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Using microRNAs to Promote Inflammation Resolution in Cardiovascular Research

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DISSERTAÇÃO

Mestrado em Engenharia Biomédica

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Abstract

Cardiovascular disease is a leading cause of morbidity and mortality in developed countries. It is mainly caused by atherosclerosis, a chronic inflammatory disease that it is characterized by the formation of plaques in the arterial intima between the endothelium and the underlying smooth muscle cells of the media.

Macrophages are the first inflammatory cells to invade atherosclerotic lesions and a main component of atherosclerotic plaques. The emerging understanding of macrophage phenotypes and their functions in the atherosclerotic plaque has led to the consensus that pro-inflammatory M1 macrophages are pro-atherogenic while M2 macrophages may promote plaque stability, primarily though their tissue repair and anti-inflammatory properties. As such, modulating macrophage function to promote plaque stability is an exciting therapeutic prospect.

MicroRNAs are a class of small noncoding RNAs that have gained status as important post-transcriptional regulators of gene expression and are likely involved in key cellular functions, including macrophage polarization. Thus, certain miRNAs will be able to modulate the magnitude of an inflammatory response, by changing expression levels of cytokines, surface receptors and other inflammation-related molecules, which will have consequences for macrophage phenotype.

In this context, the goal of this study was to use miRNAs to reduce M1-like macrophages, promoting inflammation resolution. Also we aimed to evaluate the molecular mechanism underlying such effect and the functional consequences for smooth muscle cell recruitment.

In order to achieve our goal, we polarized primary monocyte-derived macrophages into M1 and M2c phenotypes, by stimulating them with LPS and IL-10 respectively. CD86 and CD206 were used as markers to confirm M1 and M2c polarizations by flow cytometry. Next, we evaluated the expression of microRNA-195 by reverse transcription - real-time quantitative polymerase chain reaction, using Taqman assays. *In silico* predictions for microRNA-195 targets involved in Toll like receptors inflammatory pathways were performed using distinct databases. To analyze the biological effect of microRNA-195 in macrophage phenotype and its effect on the expression of these predicted targets, we performed *in vitro* transfections of microRNA-195 mimics followed by an M1 stimulus with LPS+IFN-γ in THP-1 macrophages. At a functional level, the impact of microRNA-195 expression in THP-1 macrophages was also

evaluated. Migration assays were performed using inserts with PET membranes of 8 μm pore size in which human umbilical artery smooth muscle cells were allowed to migrate for 24 hours over differently stimulated THP-1 macrophages.

The results obtained showed that microRNA-195 was upregulated in macrophages treated with IL-10 compared with LPS treated macrophages and control (untreated) group. *In silico*, TLR2 and several Mitogen Activated Protein Kinases (MAPK) were predicted to be targets of this miRNA. Importantly, further experiments confirmed that TLR2 expression in LPS+IFN-γ stimulated THP-1 macrophages was significantly reduced by the transfection of microRNA-195 mimics compared with scrambled-transfected and non-transfected cells. Moreover, phosphorylated forms of proteins p54 (JNK1), p46 (JNK2/SAPK) and p38 - MAPK that are part of the downstream TLR signaling pathway - were all downregulated by microRNA-195. Functionally, migration assays results revealed that microRNA-195-THP-1 macrophages have significantly less capability to recruit human umbilical artery smooth muscle cells compared with scrambled-transfected or non-transfected pro-inflammatory THP-1 macrophages.

In conclusion, we show that microRNA-195 is involved in macrophage polarization and inhibits the Toll-like receptor inflammatory pathway by reducing the levels of TLR2 and the phosphorylated forms of downstream proteins p54, p46 and p38 in pro-inflammatory conditions. Moreover, at a functional level, microRNA-195 inhibits the macrophage-mediated recruitment of smooth muscle cells.

Thus, we identified microRNA-195 as a new potential anti-inflammatory and anti-atherogenic agent to promote inflammation resolution in cardiovascular research. These results are being prepared as a manuscript, to be submitted for publication.

Keywords: microRNA, macrophages, inflammation, atherosclerosis

Resumo

As doenças cardiovasculares são a maior causa de morbidade e mortalidade nos países desenvolvidos. Estas são principalmente causadas pela aterosclerose, uma doença inflamatória crónica que é caracterizada pela formação de placas na túnica íntima arterial entre o endotélio e as células do músculo liso subjacentes.

Os macrófagos são as primeiras células inflamatórias a invadir as lesões ateroscleróticas e são o maior componente das placas. O conhecimento cada vez mais aprofundado acerca dos diferentes fenótipos dos macrófagos e das suas funções na placa aterosclerótica tem levado ao consenso de que os macrófagos pró-inflamatórios do tipo M1 são pró-aterogénicos, enquanto que os macrófagos do tipo M2 promovem a reparação de tecidos e têm propriedades anti-inflamatórias.

Os microRNAs são uma classe de pequenos RNAs não codificantes que têm ganho relevância como importantes reguladores da expressão dos genes após a sua transcrição e estão envolvidos em funções celulares chave, incluindo na polarização dos macrófagos. Os microRNAs são capazes de modular a magnitude de uma resposta inflamatória afetando os níveis de expressão de citoquinas, de recetores de superfície e de outras moléculas inflamatórias e, deste modo, modificar também o fenótipo dos macrófagos.

O objetivo deste estudo foi utilizar microRNAs para reduzir a polarização de macrófagos para o fenótipo M1, promovendo assim a resolução da inflamação. Em particular, procurámos desvendar os mecanismos moleculares por detrás do efeito observado e também as consequências funcionais, ao nível do recrutamento das células do músculo liso da artéria umbilical.

Para atingir esses objetivos, polarizámos macrófagos derivados de monócitos primários para fenótipos do tipo M1 e M2c, com LPS e IL-10, respetivamente. Os marcadores de superfície CD86 e o CD206 foram utilizados para confirmar a polarização, por citómetria de fluxo. De forma a investigar microRNAs expressos de forma diferenciada nos dois estados de polarização, avaliámos a expressão do microRNA-195 por RT-PCR quantitativo. Utilizando várias bases de dados, realizámos previsões *in silico* de possíveis alvos do microRNA-195 envolvidos nas vias inflamatórias dos recetores *Toll-like*. Para analisarmos o efeito biológico do microRNA-195 nos macrófagos e o seu efeito na expressão destes alvos previstos, realizámos transfecções *in vitro*, em macrófagos THP-1, do microRNA-195 seguidas de um

estímulo pró-inflamatório com LPS+IFN-γ. A nível funcional, o impacto da expressão do microRNA-195 em macrófagos THP-1 foi também avaliado. Neste sentido, foram realizados ensaios de migração usando *inserts* com membranas PET porosas (8 μm) nos quais foram colocadas células humanas do músculo liso da artéria umbilical que migraram durante 24 horas atraídos por macrófagos THP-1 estimulados de diferentes formas.

Os resultados revelaram que o microRNA-195 é sobre-expresso em macrófagos estimulados com IL-10 por comparação com macrófagos estimulados com LPS e com o grupo de controlo (não estimulados). As previsões *in silico* indicaram o TLR2 como possível alvo deste microRNA. A expressão do TLR2 em macrófagos THP-1 estimulados com LPS+IFN-γ foi significativamente reduzida pela transfecção do microRNA-195 por comparação com as células transfectadas com o controlo negativo ou com as células não transfectadas. Além disso, os níveis das proteínas fosforiladas p54 (JNK1), p46 (JNK2/SAPK) e p38, que fazem parte da via de sinalização dos recetores *Toll-like*, foram diminuídos pelo microRNA-195. Os resultados dos ensaios de migração revelaram que os macrófagos THP-1 transfectados com o microRNA-195 têm menos capacidade para recrutar células humanas do músculo liso da artéria umbilical do que os macrófagos THP-1 transfectados com o controlo negativo ou não transfectados.

Em conclusão, mostramos que o microRNA-195 está envolvido na polarização dos macrófagos e na inibição, em condições pró-inflamatórias, da via de sinalização dos recetores *Toll-like*, reduzindo os níveis do TLR2 e das formas fosforiladas das proteínas subjacentes p54, p46 e p38. Para além disso, observamos igualmente um papel anti-aterogénico, uma vez que o microRNA-195 inibe o recrutamento de células do músculo liso por parte dos macrófagos.

Assim, identificamos o microRNA-195 como um novo potencial agente anti-inflamatório e anti-aterogénico para a resolução da inflamação na investigação cardiovascular. Está a ser preparado um manuscrito com os resultados aqui apresentados, para ser submetido para publicação.

Palavras-chave: microRNA, macrófagos, inflamação, aterosclerose

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Abbreviations

BSA	Bovine serum albumin
CD	Cluster of differentiation
CO_2	Carbon dioxide
CVD	Cardiovascular disease
DMEM F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
EC	Endothelial cell
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
HDL	High density lipoprotein
IL	Interleukin
JNK	c-Jun N-terminal kinase
LDL	Low density lipoprotein
LP	Lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
NF-kB	Nuclear factor kappa B
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PEI	Polyethylenimine

PET	Polyethylene terephthalate
PMA	Phorbol 12-myristate 13-acetate
RPMI 1640	Roswell Park Memorial Institute 1640
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SR	Scavenger receptor
RBC	Red blood cell
RT	Room temperature
TLR	Toll-like receptor
VSMC	Vascular smooth muscle cell

Chapter 1

Introduction

1.1 Inflammation in cardiovascular disease

Cardiovascular disease (CVD) remains the leading cause of death among Europeans and around the world [1]. Cerebrovascular accident, acute myocardial infarction and coronary heart disease are the most common forms of CVD. A recent study by Nichols *et al*. reporting the burden of CVD across European countries concluded that, despite recent decrease in mortality rates, CVD is still responsible for over 4 million deaths per year, close to half of all deaths in Europe [1]. Globally, CVD caused 51% of deaths among women and 42% among men [1]. In Portugal, data released by "Direcção Geral de Saúde" shows that nearly 30% of deaths in 2012 were related to circulatory system diseases, which also configures the first cause of death in the country [2].

Atherosclerosis is frequently the underlying pathological feature of CVD. Atherosclerosis is a slowly progressive chronic disease, which may start developing during childhood, and it is characterized by the formation of plaques in the arterial intima, between the endothelium and the underlying smooth muscle cells of the media [3]. These plaques are formed by foam cells, immune cells, vascular endothelial cells (ECs), migrated smooth muscle cells (SMCs), platelets, extracellular matrix, and accumulated modified lipids [4]. Morphologically, advanced plaques are composed of a necrotic core and overlying fibrous cap and those with a relatively large core and thin cap are considered unstable and are vulnerable to rupture [5]. Upon cap rupture, the blood is exposed to thrombogenic material, which leads to vessel occlusion.

Hypertension, diabetes, high levels of cholesterol associated with tobacco use and obesity are considered the major atherosclerotic and CVD risk factors [1]. For that reason the treatment of these diseases may firstly include lifestyle changes and then all the medical and surgical procedures and cardiac rehabilitation. Currently, treatment of atherosclerosis focuses on the relieving symptoms, reducing risk factors in an effort to slow or stop the

buildup of plaque, lowering the risk of blood clots forming, widening or bypassing plaque-clogged arteries and preventing atherosclerosis-related diseases [6]. To slow the progress of plaque buildup, doctors usually prescribe drugs to lower cholesterol levels, blood pressure or to prevent blood clots from forming. Statins are considered the best drugs for lowering LDL cholesterol while blood thinners, as anti-platelets, are used to prevent clots formation, reducing the risk of heart attacks or strokes [6]. In the cases of severe atherosclerosis other medical procedures or surgeries may be necessary. Angioplasty is a procedure that is used to open blocked or narrowed arteries [7]. Angioplasty can improve blood flow to the heart and relieve chest pain. Coronary artery bypass grafting (CABG) is another type of surgery, in which arteries or veins from other areas of the body are used to bypass narrowed coronary arteries. The healthy blood vessel redirects blood around the blocked artery, improving blood flow [8]. Finally, carotid endarterectomy is a surgery to remove plaque buildup from the carotid arteries in the neck, in a procedure which restores blood flow to the brain, helping to prevent a stroke [6].

These are the most common and efficient treatments used to overcome late atherosclerotic lesion problems; however, researchers continue to study the pathology of the lesion in order to better understand which mediators could be used to control the inflammation during the development of the disease. Inflammation plays a role in all stages of atherosclerosis [9]. This disease involves chronic inflammation at every stage, from initiation to progression, and eventually plaque rupture. In atherosclerosis, the normal homeostatic functions of the endothelium are altered, promoting an inflammatory response [10]. Initially, adhesion molecules expressed by inflamed endothelium recruit leukocytes, including monocytes, which then penetrate into the intima, predisposing the vessel wall to lipid accretion or vasculitis [3]. Inflammatory mediators enhance uptake of modified lipoprotein particles and formation of lipid-laden macrophages [11]. T cells also enter the intima and, simultaneously with macrophages, secrete cytokines, which subsequently amplify the inflammatory response and promote the migration and proliferation of intimal smooth muscle cells, promoting fibrous cap formation [12]. Later in the process, inflammatory mediators can weaken the protective fibrous cap of the atheroma, possibly leading to thrombosis and the occurrence of acute coronary syndromes such as myocardial infarction [13]. All these aspects enhance the need to control inflammation in every single step of the disease progression, in order to prevent later problems. Therefore, inflammatory processes may be potential therapeutic targets to prevent or treat atherosclerosis and its subsequent diseases.

1.1.1. Lesion initiation and early progression of atherosclerosis

Although the exact causes of atherosclerosis are still unclear, studies show that it initiates with subendothelial accumulation of apolipoprotein B-containing lipoproteins (apoB-LPs), responsible for carrying lipids, including cholesterol, around the body [14]. The retention is partially mediated by interaction with extracellular matrix (ECM) proteins, primarily proteoglycans [15]. ECM binding makes lipoproteins susceptible to modifications, such as oxidation [16]. This leads to the transiently activation of endothelial cells which secrete chemokines that promote recruitment of circulating monocytes [17].

The adhesion cascade of monocytes involves capture, selectin-dependent rolling, activation by endothelial surface-bound chemokines, integrin-mediated adhesion and transendothelial migration [18]. During this process, several adhesion molecules and chemokines play a crucial role, including E- and P-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and tethered chemokines on the luminal surface of the endothelium [19, 20]. Notably, selectins interact with P-selectin glycoprotein ligand-1 (PSGL-1) expressed on monocytes allowing them to roll on the endothelium. Then, monocytes use lymphocyte function-associated antigen-1 (LFA-1), very late antigen-4 (VLA-4), macrophage-1-antigen (Mac-1) and their respective endothelial cell ligands, including vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), to slow rolling and form tighter adhesions [21]. Monocyte firm adhesion to the endothelium is coordinated by chemokines such as CCL2 and IL-8 [22]. Finally, firm adhesion of monocytes is followed by their entry into the subendothelial space - diapedesis [23]. In the intima, monocytes quickly differentiate into macrophages due to the production of colony stimulating factor (M-CSF) by vascular cells such as endothelial cells (EC) and smooth muscle cells (SMC) [24].

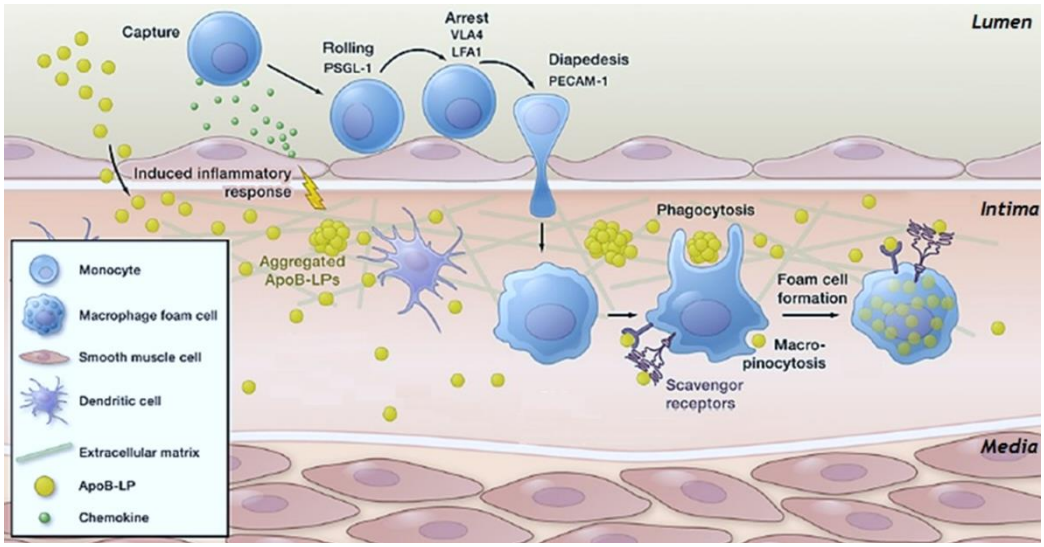


Figure 1- Lipoproteins retention, monocyte recruitment and foam cell formation *(adapted from* [25]*).* Monocytes use different chemokine–chemokine receptor pairs to infiltrate the intima, which is facilitated by endothelial adhesion molecules, including selectins ICAM1 and VCAM1. The recruited monocytes differentiate into macrophages in the intima, where take up native and modified (for example, oxidized) low-density lipoprotein (LDL) via macropinocytosis or scavenger receptor-mediated pathways (including via scavenger receptor A1 (SR-A1) and CD36), which results in the formation of the foam cells that are a hallmark of the atherosclerotic plaque. These foam cells secrete pro-inflammatory cytokines (including interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF)) and chemokines (such as chemokine (C-C motif) ligand 2 (CCL2)) that amplify the inflammatory response.

1.1.2 Macrophages in the pathogenesis of atherosclerosis

- Monocyte differentiation and macrophage polarization

Under homoeostatic conditions, the majority of the organs, including the heart, contain a small population of resident macrophages [26, 27]. Upon a pro-inflammatory injury, an increased number of blood monocytes extravasated into the tissue and differentiate into M1 macrophages (pro-inflammatory). In the following resolution phase, M1 macrophages are replaced by M2 macrophages (pro-resolution, pro-regeneration). Therefore, in response to inflammatory damage monocytes can extravasate into the heart and differentiate into M2 macrophages, thus promoting cardiac repair and remodeling [28, 29].

