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Design Strategies for Hyaluronic Acid-based Drug Delivery Systems in Cancer Immunotherapy

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ABSTRACT

Despite its robust therapeutic potential, cancer immunotherapy has provided little progress towards improved survival rates for patients bearing immunologically refractory tumors. The implementation of advanced drug delivery systems represents a powerful means of improving cancer immunotherapy by relieving immunosuppression and promoting immune response; however, the overall impact of these systems on immunotherapy currently remains modest. Hyaluronic acid represents a widely used polymer in drug delivery; meanwhile, recent studies linking hyaluronic acid to the immune system make this polymer an attractive component in the design of next-generation cancer immunotherapies. Herein, we review our current understanding of the immunological properties of hyaluronic acid and discuss them in the context of bioactive functions and immune-related interactions with receptors, immune, and cancer cells. We analyze the potential of hyaluronic acid as a component in advanced drug delivery systems, highlighting strategies for the design of more effective vaccines and cancer chemo-immunotherapies. Finally, we discuss critical considerations to facilitate design and clinical translation to overcome existing challenges and maximize therapeutic potential.

1. Introduction

Immunotherapy represents a potential future cornerstone of cancer treatment due to the capacity of this treatment strategy to enhance the recognition and eradication of cancer cells by the immune system [1], the primary line of protection against foreign pathogens or diseases. When immune cells detect harmful signals, they respond through an inflammatory reaction to prevent further damage and maintain homeostasis [2]. Inflammatory activity, which plays a crucial role in immunological function, can be modulated by bioactive molecules that promote or control immune reactions [3]. Immunotherapeutic approaches for cancer treatment include cancer vaccines, oncolytic viruses, immune checkpoint inhibitors, and chimeric antigen receptor (CAR) T cells [4]. The encouragement provided by these insights has established immunotherapy as the fourth pillar of cancer care after surgery, chemotherapy, and radiation [1,4]. The tumor immune microenvironment (TIME) represents the main cause of immunotherapy failures; most tumors possess a characteristic immune-suppressive TIME presenting *(i)* a lack of immune cell infiltration, *(ii)* exhausted immune cells that cannot infiltrate the tumor parenchyma, *(iii)* the overexpression of immune checkpoint factors, and *(iv)* the presence of cancer stem cells (CSCs) [5]. Despite the vast overall potential, cancer immunotherapy displays a lack of efficacy in 80 % of immunologically "refractory" tumors (e.g., glioblastoma, pancreas, and breast cancers) associated with immune-related adverse events [6–8].

Implementing drug delivery systems (DDS) to improve cancer immunotherapy provides advantages that favor selective targeting, thus relieving immunosuppression and promoting immune reactions [9]. Indeed, biomaterial engineering, which enables spatial and temporal functions, has emerged as a promising strategy for cancer immunotherapy. Modifications of DDSs include alterations to their chemical structure, surface characteristics, shape, self-association, or dissociation behaviors in response to endogenous stimuli (e.g., pH and overexpressed enzymes); however, most DDSs fail to overcome drug-related side effects, and several issues have hampered the widespread implementation of this therapeutic option in the clinic. The possible

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underlying "hampering" factors include a lack of biodegradability (related to using non-biodegradable materials that can induce lysosomal storage diseases), uncontrolled drug release, and/or low antigen/drug loading [10,11]. Another limitation of current DDSs relates to a lack of antigen-presenting cell (APC) targeting, which reduces effective antigen presentation and consequent induced adaptive immunity [12]. This latter point associates with a lack of specific distribution in lymphoid tissues to support adaptive immunity and boost immune responses [13].

An intriguing biomolecule with the potential to significantly improve DDS-based cancer immunotherapy is hyaluronic acid (HA), a linear, non-sulfated polysaccharide composed of D-glucuronic acid and *N*-acetylglucosamine connected by β-1,3 and β-1,4 glycosidic bonds [14]. The particular advantages of HA, including biocompatibility, safety, biodegradability, and chemical multivalency, make this polysaccharide a viable candidate for a range of pharmaceutical and biotechnological applications. HA has been employed in a wide variety of drug delivery applications: *(i)* as a backbone for drug delivery, offering controlled and sustained release of conjugated/encapsulated therapeutics, or *(ii)* as a targeting agent to decorate nanocarriers and achieve selective targeting properties, owing binding to receptors such as Cluster of Differentiation 44 (CD44). HA applications have been extensively reviewed elsewhere [14–16] and will not be addressed here.

Recently, a growing body of evidence has linked HA to the immune system. First, the ability to bind receptors involved in the regulation of immune reactions (e.g., CD44, Hyaluronan-mediated motility receptor (RHAMM), Toll-like receptors (TLRs), and Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)), which transduces in varying intracellular responses in immune cells [17]. As a mode of molecular weight (MW), HA can also induce pro- and anti-inflammatory outcomes (e.g., as occurs in the wound healing process) [18]. Despite a close connection with immune cells, HA has been mainly employed as a targeting agent, nanocarrier, or excipient in therapeutic formulations, and we lack reports of the widespread exploration of the polysaccharide's immunological potential. The rational design of HA-based DDSs for cancer immunotherapeutics may improve outcomes through various mechanisms. This review will provide a global overview of the physiological functions of HA and interactions with receptors and immune cells, emphasizing immunomodulatory properties as a step towards developing HA-based DDSs and designing next-generation cancer immunotherapies.

2. Physiological Functions of Hyaluronic Acid

2.1. Hyaluronic Acid Biosynthesis

HA is a relatively simple and atypical glycosaminoglycan, that forms a linear polymer with an MW from 5×10^5 to $4–5 \times 10^6$ Da (Fig. 1) [19]. HA is ubiquitously present throughout the body (an adult human body contains around 12–15 g) with a greater prevalence in the skin, connective tissue, extracellular matrix (ECM), the vitreous body of the eye, synovial fluid within joint cavities, and in fetal structures that develop during embryogenesis (e.g., the umbilical cord). HA biosynthesis is regulated by three hyaluronan synthases (HAS1–3) that use uridine diphosphate glucuronic acid (UDP-GlcUA) and uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) as substrates [19]; their enzymatic activity dictates the HA MW range [16]. Each HAS enzyme has specific functionality in synthesizing an MW range for resultant HA polymers; HAS1 and 2 produce longer chains (0.2 to 2 MDa or more), while HAS3 mainly produces smaller chains (0.1–0.2 MDa) [20]. Unlike other polysaccharides (e.g., chondroitin and heparan sulfate), HA does not

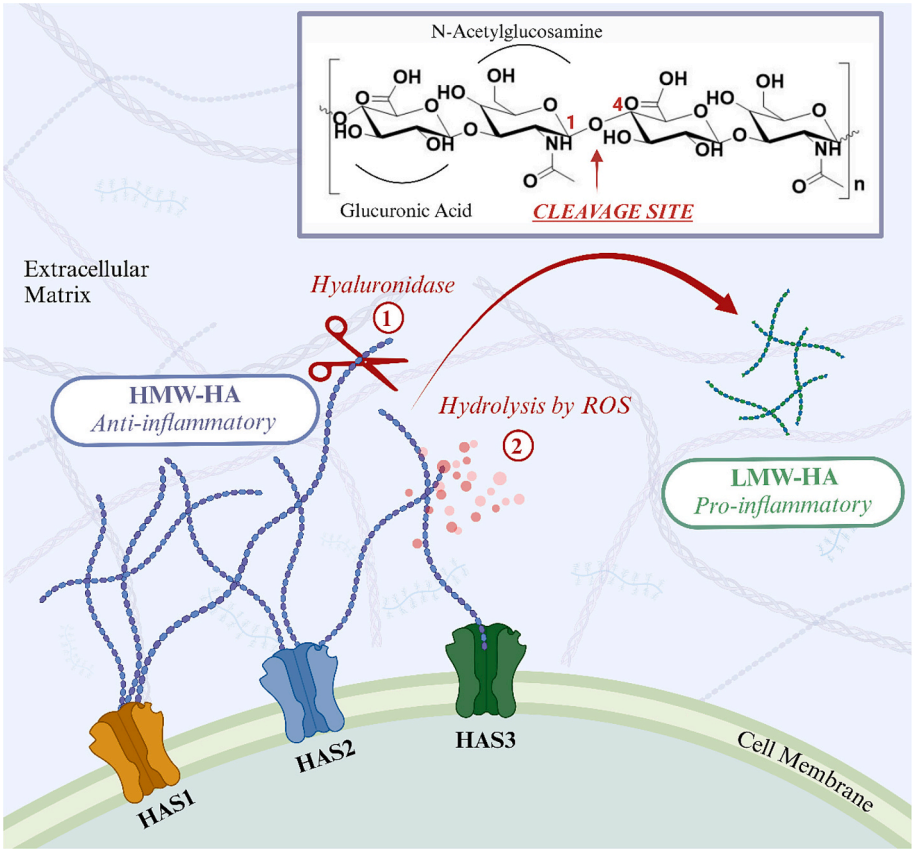


Fig. 1. Synthesis and Degradation of Hyaluronic Acid. The regulation of high molecular weight hyaluronic acid (HMW HA) synthesis by HA synthases (HAS1–3) and degradation by enzymatic (hyaluronidases) or non-enzymatic (e.g., in response to reactive oxygen species [ROS]) processes to obtain low molecular weight (LMW) HA. While HMW HA represents a critical component of the ECM and has anti-inflammatory properties, the LMW HA plays pro-inflammatory roles.

link to any core proteins, and synthesis occurs at the cell plasma membrane (rather than the Golgi apparatus) with immediate extrusion as part of the ECM [14]. The overall amount of HA in tissues results from a finely tuned balance between synthetic and catabolic processes. We direct the reader to resources that provide more detailed information on endogenous HA synthesis [3,19]).

2.2. Hyaluronic Acid Degradation

HA degradation occurs through non-specific or enzyme-driven processes that depend on bodily location [21]; for example, vitreous body HA has a half-life of 70 days, while skin HA has a much shorter half-life of approximately 24 h. The non-specific degradation pathway describes extracellular spontaneous hydrolysis in the presence of reactive oxygen species (ROS), mainly taking place in the skin or skeletal muscle [15]. The enzyme-driven degradation pathway is mediated by a class of enzymes called hyaluronidases (Hyals) - specialized endoglycosidases that can breakdown HA into small fragments by targeting the β-1,4 linkage [22]. Humans possess six Hyal subtypes (Hyal1–6), which can be located intracellularly in the membrane or in the lysosomes to regulate pH-controlled HA turnover [15]. While Hyal-mediated degradation of HA remains an essential and highly regulated process in biological systems, Hyals play additional critical roles in tumor growth, angiogenesis, and metastatic processes during the development of cancer (as in the case of head and neck, brain, lung, bladder, and metastatic breast cancer) [23–26]. We direct the reader to resources that provide more detailed information on the degradation of endogenous HA [19,27].

2.3. Molecular Weight of Hyaluronic Acid and Structure-Function Relationships

Degradation processes provide a variety of HA polymers of differing MWs that possess wide-ranging functional properties; notably, HA MW remains pivotal in determining the variety of physicochemical properties and downstream pathways [19]. We lack a strict classification for HAs regarding MW; however, the consensus places high MW (HMW) as higher than 1000 kDa, mid-MW HA (MMW) between 250 and 1000 kDa, and low MW HA (LMW) as lower than 250 kDa [18]. HA size remains a significant determinant of activity. A growing body of literature describes the anti-inflammatory effect of HMW HA and its pro-inflammatory role for LMW HA, although the reasons for this size dependency remain unclear. Hypotheses have included alterations to receptor clustering and engagement, cellular uptake, intra- vs. extracellular signaling, interactions with HA ligands, and spatial conformation of HA during the receptor(s) binding (e.g., linear vs. globular structures) [28].

High Molecular Weight HA. Due to the presence of the carboxyl group on each of the glucuronic acid units, HA naturally displays a negative charge, allowing this polymer to absorb a large amount of water (swelling up to 1000 times) and generate an expanded random-coil structure in aqueous physiological solution [29]. In an aqueous solution, HMW HA induces a state of extremely high viscosity and elasticity; for instance, 1 % solutions of >1000 kDa HA appear like jelly due to the presence of strong hydrogen bonds, interactions with water and intramolecular interactions. This beneficial shear-thinning behavior and lubricant properties open the application of HA to various formulations, including fillers, creams, gels, and drops. HMW HA can be found in vascular endothelial cells, the cumulus cells surrounding the oocyte, and surrounding chondrocytes (providing the biomechanical structure that protects cartilage) [30–32]. HMW HA also represents the main structural and functional component of the ECM, particularly in connective tissues, skin, cartilage and synovial fluid of joints [27,33]. HMW HA suppresses pro-inflammatory effects by reducing cyclooxygenase-2 (COX-2) and matrix metalloproteinase (MMP) expression and inhibiting the activation of mitogen-activated protein kinases (MAPKs) and Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [34]. HMW-HA also protects against mitochondrial DNA damage induced by exposure to pro-inflammatory cytokines [35] and acts as an anti-inflammatory and anti-fibrotic agent in rheumatoid arthritis and osteoarthritis and the repair of tympanic membrane perforations [36,37].

Low Molecular Weight HA. Shorter bioactive LMW HA possesses lower viscosity and higher solubility in water. LMW HA interacts with cancer and immune cells to alter their activity, transmitting a pro-inflammatory effect. LMW HA induces the increased expression of pro-inflammatory cytokines and chemokines (Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-1β), ROS, and inducible nitric oxide synthase (iNOS) in macrophage cell lines in an NF-κB-dependent manner [38]. LMW HA participates in cell migration and growth with a supportive microenvironment. It also contributes to signals transmission, tissue morphogenesis, and wound healing processes [19]. For example, in the case of joint inflammation, LMW HA originates from ROS-driven HMW HA cleavage, resulting in a loss of lubricant characteristics; this behavior could demonstrate the structure-function relationships of HA [27].

Interplay between Low and High Molecular Weight HA. The wound-healing process provides a key example of the precise regulation of HA according to biological function. Tissue injury impairs HA synthesis/degradation balance, increasing HA accumulation at the injured site. HMW HA can bind fibrinogen in this early phase and facilitate clot formation to reduce initial bleeding events [39]. HMW HA extrusion from cells in the form of elongated cables and fibrils allows the capture of neutrophils and inflammatory mediators (e.g., TNFα, IL-1β, IL-8), their maintenance in an inactivated state and establishes a protective shield that preserves tissue integrity. At this stage, HMW HA exerts anti-inflammatory properties. Neutrophils represent the first line of defense and, during the inflammatory stage, they start to produce myeloperoxidase (MPO) to help killing invading pathogens via ROS production; this supports also the degradation of the highly dense and cross-linked HA network, leaving space for their pro-inflammatory LMW HA counterparts [27]. The released chemokines and cytokines from the damaged tissues and immune cells create a chemical gradient that attracts monocytes and leukocytes to the wound site; here, the interactions with LMW HA prompt their activation and ability to release inflammatory cytokines (e.g., TNFα, IL-8).

