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FOCUSSED RESEARCH REVIEW

Cholesterol metabolites and tumor microenvironment: the road towards clinical translation

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Abstract Targeting the tumor microenvironment focusing on immune cells has recently become a standard of care for some tumors. Indeed, antibodies blocking immune checkpoints (e.g., anti-CTLA-4 and anti-PD1 mAbs) have been approved by regulatory agencies for the treatment of some solid tumors based upon successes in many clinical trials. Although tumor metabolism has always attracted the attention of tumor biologists, only recently have oncologists renewed their interest in this field of tumor biology research. This has highlighted the possibility to pharmacologically target rate-limiting enzymes along key metabolic pathways of tumor cells, such as lipogenesis and aerobic glycolysis. Altered tumor metabolism has also been shown to influence the functionality of the tumor microenvironment as a whole, particularly the immune cell component of thereof. Cholesterol, oxysterols and Liver X receptors (LXRs) have been investigated in different tumor models. Recent in vitro and in vivo results point to their involvement in tumor and immune cell biology, thus making the LXR/oxysterol axis a possible target for novel antitumor strategies. Indeed, the possibility to target both tumor cell metabolism (i.e., cholesterol metabolism) and tumor-infiltrating immune cell dysfunctions induced by oxysterols might result in a synergistic antitumor effect generating long-lasting memory responses. This review will focus on the role of cholesterol metabolism with particular emphasis on the role of the LXR/oxysterol axis in the tumor microenvironment, discussing mechanisms of action, pros and cons, and strategies to develop antitumor therapies based on the modulation of this axis.

Keywords Metabolism · Tumor · Microenvironment · Immune cells · Immunotherapy · NIBIT 2014

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Abbreviations

ABC	ATP-binding cassette
AIM/SPα	Apoptosis inhibitor of macrophages
BRAF	BRAF proto-oncogene, serine/threonine kinase
CCR	Chemokine receptor
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CXCR	CXC chemokine receptor
DC	Dendritic cell
ER	Endoplasmic reticulum
HDL	High-density lipoprotein
HMGCR	Hydroxyl-methyl glutaryl-coenzyme A reductase
INSIG	Insulin-induced gene
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LXR	Liver X receptor
mTOR	Mammalian target of rapamycin
PD1	Programmed cell death protein 1
RCT	Reverse cholesterol transport
SCAP	SREBP-cleavage activation protein
SREBP	Sterol response element binding protein

SULT2B1b   Sulfotransferase 2B1b
Th   T helper cell

Introduction

Immunotherapy of cancer has recently achieved clinical success due to antitumor activity resulting from the use of antibodies blocking immune checkpoints, such as the CTLA-4 and PD-1 molecules expressed on activated T cells [1]. The recent clinical success of these drugs has not only formally elevated cancer immunotherapy to the Olympus of neoplastic treatments [2], but also revealed the clinical importance of targeting the microenvironment to kill human tumors. As a consequence, several preclinical studies demonstrating the efficacy of strategies targeting the cells forming the tumor microenvironment have paved the way to clinical experimentation with a large panel of molecules endowed with immune stimulatory properties [3]. The introduction of these molecules into the clinic is changing some therapeutic paradigms. As a matter of fact, physicians can currently choose among a variety of antitumor treatments: (1) drugs targeting specific oncogene mutations (i.e., BRAF inhibitors in BRAF-mutated melanomas) [4], (2) chemotherapy for tumors particularly responsive to these non-specific antitumor drugs and (3) immunotherapy to block immune checkpoints and awaken preexisting antitumor T cells or eliciting de novo antitumor T cells [5, 6]. This new scenario highlights the weapons we currently have to fight cancer and how complicated is the choice of oncologists in some specific tumor conditions where precise clinical indications for the selection of a given drug are lacking. Finally, it fosters the investigation of biologic processes favoring the interaction of tumor cells and other cells of the tumor microenvironment in order to identify therapeutic strategies targeting directly all the components of tumor microenvironment. In this context, recent data from the literature put the emphasis on differences between the metabolism of normal and tumor cells [7] and on the possible influence of tumor-derived metabolic products on the phenotype and function of cells contributing to the formation of the tumor microenvironment [8].

