THE ROLE OF HIGH-DENSITY LIPOPROTEINS IN THE ACTIVATION OF T LYMPHOCYTES AND REGULATION OF IMMUNE RESPONSE

Marisa Alexandra Fernandes das Neves

A thesis submitted in partial fulfillment of the requirements for the Doctoral Degree in Medicine, in the specialty of Clinical Investigation

at Faculdade de Ciências Médicas | NOVA Medical School of NOVA University Lisbon

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“We shall not cease from exploration, and the end of all our exploring will be to arrive where we started, and know the place for the very first time.” (TS Eliot, 1888-1965).

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To Filipe, Luís and Leonor.

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Abstract

High-density lipoproteins (HDL) are the densest plasma lipoproteins and their complex protein and lipid constitution interferes with several physiological mechanisms. The most well characterized HDL function is reverse cholesterol transport, but other functions have also been attributed to HDL, namely anti-oxidative, vasodilatory and anti-inflammatory properties. However, the HDL immunomodulatory function is poorly understood. HDL mediation of cholesterol efflux and consequent lipid raft disruption is one of the main mechanisms by which HDL modulates the immune response, but other mechanisms are also involved. Most of the existent knowledge comes from animal studies, with limitations related to the animal model lipid metabolism. The few human studies are heterogeneous and demonstrate both anti-inflammatory and pro-inflammatory effects. Paradoxically, HDL can also suffer transformation to dysfunctional particles, by many different mechanisms. One of these mechanisms is related with the production of antibodies to HDL particles, with the main target being apolipoprotein A-I (ApoA-I).

In this thesis, I questioned the role of HDL in immune function with a special focus on the HDL effects on T cell lipid metabolism and T cell response, using cells from patients with systemic lupus erythematosus and healthy controls. This work studied: 1) the conditions in which HDL induces cholesterol depletion from $CD4^+$ T cells in vitro; 2) the HDL influence on ABCA1 and lipid raft organization in the plasma membrane (PM) of cultured $CD4^+$ T cells; 3) the HDL effect on immune conjugate formation; 4) $CD4^+$ T cell lipid metabolism in relation to anti-HDL antibodies; 5) the presence of anti-ABCA1 antibodies; 6) the HDL modulation of T cell response *in vitro.*

The main methodologies used in this thesis were peripheral blood mononuclear cells (PBMCs) isolation by density gradient separation, immune-based assays such as ELISA, cell culture experiments and flow cytometry.

The results give important clues to the importance of HDL to T cell metabolism and response: 1) HDL depletes cholesterol from the PM of $CD4^+$ T cells in 24 hour cultures, suggesting that this is the ideal time-lapse for *in vitro* studies; 2) cholesterol content in the PM of healthy $CD4^+$ T cells varies between different T cell subsets, with effector memory (EM) T cells showing the highest levels of PM cholesterol and less abundant glycosphingolipids (lipid raft constituents); 3) anti-HDL antibodies associate with an increase in the prevalence of EM T cells and a decrease in naïve and regulatory T cells (Treg). The presence of anti-HDL antibodies is also associated with an increased expression of lipid rafts in $CD4^+$ T cells from SLE patients and with a deregulated membrane cholesterol and lipid transporter ABCA1; 4) anti-ABCA1 antibodies are present in some patients with SLE; 5) 24 hour culture with HDL induces the expression of TGF-β1 in $CD4^+$ T cells without affecting inflammatory cytokines; 6) HDL inhibited the proliferation of $CD4^+$ T cells from patients with SLE but did not affect the proliferation in healthy T cells; 7) in the presence of HDL, TCRzeta phosphorylation is reduced.

In conclusion, this work supports the concept that HDL has essentially regulatory functions in the immune system, with a notorious effect in the production of TGF- β1 in $CD4^+$ T cells. HDL seems to reduce exaggerated inflammation and maintain normal immune function, with immune modulatory effects that are context dependent. Modifications in lipid metabolism occur in lymphocytes from patients with SLE, reinforcing the importance of the lipid metabolism for the immune response. These discoveries add new information

to the current knowledge on HDL immune function in humans that will hopefully be further studied in the research of atherosclerosis, autoimmune diseases and other pathologies in which lipid metabolism anomalies concur with immune dysfunction.

Resumo

As lipoproteínas HDL (*high-density lipoproteins*) são as lipoproteínas plasmáticas mais densas, com uma constituição proteica e lipídica complexa que está envolvida em vários mecanismos fisiológicos. A função das HDL melhor caracterizada é o transporte reverso de colesterol, embora outras funções lhes sejam atribuídas, nomeadamente as funções anti-oxidante, vasodilatadora e anti-inflamatória. Contudo, a função imunomoduladora das HDL ainda é pouco conhecida. O efluxo de colesterol promovido pelas HDL, com consequente desagregação das plataformas lipídicas da membrana (*lipid rafts*) é um dos principais mecanismos de modulação da resposta imunológica pelas HDL, embora outros mecanismos menos conhecidos estejam também envolvidos. O conhecimento existente provém principalmente de estudos animais com limitações relacionadas com o metabolismo lipídico do modelo animal. Por outro lado, os escassos estudos em humanos são heterogéneos e demonstram efeitos anti-inflamatórios e pro-inflamatórios. Paradoxalmente, as HDL podem tornar-se disfuncionais através de diferentes mecanismos. Um destes mecanismos é a produção de anticorpos dirigidos às partículas de HDL, sendo a apolipoproteína A-I (apoA-I) o alvo principal.

Nesta tese, foi questionado o papel das HDL na função imunológica, com enfoque especial nos efeitos das HDL no metabolismo lipídico e na resposta mediada por linfócitos T, utilizando as células mononucleadas do sangue periférico de dadores saudáveis e de doentes com lúpus eritematoso sistémico. Este trabalho estudou: 1) as condições em que a HDL induz a depleção de colesterol de linfócitos T $CD4^+$ *in vitro*; 2) a influência das HDL na organização dos *lipid rafts* e ABCA1 na membrana plasmática de linfócitos T $CD4^+$; 3) efeitos das HDL na formação de conjugados imunes; 4) metabolismo lipídico dos linfócitos

T $CD4^+$ e sua relação com os anticorpos anti-HDL; 5) presença de anticorpos anti-ABCA1; 6) modulação pelas HDL da resposta por linfócitos T *in vitro*.

As principais técnicas laboratoriais utilizadas nesta tese foram o isolamento de células mononucleadas do sangue periférico através de separação por gradiente de densidade, ensaios imuno-enzimáticos (ELISA), experiências de cultura celular e citometria de fluxo. Os resultados obtidos dão informação relevante acerca da importância das lipoproteínas HDL para o metabolismo e resposta dos linfócitos T: 1) as HDL depletam o colesterol da membrana plasmática de linfócitos T $CD4^+$ em culturas de 24 horas, sugerindo que este é a duração ideal dos estudos *in vitro*; 2) o conteúdo de colesterol na membrana plasmática de linfócitos T $CD4^+$ de dadores saudáveis varia entre as diferentes subpopulações de linfócitos T, com as células T memória efectoras a apresentar níveis mais elevados de colesterol na membrana plasmática e menos *lipid rafts*; 3) os anticorpos anti-HDL associam-se a aumento da prevalência de células T memória efectoras e diminuição da prevalência de células T *naïve* e reguladoras. A presença de anticorpos anti-HDL está também associada ao aumento de *lipid rafts* nos linfócitos T $CD4^+$ de doentes com lúpus eritematoso sistémico e a uma relação desregulada de colesterol de membrana e expressão de ABCA1; 4) anticorpos anti-ABCA1 estão também presentes em alguns doentes com lúpus eritematoso sistémico; 5) a cultura de linfócitos T $CD4^+$ com HDL durante 24 horas induz a expressão de TGF-β1, sem afetar a produção de citocinas inflamatórias; 6) as HDL inibem a proliferação de células T $CD4^+$ de doentes com lúpus eritematoso sistémico mas não afetam a proliferação de linfócitos T de dadores saudáveis; 7) na presença de HDL, a fosforilação de TCRzeta é suprimida. Em conclusão, este trabalho reforça o conceito de que as HDL têm funções essencialmente reguladoras do sistema imunitário, com um efeito

mais notório na produção de TGF-β1 pelos linfócitos T $CD4^+$. As HDL parecem reduzir a inflamação exagerada e manter a resposta imune normal, com efeitos imunomoduladores que dependem do contexto em que ocorrem. Nos linfócitos T de doentes com lúpus eritematoso sistémico ocorrem modificações do metabolismo lipídico, o que reforça a importância do metabolismo lipídico para a resposta imunitária. Estas descobertas adicionam nova informação ao conhecimento atual sobre a função imunológica das HDL em humanos, podendo no futuro ser alvo de estudo no âmbito da investigação sobre aterosclerose, doenças autoimunes e outras doenças em que anomalias do metabolismo lipídico surgem em simultâneo com a disfunção imunológica.

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List of abbreviations

Abbreviation	Definition
ABCA1	Adenosine triphosphate binding cassette transporters A1
ABCG1	Adenosine triphosphate binding cassette transporters G1
ApoA-I	Apolipoprotein A-I
ATF3	Activating transcription factor 3
BAFF	B-cell activating factor
BCR	B cell receptor
CETP	Cholesteryl ester transfer protein
DC	Dendritic cell
HDL	High-density lipoprotein
IFN	Interferon
IL	Interleukin
IRF1	Interferon regulatory factor 1
JNK	Janus kinase
LCAT	Lecithin cholesterol acyltransferase
LDL	Low-density lipoprotein
NK	Natural killer
LN	Lymph node
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LXR	Liver X receptor
MCP-1	Monocyte chemoattractant protein 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Factor nuclear kappa B
NLRP3	NOD-like, LRR-like and pyrin domain-containing protein 3

oxLDL	Oxidized low-density lipoprotein
PMN	Polymorphonuclear
PON	Paraoxonase
PAFAH	Platelet-activating factor-acetyl-hydrolase
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
RA	Rheumatoid arthritis
RCT	Reverse cholesterol transport
S1P	Sphingosine-1-phosphate
SAA	Serum amyloid A
SR	Scavenger receptor
SR-BI	Scavenger receptor class-B type I
STAT1	Signal transducer and activator 1
TACI	Transmembrane activator and calcium-modulating cyclophilin ligand interactor
TCR	T cell receptor
TGF-β	Transforming growth factor β
TNF	Tumour necrosis factor
TLR	Toll-like receptor

Chapter I.

Introduction

The progressive advances in medical knowledge allowed to extend life-expectancy in the developed world to a point where age related diseases (of which atherosclerosis is one of the most important) are greatly responsible for morbidity and mortality. The continuous pursuit for improved medical care and prevention of disease motivated the development of a large amount of research to understand atherosclerosis-associated mechanisms in the last century. However, although the first description of atherosclerosis made by Rudolf Virchow in 1856 mentioned a "fatty process as a direct product of inflammation" (Virchow 1860), it was only more than a century later that the immune mechanisms involved in atherosclerosis have come to the spotlight (Ross 1993). Along with the study of atherosclerosis mechanisms came the knowledge about risk factors. Efforts were put to reverse modifiable risk factors through lifestyle changes and pharmacological interventions. Among these, the one that most impacted on atherosclerosis was the use of statins to treat dyslipidemia and stabilize the atherosclerotic plaque. Today we have a vast knowledge in the field of atherosclerosis, but there are still many unsolved puzzles. One of them is the association of high-density lipoproteins (HDL) with a decreased risk of atherosclerosis-associated vascular events that was not reproduced in clinical trials of HDL-increasing drugs. Driven by the need to find better ways to reduce atherogenesis, the study of HDL and its actions has provided new insights on how it interferes with multiple physiologic and pathologic pathways, from inflammation to oxidative stress, from apoptosis to coagulation activation, granting it an ubiquitous protective role. Amongst these, the HDL influence on the immune response of which little is still known due to its complexity and sometimes paradoxical effects may be crucial to clarify its interaction with

the atherosclerotic disease, which is supported by the intersection of a low or dysfunctional HDL in the context of immune-driven diseases with an increased risk of atherosclerosis.

1. Atherogenesis as a multifactorial process

1.1. Plasma lipids in atherosclerosis

Plasma lipoproteins are the particles that allow the circulation of hydrophobic lipids in blood. The main plasma lipids are cholesterol (the most abundant), triglycerides (TG) and phospholipids. Mainly synthesized in the liver but also obtained from diet, cholesterol is crucial to build cell membranes, bile acids and steroid hormones. TG consist in a glycerol attached to three molecules of fatty acids and allows the storage of fatty acids in the adipose tissue. Phospholipids have a polar head group attached to two fatty acids and are the main constituents of cell membranes.

Lipoproteins are a heterogeneous group of particles varying in size, density and lipid and protein composition. They contain apolipoproteins, charged lipids (phospholipids and free cholesterol) on the surface and neutral lipids (triglycerides and cholesteryl ester) in the core. Plasma lipoproteins can be separated according to density by ultracentrifugation or electrophoresis. The largest and less dense particles are chylomicrons (CM), followed in crescent order of density by very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL and HDL. The density of lipoproteins decreases as the proportion of lipid to protein increases.

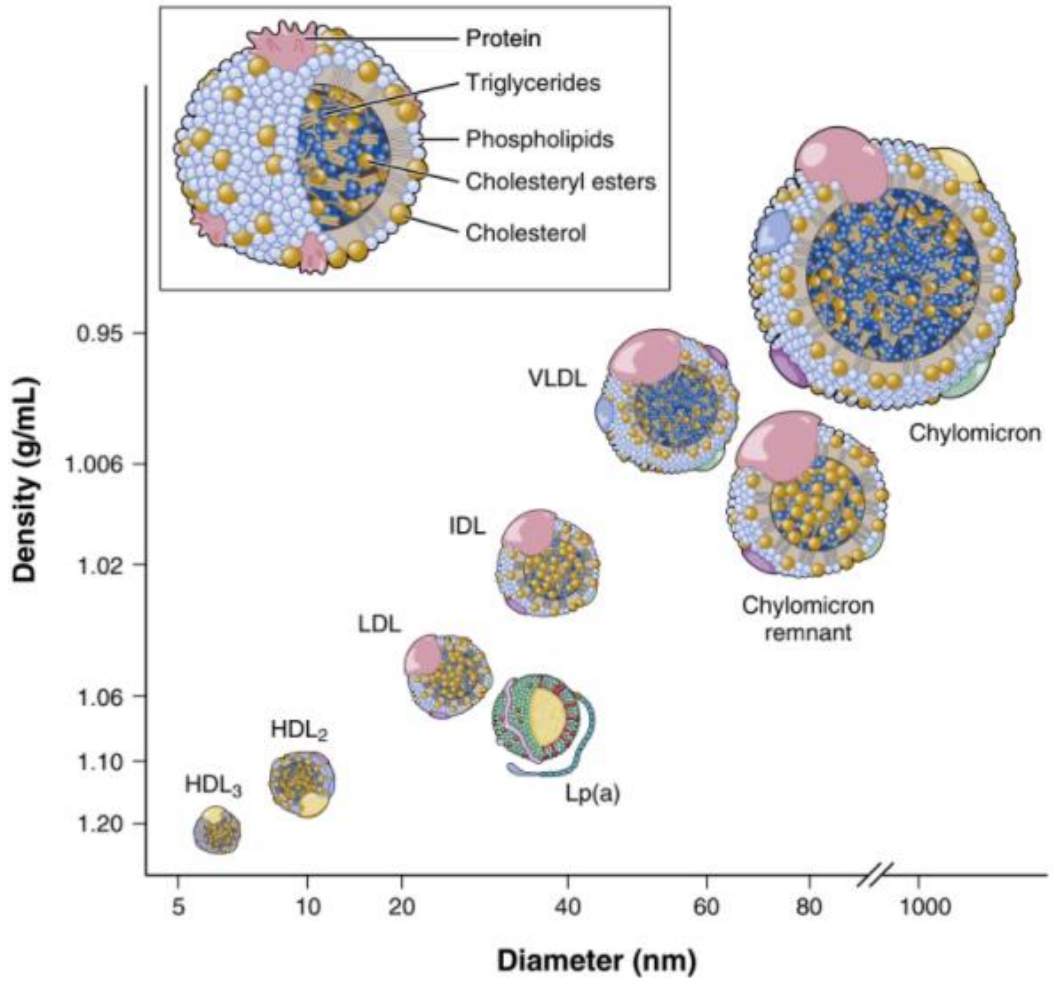


Figure 1: Relative size of plasma lipoproteins.

HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein. From Libby P, 2022.

Chylomicrons are formed within the intestinal mucosal cells to transport dietary TGs and cholesterol from within enterocytes through lymphatics into the circulation. In the capillaries of the adipose and muscle tissue, apoprotein C-II (apo C-II) on the chylomicron activates endothelial lipoprotein lipase (LPL) to convert 90% of chylomicron triglyceride to fatty acids and glycerol, which are taken up by adipocytes and muscle cells for energy use or storage. This is the so-called exogenous lipid

metabolism. VLDL, LDL and HDL are synthesized in the liver and small intestine and are responsible for the endogenous lipid metabolism. VLDL contain apo B-100 and transport excess TG and cholesterol to peripheral tissues. ApoC-II on the VLDL surface activates endothelial LPL to break down TG into free fatty acids and glycerol, which are taken up by cells. IDL are the product of LPL processing of VLDL and chylomicrons. IDL can be cleared by the liver or metabolized by hepatic lipase into LDL, which retains apo B-100. LDL, the product of VLDL and IDL metabolism, are rich in cholesterol. About 40 to 60% of all LDL is cleared by the liver in a process mediated by apo B-100 and hepatic LDL receptors. The rest is taken up by either hepatic LDL receptors or nonhepatic scavenger receptors (SRs). SRs, mainly on macrophages, take up the excess circulating LDL that was not processed by hepatic receptors. Monocytes rich in LDL migrate into the subendothelial space and become macrophages that take up more LDL and form foam cells in the atherosclerotic plaques. These mechanisms explain the association of LDL with increased atherogenesis.

High-density lipoproteins are associated with a decreased atherosclerosis risk. HDL are initially cholesterol-free lipoproteins that are synthesized in enterocytes and in the liver. The role of HDL in the reverse cholesterol transport from peripheral tissues to the liver partly explains its atheroprotective net effects. HDL is composed of a cholesterol core enriched with cholesterol esters (CE) and triglycerides (TG), and a surface lipid bilayer containing free cholesterol (FC), phospholipids (PL) and proteins. HDL is the densest plasma lipoprotein and is highly heterogeneous in size, charge, and composition.

HDL particles can be subdivided in different subclasses, using different nomenclatures according to the separation method used. Based on density determined by

ultracentrifugation, HDL particles are separated into three subfractions: HDL1 (mean density (d) 1.05 g/mL), HDL2 (1,063 < d > 1125 g/mL) and HDL3 (1,125 < d > 121 g/mL). HDL2 and HDL3 subfractions are spherical and mature particles. Electrophoresis allows to separate HDL particles based on its size: HDL2b, HDL2a, HDL3a, HDL3b and HDL3c (sizes 10,6 to 7,6 nm) (Tsompanidi et al. 2010). Immunoaffinity chromatography separates HDL into two major subpopulations according to their apolipoprotein composition: LpA-I, containing ApoA-I and no ApoA-II, and LpA-I/A-II, containing both. LpA-I are mostly found in the HDL2 subpopulation. LpA-I/A-II tend to be smaller and denser than LpA-I, and prevail in the HDL3 subfraction.

The different proteins in HDL constitute more than half of its mass and render the HDL structure its complexity. The HDL structure is modified during HDL maturation from small to large HDL particles. Apolipoproteins are the main protein constituents, followed by HDL-associated enzymes, transfer proteins, acute phase proteins and other minor proteins. Several studies have reported nearly 100 proteins in human HDL. The distribution of apolipoproteins and other proteins in HDL particles greatly varies between subjects (Asztalos et al. 2004; 2001). The protein arrangement in HDL follows certain patterns that associate with different functions. Proteomic studies showed that certain HDL proteins occur in particles in specific size ranges and are sorted in different HDL subspecies with different functional properties (Y. Zhang et al. 2019; Davidson et al. 2009).

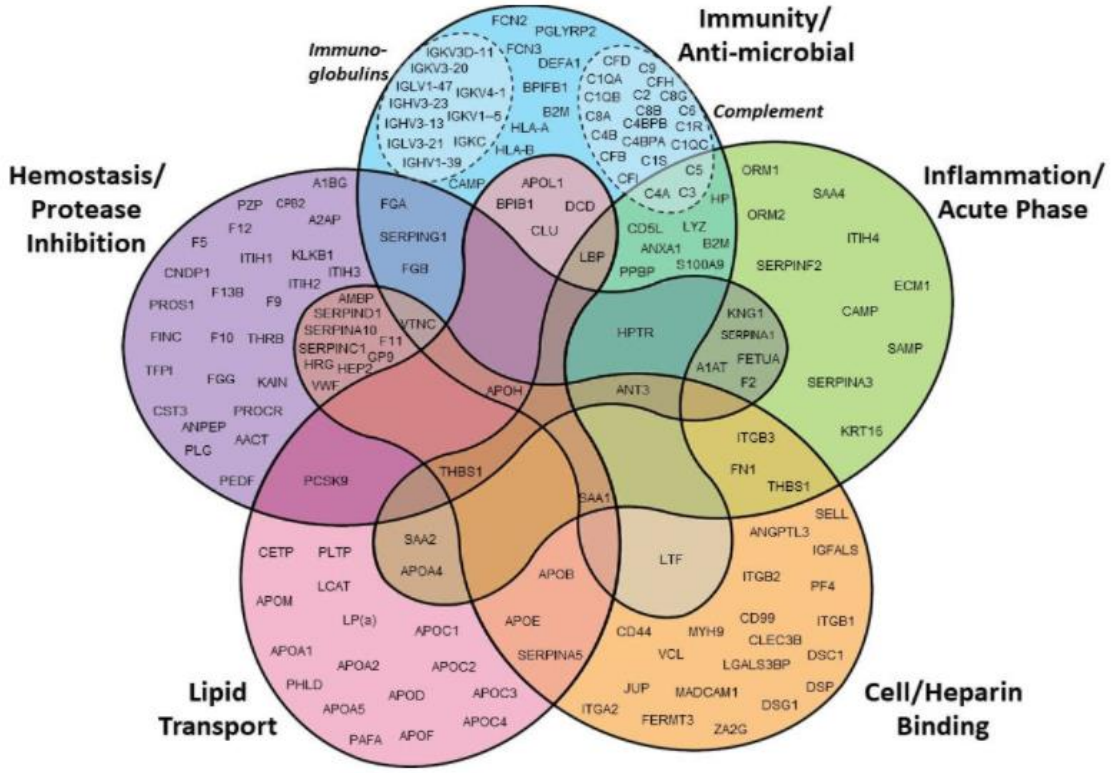


Figure 2: HDL proteomics.

Proteins detected in HDL by mass spectrometry sorted by their purported functions. From Sean Davidson 2022.

1.2. Immune mechanisms in atherosclerosis

Atherosclerosis is a chronic inflammatory disease of large and medium-sized arteries initiated by the accumulation of lipids in the vessel wall. Endothelial dysfunction and inflammation are early pro-atherogenic processes and it is becoming increasingly clear that the innate and adaptive immune responses also play important roles in atherogenesis. The development of atherosclerosis begins early in life with progressive lipid accumulation and increased arterial stiffness resulting in what is known as atherosclerotic plaques. The atherosclerotic plaque is characterized by an accumulation of lipids in the artery wall,

together with the infiltration of immune cells and the formation of a fibrous cap by vascular smooth muscle cells.

After decades of silent progression, atherosclerosis most commonly manifests as cardio or cerebrovascular diseases, the leading causes of morbidity and mortality in developed countries (Back et al. 2019). The predisposition to atherosclerosis can be potentiated by several risk factors. Modifiable risk factors are hypercholesterolemia, diet, impaired glucose metabolism, hypertension, smoking, sedentarism, hyperhomocysteinemia and infectious and inflammatory diseases. Non-modifiable risk factors are genetic factors, age and gender.

Plasma lipoproteins assume a crucial role in the progression of atherosclerosis. Circulating low-density lipoprotein (LDL) particles can accumulate in the arterial intima, where apolipoprotein B100 binds to proteoglycans in the extracellular matrix. This subendothelial retention promotes oxidation of the LDL particles. These deposits, with the help of the turbulent blood flow at arterial branching points, lead to the expression of adhesion molecules such as E-selectin and VCAM-1 on the endothelial surface. Concomitantly, chemokines attract monocytes, dendritic cells (DCs) and T cells into the intima (Zernecke, Shagdarsuren, and Weber 2008). Monocytes in the intima are then stimulated by macrophage colony-stimulating factor produced by activated endothelial cells to differentiate into macrophages (J. D. Smith et al. 1995). In turn, macrophages upregulate their scavenger receptors and increase the uptake of oxLDL to become foam cells. Following the initial inflammatory mechanisms, the innate and adaptive immune systems can also be activated and influence the development of the atherosclerotic plaque (Lacy et al. 2019).

