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Review

Albumin-based strategies to effectively prolong the circulation half-life of small immunomodulatory payloads in cancer therapy

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Small immunomodulatory payloads (IMMs) such as peptide vaccines and cytokines have the capability to activate and boost the immune response against cancer. However, their clinical use has often been hindered by their poor stability and short circulating half-lives. To enhance the pharmacokinetic properties of small IMMs and promote their trafficking and accumulation in lymphatic and tumor tissues, a large variety of strategies have been developed. One of the most successful relies on the use of serum albumin (SA), the most abundant protein in the circulatory and lymphatic system. Here, we report a comparative analysis of the different covalent and noncovalent SA-based strategies applied so far to improve the efficacy of small IMMs in cancer therapy.

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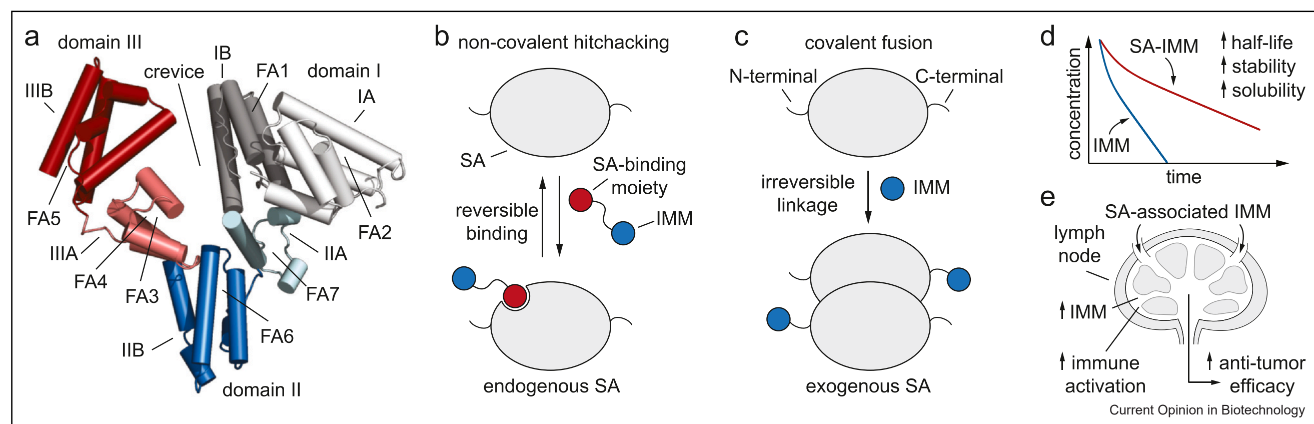
Introduction

Cancer immunotherapy (CI) has transformed the field of oncology by prolonging the survival of patients with cancer [1]. CI functions by activating and boosting potent host immune responses to eradicate tumor cells [1–3]. Current CIs include monoclonal antibodies (mAbs), immune checkpoint inhibitors (ICIs), adoptive cell transfer (ACT), cytokines, and peptide vaccines [2–4]. While several mAbs, ICIs, and ACT-based therapies have shown durable clinical responses, with numerous ongoing clinical trials and approved products [2,5], the use of small immunomodulatory payloads (IMMs), such as cytokines and peptide vaccines, in cancer therapy remains modest [6,7]. The clinical use of small IMMs has been hampered by their poor pharmacokinetic and biodistribution properties [8–12]. The small size and limited stability of peptide vaccines hinder their efficient accumulation in lymph nodes (LNs) [12–16]. The rapid clearance of cytokines limits their exposure to immune cells, thus maintaining effective concentrations requires high dosages and frequent injections that may overshoot the narrow therapeutic window, resulting in adverse toxicities that affect patient compliance [8–11]. To overcome these limitations, a large variety of strategies have been developed over the past few decades to enhance stability and increase the hydrodynamic diameter of IMMs to be ≥5 nm, large enough to extend their circulating half-life and promote their trafficking to LNs [17,18]. These approaches include covalent or noncovalent tethering of cytokines and peptide vaccines to a large variety of synthetic and natural polymers, including large unstructured polypeptides and globular proteins [10,14]. Since most of these strategies have been thoroughly described elsewhere [10,14,19,20], this review will focus exclusively on the use of serum albumin (SA) to effectively deliver small IMMs in cancer therapy.

SA is a nonglycosylated globular protein of 66.5 kDa with an average concentration in the bloodstream and in the lymphatic system of ~40 g L^{-1} and ~0.17 g L^{-1}, respectively (Figure 1) [21–23]. SA is characterized by a remarkable solubility and stability, and it has the ability

Figure 1



Structure of SA and strategies to prolong the circulation half-life of IMMs. **(a)** Crystal structure of hSA representing the three homolog domains (I, II, and III) whose reciprocal interactions create an asymmetric globular heart-shaped module with up to eight distinct FA-binding sites [74]. Each domain is divided into two subdomains (A and B), composed by four and six α-helices, respectively. The α-helices of hSA are represented by cylinders. The subdomains are shown in white (IA), gray (IB), palecyan (IIA), skyblue (IIB), salmon (IIIA), and firebrick (IIIB). The three-dimensional structure model of hSA (PDB identification code: 7AAE) [75] was generated and rendered using Pymol [76]; **(b)** Noncovalent tethering of an IMM to SA through the use of a specific binding moiety; **(c)** Covalent fusion of the small IMM to SA either at the N- or at the C-terminus of SA; **(d)** Pharmacokinetic properties of an IMM (blue) are enhanced by associating it, covalently or noncovalently, to SA (SA-IMM, red); **(e)** IMM, covalently or noncovalently, bound to SA can effectively traffic in the LN resulting in higher accumulation of IMM and ultimately superior immune system activation and increase antitumor efficacy.

to bind and transport a large diversity of endogenous and exogenous ligands [21–23]. Moreover, SA presents low immunogenicity and a maximum circulatory half-life of 19 days in humans [21–23]. This long half-life is mainly related to its structural properties and its ability to bind the neonatal Fc receptor (FcRn), which mediates the pH-dependent endocytic recycling and, ultimately, the rescue of SA from intracellular lysosomal degradation. All these properties make SA an ideal carrier for the delivery of diverse small IMMs, including chemical moieties, nucleic acids, peptides, and protein-based IMMs [21–24]. So far, two main strategies have been explored: (i) noncovalent tethering of a small IMM to SA using a specific binding moiety and (ii) covalent fusion of the small IMM to SA (Figure 1). While the first approach relies on endogenous SA, the second one requires preparation of exogenous SA-based genetic fusions or conjugates. These strategies and their abilities to initiate and regulate both innate and adaptive immunity in cancer are discussed in the following sections.