2.4. Hyaluronic Acid as the Building Block of the Extracellular Matrix

The ECM is the main “non-cellular” component of all tissues, responsible for the physical support; it is crucial in regulating cancer cell self-renewal, fate, death, and signaling, controlling a wide range of functions such as migration, proliferation, differentiation, tissue patterning, inflammation, and angiogenesis, as well as various homeostatic and pathological processes [40]. The complexity of ECM biology stems, in part, from the diversity of its components, which engage in highly dynamic interactions both with one another and with resident and migrating cells. In this complex context, HA participates in a broad range of biological functions. HMW HA represents the main structural component due to its excellent water retention ability and considerable viscoelasticity [27,33]. Notably, the ECM plays a critical role in cancer cell growth and tumor biochemical and biomechanical functions [41]. HA’s amphiphilic interactions with cells and receptors in an aqueous solution create the ideal hydrated environment for cells to move and proliferate [27]. The ECM comprises two main matrices characterized by distinct types of architecture and composition: *i)* interstitial matrices that surround cells, providing mechanical support, and *ii)* pericellular matrices, which closely contact cells to protect them from rupture [42]. However, tissues possess specific ECM compositions that display alterations according to the required biomechanical properties. Generally, the ECM represents a highly heterogeneous structure, comprising HMW HA crosslinked with fiber-forming proteins, including collagens, elastin, fibronectin, glycoproteins, proteoglycans, and glucosaminoglycans; this

complex, amorphous, and water-rich structure creates a 3D network that participates in extraordinarily dynamic interactions between components and resident and migrating cells. Additionally, laminins provide cell adhesion sites, which transduce signals into cells. Interstitial ECM comprises elastin, collagen fibrils, proteoglycans, and HA, which act as space fillers, lubricants, and osmotic buffers [43]. We direct the reader for additional information on the ECM and its therapeutic potential in cancer therapy here [44,45].

3. Hyaluronic Acid-Immune Cell Interactions and the Interplay with Cancer

Many studies have explored the links between HA, the immune system, and cancer; the following section discusses results from studies on HA-receptor interactions in these areas, focusing on MW-dependent pro- and anti-inflammatory functions.

3.1. HA-binding Proteins: a Focus on Immune Cells

HA can bind several hyaluronan-binding proteins, collectively known as hyaladherins. Many hyaladherins contain a common structural domain of around 100 amino acids in length (the link module) that participates in ligand binding (e.g., CD44, LYVE-1, and Tumor necrosis factor-inducible gene 6 protein [TSG-6]) [46]; however, some hyaladherins lack this domain and they bind HA through a basic coiled-coil domain (e.g., RHAMM), leucine-rich repeat domains (e.g., TLRs), and by forming stable complexes (e.g., inter-alpha-trypsin inhibitor [IαI]) [46]. The ability of HA to bind hyaladherins has been previously reviewed elsewhere [17], but the critical nature of this interaction with immune-related processes, such as inflammation and cancer, remains less explored [22] (Fig. 2). In this section, we focused on the main known hyaladherins that play a role in the immune system.

CD44. CD44 - a widely distributed transmembrane glycoprotein - represents the foremost HA receptor. CD44 has three visible domains, with the distal extracellular region responsible for HA binding and internalization [47], and exists in two forms (resulting from alternative splicing or post-translational modifications): the standard form (CD44s) and the variant form (CD44v), which includes a variable region resulting from alternative mRNA splicing [15]. Most cells possess CD44s, represented by an 85 kDa protein composed of N-terminal ligand-binding, transmembrane, and cytoplasic tail regions [17]. The propensity of CD44 to bind HA varies according to cell type/maturation state, receptor state, and HA MW. HA's repetitive polymeric structure entails multiple CD44 (or other receptor) binding sites, which allow simultaneous interaction with several receptors on the cell surface. Alterations to CD44 density and arrangement on the cell surface (e.g., clustering) can affect such multivalent interaction's overall strength and avidity [48]. HMW HA stimulates CD44 clustering through multivalent binding sites. In contrast, LMW HA has fewer binding sites and lower affinity for CD44, which results in CD44 competition that does not promote clustering; instead it can reduce the overall affinity between HMW HA and CD44 receptors, antagonizing the clustering process [48,49]. CD44 variants occur in a variety of conformations, including *1)* constitutively active (physiologically ready to interact with HA), *2)* inactive (unable to bind HA), or *3)* inducible (binds HA after contact with inducers like pro-inflammatory cytokines or growth factors) [50]. Changes in glycosylation, insertion of variant exons, clustering, and modulation by the cytoplasmic domain can regulate the conversion between these conformations [51]; for example, resting T cells express an inactive form of CD44 that does not bind HA; however, leukocyte activation induces CD44 expression and supports primary adhesion to the vascular endothelium in an HMW HA-dependent manner [15].

The interaction of CD44-HMW HA can induce cell migration [52]; for example, T-cell migration towards inflammatory sites occurs thanks to HA-CD44 interactions; the CD44 intracellular domain interacts with the cytoskeleton to create a link between the ECM and cytoskeletal structures [53]. In vitro studies showed that T cells can roll on surfaces via the CD44 receptor, with a transient HA binding that peaks around 2–3 days after T cell activation. However, in vivo, the activation and HA binding via CD44 is more sustained, reaching its peak around 5–8 days. This prolonged activation is essential for T cells to effectively migrate to sites of inflammation and perform their immune functions [54,55]. The directionality of cell migration strictly depends on the HA gradient in the ECM [19]. This gradient provides directional cues that guide the cells towards areas of higher HA concentration. Cancer cell motility results from the ability of HA fragments to induce CD44 proteolytic cleavage. The cleavage occurs in two main steps: ectodomain and intramembranous cleavage. The ectodomain cleavage is triggered by HA oligomers and results in the release of a CD44 intracellular domain fragment; this fragment then translocates to the nucleus and activates

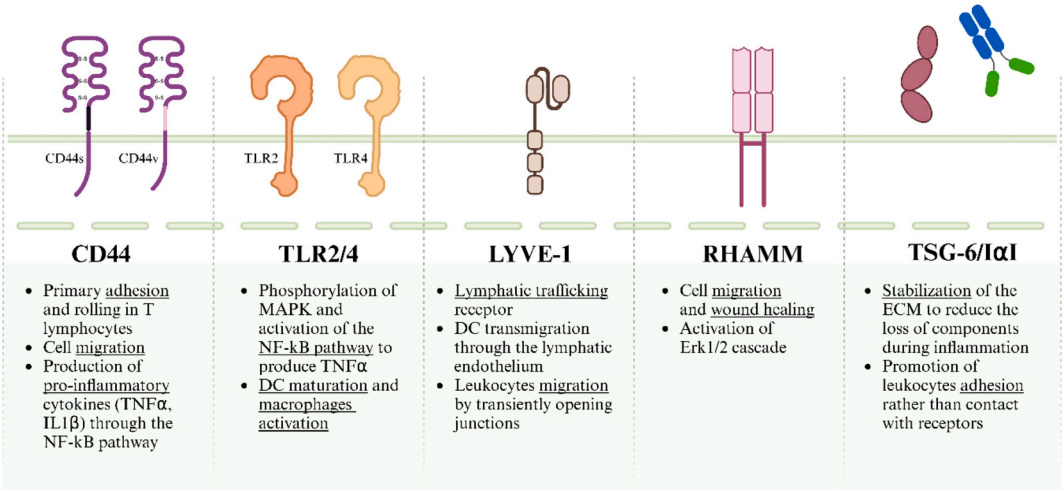


Fig. 2. Overview of the function of hyaluronic acid-binding proteins in immune-related processes. The HA-Cluster of Differentiation 44 (standard, s, or variant, v) interaction regulates T lymphocyte migration and proinflammatory cytokine production. The HA-Toll-like receptor (TLR)2/4 interaction induces dendritic cell maturation and macrophage activation. The HA-Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) interaction favors cell migration through the lymphatic system. The HA-Hyaluronan-mediated motility receptor (RHAMM) interaction triggers cancer cell migration and wound healing. The HA-Tumor necrosis factor-inducible gene 6 protein (TSG-6)/inter-alpha-trypsin inhibitor (IαI) complex forms fibril complexes that promote lymphocyte adhesion rather than contact with receptors. Abbreviations: Tumor Necrosis Factor α (TNFα), Interleukin-1β (IL1β), Nuclear Factor kappa B (NF-κB), Mitogen-Activated Protein Kinase (MAPK), Dendritic Cell (DC), Extracellular signal-regulated kinases 1/2 (Erk1/2), Extracellular Matrix (ECM).

transcription of target genes [56]. It has been shown that HA oligomers (6–40 mers) (but not HMW HA) stimulate CD44 cleavage and consequent cell motility in pancreatic cancer cell lines [57].

Once in the inflammatory site, interactions between HA fragments and CD44 on immune cells increase the levels of several pro-inflammatory cytokines, such as TNFα and IL-1β, via the NF-κB cascade [3,58]. On the other hand, HMW HA interaction with CD44 plays a crucial role in reducing lung inflammation; animal studies revealed how aerosol pre-treatments with HMW HA protected from lipopolysaccharide (LPS)-induced inflammation damage in the lungs via downregulation of TLR4 signaling [59]. Finally, HA plays a role in complement system activation and modulation via CD44 interactions. The complement system comprises part of the innate immune system, and modulation relates to autoimmunity and transplant rejection; overall, studies suggest that HA and CD44 can function to trigger the modulation of the complement system [16,60].

Toll-Like Receptors 2 and 4. TLR2 and 4 are transmembrane proteins mainly expressed by innate immune cells (e.g., dendritic cells and macrophages) that function as 'surveillance' receptors in that they selectively bind to LPS, mycobacteria, and gram-positive bacteria. For example, HA represents a natural component of the glycocalyx of *Streptococcus* and *Pasteurella multocida*, which is recognized as a pathogen-associated molecular pattern (PAMP) by TLR2/4 to initiate innate immune reactions [17]. TLR2/4 and a CD44/TLR4 complex mediate HA fragment-induced proinflammatory signaling [2]. After the binding of HA fragments to these TLRs, the transduced intracellular signal strongly depends on the presence of the downstream effector molecule MyD88 and the consequent phosphorylation of MAPK, the nuclear translocation of NF-κB, and the production of TNFα [19]; hence, the binding of small HA fragments to TLRs leads to immune cell activation and a pro-inflammatory microenvironment. Specifically, TLR4 requires the presence of HA oligomers to initiate dendritic cell (DC) maturation and proinflammatory cytokine production (e.g., TNFα) from both human and murine in vitro-derived DCs [61]. The much higher concentration of HA fragments required to induce TLR4-mediated signaling in DCs (compared to LPS) derives from the physiological presence of HA in the body and its ability to induce a proinflammatory response appearing exclusively at the site of inflammation [61]. Macrophages isolated from TLR2 or TLR4 knock-out mice, but not TLR2/4 double knockouts, remain capable of HA fragment-induced chemokine gene expression [62]. The presence of MyD88 represents a crucial requirement for macrophage activation, as HA-TLR signaling becomes abolished in MyD88-deficient macrophages [62].

LYVE-1. LYVE-1 - a type I membrane glycoprotein – is a member of the *Link module* superfamily, which is closely related to CD44. LYVE-1 binds soluble and immobilized HA present on endothelial cells of the lymphatic sinus and reticular cells of the lymph nodes, which explains the selective tropism of HA towards secondary lymphoid organs [17,63,64]. LYVE-1 is a lymphatic trafficking receptor that prefers engagement with pericellular HA rather than free HA. Indeed, DCs produce an HA cell surrounding layer (glycocalyx) on their surface, which remains pivotal for lymphatic endothelium adherence and transmigration via LYVE-1 interactions [65]. HA-mediated adhesion to DCs triggers the formation of LYVE-1 lined ring-like structures in the endothelium that gradually surround DCs, leading to transit in the undersurface [66]. Interestingly, the relatively weak interaction between LYVE-1 and HA creates a low-friction sliding interface that allows DCs to migrate along the endothelium through an amoeboid movement [67]. Besides properties related to cell migration, LYVE-1 also transduces intracellular signals that regulate endothelial junctional permeability [66]. The interaction with HA activates the MAPK/Extracellular signal-regulated kinase (Erk) and Src non-receptor tyrosine kinase signaling pathways (the latter participates in the phosphorylation of vascular endothelial-cadherin, which regulates junctional integrity to favor cell transit through the endothelium). Both LMW and HMW HA elicit the transient opening of junctions to favor the process of leukocyte migration into the vessel lumen via these pathways with differing kinetics, with LMW HA supporting a faster and more sustained process [68]. From an immunological point of view, the LYVE-1-HA axis remains crucial to the antigen presentation and migratory properties of DCs. Effector CD8 T lymphocyte responses to vaccines (viral or ovalbumin [OVA] peptides) become impaired in the case of LYVE-1 gene deletion or the administration of HA-blocking antibodies [65]. In addition, in vivo evaluations demonstrated that the absence of the LYVE-1 receptor significantly compromises the migration of dermal DCs to draining lymph nodes [65].

RHAMM. Also known as CD168, RHAMM represents another well-known hyaladherin expressed by endothelial and tumor cells; however, many RHAMM isoforms exist that are not exclusively present in the cell membrane. The RHAMM intracellular pool can be found in microtubules, centrosomes, and mitotic spindles, underscoring their functions in cell migration and behavior [69]. Extracellular HA interactions with RHAMM trigger the activation of inflammatory cascades (e.g., Erk1/2) associated with cell migration and wound healing. The RHAMM intracellular pool, which associates with the cytoskeleton and interacts with actin filaments and microtubules, participates in cell polarity and motility. Nuclear RHAMM binds MAPK, which mediates the activation of MMP-9, eliciting inflammation and cell migration [70]. HA-RHAMM interactions have important roles during inflammation and tissue repair through the activation of distinct signaling pathways in macrophages and fibroblasts [53]; for example, HA-RHAMM interaction drives the ability of macrophages to heal tissue injuries [71]. The impact of HA MW in this interaction remains incompletely understood: LMW HA (15–40 kDa) binds RHAMM in fibrosarcoma cells and induces cell adhesion, while HMW HA inhibits cell adhesion, which might reduce their ability to migrate and invade other tissues [72]. HA-mediated RHAMM activity occurs in synergy with CD44; both hyaladherins remain pivotal to wound closure stimulated by HA hexamers, which agrees with the involvement of RHAMM during stressful conditions [73].

IαI and TSG-6. Studies of inflammatory conditions such as inflammatory bowel disease, osteoarthritis, and Crohn's disease revealed the presence of pericellular cable-like structures made of HA complexed with IαI and TSG-6 during the early stage of response to inflammatory stimuli [39,74]. During inflammation, plasmin is involved in degrading components of the ECM and activates the metallo proteinases. IαI comprises two heavy chains and one light chain called bikunin, a serine protease inhibitor, which exerts antiplasmin activity and maintains homeostasis during inflammation [75]. The N-terminal of TSG-6 (link module) binds HA and is secreted by various cells after stimulation of proinflammatory cytokines (e.g., TNF or IL-1). TSG-6 mediates IαI antiprotease activity via non-covalent or covalent binding [76]. The non-covalent interaction increases the accessibility of the protease binding site by combining bikunin and link modules to promote antiplasmin activity. Covalent binding occurs between the heavy chains of IαI and HA molecules, thus creating a crosslinking complex of HA/TSG-6/IαI that can stabilize the ECM to prevent the loss of components during inflammation [17]. HA filamentous crosslinking represents a protective mechanism that promotes leukocyte adhesion rather than contact with inflammation-promoting receptors on the underlying tissues [17]. In this case, leukocytes remain inactive, and proinflammatory mediators become sequestered to reduce the inflammatory reaction [39].

