Clinical and Experimental Immunology, 2022, **208**, 181–192
https://doi.org/10.1093/cei/uxab033
Advance access publication 14 December 2021
Review

Review

The role of lipid metabolism in shaping the expansion and the function of regulatory T cells

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Summary

Metabolic inflammation, defined as a chronic low-grade inflammation, is implicated in numerous metabolic diseases. In recent years, the role of regulatory T cells (Tregs) as key controllers of metabolic inflammation has emerged, but our comprehension on how different metabolic pathways influence Treg functions needs a deeper understanding. Here we focus on how circulating and intracellular lipid metabolism, in particular cholesterol metabolism, regulates Treg homeostasis, expansion, and functions. Cholesterol is carried through the bloodstream by circulating lipoproteins (chylomicrons, very low-density lipoproteins, low-density lipoproteins). Tregs are equipped with a wide array of metabolic sensors able to perceive and respond to changes in the lipid environment through the activation of different intracellular pathways thus conferring to these cells a crucial metabolic and functional plasticity. Nevertheless, altered cholesterol transport, as observed in genetic dyslipidemias and atherosclerosis, impairs Treg proliferation and function through defective cellular metabolism. The intracellular pathway devoted to the cholesterol synthesis is the mevalonate pathway and several studies have shown that this pathway is essential for Treg stability and suppressive activity. High cholesterol concentrations in the extracellular environment may induce massive accumulation of cholesterol inside the cell thus impairing nutrients sensors and inhibiting the mevalonate pathway. This review summarizes the current knowledge regarding the role of circulating and cellular cholesterol metabolism in the regulation of Treg metabolism and functions. In particular, we will discuss how different pathological conditions affecting cholesterol transport may affect cellular metabolism in Tregs.

Keywords: Treg, lipoproteins, cholesterol, atherosclerosis, dyslipidemia

Abbreviations: AMPK: AMP-activated protein kinase; FOXP3: Forkhead Box P3; FH: familial hypercholesterolemia; FHBL: familial hypobetalipoproteinemia; HDL: high-density lipoprotein; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; LCFA: long chain fatty acids; LDL: low-density lipoprotein; LDLR: LDL receptor; LKB1: liver kinase B1; mTOR: mammalian target of rapamycin; SREBP: sterol regulatory element-binding protein; Tconv: conventional T cell; Treg: regulatory T cell; TCR: T-cell receptor; VAT: visceral adipose tissue; VLDL: very low-density lipoprotein.

Introduction

Tregs as key controllers of metabolic inflammation

Metabolic inflammation (metainflammation) is defined as a chronic low-grade inflammatory state, established by inflammatory cells, and induced by alterations in metabolism [1]. Metainflammation has been observed in obese individuals without infections and is associated with the development of type 2 diabetes (T2D) [2], nonalcoholic fatty liver disease (NAFLD) [2], and atherosclerosis [3].

A deep connection exists between metabolic disorders and the immune cell metabolism. Indeed, during metabolic diseases, all the pathways sensing nutrients such as glucose, amino acids, and lipids are commonly deregulated [4]. Recently, immunologists have started considering the importance of metabolism in T-cell homeostasis and functions, thanks to a considerable number of findings in the area of immunometabolism [5].

Based on these considerations, here we are going to focus on how whole-body and cellular cholesterol metabolism influences regulatory T cells (Tregs), a specialized $CD4^+$ T-cell subset necessary for the maintenance of self-tolerance, immune homeostasis, and repair functions [6–8]. Tregs are characterized by the expression of the transcription factor Forkhead Box P3 (FOXP3) that is recognized as the master gene for their development and function [9–11]. Murine models with congenital Treg deficiency develop a lethal autoimmunity phenotype [12]. Recently, Tregs have been described as the major sensor of the whole-body metabolic status due to their high sensitivity to external nutrient composition [13]. Also, increasing evidence emphasizes the role of Tregs in conditions of metabolic inflammation such as atherosclerosis, which

Received 31 October 2021; Revised 5 December 2021; Accepted for publication 10 December 2021

might be considered as a chronic inflammatory disease of the arterial walls characterized by the accumulation of cholesterol and the infiltration of immunocompetent cells [14]. It is well known that cholesterol contributes to the development of atherosclerosis because it accumulates in the intima and undergoes oxidative modification activating endothelial, smooth muscle and immune cells [15]. Both Treg number and activity are impaired in atherosclerosis thus potentially contributing to development of systemic inflammation [16–18]. To this regard, obesity, namely visceral obesity, represents a good example on how the derangement of lipid metabolism may promote metainflammation. Indeed, it has been reported that the frequency of Tregs decreases dramatically in proportion with increasing obesity in adipose tissue in both murine models and human subjects [19]. Mice fed with high-fat diet (HFD) present a drastic reduction of visceral adipose tissue (VAT) Tregs, which also associates with an inflammatory state [19]. In line with data from murine models, the Treg number in VAT and peripheral blood decreases in obese diabetic adults and children [20, 21]. In addition, obese patients with insulin resistance display reduced Treg number in their VAT as compared with lean controls, and Treg transfer can improve insulin resistance [22].

Lipoproteins and immunity

Circulating lipoproteins and the immune system

Cholesterol is a structural molecule essential for all cellular membranes, making its biosynthesis and regulatory pathways ubiquitous across cell types, including immune cells [23]. Cholesterol is carried through the bloodstream by complex structures called lipoproteins. Different lipoproteins can carry cholesterol and triglycerides either to the peripheral organs through chylomicrons, very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs) or inversely, from peripheral organs to the liver (mainly through high-density lipoproteins, HDLs) [24].