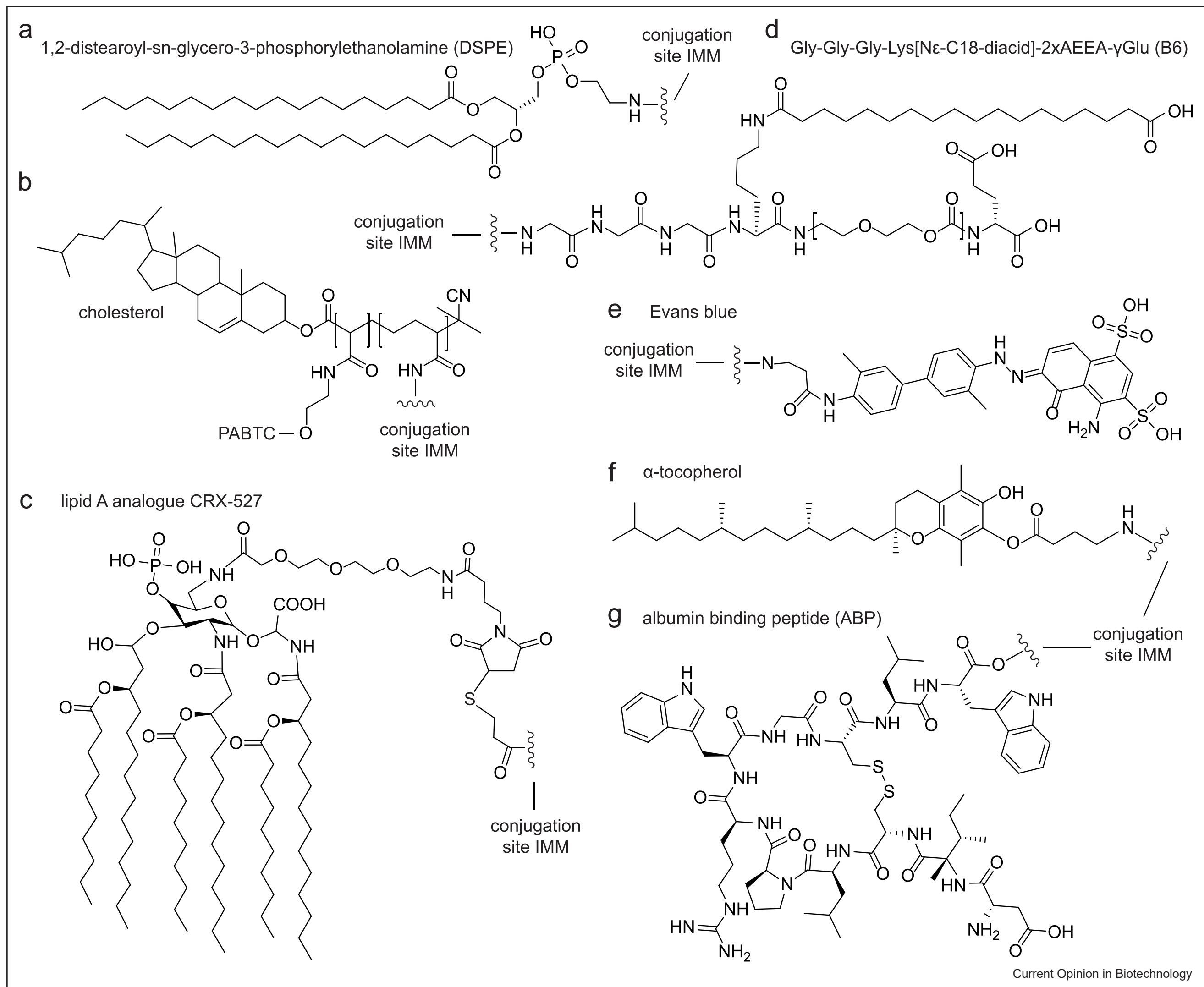
Noncovalent binding of small immunomodulatory payloads to endogenous serum albumin

The inherent ability of SA to bind a large diversity of endogenous and exogenous ligands has inspired the development of multiple chemical compound- and polypeptide-based hitchhiking strategies to enhance the mode of action of different small IMMs. The reversible noncovalent binding to SA allows detachment of the IMM, facilitating its interaction with the target, as well as its penetration and diffusion into small regions otherwise inaccessible to larger molecules. Moreover, the diversity and large number of noncovalent-binding sites distributed through the SA tertiary structure allow co-delivery of multiple IMMs concomitantly. However, the noncovalent association of the IMMs to SA could also result in a loss of the same small IMMs during FcRn endocytic recycling, ultimately decreasing the concentrations of IMMs available.

Chemical moiety conjugates

SA can bind up to seven fatty acids (FAs) simultaneously. Short- to medium-length FAs (6–12 carbons) bind SA with affinities between 0.5 and 60 μM, while longer FAs (14–18 carbons) have 10-fold higher affinities (< 50 nM) [22]. The ability of SA to bind long FAs with high affinity has led to the use of acylation as an effective and safe conjugation strategy to enhance the mode of action of small IMMs. Irvine et al. pioneered the use of the lipophilic SA-binding tail 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE) to generate innovative amphiphile (AMP) vaccines with enhanced antitumor potency (Figure 2 and Tables 1 and 2) [25]. Their AMP vaccines consisted of (i) DSPE, (ii) a polar polymeric spacer polyethylene glycol (PEG) to ensure good solubility while retaining SA-binding affinity, and (iii) an IMM, such as the antigenic melanoma tumor-–associated self-antigen tyrosinase-related protein 2 (Trp2)-derived peptide or the human papillomavirus type 16 (HPV-16)-derived cervical cancer peptide E7 antigen (HPV16-E7; Table 1) [17,25]. When tested in tumor-bearing immunocompetent mice, AMP vaccines exhibited longer half-life, enhanced stability, and higher

Figure 2



Chemical structures of moieties that bind to SA noncovalently. **(a)** Chemical structure of DSPE. The indicated conjugation site has been used to link the following IMMs: CpG, Trp-2, HPV16-E7, EGP_{20}, MUT30, PEPvIII, and KRAS/NRAS mutants; **(b)** Chemical structure of cholesterol. The conjugation sites of 2-propanoic acid butyl trithiocarbonate (PABTC) and IMM (IMDQ) are indicated; **(c)** Chemical structure of CRX-527, a lipid A analog. The indicated conjugation site has been used to append the following IMMs: OVA-HPV16, EnvH, and OVA-EnvH; **(d)** Chemical structure of Gly-Gly-Gly-Lys[Nε-C18-diacid]-2xAEEA-γGlu (B6). The indicated conjugation site has been used to covalently link IL-2; **(e)** Chemical structure of Evans blue dye. The indicated conjugation site has been used to link the following IMMs: CpG, Trp-2, Adpgk, Ntrk1, Rtn2, and Imp3. Chemical structure of **(f)** α-tocopherol and **(g)** albumin-binding peptide. The indicated conjugation site has been used to covalently link CpG and EGP20.

