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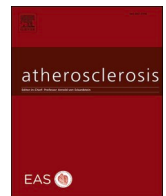
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## Macrophage metabolic regulation in atherosclerotic plaque

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### ABSTRACT

Metabolism plays a key role in controlling immune cell functions. In this review, we will discuss the diversity of plaque resident myeloid cells and will focus on their metabolic demands that could reflect on their particular intraplaque localization. Defining the metabolic configuration of plaque resident myeloid cells according to their topologic distribution could provide answers to key questions regarding their functions and contribution to disease development.

### 1. Introduction

Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of lipids and immune cells in the intima of blood vessels. Atherosclerosis is the underlying cause of cardiovascular events such as myocardial infarctions and strokes, that together are responsible for 17.9 million deaths per year worldwide (World Health Organization). Atherosclerotic plaque progression is favored in conditions of hypercholesterolemia and dyslipidemia. Its development initiates when intima-resident macrophages uptake excess lipids and form foam cells [1]. Subsequent plaque progression is dependent on a constant influx of monocytes from the blood circulation that fuels plaque macrophage accumulation [1]. In the last two decades, advances in the field of atherosclerosis have identified myelopoiesis, plaque macrophage proliferation and efferocytosis as key elements defining both atherosclerosis development and regression. More recently, it became clear that these parameters are influenced by immune cell metabolism. The atherosclerotic lesion forms a complex micro-environment defined, at least in part, by its developmental pattern. Newly recruited monocytes are highly motile cells, located close to the lumen. On the other hand, macrophages are sessile and situated deeper within the plaque [2], where specific nutrients could hypothetically be scarce, and where necrotic cores develop. Metabolite availability therefore appears as an additional potential regulator of plaque myeloid cell metabolism and

functions, depending on the cell's intra-plaque localization. Here, we discuss the metabolic regulation of macrophage functions and inflammatory properties within the plaque.

#### 1.1. Immune cell diversity in atherosclerotic plaque

Pioneering studies performed in pre-clinical models and human subjects detected the presence of immune cells in atherosclerotic lesions. Multiple immune cells including T cells, both CD4<sup>+</sup> and CD8<sup>+</sup>, B cells, macrophages, monocytes and dendritic cells (DC) were present in plaques [3–7]. These early reports used immunostaining and microscopy to determine the cellular composition of the plaque. The main limitation of this technology is the limited number of markers that could be simultaneously used to accurately define the precise nature of plaque-residing cells. More recently, flow cytometry analysis, providing the possibility to greatly extend the number of membrane markers simultaneously investigated, demonstrated that plaque cellular composition was more complex than initially described. The presence of tertiary lymphoid organs, structures enriched in T cells that develop in the vessel adventitia adjacent to plaques [8], could also contribute to plaque immune cell contamination in flow cytometry analyses. Indeed, this approach does not provide insights about the precise intra-plaque localization of the diverse immune cells. Intra-tissue localization is important since it could have an impact on oxygen and metabolites supply and therefore on

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immune cell metabolism and activation state within the plaque.

Single-cell RNA sequencing (scRNA seq) analyses further enriched our knowledge about the phenotypic diversity of mouse and human plaque-residing immune cells [1,9–13]. These studies revealed that plaque cellular composition was impressively complex and contained many diverse myeloid and lymphoid cells. Macrophages were the most abundant cells in the plaque. Several distinct macrophage and monocyte subsets were identified in the plaque of atherogenic (*LdlR*<sup>-/-</sup> and *ApoE*<sup>-/-</sup>) mice and patients. Initially described as a key feature of advanced lesions, foamy macrophages, a population of lipid-laden cells, were attributed a pro-inflammatory role mediated by the release of cytokines and chemokines [14,15]. This dogma was challenged when scRNA seq analysis demonstrated that monocytes and inflammatory macrophages, rather than foamy cells, were enriched in mRNA encoding for pro-inflammatory mediators including *il1β*, *il12*, *tnfα*, *ccl2* and *cxcl2* [10].

Two main mechanisms account for lesion growth depending on the plaque stage development. Early lesions are mainly sustained through monocyte recruitment from the blood circulation and their local retention [16–18]. In contrast, in advanced plaques, *in situ* macrophage proliferation favors plaque progression [19]. Whether these two processes require a specific metabolic rewiring and rely on different metabolic pathways remains to be established. Macrophage intraplaque proliferation correlates with plasma lipoprotein levels [20]. Lowering plasma lipid levels and genetic ablation of lipoprotein uptake receptors in macrophages (*Msr1* and *CD36*) decreased plaque macrophage proliferation rate [20]. However, the underlying molecular and metabolic mechanisms remain unknown. A recent study using multi-isotope imaging mass spectrometry found that plaque proliferating cells used preferentially glucose in comparison to neighboring non-proliferating cells [21]. Surprisingly, foamy cells were highly glucose consuming and this was correlated with increased proliferation [21]. This observation is rather surprising because foamy cells are characterized by massive lipid accumulation, and they highly express genes associated with lipid metabolism. Whether glucose or lipids serve as the main energy source for foamy cells remains to be defined. To better apprehend the metabolic configuration of plaque resident myeloid cells and foamy cells in particular, this set of data could be complemented using a new flow cytometry-based strategy named SCENITH that has recently been described [22]. This approach provides insights into cellular metabolic status with a single-cell resolution, allowing the comparison of multiple cell types contained in a given sample. Although this method requires relatively low numbers of cells, the analysis of quiescent or over-stressed aortic cells after tissue digestion could be challenging.

