The effects of triglycerides and fatty acids on T cells: role in atherosclerosis

Reilly, N.A.

CHAPTER 1

Introduction

Atherosclerosis

Atherosclerosis is considered to be a lipid-driven immune disease and is the dominant cause of cardiovascular disease (CVD), which is the leading cause of death worldwide[1, 2]. The initiation of atherosclerosis is generally attributed to excess low-density lipoprotein (LDL). This cholesterol laden lipoprotein is able to cross to the inner most layer of the arterial wall, the intima, and can accumulate there[3]. Here, the LDL particles can undergo oxidation which induces a pro-inflammatory response in the endothelial cells that make up part of the arterial intima[4]. The activated endothelial cells release chemokines, chemoattractant cytokines, that attract monocytes to the site. These monocytes mature into macrophages which can take up the accumulated LDL[1]. However, the pro-inflammatory landscape of the intima causes macrophages to upregulate the expression of scavenger receptors and downregulate the expression of cholesterol transporters and in turn, generates foam cell formation[5]. Foam cells are unable to leave the intima and aggregate, contributing to atherosclerotic plaque formation[6]. Plaque formation progresses by the continued accumulation of lipids and cells to the site and the formation of a fibrous cap[1]. As atherosclerosis advances, a necrotic core may develop as clearance of cells that underwent programmed cell death in the plaque fails[7]. Furthermore, advanced plaques may undergo calcification, in which calcium minerals accumulate, hardening the plaque and increasing the risk of rupture[8]. Complications of atherosclerosis arise as the plaque builds up, narrowing the arterial lumen and limiting blood flow[1]. Further complications include plaque rupture and erosion[9, 10]. Both plaque rupture and erosion form thrombi which can be released into the circulation and are the main cause of myocardial infarction and stroke[1].

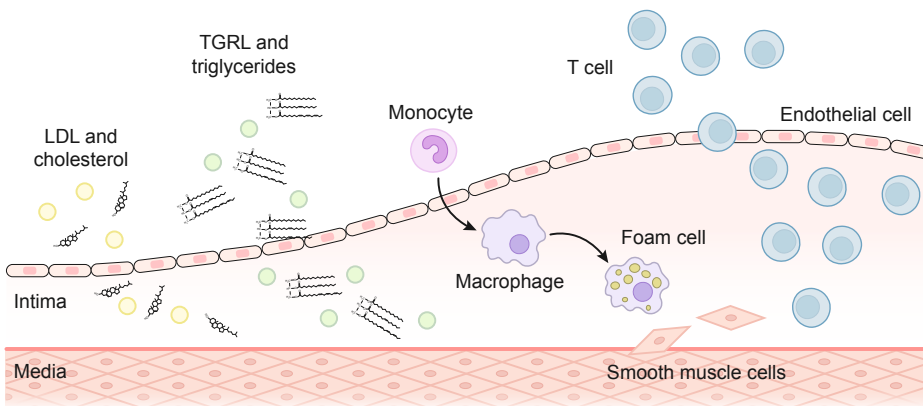

Fig. 1 | Initiation of atherosclerosis pathogenesis. Yellow circles are LDL, green circles are TGRLs, monocytes are in pink, macrophages and foam cells are purple, T cells are blue, endothelial cells are light pink, and smooth muscle cells are light red. Adapted from "Atherosclerosis" by Libby, P. *et. al.*[1].

While much of the pathogenesis of atherosclerosis is classically attributed to macrophages and LDL, it has become increasingly clear that other factors play a crucial role its development. Most notably, triglycerides and T cells have re-emerged as pivotal factors in the pathogenesis of

atherosclerosis[11-14]. Triglycerides are also contained in various lipoproteins such as triglyceride rich lipoproteins (TGRLs), which can cross the arterial intima and aggravate the disease[15]. T cells are attracted into the plaque by the inflammatory response induced by macrophages[11, 12] (Fig. 1). However, the potentially important role of the interaction between these re-emerging risk factors in atherosclerosis remains largely unknown.

