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REVIEW

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Intratumorally anchored cytokine therapy

K. Dane Wittrup[a,b,c], Howard L. Kaufman[d], Michael M. Schmidt[d] and Darrell J. Irvine[a,c,e]

[a]Koch Institute for Integrative Cancer Research at the Massachusetts Institute of Technology, Cambridge, MA, USA; [b]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA; [c]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA; [d]Ankyra Therapeutics, Boston, MA, USA; [e]Howard Hughes Medical Institute, MD, USA

ABSTRACT

Introduction: On-target, off-tumor toxicity severely limits systemic dosing of cytokines and agonist antibodies for cancer. Intratumoral administration is increasingly being explored to mitigate this problem. Full exploitation of this mode of administration must include a mechanism for sustained retention of the drug; otherwise, rapid diffusion out of the tumor eliminates any advantage.

Areas covered: We focus here on strategies for anchoring immune agonists in accessible formats. Such anchoring may utilize extracellular matrix components, cell surface receptor targets, or exogenously administered particulate materials. Promising alternative strategies not reviewed here include slow release from the interior of a material depot, expression following local transfection, and conditional proteolytic activation of masked molecules.

Expert opinion: An effective mechanism for tissue retention is a critical component of intratumorally anchored cytokine therapy, as leakage leads to decreased tumor drug exposure and increased systemic toxicity. Matching variable drug release kinetics with receptor-mediated cellular uptake is an intrinsic requirement for the alternative strategies mentioned above. Bioavailability of an anchored form of the administered drug is key to obviating this balancing act.

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1. Introduction

Despite the expanding success of immunotherapy for cancer, there remains an important gap in the therapeutic space: immune agonist antibodies [1] and cytokines [2,3] have yet to make significant inroads, despite the early approval of IL-2 for melanoma over 30 years ago. This stands in stark contrast with the broad reach and acceptance of antagonistic drugs, such as checkpoint blockade antibodies. The crux of the issue with agonist drugs is on-target (correct receptor type) /off-tumor (wrong tissue localization) activity resulting in a narrow therapeutic window, as represented schematically in (Figure 1). The high drug doses required to achieve therapeutic levels in tumor tissue inevitably activate immune cells throughout the body, producing severe systemic dose-limiting toxicities. To circumvent this challenge, strategies have been developed to: a) limit systemic activity through masking [4–8]; b) use weakened mutant cytokines either alone or targeted within a bispecific construct (NCT04250155) [9,10]; c) alter cytokine receptor subunit specificities [11,12]; d) express cytokines intratumorally [13–19]; or e) entrap cytokines within eroding biomaterial matrices [20,21]. In addition, anchoring cytokines to a pharmacologically insoluble scaffold that stably persists in a bioavailable state is a fundamentally distinct delivery strategy that we will focus on in this review. A particular benefit of this strategy is that it shifts both of the response curves in (Figure 1) in favorable directions, rather than just one of the two.

2. Intratumoral therapy

Enthusiasm for intratumoral drug administration is burgeoning, encouraged by the recognition that self-vaccinal stimulation of an adaptive anti-tumor T cell response holds potential for systemic and durable therapeutic effects [22–28]. Direct injection allows generation of *in situ* tumor-associated antigens without prior antigen identification or synthesis. Approval of talimogene laherparepvec (TVEC) oncolytic viral therapy and ongoing clinical trials for multiple others [14], together with favorable clinical results for intratumorally administered toll-like receptor (TLR) agonists [29] provide encouraging precedents. Advances in the field of interventional radiology [26] and robotic endoscopy [30] have significantly ameliorated previous concerns regarding the accessibility of visceral tumors for injection. Nevertheless, an underappreciated issue to date has been the rapidity with which soluble cytokines or other protein drugs leak out of a tumor following injection, leading to reduced efficacy, a need for frequent repeat injections, and potentially toxic systemic accumulation of drug.

