Mechanisms by which CAR-T-cell-derived extracellular vesicles induce ferrocytosis in tumor cells: a theoretical review

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Abstract

CAR-T-cell-derived extracellular vesicles (CAR-T-EVs) contain both antigen-recognition-related molecules and bioactive cargo from CAR-T cells, and are regarded as a potential cell-free extension of the anti-tumor function of CAR-T cells. This article provides a theoretical review of the possible mechanisms by which CAR-T-EVs regulate tumor cell sensitivity to ferroptosis. Based on current evidence, CAR-T-EVs may activate the IFN-JAK/STAT1-IRF1 signalling axis by delivering active molecules such as interferon, thereby downregulating SLC7A11/SLC3A2, reducing cysteine uptake and GSH synthesis, and consequently weakening GPX4-mediated antioxidant defence; concurrently, IRF1 may upregulate LPCAT3 and, in synergy with ACSL4, promote the activation of polyunsaturated fatty acids (PUFAs) and the remodelling of PUFA-PE membrane phospholipids, thereby increasing the pool of oxidisable lipid substrates. NCOA4-mediated ferritin autophagy may increase the labile iron pool, promoting lipid peroxidation via the Fenton reaction and enzymatic/non-enzymatic lipid oxidation pathways. Furthermore, the FSP1-CoQ10 pathway acts as a parallel antioxidant defence mechanism; EVs derived from the tumor microenvironment can negatively regulate ferroptosis, whilst the release of ferroptosis-associated DAMPs and membrane repair mechanisms also influence the ultimate immune response and treatment safety. This study has established an integrated mechanistic framework for how CAR-T-EVs enhance tumor sensitivity to ferroptosis; however, the relevant direct causal chains have not yet been fully established, and further experimental research is required to verify their key cargo, target molecules and cellular selectivity.

Keywords: CAR-T-cell-derived vesicles; ferrocytosis; interferon signalling; lipid peroxidation; ACSL4; GPX4; tumor microenvironment

1 Introduction

Ferroptosis is a regulated form of cell death characterised primarily by the accumulation of iron-dependent lipid peroxides; its mechanism of action differs from that of classical apoptosis and is primarily regulated by the combined effects of cell membrane lipid composition, redox status and iron metabolism[1, 2]. Recent study has shown that ferroptosis is not determined by a single molecular switch, but rather depends on the dynamic balance between the formation of lipid peroxidation substrates and the body's antioxidant defences[3]. In particular, ACSL4 is involved in the activation of polyunsaturated fatty acids, whilst LPCAT3 is involved in their re-synthesis into membrane phospholipids; together, they influence the abundance of oxidisable lipid substrates in the cell membrane[4,5]； SLC7A11-mediated cysteine uptake, glutathione (GSH) synthesis and GPX4 activity constitute an important defence system that limits lipid peroxidation[6]. Furthermore, the FSP1-CoQ10

pathway may serve as a parallel antioxidant defence mechanism alongside GPX4, whilst NCOA4-mediated ferritin autophagy can influence intracellular levels of available iron, thereby regulating sensitivity to ferroptosis[7, 8]. Consequently, by altering the supply of substrates for lipid peroxidation, compromising antioxidant defences or regulating intracellular iron homeostasis, it may be possible to increase the susceptibility of tumor cells to ferrocytosis, thereby providing new avenues for metabolic intervention in cancer treatment.

Chimeric antigen receptor T-cell-derived extracellular vesicles (CAR-T-EVs) offer a promising potential mechanism for linking tumor-targeted recognition with the regulation of ferroptosis. CAR-T cells are capable of releasing extracellular vesicles carrying bioactive components such as proteins, lipids and nucleic acids; CAR-related molecules can also be detected in some of these vesicles, and they are therefore regarded as a cell-free extension of the anti-tumor function of CAR-T cells[9]. Previous studies have shown that CAR-associated vesicles are capable of recognising antigen-positive target cells and exerting cytotoxic effects in specific models, providing experimental evidence for their potential use as therapeutic vectors[10, 11].

At the same time, there is a clear mechanistic link between immune effects and tumor ferroptosis: activated $CD8^+$ T cells release interferon-γ(IFN-γ), which downregulates the expression of SLC7A11 and SLC3A2 in tumor cells, reduces cysteine uptake and promotes lipid peroxidation, thereby enhancing the susceptibility of tumor cells to ferroptosis[12]. Furthermore, IFN-related signalling and its downstream molecules, such as STAT1 and IRF1, have also been shown to participate in the regulation of tumor ferroptosis; studies demonstrating that engineered CAR-T cells secrete IFN-κ and promote tumor ferroptosis via the IFNAR/STAT1/ACSL4 axis further suggest a potential mechanistic link between CAR-T-related immune signalling and ferroptosis[13, 14].

Against this background, this paper aims to explore the potential mechanisms by which CAR-T-EVs intervene in the reprogramming of tumor ferroptosis, based on their biological characteristics and potential targeted delivery capabilities, and in conjunction with the key regulatory networks of tumor ferroptosis. It further seeks to construct an integrated mechanistic framework for CAR-T-EVs-mediated sensitisation to tumor ferroptosis, with a view to providing a theoretical basis for subsequent experimental research and the design of therapeutic strategies. It should be emphasised that the complete causal mechanism by which CAR-T-EVs directly induce tumor ferroptosis has not yet been fully substantiated; the relevant pathways discussed in this paper should be regarded as mechanistic hypotheses proposed on the basis of existing research, rather than established biological processes.

2 The Biological Basis of Extracellular Vesicles Derived from CAR-T Cells: Formation, Cargo and Targeted Delivery

2.1 Generation and Release of CAR-T-EVs

Extracellular vesicles (EVs) are lipid bilayer-bound particles actively released by cells into the extracellular environment; they play a widespread role in the transport of substances and signal exchange between cells. Based on their biogenesis pathways,

EVs typically comprise various subclasses, including exosomes and microvesicles. Among these, exosomes primarily originate from the endosomal system; their formation usually begins with invagination of the cell membrane and the formation of early endosomes, followed by the gradual maturation of these early endosomes and the budding of intraluminal vesicles (ILVs) through the endosomal membrane[15].

When multivesicular bodies (MVBs), containing multiple ILVs, fuse with the plasma membrane, the ILVs are released into the extracellular space, becoming bioactive exosomes[16]. This process is regulated by various membrane transport and vesicle sorting mechanisms, including the endosomal sorting complexes required for transport (ESCRT) and tetraspanins. Among these, Rab proteins are involved in the intracellular transport and localisation of multivesicular bodies, whilst SNARE complexes participate in the process of membrane fusion between multivesicular bodies and the plasma membrane, jointly influencing vesicle formation and release[16].

The generation of CAR-T cell-derived EVs shares a similar cell biological basis with the fundamental processes described above; however, their release levels and molecular composition may also be influenced by the activation status of the parent T cells, CAR conformation, culture conditions and cellular metabolic state. Previous studies have indicated that T-cell activation can alter the release process of extracellular vesicles, suggesting that CAR-T-EVs are not naturally occurring particles with a fixed composition, but rather state-dependent biological products[17, 18]. Consequently, CAR-T-EVs obtained from different sources and under different preparation conditions may differ in terms of particle number, membrane protein expression and the composition of their internal cargo.

