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REVIEW ARTICLE

Metabolism and TAM functions—it takes two to tango

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From the evidence on clinical studies and experimental mouse models we now know that tumor-associated macrophages (TAMs) sustain tumor development in many different ways. They play a role in angiogenesis, tumor cell invasion, and metastasis formation. Additionally, TAMs interfere with natural killer and T-cell antitumoral activities, producing an immune-suppressive environment that protects tumor cell growth. This indicates that the tumoricidal activity of macrophages within the tumor microenviroment is lost due to an imbalance of the regulatory mechanisms underpinning these cells' function. Since metabolism is emerging as a major modulator of macrophage function, metabolic changes in response to signals coming from cancer or other immune cells might promote this imbalance, enhancing the tumorigenic activities of TAMs. In this review we describe the novel, most recent findings on how metabolism shapes TAM functions or conversely, how TAMs influence the activity of other cells through metabolic mechanisms. The complete elucidation of the metabolic switches between pro- and antitumoral properties of macrophages, now still in its infancy, is destined to provide scientists with new instruments not only to understand but also to combat cancer.

Introduction

In cancer, tumor-associated macrophages (TAMs) support different functions including the control of adaptive immunity and immune escape, regulation of vessel and matrix remodeling, promotion of cancer cell proliferation, survival, and metastasis [1,2]. In many cancer types, TAMs can represent up to 40% of the cellular content of the tumor mass. Both cancer cells and tumor microenvironmental signals hijack TAMs to sustain cancer growth. Thus, TAM-depleting strategies have been evaluated in preclinical and clinical studies, however, with not always concordant results [3–5]. This is probably due to the fact that, in principle, macrophages are supposed to protect the body against harmful agents. With this respect, a more convincing idea is to re-educate TAMs toward support their antitumor, immunostimulatory functions [1,2,5–9]. To this purpose, it is important to dissect which pathways underline the phenotypic switch in TAMs in order to be able to therapeutically target protumoral differentiation states and eventually revert their response toward their original function, namely their immune protection against nonself or foreign agents such as cancer cells. Emerging evidence suggests but does not always prove that these diverse functions are sustained by distinct metabolic programs. In general, metabolic signals and the prototypical polarizing signals such as, for instance lipopolysaccharide (LPS)/interferon-γ (IFN-γ) leading to classically activated M1 (antitumor-like macrophages), or IL-4 and IL-10 leading to alternatively activated M2 (protumor-like macrophages), are responsible for metabolic shifts that underline specific functional properties in macrophages [10–15]. However, only recently we are learning how these metabolic shifts are affecting TAM behavior and thus impact on disease outcome [16,17].

By focusing on some metabolic rheostats and a short list of defined metabolites, here we present examples of the evidence on how TAM metabolism shapes different functions in the context of tumor growth, angiogenesis, metastasis, and immune cross-talk.

Mammalian target of rapamycin (mTOR) signaling

The mTOR signaling pathway represents the central modulator of cell metabolism, growth, proliferation and survival as a result of a perfect integration of intracellular and extracellular signals. Crucial cellular processes such as insulin resistance, adipogenesis, immune cell activation and tumor development, and angiogenesis, require an active mTOR pathway. All these observations, together with the evidence that this pathway is deregulated in human diseases such as cancer and type 2 diabetes, have prompted scientists to not only investigate the mechanism of its activation but also to exploit it pharmacologically. mTOR

Abbreviations

2-OG, 2-oxoglutarate; 3-HAA, 3-hydroxyanthranilic acid; AC, adenylate cyclase; ACLY, ATP citrate lyase; AhR, aryl hydrocarbon receptor; AKT, serine/threonine-specific protein kinase; AMPc, cyclic adenosine monophosphate; AMPK, adenosine monophosphate-activated protein kinase; ARG1, arginase-1; C/EBP, CCAAT-enhancer-binding proteins; CCL13, chemokine (C-C motif) ligand 13; CCL17, chemokine (C-C motif) ligand 17; CCL22, chemokine (C-C motif) ligand 22; CD163, cluster of differentiation 163; CD206, cluster of differentiation 206; CD209, cluster of differentiation 209; CIC, citrate carrier; COX2, cyclooxygenase-2; CREB, cAMP response element-binding protein; CSF1R, colony-stimulating factor 1 receptor; CXCR4, C-X-C chemokine receptor type 4; EAAT, excitatory amino acid transporter; EP4, prostaglandin E2 receptor subtype EP4; ERK, extracellular signal-regulated kinase; FAO, fatty acid oxidation; FAS, fatty acid synthase; Foxp3, forkhead box P3; GABA, γ-aminobutyric acid; GCN2, general control nonderepressible 2; Gln, glutamine; GLS, glutaminase; Gpr132, G protein-coupled receptor 132; GS, glutamine synthetase; GSK, glycogensynthase kinase; HIF-α, hypoxia-inducible factor 1α; HSF1, heat shock transcription factor 1; IDO, indoleamine 2,3 dioxygenase; IFN-γ, interferon-γ; IL-4/10/13, interleuchin-4/10/13; iNOS, inducible nitric oxide synthase; IRF4, interferon regulatory factor 4; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; LLC, Lewis lung carcinomas; LOC, mitochondrial lactate oxidation complex; LPS, lipopolysaccharide; MBT-2, mouse bladder tumor line-2; M-CSF, macrophage colony-stimulating factor; MCT, monocarboxylate transporter; MEK, mitogen-activated protein kinase; MHC-II, major histocompatibility complex class II; MRC1, mannose receptor C-type 1; MSO, methionine sulfoximine; MSR1, macrophage scavenger receptor 1; mTOR, mammalian target of rapamycin; NAD, nicotinamide adenine dinucleotide; NFκB, nuclear factor Kappa light-chain enhancer of activated B cells; OAA, oxaloacetate; OXPHOS, oxidative phosphorylation; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PD-L1, programmed death ligand 1; PDPK1, 3-phosphoinositide-dependent protein kinase 1; PGE2, prostaglandin E2; PI3Ks, phosphatidylinositol 3-kinases; PKA, protein kinase A; PKC, phosphorylates protein kinase C; PKM2, pyruvate kinase isozymes M2; PPAR, peroxisome proliferator-activated receptor; PtdInsP3, phosphatidylinositol(3,4,5)trisphosphate; PTEN, phosphatase and tensin homolog; PUFAs, polyunsaturated fatty acids; qRT-PCR, quantitative real-time reverse transcription-PCR; RAF, rapidly accelerated fibrosarcoma; RAS, family of retrovirus-associated DNA sequences; RCC, renal cell carcinoma; REDD1, regulated in development and DNA damage response 1; rhGM-CSF, recombinant human granulocyte/macrophage colony-stimulating factor; SGK1, serum and glucocorticoid-regulated kinase 1; Sp1, specificity protein 1; TAMs, tumor-associated macrophages; TCA cycle, tricarboxylic acid cycle; TCR, T-cell receptor; T_{eff}, effector T cell; TGF-β, transforming growth factor-β; TLR, Toll-like receptor; TME, tumor microenvironment; T_{reg} cells, regulatory T cell; TSC1/2, tuberous sclerosis complex 1/2; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; VEGF, vascular endothelial growth factor.