2.1. Intratumorally administered proteins exit rapidly in the absence of a retention strategy

The speed with which cytokine or antibody-size proteins diffuse out of a tumor is faster than often recognized. However,

CONTACT K. Dane Wittrup wittrup@mit.edu Koch Institute for Integrative Cancer Research at the Massachusetts Institute of Technology, Cambridge, MA, USA

Article highlights

- Intratumoral administration is increasingly being explored to overcome poor therapeutic indices with immune agonist drugs for cancer.
- In the absence of a retention mechanism, localization of protein drugs is rapidly lost.
- Anchored, bioavailable cytokines solve the problem of matching local drug supply with cellular demand.
- Agonists have been bioavailability anchored to extracellular matrix, biomaterials, or cell surfaces.
- Systemic adaptive immune responses to intratumoral agonists have been observed preclinically and in humans.
- Tumor anchored cytokines are a critical tool for rational spatiotemporally programmed immunotherapy.

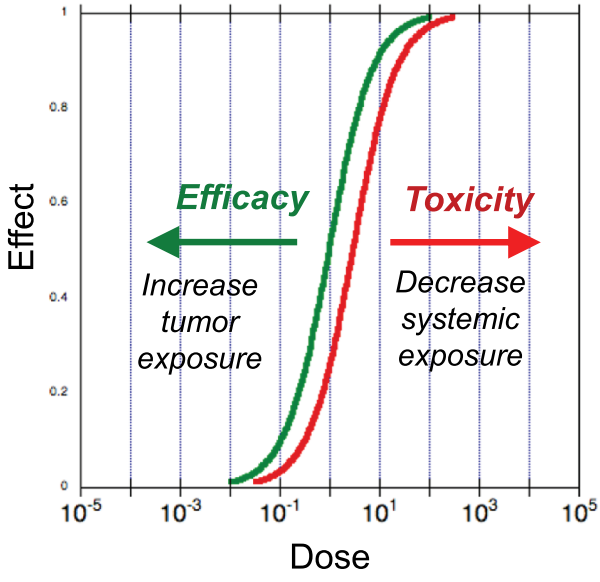

Figure 1. Framing the problem: two alternative ways to open the therapeutic window. Potency of cytokines is usually similar within and outside tumor tissue, leading to close tracking of the toxicity and efficacy dose response curves. The hypothetical curves shown here schematically represent this phenomenon. The resulting therapeutic window can then be impractically small – or even nonexistent. Multiple efforts are underway to solve this problem. Anchored cytokine immunotherapy shifts both response curves in favorable directions, greatly expanding the therapeutic window.

this phenomenon has been thoroughly demonstrated in mouse transplant tumor models [31–34]. Furthermore, this same rapid exit is observed in the clinic. An early intratumoral IL-12 clinical trial resulted in peak plasma IL-12 5–7 hours post injection, and '*remarkable toxicity … at the 100 ng/kg dose*' [35]. Inducible IL-12 expression from intratumorally administered oncolytic viruses in glioblastoma multiforme (GBM) has resulted in active levels of IL-12 in the blood [13]. These results demonstrate that if the rate of local cytokine provision does not closely approximate the local consumption rate, cytokine can be released at systemically toxic levels.

Enclosure of agonists within material depots such as hydrogels [20] or chitosan [21] prevents their rapid release, but also obscures the payload from intratumoral target immune cells until the payload is released from the depot. The release rate of soluble drug from the depot material must be matched to the client cell uptake rate lest toxic leakage occur. Similarly, approaches that rely on protein expression or proteolytic unmasking of soluble drug at the tumor site must generate sufficient drug to prime an effective response without exceeding local cell uptake and causing systemic leakage. Achieving optimal dosing is complicated by high heterogeneity in transfection efficiency or protease levels across primary tumors. As an alternative, we review here strategies for delivering bioavailable but physically anchored cytokines (Figure 2).

2.2. Alternative anchorage points

Three categories of anchored cytokine attachment points can be envisioned: extracellular matrix, cell surfaces, or exogenously administered materials. Each option offers differing spatial distribution, cytokine loading capacity, and retention kinetics, leading to numerous potential degrees of freedom for future optimization. Several examples of each type have been reported to date, and further optimization is accelerating. The most advantageous anchor type for any given payload remains to be discovered.