## 1.2. Heterogeneity and metabolic control of macrophages and monocytes during atherosclerosis

Metabolism emerged as a central regulator of macrophage and monocyte functions. Indeed, metabolic adaptations modulate key macrophage functions including cytokine production, efferocytosis and phagocytosis (for review [23–25]). *In vitro* studies, using either interleukine-4 (IL-4) or lipopolysaccharide (LPS)/interferon (IFN)  $\gamma$  stimulation led to mimicry of particular macrophage activation states (M1 and M2). These simple models were broadly used to define macrophage metabolic adaptation to external stimuli. Anti-inflammatory (M2-like) macrophages exhibit a mitochondrial oxidative metabolism while pro-inflammatory (M1-like) macrophages are characterized by a glycolytic metabolism. More than just another polarization marker, metabolic rewiring is a key player in those differentiations, and interfering with glucose flux or mitochondrial fitness prohibits M1 and M2 polarization respectively. Nevertheless, while such *in vitro* polarization models have fruitfully brought forward the importance of metabolism in immune cell decision processes, their translation to cell decisions inside complex environmental conditions is not straightforward.

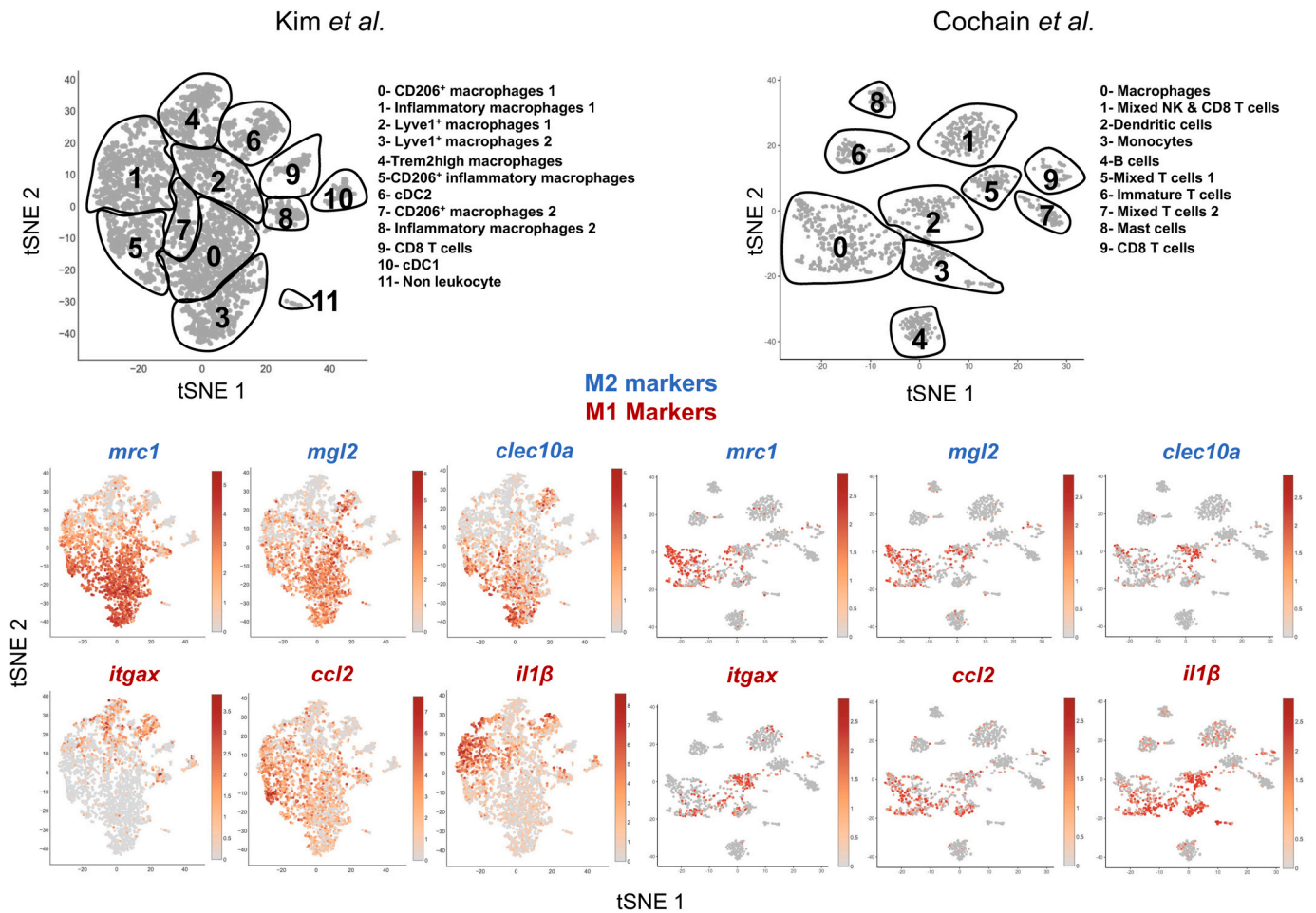
In atheromatous plaque, recently generated scRNA-seq datasets [9, 10] highlighted the co-existence of several distinct populations of pro-inflammatory and anti-inflammatory macrophages and the expression of canonical M1 or M2 macrophages was shared by one or the other population (Fig. 1). Canonical M2 markers (*mrc1*, *clec10a*, *mgl2*) prevailed in plaque resident macrophages in comparison to M1 (*cd11c*, *il1β*, *ccl2*) markers (Fig. 1). Surprisingly, M2-like macrophages, supposed to have an anti-inflammatory function, displayed a pro-inflammatory transcriptomic signature. These cells highly expressed the mRNAs encoding for pro-inflammatory cytokines (*il1β*, *tnfα*) and chemokines (*ccl2*, *cxcl2* and *cxcl1*). The clinical significance of inflammation, and namely IL-1 $\beta$ , during cardiovascular diseases was perfectly illustrated by the CANTOS trial [26]. The abundance of M2-like macrophages in plaque is surprising because local presence of IL-4 and IL-13 is limited. IL-13 levels in plaques are below the detection limit, while IL-4 concentration is also low [27]. Similarly, the impact of IFN $\gamma$  on plaque macrophage phenotype appears minimal, as myeloid-specific IFN $\gamma$  receptor deficiency did not alter atherosclerosis development in *LdlR*<sup>-/-</sup> mice [28]. Attempts have been made to explore metabolite impact on macrophage activation *in vitro*. Kratz and colleagues reported that macrophages stimulated with a combination of insulin, glucose and palmitate show an activated phenotype with traits of both M1 and M2 polarization [29]. These cells were named “metabolically activated macrophages” and showed enhanced expression of the inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$  and IL-6. On the other hand, M2 markers were globally unaffected while M2-associated lipid metabolism-related genes were induced in metabolically activated macrophages [29].

