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REVIEW

Next-generation aluminum adjuvants: Immunomodulatory layered double hydroxide NanoAlum reengineered from first-line drugs

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Metal cations

Abstract Aluminum adjuvants (Alum), approved by the US Food and Drug Administration, have been extensively used in vaccines containing recombinant antigens, subunits of pathogens, or toxins for almost a century. While Alums typically elicit strong humoral immune responses, their ability to induce cellular and mucosal immunity is limited. As an alternative, layered double hydroxide (LDH), a widely used antacid, has emerged as a novel class of potent nano-aluminum adjuvants (NanoAlum), demonstrating advantageous physicochemical properties, biocompatibility and adjuvanticity in both humoral and cellular immune responses. In this review, we summarize and compare the advantages and disadvantages of Alum and NanoAlum in these properties and their performance as adjuvants. Moreover, we propose the key features for ideal adjuvants and demonstrate that LDH NanoAlum is a promising candidate by summarizing its current progress in immunotherapeutic cancer treatments. Finally, we conclude the review by offering our integrated perspectives about the remaining challenges and future directions for NanoAlum's application in preclinical/clinical settings.

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1. Introduction

With increasing advancement over the past decades, nanomedicines have shown great potential to improve the standard of care in challenging diseases, including cancers[1−3]. A search for "nanoparticle and cancer" on PubMed yields over 60,000 manuscripts to date. However, compared to the breathtaking publications, only 100 nanomedicines have been successfully translated into commercial clinical products, and just 30 cancer nanomedicines approved globally for clinical applications[4−7]. This limited success is due to numerous challenges that must be addressed for the effective clinical transformation of nanomedicines for cancer treatments[8−13].

Many comprehensive review articles have provided scientific explanations for the failures in translating cancer nanomedicines into clinical practice[14,15]. A widely recognized viewpoint is that the successful clinical transformation of nanomedicines necessitates rigorous scientific research and close interdisciplinary collaboration among scientists, clinicians, regulatory bodies, and industry stakeholders. First, manufacturing nanomedicines is a complex process, even at the laboratory scale, and scaling up to industrial production can lead to significant batch-to-batch variations. Secondly, there is limited understanding of the molecular and cellular mechanisms underlying the interactions between nanomaterials and biological systems, which complicates the approval process for clinical use[11]. Interestingly, more than two-thirds of the approved cancer nanomedicines are based on well-established lipid or liposome formulations[6,14]. The remainders are relatively simple formulations in which small chemotherapeutic drugs are mixed or combined with well-known polymers or proteins (such as albumin). In our opinion, employing straightforward physicochemical modifications to slightly alter existing drug formulations to create new nano-formulations with well-defined structures and components could be a promising strategy to advance nano-drugs from the laboratory to clinical application.

Adjuvants are essential components in vaccine formulation, playing a critical role in enhancing immune responses. Since 1926, aluminum adjuvants (Alum) have been the most widely used ones in vaccines. However, traditional Alums often fail to elicit strong immune stimulation due to restricted local immune responses, their ineffectiveness when paired with certain antigens, and their inability to induce $CD8^+$ cell-mediated immunity. Moreover, traditional Alum adjuvants have concerns about immune disorder potential, such as augmentation of Th2 immune bias with increased immunoglobulin (Ig) E antibody production[16]. In addition, these Alum adjuvants frequently trigger severe inflammatory reactions following subcutaneous injection[17,18]. Thus, there is a pressing need to develop novel Alum that can address these shortcomings.

Layered double hydroxide (LDH), a family of hydrotalcite-like two-dimensional nanomaterials, has emerged as a promising class of novel Alum[19,20]. One of the most well-known commercial LDH products is Talcid, which was developed nearly half a century ago and is widely used as a stomach acid neutralizer[21]. The chemical composition of Talcid closely resembles that of the commercial Imject Alum, a mixture of AlOOH Alum and stomach acid neutralizer $Mg(OH)_2$. Interestingly, through a simple hydrothermal hydrolysis process, Imject Alum can be further reformulated into MgAl-LDH referred to as a novel Nano-Aluminum adjuvant (NanoAlum) and regarded as a powerful adjuvant for cancer immunotherapy[19].

In this review, we aim to explore the engineering of NanoAlums from first-line drugs, providing a comprehensive summary of their performance in immunotherapeutic treatments. We discuss the physicochemical and functional features of ideal Alum adjuvants for cancer immunotherapy, offering insights into their design and biomedical application as novel NanoAlum. Additionally, we present our perspectives on the remaining challenges and opportunities for NanoAlum from preclinical research to clinical applications, helping readers better understand its potential in cancer immunotherapy.

2. Conventional Alum and reengineered NanoAlum

Adjuvant is any material or molecule that enhances the immunogenicity of the target antigen, but should have low toxicity, excellent biodegradability and non-immunogenicity[22]. Different from many other adjuvants, such as oil-in-water emulsions, liposomes, and polymeric particles, Alums are the only inorganic adjuvant for vaccines approved for human use. However, shortcomings such as failure to induce cellular and mucosal immunity and severe inflammations limited their application against major diseases such as tumors. Thus it is pressing to develop next-generation Alum with optimized physicochemical properties, including but not limited to (1) well-defined crystal structure and simple composition with controlled particle size and colloidal stability, (2) sufficient adjuvanticity to induce balanced humoral and cellular immunity, (3) tissue microenvironment immunomodulatory functions by directly targeting B/T lymphocytes or indirectly targeting tissue physicochemical factors.

2.1. *Clinical Alum and analogues*

For almost a century, Alums, such as aluminum hydroxide (AH, which is also known as Alhydrogel) and aluminum phosphate (AP)[23,24], have been approved by the US Food and Drug Administration (FDA) and extensively used in vaccines (Table 1). These vaccines are typically designed for recombinant antigens, subunits of pathogens, and toxins[24,25]. In 2021, the FDA approved two new vaccines assisted by AH, *i.e.*, PreHevbrio® for Hepatitis B and TICOVAC® for Tick-borne encephalitis, and another two adjuvanted by AP against Pneumococcal (PREVNAR 20® and VAXNEUVANCE®)[26]. However, when these Alum-containing vaccines target intracellular microorganisms such as viruses, they are generally perceived as ineffective[26].

Alums exert their immunopotentiation effects by multifaceted mechanisms, including retention of the antigen at the injection site, recruitment of immune cells to the injection site, enhanced antigen uptake, direct and indirect stimulation of dendritic cells (DCs), promotion of specific antibody production, and induction of $CD4^+$ T cell differentiation into T helper 2 (Th2) cells (Fig. 1)[27]. Although the Alum's mechanism of action has not yet been fully established, its adjuvanticity has been closely linked with the formation of antigen depots[26,28]. On one hand, antigens are protected from degradation within the depot while being gradually released for sustained uptake by immune cells[29]. On the

Table 1 FDA-approved aluminum-based vaccines.

Type	Brand name	Approval date	Vaccine/indication	Manufacturer
AH	Biothrax	1970	Anthrax	Emergent Biosolutions
	Engerix-b	1989	Hepatitis B	Glaxosmithkline
	Havrix	1992	Hepatitis A	Glaxosmithkline
	Boostrix	2005	Tetanus toxoid, reduced diphtheria toxoid and acellular pertussis (Tdap)	Glaxosmithkline
	Kinrix	2008	Diphtheria, tetanus, and acellular pertussis (DTaP), Poliomyelitis	Glaxosmithkline
	Cervarix	2009	Human papillomavirus (HPV)	Glaxosmithkline
	Ixiaro	2009	Japanese encephalitis	Valneva Austria Gmbh
	Bexsero	2015	Meningococcal group B	Glaxosmithkline
	Prehevbrio	2021	Hepatitis B	Vbi Vaccines (delaware) Inc.
	Ticovac	2021	Tick-borne encephalitis	Pfizer Ireland Pharmaceuticals
AAHS	Recombivax HB	1986	Hepatitis B	Merck & Co., Inc.
	Pedvaxhib	1989	Haemophilus b	Merck Sharp & Dohme Corp.
	Vaqta	1993	Hepatitis A	Merck Sharp & Dohme Corp.
	Infanrix	1997	DTaP	Glaxosmithkline
	Gardasil	2006	HPV	Merck & Co., Inc.
	Gardasil 9	2014	HPV 9-valent	Merck Sharp & Dohme llc
	Vaxelis	2018	DTaP, Haemophilus b, Poliomyelitis, Hepatitis B, Haemophilus	Merck and Sanofi
AP	Tenivac	2003	Tetanus and diptheria	Sanofi Pasteur
	Adacel	2005	Tetanus Toxoid, Reduced Diphtheria Toxoid and Acellular Pertussis (TdaP)	Aventis Pasteur Limited
	Pentacel	2008	DTaP, Poliomyelitis, Haemophilus influenzae b	Sanofi Pasteur
	Prevnar 13	2010	Pneumococcal	Wyeth Pharmaceuticals, Inc.
	Trumenba	2014	Meningococcal group B	Wyeth Pharmaceuticals, Inc.
	Tdvax	2018	Tetanus and diptheria	Massbiologics
	Prevnar 20	2021	Pneumococcal	Wyeth Pharmaceuticals LLC
	Vaxneuvance	2021	Pneumococcal	Merck Sharp & Dohme LLC
AH/AP	Twinrix	2001	Hepatitis A and hepatitis B	Glaxosmithkline
	Pediarix	2002	DTaP, Hepatitis B (HBV), Poliovirus	Glaxosmithkline

AAHS, amorphous aluminum hydroxyl phosphate sulfate; AH, aluminum hydroxide; AP, aluminum phosphate.