3.2. Biointeractions of Hyaluronic Acid with Immune Cells

HA modulates the functions of various immune cells, including macrophages, dendritic cells, lymphocytes, neutrophils and natural killer cells. Depending on its molecular weight, HA can induce distinct cellular responses, ranging from pro-inflammatory to immunosuppressive effects. Understanding these interactions provides insights into potential therapeutic targets for immune-related diseases and

immunotherapeutic treatments.

Macrophages. Macrophages - innate immune cells involved in inflammation – display differing types of activation: classical activation ("M1-like") is characterized by the production and release of inflammatory mediators, resulting in the creation of an environment ready to combat a perceived threat; while alternative activation ("M2-like") is characterized by the production and release of anti-inflammatory factors, resulting in the resolution of inflammation, angiogenesis, and ECM reconstruction. HA of differing MW induces distinct reprogramming effects on macrophages: a proinflammatory response for LMW HA and an immunosuppressive response for HMW HA [77]. Additionally, treatment with HMW HA greater than 1250 kDa decreases the expression of TNFα, IL-6, IL-1β, and C—C motif Chemokine Ligand 2 (CCL2) but increases the expression of IL-10 and Arginase1 by macrophages, highlighting the ability of HMW HA to induce alternative activation with immunosuppressive features [78]. LMW HA oligomers smaller than 24-mers likely induce classical activation, with enhanced expression of proinflammatory genes and cytokines and reduced expression of genes/cytokines associated with the regulation of inflammation (e.g., Nos2, TNFα, IL-12b, and CD80) [77]. In the context of lung injury, patient serum-derived HA fragments (MW 135 kDa) stimulate the release of chemokines from extracted peritoneal macrophages through a TLR2/4-dependent mechanism [62]. MyD88 represents the key downstream pathway of NF-κB pathway: stimulating murine alveolar macrophages with HA fragments (MW 35 kDa) elicits the expression of IL-1β and TNFα [79]. Alveolar macrophages represent the only immune cells that bind HA under homeostatic conditions; the impact of HA fragments (MW not disclosed) includes the induced expression of inflammatory mediators and the inhibition of urokinase activity (that converts plasminogen in plasmin), which correlates with impaired fibrinolytic activity in acute lung injury [80]. For intermediate MW HA, findings become less clear as the response depends on the initial macrophage state, suggesting that the response could involve a population of macrophages that simultaneously express markers for classical and alternative activation or a mixed population of macrophages or that this particular MW of HA does not bind a specific receptor [77].

Dendritic cells. DCs are innate immune cells that capture and present antigens to lymphocytes to establish an adaptive immune reaction. HA-related DC activation and maturation mainly occur through the activity of HA fragments or oligosaccharides, but not MMW and HMW HAs [81]. Although we lack extensive studies of HA-mediated effects on DCs, we know that HA oligomers activate DCs via TLR4. Termeer et al. reported that bone marrow-derived DCs from mice carrying mutant TLR4 alleles appeared to remain unresponsive to HA stimulation, while DCs from TLR2-deficient mice remained susceptible to HA (fraction containing sugar units of 4–16 oligosaccharide size) [61]. Furthermore, this study revealed that the blockade of the p38/p42/44 MAPK and NF-κB pathways with specific inhibitors abrogated HA-induced DC maturation [61]. Detecting human monocyte-derived DC-maturation monitors the upregulation of specific signals (i.e., Human Leukocyte Antigen – DR isotype (HLA-DR), CD80, CD83, CD86) and downregulation of CD115 following treatment with LMW HA fragments (<20 kDa) [81]. Consequently, these fragments induced pro-inflammatory cytokines expression (e.g., IL-1β, TNFα, and IL-12). Here, any effects remained independent of the presence of CD44, as deficient C57BL/6 and wild-type mice did not exhibit different responses to HA stimulation [81]. To mimic an ECM-like microenvironment, a recent study evaluated the impact of matrix-bound HA of differing MWs on a human monocytic THP-1 DC cell line [82]. The authors confirmed the proinflammatory features of LMW HA (8–15 kDa) via increased CD11c proinflammatory maturation marker expression and the enhanced secretion of proinflammatory cytokines such as IL-1β. Intriguingly, LMW HA matrices sequestered a greater amount of IL-10, thereby suggesting the reduced availability of anti-inflammatory cytokines in the ECM. The immunomodulation potency of DCs remains independent of CD44 for all considered HA MWs [82]. Upon their activation, DC migration plays a crucial role in their function, either taking up antigens in the ECM or transitioning to lymphoid organs where they present the antigen to naïve lymphocytes [83]. Both HMW (1250–1500 kDa) and MMW (500–750 kDa), but not LMW (8–15 kDa) HA, reduce DC migratory capacity in a CD44-dependent manner. Although the reason remains unknown, one hypothesis relates to the ability of LMW HA to facilitate DC migration to scan for antigens and support the migration to nearby lymph nodes and the ability of HMW HA to restrict DC migration to allow interaction and antigen presentation to T cells within lymphoid organs [82]. The presence of the LYVE-1 receptor remains crucial for the transition of DCs into lymphatic vessels via HA-mediated interactions; targeted *LYVE-1* gene deletion, antibody blockade, or depletion of the endogenously synthesized HA-containing glycocalyx (engages LYVE-1 to mediate lymphatic migration [66]) delays lymphatic trafficking of dermal DCs and hampers their capacity to prime CD8 T cell responses [65].

Lymphocytes. T lymphocytes direct and regulate adaptive immunity via the secretion of cytokines and chemokines following antigen presentation. Naïve T cells have a very low avidity for HA since they express CD44 at low levels when inactive [84]; however, CD44 expression and their ability to bind HA increases following activation [85]. Elongated HA cables (MW not disclosed) display specific pro-adhesive features for non-activated leukocytes (monocytes and T and B cells), as demonstrated in vitro in human monocytic cells and peripheral blood mononuclear leukocytes [86]; this interaction remains crucial to avoid contact with inflammation-promoting receptors in underlying tissues [17]. When leukocytes become attracted from the blood following nearby demand, they must extravasate and establish specific interactions with endothelial cells; the HA-CD44 interaction (HMW HA, MW not disclosed) mediates this primary step of leukocyte extravasation towards the inflammation site [74] and provides a survival signal for effector T cells [2]. T regulatory cells (Tregs) – forming one part of the peripheral tolerance-related immune cells – represent another cell type involved in the immunomodulatory properties of HA. HA-CD44 binding correlates with superior Treg suppressor activity, suggesting an active role in cell regulation; in particular, HMW HA (MW 400 kDa) binds CD44 on Tregs to boost Forkhead box P3 (FoxP3) expression, which is responsible for maintaining immune tolerance; FoxP3 expression leads to subsequent production of anti-inflammatory cytokines such as IL-10 and IL-2 [16,85].

Neutrophils. Neutrophils express CD44 on their cell surface, with the HA-CD44 interaction supporting neutrophil migration and infiltration. In a lung inflammatory murine model, Hoarau et al. revealed that LMW HA (MW 200 kDa) interacts with stimulating neutrophils through TLR2/4 receptors, exerting a pro-inflammatory response via the secretion of IL-8 and MPO [87].

Natural killer cells. Natural killer (NK) cells - innate cells that orchestrate effector functions – balance the signals from activating and inhibitory receptors. Ma et al. reported that the LMW HA-CD44 axis (HA MW not reported) can prompt the enhanced production of IFN-γ by NK cells [88]. For NK cells expressing Galectin-9, interactions with CD44 promote immune activation through IFN-γ and the enhanced activation of MAPK, ERK, and mammalian target of rapamycin (mTOR) signaling pathways [89,90].

As can be appreciated, the immunomodulatory role of HA is heavily dependent on its MW, with specific size ranges inducing distinct cellular responses across the different immune cell subsets. Pro-inflammatory responses are typically achieved with LMW HA (<200 kDa), orchestrating a recall of i) classical M1-like activated macrophages, ii) DCs, iii) activated NKs, and iv) neutrophils. On the other hand, anti-inflammatory or immune-suppressive responses are defined by HMW HA (>1000 kDa); this involves the recruitment of i) M2-like macrophages, ii) Tregs, and iii) inhibition of DCs. By finely tuning HA MW, the outcomes in immune cell responses might differ, thus suggesting the potential of using LMW HA for pro-inflammatory outcomes and HMW HA for anti-inflammatory therapeutic applications.

3.3. Biointeractions of Hyaluronic Acid with Cancer Cells

This section delves into the critical roles of HA in cancer biology, focusing on its interactions with cancer cells and cancer stem cells (CSCs). HA in the tumor microenvironment influences various cancer-related mechanisms, including motility, invasion, adhesion, angiogenesis, and multidrug resistance. LMW HA often promotes pro-inflammatory responses, while HMW HA tends to have protective and anti-tumorigenic properties. The section also highlights the impact of HA on CSCs, emphasizing its role in maintaining stemness, promoting chemoresistance, and influencing tumor progression.

Cancer cells. The HA present in the tumor cell microenvironment plays a critical role in many cancer-related mechanisms. HA-CD44 and HA-RHAMM signaling derived from the interaction between the ECM and cancer cells can promote motility, invasion, adhesion, angiogenesis, or multidrug resistance [18]; furthermore, studies have highlighted the ability of the HA-mediated interaction with CD44 to upregulate MMPs activation and inhibit apoptotic cell death in a model of colorectal cancer [91,92]. Numerous studies have related HA overproduction to the overexpression of HAS1–3 and/or Hyals and a consequently unbalanced turnover of HA [93]. Interestingly, high levels of extracellular HA provoke the epithelial-to-mesenchymal transition, a critical event in invasion and cancer metastases [94]. Angiogenesis remains essential for the establishment of distal tumors, and LMW HA fragments represent the fuel for tumor neovascularization thanks to their ability to stimulate pro-inflammatory signaling [15,95]; on the other hand, HMW HA induced protective properties on angiogenesis with an apparent inhibiting effect [96]. In brief, the cancer-related effect of HAs remains contradictory and appears to be highly tumor-type-dependent; a detailed exploration of the role of HA in cancer has been described elsewhere [90,97].

Cancer Stem Cells. The CSC subpopulation of the tumor can self-renewal (displaying pluripotent properties), initiate tumorigenesis and metastasis, and induce chemo/radio-resistance. Al-Hajj et al. isolated CSCs (biomarkers: $CD44^{+}CD24^{-}$) from breast cancer that induced significant tumorigenicity in mice, while Singh et al. sorted a $CD133^{+}$ CSC population from brain tumors [98,99]; additional surface markers used to identify CSCs include CD90 and Nestin [100]. Mechanistically, the interaction between HA in the ECM with CSCs relates to CD44, which activates the Nanog-Stat3, Oct4-Sox2-Nanog, or c-Src kinase signaling pathways, leading to the upregulation of miR-21 and downregulation of miR-203 overall promoting CSC survival and self-renewal [101]. CD44 expressed by CSCs contributes to chemoresistance via specific receptor-mediated and anti-apoptotic mechanisms, including as P-glycoprotein (MDR1; encoded by the multidrug resistance (MDR) gene). MDR1 reduces drug uptake/induced drug efflux from cancer cells, thereby increasing resistance towards chemotherapeutics such as doxorubicin (DOX) [102]. As an important component of the ECM and of the tumor microenvironment, it has hypothesized that HA might have a role on CSC growth and proliferation; studies have observed contradictory findings for HA of differing MWs on cancer progression related to the presence of CSCs; for instance, HMW HA can induce the expression of CSC markers in head and neck squamous cell carcinoma with consequent promotion of cell growth and invasion, although this outcome becomes reversed by LMW HA oligosaccharides [103]. In another study, Chanmee et al. showed that HA (no differences between HMW and LMW) enables breast cancer cells to revert to a stem cell state through EMT (epithelial-mesenchymal transition, a process that can revert differentiated cells back into CSCs) by regulating the Transforming growth factor (TGF)-β network [104]. Overall, these findings are relevant suggesting an intricate connection between HA and CSCs that might have a preclinical and clinical impact on patients; thus we encourage medicinal chemist, pharmacologist, drug delivery scientist and oncologists to begin considering the inclusion of CSC-targeting therapies in their research plans.

3.4. The Interplay Between Hyaluronic Acid, Cancer Cells, and the Immune System

The well-established overexpression of receptors such as CD44 by cancer cells suggests HA as a promising approach to actively target malignant cells; however, cancer cells also express high levels of Hyals, which is linked to cancer aggressiveness, angiogenesis, and progression [105]. The role of CD44 and Hyals in cancer as a targeting strategy has been described before and falls outside the scope of this review [25,47]. Instead, we aim to discuss interactions between cancer cells and immune cells and potential targets in the TIME (Fig. 3).

HA plays a significant role in the TIME by modulating stromal cells, which include fibroblasts, immune cells, and vascular cells. HA has an intriguing effect on tumor-associated macrophages (TAMs), which are critical to cancer progression [106]. Clinical studies have shown that TAM number correlates with HA levels in tumors (although MW is generally not considered); for instance, a study of 278 breast cancer patients with poor prognosis revealed a strong association between high HA and CD44 levels and the presence of an elevated number of anti-inflammatory TAMs (M2-like type) [107]. This finding suggests that the HA-CD44 interaction may promote the activity of TAMs in the tumor stroma, inhibiting anti-cancer immune responses and fostering malignancy [107]. Moreover, HA-activated TAMs promote CSC proliferation and stemness [108]. Campo et al. also reported that LMW HA (<5 kDa) promoted proliferation, reduced apoptosis, and increased radio-resistance by binding to TLR4 expressed by colon cancer cells [109]. Similarly, the LMW HA-TLR4 interaction (MW 15–40 kDa) in glioblastoma (GBM) cells stimulates the NF-κB pathway, maintaining the proliferative activity of CSC-like cells and preventing terminal differentiation [110]. Additionally, LMW-HA augments tumor growth through lymphangiogenesis by inducing lymphatic endothelial cell proliferation/migration [111]. The ECM, of which HA comprises a significant component, facilitates interactions between specific ligands and receptors, diffusion of signaling molecules, and recruitment of immune cells [112]. Although the immunomodulatory mechanisms of HA in tumor development require further investigation, studies have revealed that HA-rich tumor microenvironments can prevent immune cell access and limit exposure to chemotherapeutic agents. For example, Singha et al. demonstrated that the depletion of HA using polyethylene glycol (PEG)-modified recombinant Hyal enhanced NK cell access to tumors and improved antibody-dependent cell-mediated cytotoxicity [113]. Overall, the interplay of HA with macrophages within the TIME highlights a role in promoting cancer progression and immune evasion, making it a critical target for cancer therapy.