Thus, one might consider that locally available metabolites, in combination with metabolic reprogramming induced by M1/M2-polarizing cytokines, could contribute to a specific and spatially defined metabolism and activation state of macrophages during plaque initiation and progression.

## 1.3. Metabolites and associated pathways mediating macrophage activation

### 1.3.1. Glucose and cellular carbohydrate metabolism

Glucose metabolism plays a central role in macrophage functional adaptation. Pioneering work revealed M1- and M2-like macrophage specific glucose fluxes into the two major pathways of cellular carbohydrate metabolism, namely glycolysis and the pentose phosphate pathway (PPP) [30]. Glucose utilization by glycolysis generates energy in form of ATP and crucial intermediates, which can act as substrates for other metabolic pathways. This includes pyruvate, which can be further converted to lactate or Acetyl-CoA, two major metabolites important for macrophage metabolic adaptations. Another example is glycolysis-derived serine generation to sustain cellular one-carbon metabolism, which is regulating epigenetic reprogramming during macrophage activation [31]. Glucose utilization by the PPP is crucial for generating the required redox power by the formation of NADPH, in order to sustain macrophage functions such as ROS handling and anti-oxidative protection by the generation of reduced glutathione. NADPH is also a critical co-factor for lipid metabolism and other metabolic branches as it acts similar to ATP as a universal energy carrier, which is used by many different enzymes throughout the metabolic networks of cells. Also, redox-sensitive protein signaling during macrophage activation is dependent on PPP activity. Another hallmark of the PPP is to provide pentose molecules, which can serve as precursors for nucleotide metabolism or become reconverted to glycolytic intermediates in the non-oxidative branch of the PPP. We recently comprehensively discussed the function and distribution of key enzymes involved in those two pathways to atherosclerosis development [32]. One critical factor for this metabolic system in macrophages, and in immune cells in general, is glucose uptake mediated by the membrane transporter *Glut1* (*slc2a1*) and subsequent phosphorylation by carbohydrate kinases (Fig. 2). Even though macrophages have been suggested



