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Ferroptosis: Emerging mechanisms, biology and hallmarks

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Abstract: Ferroptosis is a new type of cell death which is iron-dependent and caused by lipid peroxidation, distinguished from apoptosis, necrosis, pyroptosis, and autophagy. The occurrence of ferroptosis is determined by the balance between the ferroptosis execution systems and the ferroptosis defense systems. The ferroptosis execution system includes iron metabolism, lipid metabolism, mitochondrial metabolism, etc., which induces ferroptosis by promoting the production of ROS. The ferroptosis resistance system is mainly comprised of antioxidants such as GPX4 and coenzyme Q (CoQ), which eleminate ROS and suppress ferroptosis. After cells undergo ferroptosis, morphological, biochemical, genetic, immune, and other changes occur. This review focuses on the molecular mechanism and characteristics of ferroptosis, and analyzes the potential problems as well as directions of its future research.

1. Ferroptosis execution systems

The molecular mechanisms of ferroptosis are all related to the balance of the generation and elimination of peroxidized lipids, specifically PUFA-PLs. Healthy cells produce oxidized lipids and simultaneously remove them, maintaining the amount within a normal range; when production outpaces clearance, however, the accumulation of PUFA-PLs can eventually lead to ferroptosis. Ferroptosis execution system mainly comprises iron metabolism, lipid metabolism, and mitochondrial metabolism, as outlined in this section.

1.1 Iron metabolism

Ferroptosis literally means “the iron-related sinking down or prolapse”, which indicates the vital role iron metabolism (cellular iron absorption, storage, utilization and excretion, *etc.*) plays in ferroptosis execution [1]. In 1997, it was discovered that transferrin endocytosis and the binding of serum transferrin to the transferrin receptor were the main mechanisms by which cellular iron absorption was mediated.

To be specific, ferric iron ($Fe3^{+}$) is reduced to ferrous iron ($Fe2^{+}$) via iron ion reductase catalyzation, which is then transferred into cells by divalent metal transporter 1 (DMT1). The transferrin receptor 1 (TfR1) on the cell surface also facilitates the transportation of transferrin-

bound extracellular $Fe3^+$ into the cell. Heat shock protein family B member 1 (HSPB1) can inhibit cellular iron absorption by interfering with TfR1 recycling. In the cell, STEAP family member 3 (STEAP3) reduces $Fe3^+$ to $Fe2^+$ after it is delivered into the endosome [2]. DMT1 assists in bringing the reduced $Fe2^+$ into the cytoplasm where it forms the primary component of the iron pool, also known as the labile iron pool (LIP) [3]. To keep intracellular iron levels normal, ferroportin (FPN) exports intracellular iron into the extracellular matrix while simultaneously oxidizing $Fe2^+$ to $Fe3^+$.

As an integral component of enzymes oxidizing lipids and Fenton reaction, both of which peroxidizes PUFA-PLs, iron drives the process of lipid peroxidation, and the upset of iron metabolism balance can lead to lethal ramifications: a) the elevated intracellular LIP may, through the Fenton reaction, form free radicals (hydroxyl radicals), take part in the peroxidation of phospholipids to produce PLOOH, and ultimately trigger ferroptosis; b) iron catalyzes the majority of ROS production in cells, initiating lipid peroxidation [4]; c) enzymes catalyzing lipid peroxidation, including arachidonate lipoxygenase (ALOX) and cytochrome P450 oxidoreductase (POR), require iron as an necessary cofactor [5,6].

Ferroptosis, an iron-dependent form of cell death, is featured by an increase in the small pool of $Fe2^+$. To avert cell death, excess iron is stored in ferritin and converted into redox-inactive ferritin heteropolymers, which maintain redox balance and protect against ferroptosis. Nuclear receptor coactivator 4 (NCOA4) overexpression enhanced intracellular LIP via increasing ferritin degradation, namely ferritinophagy, raises iron content and encourages ferroptosis [7]. Downregulating NCOA4, conversely, suppresses ferritin breakdown and reduces the vulnerability of cells to oxidative stress [8]. Likewise, in 2016, it was reported that autophagy contributes to ferroptosis by decomposing ferritin in fibroblasts and cancer cells [7,9]. Besides NCOA4 pathway and autophagy induction, ferritinophagy is shown to be promoted by glutamate oxaloacetate transaminase 1 (GOT1) inhibition, according to a recent study [10].