T cells

T cells are a component of the adaptive immune system generally found in the lymphatic and circulatory systems in humans. These cells can broadly be split into two categories, $CD4^+$ T cells and $CD8^+$ T cells. T cell function is driven in part by strict metabolic processes that also drive cell fate[16]. Generally speaking, non-activated T cells favor the utilization of β-fatty acid oxidation, whereas activated T cells switch towards utilizing aerobic glycolysis[17, 18]. The main function of T cells is to sense and respond to their environment which, in the circulation, consists of numerous factors including lipids such as triglycerides. However, whether these interactions with lipids in the circulation can have lasting effects on T cells that could impact their function in atherosclerosis remains unknown.

It is known that throughout atherosclerotic development significant numbers of T cells are found in plaques. Specifically, through the use of single cell RNA sequencing and mass spectrometry, it was found that over half of all immune cells present in the atherosclerotic plaque were T cells[19-22]. Furthermore, these studies found that of the T cells present, half were $CD4^+$ and the other half were $CD8^+$ T cells[19] (Fig. 2). Although this technique may selectively favor the survival of T cells over that of other cell types such as macrophages and smooth muscle cells due to the inherent destructiveness of this technique, particularly during the cell isolation phase, the significance of these findings should not be overlooked. Both $CD4^+$ and $CD8^+$ T cells exhibit distinctive characteristics and functions in atherosclerosis[11, 12]. While the role of T cells in atherosclerosis has been studied to some extent, there remains a substantial knowledge gap regarding the mechanisms that govern T cell responses in the plaque and the factors that influence the preferential expression of certain subsets over others.

$CD4^+$ T cells

$CD4^+$ T cells are generally known as helper T cells and are characterized by the expression of both $CD3^+$ and $CD4^+$ on the cell surface. In atherosclerosis, $CD4^+$ T cells generally play a pro-inflammatory role as deficiency of $CD4^+$ T cell was atheroprotective[23] and adoptive transfer of $CD4^+$ T cells was pro-atherogenic[24, 25] in *Apoe*$^{-/-}$ mouse models. $CD4^+$ T cells have multiple functions including activating other immune cells such as B cells and cytotoxic T cells, releasing cytokines, and suppressing immune reactions[26]. Furthermore, upon activation $CD4^+$ T cells can differentiate into subsets, the most well-studied being T helper 1 (T_H1), T helper 2 (T_H2), T helper 17 (T_H17), and T regulatory (T_{reg}) cells[27]. Each subset has a distinguished phenotype, metabolic profile, and plays a different role in disease development and pathogenesis[18, 27, 28] (Fig. 3).

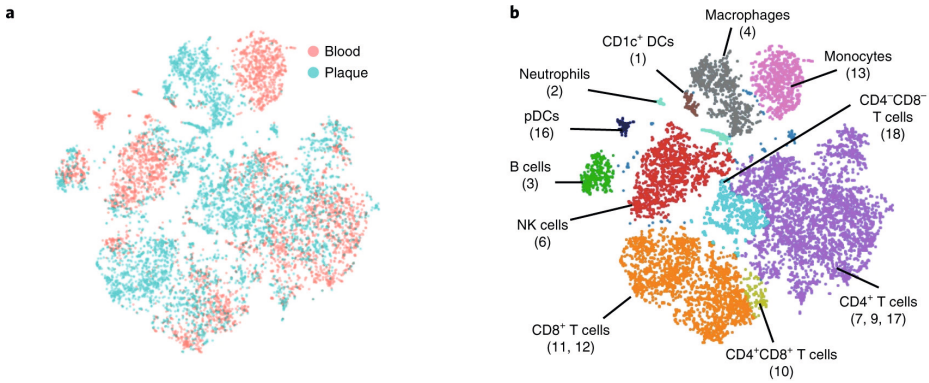

Fig. 2 | Immune cell populations in the blood and atherosclerotic plaque. Adapted from "Single-cell immune landscape of human atherosclerotic plaques" by Fernandez, D. M. *et. al.* showing the proportion of different immune cells in atherosclerotic plaques[19]. Figures are viSNE plots of $CD45^+$ immune cells (n = 9,490) derived from blood and plaque tissue of 15 patients. Clustering is based on the MetaLouvain method. **(a)** Immune cell populations in the blood (red) and in the plaque (blue). **(b)** Immune cell populations as defined by mass cytometry (CyTOF).