The interaction of HA from the ECM and CD44 expressed by CSCs promotes the release of Transforming growth factor beta (TGF-β), an immunosuppressive cytokine often elevated in the TIME. TGF-β elicits tumor-promoting effects through an increase in CSC number and the suppression of immune responses by recruiting immunosuppressive cells (such as TAMs, Tregs, and Myeloid-derived suppressor cells [MDSCs]) while controlling anti-cancer DCs and T-, B-, and NK- cells [114]. Moreover, the HA-CD44 axis acts as a critical regulator of ROS metabolism in the TIME mediated by CSCs and related to the Warburg effect, which contributes to the systemic immune response and establishes an immunosuppressive TIME [115]. The interaction between HA and receptors such as CD44 also prompts the production and secretion of the extracellular vesicles (EVs) that transport macromolecules (e.g., proteins, nucleic acids, lipids, and glycans) between cancer cells, CSCs, and immune cells. HA's role relates to filopodia formation following the induction of EV release [116]. In the TIME, EVs alter ECM-related characteristics (e.g., structural composition, stiffness), promoting cell invasion and angiogenesis and thus modifying the therapeutic outcome and the therapeutic response against the tumor; for example, pancreatic CSC-derived EVs inhibit CD8 T cell and DC maturation while supporting the proliferation of MDSCs and the recruitment of M2-like macrophages [117]. The interaction between HA and CD44 in colorectal cancer

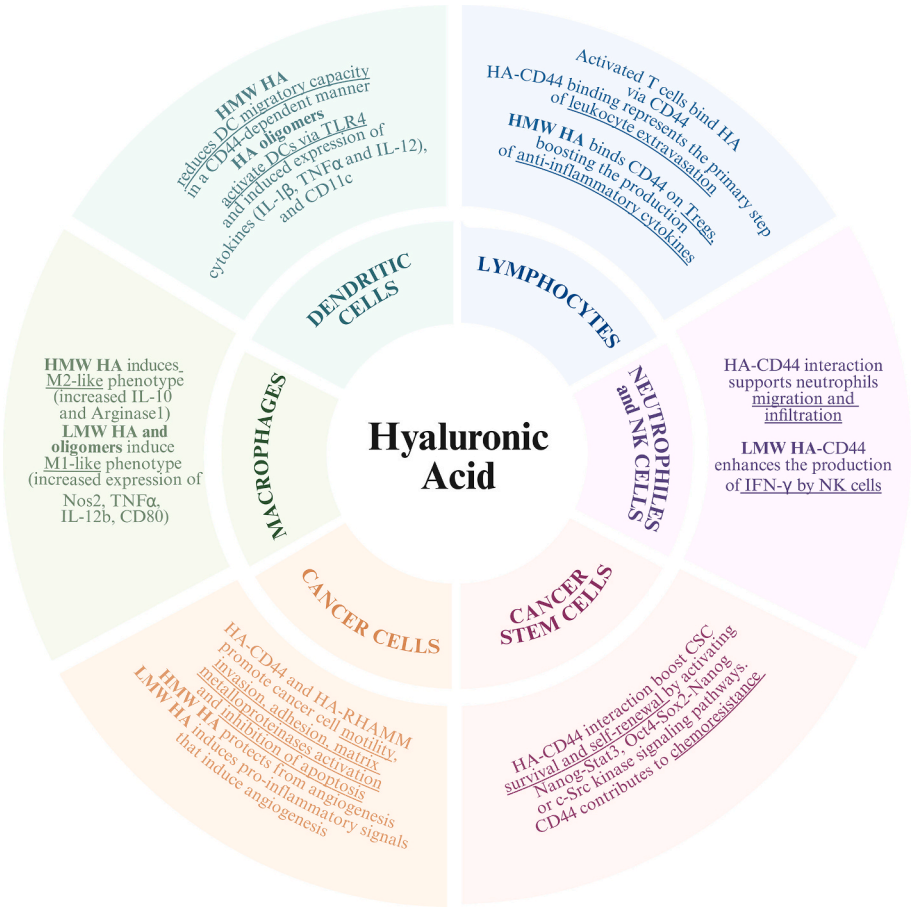


Fig. 3. The interplay between HA, immune cells, and cancer cells. Hyaluronic acid (HA) interacts with immune cells such as macrophages, dendritic cells (DCs), lymphocytes, neutrophils, and natural killer (NK) cells. Each interaction results in a different immunological outcome, mainly pro-inflammatory for low-molecular-weight (LMW) and anti-inflammatory for high-molecular-weight (HMW) HA. Interactions with cancer/cancer stem cells promote progression and development. Abbreviations: High Molecular Weight Hyaluronic Acid (HMW HA), Dendritic Cells (DC), Cluster of Differentiation 44 (CD44), Toll-Like Receptors 4 (TLR4), Interleukin-1β (IL1β), Tumor Necrosis Factor α (TNFα), Interleukin-12 (IL-12), Cluster of Differentiation 11c (CD11c), T regulatory cells (Tregs), Interferon γ (IFN-γ), Natural Killer (NK), Cancer Stem Cells (CSCs), Hyaluronan-mediated motility receptor (RHAMM), Interleukin-10 (IL-10), Cluster of Differentiation 80 (CD80).

generates EVs and human monocytes; HA, delivered by EVs and potentially serving as part of the 'corona' coating, could facilitate interactions between specific subsets of monocytes and EVs [118]. EVs derived from breast and ovarian cancer from the HMW HA-CD44 axis can also promote the differentiation of CSCs into cancer cells [119,120].

Overall, HA plays an intricate role in the immune system and cancer, through its interactions with different hyaladherins. These connections are crucial for modulating immune cell functions and influencing cancer-related processes. LMW HA generally promotes pro-inflammatory reactions, while HMW HA supports tissue-protective, anti-tumorigenic properties. However, a more in-depth investigation needs to be performed to unravel the structure-function relationship of HA, the immune system, and cancer, the affinity towards specific receptors, and how this can be harnessed for therapeutic purposes. Understanding these dynamics will offer crucial insights into potential specific targets: specific subsets of immune cells, cancer or cancer stem cells, and relative receptors leading to specific outcomes.

4. Empowering Drug Delivery Systems-Based Immunotherapies with Hyaluronic Acid

4.1. Overview of Past and Ongoing Clinical Studies Using Hyaluronic Acid as Nanocarrier for Cancer Treatment

HA already forms part of several marketed products for treating osteoarthritis, skin lesions, post-operative adhesions, and dry eye, with many other clinical studies ongoing [121–124]; however, few HA-based products have reached clinical trial phases for cancer treatment. Szoka et al. reported the first usage of HA to target cancer cells in 2001; the authors evaluated the ability of HA-decorated and DOX-loaded liposomes to target CD44-overexpressing B16F10 murine melanoma cells [125]. Since then, a range of DDSs have been designed using HA as a component. A principal hurdle slowing the transition from bench to bedside of HA-based DDSs in cancer therapeutics is their classification as new chemical entities, which requires the optimization of physico-chemical characterizations (aiming to define, for example, drug release patterns, drug loading, particle size, and morphology) [126].

The number of published studies involving HA as a targeting agent has increased exponentially over time, and anti-cancer drugs such as irinotecan and paclitaxel have been investigated in clinical trials by using HA as targeting moiety owing to their simple structure and easy fabrication processes (Table 1).

ONCOFID-P-B (hyaluronic acid-paclitaxel) - a polymer-drug conjugate comprising HA (200 kDa) conjugated to paclitaxel (drug loading 20 % *w*/w) [127,128] - was administered locally in Bacillus Calmette–Guérin (BCG) unresponsive bladder carcinoma patients with or without Ta-T1 papillary disease (which refers to papillary tumors in the inner lining of the bladder [Ta], or invading the connective tissues beneath the lining [T1]) [129]. The study evaluated tumor response by cystoscopy, cytology, and biopsy at the end of the induction and re-induction phases and by a safety follow-up visit. At the end of Phase I, 75.0 % of enrolled patients achieved a complete response to treatment,

Table 1
Examples of past/ongoing clinical trials utilizing hyaluronic acid as a nanocarrier in cancer drug delivery.

Clinical trials						
Trial	Product	MW (kDa)	Cancer	Dose (mg/kg)	Outcome	Ref
NCT05024773 Phase III	**ONCOFID-P-B** Hyaluronic acid-Paclitaxel conjugate	200	Bacillus Calmette–Guérin - unresponsive bladder carcinoma	600 mg weekly, 12 weeks (consecutively)	**Primary endpoint reached** Phase II: 45.0 % of patients displayed a complete response	[128]
NCT02216487 Phase II	**Hyaluronic acid-Irinotecan conjugate**	Not disclosed	Metastatic colorectal cancer	300 mg/m^2 irinotecan with 1000 mg/m^2 HA	**Primary endpoint not reached** This study was not adequately powered to demonstrate differences in survival	[130]
NCT01290783 Phase III	**FOLF(HA)-Iri** Hyaluronic acid-Irinotecan conjugate + folinic acid +5-fluorouracil	Not disclosed	Metastatic colorectal cancer	HA-Irinotecan (Irinotecan 180 mg/m^2); folinic acid (400 mg/m^2); 5-fluorouracil (total 2400 mg/m^2 over 46–48 h)	**Primary endpoint not reached** This study was not adequately powered to demonstrate differences in survival	[132]
NCT02216487 Phase II	**FOLF(HA)-Iri + Cetuximab**	Not disclosed	Metastatic colorectal cancer	FOLF(HA)-Iri (same dose as above) + Cetuximab (starting intravenous infusion of 400 mg/m^2 on day 1 with subsequent weekly doses of 250 mg/m^2)	**On hold after results of NCT01290783**	[131]

Abbreviations: Molecular Weight (MW), Hyaluronic Acid (HA), HA-Irinotecan, folinic acid and flurouracil (FOLF(HA)-Iri).

with no drug-related serious adverse effects or withdrawals reported. At the end of Phase II, ONCOFID-P-B induced a complete response rate of 45.0 % (with 27 complete responses in 60 patients); meanwhile, 13 of the 18 (72.2 %) patients who completed the 12-week intensive course demonstrated a complete response. ONCOFID-P-B modulates immune cells and HA receptor expression (e.g., RHAMM) in the TIME by perturbation of CD44-osteopontin axis in TAMs, which normally promotes stem cell-like properties and radiation resistance in adjacent tumor cells via the activation of CD44 signaling. Therefore, the mechanism of ONCOFID-P-B associates with TAM and Tregs FoxP3+ cell depletion, which generally exhaust CD8 T cells and, in turn, limit antitumor activity. The recruitment for Phase III clinical trials of ONCOFID-P-B remains underway.

A related clinical trial intravenously administered hyaluronic acid-irinotecan (CPT-11) (300 mg/m^2 irinotecan with 1000 mg/m^2 HA) to patients with 5-fluorouracil refractory metastatic colorectal cancer [130]. A Phase I study revealed low toxicity, with 17 % of patients displaying a partial response and 50 % displaying signs of stable disease, suggesting the maintained efficacy of irinotecan. In a Phase II trial, CPT-11 improved the progression-free survival of patients compared to the control arm (5.2 vs. 2.4 months; $P = 0.017$) and time to treatment failure (4 vs. 1.8 months; $P = 0.007$); meanwhile, CPT-11 prompted a median overall survival of 10.1 months compared to 8.0 months for patients treated with irinotecan ($P = 0.196$). Of note, additional studies must define CPT-11 efficacy and safety, with the noted study not adequately powered to demonstrate differences in survival.

A related clinical trial assessed the combination of three drugs, HA-irinotecan, folinic acid, and fluorouracil ("FOLF(HA)-Iri"), in the hope of improving the progression-free survival of metastatic colorectal cancer patients [131]. The primary endpoint was a 6-week improvement in progression-free survival in FOLF(HA)-Iri compared with free drugs; however, the top-line results of a Phase III trial revealed the failure to meet this primary endpoint. A subsequent Phase II trial of FOLF(HA)-Iri plus cetuximab aimed to explore efficacy in irinotecan-naïve second-line patients with KRAS wild-type metastatic colorectal cancer; however, the study was put on hold after the outcomes observed in the above-mentioned phase II trial.

Although the path remains long, researchers are continuously developing new HA-based DDSs with distinct types of therapeutics to generate seed data for potential translation to the clinic.

4.2. Lessons Learned from Clinical Trials

Several factors have limited the clinical success of HA-based DDSs for cancer therapy. First, the impact of HA MW on therapeutic outcomes has been underestimated; this relates to the often-disregarded immunological potential of HA and HA biodistribution and interaction with receptors. Therefore, in-depth research exploring the impact of HA MW should be considered according to the intended application. The physicochemical characterization of nanocarriers has also often been overlooked; this is of importance as biological outcome as these two factors are strictly correlated. Finally, the lack of interdisciplinary interactions between experts in different fields (e.g., drug delivery, material, and biomedical scientists, clinicians) and technical difficulties in translating preclinical results due to physical distances between research laboratories and hospitals lie among the main reasons for the limited success of HA in cancer treatment. As an example, these factors have limited any investigation of the immune system's role to only the ONCOFID-P-B trial, a peculiar status considering that all the described products that reached clinical trials promote immunogenic cell death (ICD), releasing cytokines, chemokines, and so-called damage-associated molecular patterns that elicit antigen-specific adaptive immunity. These facts highlight the underestimation of this therapeutic mode but pave the way to re-purpose these products in a cancer immunotherapy approach, exploiting immune effector mechanisms. To maximize the clinical potential of HA in the context of immunotherapy, researchers should rationally develop innovative systems capable of satisfying the medical needs identified by the academic and clinical communities.

4.3. Rationale for Implementing Hyaluronic Acid as a Component of Immunotherapeutic Drug Delivery Systems

The potential of nano-immunotherapeutics faces challenges that hinder the efficacy and safety of these innovative therapies [9] (Fig. 4). A strong rationale exists for the use of HA as a building block for DDSs as the next generation of cancer immunotherapies: this includes *i)* inherent pro- or anti-inflammatory effects of HA according to MW and the impact on DDS properties; *ii)* the close connection of HA with innate and adaptive immune responses; and *iii)* the intrinsic targeting of HA to LYVE-1 in lymphoid tissues supports the development of DDSs suitable for cancer vaccines, immunotherapy, and immunomodulation [15,18,65].

The physicochemical properties of HA are crucial for the development of novel DDSs. MW impacts the biological properties and the hydrodynamic radius of HA - when MW increases from 7 to 700 kDa, the hydrodynamic radius increases from 3 to 54 nm; moreover, a longer HA chain results in superior viscoelasticity and membrane adhesivity, features that we can exploit in DDS design [133]. HA can be chemically

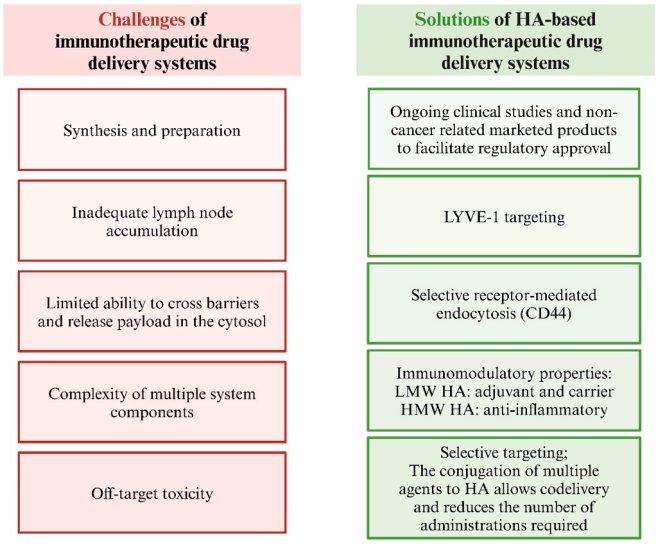


Fig. 4. Schematic overview of challenges of drug delivery system for cancer immunotherapy and solutions provided by hyaluronic acid. The physicochemical properties of hyaluronic acid (HA), which allow for straightforward production and storage, may facilitate the synthesis and preparation of complex immunotherapeutic drug delivery systems (DDSs). The inadequate lymph node accumulation of cancer vaccines represents the main reason for treatment inefficacy and off-target effects. HA-LYVE-1 targeting permits selective lymph node accumulation. Nanomedicines often have a limited ability to cross barriers and release payloads in the target cell cytosol; meanwhile, HA-based DDSs can avoid this hurdle by employing receptor-mediated endocytosis via selective receptor interaction. The complexity of multiple system components might be simplified by using low molecular weight HA as a carrier and an adjuvant for immunomodulation. Due to specific receptor binding, systemic adverse effects might be limited, and the number of administrations might be reduced.

modified through three orthogonal functional moieties - hydroxyl, carboxyl, and amide - making this polymer useful for numerous applications requiring bioconjugation, chemical crosslinking, and surface decoration of DDSs [134]. HA modification offers additional benefits, such as safeguarding the nature of sensitive payloads (e.g., biologics like mRNA and antibodies), stabilizing molecules with poor biopharmaceutical profiles, and implementing preparation methods that circumvent harsh conditions like freeze-thaw cycles. Typically involving a one-step synthetic process, HA chemical modification facilitates the production of stable, powder-form formulations that are easy to manufacture, store at room temperature, and administer [134]. These characteristics also support potential scalable production processes. For cell-based therapies, HA-based biomaterials hold promise for promoting cell proliferation, survival, and differentiation, owing to the critical role of HA in these processes [135]; hence, HA might ameliorate the efficacy of cell-based immunotherapeutic strategies.