2.2.1. Extracellular matrix (ECM) anchoring

An early application of ECM anchoring exploited the specificity of expression of the fibronectin extra domain B (EDB) in the perivascular compartment of tumors and healing wounds [36]. This specificity enables imaging from systemically administered anti-EDB antibodies [37,38]. EDB antibodies fused with cytokines (a type of 'immunocytokine') can enhance tumor localization and cytokine activity after systemic administration, but such agents also stimulate their cognate cytokine receptors on immune cells in the bloodstream [39] thereby placing a limit on dose levels that can be achieved and the corresponding therapeutic window. Instead, administering these EDB-targeted immunocytokines intratumorally may provide optimal specificity and activity with extended tumor retention through ECM binding at the injection site [40–42]. The alternatively spliced EDA domain of fibronectin has been used analogously to anchor cytokines such as IL-12 following intratumoral injection [43].

Collagen is one of the most abundant proteins in the body, almost omnipresent in solid tumors [44]. It therefore makes an appealing target for cytokine fusion proteins, which have been administered systemically [45] or intratumorally [46]. In favor of intratumoral administration, it is perhaps noteworthy that accessible collagen attachment sites in the liver and kidney can together capture a significant fraction of collagen-binding proteins if systemically administered, and hepatotoxicity is often dose-limiting for immune agonists – although this paper did not report significant immune related events in the treated mice [45]. Intratumorally administered collagen-binding IL-2 and IL-12 fusion proteins cure established syngeneic tumors, and prime a sufficient CD8 T cell response to cure contralateral noninjected tumors [46]. In a model with resection of mammary fat pad 4T1 tumors, neoadjuvant intratumoral administration of collagen-binding IL-12 essentially eliminates formation of pulmonary metastases, an indication of protective systemic adaptive immunity. Finally, a BRAF-PTEN genetically engineered mouse model of melanoma is

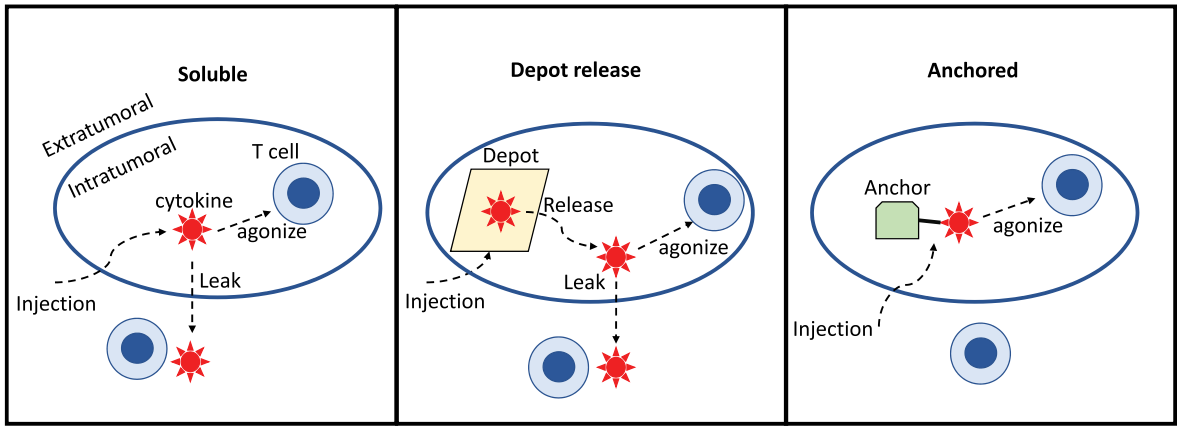

Figure 2. Schematic of alternative approaches to directly inject cytokines into tumors. Administration of soluble or depot-contained cytokine produces a kinetic competition between target cell uptake (T cells in this representation), and leakage out of the tumor. Skewing that competition toward intratumoral target-mediated consumption and away from leakage is a moving target, as both the number of target cells and depot release rate may vary patient-to-patient, by tumor microenvironment composition, and over time. Administering cytokine in a sterically accessible but anchored format obviates this issue.

cured by intratumoral collagen-retained IL-2 and IL-12 plus systemic checkpoint blockade [46].