inhibitors (rapamycin and its analogs) are currently used for the treatment of solid tumors, rheumatoid arthritis and during organ transplantation. The mTOR kinase plays an essential role in the regulation of cell growth and proliferation, since it can take part in two multiprotein complexes, mTOR complex 1 (mTORC1), which regulates protein synthesis, and mTOR complex 2 (mTORC2), which regulates cytoskeleton reorganization [18,19]. Growth factors, Toll-like receptor (TLR) ligands, or cytokines activate mTOR complex 1 (mTORC1) and mTORC2 through their cognate receptors. Receptor activation leads to the recruitment of class I phosphatidylinositol 3-kinases (PI3Ks) to the receptor complex by different adaptor molecules including the small GTPase RAB8A in macrophages [20]. PI3Ks recruit and activate the serine/threonine kinases AKT1, AKT2, and AKT3 via phosphorylation by 3-phosphoinositide-dependent protein kinase 1. This process is negatively regulated by phosphatase and tensin homolog, which dephosphorylates PtdInsP3 [21]. mTORC2 phosphorylates AKT, leading to its activation and shaping of its substrate specificity. In addition, mTORC2 phosphorylates protein kinase C and serum and glucocorticoid-regulated kinase 1 (SGK1) to control crucial cellular processes such as cytoskeletal dynamics [22]. Due to the presence of the regulatory-associated protein of mTOR (Raptor), the mTORC1 is inhibited by rapamycin. Furthermore, it is physiologically inhibited by the tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2) [18,19,23–25].

mTORC1 and inflammation

A strong body of evidence links mTORC1 to inflammation, since macrophages and other cells of the immune system regulate important modulators of inflammation such as nuclear factor kappa light-chain enhancer of activated B cells (NFκB) activity and IL-10, TGF-β, and PD-L1 expression [26] by means of mTORC1 activation. Recently, Covarrubias *et al.* [27] have shown that glucose metabolism is driven by signaling through AKT and mTORC1 to sustain IL-4-mediated M2 activation of macrophages. This suggests that alternative activation might be also mediated by the mTORC1, which could be context dependent [27], as suggested by the findings that loss of TSC1 allows enhanced M1 and diminished M2 activation [28]. TSC1 has been identified as a key modulator of macrophage polarization via mTOR-dependent and independent pathways. By exploiting mice models with myeloid-specific deletion of TSC1, Zhu *et al.* [29] show that TSC1 inhibits M1 polarization by suppressing the RAS GTPase-RAF1-MEK-ERK pathway in a mTOR-independent manner, whereas it promotes a M2-like phenotype through mTOR-dependent CCAAT/enhancer-binding protein β pathways. AKT kinases can also contribute to macrophage polarization in different ways. Blockade of AKT1 produces a M1-like phenotype, whereas blockade of AKT2 produces a M2-like phenotype. Arranz *et al.* [30] have shown that $Akt2^{-/-}$ mice were more resistant to LPS-induced endotoxin shock and to dextran sulfate sodium-induced colitis than wild-type mice, whereas $AKT1^{-/-}$ mice were more sensitive. In general, the PI3K/AKT/mTOR axis does not seem to convey a linear signal once activated as it probably integrates different stimuli from both intracellular and extracellular origin and it balances their effect to allow the cell to adapt to diverse conditions by promoting diverse basic biological processes.

mTORC2 and macrophages

At variance with mTORC1, mTORC2 is associated with the rapamycin-insensitive companion of mTOR (Rictor) and thus insensitive to rapamycin. The role of the mTORC2 in macrophages is highlighted by the findings that mTORC2 activation can be driven by macrophagic growth factors such as macrophage colony-stimulating factor (M-CSF), suggesting that mTORC2 plays key roles in the macrophage polarization and in the regulation of the inflammatory response [31,32]. Experimental evidence supports this hypothesis. Macrophages [33] and dendritic cells [34] increase their inflammatory response in the absence of the mTORC2. Murine macrophages lacking Rictor are polarized toward the M1-like phenotype [33]. However, the exact role of mTORC2 was not clearly elucidated.

Recently, Huang *et al.* [31] shed light into this mechanism, by demonstrating that increased glucose utilization is essential for IL-4-stimulated macrophages and this occurs through the activation of the mTORC2 pathway. M-CSF activates mTORC2 in a pathway that involved PI3K and AKT leading to induction of the transcription factor interferon regulatory factor 4 (IRF4). In turn, IRF4 increases glucose flux through glycolysis. In an *in vivo* parasitic nematode model, loss of mTORC2 in macrophages suppressed tumor growth and decreased immunity.

Role of mTOR in TAM functions

Few studies have highlighted the role of mTOR in TAM functions. Yang *et al.* [35] have demonstrated a

mechanism by which TAMs induce epithelial–mesenchymal transition and increase cancer stem cell-like populations via activation of AKT/mTOR signal, leading to enhanced renal carcinoma (RCC) cells invasion. The TSC2–mTOR pathway has been identified as a key modulator of the differentiation of monocytes into M2-like TAMs, with TSC2 ablation being able to increase IL-10 secretion and promote tumor angiogenesis in murine xenografts. This effect is reverted by rapamycin. Additionally, growth in murine xenografts were promoted or reduced by infusion of hosts with TSC2-deficient or TSC2-overexpressing monocytes, respectively [36]. PI3Kγ signaling through AKT and mTOR has been shown to promote immune suppression during inflammation and tumor development by a transcriptional rewiring leading to NFκB inhibition and C/EBPβ activation. Blockade of PI3Kγ restores the cytotoxic properties of $CD8^+$ activated T cell, identifying in PI3Kγ a modulator of tumor regression and increased survival in mouse models of cancer in synergy with immune checkpoint inhibitors to promote tumor regression and increased survival in mouse models of cancer [37].