2.2 Generation and Release of CAR-T-EVs

The potential therapeutic value of CAR-T-EVs depends not only on their membrane surface structure but is also closely related to the various bioactive cargoes they carry. Similar to their parent CAR-T cells, CAR-T-EVs are capable of carrying components such as proteins, nucleic acids and lipids derived from the parent cells, and delivering these molecules to recipient cells via vesicular transport, thereby influencing the recipient cells' signal transduction, gene expression and biological behaviour[17, 18]. Among these, proteins are currently a class of functional cargo receiving significant attention in CAR-T-EV research. Previous studies have detected CAR molecules and other T-cell-associated membrane proteins in CAR-related EVs, whilst also reporting that they carry cytotoxicity-related proteins such as perforin and granzymes[10, 11, 18]. Perforin contributes to changes in target cell membrane permeability, facilitating the entry of granzymes into target cells; granzymes, in turn, act on intracellular apoptosis-related molecules, thereby inducing cell death[19]. Furthermore, some studies have reported the presence of death receptor ligands, such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), on the surface of CAR-T-Exos; these may activate death receptor-related signalling pathways by binding to corresponding receptors on the surface of target cells[18].

In addition to proteins, nucleic acids are also important potential functional components of CAR-T-EVs. EVs can carry a variety of RNA molecules, including messenger RNA (mRNA), microRNA (miRNA) and other non-coding RNAs, and exert their effects by modulating the transcriptional regulation or signalling networks of recipient cells. Research by Johnson et al. found that RN7SL1, an immunostimulatory RNA associated with CAR-T cells, can participate in the regulation of both CAR-T cell-mediated and endogenous immune responses, suggesting that CAR-T cell-derived RNA may possess immunomodulatory potential that goes beyond traditional cytotoxic effects[20]. However, it is important to distinguish that the presence of a particular RNA in parental CAR-T cells does not necessarily mean that this RNA is effectively loaded into EVs, nor does it directly prove that it can be delivered to tumor cells via EVs and produce the same effects. In recent years, research into engineered CAR-T-EVs has further expanded the scope of applications for nucleic acid cargo. For example, studies have attempted to load miR-17-5p inhibitors into engineered CAR-T-derived EVs to modulate molecular processes associated with hepatocellular carcinoma, suggesting that altering the internal nucleic acid composition of EVs may confer new biological functions upon them[21].

2.3 Targeted Recognition and Intracellular Delivery of CAR-T-EVs

An important potential characteristic distinguishing CAR-T-EVs from EVs derived from ordinary T cells is that they may retain some of the chimeric antigen receptor (CAR)-associated membrane molecules from the parent CAR-T cells. A CAR typically comprises an extracellular antigen-binding domain, a transmembrane domain and an intracellular signalling domain; the extracellular antigen-binding domain is often derived from a single-chain variable fragment (scFv) and is capable of recognising specific tumor-associated antigens[22]. If CAR molecules can be presented on the EV membrane surface with the correct spatial orientation and functional conformation, this could theoretically enable CAR-T-EVs to interact with tumor cells expressing the corresponding antigen, thereby increasing the probability of their binding to the target cell surface[10, 11, 18]. Fu et al. reported that CAR-positive exosomes derived from effector CAR-T cells possess antitumor activity; recent studies have further observed that EVs derived from CD19-CAR-positive T cells are capable of expressing CAR-related molecules and exert a cytotoxic effect on CD19-positive leukaemia cells[10, 11]. These findings provide experimental evidence that CAR-associated EVs possess antigen-specific recognition capabilities.

Following interaction with target cells, CAR-T-EVs must undergo appropriate cellular uptake and intracellular transport processes before their internal cargo can exert its biological effects. Generally speaking, EVs may interact with receptor cells via membrane surface molecules, or enter receptor cells via pathways such as endocytosis; some vesicles may also release their internal cargo through membrane fusion or other membrane transport processes[16]. Once inside the cell, EVs may follow different pathways, such as endosomal transport, lysosomal degradation or the

release of their intracellular cargo. In addition to natural CAR-mediated recognition, engineered modifications also offer new possibilities for enhancing the targeting capabilities of CAR-T-EVs. For example, by altering the composition of surface molecules on the EVs, introducing specific recognition ligands, or hybridising them with other nanocarriers, attempts can be made to improve the vesicles' ability to bind to target cells and deliver drugs[23].

3 Sensitisation of tumor cells to ferrocytosis mediated by the IFN-STAT1-IRF1 signalling axis

Interferon (IFN) signalling serves as a crucial molecular bridge linking anti-tumor immunity and ferroptosis in tumor cells. Recent studies have shown that interferon released by immune cells not only regulates the immune recognition of tumor cells and the anti-tumor response, but also renders tumor cells more susceptible to ferroptosis by altering intracellular antioxidant defences and the metabolism of polyunsaturated fatty acid phospholipids[12, 13]. Among these, the JAK-STAT1-IRF1 signalling axis has attracted considerable attention: following binding of interferon to its corresponding receptor, the receptor-associated JAK kinase is activated, promoting the phosphorylation and nuclear translocation of STAT1, and further inducing the expression of downstream transcriptional regulators such as IRF1, ultimately influencing ferroptosis-related processes including cysteine uptake, glutathione metabolism and membrane phospholipid oxidation[24].

3.1 Activation of the interferon receptor and the JAK-STAT1-IRF1 signalling pathway

In the anti-tumor immune response, IFN-γ is a key cytokine linking the effector function of T cells to the susceptibility of tumor cells to ferroptosis. IFN-γ is primarily produced by activated $CD8^+$ T cells and other immune cells; it binds to the IFN-γ receptor complex (IFNGR1/IFNGR2) on the surface of tumor cells, thereby activating the receptor-associated JAK1 and JAK2 kinases, which in turn promote the phosphorylation of STAT1[25]. Activated STAT1 forms homodimers and translocates into the cell nucleus, where it regulates the expression of various interferon response genes, including IRF1[26]. Consequently, IFN-γ not only enhances the expression of immune-related genes in tumor cells but may also reshape their redox homeostasis and lipid metabolism, thereby creating conditions for subsequent iron-dependent lipid peroxidation.

It should be noted in particular that IFN-κ and IFN-γ do not exert their effects via the same receptor system. IFN-κ belongs to the type I interferon family and primarily initiates signalling via the IFNAR1/IFNAR2 receptor complex, thereby activating JAK1/TYK2 and STAT family-related pathways; whereas IFN-γ initiates signalling via the IFNGR1/IFNGR2 receptor complex[27]. Although both types of interferon may converge on STAT1-mediated transcriptional regulation, IFN-κ-mediated IFNAR signalling and IFN-γ-mediated IFNGR signalling cannot be regarded as entirely identical processes. Gao et al. reported that engineered CAR-T cells secreting IFN-κ can promote ferroptosis in tumor cells via the IFNAR/STAT1/ACSL4 axis,

suggesting that type I interferon signalling may also be involved in the regulation of ferroptosis associated with immunotherapy[14].

3.2 The STAT1-IRF1 signalling pathway suppresses antioxidant defences and lowers the ferroptosis threshold in tumor cells

The IFN-STAT1-IRF1 signalling pathway promotes ferroptosis sensitisation primarily by impairing the ability of tumor cells to maintain glutathione (GSH) homeostasis. The cysteine/glutamate antiporter system Xc^-, comprising SLC7A11 and SLC3A2, is responsible for transporting extracellular cysteine into the cell, thereby providing substrates for cysteine synthesis and GSH production[28]. GSH serves as a crucial reducing co-factor for the function of glutathione peroxidase 4 (GPX4), which limits the sustained accumulation of lipid peroxides by scavenging membrane phospholipid hydroperoxides[29]. When cysteine uptake mediated by the Xc^- system is inhibited, the intracellular supply of cysteine and GSH levels decrease; consequently, the ability of GPX4 to scavenge lipid peroxides is impaired, making tumor cells more susceptible to ferroptosis[30].

