CVR-2018-1278-R1

Immunometabolism and atherosclerosis: perspectives and clinical significance– A position paper from the working group on atherosclerosis and vascular biology of the European Society of Cardiology

Short title: *Immunometabolism and atherosclerosis*

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SUMMARY

Inflammation is an important driver of atherosclerosis, and the favourable outcomes of the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial revealed the large potential of anti-inflammatory drugs for treatment of cardiovascular disease, especially in patients with a pro-inflammatory constitution. However, the complex immune reactions driving inflammation in the vascular wall in response to an atherosclerotic microenvironment are still being unravelled. Novel insights into the cellular processes driving immunity and inflammation revealed that alterations in intracellular metabolic pathways are strong drivers of survival, growth and function of immune cells. This position paper therefore presents a brief overview of the recent developments in the immunometabolism field, focusing on its role in atherosclerosis. We will also highlight the potential impact of immunometabolic markers and targets in clinical cardiovascular medicine.

INTRODUCTION

Cardiovascular disease, including myocardial infarction and stroke, is a major cause of morbidity and mortality in the western world. Despite management of risk factors, including cessation of smoking, treatment of hypertension, and lipid lowering regimens using HMG-coA reductase inhibitors or the recently developed proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, a substantial proportion of the population still suffers from cardiovascular disease (CVD)[1]. Over the past years it became clear that atherosclerosis, the underlying cause of the majority of CVDs, is not only driven by lipids, but also by inflammation[2,3]. Research aimed at understanding the complex immune reactions driving inflammation in the vascular wall has shown that the infiltration, retention, and accumulation of lipoproteins in the arterial intima elicit a maladaptive immune response that influence the development, progression, and stability of atherosclerotic lesions[4-6].

Atherosclerotic plaques recruit many different immune cells, including macrophages, T cells, B cells, dendritic cells, neutrophils and mast cells, that change in composition during atherogenesis[7,8]. Together, they determine atherosclerotic plaque progression through the secretion of cytokines, chemokines, proteases, pro-thrombotic factors and other bioactive substances. The balance between pro-inflammatory and anti-inflammatory responses in the plaque will dictate the rate of disease development as well as the size and complexity of lesions. Large atherosclerotic lesions presenting unresolved inflammation, extensive matrix remodelling, large necrotic cores, and thin fibrous caps are at risk of rupture leading to acute thrombosis and subsequent vascular occlusion[4].

Compelling evidence that the immune system plays a pivotal role in atherosclerotic CVD in humans was reported in 2017, when the results from the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial were released[9]. Here, immunotherapy with an interleukin-1 beta (IL-1β) antibody effectively reduced CVD risk and mortality, without affecting low-density lipoprotein (LDL) cholesterol concentrations[9]. The initiation and favourable outcomes of the CANTOS trial revealed the potential of anti-inflammatory drugs to combat CVD. Although Canakinumab was not approved by the FDA for treatment of atherosclerotic CVD, CANTOS is an important proof of principle study, revealing the potential of anti-inflammatory therapies in atherosclerosis, especially in CVD patients with residual inflammation[10]. An important lesson was learned from the CIRT trial where treatment of CVD patients with methotrexate, a broad-spectrum anti-inflammatory agent, failed to reduce CVD or mortality[11]. Collectively, the data from CANTOS and CIRT have taught us that we need to develop drug targets that block atherosclerosis-specific inflammatory pathways.

Recently, it was found that alterations in intracellular metabolic pathways in immune cells occur during activation and that these pathways are key regulators of the immune responses. These data led to a novel research field in immunology, termed *'immunometabolism'* [12]. Recent data show a pivotal role for immunometabolism in disease progression, especially in cancer, but also in obesity and type 2 diabetes, diseases driving CVD atherosclerosis[13,14]. This position paper therefore presents a brief overview of the recent developments in the field, and the impact of 'immunometabolic' changes on atherosclerosis.