**Fig. 1.** Single-cell analysis of plaque immune cell activation markers. Single-Cell RNA-Seq of aortic CD45+ cells from *Ldlr*<sup>-/-</sup> mice fed a HFD for (left) 11 weeks or (right) 12 weeks. Data from (left) Kim et al. (GSM3215435) and (right) Cochain et al. (GSE97310) were analyzed using the Single-Cell Explorer software (Artyomov lab). Expression of M1 and M2 markers in both datasets is shown in tSne plots. Markers used in Kim et al.: CD206+ Macrophages: *Fcgr1*, *Itgam*, *Mafb*, *Mrc1*. Inflammatory macrophages: *Fcgr1*, *Itgam*, *Mafb*, *NLRP3*, *IL1b*, *Nfkbia*. Lyve1+ macrophages: *Fcgr1*, *Itgam*, *Mafb*, *Lyve1*. TREM2high macrophages: *Fcgr1*, *Itgam*, *Mafb*, *TREM2*, *ABCG1*, *Lpl*, *Lipa*. CD206+ Inflammatory macrophages: *Fcgr1*, *Itgam*, *Mafb*, *Mrc1*, *NLRP3*, *IL1b* (low), *Nfkbia*, *TNF*. CD8 T cells: *Lck*, *CD3*, *CD8*. cDC2: *Zbtb46*, *Itgax*, *Flt3*, *Itgam* (+), *Itgae* (-). cDC1: *Zbtb46*, *Itgax*, *Flt3*, *Itgam* (-), *Itgae*, *IRF8*. Non leukocyte: *Ptprc* (-). Markers used in Cochain et al.: Macrophages (mixed subsets): *Itgam*, *Fcgr1*, *MerTK*. Mixed NK and CD8 T cells: *CD3*, *CD8*, *Klr1c*, *Ncr1*, *Gzmb*. Dendritic cells (mixed subsets): *Itgax*, *Ciita*, *Zbtb46*. Monocytes: *Itgam*, *Fcgr1*, *Ly6C*, *CCR2*. B cells: *Ciita*, *CD19*, *CD79α/β*. Mixed T cells: *Lck*, *CD3*, *CD4*, *CD8*, *Rag1*. Immature T cells: *Lck* (-), *CD3*(+), *Rag1* (-), *CD4* (-), *CD8* (-). Mast cells: *Furin*, *Il1rl1*. CD8 T cells: *Lck*, *CD3*, *CD8*.

to express several Glut-family members, the functional significance of Glut1-mediated glucose uptake was illustrated by the selective Glut1 ablation leading to compromised glucose entry [33]. Under inflammatory conditions, more particularly in a context of atherosclerosis, macrophages display increased glucose metabolism and Glut1 expression [34,35]. Macrophage-specific Glut1-deficiency greatly affected glycolysis and the PPP, resulting in decreased metabolite content [33]. Interestingly, Glut1-deficiency also led to an increase in the level of some of the metabolites in the aforementioned pathways, including 2- and 3-phosphoglycerate, when compared to control cells [33]. This observation suggests that compensatory pathways were able to restore, at least partially, the absence of glucose entry in macrophages and generate metabolic blocks in glycolysis and the PPP independently of extracellular glucose. However, Glut1-macrophage deletion translated into defective efferocytosis and increased plaque necrotic core area [33, 36]. Whether myeloid-cell specific Glut1 deletion also affects monocyte plaque recruitment and local proliferation, on top of efferocytosis, remains to be documented. It is currently unknown if CCR2 expression, the key chemokine receptor allowing for monocyte recruitment into the growing plaque, is modulated by glucose metabolism. This information will provide a better understating on how Glut1-mediated glucose flux

contributes or prevents disease progression. Glucose metabolism was further demonstrated to increase bone marrow hematopoiesis and monocyte generation, thus leading to augmented blood monocyte counts [37–39]. Monocytosis, high circulating monocyte numbers, is an independent risk factor during atherosclerosis development and managing normal blood monocyte levels is a therapeutic avenue. However, interfering with cellular glucose metabolism of macrophages may also adversely alter efferocytosis efficiency and necrotic core development.

Another key aspect of glucose utilization for macrophage functions is the balance of glycolysis and the PPP, which appears to adapt in the course of immune activation to sustain specific metabolic demands of the cells in a timely manner [40]. These two pathways are highly interconnected by sharing crucial intermediates and their interfaces appear as highly regulated in macrophages. M1 activated macrophages specifically express isoform 3 of 6-phosphofructo-2-kinase B (PFKFB3) resulting in increased glycolytic flux [41]. Deficiency of PFKFB3 reduces glycolysis and shifts glucose utilization towards the PPP. Regulations of enzyme activities forming the oxidative branch of the PPP are critical for inflammatory activation of macrophages, as independently shown for G6PD or for PDG during hypercholesterolemia [42]. Glyceraldehyde-3-phosphate (G3P) is a key intermediate of glucose