Monocytes that arrive to atherosclerosis lesions differentiate into macrophages, which then can polarize towards different subsets, following a specific stimuli [30]. The first stage of differentiation is induced by macrophage colony stimulating factor (M-CSF) or granulocyte macrophage colony stimulating factor (GM-CSF), while the subsequent phenotypic polarization that macrophages adopt is dependent on the concentration of the various mediators that these cells are exposed to [24, 31, 32]. Nomenclature in the literature is not consistent, polarized macrophages are generally named as M1 (classically activated) or M2 (alternatively

activated) [33]. The M1 and M2 terms parallel the T helper cell th-1 and th-2 cytokines which drive macrophage polarization [33].

M1 macrophages are induced by the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) or Toll-like receptor (TLR) ligands [34]. The family of TLRs is the major and most extensively studied class of pattern recognition receptors (PRRs) and TLR2 and TLR4 are described as crucial mediators of macrophage activation [35, 36].

TLRs exhibit leucine-rich repeats (LRR) motif in the ectodomain implicated in ligand recognition (such as lipopolysaccharides (LPS) by TLR4 or lipoproteins by TLR2), a transmembrane domain, and a cytoplasmic tail containing the Toll–IL-1-resistence (TIR)-signaling domain [37, 38]. TLR signaling is initiated by ligand induced dimerization of receptors and recruitment of adaptor proteins to its TIR domain: myeloid differentiation primary-response protein 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), TIR domain- containing adaptor protein (TIRAP)/MyD88-adaptor-like (MAL) and TRIF-related adaptor molecule (TRAM) [39, 40]. Engagement of the signaling adaptor molecules activates downstream signaling pathways involving interactions between IL-1R-associated kinases (IRAKs) and the adaptor molecules TNF receptor-associated factors (TRAFs) that lead to the activation of the mitogen-activated protein kinases (MAPKs) JUN N-terminal kinase (JNK) and p38, and to the activation of transcription factors: nuclear factor-κB (NF-κB), interferon-regulatory factors (IRFs), cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1) [40–42] (Figure 2). TLR signaling finishes with the induction of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), IL -1β, IL-6, IL-12, IL-23, inducible nitric oxide synthase (iNOS) and monocyte chemoattractant protein (MCP)-1, also called CCL2, and in the case of the endosomal TLRs, the induction of type I interferon (IFN) [43, 44]. Moreover, M1 macrophages normally express increased levels of other surface molecules as CD86, CD80, MHC II, and iNOS [43].

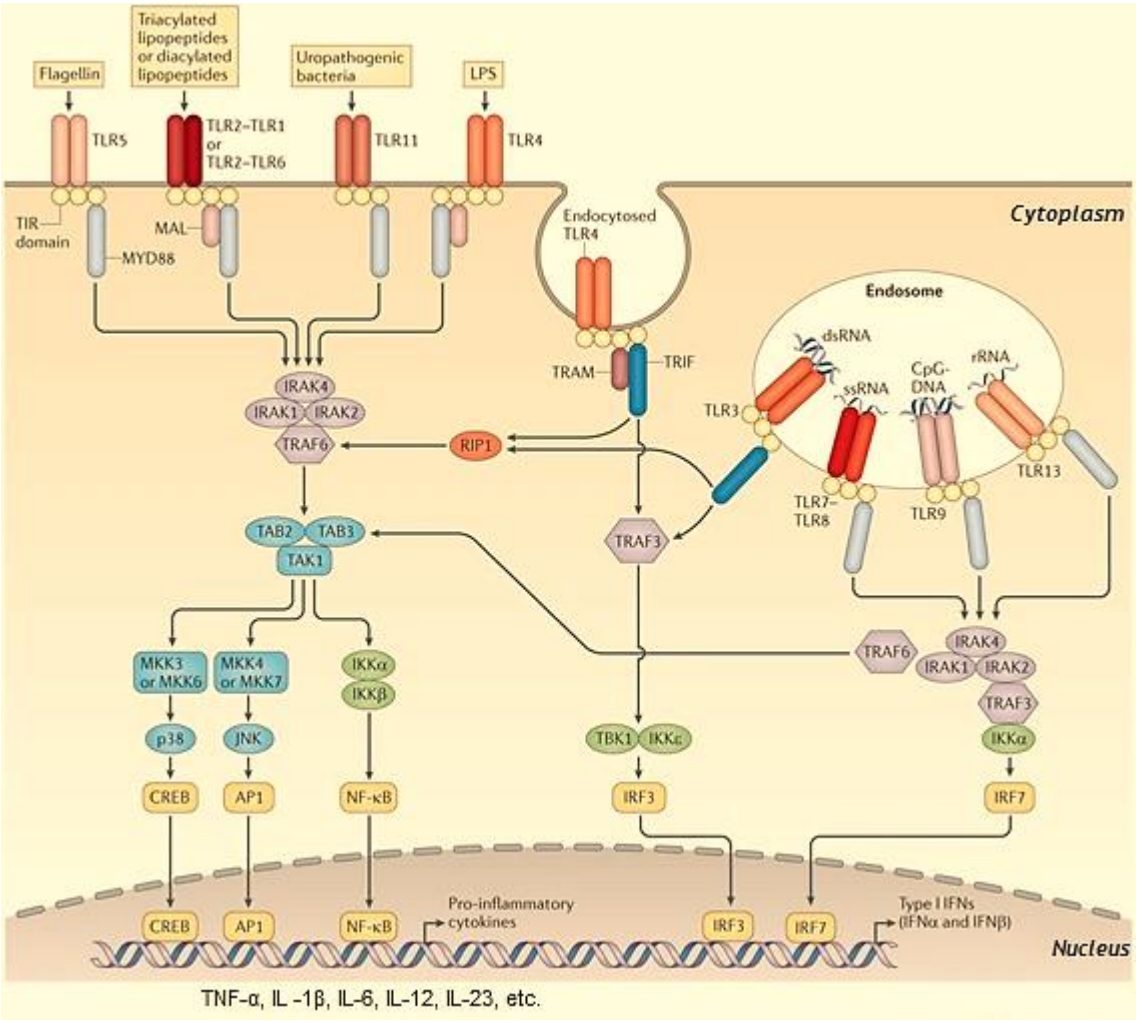


Figure 2 – Toll-Like Receptors signaling pathways *(adapted from [40])*. TLR signaling plays an essential role in the innate immune response. Recognition of pathogen-associated molecular patterns (PAMPs) activates TLR signaling leading to transcriptional activation of genes encoding for pro-inflammatory cytokines, chemokines and co-stimulatory molecules.

However, the inflammatory reaction is spatially and temporally counteracted by alternatively activated M2 macrophages, which display an anti-inflammatory phenotype and are involved in tissue remodeling and wound repair events. M2 macrophages are heterogeneous [45]. Mantovani *et al*. proposed the compartmentalization into three subtypes generated by different inflammatory and opsonic signals [43]. M2a macrophages are stimulated by IL-4/IL-13 and participate in tissue repair and extracellular matrix deposition; M2b macrophages result from activation with immune complexes (IC) and TLR agonists (like LPS) and release anti-inflammatory IL-10, as well as the pro-inflammatory cytokines TNF-α, IL-6 and IL-1, while also promoting Th-2 type adaptive immune responses with increased T-cell IL-4 production and B-cell IgG class switching; M2c macrophages which are driven by IL-10, TGF-β or glucocorticoid hormones and are characterized by high production of IL-10 levels, decrease of pro-inflammatory cytokines limiting inflammation, and increase of debris-cleaning activity [43, 46, 47]. Globally, M2 macrophages tend to produce high levels of anti-

inflammatory products such as arginase-1, IL-10, transforming growth factor-β (TGF-β), scavenger receptors (SR) and mannose receptors (MR), and express transcription factors as Krüppel-like factor 4, peroxisome proliferator activated receptor-γ (PPARγ) and STAT6 in M2a macrophages and STAT3 in M2c macrophages [48–50].

- Macrophage phenotypes in atherosclerosis

Macrophage heterogeneity is particularly important in atherosclerosis lesions, due to the opposing roles of M1 and M2 subtypes during inflammation. Plaque microenvironment is extremely rich in several molecules that can work as modulators of macrophage phenotype, such as oxidative modified lipids, lipoproteins, cytokines, chemokines and growth factors [51]. The total number of macrophages, as well as the presence of pro-inflammatory cytokines, increase with disease progression, a phenomenon associated with increased plaque vulnerability [52]. Consistent with this, M1 macrophages are shown to be the predominant population in the site most prone to plaque rupture, while in the fibrous cap regions, there are both M1 and M2 macrophages [53]. Thus, potential deleterious effects of M1 macrophages may be counteracted by protective pro-fibrotic and tissue repair effects of M2 macrophages in the fibrous cap, while the limited number of M2 macrophages cannot balance the M1-mediated effects in the unstable plaque [53].

Macrophages respond to oxLDL, cholesterol crystals, LPS and IFN-γ by polarizing towards M1 phenotype, expressing pro-inflammatory and pro-atherogenic profiles, which associates this subset to plaque vulnerability [54]; in turn, M2 macrophages display atheroprotective characteristics mainly because of the tissue remodeling capacity of M2a macrophages, primed by IL-4, and efferocytosis capacity of M2c macrophages, primed by IL-10 or TGF-β, which are responsible for the phagocytosis of apoptotic cells [55, 56]. More recently, additional plaque-specific macrophage phenotypes have been identified: Mox, induced by oxidized phospholipids and exhibit reduced phagocytic capacity and anti-oxidant properties; Mhem and M(Hb), identified in areas of intraplaque hemorrhage and characterized by anti-inflammatory and atheroprotective profiles similar to M2 macrophages; and M4, a novel human macrophage phenotype induced by platelet derived chemokine CXCL4, and reported to be irreversible and pro-atherogenic [52, 57]. Table 1 makes an overview of each macrophage subset characteristics described *in vitro* and their function in atherosclerosis.

Table 1 – Macrophages subsets characteristics described *in vitro* and functions in atherosclerosis.

Phenotype	Induction	Cell markers	Cytokines and chemokines production	Functions in atherosclerosis	References
M1	IFN-γ, TNF-α, LPS, GM-CSF	CD86, CD80, MHC II, TLR2, TLR4 and iNOS	TNF-α, IL -1β, IL-6, IL-10^{low} IL-12^{high}, IL-23, iNOS, CCL2	Th1 inflammatory response, atherogenesis, plaque rupture	[34, 43, 44]
M2a	IL-4, IL-13	MR, IL1RN	IL-10, TGF-β, CCL17, CCL22	Tissue remodeling, atheroprotective	[46, 58]
M2b	IC, TLR agonists	MR, MHCII, CD86, IL-10^{high}, IL-12^{low}	IL-6, IL-10^{high}, IL-12^{low}, TNF-α	Immunoregulation	[33]
M2c	IL-10, TGF-β, glucocorticoids	MR, CD163	IL-10, TGF-β	Efferocytosis, atheroprotective	[55, 56]
M4	CXCL4	MR, MMP7, S100A8	IL-6, TNF-α, MMP12	Weak phagocytosis, minimal foam cell formation, pro-inflammatory	[59, 60]
Mox	oxPL	HMOX-1, Nrf2, Srxn1, Txnrd1	IL-1β, IL-10	Weak phagocytosis, proatherogenic	[61]
M(Hb)	Hemoglobin/ haptoglobin	MR, CD163	ABCA1, ABCG1, LXR-α	Hemoglobin clearance, high cholesterol efflux, atheroprotective	[62–64]
Mhem	Heme	ATF1, CD163	LXR-β	Erytrophagocytosis, atheroprotective	[62, 65, 66]

Abbreviations: ABC, ATP-binding cassette transporter; ATF1, **a**ctivating transcription factor 1; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; HMOX-1, heme oxygenase-1**;** IL1RN, interleukin 1 receptor antagonist; LXR, liver X receptor; MHCII, major histocompatibility complex class II; MMP, matrix metalloproteinase; Nrf2, nuclear erythroid-2 related factor; oxPL, oxidized phospholipids; Srxn1, sulforedoxin-1; S100A8, S100 calcium-binding protein A8; Txnrd1, thioredoxin reductase 1; For the other abbreviations refer to the text.

1.1.3 Foam cell formation

In atherosclerosis, macrophages can accumulate membrane-bound lipid droplets in their cytoplasm. These lipid-loaded cells are called “foam cells” and their formation begins with the ingestion and processing of accumulated apoB-LPs [11]. This event is regulated by the balance between the uptake of the modified LDL and efflux of cholesterol and other lipids [67].

This uptake is mediated by CD36, SR-A1, oxidized LDL receptor-1 (LOX-1), TLR4/MD-2, TLR2 and a number of other receptors (Figure 3). Unmodified LDL can also enter in macrophages by micro- and micropinocytosis [31, 68–73].

To control membrane levels of sterols, cells essentially employ a feedback system that is mainly regulated at the transcriptional level by endoplasmic reticulum (ER)-bound sterol regulatory element-binding proteins (SREBPs) [74]. Under low cholesterol levels, SREBPs are transported to the Golgi complex and proteolytically processed. The generated active peptides may enter to the nucleus and activate the transcription of target genes. SREBP2 and SREBP1a activate the transcription of cholesterol-related genes expression including 3-hydroxy-3-methylglutaryl coenzyme A reductase (Hmgcr) and low-density lipoprotein receptor (Ldlr), while SREBP1c enhances the expression of fatty acid metabolism-related genes, such

as fatty acid synthase (Fas) [74–76]. Under high cholesterol levels, SREBP is unable to exit the endoplasmic reticulum and, consequently, transcription of target genes is reduced [77].

The excess of cholesterol, oxysterols and phospholipids, which accumulates in macrophages and is not degraded, can be removed by ATP-binding cassette (ABC) transporters in a process called reverse cholesterol transport [78]. ABCA1 and ABCG1 are upregulated during macrophage differentiation [79, 80]. ABCA1 efflux cholesterol to poor-lipidated APOA1 [81], while ABCG1 is mostly involved in the lipidation of mature HDL particles [82]. Peroxisome proliferator–activated receptor-γ (PPAR-γ) and liver X receptor (LXR) agonists regulate macrophage foam cell formation in atherogenesis [83–85]. PPAR-γ agonists can inhibit foam cell formation *in vivo* through ABCA1-dependent [86] and ABCA1-independent pathways [87]. Activation of PPAR-γ reduces cholesterol esterification and induces expression of ABCG1 [88]. LXR activation in macrophages reduces foam cell formation via induction of both ABCA1 and ABCG1 [89, 90].

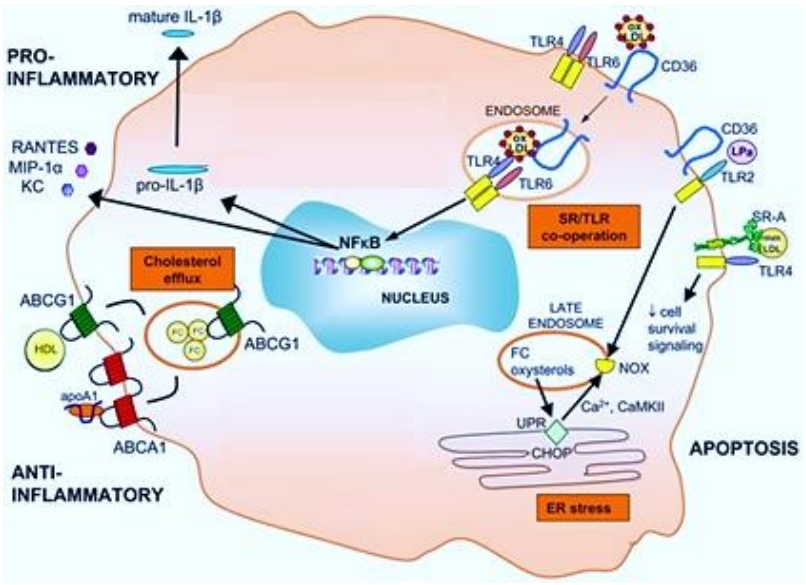


Figure 3 - Signaling pathways in macrophages following cholesterol uptake *(adapted from* [67]*).* Various pro- and anti-inflammatory forces act on macrophages in atherosclerotic lesions, leading to activation of downstream cascades, such as the inflammasome, SR/TLR cooperative signaling, endoplasmic reticulum stress, expression of the sterol responsive network, and efflux of cholesterol via ABCA1 and ABCG1 transporters.

Foam cells are important sources of chemokines (MCP-1), cytokines (TNF, IL, and IFN), enzymes (metalloproteases, cysteine and serine proteases), growth factors (platelet-derived growth factor, VEGF, and IGF), bioactive lipids and angiogenic factors. Together with proteins secreted by T cells and pro-inflammatory cytokines secreted by M1 macrophages, the inflammatory process is perpetuated, which will promote migration of vascular smooth muscle cells (VSMCs) into the intima and well as their proliferation and secretion of pro-

fibrotic agents, but also migration of endothelial cells, contributing the resulting fibrous cap formation [73, 91] .

M2 macrophages are characterized by the expression of scavenger receptors, which play a major role in the clearance of oxLDL, especially CD36 [72]. These are found to bind and internalize modified forms of LDL through mechanisms that are not inhibited by cellular cholesterol content, and these are likely responsible for macrophage cholesterol accumulation [92]. M1 macrophages express TLRs on their surface, especially TLR2 and TLR4, which are known to be part of modified LDL uptake [36, 93, 94]. For example, complex TLR4/MD-2 is described to recognize minimally oxidized LDL and induce membrane ruffling and robust micropinocytosis, resulting in uptake of native and modified LDL and foam cell formation [95]. Consistent with this, other studies have demonstrated conjugated interactions between TLRs and other proteins such as CD14 and CD36 to recognize modified lipids and promote inflammatory gene expression [96–100]. Thus, macrophages recognize modified lipoproteins through TLR2 and TLR4. Scavenger receptor CD36, which binds oxLDL, forms complexes with TLR heterodimers and in turn mediates pro-inflammatory effects of modified lipoproteins. Activation of TLR by proatherogenic modified lipoproteins leads to the activation of pro-inflammatory signaling pathways involving NF-κB, MAPKs, and reactive oxygen species (ROS) dependent signaling [101]. Expression of pro-inflammatory genes switches macrophage phenotype towards M1. In addition, oxLDL-mediated stimulation of CD36 leads to the activation of inflammasome that further aggravates vascular inflammation [101]. Michelsen *et al*. showed that mutation in either the adaptor molecule MyD88 or its upstream receptor TLR4 exhibit reduced aortic atherosclerosis, plaque lipid content and plaque macrophage infiltration [100]. They further provide evidence suggesting that MyD88 deficiency leads to decreased levels of the circulating pro-inflammatory molecules IL-12p40 and/or monocyte chemoattractant protein 1 (MCP-1/CCL2) [100, 102].

