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

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CHAPTER 6

Summary and general discussion

Summary

The pathophysiology of atherosclerosis has traditionally been understood by the interactions between macrophages, excess cholesterol laden low density lipoprotein (LDL), and the vascular wall[1]. Although these components have been extensively studied and medications like statins have successfully lowered cholesterol levels and reduced cardiovascular risk, a residual risk of cardiovascular disease (CVD) still exists[2]. Thus, it is important to consider what alternative factors might be playing a role in the pathophysiology of this disease. The aim of this thesis was to uncover how triglycerides and fatty acids interacted with and influenced T cells in the circulation and to determine whether this pre-exposure could alter the function of T cells, which could shape the role these cells play in atherosclerosis (Fig. 1).

T cells, triglycerides and fatty acids are each components of interest in further uncovering the pathophysiology of atherosclerosis. To do this, studies generally explore the role of various individual fatty acids in the context of cardiovascular outcomes, investigate how specific fatty acids influence T cell function, or examine how T cells affect atherosclerosis. Yet, there is no overall picture of the differences and commonalities between the influence of various fatty acids on T cell responses. In **Chapter 2**, we connected the findings and results of such studies in a Review to provide a generalized overview of how fatty acids, T cells, and their interactions may contribute to atherosclerosis[3]. By comparing the known effects of specific fatty acids on T cell function and the known effects of specific fatty acids on atherosclerosis, we were able to show that, by and large, the effect a fatty acid had on atherosclerosis and T cell function were similar, that the effect of fatty acids was primarily determined by the saturation level, and was similar for $CD4^+$ and $CD8^+$ T cells. Saturated fatty acids (SFAs) generally showed pro-inflammatory and pro-atherogenic effects while polyunsaturated fatty acids (PUFAs) had anti-inflammatory and atheroprotective effects. The effect of monounsaturated fatty acids (MUFAs) depended on the context of the experiment and can have both pro- and anti-inflammatory/atherogenic effects. Our review highlighted the key role for cellular metabolism in the fatty acid-induced alterations in T cell function, namely activation, proliferation and differentiation. A limitation of the studies performed so far was that they were performed during or after T cell activation, which might influence the outcomes measured as this process requires the import and use of nutrients to support cell growth and proliferation. The evaluation of current literature led us to hypothesize that the influences of fatty acids on T cells already occurs prior to activation, in a non-activated state in the circulation, which would affect the impact of T cells on the pathophysiology of atherosclerosis upon entering the plaque, where they become activated[4].

To test the hypothesis resulting from **Chapter 2**, **Chapter 3** describes the set up and use of an *in vitro* model for non-activated T cell exposure to oleic acid[5]. Oleic acid, a MUFA, is one of the most common fatty acids in the circulation[6]. The high natural abundance of this fatty acid in the circulation along with the strong effects on T cells found in previous studies make oleic acid of particular interest for further investigation[7-13]. To this end, we employed transcriptome-wide analysis of oleic acid exposed non-activated $CD4^+$ T cells. Not only have $CD4^+$ T cells been found

to aggravate atherosclerotic disease in mouse models[14, 15], but this cell type is known for its ability to differentiate into several subsets that each induce a distinct pro- or anti-inflammatory effect[16]. This makes $CD4^+$ T cells of particular investigative interest over $CD8^+$ T cells, whose role in atherosclerosis remains debated[17, 18]. We discovered that oleic acid resulted in a highly specific upregulation of cholesterol and fatty acid biosynthesis related genes and pathways. This metabolic reprogramming pre-activation resulted in increased IL-9^+ producing cells post-activation. Intriguingly, the expression of this highly pro-inflammatory cytokine was reversed when cells were exposed to the metabolic inhibitors of cholesterol and fatty acid biosynthesis during oleic acid exposure. Thus, this study supports our hypothesis from **Chapter 2**: non-activated T cells, as they occur in the circulation, can be influenced by fatty acids to become poised for preferential differentiation after activation.

Oleic acid was of interest because of its high abundance and strong effects on T cells. Yet, the interest in eicosapentaenoic acid (EPA), a PUFA, has recently increased dramatically because it was found to strongly reduce atherosclerotic CVD risk in the landmark clinical trial, REDUCE-IT[19, 20]. However, the mechanism by which EPA exerts its beneficial effects remain largely speculative. In **Chapter 4**, we test the effect of EPA on T cells to contribute understanding of the favorable outcomes observed in the REDUCE-IT trial[21]. In REDUCE-IT, patients received 4g of icosapent ethyl (IPE), a highly purified form of EPA, administered orally as 2g twice daily[22]. Upon ingestion, IPE is metabolized into EPA and absorbed into the bloodstream. Here EPA can interact with many different components of the circulation, including T cells. We employed the same *in vitro* model developed in **Chapter 3** to determine whether EPA can induce favorable transcriptomic properties in non-activated $CD4^+$ T cells. To our surprise, we observed a very strong downregulation of immune response and upregulation of antioxidant related genes through RNA sequencing. The putative effects of EPA were an order of magnitude greater than the effects we found for palmitic, a SFA, or oleic acid, a MUFA. Our data suggests that fatty acids have unique effects on non-activated T cells, as EPA, palmitic acid and oleic acid each had distinct effects on T cell transcriptomics. Our data highlights that EPA triggers a robust anti-inflammatory transcriptional profile in non-activated $CD4^+$ T cells, suggesting its potential therapeutic benefits through the modulation of immune and antioxidant genes.