We have recently shown that mTOR inhibition plays a crucial role in hypoxic TAMs by establishing a causative link between TAM metabolism and tumor vessel morphogenesis (Fig. 1) [16]. REDD1 (regulated in development and DNA damage response 1; otherwise known as RTP801 or DDIT4) is a mTOR complex1 (mTORC1) inhibitor [38], which is induced by a variety of other stress conditions, including endoplasmic reticular [39], oxidative [40], and osmotic stress [39], DNA-damaging agents [41], and cytokine stimulation such as IL-6 [42]. REDD1 is also involved in mTORC1 inhibition during hypoxia [43]. We have recently shown that metabolic changes dictated by REDD1-mediated mTOR inhibition in TAMs specifically prevents vascular remodeling and oxygen delivery, thus increasing hypoxia in a vicious cycle and fostering metastasis, without which these metabolic changes modulate the influence of TAMs on cancer cell invasion or on the immune system. TAM-specific deletion of REDD1 breaks this vicious loop and promotes tumor vessel normalization, tumor reoxygenation and metastasis inhibition (Fig. 1). Metabolically, this feature is paralleled by an increased flux through

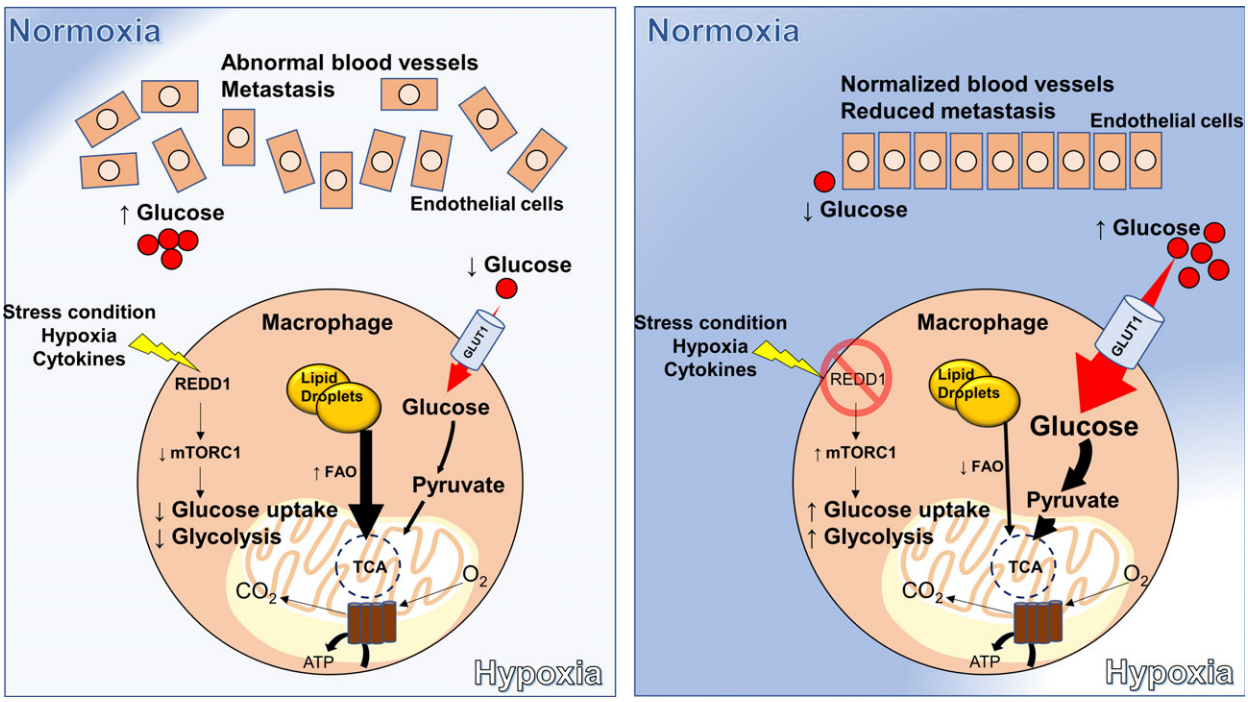

Fig. 1. Role of REDD1 in TAM metabolism and tumor angiogenesis. REDD1 induction in TAMs by tumor hypoxia (a mix of low oxygen, stress conditions and cytokines) hinders mTOR activation, resulting in decreased glucose uptake. It results in the fact that more glucose will be available for the neighboring endothelial cells that, when glycolytic, acquire a hyperactive phenotype. These endothelial cells fail to build a normal, functional vessel sprout and thus fail to rescue hypoxia (white area). REDD1 deletion by genetic means enhances glucose uptake and glycolysis in hypoxic TAMs via mTOR. Enhanced glucose uptake and glycolysis in REDD1 KO TAMs leads to glucose competition with tumor endothelial cells. As a consequence, glucose competition by REDD1 KO TAMs stabilizes tumor endothelial cells junctions and vessels, establishing tumor tissue reoxygenation (normoxia; blue area), also preventing cancer cell intravasation and metastasis.

glycolysis, which causes tumor vessel normalization by decreasing availability of glucose for endothelial cells. This is the first demonstration of metabolic competition between two different stromal compartments, namely TAMs and endothelial cells. Other examples of metabolic competition have been shown between cancer cells and T cells [44] or cancer-associated macrophages and cancer cells [45], always pointing to the direction that metabolic rivalry between cell types can drastically affect, for better (our study) [16,17] or for worse [44,45], the phenotypic features of cellular compartments and thus the disease outcome.

In conclusion, mTOR activation produces different effects. On one side, mTOR overexpression (secondary to TSC2 knockdown) switches macrophages into M2-like phenotype that promotes angiogenesis [36]. On the other side, mTORC1 activation (secondary to REDD1 knockdown) in hypoxic TAMs switches macrophages into a new metabolic phenotype that promotes tumor vessel normalization and metastasis inhibition [16]. For these reasons, the mTOR pathway is a critical regulator of monocyte differentiation to TAM and requires further investigation.

Lactate

Altered aerobic glycolysis or Warburg effect [46] is considered a key metabolic feature of cancer [47]. In normal conditions, glycolytic pyruvate enters the tricarboxylic acid (TCA) cycle for oxidative phosphorylation (OXPHOS) in aerobic conditions, whereas lactate conversion from pyruvate is enhanced in anaerobic conditions. In cancer cells, pyruvate to lactate conversion takes place even in the presence of oxygen due to metabolic alterations [47] producing metabolic acidosis with pH of the solid cancers as low as 6.0–6.5 [48]. By virtue of metabolic symbiosis, more hypoxic cancer cells would release lactate in favor of the more oxygenated cells that would then engage lactate into the TCA cycle [49,50].

Recently [51] an elegant investigation has established the mitochondrial utilization of lactate to produce pyruvate and enter the TCA cycle, although a clear localization of lactate dehydrogenase (LDH) in the mitochondrial matrix was not demonstrated. The model presented supports the hypothesis that lactate could be transported across the inner mitochondrial membrane and intramitochondrially oxidized by LDHB (Fig. 2). This important event would convey the reducing power of lactate inside mitochondria, untying cell metabolism from the malate–aspartate shuttle. In different cellular models [52–55] it has been shown that the demand for NADH to NAD^+ recycling by increased glycolytic flux overwhelms the ability of glycerol phosphate and malate–aspartate shuttles to equilibrate the NADH/NAD^+ ratio. For instance, in cancer cells it has been shown that < 20% of the amount of NADH produced by glycolysis can be oxidized by the malate–aspartate shuttle working at its maximum capacity [52]. This explains why cancer cells require high production of lactate. The transport of lactate into mitochondria would be then crucial to support cancer growth, as it would provide both carbon and reducing equivalents generated by glycolysis.

