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The role of ubiquitination in disease development, progression, and prognosis

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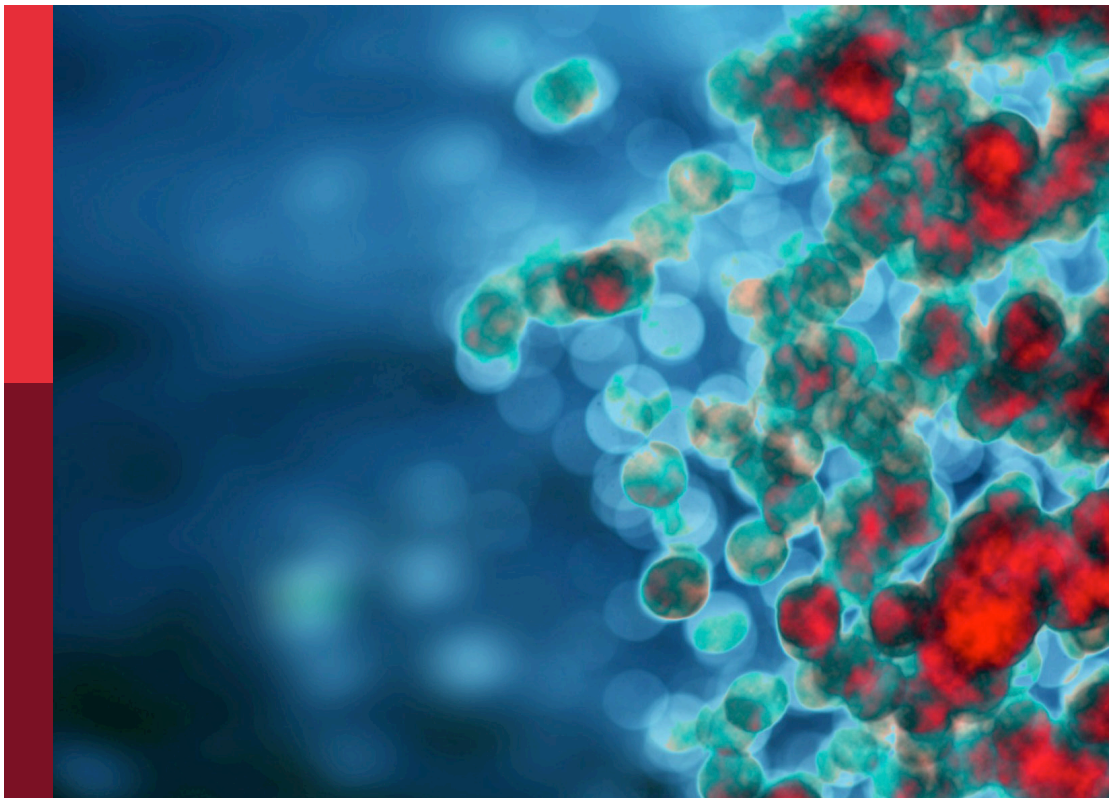

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The role of ubiquitination in disease development, progression, and prognosis

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Editorial: The role of ubiquitination in disease development, progression, and prognosis

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Editorial on the Research Topic
The role of ubiquitination in disease development, progression, and prognosis

Ubiquitination, the covalent attachment of ubiquitin to target proteins, has emerged as a pivotal mechanism governing numerous cellular processes implicated in disease onset and progression. This Research Topic set out to explore how altered ubiquitination shapes pathogenesis, influences immune and inflammatory pathways, and offers potential avenues for novel therapeutic strategies. It has brought together articles that examine these roles from multiple angles, spanning gastrointestinal malignancies, immune-related conditions, metabolic disorders, and beyond. Here, we highlight the contributions of these published works and place them into the broader context of ubiquitin biology and its clinical implications.

One of the article to this Research Topic, Huang et al. dissects the complexity of gastrointestinal tumors by exploring how mitochondria, crucial for cellular energy supply and apoptosis regulation, are tightly controlled by ubiquitin-dependent processes. Mitochondrial biogenesis, mitophagy, and fission-fusion dynamics all rely on substrate proteins whose modification by E3 ubiquitin ligases determines their stability and location. When aberrant ubiquitination perturbs these processes, tumor cells gain proliferative advantages and become more resistant to apoptosis. By illuminating how specific E3 ligases and deubiquitinating enzymes (DUBs) target mitochondrial regulatory factors, the authors shed light on new strategies that might restore correct ubiquitin-mediated signaling and improve responses to therapies in gastrointestinal cancers.

Another study in this Research Topic, Deng et al. highlight the fine-tuned mechanisms by which ubiquitination modulates the cGAS-STING pathway, a critical cytosolic DNA-sensing axis in innate immunity. This research demonstrates how over- or under-ubiquitination of cGAS, STING, or their auxiliary factors leads to either exaggerated or dampened immune activation. The authors emphasize that in cancer and autoimmune states, rebalancing cGAS-STING signaling through targeted manipulation of ubiquitin ligases or DUBs holds promise. Their work contributes to a deeper understanding of how immune homeostasis might be re-established in disorders marked by a failure to either properly mount or properly resolve inflammation.

The interlink between neuroinflammation, sensory processing, and proteostasis also appears in this Research Topic, as Zhu et al. delve into the pathobiology of migraine. By investigating relevant ubiquitinating enzymes that govern pro-inflammatory mediators and nociceptive signals, the authors reveal that the ubiquitin-proteasome system can alter the expression and turnover of calcitonin gene-related peptide and MAPK/NF-κB components. Their findings advance our grasp of the molecular underpinnings of migraine chronicity and point to promising molecular targets for treatments aimed at pain prevention rather than mere symptomatic relief.

Sepsis, a hyper-inflammatory state triggered by pathogens and sustained by damage-associated molecular patterns, is further addressed through an examination of ubiquitin's role in regulating pathways leading to cytokine storm and cell death. Li et al. illustrate how ubiquitin-dependent modifications of RIPK1 and NLRP3, central players in necroptosis and pyroptosis, allow the immune system to either escalate or temper the inflammatory reaction. They also underscore how dysregulation of deubiquitinating enzymes can tip the delicate balance, culminating in excessive or persistent inflammation that damages multiple organs. Such insights suggest that selective E3 ligase inhibitors or DUB modulators could become part of emerging therapies to minimize sepsis-related morbidity.

Contributors like Cheng et al. to this Research Topic also examine how metabolic disorders, including metabolic dysfunction-associated steatohepatitis and chronic viral hepatitis, converge on common ubiquitin-related pathways. Differential gene expression analyses in these conditions pinpoint an overrepresentation of immune and inflammatory routes, hinting that aberrant ubiquitination influences the fate of key proteins like STAT1 or CCL2. Connecting these genes to changes in protein stability not only enhances our understanding of disease progression but also opens the door to potential ubiquitin-targeted interventions. An example is the demonstration that clinically approved agents can suppress aspects of the pathogenic signature by recalibrating protein turnover, thereby offering encouraging leads for more personalized treatment strategies.

A further set of findings focuses on hepatocellular carcinoma and details how subsets of E3 ubiquitin ligases orchestrate the turnover of oncogenes, tumor suppressors, and immune-regulatory factors. The authors Wang et al. describe how these ligases, belonging to the RING, HECT, or RBR families, can directly shape tumor proliferation, apoptosis, and metastatic potential. By fine-tuning checkpoints and immune cell activation, ubiquitination also profoundly impacts how the tumor microenvironment responds to immunotherapy. These insights strengthen the rationale that targeting E3 ligases in combination with checkpoint inhibitors or other modalities could enhance treatment efficacy and perhaps overcome therapeutic resistance.

The integrative effect of ubiquitination on immune function and cellular homeostasis is showcased further in work that explores rheumatoid arthritis (RA). There, researchers Fu et al. highlight how abnormal tagging of proteins in immune and synovial cells can amplify inflammatory cascades and tissue destruction. In particular, the interplay between metabolic changes, for example lactylation, and ubiquitin-dependent degradation of cell-cycle regulators underscores that RA pathogenesis is fueled by both immunological and metabolic shifts. By pinpointing E3 ligases, such as BIRC3, which drive fibroblast-like synoviocyte proliferation and inflammatory signaling, the authors Meng et al. argue that selective inhibition of these ligases might hold the key to halting joint damage and improving disease outcomes.

Concluding the array of contributions are data on mesenchymal stem cells and inflammatory bowel disease, emphasizing how interventions that recalibrate ubiquitination can restore gut immune homeostasis. The authors Liao et al. propose that by influencing the stability of critical signaling agents in the inflamed intestine, stem-cell-based therapies may achieve a more targeted and lasting control of chronic inflammation. These findings open further discussion about how precisely timed or localized manipulation of ubiquitin pathways could complement existing immunomodulatory treatments for inflammatory bowel disease.

Collectively, the articles in this Research Topic underscore how ubiquitination has evolved from a niche protein-tagging mechanism into a unifying framework that shapes myriad facets of disease biology. In addition to unveiling novel E3 ligases and DUBs that modulate pathogenesis, these studies advocate for therapeutic strategies that correct dysregulated ubiquitination. The growing roster of small molecule inhibitors and biologics aimed at specific steps in the ubiquitination cascade offers unprecedented opportunities to alter disease trajectories in cancer, autoimmune disorders, sepsis, neurological diseases, and beyond. As the field moves forward, a deeper characterization of ubiquitin modifications in human tissues should guide the discovery of more refined diagnostic and therapeutic tools, expanding the horizon of precision medicine.

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The role of mesenchymal stem cells in attenuating inflammatory bowel disease through ubiquitination

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Inflammatory bowel disease (IBD), a condition of the digestive tract and one of the autoimmune diseases, is becoming a disease of significant global public health concern and substantial clinical burden. Various signaling pathways have been documented to modulate IBD, but the exact activation and regulatory mechanisms have not been fully clarified; thus, a need for constant exploration of the molecules and pathways that play key roles in the development of IBD. In recent years, several protein post-translational modification pathways, such as ubiquitination, phosphorylation, methylation, acetylation, and glycolysis, have been implicated in IBD. An aberrant ubiquitination in IBD is often associated with dysregulated immune responses and inflammation. Mesenchymal stem cells (MSCs) play a crucial role in regulating ubiquitination modifications through the ubiquitin-proteasome system, a cellular machinery responsible for protein degradation. Specifically, MSCs have been shown to influence the ubiquitination of key signaling molecules involved in inflammatory pathways. This paper reviews the recent research progress in MSC-regulated ubiquitination in IBD, highlighting their therapeutic potential in treating IBD and offering a promising avenue for developing targeted interventions to modulate the immune system and alleviate inflammatory conditions.

KEYWORDS

mesenchymal stem cell, exosome, inflammatory bowel disease, post-translational modification, ubiquitination

1 Introduction

Over the past 20 years, the incidence and prevalence of Inflammatory bowel disease (IBD) have increased in the newly industrialized countries of Asia, South America, the Middle East, and Africa, and the rate is particularly significant in South America and East Asia. Thus, IBD is gradually expanding to become a global disease of public health concern[1]. According to China's epidemiological data, the incidence of IBD, ulcerative colitis (UC) and Crohn's disease (CD), in the north of China is lower than in the south. For instance, the age-standardized incidence of IBD in Daqing City (Heilongjiang Province), Wuhan, and Guangzhou are 1.77/100,000, 1.96/100,000, and 3.14/100,000 respectively (1, 2). According to a study (3), the number of IBD patients in China will reach 1.5 million by 2025. Similar observations have been reported, with an estimated 6·8 million cases of IBD globally, where the USA had the highest age-standardized prevalence rate (464·5 [438·6–490·9] per 100 000 population), followed by the UK (449·6 [420·6–481·6] per 100 000) (4).

IBD has evolved from a common and fatal condition to a manageable chronic condition (5), which is divided into two broad categories, mainly UC and CD (6, 7). The main clinical manifestations are persistent abdominal pain, diarrhea, and bloody stool, accompanied by malnutrition, weight loss, mental distress, and other symptoms. If the course of the disease is prolonged, there is a possibility of colorectal cancer, a severe threat to human health. It has been observed that people with a history of digestive disease, a family history of IBD, and a functional imbalance in the body's immune system are more likely to develop IBD than the general population (8). As a chronic non-specific intestinal inflammatory disease, IBD is associated with environmental, gut microbial, genetic, and immune factors. If IBD is not correctly diagnosed and treated in time, the disease becomes severe and lesions accumulate in organs of the body, with complications for disease such as colon cancer, coronary heart disease, primary sclerosing cholangitis, and phlebitis.

Ubiquitination is an important post-translational modification of proteins, and the enzymes involved mainly include the E1 ubiquitin-activating enzyme (E1), E2 ubiquitin binding enzyme (E2), and E3 ubiquitin-ligase (E3). Ubiquitin modification can regulate the localization and function of proteins in cells, degrade proteins, and regulate life activities such as signal transmission, gene expression regulation, cell proliferation, differentiation, apoptosis, inflammation and immunity (9). Abnormal ubiquitination can cause cancer, metabolic syndrome, neurodegenerative diseases, autoimmune diseases, inflammatory diseases, infections, and muscular dystrophy. Recent studies have reported associations between ubiquitination and/or deubiquitination and the onset and development of IBD (10). A genome-wide association analysis (GWAS) study showed that rare variants of E3 ligase RNF186 were associated with IBD (11). At the same time, the expression of ubiquitin mRNA in the colonic tissue of experimental colitis rats was significantly higher than that in the normal group. Therefore, abnormal ubiquitination may be one of the important mechanisms of colonic inflammation and immune damage in IBD.

In recent years, extracellular vesicles (Evs), as a strategy of "cell-free therapy," have become a "new favorite" in research. With the development of various related technologies, more and more researchers have turned their attention to the use of Evs for disease diagnosis, prognosis, and therapeutic clinical applications. Mesenchymal stem cells (MSCs) and their derived exosomes (MSC-Exs) have been shown to play a significant role in the repair of various diseases, including IBD (12). MSCs and MSC-Exs play an important role in the information transmission process between damaged intestinal cells and can be involved in the regulation of intestinal inflammation and damage repair, showing great potential in the treatment of IBD (13). The main mechanism by which mesenchymal stem cells-derived extracellular vesicles (MSC-Evs) inhibit the activation of colon macrophages depends on the inhibition of NF-κB and iNOS transduction signals. After injection of MSC-Evs, the expression of NF-κB p65 in colon macrophages can be downregulated, and the production of NO, IL-1β, and IL-18 can be reduced, thus alleviating the symptoms of colitis (14, 15). At the same time, ubiquitination plays an essential role in the regulation of multiple biological functions, including inflammation. Studies in mice with colitis found that ubiquitin protein from inflammatory tissue is upregulated, and ubiquitin (Ub) is involved in the signaling pathway that regulates the expression of inflammatory factors (mTOR signaling pathway) (16). Wu et al. found that human umbilical cord mesenchymal stem cells-derived exosome (hucMSC-Ex) can down-regulate the expression level of ubiquitin protein. This, in turn, reduces NF-KB and mTOR activation (17). In addition, hucMSC-Ex can modulate the expression of polyubiquitination, including K48. It is concluded that MSC-Ex may play an anti-inflammatory role by regulating the level of ubiquitin modification. Currently, the product development and clinical translational application of MSCs and MSC-Exs are a hot topic in drug development, and cell-free therapy plays a unique role as a breakthrough clinical therapy technology. However, no literature currently summarizes the research progress of MSCs regulating ubiquitin modification to repair IBD. This review can provide a theoretical basis for Cell-free therapy to treat IBD through ubiquitin modification, which has important research value.

2 Mesenchymal stem cells

MSCs are a class of pluripotent stem cells with the function of self-renewal, self-proliferation, and multi-differentiation (18, 19). They positively express CD73, CD90, and CD105 and negatively express CD19, and CD45 (20). MSCs are derived from a wide range of sources, including bone marrow, adipose tissue, endometrial polyps, umbilical cord, amniotic fluid, and placenta (21). They generally possess the functions of inducing regeneration, maintaining general tissue homeostasis, and homing at target sites, which are their inherent characteristics (22). However, the differentiation and proliferation potential of MSCs from different sources may be very different (23).

MSCs can interact with cells of both the innate and adaptive immune systems. Evidence has shown that MSCs exert

immunomodulatory functions by regulating the activation, proliferation, and differentiation of immune effector cells, including natural killer cells (NK), macrophages (Mø), dendritic cells (DC), B lymphocytes, and T lymphocytes (24). MSCs can down-regulate NKp30 and natural-killer group 2, member D (NKG2D), to inhibit the cytotoxic activity of resting NK cells, and the latter is an activated receptor involved in NK cell activation and target cell killing (25). MSCs can regulate Th1/Th2 balance (T helper cells) by influencing the levels of interleukin-4 (IL4) and interferon (IFN-γ) in effector T cells (26). MSCs can also reduce inflammation, improve tissue damage, and prevent infection by secreting a variety of immunomodulatory factors, including IFN-γ, prostaglandin E2 (PGE2), and growth factors (TGF-β, VEGF) (27). In conclusion, MSCs have strong immunomodulatory properties but are less immunogenic.

At present, MSCs are widely used in the research and treatment of various human diseases such as cardiovascular diseases, osteoarthritis, metabolic disease, etc. (28)). LAI et al. believe that MSCs play a role through their secreted products (29). It has been reported that MSCs can secrete a variety of Evs, including exosomes (30). In recent years, exosomes, as a promising substitute for MSCs, have attracted more attention, and have great research significance for experimental and clinical applications.

3 Biogenesis, composition, and characteristics of MSC-derived exosomes

Extracellular vesicles (Evs) secreted by cells are divided into apoptotic bodies, microvesicles, and exosomes based on their size, content, and formation mechanism (31). Table 1 presents the classification and function of Evs. Evs are released by almost all living cells and are found in blood, urine, and bronchoalveolar lavage fluid (32). At present, exosomal research is the most attractive and constantly expanding. In 1983, the research team of Rose M. Johnstone (33), a professor in the Department of Biochemistry of McGill University in Canada, first found exosomes in sheep reticulocytes, which were considered to be cell follicles that could transport nonessential proteins between cells and were considered the "garbage" of cell metabolism. With further study, Johnstone named these small vesicles as exosomes in 1987.

TABLE 1 Classification and function of Evs.

	Exosomes	Microvesicles	Apoptotic Bodies
Origin	Endocytic pathway	Cell plasma membrane	Cell plasma membrane
Size	50–200 nm	100–1000 nm	1000–5000 nm
Content of EVs	Proteins and nucleic acids (mRNA, miRNA, lncRNA, and DNA fragments), lipids	Membrane proteins, phospholipids, RNA and other biomolecules	Cell membranes, DNA, coding and non-coding RNA, lipids, and containing specific vesicular membrane proteins,
Functions	Intercellular communication, affects the physiological and pathological state of host cells.	Cell recognition and signaling functions that help host cells transmit information and regulate immune responses	Phagocytosis, affects the cellular immune state and pathological process.

Exosomes, uniform in size and 50 to 200nm in diameter, have no cellular structure and are highly stable (34–36). Exosomes facilitate intercellular communication by carrying bioactive substances including mRNA, miRNA, lncRNA, DNA fragments, proteins, and lipids from parent cells, thereby regulating the activities of target cells. They positively express markers such as the tetraspanins protein family (CD63, CD81, and CD9), MVB biogenic proteins (Alix, TSG101, and ESCRT Complex), membrane transporters and heat shock proteins (HSP70, HSP90), lipid-associated proteins, etc. (37).

Exosome biogenesis includes three main stages of endosomes, multivesicular body (MVBs) formation and exosome release (Figure 1), which involve double invagination of the plasma membrane (38, 39). Exosomes originate in the endosomal system of cells. Extracellular substances first enter the cell through membrane invagination and endocytosis, fuse with early endosomes (ESEs), and gradually develop and mature into late endosomes (LSEs). Late endosomal invagination leads to the emergence of intracavitary vesicles (ILVs), and multiple ILVs aggregate to form MVBs. MVBs are fused with the cell membrane and released outwards as exosomes in lipid bilayers.

Because exosomes can be detected in body fluids, they are considered noninvasive or minimally invasive biomarkers for disease diagnosis. Studies have implicated exosomes in the pathophysiology of several diseases (40). For example, Gui et al. found that compared with healthy controls, the expressions of miR-1 and miR19b-3p in CSF exosomes of Parkinson's disease (PD) patients were down-regulated, while miR-153, miR-409–3p, and miR-10a5p were up-regulated (41). The exosomal miRNAs significantly correlated with the severity of PD and may be an effective biomarker for evaluating disease development in clinical PD patients. Exosomes can also be used as diagnostic markers of cancer. Roccaro et al. showed that the expression of miRNA-15a in bone marrow MSC-exosomes (BMMSC-Ex) of multiple myeloma patients is significantly down-regulated, which is closely related to the characteristics of multiple myeloma (42). In addition, the presence of high levels of Evs expressing TGF-β2 in the breast milk of normally lactating women can induce breast cancer. What is interesting is that exosomes can play a therapeutic role by delivering drugs themselves or as functional cargo for drug delivery (43, 44). For example, MSC-Exs are used in the treatment of graft-versus-host disease (GVHD), with significant therapeutic effects observed after repeated injection without serious side effects (45, 46).

MSC-Exs have similar biological functions to MSCs, playing an important role in improving tissue repair, immune regulation, inhibiting inflammatory response, and reducing apoptosis (47). They exhibit no tumorigenicity, and are easier to extract, modify, and store (48). MSC-Ex is a natural, non-toxic vesicle that can

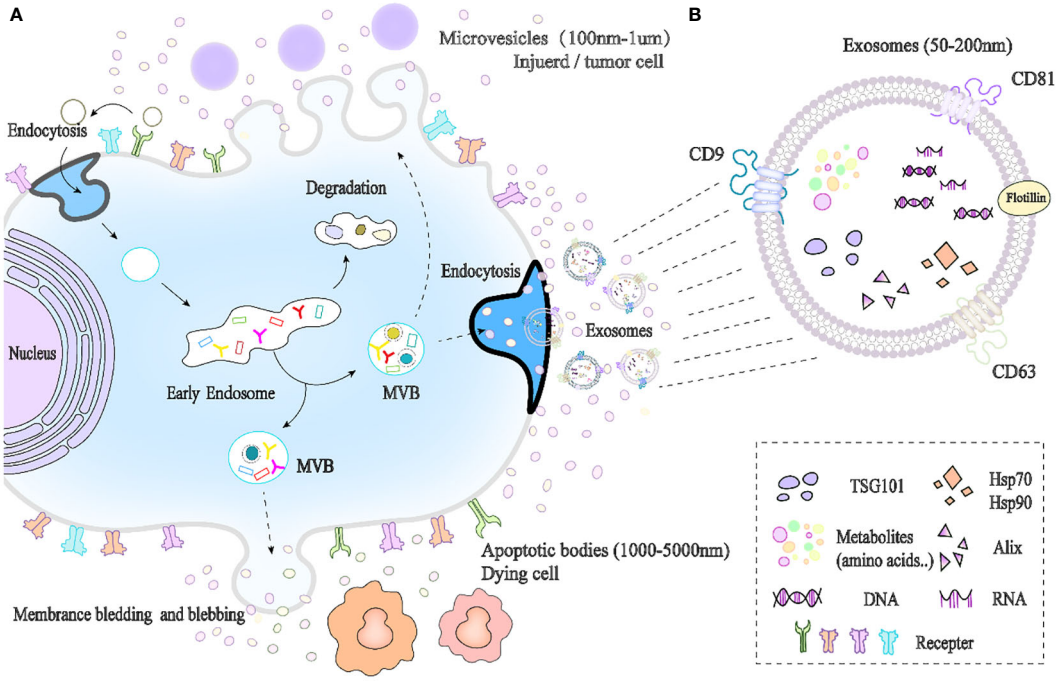

FIGURE 1
Biogenesis and composition of exosomes. **(A)** Exosomes originate from endosomal pathways, and extracellular substances enter cells through membrane invagination and endocytosis and then develop into early endosomes (ESEs), late endosomes (LSEs), and intracavitary vesicles (ILVs). Multiple intracavitary vesicles (ILVs) aggregate to form MVBs. MVBs can fuse with lysosomes to degrade and release content into the cytoplasm or be released outside the cell by budding through the cell membrane, and the latter is called exosomes. **(B)** Exosome composition.

deliver mRNA, miRNA, and protein. Therefore, it has received extensive attention as a cell-free therapeutic carrier in treating autoimmune diseases, including IBD (49). MSC-Ex research has made substantial progress in the treatment of multiple sclerosis, type-1 diabetes, and other diseases (50–52) (Table 2 shows the application of MSCs and MSC-Ex in various clinical diseases). In the DSS-induced IBD model, hucMSC-Exs treatment reduces the infiltration of macrophages in colon tissue and inhibits the expression of IL-7 (74). Moreover, hucMSC-Exs alleviate insulin secretion function in T2DM by reversing peripheral insulin resistance and alleviating β cell destruction, providing a new approach for T2DM treatment (60). In clinical treatment, MSC-Exs have obvious advantages over MSCs and may completely replace MSC therapy in the future.

4 Ubiquitination

Ubiquitination is an important process that regulates the normal expression of genes, along with phosphorylation, glycosylation, acetylation, amidation, etc. (76, 77) Ubiquitination modifies post-translated proteins (PTMs), including protein degradation, signal transduction, and DNA damage repair (78, 79). Ubiquitination modulates various cellular activities involved in inflammatory responses, innate or adaptive immune responses, and ribosomal functions, which are essential for many cell life processes (80).

Cellular processes depend on ubiquitin (Ub) and ubiquitin-like proteins (UBLs) in ubiquitination systems. Ub, a highly conserved small protein in eukaryotes, contains 76 amino acid residues and is a significant part of regulating the everyday life activities of biological proteins (81). The UBLs found so far include NEDD8 (neural-precursor-cell expressed developmentally down-regulated 8) and SUMO (1–5, small ubiquitin-like modifier). Although they have similar functions to Ub, the receptors and signaling molecules they contact are not the same and play different roles. Related studies have shown that NEDD8 which has the same homologous sequence (> 50%) as Ub, binds to Cullin, the subunit cullin of Cullin-ring ligase (the largest multi-unit E3s ubiquitase family, CRLs). After overactivation, it promotes the degradation of tumor suppressor factors (p21, p27) and the occurrence and evolution of cancer (82). Yang W et al. experimentally demonstrated that increased SUMOylating has a neuroprotective effect and seems necessary for survival, at least under certain conditions (ischemia) (83). This theory was further validated in animal models, which found that cerebral ischemia in mice leads to a significant increase in SUMO2/3 conjugates in the hippocampus and cerebral cortex, and a neuroblastoma cell model undergoing hypoxia/glucose deprivation followed by a short period of reoxygenation under the same conditions also exhibits significant increases in SUMO2/3 conjugation (84, 85).

From a more microscopic point of view, the Ub molecule itself contains seven lysine (Lys) residues, and the amino terminus of the Lys residues (K6, K11, K27, K29, K33, K48, and K63) on the

TABLE 2 Applications of MSCs and MSC-Ex in various clinical diseases.

Disease	Study type	Treatment used	Observation	Reference
Acute myocardial infarction	Case Reports	MSCs	Improved	(53)
	Clinical Trial	MSCs	Improved	(54)
	Animal model	MSC-Ex	Improved	(55, 56)
Ischemic heart disease	Clinical Trial	MSCs	Improved	(57)
Type 1 diabetes	Animal model	Both	Improved	(52, 58)
	Clinical Trial	MSCs	Improved	(59)
Type 2 diabetes	Animal model	MSC-Ex	Improved	(60)
Systemic lupus erythematosus	Clinical Trial	MSCs	Improved	(61, 62)
Graft versus host disease	Animal model	MSCs	Improved	(63)
	Case Reports	MSCs	Improved	(47)
Multiple Sclerosis	Clinical Trial	MSCs	Improved	(64)
Lung disease	Clinical Trial	Both	Improved	(65–68)
Alzheimer's disease	Animal model	MSC-Ex	Improved	(69, 70)
Kidney injury	Clinical Trial	MSCs	Improved	(71)
	Animal model	MSC-Ex	Improved	(72)
Apoplexy	Clinical Trial	MSCs	Improved	(73)
IBD	Animal model	MSC-Ex	Improved	(12, 74, 75)

substrate protein monopeptide can be labeled for monoubiquitination. Table 3 summary of the different types of ubiquitination and their functions. When the Ub molecule forms a specific isopeptide bond with the carboxyl terminus of another Ub molecule through Lys residues, it is further coupled to form a multiubiquitin chain. Ubiquitin can also bind to non-lysine residues, such as cysteine (Cys). Ubiquitin chains with different links have different cellular functions, constituting the "ubiquitin code" of diversity and complexity (86). Monoubiquitination is generally associated with receptor internalization, while polyubiquitination is usually associated with proteasome degradation signaling (87). Related studies have found that K48 and K11 connected ubiquitin chains mediate substrate proteins to be digested into amino acids by soluble peptidase (26S proteasome complex) in cytoplasm or nucleus through a series of enzymatic reactions (88, 89). Many experimental studies have shown that the polyubiquitin chain of K63 is associated with non-proteasome functions, such as DNA repair, protein sorting, immune modulation, and regulation of the activation of the NF-κB signaling pathway, and also protects target proteins from multiple signaling pathway functions, including T cell receptors, Toll-like receptors (TLRs) and RIG-I-like receptor-mediated signal transduction (90–93). The mechanisms determining whether a protein is monoubiquitinated or polyubiquitinated are not fully understood and require further studies.

Ubiquitination of proteins is accomplished through a series of continuous enzymatic reactions (94–96) (Table 4). For the enzymes required for ubiquitination, it is currently estimated that eukaryotic organisms have two E1 enzymes (UBA1 and UBA6), with approximately 30–50 E2 enzymes and more than 600 E3 enzymes. In a tertiary E1-E2-E3 enzyme-linked reaction in mammals, any two members of the E1 family can label all E2 with Ub, and 40 known E2 enzymes can further transmit Ub to the E3s family. Auxiliary E4 enzymes have been explored (97); When the substrate protein signals, the E1 enzyme activates ubiquitin and starts the ubiquitination process, which requires ATP to provide energy. Then, the E3 ubiquitin ligase specifically recognizes the substrate protein and guides the E2 conjugation-carried Ub to

TABLE 3 Summary of the different types of ubiquitination and their functions.

	Ubiquitin type	Function
Substrate + E1/E2/E3 + Ub	K11	Protein degradation, regulation of cell cycle, DNA damage, and signal transduction.
	K48	Protein degradation.
	K63	DNA repair, translation, signal transduction, and trafficking.
	Others	Protein degradation and DNA repair.

TABLE 4 Ubiquitination involves several enzymes: ubiquitin-activating enzyme (E1), ubiquitin-coupling enzyme (E2), ubiquitin-ligase (E3), and deubiquitination enzyme (DUBs).

Ubiquitin-related enzymes	Effect
E1	E1 activates ubiquitin molecules in an ATP-dependent manner by forming E1-ubiquitin thioesters.
E2	E2 dominates the determination of ubiquitination and polyubiquitination and attaches ubiquitin peptides to the substrate.
E3	E3 determines substrate specificity and covalently binds ubiquitin carried by E2 to the target protein, thereby triggering degradation or modification of the target protein.
DUBs	Reversal of the ubiquitination of the substrate, cutting the ubiquitin chain from the substrate protein into a single reusable ubiquitin fragment.

covalently bind to the substrate protein (Figure 2) (98). In the ubiquitination system, these three different types of enzymes cooperate to complete the task of modifying proteins. The ubiquitin activator E1 binds to the tail of the ubiquitin molecule, and regulates the downstream of the ubiquitination reaction. Ubiquitin coupling E2 enzyme controls the length and connection type during the assembly of ubiquitin chains, and K48 and K63-mediated polyubiquitination regulates the inflammatory development of the NF-κB signaling pathway. The specificity of the ubiquitin chain is widely believed to be determined by E2-E3 (RING-E3s) matching or substrate-E3 (HECT and with E6-APC) complexes (99).

The stability, functional activity, and interaction of the modified proteins may change, but the modification is a reversible PTM process that can be removed by the UPS (Ubiquitin-proteasome system) or DUBs (Deubiquitinating enzymes) (100). The UPS can remove proteins that have been damaged or are no longer needed in cells (tumor suppressor proteins, cell cycle regulatory proteins, etc.), which is an energy-consuming but highly efficient way to degrade proteins. When the substrate has four or more UB or UBLs, the 26s protease hydrolyzes it into peptide chains, releasing ubiquitin monomers that can be recycled (101). DUBs catalytic deubiquitination modification makes the ubiquitination process maintain the dynamic balance of the cellular process. After DUBs are bound to the ubiquitin substrate protein complex, the ubiquitin chain is broken by severing the isopeptide bond between Lys and the C-terminal of ubiquitin, and the ubiquitin monomer is free and can be collected, thus starting the next round of ubiquitination.

There are approximately over 100 types of DUBs, including ubiquitin-specific proteases (USP), ubiquitin-c-terminal hydrolases

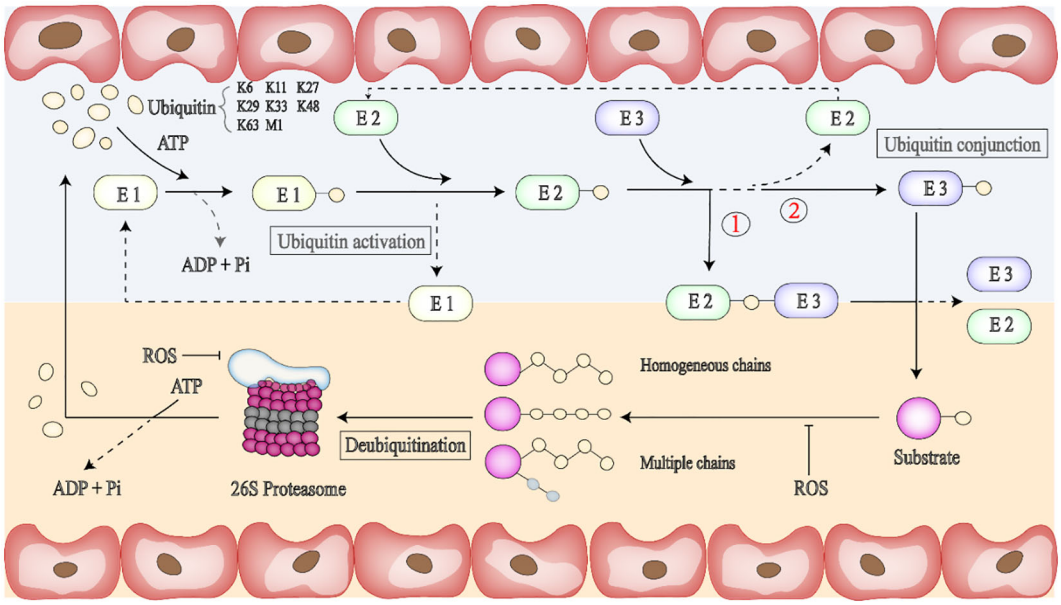

FIGURE 2
Binding of ubiquitin to protein substrates is a multi-step process. Ubiquitin molecules can be attached to ubiquitin or proteases containing lysine residues such as K6, K11, K27, K29, K33, K48, and K63. First, ATP provides energy, and the E1 enzyme activates ubiquitin and initiates the ubiquitination process. E2 enzyme can provide ubiquitin directly to the protein substrate through lysine (K) residues in the target protein. The third step is to bind the E2 ubiquitase-linked ubiquitin to the E3 ligase-linked target protein, and there are two binding forms, respectively,① E2-Ub-substrate protein-E3 or ② Ub substrate protein-E3. Ubiquitin protein ligase (E3) can promote the interaction with substrate proteins. Ubiquitination is a reversible post-translational protein modification; thus, the substrate carrying the ubiquitin chain can be deactivated by the UPS (Ubiquitin-proteasome system) or DUBs (Deubiquitinating enzymes), and free ubiquitin monomers can be re-recruited for the next round of ubiquitination. The ubiquitin chain usually determines the fate of ubiquitinated proteins.

(UCH), and so on (102, 103).Different DUBs play different roles in inflammation and cancer development by affecting their substrates' protein stability, enzyme activity, or subcellular localization (104). The USPs family is the most commonly studied DUB family. USPs regulate protein activation by dissociating single or multiple ubiquitin chains from ubiquitinated substrates (105). In malignant tumors, USP18 is elevated and activates AKT/mTOR signaling, promotes phosphorylated AKT (p-AKT) and p-mTOR protein expression, leading to cancer cell proliferation and migration (106). USP11 controls its stability by promoting deubiquitination of a residual protein (VGLL, a tumor suppressor) and exerts its tumor suppressor effect through the VGLL4/YAP-TEAD regulatory ring (107). Overexpression of USP14 inhibits I-κB and increases NF-κB phosphorylation while increasing cancer cell migration, invasion, and EMT (108). Under selective conditions, USP7, USP10, USP29, USP42, and other DUBs regulate p53 ubiquitination levels (109).

5 The relationship between ubiquitination, MSC/MSC-Ex, and IBD

The occurrence of IBD is mainly due to the abnormal amplification of the immune response of the intestinal mucosal immune system to microbial antigens from the gut in some specific populations carrying susceptible genes under certain circumstances, resulting in inflammatory damage of the intestinal mucosa. In 2001, NOD2 was identified as a susceptibility gene for CD due to its polymorphism (110). In addition, approximately 240 gene loci have been found to be associated with IBD susceptibility and occurrence, of which about 30 are CD and UC (111, 112). The loss of balance between proinflammatory and anti-inflammatory factors leads to the activation of NF-κB, TNFα, NLR, and TLR pathways to expand the range of inflammation and promote the development of IBD (Figure 3). According to mechanistic studies, a variety of signaling pathways have regulatory effects on IBD, but the specific activation, effects, and regulatory mechanisms largely remain unclear. Therefore, further exploratory studies on the pathogenesis of IBD, new targets for its treatment, and diagnostic biomarkers are needed.

The immune system is mainly composed of the innate and adaptive systems (113, 114). Ubiquitination plays a vital role in the regulation of innate immune signal transduction, but so far, only Lys residues have been identified as ubiquitination sites in innate immune signal molecules. Whether ubiquitination of non-lysine residues plays a role in innate immune signal transduction needs further study. Adaptive immunity relies on specific immune cells (T and B lymphocytes), mediating humoral and cellular immunity, and is characterized by the presence of highly specific antigen recognition receptors, namely T cell receptors (TCR) and B cell receptors (BCR). The regulatory effects of ubiquitination on immune cells are varied and not fully understood. MSCs and MSC-Exs have been shown to target the treatment of inflammatory diseases, including IBD, asthma,

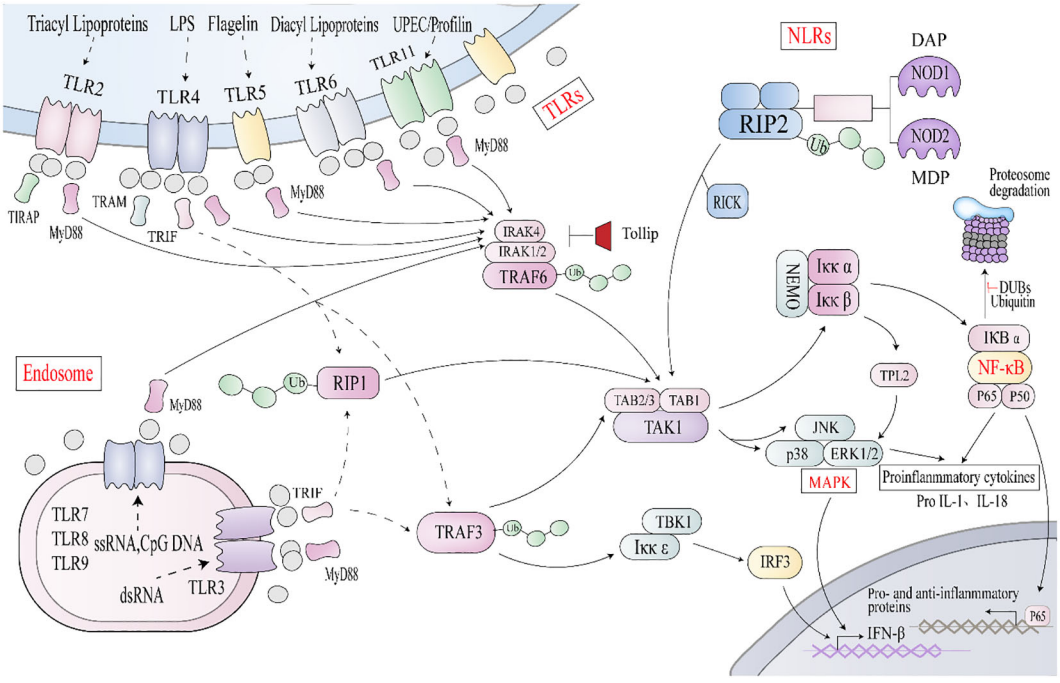

FIGURE 3
Key signaling pathways associated with IBD ① TLR stimulation triggers MyD88 to interact with IRAK4 (interleukin-1 receptor-associated kinase 4) and ② NLRs interact with RIP2, causing TRAF3, TRAF6, and Lys63 polyubiquitinizes RIP1, to recruit TAK1/TAB2/3 complex or IkK complex. This triggers the activation of NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways, promoting the transcription of proinflammatory and anti-inflammatory genes. Endosomal TLRs transmit signals through a TRIF-dependent pathway, and TRIF, along with RIP1 and TRAF3, activates TAK1 or IKKε, leading to phosphorylation of IRF3 and expression of interferon β.

and rheumatoid arthritis (115, 116). Ubiquitination is involved in the inflammatory response in IBD, serving as a potential therapeutic target through the modulation of MSC and MSC-Ex.

5.1 NF-κB signaling, ubiquitination, and MSC/MSC-Ex

Protein ubiquitination can regulate various signal-mediated inflammatory responses and plays an important role in the occurrence, development, and outcome of inflammatory diseases such as IBD. As a key component of the innate immune response, the NF-κB signaling pathway is one of the ultimate targets for regulating multiple upstream signaling pathways. Research has shown that the NF-κB signaling pathway is crucial in inducing pro-inflammatory gene expression, regulating inflammasome, activating inflammatory T lymphocytes, and innate immune cell differentiation in IBD patients. The activation of the NF-κB signaling pathway requires RIP2 and TAK1-mediated polyubiquitination of NEMO (NF-κB essential regulator kinase, also known as IKγ) and phosphorylation of the kappaB kinase inhibitor (IKK) complex consisting of NEMO, IKKα, and IKKβ. IKKs can be activated by bacterial lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), IL-1β, and various physical and chemical stresses (117). Phosphorylated IκB family proteins become targets of K48 polyubiquitination-dependent proteome degradation, releasing active NF-κB molecules into the nucleus. The classical NF-κB pathway is dominated by the action of IKKβ, which phosphorylates IKKβ family members such as IKKα and P105. When stimulated by LPS, IKK promotes nuclear translocation of p65 and p50 and induces expression of inflammatory factors such as TNF-α, IL-1β, IL-6, and IL-12, further leading to tissue damage (118).

Ub plays a crucial role in regulating the activation of NF-κB signaling. Relevant studies have shown that ubiquitin modification can affect intestinal mucosal inflammatory injury and intestinal epithelial cells' permeability and apoptosis by regulating the NF-κB signaling pathway. TRAF6, one of the E3 ubiquitin ligases, contains a highly conserved RING domain important for activating the NF-κB pathway (119). Chen et al. report that TRAF6 could only activate IKK under the condition of K63 polyubiquitination and that RING domain mutation results in the loss of ubiquitin ligase activity of TRAF6 and the failure to activate IKK (120). NIK is a central kinase in the non-classical NF-κB pathway and is also involved in the classical NF-κB pathway (121). Inhibition of the E3 ligase TRIM16 effectively increases the formation of K48-linked polyubiquitin chains on NIK (122). NEDD4 is associated with chronic inflammatory diseases, and a single rs8032158 transcription variant (TV3) in the NEDD4 genome has been found in keloid patients to activate the NF-κB signaling pathway by binding to the connexin RIP and highly selectively expressed (123, 124).

MSC and MSC-Ex regulate the NF-κB signaling pathway to influence the treatment of IBD. According to the current literature, the degradation of IκB is mainly dependent on neddylation, and when cullin1 activation is blocked, the accumulation of IκB leads to inhibition of NF-κB activity. Wang et al. verified that miR-326 in hucMSC-Ex inhibits the binding of free NEDD8 and substrate protein cullin1, preventing the expression of E1, E2, and E3 enzymes during neddylation formation (125). The hucMSC-Ex also inhibits the activation of the NF-κB signaling pathway, alleviating IBD. In addition, Qi et al. showed that serum-preconditioned adipose-derived MSCs (CM-AcMSC) significantly prevent the phosphorylation of p65 and IκB in colon cells of DSS-induced colitis model in rats, up-regulate the expression of MUC2 and tight-junction proteins such as ZO-1, claudin-1, and occludin, and protect the integrity of colon mucus (126). These results suggest that CM-AcMSC can significantly mitigate inflammation in colitis rats. A similar study showed that bone marrow MSCs (BM-MSCs) down-regulate the expression of NF-κB p65 mRNA in the colonic mucosa, suggesting that BM-MSCs may influence TNBS-induced colitis by regulating NF-κB mediated proinflammatory response (127). DC-derived exosomes activate the NF-κB signaling pathway via exosomal miR-146b to improve intestinal barrier function in DSS-induced colitis (128). Thus, a number of studies indicate that MSC and MSC-Ex play a role in the treatment of IBD by down-regulating NF-κB signaling, but the specific mechanism related to their regulation of NF-κB signaling via ubiquitination needs further study.

5.2 TLR signaling, ubiquitination, and MSC/MSC-Ex

TLRs are pattern recognition receptors. As an important part of the innate immune system, TLRs activate a series of downstream signals after recognizing pathogen-associated molecular patterns (PAMPs), inducing the secretion of inflammatory cytokines, chemokines, and type I interferons (129, 130). After binding to different stimuli, most TLRs initiate signal transduction by recruiting the adaptor protein MyD88, which contains the TIR domain and is a common important adaptor protein of most TLRs and a variety of envelope receptor-mediated signaling pathways. It plays a role in recruiting downstream kinases and regulating signal transmission; ① In the MyD88-dependent pathway, MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK4) to attract TLR, and the MyD88-IRAK4 complex recruits IRAK4 substrate IRAK2 or related IRAK1 to realize Myddosome (131, 132). This protein interacts with TRAF6, self-ubiquitination modification of TRAF6, activates TAK1, and ultimately stimulates NF-κB and JNK/P38/ERK signaling pathways, which participate in the colon inflammation in IBD; ② MyD88 signaling pathway can also be used as another pathway for TLR to induce inflammation. After TLR activation, it can activate the toll-like receptor-associated activator of interferon (TRIF) and TRAF3, resulting in NF-κB inhibiting the recruitment of protein kinase ε/tank-binding kinase 1 (IKε/TBK1), inducing the phosphorylation of IRF3 and the expression of interferon-β, and playing an antiviral role. TRIF- and MyD88-mediated signaling involve a series of ubiquitination events. TRAF3 and TRAF6, as members of the E3 ubiquitin ligase family, play an essential regulatory role in MyD88-dependent and TRIF- (non-MyD88) dependent signal transduction, which can be either a "positive signal" or a "negative signal". Moreover, TRAF3 and TRAF6 can regulate the activation of inflammation-related signaling pathways

through ubiquitin modification effects and initiate the gene transcription of many proinflammatory cytokines, such as IL-1, IL-6, TNF-α, and other transcription factors to activate a variety of immune responses, thereby playing a role in immune defense or relieving inflammation. Nrdp1 directly binds to and polyubiquitinates MyD88 and TBK1 while promoting TLR-triggered macrophages to inhibit the production of proinflammatory cytokines. In addition, Nrdp1 and Smurfp1/2 can catalyze the ubiquitination modification of MyD88 or remove the ubiquitin chain for negative regulation (133, 134). A20 and SIGIRR can also adjust the duration and/or strength of the TLR signal (135–137).

Macrophages are considered classic cells in TLR studies (138). They are the most critical cells in inducing colon inflammation, releasing large amounts of DAMP in damaged intestinal epithelial cells and activating NF-κB in intestinal macrophages signaling pathways that promote the secretion of inflammatory factors such as TNF-α and IL-1β, lymphocytes and monocytes, recruit chemokines (CCL-17 and CCL-24) and NO, and promote colon damage. Duan et al. demonstrated that LIM domain 7 (LMO7) is an important molecule that regulates macrophage polarization and inhibits intestinal inflammation in a DSS-induced IBD model (139). When proinflammatory activates macrophages, PFKFB3(6-phosphofructose-2-kinase/fructose-2,6bisphosphatase 3) promotes glycolysis by increasing the activity of phosphofructokinase-1 (PFK1). LMO7 promotes the degradation of PFKFB3 through K48-related ubiquitination, thereby effectively preventing excessive inflammatory response of macrophages and protecting tissues from inflammatory damage.

In the regulation of IBD by MSC and MSC-Ex, Liotta et al. demonstrated that the binding of TLR3 or TLR4 to MSCs can modulate their immunosuppressive activity against T lymphocyte proliferation, thereby restoring an effective T cell response during infections (140). Studies have shown that MSCs can inhibit the LPS/TLR4 signaling pathway, thereby reducing the release of inflammatory factors, improving intestinal symptoms of IBD, and reducing parenteral complications (141). Liu's team reports that metallothionein-2 in MSC-Exs, a key negative regulator of macrophage inflammatory response, plays an anti-inflammatory role in conjunction with other components of MSC-Exs to maintain intestinal barrier integrity and reduce experimental colitis in mice (142). Other studies indicate that MSC-Ev inhibits the activation of proinflammatory M1 macrophages and promotes their polarization to M2 macrophages, alleviating the inflammatory response and DSS-induced IBD (143). Deng et al. developed a technique that can sustainably release MSC-Exs for regenerative purposes using an *in situ* synthetic biotin-modified MSC-Ex (Bio-Ex) self-assembled biotinylation (144). The Bio-Ex can be taken up by macrophages and play an immunomodulatory role similar to MSC-Ex, promoting the polarization of macrophages to the M2 phenotype. Perhaps ubiquitin is involved in alleviating IBD through the TLR signaling pathway, or the regulation of macrophage inflammatory response by MSCs and MSC-Exs are linked with TLR/ubiquitination. However, the direct link to this hypothesis remains to be proven.

5.3 NLR signaling, ubiquitination, and MSC/MSC-Ex

Nucleotide-binding and oligomeric domain (NOD)-like receptors (NLRs) are a type of PRRs that are primarily distributed in the cytoplasm and have four broad classes of functions: inflammasome assembly, signal transduction, transcriptional activation, and autophagy (145–147). Cumulative data suggest that NLRs play a vital role in a variety of autoimmune diseases, such as IBD, multiple sclerosis (MS), and systemic lupus erythematosus (SLE) (148).

The C-terminal of NLRs (except NLRP10) contains a leucine-rich repeat sequence (LRR) that specifically recognizes the PAMPs' or DAMPs' molecular pattern. Based on the unique functional features of the N-terminal effector domain, NLRs can be divided into five subfamilies: NLRA, NLRB, NLRC, NLRP, and NLRX1. The structure of the NLRA subfamily includes CIITA (class II transcription activator), the activation of which is dynamically regulated by a series of post-translational modifications, such as acetylation, phosphorylation and ubiquitination.

Ubiquitination is involved in activating and terminating NOD signaling cascades. After NOD1 and/or NOD2 activation, the oligomerization of the NACHT domain between the NLRs N-terminal and the LRR domain activates and recruits the interacting proteins to form a semallome including RIP2(also known as RICK). E3 ubiquitin ligase cIAP1 forms a ubiquitin chain with RIP2, catalyzes the ubiquitination of RIP2, induces the activation of the TAK1 complex, triggers the activation of NF-κB and MAPK signaling pathway, and promotes the transcription of proinflammatory genes (IL1β, IL-18). Studies have shown that when the NOD1-RIP2 signaling pathway is activated, hybrid ubiquitin chains containing M1-Ub and K63-Ub bonds are rapidly produced, and hybrid ubiquitin chains may affect the deubiquitination rate of K63-Ub and M1-Ub chains. This affects the duration of the innate immune response (149). After NOD2-RIP2 activation, it promotes K)63-linked polyubiquitination of NEMO, thereby promoting the recruitment of TAK1 and activating the NF-κB signaling pathway (150, 151). TRAF4 is an E3 ligase that has been shown to negatively regulate NOD2 signaling (152). Moreover, autophagy-associated protein 16-like-1 (ATG16L1) negatively regulates NOD-driven inflammatory responses by interfering with RIP2 junction polyubiquitination (153).

In addition to NOD-mediated activation of NF-κB and MAPK, inflammatory bodies (NLRs) can also regulate inflammatory responses through ubiquitination modification (154). The NLRP3 inflammasome (NOD-, LRR- and pyrin domain protein 3) is the most studied, an intracellular polymeric protein signaling complex that participates in the innate immune system and plays an important role in maintaining intestinal homeostasis and preventing colitis (155, 156). NLR proteins such as NLRP3 detect pathogens or danger signals and trigger the assembly of caspase-1 inflammasome, leading to the processing and secretion of IL-1β and IL-18, promoting the proliferation and differentiation of pro-inflammatory macrophages and tissue damage. It also contributes to pyrosis, either directly or through ASC junction proteins. A study

found that in the GVHD model, activated NLRP3 inflammasome stimulates choline metabolized TMAO (trimethylamine N-oxide) to induce M1 macrophage polarization, leading to the differentiation of T1 and T17, which aggravated the disease (157). Ubiquitination also plays a vital role in the NLR signaling pathway. According to Xu et al. found that E3 ubiquitin ligase gp78 mediates the mixed ubiquitination of NLRP3 and inhibits the activity of NLRP3 by preventing the oligomerization and subcellular translocation of the NACHT domain of NLRP3, thereby reducing the activation of inflammasome and harmful effects (158). Mai et al. confirmed that promoting mitochondrial autophagy driven by E3 (Parkin) and inhibiting the activation of colonic NLRP3 inflammasome has an inhibitory effect on mouse DSS-induced colitis (159). The E3 ligase TRIM31 binds NLRP3 directly and promotes K48-linked NLRP3 ubiquitination and proteasome degradation, maintaining low NLRP3 expression and preventing unwanted inflammasome activation (160). Moreover, interference with A20 inhibits macrophage proliferation and M2-like polarization by activating the NLRP3 inflammasome pathway (161).