Beyond issues related to materials development, inadequate lymph node accumulation represents a significant barrier to effective treatment responses, often leading to inefficient antitumor immune activity [136]. Lymph nodes represent pivotal targets for cancer immunotherapies (e.g., cancer vaccines and adoptive T cell therapy), as they serve as the primary site for antigen presentation and T cell response activation [137]. While specific nanomedicine formulations display enhanced lymph node accumulation, their size (50–200 nm) often precludes effective uptake into lymph node subregions, where B and T cells are accessible only via narrow conduits (3–5 nm) [13]. Moreover, achieving sufficient vaccine drainage into lymph nodes frequently requires repeated local injections, compromising translatability and patient compliance [138]. The selective tropism of HA towards lymphoid tissue mediated by LYVE-1-based interactions represents a promising solution to these problems. HA-driven targeted delivery and enhanced lymph node retention may facilitate the controlled release of antigens and adjuvants, thus reducing the need for multiple administrations and minimizing systemic adverse effects [64,139].

Current immunotherapeutic DDSs often display greater complexity than simple small molecules, meaning many variables impact their final performance and regulatory trajectory. For instance, multiple physicochemical properties (i.e., size, structure, surface, potential, stiffness, porosity, and stability) might significantly change during synthetic processes and in vivo administration, complicating outcomes prediction and reproducibility [140]. Considering these challenges, the rational design of HA-based DDSs for cancer immunotherapeutics offers several avenues to enhance the quality and feasibility of this approach. The inherent physicochemical properties of HA, including biocompatibility, biodegradability, and high-water solubility, improve the solubility and physicochemical stability of immunostimulatory compounds or antigens that remain insoluble or unstable in aqueous environments [14,141,142].

The inability to cross various physiological barriers to reach desired targets, like the tumor ECM and/or blood-brain barrier, represents another major limitation of DDSs. In tumors, factors such as pH, interstitial fluid volume, abnormal vasculature, and mechanical properties can hamper drug/DDS penetration and diffusion [9]. After overcoming these barriers (via targeting, controlled release mechanisms or enhanced permeability), antigens, immunostimulants, or drugs can undergo selective release into the cell cytosol without degradation in lysosomal compartments; therefore, DDS design should favor adequate release kinetics in antigen-presenting cells (macrophages and DCs) [11]. HA addresses these challenges by targeting CD44 in cancer and immune cells. For cancer vaccines, selective HA targeting might tune uptake by APCs via receptor-mediated endocytosis and promote endosomal release into the cytosol to favor antigen cross-presentation and activation of adaptive immune cells [63,139]. Similarly, HA-based strategies have been developed to promote the cytosolic delivery of genetic material encoding immunostimulants or immune checkpoint inhibitors [143]. HA also enables the loading or conjugation of multiple components within the same formulation; this capacity ensures simultaneous delivery of antigens and adjuvants to the same APCs, thereby optimizing antigen presentation and eliciting a robust immune response [144,145]. We can exploit the immunostimulatory properties of LMW HA to obtain a nanocarrier that acts as an adjuvant to a vaccine [139,146]; this dual functionality simplifies formulation complexity, reduces the number of administrations, and minimizes off-target effects resulting from multiple system components [146]. Therefore, understanding the fate of the HA-based DDSs following administration remains of extreme importance for clinical translation. Although in vivo studies using HA-based DDSs have shown preferential accumulation in the liver due to the presence of CD44 and RHAMM on liver endothelial cells, strategies such as conjugation with PEG or polysarcosine (and other polymers) represent solutions to overcome undesired liver uptake [29,147,148].

Although the number of clinical investigations of HA-based drug delivery systems is still limited, there is a strong rationale for the use of HA for next-generation cancer immunotherapies, which could address current challenges of DDSs designed to deliver immunotherapeutic payloads. Indeed, the selective lymph-tropism and targeting properties, the reduction of side and stealth effect, and the MW-related immunomodulatory effects might be beneficial towards superior efficacy. Additionally, the presence of already marketed HA products could facilitate clinical transition and the regulatory processes. Overall, HA might have all the requirements to be a promising candidate for future DDS-based cancer immunotherapies.

5. Hyaluronic Acid-based Drug Delivery Systems: Applications for Cancer Immunotherapy and Immunomodulation

While immunotherapy represents an appealing therapeutic option in

cancer treatment, most patients do not benefit from currently available treatments [149]. The materials employed to develop potential DDSs for novel cancer immunotherapies should be carefully selected according to the required application [150]. The rational design of HA-based DDSs represents an appealing strategy to improve the efficacy of immunotherapies [9]; the next section of this review focuses on immunotherapy and the immunomodulatory applications of HA-based DDSs.

5.1. Hyaluronic Acid-based Conjugates

Owing to the immunosuppressive nature of most tumors, making cancer cells visible to the immune system via the delivery of non-self-antigens represents a potentially exciting strategy to induce cytotoxic T lymphocyte (CTL) responses. The conjugation of tumor antigens (molecules overexpressed or expressed only in cancer tissues) to the carboxylic and hydroxyl groups in HA can support recognition by effector T cells through a higher cell uptake, targeted delivery and consequent antigen presentation [134]. In this section, HA-based conjugates developed for immunotherapeutics purposes will be described with specific focus on their immune-related activities (Table 2).

HA as a component of cancer vaccines has been considered only in a few studies, leaving space for further investigation. An HA-based

Table 2
Relevant examples of HA-based conjugates (including the HA molecular weight, the target receptor, the cancer type and relevant outcomes) that have reached preclinical stages.

Hyaluronic acid-based conjugates					
Formulation		Target receptor	Cancer type	Outcomes	Ref
Drug delivery system	MW (kDa)				
HA-conjugate of TRP2 and gp100 antigens	100	LYVE-1	Melanoma B16-F10, Orthotopic	• Superior ability to delay tumor growth and extend survival in prophylactic and therapeutic settings in melanoma-bearing mice • Increased T-cell tumor infiltration • HA preferential trafficked conjugate to secondary lymphoid tissues (inguinal lymph nodes and spleen) by targeting LYVE-1	[63]
HA-conjugate of TLR7 and TLR8 agonists	Not disclosed	LYVE-1	Not performed	• TLR7/8 selectively released in the lymph nodes in murine immunization models, inducing negligible systemic exposure • HA enhances the bioavailability of conjugated antigens in the lymph nodes by recognizing LYVE-1 and promoting lymphatic drainage and interactions with DCs	[64]
HA-conjugates of OVA, SOD, BSA, hGH, and H5N1, antigens	200	Not disclosed	Not performed	• Covalent binding crucial for the pro-adjuvant effect • Standard immunization schedule revealed that HA-conjugates induced a stronger antigen-specific antibody response than free antigens • Only HA 200 kDa induced significant immunization, while other HAs displayed lower (50 kDa) or no efficiency (500 and 15 kDa) • The immunostimulant effect of HA compared well to active experimental adjuvants (CFA-IFA) but displayed an increase compared to those approved for clinical applications (alum and AddaVax) • CD44-mediated cell uptake led to efficient antigen presentation via MHC class I	[139]
HA-OVA conjugates	35	CD44	Lung cancer TC-1 Xenograft	• HA-conjugates selectively accumulated in tumor tissues following systemic administration in mice immunized with vaccinia virus expressing OVA • Specific anti-OVA CTL response inhibited tumor growth	[151]
PEG-coated HA-OVA conjugates	35	CD44	Lung cancer TC-1 Xenograft	• Protection of HA-OVA conjugates with PEG provided higher stability • HA-induced receptor-mediated endocytosis in cancer cells, prompting the successful exposure of foreign antigens to induce an efficient CTL response	[153]
HA-PEG-OVA polyplexes with PD-L1 siRNA	35	CD44	Lung cancer TC-1 Xenograft	• HA-PEG-OVA polyplexes delivered into CD44-expressing tumor cells and DCs effectively downregulated PD-L1 (reducing immune suppression) and cross-presented OVA • Synergistic effect on multiple arms of cancer immunity cycle: increased T cell response against antigen and TIME reprogramming induced long-lasting protective immunity	[143]
HA conjugates of DOX and poly(L-histidine) nanocores of R848	47	CD44	Breast cancer 4 T1 and 4 T1-Luc Orthotopic	• Significant improvement in tumor growth inhibition via tumor immunity regulation	[155]
HA conjugates of DOX and CpG (TLR9 agonist)	100	CD44	Glioblastoma GL261 Orthotopic	• HA-DOX and HA-CpG induced long-term survival and increased CD8+ T cell and reduced TAM numbers • *Re*-education of TAMs towards an immune-inflammatory setting (HA-CpG) and induction of ICD (HA-DOX) ascribed to CD44-mediated interactions with cells, leading to a rapid cell uptake and more pronounced effect	[158]
HA conjugates of DOX and GEM	100	CD44	Glioblastoma GL261 and SB28 Orthotopic	• Decrease in M2-like macrophages and monocytic MDSCs, in favor of the pro-inflammatory counterpart, with consequent better control of the tumor growth	[160]
HA-MSA2 (STING agonist)	100	CD44	Glioblastoma SB28 Orthotopic	• HA-MSA2 reduced immune suppression by significantly increasing the number of inflammatory cells (NK cells, DCs, and T cells) compared to MSA2 • HA conjugation overcame the poor water solubility of MSA2, enhanced its delivery to the tumor via CD44-expressing cells, and modulated immune surveillance and responses	[161]

Abbreviations: Molecular Weight (MW), Hyaluronic Acid (HA), kilo Dalton (kDa), Tyrosinase Related Protein 2 (TRP2), glycoprotein 100 (gp100), Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (LYVE-1), Toll-Like Receptor7/8 (TLR7/8), Dendritic Cells (DC), ovalbumin (OVA), superoxide dismutase (SOD), Bovine Serum Albumin (BSA), Human Growth Hormone (hGH), and influenza A virus subtype (H5N1), Freund's Complete Adjuvant (CFA), Freund's Incomplete Adjuvant (IFA), Cluster of Differentiation 44 (CD44), Major Histocompatibility Complex class I (MHC class I), Cytotoxic T Lymphocytes (CTL), Polyethylene glycol (PEG), Programmed Death Ligand 1 (PD-L1), small interference RNA (si-RNA), Tumor Immune Microenvironment (TIME), doxorubicin (DOX), cytosine phosphate guanine (CpG), Tumor-Associated Macrophages (TAMs), immunogenic Cell Death (ICD), Gemcitabine (GEM), Myeloid-Derived Suppressor Cells (MDSCs), Natural Killer (NK).

vaccine (MW 100 kDa) carrying two melanoma antigens (MHC I: TRP2 and MHC II: gp100) delayed tumor growth and extended survival following subcutaneous injection in prophylactic and therapeutic settings in melanoma-bearing mice [63]. The authors observed increased T cell infiltration in the tumor, highlighting HA as an attractive material for the formulation of cancer vaccines; moreover, HA guided preferential trafficking to secondary lymphoid tissues (i.e., inguinal lymph nodes and the spleen) by targeting LYVE-1 [63].

Yoo et al. took advantage of HA tropism to efficiently deliver TLR7/8 agonists to lymphoid tissue, via the synthesis of HA-conjugates [64]. In murine immunization models (IFN-β mice, injected in the hock or footpad), the lymph node-specific release of the TLR7/8 agonists from HA-conjugates helped to reduce systemic exposure in a strategy that might be important for developing vaccines delivering immune adjuvants directly to lymph nodes.

Another study investigating HA as an adjuvant in protein-based vaccines revealed HA's ability to enhance the bioavailability of conjugated antigens in lymph nodes. Indeed, the HA-LYVE-1 interaction promoted lymphatic drainage and the consequent interaction of protein-based vaccines with DCs [139] (Fig. 5). The authors chemically conjugated different antigens (e.g., OVA, superoxide dismutase [SOD], bovine serum albumin [BSA], human growth hormone [hGH], and influenza A virus subtype H5N1) to LMW HA (200 kDa) and revealed a requirement for covalent binding for the adjuvant effect. The standard immunization schedule, including a primary intramuscular injection and two boosters, revealed that the HA-antigen conjugates induced a stronger antigen-specific antibody response than free antigens. Mice immunized with a mixture of "free" OVA and HA antigens did not display any adjuvanticity, showing a comparable IgG titer to OVA alone. The authors also confirmed the role of HA MW in the observed biological effects: only conjugates containing HA of 200 kDa induced significant immunization, while the others remained less efficient (50 kDa) or inefficient (15 and 500 kDa). Interestingly, a comparative study with commercially available adjuvant compounds highlighted that the immunostimulant effect of HA compared well to active experimental adjuvants (Freund's Complete Adjuvant [CFA] and Freund's Incomplete Adjuvant [IFA]), but displayed increased activity when compared to those approved for clinical applications (Alum and AddaVax). This study did not describe the use of HA in vaccines for cancer specifically; however, they provide valuable insights for the formulation of next-generation cancer immunotherapies considering HA as an adjuvant.