Another route for lactate is the extracellular space, where it has been considered for a long time a waste product (Fig. 2). Recently, several findings point to secreted lactate as a signal going from cancer cells to TAMs. Colegio *et al.* [56] identified lactate as a soluble factor influencing recruitment of macrophages by cancer cells. Lactate produced by cancer cells is capable of inducing the expression of vascular endothelial growth factor and the M2-like polarization of TAMs by a mechanism mediated by hypoxia-inducible factor 1α (HIF-1α). Additionally, TAMs respond to lactate by inducing the expression of Arginase-1 which helps tumor growth by suppressing T-cell responses (Fig. 2).

The mechanism by which macrophages sense lactate in the tumor milieu has been highlighted in breast cancer, in which it has been shown to occur through G protein-coupled receptor 132 (Gpr132) [57,58]. Activation of Gpr132 by lactate promotes the alternatively activated macrophage M2-like phenotype, enhancing cancer cell adhesion, migration, and invasion, whereas its deletion reduces M2 macrophages and lowers breast cancer lung metastasis in mice. This target is under the suppressive regulation of PPARγ and this has prompted a pharmacological strategy aiming at interrupting the lactate-Gpr132 axis, through Gpr silencing or treatment with PPARγ agonists [57,58]. A further pharmacological approach includes inhibition of LDH by oxamic acid that has been shown to interrupt the flux of lactate from cancer cells [57,58]. The excess of extracellular lactate results also in the disruption of aerobic glycolysis, proliferation and survival of T cells and natural killer (NK) cells. In contrast, Foxp3-expressing T_{reg} cells are able to proliferate and to maintain redox balance under low-glucose/high-lactate concentrations because NAD^+ is regenerated by the TCA cycle (Fig. 2) [56,59–61].

Amino acids

Arginine

The specific adaptations involving amino acid metabolism of TAMs within the tumor are still poorly

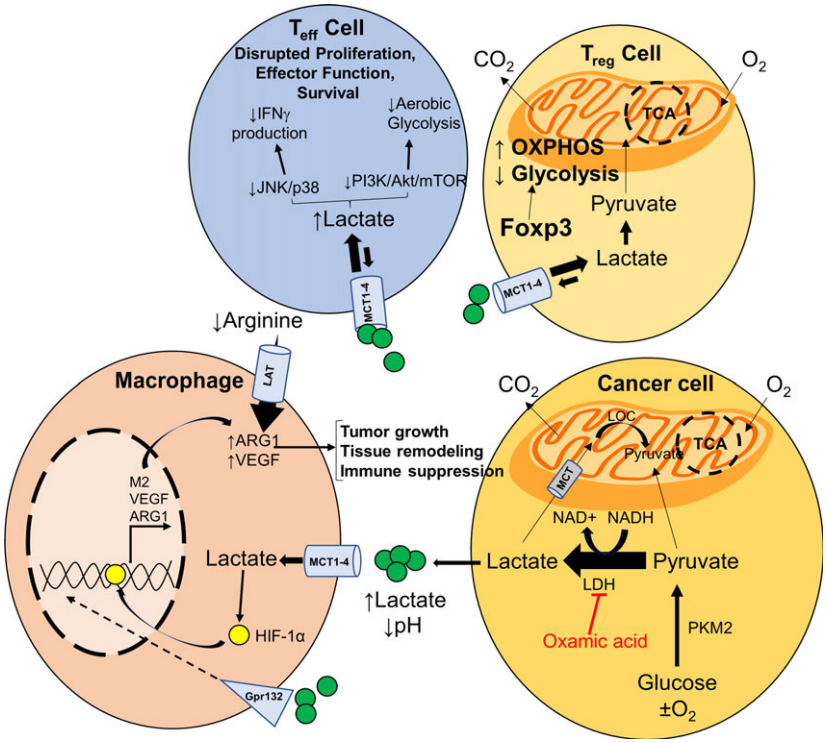

Fig. 2. Role of lactate in TAM function. Lactate can be secreted and also directly used as fuel, it enters mitochondria by mitochondrial monocarboxylate transporters (MCT) where it is oxidized to pyruvate and then acetyl CoA (A-CoA) through mitochondrial lactate oxidation complex (LOC). The enhanced production of lactate is associated with an increased acidity in the TME. Tumor-derived high-lactate concentrations interfere with the export of intracellular lactate by T cells *in vitro*, and disrupts aerobic glycolysis through the PI3K/Akt/mTOR pathway. Furthermore, lactic acidosis selectively inhibits Janus Kinase (JNK) and p38-mediated stimulation of IFNγ production by T cells and NK cells. In T_{reg} cells, Foxp3 promotes OXPHOS, facilitating proliferation under high-lactate concentrations because NAD^+ is regenerated by the TCA cycle. T_{reg} cell function coupled with impaired T effector (T_{eff}) proliferation results in amplified T_{eff} response suppression. Macrophages are polarized in an immunosuppressive phenotype M2 by Gpr132 receptor. Furthermore, lactic acid is sufficient to induce VEGF and Arg1 via HIF-1α stabilization.

elucidated. The metabolism of arginine by inducible nitric oxide synthase (iNOS) or Arginase-1 results in different biological effects [62,63]. Nitric oxide (NO) production by iNOS under physiologic conditions plays an important role in killing parasites, bacteria, viruses, and cancer cells and producing vasodilatation [64]. Ornithine and urea are the main products generated by the catabolism of arginine by Arginase-1. Ornithine is a precursor of different products, including polyamines and proline, promoting cell proliferation [65] and wound healing [66,67]. It is known that TAMs express Arginase-1. This consumption of arginine due to Arginase-1 upregulation in TAMs can stimulate nearby cancer cells [68]. Furthermore, by upregulating Arginase-1, M2 TAMs interfere with the anti-tumor activity of T cells as this depletes the arginine pool for NO and protein synthesis, which impairs T-cell receptor (TCR) function (Fig. 3) [69,70] and T-cell differentiation [71].