In **Chapters 3** to **4**, we showed that individual fatty acids have distinct effects on T cell function *in vitro*. Yet, *in vivo*, a mix of lipids are found in the circulation, including the composite of fatty acids, triglycerides, which have reemerged as a risk factor for CVD, especially at moderately increased levels[23, 24]. Although the exact role of triglycerides in CVD is still debated, individuals with moderate hypertriglyceridemia are at an increased risk for developing CVD[25-27]. However, it remains unknown if increased triglyceride levels can also influence T cells, and whether that may play a role in the increased risk of CVD observed. In **Chapter 5**, we aimed to translate our *in vitro* findings to an *in vivo* situation by determining the influence of increased circulating triglycerides on $CD4^+$ and $CD8^+$ T cells. The addition of $CD8^+$ T cells is relevant because these cells are the T cell type whose relative abundance is most increased in plaque as compared with the circulation[28].

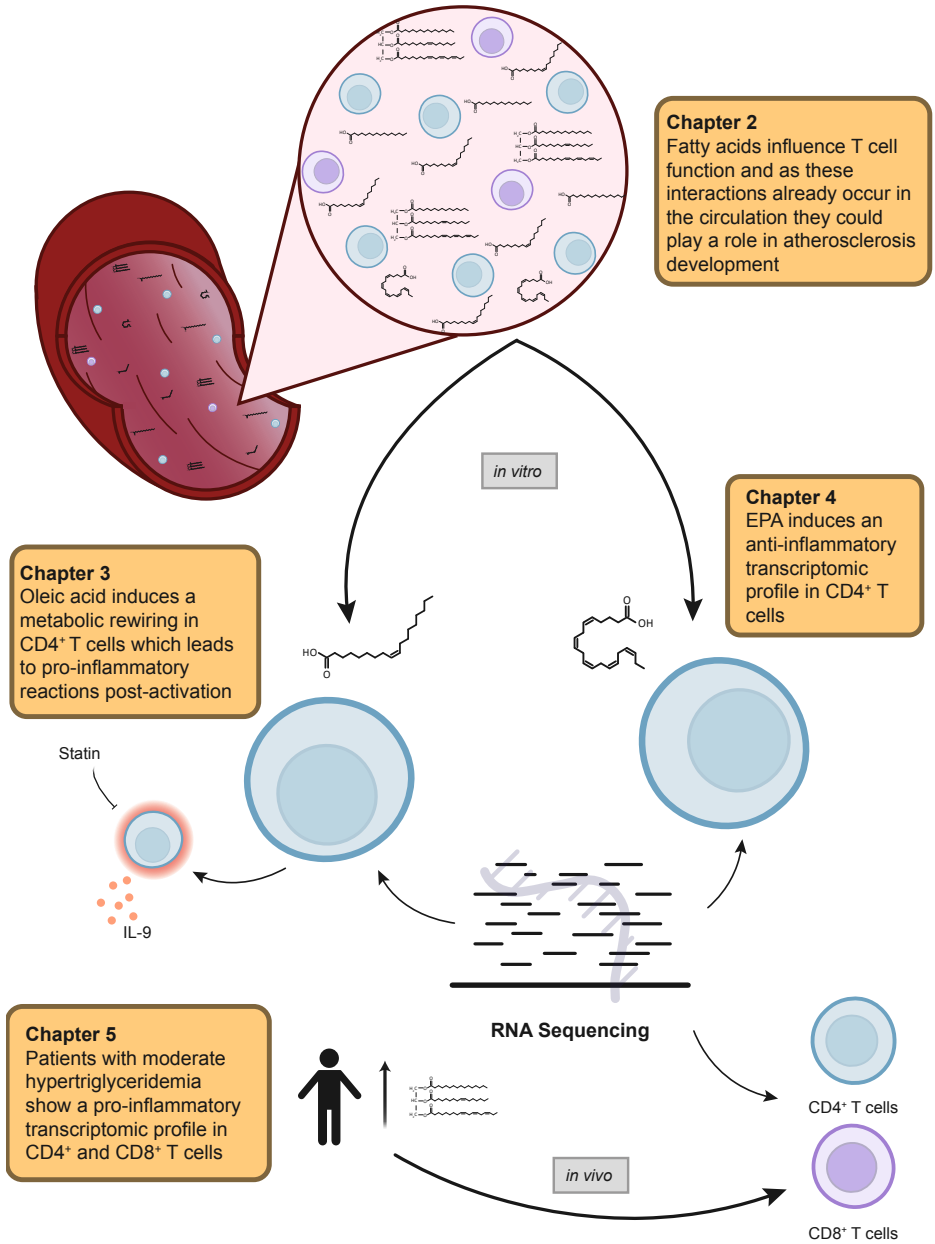

Fig. 1 | Overview of chapter progression and interrelation. This thesis starts in Chapter 2 by reviewing existing literature on the influence of fatty acids on T cells and their potential role in atherosclerosis development and progression. This chapter concludes by formulating a hypothesis that lays the groundwork for all subsequent research performed in this thesis. Chapters 3 and 4 focus on investigating the effects of a specific fatty acid on non-activated $CD4^+$ T cell transcriptomics *in vitro*. Chapter 3 additionally examines cellular function post-activation and finds that metabolic inhibitors can block the observed changes. Lastly, Chapter 5 aims to translate the *in vitro* findings to an *in vivo* context by analyzing the transcriptomic profiles of $CD4^+$ and $CD8^+$ T cells derived from patients with hypertriglyceridemia.

While the role of $CD8^+$ T cells in atherosclerosis pathogenesis remains disputed, investigating their function may offer valuable insights into how triglycerides influence T cell responses[17, 18]. We investigated patients with primary moderate and secondary moderate hypertriglyceridemia, and discovered that T cells derived from these individuals have a more pro-inflammatory transcriptomic landscape as compared to the control group. In addition, this effect was attenuated in patients with primary severe hypertriglyceridemia and reversed in hypotriglyceridemia, showing that different triglycerides levels may shape T cell transcriptomics towards a more pro- or anti-inflammatory profile. This reveals that the circulatory landscape of an individual may have profound effects on T cell function, which may influence the role these cells play in atherosclerosis and CVD.

Fatty acid induced effects on T cells