IMMUNE CELL METABOLIC PATHWAYS – BRIEF OVERVIEW

Immune cells are highly dynamic and face different metabolic demands in an inflammatory environment. Upon activation, immune cells switch between different metabolic traits (metabolic reprogramming) and can adapt to variations in environmental cues (e.g. oxygen, nutrients, growth factors), as well as to energy and biosynthetic requirements.

At least seven major cellular metabolic pathways have been described in immune cells. The interconnecting pathways of glycolysis, the pentose phosphate pathway (PPP), the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), mitochondrial fatty acid β-oxidation (FAO), fatty acid synthesis and the metabolism of amino acids regulate the survival, growth and activation of immune cells[12].

The interconnectivity of these pathways was reviewed by O'Neill et al.[12] In brief, immune cells take up glucose via the GLUT1 receptor, which is then converted in to glucose-6-phosphate and metabolized via *glycolysis*, thereby converted into pyruvate and 2 molecules of ATP. Pyruvate is converted to lactate by lactate dehydrogenase or enters the TCA cycle.
The *PPP* runs parallel to the glycolytic pathway and is responsible for nucleotide and NADPH production. NADPH is used by NADPH oxidase to generate reactive oxygen species (ROS), which is counter-balanced by the generation of glutathione and other antioxidants. The latter are major mediators of antimicrobial immunity and prevent tissue damage induced by activated macrophages and neutrophils. NADPH generated by the PPP is also required for de novo fatty acid synthesis for expansion of the endoplasmatic reticulum (ER) and the golgi to facilitate enhanced cytokine secretion[12,14].

After glycolysis, pyruvate enters the *TCA cycle* where it can be converted, together with fatty acids, into acetyl-coA. The TCA cycle results in the generation of NADH and flavin adenine dinucleotiode (FADH2), that transfer electrons to the electron-transport chain, where *OXPHOS* takes place to yield ATPs. *FAO* is the most effective way to produce large amounts of ATP. Short chain fatty acids diffuse into mitochondria where they become oxidized, whereas long-chain fatty acids conjugate to palmitoyltransferase 1 for transport. FAO yields acetyl-coA, NADH and FADH2 to enter the TCA cycle/OXPHOS to generate ATP[12,14].

Via *fatty acid synthesis*, metabolic intermediates are converted into triacylglycerols and phospholipids, needed to (re)build cellular structures. *Amino acid metabolism* yields important mediators to exert effector and regulatory functions on immune cells. Examples include glutamine, arginine and tryptophan[12,14].

IMMUNOMETABOLIC SIGNALLING AND REGULATION: MACROPHAGES AND T CELLS

Atherosclerotic plaques are characterized by a plethora of macrophage and T cell subtypes are present. The entire spectrum between classically activated M1, and alternatively activated M2 macrophages is present[7,8,15]. Likewise, the majority of T cell subsets including Th1, Th17, and regulatory T cells have been detected within the atherosclerotic plaque[5]. Activated macrophages and T cells, display a stronger metabolic bias toward aerobic glycolysis than to mitochondrial metabolism, while immune regulatory cells including M2 macrophages and Tregs exhibit a mixed metabolism involving glycolysis, fatty acid oxidation, and OXPHOS[16,17]. We here provide a brief overview on the metabolic alterations that go hand in hand with the different macrophage and T cell activation states (summarized in Fig. 1).

MACROPHAGES

Macrophages form an important plaque constituent. Plaque macrophages take up modified low-density lipoprotein and most of them have become lipid-laden foam cells but can also be activated by lipoprotein-derived antigens, e.g. phospholipids, cholesterol crystals, and apolipoprotein B peptides. In addition, part of the macrophage population in the plaque also has anti-inflammatory properties[18,19]. To fulfill this broad range of functions, macrophages are highly plastic cells and able to acquire a wide array of activation states. For instance, during inflammation their high plasticity enables macrophages to initiate inflammatory responses and to switch off inflammatory responses when no longer needed. Recent studies have elucidated how some of the metabolic processes in macrophages are wired and how metabolism shapes macrophage inflammatory responses[14].