The extensive history of tumor targeting via systemic administration of antibodies and other binders motivated development of a predictive theory to provide design guidance with respect to tumor-targeting drug size and binding affinity as they determine the efficiency of tumor drug uptake [47]. This 'outside-in' model describes principally the tradeoff between decreased renal clearance with increasing size, vs. decreased extravasation with increasing size. An analogous model was recently reported for 'inside-out' intratumoral drug delivery to an ECM anchorage point such as collagen [48], and was validated with quantitative PET biodistribution imaging. Key predictions of this model are that for ECM-anchored agents, molecular weights above approximately 60 kDa are preferable to decrease the rate of diffusion out of the tumor, while improved matrix-binding affinity monotonically increases retention. Consistent with these model predictions, tighter collagen-binding IL-2 fusion proteins gave progressively more beneficial therapeutic effects in this system [48].

2.2.2. Cell surfaces

Cell surface receptors are another point of attachment for intratumorally administered cytokines. The immunocytokine drug class was designed to accomplish tumor localization following systemic administration [49]. However, uptake by circulating cytokine receptor-positive immune cells creates a significant sink for such drugs [39,50] – a sink which is then expanded in a positive feedback loop by amplification of the number of cytokine-receptor-expressing cells [50,51]. This amplification and activation of circulating client cells also generates dose-limiting toxicity, in addition to consuming a significant proportion of the drug dose. One way to circumvent this issue is to intratumorally administer immunocytokines originally intended for intravenous administration. The high local concentration of drug after intratumoral administration rapidly binds to target antigen in the tumor leading to prolonged retention and a potent immune response while avoiding systemic clearance and toxicity [41,52,53]. Key design parameters include target cell and receptor numbers, as well as the endocytosis kinetics driving target-mediated drug disposition (TMDD). One might expect that endocytic consumption could limit target exposure duration, but nevertheless this approach has been efficacious in tumor models.

2.2.3. Exogenous depot

Rather than rely on endogenously expressed attachment points on the ECM or cell surfaces, one can attach cytokines to an insoluble or particulate material, in such a fashion as to not sterically occlude the cytokine. An early example of this approach was the intratumoral administration of liposomes with lipid-tethered cytokines on the surface [32,33] – however, here the longevity of stimulation is limited by rapid biodegradation/endocytic clearance of lipid vesicles. Interestingly, even systemically administered nanoparticles of this type can deliver cytokines to the tumor volume via the enhanced permeability and retention (EPR) effect, with residual circulating particles being rapidly cleared by the reticuloendothelial system, thereby diminishing systemic toxicity [54]. Exosomes have also been engineered to surface-display IL-12 for intratumoral administration in a similar manner [55] and have entered early stage clinical trials (NCT05156229).

It has been shown recently that the common vaccine adjuvant alum can serve as an attachment point for presentation of cytokines in the tumor space. Although physisorption to the material can be rapidly reversed *in vivo*, one can instead exploit a strong ligand exchange reaction between phosphorylated amino acids and the aluminum hydroxide matrix [56]. When IL-12 is attached to alum in this fashion, it persists in active form at the injection site for at least one to two weeks, extensively activating the myeloid compartment, and stimulating priming of anti-tumor CD8 + T cells [57]. Single intratumoral administrations of alum-anchored IL-12, alone or in combination with systemic PD-1 antibody blockade, cure established syngeneic tumors in mice with negligible toxicity.

2.3. Priming systemic adaptive immunity

A common concern raised for intratumoral therapy is how to treat disseminated, metastatic disease. This is because the

classic paradigm in oncologic pharmacology has been to design drugs intended to kill every cancer cell in the patient, hoping to eliminate resistance and recurrence. With the advent of immunotherapy, drugs are instead targeted to immune cells to foster potentially curative adaptive immune memory in the form of CD8 + T cells, whether exogenously administered or primed endogenously by therapy. The adaptive immune response can proceed to track tumor antigenic adaptations in a fashion that static therapies do not.

