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The aptamer–siRNA conjugates: reprogramming T cells for cancer therapy

“Oligonucleotide agents combining the cell selectivity of aptamers with the molecular target specificity of siRNAs are likely to provide a new class of highly precise and safer therapeutics.”

Keywords: 4-1BB • aptamers • cancer • CTLA-4 • immunotherapy • oligonucleotides • siRNA • STAT3 • T cells

Harnessing the body's own immune system has the potential to generate diverse, yet highly specific antitumor responses with protective long-term effects. Recent clinical advances demonstrated that new cancer immunotherapies can achieve impressive therapeutic effects, even in patients with advanced tumors of various types [1]. The rapid progress in this area was enabled by our improved understanding of the mechanisms underlying tumor immune evasion. Cancers evolved multiple strategies to block the activity of T lymphocytes and adaptive immune responses. These include upregulation and the triggering of inhibitory receptors on CD8 T cells, such as CTLA-4 or PD-1, or direct induction of effector T-cell death, which altogether shift the balance of T-cell-mediated responses from cytotoxic T cells to Tregs [2,3]. Targeting of the CTLA-4 and PD-1 immune checkpoint pathways in T cells provided the first effective tools to disengage tumor defense systems [1]. Nevertheless, the antibody-mediated neutralization of critical immunoregulatory cell populations carries a potential risk of collateral damage or autoimmune manifestations [1]. Growing evidence suggests that targeting intracellular signaling and transcriptional regulators can provide safer methods to correct T-cell dysfunctions, such as Th2 or Treg bias [4]. Transcription factors, such as STAT3, FoxP3, SMAD2/3 or HIF-1 α , are commonly activated in the tumor microenvironment and promote Treg functions [5–9]. Due to their intracellular localization and lack of enzymatic activity, the targeting of

transcriptional regulators using antibodies or small-molecule drugs is challenging. Oligonucleotide-based therapeutics provide alternative strategies to target the expression of transcription factors (siRNA and antisense oligonucleotides) or their binding to target DNA sequences (decoy oligonucleotides) with high specificity. However, the broad application of oligonucleotide therapeutics is limited by the difficulty in efficient and cell-specific delivery. T cells resist the delivery of formulated oligonucleotides using conventional transfection methods. The delivery of oligonucleotides using viral vectors, which has been successfully employed in some T-cell-specific gene therapies, is less suitable for cancer therapy due to potential carcinogenic effects, broad tropism and difficulty in large-scale production [10].

T-cell-specific aptamer–siRNA chimeras

The development of more stable, chemically modified oligonucleotides created an opportunity to deliver naked molecules as conjugates equipped with cell-specific targeting moieties, such as receptor ligands, monoclonal antibodies or aptamers [10]. The aptamers, compared with two other types of moieties, are DNA or RNA molecules, and this simplifies and lowers the cost of conjugate synthesis. At the same time, their 3D structure allows aptamers to bind their targets with high affinity and specifically matching the antibodies. The selection of aptamers through systematic evolution of ligands by exponential enrichment allows for the identification

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of cell type-specific oligonucleotide sequences with high affinity to known as well as unknown cell surface markers [11]. This is a considerable benefit for immunotherapeutic strategies that seek to target immune cell populations with a limited or very complex set of surface markers. The smaller size of aptamers compared with antibodies is a further advantage that can improve the organ and tissue biodistribution of conjugate molecules. McNamara *et al.* were the first to demonstrate that prostate cancer cell-specific aptamer–siRNA chimeras can successfully deliver siRNA into specific target cells *in vivo*, thereby reducing tumor growth [12]. This ground-breaking study provided a blueprint for the design of multiple other aptamer conjugates for the delivery of therapeutic siRNAs and miRNAs [11,13]. The majority of these strategies focused on targeting cancer cells; however, over the last 4 years, several research groups have attempted to use T-cell-targeting aptamer–siRNAs for the treatment of HIV and, very recently, for cancer immunotherapy. To target HIV-infected CD4⁺ T cells, Zhou *et al.* selected an aptamer that was specific for the viral envelope protein gp120 [14]. In addition to a direct neutralizing effect on viral infection, the gp120 aptamer was utilized for the delivery of siRNAs that silenced viral genes. This clever dual-function approach significantly improved the overall antiviral effect of the aptamer–siRNA chimera in comparison with the aptamer alone as tested in a humanized mouse model of HIV infection [15]. The alternative anti-HIV strategy focused on targeting CD4, an important coreceptor expressed on a subset of helper T cells. CD4 was previously shown to undergo endocytosis together with any bound cargo, thus allowing for the intracellular delivery of various molecules [14]. Wheeler *et al.* used a CD4 binding aptamer for the delivery of therapeutic siRNAs targeting the *CCR5* gene or HIV RNAs in an attempt to prevent HIV transmission into CD4⁺/CCR5⁺ T cells [16]. The CD4 aptamer–siRNA conjugates were shown to induce target gene silencing in a cell-specific manner and prevented viral transmission in mice. There are no reports on the feasibility of utilizing this strategy for cancer immunotherapy. However, it is conceivable that this approach could also allow for modulating functions of tumor-associated CD4⁺ T cells that limit adaptive antitumor immunity, such as Tregs or Th2 cells. CD4 aptamer-conjugated siRNAs for the silencing of T-cell subset-specific genes, such as *FoxP3* and *STAT5* in Tregs or *STAT6* in Th2 cells, could shift the balance towards Th1 antitumor immune responses while minimizing off-target effects [4].

