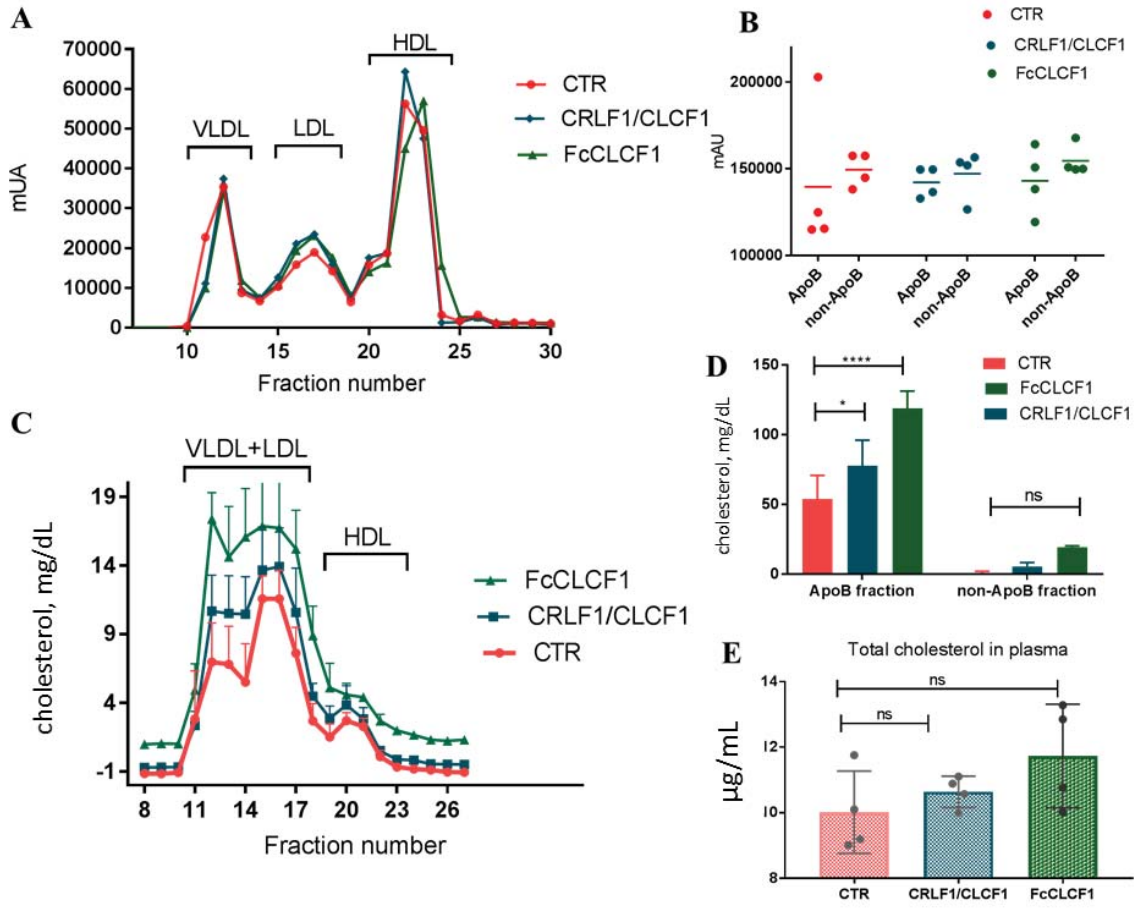


Figure 17. Effect of CLCF1 on lipoprotein metabolism.