T cell responses that protect against tumor re-challenge are more the norm than the exception for curative immunotherapies in mice [15,33,39,41,46,48,57–63]. Of greater interest is whether such responses happen in treated humans. The frequency of occurrence of spontaneous protective immunity (an 'abscopal' effect) from external beam radiation therapy alone has been vanishingly small [64], perhaps unsurprisingly so given collateral damage to infiltrating immune cells and the tumor draining lymph nodes essential for an immune response. Promisingly, there are a growing number of clinical reports of responses in uninjected lesions following intratumoral immunotherapy. The extensive (but not exhaustive) list of examples of clinical abscopal effects from intratumoral therapies summarized below strongly supports the contention that this form of therapy can prime tumor-specific and clinically meaningful systemic T cell responses. Intratumoral administration of ECM-anchored IL-2 and TNF-alpha led to complete responses in over half of the noninjected lesions in 20 melanoma patients [40]. A clinical trial of intratumoral Flt3L and TLR agonist, plus external beam radiation therapy, recruited cross-presenting DCs and induced an anti-tumor CD8 + T cell response leading to systemic cancer remission in patients with advanced indolent Non-Hodgkin's Lymphoma [27]. Local radiotherapy and anti-CTLA-4 antibody treatment together induced systemic anti-tumor T cells in metastatic NSCLC patients, achieving an 18% response rate [65]. In a trial of intratumoral oncolytic virus combined with systemic anti-CTLA-4, 52% of the patients showed decreases in non-virally-injected visceral lesions (by comparison to 23% for those treated with anti-CTLA4 as monotherapy) [66]. Phase I studies of intratumoral CpG combined with local radiation therapy elicited partial responses in uninjected lesions of both lymphoma [67] and mycosis fungoides [68] patients. Intratumoral electroporation of an IL-12 expression plasmid led to regression of at least one uninjected lesion in 46% of treated metastatic melanoma patients [69]. In a trial of neoadjuvant checkpoint blockade plus primary NSCLC lesion stereotactic irradiation, 66% of patients downgraded previously tumor-positive tumor-draining lymph nodes to 'N0' stage following therapy [70].

This growing list of successful clinical abscopal effects from intratumoral immunotherapy provides solid evidence that it is possible in some patients to foster an efficacious adaptive immune response. Such 'existence proofs' are perhaps analogous to the dawn of immunotherapy, when the first robust responses obtained with anti-PD-1 therapy were observed in only a minority of patients, yet were indicative that, with further engineering optimization, such successes could be expanded to a broader patient population.

2.4. Technical advancements in intralesional therapy

A common question is: how readily can intralesional therapy be applied to nonsuperficial lesions? In fact, there have been numerous advancements in the fields of image-guided interventional radiology [25,26] and robotic endoscopy [30] that make most lesions injectable. Further demonstration of this feasibility is the growing prevalence of needle biopsies of tumors. In a survey of Medicare patients, over 700,000 needle biopsies were performed in 2010 [71]. Many such biopsies are performed annually in breast [72], lung [73], prostate [74], and liver [75] cancer. Even so, such procedures are sufficiently invasive that it is desirable to limit their repeat frequency. To quote Hong & Levy [76]: '*due to increased availability of interventional radiologic, endoscopic and laparoscopic procedures, most if not all lesions can now be accessed with or without the assistance of imaging modalities such as ultrasound, CT, etc … Therefore, the challenge in intratumoral therapy now lies not in initial accessibility, but in optimization of drug delivery technologies to enhance intratumoral delivery and reduce repeat injections*.' It is realistic to expect that safe and effective intratumoral immunotherapies that do not require frequent administration will find a straightforward path to acceptance in clinical practice.