The activation of the innate immune system in atherosclerosis occurs mainly through the detection of danger signals by sensor proteins in the cytoplasm of innate immune cells (Christ et al. 2018). These sensor proteins are constituents of multiproteic complexes named inflammasomes that are responsible for a rapid inflammatory response through the cleavage of IL-1β and IL-18 into their pro-inflammatory active forms. The nucleotide binding domain leucine-rich repeat receptor protein 3 (NLRP3) is the inflammasome associated with chronic inflammation in atherosclerosis (Varghese et al. 2016). Toll-like receptors (TLRs) mediate the interaction between danger signals and inflammasomes. The NLRP3 inflammasome activation occurs through the actions of the TLR4/TRIF (TIR-domain-containing adapter-inducing interferon-β) axis (Fernandes-Alnemri et al. 2019).

In the orchestration of innate and adaptive immune responses, DCs play a central role. Danger signals in the arterial wall may activate DCs, leading to a switch from tolerance to activation of the adaptive immunity. In fact, DCs are clustered in arterial branch points, where they co-localize with T cells and macrophages and can uptake cholesterol (Subramanian and Tabas 2013). Macrophages assume a relevant role in the development of the atherosclerotic lesions through the uptake of oxLDL, mainly by SRs, and then transforming themselves into foam cells (Boullier et al. 2001). OxLDL also binds to TLRs 2 and 4, activating the inflammasome and inducing the excretion of pro-inflammatory cytokines (Chávez-Sánchez et al. 2010).. During inflammation, oxLDL can be internalized by macrophages independently of SRs and macrophages can polarize to a M1 phenotype with the suppression of the lipid sensors LXR and PPAR (Dushkin 2012). These mechanisms perpetuate the inflammatory response in the vessel wall, with the consequent

activation of a type 1 T helper (Th1) response (Siamon Gordon and Martinez 2010), that often becomes chronic (Tabas and Lichtman 2017).

In experimental (murine) models of atherosclerosis, there is a macrophage/T cell ratio of approximately 4:1 to 10:1 in the vascular lesions (Hansson and Hermansson 2011). Most T cells in those atherosclerotic plaques are $CD4^+$ and the remaining are $CD8^+$. The $CD4^+$ T cells have a predominant Th1 profile that activate macrophages and increase pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-2. IFN-γ, the main pro-inflammatory cytokine involved, increases the expression of other inflammatory cytokines, such as IL-1 (Szabo et al. 2003). Th2, Th17, regulatory T cells (Treg) and NK T cells are also present but whilst the Th1 profile is associated with atherosclerosis progression, the role of the other T cell subsets is less clear. In fact, the role of Th2 cells is controversial (Binder et al. 2004; Cardilo-Reis et al. 2012; Davenport and Tipping 2003) and the Th17 impact seems to be context-dependent: if IL-17 expression is accompanied by high levels of IFN-γ it is atherogenic but in the presence of IL-10 it becomes protective (Taleb, Tedgui, and Mallat 2015).

Treg are a subset of $CD4^+$ T cells that protect against atherogenesis through the inhibition of proinflammatory T cells (Foks et al. 2011), the suppression of macrophages and the activation of endothelial cells. The principal inhibitory cytokines secreted by Treg are IL-10 and TGF-β (Mallat et al. 2008). Hypercholesterolemia was shown to impair Treg but not the effector T cell accumulation in lesions, thus contributing to the decrease of Treg:Th1 cell ratios in the atherosclerotic lesions (Maganto-García et al. 2011; Z. Wang et al. 2014). A negative correlation between Treg and Th17 cells was also found in patients with unstable carotid artery lesions (Z. D. Liu et al. 2012). Additionally, Treg from patients

with acute coronary syndrome were shown to have a reduced suppressive function (Jia et al. 2013).

The role of B cells in atherosclerosis is related with both the humoral and cellular responses and with antiatherogenic and proatherogenic effects. B1 cells produce naturally occurring IgM antibodies directed to oxidation-specific epitopes that neutralize them and limit endothelial activation and foam cell formation. In contrast, B2 cells produce IgG antibodies that are proatherogenic through the formation of immune complexes, (for example with oxidized LDL) and promotion of macrophage inflammatory responses (Sage et al. 2019). IgE are also proatherogenic by stimulating macrophages and mast cells (Tsiantoulas et al. 2017). The role of IgA in atherosclerosis is less understood, but it was reported a positive correlation between IgA antibodies and cardiovascular disease (Muscari et al. 1988). Additionally, B cells can produce proatherogenic (e.g. TNF) and antiatherogenic (e.g. IL-10) cytokines (Tay et al. 2016; Rosser and Mauri 2015). Studies on B cell-mediated regulation of the immune responses showed that B cell depletion decreases T cell activation. However, blocking the B-cell activating factor (BAFF) seems to worsen atherosclerosis in mouse models, since the ligation to its main receptor (BAFFR) leads to B2 cell differentiation and increases IgG levels in mice. Interestingly, the ligation of BAFF to its alternative receptor (TACI) decreases the TLR9-interferon regulatory factor responses in macrophages, which explains its antiatherogenic net effect (Tsiantoulas et al. 2018). Indeed, the pharmacologic CD20 blockade showed to be atheroprotective, partly because of a selective depletion of IgG-producing B cells with an incomplete depletion of IgM-producing B cells and partly through an increase in BAFF levels (Ehrenstein and Wing 2016).

The reported mechanisms were observed mainly in studies using mouse models. In humans, cells in atherosclerotic plaques also express TLR family members. However, the role of TLRs in the pathogenesis of atherosclerosis seems to be different among human studies: in one study loss of function mutations in the TLR4 gene decreased cardiovascular disease, but subsequent studies did not show a protective effect of a decreased response from the TLR4 allele (K. Zhang et al. 2012).

The role of the different immune cells in atherosclerosis may also vary between mice and human: polymorphonuclear (PMN) leukocytes are fewer in the human atherosclerotic plaque, when compared to the murine one; DCs increase in atherosclerotic lesions, both in mice and humans (Ozmen et al. 2002) and T cells in human and mice atherosclerotic plaques predominantly exhibit a Th1 cell-associated cytokine secretion pattern, but with a lesser degree of polarization in humans (Frostegård et al. 1999).

The knowledge of atherosclerosis immune mechanisms provided the rational to develop clinical trials using immunosuppressors in patients with atherosclerotic disease. The Canakinumab Antiinflammatory Thrombosis Outcomes Study (CANTOS) proved that targeting the immune system, specifically IL-1β, prevents cardiovascular events in patients with atherosclerosis (Ridker et al. 2017). Colchicine, an inhibitor of tubulin polymerization that decreases the microtubules necessary for the assembly of the NLRP3 inflammasome has also shown to prevent cardiovascular events in patients with recent myocardial infarction (Tardif et al. 2019).

1.3. Plasma membrane metabolism and immune response

Plasma membrane (PM) organization was firstly described as the fluid mosaic membrane model by Singer and Nicholson, in 1972 (Singer and Garth L Nicolson 1972). This model incorporates two major concepts: the fluidity and dynamics of the membrane components and their mosaic nature, whose components diffuse laterally and mix rapidly at a physiological temperature. Later, the fluid mosaic model included the notion that both the cytoskeleton and extracellular matrix could influence the diffusion of membrane molecules and that different ordered areas of the membrane, or even solid lipid phases, could exist. The principal components of the plasma membrane are lipids (phospholipids and cholesterol), proteins and carbohydrate groups that are attached to some of the lipids and proteins.

In the immune cells, lipids are an important source of energy and are important membrane components, critical for cell proliferation and migration and membrane expansion amongst other functions (Howie et al. 2017; Owen, Gaus, and Magee 2010). In T cells, PM lipids regulate signalling, differentiation and effector functions, and increased levels of plasma membrane cholesterol promote an inflammatory T helper response (Surls et al. 2012). The lipid organization in the PM determines the constitution of micro-domains called lipid rafts, that consist in small (10-200nm) sterol- and sphingolipid-enriched domains that are very dynamic and are more ordered and less fluid than the surrounding membrane (Drevot et al. 2002; Dinic et al. 2015). The assembly of critical mediators in lipid rafts trigger signalling pathways which are crucial to the activation of immune cells. T cell function is affected by the disruption of lipid rafts, which alters TCR signalling (McDonald et al. 2014; Molnár et al. 2012). The role of cholesterol on T cells is further supported by studies showing that

statins, the inhibitors of HMG-CoA reductase, the enzyme responsible for catalysis in the rate-limiting step of cholesterol biosynthesis, suppress naïve T cells differentiation (Ghittoni et al. 2006).

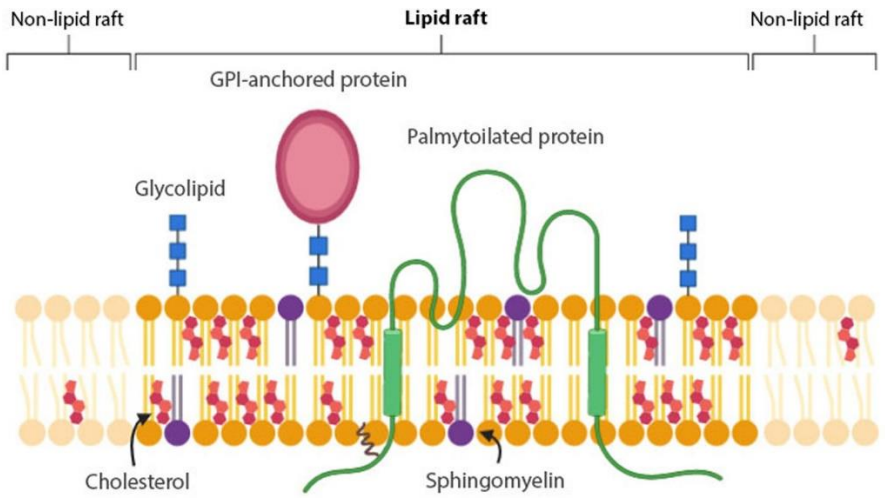


Figure 3: Lipid raft structure.

Lipid rafts are composed of cholesterol, saturated phospholipids and sphingolipids. Glycosylphosphatidylinositol-anchored and lapidated proteins have a higher affinity for lipid rafts than non-lipid rafts. From Ripa, Andreu, and López-guerrero 2021.

The immunologic synapse (IS) is a specialized junction between a T cell and an antigen-presenting cell (APC) that forms within seconds of engagement between the TCR and the cognate peptide-MHC complexes on the APC. T cells accumulate a multitude of their surface receptors, intracellular signalling and scaffolding molecules in the contact zone with APCs, resulting in the formation of a mature immune synapse that initiates an organized intracellular signalling cascade that results in T cell activation and proliferation (Kane, Lin, and Weiss 2000). The clustering of lipid rafts is central to the formation of the IS (Zumerle, Molon, and Viola 2017). The increase in lipid rafts resultant from cholesterol accumulation in T cells predispose to a higher inflammatory reactivity (Guo et al. 2017).

Furthermore, lipid rafts are thought to promote BCR signalling in B cells (Pierce 2002) and membrane cholesterol influences BCR endocytosis (Bléry et al. 2006).

Membrane lipid transporters have been hypothesized to be pivotal in the maintenance of homeostasis in the membrane lipid composition. The adenosine triphosphate binding cassette transporters A1 (ABCA1), one of the main cholesterol transporters in plasma membrane, mediates cholesterol efflux to lipid-poor ApoA-I and transports phosphatidylcholine (PC), phosphatidylserine (PS) and sphingomyelin (SM) from the inner leaflet to the outer leaflet of the plasma membrane (Vedhachalam et al. 2007). In addition, ABCA1 redistributes PM cholesterol and sphingomyelin from lipid rafts to non-raft regions (Landry et al. 2006). ApoA-I preferentially associates with non-raft membranes in ABCA1-expressing cells. The ApoA-I binding protein (AIBP) is a ubiquitously expressed protein that has anti-inflammatory effects. It is thought that its anti-inflammatory effects occur due to the promotion of cholesterol efflux (to particles containing ApoA-I) and to the regulation of lipid rafts, but it is not clear if these effects are directly related. It is known that AIBP binds to a raft-associated receptor, most likely TLR4 (Woller et al. 2018). After binding to the rafts, AIBP binds ApoA-I or HDL, brings them to the rafts and facilitates their interaction with ABCA1. The cholesterol efflux results in cholesterol depletion and disintegration of lipid rafts (Fang and Miller 2019). Additionally, ABCA1 is capable of regulating the lipid rafts not only through potentiating cholesterol efflux but also through changes in the cytoskeleton. It is present in all cell types, although with variable levels of expression, being highly expressed in macrophages, liver cells, intestinal cells, adrenal gland, endothelial cells, and placental trophoblast (Attie 2007). However, the role of ABCA1 in immune cells is poorly addressed. The infiltration of inflammatory cells and

cholesterol accumulation observed in ABCA1 knockout mice appear to be restricted primarily to the macrophage, although the expression of ABCA1 is ubiquitous. Moreover, macrophages isolated from ABCA1-deficient mice have an increased secretion of chemokines, growth factors and cytokines (Abca et al. 2005). Zhu et al. showed that the hypersensitivity of ABCA1-deficient macrophages to LPS depended on subtle increases in cell membrane cholesterol and lipid raft content, suggesting an important role of ABCA1 in the regulation of the innate immunity through modulation of plasma membrane cholesterol (X. Zhu et al. 2008). Two candidate STAT3 docking sites in ABCA1 were found to be required for the apoA-I/ABCA1/JAK2 activation of STAT3, suggesting that the macrophage cholesterol exporter ABCA1 functions as a direct anti-inflammatory receptor (Y. Liu et al. 2009).

1.4. Systemic lupus erythematosus: deregulation of lipid metabolism and immune response

SLE is an autoimmune disease in which genetic, epigenetic, and environmental factors induce diverse manifestations with a wide range of severity. The intricate pathogenic mechanisms result in the activation of autoreactive T and B cells with a consequent massive production of autoantibodies. The role of T cells in the development of SLE has been increasingly recognized. In addition, to promote B cell proliferation and autoantibody production (X. Zhang et al. 2015), T cells exert pathogenic effects through the increase of pro-inflammatory memory T cells and a biased T helper (Th)17 differentiation (Talaat et al. 2015). Furthermore, there is a distortion of the Treg response together with TCR signal modifications and cytokine imbalance, the most prominent feature being the IFN signature

in peripheral lymphocytes. It was observed that Th1, Th2 and Th17 cytokines are increased in SLE, with the exception of IL-2, IL-4 and TGF-β1, that are downregulated (Talaat et al. 2015; Muhammad Yusoff, Wong, and Mohd Redzwan 2020).

Nevertheless, T cell function might be highly affected by different metabolic aspects with modifications of lipid metabolism being associated with disease activity and damage in patients with SLE (Romo-tena and Kaplan 2020). The importance of lipid metabolism in the pathogenesis of SLE is further supported by the increased cardiovascular risk observed in these patients (Bruce 2005). The most important mechanisms implicated in atherosclerosis in SLE are excessive inflammation, LDL and HDL oxidation, production of anti-lipoprotein antibodies and a prothrombotic environment. Some authors have also reported alterations in the plasma lipid profile in patients with SLE, with increased VLDL and triglycerides but reduced HDL (Borba, Carvalho, and Bonfá 2006). This altered lipid profile has been related with adverse renal outcomes in patients with SLE (Tisseverasinghe et al. 2006), but conclusive studies on lipid metabolism in SLE are still lacking.

The cholesterol efflux capacity through ABCA1 and ABCG1 is reduced in SLE (Ronda et al. 2014; Vuilleumier et al. 2019) but more conclusive studies are still lacking. Lipid rafts are increased in $CD4^+$ T cells from patients with active SLE but the exact mechanisms that induce the lipid raft formation in this condition are not completely understood. The endogenous production of cholesterol and glycosphingolipids due to the activation of LXRs pathways might have a role in lipid raft formation, since LXRβ is increased in T cells of patients with SLE (McDonald et al. 2014). LXRs are transcription factors that upon oxysterol binding, upregulate the transcription of, for example, ABCA1 and ABCG1 genes (Rigamonti et al. 2005; Waddington, Jury, and Pineda-Torra 2015). HDL/ApoA-I

dysfunction also occurs in SLE, due to alterations related to increased inflammation but also due to the presence of anti-HDL antibodies, which are believed to contribute to the increased atherosclerosis burden in these patients (Joana R. Batuca et al. 2018). Other autoantibodies such anti-oxLDL (V. J. van den Berg et al. 2019), anti-endothelial cells antibodies (Varela et al. 2011) and anti-phospholipid antibodies (Ames, Margarita, and Delgado Alves 2009) may alsi have an important role.

The interaction between HDL/ApoA-I and ABCA1 is probably affected by the presence of autoantibodies directed to HDL components or to ABCA1. Both were described in patients with SLE. SLE, despite its complexity, is therefore one of the best clinical models linking immune activation and accelerated vascular disease. For that reason, the understanding of the pathophysiology of this condition might shed some light in the immunoinflammatory mechanisms present in atherosclerosis.

2. HDL: a major link between lipids and the immune response

The regulation of the immune response is complex and has multiple players beyond immune cells and mediators. The neuroendocrine modulation of the immune system, for example, is evident in clinical studies where a correlation between psychological stress and immune dysfunction is clearly shown (Shanahan and Anton 1988). Commensal gut bacteria have also been implicated in immune modulation. It was demonstrated that many bacterial commensals block some inflammatory pathways and promote immunologic tolerance (Kelly, Conway, and Aminov 2005). The modulation of inflammatory and

immune responses by plasma lipoproteins also occurs, with evidence showing that LDL and HDL protect from sepsis (Chien et al. 2015; York et al. 2015; Guirgis et al. 2016).

The recognition that a lipid particle could be an immune mediator opened a vast area of research, however, the complexity of lipoproteins combined to the complexity of immune responses render this area of knowledge very challenging. Some *in vitro* and *in vivo* studies of lipoprotein immune effects have shed some light into this field but the heterogeneous experimental conditions further contributed to the overall complexity in many situations. In this context, HDL seems to be particularly relevant as it has been implicated in several immune-mediated diseases based on a multitude of demonstrated physiologic and pathophysiologic effects.

2.1. HDL in physiologic conditions

HDL is responsible for the transport of cholesterol from peripheral tissues to the liver, a process named reverse cholesterol transport (RCT). This pathway consists in the removal of cholesterol from cell membranes, including macrophages and endothelial cells and its transport to the liver where it is secreted in the bile. Lipid-free/lipid-poor apoA-I is crucial as the first cholesterol acceptor in the RCT. There are four different mechanisms for efflux of cell cholesterol: through aqueous diffusion, SR class-B type I (SR-BI) and adenosine triphosphate binding cassette transporters A1 (ABCA1) and G1 (ABCG1), . Efflux of free cholesterol via aqueous diffusion is ubiquitous but seems to be inefficient. Cholesterol efflux occurs at higher rates through the SR-BI to large HDL particles according to concentration gradients (Trigatti, Krieger, and Rigotti 2003). ABCA1 and ABCG1 are transmembrane domains that mediate the efflux of both cellular cholesterol and

phospholipids to lipid-free/lipid-poor apoA-I and HDL, respectively. Cholesteryl esters in mature HDL particles are selectively taken up by the liver through SR-BI and are subsequently transferred to the bile or to apoB-containing lipoproteins (VLDL, IDL, LDL) by CETP in exchange for TGs (Zannis, Chroni, and Krieger 2006).

ApoA-I and apoA-II (70 and 20% of HDL content, respectively) are the major apolipoproteins of HDL. ApoA-I has a molecular weight of about 28KD and is secreted by the liver (about 70%) and the small intestine (about 30%) as a monomer in a lipid-free form (Krimbou, Marcil, and Genest 2006). Two apoA-I molecules associate in an anti-parallel way to form a belt-shaped disc when its circulating concentration reaches > 10 µg/mL (Phillips et al. 1997; Segrest et al. 1999). This apoA-I belt harbors phospholipids and trace amounts of free cholesterol. Lipidation of apoA-I dimers will then form discoidal HDL particles, which occurs after interaction with ABCA1. The maturation of HDL from the small, lipid-poor, to the larger HDL particles occurs after cholesterol esterification by lecitin:cholesterol acetyl transferase (LCAT). LPL hydrolyses triglycerides in apoB-containing lipoproteins, resulting in the release of surface phospholipids, free cholesterol and apolipoproteins, that will participate in HDL biosynthesis. ABCG1-mediated cholesterol efflux promotes lipidation of the larger HDL particles. The mature spherical HDL contains 45-55% apolipoproteins, 26-32% phospholipids, 15-20% esterified cholesterol, 3-5% cholesterol and about 5% TGs (Tsompanidi et al. 2010).

Although cholesterol removal from cells through RCT is the most well-known atheroprotective function of HDL, there is evidence that HDL has additional atheroprotective roles beyond RCT. Nevertheless, many of these functions are related to the HDL participation in RCT. The most fundamental are anti-oxidative, anti-thrombotic

and anti-inflammatory qualities. Other atheroprotective functions are anti-apoptotic, vasodilatory, anti-infectious and antidiabetic activities (Yamashita et al. 2010).

The anti-oxidant functions of HDL are attributed to ApoA-I and enzymes such as PON, PAF-AH and glutathione peroxidase that prevent LDL oxidation (Negre-salvayre et al. 2006). In addition to ApoA-I, other apolipoproteins have anti-oxidant effects, such as ApoE (Miyata and Smith 1996), ApoJ (Navab et al. 1997) and Apo-IV (Wong et al. 2007). HDL particles also contain small amounts of lipophilic antioxidants, mainly tocopherols (Goulinet and Chapman 1997). The main anti-thrombotic effects of HDL are the inhibition of platelet aggregation and the inhibition of coagulation factors, including tissue factor and factors X, Va and VIIIa (Carson 1981; Calabresi, Gomaraschi, and Franceschini 2003). HDL also inhibits thrombus formation through the tissue factor pathway inhibitor in its proteome (Lesnik et al. 1993) and ApoA-I has been show to neutralize the procoagulant properties of anionic phospholipids (Oslakovic et al. 2009).

The HDL vasodilatory effects are mainly explained by the induction of endothelial nitric oxide synthase (eNOS) and consequent stimulation of nitric oxide production (Mineo et al. 2003). HDL can also stimulate the production of PGI2 and improves endothelial function by promoting the repair of damaged endothelium by endothelial progenitor cells (Tso et al. 2006).

One of the main HDL proteins responsible for the HDL immune effects is sphingosine-1-phosphate (S1P). S1P is the main sphingolipid in HDL with HDL being its principal transporter in plasma (Okajima 2002). S1P binds to cells through its own cell surface receptors contributing to immune cell trafficking and endothelial barrier function (Rivera, Proia, and Olivera 2008). ApoM, a negative acute phase protein, is the carrier of S1P in

HDL. S1P is responsible of many of the HDL biological effects that are not related with reverse cholesterol transport (Egom, Mamas, and Soran 2013). These include immunological effects such as the inhibition of TNFα-induced adhesion molecules expression in endothelial cells (Kimura et al. 2006), the induction of long pentraxin 3 (Norata et al. 2008) and TGF-β in endothelial cells (Norata et al. 2005), the inhibition of TLR2 activation in macrophages (Dueñas et al. 2008), the reduction of pro-inflammatory and increase of anti-inflammatory cytokine production by DCs (Idzko et al. 2002), the migration of T lymphocytes from lymphoid organs (Mandala et al. 2002) and the control of T-cell lineage determination (G. Liu et al. 2010).

2.2. HDL and the immune system

The plasma membrane is highly influenced by the presence of HDL through its role in RCT, which in turn influences the immune cells response. Intracellular cholesterol, in the form of oxysterols, also bind to the liver X receptors (LXRα and LXRβ) thus participating in the regulation of various pathways linked to inflammation and immune response (Bensinger et al. 2008).

In macrophages, the cholesterol efflux mediated by ABCA1 and ABCG1 limits the cholesterol availability to constitute lipid rafts, which inhibits MyD88-dependent TLRs trafficking (X. Zhu et al. 2010). On the contrary, mouse models deficient for ABCA1 and ABCG1 were shown to accumulate cholesterol in peritoneal macrophages and have increased inflammatory responses to TLR agonists (Yvan-charvet et al. 2008). In T cells, the promotion of cholesterol efflux through ABCA1 and ABCG1 by LXR is associated with a decrease in proliferation (Bensinger et al. 2008). In addition to affect proliferation,

lipid metabolism can also influence T cell differentiation into Th17 and Treg cells (Berod et al. 2014).