HA-CD44 also represents an appealing interaction that can increase local drug availability and efficacy while reducing toxic side effects. Several formulations have exploited HA as a targeting agent, but only a few studies have used the HA-CD44 interaction to provide additional value to cancer immunotherapy. For example, Lee et al. employed polymer conjugates of HA (as targeting moiety) (35 kDa) and OVA (as model antigen) to make tumors visible to the immune system and study CTL-based cancer immunotherapy [151]. After systemic administration, the authors revealed a CD44-mediated cell uptake, leading to an efficient OVA presentation via MHC class I on the tumor cell surface. HA-OVA conjugates selectively accumulated in tumor tissues in mice immunized with a vaccinia virus expressing OVA; they also observed a specific generation of an anti-OVA CTL response against the virus directed to tumor cells expressing OVA, finally leading to tumor growth inhibition. Related studies demonstrated that adding a PEG shell to HA-OVA conjugates provided more significant formulation stability, thanks to the surface shielding from aggregation, opsonization, and the prolongation of circulation time [152]. In another study, conjugating PEG via a MW of 5 kDa using a MMP-9-responsive linker allowed the

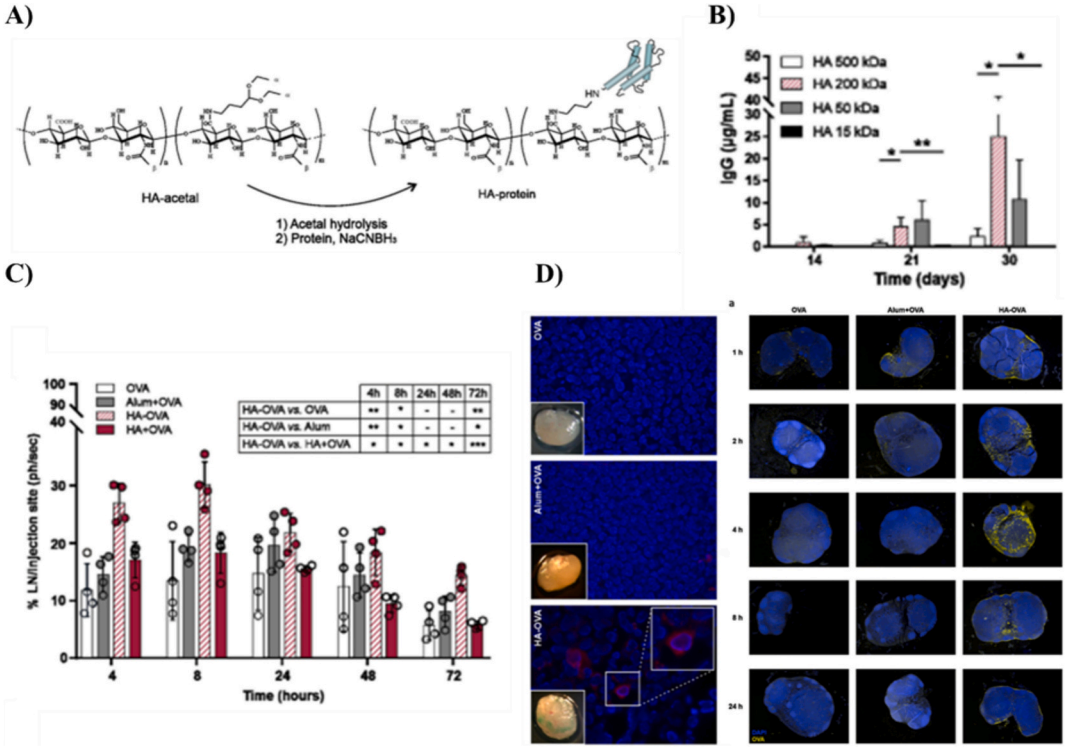


Fig. 5. Hyaluronic acid as an effective immunological adjuvant for protein-based vaccines. A) Scheme for the synthesis of a hyaluronic acid-based ovalbumin antigen conjugate (HA-OVA) starting from HA-acetal molecules. **B)** Total IgG concentration at different time points in the sera of mice immunized with HA-OVA conjugates with different MWs of HA (500, 200, 50, and 15 kDa). **C)** HA-OVA favors lymph node accumulation in terms of the percentage ratio of lymph node to muscle photons detected at different time points after intramuscular injection and according to the percentage of dye-labeled OVA^{+} cells in lymph nodes harvested 4 h post-injection. Representative fluorescence micrographs of lymph node specimens. **D)** Time course analysis of OVA distribution in lymph nodes from immunized mice: only OVA+ cells (yellow) and nuclei (blue) are visualized. Reproduced with permission from [139]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

selective detachment of the HA-OVA conjugates at the tumor site, where HA-induced receptor-mediated endocytosis; hence, the foreign antigen OVA became successfully exposed to cancer cells for recognition and an efficient CTL response [153].

Another study aimed at strengthening antitumor immunity by enhancing tumor cell recognition by the immune system via the blockade of programmed cell death ligand (PD-L1) expression [143]. The authors developed siPD-L1- polyethyleneimine-based polyplexes using PEGylated HA (35 kDa) for targeting and OVA as a model antigen [143]. The rationale behind this strategy relates to the preferential expression of PD-L1 in $CD44^{high}$ tumor cells in breast and lung carcinomas that prompts the suppression of T-cell-mediated immunity [154]; moreover, the expression of CD44 by DCs plays a key role in co-stimulation for T cell activation in tumors. Therefore, they employed HA as a targeting tool to support the dual blockade of the PD-L1 pathway in $CD44^{high}$ DCs and tumor cells [143]. The systemic injection of PEG-HA-OVA polyplexes effectively targeted $CD44^{high}$ tumor cells and DCs, inducing the downregulation of PD-L1 (reducing immune suppression) and the cross-presentation of OVA. The synergistic effect on multiple arms of the cancer immunity cycle represents the main advantage of this approach: increasing T cell response against the antigen and reprogramming of the TIME, leading to long-lasting protective immunity.

This multimodal targeting of different arms of the TIME may also achieve successful outcomes through the combination of immunotherapy and chemotherapy. A recent study combined HA-DOX (47 kDa) conjugates with poly(L-histidine) (PHIS) nanocores of resiquimod R848 (TLR7/8 agonist used as anti-tumor immune regulator) for intravenous treatment of 4T1 breast cancer (HA-DOX/PHIS/R848) [155]. R848 promotes DC maturation and plays a crucial role in anti-tumor immunity [156], while HA allows the active targeting of the high levels of CD44 expressed by breast cancer cells. Selective HA-CD44 interactions with cancer cells and immune-regulatory activity targeted to immune cells with R848 provided remarkable improvements in tumor growth inhibition via the regulation of tumor immunity [155]. Compared to control, intra-tumoral CD3+ and CD8+ T cell numbers increased in DOX-treated groups. Interestingly, the team observed a higher number of CD8+ T cells in the HA-DOX/PHIS/R848 nanoparticle-treated group compared to the remaining treatments, highlighting the strong anti-tumor immune response of the developed system.

Immunomodulation induced by local administration appears to induce enhanced therapeutic outcomes compared to systemic administration, with higher drug doses reaching the tumor site and reducing side effects [157]. The immunosuppressive nature of most tumors renders TIME reshaping a potential anticancer strategy with the induction of immune cell infiltration; for example, the anatomical location and the TIME render GBM cases refractory to current chemo-immunotherapeutics. Two studies investigated the intracranial administration of a combination of HA-DOX (100 kDa) and HA-CpG (TLR9 agonist) conjugates [158,159] to explore potential tumor immune synergism between the ICD induced by DOX and with the adjuvant properties of CpG. Encouragingly, the combination of HA-conjugates induced long-term survival, increased numbers of CD8+ T cells, and reduced numbers of TAMs. The presence of HA contributed to the re-education of tumor-associated microglia towards an immune-inflammatory setting (HA-CpG) and in the induction of ICD (HA-DOX) thanks to the expression of CD44 by immune and cancer cells, leading to more rapid cell uptake and more pronounced therapeutic effects [158]. Recently, our group proved that the combination of HA (100 kDa) conjugates of DOX and gemcitabine (GEM) might affect the local tumor-associated myeloid cells, including macrophages and MDSCs. We highlighted a decrease in M2-like macrophages and monocytic MDSCs, in favor of the pro-inflammatory counterpart represented by M1-like phenotype, with consequent better control of the tumor growth [160].

In addition to this example of HA-based conjugates of chemotherapeutics impacting the GBM TIME, a related study by Chellen et al. reported the conjugation of HA (100 kDa) to a STimulator of INterferon Genes (STING) agonist (MSA2), which reduced immune-suppression by significantly increasing the number of inflammatory cells (e.g., NK cells, DCs, and T cells) when compared to free MSA2 treatment [161]. HA conjugation overcame the poor water solubility of MSA2 to enhance delivery into CD44-expressing tumor cells and modulate immune surveillance and responses.

5.2. Hyaluronic Acid-coated Nanoparticles

The immune-adjuvant activity of HA also occurs through the improvement of drug delivery to immune cells with surface HA coating of nanoparticles (Table 3). For example, pH-responsive HA-modified liposomes displayed enhanced uptake by APCs via CD44 targeting and the selective delivery of antigens into the DC cytosol [162]. The surface modification of liposomes with HA through a pH-responsive linker prompted membrane fusion in acidic endo/lysosomes, allowing the cytoplasmic release of antigens in DCs, eventually leading to cross-presentation. Liposome derivatives with LMW HA (8–50 kDa) displayed higher production of T helper 1 cytokines (TNFα and IL12) from DC cells than HA only; these cytokines play crucial roles in activating macrophages and enhancing their ability to kill foreign pathogens. Subcutaneous administration of HA-modified liposomes encapsulating OVA as a model antigen to lymphoma-bearing mice (E.G7-OVA cells) led to antigen-specific immune responses in the spleen and tumor regression, suggesting this multifunctional system represents a promising strategy to achieve efficient antigen delivery and consequent immune responses.

Another study by Nie et al. employed HA-mannose-decorated iron oxide (HA-man@Fe_3O_4) nanoparticles as a nanovaccine, with mannose a targeting agent for TAMs and HA and iron oxide inducing M2-like immune cell polarization [163]. The authors performed a two-step vaccination: HA-man@Fe_3O_4 nanoparticles induced local TAM polarization followed by free neoantigen peptides or HA-man@Fe_3O_4-conjugated peptides. This intratumorally injected nanovaccine platform converted the TIME by increasing CD8+ T cell infiltration in the human papillomavirus-related tumor (TC-1) subcutaneous model and inducing DC maturation in sentinel lymph nodes. The HA-man@Fe_3O_4-conjugated peptides induced more significant tumor inhibition than HA-man@Fe_3O_4 and peptides, suggesting the effectiveness of the conjugation and confirming the ability of HA to preferentially activate pro-inflammatory pathways in TAMs [77].

Song et al. also exploited the ability of HA to reprogram TAMs in the development of HA-coated manganese dioxide nanoparticles to reduce tumor hypoxia and modulate chemoresistance [164]. Combination treatment with DOX induced the significant inhibition of breast tumor (4 T1 murine breast cancer) growth and proliferation and an HA-driven shift of the immune cells with an M2-like phenotype towards their M1-like counterparts.

5.3. Micelles

The conjugation of HA to hydrophobic compounds can prompt the formation of self-assembling nanoaggregates called micelles (Table 4); for example, Zhou et al. evaluated HA (10 kDa)-vitamin E succinate micelles for their potential antitumor activity [165]. The authors included Pluronic F68 in the formulation as a copolymer to improve stability and selected docetaxel as a chemotherapeutic agent. Systemic administration combined with an anti-PD-L1 antibody revealed micelle accumulation in melanoma tumors (B16 tumors) via the HA-mediated targeting of cancer cells and demonstrated that the anti-PD-L1 antibody restored immunological potential.

Yang et al. employed HA (35 kDa) to formulate micelles used for the intranasal delivery of short interfering (si)RNA for glioma therapy [166]. The authors enveloped a cell-penetrating peptide DP7-C modified with cholesterol and enveloped by HA to prolong retention time in the nasal cavity, increase drug absorption, and enable specific targeting of

Table 3
Relevant examples of HA-coated nanoparticles (including the HA molecular weight, the target receptor, the cancer type and relevant outcomes) that have reached preclinical stages.

Hyaluronic acid-coated nanoparticles					
Formulation		Target receptor	Cancer type	Outcomes	Ref
Drug delivery system	MW (kDa)				
HA-coated pH-responsive liposomes	8–50	CD44	Lymphoma E.G7-OVA Xenograft	• Enhanced APC uptake via CD44 and selectively delivered antigens to the DC cytosol, with successful endosomal escape through a pH-sensitive linker and increased Th1 cytokine production • Subcutaneous administration prompted an antigen-specific immune response in the spleen and tumor regression	[162]
HA-mannose decorated iron oxide nanoparticles	Not disclosed	Not disclosed	Lung cancer TC-1 Xenograft	• Locally injected nanovaccine converted the TIME by increasing CD8+ T cell tumor infiltration and inducing DC maturation in sentinel lymph nodes	[163]
HA-coated manganese dioxide nanoparticles	40	Not disclosed	Breast cancer 4 T1 Xenograft	• Combination treatment with DOX induced the significant inhibition of breast tumor growth and proliferation and an HA-driven shift of immune cells with an M2-like phenotype towards their M1-like- counterparts	[164]

Abbreviations: Molecular Weight (MW), Hyaluronic Acid (HA), kilo Dalton (kDa), Antigen Presenting Cells (APC), Dendritic Cells (DC), T helper 1 (Th1), Tumor Immune Microenvironment (TIME).

Table 4
Relevant examples of HA-based micelles (including the HA molecular weight, the target receptor, the cancer type and relevant outcomes) that have reached preclinical stages.

Micelles					
Formulation		Target receptor	Cancer type	Outcomes	Ref
Drug delivery system	MW (kDa)				
HA-based copolymer micelle in combination with anti-PD-L1 antibody	10	CD44	Melanoma B16 Xenograft	• Systemic administration of micelles and an anti-PD-L1 antibody led to micelle accumulation in melanoma tumors via the HA-mediated targeting of cancer cell • anti-PD-L1 antibody restored immunological potential	[165]
Core-shell nanomicelle HA/DP7-C encapsulating siRNA targeting VEGF and PLK1	35	CD44	Glioma GL261 Orthotopic	• High in vitro cell uptake associated with the HA-CD44 interaction • Intranasal delivery of the siRNA-based system prompted increased tumor accumulation and the prolonged survival of GL261 tumor-bearing mice	[166]
HA-bilirubin micelles loaded with SC144	100	CD44	Melanoma MC38 Orthotopic, Breast cancer 4 T1 Orthotopic	• Systemic administration of SC144-loaded HABN induced apoptosis and ICD of tumor cells and reprogramming of M2-like into M1-like macrophages • Powerful result of combination therapy when administered anti-PD-L1	[167]

Abbreviations: Molecular Weight (MW), Hyaluronic Acid (HA), kilo Dalton (kDa), Programmed Death Ligand 1 (PD-L1), Vascular Endothelial Growth Factor (VEGF), Polo-Like Kinase 1 (PLK1), Small Interference RNA (si-RNA), bilirubin (BN), Immunogenic Cell Death (ICD).

cancer cells. The core-shell nanomicelle HA/DP7-C encapsulating siRNA (targeting *VEGF* and *PLK1*) supported high levels of in vitro cell uptake associated with the HA-CD44 interaction. The intranasal delivery of this siRNA system led to increased tumor accumulation and prolonged survival time in a murine GBM GL261 model.

Combining HA-based micelles with immune checkpoint blockade to re-educate the TIME represents an appealing strategy. Moon et al. employed HA (100 kDa) conjugated to bilirubin (HA-BN) to encapsulate SC144, a gp130 inhibitor that blocks the IL-6/gp130/STAT3 pathway, induces ICD of tumor cells, and polarizes macrophages to the M1-phenotype in vitro (Fig. 6) [167]. Systemic administration of SC144 loaded within HABN nanoparticles (SC144@HABN) promoted ICD of cancer cells and increased the ratio of M1-like to M2-like macrophages; moreover, this approach decreased the frequency of MDSCs and CD4+ regulatory T-cells and increased the number of anti-tumor CD8+ T-cells. Moreover, SC144@HABN combined with an anti-PD-L1 antibody efficiently eliminated melanoma MC38 tumors and immune refractory breast cancer 4 T1 tumors in vivo.

