

Review

***In Vivo* Secretion of Bispecific Antibodies Recruiting Lymphocytic Effector Cells**

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Abstract: Engineered Fc-lacking bispecific antibodies have shown an exceptionally high potency for recruiting lymphocyte effector cells and enhancing antitumor activity, which is under evaluation in several clinical trials. However, current treatment regimens raise some issues that should be considered, such as the high cost of clinical-grade bispecific antibodies and the achievement of sustained therapeutic plasma levels. The use of gene transfer methods may circumvent problems related to large-scale production and purification, and result in sustained therapeutic plasma concentrations of the Fc-lacking bispecific antibodies. In fact, terminally differentiated cells and non-terminally differentiated cells can be genetically modified to secrete functionally active bispecific antibodies exerting clear anti-tumor effects. This review highlights the relevance of different promising strategies for *in vivo* delivery of therapeutic bispecific antibodies.

Keywords: bispecific antibodies; *in vivo* secretion; gene therapy; cell-based therapy; immunotherapeutic organoids; immunotherapeutic neovessels

1. Introduction

Antibody engineering has greatly contributed to the success of antibody-based therapies for cancer, through designing different formats with enhanced effector functions, improved pharmacokinetic properties and decreased immunogenicity [1]. Efforts to further improve the clinical efficacy and safety of antibody-based therapies are ongoing. In this regard, novel antibody-based strategies to

redirect immune effector functions represent promising approaches to cancer treatment [2]. These include the genetic engineering of T lymphocytes through the introduction of a chimeric antigen receptor (CAR), composed of antibody binding domains connected to T cell activating domains, and the use of bispecific antibodies (bsAbs). Both strategies combine the high specificity of antibody molecules with the efficient trafficking properties and effector functions of T cells [2,3].

BsAbs simultaneously targeting tumor-associated cell surface antigens and effector cell trigger molecules have been developed and shown to redirect cellular cytotoxicity [4–6]. Although most bsAbs are in early clinical stage study (phase 1 and phase 2), catumaxomab, an anti-EpCAM x anti-CD3 half mouse/half rat full-length IgG, has been approved by the EMA for intraperitoneal treatment of malignant ascites [7,8]. However, in a phase 1 study for the treatment of non-small cell lung cancer, it was established that the maximum tolerated dose for multiple intravenous administration of catumaxomab was 5 µg [9]. This is probably a consequence of the tumor cell independent cross-linking of T cells with Fc receptor (FcR)-bearing accessory cells, followed by cytokine release-related symptoms [8].

Furthermore, clinical-grade antibodies for therapeutic use are extremely expensive to produce, and carry non-human glycan epitopes, that can potentially affect immunogenicity and/or therapeutic efficacy, since all patients and healthy controls tested had circulating antibodies against them [10].

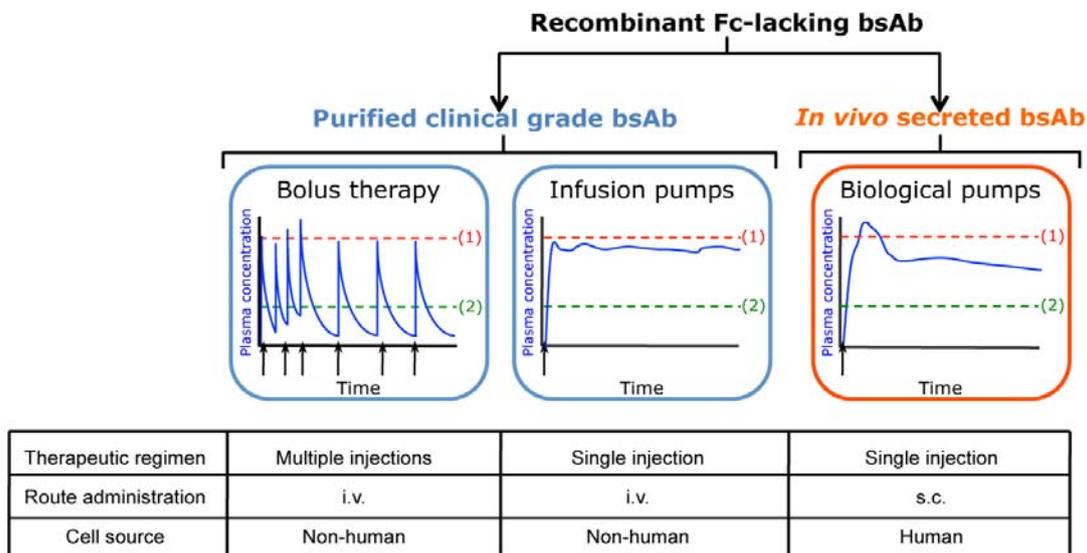
For these reasons, it is highly recommended to use engineered Fc-lacking bsAbs such as tandem scFv and diabodies. Tandem scFvs, also known as (scFv)₂, consists of two different scFvs connected by a flexible peptide linker on a single protein chain [11]. By reducing the linker length between variable domains (V_H and V_L) it is possible to force the pairing of domains between two different polypeptides, leading to a compact antibody called diabody [12]. Bispecific diabodies are formed by the association of two V_HA-V_LB and V_HB-V_LA fragments expressed in the same cell. This leads to the formation of heterodimers with two different binding sites [13].