Studies regarding the ability of macrophages with different polarization status to uptake lipids are not consistent [103]. However, the increase of pro-inflammatory environment may drive monocyte to macrophage differentiation towards M1 phenotype. Accordingly, this would account for the reported absence of M2 foam cells in advanced human lesions, or their location distant from the core [104, 105].

1.1.4 Macrophages in advanced atherosclerosis

Atherosclerosis can be considered a nonresolving inflammatory condition. As atherosclerotic lesions progresses, monocytes continue to enter plaques and differentiate into macrophages [25]. Consequently, in advanced lesions, a pro-inflammatory environment ensues with greater levels of Th1 cytokines (such IFN-γ) than Th2 cytokines. Macrophages contribute to the plaque morphology, thinning the fibrous cap, and necrotic core, which can lead to increased pro-inflammatory responses and further apoptotic signals for SMCs, ECs and

leukocytes within the plaques [106, 107]. Furthermore, apoptotic macrophage foam cells also contribute to this scenario [108].

One important function of macrophages is the clearance of apoptotic cells in a process called efferocytosis. Efferocytosis is primarily a function of alternatively activated (M2) macrophages [109]. As macrophages engulf oxidized lipids and other cellular debris in the arterial wall during early stages of atherogenesis, many of these macrophages undergo apoptosis [108]. In early atherogenesis, macrophage apoptosis is associated with reduced atherosclerosis progression [110]. Efficient efferocytosis of apoptotic macrophages results in at least three critical protective effects: (1) it clears the cells before membrane damage, preventing extracellular leakage of toxic intracellular material; (2) it triggers an IL-10- and TGFß-mediated anti-inflammatory response in the efferocytes; and (3) it promotes survival of the efferocytes themselves so that they do not succumb to potentially toxic factors of apoptotic cells [25, 111]. Therefore, macrophage apoptosis by itself will not trigger plaque necrosis. Rather, uptake of excessive apoptotic cells by macrophage effecrocytes induces endoplasmic reticulum stress and the unfolded protein pathway response, which increases the expression of a pro-apoptotic protein, called CEBP homologous protein (CHOP) [112]. As atherosclerosis progresses, efferocytosis is thought to become impaired [113]. With the plaque adopting an increasingly inflammatory environment, macrophage are differentiated towards the M1 form and consequently, M1 foam cells predominate. As M1 macrophages have low efferocytosis capability, and there is a decreasing number of M2 efferocytes, apoptotic foam cells undergo secondary necrosis [114]. Thus, failure of efferocytosis leads to a secondary necrosis, not only by macrophages which release their cellular contents, including debris, oxidized lipids and pro-inflammatory mediators, but also by smooth muscle cell-derived foam cells, amplifying the inflammatory response and leading to the development of a necrotic core and accumulation of extracellular cholesterol in the plaque [115]. At this stage, macrophages also secrete matrix metalloproteinases (MMPs) which promote neovascularization and are capable of degrading the collagen produced by SMCs, reducing the strength of the plaque's protective fibrous cap, rendering that cap thin, weak and susceptible to rupture [116]. When plaque ruptures, blood components are exposed to tissue factor, the major pro-coagulant and trigger of thrombosis, which is also produced by the macrophages [13], initiating coagulation, recruitment of platelets and formation of a thrombus, which narrows the arterial passage, restricting blood flow (Figure 4). These events are regulated by the inflammatory mediators, which demonstrate an essential link between arterial inflammation and thrombosis.

Thus, to prevent atherosclerosis progression and severity it is important to get a balance between apoptosis, efferocytosis and secondary necrosis. Many possibilities have been studied but, given the association of M1 macrophage with plaque instability, decreasing the levels of these pro-inflammatory macrophages in the plaque is a promising avenue for plaque stabilization. Modulation of macrophage phenotype in the arterial wall during atherogenesis, which would lead to the accumulation of poorly phagocytic macrophages, is being considered

a key factor in atherosclerosis research. As macrophages show a high degree of plasticity in early and advanced atherosclerotic plaques, with the ability to undergo dynamic transition between M1 phenotype and M2 phenotype, we hypothesize that modulating the balance between M2 macrophages (characterized by high IL-10 production) and pro-inflammatory M1 macrophages will increase the efficient clearance of early apoptotic cells and decrease the progression of the disease. To achieve this goal, we propose to use microRNAs (miRNAs) that have emerged as therapeutic targets for several biological mechanisms, including macrophage polarization. In cardiovascular diseases dysregulation of miRNAs has been linked to the development and progression of the pathology [117, 118]. The forced expression or suppression of a single miRNA is sufficient to cause or alleviate pathological changes. In the next section this will be discussed in detail.

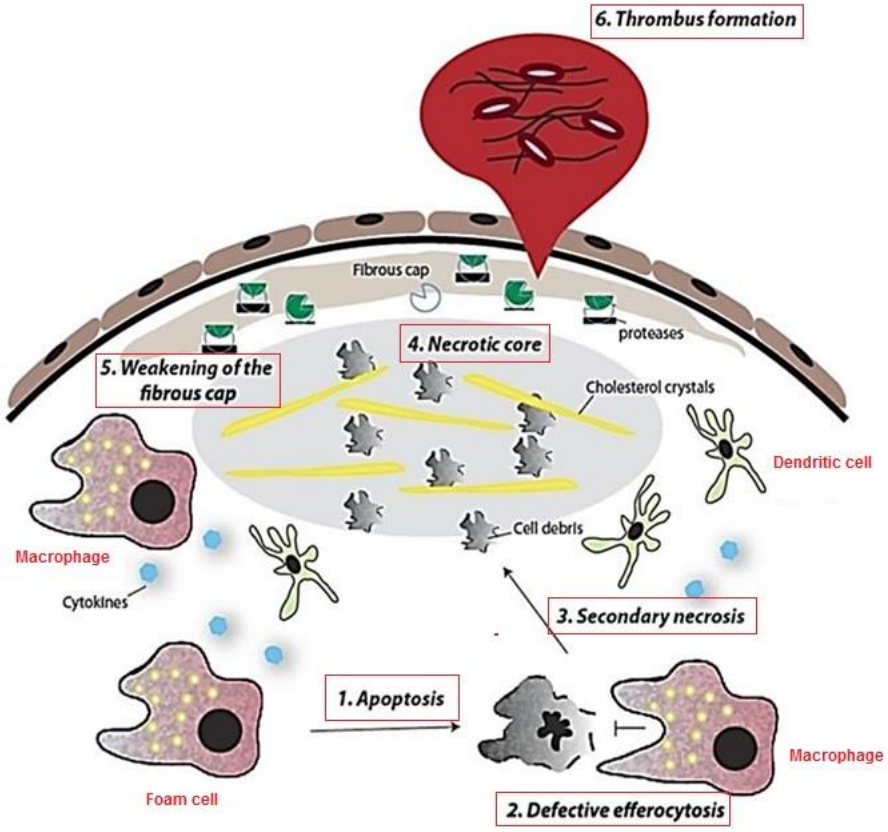


Figure 4 - Forces in the advanced lesion that destabilize the atherosclerotic plaque *(adapted from* [67]*).* (1) Macrophage foam cells undergo apoptosis as a result of prolonged endoplasmic reticulum stress and other stimuli; (2) apoptotic cells are not effectively cleared by advanced lesional macrophages (defective efferocytosis); (3) accumulation of apoptotic cells induces secondary necrosis and (4) contributes to the formation of a necrotic core. (5) Smooth muscle cell death and protease degradation of extracellular matrix weakens the fibrous cap, making it susceptible to rupture. (6) Exposure of the thrombogenic material of the lesion causes platelet aggregation and thrombus formation.

1.2 MicroRNAs

1.2.1 MicroRNA biogenesis

MicroRNAs (miRNAs) are single-stranded RNAs typically 15-25 nucleotides long, which can regulate gene expression at a post-transcriptional level and play a central role in tissue development and in the pathogenesis of several diseases [119]. miRNAs originate from long RNAs called primary miRNAs that are regulated by conventional transcription factors and transcribed by RNA polymerase II [120]. Primary miRNAs are hundreds to thousands of nucleotides long and are processed in the nucleus into an approximately 70- to 100-nucleotide hairpin-shaped precursor miRNA, by the RNase III enzyme Drosha and the double-stranded RNA binding protein DGCR8. Then, the miRNA precursor is transported into the cytoplasm by the nuclear export factor exportin 5 and further processed into an 15- to 25-nucleotide double-stranded RNA by the RNase III enzyme Dicer. This duplex miRNA is then incorporated into the RNA-induced silencing complex. Subsequently, one strand of the mature miRNA is retained in the RNA-induced silencing complex, whereas the other strand is often degraded (Figure 5) [121]. Once loaded into the RNA-induced silencing complex, the mature miRNA associates with target mRNAs and acts as a negative regulator of gene expression by promoting translation inhibition or mRNA degradation. In mammals, the predominant mechanism is translation inhibition; however, targeted genes that are strongly downregulated at the protein level often show reduced mRNA levels [122], suggesting that mRNA destabilization gives also a major contribution to gene silencing.

A mature miRNA typically regulates gene expression via an association with the 3' untranslated region (UTR) of an mRNA with complementary sequence, although emerging evidence suggests that miRNAs may also target 5'UTRs or exons and may potentially undergo base pairing with regulatory DNA sequences to regulate transcription [121]. Once miRNA bind to a 3' UTR, the degree of mRNA degradation and/or translational repression is affected by multiple mechanisms, including the overall complementarity between the miRNA and target mRNA, the secondary structure of the adjacent sequences, the distance of the miRNA binding site to the coding sequence of the mRNA, and the number of target sites within the 3' UTR [123]. Complementarity between mRNA and nucleotides 2 through 8 of the miRNA, called the "seed" region, appears to be essential for 3' UTR identification. Therefore, miRNAs with high sequence homology and identical seed region are commonly grouped into miRNA families that are likely to target similar sets of mRNAs [124].

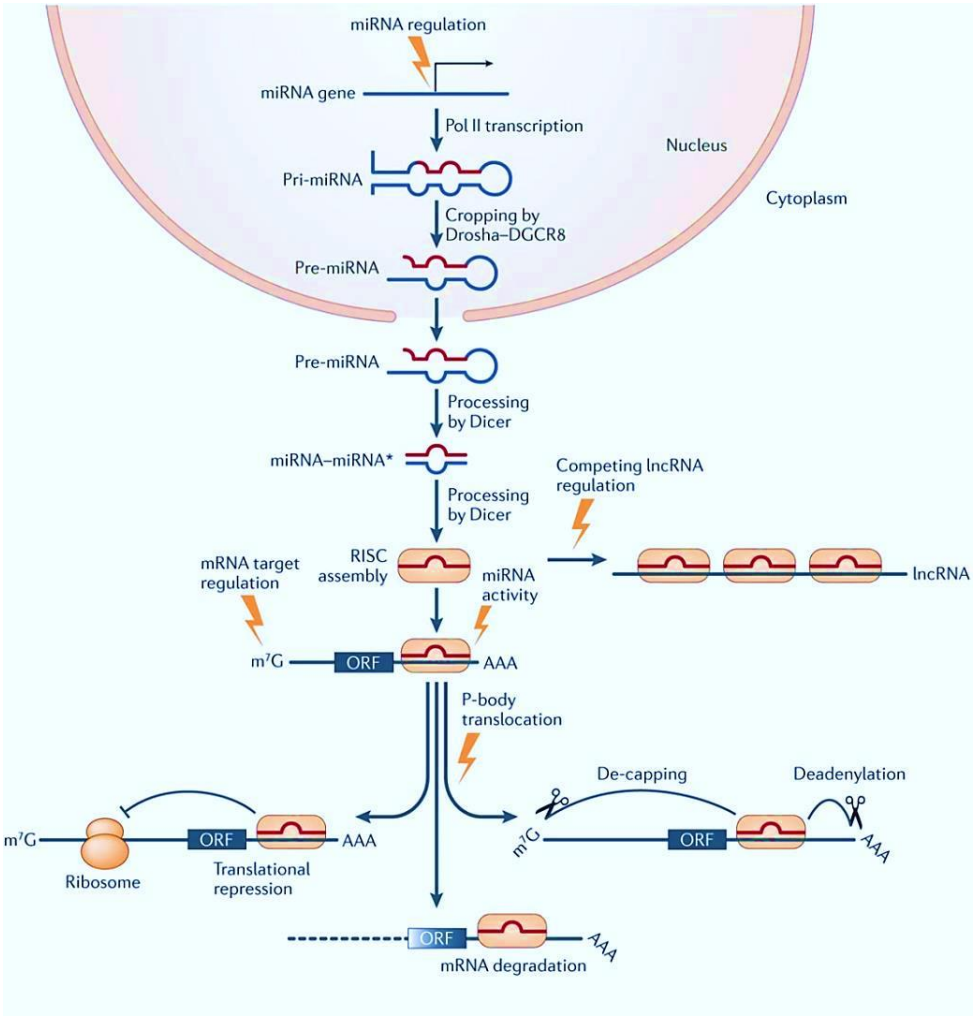


Figure 5 - Mechanism of microRNA biogenesis *(adapted from* [125]**).** In the nucleus, a primary microRNA is transcribed from the genome and processed by Drosha. Then, the precursor microRNA is exported to the cytoplasm where it is further processed by Dicer. Mature microRNA is then incorporated into RISC complex.

Accordingly to mirBase (www.mirbase.org), around 2000 miRNAs are predicted in the human genome, each one of them could potentially target several mRNAs. On the other hand, most 3' UTRs contain potential binding sites for a large number of individual miRNAs, allowing redundancy or cooperative interactions between various seemingly unrelated miRNAs [120]. Furthermore, the targets of many miRNAs can modulate the expression of additional miRNAs or groups of miRNAs, generating positive or negative feedback loops. Finally, miRNA maturation seems to be post-transcriptionally regulated in a sequence-specific manner, potentially explaining why genetically clustered and cotranscribed miRNAs are often expressed at different levels [126].

During the last decade nomenclature of the miRNAs has evolved. For that reason, a brief description of the most commonly used nomenclature is presented in Table 2.

Table 2 - Nomenclature of microRNAs. *(adapted from* [124]*)*

Nomenclature format	Description
***mir* or miR**	Genes that encode miRNAs, primary transcripts of miRNAs, and stem-loop precursor miRNAs are all named using the italicised prefix 'mir'. Mature miRNAs are named using the non-italicised prefix 'miR'.
***mir-X* or miR-X**	With the exception of a few early miRNAs, miRNAs are sequentially assigned unique identifying numbers, depending on when they are first published, for example, mir-31 or miR-31.
***mir-Xa*, *mir-Xb*, …**	Similar miRNA sequences within a species are distinguished by a lettered suffix, for example, mir-181a and mir-181b. However, this does not imply shared targets or functions.
***mir-X-1*, *mir-X-2*, …**	Identical miRNA sequences within a species are distinguished by a numerical suffix, for example, mir-7-1 (chromosome 9), mir-7-2 (chromosome 15) and mir-7-3 (chromosome 19), can all produce identical mature miRNAs.
miR-X-5p or miR-X-3p	The latest convention is to name mature miRNAs by the arm of the pre-miRNA from which they are derived, regardless of their abundance—those from the 5' arm are named miR-X-5p and those from the 3' arm as miR-X-3p.
has-miR-X, mo-miR-X, …	All of the above naming conventions can be preceded by a three-letter code which identifies the species the miRNA is from, for example, hsa=homo sapiens (human); rno=rattus norvegicus (rat).

1.2.2 MicroRNAs and macrophage phenotypes

miRNAs do not encode peptides; instead, those non-coding RNAs emerged as regulators of the expression of proteins encoded by their mRNA targets. Since the initial findings, many studies have shown that miRNAs are indeed meaningful regulators of cellular function in different tissues/organs [127]. From the perspective of inflammatory responses, miRNAs have been shown to affect directly the magnitude of the response, by targeting proteins involved in modulation of inflammation [125]. Furthermore, several studies reported a crucial role of miRNAs in the control of gene expression during macrophage polarization [128, 129]. On one hand, miRNAs regulate macrophage polarization acting as posttranscriptional inhibitors; on the other hand, miRNA levels are differently expressed in M1 and M2 phenotypes [126, 128].

miR-155 is one of the miRNA most described to be associated to M1 macrophages profile and pro-inflammatory events. O'Connell *et al*. recently observed that stimulation of macrophages with TLR2, TLR3, TLR4 and TLR9 ligands remarkably upregulated the expression of miR-155 [130]. Such an elevation of miR-155 was hampered by the knockdown of MyD88 and TRIF, two factors known to activate the NF-κB signalling [130]. Moreover, the use of an JNK inhibitor diminished the upregulation of miR-155 and production of TNF-α, suggesting that miR-155-mediated activation of M1 polarization are due to induction of the JNK pathway [130]. Other findings revealed that miR-155 targets and downregulates SHIP1 and SOCS1, leading to increased activation of AKT and IFN response genes [131, 132]. Another miRNA reported to augment classical activation of macrophages is miR-127 [133]. Inhibition of miR-127 expression by antagonist suppressed the expression of M1 signature genes and promoted transcription of M2 marker genes [133].

Conversely, there are miRNAs overexpressed on M2 macrophages, suggesting that these promote M2 macrophage polarization [134]. Overexpression of miR-125a-5p has been associated to the reduction of M1 profile induced by LPS and to the promotion of M2 profile induced by IL-4. miR-125a-5p targets KLF13, a transcriptional factor that has an important role on T lymphocyte activation and inflammation [135]. Several reports also found that miRNA let-7c regulates macrophage polarization by promoting M2 phenotype expression and inhibiting M1 phenotype, by targeting C/EBP-δ [136]. miR-124 is also described to modulate LPS-induced cytokine production in macrophages by targeting signal transducer and activator of transcription 3 (STAT3) decreasing IL-6 production and TNF-α converting enzyme (TACE) reducing TNF-α release, which suggest an anti-inflammatory role for this miRNA [137].