5.4. Hydrogels

Implantable scaffolds have been considered an efficient reservoir of immunotherapeutics such as antibodies, cytokines, antigens, chemokines, and immune cells [168]. Biomaterials such as HA possess inherent activity and thus can also promote antitumor immune responses that increase survival [169]. The viscoelastic properties of HA are ascribed to interactions between chains of hydrogen bonds: as the concentration of HA increases, so does the viscosity, owing to chain waving and the formation of 3D matrices [135], which represents the basis of the gelation exploited for the formation of HA-based scaffolds (Table 5). HA can bind growth factors, recognize CD44 receptors on lymphocyte surfaces, promote cell migration, and stimulate the activation of TLR2/4 with consequent production of IL-12.

These characteristics prompted Pinon-Zarate et al. to develop a scaffold made of gelatin and HA (1500 kDa) coupled to CpG and the tumor antigen MAGE5 for the prophylactic treatment of melanoma [170]. This matrix-based immunotherapy mediated DC and macrophage activation, reduced tumor growth rate, and increased survival in mice challenged with B16F10 murine melanoma tumors; of note, only the scaffold containing MAGE5 induced CD8+ T cell activation.

As the main component of the ECM, HA has a robust supportive effect on cell proliferation and migration and, as such, represents a good scaffold candidate for cell-based cancer immunotherapy; for instance, the peri-operative placement of Chimeric Antigen Receptor (CAR)-NK

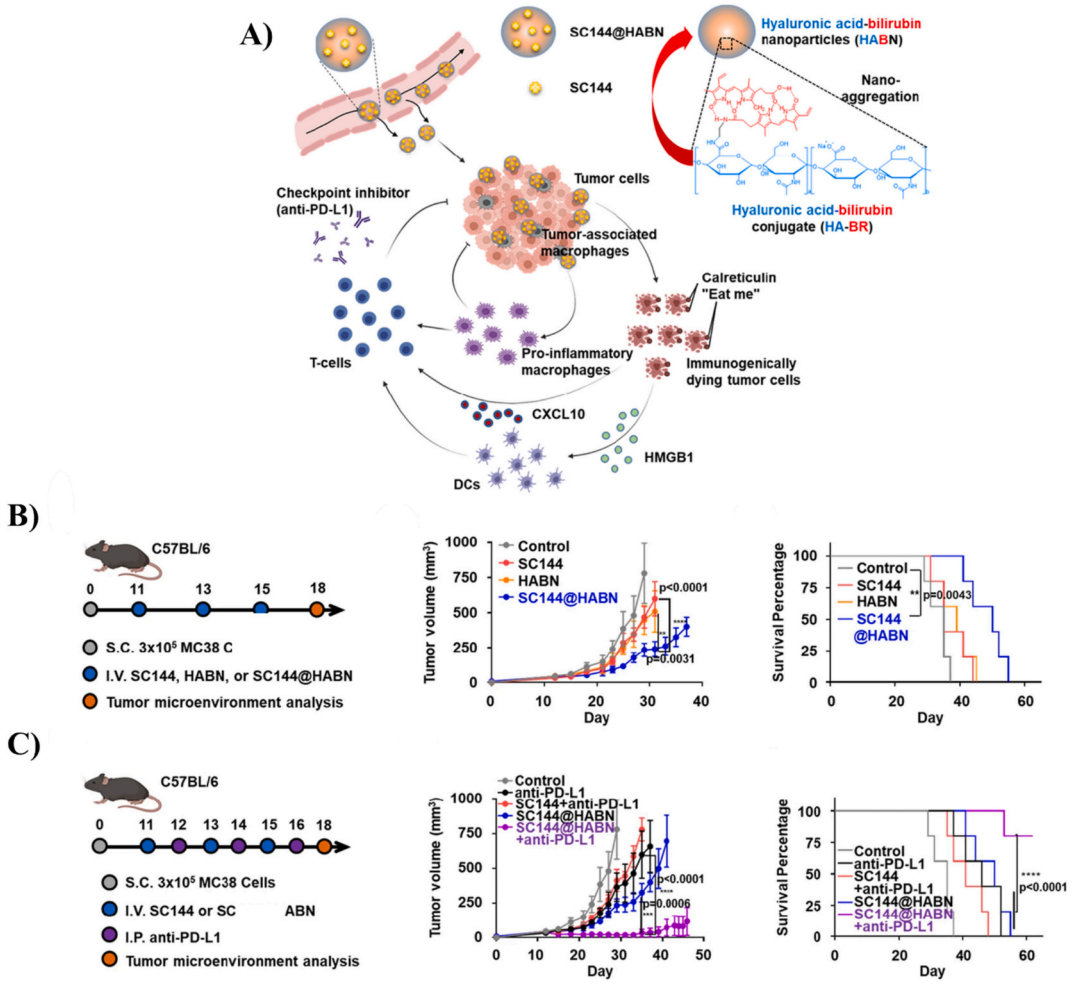


Fig. 6. Hyaluronic acid-bilirubin-mediated chemo-immunotherapy. A) Schematic representation of the hyaluronic acid (100 kDa)-bilirubin conjugate (HABN) formulation and the mechanism of action in tumor cells and tumor-associated macrophages. **B)** Scheme of in vivo experiments in MC38-bearing mice treated with SC144, HABN, or SC144@HABN, with the latter improving tumor growth graphs and survival curves compared to the remaining groups. **C)** Scheme of in vivo experiment in MC38-bearing mice treated with SC144, SC144@HABN, and SC144@HABN combined with an anti-PD-L1 antibody, with the latter significantly improving tumor growth graphs and survival curves. Reproduced with permission from Lee et al. [167].

cells embedded within HA scaffolds prevented relapse and metastases in the incomplete resected MDA-MB231 breast cancer model [171]. Similarly, an HA-based hydrogel containing CAR-T cells, IL-15, and an anti-PD-L1 antibody conjugated to human platelets facilitated cell distribution within the surgical bed, inhibiting local tumor recurrences and distant malignancies [172].

Besides an active role in cell recruitment and proliferation, HA has well-known physicochemical properties, which include biocompatibility, biodegradability, and the possibility of supporting controlled drug release, which makes it advantageous when developing scaffolds. Jia et al. used a dynamic HA-based hydrogel to protect mRNA (mOVA)-R848 lipid nanoparticles from degradation at room temperature, preventing mRNA hydrolysis and fusion of lipid nanoparticles by interchain interactions [173]. Gel-like HA undergoes state transition under physiological conditions, thus allowing long-term cargo release in vivo with enhanced lymph node-targeting abilities. In this study, the lipid nanoparticles successfully inhibited tumor growth and metastases due to prolonged exposure of mRNA and adjuvants to immune cells.

In a related study, Ren et al. proposed a degradation-regulatable HA-based scaffold as a potential strategy to reshape the TIME [174]. They fabricated the scaffold with a stable 'bulk' material (methacrylate-modified HA) and hydrolytic-labile 'sacrificing' component (methacrylate-modified oxidized HA) with different blending ratios to induce degradation from 10 to 28 days in vivo. They loaded the implantable scaffold with paclitaxel, R837 (adjuvant), and two immune checkpoint inhibitors (anti-CTLA-4 and anti-OX40 antibodies). This strategy depleted MDSCs and M2-like macrophages, enhancing DC and effector T cell tumor infiltration. The authors hoped that the implementation of this HA-based scaffold with degradation regulatability could convert the immunosuppressive TIME in antitumor immunity niches, thus improving therapeutic efficacy.

Different HA-based DDSs have been employed for preclinical studies in cancer immunotherapy and immunomodulation by exploiting various strategies, including HA-conjugates, NPs surface coatings, micelles, and HA-based scaffolds. In most cases, LMW HA has been used (<250 kDa) to target cancer or immune cells, facilitating preferential lymph node trafficking, enhancing cell uptake, and reprogramming macrophages and the tumor microenvironment. On the other hand, HMW HA (>1000 kDa) has been employed for creating HA scaffolds for cell therapy and modulation of the TIME. These approaches highlight the versatility and potential of HA, whose application can be tailored according to the intended application.

6. Critical Considerations and Future Challenges of Hyaluronic Acid-based Drug Delivery Systems

The application of HA as a component of DDSs represents an appealing opportunity in cancer immunotherapy; however, MW,

Table 5
Relevant examples of HA-based hydrogels (including the HA molecular weight, the target receptor, the cancer type and relevant outcomes) that have reached pre-clinical stages.

Hydrogels					
Formulation		Target receptor	Cancer type	Outcomes	Ref
Drug delivery system	MW (kDa)				
HA-gelatin scaffold containing CpG and MAGE5	1500	CD44 TLRs	Melanoma B16-F10 Xenograft	• Activated DCs/macrophage, reduced tumor growth, and increased survival • Only the scaffold containing MAGE5 induced CD8 T cell activation	[170]
HA-based scaffold for CAR NK cells	Not disclosed	Not disclosed	Breast MDA-MD-231 (incompletely resected) Xenograft	• Peri-operative placement of the scaffold prevented relapse and metastasis	[171]
HA-based hydrogel containing CAR-T cells, IL-15, and anti-PD-L1 antibody conjugated to human platelets	77	Not disclosed	Melanoma luciferase-labeled WM115 Xenograft	• Scaffold facilitated cell distribution within the surgical bed, inhibiting local tumor recurrences and distant malignancies	[172]
HA hydrogel containing mRNA-R848 lipid nanoparticles	40–100	Not disclosed	Melanoma B16-F10 and MC38 Orthotopic	• Protected lipid nanoparticles from degradation at room temperature, preventing mRNA hydrolysis and fusion of lipid nanoparticles by interchain interactions • Gel-like HA allows a long-term release in vivo with enhanced lymph node targeting ability • Successfully inhibited tumor growth and metastasis due to prolonged exposure of mRNA and adjuvants to immune cells	[173]
Degradation-regulatable architectured implantable macroporous scaffold (Dr-AIMS) with modified HA, paclitaxel, R837, anti-CTLA-4 and anti-OX40	500	Not disclosed	Breast Cancer 4 T1 (resection) Orthotopic	• Controlled degradation from 10 to 28 days in vivo • Depletion of MDSCs and M2-like macrophages enhanced DC and effector T cell tumor infiltration	[174]

Abbreviations: Molecular Weight (MW), Hyaluronic Acid (HA), kilo Dalton (kDa), Cytosine Phosphate Guanine (CpG), Melanoma Antigen Family 5 (MAGE5), Toll-Like Receptor (TLR), Dendritic Cell (DC), Chimeric Antigen Receptor (CAR), Natural Killer (NK), Interleukin-15 (IL-15), Programmed Death Ligand 1 (PD-L1), Resiquimod (R848), Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) Myeloid-Derived Suppressor Cells (MDSCs).

density, form (e.g., polymer-drug conjugates, nanocarriers or hydrogels), linking chemistry, and synthetic and purification processes significantly impact metabolism, interaction with receptors, and outcomes leading to multiple (and/or opposite) biological effects. This section will overview the challenges associated with the use of HA - from chemical design to regulatory approval and scaling-up processes - and offer recommendations for fostering the development of HA-based DDSs (Fig. 7).

6.1. Design and Optimization of Bioresponsive Linking Chemistries

The interaction between HA and the remaining components of the formulation remains crucial to treatment outcomes. Antigens or drugs can be physically encapsulated by HA or conjugated to HA; the inadequate selection of a linking chemistry could allow cargo release before reaching the desired site of action, strong entrapment with a lack of release, or enzyme degradation, causing system deterioration [142]. The molecular structure of HA contains a range of reactive groups (including

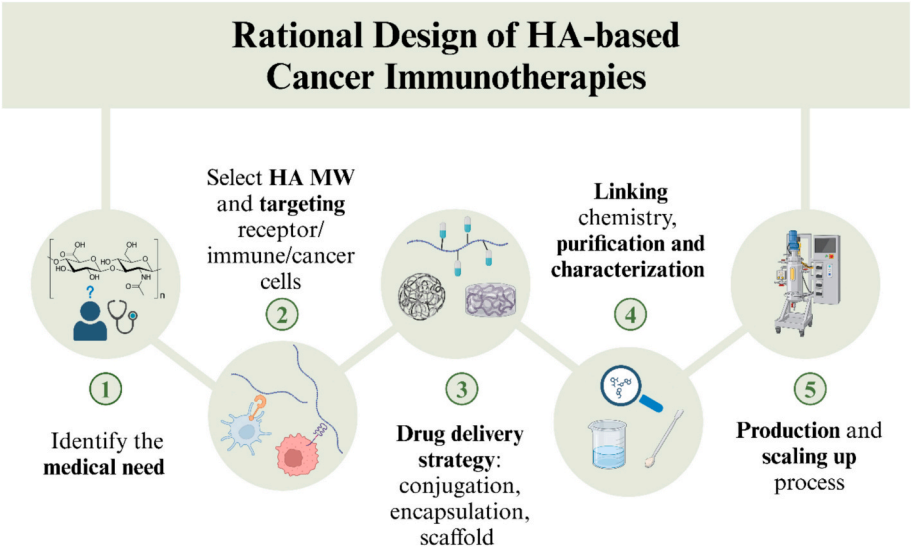


Fig. 7. Rational design of Hyaluronic Acid-based cancer immunotherapies. First, the medical need to use a hyaluronic acid (HA)-based drug delivery system must be identified. The HA molecular weight (MW) and the consequent target are selected according to desired outcomes. The drug delivery strategy (i.e., conjugation, encapsulation, or scaffold) is also chosen, specifically linking chemistry and characterization analyses. Finally, the production and scaling-up process are considered for efficient clinical translation.

carboxyl, hydroxyl, and acetylamino groups) that can easily be derivatized to form various DDSs. The selection of the linking moiety relies on the type of the nanocarrier, the match between the functional group of the HA backbone and the selected drug, the intended administration route, and the biological barriers that the DDS must overcome. The rational selection of linking chemistries allows for prolonged drug/antigen plasma stability in circulation, followed by efficient, controlled release kinetics under the desired conditions after reaching the desired site of action [175]. The linking chemistry must be designed according to the target; drugs targeting intracellular sites or transcytosis processes could require bio-responsive linkers to release the targeting ligand from the conjugate, thus securing cargo release without cell recycling. Furthermore, the inherent degradability of HA gives rise to an additional stimuli-responsive activation of the desired functions mediated by Hyals, which confers enhanced nanocarrier biodegradability and subsequent cargo release. Linkers might involve simple one-step conjugation using ester, amide, hydrazone, disulfide, thioketal, and "click" chemistries or advanced linkers and spacers that require multiple steps and procedures. The selection of the linker should be designed to modulate drug release/accessibility/target exposure, significantly improving therapeutic outcomes; however, linkers requiring several synthetic steps suffer from increased costs and scalability limitations.