Tryptophan

Tryptophan metabolism is an emerging route in macrophages. The indoleamine 2,3 dioxygenase (IDO), which is strongly expressed in TAMs, catalyzes the first and rate-limiting step in the kynurenine pathway by converting tryptophan to formylkynurenine [72,73]. IDO activity can significantly decrease tryptophan concentration limiting its availability for T cells (Fig. 3) [72,74]. Furthermore, tryptophan depletion induces the stress kinase general control nonderepressible 2, which in turn downregulates the CD3 zeta-chain in $CD8^+$ cytotoxic T cells and inhibits Th17 cell differentiation [75,76]. In addition, kynurenine itself is a potent and active suppressor of T-cell activation since it can interfere with T-cell receptor (TCR) signaling by downregulating CD3 expression or can induce T-cell death. Mechanistically, kynurenine has been shown to be an endogenous ligand of the aryl hydrocarbon receptor

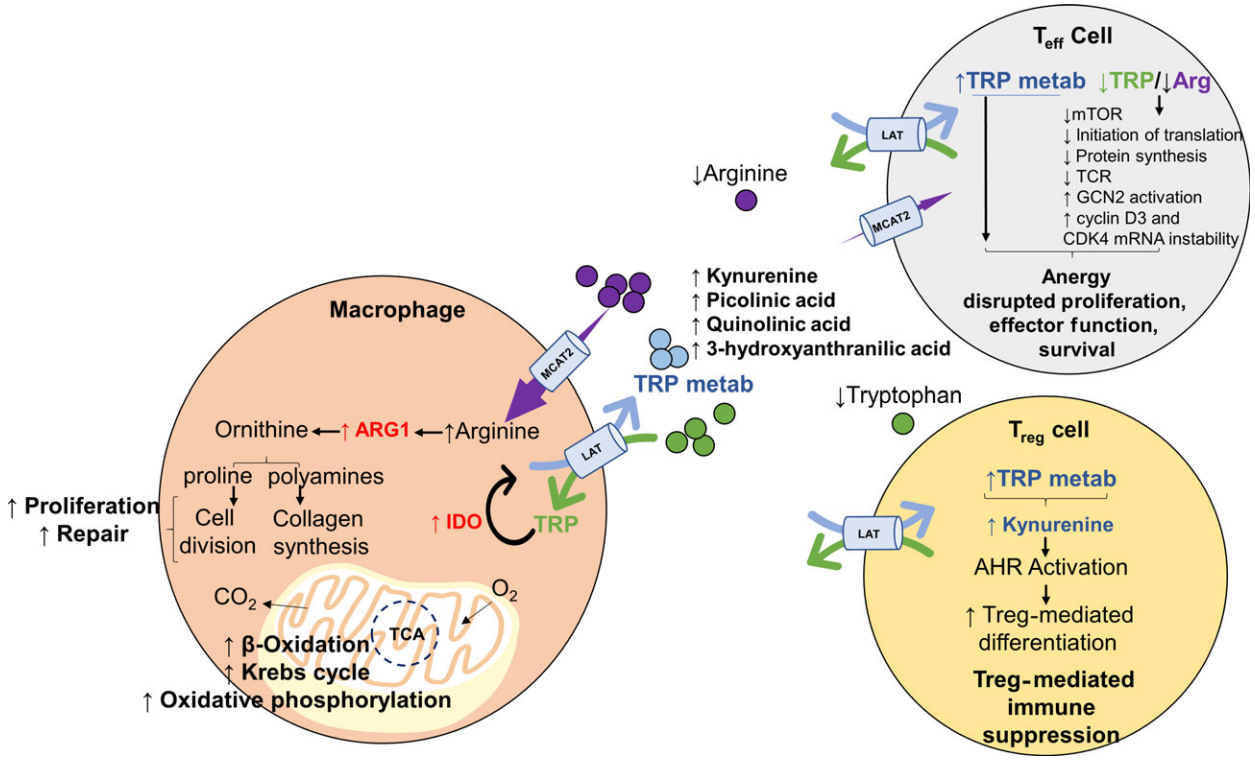

Fig. 3. Role of arginine and tryptophan in TAM function. In TAMs, Arginase-1 (Arg1) is strongly expressed and has an important role in producing polyamines, which are substrates that have a critical role in cell proliferation and wound healing. TAMs through Arg1 deplete Arginine, which is an important substrate in T-cell activation. IDO is also strongly expressed in TAMs. IDO metabolizes tryptophan to kynurenine and limits T-cell function by reducing tryptophan. Kynurenine itself can interfere with TCR signaling by downregulating CD3 expression or induce T-cell death. Mechanistically, kynurenine is a ligand of the AhR, which can induce T_{reg} cells.

(AhR), which can induce T_{reg} cells (Fig. 3) [72]. Tryptophan catabolites including picolinic acid, quinolinic acid [77], and 3-hydroxyanthranilic acid [78] has been shown to inhibit T-cell proliferation via mechanisms that are not yet fully understood. The decrease in perforin noticed in IDO overexpressing CD8 T cells might indicate a role of IDO in modulating their cytolytic capacity [79].

Glutamine

Glutamine metabolism is crucial in cancer cells, but recent evidences are linking glutamine to macrophages in general and TAM functions. The role of glutamine has been always referred as proinflammatory, as the amino acid has been widely recognized as an important metabolic fuel for immune cells [80]. Several studies have shown that glutamine may become 'conditionally essential' during inflammation as it can not only be a respiratory fuel but also an enhancer of the immune function [80], with some interesting evidences on the role of extracellular glutamine concentration on lymphocyte and macrophage activities [80]. Our study on glutamine synthetase (GS) in adipocytes has allowed to define a novel path in the interpretation of the role of glutamine metabolism in cell function. GS is a key enzyme involved in nitrogen metabolism, acid–base homeostasis, and cell signaling across multiple species of prokaryotes and eukaryotes [81]. One of the main roles of GS in vertebrates is to produce glutamine from glutamate and ammonia, which are toxic to the central nervous system [82–84]. Moreover, a continuous supply of glutamine is required for several physiological processes, including synthesis of glutamate, synthesis of proteins, and osmoregulation [85]. Since GS is the only known enzyme in humans capable of synthesizing glutamine, alterations in its expression and activity are likely to have significant biological effects. Furthermore, brain GS holds the important task of removing excitotoxic glutamate, and with this respect, its susceptibility to oxidative stress has been extensively studied. Indeed ROS-mediated loss of function of GS has been demonstrated in many neurodegenerative disorders [86–88].