Based on this, we will focus this review on metabolic aspects of the tumor microenvironment as a whole, putting the emphasis on cholesterol and lipid metabolites produced by tumor cells that have been shown to influence the phenotype and function of cells forming the microenvironment, especially immune cells. As the goal of this review is to discuss aspects of tumor metabolism having immunosuppressive consequences on the tumor microenvironment, we will remind readers of exhaustive reviews for a more comprehensive understanding of single aspects of tumor [9] and stromal metabolism [10].

Cancer metabolism: cell-intrinsic and cell-extrinsic advantages

Oncogenic and metabolic pathways are tightly connected to regulate tumor cell proliferation and survival [7]. The connection between metabolism and tumors was primarily evidenced by Otto Warburg, who showed that cancer cells produce their own energy (i.e., ATP) through aerobic glycolysis, meaning that pyruvate originated by the glycolysis is not degraded in mitochondria, even in the presence of sufficient oxygen (the so-called Warburg effect) [11]. This glycolytic switch is made possible by the high rate of glucose uptake by cancer cells that compensates for the enormous difference in terms of molecules of ATP produced by oxidative phosphorylation as compared to aerobic glycolysis [12]. Moreover, this form of metabolism supplies tumor cells with macromolecular requirements for cell growth [12]. The PI3K/Akt signaling promotes the aerobic glycolysis by increasing the expression and the membrane translocation of glucose transporters and by phosphorylating key metabolic enzymes, such as hexokinase and phosphofructokinase 2 [13]. Akt also stimulates mTOR, which in turn promotes protein and lipid biosynthesis, thus fostering tumor growth [14]. Normal cells mainly rely on dietary lipid uptake for the synthesis of new structural lipids [15]. In contrast, tumors frequently exhibit an increased ability to synthesize new fatty acids even in the presence of exogenous lipids; a process referred to as de novo lipogenesis [15]. Fatty acids support the synthesis of membrane phospholipids needed to support high-rate proliferation [15]. Saturated and monounsaturated fatty acids makes tumor cells more resistant to oxidative stress and importantly, also to chemotherapy [16]. Moreover, fatty acids support the synthesis of lipid-signaling molecules promoting tumor growth, such as the phosphatidylinositol-3,4,5-triphosphate [17]. Lipogenesis mainly occurs through the induction of sterol regulatory element binding protein (SREBP)-1c, which regulates genes involved in de novo fatty acid biosynthesis, including acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase 1 (SCD1) and fatty acid synthase (FAS) [15, 18].

Increased glucose uptake and de novo lipogenesis primarily satisfy the high nutrient demand of cancer cells (tumor cell-intrinsic advantage). However, increased glycolytic metabolism and de novo lipogenesis are also detrimental for the cells forming the tumor microenvironment, such as immune cells (tumor cell-extrinsic advantage). Indeed, glucose depletion from the tumor microenvironment attenuates glycolysis in T cells, which in turn affects intratumor T cell effector functions [19, 20]. This pathway can be blocked by anti-PD-L1 antibodies, which decrease Akt/mTOR signaling sustained by constitutive PD-L1 activation [19, 20]. Additionally, lactic acid produced by tumor cells, as a by-product of aerobic or anaerobic glycolysis,

has been reported to mediate M2-like polarization of tumor-associated macrophages, which support tumor growth [21]. Concerning lipid metabolism, it has been reported that enhanced accumulation of lipids in tumor-infiltrating DCs dampens their ability to induce effective priming of antitumor immune responses [22]. This process could be mediated by XBP1 protein, which increases the expression of multiple genes involved in lipid biosynthetic pathways [23].