Six-weeks old LDLR$^{-/-}$ mice transfected with AAV8CRLFCLCF1, AAV8FcCLCF1 or AAV8CTR (n=4 per group) were placed on a HFHC diet for 12 weeks. After 8 hours starving, mice were sacrificed and plasma was isolated by centrifugation and samples were analysed individually. Plasma samples for each mouse (four per group) were run on FPLC using Superose 6HR 10/column in order to obtain 30 fractions. **A**, Protein content of eluted fractions was determined at 280 nm. **C**, Eluted fractions were tested for cholesterol level, from which fractions 10 to 27 were used to calculate protein (**B**) and cholesterol level (**D**) in ApoB (10 to 19 fraction) et non-ApoB fractions (20-27). **E**, Individual plasma samples were analysed for total cholesterol level. P value was calculated with two-way ANOVA, * P value < 0.05; **** P value < 0.0001. P value-ns (non-significant). Error bar shows S.D.; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein. ApoB fraction include VLDL and LDL peaks; non-ApoB fraction include HDL peak.

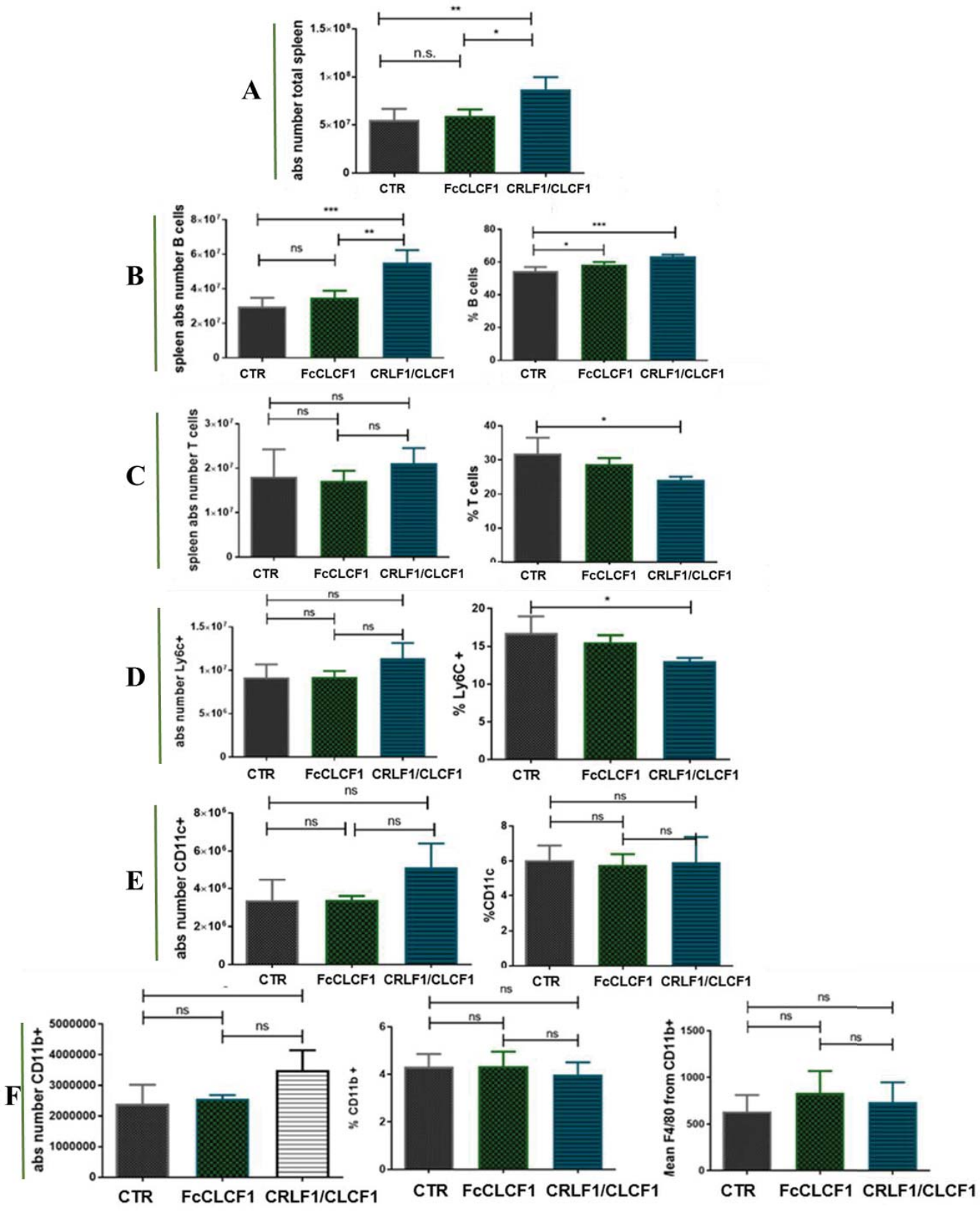
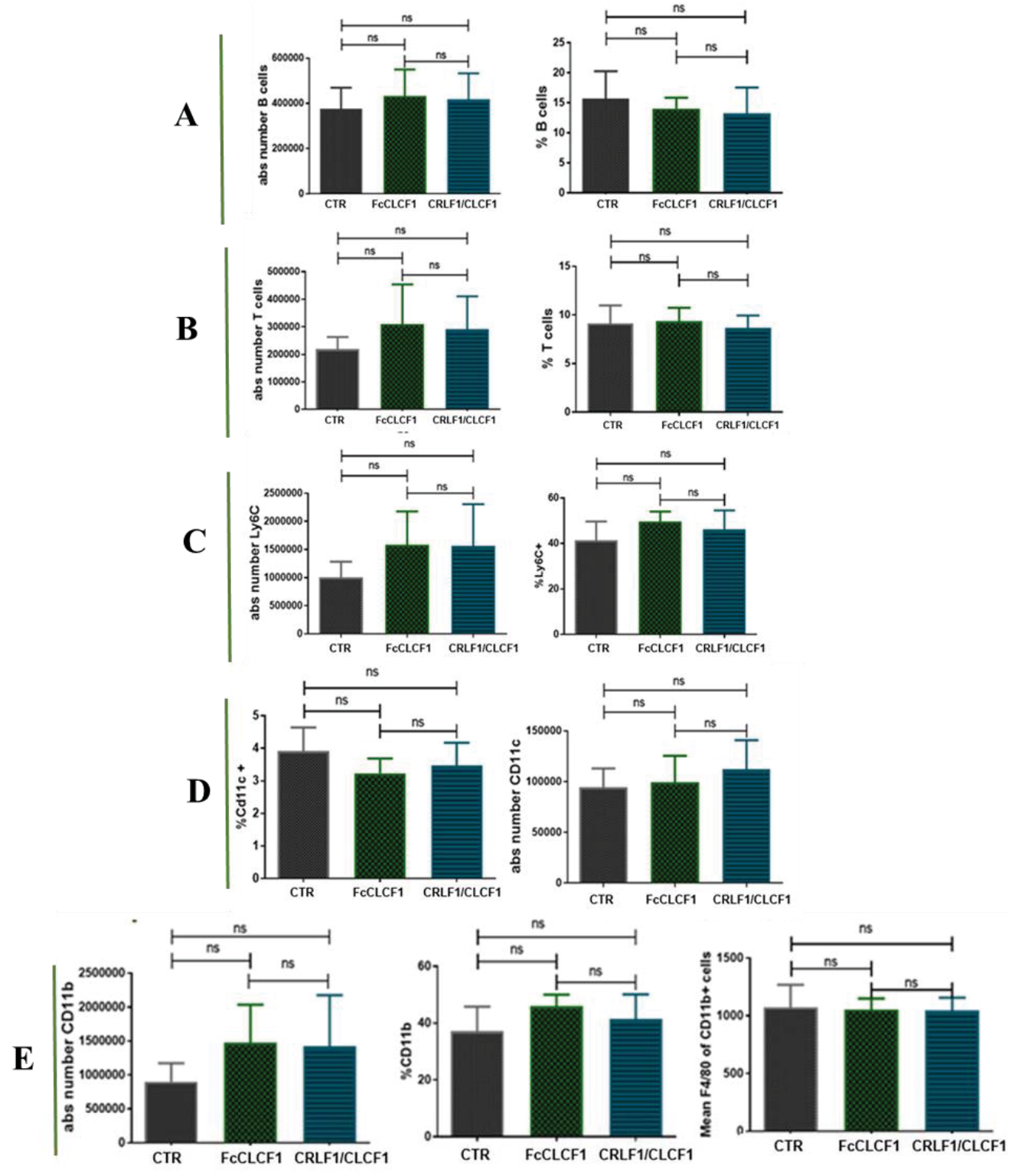