In their research on tumor immunity, Wang et al. found that IFN-γ released by $CD8^+$ T cells can suppress the expression of SLC7A11 and SLC3A2 in tumor cells, thereby reducing cystine uptake and increasing the susceptibility of tumor cells to lipid peroxidation and ferrocytosis [12]. This finding establishes a direct functional link between immune cell-derived IFN-γ and tumor ferroptosis, and also suggests that interferon signalling may promote ferroptosis by compromising the tumor cells' antioxidant defences, rather than simply increasing intracellular iron levels. This mechanism is of significant importance, as it demonstrates that the susceptibility of tumor cells to ferroptosis depends not only on the enhancement of pro-oxidative factors, but also on the cells' ability to maintain the critical defence system comprising System Xc^- -GSH-GPX4. Building on this, STAT1 and its downstream target IRF1 may be further involved in regulating the transcriptional programmes associated with tumor cell ferroptosis. The IFN-γ-induced STAT1-IRF1 signalling pathway is capable of translating immune stimulation into an intracellular transcriptional response within tumor cells, which is linked to changes in lipid metabolism and antioxidant defence.

3.3 The IRF1-LPCAT3 axis promotes the oxidation of membrane phospholipids

In addition to inhibiting the antioxidant defence mediated by the Xc^- system, interferon signalling can also increase the substrate pool for lipid peroxidation in tumor cells by regulating phospholipid metabolism. Lysophosphatidylcholine acyltransferase 3 (LPCAT3)is involved in the acyl reorganisation of polyunsaturated fatty acids into membrane phospholipids, thereby promoting the formation of phospholipids containing polyunsaturated fatty acids[31, 32]. Such phospholipids are susceptible to attack by reactive oxygen species and iron-dependent oxidative reactions; the continuous accumulation of their oxidation products can disrupt the structure and function of the cell membrane[33]. Consequently, changes in LPCAT3

expression and activity can influence the composition of cell membrane lipids and alter the susceptibility of tumor cells to ferrocytosis.

Tao et al. further elucidated the link between the IFN-γ-STAT1-IRF1 signalling pathway and LPCAT3 in melanoma and lung cancer models. The study found that co-treatment with IFN-γ and the drug mefloquine enhanced STAT1 phosphorylation and IRF1 expression, and up-regulated LPCAT3; chromatin immunoprecipitation experiments demonstrated that IRF1 is capable of binding to the LPCAT3 promoter region. Upon further knockdown of LPCAT3, both the lipid peroxidation and tumor cell death induced by the combined treatment were suppressed, suggesting that LPCAT3 is a key downstream effector molecule promoting ferroptosis in this model[13]. This study provides further insight into the mechanism of action of the IFN-γ-STAT1-IRF1 axis at the level of transcriptional regulation: interferon signalling may not only weaken the system Xc^- -GSH-GPX4 antioxidant defence system in tumor cells, but also regulate LPCAT3 via IRF1, thereby increasing the levels of easily oxidised membrane phospholipid substrates and creating an intracellular environment that promotes ferroptosis.

It is worth noting that there is a functional link between LPCAT3-mediated lipid remodelling and ACSL4-mediated activation of polyunsaturated fatty acids. ACSL4 promotes the conversion of polyunsaturated fatty acids, such as arachidonic acid, into the corresponding lyso-coenzyme A, whilst LPCAT3 is involved in their subsequent incorporation into membrane phospholipids. Together, they influence the composition of oxidisable polyunsaturated fatty acid phospholipids in the cell membrane. Consequently, IFN signalling that regulates either ACSL4 or LPCAT3 may alter the lipid peroxidation sensitivity of tumor cells; however, the specific regulatory mechanisms depend on factors such as the interferon subtype and the tumor cell background.

4. The ACSL4-LPCAT3-PUFA-PE lipid metabolic pathway and the regulation of ferrocytosis sensitivity

Unlike a simple increase in reactive oxygen species, ferroptosis also requires the presence of sufficient oxidisable lipid substrates in the cell membrane. Among these, phospholipids containing polyunsaturated fatty acids (PUFAs) are key targets of lipid peroxidation, particularly phosphatidylethanolamine (PE) containing arachidonic acid (AA) and adrenic acid (AdA)[34]. ACSL4 and LPCAT3 are key metabolic enzymes that regulate the formation of these membrane lipids: ACSL4 is responsible for activating free PUFAs into lyso-coenzyme A, whilst LPCAT3 is involved in incorporating the activated fatty acids into phospholipid molecules, thereby influencing the composition and content of PUFA-PE in the cell membrane[35, 36]. When these highly oxidisable phospholipids undergo further oxidation, and intracellular antioxidant systems such as GPX4 are unable to clear the oxidation products in a timely manner, lipid peroxidation may continue to escalate, ultimately inducing ferroptosis[37]. Consequently, the ACSL4-LPCAT3-PUFA-PE pathway primarily determines whether cells possess sufficient lipid substrates for ferroptosis and serves as a crucial link between lipid metabolism and ferroptosis sensitivity[2, 4].

4.1 ACSL4-mediated activation of polyunsaturated fatty acids: providing substrates for membrane phospholipid remodelling

ACSL4 (acyl-CoA synthetase long-chain family member 4) belongs to the long-chain fatty acid coenzyme A synthetase family; its primary function is to catalyse the binding of long-chain fatty acids to coenzyme A, thereby converting free fatty acids into the corresponding acyl-CoA[38]. In ferroptosis-related metabolic processes, ACSL4 is particularly involved in the activation of polyunsaturated fatty acids such as arachidonic acid and adrenic acid, generating metabolic intermediates such as AA-CoA and AdA-CoA[39]. This process does not directly produce lipid peroxides, but rather provides the necessary activated substrates for subsequent membrane phospholipid synthesis and fatty acid remodelling.

The regulatory role of ACSL4 in iron death susceptibility has been extensively studied. Doll et al. found that ACSL4 can determine a cell's susceptibility to iron death by altering its lipid composition; loss of ACSL4 function alters the composition of polyunsaturated fatty acid phospholipids in the cell membrane and renders cells resistant to iron death-inducing stimuli[4]. This suggests that ACSL4 is not merely a molecule undergoing changes as a consequence of ferrocytosis, but rather one that influences cellular susceptibility to ferrocytosis at an upstream stage of lipid metabolism.

From a mechanistic perspective, increased ACSL4 expression or activity can enhance the formation of PUFA-CoA, thereby providing substrates for the subsequent incorporation of PUFAs into membrane phospholipids; conversely, reduced ACSL4 expression or functional inhibition may limit the incorporation of oxidisable fatty acids into membrane phospholipids. Consequently, upregulation of ACSL4 generally favours the creation of a lipid environment that promotes ferroptosis, whilst downregulation of ACSL4 may reduce the lipid peroxidation potential of the cell membrane. However, elevated ACSL4 levels do not in themselves imply that ferroptosis has occurred; its actual effects still depend on the supply of PUFAs, the capacity for downstream phospholipid remodelling, and the state of the cell's antioxidant defences.

4.2 LPCAT3-mediated phospholipid reorganisation: promoting the formation of oxidisable PUFA-PE

PUFA-CoA generated by ACSL4 must subsequently enter the membrane phospholipid metabolic pathway in order to be converted into lipid peroxidation substrates associated with ferroptosis. LPCAT3 (lysophosphatidylcholine acyltransferase 3) is one of the key acyltransferases involved in phospholipid acylation and is a crucial regulatory molecule in the Lands' cycle[40]. Its primary function is to utilise acyl-CoA as an acyl donor to reattach fatty acids to lysophospholipid molecules, thereby altering the composition of the fatty acid side chains of the phospholipids.