Cholesterol metabolism, LXRs and oxysterols

Cholesterol is synthesized via the mevalonate pathway, an enzymatic cascade mainly controlled by the SREBP family of transcription factors [24]. SREBPs are critical regulators of fatty acid and cholesterol biosynthetic gene expression [24]. The SREBP family is comprised of three isoforms: SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c are encoded by the same gene, SREBP-1, and differ in their first exon; SREBP-2 is a distinct gene [24]. Studies in knockout and transgenic mice have shown that SREBP1 (SREBP-1a and c) preferentially regulates genes involved in fatty acid biosynthesis, while SREBP2 mainly regulates genes of the cholesterol pathway [25].

Inactive SREBP protein precursors are located in the ER membrane and associate with the SREBP-cleavage activation protein (SCAP), a cholesterol sensor membrane protein [26]. Another ER protein, i.e., the insulin-induced gene (INSIG), binds SCAP under sterol-rich conditions, thus influencing the conformational state of the INSIG-SCAP-SREBP complex, ultimately occluding the binding site of SREBP for coat protein complex (COPII) proteins and preventing the Golgi processing of SREBP [26]. In sterol-poor conditions, INSIG dissociates from the SCAP–SREBP complex and free-cholesterol SCAP–SREBP is transported into the Golgi, where SREBP maturation occurs through a series of proteolytic steps [26]. Mature SREBP then migrates into the nucleus and activates the expression of hydroxyl-methyl glutaryl-coenzyme A reductase (Hmgcr) and low-density lipoprotein receptor (Ldlr) genes, among others [26].

Dietary cholesterol delivery to peripheral tissues is mediated by lipoproteins via the circulation. In particular, LDLs deliver cholesterol to most peripheral tissues through a mechanism mediated by LDL receptors (LDLR) [26], which are present on the plasma membrane of most cells. Once degraded into the lysosomes, LDL-derived cholesterol inhibits the transcription of the Hmgcr gene through the SREBP pathway and activates the acyl-CoA cholesterol acyl transferase (ACAT) gene, allowing cholesterol esterification and storage in lipid droplets [27]. Moreover, LDL suppresses transcription of the Ldlr gene [26], thus

reducing cholesterol up-take. Dietary and biliary cholesterol can be also absorbed from the intestinal epithelium through the Niemann Pick C1-like 1 (NPC1L1) protein, which is localized at the brush border membrane of enterocytes [28].

Any excess cholesterol needs to be eliminated from cells to prevent cell damage. ATP-binding cassette (ABC) transporters mediate reverse cholesterol transport (RCT), a process by which excess cholesterol from peripheral tissues is returned to the liver by high-density lipoproteins (HDL). The ABCA1 transporter promotes cholesterol efflux to lipid-poor apoA-I lipoprotein leading to HDL formation [27]. ABCG1 cooperates with ABCA1 in macrophages, further maturing HDL [29]. ABCG5/G8 transporters inhibit, in the gut, the absorption of cholesterol and plant sterols from the diet [30].

Overall, the concerted action of these transporters together with the inhibition of the enzymes controlling cholesterol synthesis and the inhibition of receptors involved in cholesterol uptake (LDLR), maintain the whole-body cholesterol homeostatic state.