Numerous studies have demonstrated the potency of these Fc-lacking bsAbs recruiting lymphocytic effector cells in preclinical studies [14]. Although no bispecific diabodies have been administered to humans, ongoing phase 1 and 2 clinical trials with the anti-CD19 x anti-CD3 tandem scFv blinatumomab revealed impressive clinical results in relapsed B-cell non-Hodgkin lymphoma and chronic lymphocytic leukemia [15].

However, these bsAbs lacking in Fc domains present a very short serum half-life and must be administered by continuous intravenous infusion by portable minipumps (Figure 1). Although different strategies have been successfully used to increase the circulation time of recombinant bsAbs: PEGylation, N-glycosylation and fusion to human albumin or albumin-binding domains [16,17], these modifications might also affect bsAb-mediated cytotoxicity and can potentially affect immunogenicity [18].

One way to overcome these limitations would be the use of gene transfer technologies [19]. *In vivo* secretion of Fc-lacking bsAbs might result in effective and persistent levels of bsAb molecules. This could compensate for the rapid blood-pool clearance and make the bsAbs better tolerated [19].

Figure 1. Schematic chart depicting advantages and limitations associated with the use of systemic administered purified clinical-grade bsAbs (bolus therapy or infusion pumps) and *in vivo* secreted bsAbs. Simulated pharmacokinetic profile of plasma bsAbs, ranging from subtoxic (1) to subtherapeutic (2).



2. *In Vivo* Secretion of Fc-lacking Bispecific Antibodies

The feasibility of *in vivo* secretion of full-length mAbs and engineered antibodies by different cell types has now been demonstrated using different techniques, such as genetic modification of *ex vivo* expanded terminally differentiated or precursor cells and *in vivo* gene transfer using viral vectors [20–22]. BsAbs have not remained on the margins of these gene therapy strategies, and several papers describing the *in vivo* secretion of bsAbs have been published (Table 1). In 2003, a seminal work by Blanco *et al.* [23] demonstrated that non-hematopoietic human cells can be genetically modified to secrete a functionally active bispecific two-chain diabody directed against the carcinoembryonic antigen (CEA) and the CD3 ϵ chain of the TCR/CD3 complex (α CEA x α CD3). Diabody molecules were secreted at high levels and were very efficient at activating human peripheral blood T lymphocytes to proliferate and eliminate human cancer cells expressing CEA *in vitro*. Furthermore, intratumoral inoculation of diabody producer cells efficiently delayed the growth of human colon carcinoma xenografts.

Table 1. Different strategies for *in vivo* production of bispecific antibodies.

Target antigens	Antibody format	BsAb secreting cells			Effector cells		Disease model	Ref.
		Cell Vehicle	Transfer Vector	Route of Administration	Cell type	Route of Administration		
CEA x CD3	diabody	293T (h)	plasmid	s.c. co-implant	T cells	i.t.	colon cancer (h) HCT-116	[23]
CEA x CD3	diabody	T cells (h)	LV	s.c. co-implant	T cells	s.c. co-implant	colon cancer (h) HCT-116	[26]

Table 1. Cont.