Chylomicrons

Chylomicrons are the largest circulating lipoproteins, involved in the transport of dietary triglycerides and cholesterol from the intestine to peripheral tissues and liver. The structural component of these particles is the apolipoprotein B-48 and other functional apolipoproteins of the apoA-I, apoA-II, apoA-IV, apoA-V, apoC-II, apoC-III, and apoE series [24]. Once secreted, the half-life of chylomicrons is very short (around 5 h) [24] and they reach systemic circulation through lymphatics, thus bypassing the liver and facilitating nutrient delivery to the skeletal muscle and adipose tissue [25]. The triglycerides content of chylomicrons is hydrolyzed through the activity of lipoprotein lipase (LPL) allowing the delivery of free fatty acids to muscle and adipose tissue. LPL, an enzyme mainly expressed in the skeletal muscle and adipose tissue, is regulated by both apolipoproteins carried by the chylomicrons and circulating factors such as angiopoietin-like proteins (ANGPTL3, ANGPTL4, and ANGPTL8) [26]. As a result of chylomicron delipidation, a new particle called a chylomicron remnant is formed. Chylomicron remnants are finally cleared by the liver, where they deliver cholesterol and triglycerides [25].

Though short-lived, chylomicrons show several interactions with the immune system. De Sanctis *et al.* found that interleukin-2-activated T lymphocytes and NK cells are both able to internalize chylomicrons and this increases their proliferative response [27]. In addition, human macrophages produce the LPL protein thus potentially contributing to chylomicron clearance [28]. Interestingly, LPL secretion shows a steep reduction when human macrophages are stimulated *in vitro* with interferon (IFN) gamma, suggesting that, during inflammation processes, chylomicron clearance is impaired thus favoring dyslipidemia and atherosclerosis [28].

Very low-density lipoproteins

VLDL particles are produced by the liver and are rich in triglycerides. They contain apoB-100, apoC-I, apoC-II, apoC-III, and apoE. Apolipoprotein B-100 is the structural core protein. It contains several highly hydrophobic areas that serve as strong lipid-binding domains and are necessary for the assembly and secretion of VLDLs. After removal of their triglyceride content by LPL activity, expressed in the skeletal muscle and adipose tissue, VLDL remnants (called IDLs) are further metabolized to LDLs [24, 29].

Like chylomicrons, VLDLs can be internalized by activated lymphocytes and NK cells with a proliferative response [24, 27]. The greatest impact in the immune system is shown by the VLDL-derived LDLs.

Low-density lipoproteins

LDL particles are the main cholesterol carriers in the blood. They are constituted by a single apoB-100 molecule folded around a hydrophobic core and surrounded by a single layer of phospholipids. Seventy percent of circulating LDLs are cleared by the liver, while the remaining part is cleared by peripheral organs through the specific LDL receptor (LDLR). Plasma concentration of LDL is highly dependent on LDLR activity: low LDLR activity corresponds to a longer LDL particle half-life and higher plasma levels [30]. Normal concentrations of plasma LDLs range from 2.6 to 3.4 mmol/l, and elevated concentrations of LDL are tightly associated with atherosclerotic cardiovascular disease. Conversely, the reduction of LDL levels are directly proportional to reduction of cardiovascular risk, which becomes minimal when LDL levels are below 1.4 mmol/l [31]. Cells of the immune system also express the LDLR and internalize cholesterol for metabolic purposes. T cells in particular are the greatest LDL consumers: being cholesterol crucial for T-cell activation and survival [32]. The internalization and catabolism of LDL-derived cholesterol in these cells was found to be three times faster than in B lymphocytes [33].

The LDL particles become immunogenic particles when oxidized (oxLDL), thus linking cholesterol transport and accumulation to metabolic inflammation. Interestingly, it has been recently described how LDL particles, differing in size and lipid composition, show a different susceptibility to oxidation and aggregation [34]. The pro-inflammatory mechanism of oxLDLs on endothelium is still not clear [35]. One hypothesis is that they may be recognized as damage-associated molecular patterns (DAMPs) by the Toll-like receptor 4 (TLR4) and may activate the nuclear factor-κB (NF-κB) pathway [24]. Then, the inducible nitric oxide synthase (iNOS) is hyperactivated while the endothelial nitric oxide synthase (eNOS) is downregulated, thus producing an imbalance in NO levels [36]. The subsequent increase in cellular oxidative stress activates the NLR family pyrin domain containing 3 (NLRP3) inflammasome [37].

Low-grade chronic vascular inflammation, determined by oxLDL accumulation in endothelial and subendothelial space, causes vascular dysfunction that manifests through increased endothelial permeability and increased leukocyte adhesion, chemokine secretion, and immune cell trafficking [38]. Both innate and adaptive immune cells, particularly dendritic cells (DCs), macrophages, and T cells, dominate the plaque environment throughout its evolution [39]. Newton *et al.* described how oxLDL may also influence T-cell differentiation into a pro-inflammatory Th1 phenotype, enhancing vascular inflammation and damage progression in the atheroma microenvironment [40].

Increasing evidence in mice and humans correlates the exposure to high levels of LDL with impaired Treg frequency, function, stability, and migration, and such alterations are thought to contribute to the progression of the inflammatory state in the arterial wall (Fig. 1). In two different mouse models, Treg number was shown to be reduced in the atherosclerotic plaque. Mor *et al.* have demonstrated that apolipoprotein E-deficient (*ApoE–/–*) mice had a lower number of Tregs compared to wild-type littermates [41]; LDLR null (*Ldlr–/–*) mice, fed an atherogenic diet, showed a drastic reduction of Tregs at 8 and 20 weeks of age, concomitant to the rise in the number of CD4 effector cells [42]. In humans, Tregs are present at lower proportion in the atherosclerotic plaques at all developmental phases [16]. In contrast to these pieces of evidence, some studies have shown that, in mice, dietary hypercholesterolemia rather enhances Treg frequency *in vivo*, promoting their expansion in thymus and peripheral lymphoid organs, and inducing *de novo* differentiation in the liver [43, 44]. Mechanistically, cholesterol may stabilize lipids raft integrity facilitating T-cell receptor (TCR) dimerization and activation [43, 45], and it is well known that TCR homeostatic signaling is essential for Treg development in the thymus and in the periphery [46]. Such hepatically differentiated Tregs can be efficiently recruited into the aorta [44]; however, they may be exposed to local signals undermining their suppressive function and/or stability. Indeed, also a reduced Treg function appears to be linked to the atherogenic process [42]. Tregs may exert their atheroprotective properties by suppressing inflammation through the secretion of high levels of interleukin-10 (IL-10) and the transforming growth factor-β (TGF-β) [47]. IL-10 synthesis can inhibit inflammatory cytokine production by macrophages in the atherosclerotic plaque [48], whereas IL-10-deficient mice show increased effector T-cell infiltration and disease exacerbation [49]. Regarding Treg stability, Butcher *et al.* have demonstrated that the atherosclerotic plaque of *ApoE–/–* deficient mice stimulates Treg plasticity, determining the development of a dysfunctional subset of IFN-γ-producing Tregs, called Th1-like Tregs, which express low levels of co-inhibitory molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA 4) and the T-cell immunoreceptor with Ig and ITIM domains (TIGIT) [50].