Liver X receptor α (LXRα) and LXRβ are ligand-activated transcription factors belonging to the nuclear receptor (NR) superfamily [18]. They regulate cholesterol and fatty acid metabolism, the latter through the induction of SREBP-1c [18]. The activation of LXRα and β receptors is mediated by cholesterol precursors such as desmosterol [31] and by oxysterols [32]. Oxysterols, oxidized products of cholesterol, are generated through enzymatic reactions by means of several enzymes, such as cholesterol 24-hydroxylase (24S-HC), sterol 27-hydroxylase (27-HC), cholesterol 25-hydroxylase (25-HC), CYP7A1 (7α-HC), CYP3A4 (4β-HC) and CYP11A1 (22R-HC) [33, 34], and also by autoxidation [34]. Oxysterols are natural ligands of LXRs both in vitro [32] and in vivo [35]. There are several lines of evidence linking LXRs and oxysterols to cholesterol metabolism: (1) LXR activation induces the expression of ABC transporters, including ABCA1, ABCG1, ABCG5 and ABCG8 [30], which mediate the RCT process; (2) LXR activation induces the expression of several apolipoproteins, such as APOE, APOC1, APOC2 and APOC4 [36], which act as cholesterol acceptors; (3) LXR activation inhibits the uptake of LDL-associated cholesterol by increasing the expression of the inducible degrader of LDLR (IDOL), an E3-ubiquitin ligase that promotes ubiquitylation and lysosomal degradation of LDLR [37]; and (4) oxysterol 25-hydroxycholesterol (25-HC) has been shown to bind INSIG, thus further preventing Golgi processing of SREBP proteins [38]. Additionally, intracellular oxysterol storage induces HMGCR binding to INSIG, which in turn mediates proteasome degradation of HMGCR [27].

LXRs and oxysterols have primarily been involved in the regulation of cholesterol and fatty acid metabolism [18].

Notwithstanding this, experiments performed in preclinical models of diet-induced obesity and insulin resistance have shown the involvement of LXR/oxysterol signaling also in glucose metabolism, with an improved glucose tolerance upon LXR agonist treatment [39]. Chen and colleagues have demonstrated cross talk between LXR and insulin signaling. Indeed, the inactivation of oxysterols through the activity of the sulfotransferase 2B1b (SULT2B1b) enzyme abolished the insulin-mediated induction of SREBP-1c expression in primary hepatocytes [35]. Moreover, improved insulin sensitivity in obese LXR-deficient mice has recently been reported [40].

LXRs, oxysterols and tumor microenvironment

The expression of LXR isoforms depends on the cell type or tissues analyzed, with LXRβ expressed ubiquitously, while LXRα is expressed in liver, adipose tissue, adrenal glands, intestine, lungs and cells of myelomonocytic lineage [41]. Given the role of cholesterol and fatty acids in cancer cell growth, it is not surprising that LXRs and oxysterols have been extensively investigated in tumors. However, their effects on tumor cells are still controversial. LXRs and oxysterols affect tumor cells in multiple ways, depending on the specificity of LXR ligand activity, and on the histological types of tumor investigated. Several studies have reported that LXR activation inhibits cancer cell proliferation by inducing G0/G1 cell cycle arrest. Thus, treatment of LNCaP human prostate cancer cells with LXR agonists induced the up-regulation of the cell cycle inhibitor p27 [42]. The same antiproliferative effects were observed in some lines of breast cancer treated with the synthetic LXR agonist GW3965, with a reduced expression of S-phase kinase-associated protein 2 (Skp2), cyclin D1, cyclin A2, and hypophosphorylation of RB protein [43]. In human colorectal cancer cells, ligand-induced activation of LXR or the expression of a constitutive active form of LXRα increased caspase-dependent apoptosis, reducing tumor progression in xenograft models [44]. In the above-reported results, the role of LXRs and cholesterol is instrumental to the induction of the antitumor activity of LXR ligands and is associated with the expression of the LXR target gene ABCA1, which is strictly connected with the activation of the homeostatic mechanisms of RCT [27]. On the other hand, Nelson and colleagues have demonstrated a dual role of the oxysterol 27-HC in breast tumor growth and lung metastasis in a spontaneous mouse model [45]. In fact, they showed tumor growth induction by the oxysterol 27-HC following its interaction with the estrogen receptor, whereas it promoted lung metastasis through LXR activation [45]. Concerning the specific role exerted by LXRs on lipid and glucose metabolisms, Flaveny and

colleagues have recently reported that a newly developed synthetic LXR inverse agonist (i.e., a drug binding LXRs as an agonist but eliciting negative molecular and cellular responses), able to stabilize the co-repressor complex on the promoter of LXR target genes, induced tumor cell death by dampening either lipogenesis, or aerobic glycolysis, or both pathways in human tumor cell lines [46]. Noteworthily, this effect was not associated with hepatotoxicity, which is frequently observed upon the chronic exposure to LXR agonists [46].