Target antigens	Antibody format	BsAb secreting cells			Effector cells		Disease model	Ref.
		Cell Vehicle	Transfer Vector	Route of Administration	Cell type	Route of Administration		
CEA x CD3	diabody	MSC (h)	LV	s.c. organoid	T cells	i.v.	colon cancer (h) HCT-116	[35]
HER2 x CD16	(scFv) ₂	MSC-like (m)	RV	i.v.	monocytes	i.p.	breast cancer (h) MDA-MB-453	[42]
CEA x CD3	diabody	HUVEC	LV	s.c. neovessels	T cells	i.v.	colon cancer (h) HCT-116	[51]

Abbreviations: h, human; m, mouse; LV, lentivirus; RV, retrovirus; s.c., subcutaneous; i.v., intravenous; i.t., intratumoral; i.p., intraperitoneal.

Since T lymphocytes have the capacity to home to the tumor deposits [24,25] and are the effector cells of anti-CD3-based bsAbs, a logical strategy was the genetic modification of human T cells with a lentiviral vector encoding the α CEA x α CD3 diabody. Compte *et al.* [26] demonstrated that functional α CEA x α CD3 diabody was detectable in conditioned medium from lentivirus infected peripheral blood T lymphocytes for several weeks. Furthermore, the concentration of secreted diabody was sufficient to activate primary T cells to proliferate and eliminate CEA⁺ tumor cells *in vitro*. Intratumoral secretion of α CEA x α CD3 diabody by gene-modified T lymphocytes improved survival and reduced measurable tumor burden.

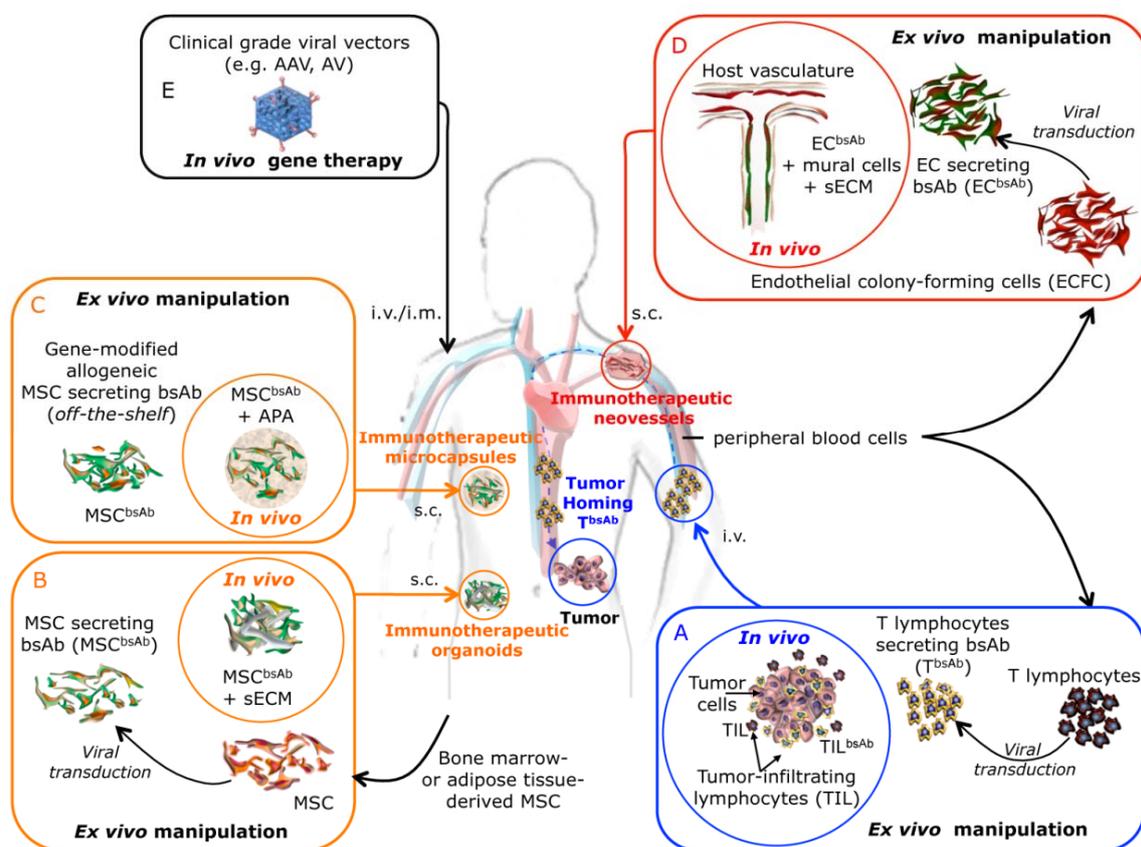
This approach would provide: (1) a selective accumulation of gene-modified bsAb-secreting T cells in tumor deposits; (2) the recruitment of both gene-modified and non-modified tumor-infiltrating lymphocytes, amplifying the effector immune response; and (3) a highly tumor site-restricted T cell activation, decreasing the toxicity inherent to systemic T-cell activation and enhancing tumoricidal activity (Figure 2a).