T_H1 cells are characterized by the expression of the key transcription factor T-bet, as well as signal transducer and activator of transcription (STAT)1 and STAT4, and generally produce interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα)[29, 30]. These cells are known to play a strong pro-inflammatory role in atherosclerosis and are the largest $CD4^+$ subset in the plaque[28]. T_H2 cells are characterized by the expression of the key transcription factor GATA3, as well as STAT5 and STAT6, and generally produce interleukin (IL)-4, IL-5, and IL-13[29, 31]. The exact role of T_H2 cells in atherosclerosis has not yet been determined as contradicting results show both pro- and anti-inflammatory effects[28]. However, a subset of T_H2 cells, T_H9 cells[32], have shown to be highly pro-inflammatory in atherosclerotic disease[33-35]. T_H9 cells are characterized by the expression of the transcription factor PU.1 and interferon regulatory factor (IRF)4 and generally produce IL-9[29]. T_H17 cells are characterized by the expression of the key transcription factor RAR related orphan receptor (ROR)γt as well as STAT3, and generally produce IL-17, IL-21, and IL-22[29, 36]. The role of T_H17 cells in atherosclerosis has not yet been defined as both pro- and anti-inflammatory effects have been found[28]. T_{reg} cells are characterized by the expression of the key transcription factor forkhead box P3 (FoxP3) as well as STAT5, and cells generally produce IL-10[29, 37]. T_{regs} are notably anti-inflammatory and atheroprotective[28]. Multiple factors play a role in subset differentiation, such as the surrounding cytokine environment and cellular metabolism, specifically lipid metabolism[27, 38, 39]. Defining how extracellular lipids may influence $CD4^+$ T cell differentiation can aid in understanding T cell responses in atherosclerotic plaques.

$CD8^+$ T cells

$CD8^+$ T cells are generally known as cytotoxic T cells and are characterized by the expression of both $CD3^+$ and $CD8^+$ on the cell surface. While $CD8^+$ T cells don't have defined subsets as $CD4^+$ T cells, they can be broadly divided into cytotoxic and regulatory $CD8^+$ T cells[40]. While the frequency of $CD8^+$ T cells is increased in plaques[19] and in the blood of patients with coronary

artery disease[41], both a pro- as well as an anti-inflammatory role has been found for CD8⁺ T cells in atherosclerosis development and progression[28, 42, 43]. Cytotoxic CD8⁺ T cells have been shown to produce IFNγ, perforin and granzyme B, which may aggravate atherosclerosis[44, 45]. On the other hand, regulatory CD8⁺ T cells may play an atheroprotective role by limiting the accumulation of T_H1 cells and macrophages and stabilizing plaques[46]. Furthermore, immunization with an ApoB- related peptide (p210) has shown an atheroprotective role mediated through CD8⁺ T cells[47, 48]. Overall, CD8⁺ T cells may play an interesting role in atherosclerosis although the exact mechanisms through which these cells induce a pro- or anti-inflammatory effect and whether lipids influence these effects have not yet been studied.