Studies report that MSC-Ex miR-378a-5p targets and blocks NLRP3 inflammasome activation in macrophages, leading to Caspase-1 cleavage and IL-1β and IL-18 reduction, delaying pyroptosis cell death and improving IBD (162). MSC-Ex alleviates colitis by increasing FXR in the colon, which binds to the NLRP3 inflammasome and inhibits the activation of inflammasome components (163, 164). The regulation of IBD inflammation by ubiquitination and the treatment of IBD with MSCs and MSC-Exs also involve NOD and NLR signaling pathways, and there may be some unknown relationships that require further studies.

5.4 T cell activation, ubiquitination, and MSC/MSC-Ex

When microbes and metabolites interact with pattern recognition receptors (PRR), such as pregnane X receptor (PXR) and TLR, there is activation of signaling pathways and key proteins that control mucosal barrier and intestinal immune functions. When pathogens invade the human body, TLRs guide mucosin-2 (MUC2) in the intestinal mucus layer to prevent intestinal pathogens and their secretions from penetrating the mucosa, thus playing an essential role in preventing inflammation (165–167). During the progression of IBD, activated T lymphocytes infiltrate the inflamed site and produce a variety of cytokines, further aggravating intestinal inflammation. T cells in IBD patients are composed of pro-inflammatory effector subsets (Th1, Th2, and Th17) and/or regulatory T (Treg) cells that have immunosuppressive effects and maintain intestinal homeostasis (168, 169). The proportion of $CD8^+$ T suppressor cells and $CD4^+$ T helper cells in the lamina propria and epithelium of the intestinal tract of IBD patients is usually normal, but the activated cells showed an increasing trend. The cytokine IL-13 secreted by Th2 cells plays a role in UC, and Th17 is involved in the pathogenesis of IBD through the production of IL-17A. On the other hand, Treg cells suppress the immune response and prevent self-hyperimmunity, and IL-10 produced by Treg cells inhibits the proinflammatory cell Th17 in the gut. In contrast, the elimination of IL-10 receptors in Treg cells leads to Th17 dysregulation and colitis (170).

Both programmed death protein 1 (PD-1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) appear to be key markers for controlling T cell tolerance and have negative regulatory effects on T cell immune function (171, 172). CTLA-4 is mainly involved in the early stages of T cell immune responses in lymph nodes (173, 174), while PD-1 is mainly involved in the late stages of T cell immune responses in peripheral tissues. PD-1 is expressed by activated T cells and downregulates T cell effector function after binding to its ligands PD-L1 and PD-L2 on antigen-presenting cells (175). Intestinal epithelial cells of IBD patients overexpress PD-L1 and PD-L2 (176), and PD-1 blocking can reverse the *in vitro* inhibition of effector T cells mediated by Treg. Most studies have shown that Tregs constitutively express CTLA-4, which is considered important for its inhibitory function (177).In a $FoxP3^+$ conditional knockout mouse model with CTLA-4 deletion, Wing et al. clearly demonstrated that CTLA-4 deficiency in $FoxP3^+$ Treg cells impairs its inhibitory function (178). Experimental data of Takahashi et al. showed that the signals sent by TCR and CTLA-4 may activate $CD25^+CD4^+$ regulatory T cells, thereby transmitting negative signals of activation and proliferation to other T cells. These possibilities are currently being investigated (179).Therefore, maintaining a balanced ratio of Th17 and Treg cell populations is essential for maintaining the intestinal immune system (180).

There is growing evidence that ubiquitination-degrading proteins play a role in suppressing immune responses by targeting the destruction of signaling proteins and pro-transcription factors. Dysfunction of E3s ubiquitin ligase, the final step in catalyzing ubiquitin attachment to substrate proteins, can lead to abnormal T cell activation and loss of tolerance to autoantigens, resulting in immune dysfunction (181), and mice lacking these proteins show significant inflammation and/or autoimmune-like symptoms. Several E3 ubiquitin ligases, most notably Itch, Roquin, and CBI-b, have been shown to regulate T cell activation. Ramon et al. found that NEDD4 family interacting protein 1(Ndfip1) is a regulatory protein of Itch, and the combination of the two can make JunB ubiquitination (182). JunB is a transcription factor that promotes the expression of Th2 cytokines IL-4 and IL-5, Therefore, blocking the production of IL-4 and IL-5 may be one of the targeted mechanisms of Th-cell associated gastroenteritis and IBD. In addition, deubiquitinating enzymes USP22 and UCHL1 have been shown to deubiquitinate and stabilize PD-L1 protein (183, 184). Proteolytic targeting chimeras (PROTACs) can recruit the E3 ligase RNF43 to the lysosomes that induce PD-L1, promoting its ubiquitination and subsequent degradation (185). Fujiwara et al. first demonstrated that Cbl-b deficiency in mice leads to functional resistance of T cells and NK cells to PD-L1/PD-1-mediated immune regulation and mild autoimmune reactions (186). To date, there has been no report of any E3 directly ubiquitinating CTLA-4 or CTLA-4 ubiquitination sites. However, multiple studies have shown a strong correlation between CTLA-4 ligation and the function and expression of Cbl-b (187). It is currently known that the main CTLA-4 inhibitory function is mediated by key T cell inhibitory

E3 ligases, namely Cbl-b, Itch, and GRAIL (188). Overall, Cbl-b not only mediates CTLA-4 signaling but also mediates PD-1-induced immunosuppression.

Exosome-mediated immune response has been shown in the pathophysiology of IBD (189). The effect of MSCs on T cells seems to depend on the MSC/T cell ratio: a high MSC/T cell ratio has a strong inhibitory effect, while a low ratio may enhance T cell proliferation. It is known that T-box (T-BET) and retinol-associated orphan receptor γ(t) in T cells (RORγT), as central regulators of Th1 and Th17 cells, respectively, are pathogenic factors for IBD progression, while MSCs may down-regulate Th1-Th17driven autoimmune and inflammatory responses by influencing the expression of T-bet and RORγt (190). HucMSC prevents experimental colitis by increasing the number of $CD5^+$ B cells and $CD5^+$Bregs that produce IL-10, and restores Treg/Th17/Th1 imbalances (191). Olfactory Ecto-Mesenchymal Stem Cell-Derived Exosomes (OE-MSC-Exs) regulate T cell responses and have a significant inhibitory function on $CD4^+$ T cells, presenting a novel cell-free therapy for IBD and other inflammatory diseases (192).

5.5 TGF-β signaling, ubiquitination, and MSC/MSC-Ex

Impairment of the TGF-β signaling pathway is associated with the development of intestinal inflammation in experimental models and IBD patients. TGF-β is an immunosuppressive cytokine produced by various cell types (immune cells, nonhematopoietic cells) and activated by integrins. There are three types of TGF-β in mammals: TGF-β1, TGF-β2, and TGF-β3. TGF-β receptors are widely expressed in body tissues and cells; therefore, members of the TGF-β family regulate the proliferation, differentiation, apoptosis, and inflammation of many cells. TGF-β signaling requires two transmembrane receptors, RI and RII types, that have serine/threonine kinase activity. When the active TGF-β ligand binds to the RII receptor, it activates the kinase activity of the RI receptor and the recruitment of Smad protein, inducing Smad complex formation, nuclear transport, and Smad DNA binding (193). Smad works with universal transcription factors (GTF), -determining transcription factors (LDTF), and other driving factors or helper proteins to regulate target gene transcription.

The TGF-β signaling pathway has been studied for its effective regulatory and inflammatory activity (194). In intestinal immunity, TGF-β inhibits intestinal bacterial antigens' inflammatory response and helps induce immune tolerance. IBD is characterized by abnormal TGF- β signaling (195). High expression of Smad7 in $CD4^+$ T cells is associated with severe colitis (196). Studies have shown that sCYLD (a short splicing form of CYLD) mediates ubiquitination of K63 junctions and nuclear translocation of Smad7 and that the sCYLD-Smad7 complex inhibits TGF-βsignaling in $CD4^+$ T cells (197). It is worth noting that Smurf1 and Smurf2, members of the E3 ubiquitase family, are critical negative regulators of the TGF-β signaling pathway (198). Smad7 recruits E3 ubiquitin ligases such as Smurf1, Smurf2, and NEDD4L to TGF-β receptors, promoting their ubiquitin-mediated degradation. RNF11 may mediate AMSH ubiquitination through the formation of the Smurf2/RNF11 complex, leading to its degradation by the 26S proteasome, negatively regulating TGF-β signaling (199).

TGF-β1 in exosomes is thought to have therapeutic potential in IBD. Exosomes produced by TGFβ1 gene-modified DCs can inhibit the development of IBD by inhibiting Th17 (200). Another study showed that TGF-β1-modified exosomes (TGF-β1-Exs) induce $CD4^+$ $Foxp3^+$ Tregs, reducing the proportion of Th17 in lymphocytes at the site of inflammation, mitigating the inflammatory response in a mouse model of colitis (201). In experimental models and IBD patients, impaired TGF-β signaling pathways have been associated with the development of intestinal inflammation. OE-MSC-Exs can regulate cell proliferation, decrease the levels of inflammatory cytokines IL-17 and IFN-γ, and increase the inhibitory cytokines TGF-β and IL-10, suggesting that OE-MSC-Exs may effectively alleviate the severity of experimental colitis by inhibiting effector T cells and enhancing regulatory T cells (192). Ma ZJ et al. found that MSC-Ex decreased the concentrations of IFN-γ, TNF-α, and IL-1β and increased the secretion of TGF-β1 and IL-10 by up-regulating anti-inflammatory response and down-regulating inflammatory response (202). It is suggested that MSC-Ex shows therapeutic power in a mouse model of DSS-induced colitis by inhibiting inflammatory mechanisms. Pd-MSC-Ev inhibits TGF-β1-induced inflammatory cytokine secretion and fibrotic marker expression, suggesting that MSC-EVs is expected to be a promising anti-fibrotic drug (203).

5.6 Deubiquitase and E3 ubiquitin ligase in IBD and MSC/MSC-Ex modulation

A variety of deubiquitases and E3 ubiquitin ligases are involved in regulating the process of IBD. USP15 binds to Lys48-linked ubiquitin chains to achieve deubiquitination, inhibiting the degradation of TAB2 and TAB3, thus hindering the selective autophagy degradation mediated by autophagy cargo receptor 1 (204). The proteasomal-associated deubiquitinase USP14 is involved in the negative regulation of the type 1 IFN signaling pathway and can also inhibit the activation of the NF-κB signaling pathway by deubiquitinating K63-linked retinoid-inducing gene I (RIG-I) (205, 206). USP19 negatively regulates the activation of TAK1-TAB1 dependent NF-κB signaling pathway by specifically removing TAK1 coupled Lys63 and Lys27 linked polyubiquitin chains, resulting in impaired TAK1 activity and destruction of the TAK1-TAB2/3 complex (207). The E3 ubiquitin ligase TRIM56 induced by type I IFN interacts with STING and targets STING for K63 junction polyubiquitination, recruitment of TBK1, and induction of IFN-β to induce innate immune response (208).TRIM32 can also interact with STING on mitochondria and ER, facilitating STING interaction with TBK1 (9). Upregulation of surface MHC Class II (MHCII) and co-stimulatory molecules such as CD80 and CD86 leads to DC maturation, E3 ligase membrane-associated RING-CH-1 (MARCH1) promotes endocytosis and lysosomal degradation of MHCII and CD86 by ubiquitinating

them. Thus limiting the antigen-presenting capacity of dendritic cells (209–211).

Sun et al. showed that USP11 plays a catalytic and non-catalytic role in regulating the stability of IκBα, thereby negatively regulating TNF-α induced NF-κB activation (212). Deubiquitination of USP26 has been found to stabilize SMAD7, leading to reduced TGF-β signaling (213). Moreover, RNF182 promotes the degradation of cytoplasmic p65 through K48 ubiquitination, inhibiting inflammatory responses (214). In patients with IBD, up-regulated USP16 expression levels can be found in macrophages. When stimulated by LPS or TNF-α, USP16 specifically removes Lys33-linked IKKs polyubiquitin chains and promotes IKK-β-mediated phosphorylation of P105, leading to autoimmune responses and the development of IBD (215). Li Y et al. confirmed that CVMSC-Exs promote trophoblast migration and proliferation by up-regulating TRIM72 expression, thereby promoting P53 ubiquitination, proteasome degradation, and reducing cell apoptosis (216). Patients with inflammatory bowel disease (IBD) have higher levels of angiotensin-converting enzyme 2 (ACE2) expression in the gut (217).ACE2 deubiquitination mediated by deubiquitination enzyme UCHL1 and ACE2 SUMO mediated by E3 SUMO protein ligase PIAS4 can increase ACE2 protein levels, while AP2-mediated lysosomal degradation can decrease ACE2 protein levels (218, 219). NEDD4 has been shown to be involved in Ev biosynthesis (exosome production), and NEDD4 is a novel GSDMD interacting protein. Bulek et al. 's data suggest that GSDMD uses selective autophagy components (including LC3+ vesicles) to mediate NEDD4-dependent sEV biosynthesis for IL-1β output, thereby supporting the interaction between autophagy and exosome biosynthesis (220), Regardless of the proposed mechanism, exosomes can alleviate DSS-induced colitis in mice by controlling ubiquitin modification levels (17). However, at present, there is little literature on the clear association between deubiquitin/E3 ubiquitin ligase and MSC/MSC-EX in IBD disease, and the specific mechanism still needs to be further explored (17, 221–223).

6 Conclusion

Scientists have been exploring more effective and easier ways to treat IBD, mainly focusing on the interaction between genetic, environmental, immunological, and gut microbial factors, to discover further fundamental mechanisms of IBD occurrence and preventive strategies. The aim is to reduce patients' medical and disease burden and improve the quality of life of affected individuals (224). Despite the best efforts, the application of MSCs and MSC-Exs remains a black box filled with unknown secrets. Studies have reported that treatment with MSCs or similar cells may promote the likelihood of cancer in patients; although this risk is unlikely to exist, there is still a chance of occurrence (225). As research continues to deepen and clinical trials advance, we hope to better understand the risks and potential benefits of exosome treatment for patients. Thus, the search for new, safe, efficient, and low-cost treatments for IBD, including MSC-Exs, is still underway.

Several experimental conclusions have proved that ubiquitin modification is involved in regulating important signaling pathways, such as NF-κB, NOD, TGF-β, and TNF-α. In addition, the dysregulation of components of the ubiquitination system often leads to various diseases such as cancer, IBD, and other autoimmune diseases. Some ubiquitin enzymes are known to directly regulate various inflammation-related transcription factors from the Smad, p53, Jun, and other families, and the ubiquitination-mediated degradation of signaling intermediates is an essential means to terminate inflammatory responses (226). However, how ubiquitination mediates the transmission and function of inflammatory signals to trigger the occurrence of IBD is largely unexplored and requires further studies. Moreover, the link between ubiquitination, IBD, and MSCs/MSC-Exs could provide an experimental basis for a novel therapeutic target and subsequent clinical application. More exploratory studies are needed in this area.

Author contributions

HL: Writing – original draft. XM: Writing – review & editing. LW: Writing – review & editing. NW: Writing – review & editing. DO: Writing – review & editing. BW: Writing – review & editing. FM: Conceptualization, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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E3 ubiquitin ligase gene *BIRC3* modulates TNF-induced cell death pathways and promotes aberrant proliferation in rheumatoid arthritis fibroblast-like synoviocytes

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovitis, degradation of articular cartilage, and bone destruction. Fibroblast-like synoviocytes (FLS) play a central role in RA, producing a significant amount of inflammatory mediators such as tumor necrosis factor(TNF)-α and IL-6, which promote inflammatory responses within the joints. Moreover, FLS exhibit tumor-like behavior, including aggressive proliferation and enhanced anti-apoptotic capabilities, which collectively drive chronic inflammation and joint damage in RA. TNF is a major pro-inflammatory cytokine that mediates a series of signaling pathways through its receptor TNFR1, including NF-κB and MAPK pathways, which are crucial for inflammation and cell survival in RA. The abnormal proliferation and anti-apoptotic characteristics of FLS in RA may result from dysregulation in TNF-mediated cell death pathways such as apoptosis and necroptosis. Ubiquitination is a critical post-translational modification regulating these signaling pathways. E3 ubiquitin ligases, such as cIAP1/2, promote the ubiquitination and degradation of target proteins within the TNF receptor complex, modulating the signaling proteins. The high expression of the *BIRC3* gene and its encoded protein, cIAP2, in RA regulates various cellular processes, including apoptosis, inflammatory signaling, immune response, MAPK signaling, and cell proliferation, thereby promoting FLS survival and inflammatory responses. Inhibiting *BIRC3* expression can reduce the secretion of inflammatory cytokines by RA-FLS under both basal and inflammatory conditions and inhibit their proliferation. Although BIRC3 inhibitors show potential in RA treatment, their possible side effects must be carefully considered. Further research into the specific mechanisms of *BIRC3*, including its roles in cell signaling, apoptosis regulation, and immune evasion, is crucial for identifying new therapeutic targets and strategies.

KEYWORDS

BIRC3/cIAP2, tumor necrosis factor, rheumatoid arthritis, ubiquitination, MAPK/NF-κB

1 Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune condition marked by synovitis, degradation of articular cartilage, and bone destruction. A notable pathological feature is the abnormal proliferation of fibroblast-like synovial cells (FLS), a common occurrence in RA. Under normal conditions, molecular mechanisms in human joints regulate FLS turnover, ensuring apoptotic clearance without excessive accumulation (1). Conversely, pathological conditions can disrupt these processes, leading to carcinoid-like deposits of FLS due to aberrant cell death dynamics. Notably, apoptosis and necroptosis serve as the principal cellular death pathways in this context (2). Apoptosis acts as a regulatory mechanism for necrotic cell death, potentially triggered in its absence, whereas necroptosis represents a form of regulated necrosis, similarly inducible without apoptotic pathways (3). Tumor necrosis factor (TNF), an inaugural cytokine capable of inducing cellular necrosis, exerts pro-inflammatory effects that enhance cell activation, proliferation, and survival (4). As progenitors of a broad family of ligands, their diverse functions have a significant impact on cellular fate. It has been determined that receptor-interacting serine/threonine kinase 1 (RIPK1) mediates inflammation and cell death, facilitating both apoptosis and necroptosis in TNF-stimulated cells (5, 6). Moreover, TNF and nuclear factor-kappa B (NF-kB) signaling pathway performs vital functions in various cancers and immune responses (7, 8). *RIPK1*-regulated inflammatory signaling, apoptosis, and programmed necrosis play crucial roles in RA-FLS. The interaction of RIPK1 with TNF receptor 1 (*TNFR1*) activates the NF-κB inflammatory signaling cascade, leading to RA inflammation. Modulation of RIPK1 activity affects FLS survival and death, thus influencing the intensity and duration of inflammation (9). *RIPK1* may further regulate the physiological and pathological functions of RA-FLS by interacting with other signaling molecules, such as MAPK. These interactions influence cell proliferation, migration, and secretion of inflammatory mediators, thereby playing a key role in the pathogenesis of RA (10). Although ubiquitination is an integral component of these signaling cascades, the ubiquitination-mediated proteasomal degradation pathway (UPP) has a vital part in the pathophysiology of RA by controlling the degradation of proteins associated with the inflammatory process. This pathway could affect the levels of inflammatory factors and other proteins, thereby influencing the inflammatory and immune responses in RA (11–14).

Ubiquitination, a vital post-translational modification, includes ubiquitin (Ub) and its target protein forming a covalent binding. Three different enzymes must function in succession for this process to occur, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) (15, 16), culminating in the formation of an isopeptide bond between the C-terminal glycine (Gly76) of Ub and a lysine residue on the target protein (16). In rheumatoid arthritis (RA), ubiquitination influences immune cells and inflammatory responses by altering critical inflammatory signaling pathways, including NF-κB. For example, synoviolin 1 (*SYVN1*) is a novel pathogenic factor in RA, inherently an E3 ubiquitin ligase, highly expressed in the synovial tissues of RA patients and mice (17). *SYVN1* elevates quantities of inflammatory cytokines like TNF-α and IL-1β, inhibits apoptosis of synovial cells in collagen-induced arthritis in mice, and significantly promotes synovial tissue proliferation (18). Furthermore, midline 1 facilitates the progression of RA by regulating the proliferation, migration, invasion, and inflammatory activities of FLS through ubiquitin-mediated proteasomal degradation of DPP4 (19). While TNF-α inhibitors have markedly revolutionized the management of RA, not all patients exhibit responsiveness, and a significant majority experience relapse upon cessation of therapy (20). The clinical use of TNF inhibitors still needs to be further improved, in which case ubiquitination could potentially be used as an adjuvant therapeutic strategy. Ubiquitination can directly affect specific signaling proteins within the TNF receptor complex, promoting their degradation and thereby mitigating TNF-induced inflammatory responses. It can also specifically degrade excessively active inflammation-related proteins without completely suppressing the immune system, potentially reducing side effects of TNF inhibitors, such as immunosuppression and infection risk (21).

As E3 ubiquitin ligase, cellular inhibitor of apoptosis protein 1 (cIAP2), and cellular inhibitor of apoptosis protein 2 (cIAP2) are involved in the proliferation and survival of FLS by regulating TNF receptor signaling and inflammatory cytokine production to influence the immune response to RA, and interacting with apoptosis-related signaling pathways to prevent apoptosis (22). In addition, cIAP1 and cIAP2 play roles in the assembly of inflammasomes, regulating the release of inflammatory mediators and affecting the function of immune cells (23). Notably, cIAP2 is encoded by the *BIRC3* gene. Studies have found that *BIRC3* mediates NF-κB activation in various regions of RA synovium, promoting inflammation and disease progression. *BIRC3* also regulates inflammation and apoptosis in RA-FLS, indicating its dual role in promoting FLS survival and inflammatory responses (24). This review comprehensively analyzes the involvement of E3 ubiquitin ligases cIAP1/2 in RA-FLS, delving into the significance of cIAP1/2 in the regulation of TNF-induced cell death pathways and RA pathophysiology, emphasizing their roles as E3 ubiquitin ligases. Additionally, we highlight the potential clinical significance of the *BIRC3* gene, which encodes cIAP2 protein, as a therapeutic target, offering new perspectives for RA treatment.

2 Inhibitors of apoptosis proteins

CIAP1 and cIAP2 are members of the inhibitor of apoptosis protein (IAPs) family and play critical roles in regulating various cellular processes (25). IAPs, akin to their counterparts in baculovirus-infected mammalian cells, play pivotal roles in cellular regulation, including apoptosis, proliferation, and differentiation. This protein family includes X-linked IAP (*XIAP*), cellular IAPs 1 and 2 (cIAP1 and cIAP2), neuronal apoptosis inhibitory protein (*NAIP*), and baculoviral IAP repeat containing 5(*BIRC5*) (26, 27). IAPs are central to the modulation of several cellular processes, including signal transduction, cytokine production, and cell survival, thereby influencing both innate and adaptive immune responses. The E3 ubiquitin ligase activity of XIAP and cIAP1 primarily governs immune regulatory functions by targeting key signaling pathways

such as NF-κB and mitogen-activated protein kinase (MAPK). Additionally, *NAIP*, cIAP1, and cIAP2 orchestrate inflammasome assembly, which is crucial for innate immune response (28). The hallmark of the IAP family is the baculovirus IAP repeat (BIR) domain, which is essential for protein-protein interactions (29, 30). In addition to the BIR domain, IAPs also possess other significant domains, including the C-terminal ubiquitin-conjugated (UBC) domain, caspase recruitment domain (CARD), and C-terminal RING zinc-finger domain, facilitating a range of functional interactions (29, 31). Originally characterized as caspase inhibitors, mammalian IAPs (cIAP1, cIAP2, and XIAP) also modulate apoptosis via E3 ubiquitin ligase activity (27). Specifically, cIAP1 and cIAP2 are integral to the receptor complexes of the TNF receptor family members, modulating signal transduction via ubiquitination of associated proteins. This includes their recruitment to TNFR1 through the TNF receptor-associated death domain (*TRADD*)/TNF receptor-associated factor 2 (*TRAF2*) pathway, leading to the ubiquitination of *RIPK1* within the TNFR1 complex (32, 33). The absence or inhibition of cIAP1 and cIAP2, particularly by the second mitochondrial activator of caspases (SMAC), renders cells vulnerable to TNF-induced apoptosis by disrupting NF-κB-mediated survival signals and facilitating the formation of pro-apoptotic TNFR1-induced complex II or *RIPK1*/*RIPK3*-dependent apoptosis (34, 35).

3 TNF-related pathway of cell death induced by cIAP1/2

3.1 TNFR1-induced complex

Binding of TNF to its receptor TNFR1 catalyzes the immediate assembly of the TNFR1 signal complex (TNFR1-SC), previously known as the TNF receptor 1 signaling complex (TNF-RSC). This complex incorporates *TNF*, TNFR1, *TRADD*, *RIPK1*, TNF receptor-associated factor 2 (TRAF2), cIAP1/2, linear ubiquitin chain assembly complex (LUBAC), inhibitor of kappa B kinase (IKK), and TGF-beta activated kinase 1 (TAK1) binding protein (TAB)-TAK complex (32). The integration of TAB-TAK and IKK complexes into TNFR1-SC relies on the recognition of lysine 63 (K63) and methionine 1 (M1) ubiquitin bonds by TAB2/3 and the NF-κB essential modulator (NEMO), respectively (36–38). The TAB-TAK complex requires only a K63 chain for recruitment, whereas the IKK complex requires both the K63 and M1 linkages. cIAP1/2, which serves as a crucial intermediary, facilitates this process by ubiquitinating several components of Complex I with K63-linked chains, including *RIPK1*. Subsequent recruitment of LUBAC enhances the M1-linked chain modification on *RIPK1* (39–41). These modifications recruit the TAK1 and IKK complexes, activating the MAPK and NF-κB pathways. Additionally, cIAP1 enhances IKK complex recruitment through the K11-linked chain modifications of *RIPK1* (42). Complex I represents the primary assembly in this signaling cascade. However, if *RIPK1* is deubiquitinated by CYLD lysine 63 deubiquitinase (*CYLD*) or remains unubiquitinated during Complex I formation (e.g., due to IAP depletion) (43), Complex IIa or IIb is generated, leading to apoptosis or necroptosis based on cellular conditions (44). Contrary to cIAP1/2's supportive role in TNFR1 signaling, its function in other TNF receptor family members, such as the tumor necrosis factor-like weak apoptosis inducer (TWEAK) and CD40 ligand (CD40L), is inhibitory. cIAP1/2, in conjunction with *TRAF2* and *TRAF3*, suppresses alternative NF-κB pathways through ubiquitination of lysine 48 (K48) linkages and degradation of NF-κB-inducing kinase (NIK), in the absence of ligand stimulation (28) (Figure 1).

3.2 TNF-related apoptosis-inducing ligand receptor–induced complex

TRAIL (tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as the APO-2 Ligand (*APO2L*), belongs to the family of tumor necrosis factors. The activation of TRAIL-R has been shown to induce the formation of complexes containing proteins similar to those induced by TNFR1, which are regulated by ubiquitin. In TRAIL-R signal transduction, pro-apoptotic proteins (FADD and Caspase-8) are first recruited to TRAIL-R and can be used as scaffolds for anti-apoptotic protein recruitment (*RIPK1*, *TRAF2*, cIAP1/2, LUBAC, TAK1, and IKK complexes) (45–47). The compound mentioned earlier is called complex I. The composition of complex **II** is very similar to that of **complex I**. It is generally believed that the former dissociates from activated TRAIL-R and forms cytoplasmic complexes (48). TRAIL-R-induced complexes **I** and **II** can activate NF-κB, MAPK pathway, and apoptosis, but only complex **II** can activate necroptosis (45). E3 ligase not only regulates apoptosis, but also the gene activation output of the TRAIL signal by participating in the two core signal components of TRAIL-induced gene activation, caspase-8 and *RIPK1* (47, 49). This process involved cIAP1/2. The E3 ligase cIAP1/2 is recruited to two TRAIL signaling complexes in a FADD-caspase-8-dependent manner. Furthermore, *TRAF2* and cIAP1/2 both promote TRAIL- and CD95L-mediated gene activation (47, 49–51). In the context of TNF signaling, TRAF2-cIAP1/2-mediated ubiquitination of RIPK1 promotes NF-kB activation (32, 42, 52, 53), the TRAF2's gene activation function is dependent on its ability to recruit cIAP1/2 (33). Consistent with the activation of *TRAF2* as a TRAIL signal transduction scaffold, cIAP1/2 depletion strongly reduced *RIPK1* ubiquitin, IKK recruitment, NF-kB activation, and inactivation of TRAIL-mediated cytokine secretion, whereas *TRAF2* recruitment remained unaffected (49, 54). As previously demonstrated for TNF signal transduction, *TRAF2* may promote TRAIL-induced cytokine production by acting as a recruitment platform for cIAPs (33). Through an unknown mechanism, cIAP1/2 is also required downstream of *TRAF2* to recruit LUBAC to track complex I (49). LUBAC mediates TNFR1-SC stabilization via linear ubiquitination of *TRADD*, *RIPK1*, NEMO, and TNFR1 and is critical for TNFR1-induced gene activation signaling (40, 55). TRAF2 depletion strongly reduced *RIPK1* ubiquitination, IKK recruitment, NF-kB activation, and TRAIL-mediated cytokine secretion, whereas *TRAF2* recruitment was unaffected (49, 56) (Figure 2).

Although several recent studies have demonstrated that the mechanism by which TRAIL promotes RA inflammation does not depend exclusively on cell death, TRAIL regulation of RA inflammation is mainly due to its ability to promote apoptosis of

synoviocytes and infiltrating lymphocytes (57). TRAIL produces various signals. TRAIL can induce migration, proliferation, and cytokine production in both cancerous and non-cancerous cells, in addition to inducing cell death via apoptosis and necroptosis. As a result, uncovering the mechanisms that regulate the complex balance between these various outputs can help us better understand TRAIL's role of TRAIL in tissue homeostasis, immunity, and cancer. Previous animal experiments have confirmed that $TRAIL_{PEG}$ ameliorates arthritis severity and significantly reduces the accumulation of inflammatory molecules (p-p65, ICAM-1, Cox-2, *MMP3* and iNOS), pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-6, IL-17) and activated macrophages (58), all of which have been shown to play a cellular and cellular component in important role in the pathogenesis of RA. The role of TRAIL in the mechanism of RA-FLS apoptosis has also been confirmed in several studies, and this role correlates with disease severity in a cell cycle-dependent manner (59–61). Blocking the expression of TRAIL and cIAP can significantly promote the apoptosis of FLS (59–61). In addition, ubiquitin and deubiquitination are key regulators of immune receptor signal transduction (45).

In comparison to TRAIL-R, the precise space and time positions of proteins in the TRAIL-R signal cascade are not well defined in TNFR1. However, it is unclear what determines whether complex I/II induces non-apoptotic or apoptotic signal transduction. However, ubiquitination of the M1 connection of Caspase-8 by LUBAC inhibits its activity (49), which may promote the spread of survival and inflammatory pathways that are usually inhibited by Caspase-8 activity (47, 62).

4 MicroRNA inhibits cIAP1/2 expression

In RA, both serum and synovial fluid (SF) samples, as well as synovial tissues and the serum and joints of rats with adjuvant arthritis, exhibit notably reduced expression of miR-17. Investigations revealed that miR-17 diminishes the expression of *TRAF2*, a cellular inhibitor of cIAP1 and cIAP2, in RA SFs when stimulated by TNF-α. Moreover, restoration of miR-17 activity enhanced the polyubiquitination of lysine 48 (K48) linkages of *TRAF2*, cIAP1, and cIAP2 in these cells, as determined by immunoprecipitation analyses. This modulation by miR-17 leads to the destabilization of TRAF2, impairing its ability to associate with cIAP2. Consequently, TNF-α suppresses the nuclear translocation of NF-κB p65, c-Jun, and signal transducer and activator of transcription 3 (*STAT3*) prompted by TNF-α. This molecular alteration results in decreased production of IL-6, interleukin-8 (IL-8), matrix metalloproteinase-1 (*MMP1*), and matrix metalloproteinase-13 (*MMP13*) in human RA SF, underscoring the pivotal regulatory role of miR-17 in inflammatory and degradative pathways in RA (63).

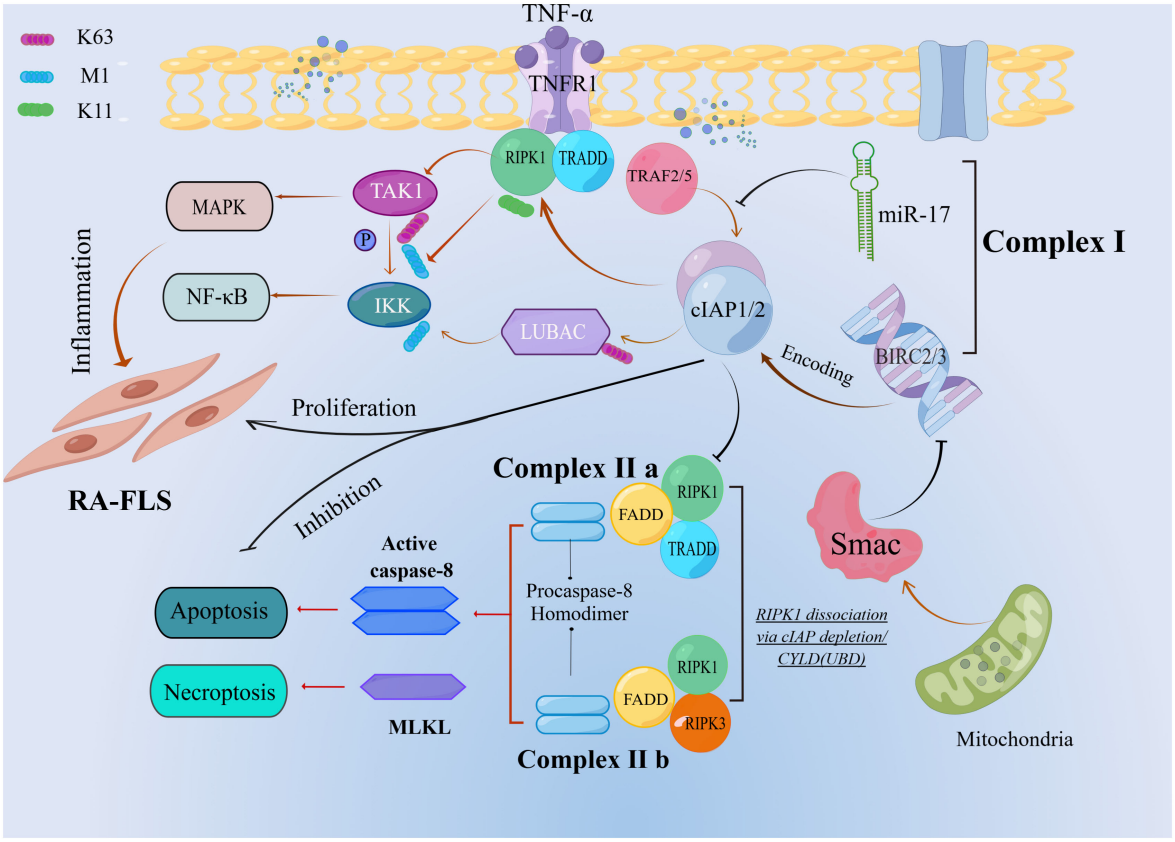

FIGURE 1
Ubiquitin-mediated regulation of TNFR1 apoptotic signaling. The cIAP1/2 mediates a series of ubiquitination in complex I. When *RIPK1* dissociation via cIAP depletion or *CYLD* is deubiquitinated, complexIIb is formed, which leads to apoptosis.

Besides RA-FLS, miRNAs also affect cIAP2 expression in other diseases. Studies have shown that *BIRC3* mRNA is upregulated in hepatocellular carcinoma (HCC) tissues, and the high expression of cIAP2 encoded by *BIRC3* promotes HCC proliferation and migration. Although miR-124 was underexpressed in HCC tissues and cell lines, *BIRC3* was identified as its target gene. Overexpression of miR-124 could target BIRC3, thereby decreasing cIAP2 protein levels and inhibiting HCC proliferation and migration through regulating the NF-κB signaling pathway (64). Circular RNA (circRNAs) are also involved in gene regulation. The oncogenic circular RNA hsa_circ_0070039 (circNUP54) is significantly upregulated in HCC. This interaction stabilizes downstream *BIRC3* mRNA by binding to the 3'-UTR region, thereby promoting HCC progression via the HuR/BIRC3/NF-κB axis (65). Furthermore studies on oral squamous cell carcinoma (OSCC) have shown that silencing circDOCK1 and upregulating miR-196a-5p results in increased apoptosis and decreased *BIRC3* formation (66).

5 Smac mimetics mediate cell death through degradation of cIAP1/2

IAPs, especially cellular cIAP1/2 and XIAP, can prevent cell death by preventing the activation of caspase-8 or inhibiting the activity of caspases-9,-3 and-7 (67–69), respectively. The E3 ubiquitin ligase domain of cIAP1/2 promotes cIAP1/2 and proteasome-dependent degradation (70, 71). A second mitochondria-derived caspase activator (Smac/DIABLO), which, along with cytochrome C, is released from the mitochondria under the induction of an inherent apoptotic pathway in response to stimuli such as genotoxic stress, relieves the inhibition of apoptosis by IAPs (72, 73). Direct binding and blocking of the interaction of XIAP with caspase-9,-3, and-7, as well as inducing proteasomal degradation of cIAP1 and cIAP2, are the main mechanisms by which Smac mimetics cause cell death (34, 35, 74, 75). The degradation of cIAP1/2 allows the release of *RIPK1* from TNFR1 and then incorporates the complex associated with caspase-8 and the fas-related death domain, which can promote cell death and cover the survival-promoting effect of NF-κB signaling (32, 76, 77). This means that any cell expressing TNFR1 should respond to Smac mimic/TNF-α therapy because the expression of TNFR1 is very common in all types of cancer cells (78). The E3 ubiquitin ligase activity of cIAP promotes cancer cell survival (32, 35, 74, 76). Furthermore, removing cIAP from cancer cells can alter cytokine signaling by converting pro-death signals into inflammatory signals, resulting in cell death via apoptosis or necroptosis (46, 79). *BIRC3* (cIAP2) is thought to be a member of the IAP family of proteins that regulate cell death and survival, according to Bai et al. (80). Previous

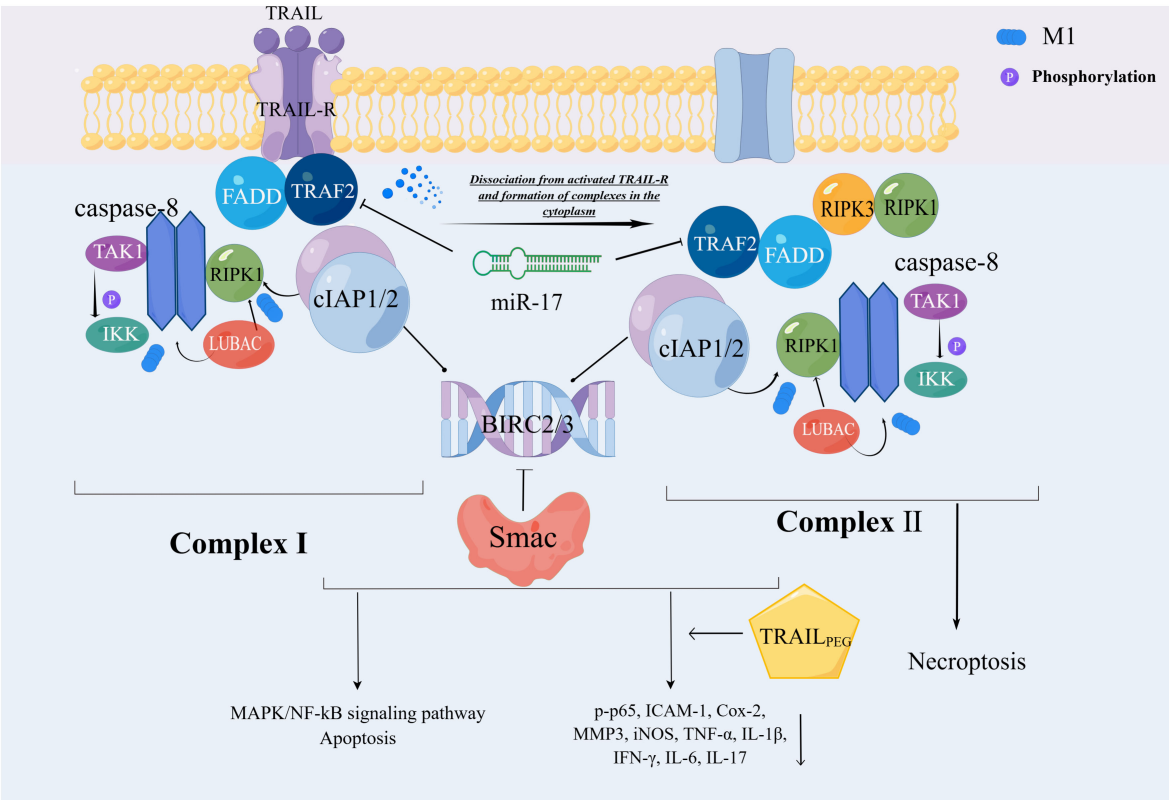

FIGURE 2
Ubiquitin-mediated regulation of TRAIL-R cell death signaling pathway. The composition of complexes I and II is relatively similar, with complexII derived from I detached from activated TRAIL-R. CIAP1/2 is involved in the ubiquitination of this process. Through the M1 chain, cIAP1/2 indirectly catalyzes the functional activation of caspase-8, which ultimately leads to the MAPK/NF-kB signaling pathway or apoptosis. This series of reactions is manifested in both complexes, but only complex 2 causes necroptosis.

studies have shown that *BIRC3* (cIAP2) promotes survival and anti-apoptosis in cancer cells and is a therapeutic target of the drug family known as "Smac mimic" (81), although not all cases support this view (81). Owing to its dual function in TNF, cIAP's regulatory role of cIAP in cancer and the immune system is critical. Abnormal expression results in uncontrollable outcomes. In the field of cancer applications, the Smac simulator has been used in clinical trials of hematological and solid cancers, but unfortunately, the results so far are inconsistent and limited (82). The role of the Smac simulator in the treatment of RA is theoretically applicable (83). However, thus far, there has been little research on Smac simulations in RA-FLS. *In vitro*, it has been confirmed that *BIRC3* has an abnormally high expression in RA-FLS compared to OA. Simultaneously, given the carcinoid proliferation of FLS and the similar TNFR1 signal transduction mechanism of cIAPs in RA, Smac mimetics may be of breakthrough significance in the treatment of RA.

6 Discussion

Rheumatoid arthritis is a chronic inflammatory autoimmune disease characterized by the infiltration of immune cells into the synovium, leading to the release of inflammatory cytokines and subsequent tissue damage (84). In addition to inflammation, fibroblast-like synoviocytes, the principal effector cells in RA, also exhibit tumor-like proliferation and invasiveness. Therefore, controlling inflammation and inhibiting FLS proliferation are crucial elements in the treatment of RA (85). CIAP1 and cIAP2, E3 ubiquitin ligases, play critical roles in the regulation of *TNFR1* and TRAIL-R signal transduction, ensuring effective signal propagation. These proteins modulate various NF-κB pathways, which are pivotal for controlling diverse cellular inflammatory and immune responses. Although cIAP1 and cIAP2 are frequently discussed collectively because of their overlapping functions in ubiquitination across different signaling pathways, their specific roles in modulating cell death in distinct cell types and tissues remain poorly understood (86). In certain pathological conditions, cIAP1 has been shown to mediate disease progression through its regulatory effects on TNF signaling, whereas cIAP2 does not participate in this process (42, 87). Conversely, cIAP2 exhibits a unique therapeutic mechanism distinct from that of cIAP1 in the context of RA, highlighting the differential contribution of these proteins to disease pathology and treatment (88). In addition, within the array of death receptor (DR) ligands, TRAIL has garnered significant interest because of its structural homology with CD95L (89, 90) and its distinct ability to selectively eliminate cancer cells without notable systemic toxicity (91, 92). This characteristic has spurred the development of TRAIL receptor agonists (TRAs), although their clinical trials have been discontinued because of issues with their efficacy (93). Consequently, the targeted modulation of *BIRC3* gene expression and ubiquitination processes in TNFR1 or TRAIL signaling mediated by *BIRC3* (cIAP2) present promising strategies for enhancing treatments for related disorders, including autoimmune diseases.

The *BIRC3* gene encodes the multifunctional protein cIAP2, which plays an important role in the regulation of various cellular processes including caspases, apoptosis, inflammatory signaling, immunity, mitogen-activated protein kinase signaling, and cell proliferation (94). We speculate that increased expression of *BIRC3* in RA promotes FLS survival and contributes to the inflammatory response. Inhibition of *BIRC3* expression can reduce the secretion of inflammatory cytokines by RA FLSs under both basal and inflammatory conditions, and impede their proliferation. Existing evidence has demonstrated that *BIRC3* plays a crucial role in promoting cancer cell survival and inhibiting apoptosis (95). The interaction between *BIRC3*, MAP3K14, and the NF-κB pathway highlights the impact of *BIRC3* inactivation on tumor cells dependent on this pathway (96). For example, in colorectal cancer cells, overexpression of *BIRC3* can lead to the activation of receptor-interacting serine/threonine protein kinase 2 (*RIPK2*), which promotes the ubiquitination of *IKBKG*, thereby inhibiting *IKBKG* protein expression and enhancing the expression of NF-κB subunits p50 and p65 (97). Recent studies have established a correlation between Fusobacterium nucleatum (Fn) and the occurrence and development of colorectal cancer (CRC), particularly noting that Fn infection upregulates *BIRC3* in CRC cells via the TLR4/NF-κB pathway (98). Moreover, the upregulation of *BIRC3* reduces the responsiveness of CRC cells to 5-fluorouracil (5-Fu), suggesting that targeting *BIRC3* may offer a promising strategy to alleviate chemotherapy resistance in advanced CRC (99).

Additionally, *BIRC2/BIRC3*, in complex with TNFR2 and TRAF2, activates the NF-κB and MAPK signaling pathways, which are crucial for inflammation and cell survival (24). Combining anti-tumor necrosis factor therapy with cIAP1/2 inhibitors by blocking the *BIRC2/BIRC3*-mediated apoptotic pathway has the potential to improve therapeutic efficacy and effectively reduce symptoms and disease progression in rheumatoid arthritis. According to researches, glioblastoma (GBM) is a highly malignant brain tumor characterized by elevated *BIRC3* expression, which is associated with tumor progression from low to high differentiation and resistance to temozolomide (TMZ) and radiation therapy, ultimately leading to reduced patient survival rates (100). Inhibiting the E3 ubiquitin ligase activity of *BIRC3* can increase tumor cell sensitivity to radiotherapy and chemotherapy, and developing *BIRC3* inhibitors may offer a promising approach for treating glioblastoma. Currently, small molecule inhibitors or antibodies targeting *BIRC3* are being developed as potential treatments to disrupt its E3 ubiquitin ligase activity and induce tumor cell apoptosis (101). A comprehensive study of the specific mechanisms of *BIRC3*, including its role in cell signaling, regulation of apoptosis, and evasion of immune responses, is crucial for identifying new therapeutic targets and strategies.

Although *BIRC3* inhibitors show promise in treating rheumatoid arthritis, potential side effects must be carefully considered, such as inducing apoptosis in normal cells dependent on *BIRC3*-regulated pathways and triggering adverse immune responses due to its role in immune regulation. FLS may activate other anti-apoptotic mechanisms, such as upregulating myeloid cell leukemia1(*MCL1*), thereby reducing sensitivity to *BIRC3* inhibitors (102). Furthermore, changes in the surrounding microenvironment, such as alterations in immunogenicity and extracellular matrix composition, may also affect the efficacy of these inhibitors.

This review discusses the critical roles of the E3 ubiquitin ligases cIAP1/2 in mediating cell death pathways induced by TNF and

promoting abnormal proliferation of fibroblast-like synoviocytes in RA, emphasizing the importance of the *BIRC3*-mediated ubiquitination process and its potential as a therapeutic target. However, the review has several limitations. Primarily, it focuses on molecular and cellular mechanisms due to the scarcity of clinical studies on *BIRC3* in RA, lacking support from large-scale, multicentric clinical trials. Additionally, it does not cover longitudinal studies to track the long-term effects of *BIRC3* inhibition on RA, which could provide deeper insights into the durability of treatment effects and potential development of resistance or adverse reactions. Although the article mentions several signaling pathways affected by *BIRC3*, it does not sufficiently explore alternative pathways that might compensate for *BIRC3* inhibition, which could aid in understanding potential resistance mechanisms and refining therapeutic strategies. Due to the limited research on targeting *BIRC3*, there is a lack of discussion on the potential side effects and safety concerns of *BIRC3*-targeted therapies. Finally, a more robust comparative analysis is needed, especially comparing the role of BIRC3 in RA with other autoimmune diseases, to provide a broader perspective on its specific functions and potential as a therapeutic target.

Author contributions

QM: Investigation, Methodology, Writing – original draft. KW: Investigation, Methodology, Writing – original draft. YS: Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

RA	rheumatoid arthritis
BIRC3	baculoviral IAP Repeat Containing 3
TNF	tumor necrosis factor
CIAP1	cellular inhibitor of apoptosis protein 1
CIAP2	cellular inhibitor of apoptosis protein 2
FLS	fibroblast-like synoviocytes
RIPK1	receptor interacting serine/threonine kinase 1
Ub	ubiquitin
Gly76	C-terminal glycine
TNF-α	tumor necrosis factor alpha
IAPs	Inhibitors of apoptosis proteins
FADD	X-linked IAP
NAIP	neuronal apoptosis inhibitory protein
BIRC5	baculoviral IAP repeat containing 5
NF-kB	nuclear factor-kappa B
MAPK	mitogen-activated protein kinase
BIR	baculovirus IAP repeat
UBC	C-terminal ubiquitin-conjugated
CARD	caspase recruitment domain
TRADD	TNF receptor-associated death domain
SMAC	second mitochondrial activator of caspases
TNFR1-SC	TNFR1 signal complex
TNF-RSC	TNF receptor 1 signaling complex
TRAF2	TNF receptor-associated factor 2
LUBAC	linear ubiquitin chain assembly complex
IKK	inhibitor of kappa B kinase
TAK1	TGF-beta activated kinase 1
K63	lysine 63
NEMO	NF-κB essential modulator
CYLD	CYLD lysine 63 deubiquitinase
CD40L	CD40 ligand
TWEAK	tumor necrosis factor-like weak apoptosis inducer
K48	lysine 48
RING	really interesting new gene
NIK	NF-kB-induced kinase
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
APO2L	APO-2 Ligand
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand

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ICAM-1	intercellular cell adhesion molecule-1
MMP3	matrix metallopeptidase 3
iNOS	inducible nitric oxide synthase
IL-1β	interleukin 1 beta
IFN-γ	interferon gamma
IL-6	interleukin 6
IL-8	interleukin-8
IL-17	interleukin 17
STAT3	signal transducer and activator of transcription 3
MMP-1	matrix metalloproteinase-1
MMP-13	matrix metalloproteinase-13
DR	death receptor TRAs, TRAIL receptor agonists
MCL1	myeloid cell leukemia1
GBM	glioblastoma

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The ubiquitin-proteasome system in the tumor immune microenvironment: a key force in combination therapy

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The ubiquitin-proteasome system (UPS) plays a crucial role in modulating the proliferation, activation, and normal functioning of immune cells through the regulation of protein degradation and function. By influencing the expression of immune checkpoint-associated proteins, the UPS modulates T cell-mediated anti-tumor immune responses and can potentially facilitate the immune escape of tumor cells. Additionally, the UPS contributes to the remodeling of the tumor immunosuppressive microenvironment (TIME) by regulating B cells, dendritic cells (DCs), macrophages, and Treg cells. Targeting the UPS in conjunction with immune checkpoint-associated proteins, and combining these with other therapeutic approaches, may significantly enhance the efficacy of combination therapies and pave the way for novel cancer treatment strategies. In this review, we first summarize the composition and alterations of the TIME, with a particular emphasis on the role of the UPS in TIME and its interactions with various immune cell types. Finally, we explore the potential of combining UPS-targeted therapies with immunotherapy to substantially improve the effectiveness of immunotherapy and enhance patient survival outcomes.

KEYWORDS

UPS, TIME, immune cells, immunotherapy, cancer

1 Introduction

The UPS is a unique intracellular protein degradation mechanism that primarily labels proteins with highly conserved ubiquitin polypeptides, facilitating the recognition and degradation of target proteins by the 26S proteasome (1, 2). Over 20 years ago, researchers identified that intracellular proteins are conjugated to highly conserved small ubiquitin polypeptides, forming complexes that bind to the 26S proteasome and are ultimately recognized and degraded into small molecular compounds. As scientific understanding of the UPS's structure and function has deepened, it has been revealed that ubiquitination is a process in which a series of ubiquitinating enzymes (E1, E2, and E3) sequentially transfer

ubiquitin molecules to specific intracellular targets (3). In this process, E1 first activates the ubiquitin molecule through ATP hydrolysis and uses the released energy to generate a unique ubiquitin (Ub)-E1 complex. The E1 complex is then transferred to a cysteine residue at the E2 active site (4). Assisted by E3 ubiquitin ligase, the E2 complex facilitates the conjugation of ubiquitin to the substrate, thereby ensuring substrate specificity (5). The typical ubiquitination process involves the C-terminal glycine of ubiquitin (Glycine 76) actively recognizing and binding to lysine residues of the target protein, forming an isopeptide bond that subsequently affects normal protein degradation. Atypical ubiquitination, such as that formed through ester or thioester bonds, while less common, has also demonstrated unique biological significance in regulating certain cellular processes (6). Moreover, ubiquitin itself has eight potential ubiquitination sites, including K6, K11, K27, K33, K29, M1, K48, and K63. This allows ubiquitin to form various ubiquitin chains, further adding to the complexity and regulation of the ubiquitination process (7).