However, one of the most important concerns when considering cells as *antibody factories* is the ability to get sustained antibody plasma levels. The extended life span of stem cells makes them an attractive alternative for the long-term antibody delivery. In several cancer treatment strategies, either neural stem cells (NSC) or mesenchymal stem cells (MSC) have been used as delivery vehicles of different therapeutic proteins [27], taking advantage of their inherent tumor-tropic properties [28,29]. In fact, Frank *et al.* [30] demonstrated that NSC can be engineered to secrete a full-length anti-HER2 human mAb, and can deliver functional antibody to tumor foci *in vivo*. However, most gene therapy protocols have been focused on the use of MSC, which present certain advantages as therapeutic vehicles: they are readily isolated, easily transduced and exhibit a unique *in vitro* proliferative capacity using a simple media formulation [29].

Notwithstanding, the use of MSC in cancer-targeting approaches raises concerns, due to their pro-angiogenic properties [31–33] and their potential role in tumor progression and metastasis [34,35]. Moreover, the ability of MSC to specifically home to tumors after systemic administration is questionable [22]. As an alternative to systemic administration, MSC can be confined to a specific location, where they will act as *biological minipumps* avoiding cell dissemination and allowing systemic secretion of therapeutic proteins. Moreover, subcutaneous inoculation of entrapped MSC would provide an easily accessible implant (*immunotherapeutic organoid*) that could be retrieved once

the therapeutic effect is accomplished (Figure 2b). Several works supported the safety, utility and efficacy of this approach to secrete therapeutic proteins that exert a variety of different biological effects *in vivo* [36–41].

Figure 2. Schematic diagram illustrating the different strategies for *in vivo* secretion of bsAbs recruiting lymphocytic effector cells. **(A)** *Ex vivo* generation of bsAb-secreting T lymphocytes (T^{bsAb}) for the immunotherapy of cancer. Adoptive transferred T^{bsAb} lymphocytes home to the tumor deposits after reinfusion into the patient, secrete bsAbs and recruit both gene-modified and non-modified tumor-infiltrating T lymphocytes. **(B)** *Ex vivo* gene-modified autologous MSC secreting bsAbs (MSC^{bsAb}) embedded in a biological synthetic extracellular matrix (sECM) are implanted subcutaneously to generate a *immunotherapeutic organoid* acting as *biological minipumps* ensuring the systemic secretion of bsAbs. **(C)** “Off-the-shelf” stocks of gene-modified allogeneic MSC secreting bsAbs (MSC^{bsAb}). MSC^{bsAb} can be APA (alginate/poly-L-lysine/alginate) microencapsulated and implanted subcutaneously to generate *immunotherapeutic microcapsules* acting as *biological minipumps* ensuring the systemic secretion of bsAbs. **(D)** Autologous endothelial colony forming cells (ECFC) *ex vivo* transduced for the expression of a bsAbs (EC^{bsAb}), are mixed with autologous mural cells, embedded in a biocompatible scaffold and subcutaneously implanted. *In vivo* engineered *immunotherapeutic neovessels* connect with the host vascular bed and allow the release into the bloodstream of the bsAbs. **(E)** Direct injection of clinical grade viral vectors carrying bsAb genes.