Findings from these studies and the successful development of new immune receptor-specific aptamer sequences prompted the rational design of several

aptamer–siRNA conjugates specifically for cancer immunotherapy. Two very recently published studies utilized aptamers targeting either stimulatory (4-1BB) or inhibitory (CTLA-4) receptors on T-cell subsets in order to augment the efficacy and persistence of antitumor immune responses [17,18]. The first strategy focused on improving the long-lasting effects of cancer immunotherapies using T-cell-specific inhibition of mTOR signaling [17]. mTOR signaling is known to promote the differentiation of activated CD8⁺ T cells into short-lived effectors rather than memory cells, thereby resulting in potent but only transient immune responses. Gilboa and collaborators conjugated siRNA for a critical regulator of mTOR signaling – *Rptor* – with an aptamer recognizing the 4-1BB molecule, which is transiently expressed on activated T cells [17]. This ingenious aptamer–siRNA design enabled the targeting of mTOR signaling in a highly defined population of activated CD8⁺ T cells, thus preventing the unwanted immunosuppressive side effects that are typical of mTOR inhibitor drugs. Further experiments *in vivo* demonstrated that aptamer–*Rptor* siRNA increased the levels of CD8⁺ memory T cells with full potential to respond to antigen challenge. The strategy was remarkably effective at enhancing the efficacy of preventative and therapeutic vaccinations against several cancer types.

The outcome of immune therapies relies on a balancing act between positive and negative signaling cross-talk between immune cell populations [2,3]. Targeting inhibitory receptors on T cells, such as CTLA-4, creates an opportunity to reprogram exhausted cytotoxic CD8⁺ T cells and Treg populations using oligonucleotide agents [19]. A recent study by Herrmann *et al.* utilized the CTLA-4-specific aptamers for the silencing of *STAT3*, an immunosuppressive molecule that is activated in both tumor-associated CD8⁺ T cells and Tregs [18]. The conjugate consisted of an RNA aptamer linked to a Dicer substrate siRNA in order to allow for the intracellular processing and separation of both parts of the molecule, as shown in our previous study [20]. In fact, the CTLA-4 aptamer–*STAT3* siRNA conjugates were readily internalized and exerted *STAT3* gene silencing both *in vitro* and *in vivo* in CTLA-4-expressing T cells. Compared with the previously discussed chimeras, the CTLA-4 aptamer had apparently broader specificity. Targeting multiple subsets of immunosuppressive T cells was a plausible cause of the potent antitumor effects of this aptamer–siRNA against several tumor models. The conjugate did not result in detectable autoimmune effects that could result from the combined blockade of two essential immune checkpoints, such as CTLA-4 and *STAT3*. However, monomeric aptamer is not an effective CTLA-4 antagonist, even though it shows

sufficient receptor affinity to ensure cargo delivery [19]. Secondly, STAT3 silencing does not affect the viability of nonmalignant cells, while it also interferes with the function of tumor-associated immune cells and the survival of cancer cells [18].

Aptamer-based therapeutics: new class of smart drugs?

The application of aptamer conjugates with siRNA or other therapeutic oligonucleotides to T-cell-targeted cancer immunotherapies still holds challenges. The substantial cost of the large-scale production of chemically modified oligonucleotides still needs to be reduced through ongoing technological progress. Further studies should also expand the set of immune subset-specific aptamer sequences that are suitable for the delivery of therapeutic molecules. These efforts will be fueled by our growing understanding of the checks and balances in the immune system during tumor progression. Oligonucleotide agents combining the cell selectivity of aptamers with the molecular target specificity of siRNAs are likely to provide a new class of highly precise and safer therapeutics. With such a tool

kit, we may finally be adequately outfitted to repair the dysfunctional immune machinery in cancer and many other diseases.

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