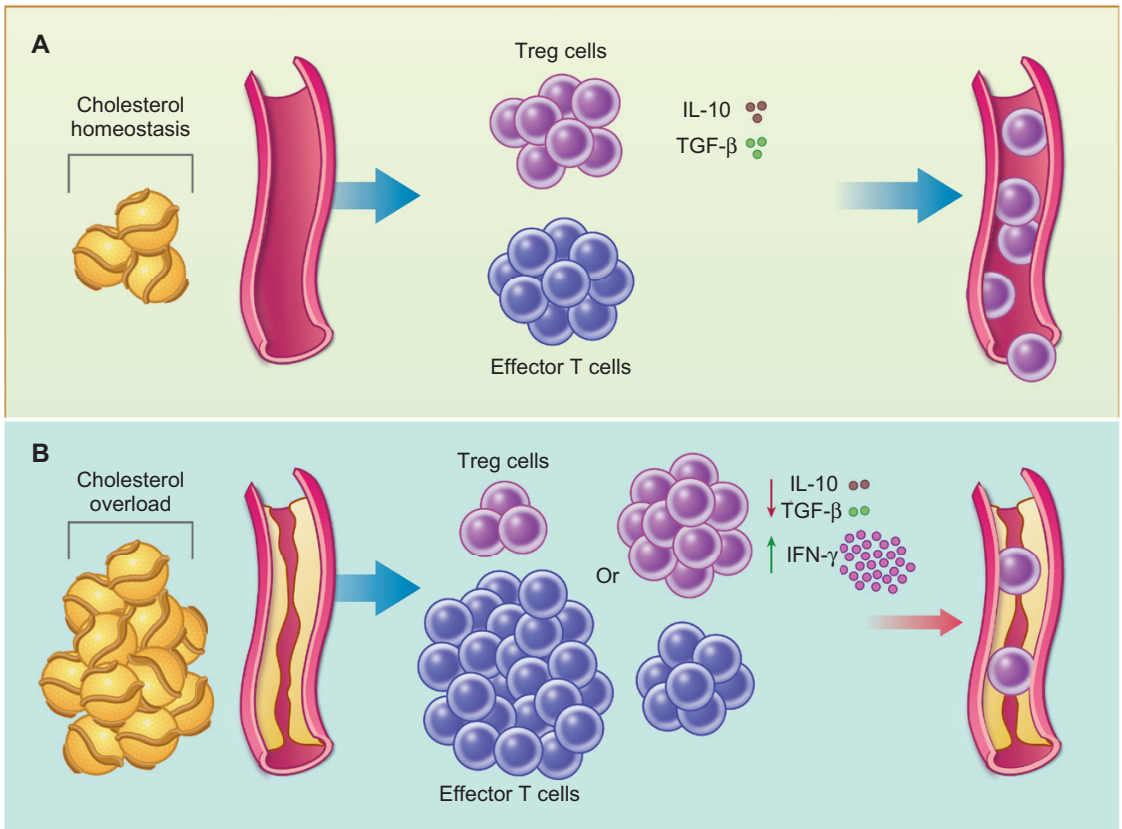

Fig. 1 Tregs are metabolic sensors and systemic cholesterol levels strongly influence Treg features. (A) In a condition of cholesterol homeostasis Tregs are proliferative, secrete cytokines, and maintain the capacity to migrate to the endothelium. (B) Pathological cholesterol overload, such as in atherosclerosis, has been shown to affect Treg frequency and function in several fashions: reduced or increased percentages, reduced ability to secrete IL-10 and TGF-β, higher differentiation into Th1-like pro-inflammatory cells, or reduced expression of selectin ligands impairing Treg capacity to migrate to the atherosclerotic plaque.

The impact of hypercholesterolemia on Treg ability to migrate into the plaque is controversial. Maganto-García *et al.* have showed a reduced migratory capacity of Tregs under prolonged hypercholesterolemia, due to a lower expression of selectin ligands [42]. Conversely, Amersfoort *et al.* have demonstrated that dyslipidemia induced a series of metabolic changes in Tregs that rather increased their capacity to migrate in the atherosclerotic plaque, suggesting that dyslipidemia-induced alterations are not due to impaired migratory capacity but to reduced suppressive function of Tregs [51].

High-density lipoproteins

HDL particles are enriched in cholesterol and phospholipids. The ability of HDL to stimulate efflux of cholesterol from peripheral tissues, to transport it into the plasma, to favor its uptake by the liver and the excretion into the bile is, comprehensively, termed reverse cholesterol transport (RCT), which is considered one of potential mechanisms by which HDLs may be anti-atherogenic. In addition, HDL particles have antioxidant, anti-inflammatory, anti-thrombotic, and anti-apoptotic properties, which may also contribute to their ability to reduce atherosclerosis progression. Apolipoproteins A-I, A-II, A-IV, C-I, C-II, C-III, and E are associated with these particles. ApoA-I is a major component of HDL responsible for both structure and function of this lipoprotein [24]. ApoA-I is mainly synthesized by the liver and the intestine. The main molecules involved in the efflux of cholesterol from macrophages are adenosine triphosphate (ATP)-binding cassette transporter gene A1 (*ABCA1*) and ATP-binding cassette transporter gene G1 (*ABCG1*). ABCA1 promotes efflux of cholesterol and phospholipids onto lipid-poor apoA-1. ABCG1 promotes cholesterol efflux from macrophage foam cells into HDL particles but the activity of ABCG1 does not influence overall HDL levels [52]. The HDL particle also binds to the scavenger receptor class B type 1 (SR-BI), through which the cholesterol contained in the HDL particle is transported into the liver without internalization [53].