Our study in 3T3-L1 adipocyte cell cultures show that modulation of intracellular glutamine levels by

GS expression represents an endogenous mechanism through which mature adipocytes control the inflammatory response. GS expression at the late stages of differentiation desensitized mature adipocytes to bacterial LPS by increasing intracellular glutamine levels [89] and this effect is reverted by GS inhibition. Interestingly, supraphysiological levels of extracellular glutamine (10 mM) rescue the adipocytes' insensitivity to LPS that was lost following GS inhibition. This observation suggests that an increase in intracellular glutamine due to GS activity (and mimicked by incubating cells with 10 mM glutamine) is responsible for the effect above described, pointing to the intriguing possibility that glutamine could exert a regulatory or signaling role. This concept is actually not novel to the scientific community, since glutamine has been described as a transcriptional modulator in many cases. For instance glutamine mediates heat shock transcription factor 1 [90], argininosuccinate synthase [91] and PPARγ [92] gene expression. Furthermore, glutamine has been reported to induce autophagy [93]. The role of glutamine has been always referred as proinflammatory, as the amino acid has been widely recognized as an important metabolic fuel for immune cells [80]. However, there are many cases in which treatment with glutamine is associated with reduction in the pro-inflammatory response [94–96]. The evidence that GS is expressed also by human macrophages and microglia [97,98] prompted us to evaluate the physiological role also in these cells. In microglia, we show that GS inhibition strongly enhances the response to a proinflammatory stimulus leading to perturbation of the redox balance and decreased viability of cocultured neurons [99]. Based on these encouraging findings we investigated the role of GS in human macrophages also in the context of the tumor microenvironment. With this respect, we took advantage of the availability of the GS conditional knockout (cKO) mouse, which is GS floxed and expressing a tamoxifen-induced Cre under the macrophage promoter Csf1r [17,99]. GS cKO bone marrow-derived macrophages stimulated with IL-10 display a unique metabolic feature that was also confirmed in blood-derived human macrophages treated with IL-10 in the presence of methionine sulfoximine, a GS inhibitor. GS inhibition induces a strong increase in intracellular glutamate, which is synthesized preferentially from glucose rather than glutamine. Glutamine metabolism is rerouted to succinate synthesis through γ-aminobutyric acid, and this could be responsible to the clear revert from M2- to M1-like phenotype, at least in part, through HIF-1α (Fig. 4). This switch toward the M1 phenotype was evident in resting macrophages treated with 10 mM glutamate, indicating that the glutamine/glutamate intracellular ratio is responsible for this revert. These effects were also evident in TAMs isolated from tumors developed in GS cKO mice implanted with Lewis lung carcinomas, which were

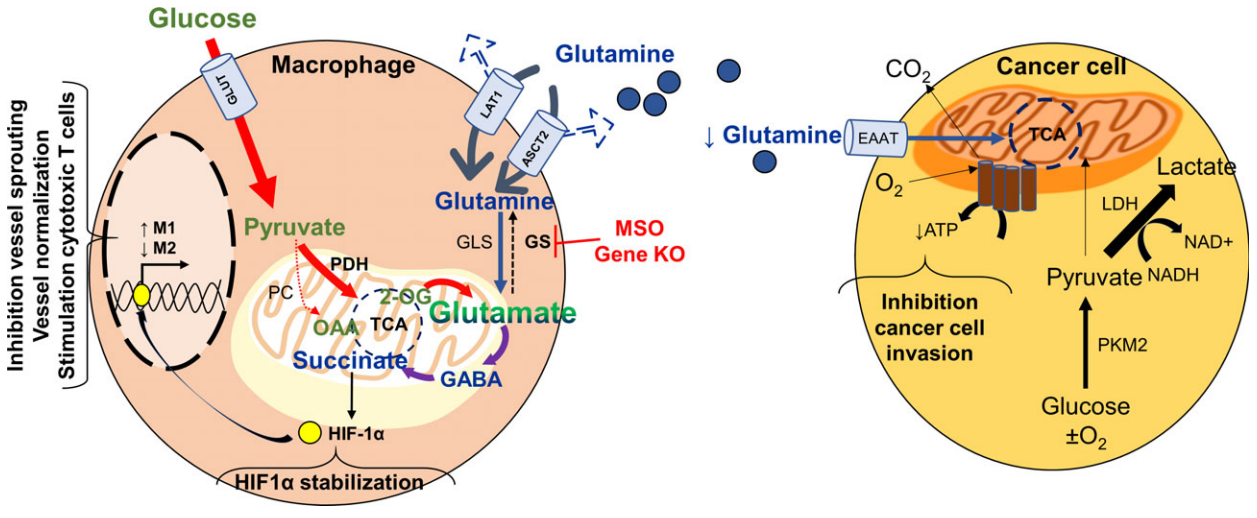

Fig. 4. Role of GS- related glutamine synthesis in TAMs. *In vitro* GS inhibition lowers intracellular glutamine while increasing glutamate levels. Reduction of glutamine levels increases glucose flux toward glutamate, which strongly accumulates in the cell. Glutamine uptake is not decreased but channeled toward succinate synthesis through GABA. Functionally, GS inhibition or gene deletion reduces M2 and increases M1 markers through stabilization of HIF-1α by succinate, leading to lower T-cell suppression, reduction in angiogenesis with features of tumor vessel normalization, and inhibition of cancer cell invasiveness. Altogether, these findings translate in a strong inhibition of metastasis formation in mice.

prevalently MHC-II^{high} and CD206^{low} (M1-like) compared with the WT controls, and displayed similar metabolic features as the pharmacologically GS-inhibited macrophages. Their reduced expression of M2-specific markers such as Arg1, CD206/Mrc1, Ccl17, and Ccl22 further confirmed that GS KO TAMs were skewed away from the M2-like phenotype. The effect of the acquired phenotypic switch of GS-deficient TAMs strongly impacts tumor metastasis, that were twofold decreased in GS cKO versus GS wild-type (WT) mice, with an increase of 75% cytotoxic CD8$^+$ T cells upon GS deletion in TAMs. In line with this shift in macrophage phenotype we found a decrease in vessel formation in tumors from GS cKO versus WT mice, although in GS cKO versus GS wild-type (WT) mice tumors displayed functionality and vascular integrity as indicated, respectively, by reduced tumor hypoxia and decreased accumulation of leaked red blood cells in the perivascular space [17]. All these results point to fundamental role of metabolism in shaping TAM function, thus influencing tumor development. Intracellular, GS-mediated, glutamine synthesis appears to be a fundamental step during M2-like differentiation of macrophages. Besides its channeling into the TCA cycle, glutamine contributes to nucleotide and uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) synthesis for support of protein folding and trafficking [100]. In M2 macrophages, the glutamine route toward UDP-GlcNAc is particularly enhanced [101] as the molecule represents the building block for the synthesis of glycosylation moieties of lectin/mannose receptors, which, in their highly glycosylated form, are among the most typical M2 polarization markers [102]. However, the large body of evidence on the regulatory role of glutamine [17,89] prompted us to hypothesize that glutamine could exert also a regulatory role inside the cell through GS activity, that may increase the levels of the amino acid above a threshold level capable of promoting regulatory effect on anti-inflammatory genes. Our results on the effect of extracellular supraphysiological glutamine is clearly evident on M1-like macrophages, in which administration of 10 mM glutamine strongly increases the expression of M2-like markers (Fig. 5). Based on these findings, we propose a signaling role of intracellular glutamine due to GS activity, the expression of which may represent a cellular commitment to specific functions. With this respect glutamine and succinate might be defined as two opposite mediators of the M2- and M1-like phenotypes, respectively, and the swinging between the two different states might be driven by perturbations in the synthesis of the two molecules.