The importance of metabolism in macrophage activation is illustrated by the fact that metabolism of the amino acid arginine formed the basis of the dichotomous M1/M2 classification. Whereas anti-inflammatory M2 macrophages use arginase to convert arginine to urea and ornithine, inflammatory M1 macrophages metabolize arginine using inducible NO synthetase to covert arginine into the pro-

inflammatory NO and citrulline[20]. Once M1 macrophages have formed, NO damages the mitochondrial electron transport chain, and M1 macrophages cannot repolarize towards M2 macrophages, whereas M2 macrophages can easily switch to an M1 phenotype[21]. The arginine pathway is only one of many metabolic pathways that drive macrophage activation states.

Inflammatory macrophages require a rapid supply of energy and biosynthetic products as they need to release their inflammatory contents fast, whereas anti-inflammatory macrophages need a more sustained source of energy for long-lasting repair responses [14]. Therefore, inflammatory macrophages have an increased glucose uptake via the glucose transporter GLUT1 and exhibit enhanced aerobic glycolysis, whereas OXPHOS via the TCA cycle is impaired[22]. During this process, pyruvate, produced by the glycolytic pathway, and generated through dimerization of pyruvate kinase iso-enzyme 2 (PKM2)[23], which catalyzes the final step of glycolysis, is converted to lactate, resulting in 2ATP molecules and induction of ROS[12]. At the same time, the PPP is enhanced through increased flux of glucose intermediates, resulting in increased NADPH synthesis, which is key for cholesterol and fatty acid synthesis, needed for phagocytosis as well as expansion of the ER and golgi, which results in enhanced production of inflammatory cytokines[24].

Other metabolic intermediaries can regulate macrophage function, including succinate, an intermediate of the TCA cycle, succinate, which accumulates in M1 macrophages and drives inflammation via succinylation of intracellular proteins, including the hypoxia-inducible factor 1-alpha (HIF-1α) that augments IL-1β production[25]. Succinate can also propagate inflammatory responses extracellularly through directly binding to the succinate receptor GPR91[26]. The endogenous metabolite itaconate has been recently shown to oppose the deleterious metabolic rewiring, allowing nuclear factor (erythroid-derived)-like 2 (NRF2) to induce downstream anti-inflammatory and anti-oxidant genes [27]. Other TCA cycle intermediates, including fumerate and citrate can contribute histone acetylation and methylation, thereby affecting epigenetic marks that drive innate immune memory[28]. Increased fatty acid synthesis is also associated with macrophage activation, as fatty acid synthesis activates the NLRP3 inflammasome, thereby promoting the release of IL-1β[6,28].

Anti-inflammatory macrophages have a less well understood phenotype but are characterized by increased rates of OXPHOS and FAO. In these macrophages, both pyruvate and fatty acids enter the intact TCA cycle as acetyl-coA, resulting in sustained ATP production via OXPHOS, resulting in upregulation of genes associated with tissue repair[22]. Interestingly, when OXPHOS prevails, the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a crucial glycolytic enzyme, moonlights and represses TNF and IFNγ through the binding to their mRNA[29]. FAO provides most ATPs and is an effective way of anti-inflammatory macrophages to provide a long-lasting energy source. However, the importance of FAO in anti-inflammatory macrophage function has recently been questioned, as etoximir mediated inhibition of FAO or deficiency in CPT2, an enzyme required for fatty acid import does not alter the M2 phenotype[30,31].

The availability and metabolism of various amino acids has also been shown to regulate innate immune cell responses. In macrophages, glutamine can regulate IL-1 secretion, the production of NO, as well as M2 polarization[22,32-34]. Arginine metabolism, via the citrulline pathway and iNOS induction, leading to nitric oxide formation is associated to the M1 phenotype[35]. Through a different mechanism, acetyl-CoA and S-adenosylmethionine can regulate epigenetic enzymes to enable histone acetylation and methylation, thereby translating metabolic rewiring into regulation of gene expression and macrophage function[36].