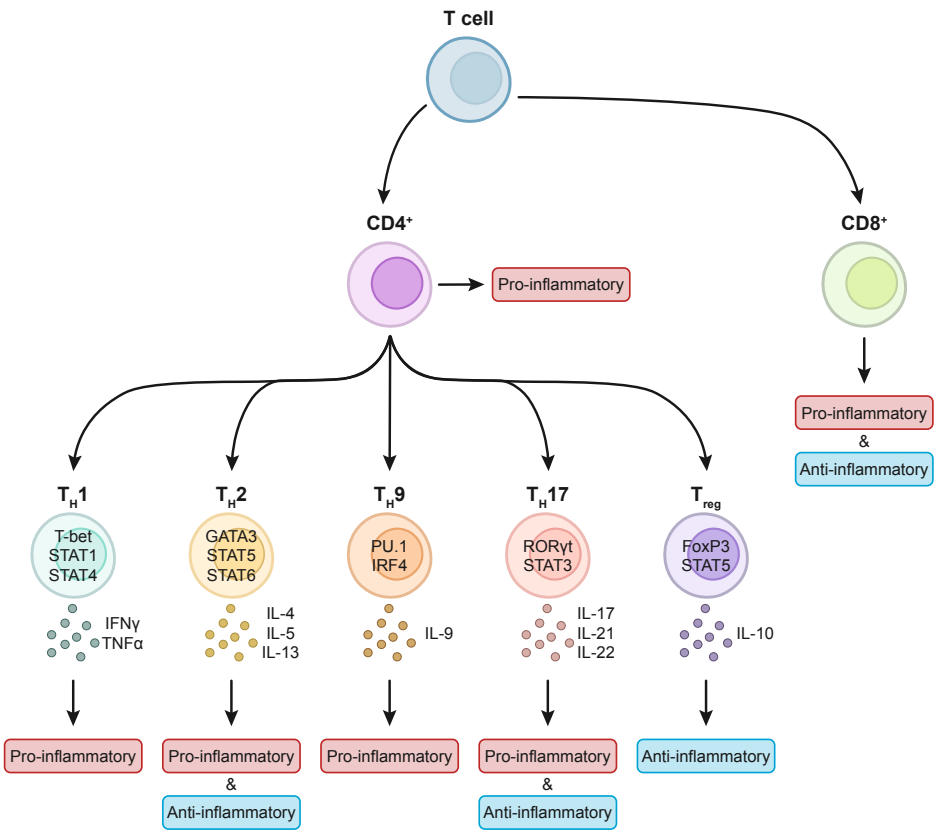

Fig. 3 | Defining transcription factors, cytokines, and role in atherosclerosis pathogenesis of different T cell subsets. T cell subsets are labelled in bold above the cell, main transcription factors are labelled inside of the cells, main cytokines produced are labelled below and to the right of the cell, and the function in atherosclerosis pathogenesis can be found below the main cytokines excreted and is either pro- or anti-inflammatory, or both.

Triglycerides and fatty acids

Triglycerides have re-emerged as a potential risk factor for cardiovascular disease. Population studies showed that increased circulating levels of triglycerides are associated with an increased risk of CVD[49-53] (Fig. 4). Furthermore, this lipid group remains a risk factor for CVD also after accounting for the effect of total cholesterol and high-density lipoprotein (HDL) cholesterol[13, 14]. This can, in part, explain why a residual risk of CVD is still present even in individuals with substantially reduced LDL cholesterol levels[54]. Elevated triglycerides are the characteristic attribute of a lipid disorder called hypertriglyceridemia[55]. This disorder may occur due to primary factors such as one or more mutations in the *LPL*, *APOA5*, or *APOE2* genes, or due to secondary factors such as diabetes, obesity, or excessive alcohol use. As such, hypertriglyceridemia has been linked to a higher risk of CVD, specifically in individuals with moderately elevated triglyceride concentrations (between 2–10 mmol/L)[56, 57]. Hypertriglyceridemia patients with extremely elevated triglyceride concentrations (>13.0mmol/L) often are no longer at risk for CVD because their excessive triglyceride levels get stored in large chylomicron lipoprotein particles that cannot cross the arterial wall[58]. Reduced triglyceride concentrations may protect from atherosclerosis, a characteristic of individuals with hypotriglyceridemia with triglyceride concentrations below 0.3 mmol/L[59]. Individuals with these disorders may offer interesting insights into the effects of elevated or depressed triglycerides on circulating T cells.

The exact contribution of triglycerides in CVD is still widely debated. This is, in part, due to the fact that triglyceride lowering therapies, such as fibrates and niacin, have not demonstrated a convincing benefit for CVD risk[60-64]. Yet, there has been one drug that has shown triglyceride lowering and substantial CVD risk reduction in patients with hypertriglyceridemia, namely icosapent ethyl (IPE)[65-67]. IPE is a highly purified and esterified form of the fatty acid eicosapentaenoic acid (EPA). IPE contains an ethyl group which is cleaved off as the drug is metabolized after ingestion in the human body, exposing cells directly to EPA[68]. The REDUCE-IT trial showed a 25% decrease in primary composite end points and a 26% decrease in secondary composite end points, even when correcting for factors such as the use of mineral oil in the control group[65, 69, 70]. The mechanism of action for this risk reduction remain largely unknown as the beneficial effects observed occurred independently of triglyceride lowering in REDUCE-IT[65], and studies into this research question are thus far limited to model membranes or in whole blood[71-73].