trafficking to draining LNs and induced superior antigen presentation and tumor control, compared with IMMs alone (Table 2) [25]. Activity of AMP vaccines did not depend on SA binding to FcRn but instead required Batf3-dependent dendritic cells (DCs), known to mediate the cross-priming of $CD8^+$ T cells (Table 2) [26]. Though, subsequent studies showed that the activity of AMP vaccines depended on SA binding to FcRn and that FcRn-mediated transcytosis of SA-bound AMP vaccines through the nasal mucosa is important for promoting stronger mucosal immunity [27,28]. Additional studies showed that AMP vaccines accumulate in draining LNs and prolong the availability of peptide antigens and adjuvant, which correlate with massive expansion of functional antigen-specific T cells that provide protection against viral or tumor challenges [27,29]. Moreover, administration of AMP vaccines to immunocompetent mice bearing tumor treated with chimeric antigen receptor T cells (CAR-T) further promoted their expansion and tumor infiltration, triggered DC recruitment to tumors, increased tumor antigen uptake by DCs, elicited the priming of endogenous

Table 1

Noncovalent binding of IMMs to endogenous SA. The name of the moiety binding noncovalently to endogenous SA is reported in the first column, whereas the name and the amino acid sequence (from N- to the C-terminus) of the IMMs linked to it are reported in the second and third columns, respectively. SA-binding affinities indicated in the fourth column are reported as published dissociation constant (K_D). Fold improvement of the terminal half-lives ($\tau_{1/2}$) of each IMM upon linkage to the SA-binding moiety is reported in the fifth column. Fold enhancement of the area under the curve (AUC) of each IMM upon linkage to the SA-binding moiety is reported in the sixth column. Legend: Nb^{SA} = albumin-binding nanobody; MUT30 = MHC II–restricted neoantigen peptide derived from the K739N mutant murine kinesin family member 18B (KIF18B); PEPvIII = epidermal growth factor receptor (EGFR) class III variant (EGFRvIII); EnvH = T helper epitope peptide derived from the envelope (Env) protein of Moloney murine leukemia virus; *a* = mouse serum albumin, *b* = human serum albumin; *c* = half maximal effective concentration (EC50); n.a. = not available; Ref = reference.

Albumin-binding moiety	IMM		K_D	$\tau_{1/2}$	AUC	Ref
	Name	Amino acid sequence				
DSPE	CpG	TCCATGACGTTCCTGACGTT (DNA sequence)	125 nM^a	3 days	↑12-fold	[25,26,31–34]
	Trp-2[181–188]	CVYDFFVWL				
	HPV16-E7[43–62]	GQAEPDRAHYNIVTFCCKCDCRAHYNIVTF				
	HPV16-E7[49–57]					
	EGP20[20–39]	VGALEGPRNQDWLGVPRQL				
	KIF18B[735–749] (MUT30)	VDWENVSPELNSTDQ				
	EGFRvIII[25–37] (PEPvIII)	LEEKKGNYVVTDHC				
	PEPvIII–OVA[257–264]	LEEKKGNYVVTDH – SIINFEKL				
	KRAS/NRAS[5–21] G12D	CYKLVVVGADGVGKSALTI				
	KRAS/NRAS[5–21] G12R	CYKLVVVGARGVGKSALTI				
	KRAS/NRAS[5–21] G12V	CYKLVVVGAVGVGKSALTI				
	KRAS/NRAS[5–21] G12A	CYKLVVVGAAGVGKSALTI				
	KRAS/NRAS[5–21] G12C	CYKLVVVGACGVGKSALTI				
	KRAS/NRAS[5–21] G12S	CYKLVVVGASGVGKSALTI				
	KRAS/NRAS[5–21] G13D	CYKLVVVGAGDVGKSALTI				
	NY-ESO-1[157–165]	SLLMWITQC				
Cholesterol	IMDQ	$C_{22}H_{25}N_5$ (chemical formula)	9.2 μM^b	n.a.	n.a.	[37,71]
CRX 527	OVA[248–265] – HPV16[742–770]	DEVSGLEQLESIINFEKLAAAAAK – GQAEDRAHYNIVTFBBKBDSTLRLBVK	1 and 6 μM^b	n.a.	n.a.	[38,40]
	OVA[323–341] – HPV16[742–770]	ISQAVHAAHAEINEAGR – GQAEDRAHYNIVTFBBKBDSTLRLBVK				
	EnvH[118–135]	EEPLTSLTPRCNTAWNRL				
	OVA[248–265] – EnvH[118–135]	DEVSGLEQLESIINFEKLAAAAAK – EEPLTSLTPRCNTAWNRL				
	OVA[323–341] – EnvH[118–135]	DEVSGLEQLESIINFEKLAAAAAK –EEPLTSLTPRCNTAWNRL				
B6	IL-2	UniProtKB accession number: Q0GK43	n.a.	↑13-fold	↑14-fold	[41]
Evans blue	CpG	TCCATGACGTTCCTGACGTT (DNA sequence)	1 μM^a	2 days	↑43-fold	[23,44]
	Trp-2[181–188]	CVYDFFVWL				
	Adpgk[318–344]	CGIPVHLELASMTNMELMSSIVHQQVFPTC				
	Ntrk1[57–64]	CSSMSLQFMTL				
	Rtn2[472–480]	CSSGAIFNGFTL				
	Imp3[77–85]	CSSAALLNKLYA				
α-Tocopherol	CpG	TCCATGACGTTCCTGACGTT (DNA sequence)	7 μM^b	n.a.	n.a.	[26]
	EGP20[20–39]	AVGALEGPRNQDWLGVPRQL				
ABP	EGP20[20–39]	AVGALEGPRNQDWLGVPRQL	8.5 μM^{b,c}	n.a.	n.a.	[26,72]
ABD	iTEP	$(GVGVPG)_{35}$ – $(GVLPGVG)_{16}$	n.a.	↑2-fold	↑4-fold	[45]
	iTEP – OVA[257–264]	$(GVGVPG)_{35}$ – $(GVLPGVG)_{16}$ – SIINFEKL				
	IL-15	UniProtKB accession number: P40933	2.8 nM^a; 3 nM^b	↑26-fold	↑ 180-fold	[46]
	cIFN-α	UniProtKB accession number: Q6QNB6	9.8 μM^a	↑6.4-fold	n.a.	[47]
ABD094	G-CSF	UniProtKB accession number: P09919	n.a.	↑8-fold	↑3-fold	[48]
Nb^{SA}	IL-21	UniProtKB accession number: Q9HBE4	< 1 μM^a	↑30-fold	↑30-fold	[49,73]
Nb80	IL-2	UniProtKB accession number: Q0GK43	166 pM	↑46-fold	n.a.	[50]