HDL mediation of cholesterol efflux and consequent lipid raft disruption is one of the main mechanisms by which HDL modulates the immune response, but other mechanisms are also involved. However, the study of those mechanisms frequently produces conflicting findings as a consequence of different methodologies being used, adding complexity to the analysis and interpretation of the results. In fact, although mice and human HDL show similar proteomics (Scott Gordon et al. 2015), major species differences difficult the translation of data to the clinical context: LDL represents the major fraction of plasma cholesterol in humans, while mice essentially have HDL (Minniti et al. 2020) and CETP is expressed in humans but not in mice (which possibly explains the differences in cholesterol lipoprotein distribution between species) (Blauw et al. 2019). These are mere examples of how translational research trying to integrate mice-based studies with human known mechanisms can be confusing and often misleading. Figure 1 summarizes the existent studies of HDL/ApoA-I immune effects.

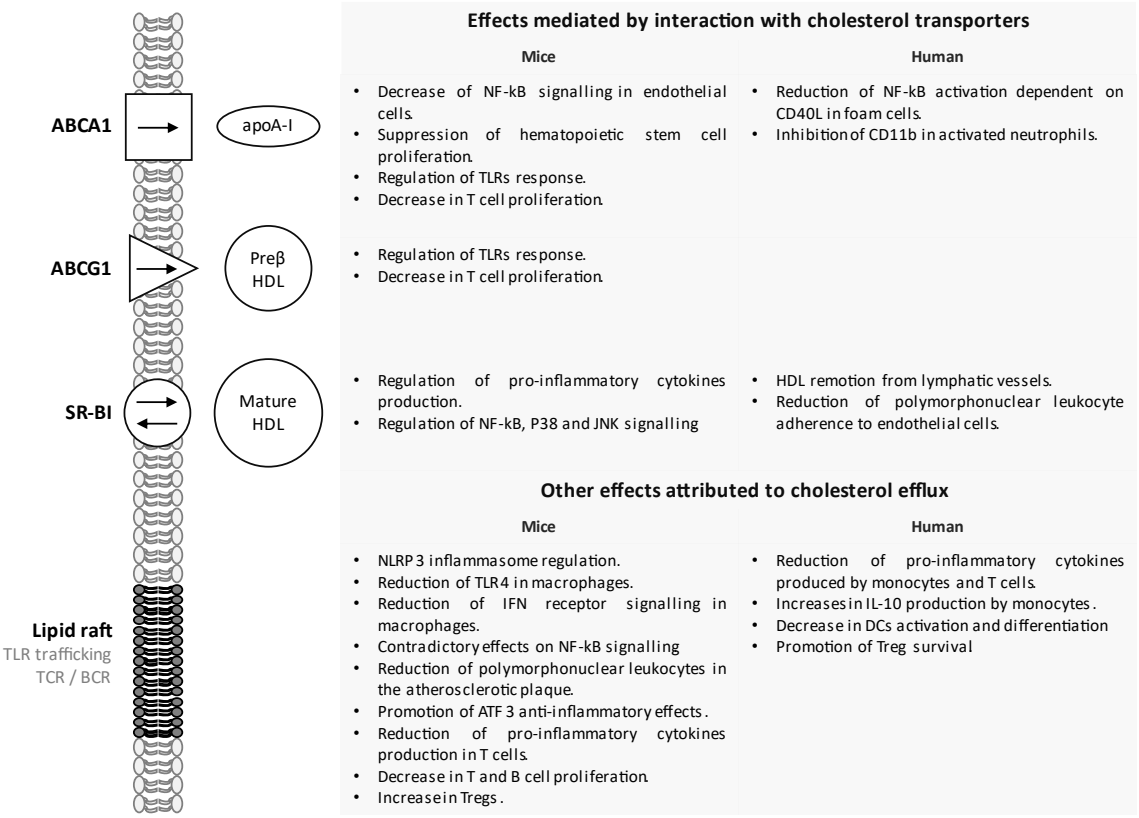


Figure 4: HDL immune effects mediated by cholesterol efflux.

The different cholesterol efflux pathways may have diverse effects on immune response. However, in many studies the specific involved pathway was not determined. Lipid raft disruption in consequence of cholesterol efflux is related with many of the HDL effects on immune response. DC: Dendritic cell. TLR: Toll-like receptor. NLRP3: nucleotide binding domain leucine-rich repeat receptor protein 3. NF-kB: nuclear factor kappa B. JNK: janus kinase. IFN: interferon. ATF3: activating transcription factor 3.

2.2.1. Immune effects of HDL: data from animal studies

Mice studies have shown several anti-inflammatory effects of HDL, mainly on endothelial cells and the innate immune system. Mice overexpressing ApoA-I had lower NF-kB signalling (IL-6, MCP-1, TNF-α) in endothelial cells, in response to palmitate (A. M. Cheng et al. 2012). However, conflicting results arose from other studies showing no influence of HDL in NF-kB in mice macrophages (De Nardo et al. 2014) or the activation

of the PKC-NK-kB/STAT1-IRF1 axis due to cholesterol depletion in the presence of HDL in murine and human primary macrophages (van der Vorst et al. 2017).

The anti-inflammatory effects of HDL on mice macrophages seem to be related to a decrease in lipid rafts consequent to the cholesterol efflux from cells, a reduction of reactive oxygen species generation through its anti-oxidant properties and an inhibition of the TLR4 and NADPH oxidase 2 translocation into the lipid rafts (Han et al. 2020). In turn, cholesterol accumulation in mice myeloid cells activates the NLRP3 inflammasome (Westerterp et al. 2018) which is linked with the HDL induced reduction of polymorphonuclear (PMN) leukocyte infiltration in the atherosclerotic plaque (Nicholls et al. 2005; Puranik et al. 2008). In fact, mice lacking the cholesterol transporters ABCA1 and ABCG1 showed leucocytosis and a myeloproliferative disorder that resolved after bone marrow transplantation into transgenic mice with high levels of HDL (Yvan-Charvet et al. 2010), demonstrating that cholesterol transporters and HDL suppress hematopoietic stem cell proliferation. ABCA1 has additional anti-inflammatory effects (that rely on ApoA-I ligation but are independent of cholesterol efflux) through the activation of the JAK2/STAT3 pathway, which suppresses the production of pro-inflammatory cytokines (Y. Liu et al. 2009). Furthermore, HDL was also reported to promote activating transcription factor 3 (ATF3)-mediated anti-inflammatory effects at supra-physiological concentrations in mice macrophages (De Nardo et al. 2014), but this effect was not observed when treating cells with HDL at physiological concentrations nor in human macrophages (Inoue et al. 2018). Interestingly, HDL was also shown to exert pro-inflammatory effects in mice macrophages. Fotakis *et al* verified that the infusion of reconstituted HDL into atherosclerotic mice induces anti-inflammatory effects related to

reduced TLR4 levels and reduced IFN receptor signalling and late pro-inflammatory effects due to a modified endoplasmic reticulum stress response in the context of extreme cholesterol depletion (Fotakis et al. 2019). However, in lesion macrophages the anti-inflammatory effects predominate.

With respect to the adaptive immune response, the capacity of mice antigen presenting cells to activate T cells is reduced by HDL and ApoA-I through cholesterol efflux and lipid raft disruption (S. hui Wang et al. 2012) and by the inhibition of the production of some inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, CCL3 and CCL4) (Gruaz et al. 2010). Moreover, increased levels of plasma membrane cholesterol have been reported to promote an inflammatory T helper response (Surls et al. 2012). Finally, mice splenocytes stimulated with TCR or BCR ligands showed a decreased T and B cell proliferation rate in the presence of HDL (Feng et al. 2011) and ApoA-I was shown to decrease lymph nodes immune cells whilst increasing Tregs in LDLr -/-, apoA-I -/- mice fed with an atherogenic diet (Wilhelm et al. 2010).

2.2.2. Immune effects of HDL: data from human-based research

Studies with human cells showed that HDL and ApoA-I reduce PMN leukocyte adherence to endothelial cells in vitro through blocking lipopolysaccharide activity and modifying CD11b/CD18 (Moudry R, Spycher MO 1997) whilst protecting against the neutrophil respiratory burst (Kopprasch, Pietzsch, and Graessler 2004; Liao et al. 2005). The ApoA-I inhibitory effect on CD11b of activated neutrophils is mediated through ABCA1 and the HDL effects are mediated by SR-BI. SR-BI assumes a relevant role in cholesterol efflux from macrophages as it mediates anti-inflammatory signalling in macrophages and

endothelial cells (Mineo and Shaul 2012) and mediates the efferocytosis of apoptotic cells (Linton et al. 2017). Endothelial cells SR-BI mediates the uptake and transcytosis of HDL in lymphatic vessels to effectively remove cholesterol from the peripheral tissue, thereby raising the possibility that lymphatic SR-BI may also reduce the foam cell formation in atherosclerotic lesions (Bess et al. 2011).

Cholesterol efflux mediated by HDL and ApoA-I results in the decrease in lipid raft abundance. In macrophages, the reduction of available lipid rafts correlates with the decrease in CD11b activation. These results are corroborated by the observation that the infusion of rHDL in patients with peripheral vascular disease attenuate neutrophil activation (Murphy et al. 2011). There is also a negative correlation between HDL and leucocyte levels in patients with coronary artery disease (Gao et al. 2014).

However, HDL from healthy young individuals demonstrated to increase the proliferation of stimulated T cells (Larbi et al. 2014). In fact, the effects of HDL on lymphocyte proliferation are not clarified, with conflicting results arising from different studies. It is very likely that variations in HDL function, concentration and lipid composition can exert different effects. Apart from the effects of HDL on immune cell proliferation, other aspects of the immune response have also been studied in human cells and in patients. Both HDL and ApoA-I inhibit the expression of inflammation markers, increase IL-10 production and promote spreading in primary human monocytes (Diederich et al. 2001; Smythies et al. 2010). In human macrophages infected with mycobacteria, HDL reduced TNF-α production through the downregulation of TLR2 and the consequent suppression of the NF-kB, p38 and JNK mitogen-activated protein kinase (MAPK) pathways. In these macrophages, HDL also reduced the production of IL-6, IFN-γ and IL-4 in a dose-

dependent manner and increased the production of IL-10 (Inoue et al. 2018). ApoA-I inhibited the soluble CD40L-stimulated activation of NF-kB in human THP-1 macrophage-derived foam cells, which seems to depend on the interaction with ABCA1 (K. Yin et al. 2012). Furthermore, HDL and ApoA-I decrease the production of inflammatory cytokines (TNF-α, IL-1β) by human T cell lines (Hyka et al. 2001). The interaction of antigen presenting cells and T cells in human peripheral blood mononuclear cells (PBMCs) was shown to be affected by ApoA-I as it may decrease DCs activation and differentiation and reduce the production of IFN-γ by T cells in response to DCs interaction (Kim et al. 2005). HDL also affects the human DCs ability to induce Th1 response upon TLR stimulation (Perrin-Cocon et al. 2012). Furthermore, HDL binds to stimulated T lymphocytes through the ApoA-I interaction with cell surface factors and inhibits the contact-mediated activation of monocytes with a consequent lower production of TNF-α and IL-1β (Hyka et al. 2001).

Regarding the T cell repertoire, HDL seems to play an essential role in Treg modulation. Treg differ from other lymphocytes in its metabolic requirements, as they rely mostly on lipids as the source of energy to survive (Shi et al. 2011; Michalek et al. 2011). Treg counts increase in the presence of HDL at physiologic concentrations in a dose-dependent manner, with a decrease in the percentage of apoptotic Treg, suggesting that HDL promotes the survival of human Treg. This effect was not seen in other T cell populations (Rueda et al. 2017). In a study that showed an increase in the frequency and absolute numbers of Treg in healthy individuals taking statins, the increase in HDL-C levels showed a positive correlation with the Treg counts (Rodríguez-Perea et al. 2015).

In summary, both HDL and its main apolipoprotein ApoA-I decrease inflammation mediated by monocytes/macrophages and neutrophils. The existent studies point TLR signalling and NF-kB pathways as the principal targets in the innate immune system. In the adaptive immune system, HDL affects T cell proliferation, increases the prevalence of Treg and modulates cytokine production. Overall, the disruption of lipid rafts seems to be crucial to the modulation of the immune response by HDL, bearing in mind that HDL can also exert pro-inflammatory effects under specific circumstances.

2.3. HDL in pathologic conditions

2.3.1. Modifications in HDL function

The function of HDL can be affected by oxidant stress, endothelial function, inflammation, thrombosis, and glucose metabolism. Several studies demonstrated the association of dysfunctional HDL with atherosclerosis (Ansell et al. 2003) due to a reduced capacity to promote RCT and to impaired anti-oxidant and anti-inflammatory properties.

HDL functional measures are not easily available. The HDL inflammatory index (HII) is the most often employed and it is calculated based on the anti-inflammatory properties of HDL such as its inhibitory role in a monocyte chemotaxis assay or the inhibitory effect on LDL oxidation. A high HDL inflammatory index has been correlated with poor survival in hemodialysis patients (Kalantar-Zadeh et al. 2007), predicts severity of organ failure in patients with sepsis (Guirgis et al. 2018) and is associated with the degree of metabolic derangement in diabetes.

2.3.2. Anti-HDL antibodies

Antibodies against HDL were shown to decrease the HDL anti-oxidant capacity and protective effects on endothelial cells (Joana R. Batuca et al. 2018) whilst promoting inflammation (Pagano et al. 2012). These antibodies occur approximately in 21,4% of SLE patients and 9% in HC, in a study from our group (J R Batuca et al. 2007). Interestingly, the presence of anti-HDL antibodies seems to be related with the SLE disease activity (J. R. Batuca et al. 2009; O'Neill et al. 2010). In fact, several studies show that anti-HDL antibodies correlate with HDL dysfunction in different autoimmune diseases (Rodríguez-Carrio et al. 2018). Additionally, anti-HDL antibodies correlate negatively with the serum levels of HDL (Rodr et al. 2020). Most of anti-HDL antibodies are directed against ApoA-1 and decrease the cholesterol efflux capacity (Vuilleumier et al. 2019; Dullaart et al. 2019). A minority of anti-HDL antibodies are directed to other HDL components, such as PON-1 and ApoE (Paiva-Lopes et al. 2020).

2.3.3. HDL in cardiovascular diseases

HDL has been regarded as a protector factor in the progression of atherosclerosis, based on population-based studies that showed an inverse relationship between HDL cholesterol levels and the risk of atherosclerosis (D. Gordon et al. 1989). Furthermore, low HDL level has long been regarded as a risk factor for coronary artery disease (K. Berg, B o rresen, and Dahlén 1976). Low HDL is also a hallmark of metabolic syndrome and half of patients with type 2 diabetes have low HDL cholesterol concentrations (< 1 mmol/L for men and <1.3 mmol/L for women) (Grant and Meigs 2007). Furthermore, HDL levels are affected by various lifestyle-related factors that also influence the risk of atherosclerosis such as

smoking, alcohol consumption, lack of physical exercise, diet and body weight (Bajer et al. 2019).

Several studies suggest that HDL functionality, mainly through the capacity to promote cellular cholesterol efflux, is probably more important in the cardiovascular protection than its plasma levels (Navab et al. 2006). Mendelian randomization genetic studies have shown no direct relation between genetically determined HDL concentrations and cardiovascular events (Voight et al. 2012; Holmes et al. 2015) or type 2 diabetes (Haase et al. 2015). These studies suggest that the association of low HDL plasma concentrations with cardiovascular disease is due to confounding factors and/or low HDL is a marker of the underlying pathophysiology. In fact, patients with low HDL levels associated with heterozygoty for loss-of-function mutations in *ABCA1* gene do not have an increased risk of ischemic heart disease (Frikke-Schmidt et al. 2008). Furthermore, carriers of a mutation in the apoA-I gene (apoA-I Milano) exhibit very low HDL levels and are more protected from atherosclerosis (Chiesa and Sirtori 2003) whilst patients with CETP deficiency who have high levels of HDL are not protected from atherosclerosis (Nagano et al. 2004). Similarly, elevated HDL levels due to hepatic LPL mutations do not reduce cardiovascular disease risk (Fazio and Linton 2015). Very high HDL cholesterol is associated with increased mortality (Madsen, Varbo, and Nordestgaard 2017; Steeg et al. 2008) and the raise in HDL cholesterol due to a variation in SR-BI increases the risk of ischemic heart disease (Zanoni et al. 2016). In addition, several HDL-raising therapies have been tried in cardiovascular diseases late-phase clinical trials, with mixed results (Keene et al. 2014; Kingwell et al. 2014). This is probably related to variations in HDL function. Niacin (Toth 2012) and the CETP inhibitors, torcetrapib (Barter, Caulfield, and Eriksson 2008) and dalcetrapib

(Schwartz et al. 2009), do not decrease cardiovascular risk, although increasing HDL and decreasing LDL. CETP inhibition significantly increases the size and protein composition of HDL particles, which probably renders HDL particles dysfunctional (Klerkx et al. 2006). It seems that apoA-I is more associated with cardiovascular protection than larger HDL particles (Steeg et al. 2008) and smaller HDL3 particles were shown to be the responsible for the inverse correlation between HDL levels and coronary heart disease (Joshi et al. 2016). Therefore, HDL dysfunction is probably more crucial than low HDL levels in the physiopathology of cardiovascular diseases (Rosenson et al. 2016).

2.3.4. HDL in autoimmune systemic diseases

Altered plasma lipoproteins profiles are commonly described in autoimmune systemic diseases, with several studies reporting a decrease in HDL plasma concentrations (Toms 2011), although not unanimously. This is thought to be one of the factors contributing to the high prevalence of atherosclerotic plaques and cardiovascular disease among these patients (Hollan et al. 2013): patients with chronic polyarthritis and SLE have increased risk for myocardial infarction even after controlling for traditional risk factors (Roman et al. 2003).

The HDL levels seem to be inversely correlated with SLE disease activity in some studies (B. Zhou, Xia, and She 2020) but results can be somehow conflicting: high HDL-levels were correlated with nephritis progression to end-stage kidney disease, but low HDL levels were associated with increased risk of all-cause mortality in lupus nephritis (P. Yin et al. 2017). Additionally, HDL from patients with SLE and rheumatoid arthritis (RA) was shown to be pro-inflammatory and associated with increased levels of oxidized LDL

(McMahon et al. 2006). HDL from patients with ankylosing spondylitis and psoriasis also showed to have several functional modifications (Holzer et al. 2012; Gkolfinopoulou et al. 2015).

There are many factors leading to modifications in HDL function in the context of inflammation (Ormseth et al. 2016; Ronda et al. 2014; Charles-schoeman et al. 2012). Inflammation itself can reduce HDL levels due to the upregulation of proinflammatory cytokines by hepatocytes, resulting in the reduced expression and secretion of ApoA-I (Ettinger et al. 1994). Additionally, oxidation dissociates ApoA-I from its lipid cargo (DiDonato et al. 2013). HDL can also be modified by the binding of acute phase proteins such as serum amyloid A, complement factors and other inflammatory proteins (Watanabe et al. 2012; McMahon et al. 2006).

The humoral response against HDL components associated with an increased cardiovascular risk, may also be an important cause of HDL dysfunction (J R Batuca et al. 2007; Dullaart et al. 2019). Conversely, dysfunctional HDL can promote inflammation (C. K. Smith et al. 2017) hence closing a positive feedback loop towards an increasing oxidative and inflammatory status.

In mouse models of SLE, the use of the ApoA-I mimetic peptide, L-4F, improved disease manifestations (Woo et al. 2010), and the HDL mimetic ETC-642 suppressed macrophage activation (C. K. Smith et al. 2017). The clarification of the HDL-related mechanisms in immune response will hopefully provide important knowledge to develop new treatments for atherosclerosis in and outside the context of immune-mediated diseases.

3. T cell response

T cells are divided in two major lineages: $CD8^+$ T cells, which produce cytokines and mediate direct target cell lysis and $CD4^+$ T cells, that following cross-linking of the TCR to antigen-bound major histocompatibility complex (MHC) class II on the surface of APCs, secrete cytokines to orchestrate the immune response.

$CD4^+$ T cells play a central role in the regulation and overall performance of the immune system: they help B cells in antibody production, enhance and maintain the responses of $CD8^+$ T cells, regulate macrophage function, orchestrate immune responses against a wide variety of pathogenic microorganisms and regulate/suppress inadequate immune responses both to control autoimmunity and to adjust the magnitude and persistence of the immune response itself. $CD4^+$ T cells are also important mediators of immunologic memory.

Upon recognition of their specific antigen via TCR, in the context of appropriate co-stimulatory signals, T cells clonally expand and traffic to tissues, where they perform effector functions. Following antigen clearance, most of the expanded effector T-cell pool contracts by apoptosis, but a residual population remains and forms the memory compartment, with an enhanced capacity to respond to a secondary antigen encounter.

The TCR is a multisubunit complex that comprises at least six polypeptides. When the TCR is activated, the immunoreceptor tyrosine-based activation motifs (ITAMs) in the TCR subunits are phosphorylated and the ζ-associated protein of 70kDa (ZAP-70) is recruited to the TCR/CD3-complex. ZAP-70 is then phosphorylated at Tyr-493 by a lymphocyte cell-specific protein tyrosine kinase (Lck) (Mustelin and Taskén 2003). The physiological substrate of the phosphorylated ZAP-70 is a linker for activation of T cells

(LAT) (Au-Yeung et al. 2009). The phosphorylated LAT then binds to the supramolecular activation complex (SMAC) at the interface between APCs and T cells, which signals for the ERK and NF-κB pathway activation (J. Cheng et al. 2011).

The best characterized costimulatory molecule is CD28, which promotes T cell proliferation and production of IL-2 by engaging either B7-1 or B7-2 (CD80 or CD86, respectively) on APCs (Lim et al. 2012). CD80 and CD86 also interact with the co-inhibitory molecule, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which shares a similar structure to CD28 but has a much higher affinity for these counter receptors (Gardner, Jeffery, and Sansom 2014) and the programmed-death ligand 1 (PD-L1), which expression in induced on T cells and interacts with CD80 with an affinity intermediate to that of CD28 and CTLA-4 (Butte et al. 2008). Both CTLA-4 and PD-L1 inhibit the T effector response and increase the suppressive function of Treg (Dilek et al. 2013). CD28 is expressed in resting and activated T cells in contrast to CTLA-4 which is not expressed on the surface of resting T cells (excluding Treg, where it is constitutively expressed), but is upregulated following activation. CTLA-4 has been associated with function, maintenance and generation of regulatory T cells (Chan et al. 2014). Treg cells constitutively express CTLA-4 which is important for the suppressive function of Treg cells and expression of the Treg transcription factor Foxp3 (Barnes et al. 2013). In addition, CTLA-4 also regulates the conventional T cell response. CTLA-4 is located in lipid rafts: in healthy activated T cells, co-ligation of CTLA-4 during TCR stimulation strongly inhibits the upregulation of lipid rafts and the TCR retention, diminishing the immune synapse (Darlington et al. 2002).

CD3ζ is an integral part of the signalling pathway involved in TCR signalling and its downregulation has been reported in numerous pathologies and conditions associated with chronic inflammation. Down regulation of CD3ζ leads to the impairment of the immune responses including reduced cell proliferation and cytokine production. Thus, CD3ζ is thought to be crucial in situations of chronic inflammatory immune responses (Baniyash 2004).

During TCR activation in a particular cytokine milieu, naive $CD4^+$ T cells may differentiate into one of several lineages of T helper (Th) cells, including Th1, Th2, Th17 and inducible Treg (J. Zhu 2018). The different Th subsets are defined by patterns of cytokine production and function (Figure 5). IFN-γ, IL-4 and IL-17A are the signature effector cytokines for the Th1, Th2 and Th17 cells, respectively, although many other cytokines can be preferentially produced by each Th subset, such as lymphotoxin α for Th1; IL-5, IL-9, IL-13, and IL-24 for Th2; and IL-17F and IL-22 for Th17 cells. All the Th subsets can produce IL-2, IL-6, IL-10, IL-21, tumour necrosis factor α (TNF-α), and granulocyte macrophage colony- stimulating factor (GM-CSF). Some regulatory functions of Tregs are mediated through the production of anti-inflammatory cytokines such as TGF-β, IL-10, and IL-35.

Th1 cells play an important role in host defence against intracellular pathogens including viruses and bacteria, being also responsible for the development of some autoimmune diseases (Szabo et al. 2003). Th2 cells are important for the response against parasites and are central in the pathogenesis of asthma and allergic diseases (Paul and Zhu 2010). Th17 cells play an important role in the defence against extracellular pathogens including bacteria and fungi and also participate in the pathogenesis of several autoimmune and inflammatory diseases (Sandquist and Kolls 2018).