In **Chapter 2** we presented an overview of the effects that various studies have identified regarding the impact of fatty acids on T cells from experimental studies and linked these outcomes to the effects of these fatty acids on atherosclerosis as reported by epidemiological studies. In each experimental study the effect of 1–9 different fatty acids was evaluated and maximally 2 of the 4 functional outcomes: metabolism, activation, proliferation, and differentiation. The four categories defined each characterize a different aspect of the T cell immune response starting from activation and ending at differentiation, with metabolism underlying each of the other three categories[29, 30]. By determining how fatty acids influence T cells during or post-activation, we can gain an understanding of how T cells may react in the lipid rich core of the atherosclerotic plaque, where T cells are in an activated state[4]. However, the method for measuring the same functional outcome differed between studies[9-12, 31-34]. The diversity in the methods used to expose T cells to fatty acids as well as to determine T cell function, suggest a need for a standardized model of T cell fatty acid exposure. Furthermore, the lack of a consensus of what a proper control is for these types of studies, whether it is nothing, a solvent, or another fatty acid, requires careful consideration[9, 32, 35]. Most importantly, measuring T cell outcomes post-activation gives no indication of whether T cells can be influenced by fatty acids encountered in the circulation, where T cells remain in a non-activated state, and whether pre-exposure to a fatty acid can impact T cell function at a later time.

The overview created in **Chapter 2** allowed us to discern which fatty acids would be of interest for future investigation. In particular, fatty acids that have a high relative abundance in blood and those with variable or strong effects on T cell function, such as palmitic acid, oleic acid and/or EPA, were of interest[6-13, 31-34, 36-48]. Therefore, these fatty acids were chosen to continue our investigations with in **Chapter 3** and **4**. We started by investigating oleic acid in **Chapter 3**. This was because previous research found both pro- or anti-inflammatory effects of monounsaturated fatty acids, including oleic acid, despite it being one of the most abundant fatty acids in circulation[6-13]. We found that oleic acid induced a pro-inflammatory profile $CD4^+$ T cells, likely through rewiring cellular metabolic pathways pre-activation, as marked by distinct and specific changes in gene

expression. By establishing a standardized model for fatty acid T cell exposure in **Chapter 3**, we were able to extrapolate its use to investigate multiple fatty acids at once in **Chapter 4**. Here, we focused primarily on EPA because of its strong anti-inflammatory properties particularly as observed in the REDUCE-IT trial[19, 20]. It was conceivable that non-activated T cells respond to all fatty acids in a similar way, inducing changes in metabolic pathways. However, this was not the case as **Chapter 4** showed almost no changes in gene expression of metabolic pathways due to EPA exposure. Instead, immune response related genes were strongly downregulated. Oleic acid once again showed a strong upregulation of metabolic genes. Palmitic acid exposed non-activated T cells downregulated metabolic genes, and there was little overlap between differentially expressed genes (DEGs) between the three different fatty acid exposed T cells. Therefore, the specific fatty acid a T cell comes into contact with in the circulation can induce a unique response in that T cell, which may dictate the responses that T cell has upon entering the lipid environment of the atherosclerotic plaque.