Another important element regarding GS expression is the fact that in blood-derived macrophages GS is specifically expressed following stimulation with IL-10, whereas it responds to a lesser, almost marginal extent to IL-4 and IL-13 [17]. Several evidences highlight the importance of IL-10 signaling in the anti-inflammatory phenotype of macrophages. IL-10 inhibits inflammatory cytokine release from macrophages and the expression of major histocompatibility complex II [103]. Recently, IL-10 has been shown to inhibit LPS-induced glucose uptake and glycolysis and promotes OXPHOS [103]. IL-10 induces mitophagy and reduces LPS-dependent IL-1β production by preventing excessive ROS release from complex II in damaged

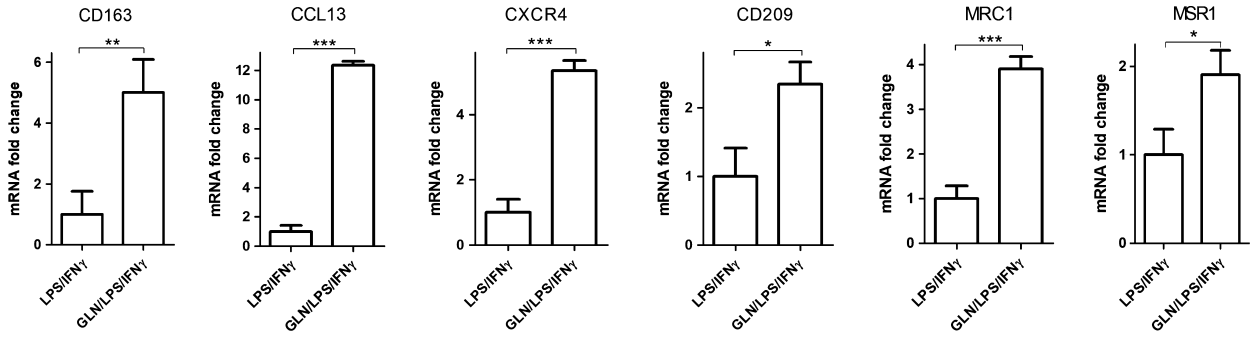

Fig. 5. Strongly supraphysiological extracellular glutamine levels polarize LPS/IFNγ macrophages toward a M2-like phenotype. Human monocytes were obtained from healthy blood donor buffy coats under an Institutional Review Board approved protocol and isolated with CD14 MicroBeads (Miltenyi Biotec Inc., Bergisch Gladbach, Germany). After differentiation for 6 days with 1000 U·mL^{-1} rhGM-CSF, macrophages were stimulated for 24 h with 100 ng·mL^{-1} LPS and 20 ng·mL^{-1} IFNγ for M1 polarization, with and/or without 2 h preincubation with 10 mM glutamine. RNA isolation and subsequent qRT-PCR analysis was performed on M2 marker genes: CD163, CCL13, CXCR4, CD209, MRC1, and MSR1 ($n = 3$). Interestingly, strongly supraphysiological levels of extracellular glutamine increased the expression of M2-like markers. These findings suggest the intriguing possibility that glutamine could control macrophage response to proinflammatory stimuli and exert a regulatory or signaling role. Results are shown as means ± SEM. Statistical significance was calculated by one-way ANOVA with Tukey *post hoc* test and considered statistically significant as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

mitochondria and limiting inflammasome activation [103]. This event is accompanied by mTORC1 inhibition, by means of a specific activation of REDD1 (Fig. 6) [103].

As glutamine synthesized from GS is known to inhibit mTOR activity [93,104] (in apparent contrast to extracellularly uptaken glutamine), the effect of IL-10 on macrophages could be unified in a comprehensive model in which the specificity protein 1 (Sp1) transcription factor may play a crucial role. Our hypothesis is that GS could represent the link mediating IL-10-driven REDD1 expression. Supraphysiological levels of glutamine are known not only to induce the *O*-glycosylation of Sp1, increasing its activity [105,106], but also to promote its nuclear translocation [105]. Since REDD1 is also a Sp1 target [107,108], it is conceivable that the increase in intracellular levels of glutamine due to IL-10-mediated GS expression could in turn promote the anti-inflammatory events typical of M2-like macrophages in synergy with REDD1 expression through Sp1, which inhibits mTOR (Fig. 6). Incidentally, GS inhibition, similar to REDD1 ablation, leads to enhanced glycolytic metabolism [16,17]. GS and REDD1 might then represent a 'consistent pair' that, under the effect of IL-10, might coherently polarize TAMs toward the multiple tasks typical of M2-like macrophages.

Lipids precursors and lipids

Studies on M2-like macrophages have demonstrated that M2-polarized cells enhance their metabolism toward oxidative metabolism and fatty acid oxidation [109,110], to sustain the typical energetically demanding secretory program. In contrast, M1-like macrophages promote *de novo* synthesis of fatty acids to respond to the cellular increase in biosynthetic demand for prostaglandin production [12]. With this respect, the contribution of the citrate carrier (CIC), a mitochondrial transport protein involved in the channeling of citrate in exchange with malate, has been established. Transport of citrate via the CIC and citrate metabolism appear to be critical for the activation of macrophages [111–113]. A large body of evidence has shown a role for CIC and ATP citrate lyase in tumorigenesis suggesting that lipid biogenesis play a significant role in cancer cell proliferation and progression [114,115].

Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are present at high concentrations in the tumor microenvironment (TME) [116]. These fatty acids are not only associated with cancer cells migration and survival but also with