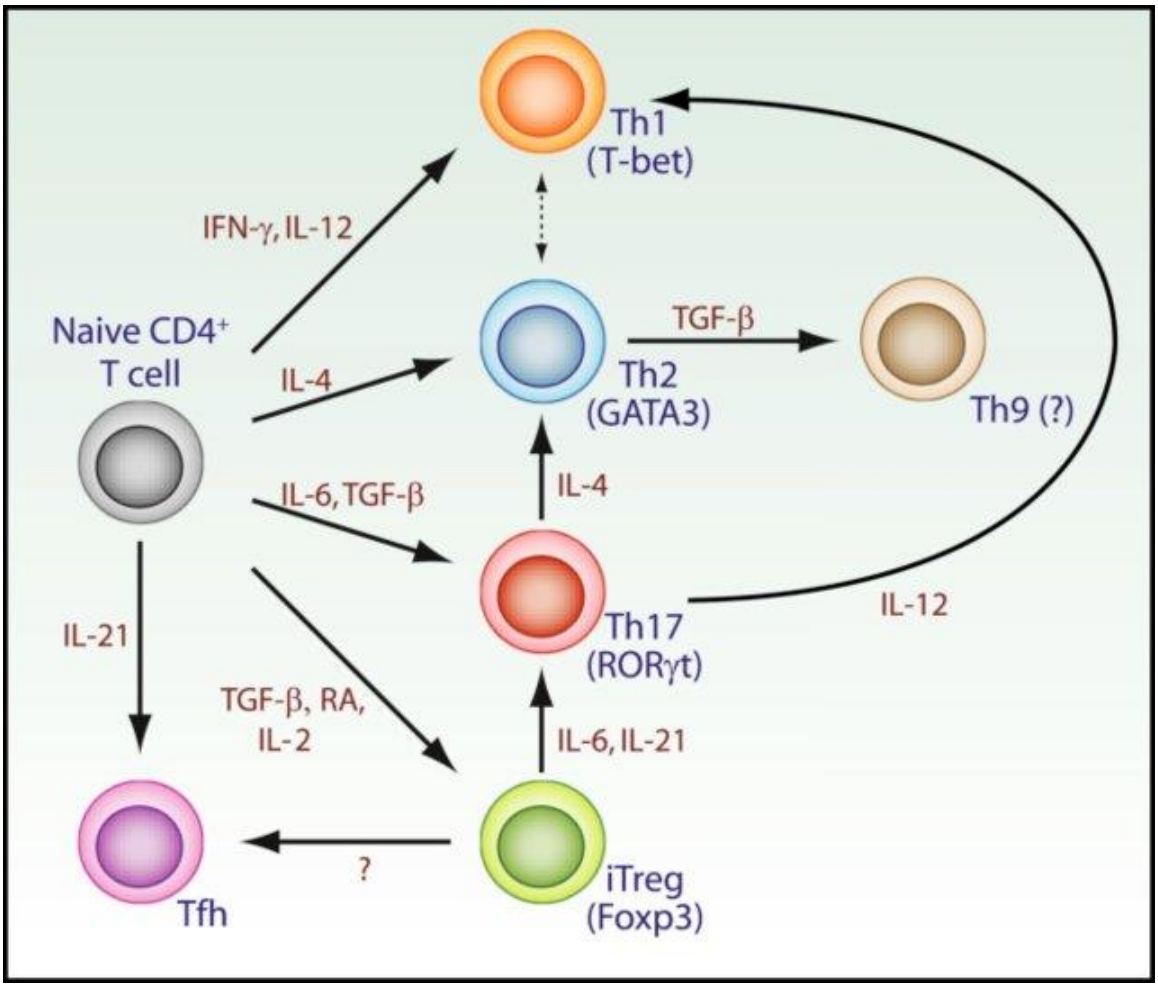


Figure 5: Differentiation of CD4$^+$ T cells.

The naïve helper T (Th) cells can be Th1, Th2, Th9, Th17, or inducible Treg (iTreg), depending upon the pathogen and types of cytokines present in the cellular microenvironment. From (L. Zhou, Chong, and Littman 2009).

Treg were firstly described as a population that constitutively expressed the interleukin 2 receptor α-chain (CD25) (Sakaguchi et al. 1995). Forkhead protein 3 (Foxp3) was subsequently identified as the master regulator that determines the phenotype and function of CD4$^+$ CD25$^+$ Treg cells (Brunkow et al. 2001). Activated human conventional T cells also upregulate FoxP3, but only transiently and at a lower level (Gavin et al. 2006). Both in mice and humans the Foxp3 gene transfer to naïve human CD4$^+$ T cells results in the

acquisition of suppressor functions (Yagi et al. 2004), although differentiation of regulatory T cells is not solely dependent on FoxP3 (W. Lee and Lee 2018). $CD4^+$ $CD25^+Foxp3^+$ Treg cells constitute a specialized T cell population that suppresses the activation, proliferation, differentiation and effector functions of many types of immune cells, including T, B, NK and dendritic cells. These functions are crucial for the maintenance of immune tolerance and are the basis for the negative regulation of inflammation. Treg are divided according to their development mechanism into natural or naïve Treg (nTreg) and peripherally derived Treg (pTreg) (Josefowicz, Lu, and Rudensky 2012). nTreg develop during the process of T cell maturation in the thymus under TCR engagement with self-antigens (H. M. Lee et al. 2012). pTreg are generated *de novo* from conventional $CD4^+FoxP3^-$ T cells in peripheral tissues in response to TGF-β (Wahl and Chen 2005) and continuous exposure to antigen (Shevach and Thornton 2014). In mice, pTregs show *in vitro* and *in vivo* functions similar to those of nTregs (DiPaolo et al. 2007) but pTregs in humans have thus far failed to demonstrate activity in an *in vitro* Treg functional assay (Tran, Ramsey, and Shevach 2007).

Three distinct subpopulations of $CD4^+CD25^+Foxp3^+$ Treg cells were described based on the expression levels of CD45RA and Foxp3: $FoxP3^{low}CD45RA^+$ resting or naïve Treg cells (subpopulation I), $FoxP3^{hi}CD45RA^-$ effector Treg cells (subpopulation II), and $FoxP3^{low}CD45RA^-$ cytokine-secreting nonsuppressive cells (subpopulation III) (Miyara et al. 2009).

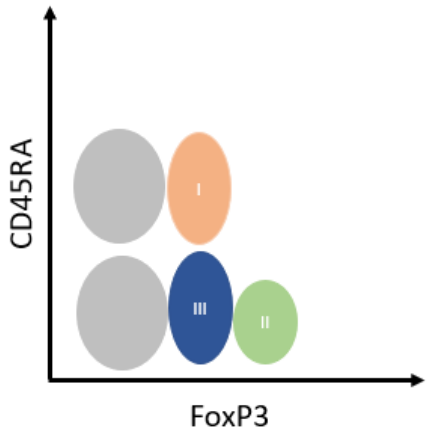


Figure 6: Schematic representation of the subpopulations of human Treg cells in flow cytometry.

Three subpopulations of human Treg cells are defined based on the expression of CD45RA and FoxP3: FoxP3^{low}CD45RA^{+} resting or naïve Treg cells (subpopulation I), FoxP3^{hi}CD45RA^{-} effector Treg cells (subpopulation II), and FoxP3^{low}CD45RA^{-} cytokine-secreting nonsuppressive cells (subpopulation III).

Among the different cytokines that participate in the T cell response, TGF-β and IL-10 are the main responsible for immune regulation. Both TGF-β and IL-10 are highly produced by Treg. TGF-β is a pleiotropic cytokine from the transforming growth factor beta superfamily that regulates embryonic development, adult stem cell differentiation, wound healing, inflammation and the overall immune response. Three TGF-β isoforms have been described: TGF-β1, TGF-β2 and TGF-β3. Most immune cells secrete TGF-β1. TGF-β promotes the differentiation of naïve T cells to Th17 cells when in the presence of IL-6 or to pTreg cells in the absence of IL-6 (Hori, Nomura, and Sakaguchi 2017; W. J. Chen et al. 2003; Tone et al. 2008). The induction of pTreg is also related to the suppression of Th1 (Gorelik, Constant, and Flavell 2002) and Th2 (Wu et al. 2017; Kuwahara et al. 2012) differentiation. As above described, TGF-β also contributes to Treg formation in the thymus by preventing their negative selection (Ouyang et al. 2010). TGF-β also inhibits the activity of effector T cells (Brabletz et al. 1993; Thomas and Massagué 2005; Budhu et al. 2017) and seems to be crucial for the eviction of autoimmune processes as shown in

TGF-β1 KO mice that exhibit a spontaneous initiation of autoimmunity (Weiner et al. 2003).

IL-10 is produced almost by every cell in the innate and adaptive immune system (Couper, Blount, and Riley 2008) and has suppressive effects on myeloid cells (Williams et al. 2004), APCs (Mittal and Roche 2015) and memory T cells (Tian et al. 2016) while promoting the survival and action of Treg (Hsu et al. 2015). IL-10 also promotes B cell response (Heine et al. 2014) and IL-10 producing B cells seem to exert immunoregulatory feedback on other immune cells (Carter, Rosser, and Mauri 2012).

In short, the interaction between plasma lipids and the immune system follows many different pathways, adding to the complexity of the regulation of the immune system. The differences between the various mechanisms explored in multiple experimental models promote the proliferation of different perspectives and theories on the influence of plasma lipids in general, and HDL in particular, in the regulation of the immune response, producing different and often conflicting results.

4. Aims of this thesis

4.1. Overall aim

There is crescent evidence of the influence of lipid pathways in T cell response. Membrane lipids are crucial for T cell response and seem to be deregulated in autoimmune diseases such as SLE. While the complex interactions between lipids and T cell response are not clarified, the search for linking factors is important to understand the intricated mechanisms

involved. In this regard, HDL seems to be an obvious link between lipid metabolism and immune regulation.

This thesis works aims at identifying the main HDL effects on $CD4^+$ T cell metabolism and function. The concomitant study of HDL effects on T cells from healthy donors and patients with SLE intends to find differences in HDL function in the context of health and disease, having SLE as a condition with overly deregulated adaptive immune response and an enhanced atherogenesis.

4.2. Specific aims

1. To identify the experimental conditions in which HDL induces cholesterol depletion from $CD4^+$ T cells *in vitro.*
2. To explore if HDL alters ABCA1 and lipid rafts detection, as well as their interaction *in vitro*.
3. To determine the propensity to form immune conjugates *in vitro* in the presence of HDL.
4. To study the correlation between the presence of anti-HDL antibodies, as a marker of HDL dysfunction, and $CD4^+$ T cells differentiation and lipid metabolism, through the quantification of plasma membrane cholesterol, lipid rafts and ABCA1 expression in the different T cell subsets.
5. To confirm that ABCA1 can be targeted by autoantibodies that could indirectly influence HDL effects.
6. To develop an ELISA protocol to detect anti-ABCA1 antibodies in serum.
7. To determine if HDL alters the prevalence of the main helper T cells subsets *in vitro*.

8. To access the effects of HDL in $CD4^+$ T cells response, in terms of proliferation, TCR activation and cytokines production (IFN-γ, TNF-α, IL-6, IL-10, TGF-β).

Chapter II

Materials and Methods

This chapter describes the methods that were used throughout the thesis. The laboratory experiments were executed at the Chronic Diseases Research Centre (CEDOC) in Nova Medical School of Lisbon and in the Centre for Rheumatology Research of University College London (UCL), London, UK.

1. Patients and controls

Patients with SLE attending the Lupus Outpatients Clinic of University College London Hospital, patients with SLE attending Lupus Outpatients Clinic of the Systemic Immunomediated Diseases Unit of Fernando Fonseca Hospital, Amadora, Portugal and healthy individuals were invited to participate. One hundred and three patients with SLE, who met the 2004 revised criteria for SLE of the American College of Rheumatology (Isenberg et al. 2005), and 61 healthy individuals were enrolled (Table 1). Informed consent was obtained from all patients and controls. This research was approved by the University College London Hospitals Research and Development Directorate (reference 00/0241) and London - City & East Research Ethics Committee (reference 15/LO/2065), by the Nova Medical School Ethics Research Committee (reference 01/2016/CEFCM), by the Fernando da Fonseca Hospital Ethics Committee, and by the *Comissão Nacional de Protecção de Dados* (authorization number 4186/2016).

Table 1: SLE patient and healthy donor demographic characteristics.

	Healthy donors (n = 61)	SLE patients (n = 103)	Statistical analysis
Mean age (range)	35 (22-59)	40 (20-66)	Non-significant
Sex: female: male	34:27	91:12	$p < 0.00001$

Groups were compared using Mann-Whitney test for significance using a 95% confidence interval. SLE: systemic lupus erythematosus.

2. Whole blood staining for *ex vivo* studies

Blood samples were collected aseptically by venipuncture to serum tubes and tubes containing EDTA. Whole blood in EDTA tubes was distributed into three polystyrene tubes (BD Biosciences), 100uL each, and incubated with fluorescent antibodies for surface proteins for 30 minutes: CD4 brilliant violet 605, CD25 APC, CD127 PE, CD27 APC/Cy7, CD45RA PE/Cy7 in all tubes, and ABCA1 FITC (Abcam) in one tube. Red cell lysis was performed with lysing solution, according to the manufacturer protocol (BD Biosciences). After washing, samples stained with ABCA1 FITC were fixed with paraformaldehyde (dissolved to 2% in PBS). The remaining samples were stained with FITC labelled cholera toxin subunit B (CTB) which binds to ganglioside M1 (GM1), a surrogate membrane glycosphingolipid marker (McDonald et al. 2014; Waddington et al. 2021), or Filipin, a cholesterol chelator that naturally fluoresces under ultraviolet light, before fixing.

3. Peripheral blood mononuclear cell (PBMC) isolation, storage, and thawing

Blood samples were collected aseptically by venipuncture using tubes containing sodium heparin. Each sample was maintained at room temperature and processed within 6 h. Peripheral blood mononuclear cells (PBMCs) were purified by density gradient separation according to standard protocols (English and Andersen 1974). Blood was diluted 1:1 in RPMI-1640 culture medium (Gibco BRL) before gently layering onto 15ml Biocoll (Biochrom) in 50ml falcon tubes (Sepmate). Samples were then centrifuged at 800g for 30 minutes at 21ºC. Recovered PBMCs were diluted twice in 1:1 RPMI 1640 culture and centrifuged at 400g for 10 minutes at 4ºC. The supernatant was discarded and the pellet resuspended in 10ml RPMI. Cell concentration was determined by diluting 1:10 in trypan blue (Sigma) and counting cells using a haematocytometer under a light microscope. After repeating cell centrifugation using the same setting, isolated PBMCs were frozen in cryovials with 1 x 10^7 cells/mL and stored in a cryoprotective media containing 10% dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS). PBMCs were gradually brought to -80ºC using Nalgene™ Mr Frosty freezing containers containing isopropanol to achieve a freezing rate of -1ºC/minute. For long-term storage, samples were transferred into a -150ºC freezer after 24-72h.

For each experiment, frozen PBMCs were removed from the -150ºC freezer and quickly thawed by pre-warming 20ml RPMI per sample to 37ºC in a water bath. A sterile Pasteur pipette was used to thaw each vial individually before diluting in pre-warmed RPMI and

wash twice to remove traces of DMSO. The cells were then resuspended in complete RPMI (RPMI-1640 medium; 10% FBS, 1% penicillin/streptomycin), as in Figure 7.

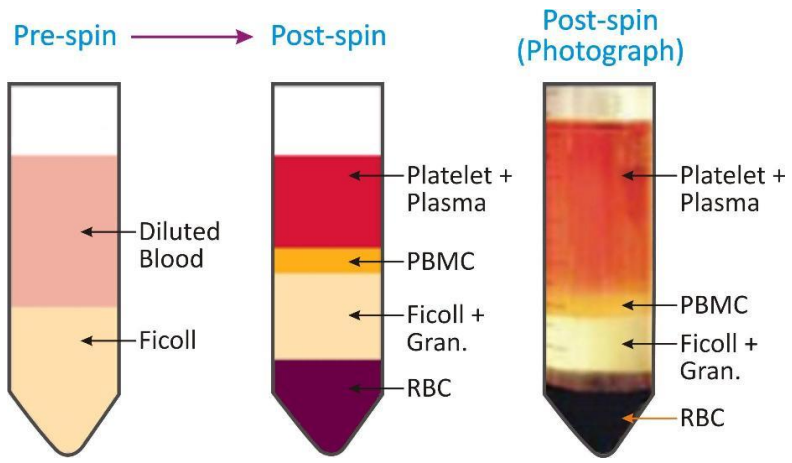


Figure 7: Isolation of PBMCs using density gradient centrifugation.

Illustration of the layering of blood before and after centrifugation.

4. Surface staining for flow cytometry

Cell staining was carried out in a 96 well plate at a concentration of 1-2x10^6 PBMCs per well. After culture, cell culture supernatant was removed and cells were washed once with PBS. Cells were incubated with a death cell marker 50µl/well diluted 1:200 in PBS (LIVE/DEAD® Fixable Violet Dead Cell Stain Kit, TermoFisher Scientific – Life Technologies) for 20 minutes at 4ºC before washing with PBS. Cells were incubated with monoclonal antibodies for extracellular proteins for 20 minutes at 4ºC. Cells were subsequently washed with PBS and fixed (eBiosciences, TermoFisher Scientific, Portugal) for 30 minutes at room temperature in the dark. Cells were then incubated in

permeabilization buffer with antibodies to intracellular targets for 30 minutes at 4ºC. Antibodies were titrated for optimal dilution (Table 2).

5. Intracellular cytokine staining for flow cytometry

After surface staining, cells were washed with PBS and fixed (eBiosciences, TermoFisher Scientific, Portugal) for 30 minutes at room temperature in the dark. Cells were subsequently incubated in permeabilization buffer and antibodies to intracellular targets. Cells were washed with PBS and resuspended in FACS buffer for analysis. Samples were collected on a BD Canto flow cytometer. The flow cytometry data were analysed by the conventional manual method of visual inspection using commercial software (FACSDiva, BD Bioscience, Inc., San Jose, CA, USA).

Table 2: Cell surface antibodies and markers used.

Marker	Fluorochrome	Clone	Species/Isotype	Manufacturer
CD3	FITC	UCHT1	Mouse IgG1, κ	Biolegend
CD4	FITC	OKT4	Mouse IgG2b, κ	Biolegend
CD4	APC	RPA-T4	Mouse IgG1, κ	Biolegend
CD4	APCCy7	RPA-T4	Mouse IgG1, κ	Biolegend
CD14	PECy7	HCD14	Mouse IgG1, κ	Biolegend
CD19	PE Dazzle	HIB19	Mouse IgG1, κ	Biolegend
CD25	PECy7	BC96	Mouse IgG1, κ	Biolegend
CD127	PECy5	A019D5	Mouse IgG1, κ	Biolegend
CD45RA	BV510	HI100	Mouse IgG2b, κ	Biolegend
CD27	APCCy7	O323	Mouse IgG1, κ	Biolegend
FoxP3	PE	259D	Mouse IgG1, κ	Biolegend
Ki-67	PE	Ki-67	Mouse IgG1, κ	Biolegend
TCRzeta (CD247)	Alexa488	K25-407.69	Mouse IgG2a, κ	BD Pharminogen
IL-6	PECy7	MQ2-13A5	Rat IgG1, κ	Biolegend
TNFα	APC	MAb11	Mouse IgG1, κ	Biolegend
IFNγ	APC	4S.B3	Mouse IgG1, κ	Biolegend
IL-17A	APC	BL168	Mouse IgG1, κ	Biolegend
IL-4	PE	8D4-8	Mouse IgG1, κ	Biolegend
IFNγ	FITC	4S.B3	Mouse IgG1, κ	Biolegend
IL-10	APC	JES3-9D7	Rat IgG1, κ	Biolegend
TGF-β	APC	TW7-16B4	Mouse IgG1, κ	Biolegend
Cholera Toxin subunit B (CTB)	FITC	N/A	N/A	Sigma
Filipin	Emission at 470nm	N/A	N/A	Sigma
ABCA1	FITC	Ab18180	Mouse IgG1, κ	Abcam
Live/dead	Violet (emission at 451 nm)	N/A	N/A	Life Technologies

6. Lipid detection

Staining for lipid rafts was carried out following staining for T cells using a surrogate glycosphingolipid marker CTB (McDonald et al. 2014). 50 µl CTB-FITC (1:100 FACS buffer) was added following re-suspension of PBMCs before incubating at 4ºC in the dark for 30 minutes. Cells were then washed and fixed in 2% PFA, then analysed by flow cytometry as previously described.

Filipin staining was carried out after staining for T cells. Cells were fixed with 2% PFA for 1 hour at room temperature before washing in FACS buffer and staining with 50 µg/ml filipin for 2 hours at room temperature in the dark. Cells were then washed and resuspended in FACS buffer before proceeding to analysis as previously described.

PBMCs from three healthy donors were cultured with and without HDL at the concentration of 20 µg/mL or ApoA-I at the concentration of 10 µg/mL for 24, 48 and 72 hours. After incubation, PBMCs were stained for Live/Dead FITC, CD3 APCeFluor 780, CD4 BV605, CD27APC, CD45RA PE-Alexa610, CD25 PE, CD127 BV421 and filipin before fixing. Flow cytometry analysis was performed on a BD LSRII flow cytometer.

7. ImageStream analysis of ABCA1 and lipid rafts colocalization

Cells were stained for B cells (CD19), monocytes (CD14), T cells (CD3), ABCA1 and lipid rafts (CTB) as previously described. Acquisition of data was carried out using Amnis® ImageStreamX Mk II, where $1x10^5$ single cells were collected for each sample,

based on object diameter and aspect ratio. Data was analysed using Amnis IDEAS software version 6.0. Focused cells were gated according to gradient RMS, and single cells or conjugates were gated according to area and aspect ratio.

PBMCs from healthy donors were incubated during 24 hours with and without HDL at the concentration of 20 µg/mL or ApoA-I at the concentration of 10 µg/mL to determine ABCA1 and CTB (cholera toxin subunit B) colocalization as CTB interacts with ganglioside GM1, a component of lipid rafts. ABCA1 was stained using an anti-ABCA1 antibody with biotin and PE-streptavidin. CTB was stained using FITC. After staining, the colocalization of ABCA1 and CTB were analysed in the imaging flow cytometer ImageStream (EMD Millipore).

8. ImageStream analysis of immune synapse formation

PBMCs were thawed as previously described and cultured with complete RPMI. Following incubation, cells were stained for CD4, CD14 and CD19, before being sorted using BD FACS Aria. After sorting, T cells were incubated with and without HDL 50 µg/mL and B cells and monocytes were stimulated with super-antigen (staphylococcal enterotoxin B) for 1 hour.

After the incubation period, T cells were mixed with B cells or monocytes at a 1:1 ratio. Then 2 x 10^6 cells were incubated for 5 and 15 minutes at 37ºC in complete RPMI tissue culture medium supplemented with 10% FCS, to allow the formation of T-B cell and T cell-monocyte conjugates. After incubation, cells were fixed with 2% paraformaldehyde.

To induce conjugate formation, purified B cells or monocytes were added to the appropriate wells and incubated at 37°C (5%CO2) for 5, 10 and 15 minutes. At each time point, 2 x Fixation/ Permeabilization Buffer was added to the appropriate wells to stop the reaction. Samples were finally incubated in the dark at 4°C for 20 minutes before staining for cell signalling molecules. ImageStream analysis of immune conjugates enables automated collection and quantitative image analysis of thousands of conjugates per sample, thereby providing a statistically robust analysis of the IS. As many as 10000 images per sample were acquired with the ImageStream system (Amnis) as described previously (Hosseini et al. 2009).

9. Proliferation assays

9.1.1. Ki-67 expression

Ki-67 is a nuclear protein that is expressed in all phases of the cell cycle. This nuclear protein is best detected during the interphase of the cell cycle within the nucleus of the actively dividing cells and is used as a proliferation marker. PBMCs pre-incubated with and without ApoA-I or HDL at different concentrations were stimulated for 72 hours with anti-CD3 (5 µg/ml) and anti-CD28 (5 µg/ml) antibodies to measure Ki-67 through flow cytometry.

Monoclonal antibodies used for surface staining included FITC conjugated anti-CD4 (Biolegend), PE/Cy7 conjugated anti-CD25 (Biolegend), PE/Cy5 conjugated anti-CD127 (Biolegend), APC/Cy7 conjugated anti-CD27 (Biolegend) and BV510 conjugated anti-CD45RA (Biolegend). Intracellular staining with mouse anti-human monoclonal antibody

against Ki-67 was performed after permeabilization and fixation with a Cytofix/Cytoperm Kit (BD Biosciences) according to the manufacturer's protocol. Cells were measured by using a FACSCanto (BD Biosciences). Data were analysed using CellQuest (BDIS) and FlowJo Software. Cells expressing high levels of Ki-67 and forming a distinct population were considered to be Ki-67^{+}.

10. CellTrace Far Red

CellTrace Far Red (CellTrace^{TM} Far Red Cell Proliferation kit, Life Technologies) is a red laser-excitable fluorescent dye that crosses the plasma membrane and covalently binds proteins inside cells. The well-retained fluorescent label offers a consistent, reliable fluorescent signal without affecting morphology or physiology and has low cellular toxicity. CellTrace Far Red was demonstrated to be an excellent probe for cellular proliferation studies using flow cytometry, allowing measurement of up to eight generations of proliferating cells. It is excited by the red laser with narrow far-red emission (excitation/emission max of 630/633 nm) and can be combined with other fluorophores.