Cancer is an extremely complex disease, involving a combination of processes such as genetic mutations, DNA damage, immune escape, and aging. The high expression of tumor immune checkpoint proteins inhibits immune recognition and normal immune responses (8). In particular, programmed death receptor 1 (*PD-1*) and programmed death ligand 1 (*PD-L1)* significantly suppress immune responses in the TME by interacting with T cells (9), thereby promoting tumor proliferation and metastasis (10). The UPS plays a critical role in regulating PD-1/PD-L1 expression and function, providing novel regulatory targets for cancer immunotherapy (11).

This review aims to summarize the intricate relationship between the UPS and the TIME. The focus is on detailing the interactions between the UPS and various immune cell types, particularly the alterations in the PD-1 pathway in T cells. By exploring the mechanisms of UPS action in TIME and its role in PD-1/PD-L1 expression and stability, we can better leverage these targets to develop novel therapeutic strategies, enhancing the efficacy of cancer treatment and improving patient survival outcomes.

2 Composition and function of immune cells in the TIME

In the TIME, various immune cells interact to form a complex network comprising both anti-tumor immune cells, which can attack tumor cells, and pro-tumor immune cells, which promote tumor proliferation and spread. The homeostatic balance of these cells is crucial in regulating tumor progression and response to therapy.

2.1 Anti-tumor immune cells in TIME

Anti-tumor immune cells are well-known for their ability to kill tumor cells through complex regulatory mechanisms. These cells include cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, classically activated macrophages (M1 macrophages), and helper T cells (Th cells) (12). However, within the TIME, the normal activation and function of these anti-tumor immune cells can be significantly inhibited.

With the advancement of single-cell sequencing technology, the roles of T cells in anti-tumor immune responses differ across various subtypes (13). Naive T cells: Naive T cells themselves are not directly anti-tumor cells, but they can differentiate into effector T cells upon antigen stimulation, thereby participating in the anti-tumor immune response. Effector T cells: Effector T cells are among the primary anti-tumor immune cells, particularly effector CD8+ T cells (cytotoxic T lymphocytes, CTLs), which can directly recognize and kill tumor cells (14). CTLs recognize antigens presented by MHC-I molecules on the surface of tumor cells (15). Guided by chemokines, CTLs kill tumor cells directly by releasing granules containing granzymes A and B, or by inducing apoptosis through interactions with death ligands, such as Fas ligand (16). CTLs also enhance tumor cell killing by secreting cytokines like interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (17). Effector T cells: Effector T cells mediate tumor cell apoptosis by secreting cytotoxic molecules such as perforin and granzymes, as well as cytokines like IFN-γ (18). Memory T cells persist after the initial immune response and can rapidly respond upon re-exposure to the same antigen. CD8+ memory T cells provide strong immune protection in the event of tumor recurrence, effectively suppressing the regrowth of tumors (19). CD4+ Helper T Cells (Th Cells): CD4+ T cells promote CTL proliferation and activation by secreting cytokines, enhance the antigen-presenting capability of DCs, and contribute to the formation of memory CTLs, thus playing a key role in long-term anti-tumor immunity (20, 21). DCs: As the most potent antigen-presenting cells (APCs), DCs are critical in initiating adaptive immune responses. They greatly enhance T-cell activation and function by expressing co-stimulatory molecules such as CD80 and CD86, which interact with CD28 on T-cells (22, 23). Additionally, DCs secrete a variety of cytokines (e.g., TNF-α, IL-6, IL-8, and IL-12), which play a key role in regulating local immune responses (24). NK Cells: The absence of MHC-I molecules is a common strategy employed by tumor cells to evade CTL surveillance. NK cells can directly lyse and kill tumor cells lacking MHC-I molecules by recognizing these tumor cells and releasing perforin and granzymes (25). M1 Macrophages: M1 macrophages contribute to the Th1-type immune response by secreting pro-inflammatory cytokines and anti-tumor factors such as TNF-α, IL-1β, and IL-12 (26, 27). They also directly kill tumor cells by releasing reactive oxygen species (ROS) and nitrogen species (RNS) (28).

The synergistic actions of these cells constitute the body's first line of defense against tumor growth and metastasis. Their activities are extensively regulated by intercellular signaling exchanges within the TIME. Understanding these processes is crucial for developing effective immunotherapeutic strategies against tumors.

2.2 Immunosuppressive cells

Regulatory T Cells (Tregs): In the TIME, the substantial enrichment of Tregs suppresses NK cell and CD8+ T cell activity through the massive secretion of immunosuppressive cytokines

such as TGF-β, IL-35, and IL-10, which in turn suppress NK cell and CD8+ T cell activity (29, 30). Tregs also induce apoptotic signaling by engaging with death ligands on the surface of effector T cells, such as FasL and PD-L1 (31). In cell contact-dependent effects, Tregs inhibit IL-2 secretion and T-cell activation by blocking CD28-mediated costimulatory signaling via *FBXO38* (32). Additionally, Tregs inhibit the antigen-presenting function of DCs by binding LAG-3 protein on their surface to MHC class II molecules on DCs (33, 34). Myeloid-Derived Suppressor Cells (MDSCs): MDSCs inhibit the immune response of effector T cells through multiple mechanisms. They disrupt T cell signaling and metabolic pathways by producing reactive species such as arginase, nitric oxide synthase, and ROS, which degrade intracellular signaling molecules and inhibit cell surface receptor expression (35). MDSCs also diminish T cell proliferation and survival by depleting key immune-stimulating amino acids such as L-arginine. Furthermore, MDSCs inhibit T cell activity by expressing PD-L1 and interacting with Tregs to foster an immunosuppressive environment (36, 37). M2 Macrophages: M2 macrophages suppress inflammation and promote tumor cell survival and proliferation by secreting immunosuppressive factors such as TGF-β and IL-10. They also enhance local immunosuppression by producing chemokines like CCL22, which attract Tregs to the TME (38, 39). M2 macrophages support tumor growth and metastasis by enhancing the tumor cells' capacity for angiogenesis and tissue remodeling (e.g., through the secretion of VEGF and PDGF) (40).

These immunosuppressive cells interact through intricate mechanisms, forming a complex immunosuppressive network in the TME, which is crucial for tumor growth, progression, and resistance to immunotherapy. Understanding these interactions helps identify new therapeutic targets that may enhance the efficacy of tumor immunotherapy by disrupting these cells' functions or blocking their signaling pathways.

3 Crosstalk between the UPS and various immune cells

3.1 T cells

T cells are critical components of anti-tumor immunity, and their normal development and activation are essential for effective immune responses. Recent research has highlighted the pivotal role of immune checkpoints, such as PD-1/PD-L1 and CTLA-4, in enabling tumor immune evasion (41). E3 ubiquitin ligases regulate these specific molecular mechanisms, thereby influencing T cell activity and anti-tumor immune function. These E3 ligases affect the stability, localization, and function of immune checkpoint proteins through specific ubiquitination events (42). FBXO38: FBXO38, a component of the SCF (Skp1-Cullin-F-box) complex, specifically targets the Lys233 residue in the cytoplasmic domain of PD-1 for polyubiquitination (43, 44). This polyubiquitination, typically Lys48-linked, signals for proteasome-dependent degradation of PD-1. Consequently, FBXO38 directly reduces PD-1 expression, inhibiting PD-1/PD-L1 interaction. This reduction significantly enhances T cell activity, thereby improving their cytotoxic effects on tumor cells (45). Cbl-b: Cbl-b inhibits CD28-mediated signaling pathways in T cells. Specifically, Cbl-b actively binds to and ubiquitinates the p85β subunit of PI3K, thereby inhibiting CD28-dependent PI3K pathway signaling (46). CD28 counteracts this by inhibiting Cbl-b's pathway, with *NEDD4* ubiquitinating Cbl-b under CD28 co-stimulation, leading to Cbl-b's degradation via the proteasome, thus suppressing its signaling pathway (47, 48). However, within the TME, PD-1 and CTLA4 enhance Cbl-b activity, subsequently inhibiting the expression of Cbl-b downstream effectors and promoting TIME formation. CTLA4, through Cbl-b, inhibits the activity of key proteins such as *PKCθ*, *Vav1*, and *PLCγ*, leading to diminished anti-tumor immune responses by T cells (49). Modulating the ubiquitination status of relevant signaling proteins can therefore enhance T cell activity and improve their tumoricidal capacity. β-TrCP and GSK3β: GSK3β phosphorylates unglycosylated PD-L1, creating a binding site for β-TrCP (50, 51). This phosphorylation is necessary for β-TrCP to mediate the ubiquitination and subsequent degradation of PD-L1 (52). β-TrCP, part of the SCF complex, recognizes and binds phosphorylated PD-L1, leading to its ubiquitination and proteasomal degradation. This reduces PD-L1 levels on the cell surface, thereby diminishing its inhibitory effect on T cells (53). COP9 Signalosome (CSN) and CSN5: CSN5, a component of the COP9 signalosome, functions as a deubiquitinating enzyme that removes ubiquitin chains from PD-L1, preventing its degradation. This leads to sustained high expression of PD-L1 on the cell surface, which in turn enhances the immunosuppressive response (54). Beyond its direct effect on PD-L1, CSN5 modulates the inflammatory response and immune evasion mechanisms by deubiquitinating other key regulatory proteins, such as downstream factors of NF-κB (55, 56).

Tregs play crucial immunosuppressive roles within the TIME, and their functions are directly influenced by the regulation of various ubiquitinating enzymes. Several key deubiquitinating enzymes (DUBs) and E3 ubiquitin ligases are instrumental in regulating Treg stability and function. USP7: FOXP3, a key transcription factor in Treg differentiation, is stabilized and functionally expressed by USP7 (57). USP7 enhances Treg stability and immunosuppressive function by deubiquitinating FOXP3, thereby preventing its degradation. In the TME, this contributes to maintaining Treg-mediated immune suppression, aiding tumor immune evasion (58). USP21: In Tregs, USP21 depletion correlates with a significant reduction in FOXP3 expression and other Treg signature genes, indicating its critical role in maintaining Treg function. Inactivating USP21 may weaken Treg immunosuppression by reducing FOXP3 stability, presenting a potential target for immunotherapy (59). USP22 and USP9X: These DUBs significantly influence T cell activity by regulating PD-L1 deubiquitination (60). Within the TIME, these enzymes help maintain high levels of PD-L1, thereby contributing to tumor-mediated immune suppression and enhancing Treg functionality (61). GRAIL: GRAIL, a RING-type E3 ubiquitin ligase, regulates CD4+ T cell function. Overexpression of GRAIL in Tregs transforms normal CD4+ T cells into regulatory phenotype cells (62). GRAIL also regulates Treg function by degrading the TCR-

CD3 complex, thereby reinforcing tumor immune escape. VHL: VHL, another E3 ubiquitin ligase, regulates thymus size and cell number by targeting HIF-1α for ubiquitination and proteasome-dependent degradation (63). Accumulation and enhanced activity of HIF-1α may affect Treg production and function, thereby impacting immune homeostasis in the TME (64). TRAF Family Proteins (TRAF3 and TRAF6): These adaptor E3 ubiquitin ligases regulate T cell and Treg development by influencing IL-2 signaling and thymic stroma development. In mouse models, TRAF3 deletion induces massive Treg proliferation in the thymus, while TRAF6 deletion impedes thymic epithelial cell development, both affecting central tolerance establishment (65).

By elucidating the specific mechanisms of action of these E3 ligases and their impact on T cells, we gain deeper insight into how they regulate tumor immune evasion and T cell activity. These insights provide valuable targets for therapeutic strategies aimed at modulating or interfering with these ubiquitination processes.

3.2 DC cells

In the TIME, DC function plays a pivotal role in tumor immunosurveillance and immune escape mechanisms, primarily through the precise regulation of ubiquitination. Ubiquitination modulates DC maturation, antigen-presenting capacity, and interactions with T cells within a complex network of molecular interactions and signal transduction pathways (66). Ubiquitination in DC Maturation and Antigen Presentation: During the immature state of DCs, MARCH1 drives lysosomal degradation and endocytosis by ubiquitinating co-stimulatory molecules like CD86 and the β-subunit of MHCII, thereby maintaining an immune-tolerant state in DCs (67). In the TME, this mechanism may facilitate tumor cells in evading immune surveillance. Upon receiving maturation signals such as TLR agonists, MARCH1 expression is downregulated, reducing the ubiquitin-mediated degradation of MHCII and CD86. This reduction allows these molecules to accumulate on the cell surface, thereby enhancing the DC's ability to present antigens to T cells and provide co-stimulatory signals essential for activating an anti-tumor immune response (68). Furthermore, CD83 interacts with MARCH1 to inhibit CD86 ubiquitination, promoting stable CD86 expression on the DC surface and enhancing its T cell activation capability (69). This regulatory mechanism is particularly critical in the TIME, as it directly influences the activation state of DCs. Regulation of the NF-κB Pathway by Ubiquitination: NF-κB is a key transcription factor regulating DC function, with its activation tightly controlled by ubiquitination and deubiquitination processes (70). In the TME, inflammatory signals received by DCs, such as TLR agonists, lead to the recruitment of MyD88 and IRAK family kinases, which subsequently interact with the E3 ubiquitin ligase TRAF6 (71). TRAF6, in cooperation with the E2 ligase Ubc13, promotes K63-linked ubiquitination of IRAK1/4, a critical step in activating the IKK complex and NF-κB. During this process, LUBAC (linear ubiquitin chain assembly complex) is crucial for full NF-κB activation (72). LUBAC extends the K63-linked ubiquitin chain through M1-type ligation, which further enhances IKK complex expression and recruitment, ultimately promoting NF-κB release, transcriptional activity, and the pro-inflammatory, activation state of DCs in the TME (73). Ubiquitination in Regulating Tumor Escape Mechanisms: Within the TIME, ubiquitination not only regulates internal signaling and functions of DCs but also influences interactions between tumor cells and DCs by modulating surface molecules on tumor cells (74). MARCH1 is an E3 ligase that can ubiquitinate MHC-II molecules on the surface of tumor cells, leading to their endocytosis and degradation. MHC-II molecules are crucial proteins for antigen presentation, and dendritic cells (DCs) recognize and capture these molecules to present antigens and activate T cells. When MHC-II molecules are degraded by ubiquitin ligases such as MARCH1, the ability of DCs to capture tumor antigens is reduced, thereby suppressing DC function and the anti-tumor immune response (75).

Ubiquitination orchestrates DC function in the TIME through a series of intricate molecular mechanisms. The precise regulation of these mechanisms is vital for activating effective anti-tumor immune responses.

3.3 Tumor-associated macrophages

In the TIME, E3 ligases play a crucial role in recruiting TAMs to the tumor site and facilitating their functional expression, thereby promoting tumor growth and immune escape.

CRL: CRL is a large E3 ligase complex composed of multiple subunits, involved in the ubiquitination and degradation of various proteins (76). In the regulation of TAMs, CRL mediates the ubiquitination and subsequent degradation of IκBα, thereby relieving its inhibitory effect on NF-κB. This allows NF-κB to translocate into the nucleus, where it activates the expression of genes associated with cell survival, inflammation, and immune regulation, including CCL2 (77). This process enhances TAM recruitment and their survival within the TME. The activity of CRL is critically dependent on neddylation, a modification of cullin proteins. Inhibition of neddylation leads to inactivation of the CRL E3 ligase complex, reducing the expression of key inflammatory regulators and thereby inhibiting TAM recruitment and tumor cell immune escape (78). COP1 E3 Ligase: COP1 is an E3 ligase with a RING finger domain, responsible for ubiquitinating and degrading several proteins, including the tumor suppressor protein P53 and transcription factors such as C/EBPδ (79). COP1 interacts with C/EBPδ through the adaptor protein Trib, promoting the ubiquitination and degradation of C/EBPδ. In many tumor cells, C/EBPδ functions to inhibit the expression of chemokines and chemotactic factors (80). The degradation of C/EBPδ enhances the secretion of chemokines, such as CCL2, by TAMs, thereby increasing TAM recruitment. Modulating COP1 activity can influence the polarization and function of TAMs towards the M2 phenotype, which is crucial for reinforcing immunosuppression within the TIME (81). KCP1 E3 Ligase: KCP1 mediates the ubiquitination and conversion of p105, the precursor of the NF-κB protein, into the active p50 subunit. p50 is a key regulator of the NF-κB pathway, which governs the expression of a broad range of genes involved in inflammatory responses and immunity (82, 83).

Overexpression of p50 has been shown to promote the expression of chemokines such as CCL3 and CCL4, which are vital mediators of TAM recruitment. Additionally, the p50 subunit influences PD-L1 expression, and its downregulation may enhance anti-tumor immune responses within the TIME (84). In tumors, loss of function or downregulation of KCP1 has been observed, which may disrupt the NF-κB signaling pathway and alter the TIME.

The UPS in the TIME influences the growth, activation, and functional expression of various immune cells by precisely regulating the stability and activity of key proteins (Figure 1). Targeting these mechanisms has led to several new clinical trials with promising outcomes (Table 1). In the future, focusing on the interaction between the UPS and immune cells is expected to provide a robust theoretical foundation for developing novel combination therapies against tumors.

4 Clinical applications of the UPS in tumor therapy, challenges and perspectives

4.1 The role of E3 ligase inhibitors in immunotherapy

Inhibitors targeting E3 ligases have demonstrated significant potential in cancer therapy. SPOP is an E3 ligase that ubiquitinates and degrades PD-L1 protein, thereby inhibiting tumorigenesis. Studies have shown that CDK4/6 can inhibit the expression of Cyclin D-CDK4, leading to the phosphorylation of Speckle-type POZ protein (SPOP), which ultimately stabilizes PD-L1 expression and increases its protein levels (85). Therefore, CDK4/6 inhibitors can enhance the ubiquitination function of SPOP, promoting anti-tumor immune responses. Research by Pan et al. has confirmed that CDK4/6 inhibitors, when combined with anti-PD-1 inhibitors, significantly inhibit tumor growth and proliferation in mice, resulting in a marked increase in overall survival.

ARV-471 is an orally administered drug that utilizes PROTAC technology to target estrogen receptor (ER) α. In mouse models of breast cancer, the combination of ARV-471 with the CDK4/6 inhibitor Palbociclib significantly suppressed tumor progression (86). Additionally, FBXO22 is a typical F-box protein and a component of E3 ligase complexes. Similar to the action of SPOP, FBXO22 directly ubiquitinates and degrades PD-L1, thereby increasing the sensitivity of tumor cells to immunotherapeutic agents. Among the upstream regulators of FBXO22, CDK5 is particularly noteworthy (87). CDK5 directly influences the ubiquitination and degradation of PD-L1, thereby modulating anti-tumor immune responses. Studies have shown that combining CDK5 inhibitors with immune checkpoint inhibitors (ICIs) can significantly enhance the efficacy of immunotherapy, effectively inhibiting tumor initiation and progression.

4.2 Clinical applications of DUB inhibitors in immunotherapy

DUBs are primarily responsible for removing ubiquitin molecules from protein substrates, thereby reversing ubiquitination modifications. This process is crucial for maintaining protein homeostasis within the cell, regulating signal transduction

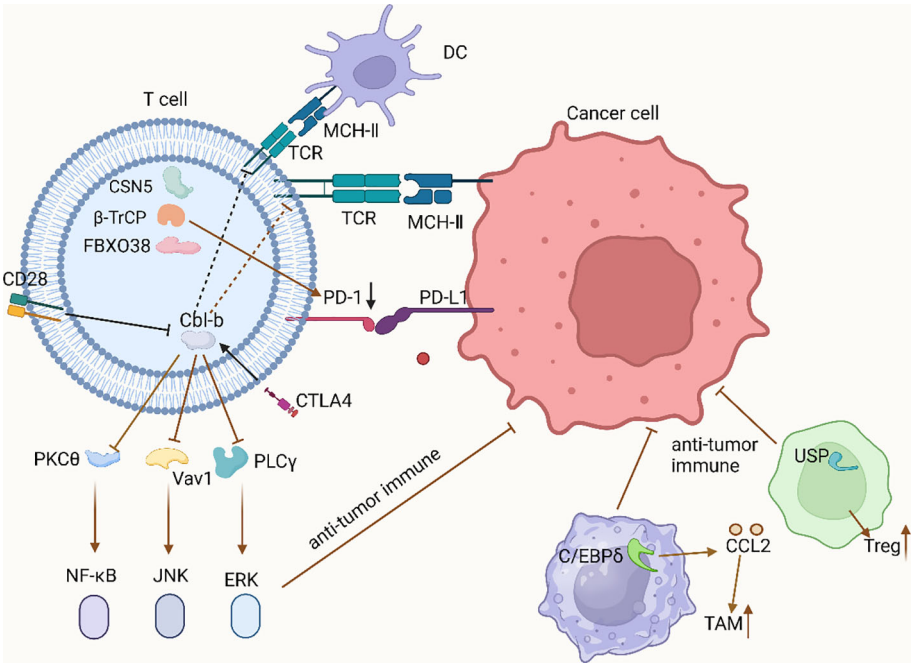

FIGURE 1
UPS regulates various types of immune cells in TIME.

TABLE 1 Clinical trials related to ubiquitinating enzymes in tumor therapy.

NCT Number	Title	Status	Conditions	Interventions	Characteristics
NCT06223542	Studying TAK-243 in Patients With Advanced Cancer	Not yet recruiting	• Advanced Lymphoma • Advanced Malignant Solid Neoplasm • Metastatic Malignant Solid Neoplasm	• Procedure: Biopsy • Procedure: Biospecimen Collection • Procedure: Computed Tomography • Procedure: Magnetic Resonance Imaging • Drug: UAE Inhibitor TAK-243	Phase: Phase 1
NCT05107674	A Study of NX-1607 in Adults With Advanced Malignancies	Recruiting	• Ovarian Cancer, Epithelial • Gastric Cancer • GastroEsophageal Junction (GEJ) Cancer • Head and Neck Squamous Cell Carcinoma • Metastatic or Unresectable Melanoma • Non-small Cell Lung Cancer (NSCLC) • Metastatic Castration-resistant Prostate Cancer (mCRPC) • Malignant Pleural Mesothelioma (MPM) • Triple Negative Breast Cancer (TNBC) • Metastatic Urothelial Carcinoma • and 4 more	• Drug: NX-1607 • Drug: Paclitaxel	Phase: Phase 1
NCT02256241	The Role of RING Ubiquitin Ligases in Biologic and Oncologic Processes in Tissues of Mesenchymal Origin	Unknown status	• Group 1: Trauma Operation for Otherwise Healthy Patients • Group 2: Primary Tumors of Mesenchymal Origin		
NCT02045095	A Dose Escalation Study of MLN7243 (TAK-243) in Adult Participants With Advanced Solid Tumors	Terminated	• Advanced Malignant Solid Tumors	• Drug: MLN7243	Phase: Phase 1
NCT01358617	Prognostic Biomarkers in Tumor Tissue Samples From Young Patients With Neuroblastoma	Completed	• Disseminated Neuroblastoma • Localized Resectable Neuroblastoma • Localized Unresectable Neuroblastoma • Recurrent Neuroblastoma • Stage 4S Neuroblastoma	• Other: diagnostic laboratory biomarker analysis	
NCT00216697	An Extension Study to Provide Bortezomib to Patients With Relapsed or Refractory Multiple Myeloma Who Previously Participated in a Bortezomib Phase I/II Study and Who May Benefit From Re-Treatment With or Continuation of Bortezomib Therapy	Completed	• Multiple Myeloma	• Drug: bortezomib	Phase: Phase 2

pathways, and modulating various cellular functions. DUBs play an essential role in maintaining immune homeostasis and modulating immune responses by regulating the ubiquitination status of key immune-related proteins (88). For example, DUBs remove ubiquitin chains from PD-L1, preventing its degradation by the proteasome and thereby maintaining high levels of PD-L1 expression on the surface of tumor cells. This elevated PD-L1 expression binds to PD-1 on T cells, inhibiting their activity and leading to immune evasion.

DUBs, such as USP7, have been found to enhance the stability and function of Tregs by deubiquitinating FOXP3. Tregs exert immunosuppressive effects in the TME, limiting the anti-tumor activity of effector T cells. Targeting DUBs to modulate their activity has emerged as a potential strategy to enhance anti-tumor immune responses and inhibit tumor progression. DUB inhibitors are of great significance in the clinical treatment of tumors. Up to now, several clinical trials have made some progress. A clinical trial, NCT02372240, showed that VLX1570, as the first DUB inhibitor to enter a clinical trial, was effective in killing tumors by mainly targeting USP14/UCH-L5. However, unfortunately, the clinical trial was terminated due to severe pulmonary toxicity (89). KSQ-4279, as this most advanced USP1 inhibitor reported so far, is undergoing phase I clinical trials (NCT05240898). It is primarily used for the treatment of advanced solid tumors, including ovarian and triple-negative breast cancers. KSQ-4279 exhibits excellent pharmacokinetic properties *in vitro* and shows significant antitumor activity in animal models. In particular, when combined with PARP inhibitors, it was able to kill tumors in multiple models, demonstrating in addition to excellent clinical efficacy (90). In addition, the first-generation USP7 inhibitor P5091 induces apoptosis in multiple myeloma cells by promoting ubiquitination of MDM2 and MDMX, which in turn activates the p53 pathway and ultimately induces apoptosis. It has achieved good efficacy in the clinical treatment of myeloma. The second-generation covalently bound inhibitor P22077 inhibits the enzymatic activity of USP7 by modifying cysteine 223 in its catalytic center. It has gained some efficacy in the treatment of many tumors (91). However, since there are still obvious side effects, its therapeutic mechanism still needs to be further explored in the future. The natural product berberine, as a novel USP7 inhibitor, promotes ubiquitination and degradation of MDM2 by disrupting the MDM2-DAXX-USP7 complex, and has also achieved good efficacy in the clinical treatment of tumors with fewer side effects. Other DUB inhibitors, such as USP21, USP14 and OTUB2, have also shown great therapeutic potential in a variety of solid tumors. USP21 inhibitors are able to inhibit the deubiquitylation of MEK2 and FOXM1, which in turn down-regulates the expression of the ERK pathway and ultimately inhibits the growth of tumor cells (92). Despite the challenges of toxicity, targeting, and drug resistance, DUB inhibitors will be a key component in the clinical treatment of cancer in the future.

USP7 enhances the stability and function of Tregs by deubiquitinating FOXP3. Tregs play an immunosuppressive role in the TME, limiting the anti-tumor activity of effector T cells. Additionally, studies have found a significant positive correlation between the expression of USP7 and PD-L1. Further research revealed that USP7 can assist tumor cells in immune evasion by increasing the expression of PD-L1 protein. The combination of USP7 inhibitors with PD-1 or PD-L1 inhibitors can significantly enhance the efficacy of immunotherapy. Moreover, combining USP7 inhibitors with anti-PD-1 monoclonal antibody therapy has shown promising therapeutic effects in lung cancer models (93). USP22 also has the ability to regulate PD-L1 stability through deubiquitination. USP22 increases PD-L1 protein levels by regulating the CSN5/PD-L1 axis. CSN5 has been confirmed as a key protein that promotes the deubiquitination of PD-L1. USP22 stabilizes CSN5 protein by deubiquitinating polyubiquitin chains, thereby enhancing PD-L1 expression. Knocking down USP22 can enhance T cell and NK cell activity, and when combined with ICIs, it significantly improves the therapeutic efficacy of ICIs (94). USP14 promotes IDO1 expression by enhancing its deubiquitination, preventing its degradation by ubiquitin ligases. High expression of IDO1 protein significantly suppresses CD8+ T cell activity and levels, facilitating immune evasion. In colorectal cancer (CRC), knocking down USP14 can inhibit IDO1 expression, enhance CD8+ T cell activity and numbers, and make CRC cells more sensitive to immunotherapy. Furthermore, clinical studies have shown that the first-generation USP14 inhibitor IU1 can significantly reduce IDO1 protein expression and inhibit IDO1-induced immune suppression. Additionally, the combination of IU1 with ICIs and IDO1 inhibitors can significantly reduce the "off-target" effects associated with these inhibitors and enhance therapeutic efficacy (95). The combined treatment of IU1 and anti-PD-1 has been shown to significantly reduce tumor proliferation and progression, offering a new therapeutic approach for future cancer patients.

4.3 Challenges and perspectives

USP7, USP22, and USP14 remain pivotal targets for the combination of UPS inhibitors and immunotherapy. In addition, USP8, USP15, USP9X, and USP18 can directly bind to PD-L1, stabilizing PD-L1 expression and promoting immune evasion by tumor cells. Inhibitors targeting these USPs, when combined with ICIs, can significantly enhance the efficacy of immunotherapy. However, PD-1/PD-L1 regulation is not solely dependent on ubiquitination; other post-translational modifications (PTMs), such as phosphorylation, acetylation, lactylation, and palmitoylation, also play critical roles in modulating PD-1/PD-L1 protein expression. Future research should, therefore, take a comprehensive approach to protein degradation pathways and explore multi-target combination therapies. Despite the immense potential of DUB inhibitors in the clinical treatment of malignant tumors, several challenges remain in developing specific inhibitors. First, the complex structural features of DUB catalytic domains, coupled with the high similarity among DUB family members, present significant challenges for targeted drug design. Second, the large molecular weight of DUBs complicates crystal formation, making it difficult to obtain complete crystal structures—an essential requirement for structure-based drug design. Additionally, DUBs may undergo conformational changes upon ubiquitin binding, which further complicates small molecule prediction and computer simulation. Moreover, the intricate regulatory mechanisms of DUBs, which involve both catalytic activity and substrate-mediated conformational modulation, add another layer of complexity to the development of specific inhibitors. Finally, given the critical role of the UPS in normal cellular functions, inhibiting UPS components may lead to severe toxicity and side effects, such as peripheral neuropathy and hematologic toxicity observed with proteasome inhibitors.

Nevertheless, the potential clinical application of DUB inhibitors in cancer treatment remains promising. Future research could focus on identifying and optimizing novel small-molecule

DUB inhibitors through high-throughput screening and computer-aided drug design (CADD). Advances in structural biology techniques, such as cryo-electron microscopy (Cryo-EM), may provide clearer crystal structures of DUBs, thereby facilitating the rational design of inhibitors. Additionally, developing multi-target inhibitors that can simultaneously target multiple DUBs may enhance therapeutic efficacy and reduce tumor drug resistance by inhibiting multiple signaling pathways concurrently. The combined use of DUB inhibitors with immunotherapy, chemotherapy, and other therapeutic modalities could significantly improve cancer treatment outcomes. The integration of genomics, proteomics, and metabolomics technologies, alongside a deeper understanding of the specific mechanisms of DUBs in various cancers, will offer more personalized treatment options for patients. Ultimately, the continued development and clinical application of DUB inhibitors hold the potential to substantially improve the survival rates and quality of life for cancer patients.

5 Conclusion

The ubiquitin-proteasome system regulates immune system responses by interacting with various types of immune cells in TIME. In this review, we aim to summarize the essential components of TIME, focusing on the normal function of T cells, DC cells, NK cells, MDSC, M2-type macrophages, and Tregs. UPS plays an important role in facilitating immune evasion by modulating these immune cells. crucial role in helping tumor cell immune evasion by regulating these immune cells. In the future, targeting relevant ubiquitinating enzymes and combining them with immunotherapy will greatly promote the efficacy of tumor therapy and significantly improve the quality of patient's survival.

Author contributions

YW: Data curation, Writing – original draft, Conceptualization, Investigation, Validation. SL: Conceptualization, Data curation, Formal Analysis, Writing – review & editing. WW: Funding acquisition, Methodology, Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

UPS	Ubiquitin-Proteasome System
TIME	Tumor Immunosuppressive Microenvironment
TME	Tumor Microenvironment
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
SCF	Skp1-Cullin-F-box
PI3K	Phosphoinositide 3-Kinase
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
β-TrCP	Beta-Transducin Repeat Containing Protein
GSK3β	Glycogen Synthase Kinase 3 Beta
COP9	Constitutive Photomorphogenesis 9
CSN	COP9 Signalosome
DUBs	Deubiquitinating Enzymes
USP7	Ubiquitin-Specific Protease 7
USP21	Ubiquitin-Specific Protease 21
USP22	Ubiquitin-Specific Protease 22
USP9X	Ubiquitin-Specific Protease 9 X-Linked
GRAIL	Gene Related to Anergy in Lymphocytes
TCR	T-Cell Receptor
CD3	Cluster of Differentiation 3
VHL	Von Hippel-Lindau Tumor Suppressor
HIF-1α	Hypoxia-Inducible Factor 1-alpha
TRAF	TNF Receptor Associated Factor
MARCH1	Membrane-Associated RING-CH-type Finger 1
MHCII	Major Histocompatibility Complex Class II
CD86	Cluster of Differentiation 86
TLR	Toll-Like Receptor
MyD88	Myeloid Differentiation Primary Response 88
IRAK	Interleukin-1 Receptor-Associated Kinase
IKK	IκB Kinase
LUBAC	Linear Ubiquitin Chain Assembly Complex
CRL	Cullin-RING Ligase
IκBα	Inhibitor of kappa B alpha
C/EBPδ	CCAAT/Enhancer-Binding Protein Delta
KCP1	Kelch-Like ECH-Associated Protein 1
p105	Precursor of NF-κB Protein
p50	Active Subunit of NF-κB Protein
DC	Dendritic Cell
Tregs	Regulatory T Cells
Th17	T Helper 17 Cells
CTL	Cytotoxic T Lymphocytes
APC	Antigen-Presenting Cells
TNF-α	Tumor Necrosis Factor Alpha
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-12	Interleukin 12
CCL2	C-C Motif Chemokine Ligand 2
CCL3	C-C Motif Chemokine Ligand 3
CCL4	C-C Motif Chemokine Ligand 4
TGF-β	Transforming Growth Factor Beta
IL-10	Interleukin 10
IL-35	Interleukin 35
VEGF	Vascular Endothelial Growth Factor
PDGF	Platelet-Derived Growth Factor
LAG-3	Lymphocyte-Activation Gene 3

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The role of lactylation in plasma cells and its impact on rheumatoid arthritis pathogenesis: insights from single-cell RNA sequencing and machine learning

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Introduction: Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by persistent synovitis, systemic inflammation, and autoantibody production. This study aims to explore the role of lactylation in plasma cells and its impact on RA pathogenesis.

Methods: We utilized single-cell RNA sequencing (scRNA-seq) data and applied bioinformatics and machine learning techniques. A total of 10,163 cells were retained for analysis after quality control. Clustering analysis identified 13 cell clusters, with plasma cells displaying the highest lactylation scores. We performed pathway enrichment analysis to examine metabolic activity, such as oxidative phosphorylation and glycolysis, in highly lactylated plasma cells. Additionally, we employed 134 machine learning algorithms to identify seven core lactylation-promoting genes and constructed a diagnostic model with an average AUC of 0.918.

Results: The RA lactylation score (RAlac_score) was significantly elevated in RA patients and positively correlated with immune cell infiltration and immune checkpoint molecule expression. Differential expression analysis between two plasma cell clusters revealed distinct metabolic and immunological profiles, with cluster 2 demonstrating increased immune activity and extracellular matrix interactions. qRT-PCR validation confirmed that NDUFB3, NGLY1, and SLC25A4 are highly expressed in RA.

Conclusion: This study highlights the critical role of lactylation in plasma cells for RA pathogenesis and identifies potential biomarkers and therapeutic targets, which may offer insights for future therapeutic strategies.

KEYWORDS

rheumatoid arthritis, lactylation, plasma cells, single-cell RNA sequencing, machine learning

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by inflammation and progressive joint destruction (1, 2). Affecting approximately 0.5-1% of the global population, RA poses a significant burden due to its debilitating nature, reduced quality of life, and increased mortality rates (3–5). Despite extensive research, the precise mechanisms underlying the pathogenesis of RA remain incompletely understood, necessitating ongoing investigations to uncover novel therapeutic targets and biomarkers for early diagnosis and treatment (6).

RA is characterized by persistent synovitis, systemic inflammation, and autoantibody production (7). The disease typically manifests as symmetrical polyarthritis, primarily affecting the small joints of the hands and feet, although large joints and other organs can also be involved (8). The hallmark of RA pathology is the formation of pannus, a hypertrophic synovial tissue that invades and destroys adjacent cartilage and bone (9). This destructive process is driven by a complex interplay of genetic, environmental, and immunological factors.

The immune system plays a central role in the development and progression of RA. A breakdown in immune tolerance leads to the activation of autoreactive T and B cells, which produce autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) (10–12). These autoantibodies form immune complexes that contribute to the activation of complement and the recruitment of inflammatory cells into the synovium (9, 13). T cells, particularly CD4+ T helper cells, are pivotal in RA pathogenesis (14, 15). They interact with antigen-presenting cells and produce a variety of cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), which propagate the inflammatory response (16–18). B cells also contribute to RA through autoantibody production, antigen presentation, and cytokine secretion (19, 20).

Recent studies have highlighted the role of metabolic reprogramming in immune cells during RA. Activated immune cells undergo metabolic shifts to meet the increased energy demands and biosynthetic needs required for proliferation, differentiation, and effector functions (21–23). These metabolic alterations include increased glycolysis, oxidative phosphorylation, and lipid metabolism (24–26). Lactylation, a post-translational modification involving the addition of lactate-derived lactyl groups to lysine residues, has emerged as a significant regulatory mechanism in cellular metabolism and gene expression (27). Lactylation can modulate protein function and influence various biological processes, including immune responses and inflammation (28, 29). Given the importance of metabolic reprogramming in RA, understanding the role of lactylation in immune cells, particularly in the context of RA, is of great interest.

Plasma cells, the terminally differentiated form of B cells, are crucial for antibody production (30). In RA, plasma cells are abundant in the synovium and produce autoantibodies that contribute to disease pathogenesis (31, 32). The persistence of plasma cells in the inflamed synovium and their role in sustaining chronic inflammation underscore their importance in RA (33). Recent evidence suggests that plasma cells exhibit distinct metabolic profiles compared to other immune cells (7). The high metabolic activity of plasma cells, characterized by enhanced oxidative phosphorylation and glycolysis, supports their robust antibody production. However, the role of lactylation in plasma cells and its impact on RA progression remain largely unexplored.

Despite advances in understanding RA pathogenesis, current therapeutic strategies remain inadequate, often failing to achieve sustained remission in many patients. The heterogeneity of RA, coupled with its complex molecular and immunological mechanisms, underscores the need for novel biomarkers and therapeutic targets. Recent research has highlighted the importance of lactylation in regulating immune cell function and metabolism, suggesting its potential role in RA pathogenesis. By integrating cutting-edge technologies such as single-cell RNA sequencing (scRNA-seq) and machine learning, we can now analyze large-scale datasets with greater precision. machine learning algorithms, in particular, enable the identification of hidden patterns in gene expression data, the prioritization of key regulatory genes, and the construction of predictive models for disease diagnosis and treatment response. The aim of this study is to investigate the role of lactylation in plasma cells and its impact on RA pathogenesis. By utilizing scRNA-seq data and integrating bioinformatics and machine learning techniques, we seek to identify core lactylation-promoting genes and develop a robust diagnostic model, providing novel insights into RA's metabolic and immunological mechanisms while highlighting potential biomarkers and therapeutic targets for future intervention.

Methods

Data collection

Single-cell RNA sequencing data for RA was collected from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), specifically dataset GSE159117, which includes one RA sample sequenced using the 10X Genomics platform. This dataset was selected because it provides high-resolution insights into the cellular heterogeneity of RA, allowing for the detailed analysis of specific cell types, such as plasma cells, that are crucial for understanding RA pathogenesis. The single-cell data enables us to explore gene expression at an individual cell level, which is essential for investigating the role of lactylation in plasma cells.

In addition, four bulk RNA sequencing datasets were selected from GEO for machine learning purposes to enhance the robustness and generalizability of our findings. These datasets were chosen based on their relevance to RA, their sample sizes, and their inclusion of both RA patients and normal controls, allowing for comprehensive model training and validation. The dataset GSE89408, which includes 28 normal samples and 152 RA samples, was used as the training set due to its large sample size, providing a solid foundation for model development. GSE12021 (9 normal and 12 RA samples), GSE55235 (10 normal and 10 RA samples), and GSE55457 (10 normal and 13 RA samples) were

selected as validation sets to ensure that our model could be validated across multiple independent datasets, further strengthening the reliability of the diagnostic model developed in this study. The selection of these datasets was also guided by their representation of both peripheral blood and synovial tissue, offering a broader perspective on the mechanisms of RA across different tissue types.

qRT-PCR analysis

RNA was extracted from tissues (tumoral and non-tumoral) using an RNA isolation kit (Bioneer, Korea, Cat.No: K-3090) per the manufacturer's instructions. The extracted total RNA was treated with DNase I to digest the genomic DNA. By using gel electrophoresis and spectrophotometry, the quality and quantity of RNA were determined. After treating the samples with an RNase inhibitor, RNA was converted to cDNA using the PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan Cat.No. RR037A). Lastly, qRT-PCR analysis was performed on a Rotor-Gene Q instrument (QIAGEN, Germany) using SYBR Green Premix Ex Taq (TaKaRa, Otsu, Shizuoka, Japan, Cat.No: RR420A). The qRT-PCR conditions were 95°C for 10 min (pre-denaturation) and forty cycles of 95°C for 10 s (denaturation), 61°C for 20 s (annealing), and 72°C for 25 s (extension). As an internal control, beta-actin (β-Actin) was utilized.

Single-Cell RNA sequencing data processing

Single-cell RNA sequencing data analysis was conducted using Seurat version 4.2.2. The dataset was first loaded and subjected to quality control, removing cells with fewer than 300 or more than 4000 RNA features, or with mitochondrial gene content greater than 10%. Data normalization and scaling were performed using the SCTransform function. Dimensionality reduction and clustering were conducted by selecting the top 15 principal components, followed by clustering with the RunUMAP function. Cell types were annotated using SingleR and previously reported literature. Marker genes for each cell type were identified using the FindAllMarkers function.

Cell communication analysis

The "CellChat" R package (version 1.5.0) was used to reveal potential intercellular communication mechanisms at the single-cell level. The createCellChat function was employed to construct the CellChat object, and the aggregateNet function was used to describe the signaling emitted from each cell type. Intercellular communication quantities and weights were visualized using the netVisual_circle function, and the netAnalysis_computeCentrality function was used to infer the input and output weights of specific signaling pathways.

Lactylation scoring

Lactylation-related gene sets were obtained from the GSEA website, comprising a total of 10 pathways. After removing duplicates, 329 lactylation-related genes (LRGs) were identified to form the lactylation gene set. To calculate lactylation scores, we applied the AUCell package, which quantifies the activity of a gene set in individual cells by evaluating whether the expression of genes from the set is enriched in the top-ranking genes of each cell. Specifically, we used the AUCell_calcAUC function to calculate the Area Under the Curve (AUC) for each cell, based on the ranking of gene expression levels. This method allows us to assess the relative expression of the lactylation gene set within each cell, resulting in a lactylation score that reflects the activity of lactylation-related pathways in individual cells.

To ensure robustness, the lactylation scores were calculated for all cells, and we compared these scores across different cell subpopulations to identify those most associated with lactylation activity. The AUCell algorithm's non-parametric approach makes it ideal for single-cell RNA sequencing data, as it allows for accurate scoring even in cases of sparse gene expression. The resulting lactylation scores were visualized using violin plots generated by ggplot2, providing a clear comparison of lactylation activity across various cell types.

scMetabolism metabolic analysis

Metabolic characteristics of various cell types within the scRNA-seq data were assessed using the R package scMetabolism. This analysis employed the AUCell scoring principle to evaluate 85 metabolic pathways. Key pathways related to glucose metabolism and lipid metabolism were visualized using bubble plots.

Pathway and enrichment analysis

Pathway enrichment analysis was conducted using the irGSEA package, a robust R package equipped with multiple scoring functions to assist in pathway scoring. This study integrated AUCell, UCell, and GSVA algorithms, and combined the pathway activation results using the RAA method to calculate the upregulation and downregulation of HALLMARK gene sets. Additionally, the "clusterProfiler" package was utilized for KEGG and GOBP enrichment analyses of plasma cells, with the results visualized using ggplot2.

Machine learning

To identify genes associated with RA and construct a robust diagnostic model, we employed 12 machine learning algorithms for screening and modeling. These algorithms—Lasso, NaiveBayes, SVM, glmBoost, Enet, plsRglm, XGBoost, LDA, Stepglm, Ridge, RandomForest, and GBM—were selected based on their

complementary strengths in handling high-dimensional gene expression data and diverse model requirements. Lasso, Ridge, and Enet are effective for regularization and preventing overfitting, crucial for gene selection. SVM is robust for non-linear classification tasks, while RandomForest and GBM, as ensemble methods, improve predictive accuracy and reduce variance. XGBoost was chosen for its efficiency in handling large datasets with strong predictive performance. Simpler methods like NaiveBayes and LDA were included for their ability to capture probabilistic relationships, while plsRglm and glmBoost provide flexibility with regularization and boosting (34). Stepglm was specifically used for variable selection, identifying the most relevant lactylation-promoting genes. The use of 134 algorithmic combinations ensured comprehensive variable selection and model construction. Diagnostic performance was evaluated using average AUC values from the training and validation sets, ensuring we selected the most predictive and reliable model for RA diagnosis.

Immune-related analysis

Relative enrichment scores for 29 immune cell types and immune processes were calculated using the GSVA and GSEABase packages, following the ssGSEA strategy. Correlation analyses were performed between immune cell types and the RA lactylation score (RAlac_score). Samples were divided into high RAlac_score and low RAlac_score groups based on the median RAlac_score, and the activation of immune processes was compared between these groups. The expression of immune checkpoint molecules was analyzed for correlation with RAlac_score, and correlation bubble plots were generated using ggplot2.

Consensus clustering

To further explore the functions of core lactylation genes in RA, unsupervised clustering was performed using the "ConsensusClusterPlus" package, with K=2 selected as the optimal parameter. PCA plots were created using the "FactoMineR" and "factoextra" packages. Differential analysis between the two subgroups was conducted using the Limma package, and heatmaps were generated using the pheatmap package. Enrichment analysis was performed with the "clusterProfiler" package, and visualization was done using the aPEAR package.

Clinical prediction model construction

To apply the RA lactylation diagnostic model in clinical decision-making, a clinical prediction model was constructed using a multivariate logistics algorithm. The rms package was used for model construction and calibration curve calculation, and ROC curves were plotted using the ROCR package.

Results

Integration and quality control of single-cell data

Before analyzing the lactylation in RA, single-cell data was integrated and quality controlled according to methodological standards, retaining 10,163 cells for downstream analysis. Based on the ElbowPlot results, the top 15 principal components were selected for dimensional reduction clustering, resulting in 13 clusters labeled from 0 to 12 (Figure 1A). These clusters were annotated into six cell types according to SingleR and previous literature (Figure 1B). The markers for each cell type were as follows: T cells (CD3D, CD3E), monocytes/macrophages (LYZ, CD14, CD68), NK cells (NKG7, GZMB), B cells (CD79A, MS4A1), plasma cells (CD38, XBP1), and granulocytes (CTSB, IRF7) (Figure 1C).

Cellular communication and activation in RA

Evaluation of cell-to-cell communication revealed that B cells and plasma cells sent a large number of signals to other cells, suggesting their activation is related to the development of RA (Figures 1D, E). Using the AUCell algorithm, lactylation scores were calculated for each cell type, showing the highest scores in plasma cells (Figure 1F). This implies that plasma cells might activate the lactylation process through extensive signaling, altering the surrounding microenvironment and contributing to the onset of RA. Since lactylation involves metabolic processes, the metabolic changes in various cell types were further assessed, with oxidative phosphorylation, glycolysis/gluconeogenesis, and glycerolipid metabolism significantly upregulated in plasma cells, indicating a strong correlation with metabolism (Figure 1G).

Pathway enrichment analysis

HALLMARK pathway enrichment analysis of all cells indicated a significant activation of the unfolded protein response in plasma cells, closely related to endoplasmic reticulum stress, while TNFα and TGFβ pathways were significantly downregulated (Figure 2A). Consistent with previous analysis, GOBP analysis showed significant upregulation of oxidative phosphorylation, glycosylation, and endoplasmic reticulum stress, and downregulation of immune activation and differentiation (Figure 2B). KEGG analysis similarly revealed significant upregulation of oxidative phosphorylation and downregulation of immune chemotaxis (Figure 2C). These results suggest that plasma cells do not accelerate the development of RA through immune activation but rather by activating their metabolic pathways to promote autoantibody production.

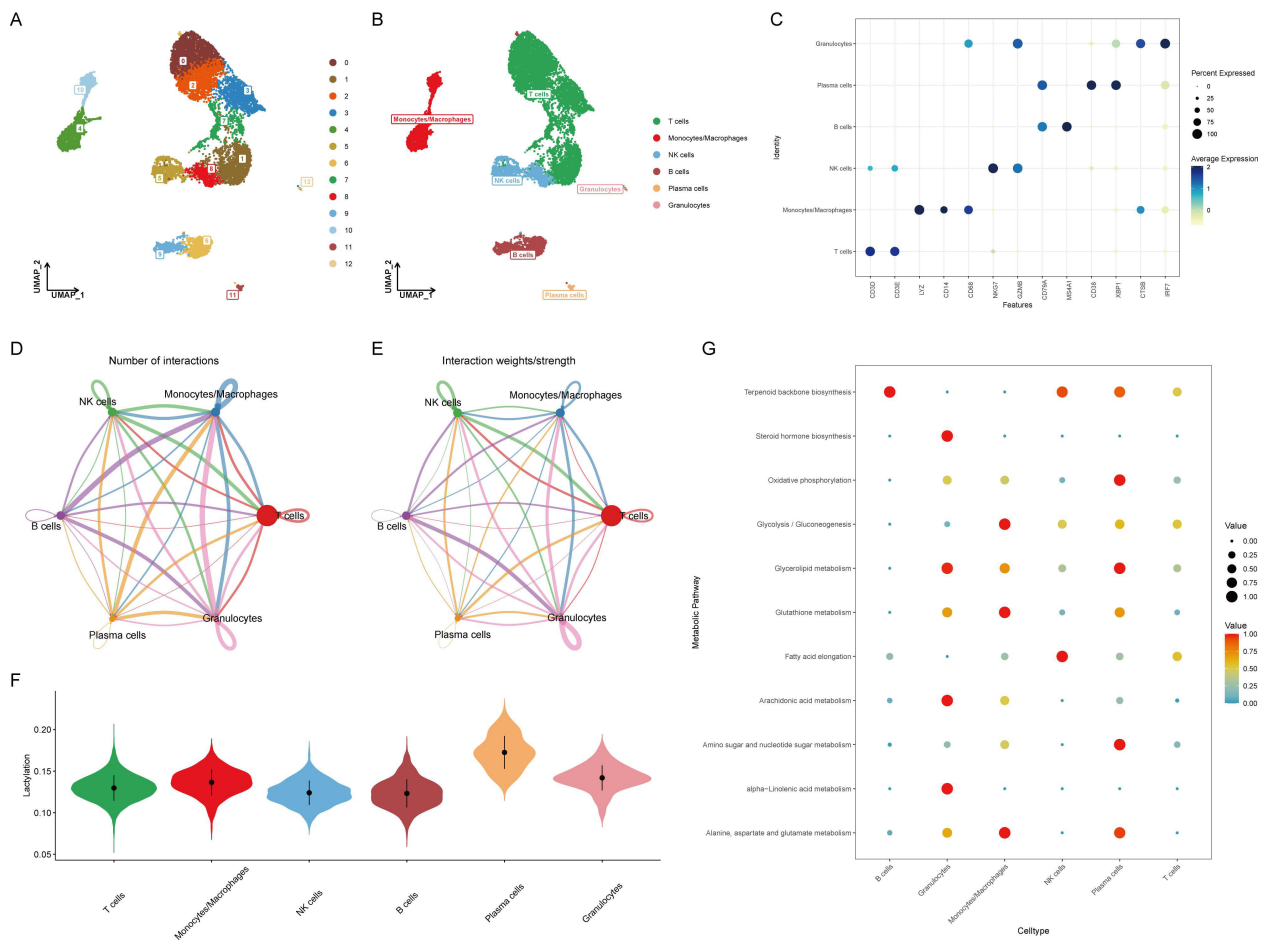

FIGURE 1
Identification and annotation of cell clusters in RA single-cell data. **(A)** ElbowPlot showing the variance explained by each of the principal components. The top 15 principal components were selected for clustering analysis. **(B)** UMAP plot displaying 13 clusters (0-12) identified from the single-cell RNA sequencing data. Clusters were annotated into six cell types based on SingleR and literature. **(C)** Dot plot showing the expression of marker genes for each annotated cell type: T cells (CD3D, CD3E), monocytes/macrophages (LYZ, CD14, CD68), NK cells (NKG7, GZMB), B cells (CD79A, MS4A1), plasma cells (CD38, XBP1), and granulocytes (CTSB, IRF7). **(D)** Heatmap illustrating the cell-cell communication network, with B cells and plasma cells sending numerous signals to other cell types. **(E)** Bar graph depicting the number of communication signals sent and received by each cell type. **(F)** Violin plot comparing lactylation scores across different cell types, calculated using the AUCell algorithm. Plasma cells show the highest lactylation scores. **(G)** Heatmap showing the expression of key metabolic pathways, including oxidative phosphorylation, glycolysis/gluconeogenesis, and glycerolipid metabolism, across different cell types. Plasma cells exhibit significant upregulation of these pathways.