The relationship between LXR/oxysterol signaling and cancer is even more complicated considering the effects exerted by LXR/oxysterol signaling also on the stromal component of the microenvironment, as evaluated by Pencheva and colleagues. These investigators demonstrated that the activation of tumor and stromal LXRβ by LXR agonists suppresses melanoma invasion, angiogenesis, and metastasis [47] through the transcriptional induction of apolipoprotein-E, a potent metastasis suppressor in melanoma [48], and by our work investigating the effects of LXR/oxysterol signaling on tumor-infiltrating immune cells [49]. Notably, immunodeficient mice (NOD-SCID mice) transplanted with mouse tumors and treated with drugs inhibiting cholesterol/oxysterol formation failed to control tumor growth, indicating the absolute requirement for an intact immune system to induce protective antitumor immune responses, when LXR signaling was shut off [49].

Before discussing the effects of LXR/oxysterol signaling on tumor-infiltrating immune cells, it is important to briefly introduce what is known about the role of LXR/oxysterol signaling in the immune system. The pioneering work of Tontonoz and colleagues has shown the anti-inflammatory activity of LXRs and their ligands in macrophages, also documented by the transcriptional profiling of LPS-induced macrophages treated with LXR synthetic agonists [50]. LXRs and oxysterols regulate not only innate immune cells, such as macrophages and dendritic cells (DCs), but also adaptive immune cells, such as T and B cells [51]. As a consequence, LXR/oxysterol signaling plays a key role in inflammatory, infectious and autoimmune diseases [51]. Studies performed in LXR-deficient mice have highlighted the central role of LXR in the clearance of *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium* infections in vivo [52, 53]. The lack of bacterial clearance in these mice depends on the loss of activation of the antiapoptotic factor AIM/SPα [52, 53], which was identified as an LXRα target gene. In contrast to the protective role of LXRs in bacterial infections, LXR-deficient macrophages and DCs under steady-state conditions are unable to up-regulate the receptor tyrosine kinase Mertk, which is associated with the phagocytosis of apoptotic cells [54]. As a consequence, LXR-deficient mice develop autoantibodies and autoimmune glomerulonephritis due to self-tolerance

breakdown [54]. As mentioned above, LXR/oxysterol signaling also regulates adaptive immune cells, such as T and B cells. Indeed, the engagement of LXRs blocks the proliferation of T and B cells undergoing activation [55]. Moreover, LXR activation has been shown to inhibit Th17 differentiation through the SREBP-1-mediated inhibition of aryl hydrocarbon receptor-mediated IL-17 transcription [56]. The involvement of LXR agonists in Th17 differentiation, however, is more complicated in light of recent results showing that this differentiation program is controlled by the accumulation of desmosterol and specific sterol-sulfate conjugates in naive CD4+ T cells, which function as potent endogenous agonists of retinoic acid receptor-related orphan receptor γ (RORγt) [57]. Interestingly, the authors report that desmosterol and sterol-sulfate conjugate accumulation during Th17 differentiation is achieved by the induction of cholesterol biosynthesis and uptake programs [57].

In tumors, LXR/oxysterol signaling has been reported to affect tumor-infiltrating immune cells [41]. We reported that human and mouse tumors produce LXR ligands/oxysterols, which create an immunosuppressive microenvironment favoring cancer progression [49]. Tumor-derived LXR ligands bind and activate the NR LXRα on maturing DCs, thereby inhibiting the functional up-regulation of the chemokine receptor CCR7 on their surface [49]. Since CCR7 is a key receptor driving DCs to secondary lymphoid organs, where they activate naive T and B cells, this mechanism would account for the dampening of effective antitumor immune responses [49]. LXR-mediated dampening of tumor-infiltrating DCs has also been reported by Flaveny and colleagues, with the induction of DC activity in the presence of an LXR inverse agonist [46].