CellTrace Far Red was made at the concentration of 5 µM (20 µl DMSO). This stock solution was diluted into 20 mL of PBS (warmed to 37ºC) for a 1 µM staining solution. The prepared cells (10 ml) were centrifuged at 400 × g for 5 min. The supernatant was carefully removed. The CellTrace Far Red staining solution (10 mL) was added to the cells and incubated for 20 minutes in a 37°C water bath, protected from light. Then, 2mL of cold FCS were added to every 10 mL of sample and incubated for 5 minutes. Cells were

centrifuged at 400 × g for 5 min and the cell pellet was resuspended in complete medium (2 x 10^6 cells per mL).

PBMCs pre-labelled with Cell Trace Far Red were distributed into 96-well culture plates. PBMCs were pre-incubated with or without HDL at the concentration of 600 µg/mL and then stimulated with CD3CD28 antibodies for 6 days, except in the unstimulated control sample. After incubation for 6 days, samples were stained with a viability dye (LIVE/DEAD ^{TM} Fixable Violet Dead Cell Stain Kit, Life Technologies) and lymphocytes were surface immunophenotyped for CD14 and CD4. Activated lymphocytes were then analysed for cell proliferation signal by computer analysis using FlowJo software.

11. TCRzeta phosphorylation experiments

CD3 zeta phosphorylation is an indicator of T cell activation via the TCR complex and is studied as a model for antigen-induced activation. Healthy PBMCs pre-incubated with or without HDL at the concentrations of 50 and 600 µg/mL for 24h were treated on ice with anti-CD3 and anti-CD28 antibodies for 5, 10, 15 and 20 minutes. This was followed by a secondary antibody incubation (goat anti-mouse IgG F(ab')$_2$, Sigma-Aldrich, USA) that recognizes the host IgG of the anti-CD3 and anti-CD28 antibodies, to cross-link the subunits of the TCR complex for optimal signal transduction and T cell activation. Immediately after the defined timepoints, cells were fixed with a fixation/permeabilization kit (eBiosciences, USA), according to the manufacturer's protocol. Staining with CD4 APC (Biolegend) and TCRzeta (CD247) AF488 (Becton Dickinson, USA) was followed by flow cytometry acquisitions using FACS Canto II flow cytometer (Becton Dickinson,

USA) and FACS Diva Software (Becton Dickinson, USA). Data were subsequently analysed using FlowJo software.

12. PBMC culture for helper T cell subsets and cytokine expression

Cells were cultured for 24h in RPMI 1640 culture medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with penicillin 10 U/ml, streptomycin sulphate 10 µg/ml, L-glutamine 2 mmol/l and 10% FCS (all obtained from Gibco), with and without HDL at the concentrations of 50, 300 and 600 µg/mL. To study cytokine expression, cells were stimulated either with or without 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (VWR, Portugal) and 500 ng/mL ionomycin (VWR, Portugal) for 12h, in the presence of the secretion blocker brefeldin A 10 µg/mL (Biolegend) for the last 6h of stimulation. To study helper T cell subsets, cells were stimulated with anti-CD3 and anti-CD28 antibodies (eBioscience, USA) and PMA/ionomycin for 12 h in the presence of the secretion blocker brefeldin A 10 µg/mL (Biolegend, USA) during the last 6h of stimulation.

Cell culture supernatant was removed, and cells were washed once with PBS. Cells were then incubated with a death cell marker (LIVE/DEAD® Fixable Violet Dead Cell Stain Kit, TermoFisher Scientific – Life Technologies, Portugal) for 20 minutes at 4ºC. Cells were incubated with CD4 APC-Cy/7 for 20 minutes at 4ºC. Cells were subsequently washed with PBS and fixed (eBiosciences, TermoFisher Scientific, Portugal) for 30 minutes at room temperature, in the dark. Cells were subsequently incubated in permeabilization buffer and with the following antibodies to intracellular cytokines: CD4 APC-Cy/7 and

IFN-γ APC, IL-6 PE-Cy/7 and TNF-α APC, IL-10 APC (Biolegend) and TGF-β1 APC (Biolegend) for 30 minutes at 4ºC. Cells were then washed with PBS and resuspended in FACS buffer for analysis. Samples were collected on a BD Canto flow cytometer.

The flow cytometry data was analysed using CellQuest software (BDIS). Cell populations were identified visually and were gated. The gates were then used to filter the population for subsequent analysis. The first step of analysis consisted of filtering out the doublets. For this, a single gate was defined using the FSC-H and forward scatter (FSC-A) plot. Lymphocytes were gated. Debris and dying or dead cells were identified by their relatively lower FSC and SSC values. The dead cell marker was plotted against CD4 for the discrimination of the $CD4^+$ T cells (living cell populations). Final analysis of data was performed using FlowJo Software.

For detection of Treg, PBMCs from healthy donors were cultured for 24 hours with and without HDL at the final concentration of 50 µg/mL. Treg and their subgroups were determined using FITC conjugated anti-CD4 (Biolegend), PE/Cy7 conjugated anti-CD25 (Biolegend), PE/Cy5 conjugated anti-CD127 (Biolegend), APC/Cy7 conjugated anti-CD27 (Biolegend), BV510 conjugated anti-CD45RA (Biolegend) and PE conjugated anti-FoxP3 (Biolegend) antibodies, after excluding death cells from analysis with a viability dye. Intracellular staining was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) following the manufacturer's instructions. Fluorescence of labelled cells was recorded using a FACS Canto II flow cytometer (Becton Dickinson, USA) and analysed using CellQuest software (BDIS). Lymphocytes were gated based on forward and sideward scatter (FSC and SSC). Treg cells were determined as a proportion of $CD4^+$ cells. $CD4^+$ T cells were analysed for CD25 and CD127 expression and then the

$CD25^{+}CD127^{-}$ cells were analysed for CD45RA and Foxp3 expression. The final analysis of the data was performed using FlowJo Software.

13. Enzyme-Linked Immunoabsorbent Assays (ELISAs)

13.1.1. Anti-high density lipoproteins (anti-HDL) IgG antibodies

IgG anti-HDL antibodies were measured by ELISA using 96-well microtiter plates (Polysorp, Nunc, VWR, Portugal) half-coated with 20 µg/mL human HDL (Sigma-Aldrich, Sintra Portugal) in 70% ethanol up for evaporation at 37ºC. The plates were then blocked (non-specific binding) with the addition of 100 µL/well of 1% of bovine serum albumin (BSA from Sigma-Aldrich) – 10 mM phosphate buffer saline (PBS from Sigma-Aldrich) pH 7.4, over one hour at 37ºC. The unbound blocking agent was removed by washing the plates four times with PBS. Then, positive and negative controls and samples were diluted (1:100) in the blocking buffer (1% BSA – 10mM PBS pH 7.4) and added in both halves of the plate for one hour at 37ºC. The unbound antibodies were removed by repeated washes. Secondary antibodies (Sigma-Aldrich) alkaline phosphatase (AP) conjugated anti-human IgG (1:1000 in blocking buffer) were added for one hour at 37ºC. Following three washes with PBS and three washes with bicarbonate (BIC) buffer pH 9.8 the colorimetric reaction was performed by the addiction of 100 µL/well p-nitrophenyl phosphate (pNPP from Sigma-Aldrich) 1:5000 in BIC buffer and the reaction was allowed to proceed for one hour at 37ºC in the dark. Absorbance of the resultant yellow colour was measured by Biotrak II plate reader (Amersham Biosciences) at 405 nm.

13.1.2. Anti-ABCA1 IgG antibodies

IgG anti-ABCA1 antibodies were measured by ELISA using 96-well microtiter plates (Polysorp, Nunc, VWR, Portugal), half-coated with ABCA1 peptide (ab14148, Abcam) 5 µg/mL in carbonate-bicarbonate (BIC) coating buffer (pH 9.8) and left overnight at 4ºC. The plates were then washed twice with phosphate buffer saline tween (PBS-T) 0.1% and blocked (non-specific binding) with the addition of 100 µL/well of 2% of bovine serum albumin (BSA from Sigma-Aldrich) – 10 mM PBS, pH 7.4, over one hour at 37ºC. The unbound blocking agent was removed by washing the plates four times with PBS-T 1%. Then, the samples were diluted (1:100) in blocking buffer (1% BSA – 10mM PBS pH 7.4) and added in both halves of the plate for one hour at 37ºC. Serial dilutions of an anti-ABCA1 antibody (rabbit polyclonal ab7360, Abcam) were performed to obtain the standard curve. The unbound antibodies were removed by repeated washes. Secondary antibodies (Sigma-Aldrich) alkaline phosphatase (AP) conjugated anti-human IgG (1:1000 in blocking buffer), or anti-rabbit IgG (1:5000 in blocking buffer) for the standard curve dilutions were added for one hour at 37ºC. Following two washes with PBS and two washes with BIC buffer the colorimetric reaction was performed by the addiction of 100 µL/well p-nitrophenyl phosphate (pNPP from Sigma-Aldrich) 1:5000 in BIC buffer and the reaction was allowed to proceed for one hour at 37ºC in the dark. Absorbance of the resultant yellow colour was measured by Biotrak II plate reader (Amersham Biosciences) at 405 nm.

14. Data analysis

Data was expressed as means ± SD, unless otherwise stated. A t-test or Mann-Whitney test was used to compare individual groups depending on the variable distribution. Relationships between variables were assessed by Spearman correlation. All reported probability values are two-tailed, with values of $p < 0.05$ being considered statistically significant. Statistical analysis and graphical illustration were carried out using the GraphPad Prism software, version 8 (GraphPad Software Inc., San Diego, USA).

Chapter III

Results

1. T cell membrane studies

1.1. T cell subsets characterization in healthy donors and patients with SLE

The phenotyping of $CD4^+$ T cells showed that in healthy $CD4^+$ T cells (n = 11) the subpopulation of naïve T cells is the more prevalent (48.4 ± 8.8%) followed by central memory (CM) T cells (32.5 ± 10.8%). Effector memory (EM) T cells (7.1 ± 3.3%) and Treg (4.9 ± 1.6%) are the less prevalent subpopulations (Figure 8). The study of PM showed that cholesterol content, measured by filipin binding, was slightly higher in EM T cells than in the other healthy $CD4^+$ T cells subpopulations. Lipid rafts were less abundant in EM T cells, suggesting a resting state. There was no correlation between ABCA1 expression and PM cholesterol or lipid rafts in the membrane of healthy $CD4^+$ T cells (Figure 9).

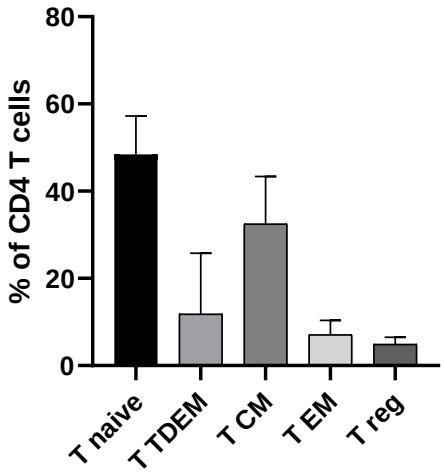


Figure 8: T cell subsets distribution in $CD4^+$ T cells from healthy donors.

Naïve T cells are the most prevalent $CD4^+$ T cell subset in healthy donors, followed by central memory T cells (n = 11).

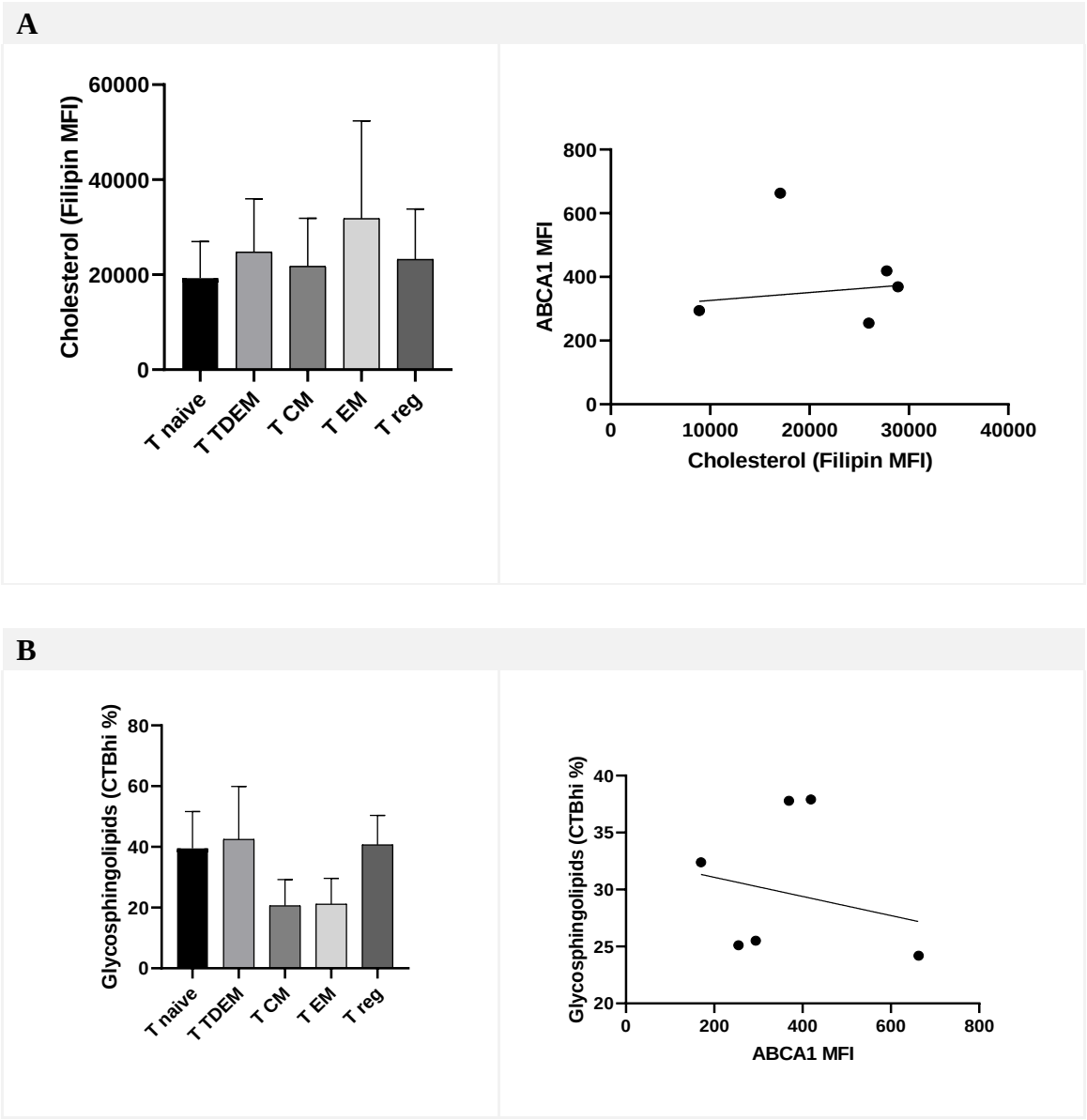


Figure 9: Cholesterol, lipid rafts and ABCA1 in the plasma membrane of CD4$^+$ T cells.

Membrane cholesterol assessed by filipin binding (A) and CTB, a surrogate marker of glycosphingolipids that compose lipid rafts (B) in CD4$^+$ T cells subsets, and correlation with ABCA1 expression. Correlations were obtained using Spearman's rank correlation.

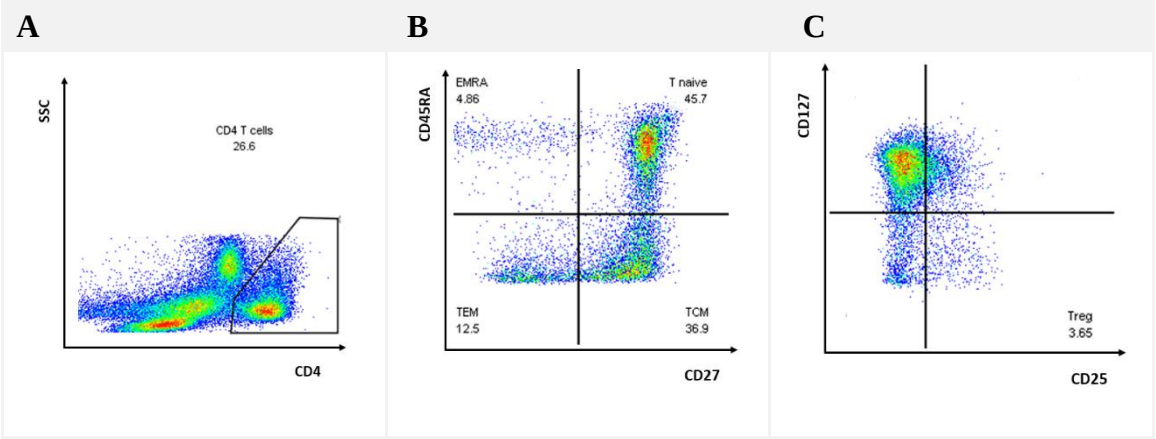


Figure 10: Flow cytometry plots showing the gating strategy for CD4$^+$ T cells subsets.

A) Gating on lymphocytes; B) Gating on CD4$^+$ T cells for naïve T cells, T central memory (TCM), T effector memory (TEM) and terminally differentiated effector memory T cells (EMRA); C) Gating on CD4$^+$ T cells for regulator T cells (Treg).

The T cell population from patients with SLE showed a slight lower prevalence of $CD4^+CD45RA^+CD27^-$ naïve T cells (48.42% vs 40.01%, p = 0.206) and a higher prevalence of $CD4^+CD45RA^-CD27^-$ effector memory (7.12% vs 18.12%, p = 0.035) and $CD4^+CD25^+CD127^-$ regulator T cells (4.92% vs 9.34%, p = 0.005) when compared to healthy controls (Figure 11). The presence of anti-HDL antibodies in sera from SLE patients was associated with a further decrease in naïve T cells (48.42% vs 26.78%, p = 0.001) and an increase in the prevalence of EM T cells (7.12% vs 25.3%, p = 0.002). The prevalence of Treg was lower in the presence of anti-HDL antibodies than in patients without detectable anti-HDL antibodies (6.47% vs 9.34%, p = 0.18).

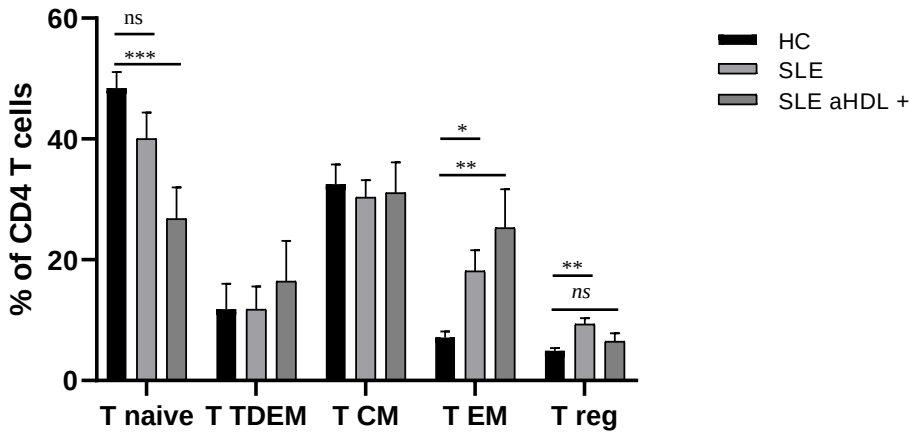


Figure 11: Prevalence of CD4⁺ T cells subsets.

The prevalence of $CD4^+$ T cell subsets differs in healthy controls (n = 11), SLE patients without detectable anti-HDL antibodies (n = 22) and SLE patients with anti-HDL antibodies (n = 6). Bars show mean and SEM. Statistical significance was considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). T naïve: naïve T cells; T TDEM: terminally differentiated effector memory T cells; T CM: Central memory T cells; T EM: Effector memory T cells; T reg: regulator T cells; aHDL: anti-HDL antibodies.

1.2. HDL *in vitro* effect on T cell membrane cholesterol

24 hour culture of $CD4^+$ T cells with HDL and ApoA-I reduced membrane cholesterol assessed by filipin binding (Figure 12). At 48 and 72 h this effect was lost, suggesting that cholesterol can possibly return to the plasma membrane. However, the 24 h reduction in membrane cholesterol was more accentuated with HDL (68,5%) than with ApoA-I (31.1%). The analysis of T cell subsets showed that the reduction of membrane cholesterol promoted by HDL and ApoA-I occurred in the same proportion all CD4+ T cell subsets (data not shown).

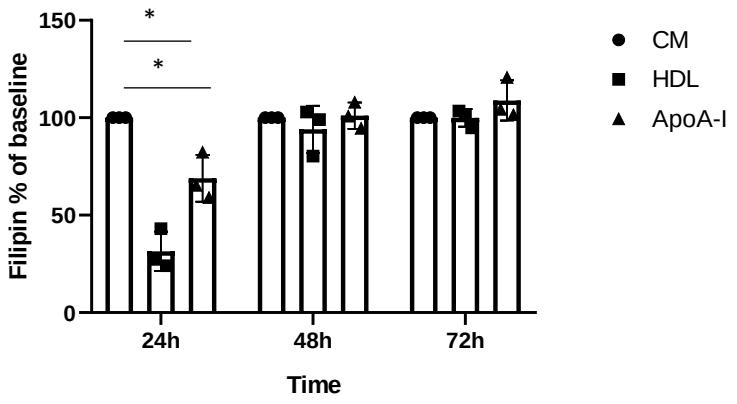


Figure 12: Plasma membrane cholesterol in CD4$^+$ T cells.

Membrane cholesterol measured by filipin binding (n = 3) show that HDL and ApoA-I reduced membrane cholesterol of in CD4$^+$ T cells after 24 hour incubation. Statistical significance was considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

1.3. HDL effect on T cell lipid rafts and ABCA1

Incubation of PBMCs with HDL or ApoA-I did not alter the colocalization of glycosphingolipids (using CTB as a surrogate marker) or ABCA1 in the PM of monocytes and T lymphocytes. ABCA1 and CTB showed low colocalization both in monocytes and T cells and this was not affected by the presence of HDL or ApoA-I. Monocytes showed higher expression of ABCA1 than T cells, but the same pattern of no colocalization with CTB (Figure 13).

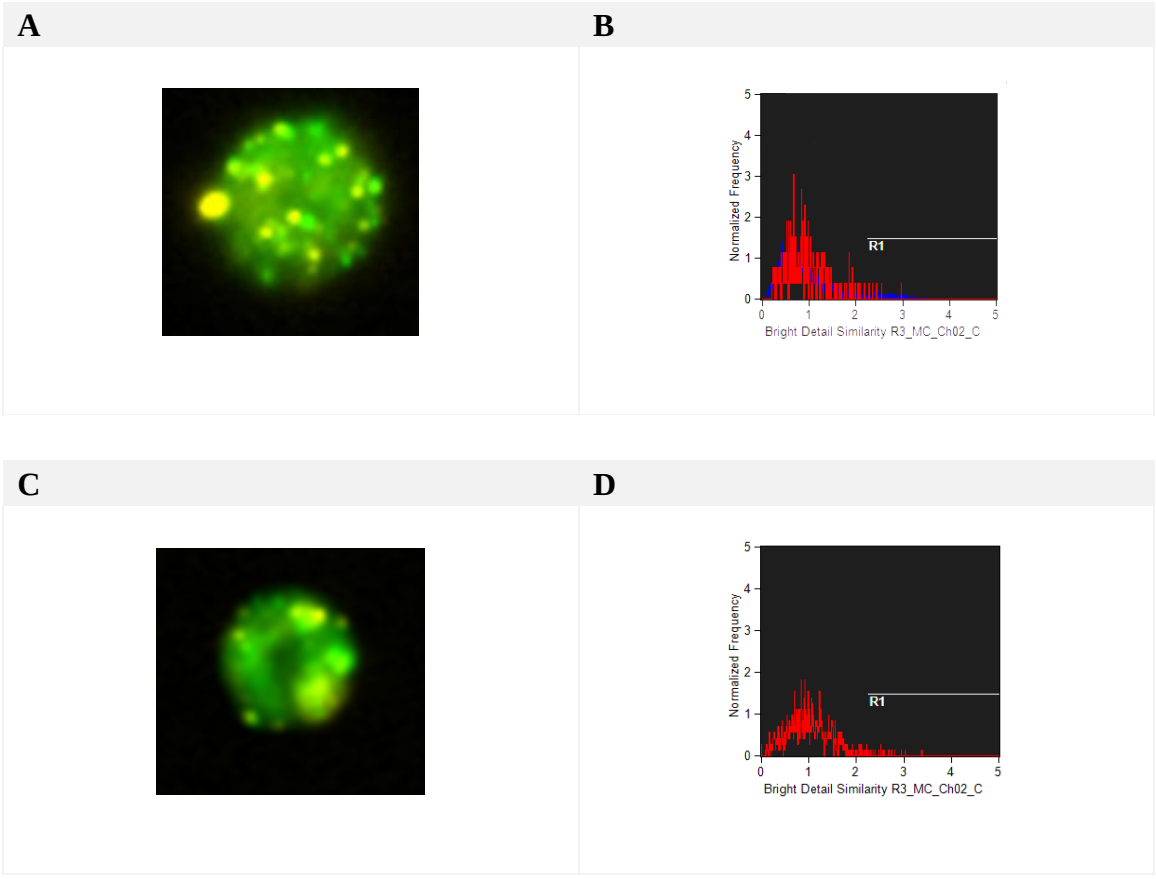


Figure 13: Colocalization of ABCA1 and lipid rafts.