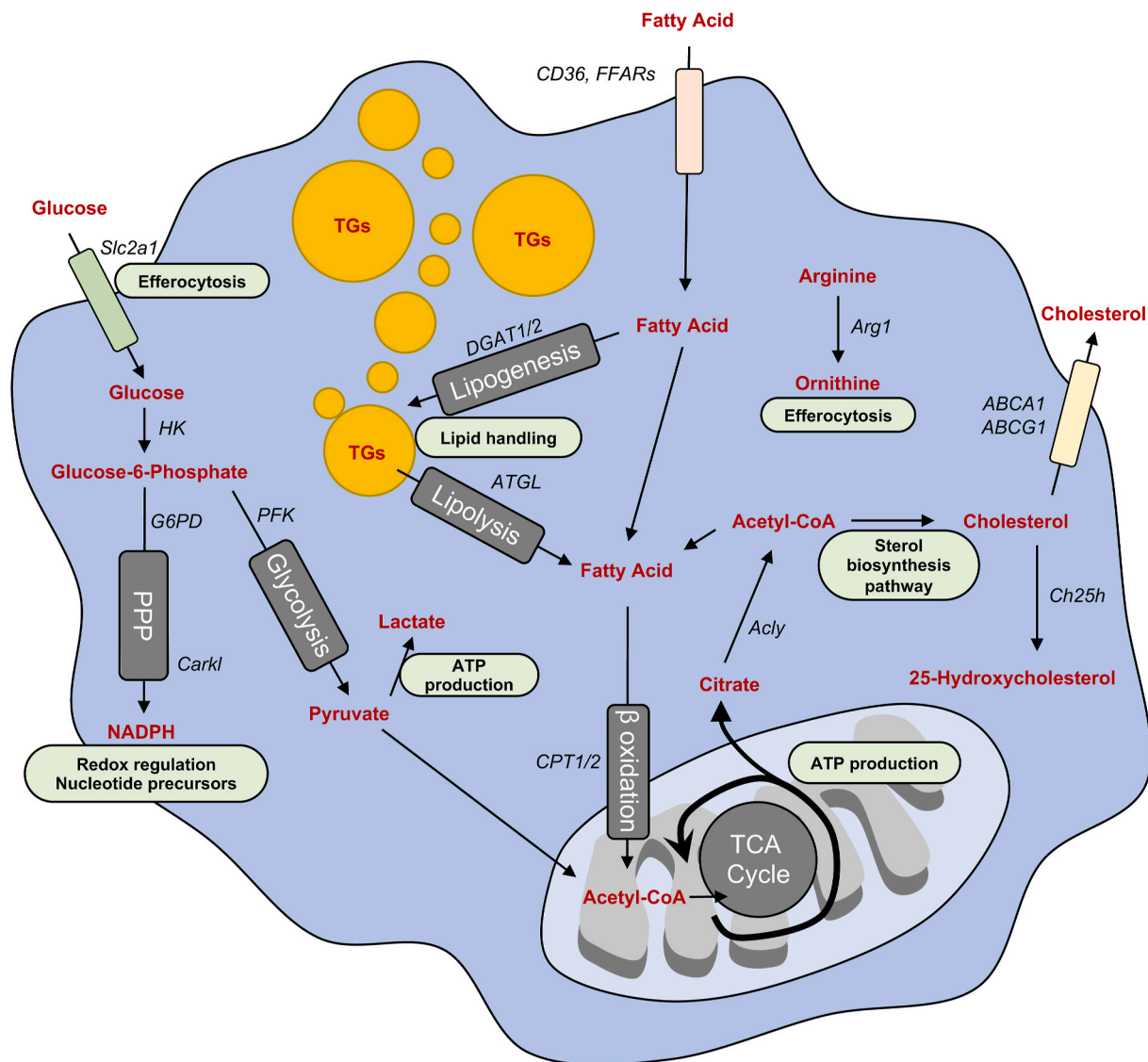


Fig. 2. Schematic representation of macrophage metabolic pathways discussed in the manuscript.

metabolism and is formed in glycolysis and the non-oxidative PPP. Both pathways seem to share this molecule to allow reflux and cycling of carbohydrates within these pathways. This mechanism allows cells to selectively set metabolism to respective needs, which may differ between macrophages or monocytes undergoing oxidative bursts, proliferation or even both at the same time. In M1-like activated macrophages expression of *Car1* (now known as *Shpk*), a heptose-specific kinase of the PPP forming sedoheptulose-7-P (S7P), is reprimanded [30]. *CARKL*-derived S7P acts as co-substrate to incorporate glycolytic-derived G3P into the PPP and its bioavailability appears rate-limiting for this process. *Car1* regulation shapes macrophage activation-specific effector functions and sets cellular redox-states [30]. An independent study reported increased S7P levels as metabolic feature of efferocytotic macrophages [43]. Taken together these observations suggest that fine-tuning of glucose flux into glycolysis and the PPP is a tightly regulated process and linked to specific macrophage phenotypes. Thus, considering approaches to modulate glucose intracellular utilization in macrophages could provide an efficient way to influence macrophage inflammatory status and the subsequent cytokine release. High Fat Diet (HFD) feeding deflected glucose flux into the PPP in macrophages, and this might be part of the metabolic reprogramming in plaque [42]. To validate whether this process occurs *in vivo* during

plaque development will require further investigation.

Whether monocytes, pro-inflammatory macrophages and foamy cells within the atherosclerotic plaque rely on specific and exclusive glucose-dependent pathways remains to be established. It will be of importance to determine whether metabolic rewiring of macrophage glucose metabolism occurs at a specific stage of disease progression, in a specific localization, and in a specific cell type. The emergence of new single-cell technologies providing the unique opportunity to define the metabolic configuration of cells in their native environment and locally available metabolites, could offer solutions to solve the aforementioned questions [44].