Modeling triglyceride, fatty acid, and T cell interactions

We showed in **Chapter 2** that a number of different models were used to examine fatty acid effects on T cells and thus we first aimed to devise a standardized model of fatty acid T cell exposure. Although animal models are commonly used for disease modelling and have previously also been used to determine fatty acid effects on T cells, *in vivo* models such as these come with the disadvantages of cost, duration, ethics, and genetic differences that make human translation difficult[49, 50]. Instead, we developed an *in vitro* model of fatty acid T cell exposure in **Chapter 3**. Developing an *in vitro* model allowed us to use $CD4^+$ T cells derived from human subjects as well as tightly control the concentration and duration of fatty acid exposure in both **Chapter 3** and **4**. While *in vitro* models do simplify the environment and present limitations such as static conditions, limited microenvironmental factors, and absence of tissue architecture, our goal was to closely mimic the conditions of the circulation[51]. We ensured our model mimicked the conditions of the circulation by using bovine serum albumin (BSA) bound fatty acid and non-activated cells cultured in the presence of fetal calf serum (FCS). Furthermore, we were also able to establish a proper control for these types of studies. The model developed used the solvent, ethanol, in which oleic acid was diluted as the control and compares the results to medium only, this eliminates the possibility of the solvent having an effect on the T cells. For these reasons, we are confident that our model reflects the most optimal conditions to study individual fatty acid effects on T cells in the human setting. The data derived from this model are highly reliable, translational, and can aid in reducing the need for the use of animal models.

In vitro modelling allowed for a controlled fatty acid exposure to T cells. The model was designed to simplify the biological system of the circulation and aid in uncovering the specific mechanisms of actions that increased fatty acid exposure had on non-activated T cells. However, the human circulation is a complex environment that an *in vitro* model could never fully mimic. Furthermore, T cells in the circulation are exposed to a myriad of lipids, including fatty acids and triglycerides, at

the same time. Therefore, it was important to measure T cell responses to increased triglycerides *in vivo*. Instead of turning towards the use of animal models, we opted to use a human model in **Chapter 5**. This was possible because the condition of moderately elevated triglycerides exists naturally in humans, called hypertriglyceridemia[52]. This condition provides a natural exposure model to study T cell reactions to increased triglycerides. In this regard, human *in vivo* testing is possible because the T cells only needed to be collected from the blood of these individuals and no other intervention was necessary. This allowed us to investigate how circulating triglycerides could influence T cells in a human system. While our *in vivo* model could not be used to verify our *in vitro* findings and the complexity of this system did not allow us to draw causative conclusions from our analysis, it did provide a starting point from which future work can build and extrapolate.

The choice between utilizing an *in vitro* or *in vivo* model depends on the specific circumstances and the particular question being addressed. In **Chapter 3** and **4** utilizing an *in vitro* model came with the advantage of being able to isolate the impact of a specific fatty acid on $CD4^+$ T cell gene expression and function. This allowed us to draw causative conclusions as we compared what the addition of one particular substance had on T cells while the rest of the conditions remained constant. Drawing causative conclusions *in vivo* is more difficult as the complexity of the system does not allow for a direct link to be laid between exposed and not exposed individuals. However, the use of a human *in vivo* model allowed us to directly study a complex biological system. Furthermore, our *in vivo* model also allowed us to measure how prolonged exposure to elevated triglycerides can influence T cells, which is not possible *in vitro*, due to the relatively short-lived nature of T cells. Both models did allow for the inclusion of multiple concentrations of fatty acids and triglyceride levels in the analysis, although the *in vitro* model only moved forward with one concentration, while the *in vivo* model was able to compare the effects different levels of triglycerides had on T cell gene expression. By using a mix of both *in vitro* and *in vivo* models this thesis was able to better understand how lipid T cell interactions in the circulation may be able to shape immune responses in diseases such as atherosclerosis.

Identifying T cell responses via RNA sequencing

Chapter 2 showed that studies investigating the effects of fatty acids on T cells often employs functional outcomes, such as metabolism, activation, proliferation or differentiation, as the readout. However, changes to the T cell may already occur prior to the outward result, post-activation, of the fatty acid exposure. Instead, looking inward, towards what is occurring within the cell may shed a new light on the processes involved in fatty acid exposure in T cells. One place to look within the cell is towards changes in gene expression. Examining changes in gene expression allows us to pinpoint specific biological processes and key genes involved in cellular responses. Additionally, it enables us to predict subsequent alterations in cell function, as changes in gene expression typically precede functional changes in cells.