1.2 Lipid metabolism

Lipid peroxides trigger ferroptosis in a variety of ways. First, the further breakdown of lipid peroxides into ROS, molecules with partially reduced oxygen like peroxides or free radicals, causes severe damage to DNA or proteins. Second, lipid peroxidation modifies the membrane's physical properties (such as thickness and degree of bending), or create holes in the membrane that allow toxic compounds to leak out of the cell. Third, aldehydes (by-products of lipid peroxidation) have intense cytotoxicity, including malondialdehyde (MDA) and 4-HNE. Therefore, increased lipid peroxidation, which results in the esterification of free PUFA into membrane phospholipids, and are then further oxidized, is considered to be the distinctive characteristic of ferroptosis [11].

Through genome-wide haploid and CRISPR-Cas9-mediated screening, acyl-coenzyme A synthetase long chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) have already been unveiled as key regulators of PUFA-PL peroxidation. Mechanically, ACSL4 ligates free PUFAs with CoAs to produce PUFA-CoAs, while LPCAT3 subsequently re-esterified PUFA-CoAs into PLs to produce PUFA-PLs. As a result, ferroptosis sensitivity can be determined by the modulation of ACSL4 and LPCAT3 [12].

PUFA-PLs, such as adrenic acid-phosphatidylethanolamine or arachidonic acid-phosphatidylethanolamine, possess bis-allylic moieties that are excessively sensitized to peroxidation. PUFA-PLs are mainly peroxidized to PLOOH *via* a non-enzymatic, iron-catalyzed autoxidation process termed Fenton reaction (as discussed in the previous section). Lipoxygenases (LOX) and cytochrome P450 oxidoreductase (POR) have also been shown to generate PLOOH during enzymatic lipid peroxidation, although POR accelerates the oxidation of PUFAs indirectly by donating electrons to P450. The role of LOX in ferroptosis is still in controversy though, as

research findings of a glutathione peroxidase 4 (GPX4)-knockout murine model showed that LOX-15 deletion alone failed to rescue cell death induced by GPX4 silencing[13].

1.3 Mitochondrial metabolism

Mitochondria are renowned as powerhouse that produce ATP for cell function, and the central hub of metabolism pathways that generates building blocks for biomacromolecules as well as lipids. Mitochondrial functions have crucial roles in initiating ferroptosis. As a rich source of ROS, mitochondria generate superoxides due to electron leakage from complexes I and III of the electron transport chain. Superoxides are subsequently catalyzed to H_2O_2 by superoxide dismutase, followed by their conversion to hydroxyl radicals via Fenton reaction and acceleration of PUFA-PL peroxidation [14]. Furthermore, mitochondria promote ferroptosis also via ATP biosynthesis. Mechanically, when ATP is depleted, AMP-activated protein kinase (AMPK) phosphorylates and deactivates Acetyl-CoA carboxylase (ACC), thereby blocking PUFA-PL production and ferroptosis; under ATP-sufficient conditions, conversely, AMPK is underactivated while ACC is activated, triggering PUFA-PL composition and ferroptosis [15,16]. Finally, the functions of mitochondria in biosynthetic pathways also have a hand in ferroptosis promotion. Specifically, tricarboxylic acid (TCA) cycle and its numerous anaplerotic processes are shown to drive ferroptosis, presumably through an ample supply of ROS, ATP and PUFA-PL [17]. Taken together, the multifaceted capabilities of mitochondria in terms of bioenergetics, biosynthesis as well as ROS composition contribute to lipid peroxidation and ferroptosis [18]. Nevertheless, the role of mitochondria in ferroptosis has been challenged by other studies, which proved that mitochondria is crucial in ferroptosis induced by cysteine depletion, rather than GPX4 inhibition [17].

2. Ferroptosis defense systems

The anti-ferroptosis systems, on the contrary to ferroptosis execution mechanisms, mainly entails antioxidant systems that suppress lipid peroxidation. Since the discovery of the GPX4-centered mechanism against ferroptosis in 2014 [19], a growing number of studies have been done to identify novel ferroptosis defense mechanisms, which provided a potent theoretical framework for this rapidly evolving realm in ferroptosis research. At least 4 defense systems have been already identified, as discussed in the sections below.