Similarly to LDLs, also HDLs show a wide range of interactions with the immune system, and most of them tend to favor an anti-inflammatory environment. Human lymphocytes are known to express the SR-BI receptor [54], which is overexpressed during clonal expansion and cell growth [55]. In a murine model, intact HDLs as well as apoA1 were found to impair antigen presentation from antigen presenting cells to T cells and thus to reduce T-cell activation, hence attenuating the pro-inflammatory reaction [56]; HDLs were also found to inhibit the interaction between activated T cells and monocytes, thus hampering the pro-inflammatory signaling carried by TNF, IL-6, IL-8, CCL3, and CCL4 [57].

Some recent evidence in mice and humans has highlighted the crucial role of HDL particles in promoting Treg survival and proliferation [58, 59]. Tregs are able to internalize HDL particles through the SR-BI/II receptor more than conventional CD4 T cells; HDL internalization increases survival through enhancement of mitochondria activity and beta-oxidation [59]; hence, the anti-inflammatory effect associated with high levels of circulating HDLs could be mediated by enhanced Treg survival.

Genetic dyslipidemias and the immune system

Genetic dyslipidemias are heritable diseases of lipid metabolism. They include at least 25 different monogenic diseases caused by mutations in 23 candidate genes following patterns of dominant, co-dominant, or recessive inheritance [60]. Familial hypercholesterolemia (FH) is the most common genetic hypercholesterolemia showing a co-dominant pattern of inheritance. The rarest form is homozygous familial hypercholesterolemia (HoFH) (OMIM #143890) affecting 1 over 300 000 individuals in the general population [61]. Typically, it is caused by biallelic mutations in the gene coding for the LDLR. Less frequently, the HoFH phenotype is caused by biallelic mutations in other two genes that also regulate plasma clearance of LDL: the *APOB* gene and the *PCSK9* gene [62]. In addition, HoFH may also show a recessive pattern of inheritance, the so-called autosomal recessive hypercholesterolemia (ARH), which is due to mutations in the adaptor protein, LDLRAP1 [63, 64]. The heterozygous form of familial hypercholesterolemia (HeFH) is far more common, affecting 1:250–1:300 individuals. These patients have typically increased levels of plasma cholesterol (with typical LDL cholesterol concentration > 190 mg/dl or 4.9 mmol/l). Mild hypercholesterolemic patients in the absence of a monogenic condition might suffer from polygenic hypercholesterolemia, caused by the accumulation of deleterious single-nucleotide polymorphisms (SNPs) in genes involved in cholesterol metabolism [65, 66].

Among dyslipidemias, low-cholesterol syndromes, such as the familial type 1 (FHBL1) and type 2 (FHBL2) hypobetalipoproteinemias, are far less common [67]. These conditions are characterized by a substantial reduction of both LDL and serum triglycerides. FHBL1 patients carry loss-of-function mutations in the *APOB* gene or in the microsomal triglyceride transfer protein (*MTP*) gene. Therefore, the intestine and the liver are unable to produce chylomicrons or VLDLs, respectively, resulting in lipid malabsorption, steatorrhea, and high-grade liver steatosis and fibrosis [68]. Individuals affected by FHBL2 have a loss-of-function mutation in the *ANGPTL3* gene, determinant of the hypobetalipoproteinemia phenotype and responsible for a relevant reduction in the cardiovascular risk of these individuals [69, 70].

Although cholesterol and serum lipoproteins interact widely with the immune system, little is known about the relationship between dyslipidemias and the immune system, being most of the published studies limited to FH patients. Moreover, the knowledge about the impact of rarer dyslipidemic conditions are almost completely lacking. A summary of published evidence concerning the immune system activity in genetic dyslipidemias are reported in Table 1.

The immune system in FH

As already mentioned, FH is characterized by markedly increased concentrations of LDL particles, which are able to interact with T cells and monocytes [70]. Fadini *et al.* found that hypercholesterolemic patients show an increased proportion of pro-inflammatory monocytes expressing CD68 and CCR2 markers, and their number was directly correlated with LDL-c levels [81]. A similar immune phenotype was found in children suffering from FH. In this case, the imbalance in the monocyte phenotype and monocytosis was modulated by the HDL-c levels. Indeed, the shift toward pro-inflammatory monocytes was only present in children having HDL-c < 1.3 mmol/l (or 50 mg/dl). Composition of lymphocyte populations was comparable, except for a trend toward increased

Table 1. Immune system alterations in genetic dyslipidemias

Lipid profile alteration	Disease	Gene(s)	Effect on immune system	Ref.
↑ LDL-c	Familial hypercholesterolemia	*LDLR* *PCSK9* *LDLRAP1* *APOB*	↑ M1/M2 ratio Th1-like inflammation[a] ↑ Treg number[a] ↓ Treg suppressive function[a]	[71, 72]
	Sitosterolaemia	*ABCG5* *ABCG8*	↑ Sitosterol accumulation in macrophages, macrophage death, and vascular inflammation[b]	[73, 74]
	Lysosomal-acid lipase deficiency	*LIPA*	Impaired T-cell maturation and function ↑ regulatory T-cell ratio ↑ IL-2, IFN-γ, IL-4[b]	[75]
↓ LDL-c	Abetalipoproteinemia	*APOB*	Impaired function of CD1-mediated antigen presentation[b]	[76]
	Familial hypobetalipoproteinemia type 1	*MTTP*		
	Familial hypobetalipoproteinemia type 2	*ANGPTL3*	Reduced population of hematopoietic stem cells in the bone marrow[b]	[76]
↑ Chilomicrons	Familial chylomicronemia syndrome	*LPL* *APOC2* *APOC5* *LMF1* *GPIHBP1*	Reduced myelopoiesis and adipose macrophage lipid content[b]	[77, 78]
↑ HDL-c	Cholesteril ester transfer protein deficiency	*CETP*	No effect on macrophage phenotype or infiltration into the plaque[b]	[79]
↓ HDL-c	Tangier disease	*ABCA1*	Increased cholesterol content in myeloid cells, activation of NLRP3 inflammasome, and increased IL-1 and IL-18 levels[b]	[80]
	Lecitin:cholesteril ester acil transferase	*LCAT*	NA	

NA, literature not available.
[a]Human studies.
[b]Animal model studies.