EPA is a polyunsaturated fatty acid, one that has more than one double bond in its carbon chain. Specifically, EPA has 5 double bonds in its 20-carbon chain denoting it as C20:5. Fatty acids may also be saturated, with no double bonds such as palmitic acid (C16:0), or monounsaturated, with one double bond such as oleic acid (C18:1). Palmitic and oleic acid are some of the most common fatty acid in the human circulation[74] and have both been shown to have pro-atherogenic effects[75-79]. Interestingly, triglycerides, which are partially comprised of fatty acids, are hydrolyzed into the fatty acid components inside cells which can be further metabolized and used by the cell[80, 81]. As such, fatty acids may also have a noteworthy influence on T cell function, potentially through changes in cellular metabolism, that could determine T cell effects in atherosclerosis.

However, the exact relationship between different fatty acids, atherosclerosis and T cells has yet to be explored.

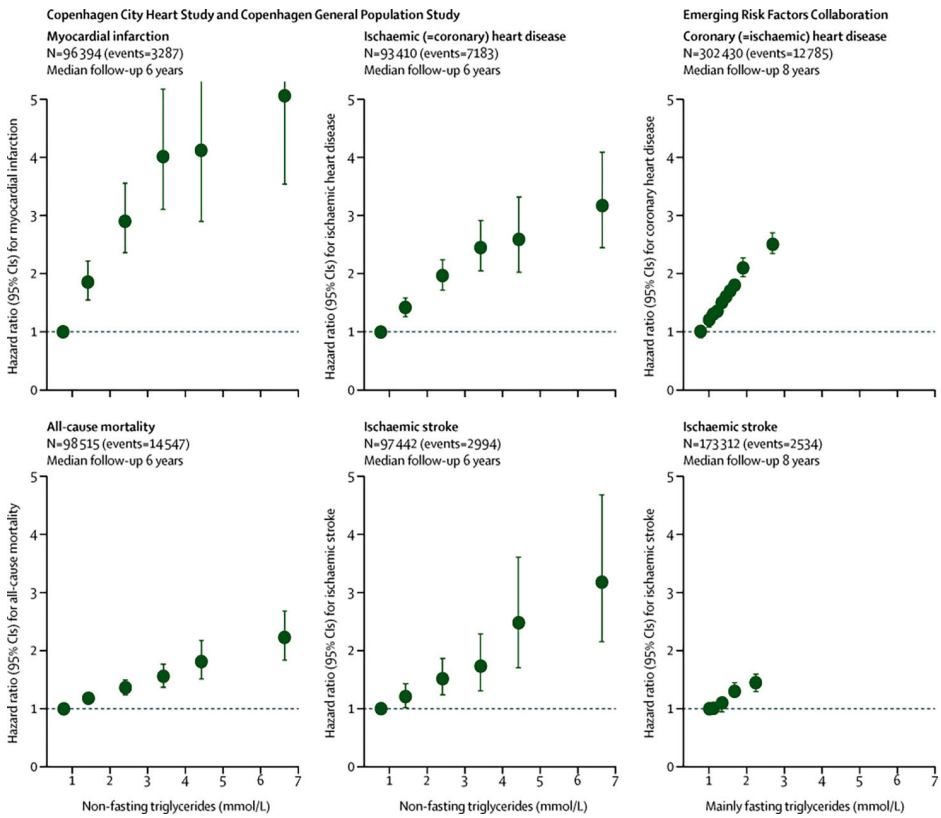

Fig. 4 | Increased hazard ratio with increased non-fasting triglycerides. Adapted from "Triglycerides and cardiovascular disease" by Nordestgaard, B. G. and Varbo, A. showing that increasing triglyceride levels increase the hazard ratio for myocardial infarction, ischemic heart disease, all-cause mortality, and ischemic stroke[56].