#### 1.4. Lipids, lipogenesis, lipolysis and lipid oxidation

Recent reports showed that pathways involved in lipid synthesis are modulated in classically activated macrophages. For instance, LPS-stimulated macrophages display an increased phosphorylation of ATP citrate lyase (*Acly*), the enzyme that converts TCA-derived citrate into acetyl-CoA and oxaloacetate (Fig. 2) [45]. Acetyl-CoA can in turn favor cholesterol and lipid biosynthesis. *Acly*-deficient macrophages showed increased expression of genes involved in fatty acid and cholesterol biosynthesis. However, this did not translate into intracellular lipid

accumulation, suggesting that Acyl-dependent lipid synthesis is not involved in lipid droplet formation [45]. Acyl-deficient macrophages showed an increased inflammatory response to LPS and impaired alternative polarization in response to IL-4. Surprisingly, transplantation of *Lyz2<sup>cre</sup> x Acyl<sup>fl/fl</sup>* bone marrow into *Ldlr<sup>-/-</sup>* mice did not affect plaque size, but rather promoted a more stable, collagenous phenotype [45]. Another recent report demonstrated that *in vitro* macrophage stimulation with LPS and IFN $\gamma$  favors lipid droplet formation [46]. Triglyceride (TG) generation in macrophages relies on two key enzymes that catalyze the addition of a fatty acyl chain to diacylglycerol. These two enzymes are named acyl-CoA diacylglycerol acyltransferases 1 and 2 (DGAT1 and DGAT2) (Fig. 2). LPS and IFN $\gamma$  treatment increased the expression of DGAT1 and 2 in macrophages and this was paralleled by increased intracellular lipid content [46]. In agreement with a role for these enzymes as drivers of lipid droplet formation, the genetic and pharmacological DGAT inhibition decreased macrophage lipid content [46]. This is consistent with the observation that plaque lipid-laden cells express CD11c. Nevertheless, this inhibition led to decreased production of pro-inflammatory cytokines (IL-1 $\beta$  and IL-6). Thus, one could speculate that the generation of foamy cells in the plaque is a biphasic process where the early accumulation of TGs is associated with a pro-inflammatory program which is later replaced by anti-inflammatory signature in “terminally-differentiated” foamy cells. Such mechanism could resemble CD8<sup>+</sup> T cells exhaustion, a process where cells lose effector ability including cytokine secretion, during persistent high antigen stimulation. Interestingly, the observed metabolic dysfunction precedes the development of exhaustion itself and does not simply reflect secondary changes in metabolism. Exhaustion is suggestive of glucose deprivation, with suppression of both glycolysis and OXPHOS and altered levels of the glycolytic metabolite phosphoenolpyruvate in exhausted cells that contribute to effector dysfunction [47]. It will be interesting to follow metabolic perturbations in lipid-accumulating macrophages over time to investigate whether similar mechanisms are at play.

Several studies demonstrated that alternatively activated (M2) macrophages exhibit a preference towards fatty acid oxidation metabolism [48]. Nevertheless, the causal link between lipid oxidation and alternative activation was challenged by a recent study using Cpt2-deficient macrophages [49]. Cpt2 is a mitochondrial enzyme involved in fatty acid mitochondrial metabolism. Cpt2-deficient macrophages have compromised fatty acid oxidation, but their IL-4 response is intact [49]. Early reports suggested that Cpt1 pharmacological inhibition with Etomoxir, using a high inhibitor concentration, prevents alternative macrophage activation [48,50]. However, lowering Etomoxir concentration demonstrated no effect on the expression of canonical M2 markers upon IL-4 stimulation while preserving Cpt1 inhibition [51–53]. The use of genetic models allowing for macrophage-selective Cpt1 and Cpt2 ablation (*Lyz2<sup>cre</sup> x Cpt1<sup>fl/fl</sup>* and *Lyz2<sup>cre</sup> x Cpt2<sup>fl/fl</sup>* mice) revealed that high Etomoxir concentration decrease macrophage M2-polarization independently from long chain fatty acid mitochondrial entry and oxidation [51]. Thus, how precisely fatty acids modulate macrophage activation state is still to be elucidated. Whether Cpt1 and Cpt2 play a role in plaque macrophage and blood monocyte metabolism and activation is yet to be established. Modulating macrophage lipid metabolism could have an impact on plaque myeloid cell content and therefore on its stability. A recent study, using a Bodipy staining, allowed to separately analyze plaque foamy macrophages [10]. The authors were able to define a clear transcriptomic signature associated with foamy cells. Those cells were enriched for genes encoding for proteins involved in lipid metabolism including *Lipa*, *Fabp4*, *Fabp5*, *CD36* and *Abca1* among others [10]. The relative contributions of these genes to atherosclerosis development were investigated in previous studies [54–60], but how precisely they affect foamy macrophages formation remains to be defined. Bodipy staining revealed that foamy cells were present in plaques after 4 weeks of Western Diet in both *Ldlr<sup>-/-</sup>* and *ApoE<sup>-/-</sup>* mice [10]. What precisely leads to foamy cells

formation and expansion remains to be determined. Surprisingly, non-foamy macrophages displayed a pro-inflammatory phenotype in comparison to foamy cells [10]. Thus, one might expect that newly recruited plaque monocytes and monocyte-derived macrophages adopt a pro-inflammatory phenotype and that they contribute to inflammation amplification by secreting inflammatory cytokines and chemokines. A recent study investigating monocyte and macrophage dynamics in plaque development, demonstrated that monocytes progress in deeper plaque layers during atherosclerosis progression [2]. Over a long period of time, these cells can uptake locally available lipids and might become foamy cells. During this process their inflammatory phenotype progressively diminishes. The critical checkpoints leading to foamy macrophages establishment are yet to be validated.