CD4 T lymphocytes in FH [82]. Narverud *et al.* have recently published an mRNA profiling of peripheral mononuclear cells from FH children. Interestingly, the authors found that, among the 587 genes screened, 176 were found to be significantly deregulated, in particular those involved in T- and B-cell activation and IFN signaling. The vast majority of the deregulated genes were associated with the plasma concentration of total and LDL cholesterol as well as with oxLDL. Moreover, statin therapy was found to revert the deregulation of 13 of these genes [71].

Finally, Bonacina *et al.* have recently demonstrated that FH patients show increased frequencies but decreased suppressive function of circulating Tregs [72]. Moreover, they proved that, in a murine model of hypercholesterolemia (*Ldlr–/–*), the adoptive transfer of Tregs, engineered to selectively migrate to the atherosclerotic plaque, reduces atherosclerosis progression and plaque inflammation in the aorta. This study demonstrates that induction of tolerance in the plaque microenvironment reduces its progression despite the high levels of plasma LDLs, highlighting the importance of the immune function in the progression of atherosclerosis [72].

Cellular metabolism of cholesterol in T cells

Cholesterol metabolism in T cells

In recent years, an increasing number of findings have highlighted the link between intracellular metabolism and immune cell function and dynamics. This intricate connection is known as 'immunometabolism' [5]. However, why T cells adopt specific metabolic programs is still unclear. Tregs can be considered a peculiar T-cell subset that is highly sensitive to the microenvironment, thanks to the wide array of immune and metabolic sensors, which allow Tregs to adapt to perturbations and external cues.

Different stages of T-cell activation can be characterized by different metabolic states. Activated T cells are highly glycolytic, and the mammalian target of rapamycin (mTOR) plays an important role in supporting the glycolytic–lipogenic metabolism [83]. The PI3K/AKT/mTOR axis is the major sensor of nutrient availability and has the critical role of promoting T-cell differentiation, homeostasis, and effector functions inducing the switch from quiescence to a metabolically active state. In conditions of poor nutrient availability, AMP-activated protein kinase (AMPK) is activated and inhibits mTOR and, thereby, a variety of biosynthetic pathways of fatty acid and cholesterol synthesis [84].

Our understanding of how cholesterol metabolism within immune cells is connected to the immune response is just beginning to emerge. Cholesterol and its derivatives are crucial to maintain plasmatic membrane fluidity, lipid raft formation, and downstream TCR signaling [45, 85]. The metabolic pathway responsible for the intracellular cholesterol availability is the mevalonate pathway, which is essential for multiple cellular processes. The third step of the mevalonate

pathway, catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), an endoplasmic reticulum resident enzyme, is generally regarded as the rate-limiting step in cholesterol synthesis [86]. This enzyme catalyzes HMG-CoA conversion to mevalonate and is inhibited by statins such as lovastatin, mevastatin, pravastatin, simvastatin, or synthetic statins [87].

The mevalonate pathway, in addition to cholesterol, generates the isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) [88]. These are the substrates of posttranslational lipid modification of cellular proteins, named prenylation, mediated by the farnesyltransferase (FT) and geranyl-geranyltransferase type 1 (GGTI). Prenylation is a lipid modification involving the covalent addition of farnesyl or, more commonly, geranyl to prenylated proteins, which plays crucial roles in cellular processes such as protein–protein interactions, intracellular trafficking, and signal transduction [89].

A tight link exists between intracellular lipid metabolism and T-cell activation, which requires a transcriptional reprogramming [90]. During T-cell activation, liver X receptors (LXRs), transcriptional factors that control different target genes involved in the efflux, transport, and excretion of cholesterol [91, 92], are downregulated, providing a proliferative advantage to T cells [90]. Concomitantly to the downregulation of LXRs, sterol regulatory element-binding proteins (SREBPs), a class of transcriptional factors involved in T-cell activation and cholesterol homeostasis [93], are upregulated [90]. SREBPs act as sensors of cholesterol content and, upon TCR stimulation, promote cholesterol biosynthesis by inducing the gene encoding for HMGCR [93]. T-cell activation is sustained not only by cholesterol, but also by other metabolites produced by the mevalonate pathway, such as FPP and GGPP. Indeed, these metabolites represent the units that are attached to the members of Ras superfamily of guanosine triphosphatases (GTPases) to mediate their membrane localization upon TCR stimulation [94]. Hence, prenylated proteins have a pivotal role in T-cell proliferation and differentiation [95].

Treg metabolism

As we have already discussed in detail in our recent review [96], Treg metabolism is highly dynamic, and its plasticity is the consequence of different origin (thymic or peripheral), type, developmental stage, and/or activity of Tregs [97]. Moreover, Tregs can display different features depending on the context in which they are studied. *In vitro*, Tregs are anergic, fail to proliferate upon TCR ligation alone, and their hyporesponsiveness can be reverted in presence of TCR ligation and high doses of interleukin-2 (IL-2) [98, 99]. Conversely, *in vivo* Tregs are highly proliferative relying on glycolysis–lipogenesis pathway [100].

Immune and metabolic signaling intersect at key molecular hubs, such as the mTOR complex [101]. Chronic overactivation or mTOR downregulation impair Treg stability and proliferation, while an oscillatory mode of mTOR activity supports Treg fitness [102, 103]. Treg expansion is also influenced by another metabolic sensor, the liver kinase B1 (LKB1). Indeed, the Treg-specific deletion of LKB1 results in reduced Treg number and function due to a defective mitochondrial metabolism [104]. Nevertheless, the downstream kinase of LKB1, AMPK, does not have a critical role for Treg proliferation and homeostasis, and mice with AMPK-deficient Tregs show no signs of spontaneous autoimmunity [105].