Intersection of Differentially Expressed Genes and Lactylation-Related Genes

Venn diagram analysis showed 25 intersecting genes between highly expressed differential genes in plasma cells and lactylation-related genes. The following analysis will focus on the elucidation of these intersecting genes (Figure 2D).

Identification of Core Lactylation-Promoting Genes in Plasma Cells for RA

The role of lactylation in plasma cells is significant in the development of RA. However, the core lactylation-promoting genes remain unidentified. To address this, 134 algorithms across 12 machine learning models were used to screen for key lactylation-promoting genes and construct diagnostic models for RA. Results showed that the Stepglm[forward] model built after gene selection by glmBoost had the highest diagnostic performance, with an average AUC of 0.918 (Figure 3A). This diagnostic model retained seven core lactylation genes for RA, allowing the calculation of the RAlac_score for each sample. Both training and validation sets showed significantly higher RAlac_scores in the RA group compared to the normal group, indicating a higher likelihood of developing RA with an elevated RAlac_score (Figures 3B-E). Violin plots revealed that CALR, NDUFB3, NGLY1, and TMEM70 were highly expressed in the RA group, while NDUFAF3, SIL1, and SLC25A4 were highly expressed in the control group (Figure 3F).

Correlation between RAlac_score and immune overactivation

Given the association of RA with immune-inflammatory responses, the relationship between RAlac_score and immune

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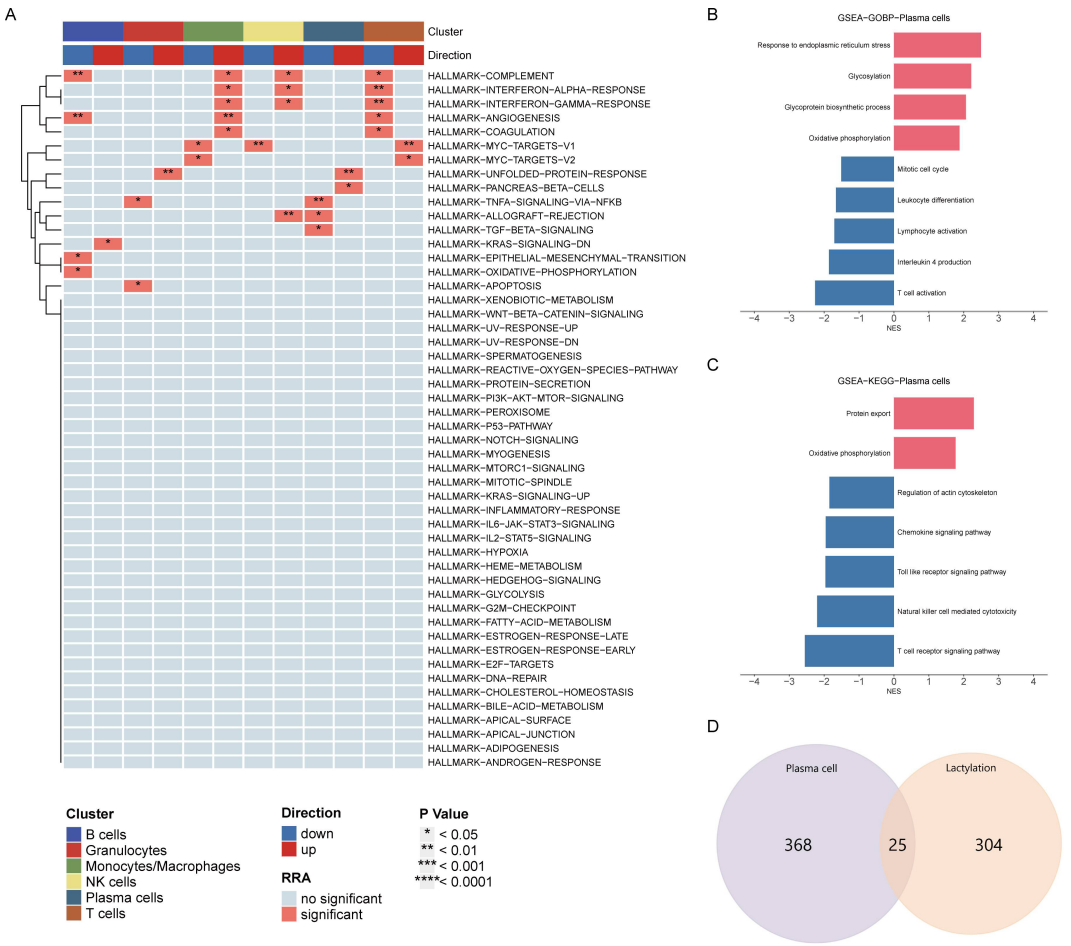

FIGURE 2
Pathway enrichment analysis of plasma cells. **(A)** HALLMARK pathway enrichment analysis indicating significant activation of the unfolded protein response and downregulation of TNFα and TGFβ pathways in plasma cells. **(B)** GOBP analysis showing upregulation of oxidative phosphorylation, glycosylation, and endoplasmic reticulum stress, with downregulation of immune activation and differentiation in plasma cells. **(C)** KEGG pathway analysis highlighting significant upregulation of oxidative phosphorylation and downregulation of immune chemotaxis in plasma cells. **(D)** Venn diagram displaying the intersection of differentially expressed genes in plasma cells and lactylation-related genes, identifying 25 overlapping genes for further analysis.

overactivation was evaluated. The results demonstrated a significant positive correlation between RAlac_score and various immune cells, including Treg, Th1, Th2, B cells, NK cells, neutrophils, Tfh cells, CD8+ T cells, pDC, and macrophages, indicating that patients with higher RAlac_scores had more intense immune infiltration and immune responses (Figures 4A-J). Similarly, all immune processes, including immune co-stimulation, were upregulated in patients with high RAlac_scores (Figure 4K). Most immune checkpoint molecules were positively correlated with RAlac_score, suggesting that RA patients are generally in a state of high immune activation, and immune checkpoint inhibitors (ICI) might be a potential therapeutic approach (Figure 4L).

Diagnostic efficacy of core RA lactylation genes

The diagnostic efficacy of the seven core lactylation genes for RA showed significant differences. Among the four RA gene sets, NDUFB3, NGLY1, and SLC25A4 had the highest diagnostic efficiency, with AUCs ranging from 0.7 to 0.91 (Figures 5A-D). The expression correlations between genes also displayed unique characteristics, with significant positive correlations observed between NGLY1 and CALR, NDUFB3 and CALR, and NDUFB3 and NGLY1. In contrast, NDUFB3 and SIL1, and NDUFAF3 and NGLY1 had significant negative correlations (Figure 5E). GENEMANIA analysis showed that the proteins expressed by these genes primarily had co-expression relationships (Figure 5F).

Consistent clustering using core RA lactylation genes

Using the expression matrix of the seven core lactylation genes in RA, consistent clustering was performed. Based on the CDF results and the clustering heatmap, k=2 was selected for stable clustering results (Figures 6A, B). PCA analysis revealed significant characteristic differences between the two clusters (Figure 6C). Differential analysis between the two clusters identified 1,621 differentially expressed genes

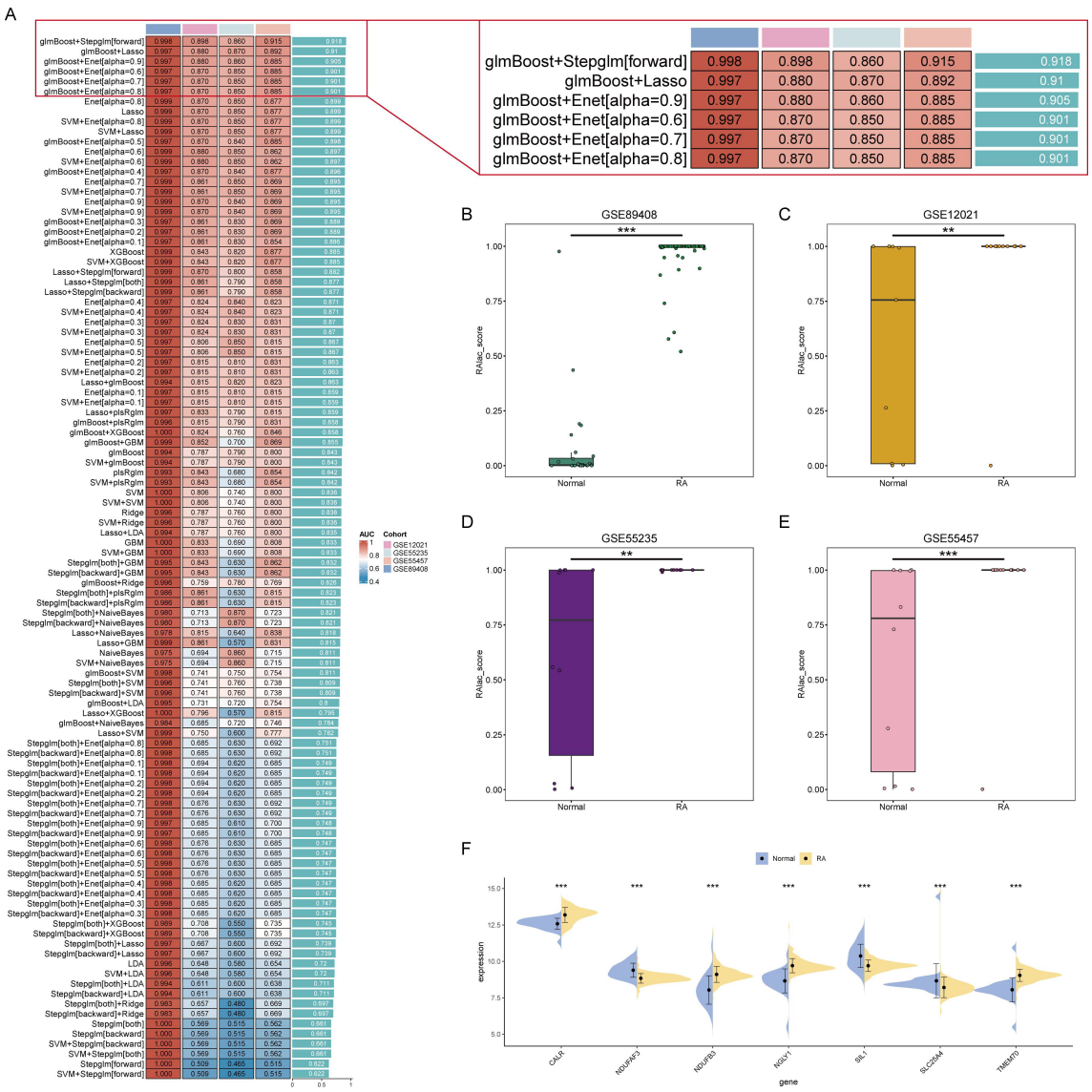

FIGURE 3
Identification of core lactylation-promoting genes in RA. **(A)** Bar graph showing the average AUC values of various machine learning models. The Stepglm[forward] model, built after gene selection using glmBoost, demonstrates the highest performance with an average AUC of 0.918. **(B-E)** Box plots comparing RAlac_scores between normal and RA groups in the training set and three validation sets. RAlac_scores are significantly higher in the RA group across all datasets. **(F)** Violin plots depicting the expression levels of the seven core lactylation-promoting genes (CALR, NDUFB3, NGLY1, TMEM70, NDUFAF3, SIL1, SLC25A4) in normal and RA samples. CALR, NDUFB3, NGLY1, and TMEM70 are highly expressed in the RA group, while NDUFAF3, SIL1, and SLC25A4 are highly expressed in the control group. ** mean P < 0.01, *** mean P < 0.001.

with logFC absolute value greater than 1 and adjusted P value less than 0.05 (Figure 6D). A heatmap displayed the top 50 upregulated and downregulated genes (Figure 6E). Enrichment analysis highlighted the GO and KEGG pathways activated in cluster 2, associated with immune activity and extracellular matrix interaction (Figures 6F, G).

Differential expression and pathway activation in clusters

The core lactylation genes showed different expression patterns between the two subgroups, with NDUFAF3 and SIL1 highly expressed in cluster 1, and CALR, NDUFB3, NGLY1, and TMEM70 highly expressed in cluster 2 (Figure 7A). Cluster 1 primarily expressed MHC-I molecules such as HLA-A and HLA-C, whereas cluster 2 primarily expressed MHC-II molecules (Figure 7B). Most chemokines and TNF family molecules were highly expressed in cluster 2, indicating that cluster 2 is an inflammatory and chemotactic subtype related to immune activation (Figures 7C, D). The Estimate algorithm was used to assess the activation of the microenvironment, showing that the stromal score, immune score, and microenvironment score were all significantly higher in cluster 2 (Figure 7E).

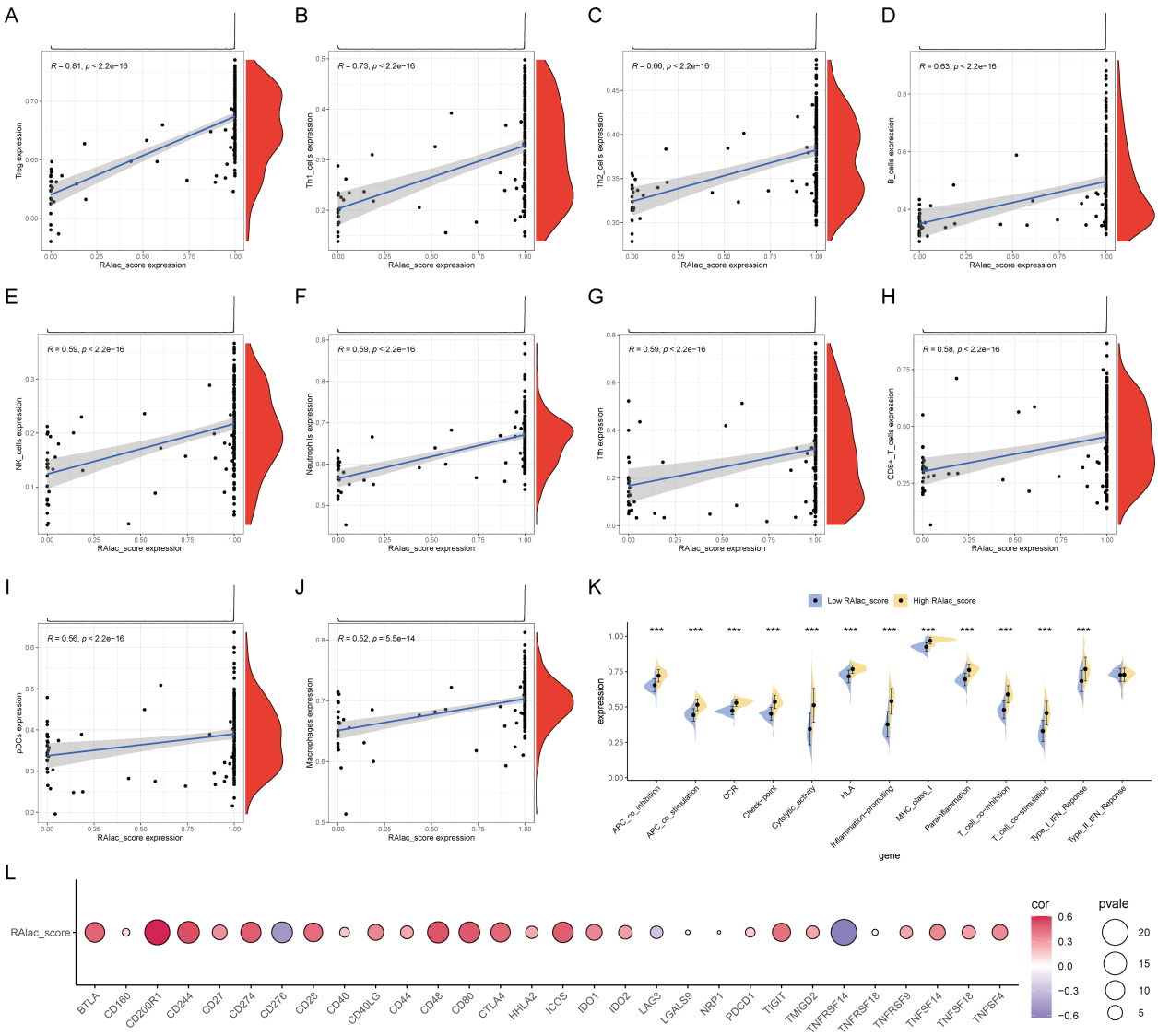

FIGURE 4
Correlation between RAlac_score and immune activation. **(A–J)** Scatter plots showing significant positive correlations between RAlac_score and various immune cell types, including Treg, Th1, Th2, B cells, NK cells, neutrophils, Tfh cells, CD8+ T cells, pDC, and macrophages. **(K)** Box plots comparing the activation of various immune processes, including immune co-stimulation, between high RAlac_score and low RAlac_score groups. All immune processes are upregulated in the high RAlac_score group. **(L)** Correlation bubble plot illustrating the positive correlation between RAlac_score and the expression of various immune checkpoint molecules, indicating a state of high immune activation in RA patients. ***P w 0.001.

Single-cell expression and clinical prediction model

At the single-cell expression level, all seven RA lactylation genes were distinctly expressed in plasma cells (Figure 8A). The aggregated expression density of these seven genes also indicated their presence in plasma cells (Figure 8B). Based on the core RA lactylation genes, a clinical prediction model for RA was constructed to evaluate the probability of developing rheumatoid arthritis (Figure 8C). This clinical prediction model demonstrated good predictive performance and calibration, with an area under the ROC curve exceeding 0.9 (Figures 8D, E). Finally, we found through qRT-PCR experimental analysis that NDUFB3, NGLY1, and SLC25A4 are highly expressed in rheumatoid arthritis compared with normal tissues (Figure 9).

Discussion

The findings of this study highlight the significant role of lactylation in plasma cells in the pathogenesis of RA. By integrating scRNA-seq data and employing advanced bioinformatics and machine learning approaches, we identified core lactylation-promoting genes and constructed a diagnostic model for RA. This discussion will contextualize our findings within the broader landscape of RA research, comparing them

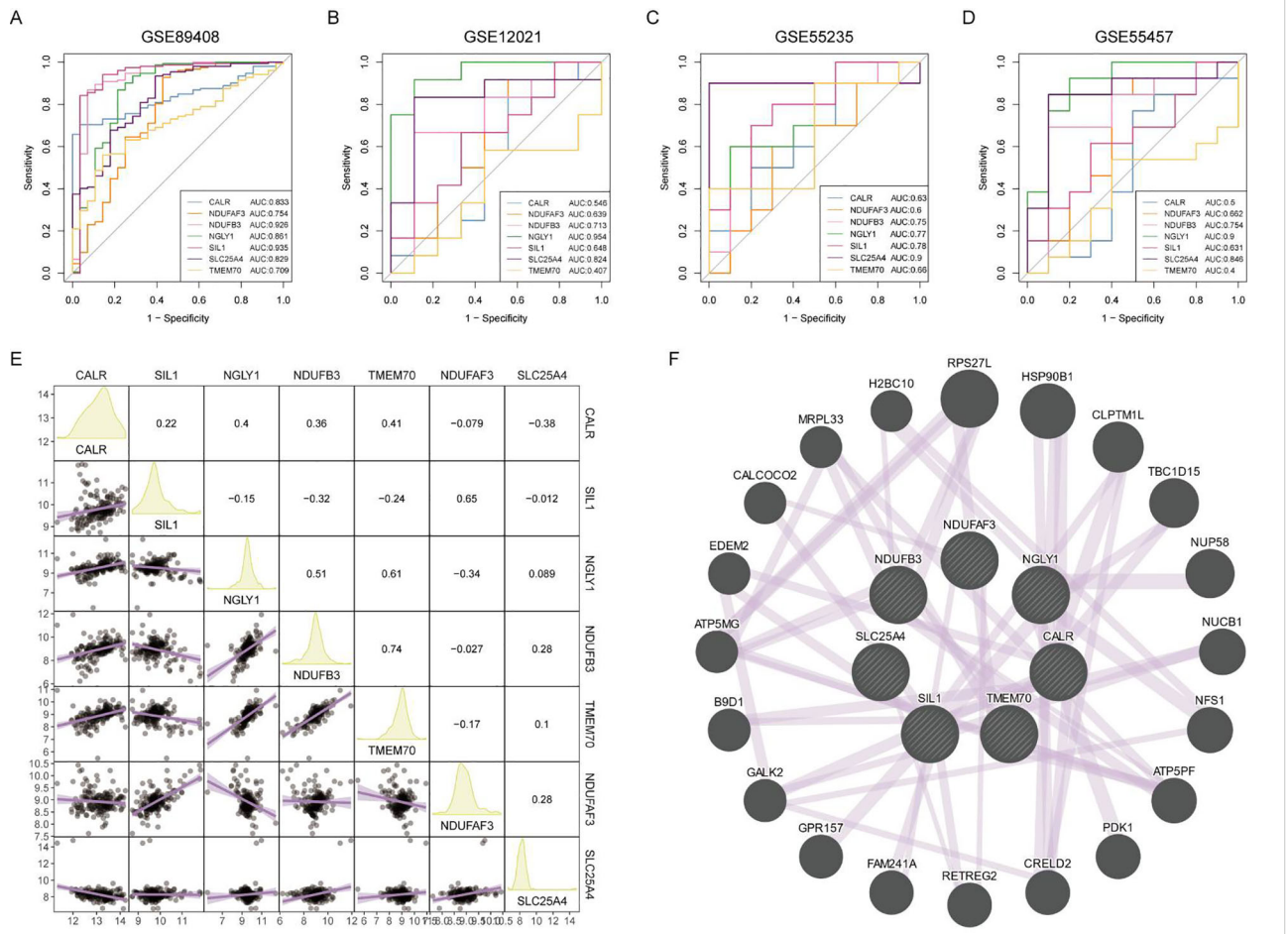

FIGURE 5
Diagnostic efficacy and expression correlation of core RA lactylation genes. **(A-D)** ROC curves showing the diagnostic efficacy of NDUFB3, NGLY1, SLC25A4, and other core RA lactylation genes, with AUC values ranging from 0.7 to 0.91. **(E)** Correlation heatmap showing the expression relationships between core RA lactylation genes. NGLY1 and CALR, NDUFB3 and CALR, and NDUFB3 and NGLY1 exhibit significant positive correlations, while NDUFB3 and SIL1, and NDUFAF3 and NGLY1 show significant negative correlations. **(F)** GENEMANIA network analysis demonstrating that the proteins expressed by these genes primarily have co-expression relationships.

with existing studies and elucidating their implications for diagnosis and therapy.

Our study aligns with the growing body of literature that emphasizes the importance of metabolic reprogramming in immune cells during RA. Previous studies have demonstrated that immune cells, particularly T cells and macrophages, undergo metabolic shifts to support their effector functions during inflammation (35). However, the role of plasma cells and their metabolic adaptations in RA has been less explored. Our results add to this emerging understanding by identifying lactylation as a crucial post-translational modification in plasma cells, implicating it in the disease's metabolic landscape.

Previous research has shown that plasma cells are abundant in the RA synovium and are responsible for the production of autoantibodies such as rheumatoid factor (RF) and ACPAs, which contribute to the disease's pathogenesis (31, 36). However, the specific metabolic pathways and regulatory mechanisms governing plasma cell function in RA have remained unclear. Our study provides evidence that lactylation is significantly upregulated in plasma cells, suggesting a novel regulatory axis that might influence autoantibody production and immune activation in RA.

The identification of core lactylation-promoting genes and their association with immune activation pathways provides novel insights into the mechanisms underlying RA. Lactylation, as a post-translational modification derived from lactate, has been shown to regulate gene expression and protein function, thus influencing various cellular processes (27). Our findings indicate that plasma cells with high lactylation scores exhibit enhanced metabolic activities, including oxidative phosphorylation and glycolysis, which are essential for sustaining the high energy demands of these cells during antibody production.

Interestingly, our study found a significant positive correlation between the RAlac_score and immune cell infiltration, including Treg, Th1, Th2, B cells, NK cells, and others. This suggests that lactylation in plasma cells might be driving a pro-inflammatory environment in RA. These findings are consistent with recent

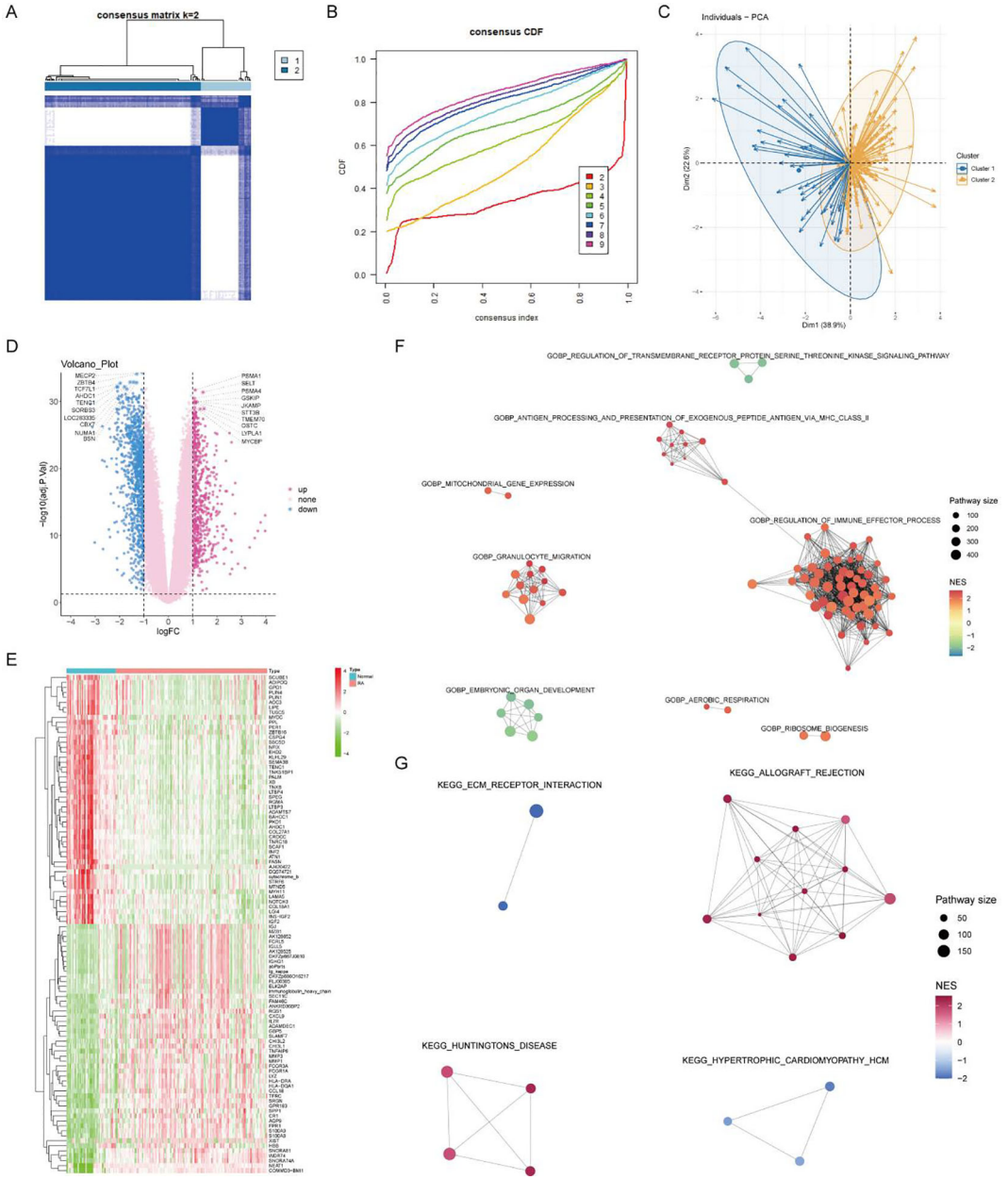

FIGURE 6
Consensus clustering and differential expression analysis. **(A, B)** CDF plot and clustering heatmap showing stable clustering results with K=2 for the expression matrix of the seven core RA lactylation genes. **(C)** PCA plot demonstrating significant characteristic differences between the two clusters. **(D)** Volcano plot of differential gene expression analysis between the two clusters, with 1621 differentially expressed genes identified (logFC >1 and adjusted P <0.05). **(E)** Heatmap showing the top 50 upregulated and top 50 downregulated genes in the differential expression analysis between the two clusters. **(F, G)** Enrichment analysis plots displaying the GO and KEGG pathways activated in cluster 2, associated with immune activity and extracellular matrix interactions.

studies highlighting the role of metabolic reprogramming in supporting the inflammatory functions of immune cells (37). The upregulation of chemokines and TNF family molecules in cluster 2, which had higher lactylation scores, further supports this notion, indicating that these cells are likely contributing to the inflammatory milieu characteristic of RA.

Pathway enrichment analyses revealed that genes upregulated in cluster 2 were associated with immune activity and extracellular matrix interactions, which are critical in the pathogenesis of RA. The involvement of pathways related to oxidative phosphorylation, glycolysis, and endoplasmic reticulum stress in cluster 2 aligns with previous studies that have shown these metabolic processes are crucial for the function and survival of activated immune cells. Moreover, our study identified significant upregulation of MHC-II molecules in cluster 2, which is known to play a role in antigen presentation and T cell activation. This finding suggests that plasma

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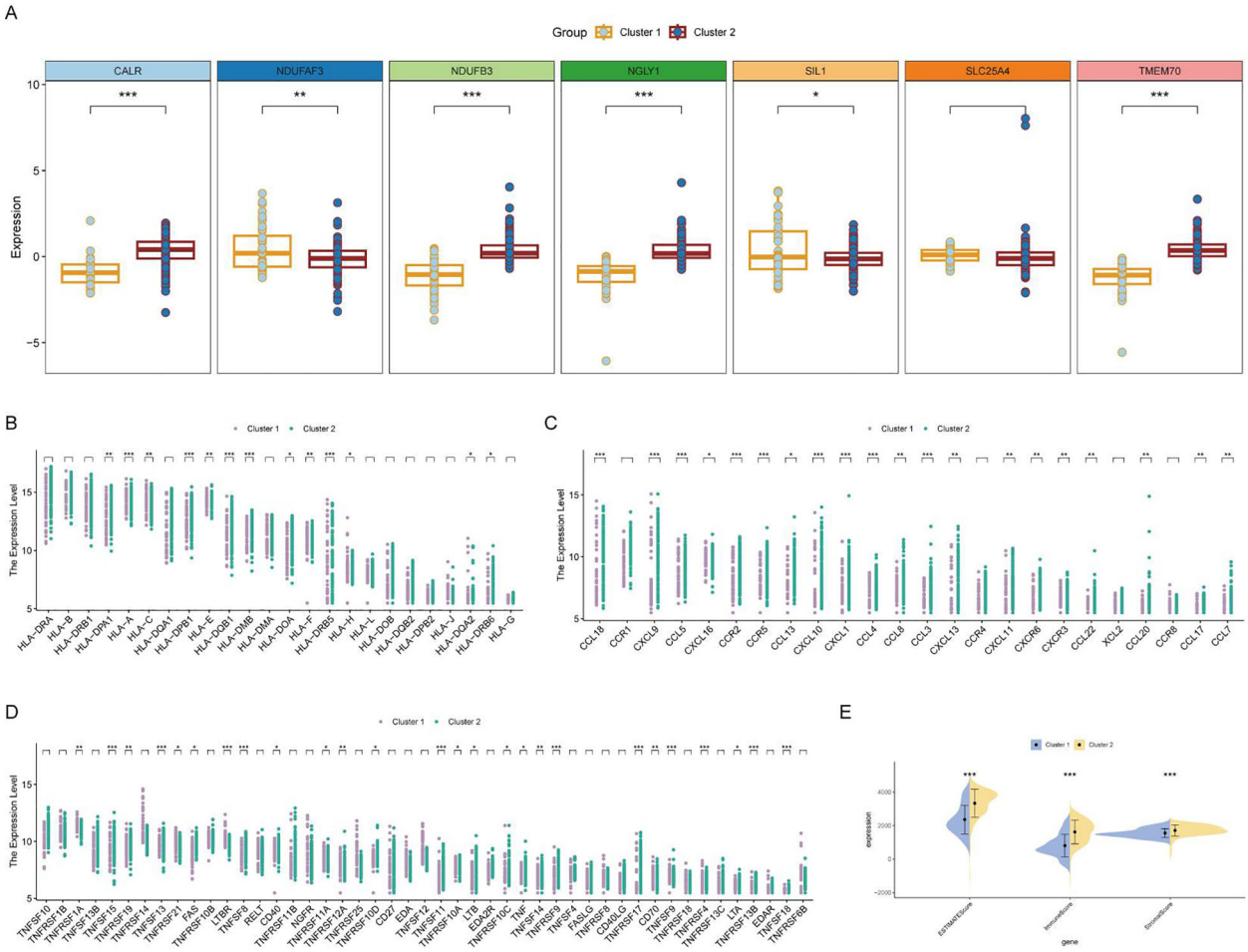

FIGURE 7
Differential expression and immune activation in RA clusters. **(A)** Box plot showing the differential expression of core lactylation genes between the two clusters. NDUFAF3 and SIL1 are highly expressed in cluster 1, while CALR, NDUFB3, NGLY1, and TMEM70 are highly expressed in cluster 2. **(B)** Heatmap illustrating the expression of MHC molecules. MHC-I molecules (HLA-A, HLA-C) are primarily expressed in cluster 1, whereas MHC-II molecules are highly expressed in cluster 2. **(C, D)** Heatmaps showing the expression of various chemokines and TNF family molecules, which are predominantly expressed in cluster 2, indicating an inflammatory and chemotactic subtype associated with immune activation. **(E)** Box plots comparing the stromal score, immune score, and microenvironment score between the two clusters, calculated using the Estimate algorithm. All scores are significantly higher in cluster 2, suggesting enhanced microenvironment activation. * mean $P < 0.05$, ** mean $P < 0.01$, *** mean $P < 0.001$.

cells with high lactylation scores might be enhancing antigen presentation and subsequent T cell activation, thus perpetuating the inflammatory response in RA. The positive correlation between immune checkpoint molecules and RAlac_score further indicates a state of heightened immune activation, which could be targeted by immune checkpoint inhibitors (ICI) as a potential therapeutic strategy.

The identification of core lactylation-promoting genes and the development of a diagnostic model based on these genes have significant clinical implications. Our diagnostic model, with an area under the ROC curve exceeding 0.9, demonstrates high predictive performance and calibration, suggesting its potential utility in clinical settings for early diagnosis and risk stratification of RA patients. The core lactylation-promoting genes identified in our study, including NDUFB3, NGLY1, SLC25A4, and others, could serve as potential biomarkers for RA. Their expression patterns in plasma cells and their association with metabolic reprogramming and immune activation highlight their relevance in the disease's pathogenesis. Future studies should validate these findings in larger cohorts and explore their potential as therapeutic targets. Targeting lactylation pathways in plasma cells might modulate their metabolic activities and reduce autoantibody production, thereby mitigating the inflammatory response in RA.

Despite the significant findings, our study has some limitations. The use of scRNA-seq data, while providing high-resolution insights into cellular heterogeneity, is limited by its snapshot nature, capturing gene expression profiles at a single time point. Longitudinal studies are needed to understand the dynamic changes in lactylation and metabolic reprogramming in plasma cells during the progression of RA. Furthermore, while we employed a variety of

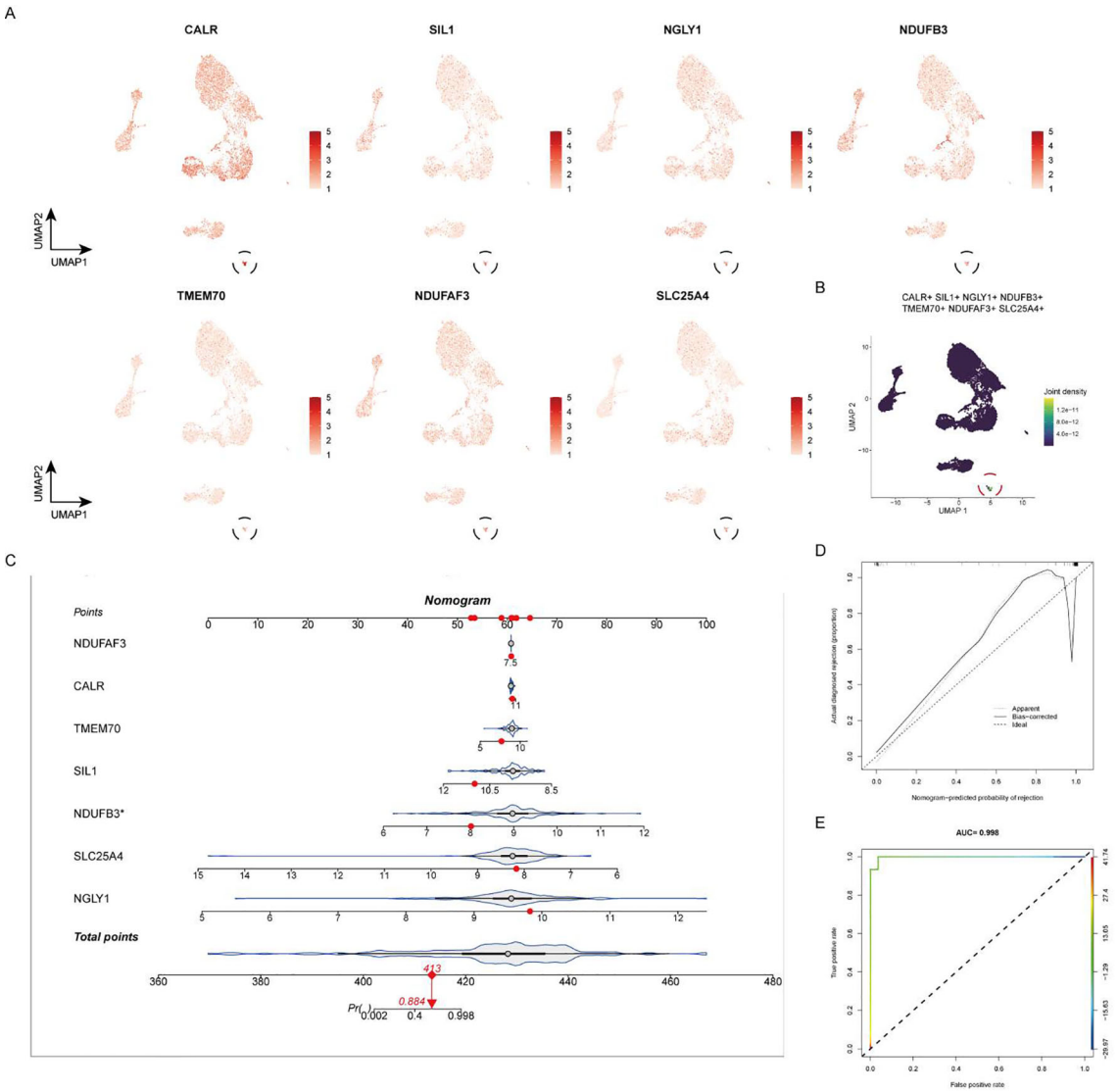

FIGURE 8
Expression of Core RA Lactylation Genes and Clinical Prediction Model. **(A)** Violin plot depicting the expression of the seven core RA lactylation genes in plasma cells at the single-cell level. **(B)** Density plot showing the aggregated expression of the seven core RA lactylation genes in plasma cells. **(C)** Flowchart illustrating the construction of the clinical prediction model for RA based on core lactylation genes. **(D, E)** ROC curves displaying the performance of the clinical prediction model, with an area under the ROC curve (AUC) exceeding 0.9, indicating good predictive efficacy and calibration.

machine learning algorithms, it is important to acknowledge the limitations associated with these approaches. Different machine learning systems may produce varying results depending on the dataset and parameters used, and the complexity of integrating multiple algorithms can introduce potential biases. Although we aimed to mitigate this by using a comprehensive set of algorithms, further optimization and validation are required to ensure robustness and generalizability. Additionally, functional validation of the diagnostic and therapeutic targets identified by these models is essential. Experimental studies should investigate the specific roles of lactylation-promoting genes in plasma cell function and their contribution to RA pathogenesis. Moreover, the therapeutic potential of targeting lactylation pathways should be explored in preclinical models of RA to assess their efficacy and safety.

In conclusion, our study underscores the significant role of lactylation in plasma cells in the pathogenesis of RA. By integrating scRNA-seq data with advanced bioinformatics and machine learning approaches, we identified core lactylation-promoting genes and developed a highly predictive diagnostic model for RA. These findings provide new insights into the metabolic and immunological mechanisms driving RA and highlight potential biomarkers and therapeutic targets for this debilitating disease. Future directions for research should focus on elucidating the precise molecular mechanisms of lactylation in plasma cells,

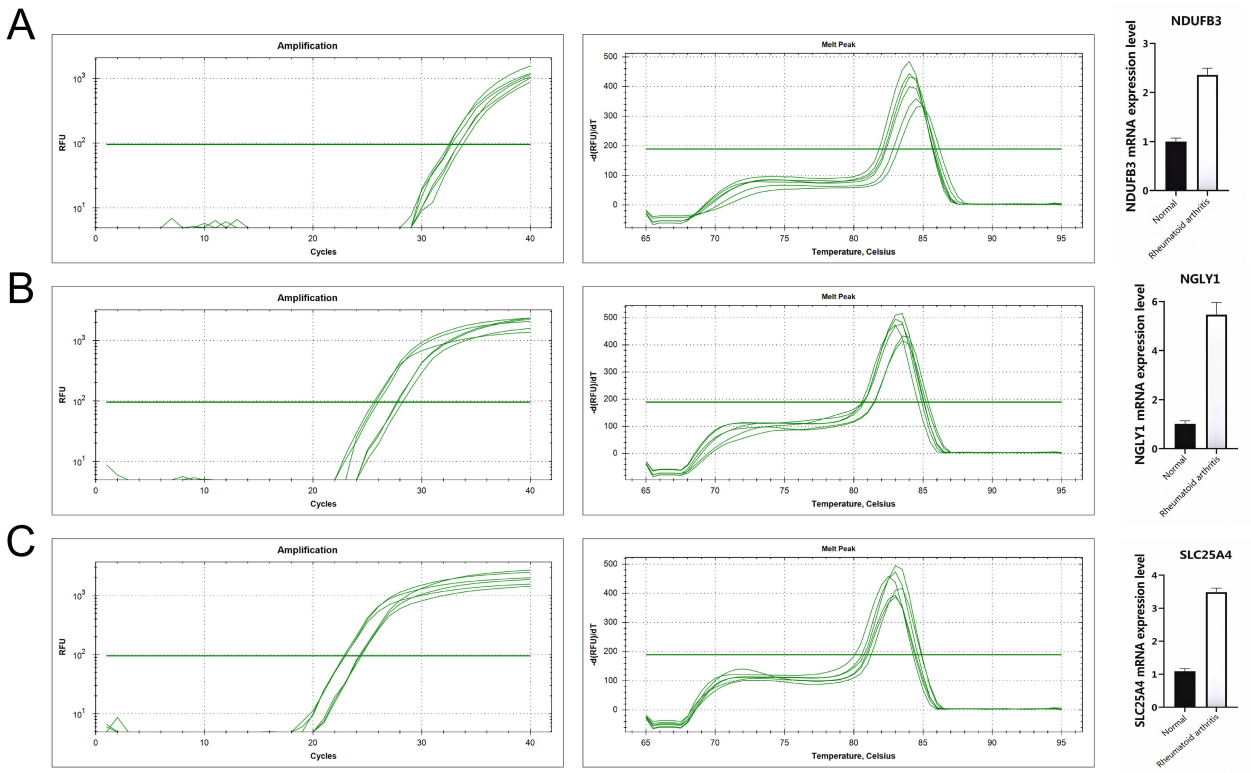

FIGURE 9
All genes are highly expressed. **(A)** NDUFB3 gene expression. **(B)** NGLY1 gene expression. **(C)** SLC25A4 gene expression.

particularly its role in regulating immune responses and metabolic pathways. Additionally, further experimental studies are needed to validate these findings in larger patient cohorts and explore the therapeutic potential of targeting lactylation pathways in RA treatment.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Author contributions

WF: Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. TW: Conceptualization, Investigation, Methodology, Writing – review & editing. YL: Supervision, Validation, Writing – review & editing. TS: Formal analysis, Methodology, Writing – review & editing. QY: Investigation, Methodology, Project administration, Resources, Writing – review & editing.

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Role and therapeutic potential of E3s in the tumor microenvironment of hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is a high-incidence, poor-prognosis malignancy worldwide, requiring new strategies for treatment. Ubiquitination, especially ubiquitination through E3 ubiquitin ligases, plays an indispensable role in the development and progression of HCC. E3 ubiquitin ligases are crucial enzymes in ubiquitination, controlling the degradation of specific substrate proteins and influencing various cellular functions, such as tumor cell proliferation, apoptosis, migration, and immune evasion. In this review, we systematically summarize the mechanisms of E3 ubiquitin ligases in HCC, with a focus on the significance of RING, HECT, and RBR types in HCC progression. The review also looks at the potential for targeting E3 ligases to modulate the tumor microenvironment (TME) and increase immunotherapy efficacy. Future studies will optimize HCC treatment by formulating specific inhibitors or approaches that will be based on gene therapy targeting E3 ligases in order to overcome resistance issues with present treatments and create optimism in the journey of treatment for HCC patients.

KEYWORDS

HCC, ubiquitination, E3 ubiquitin ligase, tumor microenvironment, MMP

1 Introduction

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer worldwide and the third leading cause of cancer-related death (1, 2). And since liver cancer is difficult to diagnose early and has less therapeutic effectiveness, making its prognosis dismal (3–5). Indeed, the application of targeted therapies and immunotherapies strongly increases overall survival in some HCC patients, whereas many remain resistant to

these therapies, partly due to TME complexity and heterogeneity (6–11). Recent development in other therapies targeting the liver tumor microenvironment likely means we will need to further characterize the liver cancer microenvironment to design new combination therapies that effectively suppress tumorigenesis or restore the sensitivity of immunotherapy-resistant tumors (9, 12–15).

The ubiquitin-proteasome system (UPS) is the major pathway for proteins to be ubiquitinated and degraded in the cell (16). In fact, ubiquitination represents a dynamic and finely regulated class of PTM; it is realized by a three-enzyme cascade reaction that includes Ub-activating enzymes (E1s), Ub-conjugating enzymes (E2s), and Ub-ligases (E3s) (17, 18). The reaction pathway comprises ATP-dependent activation of Ub by E1, transfer to a cysteine residue of E2, and covalent binding to the amino group of a lysine residue of the substrate protein via E3 (19, 20). (Figure 1A1) E3 ubiquitin ligases are particularly important in this process, as they play a pivotal role in the specific recognition and labeling of substrates. Abnormal expression or malfunction of these ligases may cause signaling pathway disruptions, leading to the build-up of misfolded or dysfunctional proteins and incorrect protein complex assembly, ultimately driving the onset and development of HCC (21, 22).

E3 ligase also takes a significant role in the TME of HCC (23). TME comprises a diverse array of cellular components, including immune cells, stromal cells, and blood vessels, along with non-cellular elements such as the extracellular matrix and secreted factors (24, 25). E3 ligases have been shown to influence the invasion and metastasis of tumor cells by regulating key proteins in the TME, such as matrix metalloproteinases (MMPs). Thus, targeting E3 ligases not only holds promise in reducing tumor burden but may also open new avenues for enhancing the efficacy of existing therapies and overcoming drug resistance.

This review aims to provide a comprehensive and updated overview of the role of E3 ubiquitin ligases in HCC, with a particular focus on their impact on the TME and immunotherapy. It offers new insights by emphasizing recent findings on how E3 ligases modulate immune cell activity and therapeutic responses within the TME, distinguishing it from previous reviews in this field.

2 Expression and function of E3 ligase in hepatocellular carcinoma

The four identified isoforms of E3 ligase (HECT-, RING-, U-box-, and RBR-type) promotes ubiquitin transfer through different mechanisms (Figure 1A2). The HECT structural domain of HECT E3 ligase transfers ubiquitin to its C-terminal leaflet by binding to the E2 enzyme, first by a process of trans-sulfurylation, followed by further delivery of ubiquitin to the target substrate (26, 27). Upon

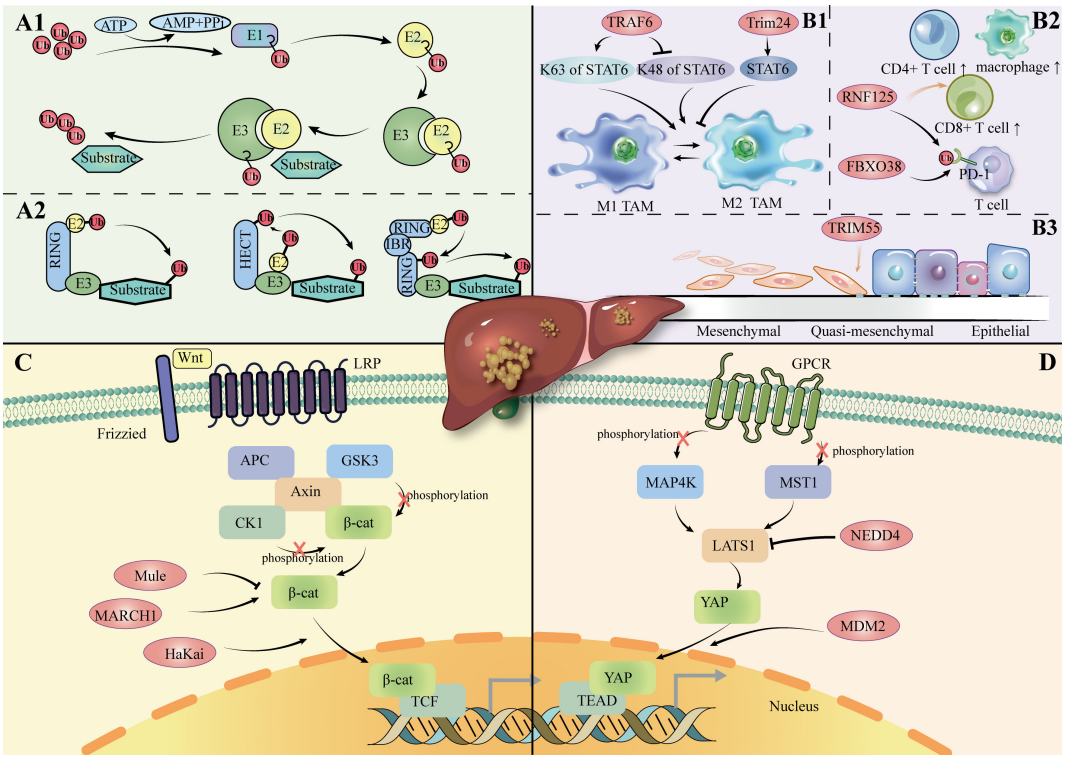

FIGURE 1
(A1): Ubiquitination process. **(A2):** Overview of the E3 ligase family. **(B1):** E3 ligase regulates the polarization of TAM in HCC. **(B2):** E3 ligase regulates immune cells in HCC. **(B3):** E3 ligase regulates the EMT process in HCC. **(C)**: Role of E3 ligase in the Wnt/β-catenin channel in HCC. LRP: Low-Density Lipoprotein Receptor-Related; APC: Adenomatous Polyposis Coli; CK1: Casein Kinase 1; GSK3: Glycogen Synthase Kinase 3; TCF: T-cell Factor. **(D)**: Role of E3 ligase in Hippo channels in HCC. MAP4K: Mitogen-Activated Protein Kinase Kinase Kinase Kinase; MST1: Mammalian Sterile 20-like kinase 1; TEAD: TEA Domain Family Member; GPCR: G-Protein-Coupled Receptor; Protein.

binding of the ubiquitin-loaded E2 to the RING1 domain of the RBR E3 ligase, ubiquitin is transferred to the RING2 domain through a trans-thioesterification reaction. The RING2 domain then facilitates the transfer of this ubiquitin to the substrate (28). In summary, HECT- and RBR-type E3 ligases transfer ubiquitin (Ub) to substrate proteins after forming a thioester bond between their active site cysteine and Ub. In contrast, RING- and U-box-type E3 ligases directly facilitate the transfer of Ub from E2 enzymes to substrate proteins (29–32).

2.1 RING E3 ligase

The RING-type subgroup represents the primary family of E3 ligases, characterized by two distinct types of RING structural domains: the RING fold structure with a zinc-binding site and a U-box domain. Structures in which both domains can function via monomers, homodimers, heterodimers, or multiple subunits (33, 34). Cullin-RING ligases (CRLs) are a type of multisubunit RING E3 ligases, with F-box proteins serving as an essential part of their structure (35). F-box proteins are categorized into three types: FBXW, FBXL, and FBXO. Studies have shown that the expression of FBXO17 is significantly elevated in the tumor tissues of hepatocellular carcinoma (HCC) patients compared to adjacent normal tissues. FBXO17 may contribute to the malignant progression of HCC by inhibiting the Wnt/β-catenin pathway (36).

Different structures of E3 ligases may be potential tumor promoters in HCC. *In vitro* and *in vivo* experiments have demonstrated that monomeric MARCH1 upregulates the PI3K-AKT-β-catenin pathway, thereby promoting the growth and progression of HCC (37). The HaKai heterodimer has been shown to promote the degradation of E-calmodulin, resulting in the nuclear translocation of β-catenin proteins and ultimately driving epithelial-mesenchymal transition (EMT) in HCC (38).However, the homologous structural domain type MDM2 may be a repressor of HCC. MDM2 was shown to diminish YAP's interaction with other proteins and promote its cytoplasmic translocation and degradation, thereby inhibiting tumorigenesis in HepG2 cells (39).