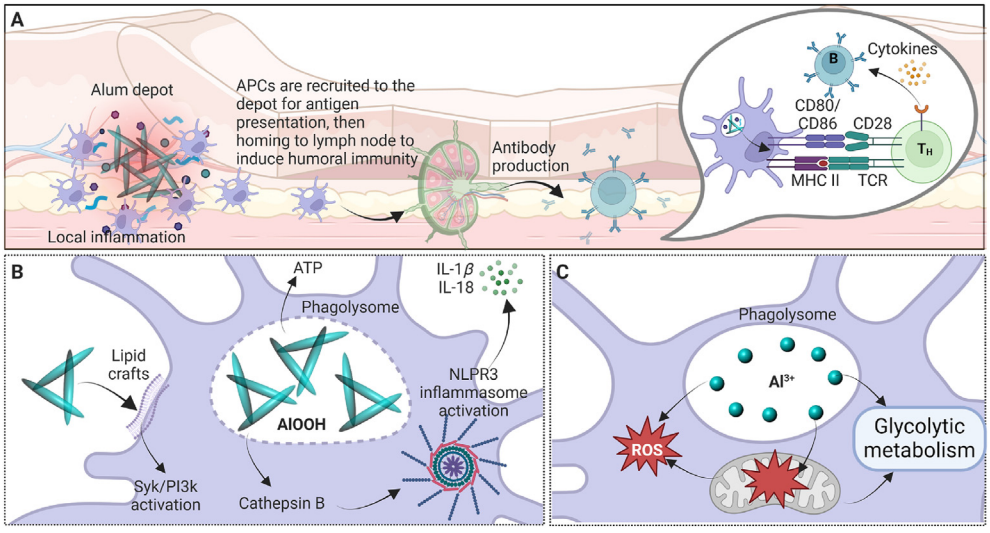

Figure 1 Adjuvant mechanism of conventional Alum. (A) Alum forms a depot at the injection site, facilitating the recruitment of APCs from the periphery for antigen uptake. The internalized antigens are processed within the phagolysosome, complexed with major histocompatibility complex (MHC)-II molecules, and presented on the APC surface, interacting with Th cells to initiate the humoral immune response. (B) Alum interacts with the lipid rafts of APCs, activating Syk and PI3-kinase signaling pathways. Meanwhile, Alum destabilizes and ruptures phagolysosomes, leading to cathepsin B activation. This, in turn, triggers the assembly of the NLRP3 inflammasome and subsequent secretion of cytokines. (C) Al^{3+} released from Alum disrupts phagolysosomal homeostasis, affecting the activation and polarization of APCs. Effects on phagolysosomal pH, ROS formation, membrane stability, and metabolic reprogramming mediate this disruption. Created with BioRender.com.

other hand, Alum particles are sensed by NOD-like receptors (NLRs) *via* interactions with NLRP3 inflammasome, inducing cell apoptosis and releasing cytokines and chemokines to recruit and activate immune cells at the injection site. This may explain the sustained stimulation of the immune responses mediated by Alum. Immunologically, Alums preferentially induce strong Th2 responses, *i.e.*, humoral responses. The immunomodulation triggered by Alums is marked by interleukin (IL)-4 production, which subsequently leads to a dominant Th2 antibody response characterized by IgG1. Alums also release Al^{3+} to attract and regulate the polarization/activation of DCs and macrophages and enhance antigen presentation to B and T lymphocytes[24,30]. However, it has been recognized that Alum cannot initiate effective T helper 1 (Th1) immune responses. In other words, they can only increase the antibody production rather than activate cytotoxic $CD8^+$ T cells[31]. Generally, Alums are generally considered well tolerated and safe due to their long history of use although the local inflammation may persist for up to 8 weeks or may be very severe[32]. Despite this, concerns regarding the safety of Alum, including agglomeration at the vaccination site and local irritation, potential neurotoxicity, and limitations in effectively triggering cellular immunity, have been raised in recent years[24,33]. Thus, overcoming these drawbacks motivates modifications of Alums in the structure and composition.

2.2. *Engineered NanoAlum for better immunotherapy*

In general, Alums interact with antigens *via* electrostatic interactions and H-bonds irregularly. Alum normally consists of fibrous AH or disc-shaped AP particles[34,35], while both form gel-like porous agglomerates in the size range of 1−20 μm, in particular after formulation with antigens[36]. The agglomeration often causes local irritation at the vaccination site. Thus Alum undergoes crystal structure optimization through a reengineering process, which produces AlOOH adjuvant with considerably enhanced humoral immune responses[37]. Reengineering of Alum to AlOOH has produced an innovative adjuvant with improved humoral immunostimulatory properties. Besides, Alum has been improved by modifying the composition. For example, Alhydrogel mixed with clinical antacid $Mg(OH)_2$ results in another commercial Imject Alum, which has been shown to potently promote humoral immune responses and moreover, stimulate a low level of cellular immune responses[25,35].

Although there are limited data available on NanoAlum in clinical trials or applications, several preclinical studies have demonstrated promising results that suggest the potential superiority of NanoAlum over conventional Alum. These studies have shown that NanoAlum exhibits enhanced immunostimulatory properties and leads to improved antigen presentation, increased immune cell activation, and heightened immune responses compared to conventional Alums.

To enhance the adjuvanticity of Alum, the facile approach is to optimize its physical properties. For instance, Sun et al.[37] reengineered the microscale Alum aggregates into nanoscale ones. Their study demonstrated that engineered AlOOH's adjuvanticity was greatly depended on their shape, crystallinity, and hydroxyl content. In comparison to the nanoplates or nanopolyhedra shape, the nanorods like AlOOH with the lowest crystallinity and highest hydroxyl content have shown the most effective in NLRP3 inflammasome activation, resulting in the highest IL-1β production in antigen presenting cells (APCs), thereafter boosting enhanced humoral immune responses in mice. Bi et al.[38] developed a series of rice-shaped AlOOH adjuvants by intrinsically controlled crystallization and atomic coupling-mediated aggregations, which elicited more potent humoral immune responses as the aspect ratios increased. Interestingly, Liang et al.[39] showed that the hydroxide supersaturation level during crystal growth changed the surface free energy of AlOOH nanorods by modulating the aspect ratios, thereby affecting the adjuvanticity of AlOOH. This study showed that AlOOH nanorods with higher aspect ratios exhibited lower surface free energy and more hydrophobic surface, thereby more efficiently promoting DC maturation. As a result, the *in vivo* studies showed that model antigens such as hepatitis B surface antigen or SARS-CoV-2 spike protein receptor-binding domain formulated with such an AlOOH nanoadjuvant successfully elicited more potent humoral immune responses.

Moreover, the nanoscale Alum particles also offer a larger surface area for better interactions with immune cells[40]. Gan et al.[41] fabricated microscale AP to the nanoscale platform to load the tumor cell membrane and CpG, promoting APCs to more efficiently internalize both antigens and CpG while enhancing their lymph node homing efficiency. The nanoscale strategy also improves the immunostimulatory capacity of AlOOH to more potently activate DCs, macrophages and B cells, resulting in better antigen presentation and increasing antibody production and cellular immune responses[42]. Such a modified Alum shows promise in immunotherapy against infectious diseases and cancer by producing antigen-specific antibodies[43]. Li et al.[44] fabricated alpha-alumina nanoparticles to deliver antigens to autophagosomes of dendritic cells, enabling autophagy-dependent cross-presentation. When mice were immunized with alpha-alumina nanoparticles conjugated with either a model tumor antigen or autophagosome derived from tumor cells, tumor regression was observed. Orr et al.[45] utilized a high shear force microfluidizer to extract well-dispersed rod-shaped $Al(OH)_3$ nanoparticles (∼60 nm) from Alhydrogel® and subsequently stabilized with an anionic polymer, polyacrylic acid (PAA) to ensure the stability. The resulting particles were found to enhance Th1 immunity. In an effort to determine whether this immunostimulatory effect is an inherent characteristic of the developed adjuvant, they substituted the negatively charged PAA with neutral polyethylene glycol (PEG) and found that the PEG-nano-Alhydrogel® did not induce significant Th1 responses. Their findings led to the conclusion that the promotion of Th1 immunity was a distinct property of PAA-nano-Alhydrogel®.

Besides, optimizing the chemical composition is another effective strategy to enhance Alum's adjuvant function. As mentioned above, Imject Alum comprises amorphous AlOOH and crystalline $Mg(OH)_2$[46]. In a comparative study with Alum, Imject Alum has been shown to promote relatively weak humoral immune responses[47]. Interestingly, hydrothermal hydrolysis of Imject Alum results in another nanoscale LDH NanoAlum (Fig. 2), which shows much stronger adjuvanicity[35]. In fact, LDH NanoAlum has a composition highly similar to the clinical gastric disease antacid Talcid that is chemically termed as $Mg_6Al_2(OH)_{16}CO_3{\cdot}4H_2O$ or $Mg_{0.75}Al_{0.25}(OH)_2(CO_3)_{0.125}{\cdot}0.5H_2O$ and has been clinically used for nearly 30 years[48,49]. The general formula of LDH is represented as $[Mg^{2+}{}_{1-x}Al_x{}^{3+}(OH)_2]Cl^-{\cdot}mH_2O$ (x denotes the molar ratio of trivalent metal cations to

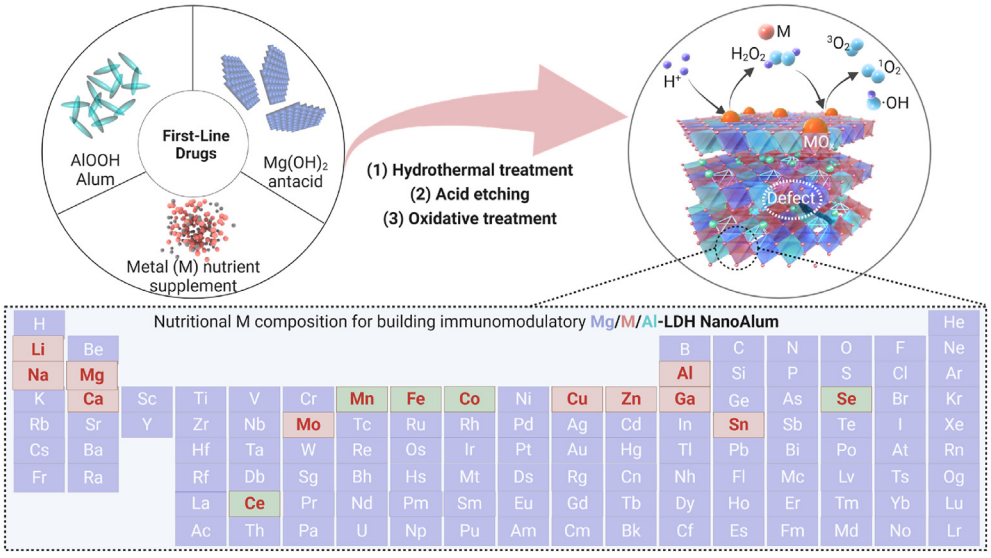

Figure 2 LDH NanoAlum reengineered from first-line drugs. LDH NanoAlum recombined from first-line AlOOH adjuvant, $Mg(OH)_2$ antacid and metal nutritional supplements has a flexible metal composition and controllable crystal structure. Created with BioRender.com.