T CELLS

T cells migrate to tissues followed by tissue-specific adaptations and carry the ability to respond to environmental and metabolic signals. In this context, high calorie consumption, obesity or T2D have

been characterized by infiltration of T cells in metabolically relevant organs in both mice and humans. The adipose tissue, which stores and senses the availability of nutrients, is an important site of immunometabolic crosstalk. While lean visceral adipose tissue (VAT) is usually enriched in Th2 cells and Tregs, VAT from obese individuals has been associated with reduced Treg and increased effector T cell numbers[37,38]. Brown adipose tissue (BAT), which has a high-energy expenditure, as well as browning/beiging of VAT have been associated with increased Treg numbers[37,38]. Interestingly, nutrient metabolism as well as adipokines such as leptin have been linked with different T cell subset differentiation[39]. Leptin has been implicated to directly induce glycolysis in T cells, promoting effector responses and increase inflammation[40]. Leptin-deficiency results in an increase in Tregs that are also more suppressive than Treg from wild type mice. Transfer of leptin deficient Tregs into an experimental model of atherosclerosis caused a significant reduction of plaque size and a marked reduction of IFNγ production, compared to transfer of wild type Tregs[41].

Similar to macrophages, glycolysis plays an important role in T cell responses. Inhibition of glycolysis with 2-deoxyglucose (2DG) shifts the polarization of naïve T cells from Th17 towards Tregs [42]. Hence, increased OXPHOS metabolism has been linked to the induction Tregs[43]. Interestingly, the glycolytic enzymes enolase has been shown to promote Foxp3 splicing and the generation of Treg cells through a non-anticipated 'moonlight' function [43], and 3-phosphate dehydrogenase (GAPDH) has been shown to modulate Th1 responses through repression of interferon-γ (IFNγ) mRNA in a low glycolic activity status[44]. Hence, only upon increased glycolytic activity Th1 cells can mount full pro-inflammatory response and secrete IFNγ [45].

The balance between effector and Treg is also influenced by FAO and fatty acid synthesis. Effector T cells have been shown to present reduced FAO upon activation[46], while Tregs present increased expression of the FAO enzymes, including carnitine palmitoyltransferase 1A (CPT1A)[47]. Inhibition of acetyl-CoA carboxylase 1 (ACC1), the rate-limiting enzyme in fatty acid synthesis, restrains Th17 polarization and promote the development of Tregs[48,49].

Recent studies reveal that specific amino acids and amino acid transporters regulate homeostasis and activation of the adaptive immune system[48,49]. Downregulation of large neutral amino acid transporter (LAT1) can impair Th1 and Th17 differentiation in vivo[50]. Moreover, indoleamine 2,3-dioxygenase-1 (IDO1), the rate-limiting enzyme catalyzing tryptophan (Trp) degradation can modulate T cell effector responses, the expansion of Tregs, and the degree of vascular inflammation and atherosclerosis [51-53]. Upon activation, T and B cells increase glutamine usage [54]. Glutamine metabolism has been implicated to the balance between effector T cells, Th1 and Th17, and Tregs[55]. Moreover, arginine, as well as glutamine and Trp availability are known regulators of immune function via the mTOR pathway [56].

Several studies demonstrate an essential role for cholesterol and fatty acids in activation, differentiation, and function of T cells[49,57,58]. Hypercholesterolaemia can influence TCR signalling and Treg numbers while Tregs can tightly regulate lipoprotein metabolism and influence hepatic inflammation[59-61]. Recent data suggest that ApoAI, the main protein HDL, modulates the conversion of Tregs into T follicular helper cells influencing atherosclerosis [62]. The crosstalk between the immune system and lipid metabolism is an area of increasing interest due to increasing prevalence of the metabolic syndrome, together with chronic inflammatory liver diseases such as non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH)–all representing additive risk to CVD.

IMMUNOMETABOLISM IN ATHEROSCLEROSIS

PLAQUE METABOLISM

The metabolic blueprints for macrophages and T cells described above are obtained from experimental model systems. Although they are of utmost importance to understand metabolic

rewiring of immune cell types in activated and modulatory states, these data may not fully reflect the changes that occur in tissues during pathogenesis. Immunometabolic data in atherosclerotic CVD are still sparse, but recent reports have revealed insights into some of the immune-metabolic patterns that mediate atherosclerosis.