A) ImageStream capture of CTB (green) and ABCA1 (yellow) in a monocyte; B) ABCA1 and CTB do not colocalize in unstimulated monocytes; C) ImageStream capture of CTB (green) and ABCA1 (yellow) in a CD4+ T cell; D) ABCA1 and CTB do not colocalize in unstimulated $CD4^+$ T cells.

1.4. HDL effect on immune conjugates formation

To verify if HDL could inhibit or delay the formation of immune conjugates, $CD4^+$ T cells were incubated with antigen presenting cells (APCs, monocytes or $CD19^+$ B cells) in the presence of super-antigen to examine immune conjugates formation for 5 and 15 minutes, in the presence and absence of HDL. However, incubation with HDL did not affect T cell-APC conjugates formation (Figure 14).

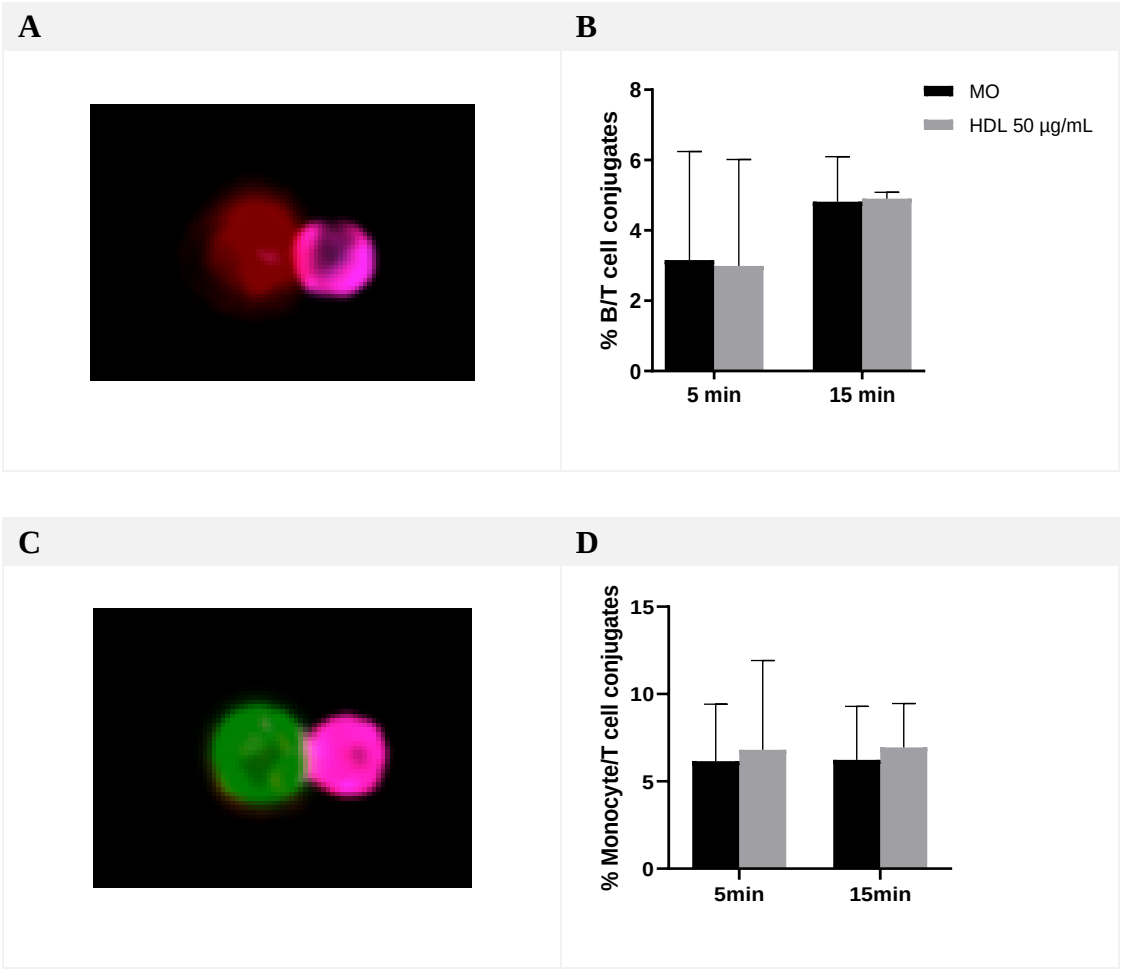


Figure 14: Immune conjugates formation.

A) ImageStream capture of $CD4^+$ T cell and B cell synapse (red – CD19, magenta – CD3); B) Percentage of B/T cell conjugates in the total amount of B cells; C) ImageStream capture of $CD4^+$ T cell and monocyte synapse (green – CD14, magenta – CD3); D) Percentage of monocyte/T cell conjugates in the total amount of monocytes. MO – medium only.

1.5. Anti-HDL antibodies associate with an increased expression of lipid rafts in $CD4^+$ T cells from SLE patients and with a deregulated membrane cholesterol and ABCA1 expression

The determination of anti-HDL levels in the sera of healthy donors and patients with SLE was used as a tool to identify the donors that might have more dysfunctional lipid metabolism and immune response. Patients with SLE showed higher levels of IgG anti-

HDL antibodies in serum (median 0.2069 µg/mL, interquartile range (IQR) 0.134 to 1.1561 µg/mL) than healthy donors (median 0.1495 µg/mL, IQR 0.0775 to 0.8308 µg/mL), although the small sample size did not allow to obtain statistical significance. The prevalence of positive titres of IgG anti-HDL antibodies was 21,4% in SLE (6 out of 28) and 9% in HC (1 out of 11). The cut-off for antibody positivity was defined as antibody concentration equal or superior to the mean plus three standard deviations of the HC group (Figure 15).

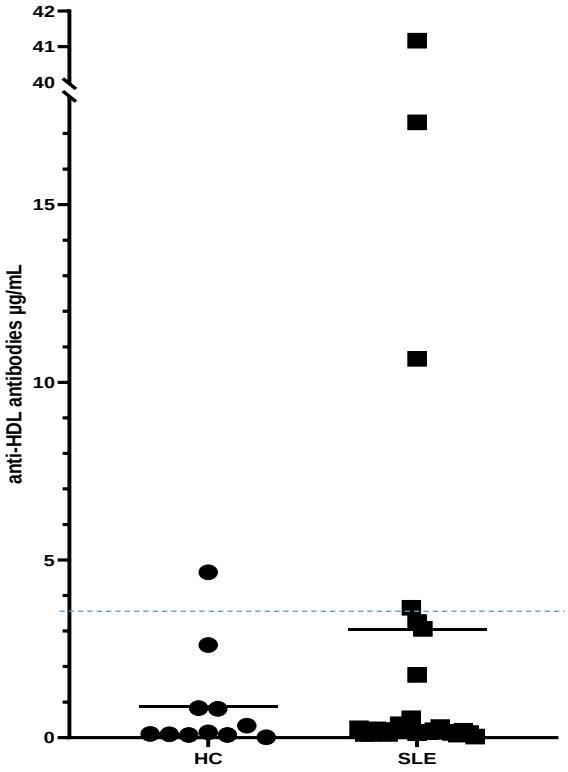


Figure 15: IgG anti-HDL antibodies.

Levels of IgG anti-HDL antibodies in healthy controls (HC), n = 11, and patients with systemic lupus erythematosus (SLE), n = 28. Bars show the means. Dashed line marks the positivity cut-off (mean plus three standard deviations of the HC group).

Plasma membrane cholesterol, measured through filipin binding, did not vary between $CD4^+$ T cells of healthy controls and SLE patients (filipin binding MFI median 25240 vs 20879, p = 0.34), except for the subpopulation of EM T cells, although without statistical significance. EM T cells showed lower PM cholesterol in SLE (filipin binding MFI median 36938 vs 14778, p = 0.15), and even lower in the presence of anti-HDL antibodies (filipin binding MFI median 36938 vs 11262, p = 0.11).

An increase in ABCA1 expression was observed in all $CD4^+$ T cells subsets (ABCA1 MFI median 369 vs 1217, p = 0.0036) but was more pronounced in the naïve population (ABCA1 MFI median 525 vs 2078, p = 0.00246). This pattern was observed in $CD4^+$ T cells from patients with SLE with and without anti-HDL antibodies.

CTB binding was increased only in $CD4^+$ T cells from the group of SLE patients with anti-HDL antibodies (CTBhi % of $CD4^+$ T cells median 29.8 vs 37.7%, p = 0.0103) – Figure 16.

SLE $CD4^+$ T cells showed a positive correlation between membrane cholesterol and ABCA1 in the naïve (r = 0.49, p = 0.0256) and Treg subpopulations (r = 0.54, p = 0.0079), which did not occur in healthy controls (Figure 18). The group of SLE patients with anti-HDL antibodies showed the strongest positive correlation between membrane cholesterol and ABCA1 (r = 0.83, p = 0.0583) – Figure 19.

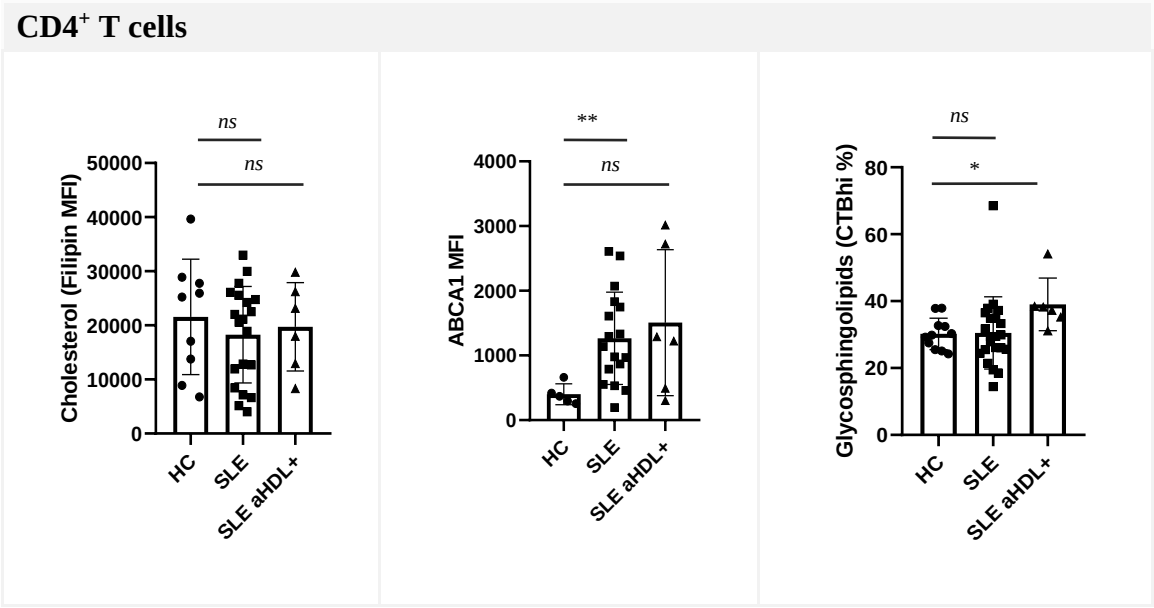


Figure 16: Membrane cholesterol, ABCA1 and lipid rafts in CD4$^+$ T cells.

Membrane cholesterol measured by filipin binding, ABCA1 and lipid rafts assessed by CTB, as a surrogate marker of glycosphingolipids, in CD4$^+$ T cells from healthy controls (HC) and patients with systemic lupus erythematosus (SLE), with and without anti-HDL antibodies. Statistical significance was considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). HC: healthy controls; SLE: systemic lupus erythematosus; aHDL: anti-HDL antibodies; MFI: mean fluorescence intensity.

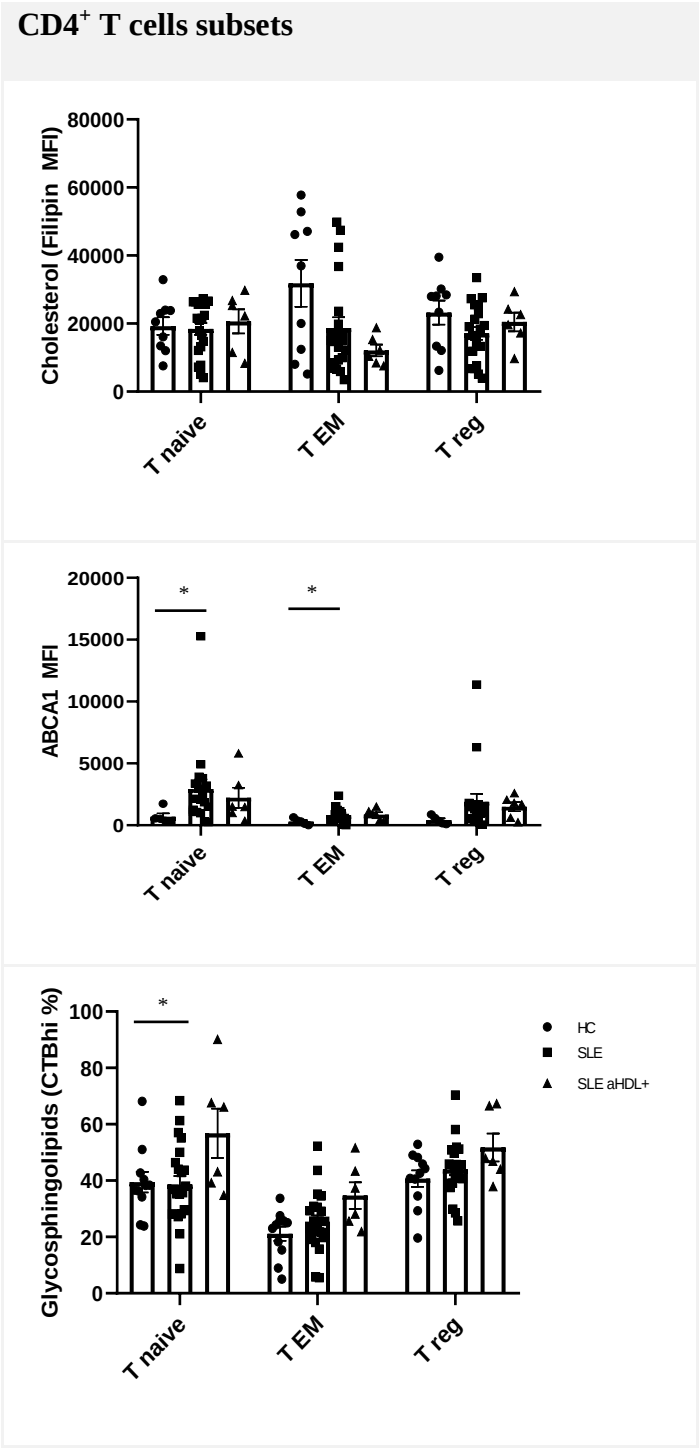


Figure 17: Membrane cholesterol, ABCA1 and lipid rafts in CD4+ T cells subsets.

Membrane cholesterol measured by filipin binding, ABCA1 and lipid rafts assessed by CTB, as a surrogate marker of glycosphingolipids, in $CD4^+$ T cell subsets from healthy controls (HC) and patients with systemic lupus erythematosus (SLE), with and without anti-HDL antibodies. Statistical significance was considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). T naïve: naïve T cells; T EM: Effector memory T cells; T reg: regulator T cells.

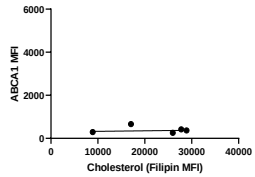
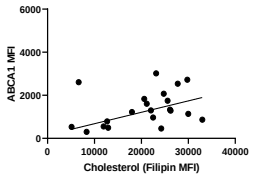
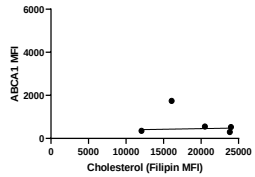
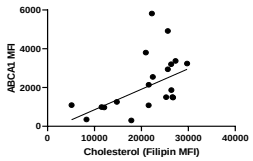
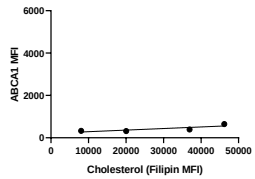
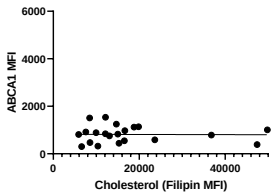
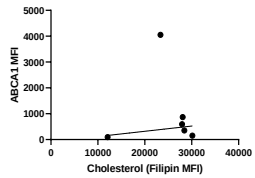
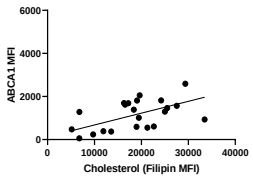
	Healthy controls	SLE
CD4$^+$ T cells	 r = 0.1 ns	 r = 0.3 ns
Naïve T cells	 r = -0.2 ns	 r = 0.49 p = 0.0256
Effector memory T cells	 r = 0.8 ns	 r = 0.06 ns
Regulator T cells	 r = -0.09 ns	 r = 0.54 p = 0.0079

Figure 18: Membrane cholesterol and ABCA1 in CD4$^+$ T cells subsets.

Spearman rank correlations between membrane cholesterol and ABCA1 among CD4$^+$ T cells subsets show that ABCA1 expression in the plasma membrane correlate positively with cholesterol measured by filipin binding in naïve and regulator T cells from patients with systemic lupus erythematosus (SLE).

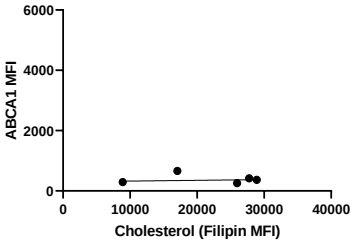
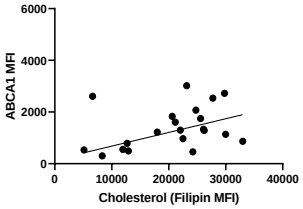
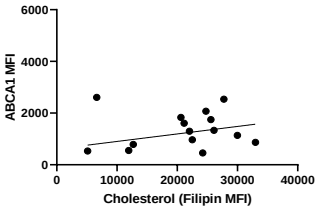
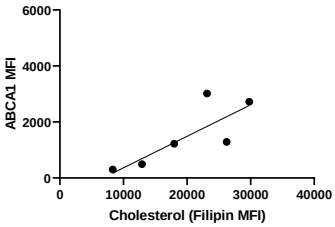
	CD4$^+$ T cells
Healthy controls	 r = 0.1 ns
SLE	 r = 0.3 ns
SLE without anti-HDL antibodies	 r = 0.07 ns
SLE with anti-HDL antibodies	 r = 0.83 ns (p = 0.0583)

Figure 19: Membrane cholesterol and ABCA1 in CD4$^+$ T cells subsets.

Spearman rank correlations between membrane cholesterol and ABCA1 among CD4$^+$ T cells from healthy donor and patients with SLE, with or without detectable anti-HDL antibodies.

1.6. Humoral response against ABCA1

Sera from 65 patients with SLE and 38 healthy volunteers were tested. SLE patients had higher anti-ABCA1 antibody titres than healthy controls ($p = 0.011$). Values superior to 3 standard deviations above the mean of healthy controls were considered positive. Seven patients showed positive anti-ABCA1 titres (11.7%). Anti-ABCA1 antibody titres did not correlate with the presence of anti-HDL antibodies. Serum levels of HDL and ApoA-I were not correlated with anti-ABCA1 antibody titres.

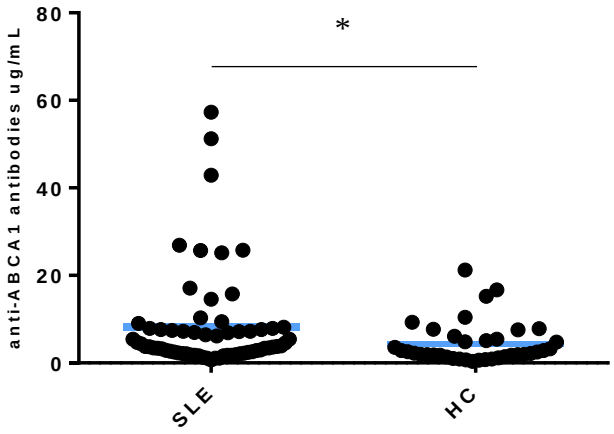


Figure 20: Levels of anti-ABCA1 antibodies.

Levels of anti-ABCA1 antibodies. in patients with systemic lupus erythematosus (SLE) and healthy controls (HC). Statistical significance was considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

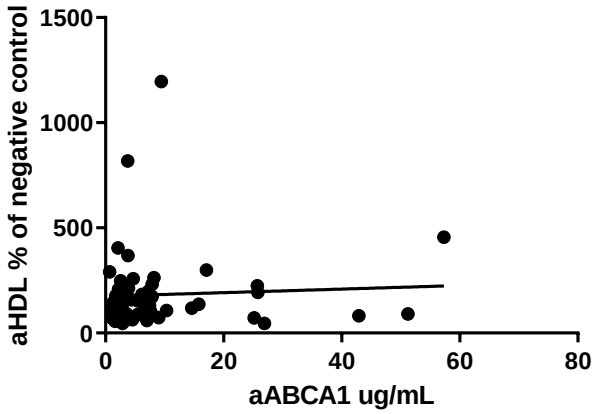


Figure 21: Correlation between anti-HDL and anti-ABCA1 antibodies.

The detection of anti-ABCA1 antibodies does not correlate with the presence of anti-HDL antibodies in patients with systemic lupus erythematosus. aHDL: anti-HDL antibodies; aABCA1: anti-ABCA1 antibodies.

1.7. Discussion

The effect of HDL on PM cholesterol *in vitro* was maximal at 24h incubation. Although cholesterol efflux occurs earlier, it is plausible that the cholesterol depletion from PM occurs later due to the cell capacity to balance the PM cholesterol content in the first 24h. It is worth noting that the presence of HDL *in vitro* decreases PM cholesterol equally in the different T cell subsets. Considering that cholesterol efflux is the core mechanism of immunomodulation by HDL, the 24h pre-culture is probably the optimal time lapse to treat immune cells with HDL. The immune conjugates formation did not change when HDL was added simultaneously with APCs to the T cell culture, suggesting that HDL would only influence the immune synapse if T cell cholesterol depletion occurred, which seems to be relevant only after 24h culture with HDL.

Interestingly, ABCA1 did not colocalize with CTB (the marker for GM1, one of the main constituents of lipid rafts) neither in T cells nor in monocytes in a resting state. It is not

known if ABCA1 can colocalize with lipid rafts in stimulated T cells or in macrophages. The presence of HDL did not influence the surface expression of ABCA1 or the CTB staining and did not induce colocalization of ABCA1 and CTB. These results suggest that the HDL immune modulatory effects do not occur through direct interaction with ABCA1 and are most probably secondary to modulation of the PM cholesterol content.

The *ex vivo* study of the T cell lipid metabolism is complex as it is affected by intracellular and extracellular factors. The characterization of T cells from patients with SLE in comparison with T cells from healthy donors showed differences in T cell subsets. Filipin binding was higher in EM T cells than in the other healthy $CD4^+$ T cells subpopulations, suggesting that the accumulation of cholesterol might be relevant for T cell effector responses. PM cholesterol and ABCA1 expression in the membrane of healthy $CD4^+$ T cells was not correlated, which suggests that T cell lipid metabolism is complex and depends on several factors other than cholesterol efflux through ABCA1.

The SLE group had 21.4% patients positive for anti-HDL antibodies. The presence of anti-HDL antibodies is associated with the SLE T cell differentiation pattern, towards an increase in EM T cells and a decrease of naïve T cells. The aberrant activation of the T cell response in patients with SLE is partially explained by a biased differentiation of naïve T cells into effector T cells (Fritsch et al. 2006). As such, EM T cells are expanded in SLE (Piantoni et al. 2018) and correlate positively with disease activity and damage. Alterations in the Treg pool were also described in SLE, although the published studies show a high degree of heterogeneity in Treg prevalence (Li et al. 2019). The majority of studies report a decrease in Treg in patients with SLE (Miyara et al. 2005; S. Hu et al. 2008; Bonelli et al. 2008; Yang et al. 2009; Atfy et al. 2009; Suen et al. 2009; Tselios et al. 2014), but some

studies also describe an unchanged (Żabińska et al. 2016) or increased Treg population (Venigalla et al. 2008; Yan et al. 2008; Handono et al. 2016; Mesquita et al. 2018; Hanaoka et al. 2020). There is also controversy regarding Treg function in SLE (B. Zhang et al. 2008; Yates et al. 2008; Alvarado-Sánchez et al. 2006). In this study, the SLE group showed an increased prevalence of Treg in comparison with healthy donors. In the subgroup of SLE patients with positivity for anti-HDL antibodies the increase in Treg numbers was less pronounced. However, it is not possible to conclude in a definitive fashion if there is a pathogenic effect of anti-HDL antibodies in the differentiation of T cells.