Whether an improved metabolic fitness of Tregs corresponds to higher suppressive function is still matter of debate. Some data indicate that glycolysis can impair Treg suppressive function *in vivo*. In mice overexpressing the main glucose transporter GLUT1, Tregs display an increased mTOR-dependent glycolysis and high frequency but reduced suppressive activity [106]. Conversely, other data show that the mTOR-driven glycolytic–lipogenic pathway supports Treg suppressive function by promoting the expression of IFN regulatory factor 4 (IRF4) and mitochondrial metabolism [107].

At the steady state, it has been reported that Foxp3 drives a metabolic program based on fatty acid oxidation and oxidative phosphorylation (OxPhos). Indeed, Tregs have a greater capacity to uptake long chain fatty acids (LCFA) than conventional T cells (Tconvs) and their ability to oxidize fatty acids allows them avoiding lipotoxicity [108]. In this setting, Foxp3 upregulates the genes encoding for electron transport complexes and confers survival advantage to Tregs in presence of high levels of LCFA [108]. Another mechanism that Tregs exploit to tolerate high fatty acid concentration is the conversion of fatty acids into triglycerides and their storage in lipid droplets (LD). Foxp3 promotes the conversion of toxic fatty acids in triglycerides via the upregulation of the two enzymes needed for triglyceride synthesis, namely DGAT1 and DGAT2, resulting in a high content of LD, which are used to fuel mitochondrial OxPhos [109]. Fatty acids can be actively imported by Tregs from lipid-rich microenvironment. For instance, intra-tumoral Tregs enhance lipid uptake through the upregulation of the transporter CD36. The internalized lipids fuel oxidative metabolism and boost Treg stability and suppressive function by modulating mitochondrial fitness [110].

As mentioned above, Tregs are highly flexible; they adjust their metabolism in response to the environment and the stage of activity: while Treg quiescence is sustained by Foxp3 that promotes lipid-fueled OxPhos [108], Treg replication *in vivo* is supported by glycolysis–lipogenesis that is triggered by mTOR and SREBP activation and increases biomass production [100, 111, 112]. Nevertheless, Foxp3 expression is maintained during proliferation despite the mTOR-induced glycolysis activation. In addition, glycolysis is engaged when Tconvs are polarized to induced Tregs through the induction of specific FOXP3 splicing variants [113]. These findings summarize how several metabolic pathways fit together to allow Tregs adapting to different nutritional and immunological signals.

Increasing evidence highlights the role of cholesterol metabolism in Treg functions (Fig. 2). Zeng *et al.* have shown that the mevalonate pathway is the most affected pathway in a mouse model of Treg-specific mTOR deletion, and this determines altered proliferation, suppressive function, and expression of effector molecules such as CTLA-4 and ICOS, resulting in a scurfy-like autoimmune disease [112]. The key role of the mevalonate pathway in Treg homeostasis is demonstrated by the observation of a scurfy-like disease in mice bearing a Treg-restricted deletion of the HMGCR gene [114].

The upregulation of the mevalonate pathway, which is required for Treg suppressive function and stability, can be controlled by the activity of LKB1 [105, 115]. LKB1-deficient Tregs exhibit impaired suppressive activity that could be reconstituted by the addition of mevalonate. In these cells, the