RNA sequencing has emerged as a revolutionary tool for studying changes in gene expression in the transcriptome and is one of the most cited next generation sequencing methods available[53]. This method involves sequencing the entirety of RNA in one single assay. By mapping these transcripts back to the human genome, it facilitates the identification of which genes come to expression under specific circumstances[54]. Additionally, it allows for the detection of novel features without the limitation of prior knowledge, as is necessary for methods such as real time quantitative polymerase chain reaction (RT-qPCR), which can only identify the expression of one singular chosen gene at a time[55]. We employed this technique in **Chapter 3**, **4**, and **5** to gain novel insights into T cell responses to fatty acids and triglycerides.

In **Chapter 3**, we identified 544 DEGs in non-activated $CD4^+$ T cells exposed to oleic acid across time using RNA sequencing. In doing so, we identified the upregulation of cholesterol biosynthesis and *de novo* fatty acid biosynthesis, each a key metabolic pathway in T cell activation and the development of pro-inflammatory $CD4^+$ T cell subsets[30]. What made this observation so remarkable, is that the T cells measured were not in an activated state, but rather non-activated. Thus, through RNA sequencing of non-activated $CD4^+$ T cells, we were able to predict that oleic acid exposure may induce a metabolic reprogramming in the cell, priming them for T cell activation and pro-inflammatory subset development post-activation. This line of thought was proven to be accurate as we later found increased frequencies of IL-9^+ producing $CD4^+$ T cells post-activation. Furthermore, we were able to identify similar genes in the 60 DEGs of oleic acid exposed non-activated $CD4^+$ T cells in **Chapter 4**, demonstrating the robustness of our approach. We also identified 1170 DEGs in EPA exposed and 33 DEGs in palmitic acid exposed non-activated $CD4^+$ T cells. The overlap between genes of each fatty acid exposed cell group was very small. This continues to show the unique signature each fatty acid has on $CD4^+$ T cells based on the degree of saturation, keeping in line with the conclusions drawn in **Chapter 2**. Additionally it also shows the precision of RNA sequencing as a technique. Lastly, RNA sequencing can also provide relevant information related to the *in vivo* situation as shown in **Chapter 5**, where the changes in gene expression could be mapped to deduce information about T cell profiles in different patient groups. For example, while there were no DEGs detected in patients with secondary moderate hypertriglyceridemia group, examining the expression of the primary moderate DEGs in the secondary moderate group revealed a highly similar profile between the two groups. Moreover, examining the same DEGs in the primary severe and hypotriglyceridemia group showed an attenuated and reversed, respectively, transcriptomic landscapes. Hence, RNA sequencing can provide meaningful insights into the changed biological processes upon fatty acid and triglyceride exposure, which can aid in predicting functional T cell outcomes.

DNA methylation and ATAC sequencing

The changes in transcriptomics correspond to the measured changes in function post-activation in **Chapter 3**, hinting that there may be a memory component to fatty acid exposure. However, to the best of our knowledge, no studies have delved into how a fatty acid exposure is able to

be retained by $CD4^+$ T cells. This is particularly important because the cells in **Chapter 3** and **4** were exposed to the fatty acid prior to activation. As epigenetics often underlies this type of cellular memory[56], we aimed to identify whether epigenetic processes played a role in the cellular recollection of fatty acid exposure.

First, we checked for changes in DNA methylation. This was originally performed as part of the work related to **Chapter 3**. Interestingly, no differences in methylation status between the oleic acid exposed and control conditions were found (Table 1-2) and the work was excluded upon peer review. DNA methylation entails the transfer of a methyl group onto the C5 position of the DNA base cytosine, which forms 5-methylcytosine. This inhibits transcription factor binding or recruits proteins implicated in gene repression to the DNA, limiting gene expression[57]. Thus, although epigenetic reprogramming is likely occurring in the cells, the mode of action is not via DNA methylation. However, DNA methylation is just one form of epigenetic gene regulation. Therefore, we performed Assay for Transposase-Accessible Chromatin (ATAC) sequencing on our EPA exposed non-activated $CD4^+$ T cells in **Chapter 4**. ATAC sequencing assesses genome-wide chromatin accessibility[58]. Again, no differences in open chromatin at individual regions between the EPA exposed and control conditions were detected. ATAC sequencing is done by sequencing open regions of chromatin by exposing DNA to the highly active transposase, Tn5, which preferentially inserts into open chromatin regions, which get sequenced. This method can aid in determining how chromatin packaging affects gene expression[59]. In remains unclear whether this is a true negative result or relates to limitations of the sensitivity of the method that detected >300 thousand peaks throughout the genome of uncertain functional relevance at varying sequencing depths. Alternative assays targeting other layers of epigenetic regulation may be adopted in future studies to resolve the memory component of fatty acid exposure in T cells.