all cations generally between 0.20 and 0.40). In the early 2000s, we also established a unique one-step hydrothermal treatment method for controllable preparation of LDH by hydrolyzing the Imject Alum-similar salts mixture of $Al(OH)_3$ and $Mg(OH)_2$[50,51]. Interestingly, Mg^{2+} in the LDH hydorxide layers can be controllably substituted by other divalent cations (*e.g.*, Zn^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}) and while Al^{3+} can be replaced by other trivalent cations (*e.g.*, Fe^{3+}, Gd^{3+}, In^{3+}, Cr^{3+}, Ga^{3+}, Ce^{3+}). Cl^- located between the interlayer galleries can be exchanged by other anions (*e.g.*, NO_3^-, CO_3^{2-}, SeO_3^{2-}) or negatively charged organic anions or biomolecules[49,52–55]. Therefore, the chemical composition of LDH NanoAlum is highly flexible, enabling the adjuvanticity of LDH to be controllable and dynamically adjustable according to immune needs.

Similarly, optimization of the physical properties like conventional Alum also enhances the antigen delivery efficiency of LDH NanoAlum. For instance, reduction of the particle size[56] and enhancement of *in vivo* colloidal stability[29] significantly enhanced the delivery efficiency of antigens and activation of DCs within lymph nodes. Compared with conventional Alum, LDH NanoAlum obtained through rational physicochemical properties optimization formed loose vaccine depots at the injection sites[29,57], thereby promoting both potent humoral and cellular immunity, showing great potential for immunotherapy against cancers or infections.

Overall, novel Alum represented by NanoAlum developed with optimized physicochemical properties is believed to greatly enhance the induction of both humoral and cellular immunity. It provides a paradigm for constructing easy-to-fabricate adjuvants with a clear crystal structure and controllable physicochemical properties, bridging the gap to speed up the laboratory manufacture to industrial products in the field of nanomedicine, showing great potential for easier clinical use.

3. The next-generation Alum: LDH NanoAlum

LDH has garnered significant attention in the past two decades for their diverse biomedical applications. Their unique physico-chemical properties, including efficient antigen loading and delivery, weak alkalinity, metal ion exchange capability, and responsiveness to various stimuli, render them promising vaccine adjuvants for immunotherapy (Fig. 3). In this section, we delve deeply into these key features.

3.1. *LDH NanoAlum for enhanced vaccination*

3.1.1. *Features of LDH NanoAlum*

Unlike conventional alums, which lack a well-defined chemical composition and crystalline structure and exist as aggregates of amorphous salts, LDH NanoAlum possesses a well-defined layered structure and known chemical composition. According to other review papers, there is a variety of approaches that have been developed for the preparation of LDH NanoAlum, including co-precipitation, hydrothermal synthesis, reconstruction and ion-exchange, so that the physical and chemical properties (size, surface potential, metal composition, etc.) of LDH NanoAlum are precisely controllable[48,49,52,53,58]. Thus, it exhibits controlled particle size (ranging from 20 to 2000 nm) and colloidal stability, enabling efficient loading and delivery of antigens to lymphoid organs and APCs[50,59–62]. As described previously, LDH is formed by the chemical hydrolysis of a mixture of $Al(OH)_3$ and $Mg(OH)_2$. Doping Al^{3+} into the $Mg(OH)_2$ nanosheets introduces additional positive charges, enabling the adsorption of negatively charged macromolecules like proteins, peptides, DNA plasmids, or RNA onto the LDH surface. Furthermore, anions intercalate within the LDH layers and balance the positive charges in the hydroxide layer, and these anions can be exchanged with other negatively charged small molecules or short RNA/DNA[46]. The unique layered crystal structure of LDH confers a high surface-to-volume ratio, enabling it to load significantly more antigens and immunomodulatory molecules compared to conventional AlOOH and Imject Alum. Notably, LDH can accommodate up to 150% (*w*/*w*) of proteins/peptides and 10%−30% (*w*/*w*) of nucleic acids[46].

The adsorption of biocompatible proteins, electrolytes, polymers, or cell membranes offers a strategy to modulate the *in vivo* colloidal stability of LDH, thereby ensuring efficient delivery of

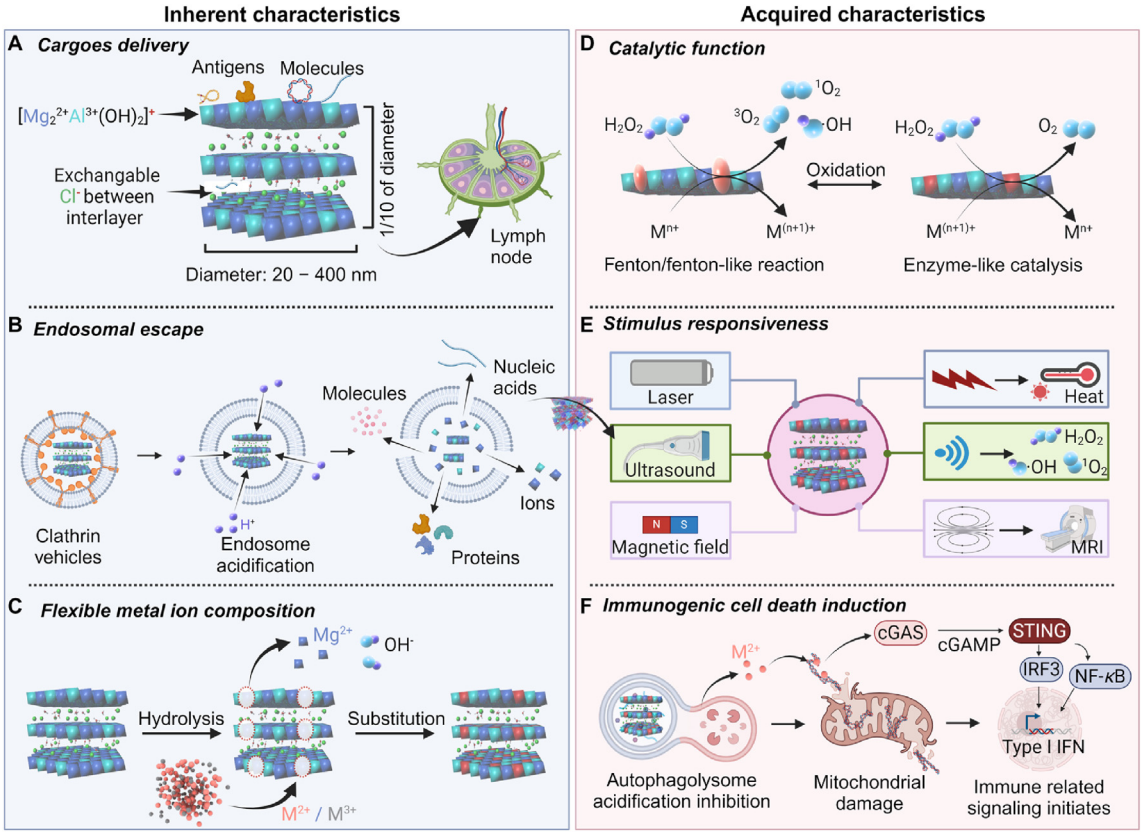

Figure 3 Features of the next generation LDH NanoAlum. (A) The positively charged layers, exchangeable interlayer anions and the adjustable particle size and colloidal stability enable LDH to efficiently deliver antigens into lymph node. (B) The weak alkalinity enables LDH to rapidly neutralize the acidification of endosomes to promote the endosomal escape of cargoes. (C) Divalent or trivalent metal ions can be readily doped into the crystal structure of LDH by isomorphic substitution of Mg^{2+}/Al^{3+}, ensuring the adjuvanticity of LDH. (D) The specific ion-doped LDH can respond to external stimuli for enhanced CDT, PTT/PDT, SDT, and MRI. (E) Adjustment of the species and oxidation status of metal ions in the crystal structure endows LDH with enzyme-like and Fenton/Fention-like catalytic activities. (F) By synergizing with the immunomodulatory metal ions, LDH can effectively induce tumor ICD by blocking autophagy, causing mitochondrial damage and immune-associated signaling pathway activation. Created with BioRender.com.

lymph node-targeted vaccines[54,63]. Exemplifying this strategy, surface coating with albumin effectively prevents LDH aggregation in biological environments[63,64]. For example, bovine serum albumin (BSA) is often used as the model albumin for surface coating of LDH, but with low immune responses[65]. Notably, BSA can be replaced with mouse serum albumin to avoid any potential immune response. For human use specifically, we can use human serum albumin instead of BSA to avoid any possible side effects. Moreover, immunological compatible albumin can be further conjugated with tumor-targeting ligands, enabling targeted delivery[66]. LDH can be also functionalized to enhance their *in vivo* performance. Cao et al.[67] described PEGylation, achieved by grafting phosphonic acid-terminated polyethylene glycol onto the LDH surface *via* electrostatic interactions. Similarly, coating with PEG improves colloidal stability and prolongs blood circulation, facilitating delivery to tumor tissues and lymph nodes[59,68]. Additionally, LDH can be coated with cell membranes derived from specific tissues, such as tumors. After surface modification with targeting ligands, these cell membrane-coated LDH nanoparticles efficiently accumulate in targeted tissues like tumors and lymph nodes[69,70].