2.1 SLC7A11-GSH-GPX4 axis

GPX4, an antioxidant enzyme of the GPX family, was first identified as a prime suppressor of ferroptosis in 2014. Back then, ferroptosis were triggered using ferroptosis-inducing chemicals (FINs), and it was found that all FINs suppressed GPX4 by depleting glutamate (GSH). Subsequent studies revealed that lipid peroxidation and ferroptotic cell death went unchecked under GPX4 inhibition condition both *in vitro* and *in vivo*.

GPX4 is the only member in the GPX family that reduces PL hydroperoxide to PL hydroxyphospholipid [20], and only have a role in avoiding ferroptosis when combined with its cofactor GSH, a thiol-containing antioxidant [21]. Specifically, each GPX4 employs 2-molecule GSH as a donor of electron, and cytotoxic lipid peroxides (L-OOH) that receive electron are reduced to non-toxic fatty alcohols (L-OH), while GSH is converted into oxidized glutathione (GSSG) in the meantime [22]. GSH is a tripeptide synthesized from glutamate, cysteine and glycine, among which cysteine limits the rate of GSH production [23]. In order to obtain intracellular cysteine, most cancer cells require specific transporter called system xc- to import cystine (the oxidized form of cysteine), followed by cystine conversion to cysteine via a NADPH-dependent reduction process

[24]. In various tumor cell lines, cystine depletion or pharmacological inhibition of SLC7A11-regulated cysteine uptake triggers potent ferroptosis [25]. These findings suggest a key function for the canonical SLC7A11-GSH-GPX4 axis in ferroptosis defense system. However, it is reported that some cells under cystine deficiency are capable of generating cysteine without xc-system, utilizing methionine transsulfuration pathway instead.

2.2 GCH1-BH4 axis

Some cancer cell lines retain resistance to ferroptosis upon GPX4 inhibition [26], which suggests the existence of anti-ferroptosis mechanisms operating in parallel to GPX4. Tetrahydrobiopterin (BH4) serves as another cofactor for aromatic amino acid hydroxylases as well as other enzymes, and a radical-trapping antioxidant eliminating lipid peroxyl radicals [27]. GTP cyclohydrolase 1 (GCH1) is recently identified as a critical regulator in the rate-limiting reaction in BH4 synthesis, which attenuates ferroptosis by producing BH4 as a radical-trapping antioxidant and regulating the generation of CoQH2 and PLs [28]. Nevertheless, the subcellular localization of BH4-GCH1 axis remains to be studied.

2.3 FSP1-CoQH2 axis

Ferroptosis suppressor protein 1 (FSP1; also known as AIFM2) was identified accidentally as a negative regulator of ferroptosis by two research teams almost simultaneously. FSP1 is recruited to the plasma membrane, lipid droplets and other cellular compartments via myristoylation, among which the plasma membrane localization is essential for defending ferroptosis [29]. Mechanistically, FSP1 facilitates the NADH-dependent conversion of coenzyme Q (CoQ) [30,31] to ubiquinol (CoQH2) [29], a renowned mitochondrial electron transporter that also traps free radicals and inhibits the biosynthesis of lipid peroxides. A crucial step in the manufacture of CoQ is the mevalonic acid pathway, which is inhibited by the small-molecule drug FIN56 to cause ferroptosis. Adding CoQ analogue edibenzoquinone to the culture media protects against the fatal effects of ferroptosis-inducing compound FIN56 [32]. It has been therefore concluded that the FSP1-CoQH2 axis shields cells from ferroptosis via the production of non-mitochondrial CoQH2 as free radical scavengers [33]. Although CoQ is produced mostly in mitochondria, it has also been found in other membranes including plasma membrane, according to recent studies [34]. Future work is required to shed light on how non-mitochondria CoQ are transferred or synthesized before FSP1-mediated reduction.