The first proof that alterations in immunometabolism are an important feature of atherogenesis was provided by the nuclear imaging community. 18F-fluorodeoxyglucose PET imaging (FdG-PET), which reflects glucose transport into cells, is widely used clinically to detect diseased tissue conditions exhibiting increased glucose metabolism, such as tumours or infections. As a radionucleotide analogue of glucose, FdG is taken up by cells via glucose transporters (GLUTs) and phosphorylated into FdG-6-phosphate that cannot be further metabolized and accumulates inside of the cell. Increased glucose metabolism has been also considered as the basis for imaging the burden of atherosclerosis. High FDG uptake has been suggested to reflect the degree of vascular inflammation and plaque vulnerability, but may also reflect hypoxia[63,64].

A recent study has revealed that besides an increase in glucose uptake, atherosclerotic disease is characterized by changes in multiple intracellular metabolic pathways in the arterial wall. Metabolomics analysis of 159 plaques of symptomatic (TIA, stroke) and asymptomatic patients obtained during carotid endarterectomy revealed a distinct metabolite profile in inflammatory plaques of patients with symptomatic carotid artery disease. These symptomatic plaques revealed a cluster of metabolites and enzymes that are associated with increased glycolysis, elevated amino acid utilization and decreased FAO. Moreover, this cluster was highly associated with plaque inflammation[65].

SYSTEMIC CHANGES IN METABOLISM AFFECTING ATHEROSCLEROSIS

Work in experimental atherosclerosis models has confirmed the functional importance of immunometabolic pathways in CVD. For example, it was shown that atherosclerotic mice deficient in hematopoietic GLUT1 have a decreased glycolytic flux in their bone marrow and atherosclerotic plaques, resulting in a decrease in atherosclerosis[66]. Likewise, deficiency of glucose-6-phosphate dehydrogenase, a key enzyme in the PPP reduced vascular superoxide levels and also decreased atherosclerosis [67].

Amino acid metabolism also proved of importance in atherosclerosis. Ablation of IDO-dependent Trp metabolism leads to a substantial increase in vascular inflammation and acceleration of atherosclerosis in *Apoe-/-* mice[51,53]. In line with this data, IDO induction has been linked to atheroprotection and increased plaque stability[52,68,69]. Nevertheless, the role of IDO in health and disease seems to be sensitive to alterations in the gut microbiome that may impair its anti-inflammatory and anti-atherosclerotic effects [70-72].

The microbiome also plays an important role in mediating immunometabolism in atherosclerosis. Changes in composition of the microbiome also affect the release of immunopotent metabolites, that have been associated with cardiovascular disease. One example is Trimethylamine N-oxide (TMAO), a plasma metabolite that is formed through the conversion of microbiome generated trimethylamine (TMA) into TMAO via the host's hepatic flavin monooxygenase[73]. The generation of TMAO involves nutrient precursors that are highly abundant in a western diet, and TMAO was shown to induce platelet activation and vascular inflammation in experimental models and patients[73-75]. TMAO levels are associated with atherosclerosis burden[76] and predict both near- and long-term risk of major adverse cardiovascular events (MACE)[77].

MONOCYTES-MACROPHAGES

Atherosclerosis is associated with several changes in monocytes and macrophages. Analysis of monocytes isolated from healthy individuals or individuals suffering from atherosclerotic CVD revealed that monocytes obtained from patients had a higher oxygen consumption rate, a higher glycolytic acidification rate and glycolytic flux. These monocytes had an enhanced glucose uptake, produced more mitochondrial ROS, and had enhanced inflammatory signalling. It was found that monocytes from atherosclerotic CVD patients switched to the glucose-ROS-PKM2-STAT3 pathway through which glucose utilization led to unbalanced ROS generation from the mitochondrial chain, that induced translocation of the enzyme PKM2 and induction of STAT3 signaling, resulting in inflammation[23].