Internalized by APCs, LDH efficiently escapes from endosomes, facilitating controlled release of antigens into the cytoplasm[57,71,72]. This mechanism is triggered when LDH-based vaccines, internalized *via* the clathrin-mediated pathway[73], leverage the inherent alkalinity from $Mg(OH)_2$ to rapidly neutralize the acidic endosomal environment. The subsequent release of Mg^{2+} ions elevates the endosomal ionic strength, and leads to water influx and ultimately endosome rupture, promoting cargo release into the cytoplasm[73,74]. Unlike conventional Alums, which primarily deliver antigens for degradation in lysosomes and presentation on MHC-II molecules, LDH NanoAlum facilitates antigen escape to the cytoplasm, enabling binding to both MHC-I and MHC-II molecules and promoting efficient cross-presentation[75]. This dual pathway contributes to the robust induction of both humoral and cellular immune responses.

3.1.2. *Mechanism and application of LDH NanoAlum*

3.1.2.1. Metal composition greatly affects the adjuvanticity. The adjuvanticity of LDH NanoAlum is greatly influenced by the chemical composition (ion species and ratio). Our understanding of the immunomodulatory effects of pristine LDH has deepened significantly through the continuous investigation during the past decade, and several intracellular signaling pathways, including PI3K/AKT, MAPK, NF-κB, Wnt/β-catenin, and JAK/STAT, have been implicated in this process[58]. Li et al.[76] firstly demonstrated

that the ratio of Mg and Al of the pristine LDH NanoAlum greatly affected the adjuvanticity of LDH NanoAlum, as decreasing Mg and Al ratio could effectively activate DCs *via* the NF-κB signaling pathway. Subsequently, Williams et al.[77] reported the potential linear relationship of the adjuvanticity with the physicochemical parameters (*i.e.*, ionic radius of metal cations, interlayer spacing and zeta potential). Their results showed that introducing metal ions such as Li^+, Ca^{2+}, and Fe^{3+} and modulation of the metal cation ratio may change the adjuvanticity of LDH, thereby increasing the magnitude and polarization of the host immunity. Recently, Zhao et al.[78] further demonstrated that substituting Mg^{2+} with Zn^{2+} significantly enhanced the adjuvanticity of LDH. Their study revealed that ZnAl−LDH promoted DC autophagy, facilitated cross-presentation of antigens and elicited potent antigen-specific $CD8^+$ T cell immunity against solid tumors. Interestingly, the change of anions between the layers also changes the adjuvanticity of LDH. Niu et al.[79] found that MgAl−LDH−NO_3 had superior anti-inflammatory ability, which promoted the differentiation of macrophages, reduced the number of Th17 cells, and inhibited IL-17 signaling.

3.1.2.2. Particle size and colloidal stability affects the antigen adsorption and delivery. LDH NanoAlum as an adjuvant for plasmid DNA delivery to lymph nodes[76], successfully eliciting antigen-specific T-cell immunity against solid tumors. This has marked the initiation of LDH-based immunotherapy applications. Subsequently, Yan et al.[80] first compared the adjuvanticity of LDH NanoAlum and conventional Alum. In their study, ovalbumin was used as the model antigen and the *in vivo* results indicated that pristine LDH NanoAlum had comparable adjuvanticity to conventional Alum in inducing Th2 humoral immunity. However, when both LDH NanoAlum and conventional Alum were combined with CpG, LDH NanoAlum rather than conventional Alum significantly switched the immunity from Th2- toward Th1-biased immunity. Similarly, Zhang et al.[81] showed that the LDH NanoAlum efficiently delivered antigens into lymph nodes to induce potent $CD8^+$ T cell immunity. In contrast, conventional Alum-based vaccines failed to induce effective T-cell immunity against solid tumors. The enhancement of immunity induced by LDH NanoAlum may be involved with its excellent antigen loading/delivery and endosomal escape capacities.

The positive surface charge enables LDH NanoAlum to efficiently adsorb negatively charged biomacromolecules through electrostatic interactions between $-COO^-$ groups of biomacromolecules and the positive charges on the LDH NanoAlum surface[56]. The adsorption capacity of LDH NanoAlum is greatly affected by its particle size, which directly affects the amount of protein adsorbed by changing the specific surface area of the particles[82]. Gu et al.[63] showed that the maximum adsorption capacity of LDH NanoAlum with particle sizes of 110 and 50 nm for BSA was 0.70 and 1.34 mg per mg LDH NanoAlum, respectively. A similar phenomenon was also observed in another study, which shows increased particle size of LDH NanoAlum from 77 to 285 nm significantly decreased the ovalbumin adsorption capacity[83]. The superior cargo loading capacity enables LDH NanoAlum to successfully formulate multivalent vaccines. For example, Chen et al.[57] successfully loaded three recombinant antigens, intimin β, proprietary antigen 1, and proprietary antigen 2, onto the surface of LDH NanoAlum. Their study demonstrated induction of antigen-specific humoral immunity against all three antigens in mice, without evidence of antigenic competition. Notably, the antibody levels were significantly higher compared to that elicited by a QuilA formulation. Similarly, Zhang et al.[59] conjugated three melanoma-derived antigen epitope peptides (Trp2, M27, and M30) individually with BSA and then loaded onto LDH NanoAlum. Their study demonstrated that this trivalent LDH nanovaccine effectively induced Trp2-, M27-, and M30-specific $CD8^+$ T cell immunity, leading to significantly greater melanoma growth inhibition than vaccines containing only one or two epitope peptides.

The tunable particle size and *in vivo* colloidal stability are critical factors that affect the delivery efficiency of LDH nanovaccines towards lymphoid organs. Chen et al.[56] synthesized LDH NanoAlum with the average particle size of 115, 243 and 635 nm for vaccine formulations through subcutaneous injection, finding that the smaller particle size enabled LDH vaccines to more efficiently induce antigen-specific antibody immune responses than the larger ones. In a further study, Chen et al.[29] compared Alum and LDH NanoAlum in promoting humoral and cellular immune responses *via* deciphering the depot effect upon subcutaneous injection. They found that LDH NanoAlum stimulated higher levels of specific antibodies and more antigen-specific IFN-γ-secreting immune cells. Moreover, the plate-like LDH NanoAlum-based depots presented a much looser and more porous structure than Alum-based ones, which enabled more quickly release of antigens than Alum (50%−60% *vs.* <10% after 35 days post subcutaneous injection) and less local inflammation. Interestingly, Yan et al.[84] further reported that reduction of the thickness of LDH nanoplates promoted a higher level of specific antibodies yet a similar level of cell-mediated immune response, leading to better inhibition of solid tumor progression and extension of mice survival. Based on the controllable colloid stability technology developed by Gu et al.[63], a further study conducted by Zhang et al.[59] showed that 108 nm LDH NanoAlum in a monodispersed (saturated with albumin) or aggregated (induced by the bridging effect of albumin) state exhibited completely different infiltration abilities to lymph nodes after subcutaneous injection. *In vivo* data showed that monodispersed LDH vaccines infiltrated into lymph nodes more quickly and induced a strong T cell immune response to inhibit the progression of solid tumors. In contrast, aggregated LDH vaccines were mainly retained at the injection site to form nodules and slowly induced immune responses. In another study, Zhang et al.[83] further formulated and co-loaded with the model antigen ovalbumin (OVA) and bioadjuvant CpG on LDH NanoAlum, with sizes varying between 77 and 285 nm. This study revealed that LDH nanovaccines with the average size of 215 nm exhibited the highest accumulation in the mouse spleen, representing approximately 1.67% of the injected dose after intravenous injection. Compared to nanovaccines targeting lymph nodes (*via* subcutaneous injection), the nanovaccines within the spleen activated more APCs, leading to a more rapid and robust $CD8^+$ T cell response against solid tumors. Notably, a complementary study showed that simultaneous delivery to both the spleen and lymph nodes elicited a potent and durable $CD8^+$ T cell response[85]. *In vivo* studies revealed that LDH nanovaccines administered *via* this combined route effectively inhibited the growth of advanced melanoma and lymphoma in mice.

3.2. *Immunomodulatory LDH NanoAlum for enhanced immunotherapy*

The basic immunomodulatory function of LDH NanoAlum stems from its inherent weak alkalinity due to the autohydrolysis of $Mg(OH)_2$. This process accelerates within acidic microenvironments, such as tumor microenvironment (TME) and lysosomes, leading to the release of Mg^{2+} ions and neutralization of protons (H^+). Notably, hydrolysis of $Mg(OH)_2$ within the LDH structure generates vacancies, allowing for the subsequent isomorphic substitution of other divalent or trivalent metal cations like Zn^{2+}, Cu^{2+}, Mn^{2+}/Mn^{3+}, Cr^{3+}, Ni^{2+}, Co^{2+}, Fe^{2+}/Fe^{3+}, and Ce^{3+} [19,86–88]. This versatile cation substitution strategy enriches the physicochemical and biological properties of LDH, enabling it to systematically remodel the physicochemical properties of TME[89–92].

3.2.1. *Metal cation-mediated microenvironment remodeling*

Neutralization of acidic TME by LDH NanoAlum has been shown to promote a proinflammatory TME for enhanced immunotherapy. Zhang et al.[93] recently employed a peritumoral injection strategy, mimicking vaccination, to deliver LDH NanoAlum around solid tumors. This approach led to depot formation at the injection site and sustained neutralization of the acidic TME for at least 14 days. Consequently, the immune cell landscape within the TME was significantly reprogrammed. Notably, the levels of pro-tumorigenic immune cells, including M2-like tumor-associated macrophages (TAMs), T regulatory cells (Tregs), and myeloid-derived suppressor cells (MDSCs), were markedly reduced. In contrast, the levels of anti-tumorigenic immune cells, such as $CD8^+$ T cells, $CD4^+$ T cells, and M1-like TAMs, were significantly increased (Fig. 4A). Besides, the weakly alkaline LDH NanoAlum also inhibited the acidification of autophagolysosome in tumor cells, causing the blockade of tumor cell autophagy and finally leading to cell apoptosis. As expected, the *in vivo* data showed peritumoral injection of LDH NanoAlum significantly inhibited the progression of both melanoma and colon tumors at early or advanced stages. Notably, the underlying mechanism of TAM polarization within the TME is also involved with the blocked autophagy. Jing et al.[94] found that MgAl−LDH exhibited stronger ability to switch TAMs from M2-to M1-phenotype by more efficiently blocking the autophagy of TAMs than other types of LDH (*e.g.*, MgFe−LDH). Meanwhile, the blockade of autophagy in M2-TAMs by MgAl−LDH also resulted in more expression of MHC II molecules, which subsequently stimulated the activation of $CD4^+$ T cells. Moreover, the regulation of T cells within the TME is correlated with the supplementation of Mg^{2+} rather than Al^{3+}, which has been demonstrated to be critical for restoring the cytotoxic function of $CD8^+$ T cells[95,96]. Jia et al.[35] showed that Mg^{2+} released from LDH NanoAlum significantly activated the NKG2D receptor of T cells. As a result, more $CD8^+$ T cells were recruited and activated within the TME to inhibit tumor growth (Fig. 4B and C).