Metabolic transformations during T cell activation have been found to be interconnected with epigenetics[60]. This makes the potential role of epigenetics in shaping cellular memory of interest for future investigation. Although we did not find differences in DNA methylation or open chromatin, via ATAC sequencing, there is some indication that histone acetylation may be an intriguing epigenetic modification to study. In **Chapter 3**, our RNA sequencing found increased expression of *SLC25A1*, a carrier that transports mitochondrial citrate to the cytosol. This carrier is required for cytosolic metabolism of citrate to regenerate acetyl-CoA, which becomes the main source for histone acetylation[61]. Histone acetylation is necessary not only for T cell activation, but also for T cell polarization[62]. Decreased cytosolic Acetyl-CoA production decreased histone acetylation at the *ifng* promoter and resulted in less IFNγ producing T_H1 cells[63]. Thus, it will be particularly interesting to measure histone acetylation in fatty acid exposed cells.

Table 1 | Top 10 CpGs ordered by P adjusted value. Differentially methylated CpGs in oleic acid exposed non-activated $CD4^+$ T cells. No evidence for differential DNA methylation after oleic acid exposure was observed across any time-point.

CpGs	Beta	Standard Error	T Statistic	P value	P adjusted
cg18478105	-3E-04	0.0004	-0.6571	0.51	0.95
cg14361672	-0.003	0.0026	-1.2504	0.22	0.95
cg01763666	-0.003	0.0067	-0.3766	0.71	0.95
cg02115394	-8E-04	0.0031	-0.2614	0.8	0.95
cg13417420	0.0011	0.0031	0.3553	0.72	0.95
cg26724186	0.0012	0.0012	0.9982	0.32	0.95
cg24133276	-0.002	0.0024	-0.9594	0.34	0.95
cg13773083	0.0094	0.0056	1.697	0.09	0.95
cg17236668	0.0009	0.0008	1.0411	0.3	0.95
cg19607165	-0.001	0.0033	-0.436	0.66	0.95

Table 2 | Top 10 genes ordered by P adjusted value. Differentially methylated CpGs in oleic acid exposed non-activated $CD4^+$ T cells mapped to the nearest gene. None of the top-10 differentially methylated CpGs mapped to genes were involved in metabolism or T cell function, neither did any of the nearest genes overlap with genes identified in the transcriptome analysis.

CpGs	Ensembl ID	Gene Name
cg18478105	ENSG00000149658	YTHDF1
cg14361672	ENSG00000160447	PKN3
cg01763666	ENSG00000176155	CCDC57
cg02115394	ENSG00000130177	CDC16
cg13417420	ENSG00000111276	CDKN1B
cg26724186	ENSG00000186111	PIP5K1C
cg24133276	ENSG00000123213	NLN
cg13773083	ENSG00000182698	RESP18
cg17236668	ENSG00000239857	GET4
cg19607165	ENSG00000137203	TFAP2A

Transcription factor footprinting

RNA and ATAC sequencing can also provide information on transcription factor binding. Transcription factors are proteins that control transcription, or the process of converting DNA into RNA. However, transcription factors commonly do not only bind directly to the promoter to have an effect on gene transcription, it can also bind several thousands of base pairs up- or downstream, to regulatory sequences such as enhancers or suppressors, through which it can stimulate or repress transcription[64]. Transcription factor footprinting is a technique that predicts which transcription factors may be active or inactive based on the known binding locations within the genome[65]. RNA sequencing data can resolve transcription factor binding information based on which sequences are present in the promotors of DEGs, as was done in **Chapter 3**. On the other hand, ATAC sequencing can identify likely transcription factors, based on the open chromatin segments that are sequenced, meaning it can also resolve information on transcription factor binding to enhancer and suppressor regions. In **Chapter 4**, we used the generated ATAC sequencing data to identify the downregulation of the key T_H2 and T_H9 transcription factors, GATA3 and PU.1, respectively, as well as the upregulation of the T_H17 antagonist REV-ERB, supporting the anti-inflammatory profile induced by EPA exposure. It is notable that our ATAC sequencing analysis did not yield any differentially expressed peaks, but the transcription factor footprinting did uncover results. Usually, ATAC sequencing generates a large number of differentially expressed peaks, which can be refined by focusing on peaks near transcription factors or DEGs. However, the reverse process is not feasible because changes in transcription factor binding does not necessarily imply relevance for nearby peaks. This unexpected outcome might stem from the inactive state of the exposed T cells or the quality of the ATAC sequencing. With more time, attention, and relaxed thresholds on peak calling, disparities in open chromatin could still emerge. Despite the initial negative findings from the ATAC sequencing analysis, the data could still be used to extract meaningful insights into cell function through transcription factor footprinting.