Moreover, other miRNAs are known to be differently expressed in M1 *versus* M2 macrophages phenotype, as miR-181a, miR-204-5p and miR-451 that are upregulated, and miR-146a-3p, miR-143-3p and miR-145-5p that are downregulated in M1 macrophages, suggesting a key role on regulation of inflammation [128, 129].

1.2.3 MicroRNAs in cardiovascular diseases

The involvement of miRNAs in the pathological process of the cardiovascular system has recently been described. Research on miRNAs in cardiovascular diseases is a rapidly evolving field. Recent studies have demonstrated that miRNAs are aberrantly expressed in the cardiovascular system under some pathological conditions [118]. Gain- and loss-of-function studies using *in vitro* and *in vivo* models have revealed distinct roles for specific miRNAs in cardiovascular development and physiological function.

miR-1 and miR-133 are two widely conserved miRNAs derived from a common precursor transcript that have been described to display cardiac- and skeletal-muscle specific expression during cardiac development [138–143]. In addition, other miRNAs have been associated to several CVDs, as cardiac hypertrophy, myocardial infarction and cardiac fibrosis [118].

miR-21, miR-23a and miR-208 have often been found to be upregulated in hypertrophy, whereas miR-133 has often been found to be downregulated. Sayed *et al*. proposed that an increase in miR-21 enhances the formation of various types of cellular protrusions through direct targeting and down-regulating of SPRY2, an inhibitor of branching morphogenesis and neurite outgrowths [144]. In turn, Lin *et al*. reported that miR-23a is a pro-hypertrophic miRNA, and its expression is regulated by the transcription factor, nuclear factor of activated T cells (NFATc3) [145]. Also, miR-208 is associated to cardiac hypertrophy as the overexpression of this miRNA in the heart was sufficient to induce hypertrophic growth in mice, which resulted in pronounced repression of the miR-208 regulatory targets thyroid hormone–associated protein 1 and myostatin, two negative regulators of muscle growth and hypertrophy [146, 147]. Conversely, Carè *et al*. observed that overexpression of miR-133 inhibited cardiac hypertrophy [148].

Moreover, in acute myocardial infarction (AMI) several miRNAs are proposed to be involved in the cardiomyocyte death/apoptosis. MiR-1 is thought to be involved in the promotion of cell apoptotic events as it was significantly increased on apoptosis induced by oxidative stress in H9c2 rat ventricular cells [149]. In addition, miR-1 is also associated with the cell death pathway by inhibiting the translation of insulin like growth factor 1 (IGF)-1 [150]. In turn, Dong *et al*. identified that miR-21 had a protective effect on ischemia-induced cell apoptosis [151]. The protective effect of miR-21 against ischemia-induced cardiac myocyte damage was further confirmed *in vivo* by decreased cell apoptosis in the border and infarcted areas of the infarcted rat hearts after treatment with an adenovirus expressing miR-21 [151]. MiR-199a was reported to be downregulated in cardiac myocytes on a decline in oxygen tension. This reduction is required for the rapid upregulation of its target, hypoxia-inducible factor (Hif)-1alpha. Replenishing miR-199a during hypoxia inhibits Hif-1alpha expression and its stabilization of p53 and, thus, reduces apoptosis [152].

In cardiac fibrosis, an important miRNA family has emerged as inhibitor of fibrosis in the border zone of the infarcted area. miR-29, which is fibroblast enriched, targets mRNAs

encoding ECM-related proteins involved in fibrosis, including multiple collagens, fibrillins and elastin. MiR-29 is dramatically repressed in the border zone flanking the infracted area in the mouse model of AMI [153]. Downregulation of miR-29 would be predicted to counter the repression of these mRNAs and enhance the fibrotic responses.

Regarding to atherosclerosis, several studies have been performed in order to better understand the role of miRNAs in key steps of the disease initiation and progression as endothelial dysfunction, cholesterol homeostasis and reverse cholesterol transport, plaque development and plaque rupture [117].

As detailed in section 1.1.1, during endothelial dysfunction several endothelial adhesion molecules (e.g. ICAM-1, VCAM-1, E-selectin) and chemotactic factors are overexpressed, allowing the recruitment and entry of monocytes and other leukocytes into the intima. miR-10a is one of the miRNAs that is thought to be involved in endothelial dysfunction as it inhibits a number of pro-inflammatory genes in ECs, including VCAM-1 and E-selectin, and the NF-κB pathway [154]. miR-126, miR-31 and miR-17-3p also regulate vascular inflammation by controlling the expression of the adhesion molecules [155, 156]. In addition, ECs transfected with miR-155 mimic were shown to be less migratory compared with control ECs [157]. Relatively to endothelial aging, miR-217 and miR-34a are described to promote EC senescence [158, 159], whereas Vasa-Nicotera *et al*. suggested miR-146a as a target of the NOX4, a protein implicated in cell senescence and aging [160].

Cholesterol homeostasis is also regulated by miRNAs. A recent study identified miR-33a and miR-33b as intronic miRNAs located within the *Srebp2* and *Srebp1* genes respectively [161]. Both miRNAs are co-transcribed with their host genes and regulate cholesterol and fatty acid metabolism [162, 163]. Also miR-122 is involved in the regulation of cholesterol metabolism. Élmen *et al*. showed that inhibition of miR-122 levels in the liver results in a significant reduction of plasma cholesterol levels in mice and non-human primates [164].

Interestingly, miR-33 is also described to have a crucial role in the inhibition of reverse cholesterol transport, also known as cholesterol efflux. This miRNA is known to target ABCA1 and ABCG1 *in vivo*. Rayner *et al*. showed that it inhibits cellular cholesterol efflux to APOA1 and mature HDL and reduces the circulating HDL-cholesterol (HDL-C) levels in mice and non-human primates [161]. Importantly, genetic deletion of miR-33 in mice increases plasma HDL-C levels and reduces the progression of atherosclerosis [165]. As ABCA1 has a very long 3'UTR chain, several reports have found multiple miRNAs that can regulate this transporter including, miR-26, miR27-a/b, and miR-758, among others [166–168].

Inside the intima, monocytes/macrophages and VSMCs are loaded with modified LDLs through scavenger receptors, TLRs and LDL receptors giving rise to foam cells and activating VSMCs, which then lead to plaque development. Lipid uptake and inflammatory responses in monocytes/macrophages are regulated by miRNAs such as miR-155 or miR-125a-5p. miR-125a-5p was found to mediate lipid uptake and to decrease the secretion of some inflammatory cytokines (IL-2, IL-6, TNF-α) in oxLDL-stimulated monocyte-derived macrophages [169]. However, data regarding miR-155 is ambiguous since there are studies associating this

microRNA to atherosclerosis progression while others emphasize its role in the prevention of disease development [170, 171].

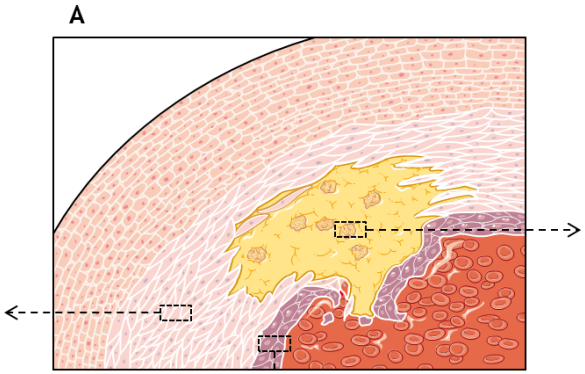
The evolution from fatty streaks to a fibrous atheroma is also promoted by VSMCs proliferation in the neointima. VSMC differentiation and apoptosis is regulated by transforming growth factor-ß (TGF-ß), which is a known target of miR-26a [172]. Metalloproteinases (MMP) expression, such as MMP2/9, controls VSMCs proliferation. MMPs expression is regulated by DNMT3b, a methyltransferase enzyme that silences the expression of these genes [172]. Interestingly, Chen *et al*. showed that miR-29b is upregulated in VSMCs treated with ox- LDLs and targets MMP2 thereby reducing VSMC migration [173].

Wang *et al*. showed that miR-195 inhibits VSMCs proliferation and migration and reduces the synthesis of IL-1ß, IL-6, and IL-8 [174]. Using bioinformatics prediction and experimental studies, the authors showed that miR-195 could repress the expression levels of Cdc42, CCND1, and FGF1 genes, which are involved in cell cycle control, migration, and proliferation. Moreover, the authors found that the miR-195 introduced by adenovirus substantially reduced neointimal formation in a balloon-injured carotid artery, which confirms the therapeutic potential of this miRNA [174].

As the intima wall thickens, the oxygen effective diffusion from the lumen becomes more difficult and *vasa vasorum* proliferates in the inner layers of the vessel wall. The promotion of neovessels formation has been linked to cholesterol-loaded macrophages which produce cytokines responsible for this proliferation [175, 176]. In advanced lesions, the microenvironment becomes enriched in pro-inflammatory, pro-oxidant and proteolytic factors, as the neoangiogenesis drive more blood components, specially monocytes and macrophages, to the plaque. This pro-inflammatory scenario has been studied in order to understand miRNAs role in this context. miR-146a and miR-21 were described to target TLR4, negatively regulating LPS-induced lipid accumulation and inflammatory responses in macrophages by the TLR4-NF-κB pathway [177, 178]. The loss of collagen, endothelial and VSMCs due to the high expression of proteolytic enzymes and apoptosis also favours plaque instability and further rupture. At this regard, miR-29 inhibits the expression of Col3A1, and elastin (ELN), thus reducing vascular integrity [153, 179]. miR-365 can function as a pro-thrombotic factor by stimulating endothelial apoptosis [180], whereas miR-21 protects VSMCs from H_2O_2-induced apoptosis [181].

Figure 6 stresses the relationship between miRNAs and atherosclerosis, by showing their effect on the three main types of cell involved in the pathogenesis of the disease.

In summary, studies have unravelled the role of miRNAs in inflammation and in atherosclerosis development and progression. Consequently, miRNAs are potential therapeutic targets. In this context, modulation of macrophages inflammatory response using miRNAs may lead to the development of novel therapeutic approaches for arthrosclerosis and cardiovascular diseases.



B

Vascular Smooth Muscle Cells

microRNA	Target(s)	Function	Reference
miR-1	KLF4, PIM-1	Differentiation and proliferation	[140, 141]
miR-21	BCL-2, PTEN, PDCD4	Proliferation and apoptosis	[183, 184]
miR-26a	SMAD1,SMAD4	Differentiation and apoptosis	[172]
miR-29b	DNMT3b	Migration	[173]
miR-125b	SP7	Calcification	[186]
miR-143/145	KLF4, KLF5, myocardin, ELK	Differentiation, proliferation, migration and phenotype	[192-195]
miR-195	CDC42	Proliferation, migration, and proinflammatory biomarkers	[174]
miR-221/222	cKit, p27(Kip1), p57(Kip2)	Proliferation, migration and apoptosis	[200-202]

C

Endothelial Cells

microRNA	Target(s)	Function	Reference
miR-10a	HOXA1, MAP3K7, BTCR	Inflammation and dysfunction	[154]
miR-21	PPARα	Inflammation and dysfunction	[181]
miR-27a/b	SEMA6A	Cell repulsion and angiogenesis	[185]
miR-34a,217	SIRT1	Cell senescence	[158, 159]
miR-92a	KLF2, KLF4, SOCS5	Dysfunction	[187-189]
miR-146a	NOX4	Aging	[160]
miR-155	AT1R, ETS-1, MYLK	Inflammation, migration, phenotype and function	[157, 198]
miR-365	BCL-2	Apoptosis	[180]

D

Monocytes / Macrophages

microRNA	Target(s)	Function	Reference
miR-15a	CARM1	Inflammation	[182]
miR-21	TLR4	Regulation of LPS-induced lipid accumulation and inflammation	[178]
miR-27a/b	ABCA1, ACAT1, LPL	Cholesterol efflux, influx, esterification and hydrolysis	[167]
miR-33a/b	ABCA1, ABCG1, AMPKα, CROT, CPT1A, HADHB, SRBP-2	Cholesterol efflux, influx and metabolism	[161-163, 165]
miR-125a-5p	ORP6	Lipid uptake and inflammation	[169]
miR-146a	TLR4, IRAK1, TRAF6	Regulation of LPS-induced lipid accumulation and inflammation	[177, 190, 191]
miR-155	HBP1, BCL6, FADD, MAP3K10, SHIP1, SOCS1	Foam cell formation, apoptosis and inflammation	[170, 171, 196, 197]
miR-342-5p	AKT1	Inflammation	[199]

Figure 6 - MicroRNAs in atherosclerosis. (A) Some of the relevant factors involved in atherosclerosis development and plaque rupture: cell types (leucocytes/macrophages, SMCs, ECs, RBCs, platelets), cholesterol particles (VLDL, LDL and HDL), collagen, necrotic and lipid core. (B–D) Systematization of microRNAs and microRNAs-targets in the most relevant cell types involved in atherosclerosis.

1.3 Objectives

The aim of this work was to promote inflammation resolution by reducing M1-like and/or enhancing M2-like macrophage polarization, using miRNAs.

Specifically, we intended to:

1. Investigate miR-195 for its potential to modulate the polarization of human primary macrophages;

2. Determine potential miR-195 targets involved in inflammatory pathways by silico predictions;

3. Analyze the biological effect of miR-195 on macrophage activation and differentiation and unravel the downstream molecular targets;

4. Evaluate the functional impact of miRNA delivery to macrophages regarding their interaction with smooth muscle cells, namely by inducing alterations in their migratory profile.

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Human monocyte isolation, differentiation and polarization

Human primary monocytes were obtained from healthy blood donors buffy coats (BC), kindly donated by *Serviço de Hemoterapia, Centro Hospitalar de São João (CHSJ)* - Informed consent was obtained from all subjects before blood donation. *RosetteSep* human monocyte enrichment isolation kit (StemCell Technologies) was used, according to Oliveira *et al*. [203]. Firstly, BC were transferred to 50 ml tubes and centrifuged at room temperature (RT) for 20 minutes (min) at 1200 g, without active acceleration or brake (Centrifuge 5810/ 5810 R, Eppendorf). Blood components were separated into three fractions, namely red blood cells (RBCs) on the top, peripheral blood mononuclear cells (PBMCs) in the thin middle layer, and plasma on top. PBMCs were collected, together with RBCs (approximately 3 mL) necessary to the formation of immunorosettes, and incubated with *RosetteSep* human monocyte enrichment isolation kit (67 µL/mL) for 20 min in a horizontal shaker. The mixture was diluted at a 1:1 ratio with 2% fetal bovine serum (FBS, Lonza) in PBS, gently layered over Histopaque-1077 (Sigma) to avoid mixture and then centrifuged as previously. The enriched monocyte layer was carefully collected, washed with 45 ml PBS, followed by centrifugation at 100 g for 17 min with maximum acceleration and brake. Finally, the pellet was resuspended in complete culture medium [RPMI 1640 (Life Technologies) supplemented with 10 % FBS and 1 % penicillin streptomycin (P/S) (Invitrogen) and Glutamax (Invitrogen)]. Cell count was performed in an optical microscope (Compact inverted microscope CKX41, Olympus) using a Neubauer chamber with trypan blue dye (Sigma) – exclusion assay. Finally, 0,5 x 10^6 cells per well were plated on top of sterile 13 mm glass coverslips in 24-well plates and cultured in a humidified 37ºC/5% CO_2 incubator (Figure 7).

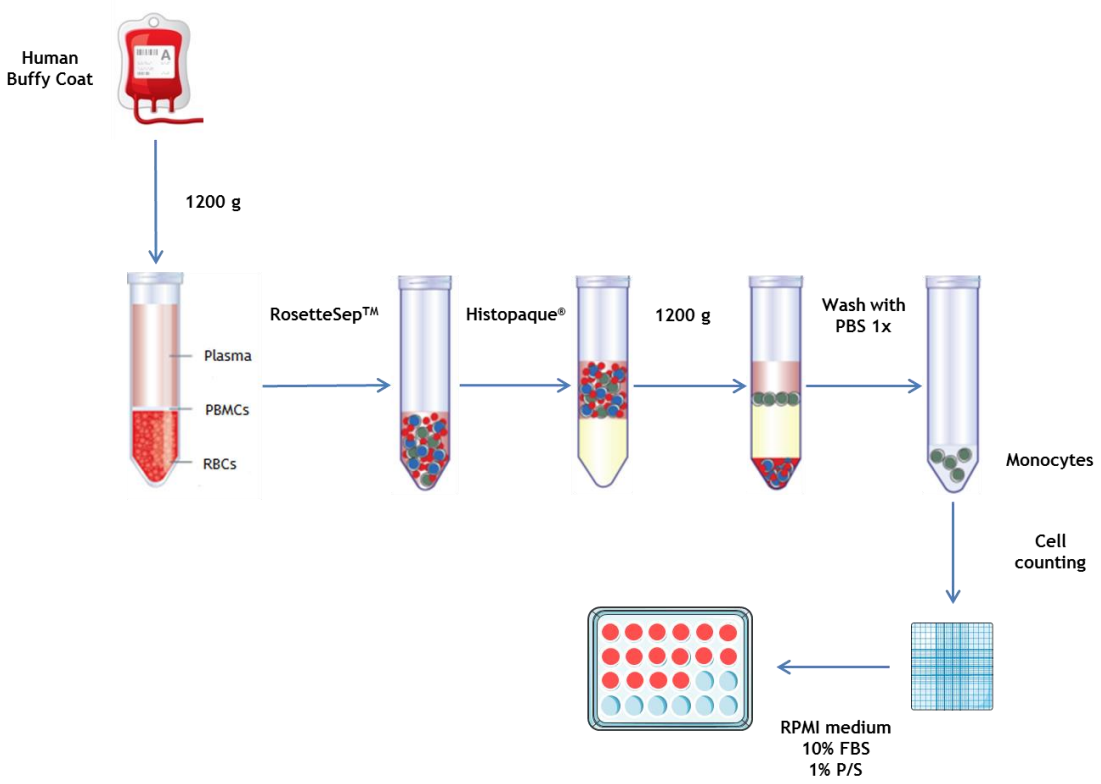


Figure 7 - Monocyte isolation from human buffy coats using RosetteSep. PBMCs and RBCs are incubated with RosetteSep Human Monocyte Enrichment cocktail that is designed to separate monocytes from other blood cells, by negative selection. It binds cell surface antigens on human hematopoietic cells, except monocytes, crosslinking unwanted cells with RBCs in immunorosettes that pellet along with the free RBCs when centrifuged over a density medium such as Histopaque. Following centrifugation, monocytes were collected, washed and cultured *in vitro*.