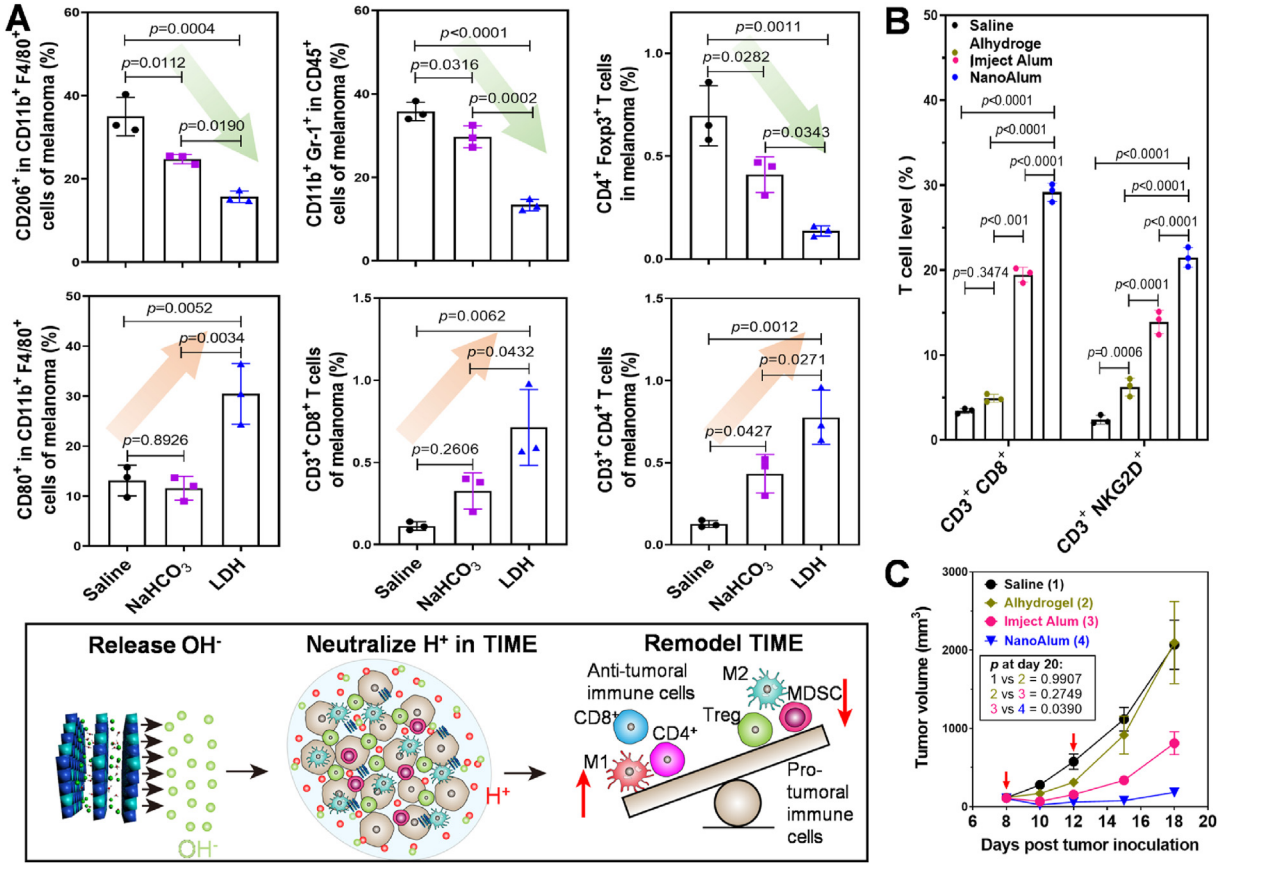

Figure 4 LDH NanoAlum remodels tumor immune microenvironment (TIME) by acid neutralization. (A) LDH nanoparticles efficiently remodel the immunosuppressive TME of melanoma. Reprinted with permission from Ref. 93 Copyright © 2022 American Chemical Society. (B, C) NanoAlum significantly increased the level of $CD8^+$ $NKG2D^+$ T in tumor-resident T cells and inhibit the growth of colon tumor and melanoma. Reprinted with permission from Ref. 35. Copyright © 2023 Elsevier B.V.

Interestingly, the change in metal ion composition also changes the immunomodulatory function of LDH NanoAlum. Zhang et al.[97] developed a ZnMgAl−LDH NanoAlum by partially replacing Mg^{2+} with Zn^{2+} (Fig. 5A). Their study showed that the introduction of Zn^{2+} not only promoted the formation of the proinflammatory immune network in TME, but also caused tumor immunogenic cell death (ICD) by activating tumor cyclic guanosine monophosphate-adenosine monophosphate synthase-stimulator of interferon genes (cGAS−STING) signaling pathway and downregulating the expression of immune checkpoints (*i.e.*, PD-L1, CD47). As a result, the peritumoral injection of ZnMgAl−LDH significantly inhibited the growth and metastasis of breast cancer tumors and prevented tumor recurrence after surgery. Liu et al.[98] synthesized an ultrathin MnMgAl−LDH NanoAlum loaded with pro-inflammatory cytokine interferon (IFN-γ) (Fig. 5B). The introduction of Mn^{2+} significantly depleted glutathione (GSH) and generated hydroxyl radicals to cause tumor ICD, which was further enhanced by IFN-γ-mediated SLC7A11 downregulation. The synergy between tumor ICD-caused cell ferroptosis and the intrinsic immunomodulatory property of MnMgAl−LDH facilitated the maturation of DCs and the priming of T cells. Moreover, Zhao et al.[78] completely replaced Mg^{2+} with Zn^{2+} to obtain ZnAl−LDH NanoAlum (Fig. 5C). Their study showed that the increased Zn content enabled ZnAl−LDH NanoAlum to induce more reactive oxygen species (ROS) within tumor cells to initiate tumor ICD. Similarly, Chang et al.[99] recently utilized Fe^{2+} to completely replace Mg^{2+} and obtained another FeAl−LDH NanoAlum for delivering dihydroartemisinin (DHA, Fig. 5D). Within the TME, Fe^{2+} released from FeAl−LDH aggravated intratumoral oxidative stress injury by synergizing the Fenton reaction and DHA activation, thereby causing tumor apoptosis, ferroptosis and ICD. Another study conducted by Li et al.[100] constructed a MgFe−LDH NanoAlum by replacing Al^{3+} with Fe^{3+}, which greatly promoted the activation, glycolytic activity, and cytotoxic killing of $CD8^+$ T cells. Their study showed that the cell-surface molecule lymphocyte function-associated antigen 1 (LFA-1)-driven signaling activity (*i.e.*, phosphorylated FAK and ERK1/2) was augmented upon the stimulation of free Mg^{2+} or Mg^{2+}-containing MgFe−LDH. At the same time, the ROS-promoter Fe^{3+} or