A
abs number total spleen
n.s.
CTR
FcCLCF1
CRLF1/CLCF1
B
spleen abs number B cells
% B cells
C
spleen abs number T cells
% T cells
D
abs number Ly6c+
% Ly6C +
E
abs number CD11c+
%CD11c
F
abs number CD11b+
% CD11b +
Mean F4/80 from CD11b+

Figure 18. Effect of CRLFCLCF1 and FcCLCF1 expression on $LDLR^{-/-}$ mice spleen cells population measured by flow cytometry.

A, Absolute number of total spleen cells population**. B,** B cell percentage and absolute number in spleen. **C,** T cell percentage and absolute number in spleen. **D,** Absolute number and percentage of Ly6C+ spleen cells**. E,** Absolute number and percentage of CD11c+ spleen cells**. F,** Absolute number, percentage of CD11b+ spleen cells and F4/80+ cell of CD11b+. For absolute cells counting 123 count ebeads (eBioscience) were used. P value was calculated using one-way ANOVA test. * $p< 0.05$; ** $p< 0.005$; *** $p<0.0005$; p value-ns (non-significant).


A
abs number B cells
600000
400000
200000
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CTR
FcCLCF1
CRLF1/CLCF1
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20
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300000
200000
100000
% T cells
C
abs number Ly6C
2500000
2000000
1500000
1000000
500000
%Ly6C+
60
40
20
D
%Cd11c +
abs number CD11c
150000
100000
50000
E
abs number CD11b
%CD11b
Mean F4/80 of CD11b+ cells
1500
1000
500

Figure 19. Effect of CRLFCLCF1 and FcCLCF1 expression on $LDLR^{-/-}$ mice bone marrow cells population measured by flow cytometry.

A, B cell percentage and absolute number in bone marrow. **B,** T cell percentage and absolute number of bone marrow cells. **C,** Absolute number and percentage of Ly6C+ bone marrow cells**. D,** Absolute number and percentage of CD11c+ of bone marrow cells**. E,** Absolute number, percentage of CD11b+ bone marrow cells and F4/80+ cell of CD11b+. For absolute cells counting, 123 count ebeads (eBioscience) were used. P value was calculated using one-way ANOVA test. P value-ns (non-significant).

Acknowledgements:

I would like to thank:

- Platform of histology and immunology (Dr. M. Sirois and Dr. J.F. Tanguay), Marie-Élaine Clavet-Lanthier and Cynthia Torok for technical support and ImagePro Plus software analyse;
- Louis Villeneuve for microscopy confocal imaging;
- Teodora Mihalache-Avram for great support and helping in aortas sampling, microscopic imaging analyses;
- Dre. Catherine Martel for her advice and support during all my master internship;
- Annie Demers, PhD for helping in adeno-virus preparations and RT-qPCR;
- Sarah Pasquin, PhD candidate for help with flow cytometry and statistical analyses;
- Audric Moses, M.Sc. operations manager Lipidomics Core Facility from Alberta for FPLC;
- Steve Poirier, PhD for FPLC assistance and scientific inspirations;
- Dr. Tomoya Yamashita and Namrata Chhabra, MD for authorising to use the figure N4 and figure N5;

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