2.2 HECT E3 ligase

HECTs are second only to human RING E3 ligases in number, and their HECT structural domains consist of an N-terminal lobe, a C-terminal lobe, and a flexible chain (26).Knockdown of WWP2 (HECT-type) significantly elevated the expression levels of apoptosis-related markers in HCC) cell lines, including caspase-7, caspase-8, and Bax, suggesting that inhibition of WWP2 may be a therapeutic tool to negatively regulate HCC overproliferation and escape apoptosis (40). Mule, a member of the HECT E3 ligase family, functions as a tumor suppressor in HCC by inhibiting the Wnt/β-catenin signaling pathway. Specifically, Mule directly targets β-catenin for degradation in HCC, thereby suppressing β-catenin-mediated cancer stem cell (CSC) activity (41).

HECT E3 ligase was shown to mediate the Hippo pathway in HCC cells, including its participation in the Wnt/β-catenin pathway (Figures 1C, D). LATS1 is one of the core components of the Hippo pathway. NEDD4 acts as a direct targeting factor for LATS1, which causes its ubiquitinated degradation and increases the transcriptional activity of YAP. In QGY7703 and SMMC7721 hepatoma cell lines, siRNA-mediated NEDD4 knockdown assays showed that decreased expression of NEDD4 inhibited cell proliferation, invasion, and migration, promoted apoptosis, and further supported the role of the NEDD4-LATS1 pathway in HCC progression (42).

2.3 RBR E3 ligase

The RBR E3 is composed of two RING structural domains (RING1, RING2) and IBR structural domain. Parkin was known to play an oncostatic role in a wide array of tumors including HCC and breast cancer (43). Through direct degradation of TRAF 2 and TRAF6, parkin drives HCC cell apoptosis by inhibition of the NF-κB pathway (44).

3 E3 ligase regulates TME in hepatocellular carcinoma

Given the plasticity of TME and its involvement in the progression of multiple cancers, the modification of TME into an anticancer environment is a promising therapeutic strategy (45–49). Currently, most drugs for TME, such as immunotherapies and antiangiogenic drugs, have limited or unmet efficacy (50, 51).This phenomenon may stem from the complexity of TME and the diversity of its responses to drugs, thus making it difficult to achieve significant clinical results with these therapies in practice. With increasing evidence that ubiquitin signaling cascades modulate immune cell activity and the stability of soluble factors in the TME, a permissive or inhibitory environment for tumor growth can be provided. Moreover, as the first major family of ubiquitinating enzymes, the diversity and specificity of E3 ligases endow them with roles in broadly regulating tumor signaling pathways and biological processes, so making full use of intrinsic E3 ligases to target key mediators seems to be an attractive strategy for anticancer drug development.

3.1 E3 ligase on immune cells

Typically, the immune cells infiltrating the TME CD8+ T cells, CD4+ T helper 1 (Th1), M1 macrophages and NK cells are usually antitumorigenic, whereas the opposite is true for M2 macrophages (52, 53).Regulatory T-cells (Tregs) show these two opposite effects in animal models and clinical trials (54–56) (Figure 1B2).

E3 ligase can regulate the proportion and function of immune cells in the TME by targeting the degradation of tumor suppressors. Analysis of data from The Cancer Genome Atlas (TCGA) public database revealed that RNF125 expression levels are positively correlated with the infiltration of CD4+ and CD8+ T cells, as well as macrophages, within tumors (57).WD repeat 4 (WDR4) has been

reported to be a substrate junction for CRL, which can degrade a tumor suppressor, the promyelocytic leukemia (PML) protein.In this process, the expansion of Treg cells, M2 macrophages, and the reduction of CD8+ T cells contribute to the establishment of an immunosuppressive and pro-metastatic TME (58).

In addition, E3 ligases are crucial in immunomodulation by regulating the ubiquitination of key proteins and influencing T cell differentiation. In a study on HCC, In a study on HCC, Jiang et al. found that lncRNA-EGFR binds to EGFR, inhibiting c-CBL-mediated ubiquitination and thus preventing EGFR degradation. This mechanism helps to maintain the continuous activation of the RAS/RAF/MEK/ERK signaling pathway downstream of EGFR, which ultimately promotes the differentiation of Tregs (59).

TME can induce cancer immunosuppression through the upregulation of PD-L1 protein expression. However, E3 ligase plays a role in inhibiting the ubiquitination and degradation of PD-L1, thereby assisting tumor cells in evading T cell-mediated immune surveillance. For example, the RING E3 ligase FBXO38 mediates the ubiquitination of PD-1, thereby regulating antitumor immunity in T cells (60).In hepatocellular carcinoma, RNF125 (RING type) directly ubiquitinates PD-L1 and maintains a stable protein level of PD-L1 (61).

Additionally, E3 ligases are crucial in regulating immune cell differentiation and function. Macrophages, through their M1 and M2 polarization, significantly influence tumor progression and shape the immune environment. Next, we will explore the role of E3 ligases in regulating the polarization of TAMs.

3.2 E3 ligase on TAMs

One of the important processes in which E3 ligases play a role is polarization toward tumor-associated macrophages (TAMs). (Figure 1B1) TAMs are one of the major immune cell types in the tumor microenvironment. When TAMs are exposed to different types of signaling stimuli, they polarize into two contrary functional profiles: differentiation toward M1, with an anti-tumoral effect by response to Th1; differentiation to the M2 type, with pro-tumor effects through Th2 cytokines (62, 63). Specific E3 ligases regulate key signaling pathways such as NF-κB and STAT6 to influence M1 and M2 polarization, further influencing the immune response in the tumor microenvironment.

E3 ligase is one of the mediators that regulate ubiquitination in macrophage polarization. STAT6, for instance, is one of the main transcription factors that drive M2 macrophage polarization (64). TRAF6 is an E3 ligase that is the main activator of K63-linked ubiquitination of STAT6 in M2-polarized macrophages stimulated with IL-4. In contrast, it inhibits the degradation (65).

Under hypoxic conditions, Seven in Absentia homologue 2 (SIAH2), an E3 ligase with a RING domain, is the regulator of proteasome degradation of NRF1 (Nuclear Respiratory Factor 1) and, therefore, switches TAM polarization to the tumor-promoting M2 state in breast cancer (66). The underpinning mechanisms of the SIAH2-NRF1 axis are linked to changes in mitochondria-dependent metabolic reprogramming, with an increase in lactate. Another RING E3 ligase, TRIM24, degrades the histone acetyltransferase CBP that acetylates STAT6, which inhibits TAM polarization to M2 (67).

4 E3 ubiquitin ligases target MMPs in TME

4.1 Role of MMPs in TME of hepatocellular carcinoma

MMP is a zinc-dependent endopeptidase and multifunctional enzyme that can be secreted by TAM (68, 69). MMPs are categorized into several groups: collagenase, gelatinase, stromelysin, membrane MMPs, and other unclassified MMPs (70). MMPs have the ability to degrade almost all components of the ECM, leading to structural changes in the cellular and tissue environments. TME is composed of various cellular constituents, along with the biochemical and biophysical elements of ECM, and is defined by their intricate interactions within and surrounding solid tumor masses (71, 72). In TME, MMPs play a crucial role. When MMPs are dysfunctional, they lead to the destruction of the ECM, which promotes cell migration and tumor metastasis (73–75). In tumor stem cells of HCC, MMP remodel the ECM, resulting in tumors that exhibit more aggressive and functional stemness (76). Recent studies have demonstrated that MMP9, secreted by TAMs, is particularly involved in ECM degradation, facilitating tumor invasion and metastasis in HCC. Inhibiting MMP9 activity in TAMs has been shown to reduce ECM breakdown and, consequently, limit the metastatic potential of HCC cells (77).

4.2 Mechanism of regulating the microenvironment of hepatocellular carcinoma by targeting MMPs via E3 ligase

Protein expression of MMP can be controlled by E3 ubiquitin ligases (78, 79). (Table 1) For example, TRIM55 is associated with a decrease in MMP2 (80). TRIM66 reduces MMP9 expression (81). EMT is a process through which epithelial cells acquire mesenchymal traits, facilitating cancer invasion and metastasis (82). Among them, MMP2 is the major MMP in the pathogenesis of EMT in hepatocellular carcinoma (73). It has been demonstrated that overexpression of TRIM55 (RING-type) effectively reduced the migration and invasion ability of HCC cells by modulating epithelial-mesenchymal transition and inhibiting the activity of MMP2 (80). This suggests that E3 ligase can influence the hepatocellular carcinoma microenvironment by affecting MMP protein expression and EMT (epithelial-mesenchymal transition). (Figure 1B3).

5 Potential of E3 ligase as a therapeutic target in hepatocellular carcinoma

A growing body of evidence indicates that abnormal ubiquitination expression is correlated with poor cancer

TABLE 1 Summary of E3s in HCC.

Type	Characteristic domains	E3s	Signaling pathway	Substrates in HCC	Effect	Reference
RING	RING/U-box	FBXO17	wnt/β-catenin	MMP-9, MMP-2	Promote cell metastasis	(36)
		TRIM55	–	MMP2	Promote cell migration and invasion	(68)
		β-TrCP	JNK/β-TrCP	MMP-9	Regulate cell motility and promote cell invasion	(69)
		MARCH1	PI3K-AKT-β-catenin		Promote cell proliferation, migration, and invasion	(37)
HECT	N-terminal lobe, C-terminal lobe, and a flexible tether	NEDD4	–	LATS1	Increase YAP transcriptional activity	(42)
		Mule	wnt/β-catenin	β-catenin	Inhibit CSC	(41)
		WWP2	–	caspase-7, caspase-8 and Bax	Promote cell proliferation and evasion of apoptosis	(40)
RBR	RING1, RING2, IBR	Parkin	NF-κB	TRAF2, TRAF6	Promote cell apoptosis	(44)

HECT, homologous with E6-associated protein C-terminus; RING, really interesting new gene, U-box-; RBR, RING- between-RING;MARCH1, Membrane-associated RING-CH-1; β-TrCP, β-Transducin Repeat Containing Protein; MDM2, Mouse Double Minute 2; YAP, Yes-associated protein; WWP2, WW Domain Containing E3 Ubiquitin Protein Ligase 2; Bax, Bcl-2-associated X protein; NEDD4, Neural Precursor Cell Expressed Developmentally Down-Regulated Protein 4; IBR, In-Between-RING; TRAF, TNF Receptor Associated Factor.

prognosis. Given the critical role of various E3 ligases in the tumorigenesis of HCC, targeting E3 ligase activity is considered a promising therapeutic strategy for cancer treatment. p53 is one of the most important tumor suppressors *in vivo*, and MDM2 regulates the level of P53. Antagonizing MDM2 seems to be an effective strategy to develop promoter inhibitors for HCC tumors, but further clinical trials are still needed (83–85). For example, in a mouse model, the MDM2 inhibitor APG-115 induced synergistic activity with anti-PD-1 antibody-based immunotherapy (86). Recent studies have shown that Fbxw7 increases the sensitivity of HCC cells to sorafenib (87, 88). This finding implies a potential clinical application of Fbxw7 in enhancing sorafenib efficacy in liver cancer treatment. Multiple compounds can target MMPs via E3 ligase for cancer therapy. Zhang et al. found that the natural agent ALCA upregulated NEDD4L (HECT-type) and caused ubiquitination of β-catenin, which activated Wnt-induced transcription of the MMP9 gene in lung adenocarcinoma cells (89). Gallic acid reduces MMP2 and MMP9 protein levels by inducing β-TrCP in human leukemia cells (90). Notably, MMP9 is the major MMP in the pathogenesis of EMT in hepatocellular carcinoma (73). Therefore, utilizing compounds to regulate the expression of E3 ligases and modulate MMP levels could represent a promising strategy for the treatment of HCC.

6 Conclusion

Hepatocellular carcinoma is heterogeneous at the genetic and epigenetic levels, making the development of therapeutic agents for liver cancer difficult (91, 92). Ubiquitination, a crucial post-translational modification of proteins, has been increasingly recognized on a broader scale. In this intricate environment, E3 ligases, beyond targeting substrates for proteasomal degradation, also regulate various signaling pathways such as PI3K/AKT and Wnt/β-catenin. Moreover, most E3 ligases in HCC are oncoproteins (93, 94). So E3 ligase is an attractive drug target for cancer therapy.

The role and importance of E3 ligases in hepatocellular carcinoma have been widely explored, though numerous questions still persist. As E3 ubiquitin ligases are frequently mutated, their targeting specificity may be insufficient, which leads to less accurate recognition of the target and may trigger off-target effects, ultimately leading to poor therapeutic efficacy. Recent advancements in technologies like CRISPR and PROTAC (Proteolysis Targeting Chimeras) have opened new avenues for more precise targeting of E3 ligases in cancer treatment. In particular, CRISPR can be used to knock out or activate specific genes related to E3 ligases, thereby providing a strategy to mitigate their oncogenic effects in the TME (95, 96). On the other hand, PROTACs (Proteolysis Targeting Chimeras), which are also small molecule inhibitors, degrade POIs (Cullin-RING type) in a substoichiometric manner, leading to more prolonged and potent biological effects on the target compared to SMIs. In addition, PROTAC dBET1 inhibits the pro-inflammatory response by regulating MMP9 in lipopolysaccharide (LPS)-activated microglial cells (97). As a result, PROTACs have emerged as a promising approach for developing new targeted anticancer therapies.

Interestingly, similar to the process of ubiquitination, SUMization (Small Ubiquitin-like Modifier) plays an important role in most organisms, regulating a variety of cellular processes, including DNA replication, transcription, immune response (98, 99). An increasing body of research indicates a strong association between SUMOylation and the progression of hepatocellular carcinoma (100). SUMO E3 ligase may also be a potential target for the treatment of hepatocellular carcinoma.

Author contributions

HW: Formal analysis, Writing – original draft, Writing – review & editing. QL: Visualization, Writing – original draft, Writing – review & editing. QT: Data curation, Writing – original draft, Writing – review & editing. GS: Writing – original draft. GW: Writing – original draft. XM: Writing – original draft. CW: Writing – original draft. LZ: Writing – original draft. JL: Conceptualization, Writing – review & editing, Writing – original draft. JDL: Conceptualization, Writing – review & editing, Writing – original draft. BL: Conceptualization, Writing – review & editing, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Common molecular basis for MASH and hepatitis C revealed via systems biology approach

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Background: Metabolic dysfunction-associated steatohepatitis (MASH) is characterized by liver inflammation and damage caused by a buildup of fat in the liver. Hepatitis C, caused by hepatitis C virus (HCV), is a disease that can lead to liver cirrhosis, liver cancer, and liver failure. MASH and hepatitis C are the common causes of liver cirrhosis and hepatocellular carcinoma. Several studies have shown that hepatic steatosis is also a common histological feature of liver in HCV infected patients. However, the common molecular basis for MASH and hepatitis C remains poorly understood.

Methods: Firstly, differentially expressed genes (DEGs) for MASH and hepatitis C were extracted from the GSE89632, GSE164760 and GSE14323 datasets. Subsequently, the common DEGs shared among these datasets were determined using the Venn diagram. Next, a protein-protein interaction (PPI) network was constructed based on the common DEGs and the hub genes were extracted. Then, gene ontology (GO) and pathway analysis of the common DEGs were performed. Furthermore, transcription factors (TFs) and miRNAs regulatory networks were constructed, and drug candidates were identified. After the MASH and hepatitis C cell model was treated with predicted drug, the expression levels of the signature genes were measured by qRT-PCR and ELISA.

Results: 866 common DEGs were identified in MASH and hepatitis C. The GO analysis showed that the most significantly enriched biological process of the DEGs was the positive regulation of cytokine production. 10 hub genes, including STAT1, CCL2, ITGAM, PTPRC, CXCL9, IL15, SELL, VCAM1, TLR4 and CCL5, were selected from the PPI network. By constructing the TF-gene and miRNA-gene network, most prominent TFs and miRNAs were screened out. Potential drugs screening shows that Budesonide and Dinoprostone may benefit patients, and cellular experiments showed that Budesonide effectively inhibited the expression of genes related to glycolipid metabolism, fibrosis, and inflammatory factors.

Conclusion: We extracted 10 hub genes between MASH and hepatitis C, and performed a series of analyses on the genes. Molecular docking and *in vitro* studies have revealed that Budesonide can effectively suppress the progression of MASH and hepatitis C. This study can provide novel insights into the potential drug targets and biomarkers for MASH and hepatitis C.

KEYWORDS

metabolic dysfunction-associated steatohepatitis (MASH), hepatitis C, bioinformatics analysis, differentially expressed genes, Budesonide

1 Introduction

Liver disease has emerged as a major cause of global health burden. It accounts for approximately 2 million deaths per year worldwide, including 1 million due to complications of cirrhosis and 1 million due to viral hepatitis and hepatocellular carcinoma (HCC) (1, 2). Among them, Metabolic dysfunction-associated steatohepatitis (MASH) and viral hepatitis are the main causes of liver cirrhosis and HCC (1).

Metabolic dysfunction-associated steatotic liver disease (MASLD) is a chronic condition marked by excessive fat deposition in hepatocytes, unlated to alcoholic consumption or other specific liver-damaging factors. It is usually accompanied by insulin resistance and diabetes mellitus. The prevalence of MASLD is 25% to 30% and continues to rise each year as high-fat diets become more prevalent (3). MASH is the progressive form of MASLD. In the development of MASLD, about 5% to 20% of patients progress from simple fatty liver disease to MASH, a more severe disease defined by macrovesicular steatosis, hepatocyte injury (ballooning), and liver inflammation (4). MASH may culminate in cirrhosis and HCC and is currently a leading cause of liver transplant (5).

Hepatitis C virus (HCV) is a hepatotropic RNA virus that can cause acute and chronic hepatitis, with progressive liver damage resulting in cirrhosis, decompensated liver disease, and HCC. In 2020, there were an estimated 56.8 million hepatitis C virus infections worldwide (HCV RNA viraemic prevalence 0.7%), China (9.48 million), Pakistan (7.39 million) and India (6.13 million) being the three countries with the highest disease burden (2, 6). The early stage of hepatitis C is often asymptomatic, with only 56% of patients being aware of their infection. Missing the opportunity for treatment can lead to more serious liver injury in the late stage. Compared with HBV, a greater proportion of hepatitis C progresses to cirrhosis and liver cancer.

Furthermore, indirect estimates from much research suggest that 3% to 6% of adults at the population level have MASH. With the worldwide epidemics of diabetes and obesity, the proportion of MASH patients is expected to increase over the next decade (7). Therefore, the probability for interaction between MASH and hepatitis C is significant. Several studies have shown that hepatic steatosis is also a common histological feature in HCV infected patients (8, 9). Analysis of the results of 25 studies, collectively including 6400 patients, showed that up to 55.54% of these HCV infected patients have variable degrees of hepatic steatosis (10). The average prevalence of HCV-associated MASH is between 4% and 10% (11). This is also supported by the observation that the degree of liver steatosis is directly related to the level of HCV replication, as measured by serum HCV RNA (8). Steatosis was significantly reduced in hepatitis C patients after antiviral therapy. Therefore, much more work needs to be done to explore the strict association between hepatitis C and MASH.

In this study, we first analyzed three microarray datasets downloaded from the Gene Expression Omnibus (GEO) platform to obtain differentially expressed genes (DEGs) for hepatitis C and MASH. Then we analyzed the identified common DEGs using Gene Ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, and Protein-Protein Interaction (PPI) Networks analysis. In addition, transcription factors (TFs), potential target miRNA, and associated diseases of hub genes were predicted. Finally, we identified a number of compounds that may be used in the treatment of MASH and chronic hepatitis C based on hub genes. Our results support the possibility that MASH and hepatitis C share the same molecular basis and regulatory pathways, which might serve as potential therapeutic targets for patients with MASH and hepatitis C and facilitate the development of targeted drugs.

2 Materials and methods

2.1 Gene expression profile data collection

In this study, three gene expression datasets were collected from the Gene Expression Omnibus (GEO) database. GSE14323 was derived from a hepatitis C associated hepatocellular carcinoma study, GSE89632 and GSE164760 were derived from two studies of hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. In all cohorts, no patients were diagnosed with both MASH and hepatitis C. Datasets are available at https://www.ncbi.nlm.nih.gov/geo/ for more information. Pertinent information for the selected GEO datasets used in this study was summarized in Table 1.

TABLE 1 Accession information for datasets downloaded from the GEO database.

Accession	GPL	Etiology	Sample size case/control	Country	Year
GSE14323	GPL571	HCV	**HCV: 96** (41 HCV-cirrhosis; 38 HCV-HCC; 17 HCV-cirrhotic tissues from HCC patients) **Healthy: 19**	USA	2018
GSE164760	GPL13667	MASH	**MASH: 156** (74 NASH, 29 adjacent non-tumor tissues; 53 tumor tissues from NASH-HCC patients) **Healthy: 6**	Spain	2021
GSE89632	GPL14951	MASH	**MASH: 19** **Healthy: 24**	Canada	2017

2.2 Identification of differentially expressed genes

Background expression value correction and data normalization were conducted for the raw data in each dataset using an R package (Affy; version 1.52.0). Probes in each data file were then annotated based on the appropriate platform annotation files. Probes without matching gene symbols were removed when a probe corresponded to more than one gene, the preceding gene was deleted. In instances where different probes mapped to the same gene, the mean value of all probes mapping to that gene was taken as the final expression value for that gene. Batch effects were removed using the R package (SVA; version 3.48.0) Then, the Linear Models for Microarray Analysis R package (limma; version 3.56.2) was applied for differential expression analysis. Those genes with a p-value < 0.05 and |log2FC| > 0.5 were deemed to be the DEGs. In addition, the overlapping DEGs between MASH and hepatitis C were delineated using the ggvenn R packages.

2.3 Gene ontology, pathway enrichment analyses, and gene set enrichment analysis

Gene Ontology (GO) is a commonly used bioinformatics tool that provides comprehensive information on gene function of individual genomic products based on defined features. This analysis consists of three facets: molecular functions (MF), biological processes (BP) and cellular components (CC). Enrichment analysis was performed using DEGs overlapping, and R package (clusterProfiler; version 4.8.1) was used in this analysis process. KEGG is considered as a knowledge base for systematic analysis of gene functions, linking genomic information with higher order functional information. For quantifying the top listed functional items and pathways, and a statistical threshold criterion with p-value < 0.05 and q-value < 0.05 were used to identify significant GO terms and KEGG pathways. Additionally, we performed GSEA analysis of GSE datasets by gseGO (R clusterProfiler package).

2.4 Protein–protein interaction network construction and hub gene analysis

In order to analyze the connections among the proteins encoded by identified DEGs, DEGs were uploaded to Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org/), a database of known and predicted PPI networks. The results with a minimum interaction score of 0.4 were visualized in Cytoscape. And then, we used the Cytoscape plug-in Minimal Common Oncology Data Elements (MCODE, http://apps.cytoscape.org/apps/mcode) to screen out key protein expression molecules. Furthermore, CytoHubba, a Cytoscape plugin app, providing a user-friendly interface to explore important nodes in biological networks, was utilized with the maximal clique centrality (MCC) method to explore the PPI network for hub genes.

2.5 Construction of regulatory networks of transcription factors and miRNAs

To determine major transcriptional variations, we analyzed the interaction networks of hub genes with miRNAs and transcription factors (TFs) using the NetworkAnalyst platform. Specifically, the NetworkAnalyst platform was utilized to locate topologically credible TFs from the JASPAR database that tend to bind to the hub genes. For hub genes and miRNA network construction via NetworkAnalyst platform, the TarBase and miRTarBase databases were used to extracted miRNAs with hub genes focused on topological analysis.

2.6 Screening of potential therapeutic compounds

An online resource, Drug Signatures Database (DSigDB), connects drugs/compounds to their target genes. To study the drug molecular properties of MASH and hepatitis C, we used the DSigDB library under the Diseases/Drugs function in Enrichr (https://maayanlab.cloud/Enrichr/enrich).

2.7 Gene-disease association analysis

The DisGeNET database contains one of the most comprehensive collections of genes and variants associated with human disease. Based on hub genes, we identified diseases and chronic health problems using DisGeNET database under the Diseases/Drugs function in Enrichr.

2.8 Molecular docking

Molecular docking that an established in silico structure-based method is widely used in drug discovery. Docking enables the identification of novel compounds of therapeutic interest, predicting ligand-target interactions at a molecular level, or delineating structure-activity relationships (SAR), without knowing *a priori* the chemical structure of other target modulators. In our study, key targets of MASH and hepatitis C were obtained through hub genes identification, including CD4 and SRC. Next, the crystal structures of these key proteins were downloaded from the Protein Data Bank (https://www.rcsb.org/) for further molecular docking. The molecular structures of potential drug molecules were obtained from the ZINC (https://zinc.docking.org/) database. The Autodock tools (version 1.5.4) was utilized in all docking experiments, with the optimized model as the docking target. The screening method is restricted to molecular docking, and molecular dynamics simulation has not been carried. In addition, the results were shown with binding energy (BE), a weighted average of docking score, to assess the reliability and describe the accuracy of the ligand positioning. Pymol (PyMOL Molecular Visualization System 2020) was used for 3D visualization of the docking results.

2.9 Cell culture

The human hepatoma cell line Huh7.5 and HCV full-length genomic plasmid were kept in our laboratory. Huh7.5 cells were cultured in DMEM with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 humidified atmosphere. The cells were treated with 0.2 mM PA and 0.1 mM OA for 24 h to create a hepatocyte steatosis model *in vitro*. In the drug experiment, the Budesonide powder was first dissolved in Dimethyl sulfoxide (DMSO), then formulated into an aqueous solution, and 1 mM of the aqueous Budesonide solution was added to the cell culture media. As a control group, only the same dose of DMSO aqueous solution was added to the cell culture medium.

2.10 HCV transfection

Twenty-four hours before transfection, 4.2×10^5 Huh7.5 cells per well were seeded in six-well plates. For transfections, 10 μg of HCV recombinant plasmid was linearized with XbaI, treated with mung bean nuclease, purified, and *in vitro* transcribed using T7 RNA polymerase (Promega) (100 μl total). The resulting HCV RNA transcripts were mixed with 150 μl Opti-MEM (Invitrogen) and incubated for 10 min at room temperature, mixed with 255 μl transfection complex [5 μl of Lipofectamine 2000 (Invitrogen) in 250 μl of Opti-MEM with 10-min incubation], incubated for 20 min, and added dropwise into the Huh7.5 cell cultures that had been preincubated in 2 ml of Opti-MEM for 20 min. The transfected cultures were left for ~16 h and then were subcultured every 2-3 days; the supernatant was collected, filtered (pore size 0.45 μm), and stored at -80°C. To passage virus, Huh7.5 cells grown in six-well plates were incubated with 1 mL transfection collected culture supernatant for ~16 h and then were subcultured every 2-3 days.

2.11 qRT-PCR

Total RNA was extracted with TRIzol (DP424, TIANGEN) and reverse-transcribed into cDNA using a reverse transcription kit (R323-01, Vazyme). For qRT-PCR, cDNAs were combined with SYBR master mix (Q311-02, Vazyme). qRT-PCR was performed in triplicate with a Bio-Rad CFX Thermocycler. The data were collected and analyzed with Bio-Rad real-time PCR detection systems and software. The primers are described in Supplementary 1.

2.12 Western blot

The cells were lysed with lysis buffer (Biosharp, BL504A) in the presence of protease inhibitor cocktail (87786, Thermo Fisher) according to the manufacturer's instruction. Total protein was dissolved in loading buffer (0.2 M Tris-HCl [PH 6.5], 0.4 M dithiothreitol, 277 mM sodium dodecyl sulfate [SDS], 6 mM bromophenol blue, and 4.3 M glycerol), separated by a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel, and transferred to immune-blot NC Membranes (1620115, Bio-Rad). The membrane was blocked with 5% skim milk powder in Tris-buffered saline and Tween-20 (TBST) for 1 h and probed with corresponding antibodies. The signal was detected with ECL Western Blotting Substrate (BL520B, Biosharp). Antibodies are described in Supplementary 1.

2.13 ELISA determination of IL-1β, IL-6 and TNF-α

IL-1β, IL-6 and TNF-α were determined in cell supernatant. Cell supernatant was collected and filtered by 0.45 mm strainer. Cell Extraction Buffer PTR (Abcam) containing a protease inhibitor cocktail (Roche, Switzerland). Human IL-1β (ab214025, Abcam), human IL-6 (ab178013, Abcam), and human TNF-α (ab181421, Abcam) ELISA kits were performed according to the manufacturer's instructions.

2.14 Statistical analysis

Unpaired *t*-test was used to detect the difference between two groups. And comparison among more than two groups of qRT-PCR and ELISA results was assessed by the analysis of *t*-test using the statistical software GraphPad Prism and P values < 0.05 were

considered statistically significant. Statistical significance is defined as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, or NS (not significant).

3 Result

3.1 Identification of DEGs and common DEGs among hepatitis C and MASH

To investigate the interrelationships and implications between MASH and hepatitis C, we analyzed human microarray datasets from the GEO database to identify genes that involved in the development of MASH and hepatitis C. We obtained the datasets GSE164760 and GSE89632 for MASH, and GSE14323 for hepatitis C from the NCBI GEO database. The identification of common DEGs between MASH and hepatitis C suggests a shared molecular pathological basis. In the MASH datasets GSE164760 and GSE89632, a total of 5,809 DEGs were identified. In the HCV dataset GSE14323, a total of 2,401 DEGs were identified (Figure 1A). The volcano maps were used to visualize the expression pattern of DEGs in both diseases (Figures 1B, C). In addition, the overlap of differential genes between hepatitis C and MASH was evaluated using a Venn diagram analysis. As shown in Figure 1D, there were 866 common DEGs between MASH and hepatitis C (Figure 1D).

3.2 Gene ontology, pathway analysis, and GSEA

The GO database provides a standardized description of gene products in terms of their function, participating biological pathways, and cellular localization. The results of the GO analysis of hepatitis C or MASH were shown in Supplementary Figure S1. In order to further elucidate the biological functions of these differential genes and the signaling pathways involved, GO, KEGG enrichment analyses and GSEA were performed on the above 866 genes to explore the common regulatory pathways. The GO analysis revealed that the common genes might be related to positive regulation of cytokine production, wound healing and regulation of body fluid levels. The analysis of cellular components indicated that the DEGs were mainly associated with the collagen-containing extracellular matrix, cell-substrate junction and focal adhesion. In terms of molecular function, the DEGs were significantly enriched in amide binding, peptide binding and peptidase regulator activity (Figure 2A). The Loop graphs show the correlation between the three most important GO terms and the enriched DEGs (Figure 2B). These GO terms are humoral immune response, positive regulation of cytokine production and adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains respectively. The results of the KEGG analysis of hepatitis C or

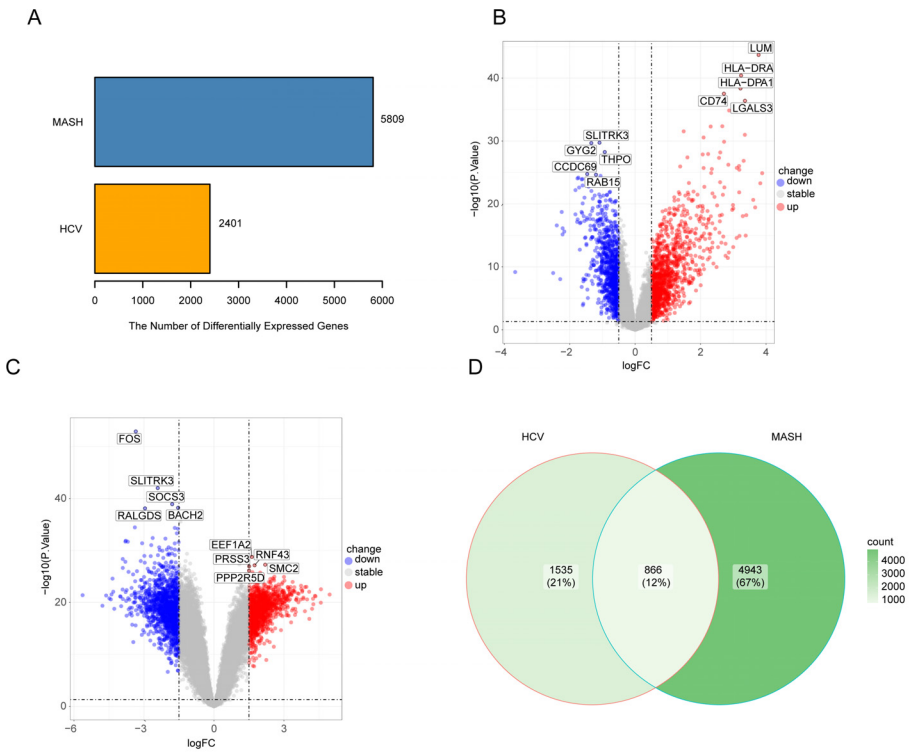

FIGURE 1
The volcano plot and venn diagram of differentially expressed genes (DEGs). **(A)**, Comparing the number of differentially expressed genes between MASH and HCV; **(B)**, volcano plot of DEGs in GSE14323; **(C)**, volcano plot of DEGs in GSE164760 and GSE89632; **(D)**, Venn diagram showing the overlap of differentially expressed genes between MASH and HCV.

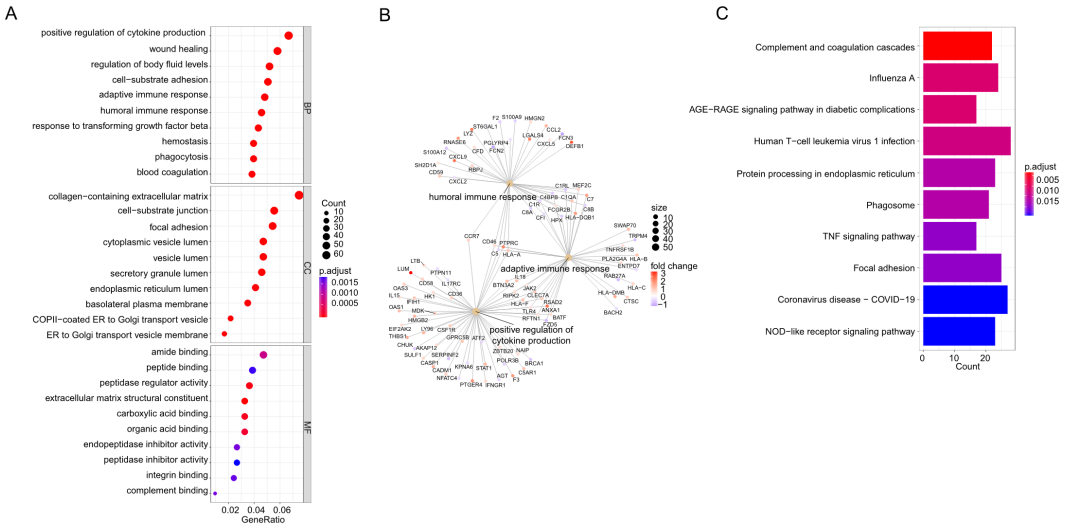

FIGURE 2
(A) Gene Ontology (GO) analysis based on the common differentially expressed genes (DEGs). **(B)** Loop graphs show the correlation between the five most important GO terms and the enriched DEGs. **(C)** Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis based on the common differentially expressed genes (DEGs).

MASH were shown in Supplementary Figure S2. The KEGG analysis showed that these overlapping DEGs might be primarily involved in complement and coagulation cascades, Influenza A, AGE-RAGE signaling pathway in diabetic complications, Human T-cell leukemia virus 1 infection, Protein processing in endoplasmic reticulum, Phagosome, TNF signaling pathway (Figure 2C). More or less, all of these signaling pathways are involved in inflammation. The GSEA was performed for the common DEGs and the result was shown in Supplementary Figure S3. The enriched pathway includes cell-substrate junction, endoplasmic reticulum, focal adhesion and regulation of inflammatory response.

3.3 PPI network construction and hub genes identification

To identify interactions among the common differential genes, a PPI network of DEGs was performed based on the STRING database and the obtained results were imported into Cytoscape for visual analysis. Finally, MCODE plug-in was utilized to identify meaningful gene cluster modules and obtain gene cluster scores (filter criteria: degree cut-off = 2; node score cut-off = 0.2; k-core = 2; max depth = 100). The two modules with the highest score were selected (Figures 3A, B). Based on MCC algorithm of CytoHubba plug-in, genes in the top ten were identified as potential hub genes: STAT1, C-C motif ligand 2 (CCL2), ITGAM, PTPRC, CXCL9, IL15, L-selectin (SELL), VCAM1, Toll-like receptor 4 (TLR4), CCL5 (Figure 3C, Table 1). The qRT- PCR result of 10 hub genes showed, most of the hub genes expression was significantly higher than the control in the cell model. But only the gene expression of *SELL* and *CXCL9* have no significantly between control and hepatitis C-MASH cellular model (Figure 3E). Many of them were related to the liver inflammation and hepatocyte injury. Studies from Grohmann M et al. were consistent with a STAT1 gene signature being of functional relevance to the development of MASH (12). Moreover, during the development of chronic viral hepatitis and MASH, CCL2 and CCL5 played an important role (13). In addition, VCAM1 plays a key role in liver inflammation in MASH and hepatocyte TLR4 determine MASH-induced fibrosis (14, 15).

3.4 Regulatory network of DEGs-related TFs and miRNAs

In order to screen out important regulatory factors of hub genes at the transcriptional level, the Network Analyst platform was used to predict target transcription factors (TFs) and miRNAs of hub genes. And Cytoscape software was utilized to construct interaction network. As shown in Figure 3D and Supplementary Table S1, Interferon regulatory factor 1 (IRF1) and ZNF175 were the most prominent TF in this network. The most prominent miRNAs were hsa-mir-26b-5p, hsa-mir-146a-5p and hsa-mir-155-5p. hsa-mir-26b-5p was interacted with three hub genes, including TLR4, CXCL9 and CCL2. hsa-mir-146a-5p was interacted with three hub genes, including STAT1, TLR4 and CCL5. hsa-mir-155-5p was interacted with three hub genes, including CCL2, STAT1 and VCAM1 (Figure 4, Supplementary Table S2). There were studies have shown that miR-26b-5p, miR-155-5p and mir-146a-5p may regulate MASLD by involving in some signal pathway or target gene (16, 17).

3.5 Disease-gene network

Different diseases may be related to each other, and their regulatory networks usually share at least one or more similar

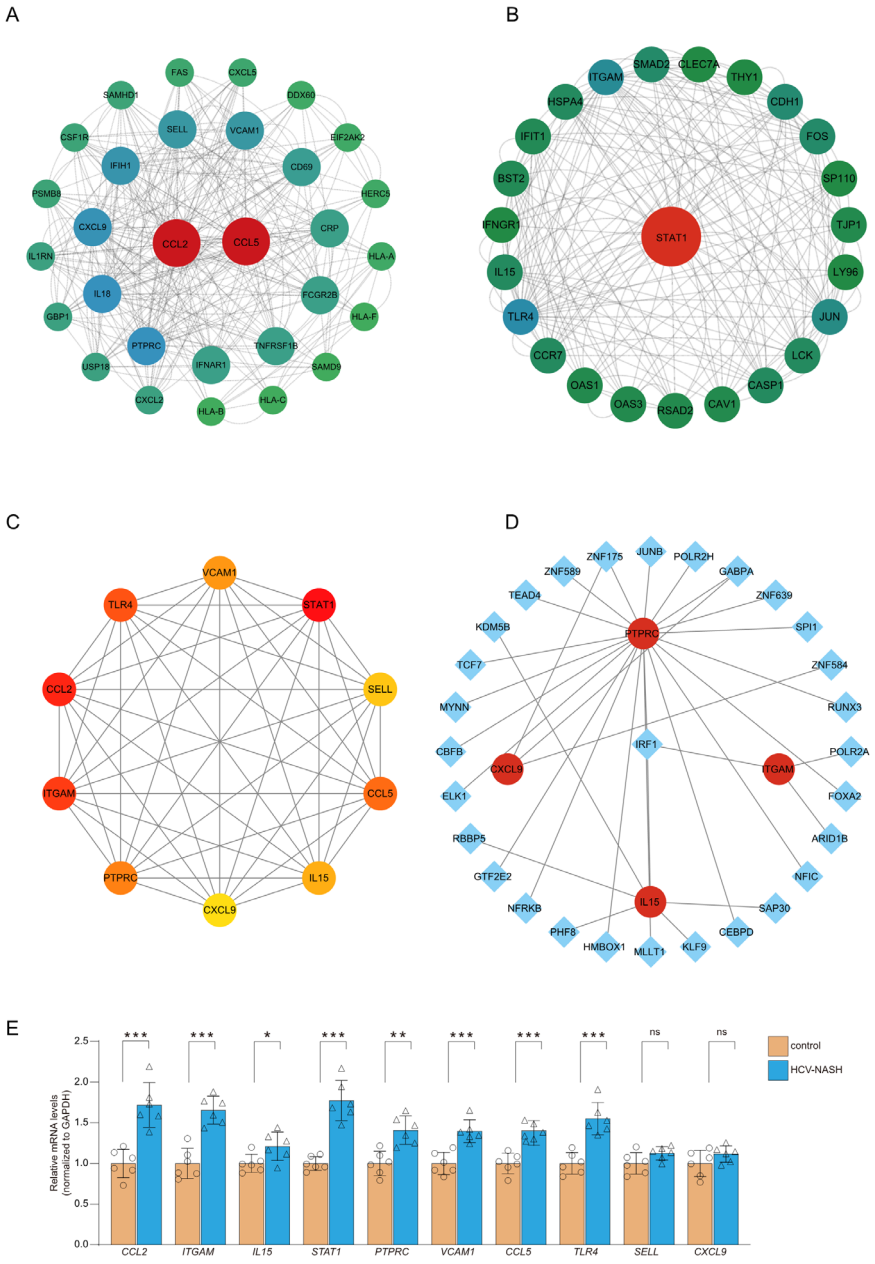

FIGURE 3
PPI network construction and module analysis. The PPI network of DEGs was constructed in Cytoscape. The most significant module was obtained by MCODE plug-in. **(A)** module score:10; **(B)** module score:8.6. **(C)** PPI network for the top 10 hub genes analyzed by cytoHubba. **(D)** The cohesive regulatory interaction network of DEGs-TFs obtained from the NetworkAnalyst. The square nodes represented TFs, and gene symbols interact with TFs as circle nodes. **(E)** qRT-PCR was performed to detect the levels of hub genes in cell model. Results were represented as mean ± SEM (n = 6, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, or NS).

genes. Using the DisGeNET database and the Diseases/Drugs function in Enrichr, we identified diseases and chronic health problems associated with the hub genes. As shown in Figure 5 and Supplementary Table S3 illustrates the relationship between hub genes and diseases. Consistent with previous research, Liver Cirrhosis was the most prominent common related diseases of MASH and hepatitis C. HCV-related cirrhosis prevalence increased by 28.7% and MASH-related liver cirrhosis has been shown to be the dominant etiology of cirrhosis, accounting for 59.5% of cases. Overall, cirrhosis accounted for 2.4% of total global deaths in 2017. Suggesting that it is very important to intervene the development of cirrhosis based on these genes (18).

3.6 Potential drugs screening and molecular docking

To explore potential drugs for MASH and hepatitis C, we utilized the Enrichr platform based on the DSigDB database to predict small molecule drugs related to the hub genes. The

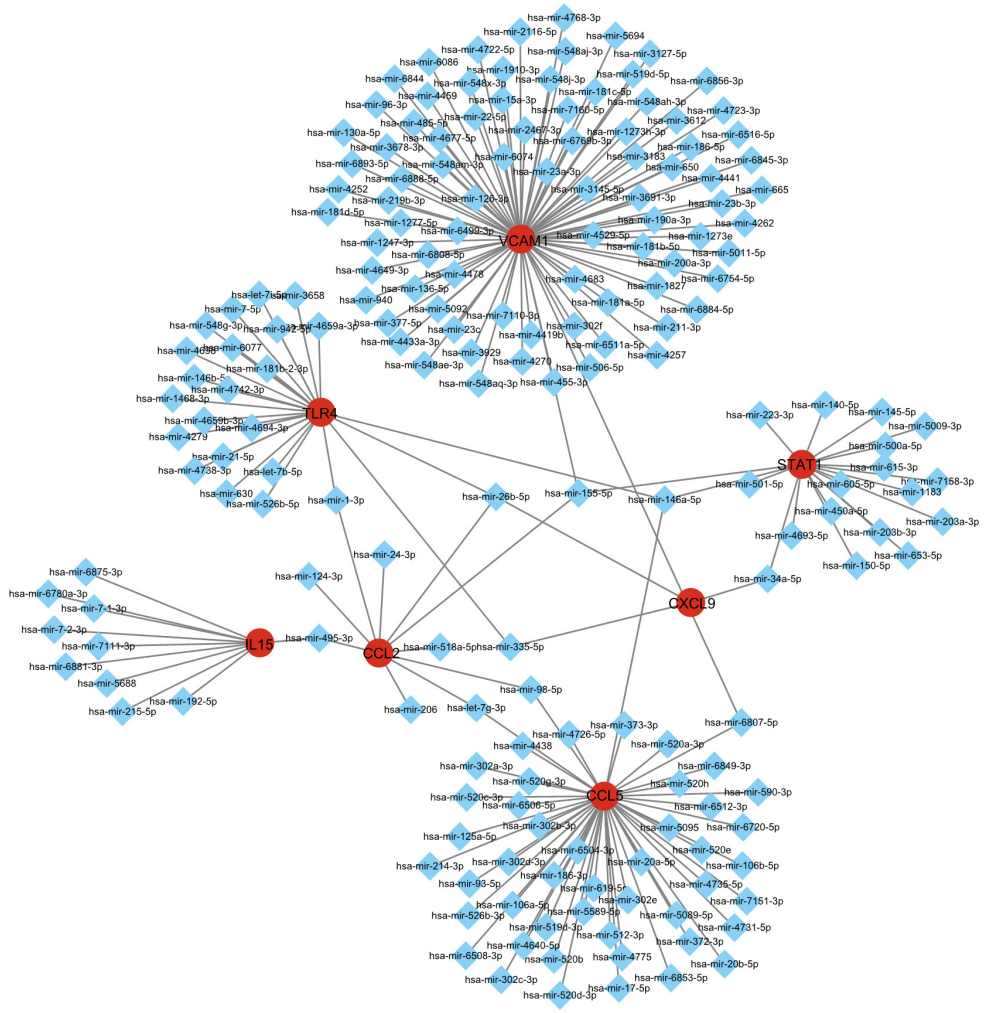

FIGURE 4
Integrated miRNA-gene interaction networks for the eight hub genes. The square node indicates miRNAs and the gene symbols interact with miRNAs in the shape of a circle.

first 10 potential compounds were finally extracted (Table 2). We selected two target proteins (CCL2 and STAT1) with the strongest regulatory interaction with NASH and hepatitis C (Supplementary Table S3) for molecular docking analysis to predict their potential therapeutic effects. The docking score between Budesonide and CCL2 was -5.28 (kcal/mol) (Figure 6A). The docking score between Dinoprostone and STAT1 was -5.54 (kcal/mol) (Figure 6B). Our results provided possible target genes for pharmacological effect of the potential drugs.

3.7 Preliminary laboratory validation of the Budesonide in a cell model of MASH and hepatitis C

Next, we selected the Budesonide to treat the cellular model of MASH and hepatitis C. The qRT-PCR results showed that Budesonide significantly inhibited the expression of genes related to glycolipid metabolism (Figure 7A), fibrosis (Figure 7B), and inflammatory factors (Figure 7C). The expression of IL-1β, IL-6 and TNF-α were suppressed after Budesonide treatment by ELISA kits (Figure 7D). Western blotting results showed that MASH-associated protein (α-SMA, CTGF, SCD1, CD36) and the NS5A, CORE of HCV, expression of which was significantly suppressed after Budesonide treatment (Figure 7E). The above results suggest that Budesonide can effectively inhibit the progression of MASH and hepatitis C, at least at the cellular level.

4 Discussion

According to the estimation from the World Health Organization (WHO), Hepatitis C is a global epidemic with approximately 58 million people worldwide are chronically infected as of 2019, and 1.5 million people were newly infected globally in 2019 (19). On the other hand, the incidence rate of MASH has been increasing in recent years, and there is a concurrent increase in the prevalence rate of hepatitis C (2). Since liver cirrhosis

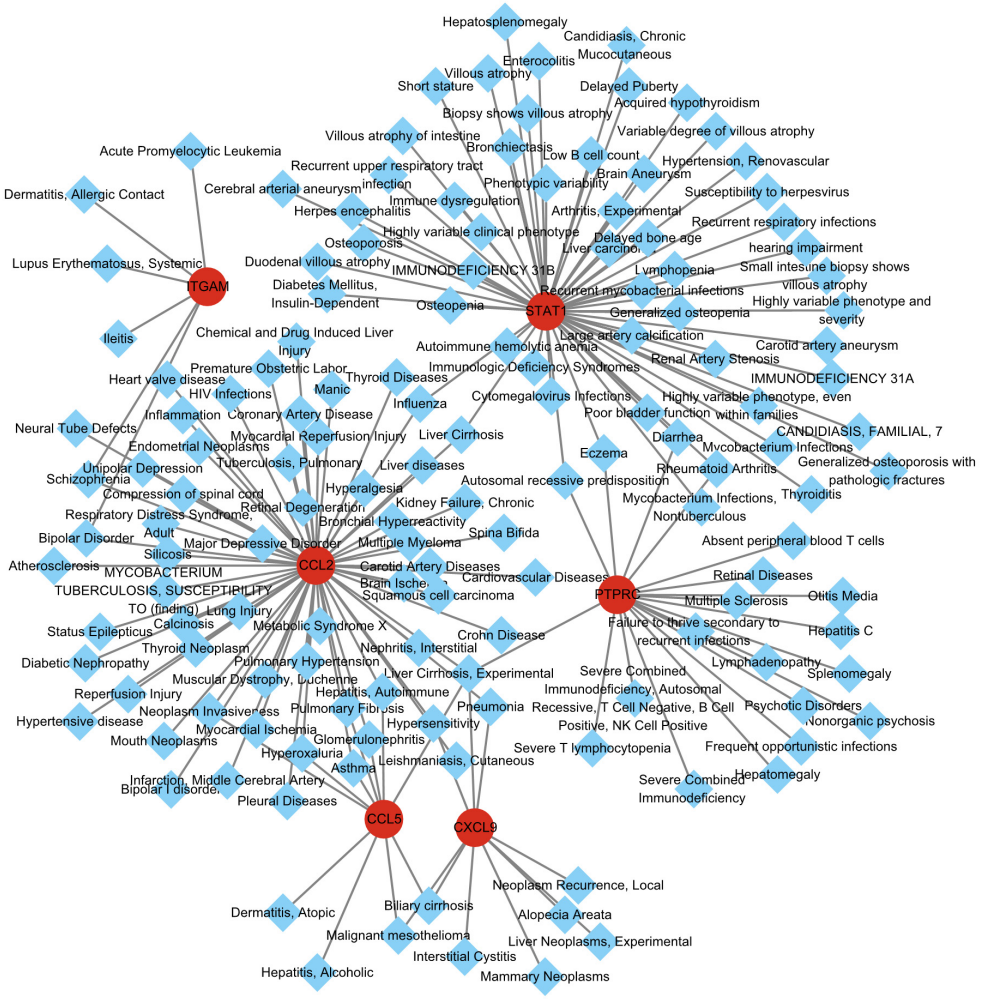

FIGURE 5
Integrated gene-disease interaction networks for the nine hub genes.

and HCC are common outcomes of hepatitis C and MASH (20), and HCV infection has a high probability of inducing hepatic steatosis (21), there is considerable interest in understanding the common mechanisms that regulate these two diseases. However, the mechanism of interaction has not been fully understood up to now.

Recently, bioinformatics analysis based on microarray data became a widely used approach to study gene expression profiles in diseases. In this study, we integrated the microarray data of hepatitis C and MASH to explore the common molecular mechanisms underlying these diseases. We identified 866 common DEGs in both diseases and performed GO analysis and KEGG analysis using these genes. The most significantly enriched biological processes was positive regulation of cytokine production, next wound healing, and then regulation of body fluid levels.

Cytokines are chemical messengers in immune system, which consist of chemokines, interferons (IFN), interleukins (IL), tumor necrosis factor (TNF), and colony-stimulating factors (CSF). Cytokines play important roles in host responses to infection, immune responses, inflammation, trauma, sepsis, and cancer. However, aberrant production of cytokines can also contribute to pathologic inflammation. For example, previous studies have shown that the expression of IFN-γ and IFN-γ-inducible chemokines CXCL10, -9, -11 in hepatocytes and lymphocytes of hepatitis C patients increased, which was related to the degree of inflammation (22). Further research found that the increased CRP, IL-1β, and TNF-α were significantly associated with MASH and hepatic fibrosis (23). Recently, a study demonstrated that TNF-α is a key step in Miz1 degradation, resulting in a further reduction in hepatocyte mitophagy, and then promote MASH progression (24). Interestingly, according to a variety of studies, IL-1β, IL-6, TNF-α, and IFN-γ are up-regulated during HCV induced MASH (25). Therefore, the cytokine regulation may be a crucial line between MASH and hepatitis C.

Significantly enriched pathways in KEGG analysis were in order as follows: complement and coagulation cascades, Influenza A, AGE-RAGE signaling pathway in diabetic complications, Human T-cell leukemia virus1 infection, Protein processing in endoplasmic reticulum, Phagosome, TNF signaling pathway, Focal adhesion, Coronavirus disease, and NOD-like receptor signaling pathway.

TABLE 2 Potential drugs targeting common DEGs among HCV and MASH.