Although inverse agonists and possible antagonists could be of benefit for the treatment of tumors producing LXR ligands, the recent elucidation of LXR-independent activity of oxysterols should take into account, as possible antitumor treatments, strategies inhibiting their generation or, alternatively, strategies inactivating oxysterols. This hypothesis arises from our work showing that tumor-released oxysterols recruit neutrophils within the tumor microenvironment, in a CXCR2-dependent, LXR-independent manner [58]. These neutrophils are endowed with pro-tumor functions, as they support cancer progression by promoting neo-angiogenesis and/or suppressing tumor-specific T cells, depending on the particular tumor model investigated [58]. The use of compounds inhibiting cholesterol and oxysterol synthesis, as well as the inactivation of oxysterols by means of SULT2B1b enzymatic activity [41], blocks the recruitment of pro-tumor neutrophils within the microenvironment, thus controlling tumor growth [58]. We argue that the above-described strategies might also be useful for targeting LXR-dependent mechanisms [49].

Notably, the pharmacologic and genetic inactivation of oxysterols is also able to restore DC functionality in vitro and in vivo [49], suggesting that a more general strategy of antitumor treatments blocking LXRs and cholesterol metabolites should take into account both LXR-dependent and LXR-independent effects of oxysterols. Finally, recent results elucidating the effects of sulfated sterols in Th17 differentiation [57] need additional efforts to analyze the role of these molecules in cancer.

Concluding remarks

Altered tumor metabolism affects the various cell components forming the tumor microenvironment, especially immune cells [8]. Oxysterols are oxidized cholesterol products that engage LXR NRs, which in turn regulate primarily cholesterol and fatty acid metabolism, as well as some aspects of glucose metabolism. The knowledge we have currently acquired on the physiology of LXRs/oxysterols points to a relevant role of this axis in the pathophysiology of tumors, as witnessed by preliminary preclinical results indicating antitumor activity exerted by synthetic agonists of LXRs [27], inverse agonists of these NRs [46], or drugs blocking their production and therefore restoring immune-mediated antitumor responses [49, 58]. However, the positive antitumor outcome obtained with targeting strategies inducing opposite effects (e.g., LXR agonists and inverse agonists both inhibiting tumor growth in vivo) also raises some issues about the multifaceted biology of LXRs and oxysterols within the tumor microenvironment. Therefore, an effort should be made to achieve a deeper understanding of the role of the LXR/oxysterol axis on the different components of the microenvironment, such as tumor cells themselves, and stromal cells. Tumor histotype and stage, immune cell subsets, non-immune stromal cells, LXR isoforms (i.e., LXRα and/or β isoforms) and other factors should be all dissected to clearly map and define cell, tissue and isoform specificity of LXR/oxysterol signaling within the microenvironment. The definition of these aspects could lead to an increased knowledge about the biology of LXR/oxysterol signaling in the pathophysiology of tumors and could add possible antitumor treatments to the current therapeutic scenario of antineoplastic molecules, which have prolonged the overall survival of patients bearing certain types of tumors [1, 4]. Finally, as anticipated in the Introduction, the possibility of inhibiting tumor cells while restoring immune responses by exploiting a single molecule/strategy is of exceptional value for a cancer treatment strategy, because it could result in synergistic antitumor effects with acceptable side effects relative to recently tested combination strategies based on the use of targeted therapy (e.g., the BRAF inhibitor vemurafenib)

and immune checkpoint blockade (e.g., the anti-CTLA-4 antibody ipilimumab) showing undesirable toxicities [59].

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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