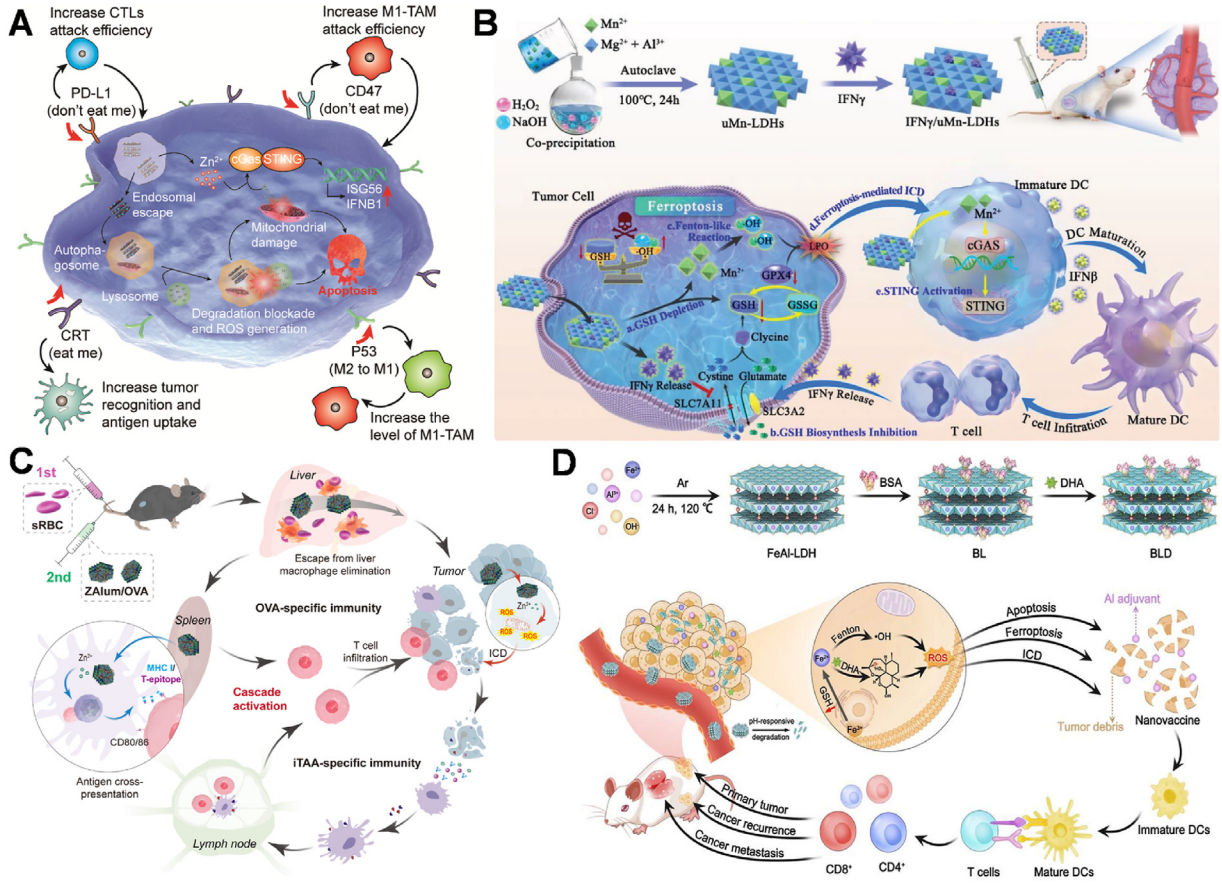

Figure 5 LDH NanoAlum induces efficient metalloimmunotherapy. (A) Design and fabrication of a Zn^{2+} doped layered double hydroxide based immunomodulating adjuvant for robust and safe cancer metalloimmunotherapy. Reprinted with permission from Ref. 97. Copyright © 2022 Wiley-VCH GmbH. (B) The synthetic process and the therapeutic mechanism of IFNγ/uMn-LDHs for co-enhancement of ferroptosis and antitumor immunity. Reprinted with permission from Ref. 98. Copyright © 2023 Wiley-VCH GmbH. (C) Erythrocyte-assisted systemic immunization of ZAlum/OVA nanovaccines promoting potent cancer metalloimmunotherapy through cascadeimmune activation. Reprinted with permission from Ref. 78. Copyright © 2023 The Authors. Advanced Science published by Wiley-VCH GmbH. (D) Steps in creating the BSA-tagged FeAl-LDH with a DHA cargo construct and intravenous injection of it leading to the release of Fe ions, Al ions, and the drug cargo dihydroartemisinin (DHA) in the weakly acidic tumor microenvironment. Reprinted with permission from Ref. 99. Copyright © 2024 American Chemical Society.

Fe^{3+}-involving MgFe−LDH increased the translocation of nuclear factor of activated T cells from the cytoplasm to the nucleus to regulate the T cell receptor signaling, thereby shaping the $CD8^+$ T cell function.

3.2.2. *Catalytic LDH NanoAlum for microenvironment remodeling*

The reconstruction of the crystal structure endows LDH NanoAlum with enzyme-like catalytic ability to remodel the physicochemical properties of TME. Ma et al.[101] fabricated a nanozyme by confining astaxanthin (AST) within the LDH interlayer to mimic superoxide dismutase (SOD) and catalase (CAT) (Fig. 6A). The data showed that the SOD-like activity of this LDH nanozyme is 16.3 times higher than the typical ROS scavenger CeO_2. The LDH-based nanoparticles can also be modified to have peroxidase-like activity. For example, Ning et al.[102] artificially constructed an enzyme based on defect-rich CoFe−LDH nanosheets, which showed superior peroxidase-like activity (Fig. 6B). Amini et al.[103] developed a Ni/Al−$Fe(CN)_6$ LDH nanozyme by intercalating hexacyanoferrate into Ni/Al LDH, decomposing H_2O_2 into H_2O and reducing the ROS level (Fig. 6C). Recently, Sun et al.[104] constructed acid-sensitive nanozymes based on peroxidized LDH NanoAlum for O_2 self-supply and self-boosted lactate depletion in the TME (Fig. 6D). In this study, partial $Mg(OH)_2$ in the crystal structure of LDH NanoAlum was peroxided into MgO_2, and the surface of peroxidized LDH was coated with partially crosslinked catalase (CAT) and lactate oxidase (LOX). Upon exposure to the acidic TME, MgO_2 gradually hydrolyzed to generate H_2O_2, which the co-loaded CAT immediately converted to produce O_2 to relieve tumor hypoxia. At the same time, produced O_2 also synergized with LOX to oxide the excess lactate, thus simultaneously reversing the acidic and hypoxic TME.

Moreover, the incorporation of multivalent metal cations such as Fe^{3+}/Fe^{2+}, Co^{3+}/Co^{2+}, Mn^{4+}/Mn^{2+}, Cu^{2+}/Cu^{+} and Ce^{4+}/Ce^{3+} endows LDH NanoAlum with Fenton//Fenton-like catalytic abilities[105–107]. For instance, CuAl−LDH nanosheets decomposed H_2O_2 and generated toxic hydroxyl radicals (·OH) *via* the Fenton reaction, leading to efficient cancer cell apoptosis[108,109]. The increase of ROS generation has been positively correlated to ICD

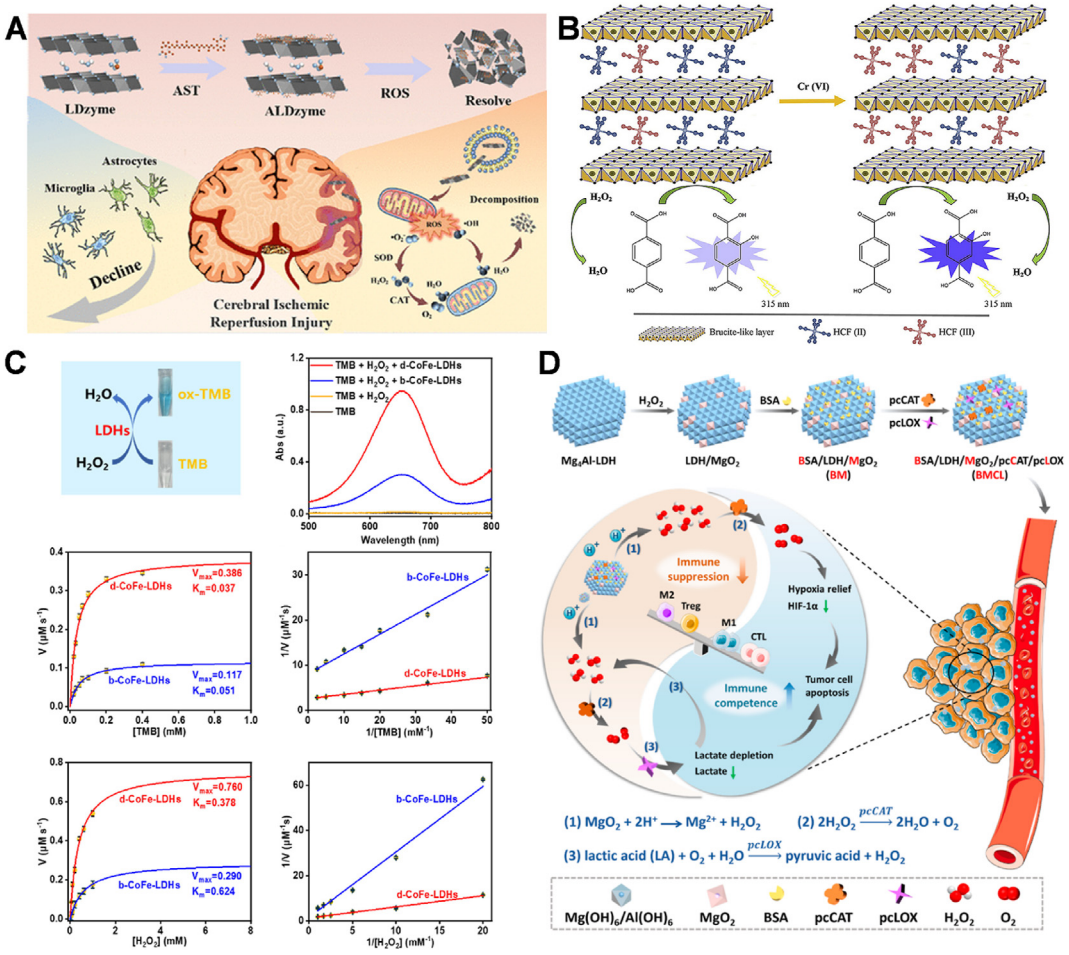

Figure 6 LDH NanoAlum normalizes the TME *via* enzyme-like properties. (A) The layered double hydroxide (LDH)-based nanozyme (denoted as ALDzyme) can mimic natural enzymes including superoxide dismutase (SOD) and catalase (CAT). Reprinted with permission from Ref. 101. Copyright © 2023 American Chemical Society. (B) Peroxidase-like activity property of LDHs for colorimetric detection. Reprinted with permission from Ref. 102. Copyright © 2023 American Chemical Society. (C) Determination of Cr^{4+} by a Ni/Al−$Fe(CN)_6$ LDH catalyzed TA−H_2O_2 system. Reprinted with permission from Ref. 103. Copyright © 2020 Elsevier B.V. (D) The synthetic procedure of BMCL nanozymes and their self-boosted cascade catalytic processes in the TME. Reprinted with permission from Ref. 104. Copyright © 2022 American Chemical Society.

induction. Cao et al.[110] constructed a PEG-conjugated FeAl−LDH to generate abundant hydroxyl radicals at a high reaction rate by catalytically disproportionating H_2O_2 under tumorous acidic conditions, resulting in death of tumor cells and suppression of tumor growth. Similarly, Pan et al.[111] produced FeNi−LDH as a biocatalyst to improve cancer therapy *via* triggering apoptosis and ferroptosis. Compared to Fe−OH and Ni−OH, FeNi−LDH showed considerably higher catalytic activity, generating large quantities of ·OH and $O_2\cdot^-$ radicals, which greatly enhanced chemo-dynamic therapy. Both *in vitro* and *in vivo* experiments demonstrated a robust pathway of cell apoptosis induced, increased intracellular oxidative stress, and accumulated lipid peroxide, inhibiting tumor growth, and promoting anti-tumor immunity.