Term	P-value	Adjusted P-value	OddsRatio	Combined Score	Genes
Acetovanillone	1.02E- 11	7.41E-09	511.5641026	12947.91105	ITGAM;VCAM1;STAT1; CCL5;CCL2
glutathione	1.82E- 11	7.41E-09	229.1538462	5667.339993	ITGAM;VCAM1;STAT1; CCL5;CCL2;TLR4
AGN-PC-0JHFVD	1.43E- 10	3.88E-08	160.5810811	3640.504172	PTPRC;VCAM1;SELL;IL15; CCL5;TLR4
budesonide	7.78E-09	0.00000159	282.8794326	5281.678992	VCAM1;STAT1;CCL5;CCL2
dexamethasone	1.64E-08	0.00000245	70.75301205	1268.246275	ITGAM;VCAM1;IL15;CCL5; CCL2;TLR4
Dinoprostone	2.08E-08	0.00000245	103.6596859	1833.578824	VCAM1;STAT1;IL15;CCL5; TLR4
Vanadium pentoxide	0.000000021	0.00000245	217.8032787	3850.285258	CXCL9;VCAM1;STAT1;IL15
Demecolcine	3.23E-08	0.00000329	51.34138857	885.5741104	CXCL9;ITGAM;VCAM1;IL15;CCL5;CCL2;TLR4
Tosyllysylchloromethane	0.000000061	0.00000553	611.5102041	10158.88265	ITGAM;VCAM1;CCL2
PD98059	8.87E-08	0.00000724	76.78210117	1246.796682	ITGAM;VCAM1;STAT1; CCL5;CCL2

These results indicated that the common pathways between MASH and HCV infection are mainly associated with immunomodulation. It is also suggested that the immune response plays an important role in the development of these two anomalies (26, 27).

After constructing the PPI network of DEGs, we identified the top ten hub genes using the MCC algorithm of CytoHubba plug-in, the top ten were STAT1, CCL2, ITGAM, PTPRC, CXCL9, IL15, SELL, VCAM1, TLR4 and CCL5.

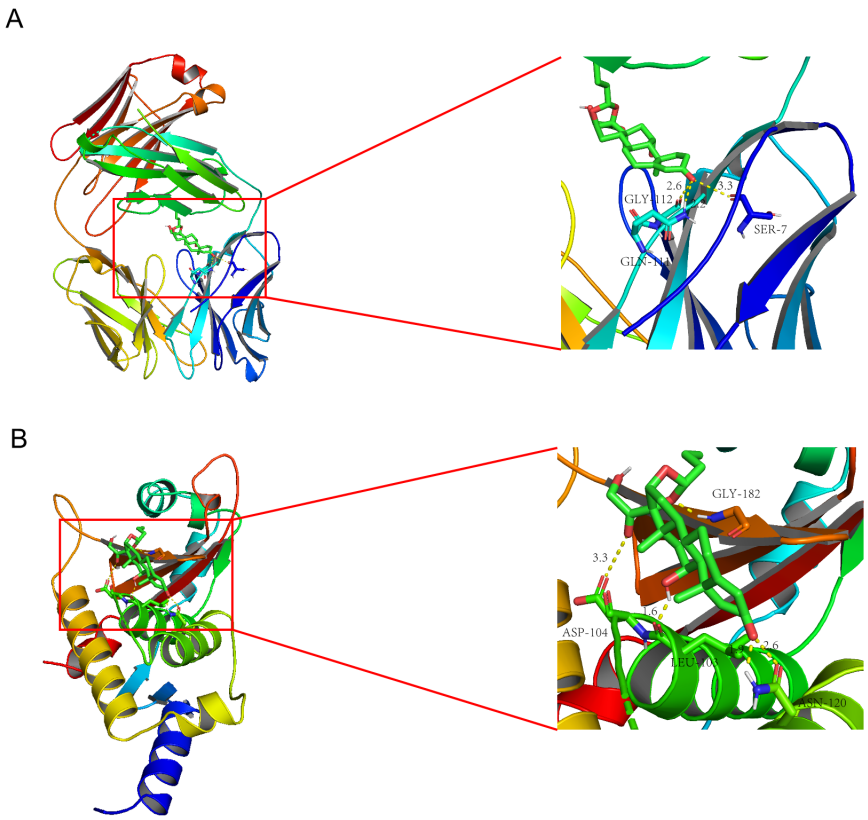

FIGURE 6
Molecular docking patterns Budesonide **(A)** with the CCL2; Molecular docking patterns Dinoprostone **(B)** with the STAT1.

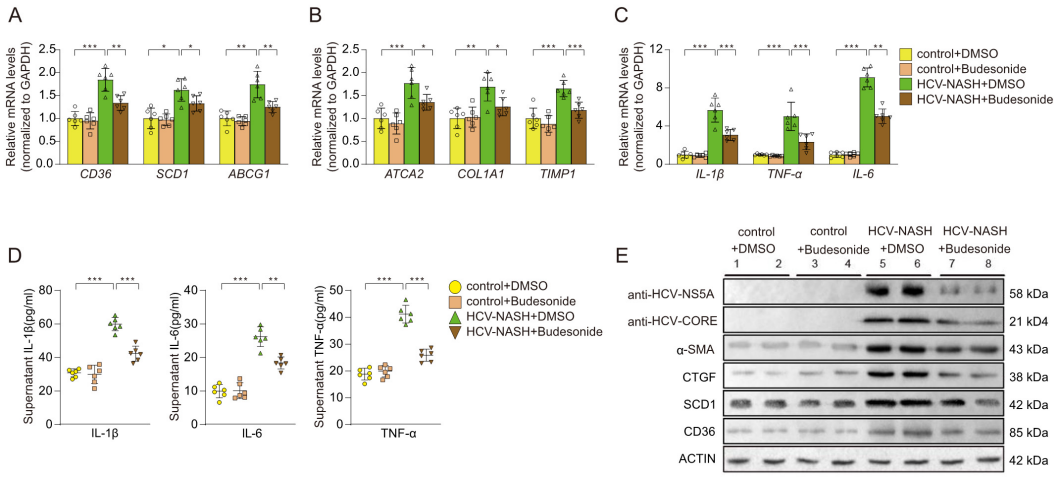

FIGURE 7
Effects on cellular models after Budesonide treatment. The expression of genes related to glycolipid metabolism **(A)**, fibrosis **(B)**, and inflammatory factors **(C)** were performance by qRT-PCR. The proteins of inflammatory were detected by ELISA **(D)**. **(E)** Immunoblot analysis of the proteins expression, which were HCV and MASH after Budesonide treatment. Data are shown as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (n=6 per group).

STAT1, belonging to signal transducer and activator of transcription (STAT) family, can be activated by IFN and thereby regulates the gene expressions involved in cell growth, differentiation, apoptosis, and immune response. Cytokine-induced tyrosine and serine phosphorylation facilitate STAT1 homo/hetero- dimerization, nuclear translocation, and transactivation of downstream gene expression. Obesity is one of the triggers of MASH. A recent study reported that the inactivation of negative regulators of the STAT1 signaling in obesity can contribute to the development of MASH and HCC. The oxidation and inactivation of the STAT1 phosphatase TCPTP and heightened STAT1 signaling were evident in MASH in both obese mice and humans. Heightened STAT1 signaling was responsible for the recruitment of activated cytotoxic T cells and the ensuing MASH and fibrosis (12). Meanwhile, another research showed that the expression levels of STAT1 were increased in hepatitis C patients, even higher than that of MASH patients (28). Combined with our analysis, STAT1 could be an important cross-talk gene between MASH and hepatitis C.

CCL2, an important proinflammatory cytokine involved in various inflammatory responses, is also known as monocyte chemoattractant protein 1 (MCP-1). Under the inflammation in the body, monocytes, macrophages, B cells, endothelial cells, and many other cells can secrete CCL2, which plays important roles in rheumatoid arthritis and glomerulonephritis. CCL2 was the first discovered and well investigated C-C chemokine, preferentially binding to its receptor CCR2. Previous studies indicated that the CCL2-CCR2 signaling axis played a role in the promotion of pathological angiogenesis, the survival and invasion of tumor cells, and the recruitment of immune inhibitory cells (29). In a recent study, compared with normal or MASLD patients, plasma CCL2 concentrations were significantly increased in MASH patients. This study showed that a secreted glycoprotein Sparcl1 can promoted the expression of CCL2 in hepatocytes through binding to Toll-like receptor 4 (TLR4) and activation of the nuclear factor kappa B (NF-κB)/p65 signaling pathway (30). It happens that there is a study showed that interaction of HCV core protein with gC1qR could induce CCL2 and CXCL10 secretion in macrophages via NF-κB signaling pathway (31). CCL2 may be a common regulator and novel therapeutic targets in hepatitis C and MASH.

CXCL9, IL15 and CCL5 are also belong to cytokines. A series of studies have shown that CXCL9, IL15 and CCL5 up-regulated significantly in hepatocytes of HCV-infected patients and MASH patients, and existed a correlation between CXCL9 levels and liver fibrosis (32–35). Furthermore, CCL5 could activate hepatic JAK-STAT1/3 and NF-κB signaling and induce hepatocyte damage evidenced (35). Therefore, combined with the results of our GO analysis, it indicated that these cytokines are important factors in chronic liver inflammation, which could be potential therapeutic targets and biomarkers of MASH in the future.

TLR4 is a member of Toll-like receptors family that are of central importance during the host defense against invading pathogens. A growing body of evidence suggests that TLRs, especially TLR4, have a key role in the pathogenesis of chronic inflammatory liver diseases. In Kupffer cells, TLR4 played a critical role in mediating the progression of simple steatosis to MASH by inducing ROS-dependent activation of XBP1 (36). Promoting the degradation of TLR4 can effectively inhibited MASH progression in monkeys (37). Meanwhile, the HCV protein NS5A has the ability to activate the TLR4 gene promoter, thus increasing TLR4 expression. So that the TLR4 expression was higher in the hepatitis C patients group than in the control group (38).

VCAM1 is a member of the immunoglobulin superfamily of cell adhesion molecules and is predominantly expressed on the surface of endothelial cells. It plays a role in firm adhesion of leukocytes to the endothelium. The expression of VCAM1 can be triggered by inflammatory signals. A series of studies reported that VCAM1 is

upregulated in murine and human MASH (14, 39). VCAM1 inhibition attenuated proinflammatory monocyte hepatic infiltration, and thereby alleviated liver fibrosis in diet-induced murine MASH models (40). HCV infection also results in upregulation of VCAM1, which are associated with advanced liver fibrosis. Therefore, VCAM1 could be a common liver-fibrosis-enhancing factor in MASH and hepatitis C.

ITGAM, PTPRC and SELL, all belong to cluster of differentiation (CD) antigens, are cell surface molecules expressed on leukocytes and other cells associated with the immune system. ITGAM (antigen-like family member B, CD11b) is an integrin that mediate macrophage adhesion, migration, chemotaxis, and accumulation during inflammation (41–43). PTPRC (CD45) is a leucocyte common antigen and a transmembrane glycoprotein expressed on almost all hematopoietic cells except for mature erythrocytes. SELL (CD62L) is a type-I transmembrane glycoprotein and cell adhesion molecule expressed on most circulating leukocytes. Further studies are urgently needed to explore the roles and detailed anti-inflammatory mechanisms.

We also anticipate target TFs and miRNAs of hub genes so as to find out important regulatory factors at transcriptional level in MASH and hepatitis C. IRF1 is the most prominent TFs. The study found that it interacted with three hub genes, including PTPRC, ITGAM and IL15. And IRF1 is a master transcription factor in the Interferon-γ pathway, playing important roles in apoptosis, inflammation, cell growth and polarization, oncogenesis, and cancers are well documented (44). The most prominent miRNAs, hsa-mir-26b-5p, hsa-mir-146a-5p and hsa-mir-155-5p were found in our study. hsa-miR-155-5p is a crucial regulator that controls cellular pro-inflammatory activities and has been implicated in both HCC and hepatitis C (45). hsa-mir-146a-5p was significantly upregulated in tissues with chronic nasopharyngitis and can target various molecules involved in the NF-κB/NLRP3 pathways (46–48). Thus, targeting these TFs and miRNAs may shed light on the treatment of MASH and hepatitis C.

Besides, we anticipate the connection of common DEGs with different disorder by constructing a hub genes-disease network. As we know that both hepatitis C and MASH cause persistent liver cell damage, which leads to liver fibrosis (2). Consistent with previous research, our results showed that liver cirrhosis was the most prominent in the visible disease network.

We further predicted potential drugs related to the hub genes in patients with MASH and hepatitis C. The results of molecular docking showed a good binding activity between the two most important components (Budesonide and Dinoprostone) and the two important target proteins (CCL2 and STAT1), and the main forms of interaction between components and targets are electrostatic and van der Waals force.

Budesonide is an orally active, second-generation corticosteroid with high anti-inflammatory effect. It has been widely used in the treatment of asthma, pneumonia, ulcerative colitis and other inflammatory diseases (49). Although Budesonide has not been used for the treatment of hepatitis C or MASH, glucocorticoids has strong anti-inflammatory properties through both genomic and non-genomic effects, are widely used in a variety of liver diseases with overactive immune and inflammatory responses, such as liver failure, autoimmune hepatitis and alcoholic liver disease (50). Therefore, we chose budesonide for further experimental verification. The results showed that budesonide significantly suppressed the expression levels of genes related to glycolipid metabolism, fibrosis, and inflammatory factors in cellular model of MASH and hepatitis C. Recent studies found that glucocorticoids could prevent immunologic injury in hepatocytes infected with HBV and might be effective in the treatment of HBV-ACLF (51). A clinical study showed that glucocorticoid can promote the expression of suppressor of cytokine signaling (SOCS) 1 and inhibit the expression level of TNFα and IL-6 in the serum of Acute-on-chronic hepatitis B liver failure (ACHBLF) patients (52). The results were similar to those of our cell experiments. Therefore, Budesonide may be a potential drug for the treatment of MASH and hepatitis C, which also confirms the reliability of the results of our analysis.

Our study has some limitations that need further in-depth work. The bioinformatics analyses were conducted based on one HCV cohort and two MASH cohorts. However, it should be noted that using different datasets would lead to different conclusions, and increasing the number of cohorts or using larger datasets will yield more robust results. Besides, all our findings are validated in cell experiments, while complementary validation using animal experiments is still needed in further studies. Importantly, to enhance the clinical relevance of our study, future efforts should focus on collecting and testing high-quality cohorts of patients with co-existing MASH and hepatitis C. Furthermore, the effects of the identified drugs should be evaluated to confirm the feasibility of our research method between different diseases, such as MASH and hepatitis C.

In conclusion, this study is the first time to use bioinformatics tools to explore the close genetic relationship between MASH and hepatitis C. We identified common DEGs and elucidated the common molecular basis, which predicts multiple common pathways closely related to the two diseases. Our study identified 10 hub genes and their common TFs and miRNAs, which may have a critical influence on the pathophysiological mechanism of hepatitis C and MASH. Next, further experiments will be needed to validate these findings.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Key Laboratory for Precision Diagnosis and Treatment of Pediatric Digestive System Diseases, Endoscopy Center and Gastroenterology Department,

Shenzhen Children's Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YC: Methodology, Software, Writing – original draft, Writing – review & editing. ZS: Supervision, Writing – review & editing. YL: Funding acquisition, Writing – original draft. XX: Investigation, Writing – original draft. DZ: Resources, Writing – original draft. YGZ: Methodology, Writing – original draft. LL: Software, Writing – original draft. YZZ: Supervision, Writing – original draft. WL: Investigation, Writing – original draft. DB: Formal analysis, Writing – original draft. DD: Formal analysis, Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1442221/full#supplementary-material

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Regulation of ubiquitination in sepsis: from PAMP versus DAMP to peripheral inflammation and cell death

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Sepsis (sepsis) is a systemic inflammatory response triggered by infection, and its pathologic features include overproduction of peripheral inflammatory factors (e.g., IL-1β, IL-6, TNF-α), which ultimately leads to cytokine storm and multiple organ dysfunction syndrome (MODS). Pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) induce strong immune responses and exacerbate inflammation by activating pattern recognition receptors (PRRs) in the host. Ubiquitination, as a key protein post-translational modification, dynamically regulates the activity of several inflammation-associated proteins (e.g., RIPK1, NLRP3) through the coordinated action of the E1, E2, and E3 enzymes, affects cell death pathways such as necroptosis and pyroptosis, and ultimately regulates the release of peripheral inflammatory factors. Deubiquitinating enzymes (DUBs), on the other hand, influence the intensity of the inflammatory response in sepsis by counter-regulating the ubiquitination process and balancing pro- and anti-inflammatory signals. This review focuses on how PAMP and DAMP activate inflammatory pathways via PRRs, and the central role of ubiquitination and deubiquitination in the development of sepsis, especially the mechanisms in regulating the secretion of peripheral inflammatory factors and cell death. By deeply dissecting the impact of the balance of ubiquitination and deubiquitination on inflammatory regulation, we further envision its potential as a therapeutic target in sepsis.

KEYWORDS

sepsis, peripheral inflammatory factors, ubiquitination, PAMP, DAMP, necrotic apoptosis

1 Introduction

Sepsis (sepsis) is a dysfunctional host response to infectiont, which is characterized by the abnormal secretion of peripheral inflammatory factors such as IL-1β, IL-6, and TNF-α (1–3). This condition is increasingly relevant in critically ill patients due to a rising incidence, which may reflect factors like an aging population and the prevalence of

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comorbidities (2, 4). These peripheral inflammatory factors act as powerful pro-inflammatory mediators, which not only drive local inflammatory responses, but also spread through the whole body, leading to the outbreak of cytokine storms, which ultimately lead to multi-organ dysfunction and failure (5) (Figure 1B). These peripheral inflammatory factors act as powerful pro-inflammatory mediators that not only drive localized inflammatory responses, but also spread systemically, leading to an outbreak of cytokine storms, which in turn triggers systemic inflammatory response syndrome (SIRS) and ultimately leads to multi-organ dysfunction and failure (5, 6).The persistent elevation of peripheral inflammatory factors is an important pathological feature in the development of sepsis, reflecting the state of immune imbalance (2, 6–8). In sub-Saharan Africa, the incidence of sepsis was reported to be 1,772 cases/100,000 people with a hospital mortality rate of 23.7% during 2013-2016 (9). The pathologic progression of this disease is triggered by pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP), which induce an inflammatory response through the activation of host pattern-recognition receptors (PRRs), forming a pro-inflammatory cascade (10, 11).

Ubiquitination, a key post-translational modification of proteins, regulates the function and localization of target proteins through the synergistic action of E1, E2, and E3 enzymes, while deubiquitinating enzymes (DUBs) reverse this regulatory process by excising the ubiquitin chain (12, 13). Ubiquitination and deubiquitination dynamically modulate necroptosis by regulating inflammation-related proteins involved in apoptosis and pyroptosis. This regulation influences the release of peripheral inflammatory mediators, ultimately shaping the inflammatory response in sepsis patients. Both PAMPs and DAMPs activate pro-inflammatory signaling pathways via PRRs, while

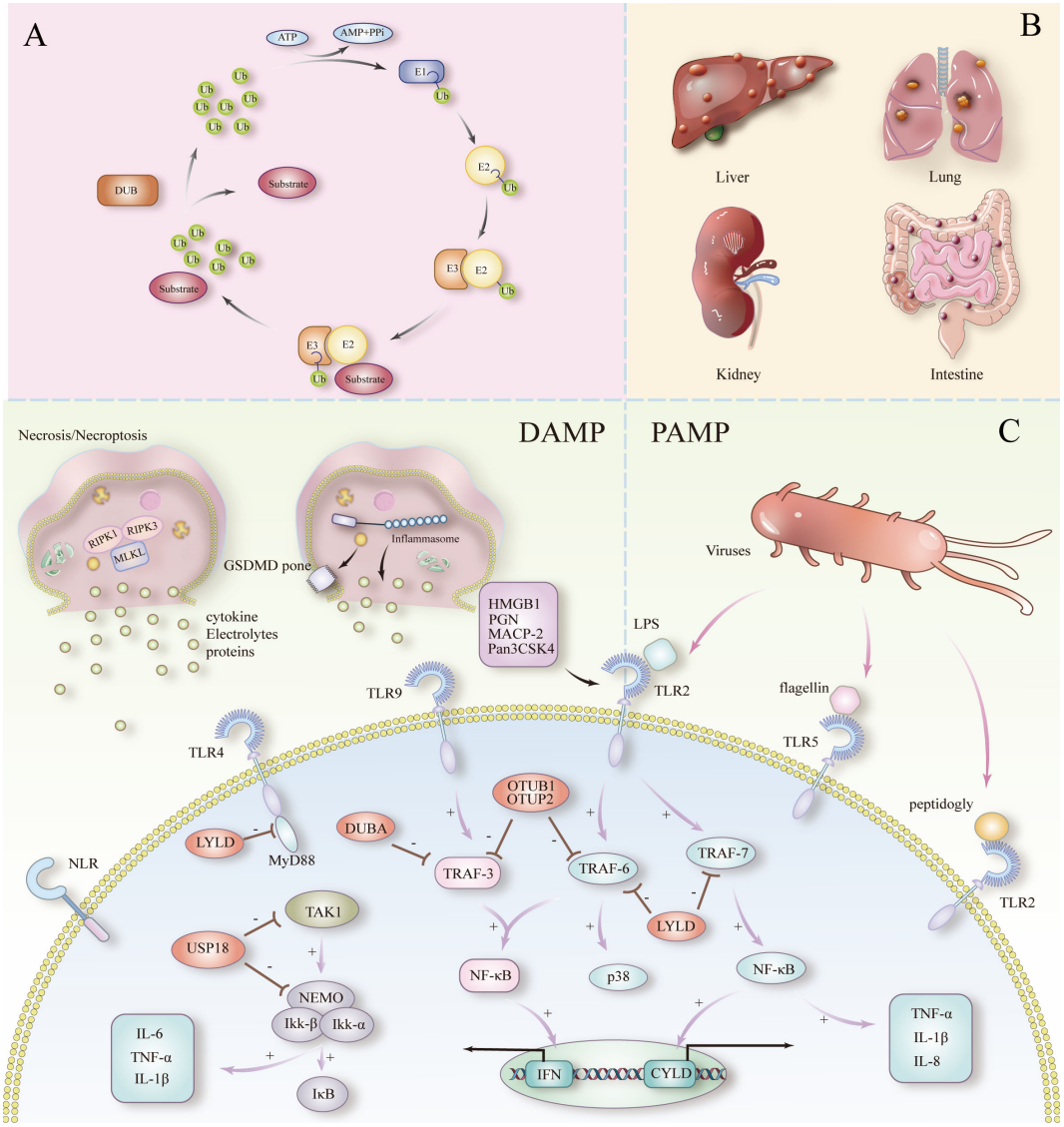

FIGURE 1
(A) Ubiquitination and deubiquitination processes. **(B)** Septicemia triggering multiple organ failure. **(C)** PAMPs and DAMPs to peripheral inflammation and cell death in sepsis.

ubiquitination finely tunes the intensity and duration of this inflammatory response by modulating key proteins (e.g., NF-κB) within these signaling pathways. DUBs such as CYLD and A20 control the inflammatory response by removing the K63 of the RIPK1 chain ubiquitin and inhibit the overactivation of downstream pro-inflammatory signaling to prevent uncontrolled inflammation, revealing that the dynamic balance between ubiquitination and deubiquitination is critical for the regulation of inflammation in sepsis (14). This process holds significant clinical promise as a potential therapeutic target.

The aim of this review is to delve into the multilevel regulatory mechanisms of ubiquitination and deubiquitination in sepsis, focusing on the process by which PAMP and DAMP trigger inflammatory responses through activation of PRRs, and how they regulate the activity and function of key inflammatory proteins (e.g., RIPK1 and NF-κB) via the ubiquitination pathway. We will elaborate how these regulatory mechanisms affect cell death and immune cell activation (15–17).

2 The ubiquitination system and its key role in sepsis

Ubiquitin is a small protein consisting of 76 amino acids and is present in all tissues of eukaryotes (18). With only three amino acid differences between mammals, yeast and plants, it shows significant evolutionary conservation. It acts as a modifier by covalently attaching to cellular proteins via an enzymatic cascade, which involves three classes of enzymes called E1 (activation), E2 (binding), and E3 (ligation) (19). The process of ubiquitin labeling of proteins is called ubiquitination, and the process involves three major steps: first, E1 activating enzymes activate the ubiquitin molecule; then, E2 binding enzymes receive and transfer the ubiquitin; and finally, E3 ligase covalently attaches ubiquitin to the lysine residues of the target protein. Ubiquitination is one of the most versatile cellular regulatory mechanisms known to be used to control cell death and immune-inflammatory responses (Figure 1A). In sepsis, E3 ligases such as TRAF6 amplify the inflammatory response by activating the NF-κB and MAPK signaling pathways through K63 chain ubiquitination. RING-type and HECT-type E3 ligases play different roles in the recognition and modification of different inflammation-associated proteins (20, 21). Dysregulation of ubiquitination often results in a dysfunctional inflammatory response, exacerbating inflammation in sepsis. Out of control, exacerbating the cytokine storm in sepsis.

The process of ubiquitination is reversible. Deubiquitination is the opposite process of ubiquitination, where mono- or polyubiquitin chains are removed from a modified protein to terminate the ubiquitination function of the protein. In simple terms, the degradation of ubiquitinated proteins is prevented and their stability is maintained (14). Currently DUBs number about 100 in humans and are classified into six structurally distinct DUB families including UCHs (ubiquitin carboxyl-terminal hydrolases) of human USPs (ubiquitin-specific proteases), OTUs (ovarian tumor proteases), MJDs (Machado- Josephin's disease protein structural domain proteases), MINDYs (motifs with containing ubiquitin (MIU) and JAMMs (Zn-JAB1/MPN/MOV34 structural domain proteases) (22–25). The first five belong to the cysteine proteases (26). Among them, the USP sub family is the most diverse member of the DUB family (27, 28). For example, DUBs in the nucleus (e.g., USP10) regulate gene transcription and DNA repair, whereas DUBs located in the cytoplasm (e.g., CYLD and A20) regulate inflammation-associated signaling pathways, such as NF-κB and MAPK. In sepsis, cytoplasmic DUBs (CYLD and A20) ubiquitylize the K63 chain of key proteins, such as RIPK1 and NEMO, by removing the inhibit overactivation of NF-κB and MAPK signaling, thereby reducing cytokine release and inflammatory spread. This distribution and functional specificity suggests that DUBs finely regulate immune responses in different cellular regions, with potential clinical implications for the maintenance of immune homeostasis in sepsis patients.

3 DAMP and PAMP production and their role in death: mechanisms of ubiquitination regulation

DAMP (damage-associated molecular pattern) and PAMP (pathogen-associated molecular pattern) are key factors in triggering inflammatory responses and cell death. DAMP is released by damaged or dead cells, whereas PAMP originates from exogenous pathogens. Both initiate a strong innate immune response through activation of host pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerized structural domain-like receptors (NLRs), initiating a strong innate immune response. This activation not only leads to localized inflammation, but also induces SIRS, exacerbating the cytokine storm and ultimately leading to multi-organ damage. In sepsis, the persistence of DAMP and PAMP contributes to an uncontrolled inflammatory response, in which ubiquitination and deubiquitination mechanisms finely regulate inflammatory intensity and cell survival by modulating key signaling molecules (e.g., RIPK1, NF-κB).

3.1 DAMP production and its role in inflammation

DAMP are endogenous molecules released by damaged or dead cells during the process of death. These molecules include HMGB1 (high mobility group protein B1), ATP, uric acid crystals, heat shock proteins, etc. (10, 29). DAMP plays a key inflammatory-activating role in sepsis, by binding to specific PRRs (e.g., TLR and NLRs), triggering massive activation of immune cells (e.g., macrophages, neutrophils) and secretion of peripheral inflammatory factors (10, 11, 30) (Figure 1C). This process allows the inflammatory reaction to spread rapidly, forming a vicious cycle. Particularly in sepsis, the sustained release of DAMP is closely associated with extensive tissue damage, and this vicious cycle ultimately triggers a cytokine storm that results in multi-organ failure.

3.2 PAMP production and its role in inflammation

PAMP are carried by exogenous pathogens, including lipopolysaccharide (LPS) and peptidoglycan from bacteria, double-stranded RNA from viruses, and β-glucan from fungi (31). These molecules activate downstream signaling pathways such as NF-κB, MAPK, etc., by binding to receptors such as TLR, NLR, and other receptors etc., triggering a strong innate immune response. In sepsis, pathogen mass multiplication and sustained PAMP stimulation keep the host's immune system in a highly activated state, leading to the overproduction of peripheral inflammatory factors and their subsequent systemic inflammatory response.

3.3 Regulatory effects of ubiquitination on key proteins and cell death

In sepsis, both DAMP and PAMP are recognized by PRRs (e.g., TLR and NLR), leading to activation of inflammatory pathways and release of mediators. These mediators amplify the inflammatory response through feedback mechanisms, often leading to SIRS (32). However, the regulation of the inflammatory response does not only depend on these exogenous stimuli, but is also finely controlled by intracellular protein regulatory mechanisms, especially the process of ubiquitination. Ubiquitination, as a post-translational modification of proteins, dynamically regulates the function of several key proteins, which in turn affects the intensity and duration of the inflammatory response. By regulating the activity of inflammation-associated proteins such as RIPK1 (receptor-interacting protein kinase 1) and NLRP3, ubiquitination plays a key role in cell death and the release of peripheral inflammatory factors (33, 34).

3.3.1 Effect of ubiquitination on necroptosis

Necroptosis, also known as programmed cell death, is a regulated, cell death mechanism dependent on the activation of RIPK1 (receptor-interacting protein kinase 1), RIPK3 and MLKL (mixed-spectrum kinase structural domain-like proteins) mediating cell death, which is characterized by a loss of cellular membrane integrity leading to the release of cellular contents into the extracellular matrix, which triggers an intense inflammatory response (35, 36). Ubiquitination controls the onset of necrotic apoptosis by regulating the activity of a series of key proteins, including RIPK1, RIPK3 and MLKL.

Ubiquitination of the K63 chain of RIPK1 by the E3 ubiquitin ligases IAPs activates pro-inflammatory downstream activity via NFKB (37). However, when RIPK1 is dysfunctional in its ubiquitination or de-ubiquitinated by CYLD, RIPK1 forms a complex with RIPK3 and further activates downstream MLKL, which induces necrotic apoptosis, leading to DAMP release, exacerbating the inflammatory response (38). In addition, deubiquitination of RIPK3 by CYLD increases its stability, making it more susceptible to forming a complex with RIPK1 and activating MLKL.

Ubiquitination of MLKL regulates its activity and membrane localization, and unubiquitinated MLKL binds more readily to RIPK3 and receives phosphorylation, resulting in oligomerization and translocation to the plasma membrane (39). Oligomerization of phosphorylated MLKL has been reported in the presence of highly phosphorylated inositol phosphates (IP6), resulting in the formation of necrosomes. translocation of MLKL oligomers in plasma membranes to phosphatidylinositol phosphate (PIP)-rich plaques and form macropores. Eventually, MLKL pores lead to necroptosis by allowing ion influx, cell swelling and membrane cleavage leading to uncontrollable release of intracellular substances (e.g. HMGB1, ATP, etc.) (40).

3.3.2 Effect of ubiquitination on pyroptosis

Pyroptosis is usually achieved in sepsis through both classical and nonclassical pathways. Both pathways involve activation of inflammatory vesicles and the action of Gasdermin family proteins. The classical pathway involves activation of caspase-1 by inflammatory vesicles (e.g., NLRP3), which triggers cleavage of GSDMD; the nonclassical pathway involves activation of caspase-4, caspase-5, or caspase-11 by intracellular LPS, which directly cleaves GSDMD (41–43).

In the classical pyroptosis pathway, the K63 chain of NLRP3 is ubiquitinated by the E3 ubiquitin ligase TRIM31, which in turn inhibits the over-assembly of inflammatory vesicles, thus limiting the excessive occurrence of pyroptosis (44). However, in sepsis, dysregulation of ubiquitination (e.g., enhancement of the deubiquitinating enzyme BRCC3) increases the activity of NLRP3, which leads to the excessive activation of inflammatory vesicles, thereby exacerbating cellular pyroptosis and further releasing large amounts of pro-inflammatory cytokines (e.g., IL-1β and IL-18) (45).

In non-classical cleavage pathways, ubiquitination affects cellular responses to endogenous and exogenous stimuli by modulating the activity of these caspases. The current study found that the E3 ligase NEDD4 ubiquitinates modified caspase-11 (46, 47).

The role of pyroptosis in sepsis is twofold. In the early stages of infection, moderate pyroptosis helps the immune system to recognize and clear pathogens. However, in the advanced stages of sepsis, hyperactivation of pyroptosis and sustained release of peripheral inflammatory factors leads to a cytokine storm that further exacerbates systemic inflammatory responses and organ failure.

4 Inflammatory signaling activated by DAMP and PAMP via PRRs

Pattern recognition receptors (PRRs) are a central part of the innate immune system. PRRs, TLRs and NLRs, are the main sensing receptors in the initiation phase of sepsis (Figure 1C). Among them, TLRs are the most studied and relevant PRRs associated with cytokine storm and sepsis.

Toll-like receptor 4 (TLR4) is triggered by LPSand is the first mammalian paradigm for innate immune signaling (48). TLRs expressed at the plasma membrane (TLR1, 2, 4, 5, and 6) recognize a wide range of lipid- and protein-like ligands in the extracellular environment (49, 50). In particular, after recognizing LPS, TLR4 activates the NF-κB and MAPK signaling pathways, initiating a pro-inflammatory response that further triggers the sepsis-associated inflammatory cascade. It has been shown that TLR4 is particularly associated with sepsis, and TLR4-deficient mice exhibit reduced responsiveness in the face of LPS from Gram-negative bacteria. Further evidence for a central role of TLR4 in the development of sepsis (51).

In patients with sepsis, changes in the expression of TLR2 and TLR4 are more active on neutrophils than on monocytes. The expression of Toll-like receptor signaling genes in monocytes is reduced in more severe disease. In contrast, in the clinical phase of sepsis, neutrophil expression of these genes is upregulated (52). Like TLR4, TLR2 responds to the internalization of bacterial IL-12 (53).

The NLR is present in the cell and recognizes several types of ligands, including bacterial cell wall components, toxins, and host-derived molecules (e.g., ATP, uric acid, and damaged cell membranes), which in turn trigger the activation of different biological pathways. This ligand specificity stems from its NH2-terminal effector structural domain (54).

5 Deubiquitination and peripheral inflammatory factor regulation in sepsis

In the initial stage of sepsis, an intense inflammatory response is ignited, leading to a surge of large amounts of inflammatory factors (e.g., IL-1β, TNF-α), which in turn creates a cytokine storm. When local inflammation cannot be controlled, inflammation can spread throughout the body, leading to SIRS. As inflammation persists, immune function becomes progressively impaired and immunosuppression occurs, leading to viral reactivation, secondary infections, and ultimately increasing the risk of death in patients.

5.1 Type I IFN

Type I interferons are produced by a variety of cells (macrophages, conventional dendritic cells (cDCs), and inflammatory monocytes in response to activation of cell-surface and intracellular pattern-recognition receptors, in particular the TLR family (55). The cytoplasmic structural domains of all TLRs share a high degree of similarity with those of the IL-1 receptor type I, and together they assist in host defense against infection. Closely associated with innate immune response (56–58). In the absence of IL-1R1 signaling, in contrast to the level of ubiquitination of TRAF3, its degradative ubiquitination was unaffected because of the upregulation of DUBA (deubiquitinating enzyme A) at this time (59).

DUBA (deubiquitinating enzyme A) selectively cleaves the K63-linked ubiquitin chain from TRAF3 (60). DUBA short interfering RNA enhances TLR9-dependent type I IFN responses (59). Mice lacking IL-1RI signaling fail to produce protective type I IFN responses after administration of TLR9 ligand (CpG), suggesting that IL-1 signaling modulation of DUBA expression attenuates TLR9-mediated pro-inflammatory responses (59). Mice lacking IL-1RI signaling fail to produce a protective type I IFN response after administration of TLR9 ligand (CpG), suggesting that regulation of DUBA expression by IL-1 signaling attenuates TLR9-mediated proinflammatory responses.

In virus-induced sepsis, the virus triggers a strong inflammatory response by activating the host's immune system, where overactivation of the type I interferon pathway and the NF-κB signaling pathway often leads to SIRS and cytokine storm (61, 62). Sustained high levels of pro-inflammatory signaling can contribute to the immune system imbalance, which in turn develops into sepsis (62, 63).

OTUB1 and OTUB2 of the OTU family specifically remove K63 chain ubiquitin on TRAF3 and TRAF6 to negatively regulate the virus-triggered type I interferon pathway. Overexpression of OTUB1 and OTUB2 inhibits the activation of IRF3 and NF-κB, which in turn attenuates the transcription of the IFNB1 gene and the cellular antiviral response (64). This deubiquitination suggests that OTUB1 and OTUB2 play a negative regulatory role in response to viral infections to prevent damage to the host from an over-activated inflammatory response.

5.2 IL family and TNF-α

The pro-inflammatory cytokines of the IL-1 family include IL-1α, IL-1β, IL-18, and IL-36, and the structure of the corresponding bound receptor consists of an extracellular Ig structural domain and an intracellular TIR structural domain (65).This process involves myeloid differentiation major response protein 88 (MyD88) and IL-1 receptor associated kinase 4 (IRAK4) recruitment, and activation of NF-κB and MAPK (66, 67). IL-8 (CXCL8) belongs to the chemokine family and is mainly involved in neutrophil recruitment (68, 69). TNF-α (tumor necrosis factor α or cachectin) belongs to the pro-inflammatory cytokine family and is secreted by a variety of immune cells (macrophages, monocytes, neutrophils) (70, 71).

Multiple deubiquitinating enzymes have been shown to be involved in TIR-mediated downstream signaling activation, thereby indirectly affecting the release of peripheral inflammatory factors. As the most studied DUB family member among USPs, CYLD (oncogene cylindromatosis) is induced by Gram-negative and Gram-positive bacterial pathogens or their products (72–74). Meanwhile, CYLD acts as a negative regulator in TLR2 signaling. When TLR2 ligands (e.g., peptidoglycan PGN, MALP-2, and Pam3CSK4) bind to TLR2, TRAF6 and TRAF7 are activated, initiating the downstream NF-κB and p38 pathways, which in turn promotes the release of TNF-α, IL-1β, and IL-8 (75). In the meantime, virtually all microbial products are also activated via the TLR ligands induced IL-1β production (76, 77). During this process, the transcriptional level of CYLD gradually increased

(75).There was a negative regulatory effect of CYLD on TRAF6 and TRAF7, which inhibited the over-activation of these pro-inflammatory pathways by removing the ubiquitylation of K63 chain on TRAF6 and TRAF7. Overactivation of these pro-inflammatory pathways, creating a negative feedback mechanism to prevent deleterious inflammatory responses. Absence or deficiency of CYLD results in uncontrolled inflammatory responses that significantly exacerbate the severity of sepsis (75).

Recent studies have further revealed the negative regulatory role of CYLD in the MyD88-mediated TLR signaling pathway. It is well known that key receptors involved in sepsis initiation include toll-like receptors (TLRs) (78). The gram-negative bacterium NTHi (non-typeable Haemophilus influenzae), an infectious trigger of sepsis, generally initiates the host immune response through the TLRs signaling pathway (79–81). MyD88 is an adaptor molecule for the majority of TLRs (TLR2, TLR4) and IL-1R signaling (82, 83). It was demonstrated that CYLD removes the K63-connected ubiquitin chain by directly interacting with MyD88, specifically the ubiquitin modification located at lysine 231, thereby negatively regulating NTHi-induced MyD88-mediated signaling. This deubiquitination effectively inhibited NTHi-triggered pro-inflammatory cytokine production and prevented excessive inflammatory responses (84). In addition, mice were able to generate strong antibody responses to T-cell-dependent antigens, despite the lack of MyD88 and TRIF, two key components of TLR signaling, and the lack of a TLR ligand response (85).

In addition, a deubiquitinating enzyme, USP18, affects TNF-α and IL-1β levels. USP18, an interferon-inducible gene, is significantly up-regulated in TLR ligand-stimulated human monocytes and macrophages. LPS combined with TLR revealed increased phosphorylation of IKK, accelerated degradation of IκB, and higher levels of TNF-α, IL-6, and IL-1β mRNA expression in THP-1-derived macrophage species transfected with USP18-specific siRNAs (86).

6 Conclusions and perspectives

DAMPs released by necrotic apoptosis amplify the immune cell response to pathogens, while focal death exacerbates the cytokine storm in sepsis by releasing proinflammatory factors (e.g., IL-1β, IL-18). Consider the role of ubiquitination and deubiquitination at various stages of sepsis and control this process to prevent necrotic apoptosis and pyroptosis. Since ubiquitination and deubiquitination are independent yet interrelated processes, identification of specific deubiquitinating enzymes (DUB) corresponding to specific ubiquitinases may provide another effective strategy for regulating ubiquitination.

Multiple deubiquitinating enzymes are negative regulators of TLR signaling, but how they accomplish their job of inhibiting TLR overactivation and the associated cytokine storm has yet to be further explored. Sustained exposure to PAMPs (e.g., LPS released by bacteria) and DAMPs (e.g., molecules released by dying cells and damaged tissues of the host) leads to excessive and sustained activation of the TLR signaling pathway. With excessive activation of the TLR signaling pathway, cytokines such as TNF-α, IL-1β, and IL-6 are released in large quantities, ultimately triggering a cytokine storm. This cytokine storm drives the development of MODS by triggering a systemic inflammatory response, which in turn leads to organ damage and high mortality in sepsis patients. Therefore, in-depth studies on the regulatory mechanisms of TLR signaling are needed, especially on how to inhibit excessive cytokine storms by modulating the negative feedback or deubiquitination pathways of TLR signaling.

Author contributions

YL: Visualization, Writing – original draft, Writing – review & editing. JY: Formal analysis, Writing – original draft. ZZ: Formal analysis, Writing – original draft. WL: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Exploring the role of ubiquitination modifications in migraine headaches

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Migraine is a complex neurovascular disorder whose pathogenesis involves activation of the trigeminal vascular system, central and peripheral sensitization, and neuroinflammation. Calcitonin gene-related peptide (CGRP) plays a dominant role and activation of MAPK and NF-κB signaling pathways regulates neuropeptide release, glial cell activation, and amplification of nociceptive signals. Aberrant activation of these pathways drives migraine onset and chronicity. The ubiquitin-proteasome system (UPS) is involved in neurological and inflammatory disorders. ubiquitination in the UPS is achieved through a cascade of enzymes, including Ub-activating enzyme (E1), Ub-coupling enzyme (E2), and Ub-ligase (E3). The aim of this review is to systematically explore the role of ubiquitination in the regulation of MAPK and NF-κB signaling pathways, with a focus on the mechanisms of ubiquitinating enzymes in neuroinflammation and pain signal amplification, and to explore their potential as diagnostics, biomarkers, predictors of response to therapy, and monitoring of chronicity in migraine disease.

KEYWORDS

ubiquitination, migraine, inflammation, biomarker, central sensitization, peripheral sensitization

1 Introduction

Migraine is now the sixth most prevalent disease worldwide and a leading cause of disability (1). According to the Global Burden of Disease Study (GBD) 2019, approximately 8-15% of migraine sufferers have at least one attack per year. The pathophysiology of migraine is thought to involve abnormal activation of the trigeminal vascular system, peripheral and central sensitization, and neuroinflammation (2). Among them, calcitonin gene-related peptide (CGRP) plays a dominant role. Related regulatory pathways, such as the mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) pathways, regulate neuropeptide release, glial cell activation, and pain signaling,

mediating inflammatory and sensitizing responses. These may drive is the development of migraine pathogenesis and chronicity.

As a type of protein post-translational modification (PTM), the ubiquitin-proteasome system (UPS) is a pathway for protein ubiquitination and degradation. Ubiquitination involves a cascade reaction of three enzymes: Ub-activating enzymes (E1s), Ub-coupling enzymes (E2s) and Ub-ligases (E3s); deubiquitination is mediated by deubiquitinating enzymes (DUBs) (3, 4). Disturbances in the UPS have been shown to be associated with the induction and severity of a variety of neurologic and inflammatory disorders, suggesting its possible involvement in the pathogenesis of migraine headaches.

This paper systematically explores the role of ubiquitination in the regulation of MAPK and NF-κB signaling pathways, focusing on the regulatory mechanisms of ubiquitinating enzymes in neuroinflammation and nociceptive signal amplification, as well as exploring its feasibility as a biomarker for disease diagnosis, prediction of therapeutic response, and chronicity monitoring.

2 Pathogenesis of migraine and association of MAPK and NF-κB signaling pathways

Migraine is a complex neurovascular disorder whose pathogenesis involves multilevel interactions between the nervous and vascular systems. Recent studies have shown that the core mechanisms of migraine include activation of the trigeminal vascular system, central sensitization, peripheral sensitization and neuroinflammation. Together, these mechanisms form the pathophysiologic basis of migraine (Figure 1A).

2.1 Trigeminal vascular system activation

The trigeminal vascular system is the anatomical and physiological basis of migraine attacks, and a key mechanism of migraine nociceptive perception (5–7) (Figure 1B). Stimulation of the trigeminal ganglion (TG) leads to vascular release of neurotransmitters and inflammatory mediators, such as CGRP, PACAP, and substance P upon activation (8). These molecules bind to receptors and induce intracranial vasodilation, neuroinflammatory activation and enhanced pain signaling. Then, it leads to sensitization of secondary neurons in the trigeminal cervical complex (TCC) of the brainstem, and ultimately activate tertiary neurons in the thalamus (9).

CGRP plays a key role in neural sensitization and amplification of pain signals in migraine by binding to its receptor, which consists of CLR and RAMP1. After binding, CGRP activates Gs proteins, initiating the production of cAMP by adenylyl cyclase (AC). It further activates protein kinase A (PKA), which regulates sodium and potassium ion channel (10–12). Currently, CGRP receptor antagonists (gepants) have become effective acute treatments (13, 14).

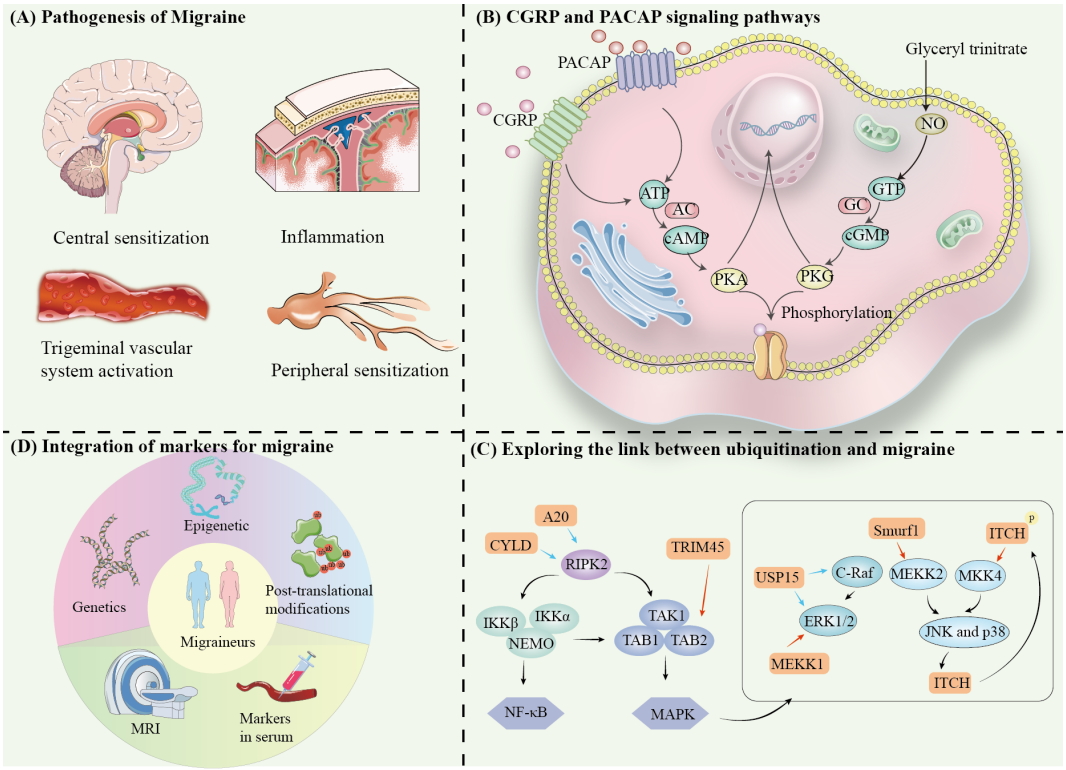

FIGURE 1
(A) Pathogenesis of Migraine. **(B)** CGRP and PACAP signaling pathways. **(C)** Exploring the link between ubiquitination and migraine. **(D)** Integration of markers for migraine.

PACAP acts mainly by binding to PAC1, VPAC1 and VPAC2 receptors (15–18). Similar to CGRP, PACAP also activates the cAMP-PKA pathway leading to migraine headaches (19). In addition, PACAP appears to have unique downstream effects, such as acting through direct activation of the EPAC pathway (20, 21). Furthermore, through the Ras-Raf-MEK-ERK signaling pathway, PKA contributes to extracellular signal-regulated kinase (ERK) phosphorylation and activation, which in turn combine peripheral sensitization and central sensitization mechanisms (15, 22).

2.2 Peripheral sensitization

Peripheral sensitization refers to a decreased threshold of damage receptors and an increased sensitivity of injury receptors. In response to repetitive stimuli or inflammatory states, excitability of sensory neurons in the periphery increases, which enhances the transmission of pain signals (23–25).

Transient receptor potential (TRP) channels, such as TRPV1, TRPA1 and TRPM8, have been shown to be closely associated with neuropathic pain. Studies have shown that blocking TRPV1 and TRPA1 significantly attenuates neurogenic hypersensitivity reaction (26–31).

In the inflammatory state, TRPV1 receptors are activated, triggering a massive inward flow of sodium and calcium ions, which triggers depolarization of injury receptors, thereby amplifying pain signals (32). Activation of TRPV1 also stimulates the release of CGRP and substance P from the nerve endings, leading to vasodilation (33).Vasodilation leads to an increase in the mechanical pressure on the local tissues, thereby further stimulating the sensory neurons of the trigeminal nerve fiber system, causing a decrease in the nociceptive threshold of injury receptors.

Notably, TRPV1 not only causes acute pain, but also persistent pain, especially pain associated with inflammation (34–36). It was found that hormone level changes may indirectly regulate the release and physiological roles of CGRP by affecting the expression and activity of TRPV1 (37). In particular, TRPV1 activation not only promotes CGRP release, but also enhances synaptic transmission efficiency and exacerbates central sensitization of neurons by promoting the release of glutamatergic vesicles (38).

Through the cAMP-PKA pathway, CGRP modulates ion channels on neuronal cell membranes, enhancing peripheral nerve sensitivity. Upon tissue injury, CGRP acts synergistically with substance P, leading to an increase in vascular permeability of local tissues and the release of inflammatory factors (39–42). Inflammatory response further activates primary afferent neurons, forming a positive feedback loop that amplifies pain signals through inflammation.

2.3 Central sensitization

Central sensitization is an important mechanism of chronic pain and is characterized by CNS hyperresponsiveness to injurious stimuli (43–45). It involves increased presynaptic neurotransmitter release and persistent neuroinflammation (45–47). Enhanced synaptic transmission in the caudate of the TNC is the neural basis for central sensitization in a CM rat model (45, 48, 49). The release of glutamate (Glu) is a key step in central sensitization, the regulation of which is dependent on the activation of ERK and p38 signaling pathways (50, 51). At presynaptic sites, the central terminals of injurious primary afferent nerves, activation of cytokine receptors and chemokine receptors leads to phosphorylation of ERK and p38 (P-ERK, P-p38) and glutamate (Glu) release. This synaptic vesicle release is associated with activation of the ion channels TRPV1, Na channel (52, 53).

At postsynaptic sites, phosphorylation of AMPA and NMDA glutamate receptors significantly enhances neuronal responsiveness to excitatory signals. Phosphorylation of AMPA receptors by PKA) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) results in increased insertion into synaptic membranes, a significant elevation in open probability, and enhanced response to glutamate response (54, 55). phosphorylation of NMDA receptors in response to Src kinase enhances calcium ion permeability and amplifies postsynaptic Ca^{2+} signaling (56, 57).

In addition, P-ERK reduces the repolarization capacity of postsynaptic neurons by inhibiting their potassium channel activity, leaving them in a state of hyperexcitability (58, 59). Notably, P-ERK translocates to the nucleus and promotes the phosphorylation of the cAMP response element-binding protein (CREB), which activates proinflammatory factors, such as c-Fos and NK-1, as well as the nociceptive regulation-related gene expression, further consolidating the molecular basis of central sensitization (60, 61).

2.4 Neuroinflammation

By activating the NF-κB signaling pathway, microglia and astrocytes are activated in response to injurious stimuli and release a series of proinflammatory factors and chemokines, including tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and chemokines (62–64). Release of these factors enhances the inflammatory response and further exacerbates neuronal excitability. It was shown that upregulation of miR-155-5p activated the NF-κB signaling pathway by inhibiting SIRT1, which in turn exacerbated the release of microglial pro-inflammatory factors and neuroinflammation by inhibiting miR-155-5p, it was able to activate SIRT1 in the TNC region of CM mice, which effectively reduced neuroinflammation (60). In addition, activation of NLRP3 inflammatory vesicles was also involved in the release of pro-inflammatory factors, which further contributed to the onset of central sensitization and the chronicity of migraine (65, 66).

Further studies revealed that autophagy plays an important role in the regulation of neuroinflammatory and oxidative stress processes in astrocytes. By inhibiting autophagy, the binding of TRAF6 to K63 ubiquitinated proteins could be promoted, which increased the activities of p-MAPK8/JNK and NF-κB, thereby exacerbating the release of pro-inflammatory factors (e.g., TNF-α, IL-1β). Conversely, activation of autophagy can significantly reduce neuroinflammatory levels (67).

3 Ubiquitination and migraine

Multiple family and twin studies have shown that common migraine is heritable, with heritability estimates ranging from 30% to 60%, suggesting the presence of genetic factors that predispose individuals to migraine (68, 69). Migraine has a heritability of 42%, and relatives of people with migraine are 2- to 3-fold more likely to have the disorder (70). Although the risk of migraine is predominantly polygenic, pathogenic variants in a single gene can lead to monogenic migraine disorders (e.g., familial hemiplegic migraine FHM) and the gene is dominant, this suggests that the susceptibility and complexity of migraines may be based on genetics and may be subject to different gene-gene and gene-environment interactions (71).