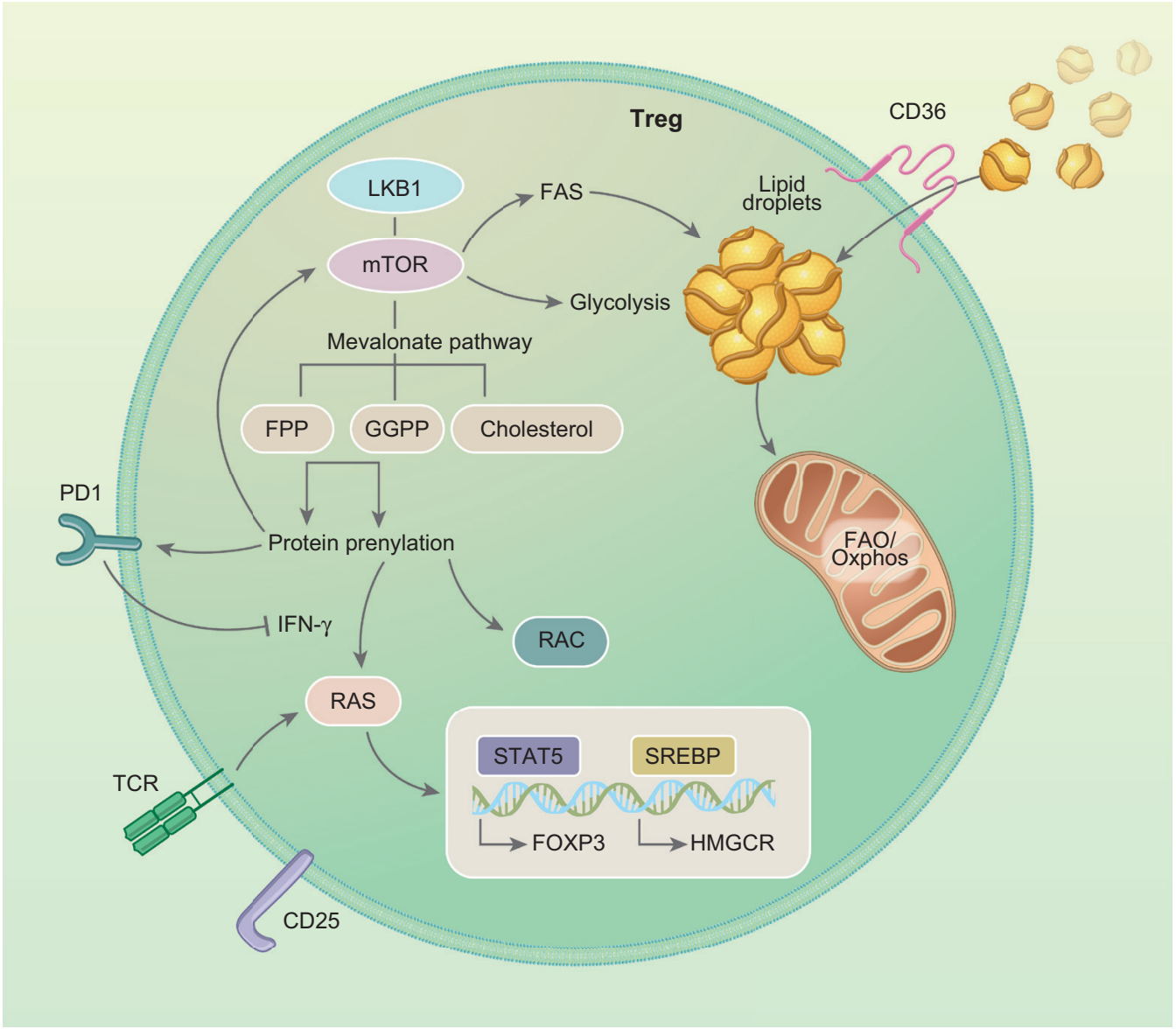

Fig. 2 Treg stability and functions heavily rely on lipid metabolism. Mevalonate pathway alteration due to the lack of LKB1-mediated signaling results in the decrease of RAC geranylation and mTOR farnesylation determining the impairment of effector Treg maintenance and differentiation, respectively. Ras geranylation sustains TCR signaling and Treg stability through STAT5 phosphorylation that induces FOXP3 expression. Moreover, in tumor, geranylation supports PD1 expression via a SREBP-dependent activation of the mevalonate pathway, preventing IFN-γ production by Tregs and sustaining their suppressive capacity. Lipids derived from fatty acid synthesis and CD36-mediated uptake can be stored as lipid droplets in the cytoplasm or can be used as substrates for fatty acid oxidation to fuel mitochondrial OxPhos. FAO, fatty acid oxidation; FAS, fatty acid synthesis; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

downregulation of the mevalonate pathway promotes the conversion to Th1- and Th17-like effector cells and, in support of this, purified Tregs from wild-type mice treated with statins gain the ability to produce IFN-γ [115].

Statins influence several Treg features beyond stability, as highlighted in several studies. In a standard *in vitro* suppression assay, in the presence of simvastatin, murine Tregs lost their ability to suppress, to proliferate, and to upregulate effector molecules such as CTLA4 and ICOS [112]. Furthermore, Treg proliferation and activation was impaired also *in vivo* in mice daily treated with simvastatin [112]. Opposing data are available regarding the effects of statins on human Tregs. In patients with acute coronary syndrome [116] or rheumatoid arthritis [117], statin treatment was shown to increase Treg numbers, an event that likely contributed to symptoms amelioration. In line with these findings, atorvastatin supplementation increased Treg survival and function in a culture of human mononuclear cells *in vitro* [118, 119]. It could be proposed that, in human chronic metainflammatory diseases, Treg expansion may be not directly related to statin exposure, but indirectly dependent on the improvement of the inflammatory status in treated patients. However, in contrast to this idea, some data show that statins increase Treg frequency also in healthy donors [120]. Interestingly, Tregs positively correlated with HDL in statin-treated donors [120], and HDL has been shown to support Treg survival [58, 59]: these data suggest that, also in the absence of an inflammatory disease, statins may promote Treg expansion indirectly, through the

modulation of circulating lipoproteins. It may thus be difficult to discriminate whether the mevalonate pathway has a direct role in Treg expansion, and a preferential role in Tregs compared to Tconvs, based on observations in statin-treated patients.

Intracellular cholesterol homeostasis must be finely balanced, and this equilibrium is regulated through the influx and efflux transport mechanisms, mediated by the scavenger receptor CD36 and the transporters ABCA1 and ABCG1 [121, 122]. LKB1 deficiency in Tregs leads to an abnormal accumulation of intracellular cholesterol due to the increased expression of CD36 and the decrease of ABC transporters, resulting in downregulation of mevalonate pathway and consequently a decrease in the levels of isoprenoids GGPP and FPP [115].

Recent data have highlighted the importance of protein prenylation in Treg fitness. Geranylgeranylation and farnesylation dictate the differentiation and the maintenance of effector Tregs through RAC geranylation and mTORC1 farnesylation, respectively [123]. These processes become particularly relevant in the context of cancer, where Tregs proliferate and acquire an effector phenotype; indeed, geranylation supports PD1 expression and represses IFN-γ production by intra-tumoral Tregs through a SREBP-dependent induction of the mevalonate pathway, while SREBP signaling is dispensable for Treg homeostasis, lipid uptake, and mitochondrial fitness under steady state [111]. It is well known that SREBP induces the mevalonate pathway [124], and indeed HMGCR-deficient Tregs showed defective PD1 expression upon TCR stimulation, which can be restored by treatment with mevalonate or with GGPP. Thus, the axis of SREBP, mevalonate pathway, and PD1 geranylation sustains Treg stability and suppressive function and prevents Treg fragility in the tumor microenvironment [111]. The pivotal role of isoprenoids in Treg fitness has been highlighted also in a mouse model of colitis, where GGPP was shown to enhance Treg generation *in vitro* and *in vivo* through the amplification of IL-2/STAT5 signaling. Mechanistically, prenylation activates Ras, which phosphorylates ERK1/2 and this results in IL-2 production by T cells and STAT5 phosphorylation, leading to increased Treg numbers [125].

Some genetic evidence linking the mevalonate pathways and susceptibility to autoimmune and autoinflammatory diseases supports the notion that this metabolic route may play a pivotal role in Treg fitness. An SNP in the gene encoding for LKB1 has been recognized as a predisposing factor for the development of multiple sclerosis in a small human study [126]. The mevalonate kinase deficiency (MKD) encompasses a spectrum of genetic autoinflammatory disorders caused by mutations in the *MVK* gene that encodes for the mevalonate kinase. This disease can be characterized by hepatosplenomegaly, lymphadenopathy, skin rash, mucosal ulcer, abdominal pain, and severe fever attacks, as a consequence of the huge production of pro-inflammatory cytokines including IL-1β [127]. Mechanistically, it is thought that isoprenoid reduction may increase inflammasome activation and IL-1β release [128]. In line with this view, Politiek *et al.* have suggested that altered protein prenylation can be the pathogenic mechanism behind the MKD [129]. However, it could be also speculated that Treg-intrinsic defects in mevalonate pathway and protein prenylation may contribute to the severe inflammatory manifestations in these patients.