Primary human monocytes were allowed to differentiate into macrophages. Cell culture media was carefully changed at day 7, ensuring minimum cell disturbance and incubated for additional 3 days. At day 10, macrophage polarization was induced. For pro-inflammatory M1 stimuli, cells were treated with 10 ng/mL lipopolysaccharide (LPS), while for anti-inflammatory M2c stimuli, cells were treated with 10 ng/mL interleukine-10 (IL-10) and incubated for additional 3 days (Figure 8).

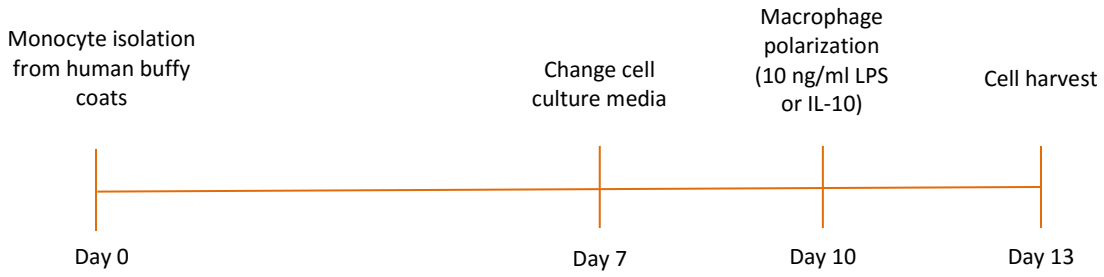


Figure 8 - Monocyte/macrophage *in vitro* culture and polarization. At day 0 monocytes were isolated from human buffy coats using RosetteSep and allowed to differentiate for 10 days. Medium was changed at day 7. Macrophage polarization was induced with 10 ng/mL of LPS or 10 ng/mL of IL-10 for M1 and M2c phenotypes, respectively. Cells were harvested 3 days after polarization, at day 13 of culture, and stained for flow cytometry or processed for RNA extraction.

2.1.2 THP-1 culture, differentiation and polarization

THP-1 monocytic cells were cultured in complete RPMI 1640 medium supplemented with 10% FBS + 1% P/S in an incubator at 37ºC/5% CO_2. In order to induce a macrophage-like phenotype, cells were plated in 24 well plates (0.250 x 10^6 cells per well) with complete RPMI 1640 medium containing 100 ng/mL of phorbol 12-myristate 13-acetate (PMA) and allowed to differentiate for 48h. Adherent cells were then rested in culture medium without PMA for 24 h to obtain the resting state of macrophages. For polarization, cells were stimulated with 100 ng/mL LPS + 20 ng/mL IFN-γ (Sigma).

2.1.3 Human umbilical artery smooth muscle cells

Human umbilical artery smooth muscle cells (HUASMC), kindly donated by Dr. Elisa Cairrão from *Centro de Investigação em Ciências da Saúde - Universidade da Beira Interior*, were cultured in Dulbecco's modified Eagle's F12 (DMEM-F12) supplemented with 5% FBS, epidermal growth factor (EGF, 5 µg/ml), fibroblast growth factor (FGF, 0.5 ng/ml), heparin (2 µg/ml) (all from Immunotools), and insulin (5 µg/ml) (Sigma). The cells were plated in T75 flasks previously coated with collagen and cultured in a humidified 37ºC/5% CO_2 incubator. The culture medium was changed every 2–3 days. Confluent cells were detached using trypsin and subcultures were performed until the eight passage.

2.2 Transfections

Differentiated THP-1 macrophages were transfected with Pre-miR miRNA Precursor miR-195-5p or with Pre-miR miRNA Precursor Negative Control (Scrambled - SCR) (Life Technologies) using Lipofectamine 2000 transfection reagent (Invitrogen) or HiPerFect Transfection Reagent (Qiagen). Briefly, miRNA mimics or SCR control were diluted in Opti-

MEM. A second reaction was prepared by incubating Lipofectamine 2000 in Opti-MEM for 5 min at room temperature (2 µL Lipofectamine 2000 per 50 µL Opti-MEM). Lipofectamine 2000 was then added to miRNA mimics and incubated for 20 min at room temperature to allow formation of complexes. Finally, solution was added to the cells with RPMI media without antibiotics. Final miRNA mimics concentration was 50 nM per well.

2.3 Flow cytometry

Differentiated and polarized macrophages derived from primary monocytes or THP-1 cells were harvested with 5mM EDTA-PBS and centrifuged at 300 g for 5 min, at 4ºC. Cells were resuspended in FACS buffer (PBS 1x, 2% FBS, 0,01% azide) and staining was performed in a final volume of 50 µL by incubating fluorochrome-conjugated antibodies with cells for 20 min at 4ºC in the dark. For primary human macrophages, CD14 was used as monocytic cell lineage marker while CD86 and CD206 were used as M1 and M2 specific markers, respectively. The following antibodies were used: anti-CD86-FITC (Immunotools, 2 µL/sample), anti-CD14-APC (Immunotools, 2µL/sample) and anti-CD206-PerCPCy5 (BD Biosciences Pharmingen, 5µL per sample). Unlabeled macrophages and labelling with isotype controls IgG1-FITC and IgG1-APC were used to define background staining.

To evaluate levels of TLRs in THP-1 transfected macrophages the following antibodies were used: anti-CD11b-PE, anti-TLR4-Alexa Fluor 488 (both from eBioscience, 2µL of each/sample) and anti-TLR2-Alexa Fluor 647 (Biolegend, 2µL/sample). Unlabeled THP-1 macrophages and isotypes IgG1-PE, IgG2-FITC and IgG1-APC stainings were used as control.

To perform HUASMC characterization by flow cytometry, adhered cells were detached using trypsin. Cells were resuspended in FACS buffer and stained as follows: single staining with anti-CD90-APC as positive marker (eBioscence, 5 µL/sample), CD10-APC and CD34-FITC as negative markers (Immunotools, 2 µL of each/sample); double staining with anti-CD90-APC and anti-CD34-FITC; unstained and IgGs (IgG1-APC and IgG1-FITC, Immunotools) as negative controls.

Fluorescence was analyzed using FACS Canto II flow cytometer (BD Biosciences Pharmingen) with BD FACS Diva software, and 10 000 events were acquired per sample. Results were analyzed using FlowJo Software (http://www.flowjo.com/).

2.4 Inverted fluorescence microscopy

Presence of two important structural proteins, α-smooth muscle actin (α-SMA) and vimentin, in HUASMC was evaluated by fluorescence microscopy. $2.5x10^3$ HUASMC were plated in top of 15 mm coverslips. Cells were then fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100 for 5 min, washed with PBS and incubated with 1% BSA for 30 min. Staining was performed by incubating the cells for 1h with primary anti-α-SMA (Sigma) and anti-vimentin (Ab-2) (Thermo Scientific), diluted 1:400 and 1:100 in 1% BSA

respectively. Following washing, cells were incubated with 10 µg/mL of secondary anti-mouse Alexa 647 for 30 min. Finally, coverslips were placed on a microscope slide with the cells facing up. Fluoroshield with DAPI (4',6-diamidino-2-phenylindole) mounting media (Sigma) was added to stain HUASMCs nuclei. Images were acquired using a 40x oil objective in an Axiovert 200M inverted fluorescent microscope (Zeiss).

2.5 RNA extraction

Total RNA was extracted from macrophages using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cell pellet was disrupted with 1ml TRIZOL and incubated for 5 min at room temperature. Next, 0,2 mL chloroform were added to the cells, inverted vigorously for 15 seconds and incubated for 5 min at RT. In order to obtain 3 phases, where the aqueous upper one contains RNA, a centrifugation 12000g for 15 min at 4ºC was performed. The upper phase was collected to a clean minicentrifuge tube. To precipitate RNA, sample was incubated for 5 min at RT with 0.5 mL of isopropanol. A second centrifugation was performed to obtain a RNA pellet which was washed with 1ml 75% ethanol. Sample was centrifuged 7500 g for 5 min at 4ºC and supernatant was discarded. Finally, RNA pellet was resuspended in 50 µL of DEPC-treated H_20.

RNA concentration was evaluated by measuring absorbance at 260 nm in NanoDrop 1000. Ratio of 260/280 nm and 260/230 nm range was between 1.8 and 2 suggesting no contamination by proteins or ethanol.

2.6 Reverse transcription and real-time quantitative polymerase chain reaction

miRNA expression was evaluated by Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) using TaqMan miRNA assays. Firstly, cDNA was synthesized using 60 ng of RNA, TaqMan MicroRNA Reverse Transcription Kit (containing buffer, dNTPs, RNAse inhibitor and reverse transcriptase) and gene specific stem-loop Reverse Transcription primers (miR-195 and small nuclear RNA U6) (Applied Biosystems). The reaction was performed in a thermal cycler (MyCycler Thermal Cycler, Bio-Rad) using the following conditions: 16ºC – 30 min; 42ºC – 30 min; 85ºC - 5 min. cDNA samples were diluted in 50 µL nuclease-free water and qPCR reactions prepared in a 96-well PCR plate using cDNA, miR-195 or small nuclear RNA U6 TaqMan probe and SsoAdvanced™ Universal Probes Supermix (Bio-Rad). RT-qPCR was carried out in iQ5 Real-Time PCR Detection System (Bio-Rad) for an initial 10 min at 95ºC followed by 40 cycles of 15 seconds at 95ºC and 1 min at 60ºC. Experiments were performed in duplicate and small nuclear RNA U6 was used as reference gene. Relative expression levels were calculated using using the quantification cycle (C_q) method, according to MIQE guidelines.

2.7 Western blot

2.7.1 Protein extraction and quantification

Expression of proteins involved in TLR pathways was analyzed by western blot (WB). After transfections and/or stimulation with 100 ng/mL LPS + 20 ng/mL IFN-γ, THP-1 macrophages were harvested. Cells were lysed for 30 min on ice using lysis buffer (50 mM Tris-HCl - pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS) supplemented with protease inhibitors (200mM pMSF, 1mg/mL Leupeptin, 1mg/mL Aprotinin) and phosphatase inhibitors (50 mM $NaVO_3$, 50 mg/mL $Na_4O_7P_2$, 10mM NaF 100x). Samples were centrifuged at 14000 rpm and supernatant containing protein was collected. Proteins were quantified by Lowry Method [204].

2.7.2 Protein electrophoresis, blotting and detection

After quantification, 20 ng of protein was added to buffer composed by 2-mercaptoethanol (ßME), (Sigma) and 4X Laemmli Buffer (BioRad) containing sodium dodecyl sulfate (SDS). Samples were denatured at 94ºC for 5 min in a thermoblock and immediately placed on ice.

Protein was run by SDS-Polyacrylamide Gel Electrophoresis (PAGE). The electrophoresis system was assembled, filling the inner space of the running module and the electrophoresis tank with running buffer 1x (prepared from 10x Tris/Glycine/SDS running buffer, Bio-Rad). Then, molecular weight standard and samples were loaded in the *stacking gel* wells. Electrophoresis was performed at 80V until the samples reach the resolving gel and at 100V until the end of the gel. Protein transfer (blotting) was performed at 100V for 1hour.

Once blotting was completed, membrane was subject to immunodetection. Firstly, membrane was stained with Ponceau solution (0.2% w/v Ponceau, 5% acetic acid in H_2O) in order to evaluate the effectiveness of the transfer. Ponceau staining was completely removed by washing with TBS 1X-Tween 0.1% (Tris-base and NaCl in H_2O and Tween 20) and the membrane was incubated in a blocking solution (Bovine Serum Albumin (BSA) 5% in TBS-Tween 0.1%) at room temperature for 1 hour. Membranes were incubated overnight at 4ºC with anti-p38 MAP Kinase, anti-phospho-p38 MAP Kinase (Thr180/Tyr182), anti-phospho-SAPK/JNK (Thr183/Tyr185), and anti-α-tubulin (all from Cell Signalling Technology), diluted at 1:1000 in 2% milk TBS-Tween 0.1%. Next, membrane was washed in TBS-Tween 0.1% for 30 min and then incubated with secondary antibody Horseradish Peroxidase (HRP) labelled anti-rabbit (total p38, ph-p38, ph-SAPK/JNK and GAPDH) or anti-mouse (α-tubulin) both diluted 1:10000 in 2% milk TBS-Tween 0.1%, for 1 hour at room temperature. The membrane was washed as previously indicated and detection performed using Amersham Enhanced Chemiluminescence (ECL) Western blotting detection reagents, from GE Healthcare (Figure 9). Membrane was transferred to an acetate sheet inside an X-ray film Hypercassette (GE Healthcare). In order

to obtain optimal detection, X-ray films were exposed for different time points in a dark room. Final developed films were analyzed using FIJI software (http://www.fiji.sc/).

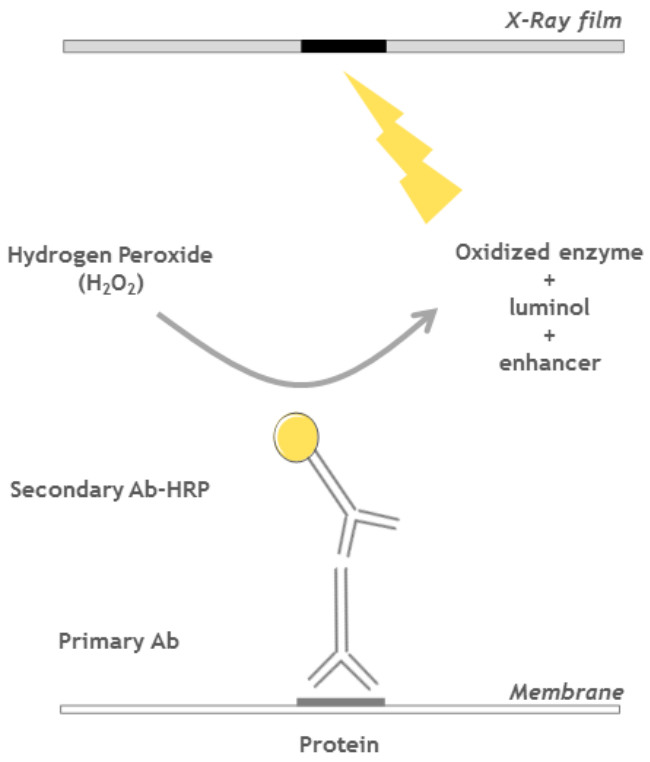


Figure 9 – Principle of ECL Western Blotting detection. Enhanced chemiluminescence is based on the emission of light during the horse radish peroxides (HRP) and hydrogen peroxide-catalyzed oxidation of luminol. The emitted light is captured on an X-ray film for qualitative or semi-quantitative analysis.

2.8 Human umbilical artery smooth muscle cells recruitment: migration assay

To evaluate miR-195 effect on recruitment of HUASMC by THP-1 macrophages, transwell migration assays were performed. Firstly, THP-1 cells were differentiated, transfected with miR-195 mimics or control and stimulated with 100 ng/mL LPS + 20 ng/mL IFN-γ in 500 µL of DMEM F12 without FBS for six hours (bottom compartment - BC). Then, $1x10^5$ HUASMC in 500 µL of DMEM F12 media without FBS were plated in Boyden chambers (inserts) with PET membranes of 8 µm pore size previously blocked with sterile 1% BSA (top compartment – TC) (Figure 10). To evaluate HUASMC recruitment, the following conditions were used: only stimulated THP-1 macrophages, THP-1 macrophages transfected with SCR and stimulated and THP-1 macrophages transfected with miR-195 and stimulated. Controls used were: only DMEM F12 with LPS+IFN-γ in the BC (without THP-1 macrophages) and only differentiated THP-1 macrophages (non-transfected neither stimulated).

HUASMC were allowed to migrate through the membrane for 24h. Then, culture media was removed and membranes washed two times with PBS 1X. Cells were fixed with 4% PFA for 15 min at RT. Insert membranes were washed again with PBS 1x and the inner side of the membrane was scrubbed with a cotton swab to remove non migrated cells. Membrane was removed and placed on a microscope slide, with the side containing migrated HUASMC facing up, and Fluoroshield with DAPI (Sigma) was used as mounting media. Migrated HUASMC nuclei were counted in the inverted fluorescence microscope. Six random fields per membrane were selected and the results represent the sum of migrated cells in all the fields.

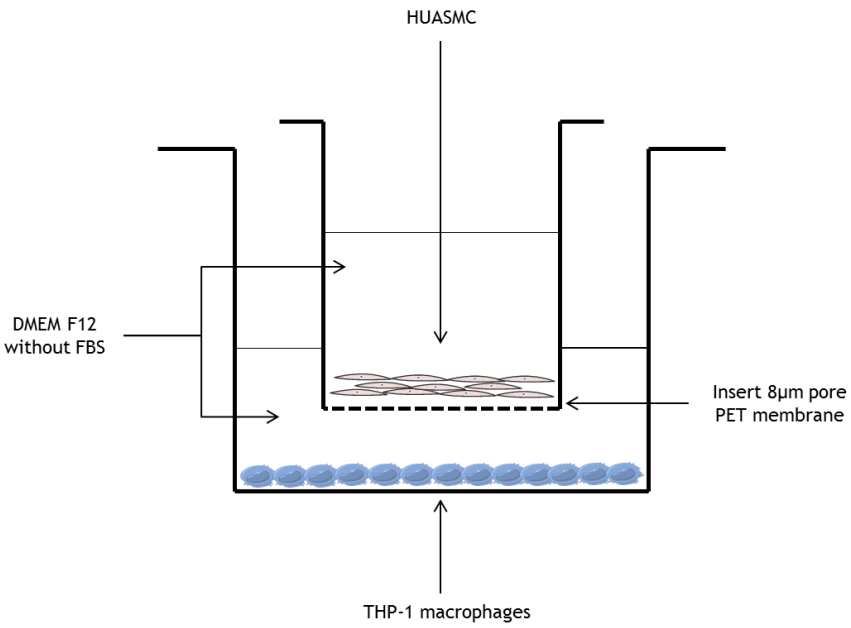


Figure 10 - Schematic representation of migration assay. THP-1 macrophages in DMEM-F12 without FBS were plated in the wells of a 24 well plate (bottom compartment) and subjected to different stimuli. HUASMC were plated in inserts with PET membranes of 8 µm pore size (top compartment) and were allowed to migrate for 24 hours.