The catalytic function of LDH NanoAlum can be further amplified by optimizing the metal cation composition and external stimuli such as laser irritation, ultrasound (US), and magnetic field, thereby amplifying tumor ICD induction and achieving visualized therapy. Li et al.[112] constructed a FeOOH-doped CuAl−LDH (FeOOH@CuAl−LDH) for enhanced cytotoxic ROS generation (Fig. 7A). In this study, it is demonstrated that FeOOH serves as a ROS inducer *via* the Fenton reaction. In contrary, the CuAl−LDH serves as a ROS generation booster by augmenting photothermal therapy. In 4T1 breast cancer-bearing mice, one treatment with the FeOOH@CuAl−LDH ICD amplifier eradicated both primary and abscopal tumors and induced higher levels of cytotoxic T lymphocytes in abscopal tumors and spleens. Moreover, Sun et al.[113] developed a defect-rich MnMgAl−LDH for alleviating tumor hypoxia by sequential oxidation of Mn^{2+} to MnO_2 and acid etching to create structural defects (Fig. 7B). Upon exposure to acidic TME, the endogenous H_2O_2 was catalyzed by MnO_2 doped on MnMgA−LDH into O_2 to relieve tumor hypoxia. At the same time, the MnO_2-associated defects enhanced US-triggered separation of e^-/h^+ pairs arising from MnO_2, thereby further amplifying the transfer of H_2O_2 into $^3O_2$, ·OH and $^1O_2$ to induce tumor ICD. By synergizing with the Mn^{2+}-mediated cGAS−STING immune activation, protective anti-tumor immunity was induced against distant tumors. Wu

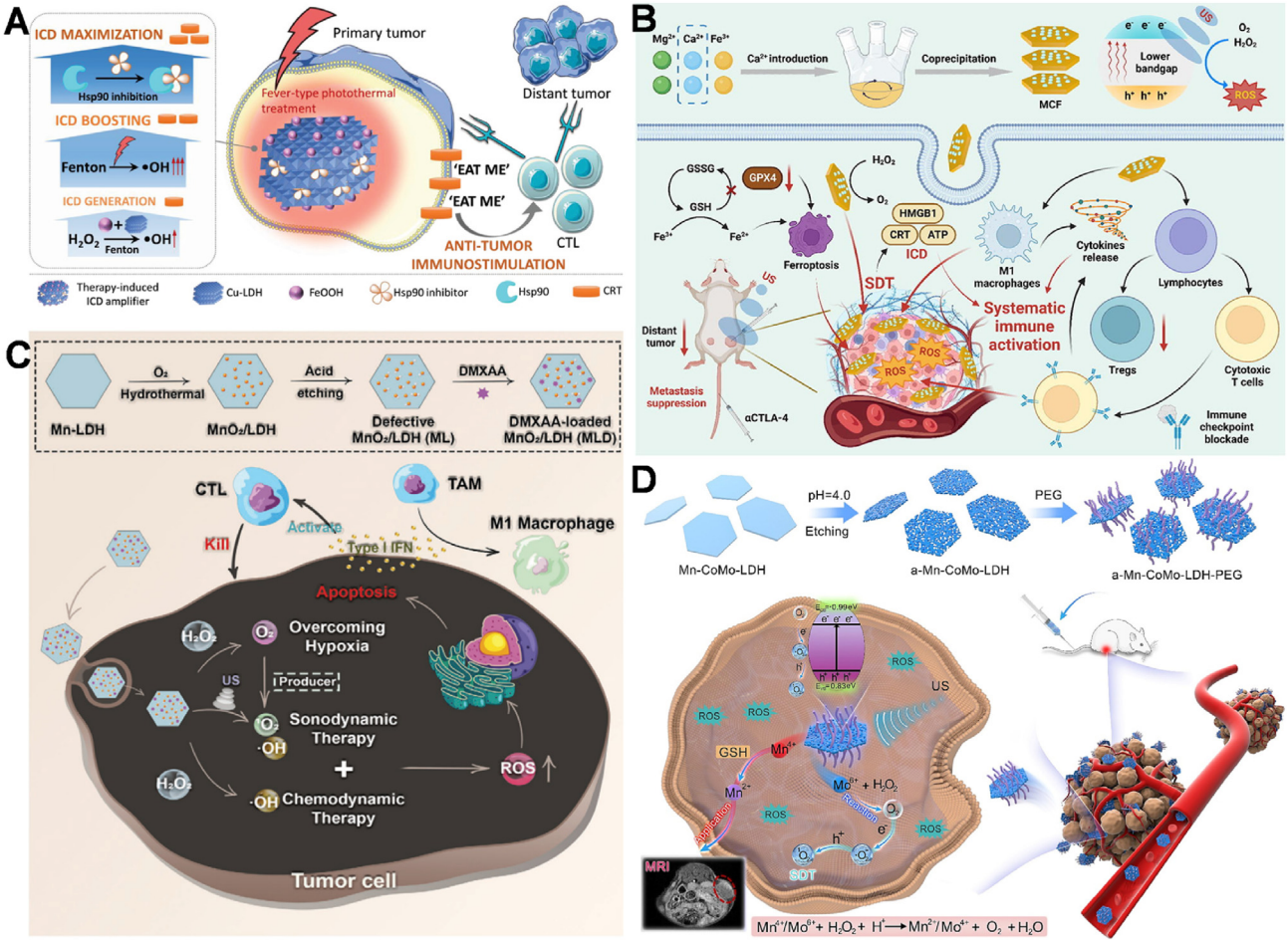

Figure 7 LDH NanoAlum promote antitumor therapeutic effects in response to external stimuli. (A) therapeutic effects induced by FeOOH@STA/Cu-LDH nanohybrid through virtue of a unique ICD maximization strategy, to maximize primary 4T1 tumoral ICD along with magnificent CRT expression, thereafter awakening CTL for systemic tumor immune elimination. Reprinted with permission from Ref. 112. Copyright © 2020 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (B) The synthetic procedure of MLD NPs and their self-enhanced SDT and CDT effect. Reprinted with permission from Ref. 113. Copyright © 2022 Wiley-VCH GmbH. (C) The construction of Ca^{2+}-introduced MgCaFe-LDH to fulfil high-performance oxidation stress and outstanding anti-tumor immunotherapy by activating T-cell mediated immunity. Reprinted with permission from Ref. 114. Copyright © 2023 Elsevier Ltd. (D) The preparation of a-Mn-CoMo-LDH-PEG as a sonosensitizer for MRI-guided sonodynamic cancer therapy. Reprinted with permission from Ref. 115 Copyright © 2023 Elsevier B.V.

et al.[114] constructed a MgCaFe−LDH for enhanced sonodynamic therapy and modulation of the TME (Fig. 7C). The increasing Ca^{2+} introduction optimized the electron structure and bandgap of MgCaFe−LDH, thereby fulfilling high-performance oxidation stress by reducing tumor hypoxia and decreasing GSH concentration. Excitedly, the synergy between tumor hypoxia relief and immunomodulatory Mg^{2+} and Ca^{2+} supplementation effectively activated $CD8^+$ and $CD4^+$ T cells and enhanced the infiltration of T cells into tumor tissues.

Notably, the existence of Mn^{2+} also enables LDH to sense the acidic TME for enhanced magnetic resonance imaging (MRI)[115−118]. Cui et al.[119] utilized Mn-doped CoMo−LDH nanosheets (NSs) for enhanced MRI-guided sonodynamic therapy (SDT) (Fig. 7D). Their study showed that the ROS production capacity of MnCoMo−LDH was 1.3 times higher than CoMo−LDH NSs and 3.9 times higher than crystalline MnCoMo−LDH under US irradiation. Moreover, the doped Mn^{4+} in MnCoMo−LDH decomposed H_2O_2 into O_2 to alleviate tumor hypoxia. It also consumed GSH to reduce its clearance of ROS, thereby enhancing the SDT performance synergistically. In addition, Komarala et al.[120] showed that Fe_3O_4-decorated LDH nanohybrid can respond to an external magnetic field for hyperthermia therapy of cervical cancer. In an alternating current (AC) magnetic field, this magnetic nanohybrid generated heat and killed Hela cells in a dose and time-dependent manner.

Overall, the past years have witnessed the wide application of LDH NanoAlum for enhanced immunotherapy against cancers and infectious diseases (Table 2[29,35,56,57,59,75,76,81,83−85,93,94,97,99,100,108,113,98,121−146]), showing that LDH NanoAlum is a better adjuvant, due to its inherent physicochemical and biological characteristics that make LDH NanoAlum an excellent vaccine adjuvant and tissue microenvironment immunomodulator to amplify cancer immunotherapy. In details, the advantages of LDH NanoAlum over conventional Alum include:

(1) Well defined crystal structure and simple composition with precisely controlled particle size and monodispersed particle status, which clearly defines the adjuvant capacity in the antigen loading/capture, lymphoid organ and APC targeting, cellular uptake and presentation/cross-presentation;
(2) Efficient induction of balanced humoral and cellular as well as mucosal immune responses, which may combine with immune modulators (agonists/antagonists and metal cations) for further immune regulation;
(3) Capacity to normalize physicochemical properties (such as pH, O_2 and ROS) in the tumor tissue, which assists to stimulate cellular immune responses and 'heat' the cold tumor to the hot one;
(4) Capacity to respond external stimuli (such as near infrared, ultrasound, and magnetic field) in the tumor tissues and stimulate specific anti-tumor immune responses.

4. Understanding the biosafety of LDH NanoAlum: Towards preclinical/clinical applications

LDHs incorporating diverse metal cations have been widely explored as promising nano-adjuvants. Despite limited direct and systematic biosafety assessments, the extensive 20-year research history of LDHs provides preliminary evidence for their favorable safety profile. However, future preclinical and clinical studies must prioritize rigorous and comprehensive biosafety evaluations to ensure responsible translation.

4.1. Comparison of cytotoxicity of LDH NanoAlum in different cell lines

Given that cells are the ultimate targets of nanomedicines, it is ideal that they should exhibit high specificity and minimal interactions with healthy tissues[147]. Extensive *in vitro* studies have explored the cytotoxicity of LDH nanomaterials using various cell lines, including immune cells (macrophages[35] and DCs[97]), vascular cells [endothelial cells[148] and smooth muscle cells (SMCs)[149]], and tumor cells (melanoma cells[35], breast cancer cells[150], colon cancer cells[151]), as well as erythrocytes[152] and neurons[149]. In general, these studies report high viability (>80%) of macrophages, dendritic cells, and endothelial cells even at high LDH concentrations (500 μg/mL) in culture media [35,97,151]. LDH (500 and 10 μg/mL) did not significantly affect the proliferation, viability, migration of SMCs and human umbilical vein endothelial cells (HUVECs). LDH (400 μg/mL) also displayed negligible hemolytic activity, and complement activation product C5a levels remained unchanged upon exposure to LDH (20 and 200 μg/mL)[152].