T cells of patients with SLE have been described as having excess PM cholesterol and lipid rafts (Jury, Flores-Borja, and Kabouridis 2007). Although I did not find an increase in PM cholesterol in the $CD4^+$ T cells of this group of patients with SLE, a different metabolic pattern in EM T cells is evident. The cholesterol content of the PM from EM T cells was lower in patients with SLE than in healthy donors. This might be explained by a higher demand of lipids in EM T cells for a proliferative response. The membrane cholesterol of EM T cells is even lower in the presence of anti-HDL antibodies, which suggests that in this case the cholesterol transport to HDL is not as relevant as the lipid requirement for cell metabolism. Additionally, the lower cholesterol in the PM of EM T cells and the presence of anti-HDL antibodies might be independently correlated with SLE disease activity.

The expression of ABCA1 is increased in all SLE $CD4^+$ T cell subsets (mainly T naïve and Treg) in comparison with healthy controls, either in anti-HDL antibodies positive and negative patients, which suggest that the mechanism for increased ABCA1 expression is intrinsic to the cell and possibly in close relation with the increase in LXRβ already

reported in $CD4^+$ T cells from SLE patients. The increase in ABCA1 expression has been shown to correlate with lower cholesterol and lipid rafts in the PM of T cells in SLE patients, which was not observed in this study. In fact, ABCA1 expression showed a positive correlation with the cholesterol content in $CD4^+$ T cells (except for the EM T cells subset) and this correlation was more pronounced in the presence of anti-HDL antibodies. This suggests that anti-HDL antibodies may affect the cholesterol efflux capacity through ABCA1 in the context of SLE. In fact, other study demonstrated that in obese healthy individuals, ABCA1-mediated cholesterol efflux correlates positively with the occurrence of anti-ApoAI antibodies. This is possibly due to ACAT stimulation and consequent inhibition of cholesterol efflux by passive diffusion (Vuilleumier et al. 2019). The finding that the lipid rafts did not correlate and did not colocalize with ABCA1 supports the theory that ABCA1 acts indirectly on lipid rafts through the extraction of excess cholesterol from the cell. In this study, only the group of SLE patients with anti-HDL antibodies had increased levels of lipid rafts. Although it is reasonable to hypothesize that HDL/ApoA-I blockage by antibodies may potentiate the lipid rafts formation due to a decreased cholesterol efflux, a causative role for the anti-HDL antibodies cannot be assumed.

The occurrence of anti-ABCA1 antibodies in patients with SLE can potentially induce ABCA1 dysfunction. These antibodies are increased in patients with SLE when compared to a healthy population. However, the prevalence of anti-ABCA1 is probably underestimated since only a polypeptide of the ABCA1 complex was used to detect these antibodies. Anti-HDL antibodies are also increased in patients with SLE when compared to healthy controls, but their presence was not correlated with the levels of anti-ABCA1 antibodies, meaning that this coincidental production could be caused by the unspecific

production of autoantibodies in the context of SLE. The absence of correlation suggests that the altered pattern of ABCA1 and membrane cholesterol seen in $CD4^+$ T cells from patients with SLE and anti-HDL antibodies is not due to the presence of simultaneously occurring anti-ABCA1 antibodies.

2. T cell response studies

2.1. HDL and ApoA-I effects on human $CD4^+$ T cell proliferation

In healthy donors, HDL in the concentrations of 50, 300 and 600 µg/mL and ApoA-I in the concentration of 10 µg/mL did not have a significant effect in $CD4^+$ T cell Ki-67 expression in ten out of twelve subjects. In two donors, HDL decreased $CD4^+$ T cells proliferation at the concentration of 300 and 600 µg/mL. In patients with SLE, there was a decrease in proliferation with the HDL concentration of 600 µg/mL on the brink of significance (p = 0.068). When comparing the proliferative response of $CD4^+$ T cells from patients with SLE in the presence of HDL 50 and 600 µg/mL, the difference was statistically significant (p = 0.019). This is attributed to the fact that at lower concentrations, HDL slightly increases proliferation.

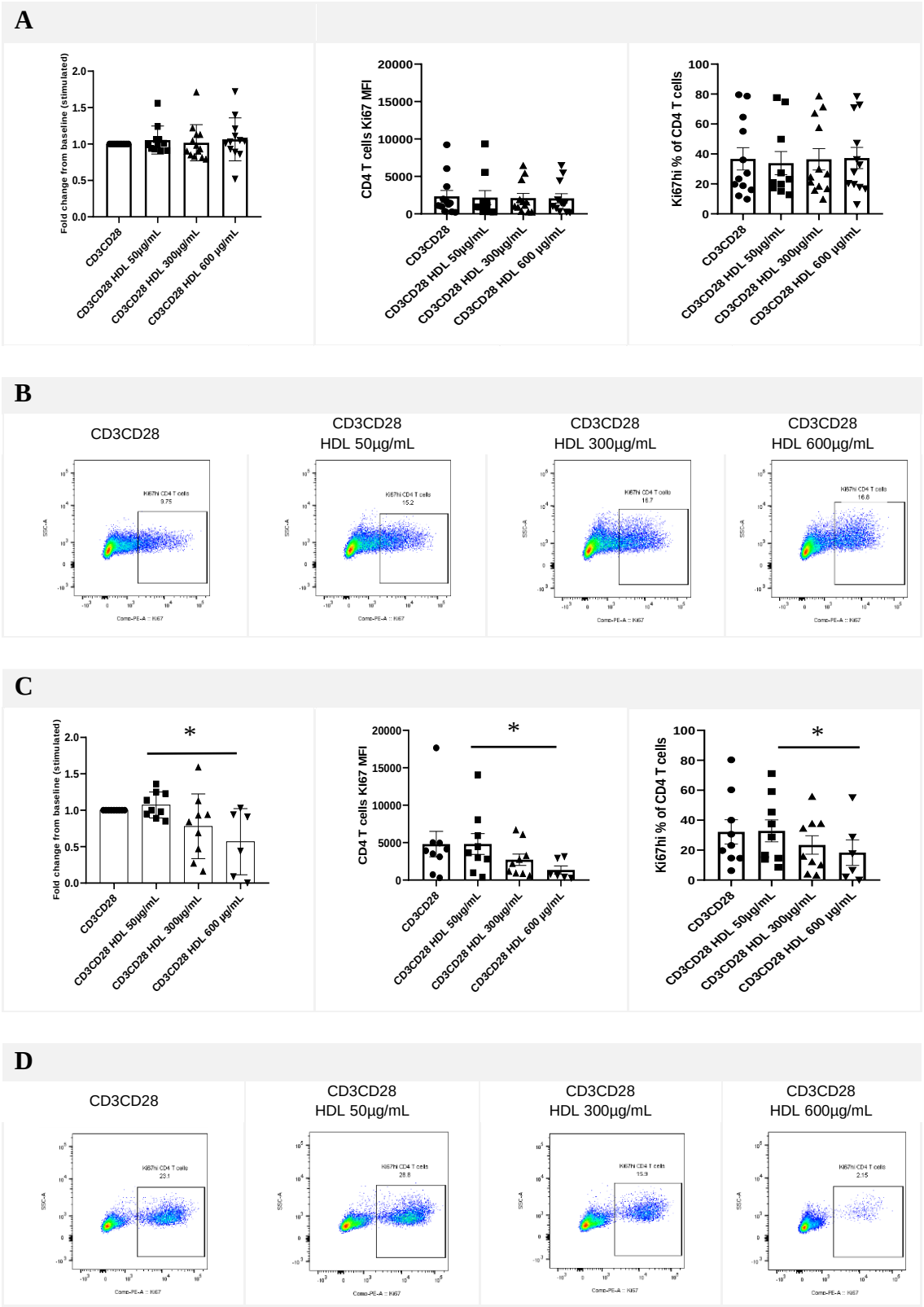


Figure 22: CD4^{+} T cell proliferation.

A) Healthy controls fold change from baseline stimulation in the percentage of Ki-67hi CD4 T cells, n = 12; B) Representative flow cytometry plots from an healthy donor; C) Fold change from baseline stimulation in

the percentage of Ki-67hi CD4 T cells from patients with SLE, n = 9; D) Flow cytometry plots showing an evident decrease in proliferation with crescent HDL concentration in $CD4^+$ T cells from a patient with SLE. Statistical significance was considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

The effect of HDL and ApoA-I on CD3CD28 stimulated $CD4^+$ T cells from healthy donors was further analysed with Cell Trace. The results showed a slight increase in proliferation in the presence of HDL, as the percentage of divided $CD4^+$ T cells increased from 15.2 to 17.8% (Figure 23).

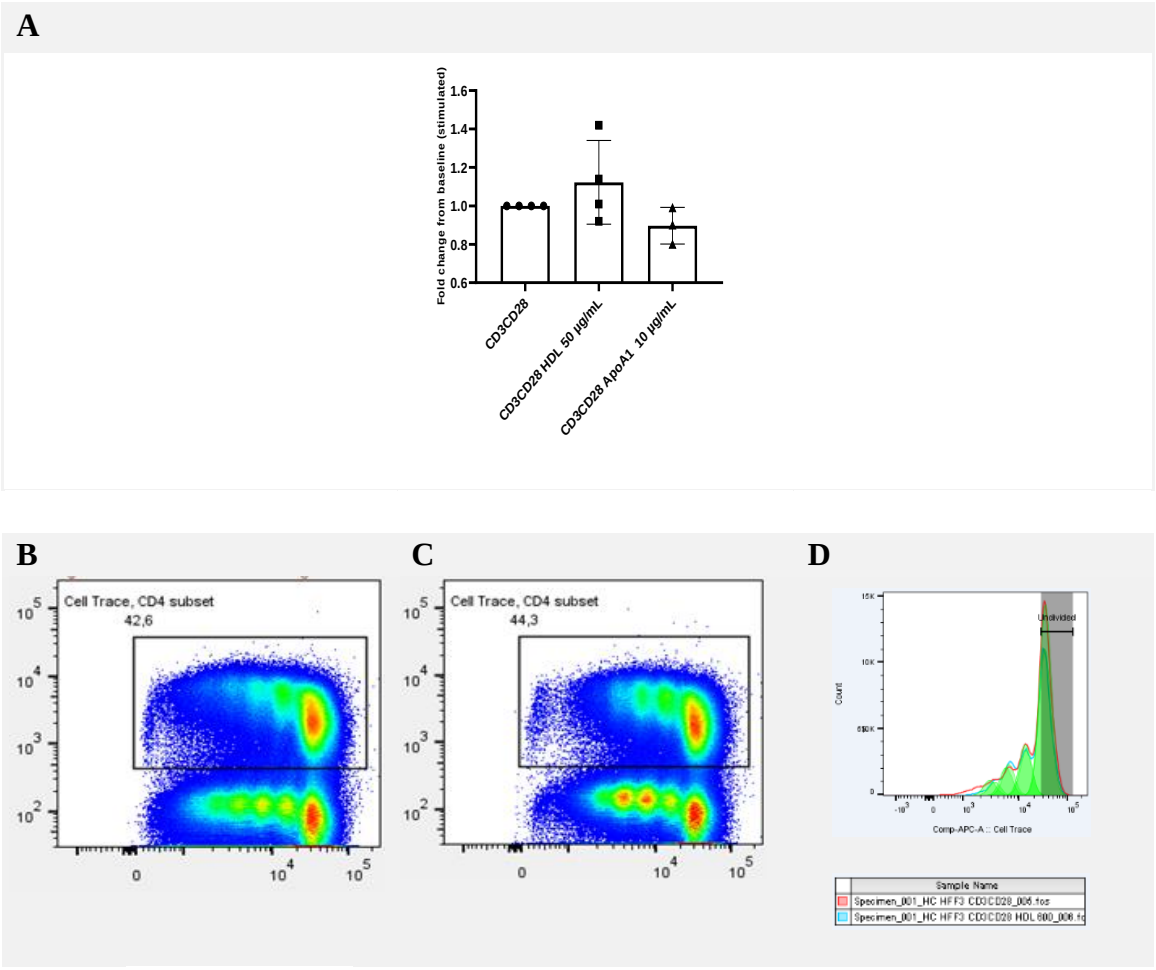


Figure 23: Cell Trace Far Red.

A) Fold change from baseline of the percentage of $CD4^+$ T cells that entered cell division, in the presence of HDL 50 µg/mL or ApoA-I 10 µg/mL (n = 4). B) Flow cytometry plot of lymphocytes without HDL. C) Flow cytometry plot of lymphocytes in the presence of HDL 600 µg/mL. Plot C shows a slight increase in $CD4^+$ T cells proliferation measured by Cell Trace in the presence of HDL (percentage of divided cells increased from 15.2 to 17.8% of $CD4^+$ T cells).

2.2. HDL effect on early T cell activation

HDL had no effect in the expression of CD25 on CD3CD28 stimulated $CD4^+$ T cells after 24 and 72 hour incubation (Figure 24).

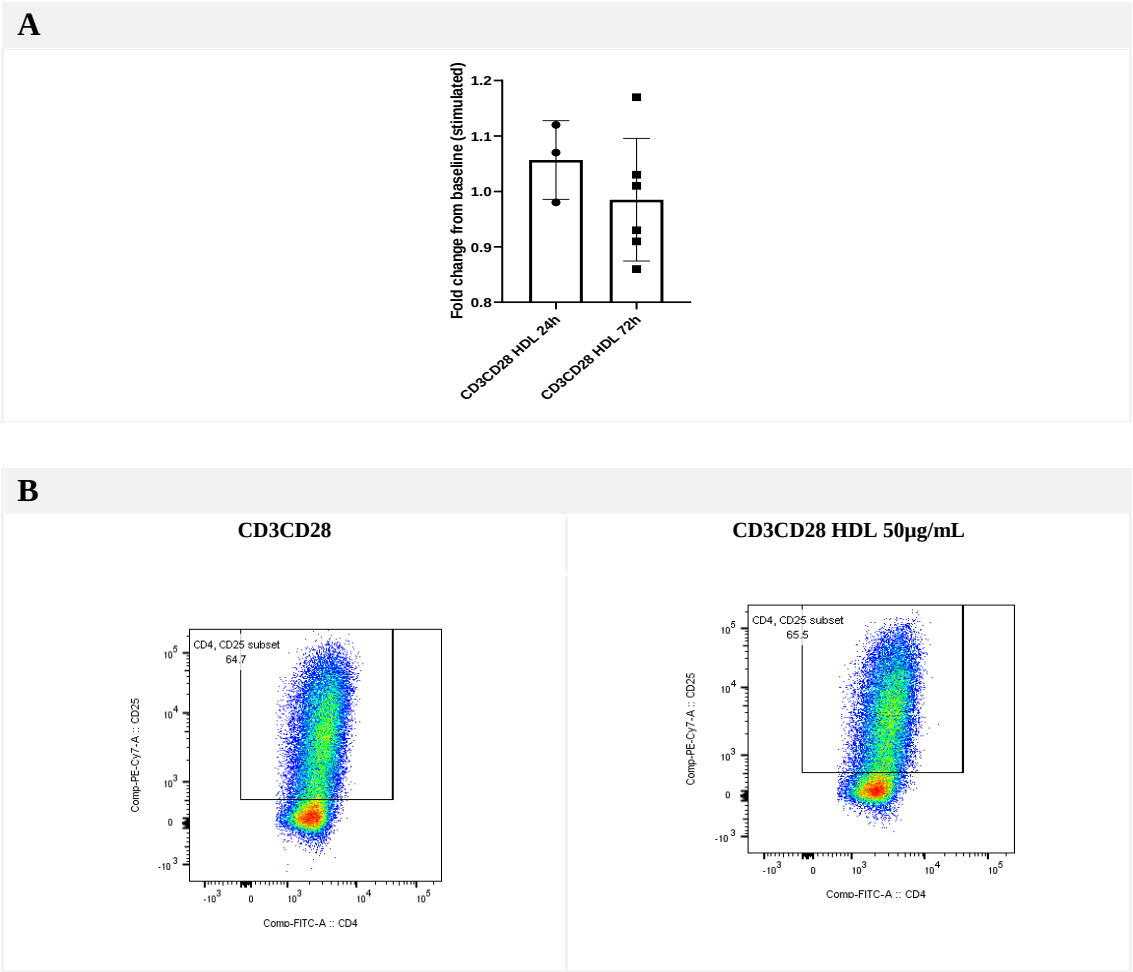


Figure 24: Expression of CD25 in $CD4^+$ T cells from healthy donors.

A) Fold change from baseline of the CD25hi subset of $CD4^+$ T cells in the presence of HDL, for 24 and 72h; B) Representative flow cytometry plots, after 72h incubation.

2.3. HDL effect on TCRzeta phosphorylation

TCRzeta phosphorylation gradually decreased in the presence of HDL at the concentration of 600 µg/mL, with a difference to baseline CD3CD28 stimulation almost significant at 15

minutes (p = 0.06) and significant at 20 minutes (p = 0.01). The mean reduction in TCRzeta phosphorylation at 20 minutes was 30,5% (Figure 25). A lower HDL concentration (50 µg/ml) did not change TCRzeta phosphorylation.

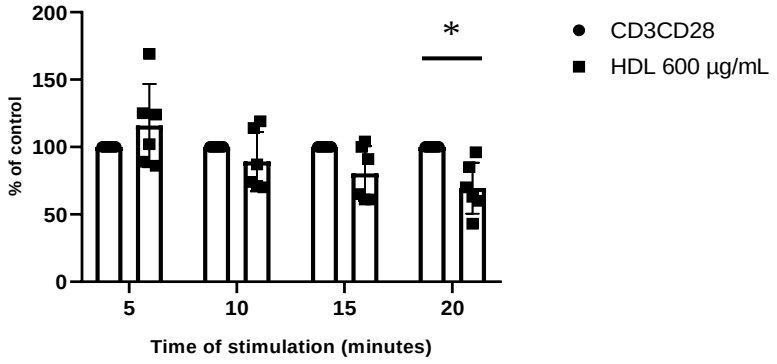


Figure 25: TCRzeta phosphorylation.

TCRzeta phosphorilation after CD3CD28 stimulation for 5, 10, 15 and 20 minutes (n = 6), with or without pre-incubation with HDL. Statistical significance was considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

2.4. HDL effect on helper T cell subsets

The polarization of $CD4^+$ T cells in Th1, Th2, Th17 and Treg subsets did not change in the presence of HDL (50, 300 and 600 µg/mL) in comparison with stimulated controls without incubation with HDL. Th1/Th2 ratio also did not vary (Figure 27 and 28).

HDL at 50 µg/mL did not change the total percentage of Treg (CD25hiCD127lo T cells) in the population of $CD4^+$ T lymphocytes after 24 hour incubation, but the phenotype of Treg was further analysed using CD45RA and FoxP3 staining to define subpopulations I, II and III (Figure 29). The Treg subpopulation analysis revealed that HDL seems to induce the differentiation of pTreg as shown by the decrease of subpopulations I:II ratio (p = 0.029), which translates into an increase in cells expressing FoxP3 (Figure 30).

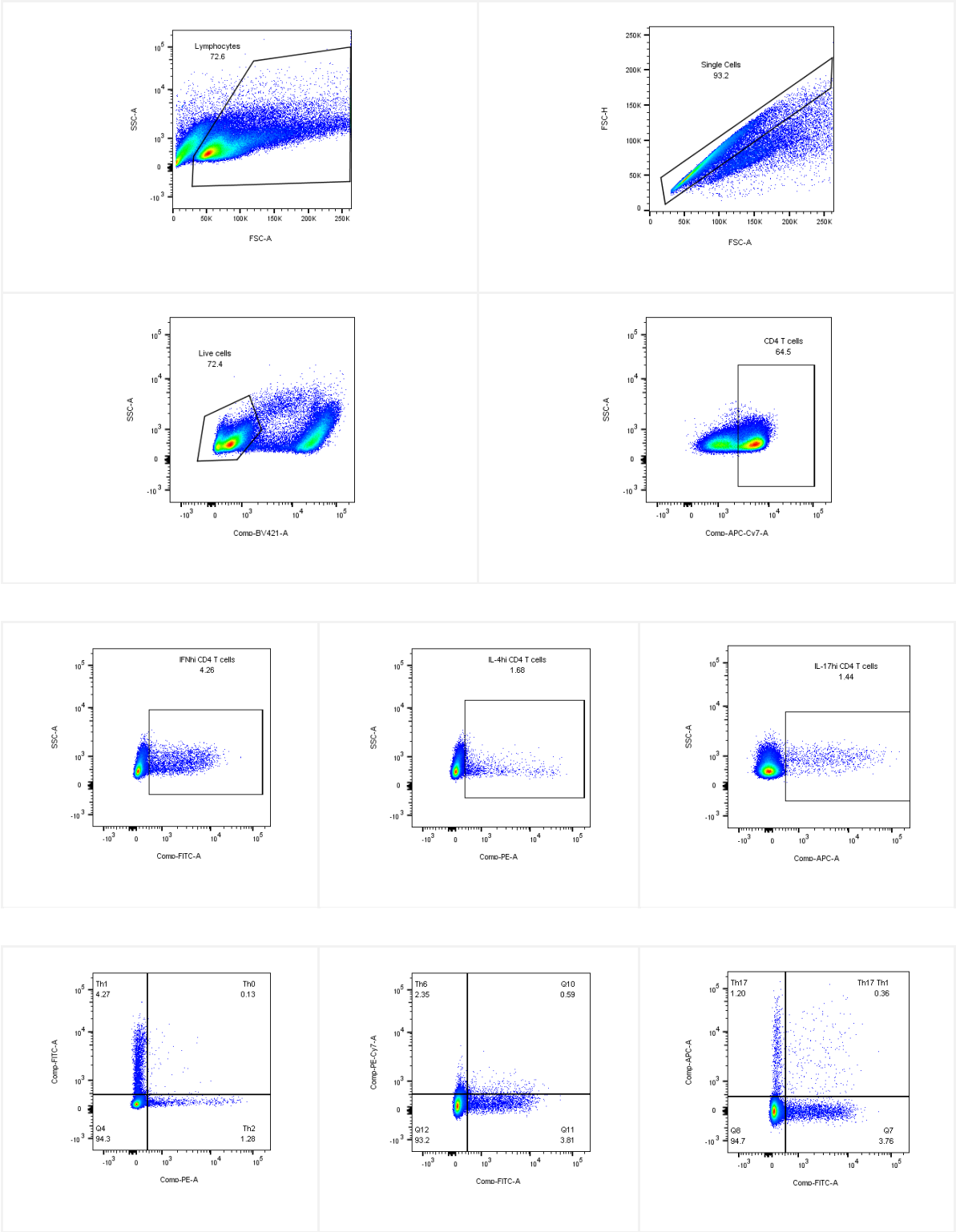


Figure 26: Flow cytometry plots showing the gating strategy for Th1, Th2 and Th17 subsets.