In the lipid metabolic processes associated with ferroptosis, PUFA-CoA activated by ACSL4 provides substrates for LPCAT3-mediated phospholipid

remodelling, enabling polyunsaturated fatty acids to incorporate into membrane phospholipids and thereby increasing the proportion of easily oxidisable lipid molecules in the cell membrane[41]. In particular, PE containing AA or AdA side chains has a polyunsaturated fatty acid structure that is prone to oxidation and is therefore considered an important lipid substrate in the ferroptosis process. It should be noted that ACSL4 and LPCAT3 are not enzymes that catalyse the same reaction: the former is responsible for fatty acid activation, whilst the latter is involved in the reincorporation of fatty acids into phospholipid molecules[42]. The two are interconnected in terms of their metabolic functions and jointly influence the formation of PUFA phospholipids in the cell membrane.

In their study on the inhibition of LPCAT3, Reed et al. found that, following inhibition of LPCAT3 function, the composition of polyunsaturated phospholipids within the cells changed, and the cells' susceptibility to ferrocytosis consequently decreased[5]. This result suggests that the process of phospholipid remodelling involving LPCAT3 influences the formation of ferrocide substrates, rather than merely representing general membrane lipid metabolism. Consequently, ACSL4 and LPCAT3 can be regarded as two key metabolic nodes in the formation of ferrocide lipid substrates: ACSL4 determines whether PUFAs can be effectively activated, whilst LPCAT3 plays a role in determining whether these fatty acids can incorporate into the corresponding membrane phospholipid structures.

4.3 PUFA-PE oxidation and the accumulation of its products: the lipid-mediated mechanism of ferroptosis

When PUFAs incorporate into membrane phospholipids to form PUFA-PE, the fatty acid side chains—which contain multiple double bonds—become key targets for lipid oxidation reactions. In an oxidative environment involving iron, PUFA-PE can undergo a series of oxidative reactions to form phospholipid hydroperoxides (PLOOH), of which oxidised PE is a particularly important class of product in ferrocide research[43].

Research by Kagan et al. has further clarified the role of oxidised PE in ferroptosis. The study found that PE containing AA and AdA can form specific oxidised phospholipid products; these oxidised PEs are closely associated with the occurrence of ferroptosis, providing direct lipidomic evidence that PUFA-PE acts as a key lipid substrate for ferroptosis[44]. This implies that the lipid remodelling mediated by ACSL4 and LPCAT3 does not merely alter the physical properties of the cell membrane, but is also capable of influencing whether cells possess the substrate basis necessary for the formation of specific ferroptosis-related oxidised phospholipids.

PUFA-PE oxidation can occur via enzymatic and non-enzymatic pathways. Enzymatic oxidation primarily involves members of the lipoxygenase (ALOX) family, which can participate in the oxidation of polyunsaturated fatty acids and related phospholipid substrates; non-enzymatic oxidation, on the other hand, is associated with iron-mediated free radical reactions and lipid free radical chain propagation[45, 46]. Both pathways may contribute to the process of ferrocytosis, with their relative contributions depending on cell type, the composition of lipid substrates and the redox

state. Consequently, not all PUFA-PE oxidation associated with ferrocytosis can be attributed to a single ALOX enzyme.

Under normal circumstances, cells can eliminate phospholipid hydroperoxides via GPX4. GPX4 utilises GSH as a reducing co-factor to reduce harmful phospholipid hydroperoxides to their corresponding phospholipid alcohols, thereby limiting the further propagation of lipid peroxidation[6]. When GSH supply is insufficient or GPX4 activity is reduced, the capacity to clear oxidised phospholipids is diminished, and PLOOH tends to accumulate in the cell membrane[47]. As the lipid peroxidation reaction continues to propagate, membrane structure and biological function are disrupted, ultimately leading to ferroptosis.

It can thus be seen that the ACSL4-LPCAT3 pathway and the GPX4 antioxidant system act on different stages of the ferroptosis process: the former increases the availability of easily oxidised phospholipid substrates, whilst the latter is responsible for scavenging lipid peroxides that have already formed. The dynamic balance between the two determines whether cells can control oxidative damage to membrane lipids and also constitutes an important basis for the sensitivity of tumor cells to ferroptosis.

4.4 Regulation of the ACSL4-LPCAT3 pathway by upstream signals

ACSL4 and LPCAT3 do not act in isolation; their expression and functional status are also regulated by upstream signalling pathways. The IFN-STAT1-IRF1 signalling axis discussed in the previous section is one of the key mechanisms linking immune stimulation to the reorganisation of lipid metabolism. Furthermore, the function of ACSL4 is also regulated by post-translational modifications.

Zhang et al. found that protein kinase CβII (PKCβII) is capable of phosphorylating ACSL4, thereby enhancing its pro-lipid peroxidation activity and promoting ferroptosis[48]. This finding suggests that the lipid metabolic effects mediated by ACSL4 are not only related to its expression levels but may also be regulated by upstream protein kinase signalling. Therefore, when studying ferroptosis in tumor cells, merely detecting total ACSL4 protein expression may not be sufficient to fully reflect its functional status; consideration should also be given to relevant post-translational modifications and their impact on lipid metabolism.

Taken as a whole, there appears to be a synergistic relationship between the IFN-STAT1-IRF1 signalling pathway and the ACSL4-LPCAT3-PUFA-PE pathway: interferon signalling promotes the expression or activity of specific lipid metabolic nodes, leading to the formation of more oxidisable membrane phospholipids in tumor cells; at the same time, the inhibition of the system Xc^- -GSH-GPX4 defence system by IFN signalling reduces the capacity to clear oxidised phospholipids. Ultimately, the combined effect of increased pro-oxidant substrates and weakened antioxidant defences drives the accumulation of lipid peroxides. This synergistic regulation may represent an important metabolic basis for the enhancement of tumor cell ferroptosis sensitivity by immunotherapy.

5 NCOA4-mediated ferritin autophagy, the active iron pool and iron-dependent

lipid oxidation

5.1 NCOA4-mediated ferritin autophagy and its role in ferroptosis

Intracellular iron does not exist entirely in a free state, but is primarily stored in forms such as ferritin to prevent oxidative damage caused by excess reactive iron. Ferritin consists mainly of a ferritin heavy chain (FTH1) and a light chain (FTL), with FTH1 possessing ferrooxidase activity, which is involved in the oxidation of Fe^{2+} to Fe^{3+} and facilitates its storage. When cells need to mobilise stored iron, ferritin can be degraded via the autophagy-lysosomal pathway, thereby releasing the iron it contains. This selective autophagy process is known as ferritinophagy.

NCOA4 (nuclear receptor coactivator 4) is a key cargo receptor that mediates ferritin autophagy. It recognises ferritin and delivers it to the autophagic apparatus, facilitating its entry into autophagosomes and subsequent lysosomal degradation. Mancias et al. were the first to identify NCOA4 as a key cargo receptor mediating ferritin autophagy through quantitative proteomics studies, providing important insights into the selective mobilisation of intracellular iron stores[8].

Hou et al. further investigated the link between ferritin autophagy and ferroptosis. Their study found that inhibiting autophagy-related processes reduced intracellular ferrous iron levels and lipid peroxidation, and attenuated erastin-induced ferroptosis; building on this, the study further confirmed that genetic inhibition of NCOA4 blocks ferritin degradation and suppresses ferroptosis, whilst NCOA4 overexpression promotes ferritin degradation and enhances ferroptosis[49]. This suggests that NCOA4-mediated ferritin autophagy not only contributes to the maintenance of cellular iron homeostasis but also provides a source of iron for iron-dependent lipid peroxidation by mobilising stored iron from ferritin.