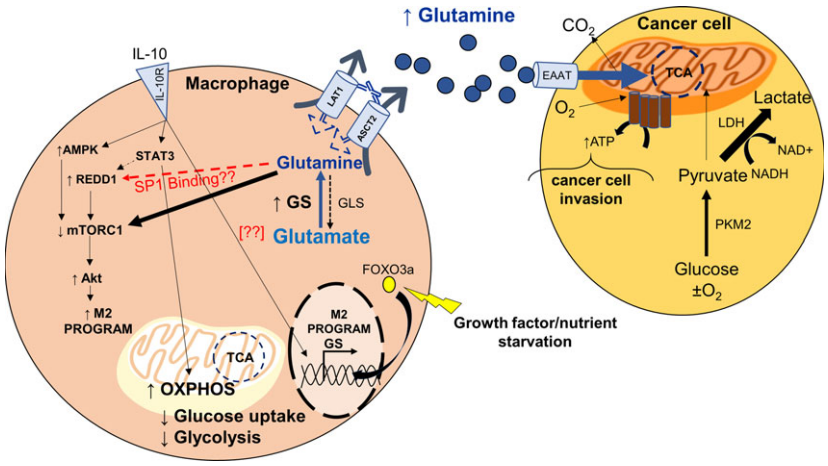

Fig. 6. The emerging role of IL-10 signaling in modulating TAM function through metabolism. IL-10 alters macrophage function by promoting the clearance of damaged mitochondria and modulating cellular metabolism to limit inflammation. IL-10 signaling via STAT3 inhibits mTORC1 activation through REDD1, and improves mitochondrial function, favoring the channeling of nutrients toward the TCA cycle for ATP production. IL-10 contributes to the inhibition of mTOR through an increased phosphorylation of adenosine 5′-monophosphate-activated kinase (AMPK). IL-10 treatment increases GS expression and activity. The increased intracellular glutamine negatively modulates mTOR activity and regulates autophagy. Glutamine increases the transcriptional activity of Sp1, which might enhance REDD1 expression leading to mTORC1 inhibition (see also Fig. 1). GS is also a downstream effector of the PI(3)K–PKB–FOXO signaling network. Under starving conditions GS expression is enhanced by the FOXO transcription factors leading to autophagy, which is a consequence of the increased intracellular glutamine levels due to GS activity. Since GS is sensitive to nutrient starvation, depauperation of nutrients by cancer cells might enhance GS expression in macrophages and force them to secrete glutamine into TME, thus acting as tumor metabolic servants.

TAM polarization (Fig. 7) [116]. The uptake of triacylglycerol substrates via CD36 and their subsequent lipolysis by lysosomal acid lipase (LAL) is important for the engagement of elevated OXPHOS, enhanced spare respiratory capacity (SRC), prolonged survival and expression of genes that together define the M2-like activation status [110]. Furthermore, these fatty acids (and in particular linoleic acid) act as potent agonists of the peroxisome proliferator-activated receptor β/δ (PPARβ/δ) in macrophages and accumulate in lipid droplets, thereby providing a reservoir of PPARβ/δ ligands. PPARβ/δ is a transcription factor associated with M2 gene expression (Fig. 7) [116].

Prostaglandin E2

Prostaglandin E2 (PGE2) is an important factor which can reprogram M1 macrophages to M2-like cells [117]. PGE2 binding to EP4 receptors activates cAMP pathway which leads to the inhibition of glycogensynthase kinase (GSK) and subsequent activation of cAMP response element-binding protein (CREB) signaling, which leads to the transcription of genes associated with the M2 macrophage phenotype (Fig. 7) [117]. PGE2 further enhances STAT3 expression and phosphorylation, controlling macrophage polarization [118]. PGE2, via EP4 signaling, suppresses the cytotoxicity and cytokine production of NK cells and can also potently induce Foxp3 expression in naïve T cells, a transcription factor that is necessary for the development of T_{reg}-associated immunosuppressive properties [119]. Cancer cell or TAM-secreted PGE2 and sphingosine-1-phosphate (S1P) mediate immunosuppressive and metastasis-promoting functions [119–122]. Blockade of PGE2-producing enzymes microsomal PGE2 synthase 1 (mPGES1) and cyclooxygenase-2 (COX-2) is known to promote M2-to-M1 polarization of TAMs in an Apcmin/+ colon cancer model [123]. Furthermore, reduced PGE2 level due to COX-2 inhibition in bone marrow cell/MBT-2 bladder cancer cell coculture reduces the expression of T-cell-suppressive programmed cell death ligand 1 (PD-L1) in an MBT-2 cancer model [122].

Conclusions and perspectives

Targeting TAMs is emerging as a strategy to improve the effects of cancer therapy. The fact that these macrophages are normal diploid cells might in some way prevent drug resistance that inevitably accompanies tumor cells with an enhanced mutation rate. Clinical trials targeting CSF1R signaling to block the protumoral role of TAMs are showing some clinical efficacy [124]. However, this general targeting might produce harmful consequences. The role of metabolism on shaping TAM function might be useful with

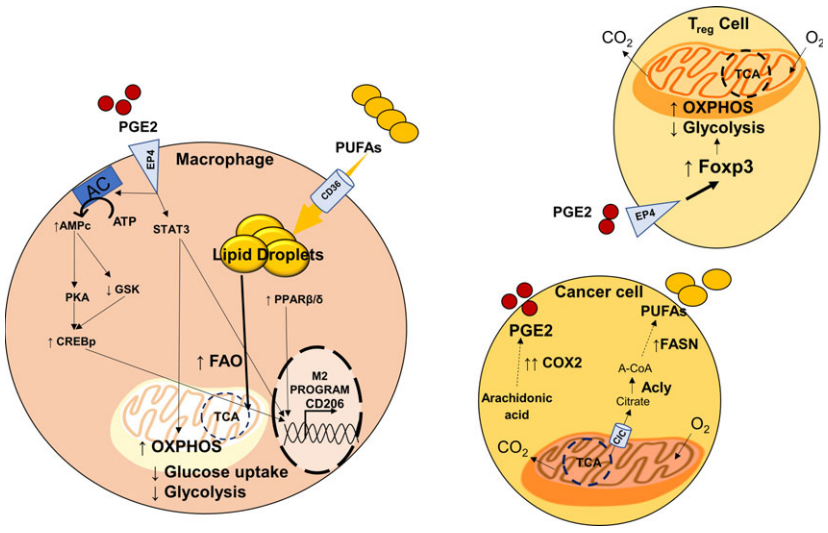

Fig. 7. The role of lipids and lipid precursors in TAM function. PUFAs are present at high concentrations in TME and interfere with TAM polarization. The uptake of triacylglycerol substrates via CD36 and their subsequent lipolysis by LAL foster M2 activation by engaging OXPHOS and SRC. They also act as potent nuclear receptor peroxisome proliferator-activated receptor β/δ (PPARβ/δ) agonists in macrophages and accumulate in lipid droplets, thereby providing a reservoir of PPARβ/δ ligands. PGE2 promotes skewing of M1 to M2-macrophages through CREB signaling, which is activated by cAMP pathway that leads to the inhibition of GSK. PGE2 further enhances STAT3 expression and phosphorylation, controlling macrophage polarization. PGE2, via EP4 signaling, suppresses the cytotoxicity and cytokine production of NK cells and can also potently induce Foxp3 expression in naïve T cells.

this respect. First of all, understanding the role of metabolic enzymes in the balance between pro- and anti-inflammatory properties of macrophages might lead to selective inhibition of the specific enzymes rather than ablation of general macrophage function [7–9]. Furthermore, use of small molecules as enzyme inhibitors, rather than antibodies, might produce important consequences with respect to both costs and efficacy. By targeting enzymatic activities linked to their protumoral phenotypes, these inhibitors might reduce the immunosuppressive activities of macrophages, leading to a more effective chemotherapeutic and/or immunotherapeutic regimen. Combined strategies are probably the future in the cure for cancer. Additional definitions of how metabolism shapes immunoregulatory mechanisms in macrophages will be very useful.

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