2.9 *In silico* target predictions

In order to analyze potential targets of hsa-miR-195-5p, *in silico* predictions were performed by screening the following databases:

miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/);

miRanda (http://www.microrna.org/microrna/home.do);

RNA22 (https://cm.jefferson.edu/rna22/Interactive/);

TargetScan (http://www.targetscan.org/vert_71/).

The search focused on genes involved in inflammatory pathways. miRNA FASTA format sequence annotations were obtained from the miRBase database (http://www.mirbase.org/). Target genes transcripts FASTA format sequence annotations were obtained from Ensemble (http://www.ensembl.org/). Only human protein coding transcript sequences were selected. The name of the target gene, the transcript ID and the interaction site were considered.

2.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GaphPad Software, Inc.). D'Agostino-Pearson omnibus normality test was used to evaluate if the values follow a Gaussian distribution. In positive cases, paired t-test or one-way ANOVA were used to compare the samples. When data no pass the normality test, non-parametric test of Kruskal-Wallis, followed by Dunns post-test comparing of user defined sets of data, were used to evaluate significant differences between the different samples. Statistical significance was considered when $p<0.05$ (* - $p<0.05$; ** - $p<0.01$).

Chapter 3

Results

3.1 miR-195 is involved in macrophage polarization

At day 10 of *in vitro* culture, primary human monocytes are differentiated into macrophages. Cells were adherent to the plate with some cells showing a typical spindle-like morphology, while others exhibit a "fried egg" shape (Figure 11A). This was confirmed by flow cytometry, where more than 90% of the cells were positive for CD14, the monocyte/macrophage-lineage cell marker (Figure 11B), considering the IgG1 negative control. Therefore, monocytes were efficiently isolated from human buffy coats and successfully cultured *in vitro* for differentiation into macrophages.

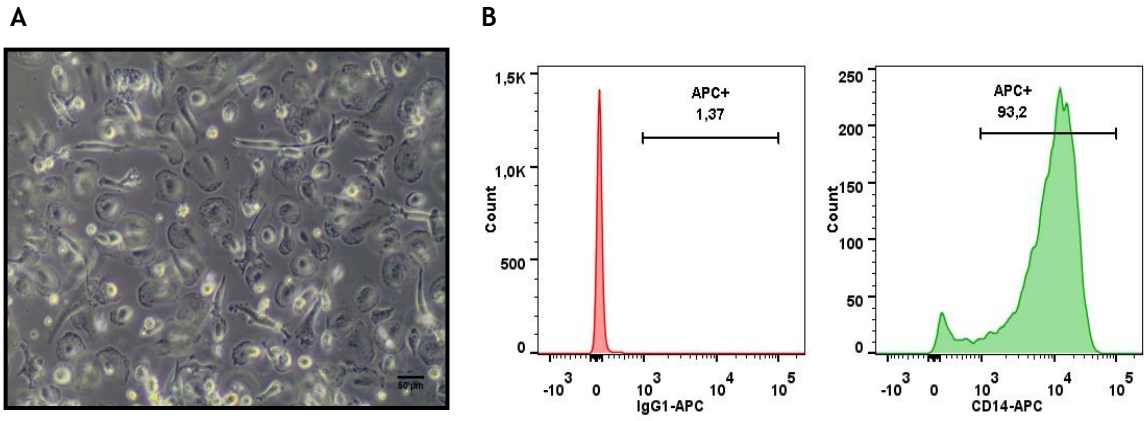


Figure 11 - Human primary monocyte isolation and differentiation. A) Microscopy image of macrophages differentiated from monocytes at day 10 of culture (magnification of 200X). Scale bar 50µm. B) Flow cytometry analysis of CD14 marker in macrophages.

Next, to induce macrophage polarization into pro-inflammatory M1 or anti-inflammatory M2c phenotypes, cells were treated with 10 ng/mL LPS or 10 ng/mL IL-10, respectively. Macrophage polarization was evaluated by flow cytometry using anti-CD86 and anti-CD206 antibodies for M1 or M2c like macrophages. A representative profile is shown in Figure 12A. When the mean fluorescence intensity was quantified, results from 9 independent donors show significantly higher levels of cell surface marker CD86 in LPS-treated macrophages (mean fluorescence intensity = 856 ± 474) compared with IL-10-treated macrophages (mean fluorescence intensity = 334 ± 219) or with the non-treated macrophages (mean fluorescence intensity = 411 ± 240) ($p<0.01$) (Figure 12B). On the other hand, LPS-treated cells show significant lower levels of the mannose receptor CD206 (mean fluorescence intensity = 3332 ± 1221) than IL10-treated cells (mean fluorescence intensity = 5099 ± 1824) ($p<0.05$). No significant differences were found between IL-10-treated macrophages and control group (mean fluorescence intensity = 5135 ± 2236) (Figure 12B). This may be partially explained if cells in the controls acquire a more M2-like phenotype.

Globally, LPS-treated macrophages show higher CD86 and lower CD206 levels than IL-10-treated macrophages, which allow to separate these cells into two subsets.

After optimization of monocyte isolation, differentiation and macrophage polarization procedures, we search for miRNA with novel roles in macrophage polarization. Total RNA from polarized macrophages from 9 different donors was isolated, but 2 donors were excluded from the analysis due to poor quality and low quantity. Particularly, we evaluated the expression levels of miR-195 in polarized macrophages because this miRNA plays a role in the cardiovascular system by inhibiting VSMC proliferation and migration [174], but its effect on inflammation modulation is unknown.

Result show that miR-195 is significantly overexpressed in IL-10 treated macrophages compared with control (2.1 fold increase) or LPS treated (1.6 fold increase) groups ($p<0.05$) (Figure 12C). Importantly, no significant differences in U6, which was used as a reference gene, were detected (Figure 12C). In conclusion, miR-195 is involved in M2 polarization following Il-10 stimulation.

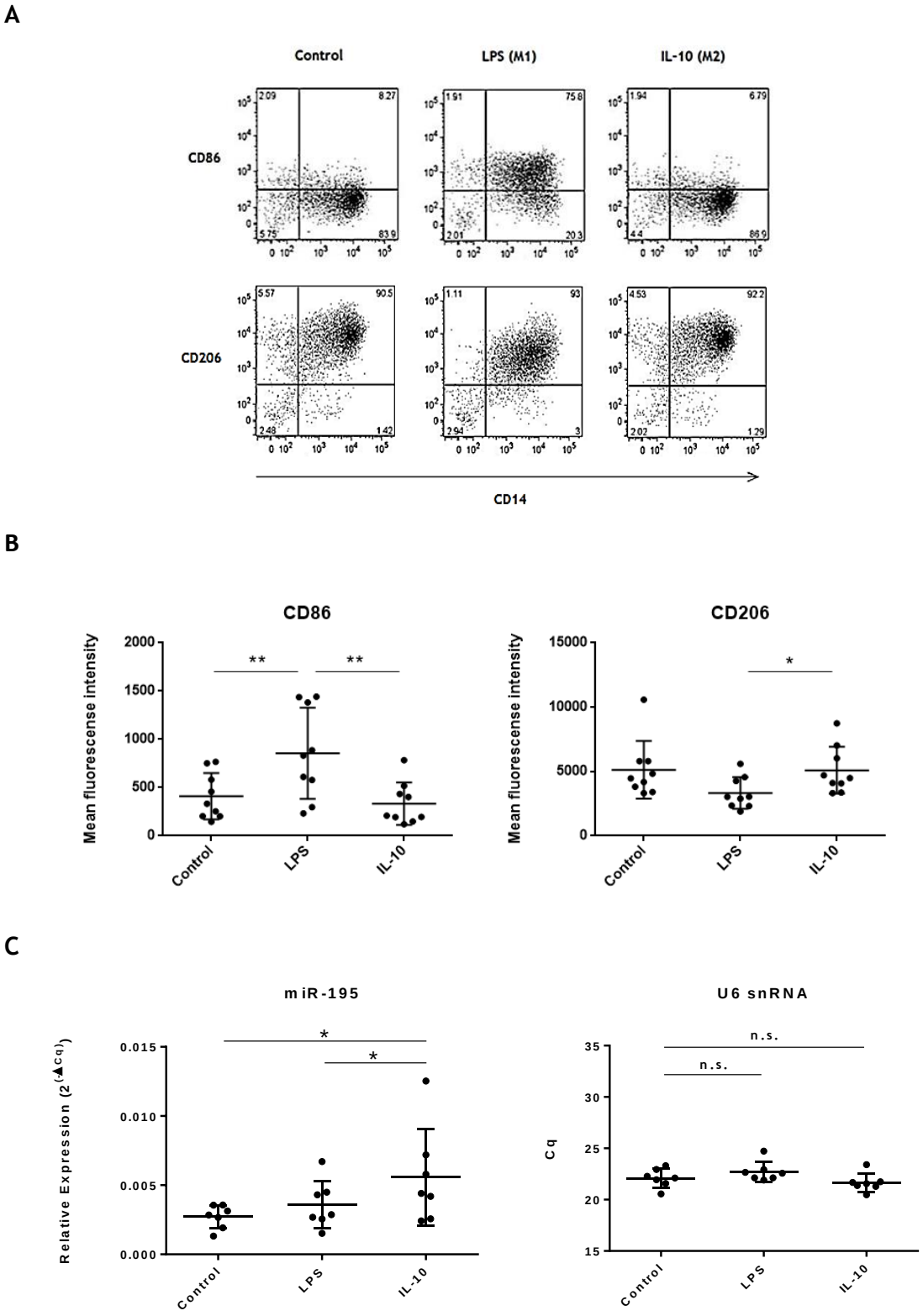


Figure 12 – miR-195 expression levels in polarized macrophages. Macrophages were differentiated for 10 days and polarized for further 3 days in presence of the indicated stimuli. A) Representative profile of macrophage polarization as analyzed by flow cytometry. B) Quantification of the mean fluorescence intensity of the M1 marker CD86 and the M2 marker CD206, across 9 different individuals, following LPS or IL-10 stimulation. Graphics represent mean fluorescence intensity values ± SD. C) Expression of miR-195 by RT-qPCR in control, LPS and Il-10 treated macrophages, across 7 different donors. U6 was used as reference gene. Cq: quantification cycle. Statistical significance was considered when $p<0.05$ (* - $p<0.05$; ** - $p<0.01$; n.s. – non-significant).

3.2 Target predictions for miR-195 in TLR inflammatory pathway

Following the previous results, we identified miR-195 as a potential anti-inflammatory mediator. Thus we determined if TLRs and other downstream proteins involved in the inflammatory pathway were predicted as a direct miR-195 target.

Complementary base pair interactions between hsa-miR-195-5p sequence and transcript sequences of selected proteins were analyzed using four databases, which evaluate predicted interactions based on algorithms and validated miRNA-target interactions. miRWalk and RNA22 databases predict TLR2 as a target of miR-195 whereas ERK, JNK and p38 - all mitogen-activated protein kinases during TLR signaling pathway - are also miR-195 target candidates by other algorithms/databases (Table 1). Moreover NFKB1 (p50) – a transcription factor downstream to the above mentioned kinases – is predicted by all the explored databases.

Table 3 - Target predictions for hsa-miR-195-5p in different databases.

Target Gene	Transcript ID (Ensemble)	Interaction site (by RNA22 and TargetScan)	Databases				
			miRWalk	miRanda	RNA22	TargetScan	SUM
TLR2	ENST00000260010	GCTG--TGCTCTGTTCCTGCTG ||:: ||||| ||||: CGGTTATAAAGACA-CGACGAT	1	0	1	0	2
MAPK1 (ERK2)	ENST00000215832	GCAAGTCTTTTTAAATGCTGCTT || |:| |||:| |||||| CGGTTATAAAGA--CACGACGAT	1	1	1	1	4
MAPK3 (ERK1)	ENST00000263025	TTCAATCTCCCGCTGCTGCTGCTG :|||| ||| ||||||: CGGTTA--TAAAGAC-ACGACGAT	1	1	1	1	4
MAPK8 (JNK1)	ENST00000374189	GTCATGCACCTTTG-GCTGCTA |:|| |:|| ||||| CGGT-TATAAAGACACGACGAT	1	1	1	1	4
MAPK9 (JNK2/SAPK)	ENST00000452135	ATCTTCCAGGTAGTGCTGCTT : : ||||||| CGGTTATAAAGACACGACGAT	1	1	0	1	3
MAPK11 (p38-β)	ENST00000330651	TCC-TGGTTTACCAGCTGCTG || :||| |||||: CGGTTATAAAGACACGACGAT	1	0	1	0	2
MAPK12 (p38-γ)	ENST00000622558	GCCTTTCCAGTCCGAGCTGTTC ||| || | ||||:| CGG-TTATAAAGACACGACGAT	1	1	1	1	4
MAPK13 (p38-δ)	ENST00000211287	ACCGG--GAGCTGCTGCTGCTG ||:: ||| |||||||: CGGTTATAAAGAC-ACGACGAT GGCAGTGATGCTGTGTTGGTT | ||:|: |||||:|| | CGGTTATAAAGACACGACGAT	1	1	1	1	4
MAPK14 (p38-α)	ENST00000229795	ACC-TGGTTTCTGTTCTGTTG || :||||||| |||:|: CGGTTATAAAGACACGACGAT	1	0	1	1	3
NFKB1 (p50)	ENST00000226574	GGCGGTG--GTAGTGGTGCTG | |::|: ||| ||||: CGGTTATAAAGACACGACGAT	1	1	1	1	4

3.3 miR-195 reduces TLR2 levels in THP-1 macrophages

To address the biological effect of miR-195 in macrophage phenotype, gain-of-function studies were performed. In order to obtain high transfection efficiency, the monocytic THP-1 cell line was used, instead of primary macrophages. THP-1 monocytes were plated with 100 ng/mL of PMA and allowed to differentiate into macrophages for 48 hours. As shown in Figure 13, differentiated cells acquire a macrophage-like morphology and become adherent to the plate.

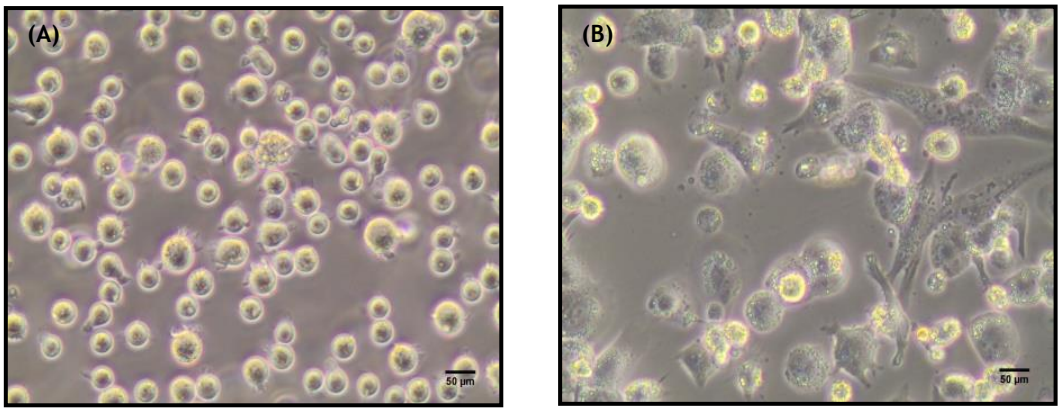


Figure 13 - THP-1 macrophages differentiation represented in two stages. (A) Cells immediately after 100 ng/mL PMA addition (magnification of 200X). Scale bar 50 µm; and (B) fully differentiated THP-1 macrophages after 48 hours of incubation with PMA (magnification of 200X). Scale bar 50 µm.

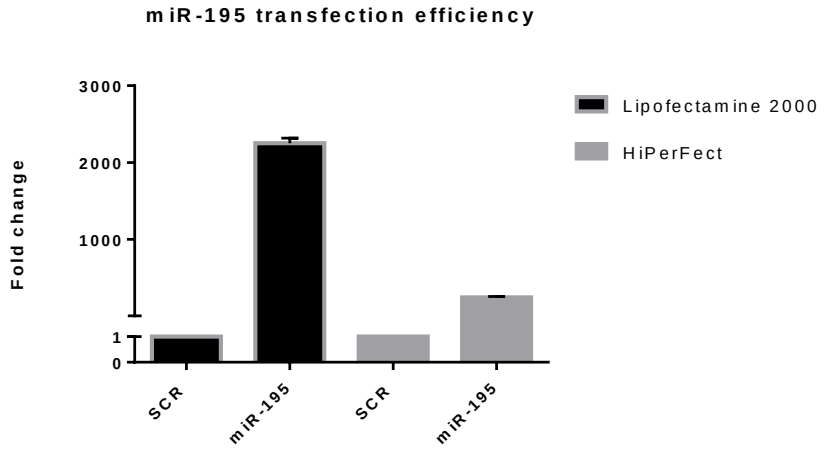
Transfections of miR-195 mimics or a negative control (SCR) were tested using two different transfection reagents namely, Lipofectamine 2000 (Invitrogen) and HiPerFect (Qiagen). RT-qPCR results show that both reagents successfully transfected the cells. However, Lipofectamine 2000 was the most efficient, leading to higher miR-195 expression levels than HiPerFect reagent (Figure 14A). For this reason all the subsequent experiments were performed using this transfection reagent.

Knowing that TLRs activation is implicated in atherogenesis, by starting signaling cascades that culminate in the production of pro-inflammatory cytokines, foam cell formation and activation of adaptive immunity, we next evaluated the expression of 2 TLRs. TLR2 that was predicted as a direct target of miR-195, and TLR4, the most studied TLR and a receptor for LPS stimulation. After transfection, THP-1 macrophages were stimulated with pro-inflammatory stimuli and harvested after 36 hours. Results illustrated in Figure 14B (left) show that levels of TLR2 increase slightly upon stimulation for M1 phenotype. Importantly, when cells were transfected with miR-195 before M1 stimulation there was a significant decrease in TLR2 levels, compared with either M1 macrophages - 27% reduction - and M1 macrophages transfected with SCR - 29% reduction ($p<0.05$). Levels of TLR4 were also evaluated. TLR4 decreased at the cell surface upon LPS (M1) stimulation, as expected due to

receptor internalization. However, despite it no longer decreasing in M1 miR195-macrophages, this result was no different from that obtained with M1 SCR-transfected macrophages (Figure 14B, right).