It should be noted that increased NCOA4 expression does not necessarily imply that ferroptosis will occur in cells. Its actual effect also depends on the extent of ferritin degradation, the cell's capacity for iron uptake and efflux, and whether antioxidant systems such as GPX4 can promptly clear lipid peroxides. Therefore, NCOA4 is best understood as an upstream iron mobilisation node regulating sensitivity to ferroptosis, rather than as an executor factor that single-handedly determines the occurrence of ferroptosis.

5.2 The Formation of Active Iron Pools and Their Dynamic Regulation

The effect of ferritin autophagy on ferroptosis is primarily manifested in altering the balance between intracellular stored iron and available iron. The iron within the cell that can participate in redox reactions is commonly referred to as the labile iron pool (LIP), which includes Fe^{2+} with redox activity[49, 50]. Unlike the relatively stable stored iron within ferritin, the iron in the LIP is capable of participating in oxidation reactions and influences the cell's sensitivity to ferroptosis-inducing stimuli.

Intracellular LIP is not determined solely by ferritin autophagy, but is regulated by a combination of multiple processes. Transferrin receptor 1 (TFRC) mediates the cellular uptake of iron bound to transferrin; divalent metal transporter 1 (DMT1) is involved in iron transport within endosomes; FTH1 and FTL are involved in iron storage; whilst the iron efflux protein SLC40A1 (ferroportin, FPN1) is responsible for

transporting intracellular iron out of the cell[51-53]. Consequently, intracellular levels of active iron are in fact determined by the dynamic balance between iron uptake, storage, mobilisation and efflux.

During ferrocytosis, NCOA4-mediated ferritin autophagy promotes the release of stored iron, allowing some of this iron to enter the utilisable iron pool and increasing the availability of intracellular Fe^{2+} [54]. When cells simultaneously harbour high levels of oxidisable polyunsaturated fatty acid phospholipids, whilst antioxidant defence capabilities—such as those of GPX4—are insufficient, the increase in reactive iron may further drive lipid peroxidation. Consequently, ferritin autophagy and lipid metabolism, as well as antioxidant defence, are synergistic rather than independent processes.

5.3 Active iron is involved in iron-dependent lipid oxidation

An increase in intracellular Fe^{2+} can promote lipid peroxidation via multiple pathways. Among these, the classic Fenton reaction is a process in which Fe^{2+} reacts with hydrogen peroxide to generate reactive oxygen species, such as hydroxyl radicals[57]. These reactive oxygen species can attack polyunsaturated fatty acid phospholipids in the cell membrane, triggering a lipid radical chain reaction and promoting the accumulation of lipid peroxides[55, 56].

However, the Fenton reaction does not fully account for the lipid oxidation process in ferrocytosis. Iron may also participate in enzyme-mediated lipid oxidation reactions involving lipoxygenases (LOX), promoting the formation of corresponding lipid hydroperoxides from polyunsaturated fatty acid phospholipids[57]. At the same time, elevated intracellular Fe^{2+} levels may also enhance non-enzymatic lipid oxidation[58].

Consequently, an increase in the pool of reactive iron can promote lipid peroxidation at multiple levels; however, its effect is still jointly influenced by the composition of membrane lipid substrates and the cell's antioxidant capacity.
In light of the preceding discussion, ACSL4 promotes the activation of polyunsaturated fatty acids such as arachidonic acid and adrenic acid, whilst LPCAT3 is involved in their incorporation into membrane phospholipids, thereby increasing the pool of oxidisable phospholipid substrates. When NCOA4-mediated ferritin autophagy further elevates intracellular available iron levels, the cell simultaneously possesses both the easily oxidisable substrates and the iron-dependent oxidative conditions required for lipid peroxidation. If the GPX4-GSH system fails to effectively scavenge the generated lipid peroxides, oxidisable phospholipids may accumulate persistently, ultimately inducing ferroptosis.

5.4 Synergistic Interaction between Ferritin Autophagy and the Antioxidant Defence System

The occurrence of ferroptosis does not depend solely on intracellular iron levels, but is rather the combined result of an imbalance between iron-dependent lipid oxidation and antioxidant defences. NCOA4-mediated ferritin autophagy primarily influences the iron supply, whilst the GPX4-GSH system is responsible for the

clearance of lipid peroxides; both act on different stages of the ferroptosis regulatory process.

When NCOA4-mediated ferritin autophagy is enhanced, intracellular iron stores may be mobilised, increasing the availability of Fe^{2+} for oxidative reactions[59]. If this is accompanied by reduced SLC7A11 expression, restricted cysteine uptake and insufficient GSH synthesis, the ability of GPX4 to scavenge phospholipid hydroperoxides will be impaired, thereby facilitating the accumulation of lipid peroxides. Conversely, if GPX4 function remains at a high level, even an increase in intracellular reactive iron may not necessarily be sufficient to trigger significant ferroptosis.

Furthermore, the FSP1-CoQ10 system is a parallel antioxidant defence pathway distinct from the GPX4-GSH system, capable of inhibiting lipid free radical chain reactions by maintaining the reduced state of coenzyme Q10[60]. Consequently, when assessing whether NCOA4-mediated ferritin autophagy truly promotes ferrocytosis, the compensatory roles of the GPX4 and FSP1 defence pathways must also be taken into account. If only elevated NCOA4 expression or reduced ferritin levels are observed, without measuring active iron, lipid peroxidation and the status of antioxidant defence, this is insufficient to determine whether the cells have entered the process of ferrocytosis.

6 The GPX4-GSH and FSP1-CoQ dual antioxidant defence system: a key inhibitory mechanism in ferroptosis

Ferroptosis does not necessarily occur simply because of an increase in intracellular iron or enhanced membrane lipid peroxidation; the key lies in whether the cell can clear lipid peroxides in a timely manner. In this regard, the GPX4-GSH system and the FSP1-CoQ system constitute two independent anti-ferroptotic defence pathways that exhibit a certain degree of compensatory interaction: the former directly reduces membrane phospholipid hydroperoxides, whilst the latter inhibits lipid radical chain reactions by maintaining reduced coenzyme Q. These two pathways jointly determine the sensitivity of tumor cells to ferrocytosis stimuli.

6.1 The System Xc^- -GSH pathway provides GPX4 with reducing substrates

Glutathione peroxidase 4 (GPX4) is one of the key antioxidant enzymes involved in cellular defence against ferroptosis. Its primary function is to reduce phospholipid hydroperoxides (PLOOH) in membrane phospholipids to relatively stable phospholipidols (PLOH), thereby preventing the continued accumulation of lipid peroxides[61]. Unlike some antioxidant enzymes that primarily clear aqueous-phase peroxides, GPX4 is capable of acting on lipid peroxides within membrane structures; it therefore plays a vital role in maintaining the redox homeostasis of cell membrane lipids[6].

GPX4 requires reduced glutathione (GSH) as a reducing substrate to exert its enzymatic activity, whilst the synthesis of intracellular GSH depends on the supply of cysteine. System Xc^- is a cysteine/glutamate antiport system located on the cell membrane, primarily comprising the light chain subunit SLC7A11 and the heavy

chain subunit SLC3A2. It is responsible for transporting extracellular cysteine into the cell whilst simultaneously exporting intracellular glutamate. Once inside the cell, cysteine is reduced to cystathionine, which subsequently participates in GSH synthesis. Therefore, SLC7A11-mediated cysteine uptake is a crucial upstream step in maintaining cellular GSH levels and the antioxidant activity of GPX4.