3. Conclusions

To date, the areas of greatest growth in cancer immunotherapy have been antagonistic antibodies that target T cells, and autologous engineered T cell therapies. There is a glaring absence of agonistic therapies in the immune oncology pharmacopoeia, despite the clear potential advantages that could be achieved by activating anti-tumor T cells or the ancillary immune cells that instruct and support adaptive cell-mediated immunity. Most clinical trials of immune agonists fail due to dose-limiting toxicity prior to attainment of immune activation in the tumor compartment. A wide variety of tactics are being deployed to circumvent this limitation, but in this review we have focused on the rather straightforward notion of simply putting the drug in the tumor and making it stay there. This strategy has demonstrated compelling efficacy and tolerability in preclinical tumor models.

4. Expert opinion

Anchored cytokines are a key tool for developing biomimetic therapeutic strategies. Biomimicry is a powerful design principle for molecular bioengineers because natural selection hones solutions that are, by definition, robust and effective. Aligning one's technical objectives with nature's survival imperative has historically led to major advances (taking as just one example the success of directed evolution in crafting new enzymes and antibodies.) Along these lines, pharmacology might benefit by borrowing from the playbook of an immune response to an invading pathogen. A series of actions limited in location, duration, and sequence reject the initial infection, and construct memory of the attacker's molecular features that leads to rapid future protection against the same

pathogen. One could say that the natural immune response is therefore 'spatiotemporally programmed.' Why should we not do the same for cancer immunotherapy?

Taking IL-2 as an example, it is transiently expressed primarily (though not exclusively) by CD4 + T cells following T cell receptor (TCR) stimulation [77]. IL-2 expression is negatively regulated by autocrine/paracrine feedback control loops [78] that strictly limit the duration and location of its expression. By contrast with this endogenous local and time-limited deployment of IL-2, high-dose IL-2 therapy utilizes extended bolus infusions that more closely resembles the exposure patterns of an endocrine factor. This distinction might well be expected to contribute to immune suppressive effects detrimental to therapy, particularly from regulatory T cells stimulated via IL-2 exposure [79].

There is a growing recognition that temporal sequencing (i.e. dose scheduling) within treatment cocktails can have definitive effects on the success or failure of therapy [80]. For example, type I interferon treatment prior to significant antigen uptake completely short-circuits a tumor immune response, while delaying the very same dosage relative to delivery of an anti-tumor antibody produces a curative therapy [61].

Meanwhile, efforts to spatially restrict immunotherapy drugs have been impeded by the widespread preference to 'soak' the patient systemically with drug at its maximum tolerated dose for as long as tolerable. The misguidedness of this approach can perhaps best be appreciated by recognizing that a vaccinal effect is essentially an absolute requirement for curative immunotherapy. Would one optimize a vaccine by maximizing antigen and adjuvant exposure throughout the body for extended periods of time? Clearly not. Rather, intense localized inflammation together with concentrated depots of antigen are found to contribute to the most successful vaccination strategies. The analogy to a 'hot' tumor microenvironment with high tumor antigen density is direct – the objective should be for the tumor itself to become a localized vaccination site. Such an approach could circumvent the on-going immunoediting process that limits many immunotherapies [81].

The optimal combination of intratumoral location, timing, and duration of exposure is likely to vary considerably amongst different cytokine drug candidates. Although first-generation anchored cytokine immunotherapies have demonstrated powerful preclinical efficacy with minimal toxicity [32,33,46,48,57], it is to be expected that still further improvements will be attainable through the development of better tools for controlling the spatiotemporal cytokine exposure profile, and identification of optimal combinations with mainstay systemic therapies such as checkpoint blockade. Providing oncologists with such tools to precisely orchestrate immune attacks on solid tumors could significantly advance the clinical management of cancer.

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Declaration of interest

KD Wittrup & DJ Irvine are inventors on patent applications filed by the Massachusetts Institute of Technology in the areas described in this review. HL Kaufman and MM Schmidt are employees of Ankyra Therapeutics, which has licensed MIT IP on alum-anchored cytokines, and is clinically translating this technology. KD Wittrup, DJ Irvine, HL Kaufman, and MM Schmidt hold equity in Ankyra Therapeutics. DJ Irvine is an Investigator of the Howard Hughes Medical Institutes. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

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