Taken together these results show that THP-1 are amenable to transfection, and miR-195 reduces levels of TLR2 in pro-inflammatory conditions.

A



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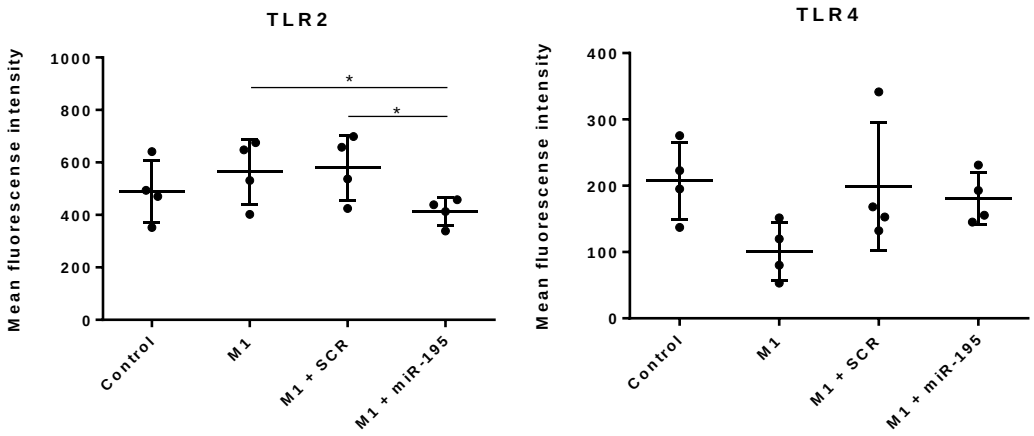


Figure 14 - miR-195 transfection and impact on TLR2 and TLR4 levels upon pro-inflammatory stimuli. A) Transfection efficiency for miR-195 mimics in THP-1 cells compared with scrambled control (SCR) was evaluated by RT-qPCR. Transfections were performed using Lipofectamine 2000 or HiPerFect Reagent. B) TLR2 (left) and TLR4 (right) levels on THP-1 were evaluated by flow cytometry after transfection with either miR-195 or SCR and after induction of M1-like macrophages with LPS and IFN-γ. Results from four independent experiments are shown (n=4). Statistical significance was considered when * - $p<0.05$.

3.4 miR-195 inhibits TLR2 signaling pathway

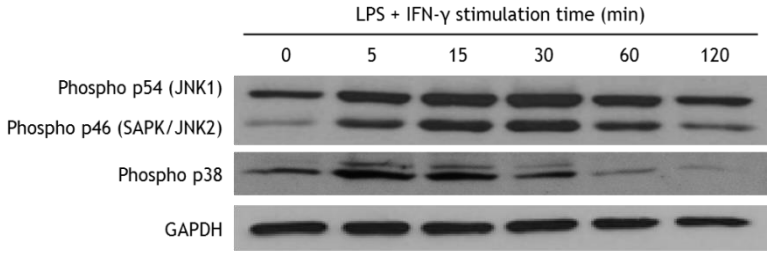
To evaluate if TLR2 downstream proteins are regulated by miR-195, protein levels of p54 (JNK1), p46 (SAPK/JNK2) and p38 were analyzed by Western Blot.

Aiming to understand the maximum expression level, we first determined the levels of the phosphorylated forms of these proteins in different time points after M1 (LPS+IFN-γ) stimulation. Thus, differentiated THP-1 macrophages were induced by pro-inflammatory stimuli and cells were collected at different time points – 0, 5, 15, 30, 60 and 120 minutes. Results show that, under M1 stimuli, phospho p54 (JNK1), phospho p46 (SAPK/JNK2) and phospho p38 are generally expressed in the highest levels in time points 15 and 30 minutes (Figure 15A). These time points were selected for the subsequent experiments.

We evaluated the levels of phosphorylated protein and total protein. Although differences in total p38 were not evident, phospho p54, phospho p46 and phospho p38 were all clearly downregulated by miR-195 in two independent experiments. As illustrated in Figure 15B, C, phospho p54 and phospho p46 are downregulated in macrophages transfected with miR-195 compared to with the respective SCR controls after M1 stimulation for 15 and 30 minutes. After 15 minutes of M1 stimulation, macrophages overexpressing miR-195 exhibit a small reduction of 17% in phospho p54 (Fold change to SCR (Fc) = 0.8347 ± 0.1274) and 41% in phospho p46 levels (Fc = 0.5776 ± 0.0057) compared with M1 macrophages transfected with SCR. After 30 min of M1 stimulation, phospho p54 levels (Fc = 0.7512 ± 0.0189) show a 25% reduction while phospho p46 (Fc = 0.5776 ± 0.0261) maintains the reduced levels in miR-195-macrophages compared with SCR control. Regarding p38, both total and phosphorilated forms of the protein were tested. No differences were detected in total p38 protein levels (Fig. 5B, Annex 1 - Figure 21), which suggests p38 is not a direct target of miR-195. Regarding phospho p38 levels, after 15 minutes of M1 stimulation no differences are detected (Fc = 0.9517 ± 0.1409); however, 30 minutes after M1 stimulation, phosphorylated levels of this protein (Fc = 0.5905 ± 0.0216) are diminished by 41% in miR-195 transfected THP-1 macrophages (Figure 15B,C).

In conclusion, miR-195 impacts an inflammatory pathway through downregulation of phosphorylated forms of intracellular proteins downstream of TLR2.

A



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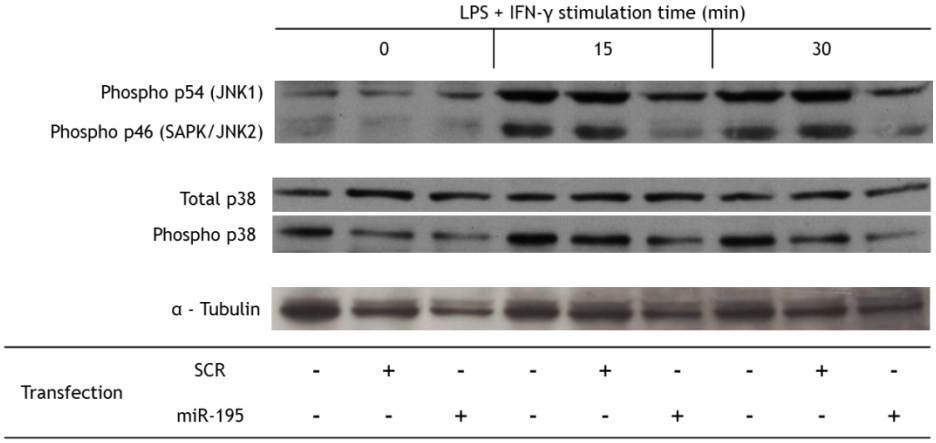

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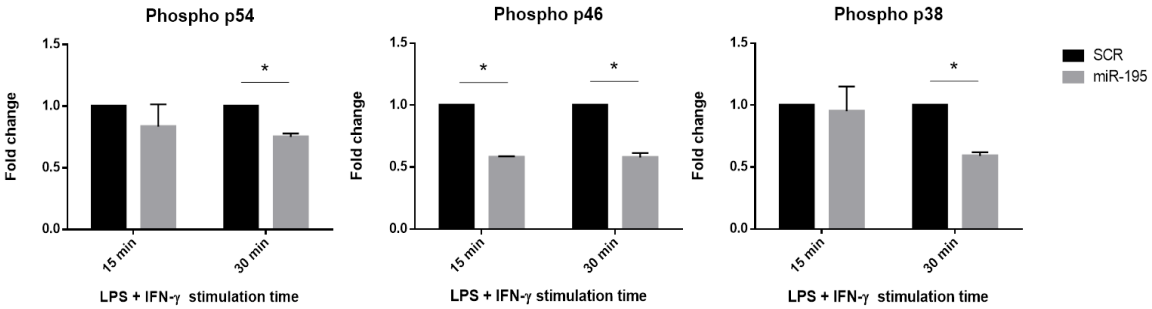


Figure 15 – miR-195 downregulates phospho p54, phospho p46 and phospho p38. A) Western blot for ph-p54, ph-46, ph-p38 after LPS+IFN-γ stimulation in different timepoints. B) Western blot for ph-p54, ph-46, total p38 and ph-p38 in 9 different experimental conditions. C) Quantification of ph-p54, ph-46, ph-p38 levels in two independent experiments. α-Tubulin was used as normalizer.

3.5 Expression of miR-195 in macrophages inhibits smooth muscle cells recruitment

The impact of miR-195 expression in macrophages was also evaluated at a functional level.

In advanced stages of atherosclerosis pro-inflammatory macrophages promote the recruitment of smooth muscle cells to the lesioned area which also become lipid loaded foam cells producing pro-inflammatory cytokines. Thus, we performed migration assays using human umbilical artery smooth muscle cells (HUASMC) to evaluate if miR-195-THP-1 macrophages under pro-inflammatory stimuli impact the capability to recruit these cells.

Firstly, culture conditions for HUASMC were optimized. Cells were cultured in T75 flasks previously coated with collagen until confluent. As shown in Figure 16, HUASMC grown in a collagen coated surface have increased proliferation and reach the confluence more easily than cells grown in non-coated surface. This was expected as it better mimics the *in vivo* growth environment of these cells.

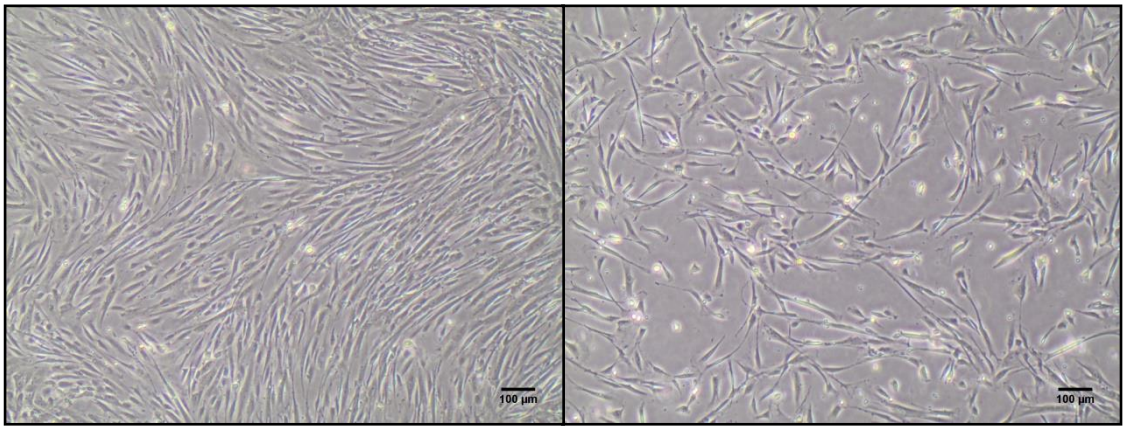


Figure 16 – Human umbilical artery smooth muscle cells culture. Left image shows HUASMC after 5 day culture in a pre-coated flask and right image represents the same cells in the same culture conditions in a non-coated flask (magnifications of 100X). Scale bar 100 µm.

We further characterized HUASMC cells evaluating by flow cytometry the levels of surface markers previously described to be part of the marker profile for the evaluation of HUASMCs [205]. Thus, CD90 was used as positive marker, CD10 and CD34 (endothelial marker) were used as negative markers. Results clearly show high levels of CD90 (99,9% $CD90^{+}$) in HUASMCs that, in turn, do not express CD10 nor CD34 (Figure 17). Furthermore, we evaluated, by inverted fluorescence microscopy, the expression of two important SMC structural proteins, α-SMA and vimentin. HUASMC positively express both markers, as shown in Figure 18A,B.

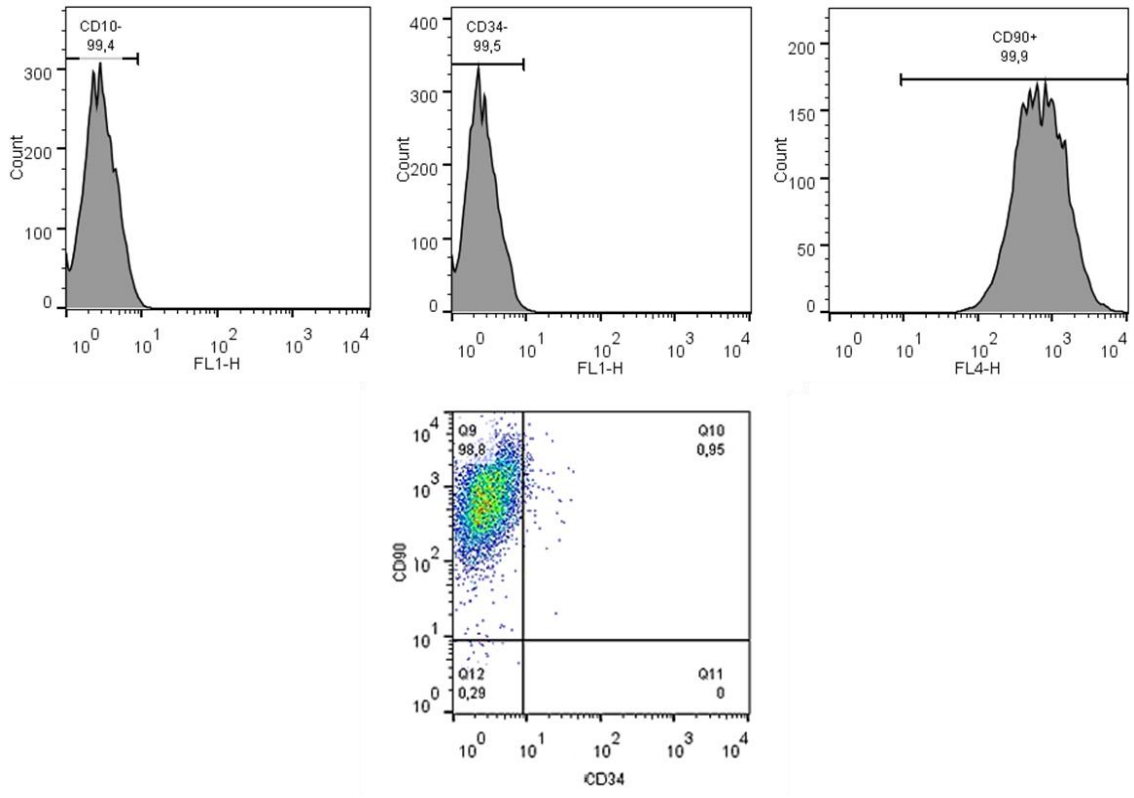


Figure 17 – Characterization of human umbilical artery smooth muscle cells by flow cytometry. Histograms represent flow cytometry results for CD10, CD34 and CD90. Dot plot graphic show double staining with CD90 and CD34.

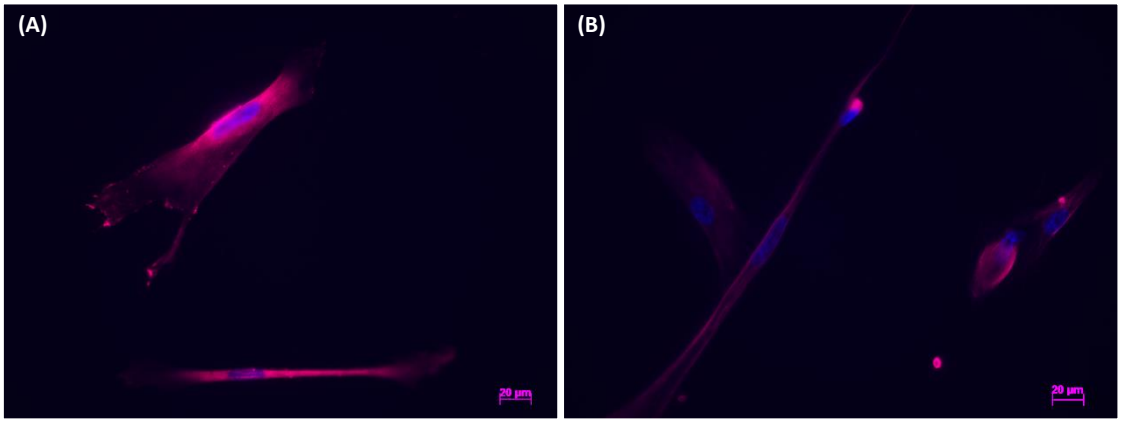
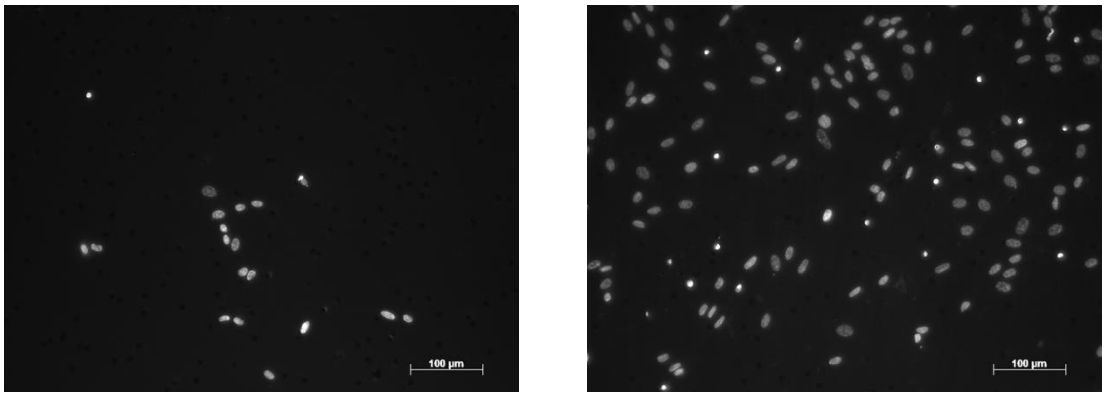


Figure 18 – Evaluation of human umbilical artery smooth muscle cells markers by inverted fluorescence microscopy. (A) Cells show positive staining for α-SMA marker. (B) Cells show positive staining for vimentin marker. Microscope images obtained using a 40x oil objective. Scale bar 20 µm.