2.4 DHODH-CoQH2 axis

DHODH is an enzyme localized on the inner mitochondrial membrane, which reduces CoQ to CoQH2 (an antioxidant detoxifying lipid peroxidation) in the pyrimidine biosynthesis pathway. DHODH-CoQH2 axis is dramatically promoted upon abrupt GPX4 suppression, and is thus believed to compensate for GPX4-GSH axis in mitochondria [35]. This results in robust mitochondrial lipid peroxidation and ferroptosis when both mitochondrial GPX4 and DHODH are inhibited. Of note, cytosolic GPX4 and FSP1 lack the capacity to suppress mitochondrial lipid peroxidation in the same way that mitochondrial GPX4 and DHODH do [35], possibly in that they fail to neutralize lipid peroxides generated in mitochondria, suggesting the crucial role subcellular localization plays in ferroptosis defense.

Caption:

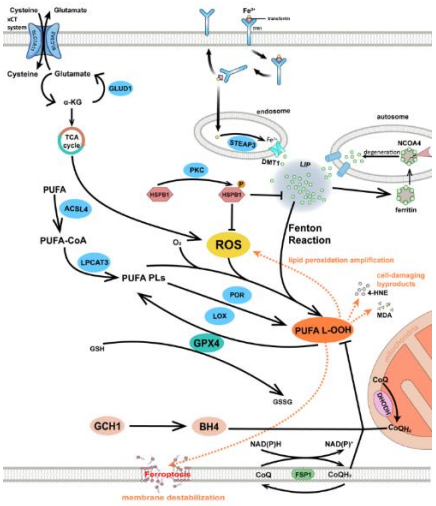

Figure 1: Execution systems and defense systems of ferroptosis.

According to these studies, ferroptosis defense systems can be classified into GPX4-reliant and CoQH2-reliant mechanisms, each of which can be further subdivided based on compartmentalization, i.e., non-mitochondrial (cytosolic GPX4 and FSP1-CoQH2 axis) and mitochondrial (mitochondrial GPX4 and DHODH-CoQH2 axis) (refer to **Figure 1**). The two layers of mitochondrial membrane prevents non-mitochondrial mechanisms from entering mitochondria whereby lipid peroxides accumulate, which justifies the division of ferroptosis defense systems. Nevertheless, it is reported that cytosolic GPX4 transfer to the intermembrane space of mitochondria [36], thus posing a challenge to the compartmentalization theory. Additional work is needed to provide supporting evidence for the compartmentalization paradigm and clarify opposing findings in previous research.

3. Hallmarks of ferroptosis

3.1 General changes of ferroptotic cells

3.1.1 Morphological hallmarks

Ferroptosis differs morphologically from other cell death modalities [37], but commonly displays necrosis-like morphological changes as a type of regulated necrosis [38], such as a decrease in plasma membrane integrity, cell and cytoplasmic organelle enlargement and moderate chromatin condensation [13,37]. At the ultrastructural level, ferroptosis is primarily characterized by pronounced morphological alterations in the mitochondria, whose shape plays a key role in determining whether a cell will survive or die and shows up as structural alterations to the mitochondrial outer membrane and inner membrane with crista-like foldings. Mitochondria exhibit swelling or condensation, overall atrophy, increased mitochondrial membrane density, decreased mitochondrial cristae, outer membrane rupture, etc. after ferroptosis is triggered [13,37], which are significant and particular to ferroptosis.

3.1.2 Biochemical hallmarks

3.1.2.1 Iron accumulation

The accumulation of iron appears to be a biochemical indicator of ferroptosis since the first study described ferroptosis as an iron-dependent RCD [39]. As a trace element, iron is distributed in

different cytoplasmic organelles. Ferroptotic cells or tissues are found to have higher levels of cytoplasmic or mitochondrial $Fe2^+$, as measured by biochemical assay kits [40], or probes (such as Phen Green SK or FerroFarRed) [41,42]. Ferroptosis inducers erastin and RSL3 elevate levels of intracellular or mitochondrial $Fe2^+$, thereby suppressing the antioxidant system [37]. Iron may cause ROS accumulation via Fenton reaction, as discussed in the previous section. Conversely, using iron chelators (such as deferoxamine) or targeting genes related to iron metabolism, limits ROS generation and prevents ferroptosis in vivo [43] or in vitro [37].An open question concerns why metals like zinc, which is also capable of generating free radicals through Fenton reaction [44], fail to induce ferroptosis as iron does [37]. One possible explanation is that zinc overload results in ROS accumulation, but the particular downstream effectors it activates fail to induce ferroptosis.