Table 2

Lymph node accumulation, immune system response, and therapeutic effect of IMMs noncovalently bound to endogenous SA. The name of the moiety binding noncovalently to endogenous SA is reported in the first column, whereas the name of the IMMs linked to it is reported in the second column. The name of the mouse model used is indicated in the third column. Both cancer type and cell line are reported in the fourth column, whereas the location of the tumor in mice is indicated in the fifth column. The route of administration of the small IMM linked to the SA-binding moiety is reported in the sixth column. The type of LN in which the small IMM linked to the SA-binding moiety is delivered and accumulated is shown in the seventh column, whereas the extent of immune response is indicated in the eighth column. Observed therapeutic effects are reported in the ninth column. Legend: dLNs = draining lymph nodes; iLNs = inguinal lymph nodes; aLNs = axillary lymph nodes; $CD4^+$ T cells = CD4-positive T cells also known as T helper cells (Th cells); $CD8^+$ T cells = CD8-positive T cells also known as cytotoxic T cells (CTLs); $CD69^+$ T cells = CD69-positive T cells; $CD3^+$ T cells = CD3-positive T cells; Tcm = central memory T cell, T_{EM} = effector memory T cells; Mo-DC = monocyte-derived dendritic cells; DC1 = myeloid-derived dendritic cells; DC2 = plasmacytoid-derived dendritic cells; MDSCs = myeloid-derived suppressor cells; M = macrophage; M1 = polarized macrophage 1; M2 = polarized macrophage 2; N = neutrophils; Tlr2 = toll-like receptor 2; Batf3 = basic leucine zipper transcriptional factor ATF-like 3; Pmel = premelanosome protein also known as glycoprotein 100 (gp100); MSLN = mesothelin; Tg = transgenic; s.c. = subcutaneous; i.v. = intravenous; i.p. = intraperitoneal, i.d. = intradermal; s.t. = stereotactic; MRD = minimal residual disease; n.a. = not available; n.i. = not investigated; # = clinical trial in human patients; Ref = reference.

Albumin-binding moiety	IMM	Animal model	Cancer type	Tumor location	Route of administration	LN accumulation	Immune response	Therapeutic effects	Ref
DSPE	CpG Trp-2[181–188] HPV16-E7[43–62] HPV16-E7[49–57]	C57BL/6 mice	n.a. Melanoma – B16F10 cell line Lung cancer – TC1 ($E7^+$) cell line	s.c. (flank)	s.c. (tail base)	↑dLNs ↑iLNs ↑aLNs	↑$CD8^+$ T cells	↑tumor growth inhibition	[25]
	EGP20[20–39] KIF18B[735–749] (MUT30)	C57BL/6 mice FcRn Knockout C57BL/6 mice Batf3 Knockout C57BL/6 mice	n.a.	n.a.	s.c. (tail base)		↑$CD8^+$ T cells ↑$CD69^+$ T cells ↑memory T cells	n.i.	[26]
	EGFRvIII[25–37] (PEPvIII) PEPvIII – OVA[257–264]	C57BL/6 mice	Melanoma – B16F10 cell line Melanoma – $Trp1^{-/-}$B16F10 cell line glioma – CT-2A cell line	s.c. (flank)	s.c. (tail base)		↑DC cells ↑$CD8^+$ T cells ↑$CD4^+$T cells ↑CAR-T cells ↑M cells	↑tumor growth inhibition	[31]
	CpG HPV16-E7[49–57] NY-ESO-1[157–165] EGP20[20–39] KRAS/NRAS[5–21] G12D KRAS/NRAS[5–21] G12V	C57BL/6 mice	Melanoma – B16F10 cell line melanoma – B16F10 ($gp100^+$) cell line Kidney fibroblast-like -Cos7 cell line	s.c. (flank)	s.c. (tail base)	↑dLNs ↑iLNs	↑DC cells ↑$CD8^+$ T cells ↑CAR-T cells	↑tumor growth inhibition ↑survival	[33]
	KRAS mutants	Clinical trial#	Patients with MRD#	n.a.	s.c.	n.i.	↑$CD3^+$ T cells ↑$CD8^+$ T cells ↑$CD4^+$ T cells	↓toxicity* ↓tumor biomarker*	[34]
Cholesterol	IMDQ	C57BL/6 mice	n.a	n.a	s.c. (footpad)	↑dLN	↑DC cells ↑M cells ↑B cells	n.i.	[37]