### 1.5. Prostaglandin and sterol metabolism

In addition to cytokines like IL-4 or IL-13, metabolites also have been shown to induce alternative activation. This is the case of inflammation-resolving lipid mediators such as prostaglandin E2 (PGE2). PGE2 independently induced a fraction of the IL-4 induced transcripts in macrophages, and broadly modified the effects of IL-4. This subtle PGE2 effect was recently demonstrated to play a crucial role in macrophage alternative polarization. This reprogramming was associated with a profound effect on alternatively activated macrophage phenotype [61]. On its own, PGE2 treatment enhanced the expression of canonical M2 markers (CD301, Arg1). The precise function of CD301 (encoded by *clec10a*) during atherosclerosis development remains to be established and recent data suggested that CD301 could be involved in macrophage fusion and the formation of multinucleated cells [62]. Arginase 1 (Arg1) converts arginine to ornithine. A recent study investigated the role of hematopoietic cell Arg1 (*Tie2<sup>cre</sup> x Arg1<sup>flox/flox</sup>*) during atherosclerosis progression [62]. *Tie2<sup>cre</sup> x Arg1<sup>flox/flox</sup>* mice had similar plaque size in comparison to control animals. However, *Arg1*-deletion in macrophages favored foamy cell generation [62]. Additionally, arginine promotes continual efferocytosis and this mechanism is crucial to eliminate apoptotic cells from the plaque [63]. PGE2 further potentiated the expression of these markers in the presence of IL-4, but also dampened the IL-4-driven activation of mitochondrial metabolism, without affecting lactate production [61]. These results suggest that surface markers alone cannot predict the cellular metabolic state. PGE2 treatment also inhibited macrophage proliferation, one of the mechanisms leading to plaque development [61]. Production of PGE2 was shown to occur in LPS-treated BMDMs [64], and plaque macrophages selectively express its receptor EP4. In plaque, PGE2 is mainly synthesized by endothelial and smooth muscle cells [65]. Whether locally produced PGE2 plays a role in macrophage metabolic reprogramming during atherosclerosis remains to be defined.

Further distancing from the classical/non classical bipolar activation state is the case of the integrin CD11c in atherosclerotic plaques. CD11c expression appears higher in the population of Trem2<sup>hi</sup> foamy cells (Fig. 1). This observation is puzzling because plaque foamy cells have an anti-inflammatory phenotype, the contrary of *in vitro* induced classically activated macrophages that display high CD11c expression. A previous study demonstrated that foamy cells are characterized by an anti-inflammatory gene signature [66]. Using peritoneal foamy cells, the authors demonstrated that these cells have decreased mRNA expression of *il1 $\beta$* , *cxcl9* and *cxcl10* [66]. The anti-inflammatory effects were mediated by desmosterol accumulation. Indeed, desmosterol was the most abundant sterol detected in foamy peritoneal macrophages [66]. The accumulation of lanosterol, the first sterol in the cholesterol biosynthetic pathway, also triggers anti-inflammatory response in macrophages [67]. Whether the aforementioned mechanisms contribute to the formation of anti-inflammatory foamy cells in plaque remains to be demonstrated. 25-hydroxycholesterol also plays an anti-inflammatory role as demonstrated using genetic ablation of Ch25h, the enzyme converting cholesterol to 25-hydroxycholesterol

(Fig. 2) [68]. 25-hydroxycholesterol addition to peritoneal macrophages isolated from Ch25h-deficient mice decreased their production of pro-inflammatory cytokines (IL-6, IL-1 $\beta$ , TNF $\alpha$ ) while promoting anti-inflammatory cytokine generation (IL-10). Ch25h<sup>-/-</sup>ApoE<sup>-/-</sup> mice developed increased atherosclerotic lesions compared to Ch25h<sup>+/+</sup>ApoE<sup>-/-</sup> control animals, further validating the anti-atherogenic role of 25-hydroxycholesterol [68]. In peritoneal and bone marrow-derived macrophages, TLR activation induced Ch25h mRNA expression and subsequent 25-hydroxycholesterol synthesis [69–71]. The transcription factor ATF3 was demonstrated to regulate Ch25h expression in macrophages [72]. Of interest, ATF3<sup>-/-</sup>ApoE<sup>-/-</sup> mice developed increased plaque lesions and accumulated 25-hydroxycholesterol in the aorta in comparison to control animals [72]. Interferons (IFN), and in particular type I interferons (IFN $\alpha$  and IFN $\beta$ ), are involved in macrophage 25-hydroxycholesterol production [71].