3.1.2.2 Lipid peroxidation

Lipid peroxidation is a crucial mediator of ferroptosis [45], mainly driven by free radicals. Fatty acids are divided into three types based on their degree of saturation in the carbon chain, namely, saturated (no double bound), monounsaturated (one double bond) and polyunsaturated (two or more double bonds). Although various fatty acids and other lipids can be oxidized, the oxidation of polyunsaturated fatty acids (PUFAs) into lipid hydroperoxides (LOOHs) is specifically important for ferroptosis [46]. In addition to LOOHs, lipid peroxidation generates reactive aldehydes subsequent to LOOH synthesis [e.g., malondialdehyde (MDA) and 4-hydroxynonenal (4HNE) [47,48]. Of note, despite the pivotal role of mitochondria and their dramatic morphological changes in ferroptosis, the peroxidation of cardiolipin (a mitochondria-exclusive phospholipid) is not yet reported in ferroptosis [49].

3.1.3 Genetic hallmarks

3.1.3.1 Upregulation of PTGS2 gene

The upregulation of ferroptosis-related genes has been recognized as a hallmark of ferroptosis, as evidenced by prostaglandin-endoperoxide synthase 2 (PTGS2/COX2), whose renowned function is to catalyze the conversion of AA into prostaglandins. *PTGS2* is most upregulated among 83 oxidative stress-related genes in BJeLR cells upon erastin or RSL3 exposure [19], whose mRNA transcription level is also identified as a biomarker of erastin or RSL3 treated murine model [19].

3.1.3.2 Upregulation of CHAC1 gene

The well-known function of prostaglandin-endoperoxide synthase 2 (PTGS2/COX2) is to decompose glutathione (GSH) into cysteinylglycine dipeptide and oxoproline [50]. Mechanically, upon treatment with certain ferroptosis inducers (e.g., erastin and artesunate), the endoplasmic reticulum (ER) stress pathways activate ChaC glutathione specific gamma-glutamylcyclotransferase 1 (CHAC1) that promotes GSH digestion as a γ-glutamyl cyclotransferase [50], followed by ferroptosis onset [51]. Transcriptional upregulation of *CHAC1* is observed following treatment with systemic xc- inhibitors, while blockers of the GPX4-GSH axis (e.g., RSL3 and buthionine sulphoximine) fail to affect *CHAC1* expression, providing a pharmacodynamic biomarker for system xc– suppression.

3.1.3.3 Activation of NFE2L2 targeted genes

The nuclear factor erythroid 2-like 2 (*NFE2L2/NRF2*) is an essential transcription factor for cell survival under oxidative stress by promoting an array of detoxification and antioxidant gene expression. The correlation between *NFE2L2* and ferroptosis was initially described in 2016, when

metallothionein 1 G (MT1G) was identified as a novel target of *NFE2L2* and a contributor to ferroptosis resistance in sorafenib-treated hepatocellular carcinoma cells [52]. Further functional studies reveals that *NFE2R2* prevents cells from ferroptotic injury both in vitro and in vivo. *NFE2L2* targets a multitude of genes, which are responsible for various functions (e.g., iron metabolism, GSH metabolism, etc.). The *NFE2L2*-targeted genes that are associated with ferroptosis are summarized in Table 1, categorized by their biological functions. Nonetheless, the upregulation of *NFE2L2*-targeted genes is also observed in other types of RCD, and the expression of *NFE2L2* target gene is not a desirable biomarker of ferroptosis.

3.1.4 Protein hallmarks of ferroptosis

The intracellular level of a protein is determined by both genetic regulation and the rate of protein decomposition. The overexpression of a few proteins corresponding to the genetic hallmarks of ferroptosis can in theory be regarded as a biomarker of ferroptosis. Enzymes (e.g., PTGS2 [19], CHAC1 [51], and ACSL4 , as well as other proteins (e.g., transferrin receptor C, TFRC [53]), are evaluators of the response of cells to ferroptosis in vitro and in vivo, which has already been confirmed by previous western blotting, immunohistochemical or immunofluorescence analysis of their expression levels and subcellular localization.