Table 2 *(continued)*

Albumin-binding moiety	IMM	Animal model	Cancer type	Tumor location	Route of administration	LN accumulation	Immune response	Therapeutic effects	Ref
CRX 527	OVA$^{[248–265]}$ – HPV16$^{[742–770]}$ OVA$^{[323–341]}$ – HPV16$^{[742–770]}$ EnvH$^{[118–135]}$ OVA$^{[248–265]}$ – EnvH$^{[118–135]}$ OVA$^{[323–341]}$ – EnvH$^{[118–135]}$	C57BL/6 mice	Melanoma – B16-OVA cell line Lung cancer – TC1 cell line	s.c. (flank)	s.c.	↑dLN	↑DC cells ↑Mo-DC cells ↑CD8$^+$ T cells ↑CD4$^+$ T cells	↑tumor growth inhibition ↑survival	[38]
B6	IL-2	BALB/c mice	n.a	n.a	i.v.	n.a.	↑CD8$^+$ T cells	n.i.	[41]
Evans blue	CpG	C57BL/6 mice	Lymphoblastoma-EL4 cell line lymphoma-EG7. OVA cell line	s.c. (shoulder)	s.c. (tail base)	↑LNs	↑CD8$^+$ T cells ↑B cells ↑DC cells ↑M cells	n.i.	[23]
	Trp-2$^{[181–188]}$ Adpgk$^{[318–344]}$		Melanoma – B16F10 cell line; Colon cancer – MC38 cell line; Lymphoma-EG7. OVA cell line	s.c. (shoulder) i.v. (orthotopic)				↑tumor growth inhibition	
	Ntrk1$^{[57–64]}$ Rtn2 Imp3	C57BL/6 mice	Glioma – GL261 cells	s.t. (orthotopic)	s.c. (tail base)	↑iLNs ↑dLN	↑CD8$^+$ T cells ↑CD4$^+$ T cells ↑DC cells ↑Tcm cells ↑T_{EM} cells	↑tumor growth inhibition	[44]
α-Tocopherol	EGP20$^{[20–39]}$	C57BL/6 mice FcRn knockout C57BL/6 mice Batf3 knockout C57BL/6 mice Tg pmel-1 C57BL/6 mice	n.a.	n.a.	s.c. (tail base)	↑iLNs ↑aLNs	↑CD8$^+$ T cells	n.i.	[26]
ABP	EGP20$^{[20–39]}$	C57BL/6 mice FcRn knockout C57BL/6 mice Batf3 knockout C57BL/6 mice Tg pmel-1 C57BL/6 mice	n.a.	n.a.	s.c. (tail base)	↑iLNs ↑aLNs	↑T cell	n.i.	[26,72]
ABD	iTEP iTEP – OVA$^{[257–264]}$	C57BL/6 mice	n.a.	n.a.	s.c. (tail base)	↑dLN	↑DC cells ↑CD8$^+$ T cells	n.i.	[45]
	IL-15	BALB/c mice	Colon cancer-CT26 cell line Melanoma – B16F10 cell line	s.c. (flank)	i.p.		↑CD8$^+$ T cells ↑NK cells ↓Treg cells ↓MDSC cells	↑tumor growth inhibition	[46]
	cIFN-α	BALB/c nude mice	Melanoma – C8161 cell line	s.c. (flank)	i.v.		n.a.	n.i.	[47]

Table 2 *(continued)*

Albumin-binding moiety	IMM	Animal model	Cancer type	Tumor location	Route of administration	LN accumulation	Immune response	Therapeutic effects	Ref
ABD094	G-CSF	Sprague–Dawley rat	Neutropenia	i.p.	s.c.	n.a	↑N cells	n.i.	[48]
Nb^{SA}	IL-21	C57BL/6 mice BALB/c mice	Colon cancer – MC38 cell line Colon cancer – CT26-MSLN cell line	i.d.	i.p.	n.a.	↑DC1 cells ↑M1 cells ↑$CD8^+$ cells ↑$CD4^+$ cells ↓DC2 cells ↓M2 cells	↑tumor growth inhibition	[49,73]
Nb80	IL-2	C57BL/6 mice	Lung cancer	s.c. (flank)	i.p.	n.a.	↑$CD8^+$ T cells ↑NK cells	↑tumor growth inhibition ↑survival	[50]

antitumor T cells ultimately circumventing antigen-negative tumor escape and enhancing antitumor efficacy (Table 2) [30,31]. Recently, conjugation of DSPE to the molecular adjuvant cytosine-phosphoguanine motif (CpG) led to AMP-CpG, a lipid-modified toll-like receptor 9 (TLR9) agonistic DNA oligonucleotide. Co-administration of AMP-CpG with a multiantigen-specific protein subunit vaccine, which included the Epstein-Barr virus (EBV)-encoded gp350 glycoprotein and an engineered recombinant EBVpoly protein bearing different conserved immunodominant $CD8^+$ T cell epitopes derived from multiple EBV lytic and latent antigens, elicited broad humoral and cellular immunity ultimately promoting effective immunity and conferring protection against EBV-associated B cell lymphoma in mice (Tables 1 and 2) [32]. An AMP vaccine, named ELI-002 2P, including the AMP-KRAS G12D and G12R mutant peptide-based antigens and an AMP-modified CpG oligonucleotide adjuvant designed to expand polyfunctional mutant KRAS-specific T cells, showed increased immunogenicity, tumor clearance, and survival in mouse models [33]. ELI-002 2P vaccine is currently in human Phase 1 clinical trial (AMPLIFY-201) as immunotherapy against mutant KRAS-driven solid tumors. The study showed no dose-limiting toxicities, treatment-related serious adverse events or cytokine release syndrome, and no maximum tolerated dose was identified (Tables 1 and 2) [34]. Recently, a new AMP vaccine, named ELI-002 7P, was designed against seven KRAS and neuroblastoma RAS viral oncogene homolog (NRAS) peptides including mutations G12D, G12R, G12V, G12A, G12C, G12S, and G13D. ELI-002 7P immunotherapy is currently being investigated in human phase 1/2 trial (AMPLIFY-7P) in subjects with KRAS- and NRAS-mutated solid tumors (NCT Number: NCT05726864).

A similar strategy was adopted by De Vrieze et al. to reduce the systemic inflammation of imidazoquinolines (IMDQs), synthetic agonists of toll-likes receptor 7 and 8 (TLR7 and TLR8) [35,36]. Toward this goal, they designed lipid–polymer amphiphile conjugates composed of a cholesterol tail coupled to a hydrophilic polymer decorated with multiple IMDQs (Figure 2 and Table 1). The cholesterol–polymer–IMDQ conjugates bound SA and induced higher DC, B cell, and macrophage activation than the control conjugate lacking the cholesterol moiety (Table 2) [37].

Analogously, Tondini et al. applied the lipid A analog CRX-527 to enhance the antitumor efficacy of different antigenic peptides [38]. CRX-527 is a toll-like receptor 4 (TLR4) ligand that binds SA at two different sites (Figure 2 and Table 1) [39,40]. Antigenic peptides conjugated to CRX-527 enhanced DC stimulation and boosted T cell activation and expansion, resulting in superior anticancer immunity (Table 2) [38].

Additionally, FA conjugation has been used to improve delivery, lower systemic toxicity, and enhance the efficacy of cytokines [8–11]. Selective bioconjugation of interleukin IL-2 to octadecanoic (C18) diacid modified with a linker including a γGlu and two units of 8-amino-3,6-dioxaoctanoic acid (AEEA) on one side and four amino acids (Gly-Gly-Gly-Lys) on the opposite side (Gly-Gly-Gly-Lys[Nε-C18-diacid]-2xAEEA-γGlu) led to B6, a FA-conjugated-IL-2 with extended half-life and enhanced cytotoxic $CD8^+$ T cells proliferation activity (Figure 2 and Tables 1 and 2) [41].