The susceptibility and pathophysiological aspects of migraine have been explained from a locus perspective, but recently there has been a developing interest in investigating the role of gene regulatory mechanisms in the predisposition and chronicity of migraine, particularly epigenetic regulation. The number of studies on the role of epigenetic mechanisms in migraine is now found to be increasing yearly. Epigenetic mechanisms regulate cell cycle development by controlling the expression of individual genes, including acetylation, phosphorylation, etc (72, 73). TRP channels can convert injurious stimuli into pain signals, and the expression of TRPA1, TRPA1 encoding gene, has been demonstrated to be affected by pain-related syndromes. Acetylation modifications, this process may enhance neural excitability and facilitate pain transmission by altering electrical activity or localization (74, 75).

Similar to acetylation, ubiquitination also acts at the protein level. Ubiquitination occurs by adding single or multiple ubiquitin molecules to a target protein, modulating its stability, activity, or degradation. Studies demonstrating the relevance of the ubiquitination system to migraine are extremely limited. Ubiquitin C terminal hydrolase 1 (UCHL1), an enzyme with both ligase and hydrolase activities, is present in almost all neurons (76, 77). Serum levels of UCHL1 were significantly elevated during acute attacks in migraine patients; also, before treatment, UCHL1 levels were significantly and positively correlated with visual analog scores (VAS) (78). This suggests that UCHL1 can be used to assess seizure severity and response to treatment. Although there is conclusive evidence providing the relevance of ubiquitination system-associated proteins in acute attacks of migraine, there is little direct evidence that the meso-ubiquitination system plays a role in migraine.

Ubiquitinating enzymes (e.g., MEKK1, Smurf1, ITCH, and TRIM45) and deubiquitinating enzymes (e.g., USP15, A20, and CYLD) may be involved in the occurrence and chronicity of migraine by regulating the ubiquitination of MAPK, JNK, and NF-κB signaling pathways.

Migraine mechanisms involve dynamic processes of central sensitization and peripheral sensitization, abnormal activation of the trigeminal-vascular system, and neurogenic inflammation. Each of the four mechanisms is associated with various enzymes of the ubiquitination system, given the important role of ubiquitination in other neuroinflammatory disorders, it is reasonable to hypothesize that ubiquitination is involved in migraine attacks and chronicity (79–81).

Various trigger molecules can induce migraine, including CGRP, PACAP, adenosine triphosphate-sensitive potassium (KATP) channel opener, and large conductance calcium-activated potassium (BKCa) channel opener. The epigenetic link of CGRP and its potential in migraine has been discussed (82). In isolated trigeminal ganglion neurons, CGRP stimulates pain-related intracellular signaling molecules such as cAMP, CREB, MAPK, p38, and ERK (83). In the following, we explore the ubiquitylation of these proteins to play a function in migraine Possibilities (Figure 1C).

3.1 Regulation of ERK1/2 by ubiquitination

The ERK1/2 signaling cascade was first identified in four MAPK signaling pathways (84, 85). ubiquitination of ERK1/2 is regulated by MEKK1, which has a RING finger structure and exhibits an E3 ligase function, and USP15, a deubiquitinating enzyme. ERK2 is deubiquitinated by USP15, but the stability of the protein is not affected. Not only associated with ubiquitination, USP15 also induces ERK1/2 phosphorylation (86). Interestingly, USP15 is also known to regulate C-Raf DUB, binding to C-Raf and protecting the protein from proteolytic degradation by the 26S proteasome 34688658. overexpression of C-Raf and activation of the ERK1/2 signaling pathway cause overexpression of USP15 expression, leading to cell proliferation and migration (87).

3.2 Regulation of p38, JNK by ubiquitination

Although MEKK1 has ubiquitinating enzyme properties, it is still a member of the MAP3K family. jNK1/2/3 and p38 signaling cascades share upstream regulators such as MEKK1-4 and MKK4. In the inflammatory response, the E3 ubiquitin ligase Smad ubiquitination regulatory factor 1 (Smurf1) ubiquitinates the K48-conjugated polyubiquitin chain of MEKK2, the same type of ubiquitinating enzyme, ITCH, participates in a negative feedback loop of JNK (88). ITCH regulates MKK4 (89). ITCH is a downstream substrate of JNK, and activation of JNK promotes ITCH phosphorylation, and phosphorylated ITCH induces ubiquitination of K140 and K143 of MKK4 (90).

3.3 Regulation of NF-κB by ubiquitination

RIPK1 is the first kinase found in the RIPK family (91). RIPK2 does not have any death structural domains and does not trigger cell death signaling, but has a cysteine asparaginase activating and recruiting structural domain (CARD) that contributes to function in the NOD-like receptor (NLR)-associated inflammatory signaling pathway (92, 93). K63 ubiquitination of RIPK2 interacts with LUBAC and the kinase complex TAK1 and promotes linear ubiquitination of RIPK2, initiating the MAPK signaling pathway (94). Interestingly, the kinase complex also triggers a separate pathway for activation of the IKK complex, which consists of NEMO, IKKα, and IKKβ, and activation of the protein complex leads to the activation of NF-kinase. The IKK complex consists of NEMO, IKKα, and IKKβ, and activation of this protein complex leads to NF-κB activation. In addition, deubiquitinating enzymes A20 and CYLD have linear

bonding specificity that can counteract RIPK2 ubiquitination (95, 96). Linear ubiquitination is much more attractive for NEMO binding than normal polyubiquitination, so ubiquitinated RIP1 also attracts NEMO/IKK and TAB/TAKI bindings, and thus activates downstream NF-KB signaling pathways. NF-KB signaling pathway and JNK, P38/MAPK signaling pathway (97).

The E3 ligase tripartite motif-containing 45 (TRIM45) constitutively interacts with TAB2 and promotes polyubiquitination of the TAB2-Lys-63 linkage, leading to the formation of the TAB1-TAK1-TAB2 complex and activation of TAK1, and ultimately the activation of the nuclear factor-carbamyl B (NF-κB) signaling pathway (98).

4 The potential of ubiquitination in migraine treatment

In recent years, the development of drugs targeting ubiquitinating and deubiquitinating enzymes has emerged as a research hotspot in the field of precision therapeutics (99). Although most studies have focused on cancer, the role of these drugs in ubiquitination regulation provides a potential reference for migraine treatment.

Inhibitors of ubiquitinating enzymes (TAK-243, an E1 ligase inhibitor, and MLN4924, an E3 ligase inhibitor) as potential drugs for precise regulation of inflammatory responses and cell signaling pathways (100–102).

The development of activators of USP family deubiquitinating enzymes could be a potential strategy in the treatment of migraine. USP25 inhibits the overactivation of the NF-κB and MAPK pathways by removing the K63 polyubiquitin chain on TAB2, thereby attenuating microglia-mediated neuroinflammation (103). Notably, it has been found that USP5 inhibits the expression of proinflammatory factors by maintaining NF-κB signaling pathway activation to promote the expression of pro-inflammatory factors, whereas its inhibitor Vialinin A was able to significantly reduce TNF-α and IL-1β-induced pro-inflammatory gene expression, suggesting that USP5 inhibitors may serve as drug candidates for the treatment of inflammatory diseases, such as Kawasaki disease (104). In addition, the metalloproteinase inhibitor THL significantly inhibited NLRP3 activation by blocking BRCC3 complex-mediated deubiquitination, alleviating inflammation and tissue damage in a variety of inflammatory disease models such as sepsis, autoimmune encephalomyelitis, and nonalcoholic fatty liver disease (105).

5 Conclusion

In recent years, studies on migraine mechanisms have revealed the important roles of inflammatory factors, neurogenic inflammation, and CGRP signaling pathways in the disease. As one of the important mechanisms of epigenetic regulation, ubiquitination plays a key role in the pathogenesis and chronicity of migraine by regulating protein degradation, cell signaling, and inflammatory responses. In terms of genetic markers, mutations in CACNA1A, ATP1A2, and SCN1A are closely associated with FHM, while variants in NOTCH3 and TREX1 genes have been linked to migraine and its associated cerebrovascular diseases (e.g., cerebral arteriolar dominant disorders) (106–109). In addition, DNA methylation analysis identified methylated regions specific to migraine patients, suggesting a role for epigenetic modifications in migraine susceptibility (110). In the blood, plasma CGRP levels were significantly elevated in patients during migraine attacks (111). On the imaging side, functional magnetic resonance imaging (fMRI) and diffusion tensor imaging (DTI) studies have shown that migraine patients have increased visual Increased thickness of the visual cortex, significantly altered functional connectivity in nociceptive pathways, and white matter hyperintensity are characteristic imaging hallmarks of migraine with aura (112, 113). These studies suggest the importance of plastic changes in neural structure and function in migraine.

Future studies need to further elucidate the specific link between ubiquitination and the above markers. For example, do ubiquitination modifications affect the degradation of key molecules in the CGRP signaling pathway? Does it mediate the chronicity of migraine by modulating inflammatory factors such as TNF and IL-6? By integrating multi-modal data from epigenetics, genetic markers, blood biomarkers, and neuroimaging, we aim to enhance the understanding of the molecular mechanisms underlying migraine, thus advancing marker-based precision medicine. This integrated approach may pave the way for more effective, individualized treatments for migraine patients (114) (Figure 1D).

Author contributions

QZ: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. JY: Visualization, Writing – original draft, Writing – review & editing. LS: Data curation, Validation, Writing – original draft. JZ: Data curation, Validation, Writing – original draft. PZ: Supervision, Writing – original draft. JL: Visualization, Writing – original draft. XS: Conceptualization, Validation, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

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The role of cGAS-STING pathway ubiquitination in innate immunity and multiple diseases

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The cGAS-STING pathway is essential in innate immunity, especially in antiviral responses and cellular stress management. cGAS acts as a cytoplasmic DNA sensor by initiating the synthesis of the second messenger cyclic GMP-AMP synthase (cGAMP), which subsequently activates the STING pathway, leading to the production of type I interferons and other cytokines, as well as the activation of inflammatory mediators. Recent studies have demonstrated that ubiquitination changes closely regulate the function of the cGAS-STING pathway. Ubiquitination modifications influence the stability and activity of cGAS and STING, while also influencing the accuracy of the immune response by adjusting their degradation and signal intensity. E3 ubiquitin ligase specifically facilitates the degradation or modulates the signaling of cGAS-STING-associated proteins via ubiquitination alterations. Furthermore, the ubiquitination of the cGAS-STING pathway serves distinct functions in various cell types and engages with NF-κB, IRF3/7, autophagy, and endoplasmic reticulum stress. This ubiquitin-mediated regulation is crucial for sustaining the balance of innate immunity, while excessive or inadequate ubiquitination can result in autoimmune disorders, cancers, and viral infections. An extensive examination of the ubiquitination process within the cGAS-STING pathway elucidates its specific regulatory mechanisms in innate immunity and identifies novel targets for the intervention of associated diseases.

KEYWORDS

CGAS, STING, ubiquitination, innate immunity, cancer

1 Introduction

Innate immunity serves as the body's primary defense mechanism against a variety of pathogens, including bacteria, viruses, fungi, and parasites (1). The classical cGAS-STING signaling pathway is an essential element of the innate immune system and significantly contributes to the body's defense against pathogen invasion (2). The identification of exogenous or endogenous DNA by cGAS activates the enzyme, resulting in the production

of significant quantities of cyclic GMP-AMP synthase (cGAMP) (3). This cGAMP then binds to STING, activating it and promoting the translocation of STING from the endoplasmic reticulum to the Golgi apparatus (4, 5). Thereafter, upon activation, STING recruits TBK1 and subsequently activates IRF3, leading to its dimerization and translocation to the nucleus, where it orchestrates the transcription of type I interferon (IFN) genes. Furthermore, the activation of STING facilitates its interaction with the NF-κB signaling pathway, promoting the nuclear translocation of NF-κB and thereby inducing the expression of pro-inflammatory cytokines, such as IL-1β, TNFα, and IL-6, which collectively augment the immune response (6).

Ubiquitination is essential for the regulation of immune pathways, serving as a post-translational modification process in which ubiquitin molecules alter target proteins within cells via specialized enzymes, including ubiquitin-activating enzymes, conjugating enzymes, ligases, and degrading enzymes (7, 8). This mechanism regulates homeostasis in the body by affecting protein activation, localization, and degradation, thus precisely coordinating immune responses. This coordination enables the host to efficiently address threats while averting pathological overactivation that could lead to autoimmune disorders (9). Recent studies have revealed that the activity of the cGAS-STING pathway is tightly regulated by ubiquitination modifications (10, 11). The review aims to provide a comprehensive overview of the ubiquitination processes involved in the cGAS-STING pathway and their roles in various diseases, providing significant therapeutic insights for autoimmune disorders, cancer and viral infections, among other diseases.

2 Ubiquitination of cGAS-STING pathway

2.1 Ubiquitination in cellular processes of cGAS

Ubiquitination is crucial for the activation, stability, and function of cGAS, necessitating multiple E3 ubiquitin ligases. K63-linked ubiquitination facilitates signal transduction, whereas K48-linked ubiquitination generally indicates degradation (12). TRIM56 induces Lys335 monoubiquitination of cGAS, leading to a significant increase in its dimerization, DNA-binding activity, and cGAMP production, which is important for cytoplasmic DNA sensing and IFNαβ production to induce anti-DNA viral immunity (13). Recent study has revealed the cGAS-based antiphage signaling system (CBASS) in bacteria, with Cap2 and Cap3 comprising around 39% of the CBASS system (14, 15). Cap2 assembles into a hexameric complex with a high affinity for cGAMP, hence obstructing the cGAS signaling pathway and hindering phage proliferation. This transpires via the establishment of a thioester bond with the C-terminal glycine of cGAS, enabling the binding of cGAS to target proteins similarly to ubiquitin attachment (16). K27-linked polyubiquitination of cGAS is predominantly mediated by RNF185, the first E3 ubiquitin ligase of cGAS, to enhance its enzymatic activity (17). K48-linked ubiquitination of cGAS is a recognition signal for p62-dependent selective autophagic degradation. The induction of TRIM14 by IFN-I accelerates cGAS stabilization by recruiting USP14 to cleave the ubiquitin chain of cGAS at lysine (K)414 (18). On the other hand, The E3 ubiquitin ligase TRIM41 positively regulates cGAMP synthesis by interacting with cGAS and facilitating its monoubiquitination (19). cGAS was initially recognized as a cytoplasmic DNA detector (5). A recent study indicated that nucleosoluble cGAS is essential for the recognition of nuclear-replicating DNA viruses (20). Furthermore, TRIM41 interacts with and ubiquitinates ORF2p, affecting its stability, whereas intranuclear cGAS enhances the binding of ORF2p to TRIM41, hence facilitating TRIM41-mediated degradation of ORF2p and restricting LINE-1 (L1) retrotransposition (21). This could offer a route for future intervention in aging and tumorigenesis. USP15 is a constituent of the deubiquitinating enzymes subfamily of cysteine proteases, which facilitate the removal of ubiquitin from substrates in a ubiquitin-specific manner (22). USP15 activates cGAS in the presence of DNA by two parallel pathways: facilitating cGAS deubiquitination and enhancing its phase separation, which contrasts with conventional cGAS deubiquitination mechanisms, such as those mediated by USP14 (18, 23). Furthermore, the deubiquitinating enzyme USP27X associates with cGAS and eliminates K48-linked polyubiquitinated chains from cGAS, leading to the stability of cGAS (24). Andrea Ablasser et al. clarified the mechanism through which the ubiquitin-proteasome system (UPS) degrades nuclear cGAS, identifying SPSB3 as a substrate receptor that targets cGAS and associates with the cullin-RING ubiquitin ligase 5 (CRL5) complex to ubiquitinate nuclear cGAS, facilitating its subsequent degradation (25).

2.2 Ubiquitination in cellular processes of STING

STING serves as a pivotal junction protein within the cGAS-STING pathway. The activation of the STING pathway involves the translocation of STING from the ER to the ER-Golgi intermediate compartment (ERGIC) and then to the Golgi apparatus, a crucial step in initiating its downstream signaling pathway (26). The distribution and function of STING are governed by several E3 ubiquitin ligases that modify STING via ubiquitination (27). TRIM56 enhances K63-linked ubiquitination of STING, facilitating STING dimerization and its accumulation in the Golgi apparatus, thereby recruiting TBK1 and stimulating IFN-1β production (28). TRIM32, RNF115, and mitochondrial E3 ubiquitin ligase 1 (MUL1) promote K63-linked polyubiquitination, hence enhancing the efficient transport of STING and the activation of its downstream pathways (29, 30). Researchers at Shandong University discovered that TRIM10 promotes the ubiquitination of lysine residues 289 and 370 of STING at K27 and K29, thereby facilitating the translocation of STING from the ER to the Golgi, enhancing its aggregation in the Golgi as well as the recruitment of the downstream kinase TBK1 (31). The E3 ubiquitin ligase complex, located in the ER and consisting of AMFR-GP78 and INSIG1, promotes K27 polyubiquitination of STING, recruits TBK1,

and triggers IFN production (32). The ubiquitination of STING at the K236 site by RNF144A is crucial for STING translocation and the subsequent control of STING-mediated antiviral responses (33). Conversely, USP21 is a significant deubiquitinating enzyme of STING, which negatively modulates the synthesis of IFN-I triggered by DNA viruses by hydrolyzing the K27/63-linked polyubiquitin chains on STING (34). In recent years, many ubiquitin-like proteins have been identified that ubiquitinate STING proteins. UFL1, the only recognized E3 ubiquitin ligase for UFM1, reduces K48-linked ubiquitination of STING at the Lys338, Lys347, and Lys370 residues by competitively binding with STING and TRIM29, thereby inhibiting its degradation, maintaining STING protein stability, and augmenting the antiviral immune response (35).

RNF5 promotes K48-linked ubiquitination and subsequent degradation of STING, hence suppressing the antiviral immune response (36). Following viral infection, RNF5 is ubiquitinated at the Lys150 site in mitochondria, resulting in the degradation of STING, which inhibits virus-induced signaling (37). RNF5 also restricts IFN-I antiviral responses in herpes simplex virus (HSV) corneal epitheliitis by suppressing STING/IRF3 signaling (37). Conversely, RNF5 and TRIM30a facilitate K48 polyubiquitination of STING, resulting in its proteasomal degradation and suppression of the DNA signaling cascade response (38). TRIM29 serves as a negative-feedback regulator of the intracellular response to DNA and DNA viral stimuli by facilitating K48 ubiquitination of STING, hence increasing its degradation (39). Reverse transport of STING to the ER and translocation of activated STING to the lysosome for degradation are critical pathways for alleviating faulty and excessive STING signaling, where in ubiquitination modifications are significant (40, 41). STING is degraded via a microautophagy mechanism that relies on an endosomal sorting and transport complex (ESCRT). During transport, STING is ubiquitinated at lysine 288 through K63 ubiquitination, a modification that is essential for its degradation to avoid excessive immunization (42). Moreover, HRD1 ubiquitinates STING proteins, mostly through K27-linked ubiquitination, facilitating the degradation of ER-neonatal STING proteins and thereby inhibiting STING-mediated immunological responses (43). These mechanisms highlight the complex regulation of STING by ubiquitination, which is crucial for maintaining the balance of the immune response and preventing excessive or abnormal activation (Figure 1).

3 Ubiquitination dysregulation of the cGAS-STING pathway is associated with multiple diseases

The dysregulation of ubiquitination in the cGAS-STING pathway is often associated with autoimmune diseases, viral infections, inflammation, and disturbances in intestinal homeostasis. cGAS identifies DNA of a specific length irrespective of its sequence, enabling DNA from any source to provoke an immune response through the activation of the cGAS-STING pathway (44). Hyperactivation of the cGAS-STING pathway contributes to autoimmune illnesses, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Aicardi-Goutieres syndrome (AGS), as well as other conditions such as cancer (45, 46). Patients with functional mutations in Trex1 trigger autoimmune disorders by persistently stimulating the cGAS-STING signaling pathway due to the accumulation of their own DNA (47). In various DNA viral infections, including Human Immunodeficiency Virus (HIV) and Hepatitis B Virus (HBV), the viral proteins, specifically HIV coat and HBV Pol, disrupt the cGAS-STING signaling pathway by targeting STING ubiquitination, thereby facilitating the modulation of the host immune response and allowing for viral immune evasion to sustain persistent infection in the host (48, 49). The papain-like protease of the RNA virus pig epidemic diarrhea virus and the papain-like protease of coronavirus play significant immunomodulatory effects. They suppress the host immune response by negatively modulating the cGAS-STING pathway, exerting deubiquitination, and exhibiting IFN antagonistic activity, enabling the virus to evade the host's innate immunological defenses and to persist for replication and transmission (50, 51). Bone marrow chimera investigations indicate that STING accumulation in intestinal macrophages and monocytes serves as a primary instigator of inflammation (11). Modifications in the cGAS-STING DNA signaling pathway influence intestinal homeostasis. Elevated STING expression was identified as a hallmark of intestinal inflammation in mice with colitis and in individuals with inflammatory bowel disease (52). The STING can be triggered by the bacterial product cyclic di-GMP in myeloid cells, leading to K63-linked ubiquitination and subsequent accumulation of STING in intestinal myeloid cells, and then triggering intestinal inflammation (11). Therefore, rigorous regulation of ubiquitination of ubiquitination activation and degradation of cGAS-STING pathway is essential.

4 The downstream effects of the ubiquitination process in the cGAS-STING pathway

The interaction between the ubiquitination of the cGAS-STING pathway and several signaling cascades, such as NF-κB, IRF3/7, autophagy and ER Stress, emphasize the intricacy of immunomodulation (Figure 2).

4.1 NF-κB

The interplay between the ubiquitination of the cGAS-STING pathway and the NF-κB pathway initiates a cascade that enhances IFN responses and robust host antiviral defenses (53). Gelsevirine, a natural substance, blocks excessive activation of the STING/NF-κB pathway by facilitating TRIM21-mediated K48 ubiquitination degradation of STING to address organ damage resulting from sepsis (54). Furthermore, the envelope glycoprotein Gn of the severe fever with thrombocytopenia syndrome virus (SFTSV) interacts with STING to impede STING dimerization and K27 ubiquitination, thereby hindering the assembly of the STING-TBK1 complex and

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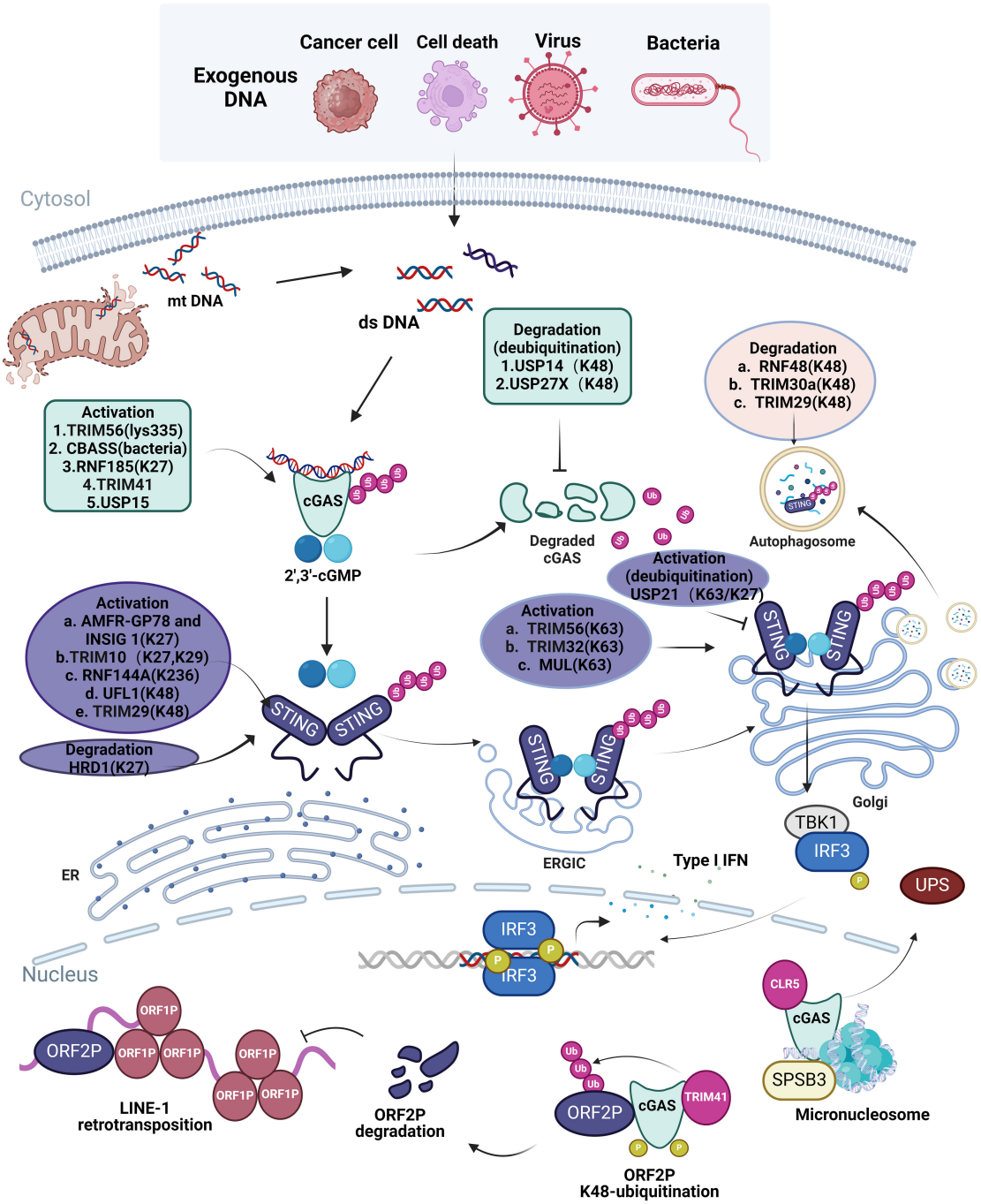

FIGURE 1
Ubiquitination in cellular processes of cGAS-STING Pathway. Exogenous dsDNA released from cancer cell, cell death, virus, bacteria and endogenous dsDNA released from mitochondria are easily recognized by intracellular cGAS, which promotes the cGAS-STING-TBK1 signaling pathway and releases IFN I to elicit innate immune response. The ubiquitination of different ubiquitinating enzymes to different sites of cGAS and STING in different organelles is listed in the figure; the ubiquitination of cGAS and STING promotes or inhibits the activation and degradation of cGAS and STING, which affects the innate immune response of the cGAS-STING pathway in cells. However, in the nucleus, TRIM41 and CLR5 also cause ubiquitination of cGAS, which restricts L1 retrotransposition as well as degradation of cGAS via the ubiquitin protease hydrolysis system. Image created with BioRender.com, with permission.

subsequent signaling, which obstructs the nuclear translocation of IRF3 and p65, ultimately attenuating downstream innate immune signaling (55). This clarifies the connection between the ubiquitination of the cGAS-STING pathway and NF-κB, alongside immunological mechanisms, including enhanced signal activation, viral protein disruption, and modulation by natural products.

4.2 IRF3/IRF7

In the context of autoimmune diseases, the ubiquitination of the cGAS-STING pathway can interact with IRF3/IRF7. Aberrant activation of the cGAS-STING pathway may lead to hyperactivation of IRF3/IRF7, thereby eliciting an excessive

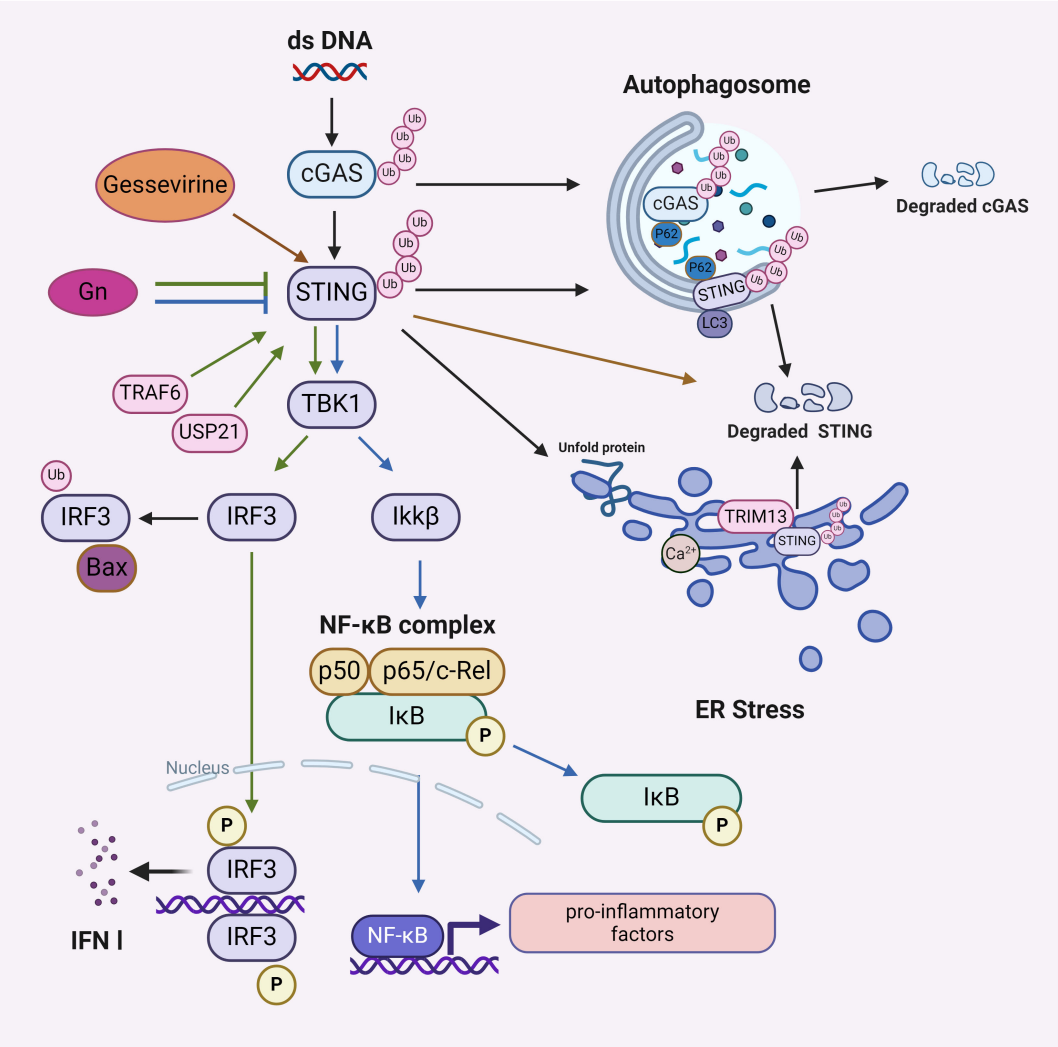

FIGURE 2
The downstream effects of the ubiquitination process in the cGAS-STING pathway. Affects NF-κB signaling pathway: Gelsevirine affects STING/NF-κB by promoting ubiquitinated degradation of STING and Gn by blocking ubiquitinated activation of STING; affects IRF3/IRF7 signaling pathway: TRAF6 ubiquitinates or USP21 deubiquitinates STING to activate or maintain STING stability, promoting downstream activation of IRF3 and IRF7; affects autophagy: p62 mediates ubiquitination of cGAS and autophagic degradation of STING; affects ER Stress: STING acts as a mediator of ER Stress, and ubiquitination of STING by TRIM13 leads to STING degradation. Image created with BioRender.com, with permission.

immune response (56). TRAF6 can ubiquitinate cGAS and the K63 chain of STING to facilitate the subsequent activation of IRF3 and IRF7, hence initiating the production of antiviral INF-I (57, 58). USP21 deubiquitinates STING, enhancing its stability and activity, which then promotes the downstream activation of IRF3 and IRF7, thereby amplifying the antiviral immune response (59). Investigating the interaction mechanism between the ubiquitination of the cGAS-STING pathway and the IRF3/IRF7 pathway may elucidate its significant involvement in autoimmune disorders and antiviral responses.

4.3 Autophagy

The cGAS-STING pathway can induce autophagy through the translocation of STING, independently of TBK1-IRF3 and conventional autophagy signaling molecules. Mechanistically, the transfer of STING across membranes and its association with ERGIC-containing membranes are essential for cGAMP-induced autophagy, which effectively eliminates intracellular DNA and viruses (26). STING-dependent signaling network is associated with health and disease by regulating autophagic degradation or various cell death modalities (60). p62 interacts with ubiquitinated STING through its ubiquitin-binding domain, subsequently binding to the autophagosome marker protein LC3, which facilitates the transport of cGAS or STING to the autophagosome for degradation, thereby inhibiting the activation of the cGAS-STING pathway and averting excessive immune responses (61). However, STING activation negatively influences the autophagy process initiated by energy deficiency. The mechanism entails STING binding to STX17, an essential protein for autophagic membrane fusion, which affects the transport of STX17 from the

ER to the intact autophagosome, thereby inhibiting the efficiency of autophagosome-lysosome fusion and downregulating cellular autophagy (62). In summary, multiple modes of ubiquitination occur in the cGAS-STING pathway, which binds to the autophagic system to regulate the immune response and maintain cellular homeostasis in organisms.

4.4 ER stress

STING was identified as a mediator of ER stress and the UPR through a novel pattern referred to as "the UPR motif" (63). Additionally, the cGAS-STING pathway may induce ER stress through an interaction between STING and the calcium sensor stromal interaction molecule 1 (STIM1) (64). Given that TRIM13 is consistently localized in the ER, it may modulate STING degradation via ER-mediated degradation (65, 66). This may differ from the ERGIC-initiated autophagosome route and the post-ER-initiated autophagy pathway (26). A proposed strategy for regulating STING homeostasis during inflammatory responses induced by pathogenic DNA involves the transmembrane ER-associated TRIM13. This ER-localized E3 ubiquitin ligase TRIM13 interacts with STING through transmembrane structural domains and facilitates the polyubiquitylation of STING's Lys6 ligand, leading to a reduction in ER exit and an increase in ER-mediated STING degradation (67).

5 Therapeutic potential on targeting ubiquitination in the cGAS-STING pathway

Targeting the ubiquitination of the cGAS-STING pathway holds significant potential in the treatment of autoimmune diseases, cancer, and viral infections. In autoimmune diseases like SLE, the overactivation of the cGAS-STING pathway can lead to excessive immune responses, with RNF185 being a key E3 ubiquitin ligase implicated in SLE pathogenesis (17). The SARS-CoV-2 PLPRO removes polyubiquitin chains from STING, inhibiting IFN-I responses and providing insights into viral interactions with the cGAS-STING pathway (68). Additionally, the deubiquitinating enzyme MYSM1 interacts with and modifies STING, preventing downstream signaling and offering new therapeutic avenues (69). In cancer research, the cGAS-STING pathway is significant for tumor development and therapy. Activating this pathway can enhance the 'tumor-immune' arousal effect, improving chemotherapy sensitivity. IDI1, a metabolic enzyme, interacts with cGAS, and TRIM41, an E3 ligase from hepatocellular carcinoma cells, promotes cGAS degradation, suggesting a role in innate immunity and a potential target for liver cancer treatment (70). TRIM29 induces STING degradation, affecting DNA viral infections, and its knockdown in airway epithelial cells enhances INF-I production, nearly eradicating EBV in nasopharyngeal carcinoma cells (39). Meanwhile, the deubiquitinating enzyme USP35 is a negative regulator of STING-associated INF-I signaling in ovarian cancer, with its silencing triggering potent anti-tumor activity and improving prognosis (71). Furthermore, TRIM29-mediated STING ubiquitination degrades STING in immune and cancer cells, and its overexpression hinders immune responses. The protein-protein interaction modulator SB24011 inhibits STING-TRIM29, upregulating cellular STING levels and showing potential as an anti-cancer therapy (72). Targeting the DTX3L-cGAS axis may also be a promising approach for pancreatic tumor treatment (73). Developed by Professor Jin Jian and Professor Wenyi Wei, the first-in-class deubiquitinase-targeting chimeras (DUBTACs) of cGAS MS7829 and MS8588 stabilize and activate cGAS, effectively inhibiting cancer cell growth by enhancing the cGAS-STING signaling pathway (74). The cGAS-STING pathway is also a key in viral infections, with TRIM30α induced by HSV-1 infection in dendritic cells, promoting STING degradation and acting as a negative feedback regulator of the innate immune response (75, 76). MARCH8 negatively regulates the cGAS-mediated natural immune signaling pathway, and ARIH1 promotes antiviral and autoimmunity responses by catalyzing mono-ISGylation and cGAS oligomerization (77, 78). Knockdown of TRIM14 impairs the antiviral response triggered by HSV-1 in a cGAS-dependent manner (18). These findings highlight the complexity of the cGAS-STING pathway in targeting ubiquitination in viral infections, offering potential research directions and therapeutic targets (79). Targeting the ubiquitination of the cGAS-STING pathway holds significant potential in the treatment of autoimmune diseases, cancer, and viral infections.

6 Discussion

Ubiquitination alterations of the cGAS-STING pathway are essential for its functionality and stability. The E3 ubiquitin ligases TRIM41 and TRIM56 augment dimerization, DNA-binding activity, and cGAMP synthesis; RNF185, and the deubiquitinating enzymes USP15 and USP27X promote cGAS stability (13, 17, 19, 23, 24). TRIM32, TRIM56, MUL1, and TRIM10 ubiquitin ligases facilitate the translocation of STING from the endoplasmic reticulum to the Golgi, augment its retention in the Golgi, and enlist TBK1 along with subsequent pathways for INF-I synthesis (28–31). Further, many ubiquitinating enzymes are present to facilitate the degradation of STING (37, 75). The interaction between the ubiquitination of the cGAS-STING pathway and other signaling cascades underscores the intricacy of immune control. Ubiquitinated cGAS or STING can be identified by the autophagy receptor protein p62/SQSTM1, which subsequently directs autophagosomes for destruction (40). The natural compound Gelsevirine prevents excessive activation of the STING/NF-κB pathway by facilitating the ubiquitin-mediated degradation of STING (54). The ubiquitination modification of the cGAS-STING pathway interacts with the IRF3/7 pathway, influencing antiviral and autoimmune responses. Furthermore, the

cGAS-STING pathway may elicit ER stress via the interaction of STING with the calcium sensor STIM1 (64). SLE, the E3 ubiquitin ligase RNF185 induces hyperactivation of the pathway associated with autoimmune disorders (17). Viral proteins in infections influence host immunological responses and facilitate immune evasion by disrupting STING ubiquitination (68). In addition, in pancreatic tumors, ovarian cancer, and nasopharyngeal carcinoma, blocking the ubiquitination of the cGAS-STING pathway may represent a novel therapeutic approach to inhibit anti-tumor immunity (39, 70, 74).

In summary, the cGAS-STING pathway is crucial for innate immunity, with ubiquitination changes meticulously regulating its function, hence influencing the stability, activity, and accuracy of the immunological responses of cGAS and STING. The cGAS-STING pathway functions differently across many cell types and engages with multiple pathways; its dysregulation can result in numerous illnesses. Focusing on the particular ubiquitin ligases and deubiquitinating enzymes implicated in the cGAS-STING pathway presents a promising approach for the creation of therapeutics for autoimmune diseases, cancer, and viral infections, highlighting the necessity of investigating these interactions for novel therapeutic targets.

Author contributions

CD: Writing – original draft, Writing – review & editing. DC: Writing – original draft, Writing – review & editing. LY: Writing – review & editing. YZ: Resources, Validation, Writing – original draft. CJ: Resources, Validation, Writing – original draft. YL: Investigation, Resources, Writing – original draft. QL: Writing – original draft. ML: Validation, Visualization, Writing – review & editing. RZ: Validation, Visualization, Writing – original draft. BH: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. SL: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Ubiquitination regulation of mitochondrial homeostasis: a new sight for the treatment of gastrointestinal tumors

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Mitochondrial homeostasis (MH) refers to the dynamic balance of mitochondrial number, function, and quality within cells. Maintaining MH is significant in the occurrence, development, and clinical treatment of Gastrointestinal (GI) tumors. Ubiquitination, as an important post-translational modification mechanism of proteins, plays a central role in the regulation of MH. Over the past decade, research on the regulation of MH by ubiquitination has focused on mitochondrial biogenesis, mitochondrial dynamics, Mitophagy, and mitochondrial metabolism during these processes. This review summarizes the mechanism and potential therapeutic targets of ubiquitin (Ub)-regulated MH intervention in GI tumors.

KEYWORDS

mitochondrial homeostasis, ubiquitination, gastrointestinal tumors, mitochondrial biogenesis, mitochondrial dynamics, mitophagy, mitochondrial metabolism

1 Introduction

Gastrointestinal (GI) tumors, including hepatocellular carcinoma (HCC), esophageal cancer (ESCA), gastric cancer (GC), colorectal cancer (CRC), and pancreatic cancer (PAAD) (1), are the leading causes of cancer-related deaths worldwide. Highlighting its severe public health burden, there are an estimated 4.9 million new cases and 3.9 million deaths annually, according to the latest data (2). Mitochondria (Mt) plays a crucial role in the occurrence and development of GI tumors (3). As a key organelle in the metabolic reprogramming of cancer cells, Mt dysfunction is one of the main drivers of cancer initiation and progression (4). Mt stress releases Mitochondrial DNA (mtDNA) into the cytoplasm and extracellular space, activating multiple innate immune signals (5). In GI tumors, the mtDNA mutation rate is higher, mainly in the D-loop region, which is the hypervariable region of mtDNA and is responsible for the regulation of mtDNA transcription and replication, which may be related to the special physiological environment of gastrointestinal cells (such as acidic environment,

frequent cell turnover, etc.) (6, 7). Concurrently, the increased electron leakage from Mt stress generates a high concentration of reactive oxygen species (ROS), which further aggravates tissue damage and inflammation and suppresses the signal presentation between dendritic cells and T cells, leading to immune cell dysfunction and further promoting the infiltration of tumor-associated macrophages and the formation of an immunosuppressive tumor microenvironment (8, 9). Additionally, DNA and ROS released by Mt can change the balance of intestinal microbiota, leading to damage to the intestinal barrier and the occurrence of inflammatory bowel disease (IBD) (10). IBD is one of the risk factors for CRC (11). In the late GI tumors stage, Mitochondrial metabolism (MM) may instead increase, promoting cancer growth (10). This may be related to the B-cell lymphoma/leukemia-2 (BCL-2) protein family promoting the increase of mitochondrial permeability transition and mitochondrial permeability transition resistance, affecting the release of cytochrome C, thereby promoting the malignant transformation and progression of tumors (12). Studies have shown that Mt can also act as strategic molecular intermediaries or transport media for targeted therapies involved in antitumor activities (13). In summary, Mt dysfunction and structural damage further complicate the mechanism of GI tumorigenesis and development. Therefore, maintaining the normal function of Mt and regulating Mitochondrial homeostasis (MH) is key to intervening in the progression of GI tumors. MH refers to a state of balance in which Mt maintains normal function and structure within cells, including processes such as Mitochondrial biogenesis (MB), Mitochondrial dynamics (MK), Mitophagy, and MM (14–16).

Ubiquitination is involved in regulating these processes to maintain normal cellular functions. Ubiquitination is one of the main post-translational modifications of proteins, involving the addition of Ub molecules to target proteins, and plays a role in regulating protein degradation, signal transduction, DNA repair, cell cycle, and apoptosis within organisms (17–19). The process of ubiquitination is primarily catalyzed by E1 Ub-activating enzymes, E2 Ub-conjugating enzymes, and E3 Ub ligases, which play roles at different stages of the enzymatic ubiquitination cascade. Deubiquitinating enzymes (DUBs) can reverse ubiquitination by removing Ub. Under normal conditions, the ubiquitination process balances MH through the action of ubiquitinating enzymes and DUBs (20). However, when the ubiquitination process is disrupted, and MH is imbalanced, the progression of GI tumors is affected (Figure 1). This study to elucidate the molecular mechanisms and therapeutic targets of ubiquitination-regulated MH in the intervention of gastrointestinal tumors by reviewing the literature on Mt, ubiquitination, and GI tumors published in the past decade from databases such as PubMed and Web of Science, thereby providing theoretical support for the clinical diagnosis and treatment of GI tumors.

2 Ubiquitination regulates MH through multiple pathways

2.1 Ubiquitination targeting MB

MB refers to the synthesis of new Mt within cells, a process activated by various physiological and environmental signals such as cellular stress, increased energy demands, exercise training, and hormonal changes (21). This process primarily involves the transcriptional activation of nuclear-encoded mitochondrial genes, the translocation of corresponding proteins to Mt, the replication of mtDNA, and the synthesis of mitochondrial phospholipids (22). We found that ubiquitination targets peroxisome proliferator-activated receptor gamma coactivator-1

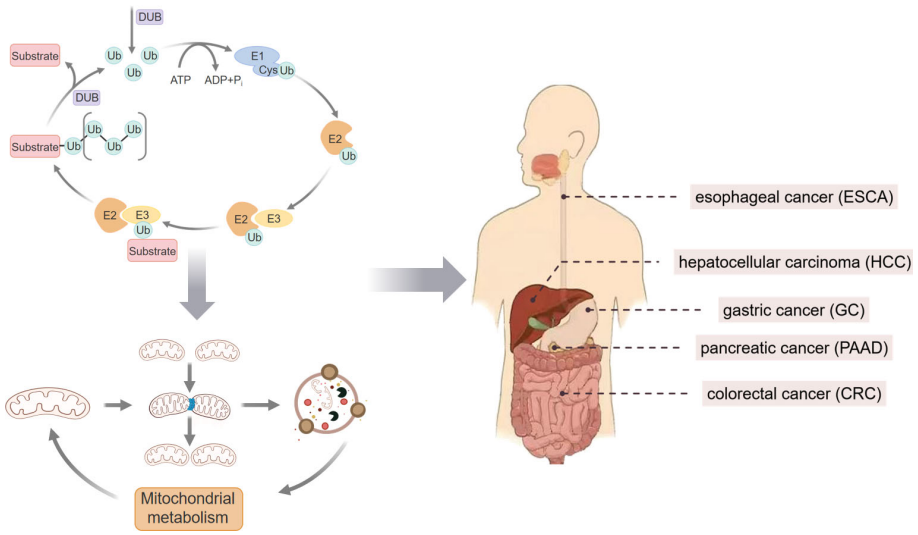

FIGURE 1
Schematic representation of MH targeted by ubiquitination affecting GI tumors.

alpha (PGC-1α) to participate in the transcriptional activation of nuclear-encoded mitochondrial genes, Mitochondrial ubiquitin ligase/the E3 ligase Membrane-associated ring-gh-type finger 5 (MITOL/MARCH5) ubiquitinates DNA polymerase gamma catalytic subunit (PolγA) to mediate mtDNA replication, and the tumor necrosis factor receptor-associated aactor 6 (TRAF6) E3 ligase restricts protein translocation, while mitochondrial phospholipid synthesis and membrane changes also reciprocally affect ubiquitination (23, 24).

2.1.1 Regulation of PGC-1α

PGC-1α is a primary regulatory factor in MB and energy metabolism, controlling both the nuclear and mitochondrial genomes (25, 26). PGC-1α regulates mtDNA replication, transcription, and translation, as well as the assembly of oxidative phosphorylation (OXPHOS), through co-activating nuclear respiratory factors (NRFs) and estrogen-related receptor alpha, thus maintaining mitochondrial quantity and function (27). Additionally, as a cellular energy responder, PGC-1α is activated via the AMP-activated protein kinase/silent information regulator 1 (SIRT1) pathway, enhancing MB (28). The heme oxygenase 1/PGC-1α pathway responds to oxidative stress by directly activating antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, thereby improving the cell's antioxidant capacity, reducing ROS production, maintaining mitochondrial adenosine-triphosphate (ATP) levels and membrane potential (MMP), and protecting Mt from oxidative stress-induced damage (29).

The mechanisms of ubiquitination regulating PGC-1α mainly include promoting PGC-1α degradation, modulating its stability, and controlling its activity. Research has shown that radiation-induced DNA-dependent protein kinase can phosphorylate serine 636 of PGC-1α, thereby enhancing the binding of the E3 Ub ligase Ring finger protein 34 to PGC-1α and accelerating its ubiquitination and degradation (25). However, this binding can be competitively blocked by lysine methyltransferase 5C, which reduces the ubiquitination level of PGC-1α and extends its half-life (30). In addition, neural precursor cell expressed, developmentally downregulated protein 4-1 (NEDD4-1) is also an E3 ligase that can mediate the ubiquitination and degradation of PGC-1α by enhancing the recognition of the "TPPTTPP" sequence in PGC-1α through phosphorylation mediated by glycogen synthase kinase 3β (GSK-3β) (31).

2.1.2 MITOL ubiquitinates PolγA

The mitochondrial membrane-integrated Ub ligase MITOL is a key regulator of mitochondrial membrane fission, fusion, and mitophagy (32). PolγA is the only DNA polymerase in Mt, and it replicates mtDNA through a process known as "D-loop replication," where the heavy strand is replicated first, followed by the light strand (33). Recent studies have shown that MITOL ubiquitinates PolγA, negatively regulating its interaction with the translocase of the outer mitochondrial membrane 20 (Tom20), thereby inhibiting its entry into Mt (34).

2.1.3 TRAF6 restricts p53

TRAF6 limits the translocation of tumor protein 53(p53) toMt by promoting the ubiquitination of p53 at lysine 24 (K24) in the cytoplasm through K63-linked Ub chains. This modification restricts the interaction of p53 with myeloid cell leukemia-1 (MCL-1)/BCL-2 Antagonist 1 (BAK), thereby inhibiting p53 mitochondrial translocation (35).

2.1.4 Ub ligases regulating mitochondrial phospholipid synthesis

Cardiolipin (CL) is a characteristic phospholipid of the mitochondrial inner membrane, playing a crucial role in cellular energy metabolism, MK, and the initiation of apoptotic pathways (36). The precursor for CL synthesis is phosphatidic acid (PA), which is synthesized in the endoplasmic reticulum and then transported to the outer mitochondrial membrane (OMM) (37). From there, it is transferred to the inner membrane for CL synthesis by the uncharacterized protein with a phosphatidic acid transfer protein-like domain1-mitochondrial distribution and morphology Protein 35 (MDM35) protein complex in the intermembrane space (38). Studies have shown that common lipids in the mitochondrial membrane interact with MITOL and influence its activity and stability depending on the *in vitro* lipid composition (39). Notably, the binding of CL to purified MITOL significantly reduces its thermal stability, whereas the presence of PA enhances its stability most strongly (32), which further confirms that lipids can directly affect the activity of Ub ligases and may control the ubiquitination-dependent mechanisms regulating MK and turnover. Phosphatidylcholine is a lipid essential for mitochondrial membrane construction, primarily synthesized through the Kennedy pathway using choline as a substrate. During phosphatidylcholine synthesis, choline kinase accelerates mitochondrial damage. The mitochondrial kinase PTEN-induced putative kinase 1 (PINK1) accumulates on the membrane of damaged Mt, activates the Parkin rbr E3 ub protein Ligase (Parkin), and promotes substrate ubiquitination to initiate mitophagy (40).

2.2 Ubiquitination regulates MK

Mt are highly dynamic organelles capable of undergoing continuous cycles of fusion and fission, thereby altering their morphology, size, and spatial distribution (41). This physiological process is referred to as MK. MK refers to the state in which Mt maintains homeostasis through fusion and fission processes in cells. This dynamic change causes Mt to assume a variety of morphologies in the cytoplasm, such as punctate, fragmented, strip, or linear. MK is closely related to the functions of Mt, such as cell proliferation, cell metabolism, and cell migration (42). This balance is maintained by various dynamin-related guanosine triphosphatase (GTPases), which play critical roles in these processes (43). Key proteins involved include dynamin-related protein 1 (Drp1), mitofusin 1 (MFN1), mitofusin 2 (MFN2), and aptic atrophy 1 (OPA1), which mediate membrane dynamics and structural changes (44).

2.2.1 The recruitment of Drp1

Drp1 is an essential protein in the mitochondrial fission process, containing a GTPase domain that enables it to bind and hydrolyze GTP (45). Drp1 primarily resides in the cytoplasm and, in response to mitochondrial fission signals, is recruited to the mitochondrial surface. There, it assembles into ring-like structures and utilizes the energy from GTP hydrolysis to divide Mt into two separate units (46). Identified Drp1 receptors include mitochondrial fission factor (Fis1), mitochondrial fission factor, and MK proteins of 49 kDa and 51 kDa (47). These receptor proteins facilitate the localization of Drp1 toMt through protein-protein interactions. Recent studies have revealed that the clustered Mt homolog gene and its drosophila homolog clueless promote the recruitment of Drp1 to the mitochondrial surface via its receptors in both human cell lines and Drosophila models, thereby enhancing mitochondrial fission (48). Current studies have shown that Drp1 can be targeted for ubiquitination and degradation by E3 Ub ligases, such as MITOL and Parkin, thereby influencing the normal mitochondrial fission-fusion process (49, 50). Knockout of either enzyme results in increased Drp1 activity and uncontrolled mitochondrial hyper-fragmentation (49). In cells lacking MITOL, re-expression of MITOL reverses the suppression of stress-induced apoptosis (51). Furthermore, emerging evidence indicates that Drp1 is not only a substrate of MITOL but also a regulatory factor of MITOL activity. Drp1 may modulate MK by influencing the functional activity of MITOL (52).