Outline of this thesis

This thesis aims to uncover how non-activated T cells can be influenced by triglycerides and fatty acids found in the circulation and whether this interaction can have a lasting effect on T cell function that may influence the role T cells play in atherosclerosis. Particularly, we focus on $CD4^+$ T cells as their role in atherosclerosis has been more well defined and explore the effects of fatty acids and triglycerides from *in vitro* to *in vivo*.

In **Chapter 2** we delve deeper into the role various fatty acids play in atherosclerosis development, how those fatty acids can influence T cell function, and whether the interactions between fatty acids and T cells in the circulation may influence the role T cells play in atherosclerosis. We review the

effects of 14 different fatty acids on four different T cell processes namely, metabolism, activation, proliferation, and differentiation. Indeed, it has been found that fatty acids can influence T cell function post activation and that the effect fatty acids have on T cells, pro- or anti-inflammatory, match the effect that fatty acid is known to have on atherosclerosis. However, interactions between fatty acids and T cells occur prior to atherosclerosis development, in the circulation, where T cells are in a non-activated state. Thus, this chapter concludes by hypothesizing that the interactions that take place between T cells and fatty acids in the circulation may have effects that influence the effect T cells have upon entering the high lipid environment of the atherosclerotic plaque.

In order to test the hypothesis provided in the previous chapter, that is, whether fatty acids can influence non-activated T cells, **Chapter 3** presents a new *in vitro* model to test the effect of fatty acids on non-activated $CD4^+$ T cells and apply it to evaluate the effects of oleic acid. This monounsaturated fatty acid is one of the most abundant fatty acids in the circulation and has been associated with an increased risk of CVD[78]. However, the role of oleic acid on T cells has shown opposing results. To elucidate the effects of oleic acid on $CD4^+$ T cells, we determined changes in gene expression in non-activated cells after exposure and measured phenotypic markers post-activation. Additionally, we test the role cellular metabolism plays in determining functional outcome by exposing the cells to metabolic inhibitors during oleic acid exposure.

Building on this work, in **Chapter 4**, we determine the effects of EPA, palmitic and oleic acid on non-activated $CD4^+$ T cells. In it purified form IPA, EPA was shown to be highly atheroprotective in the influential REDUCE-IT trial. However, the mechanism by which EPA induces anti-inflammatory effects remains unclear. Here, we aim to uncover whether EPA induces an anti-inflammatory transcriptomic profile in non-activated $CD4^+$ T cells, which could aid in explaining the beneficial effects measured in REDUCE-IT. To distinguish the unique effect of EPA on $CD4^+$ T cells, this chapter also compares the results of EPA exposure to two other fatty acids of different saturation, palmitic acid, a saturated fatty acid, and oleic acid, a monounsaturated fatty acid.

The previous chapters use an *in vitro* model to evaluate the effects of fatty acids on non-activated T cells. In **Chapter 5** we move to an *in vivo*, human setting to test whether prolonged exposure to elevated triglycerides, the form in which fatty acids are transported in the circulation, affects $CD4^+$ and $CD8^+$ T cells *in vivo*. Gene expression changes were measured in $CD4^+$ and $CD8^+$ T cells derived from patients with moderate hypertriglyceridemia, in the range that is a risk for CVD. We divide the patient groups into primary moderate, due to genetic mutations, and secondary moderate, due to lifestyle. Additionally, we tested individuals with primary severe hypertriglyceridemia, above the range at risk of CVD, and individuals with primary hypotriglyceridemia, severely depleted triglyceride levels. This was done to test whether the effects on T cells would dissipate when triglycerides are more likely stored in chylomicrons, as is the case in primary severe hypertriglyceridemia, or whether the effects on T cells would be anti-inflammatory in the absence of triglycerides, as is the case in hypotriglyceridemia.

Lastly, all results of this thesis are summarized, connected, and put into context in **Chapter 6**. Here, we discuss the findings and deliberate the implications thereof as well as comment on the future research directions. In sum, the investigations conducted within this thesis have enriched our understanding of the intricate interplay between fatty acids, triglycerides, and their impact on circulating T cells, shedding insights into the pivotal roles these components play in driving the development and advancement of atherosclerosis.

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