The mechanism for altered monocyte-macrophage metabolic activity may relate to alterations in LDL levels which are characteristic of atherosclerosis [78,79]. In vitro studies have shown that oxLDL results in an increase in glycolysis, inflammation and oxidative damage in macrophages[80-82]. In vivo studies confirm these findings. High cholesterol fed LDLr$^{-/-}$ mice indeed show epigenetic and metabolic reprogramming of myeloid (progenitor) cells, with profound upregulation of the inflammasome [83]. Exposure to high levels of modified LDL triggers epigenetic and metabolic reprogramming of macrophages and exacerbate inflammatory responses[83]. Altered macrophage metabolism can provoke prolonged responses, a phenomenon called “innate immune memory” or “trained immunity” [84]. Glycolysis, glutaminolysis and cholesterol synthesis can influence the activity of methyltransferases and demethylases, acetyltransferases and deacetylases, which by targeting DNA and histones promote increased inflammatory gene transcription.Interestingly, detailed analysis of distinct plaque macrophage subsets uncovered that non-foamy, rather than lipid-loaded foamy macrophages are pro-inflammatory and are likely the cells that drive lesion inflammation[85]. Mechanistically, increased activation of the liver-X-receptor (LXR), trying to mediate cholesterol efflux, in parallel to suppressed activity of the PPP in foam cells, may explain the reduced inflammatory responses in those lipid-loaded macrophages[86,87].

Although much work needs to be done to understand the intertwined and dynamic metabolic changes and pathways that occur in atherosclerosis, it is clear that the atherosclerotic micro-environment causes immunometabolic changes that can drive progression or regression and stabilization of atherosclerotic disease (illustrated in Figure 1). Targeting of immunometabolic pathways is therefore a promising approach to combat atherosclerotic CVD.

Unfortunately, a big gap still exists between the experimental work on immunometabolism and implementation of these experimental results in the clinic. Compelling evidence substantiates that targeting inflammation in CVD improves outcome[9], and it is increasingly known that existing therapies, including HMG-CoA reductase inhibitors[88] and anti-diabetic drugs can also reduce arterial inflammation, most likely by affecting immunometabolic pathways. Therefore, we are approaching an exciting future for scientific advances in immunometabolism which may lead to novel therapies to address residual cardiovascular risk in the clinic.

CONCLUSIONS AND PERSPECTIVES

This Position Paper illustrates the promise of immunometabolism to identify new targets for prevention and treatment of cardiovascular diseases. Although experimental data are promising, implementation of experimental therapies in the clinic presents challenges. More early stage investments should help to develop this field further. Ultimately, large scale randomized clinical trials will be necessary to evaluate immunometabolic-targeted therapies and ascertain their effectiveness and possible unwanted actions.

CONSENSUS STATEMENTS

- Metabolites are not just ‘fuels’ in their pathways, they are also effectors and signalling molecules that regulate the immune system;
- Cellular metabolic pathways are tightly regulated by several pro-atherogenic factors including lipids, glucose, amino acids as well as pro- and anti-inflammatory cytokines;
- Systemic and microenvironment-induced changes in basic metabolic pathways can skew the balance between pro- and anti-inflammatory responses in atherosclerosis;
- The identification of the key immunometabolic reactions governing plaque development and stability will give a new understanding of disease processes, and likely lead to novel therapeutic approaches to prevent and treat atherosclerotic CVDs;
- We now recognize a new frontier, immunometabolism, which presents further opportunity for the CVD field to expand fundamental understanding and furnish new therapeutic avenues for our patients.

ACKNOWLEDGEMENTS

DFJK is supported by the Swedish Heart-Lung Foundation and the Novo Nordisk Foundation (NNF15CC0018346).

EL is funded by the NWO (VICI), the European Research Council (ERC-con) and the DFG (SFB1123), and the CVON consortium GENIUS-II.

MB is funded by Swedish Research Council (2014-2312); and the Swedish Heart and Lung Foundation (20150600 and 20150683).

CB

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LON

CW is funded by the DFG (SFB1123) and by ERC (AdG °692511).

PCE is funded by the British Heart Foundation.