When SLC7A11 expression is reduced or the function of System Xc^- is inhibited, cellular cysteine uptake decreases, leading to an insufficient supply of cysteine and a consequent restriction on GSH synthesis[62] At this point, even if the GPX4 protein remains present, its effective enzymatic activity may decline due to a lack of GSH, resulting in the failure to promptly clear membrane phospholipid hydroperoxides and ultimately increasing the cell's susceptibility to ferroptosis[63, 64]. The classic ferroptosis inducer erastin exerts its effect primarily by inhibiting System Xc^-, whilst RSL3 mainly acts by directly inhibiting GPX4; the two agents target the substrate supply and key enzyme components of the antioxidant defence system, respectively[6].

Consequently, System Xc^--GSH-GPX4 does not consist of three independent molecules, but rather forms a continuous antioxidant metabolic axis: SLC7A11/SLC3A2 are responsible for cysteine uptake; GSH synthesis depends on the supply of cysteine; and GPX4 utilises GSH to scavenge membrane phospholipid peroxides. Inhibition of any key step in this pathway may impair the cell's ability to control lipid peroxidation.

6.2 A decline in GPX4 function leads to the continuous accumulation of lipid peroxides

During ferroptosis, the ACSL4-LPCAT3 pathway increases the levels of membrane phospholipid substrates containing polyunsaturated fatty acids, whilst ferritin autophagy and other iron metabolic processes may elevate intracellular levels of available iron[65]. However, these changes do not necessarily lead to cell death; whether ferroptosis occurs depends on the cell's ability to clear the resulting lipid peroxides in a timely manner. GPX4 acts as a key regulatory node linking the generation and clearance of lipid peroxides.

GPX4 catalyses the reduction of phospholipid hydroperoxides via its selenocysteine active site, converting potentially cytotoxic phospholipid peroxides into relatively stable lipid alcohols[66]. During this process, GSH provides reducing equivalents and is oxidised to glutathione disulphide (GSSG), which can be reduced back to GSH with the participation of glutathione reductase and NADPH[67]. Consequently, the cell establishes a continuous GSH regeneration cycle to maintain the antioxidant function of GPX4.

When GPX4 expression is reduced, enzyme activity is inhibited, or GSH supply is insufficient, phospholipid hydroperoxides in the cell membrane cannot be effectively cleared. As oxidised phospholipids continue to accumulate, chain reactions of lipid free radicals expand further, causing damage to membrane structure and function, ultimately triggering ferroptosis[68]. Research by Yang et al. established the

key role of GPX4 in regulating ferroptosis, demonstrating that inhibition of GPX4 can induce iron-dependent lipid peroxidation and tumor cell death[6].

It should be emphasised that the expression level of the GPX4 protein is not entirely equivalent to its actual antioxidant capacity. Even if no significant decrease in GPX4 protein levels is detected, GPX4 may still be unable to function fully if intracellular GSH is depleted. Therefore, when investigating whether CAR-T-EVs influence tumor cell ferroptosis, their protective capacity should not be assessed solely by detecting GPX4 expression via Western blot; GSH/GSSG levels, lipid ROS and the accumulation of phospholipid peroxides should also be measured concurrently.

6.3 The FSP1-CoQ antioxidant pathway: a parallel defence system independent of GPX4

Although the GPX4-GSH pathway is the central mechanism of ferroptosis defence, some cells are still able to maintain a certain degree of resistance to ferroptosis following the inhibition or loss of GPX4. This suggests that other defence systems capable of limiting lipid peroxidation also exist within cells. In 2019, Bersuker et al. and Doll et al. independently identified ferroptosis suppressor protein 1 (FSP1) through genetic screening, revealing the crucial role of the FSP1 – coenzyme Q (CoQ) pathway in the regulation of ferroptosis[7, 69].

FSP1 was originally termed AIFM2 (apoptosis-inducing factor mitochondria-associated 2); subsequent research revealed that it possesses an anti-ferroptotic function independent of GPX4[70]. FSP1 utilises the reducing power provided by NAD(P)H to reduce oxidised coenzyme Q (ubiquinone, CoQ) to reduced coenzyme Q (ubiquinol, $CoQH_2$)[71]. $CoQH_2$ is a lipid-soluble free-radical-scavenging antioxidant molecule capable of trapping lipid peroxyl radicals within membrane structures, thereby inhibiting the continued propagation of lipid free-radical chain reactions and reducing the accumulation of lipid peroxides[7, 69].

Unlike GPX4, which directly utilises GSH to reduce phospholipid hydroperoxides, the FSP1-CoQ system primarily limits the amplification of lipid radical reactions by regenerating fat-soluble antioxidant molecules[72]. Therefore, although both pathways ultimately inhibit membrane lipid peroxidation, their mechanisms of action differ. GPX4 is more directly involved in the enzymatic reduction of phospholipid hydroperoxides, whilst FSP1 exerts a free radical scavenging effect via $CoQH_2$, thereby blocking lipid oxidation chain reactions.

In terms of subcellular localisation, N-terminal myristoylation of FSP1 facilitates its recruitment to the plasma membrane, enabling it to function as a CoQ reductase in membrane-associated regions[69]. FSP1 expression levels are associated with the resistance to ferroptosis in various tumor cells, and its absence increases cellular sensitivity to GPX4 inhibitors. Related studies have also found that pharmacological

inhibition of FSP1 can act synergistically with GPX4 inhibition to enhance the ferroptotic response in certain tumor cells[7, 69].

Consequently, the FSP1-CoQ pathway is not a downstream component of the GPX4-GSH system, but rather an independent antioxidant defence mechanism that operates in parallel with it. Even when GPX4 function is inhibited, FSP1 may still limit lipid peroxidation by maintaining $CoQH_2$ levels; conversely, when FSP1 function is compromised, the GPX4-GSH system may still assume the primary role in clearing lipid peroxides. This parallel relationship helps to explain, in part, the differences in sensitivity to GPX4 inhibitors observed across different tumor cell types.

7 Iron-mediated cell death, potential immunogenic effects and therapeutic limitations

The preceding sections have discussed the mechanisms by which CAR-T-derived extracellular vesicles (CAR-T-EVs) may influence the susceptibility of tumor cells to ferroptosis, focusing on interferon signalling, membrane lipid metabolism, iron homeostasis and antioxidant defence, respectively. However, ferroptosis is not merely a process characterised by elevated levels of intracellular lipid peroxidation; its ultimate biological significance also depends on cell membrane damage, the release of intracellular contents, feedback from the tumor microenvironment, and the survival status of the effector T cells themselves. Consequently, this section further examines the final execution of ferroptosis and its potential immunological effects, discussing the negative feedback regulation mediated by EVs in the tumor microenvironment, the risk of ferroptosis in CAR-T cells themselves, and the safety concerns associated with CAR-T-EVs as a potential cell-free therapeutic platform.

7.1 The final stages of ferroptosis: lipid peroxidation and cell membrane damage

Ferroptosis begins with the oxidation of polyunsaturated fatty acid phospholipids in the cell membrane and organelle membranes; however, the formation of lipid peroxides does not in itself signify that cell death has been completed. As phospholipid hydroperoxides continue to accumulate, changes occur in the composition, fluidity and structural stability of the membrane lipids, ultimately leading to impaired cell membrane function and cell death[73]. Consequently, the final execution of ferroptosis involves not only the generation of lipid peroxides but also the propagation of oxidative damage across the plasma membrane, as well as whether the cell's ability to repair membrane damage is overwhelmed.