Besides FAs, SA can bind numerous other small molecules (SMs). For example, Zhu et al. exploited the ability of Evans Blue (EB) to bind multiple distinct sites of SA to develop innovative self-assembling SA/AlbiVax nanocomplexes (Figure 2 and Table 1). The AlbiVax was prepared by conjugating a maleimide-functionalized EB derivative (MEB) with thiol-modified peptides derived from the melanoma tumor–associated self-antigen Trp2 or from the MHC-I H-$2D^b$-restricted neoantigen peptide from murine MC38 colorectal cancer cell (Adpgk) [23]. When tested in vivo, the SA/AlbiVax nanocomplexes showed a 100-fold more efficient co-delivery of antigens to LNs and a 10-fold increase in the frequency of peripheral antigen-specific $CD8^+$ cytotoxic T lymphocytes compared to the benchmark incomplete Freund's adjuvant (Table 2) [23,42,43]. Combination of SA/AlbiVax nanocomplexes with the ICI anti-programmed cell death protein 1 (anti-PD-1) mAb and the chemotherapeutic Abraxane enhanced antitumor immunity and therapeutic efficacy [23]. To enhance the therapeutic efficacy of monovalent vaccines and to prevent tumor immune evasion, Zhu et al. have recently developed a multivalent lymph node–targeting adjuvant/antigen-codelivering albumin-binding vaccines (AAco-AlbiVax). The system is based on a Y-shaped DNA scaffold that was site specifically conjugated to (i) the adjuvant CpG, (ii) the albumin-binding MEB, and (iii) one peptide neoantigen derived from the H2-D^b-restricted mutant neurotrophic receptor tyrosine kinase 1 (Ntrk1), reticulon-2 (Rtn2), or the U3 small nucleolar ribonucleoprotein 3 (Imp3; Table 1). In mice, AAco-AlbiVax elicited antitumor immunity, including neoantigen-specific $CD8^+$ T cell responses. Further combination of AAco-AlbiVax with radiotherapy and both anti-PD-1 mAb and anti-cytotoxic T-lymphocyte antigen 4 (anti-CTLA-4) mAb significantly inhibited progression of orthotopic glioblastoma multiforme in mice (Table 2) [44].

Similarly, Irvine et al. used the low molecular weight organic molecule α-tocopherol, an active form of vitamin E capable of binding SA, to enhance the potency of the molecular adjuvant CpG and the melanoma glycoprotein 100 (gp100) antigen EGP_{20} (Figure 2). The α-tocopherol conjugates showed ~3-fold higher CpG levels in the draining inguinal and axillary LNs, and 10-fold higher frequencies of T cells response to antigenic peptides (Tables 1 and 2) [26].

Polypeptide binder conjugates

In addition to FAs and SMs, an increasing number of polypeptides have been used as SA-binding moieties. Polypeptides usually have a large interaction interface with their target, leading to high binding affinities and specificities. Moreover, polypeptides can be coupled to IMMs, either recombinantly or chemically.

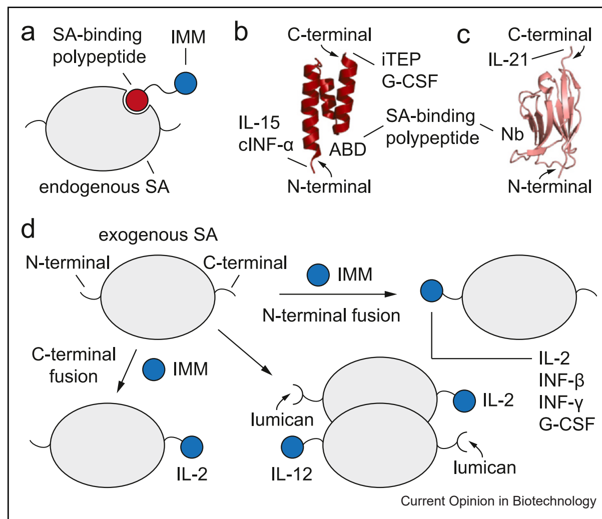
For instance, chemical linkage of the albumin-binding peptide (ABP, ^{N}DICLPRWGCLW^{C}) to the peptide antigen EGP_{20} via a PEG2000 spacer, led to ABP-PEG-EGP_{20}, a conjugated molecules with enhanced LNs accumulation (> 13-fold) and higher antigen-specific T cell activation (5-fold) than EGP_{20} alone (Figure 2 and Tables 1 and 2) [26].

Recombinant fusion of a SA-binding domain (ABD, ^{N}LAEAKVLANRELDKYGVSDFY KRLINKAKTVEGVEALKLHILAALP^{C}) to the immune-tolerant elastin-like polypeptide (iTEP) resulted in a greater LNs accumulation (> 3-fold), DCs (> 4-fold), and $CD8^+$ T cells activation than iTEP alone (Figure 3 and Tables 1 and 2) [45]. Moreover, fusion of IL-15 to ABD led to IL-15-ABD, a recombinant molecule that showed longer half-life (> 20-fold) but also the ability to overpower immunosuppressive cells (e.g. Tregs and MDSCs) while enhancing the antitumor activity of $CD8^+$ T cells and natural killer (NK) cells (Figure 3 and Tables 1 and 2) [46].

Additionally, fusion of a cyclized interferon-alpha (cIFN-α) to an ABD enabled the generation of cIFNα-ABD, a recombinant molecule with retained SA-binding affinity, longer circulatory half-life (> 4-fold), higher stability, greater tumor penetration and retention (> 4-fold), and stronger antitumor efficiency than linear or cyclic IFN-α alone (Figure 3 and Tables 1 and 2) [47]. Similarly, fusion of the granulocyte colony-stimulating factor (G-CSF) to another ABD yielded ABD-G-CSF, a recombinant molecule with improved pharmacokinetic properties (half-life > 9 hours) and higher neutrophil stimulation (Figure 3 and Tables 1 and 2) [48].

Analogously, fusion of a SA-binding nanobody (Nb^{SA}) to IL-21 extended its circulatory half-life (> 30-fold) and enhanced its antitumor effectiveness (Figure 3 and Tables 1 and 2). Combination of IL-21-Nb^{SA} with the anti-PD-1 mAb tuned the ratio of specific subsets of tumor-associated macrophage (M1 > M2) and DCs (D1 > D2), while it triggered the expression of two additional checkpoint molecules, T cell immunoglobulin mucin-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3). Indeed, combination of IL-21-Nb^{SA} with anti-

Figure 3



Noncovalent and covalent-based strategies to enhance delivery of IMMs. **(a)** Noncovalent tethering of an IMM (blue) to SA through the use of a specific binding moiety (red); **(b)** Crystal structure of a bacterial SA-binding domain (ABD; firebrick; PDB identification code: 1GJS). ABDs fold into a small (~5 kDa) and highly stable three-helix-bundle domain. ABDs are often derived from protein G of *Streptococcus* strain GI48 and from protein PAB of *Finegoldia magna* [77]; **(c)** Crystal structure of a SA-binding nanobody (Nb; salmon; PDB identification code: 5VNW). Nbs are small (12–15 kDa) and stable variable domain of the heavy-chain-only (VHH) antibodies naturally occurring in the Camelidae family [78]. Nbs present a typical immunoglobulin variable domain (IgV) fold comprising nine β-strands and three hypervariable loops. The three-dimensional structure model of ABD and Nb was generated and rendered using Pymol [76]; **(d)** Covalent fusion of a small IMM to either the N- or the C-terminus of exogenous SA.