Measuring T cell functionality

Using the results of the RNA sequencing data generated in **Chapter 3**, we were able to formulate a hypothesis on the functional changes that may occur post-activation in $CD4^+$ T cells pre-exposed to oleic acid. To show these functional changes occurred post-activation, we employed several different methods to test T cell functionality. **Chapter 2** suggested that there were four facets to look at, metabolism, activation, proliferation, and differentiation. We initially investigated proliferation through a radioactive labeling-based assay[5]. We were unable to detect any differences in cellular proliferation using this method. This method utilizes a radioactive version of the DNA base thymine so that the radiolabeled thymidine is used to synthesize DNA during cell division. Thus, every cell division generates more radiolabeled thymidine, providing an overall measure of proliferation[66]. However, this method does not provide more detailed insights into proliferative capabilities such as number of cell divisions, and as such, other methods of cellular proliferation should be considered.

We next explored metabolism and activation utilizing a Seahorse bioanalyzer. Unfortunately, it proved difficult to obtain consistent results using this method. This method measures changes in oxygen consumption (OCR) and proton efflux (ECAR) in culture media as a measurement of cellular metabolism[67]. Given the distinct shift in cellular metabolism during activation from beta oxidation (oxygen consumption) to aerobic glycolysis (proton efflux), the Seahorse bioanalyzer offers the potential to quantify the rate of T cell activation and metabolic alterations[30]. However, this method is very sensitive and requires extremely controlled conditions, small changes in the pH of the medium, number of cells per well, and concentration of substrates added can all influence the results observed. Thus, the need for a large number of replicates limits the number of conditions and donors that can be measured at once, contributing to the variable results measured.

Next, we shifted our focus to the fourth facet of T cell function, differentiation. As T cells activate, they differentiate into various subsets marked by specific cytokine production and gene expression[29]. To measure cytokine production we employed an enzyme-linked immunosorbent assay (ELISA) and to measure changes in gene expression we used an RT-qPCR. Unfortunately, these methods yielded variable or nonsignificant results, making it difficult to interpret changes in T cell function. In an ELISA assay, an antigen, in this case the cytokine of interest, is anchored to a solid surface, either directly or through a capture antibody. Then, a detection antibody linked to a detectable molecule like an enzyme or a fluorophore binds to the antigen, which can be measured via a spectrophotometer[68]. An ELISA thus provides a semi-quantitative rather than an absolute measurements as it relies on standard curves for quantitation. An ELISA as well as an RT-qPCR may have difficulties measuring low abundance analytes because of its limited sensitivity and dynamic range[69]. Furthermore, ELISA's can suffer from cross reactivity with similar analytes as cells are measured in bulk samples and RT-qPCR's can suffer from amplification biases especially for low abundance transcripts[68, 70]. Both methods typically only measure one cytokine/gene at a time, which can be time-consuming and inefficient when analyzing multiple targets simultaneously[69, 70].

Finally, we adopted flow cytometry as a means of discerning T cell differentiation. This technique allows for complex mixtures of cells to be simultaneously characterized, identified, and is generally more sensitive than, for example, an ELISA. Cytometry, both traditional flow and spectral, depends on lasers to catch the scattering of light and one or more fluorescent markers that are read by detectors as single cells in solution flow passed. Thus, this technique can discern rare cell populations and low abundance cytokines with high sensitivity because it is measured at the single-cell level. Populations of cells can then be identified based on the light scattering and fluorescent properties[71]. In traditional flow cytometry, the aim is to collect the emission of a fluorochrome through one detector channel where it is expected to be excited. However, because most fluorochromes have broad emission spectra, "spill over" into another detector channel may occur, called spectral overlap, which has to be later compensated during the analysis to obtain the pure signal[72]. Instead, spectral cytometry records small segments of light, between 38–64 parameters, across the full spectrum. By recording each fluorochrome across the entire

emission spectra, a spectral fingerprint is produced. This reduces the need for compensation as each fluorochrome can be identified by its unique fingerprint and increases the number of markers which can be measured in one sample[73]. Therefore, we opted to use spectral cytometry to measure 26 different T cell markers both extra- and intracellularly as well as intranuclearly. The adoption of spectral cytometry enabled us to accurately characterize components of T cell differentiation, leveraging its ability to discern a broad range of markers simultaneously. This approach provided the stable and reproducible identification of increased IL-9$^+$ production by oleic acid pre-exposed T cells, underscoring the value of spectral cytometry in elucidating complex cellular responses.