Hirata et al. found that lipid peroxidation can increase plasma membrane tension and promote the opening of mechanosensitive or cation channels, such as Piezo1 and TRP, leading to alterations in intracellular and extracellular ion homeostasis. These changes further exacerbate membrane damage and contribute to the final execution of ferroptosis[74]. This study suggests that the plasma membrane is not merely a passive site of damage caused by lipid peroxidation; changes in its mechanical state and ionic

permeability may also play a role in determining whether a cell crosses the execution threshold for ferroptosis. However, the roles of Piezo1 and TRP channels remain model-dependent and cannot be regarded as essential and universal executor molecules in all ferroptosis models.

Recent studies have further elucidated the regulatory role of cell membrane repair mechanisms in the final stages of ferroptosis. Yang et al. found that the transmembrane protein TMEM16F mediates phospholipid inversion, redistributing membrane lipids in damaged regions, thereby reducing local membrane tension and alleviating plasma membrane damage. In the absence of TMEM16F, cells become more sensitive to ferroptosis and are more prone to plasma membrane collapse and the release of cellular contents[75]. These findings suggest that the final outcome of ferroptosis depends not only on the extent of lipid peroxidation but is also influenced by the cell's ability to repair membrane damage. In other words, even when cells have undergone significant lipid peroxidation, if the membrane repair mechanisms remain effective, they may delay or limit the final breakdown of the membrane.

With regard to CAR-T-EV-mediated ferroptosis in tumor cells, the aforementioned mechanism offers a potential avenue for downstream research: if EV treatment can enhance lipid peroxidation in tumor cells whilst simultaneously impairing their ability to repair membrane damage, it may, in theory, promote the transition of cells from a reversible or sublethal state of oxidative stress to an irreversible stage of terminal damage[76,77]. However, there is currently insufficient evidence to demonstrate that CAR-T-EVs can directly regulate Piezo1, TRP or TMEM16F[78, 79]. Therefore, these molecules should be considered as candidate regulatory nodes in the execution phase of ferroptosis, rather than being directly incorporated into the established CAR-T-EV signalling pathways.

7.2 The release of iron-dependent DAMPs and their potential immunogenic effects

In addition to directly causing tumor cell death, ferroptosis may also influence immune cells in the tumor microenvironment through the release of damage-associated molecular patterns (DAMPs). Following disruption of cell membrane integrity, HMGB1, ATP and other intracellular components may be released into the extracellular space; certain lipid peroxides may also act as immunoregulatory signals, influencing the function of macrophages, dendritic cells (DCs) and other immune cells[80]. Consequently, ferroptosis may translate oxidative damage within tumor cells into local changes in the immune microenvironment, thereby providing a potential basis for its combination with immunotherapy.

It should be noted that the immunological effects of ferroptosis are closely related to the stage of the death process. Using an inducible ferroptosis model, Wiernicki et al. divided the ferroptosis process in tumor cells into three stages: the initial stage, characterised primarily by lipid peroxidation; the intermediate stage, accompanied by ATP release; and the terminal stage, manifested by HMGB1 release and loss of plasma membrane integrity[81]. Studies have found that cells undergoing ferroptosis in the initial phase do not effectively promote DC maturation; rather, they reduce the ability of DCs to phagocytose tumor cells and present antigens in a

cross-presentation manner. Under specific experimental conditions, cells undergoing early ferroptosis can even attenuate the immunoprotective effects induced by immunogenic apoptotic cells[83]. This suggests that lipid peroxidation alone does not necessarily indicate that tumor cells have generated effective immunogenic death signals.

In contrast, terminal-stage ferroptosis is accompanied by a loss of plasma membrane integrity and the release of a greater number of intracellular components, which may generate stronger local inflammatory and immunostimulatory signals. Yang et al. found that the absence of TMEM16F renders ferroptotic cells more susceptible to plasma membrane collapse and the release of DAMPs such as HMGB1 and ATP; in their experimental model, this terminal membrane damage was also associated with a stronger anti-tumor immune response[75]. Furthermore, Li et al. reported that the selective degradation of GPX4 in tumor cells can induce ferroptosis and enhance the anti-tumor immune response, suggesting that, in specific tumor models, the precise induction of ferroptosis may produce synergistic effects with immunotherapy[82].

Consequently, there may be a stage-dependent bidirectional relationship between ferroptosis and anti-tumor immunity: early or incomplete ferroptosis may fail to effectively activate antigen presentation and may even inhibit DC function; whereas, when tumor cells enter the terminal membrane damage stage, the release of DAMPs may enhance local immune stimulation. However, the release of DAMPs does not automatically equate to an effective anti-tumor immune response; this ultimately depends on multiple factors, including DC maturation, antigen uptake and cross-presentation, T-cell recruitment, and the immunosuppressive microenvironment.

7.3 Negative feedback regulation of ferroptosis by EVs from the tumor microenvironment

Although the CAR-T-EVs discussed above may enhance tumor cell sensitivity to ferrocytosis via pathways such as interferon signalling, lipid metabolic reorganisation, ferritin autophagy and the weakening of antioxidant defences, the tumor microenvironment does not passively undergo this process. tumor-associated fibroblasts (CAFs), tumor-associated neutrophils (TANs), tumor-associated macrophages (TAMs) and the tumor cells themselves can all release EVs, which, by transporting proteins, miRNAs and other bioactive cargo, alter the lipid metabolism, iron homeostasis and redox status of tumor cells, thereby exerting a negative regulatory effect on ferroptosis.

Firstly, EVs from the tumor microenvironment can reduce the formation of easily oxidised membrane lipid substrates by regulating ACSL4 and related lipid metabolic processes. Qi et al. found that exosomes released by pancreatic cancer-associated fibroblasts can carry miRNAs that target ACSL4, thereby reducing ACSL4 expression in tumor cells, inhibiting ferroptosis and promoting resistance to gemcitabine[83]. Dong et al., on the other hand, reported that tumor-associated neutrophil EVs derived from gastric cancer inhibit ferroptosis in tumor cells via the miR-9-3p/ACSL4 axis, thereby enhancing chemoresistance[84]. These studies suggest

that microenvironmental EVs can counteract pro-ferroptotic signals at the level of lipid substrate formation.

Secondly, EVs may also reduce the sensitivity of tumor cells to lipid peroxidation by enhancing their antioxidant defences. For example, CAF-derived EVs can enhance the antioxidant capacity of tumor cells by regulating the modification and stability of SLC7A11-associated proteins. A 2026 study further reported that CAF-derived exosomes can inhibit ferroptosis in colorectal cancer cells via GALNT14-mediated O-GalNAc glycosylation of SLC7A11[85]. Furthermore, EVs derived from hypoxic tumor cells have also been reported to promote melanoma resistance by regulating the ubiquitination and stability of SLC7A11 via HIF-1α-related mechanisms[86]. These findings suggest that EVs in the tumor microenvironment not only influence the formation of lipid peroxidation substrates but may also confer resistance to ferroptosis on tumor cells by maintaining System Xc^- function and cellular antioxidant capacity.