6.2. Strategies to Enhance the Selectivity Towards Immune or Cancer Cells

The targeted delivery of therapeutic agents to a specific subset of immune cells or cancer cells (by distinguishing them from healthy or unrelated cells) offers several advantages, such as enhancing the treatment's effects while minimizing systemic side effects. Although HA has a special affinity towards CD44, this receptor is differentially expressed across human and murine tissues. High expression is observed in the liver [176] and spleen [177], moderate levels in the lungs [178] and kidneys [179], and low expression in the brain [180,181] and heart [182]. In the intestine, CD44 shows region-specific expression and notable alternative splicing [183,184]. However, since there are significant differences between animal models and humans, an accurate mapping of CD44 expression (as well as the other HA-targeted receptors described in this review) is essential to ensure effective translational relevance. Also, in the context of CD44 targeting, the HA MW plays a role: HMW HA (700 kDa) coating of nanoparticles has been shown to bind with higher-affinity CD44 receptors than LMW HA (6.4 kDa). This might be ascribed to the increase in the avidity as a result of multivalent interactions between HA binding sites and CD44 receptors expressed on cancer cells [185]. We suggest that both the binding affinity and, consequently, the MW of HA should be carefully considered in the experimental design according to the desired application (immunomodulation/target). As discussed above, a key advantage of HA is the ease of implementation of the chemistry, a key feature for developing targeted DDS. For immune cell targeting, LMW HA fragments are more readily internalized by phagocytic immune cells [186], and they can better escape from HYAL-driven clearance [187]. Additionally, incorporating a polymeric matrix having a positive surface charge can make the system more prone to cause cytokine secretion, inducing T-cell proliferation and consequent immune response [188]. To further improve specificity, HA-based carriers can be functionalized with immune cell-specific targeting ligands to drive selective interactions and uptake by the desired immune cell populations [189]. For instance, targeting of HA-based nanocarriers towards monocytes (macrophages and DCs) can be exploited by taking advantage of the selective expression of specific receptors such as CD206 (or mannose receptor), folate receptor, or CD11b [189]. Specifically, interaction with CD206 on tumor-associated macrophages offers the potential to reprogram or deplete these cells, ultimately leading to tumor suppression [190–193]. To enhance tumor selectivity, the use of bioresponsive linkers that are rationally designed based on the features of the TIME becomes crucial. For example, pH-sensitive linkers such as hydrazone bonds can facilitate drug release in the acidic TIME, while MMP-cleavable peptides respond selectively to tumor-associated matrix metalloproteinases [194,195].

6.3. Purification and Characterization of Hyaluronic Acid-based Drug Delivery Systems

The synthesis and formulation of DDSs require adequate chemical and physical characterization to identify each intermediate after every step, which remains crucial to enhanced therapeutic outcomes and efficient translation. These factors have more significance for HA, given that this polymer is isolated from animal tissues or produced via microbial fermentation [196]. HA isolated from animal tissues (e.g., rooster combs, skin, synovial fluid, and umbilical cord) displays high MW polydispersity owing to seasonal variations, animal health, and differing animal feed. The presence of other glycosaminoglycans can hamper HA purification, potentially resulting in contamination and safety concerns. Microbial fermentation provides an alternative approach but has the potential to also involve other impurities, such as nucleic acids, proteins, or microbial toxins [197]. Immunological studies performed with non-purified extracts demonstrate that compounds and contaminants (like lipopolysaccharides) could affect immunological activity [198]. For example, pharmaceutical-grade HA and HA fragments fail to stimulate in vitro-derived or ex vivo macrophages and DCs and did not induce leukocyte recruitment after intratracheal instillation in mouse lungs [199]. The authors ascribed the stimulatory effects observed in other studies as results of endotoxin contamination that was not entirely removed by polymyxin B treatment.

Controlling MW during production remains vital to intended applications owing to the opposing immunological outcomes of HAs of differing MWs [18]. The border between pro- and anti-inflammatory HA remains controversial, with competing studies demonstrating opposing immunogenic reactions induced by HA [3]. Further investigation will determine the specific range of MWs that boost immune responses and induce anti-inflammatory effects. Although in vitro experiments may provide valuable insight with regards to identifying target cells and related pathways, most observations remain untranslatable to the in vivo setting [142]; therefore, we must address the issue of HA polydispersity after production to ensure high purity and maximize yields [71]. First, fine-tuning microbial fermentation by adjusting pH, temperature, and nutrient supply can help control MW distribution. Following production, monodisperse LMW HA can be prepared via chemical hydrolysis in ethanolic hydrochloric acid, which ensures low ethanol and water consumption and the possibility of hydrolyzing substantial amounts of HA in small solvent volumes. Controlled enzymatic degradation with Hyals, which can selectively degrade HMW HA with precise control [197], represents another approach. After obtaining the desired MW HA, subsequent synthetic steps (such as drug conjugation, encapsulation, and cross-linking) can occur under conditions associated with the selected linking chemistry. These steps require additional analyses and batch-to-batch characterization of obtained materials [200]. Important features to be determined include the chemical identity of the polymer (for novel constructs such as polymer-drug conjugates), MW, hydrodynamic diameter, surface charge, drug loading, release kinetics, free drug content, and rheological behavior (for hydrogels). The adequate characterization of the HA-based DDSs generally requires a combination of analytical techniques followed by in-depth biological evaluations:

Identification of chemical composition and identity. Techniques such as nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FT-IR), elemental analysis, high-performance liquid chromatography (HPLC, coupled with different detectors), or even more sensitive techniques such as liquid chromatography-mass spectrometry (LC-MS), size exclusion chromatography (SEC), gel permeation chromatography (GPC), circular dichroism, and matrix-assisted-laser-desorption time-of-flight mass spectrometer (MALDI-TOF MS) can all

aid in identifying the chemical identity of novel products. These techniques can provide information regarding chemical composition and structure, MW of conjugates and polymers, estimate drug loading, and identify impurities (side products). For further information regarding the methodology employed for the characterization of DDSs, we refer the reader to the relevant literature [201].

Analysis of hydrodynamic conformation. To investigate conformational features, dynamic light scattering (DLS), scanning, transmission, or cryogenic transmission electron microscopy (SEM, TEM, and cryo-TEM), atomic force microscopy (AFM), field flow fractionation (FFF) coupled with light scattering detectors, and small-angle neutron (SANS), X-ray (SAXS) and light (SALS) scattering represent state of the art techniques employed when investigating the size, surface charge, and conformation. This set of analyses offers profound insight into polymer chain conformation and thus predicts interactions with biological components such as receptors and biological outcomes [202].

Biocompatibility studies. The form and solubility of final HA-based DDSs can affect interactions with receptors on targeted cells and consequent biological outcomes [142]. Weight, conformation, functional groups, and branching may contribute to final immunomodulatory effects and pharmacokinetic and biodistribution profiles [4]. Therefore, preliminary in vitro evaluations of toxicity, drug release in relevant biological fluids (e.g., plasma, urine, cerebral spinal fluid, or gastroenteric fluid), immunogenic properties (e.g., via HPLC, LC-MS, qPCR, and enzyme-linked immunosorbent assay (ELISA) or ELI-Spot) remain imperative to ensuring the biosafety of the newly synthesized DDSs. Immunogenicity, pharmacokinetics, and biodistribution profiles should be investigated in relevant in vivo models.

6.4. Production and Scaling-up of Hyaluronic Acid-Based Drug Delivery Systems

Implementing a quality-by-design ethos in pharmaceutical development remains crucial to ensure that the final product meets desired quality standards [203]. This step involves identifying critical quality attributes, representing critical physicochemical and biological properties that must be controlled (e.g., size, encapsulation efficiency, polydispersity index, zeta potential, and drug release kinetics). Assessing production process parameters and potential risk factors remains crucial to the optimal monitoring of manufacturing performance [203]. Additionally, a design-of-experiments approach can help to understand how critical parameters affect the critical quality attributes during production setup; this structured approach ensures that a high-quality final product meets all regulatory requirements [204]. Implementing these strategies can allow the achievement of a more consistent MW distribution of HA products and well-characterized HA-based DDSs, enhancing effectiveness and safety.

The industrial scalability of HA-based DDSs poses a challenge as production must be significantly scaled up while maintaining quality and purity. Due to the remarkable level of HA production by bacteria, fermentation is considered the most suitable method for large-scale production [205]. Lancefield groups A and C *streptococci* synthesize HA as a large extracellular capsule; HA release by streptococci in industrial large-scale fermenters significantly increases the viscosity of the culture medium, thereby reducing mixing efficiency and oxygen mass transfer rates. This technical limitation prevents HA production yields from exceeding 6–7 g/L over 6–16 h under standard batch and fed-batch culture conditions (the average MW of HA under these conditions is 1–2 MDa). While glucose represents the preferred carbon source for streptococcal strains, other less expensive sources (e.g., starch, lactose, sucrose, and dextrin) can achieve similar HA productivity and reduce overall costs. The pathogenic nature of the bacteria represents a primary challenge of streptococcal HA production, requiring careful and complete separation of HA from cells during recovery and purification. The high viscosity of the growth medium complicates the separation of streptococcal cells, requiring repeated precipitation and ultrafiltration to obtain a safe and sterile product. Attaining high-purity HA for clinical use remains challenging due to high polydispersity and associated impurities, which should be monitored carefully to avoid excess inflammatory reactions [15]. While prokaryotic HA production is well established in the industry, cell-free HA production (involving the in vitro use of isolated HA synthases) remains under investigation. Although this process can produce high-purity HA with defined MWs and narrow polydispersity values, the technological feasibility and economic advantages for large-scale HA production remain undemonstrated [206]. The complex synthesis of HA-based drug conjugates, which can alter the nature of the payload, affect off-the-shelf stability, and hamper clinical translation, represents another significant challenge during the scaling-up production. The elevated level of complexity represents a critical challenge to scaling up DDS production [92]. Considering novel linking chemistries, solvent-free methods, and one-pot synthesis could represent safe and economical alternatives for the synthesis of HA-based DDSs, contributing to integrated translational considerations [121].

6.5. Regulatory Considerations

The HA implemented for medical uses is classified by North American and European regulatory agencies as a medical device to ensure safety and effectiveness. HA-based medical devices are classified as class III in both the USA and in Europe (in some cases as IIb, in EU) [207]. HA as polymeric material for medical uses must comply with procedures described in Pharmacopeia and the American Society for Testing Materials (ASTM) standards or ISO norms. Each product should possess a list of specifications and validated analytical procedures. All manufacturing processes should be performed under good manufacturing practice (GMP) and deeply characterized by previously approved analytical methods. Norm ISO 14971 imposes that all components should be included in the analysis, including their source and purity. New chemical entities (e.g., polymer-dug conjugates and hydrogels) and HA-based DDS (e.g., nanoparticles) require data from at least three independent batches to demonstrate the reproducibility of the manufacturing process.

To avoid contaminantsinducing undesired reactions, regulatory agencies prefer common sterilization methods, including exposing HA-based DDS to ethylene oxide gas, dry or wet heat, electron beams, or γ-irradiation. Common methods for removing endotoxins (e.g., lipopolysaccharides, LPS) from HA solutions include ultrafiltration, adsorptive matrices like chromatographic resins, and ion-exchange resins [208]. Filtration represents the preferred sterilization method for liquids, where sterilizing material while avoiding degradation represents the challenge [207]. Of note, sterilization methods can impact HA MW, with γ-irradiation inducing the highest decrease in MW [209]; therefore, post-sterilization analysis validation (MW determination) remains crucial to evaluate possible HA degradation.

Finally, HA-based DDS should be adequately packaged; ISO 780 and ISO 9001 norms represent the standards for handling and storing HA-based products. Given the highly hygroscopic nature of HA, related products should be stored in their packaging in a clean and dry place away from any heat sources at low temperatures (−20 or 4–8 °C) or room temperature (25 °C). The product's specific features will dictate individual packaging and storage concerns.

6.6. Potential Clinical Translation and Perspectives

All previously mentioned considerations must be addressed to fully realize the potential of HA-based DDSs in clinical settings. Stability, manufacturing, regulatory issues, and safety remain critical to efficient clinical translation. When translating these systems into human applications, additional factors such as large-scale screening, comparison with other systems, and integration with standard of care or therapeutic strategies also remain crucial.

The systematic parallel screening of numerous DDS properties (e.g., MW, drug combination, biomaterials, linking chemistry, and size) remains challenging due to the difficulty of the rapid, precise, and highly reproducible synthesis of nanomedicines with unique features. The lack of reliable in vitro models that recapitulate the immune system and the tumorigenic process further complicates this process. Implementing 3D cultures, organ-on-chip, or organoids associated with microfluidic strategies in the industrial process could offer more reliable in vitro evaluation and screening systems, working synergistically with animal models**.** Repurposing preexisting drugs and delivery systems that failed in previous applications might hold promise for novel cancer immunotherapy approaches [210]. The evaluation and comparison of the advantages of HA-based systems with commonly used standard adjuvants and immunostimulants represents another aspect requiring special attention. They should be evaluated regarding biocompatibility, ability to induce an immune response, scalability, and reduction of side effects.

Finally, most investigated immunotherapeutic strategies tackle a single arm of the immune system, such as stimulation of the immune response against a specific antigen (vaccines), reduction of the immune suppression (immune checkpoint inhibitors), activation of specific DC responses against the tumor (DC vaccines) [211]. However, these monotherapies often yield limited outcomes due to the complexity of the TIME, underscoring the need for combination therapies to achieve synergistic effects across different levels of the immune response [211]. HA-based DDSs are well-positioned to address these needs; by selectively targeting CD44-expressing cancer cells and draining in the lymphatic system, HA conjugation to chemotherapeutics facilitates tumor cell death, which releases pro-inflammatory signals to enhance immune activation [158,159,212]. HA-modified DDSs can also enhance the penetration and efficacy of chemotherapeutics while promoting ICD [158]. This dual action of chemo−/immune-therapy can improve overall antitumor immune responses and complement other immunotherapeutic approaches, representing a comprehensive and effective strategy for cancer treatment.

Overall, the rational design of HA-based cancer immunotherapies is complex but promising and must address several critical challenges and considerations. To develop successful HA-based products, it is essential to carefully analyze and evaluate various aspects, including MW-related properties, targeting efficiency, and production issues. Although it is still a long way, addressing these critical aspects will lay the groundwork for developing clinically relevant next-generation cancer immunotherapies and immunomodulatory DDSs.

7. Conclusion

HA holds immense potential in developing DDS-based cancer immunotherapies due to its unique physicochemical properties, inherent immunomodulatory activity, and selective protein interactions. In this review, we explored the role of HA as a building block for the design of the next-generation DDSs for cancer immunotherapy. Several critical challenges in drug delivery might be addressed by HA-based systems, including boosting lymph node accumulation and retention, overcoming physiological barriers, and reducing systemic side effects through selective targeting. Few clinical studies have investigated HA-conjugates for cancer treatment, although several pre-clinical applications have studied HA as a component of cancer immunotherapeutic strategies (e.g., polymer conjugates, surface coating, micelles, and scaffolds). Overall, the potential of HA in immunotherapy remains underestimated, and the natural adjuvanticity of HA should be considered an advantage for the design of novel cancer immunotherapies. Interdisciplinary collaboration and advanced evaluation models remain essential to provide meaningful insights. In conclusion, HA-based DDSs offer a promising and multifaceted approach to cancer immunotherapy by combining targeted delivery to immune cells and enhanced immune cell activation to achieve comprehensive immune responses.

CRediT authorship contribution statement

Giulia Rodella: Writing – review & editing, Writing – original draft, Conceptualization. **Véronique Préat:** Writing – review & editing, Supervision, Conceptualization. **Bernard Gallez:** Writing – review & editing, Supervision, Conceptualization. **Alessio Malfanti:** Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

Data will be made available on request.

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