Future directions

The experiments conducted in this thesis lay the groundwork for future investigations, particularly focusing on elucidating the relationship between triglycerides/fatty acids, T cells, and atherosclerosis. To advance our understanding, the next step involves providing mechanistic insights into how fatty acids influence T cell responses. Cellular proliferation, a crucial aspect of the adaptive immune response, was examined in **Chapter 3** using radioactive labeling. However, this method solely offers information on cell numbers without depicting proliferative capability, lacking data on the frequency of cell division. To address this gap, flow cytometry may be a valuable tool[74]. By labeling the cell membrane with a fluorescent dye, each cell division round results in signal dilution, allowing for the quantification of proliferative activity[75]. Moreover, alternative cytometry techniques like mass cytometry could provide deeper insights into T cell differentiation. Unlike conventional fluorescence-based methods, mass cytometry employs metal isotopes for cell labeling, overcoming spectral overlap issues. This approach enables the measurement of a larger number of markers in a single sample, facilitating the identification of cell subsets based on transcription factors and cytokines[76].

Single cell RNA sequencing may also offer additional insights into T cell subset development. Single cell RNA sequencing can not only identify subpopulations of T cells, but could also provide information about the genes and signaling pathways that are active or differentially expressed during subset development[77]. Moreover, to gain a full understanding of mechanistic outcomes of triglyceride/fatty acid exposure on T cells, examining protein production will be essential. Techniques that use mass spectrometry, such as shotgun proteomics may allow for high-throughput protein identification and quantification[78]. These techniques can assess whether transcriptomic changes translate into altered protein expression. Integrating these methods offers a comprehensive approach to elucidate how fatty acid exposure shapes T cell responses and provides a higher resolution of the underlying mechanisms.

Finally, translating the findings of this thesis into practice in the clinic will be important. By understanding how the various fatty acids of the circulation shape T cell responses, clinicians will be able to make more informed decisions in patient care. Understanding typical ranges of

fatty acids in the circulation allows clinicians to assess whether an individual deviates from these norms, potentially indicating either advantageous or detrimental effects based on the specific fatty acid. For instance, lower levels of oleic acid may suggest reduced risk of atherosclerosis due to decreased T_H9 cell development, and vice versa[5, 79]. This can be achieved through either standard lipid profiling or lipidomic profiling to provide information about concentrations of fatty acids in the circulation as well as in the atherosclerotic plaque[80, 81]. This will also show whether T cells might have a secondary response to a fatty acid encountered in the circulation and also in the plaque. This knowledge may also enable a more targeted approach to patient care, such as using statins to mitigate the inflammatory properties of oleic acid on T cells, thereby aiding this drugs anti-inflammatory and anti-atherogenic effects.

In order to do this, a comprehensive overview of T cells responses to fatty acids is imperative, especially considering variations in fatty acid concentrations in the circulation. This is particularly relevant for individuals with hypertriglyceridemia as triglycerides are the composite of three fatty acid chains linked by a glycerol group[25-27]. Understanding which fatty acids make up the triglycerides in an individual with hypertriglyceridemia could aid in understanding how to most effectively treat these patients. For example, boosting EPA concentrations with medications like IPE, to counteract the negative effects of more common fatty acids such as palmitic and oleic acid may mitigate inflammatory processes in T cells and aid in reducing atherosclerosis. Furthermore, analyzing T cells derived from individuals treated with IPE may offer additional insights into the beneficial outcomes observed of studies such as REDUCE-IT[19, 20].

Expanding this overview of fatty acid effects to other immune cell types, notably $CD8^+$ T cells, is crucial. $CD8^+$ T cells are the second most abundant immune cell type in circulation and their population increases in atherosclerotic plaques[28], yet their precise role in disease pathogenesis remains undefined[17, 18]. This lack of clarity may stem from how the circulatory landscape influences $CD8^+$ T cell responses. Furthermore, other immune cell types, such as monocytes, macrophages, B cells, and dendritic cells should also be investigated[1]. In doing so, an atlas could be created that elucidates how circulatory landscapes shape immune responses and, consequently, impact atherosclerosis development and progression. Thus, targeting triglycerides/fatty acids may offer beneficial effects on atherosclerosis by being able to shape anti-inflammatory responses in T cells.

Conclusion

This thesis represents a departure from previous research paradigms by delving into the intricate interplay between fatty acids and T cells, particularly focusing on their influence at earlier non-activated stages. By developing an innovative *in vitro* model, we not only unveiled the profound impact of fatty acid exposure on the transcriptomic landscape of T cells, but also revealed the consequential effects on responses post-activation. Moreover, this research attempted to bridge the gap between *in vitro* findings and real-world implications by investigating T cells from individuals with hypertriglyceridemia. While the exact mechanistic pathways remain to

be elucidated, these findings serve as a solid base from which new hypotheses can be generated and tested, suggesting that $CD4^+$ T cells are intricately molded by environmental interactions. Thus, targeting triglycerides and fatty acids may lead to beneficial effects on atherosclerosis by leveraging the anti-inflammatory properties of T cells. This work opens new avenues for exploration, advancing our understanding of the complex relationship between fatty acids and T cell dynamics, with implications for shaping our comprehension of human health and disease.

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