DISCLOSURES

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The remaining authors have nothing to disclose.

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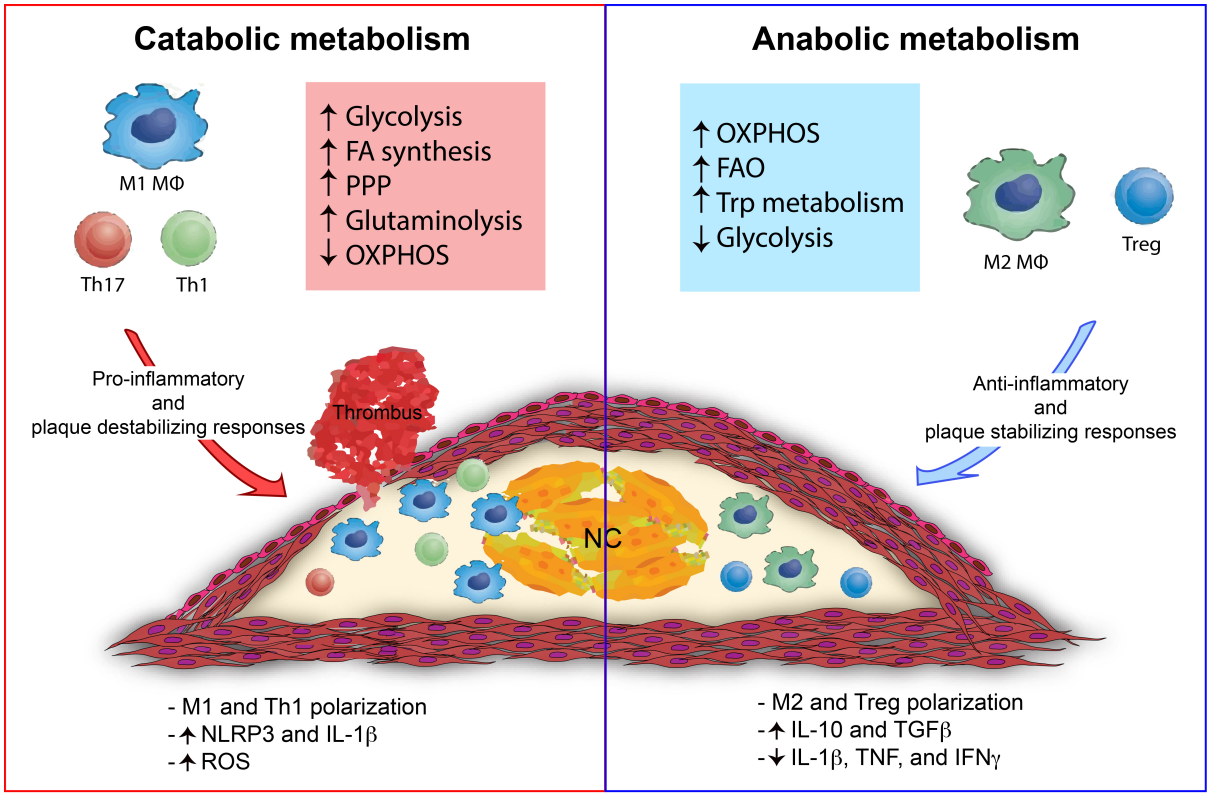

Figure 1. The role of immunometabolism in atherosclerosis.

Pro-inflammatory and anti-inflammatory immune cells present distinct metabolic phenotypes. In general, M1 type macrophages, T helper (Th) 1, and Th17 cells are characterized by more catabolic metabolism, while M2 type macrophages and regulatory T cells (Tregs) present bias towards more anabolic metabolism. Alterations in metabolism on immune cells carry the potential to influence plaque progression and stabilization. FA, fatty acid; PPP, pentose phosphate pathway; OXPHOS, oxidative phosphorylation; FAO, fatty acid oxidation; NLRP3, NACHT, LRR and PYD domains-containing protein 3; ROS, reactive oxygen species; TGFβ, growth factor beta; TNF, tumour necrosis factor; IFNγ, interferon gamma.