Knowing that the HUASMC cells were expressing the correct markers we proceeded to evaluate their ability to migrate in response to macrophages transfected with miR-195.

First we evaluated the capacity of HUASMC to migrate in response to FBS, a non-specific stimulus, to optimize migration experiments. Two densities of cells (5 x 10^4 and 1 x 10^5 cells) were plated in serum-free medium in the top compartment of a transwell and were allowed to migrate for 6 or 24 hours, using FBS as a chemoattractant. Figure 19A illustrates the nuclei of the cells that crossed the membrane pores and are found on the bottom side of the transwell membrane. As expected, results show higher number of migrated HUASMC (7-fold difference) after 24 hours compared with 6 hours (Figure 19B). Furthermore, the initial number of HUASMC placed on the top compartment influenced the final number of migrated cells (Figure 19B). Thus, 1 x 10^5 HUASMC plated on top and time point of 24h performed the best and were the selected conditions for the following experiments.

A



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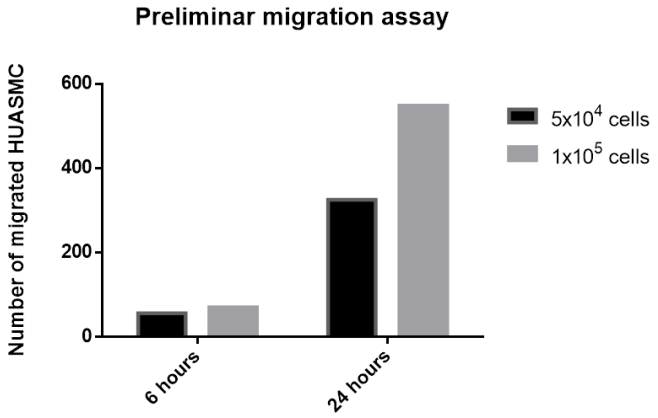
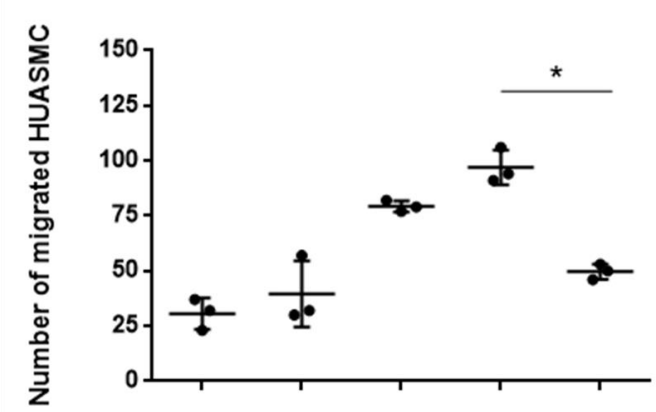


Figure 19 – Evaluation of human umbilical artery smooth muscle cells migration ability. A) Inverted fluorescence microscope images representing migrated cells stained with DAPI from an initial number of 1 x 10^5 HUASMC and allowed to migrate for 6 (left) or 24 hours (right). Scale bar 100 µm. B) Migratory profile of HUASMC in transwells following a nutrient gradient. FSB was used as a chemoattractant.

Finally, the effect of miR-195-THP1-macrophages on HUASMC migration was determined. Results show that THP-1 macrophages transfected with miR-195 in pro-inflammatory conditions have less capability to recruit HUASMC compared with non-transfected and SCR-transfected pro-inflammatory THP-1 macrophages ($p<0.05$). In fact, transfection of M1 macrophages with miR-195 reduced their capability to recruit HUASMC in almost 40% compared with non-transfected M1 macrophages and in 50% compared with M1 macrophages transfected with SCR (Figure 20).



THP-1 macrophages	-	+	+	+	+
LPS + IFN-γ stimulus	+	-	+	+	+
SCR	-	-	-	+	-
miR-195	-	-	-	-	+

Figure 20 - miR-195 reduces the capability of THP-1 macrophages to recruit human umbilical artery smooth muscle cells. HUASMC migration in different conditions. Experiments were performed in triplicates. Statistical significance was considered when * - $p<0.05$.

Chapter 4

Discussion

4.1 Primary cells *versus* cell lines *in vitro* cultures for the study of macrophage polarization

In the current work primary human monocyte-derived macrophages and macrophages differentiated from a human monocytic cell line were used. The efficiency of monocyte isolation from human buffy coats and the subsequent differentiation into macrophages can be affected by methodological procedures, but also by the biological variability associated with different individuals. In fact, this variability explains the need to test several different donors in order to establish a trend in the normal population. Primary monocytes/macrophages should provide experimental results most similar to natural biological mechanism than the use of cell lines [206]. Generally, primary cells retain normal morphology, cellular function, growth characteristics, cellular markers, signaling and genetic integrity when cultured for short periods of time, better replicating what happens *in vivo* [162, 207]. However, the short life time of these cells in culture, and their low tolerance levels to exogenous or environmental factors, such as transfections, make them difficult to manipulate. For that reason, in this study a monocytic cell line (THP-1 cell line) was used to perform transfection assays [171, 208, 209]. Cell lines by definition have been altered or "transformed" in some manner, either naturally or by manipulation in the laboratory, departing from the native cellular functions and growth characteristics found in normal primary cells [210]. However, although cell lines typically exhibit abnormal or uncontrolled metabolic function and proliferative capacities in culture as a result of intracellular changes, they were generated to answer particular research questions and have the benefits of lower cost, longer time in culture and elevated output signals for assay development [211]. In this work, we used THP-1 cell line which is a human monocytic cell line derived from acute monocytic leukemia patients. This cell line is a commonly used model in macrophages studies as these cells feature and share many monocyte/macrophages- like characteristics. When adhered and

differentiated into macrophages, these cells exhibit the heterogeneity characteristic of primary macrophages, with some cells presenting rounded shape and others presenting stretched forms [212]. Differentiated THP-1 macrophages also express surface receptors similar to primary macrophages such as CD11b, CD14, TLR2 and TLR4 [212]. The cytokine production profile of THP-1 cells and primary PBMCs can also be compared, as they produce similar levels of IL-1β and TNF-α after stimulation with LPS [213]. Moreover, they have been extensively used as model for transfections of macrophage like cells, exhibiting low cell death and high transfection efficiency [167, 177]. These similar characteristics make these cells the most attractive to substitute and overcome the practical issues associated to the primary macrophages cultures.

4.2 miR-195 as a modulator of inflammation

miRNAs play crucial roles in the development of cells of the immune system [214]. As discussed before, macrophages can present different phenotypes, depending on the cytokine environment they encounter. The gene expression profile of each macrophage subset has been lately studied in order to understand in which manner these differently expressed miRNAs influence the immune functions of polarized macrophages [215].

Previously, miR-195 was showed to play a role in cancer, by inhibiting cell cycle progression and proliferation of different cancer cell types [216–218], and in the cardiovascular system by regulating VSMC phenotype, migration and preventing neointimal formation [174]. Macrophages and SMCs represent major players in the pathogenesis of atherosclerotic vascular diseases. Activated pro-inflammatory macrophages promote pro-atherogenic functions of SMCs [219]. For this reason we evaluated if these anti-atherogenic miR-195 could act through macrophages, inhibiting their pro-inflammatory action, and conditioning their relation with SMCs.

Herein, we describe for the first time that miR-195 is overexpressed in IL-10-treated macrophages. This novel finding lead us to further explore its biological function and investigate if increased expression of miR-195 could inhibit the mechanisms involved in the M1 macrophage polarization, following other described miRNAs such as miR-125a, which is known to inhibit the classical macrophage activation and to decrease secretion of some inflammatory cytokines (IL-6 and TNF-α) [135, 169], and miR-146a known to target TLR4 and thereby inhibiting the activation of TLR4-dependent signaling pathways [135].

4.3. miR-195 in TLR signaling pathway

TLRs recognize distinct pathogen-associated molecular patterns and play a critical role in innate immune responses [40]. These surface receptors participate in the first line of defense against invading pathogens and play a significant role in inflammation, immune cell regulation, survival, and proliferation [220]. The activation of TLR-dependent signaling pathways promotes the consecutive phosphorylation of several molecules leading to activation of MAP kinases (JNK, p38 MAPK) and NF-κB. At the end of the TLR signaling cascade, in the nucleus, transcription factors are activated inducing the production of pro-inflammatory cytokines, such as IL-1ß, IL-6, IL-8 and TNF-α [40, 221].

We investigated if the miR-195 could affect these pathways, starting from the receptor and progressing to the intermediate molecules involved. The results from computational miRNA target prediction algorithms included many potential targets, but TLR2, MAP kinases, JNK/SAPK and p38 genes were selected as they yielded particularly high scores. Thus, we analyzed the expression of TLR2 and the results showed a significant reduction of this receptor in THP-1 macrophages transfected with miR-195, suggesting that the expression of this surface receptor can be regulated by miR-195. TLR2 expression regulation through miRNAs has been described by other authors. Philippe *et al*. proved by luciferase assays that Tlr2 mRNA is a direct target of miR-19 in rheumatoid fibroblast-like synoviocytes, and Benakanakere *et al*. showed modulation of TLR2 expression in human gingival epithelial cells by miR-105 [222, 223]. However, to the best of our knowledge, miRNAs targeting TLR2 in human macrophages were so far unknown.

Following in the same line, the levels of p54 (JNK1), p46 (JNK2/SAPK) and p38 MAPK proteins were investigated by western blot. Algorithms can predict hundreds of transcripts as potential miRNA targets *in silico* [224]. However, generally only a small portion is experimentally validated [225–227]. Although p38 total protein levels were not altered, the activated/phosphorylated form was visible downregulated by miR-195 as well for p54 (JNK1) and p46 (JNK2/SAPK) activated/phosphorylated forms. JNK isoforms and p38 MAPKs have similar mechanisms of activation [228, 229]. Specifically, both are activated by small GTPases of the Rho family, such as CDC42 [230]. This transcript has already been reported to be a target of miR-195 [174], which could partially explain the inhibition of ph-p38, ph-p46 and ph-p54, that we detected by western blot.

Together these results indicate that miR-195 efficiently inhibits the pro-inflammatory TRL signalling pathway, specifically p38 and JNK pathways, reinforcing the idea that this specific miRNA acts as an anti-inflammatory agent in macrophages.

4.4 miR-195 in smooth muscle cells recruitment by macrophages

In advanced states of atherosclerotic lesions, macrophages secret high levels of pro-inflammatory cytokines and chemokines which perpetuate the inflammatory process and promote migration of VSMC into the intima [91]. There, SMC proliferate and secrete pro-fibrotic mediators, resulting in the fibrous cap formation. At some stages this fibroproliferative response is believed to be a defensive, protective, physiologic response to injury, designed to wall off, contain, enclose, or sequester the injurious agent, and then to assist in resolution of the injury [231]. However, atherosclerotic SMC are described to switch to a macrophage-like phenotype also by uptake lipids [232]. As result of lipid over-ingestion, resident and recruited SMC die because of toxic chemical agents, such as oxidized LDL. The death of the SMC leads to discharge of the ingested lipids and other cellular elements into the extracellular space, and to the degeneration of non-viable fibrous tissue [16]. At this point the fibrous cap covers a mixture of apoptotic leukocytes, lipid, and debris which may form a necrotic core that thins the fibrous cap, already diseased and weakened by dysfunctional SMC, leading to plaque rupture [231]. Thus, the fibrous cap has become a pathologic component of the disease process, and not a protective structure designed to enclose the necrotic core.

Previous work had shown that miR-195 can directly inhibit SMC proliferation and migration [174]. Moreover, our migration assay results show that also macrophages overexpressing miR-195 lose their ability to promote SMC recruitment. We concluded that miR-195-overexpressing macrophages stimulated with a pro-inflammatory stimulus recruited significantly less SMC than M1 macrophages transfected with SCR or the non-transfected M1 macrophages. This is the first evidence showing that modulation of macrophage polarization thought miR-195 has a paracrine effect on SMC.

Although chemokines and cytokines secreted by miR-195-macrophages were not determined so far, it is likely that pro-inflammatory cytokines such as MCP-1, IL-1β, IL-8 and/or TNF-α, known to promote the recruitment and proliferation of SMC, are decreased [233, 234].

Chapter 5

Conclusions and future perspectives

The results discussed above indicate that miR-195 may have a dual role in atherosclerosis and other cardiovascular diseases. On one hand, it modulates inflammation by decreasing TLR2 pro-inflammatory pathway in macrophages. On the other hand, miR-195 reduces macrophage capacity to recruit SMC. Therefore, we identified miR-195 as a new potential agent for future anti-inflammatory and anti-atherogenic therapies in the context of cardiovascular research. Since macrophages action is present in several key processes of plaque development, regulation of miR-195 expression could have beneficial outcomes in the treatment of atherosclerosis. A manuscript describing and discussing these results is being prepared to be submitted for publication.

Specific modulation of miRNAs by pharmacological compounds is an attractive therapeutic approach that has been increasingly studied. Antisense oligonucleotides (ASOs) have been used to specifically target and inhibit miRNAs overexpressed in atherosclerosis [235, 236]. However, several issues related to risks of unintended targeting on other RNA molecules and to the lack of accuracy methods to test ASOs efficacy need to be overcome [237].

In order to upregulate specific miRNAs described as anti-atherogenic, an emerging therapeutic approach is the delivery of miRNAs. In particular, Lovren *et al*. described a lentiviral delivery of miR-145 which is highly expressed in human aortic SMC and regulates their fate and plasticity [238]. The SMC-targeted miR-145 treatment in apolipoprotein E knockout mice markedly reduced plaque size in aortic sinuses, ascending aortas, and brachiocephalic arteries and also promoted plaque stability by reducing the necrotic core area and increasing plaque collagen content [238].

Thus, in the future it would be interesting to optimize delivery systems for miR-195. Our collaborators from Laboratory of Macromolecular Synthesis and Functionalization (Zhejiang University – Hangzhou, China) developed a new type of non-viral PEG-detachable gene vector for redox-responsive gene delivery [239]. The mPEG–SeSe–PEI polymer efficiently condensed DNA into tightly packed spherical nanoparticles about 80 nm in size, which showed excellent

extracellular stability under physiological conditions and exhibited easily intracellular cleavage resulting in facilitated dePEGylation and DNA release [239]. We tested the ability of this polymer to condensate the negative control (SCR) used in the previous experiments, and the results were promising. DLS analysis with a Zetasizer Nano ZS (Malvern) nanoparticle analyzer revealed nanoparticles with 233.9 ± 36.9 nm diameter (Annex 2 - Figure 22), which might be suitable for *in vitro* experiments. Optimization of the procedures is still ongoing and transfection efficiency will be tested in a near future. Additionally, the impact of miR-195 in other cell types, including in endothelial cells, will be addressed in the next future. This is of crucial importance as we intend to test these nanoparticles not only *in vitro* but also in animal models. Improvement of miRNA delivery systems to impair atherogenesis, through modulation of inflammation or reduction of SMC recruitment, may unravel new future treatments for arthrosclerosis.

Annexes

Annex 1 – Quantification of total p38 protein by Western Blot

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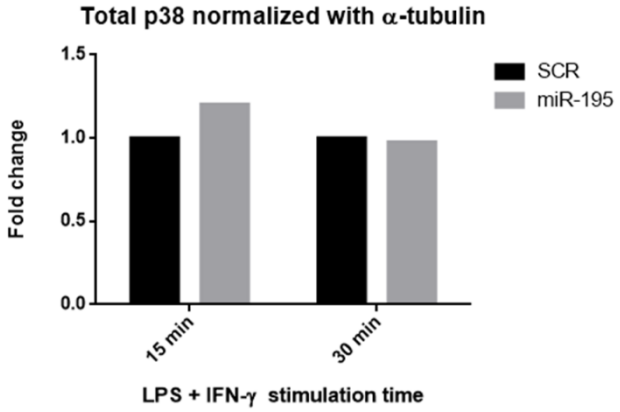

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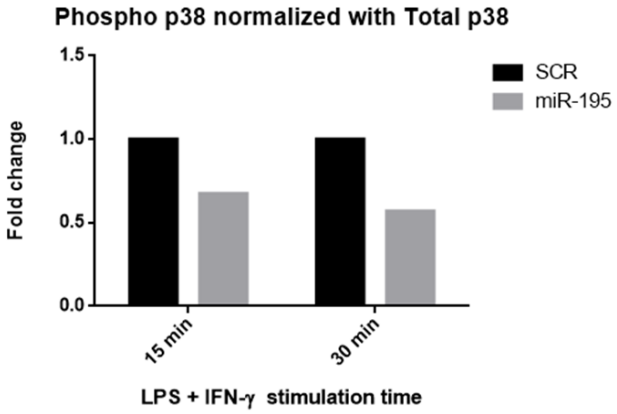

Figure 21 – Quantification of total p38 and phospho p38 levels normalized with total p38. A) Total p38 was evaluated in one experiment and their levels were normalized with α-tubulin. B) Phospho p38 levels were also quantified after normalization with total p38 levels of the same experiment.

Annex 2 – mPEG-SeSe-PEI / SCR polyplexes size evaluation by DLS

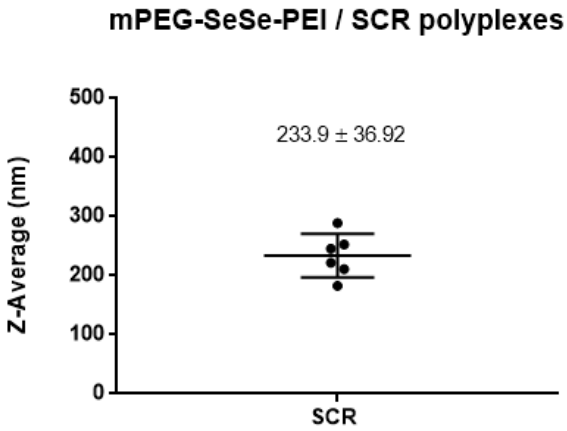

Figure 22 – mPEG-SeSe-PEI/SCR polyplexes size evaluation by Dynamic Light Scattering. mPEG-SeSe-PEI/SCR polyplexes formation were performed in Hepes buffer (pH 7.4). Particle size was determined by Dynamic Light Scattering using a Zetasizer Nano ZS. Six independent measures were performed (mean ± SD).

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