PD1, anti-TIM-3, and anti-LAG-3 mAbs resulted in a stronger antitumor response and limited toxicity (Figure 3 and Tables 1 and 2) [49]. Recently, fusion of IL-2 to Nb80, a cross-reactive nanobody that binds SA from different species, led to Duraleukin, a protein fusion with a 46-fold longer circulating half-life than IL-2 alone, that increased the numbers of tumor-infiltrating CD8$^+$ T cells and NK cells (Tables 1 and 2) [50].

Covalent fusion to exogenous serum albumin

The pharmacokinetic properties of IMMs can also be enhanced by covalent fusion to exogenous SA. Compared with the noncovalent-based hitchhiking strategies described above, covalent linkage of IMMs to SA (i) enables longer circulation lifetimes of the IMM, as the risks of IMM loss during the FcRn-mediated recycling of endocytosed SA is minimized, (ii) ensures steric access of the IMM to cell receptors, (iii) increases the overall size of the IMM, thus reducing its rate of diffusive escape from the tumor ('tumor entrapment'), which prolongs IMM persistence to provide sustained and local boosts of the immune system [51]. However, covalent linkage of IMMs to SA (i) is generally limited to only two locations, the N- and C-terminus of SA and (ii) requires recombinant protein production, which is often labor intensive and costly.

Table 3

Covalent fusion of IMM to exogenous serum albumin. The name of IMMs covalently linked to exogenous SA is reported in the first column. Fold improvement of the terminal half-life ($\tau_{1/2}$) and area under the curve (AUC) of each IMM upon linkage to SA are reported in the second and third columns, respectively. Legend: n.a. = not available; Ref = reference.

IMM linked to serum albumin	$\tau_{1/2}$	AUC	Ref
mSA-IL-2	↑21-fold	n.a.	[57,59,60]
lumican-mSA-IL-2 IL-12-mSA-lumican	n.a.	↑10-fold	[61]
hSA-IL-2	↑6-fold	↑75–35-fold	[53]
IL-2-mSA IL-12-mSA	n.a.	n.a.	[62]
hSA-IFNβ	↑10-fold	n.a.	[54]
cSA-IFNγ	↑4-fold	↑170-fold	[66]
hSA-GCSF	↑10-fold	n.a.	[67–69]

For example, fusion of human SA (hSA) to the C-terminus of IL-2 yielded IL-2-hSA, also known as Albuleukin, a molecule with 50-fold longer circulating half-life than IL-2 alone (Figure 3 and Tables 3 and 4) [52]. Though Albuleukin accumulated preferentially in the LNs, liver, and spleen, it failed to provide significant clinical benefits over conventional IL-2 antitumor therapy [53–56].

Further co-administration of untargeted IL-2 fused to the C-terminus of murine SA (mSA) with different tumor antigen–specific mAbs revealed superior tumor lymphocyte infiltration, synergistic activation of both innate and adaptive response, and production of anti-tumor Abs when tested in different isogenic murine tumor models (Figure 3 and Tables 3 and 4) [57]. Similarly, combination of the untargeted mSA-IL-2 with the anti-PD-1 mAb enabled long-term tumor clearance and creation of an immunological memory when tested on an isogenic mouse model of glioblastoma (Tables 3 and 4) [58]. Further combination of the delayed systemic clearance mSA-IL-2 with anti-PD-1 mAb, different tumor antigen–specific mAbs, and an AMP vaccine showed higher potency in multiple challenging tumor models. Efficacy relied on the activation of multiple types of adaptive and innate immune cells as well as a higher CD8$^+$ to regulatory T cell (Treg) ratio. Notably, such combination immunotherapy stimulated immune responses against antigens not included in the vaccine, thus expanding its potential application to tumor types

Table 4

Lymph node accumulation, immune system response and therapeutic effect of IMM covalently linked to exogenous serum albumin. The name of IMM covalently linked to exogenous SA is reported in the first column. The name of the mouse model used is indicated in the second column. Both cancer type and cell line are reported in the third column, whereas the tumor location is indicated in the fourth column. The route of administration of the small IMM covalently linked to SA is reported in the fifth column. The type of LN in which the small IMM linked to SA is delivered and accumulated is shown in the sixth column, whereas the extent of immune response is indicated in the seventh column. Legend: dLNs = draining lymph nodes; iLNs = inguinal lymph nodes; $CD4^+$ T cells = CD4-positive T cells also known as T helper cells (Th cells); $CD8^+$ T cells = CD8-positive T cells also known as cytotoxic T cells (CTLs); Treg cells = regulatory T cells; Mo-DC = monocyte-derived dendritic cells; MDSCs = Myeloid-derived suppressor cells; M = macrophage; M1 = polarized macrophage 1; M2 = polarized macrophage 2; N = neutrophils; Tg = transgenic; hmHER2 = human mutated epidermal growth factor receptor 2 (HER2); Trp1 = tyrosinase-related protein 1; Trp2 = tyrosinase-related protein 2; s.c. = subcutaneous; i.v. = intravenous; i.p. = intraperitoneal, i.d. = intradermal; r.o. = retro-orbital; p.t. = peritumoral; i.n. = intramodular; * = combination therapy; # = clinical trial in human patients; n.a. = not available; Ref = reference.