2.2.2 MFN1 and MFN2

The mitochondrial fusion proteins MFN1, MFN2, and OPA1 are essential GTPases responsible for the structural fusion of mitochondrial membranes. MFN1 and MFN2 regulate the fusion of the OMM, while OPA1 mediates the fusion of the inner mitochondrial membrane (IMM) (53). Studies have shown that a family with sequence similarity 73 member A/B promotes mitochondrial fusion through the regulation of phospholipid metabolism, particularly via mitochondrial phospholipase D, in collaboration with MFN1/2 on the OMM (54). Additionally, the active domain of G-protein β2 (Gβ2) undergoes structural remodeling with MFN1's binding domain, regulating MFN1 migration on the mitochondrial membrane and facilitating mitochondrial fusion. However, Gβ2 does not interact with MFN2 (55). Sudeshna Nag et al. (56) reported that under stress conditions induced by carbonyl cyanide 3-chlorophenylhydrazone (CCCP), the interaction between mitochondrial phosphatase phosphoglycerate mutase 5 (PGAM5) and MFN2 is weakened. Instead, PGAM5 shifts to interact with Drp1. Concurrently, MFN2 undergoes phosphorylation and ubiquitination by kinases and E3 Ub ligases, leading to proteasomal degradation. This results in an increased proportion of Mt failing to undergo fusion, as well as mitochondrial fragmentation and degradation. This process can be reversed by deubiquitinating enzymes such as Ub-specific protease 30 (USP30). USP30, a deubiquitinase, inhibits mitochondrial fusion by reducing the non-degradative ubiquitination levels of MFN1/2 (57). The SCFMdm30 complex, an intracellular E3 Ub ligase complex composed of S phase kinase-associated protein 1, Cullin1 (CUL1), F-box protein (FBX), and Ring-box 1, promotes the K48-linked ubiquitination of fusion of Mt protein 1 (Fzo1) (the yeast homolog of MFN1/2), leading to its degradation and subsequently impairing mitochondrial fusion (58). In contrast, USP2 specifically binds to the ubiquitinated form of Fzo1, removing the Ub modification to regulate Fzo1 stability and enhance mitochondrial fusion efficiency (59).

2.2.3 OPA1

Compared to MFN1/2, OPA1 exhibits a broader range of functions, including maintaining the respiratory chain, MMP, cristae structure, regulation of apoptosis, and mitochondrial DNA stability (60). OPA1 exists in multiple isoforms, primarily categorized into long isoforms (L-OPA1, including a and b) and short isoforms (S-OPA1, including c, d, and e). L-OPA1 predominantly regulates the fusion of IMM, while S-OPA1 is involved in IMM fission (61). Studies have shown that treatment of SH-SY5Y cells with 6-hydroxydopamine (6-OHDA) results in a decrease in the protein levels of MFN2 and OPA1. Conversely, 6-OHDA treatment increases the expression of Fis1 and Drp1, leading to excessive mitochondrial fission, thereby affecting mitochondrial morphology and function (62). Moreover, the study found that carnosic acid, a rosemary extract, enhances the ubiquitination of inhibitor of nuclear factor kappa-B kinase subunit gamma (IKKγ), activating the Parkin/IKKγ/p65 signaling pathway to upregulate OPA1 expression and maintain MK homeostasis (62). Optic atrophy 1 (OMA1) is a metalloprotease located in the IMM, with OPA1 being one of its primary substrates (63). Under physiological conditions, OMA1 remains inactive but is rapidly activated during mitochondrial stress, such as MMP loss or excessive ROS production, negatively regulating OPA1 (64). It was demonstrated that leptin increased OPA1 expression by promoting the ubiquitin-mediated degradation of OMA1 via the GSK3 pathway, thereby enhancing the anti-apoptotic capacity of these cells (65).

2.3 Ubiquitination-mediated mitophagy

Similar to MK and MB, Mitophagy is another key process in the maintenance of line MH. Mitophagy is a cellular autophagic process that involves the selective sequestration and degradation of damaged or dysfunctional Mt, thereby maintaining the integrity of the mitochondrial network and cellular homeostasis (66, 67). Ubiquitination participates in mitophagy primarily through the PINK1/Parkin pathway, the PINK1/SYNPHILIN1/SIAH1 complex, as well as the interactions of Mitochondrial E3 Ub protein ligase 1 (MUL1) (68, 69).

2.3.1 PINK1/Parkin pathway

The PINK1/Parkin pathway is the most widely studied mitophagy pathway and is a classic Ub-dependent pathway (70). PINK1, as a sensor of mitochondrial health, accumulates on the outer membrane of damaged Mt when they are compromised and activates Parkin, which in turn promotes the recruitment of Parkin to the Mt. PINK1 phosphorylates Ub on the Ser65 site of substrates

on the mitochondrial outer membrane, a process that activates Parkin, allowing it to ubiquitinate numerous substrates on the mitochondrial outer membrane, thereby triggering selective autophagy (71). In healthy Mt, PINK1 is imported into the inner membrane, where its membrane-binding portion is cleaved by the protease presenilin-associated rhomboid-like (PARL) (72). The cleaved catalytic portion exposes unstable amino acid residues at the N-terminus and is rapidly degraded by the Ub-proteasome system (UPS) (72). This process is a crucial step in mitochondrial quality control, ensuring that only functionally intact Mt remains in the cell (73). When the function of PINK1 or Parkin is impaired, the removal of damaged Mt is hindered, leading to mitochondrial dysfunction and pathological changes in the organism (74). The loss of DJ-1 inhibits the recruitment of the selective autophagy receptor, synphilin, to depolarized Mt, further blocking PINK1/Parkin-mediated mitophagy (75). In addition to removing damaged mt, the PINK1/Parkin pathway also contributes to MB development. It has been shown that loss of PINK1/Parkin in nerve cells inhibits MB and ubiquitinates Parkin-interacting substrate, thereby relieving the inhibitory effect on PGC-1α (76).

2.3.2 PINK1/SYNPHILIN1/SIAH1 complex

Similar to Parkin, seven in absentia homolog 1 (SIAH1) is also an E3 Ub ligase (77). Through forming a complex with PINK1 and synphiln1, SIAH1 promotes the recruitment of autophagic markers, Microtubule-associated protein one light chain 3 (LC3), and the lysosome-associated membrane protein 1 (Lamp1), thereby facilitating mitochondrial autophagy (78). LC3 is a key marker in the autophagy process. During autophagosome formation, the cytosolic form of LC3-I participates in a Ub-like reaction involving Autophagy-related (Atg) 7 and Atg3 (E1-like Ub-activating enzyme and E2-like Ub-conjugating enzyme), binding to phosphatidyl ethanolamine to form the lipidated form, LC3-II, which attaches to the autophagosomal membrane and serves as a structural protein of the autophagosome (79). LAMP1 is commonly used as a marker for lysosomes, and LAMP1-positive organelles are often referred to as lysosomal compartments. After the fusion of the autophagosome with the lysosome, lysosomal hydrolases can degrade the autophagosome's contents, and LAMP1-labeled organelles participate in this degradation process (80).

2.3.3 Involvement of MUL1

MUL1 participates in mitochondrial autophagy and mediates MM and MK (81). MUL1 mediates sodium selenite-induced mitochondrial autophagy and the stability of the autophagy-related protein Unc-51, Like autophagy activating kinase 1 (ULK1), during this process (82). Interacting with the ULK1/ATG13 complex, MUL1 promotes the formation of K48 polyubiquitin chains on ULK1, leading to its degradation via the UPS. In muscle cells, MUL1 mediates the degradation of MFN2 via the UPS while also inducing mitochondrial autophagy (82). Additionally, by promoting the Small Ub-like modifier conjugation (SUMOylation) of the highly dynamic protein Drp1, which is recruited to Mt, MUL1 enhances the stability of Drp1 on the mitochondrial surface, playing a critical role in the dynamic regulation of mitochondrial morphology (83). The regulatory effect of MUL1 on mitochondrial energy metabolism has also been observed. MUL1 regulates the protein levels of protein kinase Bβ and hypoxia-inducible factor 1-alpha (HIF-1α) through K48-specific polyubiquitination, and the loss of MUL1 leads to the accumulation and activation of these substrates, affecting mitochondrial respiration and resulting in a shift to a new metabolic and lipidomic state (84). This is evident in the fact that, compared to wild-type cells, MUL1(-/-) cells show impaired mitochondrial respiration and increased ATP production through glycolysis, indicating a metabolic shift from oxidative phosphorylation to glycolysis (84).

2.4 Ubiquitination in MM

Under physiological conditions, mitochondrial energy metabolism includes the tricarboxylic acid cycle, OXPHOS, and fatty acid oxidation (85). During these processes, ubiquitination plays a key role in regulating mitochondrial homeostasis through various metabolic products such as glucose, fatty acids, amino acids, and the electron transport chain (ETC) (86, 87).

2.4.1 Glucose metabolism

Currently, the mechanisms by which ubiquitination participates in mitochondrial glucose metabolism under physiological conditions are not well understood. However, in certain pathological states, ubiquitination may play a role in reshaping mitochondrial glycolysis (88). In a study using a heart-specific promoter cTnT, the deletion of NEDD8-Activating enzyme E1 impaired cardiac oxidative metabolism and mitochondrial function, leading to the down-regulation of genes related to fatty acid utilization, while genes associated with glucose utilization were significantly up-regulated (89). Another study found that inhibition of polycomb repressive complex 1 reduced histone H2A ubiquitination (H2Aub) occupancy and, by suppressing ubiquitination, promoted the expression of Hsp27 (heat shock protein 27). Hsp27 enhances glycolysis during myocardial ischemia by activating the NF-κB/PFKFB3 signaling pathway, and it also reduces mitochondrial ROS production by interacting with Coenzyme Q9, inhibiting ferroptosis during reperfusion (90). Research progress shows that E3 ligases and deubiquitinating enzymes influence the Warburg effect in tumors by regulating glycolysis-related signaling pathways and transcription factors (91, 92). Phosphofructokinase platelet (PFKP) is a gene encoding a rate-limiting enzyme of glycolysis, and its role in mediating glycolytic regulation of tumor progression has been well-established in lung cancer and advanced prostate cancer (93, 94). HMG-CoA Reductase Degradation 1 (HRD1), as a metabolic enzyme, catalyzes the ubiquitination of PFKP and promotes its degradation, thereby inhibiting the expression and activity of PFKP in cancer cells and obstructing cell invasion and proliferation (95). The gut microbiota and its derivative metabolite taurocholic acid can epigenetically promote the glycolysis of Myeloid-derived suppressor cells by enhancing the monomethylation of the target

gene H3K4 and inhibiting C-terminus of Hsc70-interacting protein-mediated PDL1 ubiquitination, which in turn suppresses the proliferation and function of effector T cells (96).

2.4.2 Fatty acid metabolism

Under energy stress conditions, tumor cells mobilize lipids stored in lipid droplets and generate energy through mitochondrial fatty acid oxidation (β-oxidation) (97). The direct contact between lipid droplets promotes the hydrolysis of triglycerides in the lipid droplets into fatty acids and glycerol, which are then transported into theMt (98). In this process, nicotinamide adenine dinucleotide kinase (NADK) regulates fatty acid synthesis by maintaining the intracellular coenzyme nicotinamide adenine dinucleotide phosphate levels, thereby controlling lipid storage and metabolic homeostasis in lipid droplets (98). The knockdown of NADK affects the levels of acetyl-CoA, thereby regulating the acetylation modification of the key transcription factor PGC-1α and MB and influencing mitochondrial function and number by affecting CL synthesis (99). A Study found that mitochondrial Signal Transducer and Activator of Transcription 3 (STAT3) could reduce the ubiquitination and degradation of carnitine palmitoyltransferase 1a, thereby inhibiting fatty acid oxidation metabolism and reducing oxidative stress formation (100). In stem cells, the fatty acid synthesis regulated by the lipid synthesis enzyme Acetyl-CoA Carboxylase 1 can influence acetylation-mediated Fis1 Ub-proteasomal degradation by consuming Acetyl-Coenzyme A. At the same time, it can produce lipid products that drive the shift of Mt from a dynamic equilibrium to fission, thereby enhancing mitochondrial fission (101).

2.4.3 Amino acid metabolism

Almost all amino acids are synthesized or degraded in the Mt (102). Impaired amino acid metabolism is associated with primary mitochondrial diseases and mitochondrial dysfunction disorders. For example, abnormalities in branched-chain amino acid metabolism are closely related to the progression of diseases such as diabetes (103), atherosclerosis (104), and cancer (105). Wang T et al. (106) conducted experiments on amino acid starvation in tumor cells, assessing the levels of K48-polyubiquitinated proteins in cultured cells after starvation. They found that short-term starvation promoted protein ubiquitination, but after prolonged treatment, there was a significant decrease. This suggests that early amino acid depletion promotes protein ubiquitination, while later stages lead to the degradation of these polyubiquitinated proteins. This phenomenon may be related to the energy depletion induced by amino acid starvation, which in turn triggers mitochondrial autophagy. Current research generally considers the UPS and autophagy-lysosome systems to have distinct functions, but the two systems can interact and influence each other. Amino acid starvation may simultaneously affect the UPS (107), protease activity in mTOR-inhibited Human Embryonic Kidney 293 cells, and polyubiquitination of the 26S proteasome (108). These pathways can all lead to autophagy to varying extents, with amino acid starvation being an important ubiquitination-mediated mechanism for regulating mitochondrial homeostasis (109).

The mechanistic target of rapamycin complex 1 (mTORC1) is a serine/threonine kinase that integrates various environmental signals to regulate cell growth and metabolism. Activation of mTORC1 requires binding to the lysosome through the Ragulator-Rag complex. One essential component of Ragulator, mTOR Activator 1 (LAMTOR1), undergoes dynamic ubiquitination modifications in response to the abundance of amino acids. The E3 ligase TRAF4 directly interacts with Late Endosomal/Lysosomal Adaptor and MAPK and LAMTOR1 and catalyzes polyubiquitination at the K151 site with K63 linkages. This ubiquitination promotes the binding of LAMTOR1 to Rag GTPases and enhances the activation of mTORC1 (110).

2.4.4 ETC

ETC in Mt consists of a series of protein complexes (NADH dehydrogenase, succinate dehydrogenase, cytochrome c reductase, and cytochrome c oxidase) located on the IMM. These complexes are responsible for transferring electrons from one complex to another, ultimately transferring electrons to oxygen, resulting in the formation of water (111). Ubiquitination plays a role in the ETC mechanism, primarily through the targeting of specific proteins. The epigenetic regulator Unfolded Protein Response Factor 1 modulates K27 ubiquitination through NLRP14, thereby maintaining the stability of the mitochondrial Na+/Ca2+ exchanger protein Mitochondrial Sodium/Calcium/Lithium Exchanger, which ensures the stability of mitochondrial morphology and function (112). The contact sites between the endoplasmic reticulum membrane and the mitochondrial membrane, known as mut-associated membranes, represent multifunctional microdomains involved in mitochondrial homeostasis (113). Mt-associated membrane-specific E3 Ub ligases can ubiquitinate nascent proteins, thereby activating TANK binding kinase 1 on the Mt-associated membrane (114), leading to the degradation of ribosomal proteins and disrupting the overall mitochondrial homeostasis. Meanwhile, the Mt-associated membrane is closely linked to Ca2+ homeostasis. Upon ubiquitination of inositol 1,4,5-trisphosphate receptor type 2, the mitochondrial outer membrane protein Fundc1 loses its binding site, promoting increased mitochondrial Ca2+, mitochondrial fragmentation, and apoptosis (112).

In addition, RNA molecules also have a significant association with ETC. BDNF-AS is a natural antisense long non-coding RNA of brain-derived neurotrophic factor (BDNF) (115). The expression of BDNF-AS is significantly positively correlated with Voltage dependent anion channel 3 (VDAC3) expression. Previous studies have shown that VDAC3 influences cellular ferroptosis by regulating mitochondrial iron ion flux (116), but the mechanism by which BDNF-AS regulates VDAC3 expression is still unclear. Circular RNA Microtubule crosslinking factor 1 can promote its development by inhibiting the Ub-mediated degradation of the mitochondrial protein complement C1q binding protein and mediating β-catenin activation (117). Under various stress-induced cellular senescence conditions, the expression of SIRT1

protein is down-regulated through Ub-mediated proteasomal degradation (118), a process typically involving Ub-dependent proteasomal degradation. However, some studies suggest that DNA damage-induced cellular senescence follows the autophagosome-lysosome pathway, which may be linked to mitochondrial homeostasis imbalance caused by DNA damage, leading to a decline in the NAD+-dependent biological function of SIRT1, affecting the ubiquitination binding process (119).

The mechanism of interaction between ubiquitination and deubiquitination targeting MH is shown in Figure 2 and Table 1.

3 Ubiquitination targeting MH in the progression of GI tumors

3.1 HCC

In HCC, In HCC, ubiquitination targeting the MH mechanism with several therapeutic agents was found (Table 2). PINK1/Parkin-mediated mitophagy plays a crucial role. Cyclin-dependent kinase 9 (CDK9) activates SIRT1 and promotes the stabilization of PINK1 protein through its mediated deacetylation, but this effect is blocked by Wogonin (122). Matrine, a traditional Chinese medicine (TCM), triggers mitochondrial dysfunction and induces the upregulation of PINK1/Parkin through phosphatase and PTEN. The elevation of the p62 and LC3-II/I ratio suggests that Matrine acts as both an inducer of autophagy and an inhibitor of autophagosome-lysosome formation, while the blockade of autophagy promotes Matrine-induced cell death (124). The TCM Quercetin (123) upregulates the expression of PINK1/Parkin in Huh7 and Hep3B cells, thereby exerting its anti-cancer effects in HCC. The hepatitis B virus (HBV)-encoded X protein (HBx) plays a key role in inducing HCC (129). Studies have found that thyroid hormone (TH) induces the ubiquitination of mitochondrial-related HBx through PINK1/Parkin and triggers selective mitophagy, thereby inhibiting HBx-promoted ROS and carcinogenesis (125). Lipid metabolism disorder is one of the important characteristics of HCC. Ubiquitin conjugating enzyme E2 O (UBE2O), as an E2 enzyme, has been found to promote HCC progression with high expression. Meanwhile, UBE2O interacts with the mitochondrial β-oxidation enzyme and mediates its ubiquitination and degradation, thereby regulating lipid metabolism reprogramming under the action of E2 and E3 enzyme activities (126). Studies have shown that the VDAC1 inhibitor Novobiocin can reduce the mono-ubiquitination level of VDAC1 K274, and subsequent mutation of this site weakens the interaction between Hsp90α-VDAC1, increases the oligomerization of VDAC1, and thus affects the progression of HCC (120). Crosstalk between some forms of cell death has also been found in HCC. During ferroptosis, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is primarily localized to the Mt, but after treatment with pyroptosis inducers, HMGCR translocates to the endoplasmic reticulum. BRCA1/BRCA2-containing complex subunit 36 deubiquitinates HMGCR through DUB activity and inhibits ferroptosis while promoting pyroptosis (127). Transarterial chemoembolization (TACE) is the main treatment method for advanced liver cancer, but postoperative hypoxia can easily worsen the patient's condition. The hypoxic environment caused by TACE leads to overexpression of S100 calcium-binding protein A9, which forms trimers as a penta-glycine motif protein, triggers mitochondrial fission and ROS production through the deubiquitination and stabilization of PGAM5, and ultimately promotes the growth and metastasis of HCC (121).

3.2 PAAD

In PAAD, the enhancement of ferroptosis or drug sensitivity through ubiquitination has garnered attention (Table 3). Due to the dense stroma of PAAD tumors, most tumor-targeting drugs are not sensitive to PAAD treatment (136). As a first-line oncology drug, gemcitabine exhibits a significant chemoresistance phenomenon in pancreatic cancer (137). Stomatin like 2 (STOML2), also known as SLP-2, is a protein located in the mitochondrial inner membrane that participates in maintaining mitochondrial stability (138). Studies have found (113) that increased expression levels of STOML2 can stabilize PARL, thereby preventing PINK1-dependent mitophagy induced by gemcitabine (131). In mammals, ULK1 is a key component of the autophagy initiation complex (139). In PAAD, the E3 ligase NEDD4 like E3 ubiquitin protein ligase NEDD4L binds to ULK1 and is involved in its ubiquitination regulation. In NEDD4L knockout cells, genetic or pharmacological inhibition of ULK1 or Solute Carrier Family 1 Member 5 (SLC1A5/ASCT2) can sensitize PAAD cells, especially under nutrient deprivation conditions (135). Ferroptosis activator imidazole erastin (IKE) upregulates its E3 Ub ligase RANBP2-type and C3HC4-type zinc finger containing 1 (RBCK1), and the knockdown of RBCK1 enhances the cytotoxic effect of IKE on PAAD cells (134). This is attributed to the interaction between RBCK1 and MFN2, leading to polyubiquitination and promoting proteasomal degradation under ferroptotic stress, which results in reduced ROS and lipid peroxidation. Another study indicates that the PINK1-PARK2 pathway-mediated degradation of SLC25A37 and SLC25A28 increases mitochondrial iron accumulation, leading to HIF-1α dependent Warburg effect and AIM2 inflammasome activation in tumor cells, promoting the release of high mobility group box 1 and further inducing the expression of CD274/PD-L1 (133). Galactan RN0D, isolated from the TCM Sanqi, has been identified as an activator of the PINK1/Parkin pathway, ultimately activating cytotoxicity in tumor cells (132). Ubiquitination-mediated Mt gene transcription has also been observed in PAAD. As a core component of endoplasmic reticulum-associated protein degradation, the HRD1-SEL1L complex, when increased, reduces the stability of the mitochondrial protein AlkB homolog 1, leading to impaired transcription of mitochondrial DNA-encoded genes (140). The SIRT4 agonist entinostat reverses this process (130), possibly by deacetylating lysine 547 of SEL1L and increasing the protein levels of HRD1.

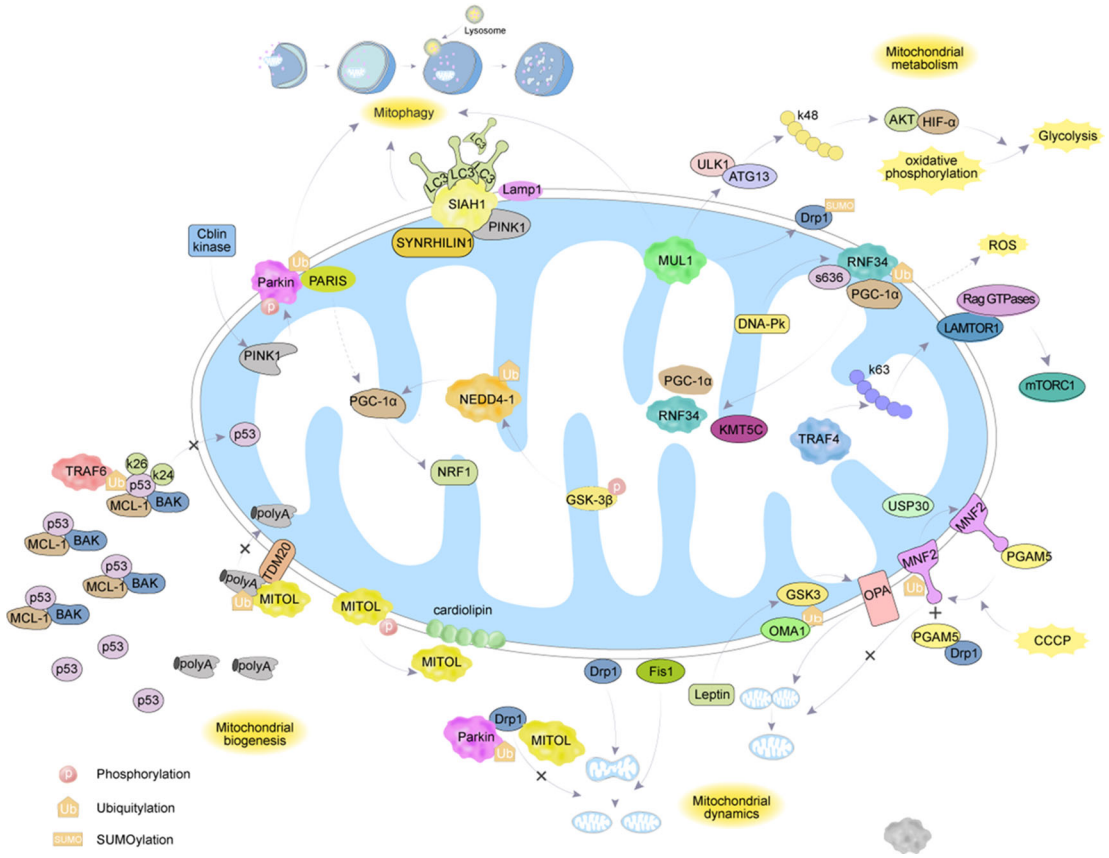

FIGURE 2
The regulatory mechanisms of ubiquitination enzymes and deubiquitination enzymes on MH.

3.3 CRC

In CRC, several ubiquitination mechanisms have also been identified (Table 4). The p53 is one of the most important tumor suppressors, which inhibits the formation and development of tumors by regulating the expression of various genes, including those that promote cell cycle arrest and apoptosis (151). In CRC, p53 ubiquitination plays a significant role. A protein named UBX domain-containing protein 2A promotes the carboxy-terminal ubiquitination of Mortalin-2 in an Hsp70 interaction-dependent manner, reducing Mortalin-2 levels, which not only inactivates p53 but also directly promotes tumor cell invasion and migration (144). The mitochondrial antiviral signaling (MAVS) protein promotes p53-dependent cell death in response to DNA damage (143). MAVS is underexpressed in CRC and inhibits p53 ubiquitination by blocking the formation of the p53-MDM2 complex (143). Lipoic

TABLE 1 Ubiquitinating enzymes targeting MH.

Ub/DUB	Full name	Type
SIAH1	Siah E3 Ubiquitin Protein Ligase 1	E3 ubiquitin ligase
Parkin	Parkin rbr E3 ub protein Ligase	E3 ubiquitin ligase
MUL1	Mitochondrial Ubiquitin Ligase Activity Factor 1	E3 ubiquitin ligase
RNF34	Ring Finger Protein 34	E3 ubiquitin ligase
NEDD4-1	Neural Precursor Cell Expressed, Developmentally Down-regulated Protein 4-1	E3 ubiquitin ligase
MITOL/MARCH5	Mitochondrial ubiquitin ligase/Membrane-Associated RING-CH-type finger protein	E3 ubiquitin ligase
TRAF6	TNF receptor-associated factor 6	E3 ubiquitin ligase
TRAF4	TNF receptor-associated factor 4	E3 ubiquitin ligase
USP30	Ubiquitin-specific Protease 30	DUBs

TABLE 2 Ubiquitination targeting the MH mechanism in HCC.

MH	Medicine	Targets	Ubiquitination	Deubiquitination	Main findings	References
MB	/	Hsp90	VDAC1(K274)	/	Hsp90 promotes cell apoptosis associated with VDAC1 oligomerization by reducing VDAC1 protein K274 monubiquitination	(120)
MK	/	S100A9	/	PGAM5	As a scaffold, S100A9 recruits ubiquitin-specific peptidase 10 and phosphoglycerate mutase family member 5 (PGAM5) to form a trimer, causing deubiquitination and stabilization of PGAM5, leading to mitochondrial fission and reactive oxygen species production, thereby promoting HCC growth and metastasis	(121)
Mitophagy	/	CDK9 targets SIRT1-FOXO3-BNIP3	PINK1-Parkin	/	CDK9 inhibition blocks the initiation of PINK1-PRK1-mediated mitophagy by regulating the SIRT1-FOXO3-BNIP3 axis and enhances the therapeutic efficacy of treatments involving mitochondrial dysfunction in HCC	(122)
	Quercetin	SIRT1	PINK1-Parkin	/	Quercetin up-regulates the expression of PINK1 and PARK2, which are the regulators of mitophagy, and enhances the colocalization of mitochondria and lysosomes to promote autophagy	(123)
	Sanguinarine	/	PTEN-PINK1-Parkin	/	Sanguinarine promoted mitochondrial apoptosis by blocking mitophagy through PINK1-Parkin	(124)
	TH	HBx	PTEN-PINK1-Parkin	/	TH simultaneously induces mitochondrial biogenesis and HBx-targeted mitochondrial autophagy, thereby inhibiting HBx-promoted ROS and carcinogenesis	(125)
MM	/	HADHA	UBE2O	/	UBE2O promotes lipid metabolism reprogramming and liver cancer progression by mediating HADHA ubiquitination	(126)
	/	BRCC36	/	HMGCR	BRCC36 deubiquitinates HMGCR through the activity of deubiquitinating enzymes, as well as inhibiting ferroptosis and promoting pyroptosis. In addition, BRCC36 acts as an oncogene in HCC, promoting cancer cell proliferation, migration, invasion, and tumor growth	(127)
	Ponicidin	Keap1-PGAM5	PGAM5	/	ponicidin targets Keap1 and promotes the formation of the Keap1-PGAM5 complex, ubiquitinizes PGAM5, and activates the cysteine-dependent mitochondrial pathway, leading to mitochondrial damage and ROS production	(128)

acid (LA) is a dithiol compound with redox activity and is an essential cofactor for mitochondrial oxidative decarboxylation (152). The p53 is found to be ubiquitinated and degraded by the proteasome mechanism after LA treatment, a process that does not involve the MDM2. Interestingly, the combined application of LA and anticancer drugs (doxorubicin, 5-fluorouracil) attenuates the stabilization of p53-mediated p21 and exerts a synergistic cytotoxic effect on CRC cells in a p53-dependent manner (153). Dihydroartemisinin downregulates the expression of the mitochondrial inner membrane scaffold protein anti-proliferative protein 2 in a Ub-dependent manner and blocks the downregulation of p53 and p21, thereby enhancing the cytotoxicity of oxaliplatin in CRC (154). TRAF6 promotes the K63-linked ubiquitination of p53 at K24 in the cytoplasm to limit the interaction between p53 and MCL-1/BAK, thereby restricting the mitochondrial translocation of p53 and spontaneous apoptosis. Additionally, TRAF6 promotes the K63-linked ubiquitination and transactivation of nuclear p53 by recruiting p300 to acetylate p53 (35). The PINK1/Parkin pathway has also been identified in CRC. SIRT3 is highly expressed in CRC with mitochondrial dysfunction, leading to PINK1/Parkin-mediated mitophagy. Targeting histone H2Aub ubiquitination at K119 reduces it, thereby enhancing DNA damage repair induced by radiation (147). Delta-valentine, as an emerging dietary metabolite, targets SIRT3 to participate in the process (146), while Aloe Gel Polysaccharides mediate the PINK1/parkin pathway in a ROS-dependent manner (148). Non-coding RNA targeting ubiquitination to regulate mitochondrial homeostasis mechanisms in CRC has been discovered for the first time. Non-coding RNA piR-823 interacts with PINK1, promoting its ubiquitination and proteasome-dependent degradation, thereby alleviating mitophagy,

TABLE 3 Ubiquitination targeting the MH mechanism in PAAD.

MH	Medicine	Targets	Ubiquitination	Deubiquitination	Main findings	References
MB	Entinostat	SIRT4	SEL1L-HRD1-ALKBH1	/	SIRT4 deacetylates lysine 547 of SEL1L and increases protein levels of the E3 ubiquitin ligase HRD1. Increased SEL1L-HRD1 complex decreases the stability of the mitochondrial protein ALKBH1. Upon down-regulation of ALKBH1, transcription of mitochondrial DNA-encoding genes is blocked, leading to mitochondrial damage.	(130)
Mitophagy	Gemcitabine	STOML2	PARL/PINK1	/	STOML2 regulates autophagy through the PARL/PINK1 pathway, thereby reducing the chemoresistance of pancreatic cancer. Overexpression of STOML2 as a targeted therapy may help sensitize gemcitabine in the future.	(131)
	RN0D	/	PTEN-PINK1-Parkin	/	RN0D is identified as an activator of the PTEN-induced kinase 1 (PINK1)/Parkin pathway, ultimately activating cytotoxic mitophagy in tumor cells.	(132)
MM	/	SLC25A37/ SLC25A28	PINK1-PARK2	/	The PINK1-PARK2 pathway mediates the degradation of SLC25A37 and SLC25A28, increasing mitochondrial iron accumulation, leading to HIF1A-dependent Warburg effect and AIM2-dependent inflammasome activation in tumor cells. AIM2-mediated HMGB1 release further induces the expression of CD274/PD-L1. Therefore, in PINK1-/- and PARK2-/- mice, pharmacological administration of mitochondrial iron chelators, anti-HMGB1 antibodies, or genetic knockout of Hif1a or Aim2 can protect against the development of pancreatic tumors. Low expression of PARK2 and high expression of SLC25A37 and AIM2 are associated with poor prognosis in patients with pancreatic cancer.	(133)
	Erastin	MFN2	RBCK1	/	The ferroptosis activator erastin (IKE) induces the upregulation of E3 ubiquitin ligase RBCK1 expression in PDAC cells at the transcriptional or translational level. *In vitro*, knockdown or absence of RBCK1 makes PDAC cells more susceptible to IKE-induced ferroptosis. In a mouse xenograft model, RBCK1 gene knockout increases the killing effect of ferroptosis inducers on PDAC cells. Mechanistically, RBCK1 interacts with and polyubiquitinates the key regulator of mitochondrial dynamics, mitofusin 2 (MFN2), to promote its proteasomal degradation under ferroptosis stress, leading to reduced mitochondrial ROS generation and lipid peroxidation.	(134)
	/	ULK1	NEDD4L	/	NEDD4L can ubiquitinate and degrade ULK1. After knockdown of NEDD4L, the autophagy activity in cells is enhanced, and the cellular oxygen consumption rate and mitochondrial membrane potential increase, maintaining the fusion state of mitochondria to cope with metabolic stress.	(135)

a mechanism reversed by Ant-823, which promotes Parkin activation (145). Regulation of the ETC and MM has also been identified in CRC. Receptor-interacting protein kinase 1 (RIPK1) interacts with the mitochondrial calcium uniporter (MCU), promoting cell proliferation by increasing mitochondrial calcium uptake and energy metabolism. The ubiquitination site of RIPK1 (RIPK1-K377) is a key site for interaction with MCU and the promotion of cell proliferation (150). As a molecular chaperone of Hsp90, Tumor necrosis factor receptor-associated protein 1 regulates the glycolytic enzyme phosphofructokinase-1 (PFK1) to maximize lactate production, balancing low OXPHOS. This depends on the interaction between TRAP1 and PFK1, which favors the glycolytic activity of PFK1 and prevents its ubiquitination/degradation (155). The interaction between membrane glycoprotein CD36 and glypican 4 (GPC4) induces proteasome-dependent ubiquitination and degradation of GPC4 in CRC cells, reducing the high addiction of

TABLE 4 Ubiquitination targeting the MH mechanism in CRC and IBD.

Cancer	MH	Medicine	Targets	Ubiquitination	Deubiquitination	Main findings	References
CRC	MB	Compound 8	PKM2	VDAC3	/	Succination-mediated translocation of PKM2 to mitochondria under glucose starvation conditions plays a role in the cell's switch from a proliferative mode to a survival mode, and vice versa. Mitochondrial PKM2 inhibits ubiquitination-mediated degradation of VDAC3 and increases mitochondrial permeability, producing more ATP to maintain cell survival under nutrient depletion. In human colorectal cancer, the upregulation of mitochondrial PKM2 is positively correlated with the upregulation of VDAC3.	(141)
		/	TRAF6	p53	/	TRAF6 promotes the ubiquitination of p53 in the cytoplasm, thereby limiting the mitochondrial translocation of p53 and spontaneous apoptosis.	(35)
		/	survivin and cIAP1	/	USP36	USP36 binds to and removes the lysine-11 linked ubiquitin chains of cIAP1 and the lysine-48 linked ubiquitin chains of survivin, thereby preventing protein degradation. Overexpression of USP36 can disrupt the formation of the XIAP-second mitochondrial-derived caspase activating factor (SMAC) complex and promote the ubiquitination of receptor-interacting protein kinase 1 (RIPK1), confirming that USP36 can inhibit both intrinsic and extrinsic apoptosis by deubiquitinating survivin and cIAP1.	(142)
		/	MDM2	p53	/	The MAVS protein can inhibit the ubiquitination of p53, promoting p53-dependent cellular damage.	(143)
		veratridine	mot-2-CHIP	UBXN2A	/	UBXN2A promotes the carboxy-terminal ubiquitination of mot-2 that is dependent on the HSP70-interacting protein (CHIP). UBXN2A increases the proteasomal degradation of mot-2. Subcellular compartmentalization experiments show that UBXN2A can reduce the expression levels of mot-2 and its chaperone HSP60. The existence of a multi-protein complex of UBXN2A, CHIP, and mot-2 indicates that UBXN2A and CHIP have synergistic tumor suppressor activity in tumors enriched with mot-2.	(144)
	Mitophagy	/	piR-823	PINK1	/	piR-823 inhibits PINK1-mediated mitophagy.	(145)
		δVB	/	PINK1-Parkin		δVB activates mitochondrial apoptosis through the PINK1/Parkin pathway, with observed upregulation of PINK1, Parkin, and LC3B protein levels.	(146)

(Continued)

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TABLE 4 Continued

Cancer	MH	Medicine	Targets	Ubiquitination	Deubiquitination	Main findings	References
		/	SIRT3	PINK1-Parkin	/	SIRT3 is highly expressed in colorectal cancer cells with mitochondrial dysfunction, leading to PINK1/parkin-mediated mitophagy.	(147)
		AGP	/	PINK1-Parkin	/	AGP induces cytotoxic mitophagy in CT26 cells through the ROS-associated PINK1/Parkin pathway and TFEB activation.	(148)
	MM	/	CD36	GPC4	/	cd36 -GPC4相The CD36-GPC4 interaction promotes proteasome-dependent ubiquitination of GPC4, thereby inhibiting the β-catenin/c-myc signaling pathway and downstream glycolytic target genes GLUT1, HK2, PKM2, and LDHA. Furthermore, the knockout of CD36 significantly increased the occurrence of colorectal tumors in inflammation-induced CRC models and ApcMin/+ mouse models.	(149)
		/	RIPK1-MCU	RIPK1(k377)	/	RIPK1-K377 is a key site for interaction with MCU, and upon binding, it promotes cell proliferation by increasing mitochondrial Ca2+ uptake and energy metabolism.	(150)

CRC cells to glucose or glycolytic inhibition through mitochondrial reduction, i.e., reducing the expression of glycolytic target genes GLUT1, HK2, PKM2, and LDHA, thereby inhibiting the energy metabolism and growth of tumor cells (149). Deubiquitination is the reverse process of ubiquitination (142). USP36 exerts its pro-apoptotic function by targeting cIAP1 and survivin, and it has been found that USP36 can be degraded through polyubiquitination, although the E3 ligase responsible for this process remains unidentified (156).

IBD is a chronic relapsing inflammatory disorder associated with an increased risk of developing CRC (157). Compared with sporadic CRC, IBD-related CRC typically occurs at a younger age, progresses more rapidly, and has a worse prognosis (158). Therefore, focusing on the pathogenesis of IBD can help prevent the development of CRC in advance. Studies have shown that mitochondrial dysfunction activates pyroptosis through multiple pathways, thereby exacerbating the occurrence of IBD (159). Mitochondrial dysfunction, combined with impaired autophagy, leads to increased ROS levels, which activate the Nucleotide-binding oligomerization domain, Leucine-rich repeat, and Pyrin domain-containing protein 3 (NLRP3) inflammasome and subsequently induce pyroptosis (160). Meanwhile, defects in mitochondrial respiration lead to cellular energy metabolism disorders, making cells more prone to pyroptosis (160). Pyroptosis of intestinal epithelial cells disrupts the intestinal barrier, rendering the gut more susceptible to attacks from pathogens and inflammatory factors. Additionally, the pore-forming action of Gasdermin proteins, which causes cell membrane rupture and the release of large amounts of inflammatory factors (such as IL-1β and IL-18), further intensifies the intestinal inflammatory state (159). Research has found that the E3 Ub ligase gp78 mediates mixed ubiquitination of NLRP3, inhibiting its activity by preventing the oligomerization and subcellular translocation of the nucleotide-binding and oligomerization domain of NLRP3, thereby reducing inflammasome activation and its detrimental effects (161). Parkin-driven mitophagy and inhibition of NLRP3 inflammasome activation in the colon exert a protective effect against DSS-induced colitis in mice (162). Moreover, in IBD, the epigenetic modifier SET and MYND Domain containing protein 5 (SMYD5) regulates toll-like receptor 4 target genes in macrophages at the K20 site (163). This regulation increases the risk of IBD progression to CRC by mediating PGC-1α ubiquitination and degradation through methylation, thereby inhibiting MB.

3.4 GC

For GC patients, clinical drug resistance has always been a limitation in late-stage treatment. X-ray repair cross-complementing 1 (XRCC1) is a key regulator of cisplatin-induced DNA damage and apoptosis (164). Thioredoxin-like 1 mediates cisplatin resistance by negatively regulating the expression of XRCC1 through the UPS (165). Myocyte enhancer factor 2A activates PGC1α transcription and inhibits Kelch-like ECH-associated protein 1, reducing the ubiquitination and degradation of NRF2, thereby regulating ROS levels and mediating GC cisplatin resistance (166). Ferroptosis

resistance is one of the key factors leading to GC drug resistance. Studies have shown that SRY-box transcription factor 13 (SOX13) promotes the protein reshaping of ETC complexes by directly transactivating Supercomplex assembly factor 1, leading to the assembly of supercomplexes, mitochondrial respiration, mitochondrial energetics, and increased chemo- and immuno-resistance (167). Zanamivir restores the ferroptosis resistance phenotype by directly targeting SOX13 and promoting the ubiquitination and degradation of SOX13 mediated by a tripartite motif containing 25 (TRIM25) (167). Research has found that LncRNA BDNF-AS can affect the ubiquitination modification of VDAC3 by FBXW7 by recruiting WD repeat-containing protein 5 (168). The USP7 can stabilize Heterogeneous nuclear ribonucleoprotein A1 in cancer-associated fibroblasts through deubiquitination, leading to increased secretion of exosomal miR-522, thereby inhibiting ferroptosis and promoting acquired drug resistance in GC (169), primarily by targeting arachidonate 15-lipoxygenase and blocking the accumulation of lipid ROS in Mt. In TCM treatment, the compound herbal medicine Huachansu induces apoptosis in GC cells by increasing ROS levels and inhibiting USP activity (170). Mechanisms and targets are shown in Table 5.

3.5 ESCA

In ESCA, OTU deubiquitinase 1 is a deubiquitinating enzyme that regulates the apoptosis-inducing factor (AIF), capable of ubiquitinating AIF at K244, impairing mitochondrial oxidative phosphorylation, and reducing cell viability. Additionally, its deubiquitination at K255 enhances AIF's binding capacity to DNA, promoting the occurrence of parthanatos (171). On the other hand, the E3 Ub ligase Itch plays a significant role in TNF-related

TABLE 5 Ubiquitination targeting the MH mechanism in GC.

MH	Medicine	Targets	Ubiquitination	Deubiquitination	Main findings	References
MB	/	MEF2A	KEAP1-NRF2	/	MEF2A activates the transcription of PGC1, increasing mitochondrial biogenesis. MEF2A inhibits the transcription of KEAP1, reducing the ubiquitination and degradation of NRF2, and activating the KEAP1/NRF2 signaling pathway, thereby regulating reactive oxygen species levels and maintaining the homeostasis of the mitochondrial biogenesis process.	(166)
	Cisplatin	TXNL1	XRCC1	/	XRCC1 is a key regulator of cisplatin-induced DNA damage and apoptosis. TXNL1, a member of the thioredoxin family, negatively regulates the expression of XRCC1 through the ubiquitin-proteasome pathway.	(165)
MM	/	BDNF-AS-WDR5-FBXW7	VDAC3	/	BDNF-AS regulates the expression of FBXW7 by recruiting WDR5, thereby affecting the transcription of FBXW7; FBXW7 ubiquitinates and regulates the protein expression of VDAC3.	(168)
	Zanamivir	SOX13	TRIM25	/	Zanamivir targets SOX13 and promotes the ubiquitination and degradation of SOX13 mediated by TRIM25, inhibiting the assembly of the mitochondrial respiratory chain supercomplex and restoring ferroptosis sensitivity.	(167)
	Cisplatin and paclitaxel	mir-522-ALOX15	/	hnRNPA1(USP7)	hnRNPA1 is found to mediate the packaging of miR-522 into exosomes, and USP7 stabilizes hnRNPA1 by deubiquitination. Cisplatin and paclitaxel promote the secretion of miR-522 from cancer-associated fibroblasts (CAFs) by activating the USP7/hnRNPA1 axis, leading to the suppression of ALOX15 and a reduction in lipid-ROS accumulation in cancer cells, ultimately leading to decreased chemotherapy sensitivity.	(169)
	CCMH	PI3K/Akt and MAPK	UPS	/	CCMH affects the ROS pathway, ubiquitin-proteasome system, PI3K/Akt, and MAPK signaling pathways. CCMH significantly increases the level of ROS in gastric cancer cells, and NAC can reverse the effect of CCMH on ROS levels in gastric cancer cells. NAC antagonizes the apoptotic induction of CCMH. CCMH can significantly reduce the activity of the 20S proteasome in gastric cancer cells. CCMH also regulates the expression of key proteins in the PI3K/Akt and MAPK signaling pathways.	(170)

apoptosis-inducing ligand-mediated apoptosis in ESCA. Knockdown of Itch leads to resistance to TNF-related apoptosis-inducing ligand-mediated apoptosis and significantly alters mitochondrial morphology, increasing mitochondrial cholesterol content. High cholesterol levels reduce membrane fluidity, further intervening in mitochondrial dynamic homeostasis (172). Apart from cholesterol, proteins are also an important factor affecting mitochondrial dynamic homeostasis. Syntaphilin (SNPH) is a static mitochondrial anchor protein primarily expressed in the brain, playing a crucial role in neurotransmitter release and MK. In various tumor cells, SNPH is downregulated or even silenced, leading to the redistribution of Mt from the perinuclear area to the cell periphery, resulting in increased tumor cell migration and invasion (173). Studies have found that CUL1 can ubiquitinate SNPH, disrupting mitochondrial dynamic homeostasis and promoting tumor metastasis and radioresistance (174). In the transformation of ESCA keratinocytes, cells with high CD44 expression exhibit a series of mitochondrial autophagy characteristics: mitochondrial fragmentation, reduced mitochondrial content, and Parkin mitochondrial translocation (175). Mechanisms and targets are shown in Table 6.

3 Discussion

In summary, we have summarized the various aspects of MH regulation by different ubiquitinases and deubiquitinases in current scientific research, including MB, mitophagy, and the MM involved in these processes. In gastrointestinal tumors, through the ubiquitination regulation of MH, we have discovered different cell death crosstalk mechanisms, such as mitophagy, ferroptosis, and apoptosis, tumor drug resistance mechanisms, metabolic reprogramming, and new targets for TCM treatment. However, the specific roles of these mechanisms and the potential crosstalk between signaling pathways in different tumor types have not yet been fully elucidated. Clinical treatment results suggest that single-target therapies may not be sufficient for gastrointestinal tumors, and the development of new drugs is needed. Proton beam therapy (PBT) has been shown to inhibit colon cancer metastasis by stimulating mitochondrial biogenesis through the upregulation of PGC-1α and its co-transcription factors (NRF1α/ERRα). Additionally, compounds like hydroxytyrosol (HTyr) can promote mitochondrial biogenesis by increasing PGC-1α expression, offering potential as adjuvant anticancer agents. Advances in gene-editing technologies, such as CRISPR/Cas9, offer the potential to directly target mitochondrial dysfunction by correcting genetic defects or modulating key regulatory pathways. Although clinical translation is still in its infancy, preclinical studies have demonstrated the feasibility of these approaches. In addition, current research has not determined the mechanisms by which ubiquitination regulation of MH is involved in the immune evasion of gastrointestinal tumors, which may require further experimental data support.

TABLE 6 Ubiquitination targeting the MH mechanism in ESCA.

MH	Medicine	Targets	Ubiquitination	Deubiquitination	Main findings	References
MK	/	CREB-SNPH	UPS	/	Ubiquitin-proteasome degradation and histone modification promote the downregulation of SNPH in RR ESCC cells. Dephosphorylation of CREB promotes the re-expression of SNPH, which induces radiosensitization. Moreover, the expression of SNPH is related to the radiotherapy efficacy in esophageal squamous cell carcinoma and is an independent prognostic factor for patients with esophageal squamous cell carcinoma.	(174)
Mitophagy	/	CD44	Parkin	/	Cells with high CD44 expression exhibit a series of mitochondrial autophagy characteristics: mitochondrial fragmentation, reduced mitochondrial content, and Parkin translocation to mitochondria.	(175)
MM	/	OTUD1	/	AIF(K244)	OTUD1 can deubiquitinate AIF at position K244, disrupt mitochondrial structure, and impair OXPHOS, promoting the function of AIF in mitochondrial respiration, and inducing a shift in cellular metabolism towards glycolysis.	(171)
	/	COP1/ ZRANB1	MITF	/	COP1 and ZRANB1 jointly regulate the ubiquitination status of MITF, maintaining the stability of mitochondrial structure and function.	(176)
	/	Itch	STARD1	/	After Itch is knocked out, the morphology of mitochondria changes significantly, and cholesterol content increases. Itch may stabilize STARD1, increase the input of cholesterol to mitochondria, thereby inhibiting Bax activation and the release of cytochrome c.	(172)

Author contributions

BH: Conceptualization, Writing – review & editing, Writing – original draft. YY: Writing – original draft. JL: Investigation, Writing – original draft. BZ: Investigation, Visualization, Writing – review & editing. NL: Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

MH	Mitochondrial Homeostasis
MB	Mitochondrial Biogenesis
MM	Mitochondrial Metabolism
MK	Mitochondrial Kinetics
GI	Gastrointestinal
Mt	Mitochondria
mtDNA	Mitochondrial DNA
HCC	Hepatocellular Carcinoma
ESCA	Esophageal Cancer
PAAD	Pancreatic Cancer
GC	Gastric Cancer
CRC	Colorectal Cancer
ROS	Reactive Oxygen Species
BCL-2	B-cell lymphoma/leukemia-2
IBD	Inflammatory Bowel Disease
Ub	Ubiquitin
DUBs	Deubiquitinating enzymes
USP	Ub-specific protease
UPS	Ubiquitin-Proteasome System
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1 alpha
OXPHOS	Oxidative Phosphorylation
NRFs	Nuclear Respiratory Factors
MITOL1/MARCH5	Mitochondrial ubiquitin ligase/Membrane-Associated RING-CH-Type Finger 5
PolγA	DNA Polymerase Gamma Catalytic Subunit
TRAF6	Tumor Necrosis Factor Receptor-Associated Factor 6
SIRT1	Silent information regulator 1
ATP	Adenosine Triphosphate
MMP	Mitochondrial Membrane Potential
GSK-3β	Glycogen synthase kinase 3β
Tom20	Translocase of the outer mitochondrial membrane 20
MCL-1/BAK	myeloid cell leukemia-1/BCL-2 Antagonist 1
p53	Tumor Protein 53
K24	Lysine 24
CL	Cardiolipin
PA	phosphatidic acid
OMM	Outer Mitochondrial Membrane
IMM	Inner Membrane Membrane
NEDD4-1	Neural Precursor Cell Expressed, Developmentally Down-regulated Protein 4-1
MDM35	Mitochondrial Distribution and Morphology Protein 35
PINK1	PTEN induced putative kinase 1
Parkin	Parkin rbr E3 ub protein Ligase
GTPase	Guanosine Triphosphatase
Drp1	Dynamin-related protein 1
MFN1	Mitofusin 1
MFN2	Mitofusin 2
OPA1	Optic Atrophy 1
Fis1	Mitochondrial fission factor
Gβ2	G-protein β2
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
PGAM5	Phosphoglycerate mutase 5
CUL1	Cullin1
FBX	F-box protein
Fzo1	Fusion of Mitochondria Protein 1
6-OHDA	6-hydroxydopamine
IKKγ	Inhibitor of Nuclear Factor Kappa-B Kinase Subunit Gamma
OMA1	Optic Atrophy 1
MUL1	Mitochondrial E3 ubiquitin protein ligase 1
PARL	Presenilin-Associated Rhomboid-Like
ULK1	Unc-51 Like Autophagy Activating Kinase 1
SIAH1	Seven in Absentia Homolog 1
LC3	Microtubule-associated protein 1 light chain 3
Atg	Autophagy-related
LAMP1	Lysosomal-associated membrane protein 1
SUMOylation	Small Ubiquitin-like Modifier Conjugation
HIF-1α	Hypoxia-inducible factor 1-alpha
ETC	Electron Transport Chain
H2Aub	histone H2A ubiquitination
Hsp27	heat shock protein 27
PFKP	Phosphofructokinase Platelet
HRD1	HMG-CoA Reductase Degradation 1
NADK	Nicotinamide Adenine Dinucleotide Kinase
STAT3	Signal Transducer and Activator of Transcription 3
mTORC1	mechanistic target of rapamycin complex 1
LAMTOR1	mTOR Activator 1
BDNF	brain-derived neurotrophic factor
VDAC3	Voltage dependent anion channel 3
CDK9	Cyclin-dependent kinase 9
TCM	traditional Chinese medicine
HBx	hepatitis B virus -encoded X protein
UBE2O	Ubiquitin conjugating enzyme E2 O
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
TACE	Transarterial chemoembolization
STOML2	Stomatin like 2
IKE	imidazole erastin
RBCK1	RANBP2-type and C3HC4-type zinc finger containing 1
MAVS	mitochondrial antiviral signaling
LA	Lipoic acid
NLRP3	Nucleotide-binding oligomerization domain, Leucine-rich repeat, and Pyrin domain-containing protein 3

RIPK1	Receptor-interacting protein kinase 1
MCU	mitochondrial calcium uniporter
PFK1	phosphofructokinase-1
GPC4	glypican 4
USP36	Ubiquitin specific peptidase 36
XRCC1	X-ray repair cross-complementing 1
SOX13	SRY-box transcription factor 13
AIF	apoptosis-inducing factor
SNPH	Syntaphilin

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