Conversely, tumor-associated cells exhibit increased susceptibility to LDH nanomaterials compared to normal cells, with significantly lower survival rates at higher LDH concentrations. For instance, melanoma cell lines demonstrated an average survival rate of 70% upon exposure to 200 μg/mL LDH, dropping below 30% at 500 μg/mL[104,109]. This enhanced sensitivity may be attributed to the inherent property of LDH, as mixed hydroxides, to dissolve more readily in acidic environments[132]. This is a more reasonable hypothesis, but of course, some LDH may also intervene in cell proliferation and/or cause damage in different cell lines, which needs to be further explored. Therefore, controlling the concentration of LDHs within a certain range can rationally design studies for different disease models.

Due to the special layered structure of LDH, different metal ions can be doped and replace the initial Mg^{2+} and Al^{3+} cations. Doping cations, including Mn^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Ce^{3+}, Gd^{3+} and Co^{2+}, may alter the LDH cytotoxicity to different degrees[153]. These ions are normally a small portion in LDH and should not dominate the biosafety of LDH, basically bringing an increase or decrease in cytotoxicity within 5%[112,151]. In addition, these ions play different functions based on their respective ionic properties, such as photothermal conversion and Fenton reaction (such as Fe^{2+}/Fe^{3+}, Cu^{2+} and Mn^{2+} ions)[151,154], immunomodulation (*e.g.*, Mn^{2+} and Zn^{2+} ions)[97,151], and bio-imaging functions (*e.g.*, Gd^{3+}, Cu^{2+} and Mn^{2+} ions) [116,117,155].

4.2. Biodistribution and maximum safe doses of LDH NanoAlum via different administration routes

LDH is also widely used as an anti-ulcer drug, and no severe side effects are reported for gastric and intestine systems, demonstrating that LDH is a very safe, orally administered drug. In published research reports, LDHs are generally administered *in vivo via* intravenous (IV)[113], subcutaneous (SC)[93,124], and intraperitoneal (IP) injection[156]. Animal testing in these studies generally involves experimental cycles of 10−20 days, 1−4 administrations, and total doses of 50−200 mg/kg. During the test period, safety-related data from animal experiments indicate that LDHs did not cause adverse effects in experimental animals,

Table 2 Preclinical research for LDH immunomodulatory.

Type of LDH	Antigen/agonist	Indication	Ref.
Cancer			
Mg/Al	$pcDNA_3$-OVA plasmid	B16-OVA melanoma	121
Mg/Al	–	DCs	76
Mg/Al	OVA	DCs	75
Mn/Mg/Al	OVA	4T1 breast tumor	122
Mg/Al	CpG	B16F10 melanoma/CT26 colon tumor	35
Mg/Al	CpG	4T1 breast tumor	123
Mg/Al	CpG	CT26 colon tumor	124
Mg/Al	CpG/OVA	E.G7-OVA lymphoma	84
Mg/Al	CpG/OVA	E.G7-OVA lymphoma	83
Mg/Al	CpG/OVA	E.G7-OVA lymphoma	85
Mg/Al	OVA/yeast cell shell vaccine	E.G7-OVA lymphoma	125
Mg/Al	Trp2, M27 and M30	B16F10 melanoma	59
Mg/Al	Trp2/CpG	B16F10 melanoma/CT26 colon tumor	93
Mg/Al	Trp2 peptide	B16F10 melanoma	81
Mg/Al	cGAMP	Hepa1-6 hepatocellular carcinoma (HCC)	126
Mn/Mg/Al	–	CT26 colon tumor	113
Mn/Mg/Al	–	4T1 breast tumor	98
Mg/Al and Mg/Fe	–	TC-1 ovarian carcinoma	94
Zn/Mg/Al	–	B16F10 melanoma/4T1 breast tumor	97
Mg/Fe	–	4T1 breast tumor	100
Cu/Al	–	4T1 breast tumor	108
Fe/Al	–	4T1 breast tumor	99
Fe/Co	–	4T1 breast tumor	127
Co/Cu/Fe	–	H22 HCC	128
Ni/Fe	–	4T1 breast tumor	129
Co/Al	–	4T1 breast tumor	130
Zn/Al	–	Lewis lung carcinoma	131
Infectious			
Mg/Al	$pcDNA_3$-HBVsAg plasmid	HBV	132
Mg/Al	Intimin β, QuilA	–	29
Mg/Al	Intimin β	Enterohemorrhagic *Escherichia coli* (EHEC) infection	56
Mg/Al	Intimin β, proprietary antigen 1 and proprietary antigen 2	*Escherichia coli*	57
Mg/Al	Foot-and-mouth disease (FMD) vaccine AKT-III	FMD	133
Mg/Al	Porcine epidemic diarrhea (PED) subunit vaccines	PED	134
Mg/Al	OVA/Pertussis toxin (PTd)	Pertussis	135
Mg/Al	Brucella outer-membrane vesicles (OMVs) associated proteins formed subunit vaccine	Brucella	136
Mg/Al	plasmid pVAX1-F(o) DNA	Newcastle disease (ND)	137
Ga/Mg/Al	–	Implant-associated infections (IAIs)	138
Zn/Al	–	Ganoderma disease	139
Others			
Mg/Al	–	Spinal cord injury (SCI) recovery	140
Mg/Al	–	Osteointegration	141
Mg/Al	–	Bone defects regeneration	142
Mg/Al	–	Osteogenic differentiation	143
Mg/Fe	–	Bone repair	144
Ga/Al	–	Osteoporosis	145
Ce/Mg/Al	–	Rheumatoid arthritis	146

–, not applicable.

which included body weight, animal vigor, alterations in blood biochemical parameters, histopathological alterations in organs, and death of undesirable groups of animals. Specifically, in one study for size-dependent toxicity of LDH, mice received IP injections at doses up to 600 mg/kg once per week for 4 weeks. The investigators did not observe any significant changes in mortality and body weight during the treatment period, suggesting that the LD50 value for LDH is much higher than 600 mg/kg[156]. Even though no data are available for both IV and SC injections, the often-used maximum doses at 50 and 100 mg/kg[59,83] are much lower than 600 mg/kg and do not cause organ damages.

The *in vivo* distribution of drugs is one of the safety indicators for evaluating drugs and their delivery systems. In related studies, LDHs have shown certain tumor-targeting properties. This targeting is probably determined by the natural hexagonal morphology and weak alkalinity, which makes it easier for LDH to accumulate in the acidic TME[155]. LDH often accumulates in the liver and kidney, which may be their general metabolic

pathway. For example, Li et al.[98] developed an LDH-based MRI contrast agent. *In vivo* imaging revealed that the nano-contrast agent could effectively accumulate at the tumor site with the tumor accumulation up to 3.5% injected dose (ID) at 24 h post IV injection. At the same time, the images of the liver and kidney suggested that LDHs were majorly metabolized through the liver and kidney. Of course, modifying the surface of LDH with different substances enhances specific targeting. For example, Wang et al.[124] and Li et al.[157] used tumor cell membranes to encapsulate LDH and adsorbed Mannose-BSA on the outer surface, and such LDH nanohybrids effectively accumulated to lymph nodes. Liu et al.[158] developed a pH-sensitive charge-reversible polymer-coated LDH nanohybrids to effectively enhance tumor accumulation (up to 6.0% ID) and cellular uptake and significantly inhibit tumor growth with only one treatment.

5. Conclusions and perspectives

Up to date, 27 of 111 human-licensed vaccines are using Alums, according to the FDA website. Here, we have summarized the progress of Alum and LDH-based NanoAlum as adjuvants over the past decades. Compared to conventional Alum, LDH NanoAlum has essential advantages as a next-generation potent vaccine adjuvant to reinforce anti-tumor immunologic responses for improved cancer immunotherapy and infection treatment. These features include excellent biosafety, clear crystal structure, simple composition, ready industrial fabrication, and controlled physicochemical properties. Since the 2000s, our group and many researchers have made great contributions to LDH-based nanovaccine to improve therapeutic outcomes in treating chronic diseases, including tumors and infections. Specifically, drug-free or drug-minimal LDH NanoAlum have shown great potential in TME reprogramming, such as activating anti-tumor immune cells (M1 type macrophages, matured DCs, $CD8^+$ CTLs and NK cells) and depleting pro-tumor immune cells (M2 type macrophages, MDSCs, and Treg cells), and evoked the body's immune system. These features make their clinical translation highly possible.

However, there are still many critical challenges in the clinical translation of LDH NanoAlum as practical adjuvants. The challenges mainly lie in two aspects. The first one is still biosafety. Because the research of NanoAlums is in infancy (~10 years), more industry/clinical protocols involving their scale-up manufacture with acceptable batch-to-batch variations, long-term *ex/in vivo* evaluation of biodegradation, biocompatibility and biosafety in large animals or even humans, and controllable preclinical quality and criteria for the clinical evaluation of safety and cost-effectiveness as well as the post-marketing surveillance, are needed to be established before they are readily applicable as efficient clinical adjuvants. The second challenge is the feasibility of LDH NanoAlum in assisting current mainstream treatment modalities for enhanced treatments. As adjuvants, LDH NanoAlum must be combined with immunotherapy, chemotherapy or even surgery, which may involve the incorporation of NanoAlum before, during and after the modal treatment. Thus, optimizing the incorporation timepoint and dose will be critical for treatment outcomes, which requires a wide range of explorations regarding these combinations. For example, LDH NanoAlum can be applied to reprogram the TME before CAR-T infusion, which may better help the infiltrating CAR-T cells eradicate the tumor cells. A similar example may be the combination of LDH NanoAlum and immune checkpoint blockage antibody therapy, where pretreatment with LDH NanoAlum may help recruit more T cells and enlarge the immunotherapeutic effect. Overall, the LDH NanoAlum is a potential next-generation adjuvant specifically for cancer immunotherapy.

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Conflicts of interest

The authors declare no conflicts of interest.

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