Several genetic defects that compromise mTOR activity have been linked to autoimmune manifestations, which are in some cases associated to reduced Treg frequency/function [130]. The genetic deficiency of lipopolysaccharide-responsive beige-like anchor protein (LRBA), which is involved in vesicle trafficking and mTOR activation, leads to a primary immune deficiency characterized by immune dysregulation and Treg defects [131]. Also mutations affecting the CARD11–BCL10–MALT1 signalosome complex, which is required for optimal mTOR activation, are associated to reduced Treg frequencies in humans, even though this does not induce spontaneous autoimmune manifestations [132].

Connections between microenvironmental and cellular metabolism in Tregs

As highlighted above, Treg development, maintenance, and functions depend on the complex interaction with different microenvironmental and systemic cues, as a systemic metabolic alteration can negatively affect Treg features and functions [133]. A perfect example of the connection between the microenvironmental and the cellular metabolism in Tregs is represented by the metabolic syndrome (MetS), defined as the co-occurrence of certain conditions as hyperglycemia, insulin resistance, atherogenic dyslipidemia, and obesity, that contribute to the development of cardiovascular disease and T2D [134]. Obesity is the major hallmark of MetS. The first evidence connecting Treg fitness and metabolism to adipose tissue dysfunctions came from the study of Feuerer *et al.*, who examined adipose tissue-resident Tregs in lean and obese mice: Tregs preferentially accumulated in healthy VAT of lean mice, thanks to the Treg response to local antigens and to the presence of specific cytokines and adipokines promoting Treg migration and survival; conversely, Tregs frequency was dramatically reduced in VAT of obese mice [19]. The peculiar program and activity of VAT Tregs are dependent on PPAR-γ, a nuclear factor related to lipid rearrangement in adipocytes [135]. Interestingly, PPAR-γ agonism induces VAT Treg expansion in obese mice, and this event is associated to a higher accumulation of lipids in VAT Tregs [135]. In humans, the scenario is more complicated, as some studies have shown increased Treg percentages or higher FOXP3 mRNA levels in the VAT of obese compared to lean individuals [22, 136–138]. A functional assessment of these cells would help understanding whether they display qualitative defects, in their suppressive ability and/or stability, in that microenvironment.

The reduction of Treg frequency is a hallmark of obesity and T2D, and these conditions are characterized by the establishment of a chronic low-grade inflammation with high concentrations of leptin and adipocytokines. Leptin is an adipokine principally produced by adipose tissue, which controls food intake, systemic metabolism, and Treg functions [139, 140]. Human and murine Tregs produce leptin and express the leptin receptor, and leptin neutralization with a monoclonal antibody reverses human Treg anergic state *in vitro*; *in vivo*, the genetic leptin deficiency determines a higher Treg frequency compared to wild-type mice [141]. Mechanistically, obesity increases the concentration of circulating leptin leading to mTOR upregulation that, in turn, affects Treg proliferation [142]. Opposite to obesity, starvation induces a systemic leptin reduction [143], results in decreased mTOR activity, and thus promotes Treg expansion [102]. Therefore, the systemic metabolic pressure, defined as a condition of prolonged nutritional overload that determines an elevated body mass index and overproduction of adipocytokines, can be linked to the

development of autoimmune disorders in industrialized countries, possibly through a chronic limitation of Treg fitness and functions. Indeed, some studies have revealed positive associations between elevated body mass index and the development and/or progression of several autoimmune diseases [144–146].

Dietary fluctuations, which occur during a balanced diet, are associated with the oscillatory mode of mTOR activity, which is essential to ensure Treg homeostasis and proliferation [102]. During nutritional overload and obesity, chronic mTOR activation due to the constantly high level of calories and nutrients results in the impairment of Treg number and functionality, determining the accumulation of autoreactive T cells that leads to the development of autoimmunity and chronic inflammation. In support of this, an elevated body mass index is a fundamental predisposing factor for the development of type 1 diabetes and multiple sclerosis in youth. In the latter case, it has been demonstrated that, in multiple sclerosis patients, impaired Treg proliferation is associated with the activation of mTOR [147].

Conclusions

We have probably only started understanding the multiple interactions between immunity and lipid metabolism. The available evidence points to a role for nutrients in shaping the metabolism of immune cells not only by directly fueling certain metabolic routes, but also acting as signaling molecules that promote a coordinated molecular, metabolic, and functional reprogramming. The latter activity is guaranteed by the presence of molecular hubs working as metabolic rheostats, such as mTOR and SREBP [101], and of cellular platforms that are exceptionally sensitive to external variations, such as the Tregs [13]. A deeper understanding of these events may come from the study of genetic dyslipidemias characterized by extreme phenotypes and may pave the way for the development of novel immunotherapeutic approaches that integrate metabolic interventions.

Acknowledgements

Not applicable.

Funding

This work was funded by Sapienza Università di Roma - Progetti di Ricerca di Ateneo (2020 RP120172A7CBD322 to S.P. and 2019 RG11916B50C85210 to M.A.); Associazione Italiana per la Ricerca sul Cancro (IG-2017 19784 to S.P.); Ministry of Education, University and Research (MIUR) [Progetti di Ricerca di Interesse Nazionale (PRIN) Grant 2017 Prot. 2017K7FSYB to S.P.]; Italian Ministry of Health (GR-2019-12368551 to S.P.).

Conflict of interest

The authors declare no conflict of interests.

Author contributions

S.P. defined the structure and content of the manuscript and supervised the manuscript drafting. A.P.G. and S.B. wrote the first draft. A.P.G. prepared the figures. S.B. prepared the table. I.P., A.R., A.D.C., I.M., L.D., S.P., and M.A. revised the manuscript providing relevant conceptual contribution. All authors read and approved the final paper.

Data availability

This manuscript does not contain original data.

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