### 1.6. Intra-plaque localization drives immune cells metabolic configuration?

The precise localization of macrophage subsets in nascent and advanced lesions remains to be fully defined. A diversity in macrophage plaque subsets was also documented in humans [73]. It was documented that plaque shoulders contain mainly a population of pro-inflammatory M1-like macrophages [74]. These regions are typically prone to rupture. Additionally, the presence of CD68<sup>+</sup>MR<sup>-</sup> (mannose receptor), M1-like macrophages, was also detected mainly in lipid-rich areas of human carotid lesions [75]. The presence of M2-like macrophages in human carotid plaques, co-expressing CD68 and MR, was initially reported in 2007 [76]. Adventitial macrophages displayed a prominent M2-like phenotype [74]. A comparative analysis of macrophage content and diversity in progressive disease stages revealed that both M1- and M2-like macrophages accumulate in plaque during atherosclerosis progression [74]. A comparison of macrophage content and activation state in asymptomatic and symptomatic carotid plaques revealed that CD11c-expressing M1-like macrophages were enriched in symptomatic tissues [77]. Inversely, CD163<sup>+</sup> M2-like macrophages were more frequent in asymptomatic plaques [77]. Indeed, M1 polarization was associated with instable plaques [78]. Most of the current studies were performed on carotid samples. Comparative analysis of macrophage content and phenotype among vessels isolated from different parts of the body is limited. Plaques in femoral arteries contained a greater proportion of M2-like macrophages when compared to plaques in carotids [79]. M1-like cells were more abundant in carotid plaques in comparison to femoral lesions [79]. The metabolic configuration of these plaque resident macrophage populations is yet to be defined. To determine plaque macrophage subsets metabolism using standard technologies like the Seahorse Analyzer, one would first need to digest the tissue and sort these cells for further analysis. This experiment requires large number of cells and seems challenging to perform due to the low relative frequency and increasing diversity of macrophages residing in advanced plaques notwithstanding that incubating tissue-derived cells with culture media and inhibitors will most likely perturb their metabolic profile beforehand. Alternative strategies to assess metabolic states of cells have now been described that could cope with these limitations [44]. Recently, mass cytometry (CyTOF) and scRNAseq analysis of human atherosclerotic plaques confirmed the presence of M1- and M2-like macrophages [80]. These state-of-the-art technologies further identified the co-existence of multiple macrophage subsets in the plaque [80,81]. Importantly, the relative abundance of these macrophage subsets differed in symptomatic and asymptomatic plaques [80]. The tissue distribution, metabolic profile and contribution to disease progression of the different macrophage subpopulations remains to be established. A metabolic imaging approach based on enzyme histochemistry was developed to assess metabolic configuration of single cells within their specific tissue microenvironments by profiling multiple key enzyme activities of cellular metabolism [82].

Defining the metabolic configuration of nascent and advanced plaques could help to understand whether a specific macrophage activation status could be linked or due to a particular plaque localization and metabolite access. One could expect that deeper plaque zones might have limited access to bloodborne nutrients. Therefore, cells residing in deeper plaque layers would need to rewire their metabolic configuration to adapt to their local microenvironment and ensure their survival or proliferation. For instance, it has been shown that deeper plaque regions, enriched in macrophage content, are characterized by a hypoxia and this leads to Hif1a stabilization in macrophages and the activation of hypoxia response element (HRE) [83]. Hif1a stabilization leads to glycolysis induction in macrophages through the increase of the expression of Glut-1, VEGF secretion and a well-defined metabolic configuration [83]. Low oxygen concentration in deep layer of the plaque stabilizes the transcription factor HIF-1 which promote a glycolytic metabolism and therefore favor macrophage efferocytosis. This observation suggests that glucose promotes efficient apoptotic cells removal from the plaque and that lowering systemic glucose level might dampen macrophage efferocytosis [33,36]. VEGF production leads to the generation of blood vessels locally. This neovascularization would likely play a role in providing local metabolites. The presence of Hif1 protein was identified in atherosclerotic plaque [84]. Hypoxia triggered cholesterol synthesis and blunted cholesterol efflux leading to its accumulation in macrophages [84]. One could expect that hypoxia might trigger foamy cell formation in the plaque and modulate their inflammatory phenotype. Thus, having more information about the metabolic milieu in different areas of the plaque could allow us to consider different strategy to modify local immune cell metabolism and prevent plaque destabilization in these hypoxic areas (Fig. 2).

## 2. Conclusions

Understanding the metabolic reprogramming of immune cells in nascent and advanced plaque might help to better apprehend the mechanisms leading to plaque progression. We are still in early stages and the information of the local metabolite distribution in plaques is still incomplete. Glucose, fatty acid and amino acid metabolism have been shown to modulate immune cell functions and disease development. However, how precisely these pathways are interconnected and how they influence the recruitment, survival and function of the multiple immune cell populations residing in plaque needs to be established. Combining the cartography of immune cell metabolite content, together with single-cell enzyme activity profiling *in situ* and *ex-vivo* metabolic profiling using SCENITH would offer some powerful new tools to investigate the regulation of immune cell metabolism within the plaque. Recently, single-cell metabolic regulome profiling (scMEP) was published and this technology offers the opportunity to associate a particular metabolic configuration with a cell type [85]. A better knowledge of the metabolic regulation of monocytes and macrophages will most certainly pave the way to new and more subtle treatments for cardiometabolic diseases.

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### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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