Furthermore, EVs from the tumor microenvironment may also inhibit ferroptosis by altering iron homeostasis and the redox status of organelles. Research by Battaglia et al. demonstrated that ovarian cancer cells are able to secrete ferritin-rich cargo via a CD63-associated EV pathway, thereby reducing the accumulation of reactive iron within the cells and conferring resistance to ferroptosis[69]. Furthermore, Zheng et al. reported that macrophages and their derived EVs can modulate resistance to ferrocytosis in tumor cells via PRDX6-mediated mitochondrial autophagy[87]. This suggests that the regulation of ferrocytosis by tumor microenvironment EVs is not limited to individual molecules such as ACSL4 or SLC7A11, but may also involve multiple levels, including iron storage and efflux, mitochondrial function, and organelle redox homeostasis.

In addition to directly inhibiting ferroptosis in tumor cells, tumor-derived EVs can also directly impair CAR-T cell function, thereby indirectly compromising therapeutic efficacy. Zhu et al. found that EVs released by CD19-positive tumor cells can carry the CD19 antigen and continuously stimulate CD19 CAR-T cells, leading to abnormal cytokine release, CAR-T cell exhaustion and reduced anti-tumor activity[88]. Zhong et al. further discovered that small EVs derived from solid tumors can simultaneously carry tumor antigens and PD-L1, preferentially interacting with corresponding CAR-T cells to inhibit their proliferation, migration and effector function[89]. Although these studies do not directly prove that tumor EVs suppress CAR-T function via the ferroptosis pathway, they demonstrate that tumor-derived EVs can establish a therapeutic negative feedback loop at both the immune cell and tumor cell levels.

Taken together, EVs in the tumor microenvironment may form a multi-level ferroptosis resistance network: on the one hand, they reduce the formation of ACSL4-related, easily oxidisable membrane lipid substrates; on the other hand, they enhance SLC7A11-related antioxidant defences and alter intracellular iron and redox states through ferritin efflux or mitochondrial regulation; simultaneously, some tumor-derived EVs can also directly induce abnormal activation or functional exhaustion of CAR-T cells. Consequently, when investigating CAR-T-EV-mediated

ferroptosis, the counteracting effects of EVs from the tumor microenvironment should be taken into account; the final therapeutic outcome should not be judged solely on the basis of the unidirectional effects of CAR-T-EVs on tumor cells.

7.4 Therapeutic Safety and Potential Advantages of Ferroptosis Mediated by CAR-T-EVs

Compared with the direct infusion of live CAR-T cells, CAR-T-derived EVs, as a cell-free therapeutic platform, offer certain developmental advantages. EVs are capable of retaining some of the parent cells' membrane surface molecules and bioactive cargo, and may be engineered to carry specific proteins, nucleic acids or drugs to achieve targeted action against tumor cells. Fu et al. reported that CAR-positive EVs derived from effector CAR-T cells possess antitumor activity and exhibited low toxicity in their experimental model[10]. Lanuti et al. also found that EVs derived from CD19 CAR-T cells are capable of expressing functional CARs and killing leukaemia cells, providing further evidence for CAR-positive EVs as potential cell-free therapeutic tools[11].

From the perspective of ferrocytosis therapy, CAR-T-EVs may possess potential value in two respects. Firstly, EVs can serve as delivery vehicles for functional cargo, delivering molecules that regulate lipid metabolism, antioxidant defence or iron homeostasis to tumor cells, thereby altering their susceptibility to ferrocytosis. Secondly, if functional CAR molecules are retained on the surface of EVs, they may enhance the likelihood of binding to antigen-positive tumor cells through tumor antigen recognition, thereby providing a basis for reducing off-target delivery. However, CAR-mediated antigen binding does not equate to absolute selectivity; the tissue distribution of EVs, non-specific uptake and the efficiency of intracellular cargo release will still influence the final therapeutic outcome.

In terms of safety, CAR-T-EVs lack the autonomous proliferation and sustained expansion capabilities of intact living cells; consequently, they are expected to reduce some of the risks associated with the continuous proliferation of activated T cells or long-term immune stimulation. However, the absence of cells does not equate to the absence of toxicity. Immunologically active molecules on the surface or within EVs may still affect normal tissues and immune cells; furthermore, if the delivered ferroptosis-modulating cargo lacks cell-type specificity, it may also damage the antioxidant defence systems of normal cells[90]. In addition, the in vivo distribution and clearance of EVs, immune responses following repeated administration, and inter-batch variations in cargo all require systematic evaluation.

Of particular concern is the fact that tumor antigen positivity does not necessarily mean that EVs act exclusively on tumor cells. Some tumor antigens may also be expressed at low levels in normal tissues or be present on the surface of other non-tumor cells[91]. Therefore, relying solely on CAR recognition for tumor targeting may not be sufficient to ensure the tissue specificity of ferrocide-inducing payloads. A more ideal design would combine CAR-mediated antigen recognition with intracellular responsive payload release, enabling the ferrocide-regulating payloads to exert their effects within the target tumor cells as far as possible.

Overall, CAR-T-EVs may offer a more flexible, cell-free delivery platform for the regulation of tumor ferroptosis; however, their potential safety advantages still need to be evaluated in conjunction with payload selectivity, tissue distribution and in vivo pharmacokinetics. In particular, for payloads that simultaneously affect GPX4, FSP1 or iron homeostasis, priority should be given to tumor cell selectivity, rather than relying solely on enhanced in vitro tumor killing as the basis for assessing efficacy and safety.

8 Summary and Discussion

Ferroptosis, as a form of regulated cell death characterised by iron-dependent lipid peroxidation, offers a new avenue of research for improving the efficacy of tumor immunotherapy. This paper focuses on the potential link between CAR-T-derived extracellular vesicles (CAR-T-EVs) and ferroptosis in tumor cells. Starting from the biological characteristics and targeted delivery capabilities of EVs, it highlights the IFN-JAK/STAT1-IRF1 signalling pathway, ACSL4-LPCAT3-mediated membrane lipid remodelling, NCOA4-mediated ferritin autophagy, as well as the dual antioxidant defence systems involving GPX4-GSH and FSP1-CoQ. These mechanisms collectively constitute a regulatory network governing tumor cell sensitivity to ferroptosis: on the one hand, they increase oxidisable membrane lipid substrates and intracellular available iron; on the other, they impair the clearance of lipid peroxides, thereby potentially driving ferroptosis in tumor cells.

At the same time, the ultimate biological effects of ferroptosis are also influenced by the tumor microenvironment and the state of the immune cells themselves. Extracellular vesicles (EVs) derived from the tumor microenvironment may exert a negative regulatory effect on ferroptosis by inhibiting lipid peroxidation, enhancing antioxidant defences, or altering iron homeostasis; whilst CAR-T cells themselves are also at risk of iron overload and damage from lipid peroxidation. Consequently, future strategies involving CAR-T-EVs must not only focus on enhancing ferroptosis in tumor cells but also take into account the survival and sustained functional efficacy of CAR-T cells, whilst further evaluating the release of ferroptosis-associated DAMPs and their actual impact on anti-tumor immunity.

It should be noted that the mechanism by which CAR-T-EVs mediate ferroptosis in tumor cells currently remains a theoretical framework proposed on the basis of existing research evidence, and a complete chain of direct causal evidence has yet to be established. In particular, whether CAR-T-EVs can effectively deliver specific functional cargo whilst simultaneously regulating tumor cell lipid metabolism, iron homeostasis and antioxidant defences remains to be verified by further experimental studies.

Overall, CAR-T-EVs offer a research approach worthy of exploration for linking tumor antigen recognition, extracellular vesicle delivery and the regulation of ferroptosis. Should it be possible in future to identify their key functional cargo, targets of action and cell selectivity, whilst enhancing ferroptosis in tumor cells whilst preserving the function of the CAR-T cells themselves, this is expected to provide new mechanistic grounds and strategic directions for CAR-T-based tumor therapy.

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