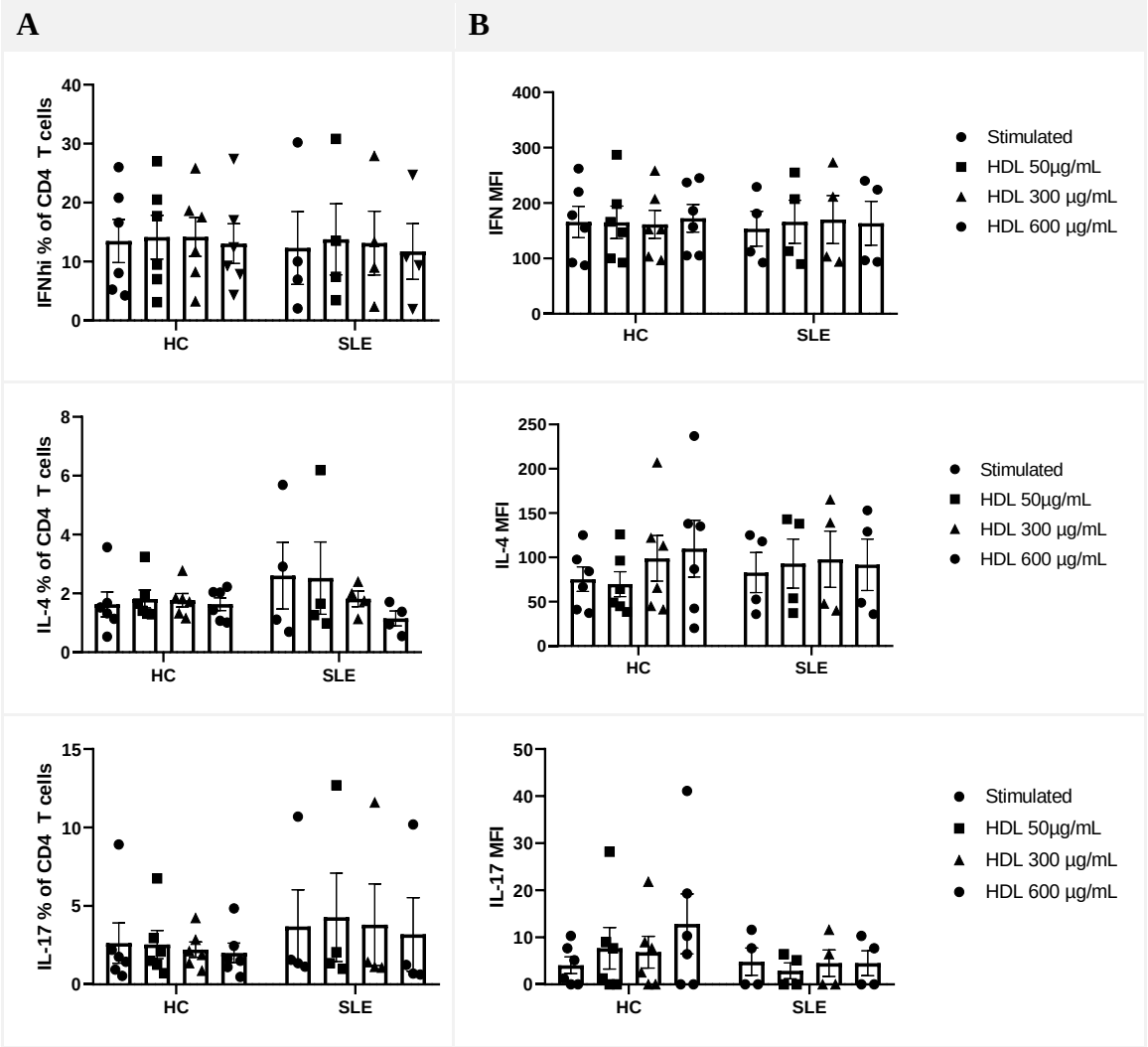


Figure 27: Cytokine expression in CD4$^+$ T cells after CD3CD28 and PMA/ionomycin simulation.

Cytokine expression in CD4$^+$ T cells after 72 hour CD3CD28 stimulation and 6 hour stimulation with PMA/ionomycin, with or without increasing HDL concentrations, showed that HDL did not influence cytokine signatures of Th1, Th2 and Th17 cells.

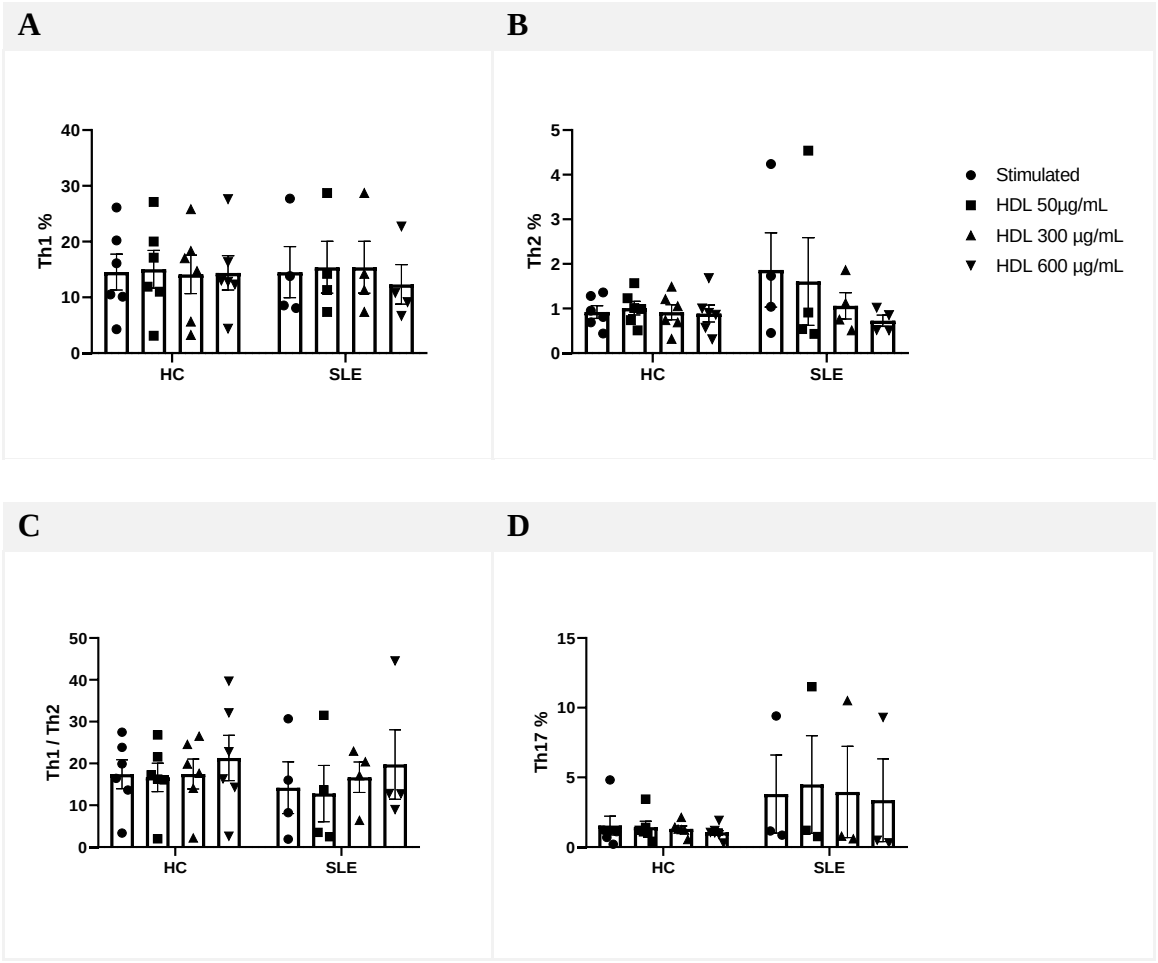


Figure 28: Prevalence of CD4⁺ T cell subsets.

The presence of HDL did not affect the polarization of helper T cells. A) T helper 1 (Th1); B) T helper 2 (Th2); C) T helper 1 and T helper 2 ratio; D) T helper 17 (Th17).

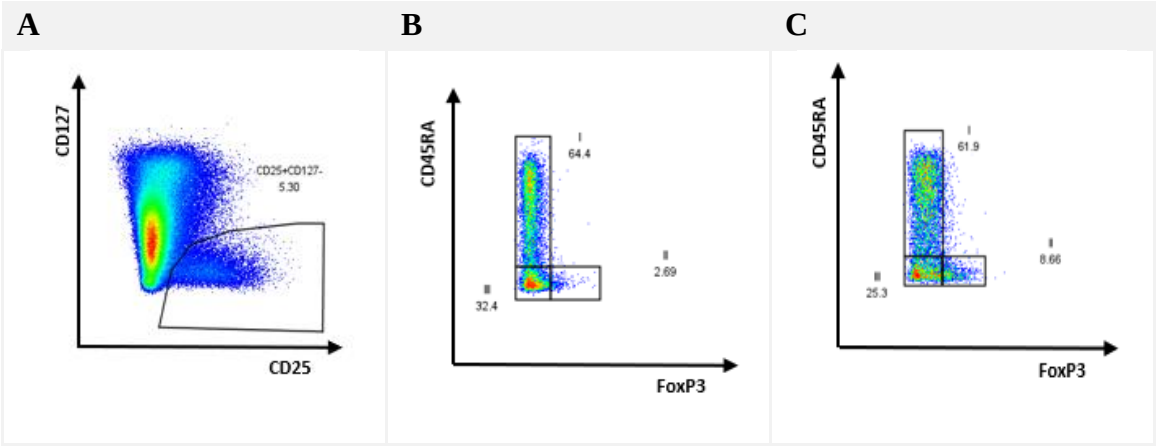


Figure 29: Flow cytometry plots showing Treg gating strategy.

A) Gating on $CD3^{+}CD4^{+}CD25^{+}CD127^{-}$ cells; B) Subpopulations I, II and III in Treg cultured in culture medium without HDL; C) Treg subpopulations after culture with HDL.

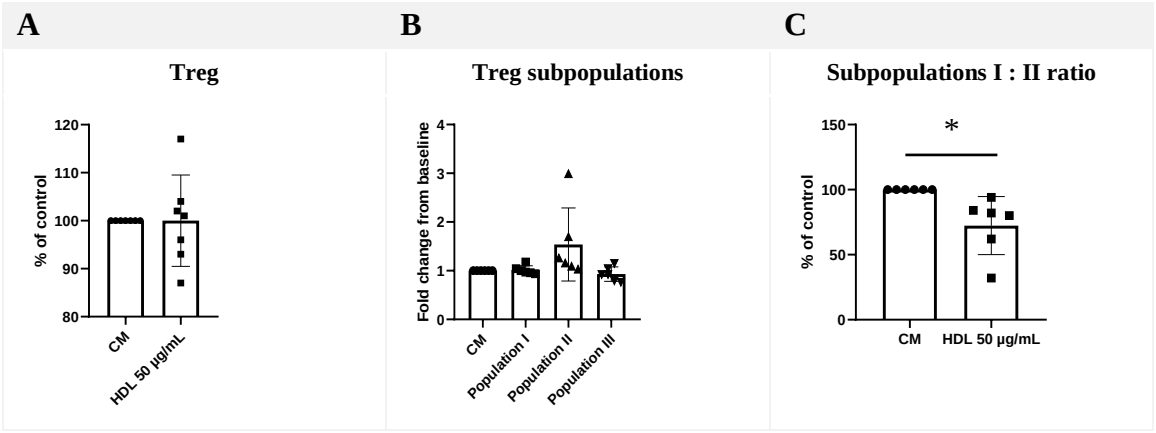


Figure 30: Treg prevalence and differentiation.

A) The prevalence of Treg did not change in the presence of HDL (n = 7). B) Treg subpopulations prevalence fold change from baseline. C) The analysis of Teg subpopulations revealed a decrease in subpopulation I:II ratio. Statistical significance was considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

2.5. HDL effect on $CD4^+$ helper T cells cytokine expression

HDL increased the production of TGF-β1 by $CD4^+$ T cells from healthy donors and patients with SLE, in a dose-dependent fashion. The expression of IFN-γ, TNF-α and IL-10 in stimulated $CD4^+$ T cells treated with HDL was not significantly different from untreated cells. However, there is a great heterogeneity among different donors in the relative frequencies of cytokine-producing cells.

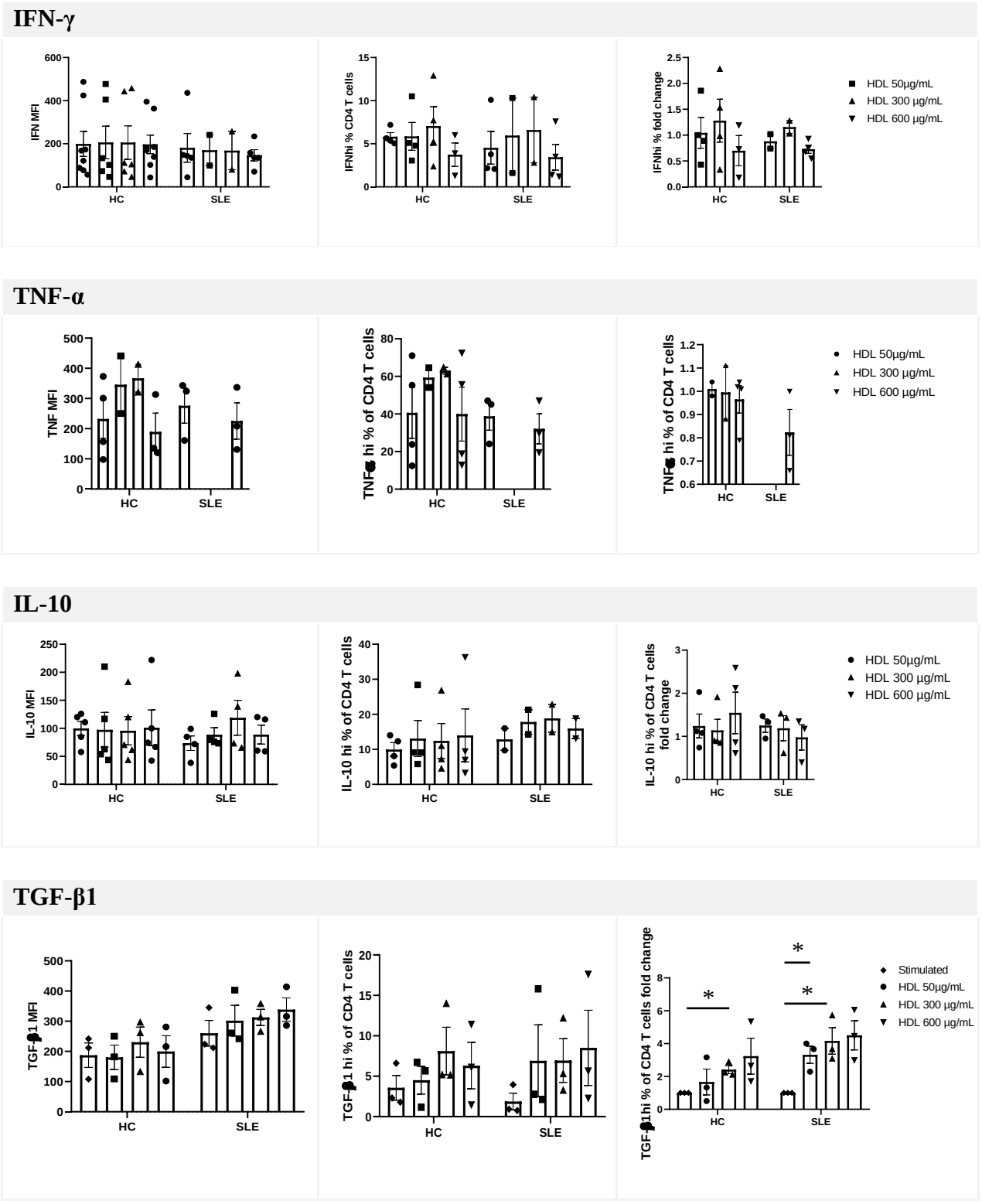


Figure 31: Cytokine expression in CD4⁺ T cells after PMA/ionomycin stimulation.

Stimulated CD4⁺ T cells increased the production of TGF-β1 in the presence of HDL, with a dose-dependent effect. The expression of IFN-У, TNF-α and IL-10 was not significantly altered. Statistical significance was considered when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

2.5.1. Discussion

This work is the first to my knowledge to report the effects of HDL in different aspects of the function of human T lymphocytes. The most striking observation was the increase of the expression of TGF-β1 in $CD4^+$ T cells from healthy donors and patients with SLE. The absence of an effect in the production of other cytokines suggests that this is a specific mechanism by which HDL regulates the immune response. Although TGF-β is produced by several cell types with redundant effects in immune regulation, T cell derived TGF-β has additional functions in controlling immune tolerance (Turner et al. 2020). In atherosclerosis, TGF-β has both atherogenic and atheroprotective effects (Toma and McCaffrey 2012). Mice studies showed that TGF-β signalling dampens T cell activation and decreases the secretion of pro-inflammatory cytokines (Robertson et al. 2003). Depending on the presence or absence of IL-6 in the surrounding milieu, TGF-β promotes the differentiation of Th17 or Treg, respectively. As such, the observation that HDL increases the expression of TGF-β1 in $CD4^+$ T cells suggests a complex regulatory role for HDL, which is in accordance with the immune modulatory and atheroprotective role of HDL. Through the increase in TGF-β1, HDL can thus decrease T cell activation. The only previous study on the effects of HDL on TGF-β demonstrated that the HDL subfraction 3 specifically induces TGF-β2 expression in human endothelial cells (HUVECs), without affecting TGF-β1 or TGF-β3 (Norata et al. 2005). Other lines of evidence point to a close interplay between the cell lipid metabolism and TGF-β expression. It was demonstrated that cholesterol suppresses TGF-β responsiveness by increasing the accumulation of TGF-β receptors in lipid raft and/or caveolae accumulation, which accelerates the TGF-β degradation (C. L. Chen et al. 2007). This and other studies also showed that cholesterol-

lowering agents (statins) and cholesterol-depleting agents (e.g. nystatin) have the opposite effect (Roy and Wrana 2005; Porreca et al. 2002). Additionally, TGF-β1 increases the expression of ABCA1, ABCG1 and SR-BI in foam cells through the up-regulation of the liver X receptor α (LXRα) pathways, thus increasing the cholesterol efflux mediated by HDL (Y. W. Hu et al. 2010).

In SLE, TGF- β1 is one of the few cytokines that is downregulated in serum, with a negative correlation between TGF-β1 levels and disease activity and organ damage (Metawie et al. 2015; Becker-merok et al. 2010; Edelbauer et al. 2012; Jin et al. 2012; Jackson et al. 2006; Hammad, Youssef, and El-arman 2006). There is also evidence that lymphocytes from patients with SLE are more resistant to TGF-β1 (Rekik et al. 2018). This data suggests that inducing an increase in TGF-β can revert part of the immune deregulation present in SLE. However, conflicting results aroused in a study showing that SLE PBMCs produce higher TGF- β1 levels than healthy controls (Yuan et al. 2017). TGF-β1 was also shown to be negatively correlated with the carotid intima-media thickness in patients with SLE (Jackson et al. 2006). Jackson et al further demonstrated that the activation of TGF-β1 is negatively correlated with PBMCs apoptosis, which corroborated animal studies in which a TGF-β1 KO mouse model had increased mitochondrial membrane potential and consequent increased apoptosis. Moreover, patients with SLE have increased mitochondrial membrane potential and apoptosis (Gergely et al. 2002) and HDL is known to have antiapoptotic effects (Mineo and Shaul 2012). Possibly, one of the mechanisms responsible for the anti-apoptotic properties of HDL is the induction of TGF-β1 production by T lymphocytes.

The results from proliferation experiments suggest that HDL does not normally prevent proliferation of healthy lymphocytes but can decrease the proliferation of $CD4^+$ T cells in pro-inflammatory conditions, such as in patients with SLE. In a previous study, HDL from healthy young individuals was able to mildly increase the proliferation of stimulated T cells (Larbi et al. 2014). The decrease in TCR zeta phosphorylation in the presence of HDL suggests that HDL potentiates TCR zeta downregulation, a negative feedback mechanism that protects from an exaggerated inflammatory response. These results support the anti-inflammatory and immunosuppressive role of HDL in the presence of ongoing inflammation. CD3CD28 stimulation induced a strong CD25 expression that was not affected by the incubation with HDL. These results also reinforce the idea of a "fine-tune" immune modulation by HDL that is context-dependent and essentially protects from chronic inflammation.

In these experimental conditions, HDL did not affect the in vitro polarization of Th subsets. However, a trend to the differentiation of pTreg could be seen, although without affecting the total prevalence of Treg. The effect of HDL in the promotion of the Treg subpopulation II (with induced expression of FoxP3) resembles the findings of Mathian A. and co-authors showing that methylprednisolone decrease the prevalence of non-regulatory $FoxP3^{low}$ T cells in patients with active SLE, without changing the quantity of nTreg (Mathian et al. 2015).

Chapter IV.

Overall Discussion and Conclusions

Since the first study showing an association between HDL and a decreased risk for cardiovascular disease, more than four decades ago, HDL has been extensively studied. The Framingham study showed that higher HDL levels confer greater protection from atherosclerotic disease. However, the correlation between HDL and protection from cardiovascular disease has shown to be non-linear due to a variety of confounding mechanisms.

Most studies on the HDL atheroprotective mechanisms have been performed in animal models or in cultured human cells with the focus on single pathways, without giving a contextualised insight about their overall interaction. Furthermore, the experimental conditions have always been highly heterogeneous, rendering comparisons and extrapolations difficult or even impossible. As such, the HDL net effect is difficult to address. Additionally, the biased publication of results highlighting the protective role classically attributed to HDL, might have led to ignoring the effects that would not fit into that category in an obvious fashion.

The scope of this thesis is to study the effect of HDL in the human immune system as a hole, considering that HDL is relevant for the immune cells lipid metabolism and to the inflammatory response. The variety of mechanisms potentially affected by HDL turns its study very challenging as it is not possible to isolate pathways that are reciprocally affected. Additionally, HDL function itself can be altered by the context, principally in an inflammatory milieu as happens in *in vitro* immunologic studies.

The use of immune cells from healthy donors and patients with SLE provided the chance to observe context-dependent modifications in lipid metabolism and the HDL effects, as in patients with SLE both vascular disease and the immune response are exaggerated, thus

providing a good clinical (real life) model for the interactions between immune activation and atherogenesis.

Therefore, e*x vivo* studies can give important clues to better understand the cholesterol-ABCA1-lipid rafts interaction. The analysis of $CD4^+$ T cells in fresh whole blood from healthy donors and patients with SLE demonstrated that PM cholesterol varies between different T cell subsets, with EM T cells showing the higher levels of PM cholesterol. On the contrary, lipid rafts were less abundant in healthy EM T cells, suggesting a resting state. Although affecting PM cholesterol, 24h incubation with HDL did not influence ABCA1 of lipid rafts in the membrane of $CD4^+$ T cells.

Following the study of PM and the humoral intervenients that could affect cell lipid metabolism and immune response, the work was focused on the interaction between HDL and T cell response. The most striking HDL effect observed was the increase on TGF-β1 production in CD4 T cells. It is probably associated with cholesterol efflux, as it was previously demonstrated that cholesterol depletion promotes TGF-β1 signalling pathways in an animal model (Shapira et al. 2018). The exclusive effect on TGF- β1 production also suggests that it is a crucial cytokine for the HDL-mediated regulation of immune response. Other effects were also seen, namely a reduction in TCRzeta phosphorylation and in the proliferation of $CD4^+$ T cells from patients with SLE, and a slight increase on the expression of FoxP3 in Treg.

Interestingly, HDL did not affect the proliferation of $CD4^+$ T cells from healthy donors, which also supports the theory that HDL has essentially regulatory functions in the immune system, reducing exaggerated inflammation and maintaining a normal immune function.

The occurrence of anti-HDL antibodies in SLE adds a tool to understand the relevance of these mechanisms *in vivo.* In fact, the presence of anti-HDL antibodies was associated with an increase in the prevalence of EM T cells and a decrease in naïve and Treg. Anti-HDL antibodies were also associated with an increased expression of lipid rafts in $CD4^+$ T cells from SLE patients and with a deregulated membrane cholesterol and ABCA1 dynamics. These findings suggest that lipid metabolism in the plasma membrane is crucial to the immune response and modifications in the HDL function might deregulate this response, as probably occur in the presence of anti-HDL antibodies.

Whilst anti-HDL antibodies are associated with decreased HDL levels and HDL dysfunction through the direct interaction with the HDL particles, other autoantibodies might indirectly influence HDL quantity and quality. The presence of anti-ABCA1 antibodies in cardiovascular and inflammatory diseases could explain the lower HDL levels and HDL loss of function in these diseases. This work demonstrated that anti-ABCA1 can be detected through ELISA and are more prevalent in patients with SLE than in healthy controls. However, the unviability of the complete ABCA1 protein probably reduces the detection of anti-ABCA1 antibodies, which might have affected the results.

Overall, this work suggests that HDL rather than being always an anti-inflammatory or anti-apoptotic, may play different roles in different contexts. It is able to assume an immune suppressive role in diseases or situations characterized by immune and vascular dysfunction such as SLE, where it will work as a protective factor for atherosclerosis and, in "steady state" situations, such as in healthy individuals, it might have a more neutral effect. This dichotomy whilst highlighting the complexity of HDL would explain the multiple contradictions found in this field of research.

Chapter V.

Future perspectives

This study showed that HDL can modulate the immune response with essentially regulatory actions. The future exploration of HDL effects in T cell function focusing on the study of Treg subpopulations in different health and disease contexts can give insight into the interaction between the lipid metabolism and the immune mechanisms implicated in the pathogenesis of different diseases such as atherosclerosis, autoimmune diseases and cancer.

The modulation of the immune response by HDL seems to be related with a promotion of TGF-β expression. As such, the study of a TGF-associated link contributing to an anti-atherosclerotic phenotype, may help to better understand the HDL effects and to clarify, for example, why patients with immunoinflammatory conditions such as SLE are more prone to accelerated forms of this disease.

From the lipid point of view, the "HDL paradox" (i.e., the absence of clinical benefit with therapies that increase HDL despite *in vitro* demonstration of their potential) is still puzzling and may be associated with the complexity of the mechanisms associated with this lipoprotein and the poor clinical relevance of the measurement of "total" HDL. This could be overcome with the analysis of its different sub-types or even the use of functional tests that may reflect more accurately the influence of HDL in atherosclerosis and immune regulation.

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