The first demonstration of the use of MSC-based *organoids* for systemic delivery of engineered antibodies *in vivo* was a study by Compte *et al.* [35]. Lentiviral-transduced bone-marrow derived MSC secreting a bispecific α CEA x α CD3 diabody were embedded in a synthetic extracellular matrix and subcutaneously implanted in nude mice, generating a long-lasting functional *immunotherapeutic organoid* (Table 1). The systemically released α CEA x α CD3 diabody activated passively transferred human T cells to mediate potent and antigen-specific lysis of CEA-expressing tumor *in vivo* (Figure 2b). This strategy demonstrated for the first time that human MSCs genetically engineered to secrete Fc-lacking bsAbs, seeded in an synthetic ECM scaffold and implanted in a location distant from the primary tumor, are able to induce an effective antitumor response and tumor regression. Later on, Kasuya *et al.* [42] demonstrated that retrovirally transduced, MSC-like 10T1/2 cells secreted a functional anti-HER2 x anti-CD16 single-chain antibody (Table 1). Furthermore, antibody produced by gene-modified 10T1/2 cells implanted in the perivascular area of pulmonary arteries exhibited antitumor effect against HER-2⁺ human breast cancer cells implanted intraperitoneally, after inoculation of human T cells.

Another approach for long-lasting bsAbs secretion has focused on endothelial cells. The utility of endothelial cells for gene therapy approaches has been extensively proved in a wide range of different pathologies [43–45]. Several works have shown the feasibility to build blood vessel networks *in vivo* using human endothelial cells and mural cells [33,46–49]. Given that engineered vessels become eventually anastomosed to the host vascular bed, the secreted protein would be directly released into the blood stream and exert a systemic effect [50].

Taking advantage of the previously developed model of human angiogenesis *in vivo*, Compte *et al.* [51] explored the potential of *immunotherapeutic neovessels* after gene-modifying human vein endothelial cells (HUVEC) for the secretion of a bsAb (Table 1), based on the permissiveness of endothelial cells to be transduced by lentiviral vectors (Figure 2d). As a proof of principle, HUVEC transduced with a lentiviral vector were used for the expression of the α CEA x α CD3 diabody. These endothelial cells, along with MSC as a source of mural cells, were embedded in a solubilized tissue basement membrane matrix (Matrigel) to generate subcutaneous vascular networks capable of systemic bsAbs release in nude mice. Furthermore, the secreted α CEA x α CD3 diabody exerted a therapeutic effect in mice bearing distant CEA⁺ tumors after inoculation of human T cells. Interestingly, secretion of diabody in mice bearing *immunotherapeutic neovessels* remained highly stable. Diabody plasma concentration dropped less than 30% relative to the maximum peak, while secretion by MSC-based *immunotherapeutic organoids* dropped more than 80% during the same lapse of time [35].

In a practical clinical setting, this strategy would require: (1) to establish a suitable source of autologous endothelial cells; and (2) to characterize the most appropriate biological support for the generation of a stable and functional vasculature. Regarding the first point, Melero-Martin *et al.* [52] have recently demonstrated the vasculogenic potential of endothelial colony-forming cells derived from peripheral blood.

3. Future Prospects

In recent years, the use of gene-modified cells for antibody delivery has shown great potential as a therapeutic tool. Autologous cells seem to be the best choice for cell-based delivery therapies, and the most practical approach might be the use of *immunotherapeutic organoids* to keep the cells at the implantation site. However, these individualized therapies imply a high economic cost. From a practical point of view, the only realistic approach to apply these strategies to the clinical setting would imply the use of stocks of gene-modified allogeneic cells ready to be used. However, immune responses against allogeneic cells represent a major barrier for development in this field. Microencapsulation systems become an interesting alternative to protect gene-modified allogeneic cells from immune system and ensure the systemic release of the antibody for a long period of time (Figure 2c). The feasibility and effectiveness of this approach for the *in vivo* secretion of therapeutic proteins has been experimentally demonstrated [53–55]. Several studies have also demonstrated that stable secretion of antibodies can be reached through *in vivo* gene therapy methods (Figure 2e). Therapeutic levels of serum antibodies have been reported after intramuscular or intravenous injection of adeno-associated virus, adenovirus or lentivirus [20,21]. These approaches could be applied to cancer immunotherapeutic regimens in which long-term secretion of Fc-lacking bsAbs *in vivo* is desirable.

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Conflict of Interest

The authors declare no conflict of interest.

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