IMM linked to SA	Animal model	Cancer type	Tumor location	Route of administration	LN accumulation/ delivery	Immune response	Therapeutic effects	Ref
mSA-IL-2	C57BL/ 6 mice C3H/ HeN mice BALB/c mice NOD scid mice NSG mice C57BL/ 6 mice BALB/c mice	Melanoma – B16F10 cell line Fibrosarcoma – Ag104A-Ld cell line Breast cancer – 4T1-Luc cell line Cervical cancer – TC1 ($E6^+$ $E7^+$) cell line Melanoma – B16F10 cell line Breast cancer DD-Her-2/neu cell line	s.c. (flank) s.c. (flank) s.c. (flank) s.c. (flank)	r.o.; i.p. r.o. i.p. i.p.	↑dLN	↑$CD8^+$ T cells ↑N cells ↑M cells ↑NK cells* ↑DC cells ↑$CD8^+$ T/Treg cells ratio* ↑$CD4^+$ cells* ↑antitumor antibodies*	↑tumor growth inhibition* ↑immunological memory* ↑tumor growth inhibition* ↓metasta-sis*	[57,59,60]
lumican-mSA-IL-2 IL-12-mSA-lumican	C57BL/6 mice BALB/c mice BALB/c mice	Melanoma – B16F10 cell line Melanoma – B16F10-$Trp2^{-/-}$ cell line Breast cancer – EMT-6 cell line Colon cancer – MC38 cell line Breast cancer – 4T1-Luc cell line	s.c. (flank)	p.t., i.n., s.c. (base tail)	↑iLNs	↑$CD8^+$ T cells ↑CAR-T cells ↑NK T cells	↑tumor growth inhibition* ↑survival* ↓toxicicty*	[61]
hSA-IL-2	BALB/c mice C57BL/ 6 mice	Renal cancer – Renca cell line[a] Melanoma – B16F10 cell line	s.c. (flank) s.c. (flank)	i.p., s.c. i.p., s.c.	↑LNs	↑$CD8^+$ T cells ↑$CD4^+$ T cells ↑B cells ↑NK cells	↑tumor growth inhibition* ↑survival* ↓metasta-sis*	[53]
IL-2-mSA IL-12-mSA	C57BL/ 6 mice	Lung cancer – KP cell line	s.c. (flank); i.v. (orthotopic)	i.v.	↑tumor dLNs	↑$CD8^+$ T cells ↓$CD4^+$ T cells ↑$CD8^+$ T/Treg cells ratio	↑survival ↓toxicicty	[62]
hSA-IFNβ	C57BL/ 6 mice	Cervical cancer – TC1 ($E6^+$ $E7^+$) cell line	s.c. (flank)	s.c.	↑dLNs	↑ $CD8^+$ T cells ↑ DC cells ↑ B cells	↑tumor growth inhibition* ↑survival*	[64]

Table 4 *(continued)*

IMM linked to SA	Animal model	Cancer type	Tumor location	Route of administration	LN accumulation/ delivery	Immune response	Therapeutic effects	Ref
cSA-IFNγ	BALB/c mice	Malignant histiocytosis – DH82 cell line	s.c. (flank)	s.c.	↑dLNs	n.a.	↑tumor growth inhibition* ↑survival*	[66]
hSA-GCSF	Clinical trial#	Breast cancer patient#	n.a.	i.v.	n.a.	↑N cells	↓number of injections ↓leukemia symptoms	[67–69]

lacking known antigens [59]. Similar results were observed when triple-negative breast cancer mouse models were treated with mSA-IL-2 in combination to an anti-PD-1 mAb and an agonist of the stimulator of interferon genes (STING) [60]. To limit the systemic dissemination of SA-cytokine fusions while prolonging their local tumor residence, Wittrup et al. developed novel tumor antigen–agnostic intratumoral injection-based immunotherapies. By appending the small leucine-rich proteoglycan lumican, a collagen-anchoring protein, to either the N- or C-terminus of mSA, and the interleukins IL-2 and IL-12 to the C- and N-terminus of mSA, respectively, they obtained fusion proteins (lumican-mSA-IL-2 and IL-12-mSA-lumican) that displayed longer intratumoral retention, no systemic exposure toxicity, enhanced tumor-targeting antibody efficacy, strong tumor-specific T cell and NK cells response, greater cancer vaccine efficacy, improved CAR-T cell treatment, and augmented neoadjuvant checkpoint blockade (Figure 3 and Tables 3 and 4) [61]. Interestingly, co-administration of untargeted IL-2-mSA and IL-12-mSA fusions led to enhanced tumor-reactive $CD8^+$ T cell effector differentiation, decreased numbers of tumor-infiltrating $CD4^+$ Treg, and increased survival of lung tumor–bearing mice (Table 3) [62].

Fusion of interferon-beta (IFN-β) to the C-terminus of hSA led to the generation of hSA-IFNβ, also known as Albuferon, a molecule with retained activity and 10-fold longer circulation half-life than IFN-β alone [63]. Pharmacokinetic and biodistribution studies revealed that hSA-IFNβ accumulated preferentially in the tumor-draining LNs. Co-administration of hSA-IFNβ with either ovalbumin (OVA) or HPV16-E7 antigenic peptides revealed enhanced DC maturation and generation of antigen-specific CD8+ T cells in tumors (Figure 3 and Tables 1 and 3) [64]. Similar antitumor efficacy has been observed in dogs with canine renal malignant histiocytosis that have been treated with a canine interferon-gamma (cIFN-γ) fused to the C-terminus of canine SA (cSA) (Figure 3 and Tables 1 and 3) [65,66].

Finally, fusion of human granulocyte colony-stimulating factor (G-CSF) to the C-terminus of hSA led to the long-acting hSA-G-CSF (CG-10639) capable of increasing leukocytes, neutrophilic granulocytes, and monocytes in a dose-dependent manner, thus preventing severe neutropenia in patients with cancer with myelosuppressive chemotherapy (Figure 3 and Tables 1 and 3) [67–70].

Conclusions

Albumin has been shown to be an effective carrier to prolong the plasma residence time of numerous small IMMs, effectively trafficking them into different lymphatic areas and enhancing their diffusion and accumulation into tumor tissues. When covalently linked to or

noncovalently associated with SA, IMMs are protected from proteolytic degradation and rapid renal filtration due to the hydrodynamic volume of SA and its ability to bind the recycling FcRn. Noncovalent and covalent strategies for SA binding have their own advantages and disadvantages, and the choice of one over another depends on many factors, including the intrinsic properties of the IMMs and their receptors, as well as the type of immune and cancer cells toward which IMMs should function. Thus, for each IMM, multiple factors should be carefully evaluated concomitantly to maximize the therapeutic efficacy while minimizing undesired toxic effects. In the case of endogenous SA-based delivery strategies, future research efforts should be oriented toward the development of novel ligands capable of binding SA with tunable affinities or recognizing different SA sites, thus enabling co-delivery of multiple IMMs at once. In the case of exogenous SA-based delivery strategies, further development should involve the use of bioengineered SA with different affinities to FcRn, enhanced fusion linkers as well as capability to transport multiple IMMs at once. Finally, synergistic combination therapies involving the use of both endogenous and exogenous SA-based delivery systems and different IMMs at once should be explored to ultimately enhance efficacy of small IMMs against multiple types of tumors.

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Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- •• of outstanding interest

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