Ferroptosis is closely related to autophagy, which mediates ferroptosis by regulating gene transcription and protein degradation process. On the one hand, monitoring the conversion of microtubule associated protein 1 light chain 3 (MAP1LC3)-I to MAP1LC3-II has been used to gauge the injury caused by ferroptosis inducers [54]. Also, the sensitivity to ferroptosis can be monitored by tracking changes in autophagic influx or efflux with autophagic flux probes (e.g., GFP-LC3-RFP-LC3ΔG [55] or RFP-GFP-LC3B [56]), which combines the application of different lysosomal inhibitors. On the other hand, the degradation of ferroptosis suppressors via autophagy pathway is also an approach in which excessive autophagy triggers ferroptosis. This type of autophagy-dependent ferroptosis was first reported in 2016 by a study conducted on mouse embryonic fibroblasts and pancreatic ductal adenocarcinoma (PDAC) cells [7]. It was observed that erastin induces degradation of ferritin in an autophagy-dependent manner, which was termed ferritinophagy and regulated by NCOA4 (see the previous section) [7,9]. In addition to ferritin, GPX4 degradation is also reported in ferroptosis-sensitive cells under treatment of ferroptosis activators (e.g., erastin [57,58], RSL3 [59], FIN56 [32], and PdPT [60]), which can be blocked by ER molecular chaperone heat shock protein family A (HSP70) member 5 (HSPA5) [57] and enhanced by heat shock protein 90 (HSP90)-dependent chaperone-mediated autophagy (CMA) in vitro [58]. Clockophagy is a novel selective autophagy process favoring ferroptosis via suppression of hypoxia inducible factor 1 subunit alpha (HIF1A), thus disturbing lipid uptake and storage [61]. This type of autophagy specifically degrades the key clock circadian regulator aryl hydrocarbon receptor nuclear translocator like protein 1 (ARNTL/BMAL1) depending on sequestosome 1 (SQSTM1/p62) [61]. Autophagic degradation of various proteins regulated is a theoretical indicator of ferroptosis vulnerability, whose sensitivity and specificity needs to be established by future studies.

3.1.5 Immune hallmarks of ferroptosis

Ferroptosis can trigger totally opposite immunological results, according to the type of cell undergoing ferroptosis. If leukocytes undergo ferroptosis, which play a critical role in immune response, ferroptosis can result in loss of function, manifested by a decline in immunity. For example, ferroptotic T cells failed to expand and to protect from viral or parasite infections in GPX4-deficient mice [62]. Nonetheless, ferroptosis in leukocyte subsets is not the main topic of this review, and we refer readers to an excellent review on ferroptosis in immune cells [63]. Perhaps more

importantly, when ferroptosis affects cells other than leukocytes, it will possibly change the way immune system deals with dying or dead ferroptotic cells, depending on the chemicals released. Generally speaking, ferroptosis is an immunogenic and inflammatory type of RCD with the secretion of DAMPs [e.g., DNA and high mobility group box 1 (HMGB1)] and lipid oxidation products (e.g., 4-HNE, oxPLs, LTB4, LTC4, LTD4, and PGE2), as well as the subsequent activation of DAMP receptors throughout the ferroptosis process.

4. Conclusions and future perspectives

Ferroptosis, as an emerging regulatory cell death modality, is closely related to iron metabolism, lipid metabolism, amino acid metabolism, and other metabolic pathways. Numerous metabolic pathways can be divided into two major systems based on their different functions: promoting and inhibiting. In healthy cells, a delicate balance is struck between the execution and defence systems, ensuring that cells do not perish due to peroxidation. If the execution system is inhibited or the defence system enhanced, intracellular lipid peroxidation and ferroptosis occur. On the other hand, the molecular mechanism of ferroptosis is closely related to its characteristics. After ferroptosis occurs in cells, unique changes occur in morphology, biochemistry, gene expression, proteins, immune characteristics, and other aspects. These features are of great significance for the detection of ferroptosis in cell biology research.

However, there are still many areas for further research on the molecular mechanisms and characteristics of ferroptosis. For example, more detailed work is required to determine the subcellular localization of the BH4-GCH1 axis and FSP1-CoQH2 axis in the ferroptosis inhibition system. In addition, further research is necessary to investigate the impact of DAMPs released by ferroptosis cells on the immune system. Considering the importance of ferroptosis in various diseases, it is necessary to incorporate ferroptosis into the entire molecular biology and immunology research scenery for in-depth research.

Author Contributions

Z.W. took the lead in writing the manuscript. D.L. and Z.W. discussed the contents and edited the manuscript. Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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