Targeted IL-15-based Protein Fusion Complexes as Cancer Immunotherapy Approaches
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ABSTRACT
This mini review provides an overview and rationale for creating IL-15-based fusion protein complexes to be used as targeted immunotherapeutic agents. IL-15 stimulates proliferation and activation of CD8+ T and natural killer cells which result in augmentation of their anti-tumor activities. We have created ALT-803, an IL-15 superagonist complex which exhibits longer serum half-life, longer retention in lymphoid tissues, and better immunostimulatory and anti-tumor activities compared to native IL-15. When used alone or in combination with other immunotherapeutic molecules in various mouse tumor models, ALT-803 effectively reduces tumor burden and prolongs survival by stimulating the innate and adaptive arms of the immune system. To evaluate whether ALT-803 could be used as a protein scaffold to create IL-15-based tumor cell-specific molecules, we genetically fused it with a single chain anti-CD20 antibody derived from the variable regions of rituximab. This novel fusion protein exhibits enhanced anti-tumor activity compared to rituximab while maintaining IL-15 immunostimulating properties. Thus, ALT-803 may be exploited as a versatile scaffold to produce multivalent targeted fusion proteins to improve the anti-tumor efficacy of other therapeutic agents in the clinic.

Interleukin-15 and ALT-803
Common gamma chain (γc) cytokines, which include interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21, have been studied extensively and shown to be promising as cancer immunotherapeutic agents1. Recombinant IL-2 (rIL-2) has been approved for treatment of patients with metastatic renal cell carcinoma and melanoma more than two decades ago. Although rIL-2 can induce durable and major responses in a subset of patients2,3, the use of rIL-2 at the effective dose level causes severe side effects, such as capillary leak syndrome and hypertension, requiring extensive in-patient care during its administration4. These treatment-induced side effects curtail the clinical utilities of rIL-2 as an immunotherapeutic drug.

IL-15, a four helix γc cytokine, is structurally related to rIL-25. The two γc cytokines use the same IL-2/IL-15 receptor βγc (IL-2/15Rβγc) displayed on the surface of natural killer (NK) and T cells for signaling1. However, IL-15 has been proven to stimulate anti-tumor immune responses of NK and T cells without the induction of IL-2-associated capillary leak syndrome, activation-induced cell death, and expansion of T regulatory cells6,7. Thus, IL-15 is considered a promising immunotherapeutic for cancer treatment8. IL-15 associates with IL-15 receptor α (IL-15Rα) on
monocytes and dendritic cells and is trans-presented to form a complex with the IL-2/15Rβγc on NK and T cells resulting in their activation. To develop an IL-15-based therapeutic agent, we created a soluble complex, ALT-803, consisting of two protein subunits of a human IL-15 variant with an asparagine to aspartic acid substitution at position 72 (N72D) associated to a dimeric human IL-15Rα sushi (IL-15RαSu) domain/human IgG1 Fc fusion protein (IL-15RαSuFc) (Figure 1A). The N72D mutation increases the binding affinity of IL-15 to the IL-2/15Rβγc complex approximately 4- to 5-fold and significantly enhances the IL-15 biological activities. Compared to native IL-15, ALT-803 exhibits increased in vivo half-life (25 hours versus <40 minutes for native IL-15) and better distribution to the lymphoid organs (i.e., lymph nodes, spleen) for prolonged immune cell stimulation. ALT-803 is capable of simultaneously activating innate and adaptive immune cells and eliciting both rapid and long lasting anti-tumor activity as well as providing protective anti-tumor immunity. In pre-clinical models, systemic ALT-803 administration leads to antigen-independent proliferation, activation, and differentiation of memory CD8+CD44high T cells into potent effector cells and upregulation of NKG2D (KLRK1), but not CD25 or PD-1. These CD8+ T cells exhibit increased cytotoxic activity against tumor cells, as shown by secretion of high levels of IFNγ, granzyme B, and perforin. In addition to T cell stimulation, ALT-803 also induces NK cell proliferation, degranulation (measured by CD107a expression), IFNγ and TNFα production, and tumor cell killing. ALT-803 was also shown to promote robust expansion of NK cells with the highest fold increase observed in “high effector” CD11b+ CD27high NK cells which are resistant to self-tolerance, are highly cytotoxic, and exhibit increased cytokine production and migratory capacity. Felices et al. demonstrated that ALT-803 can restore function of ovarian cancer patient ascribes-derived NK cells and enhance cytotoxicity against ovarian tumor cells in vitro and in vivo. In non-human primates, ALT-803 treatment results in increased peripheral blood CD4+, CD8+ T and NK cell levels following four consecutive doses with no significant treatment-dependent adverse effects.

Another mechanism by which ALT-803 enhances NK cell-mediated lysis of tumor cells is through antibody-dependent cellular cytotoxicity (ADCC). Short-term ALT-803 stimulation of human NK cells in vitro was shown to enhance rituximab-directed ADCC against Daudi and Raji tumor cell lines. ALT-803 combination treatment with rituximab in B-cell lymphoma-bearing mice results in significant reduction of tumor burden and increased survival when compared to treatment with rituximab alone. Additionally, when combined with other potential immunotherapeutic molecules, such as checkpoint inhibitors, NKG2D ligand neutralizing antibody, or MEK inhibitor, ALT-803 improves the efficacy and durability of anti-tumor responses. Combining the immunostimulatory property of ALT-803 with the blockade of inhibitory immune checkpoints in multiple in vivo tumor models has been shown to increase the therapeutic effect of various checkpoint inhibitors (i.e., PD-1, PD-L1, and CTLA-4) by promoting development and function of effector NK and CD8+ T cells and mediating a more potent anti-tumor activity versus checkpoint inhibition alone. Therefore, although ALT-803 exhibits anti-tumor efficacy as a monotherapy, it also has great potential for use in combination with other immunotherapeutic agents. Importantly, mice cured by ALT-803 treatment have been shown to be resistant to tumor rechallenge, which indicates that ALT-803 also induces long-term memory and durable anti-tumor immunity. As a result, ALT-803 is being developed as a potential cancer immunotherapeutic and is currently in multiple clinical trials for treatment of patients with advanced solid and hematologic malignancies.

ALT-803-based Protein Scaffold for Targeted Immunotherapeutics

ALT-803 may act as a versatile protein scaffold, which can be modified for the creation of novel target-specific immunotherapeutic agents. We previously showed that IL-15 binding and function are preserved when ALT-803 components are fused to single chain T cell receptor (TCR) antigen binding domains. These targeted fusion proteins consist of TCRs recognizing a peptide of human p53 protein (c264sCTCR or c149scTCR), or an OVA protein epitope (OT1scTCR), linked to either IL-15, IL-15N72D, or IL-15RαSu N termini. Dimerization through IL-15:IL-15Rα or IL-15N72D:IL-15Rα interaction was shown to maintain the individual TCR’s functional activity and increase effective binding through enhanced avidity. Additionally, TCR fusion to N termini of either IL-15:IL-15RαSu or IL-15N72D:IL-15RαSu scaffold provides the spatial orientation necessary for functionally independent heterodimer complexes while retaining flexibility to allow folding of closely paired TCR chains. These fusion proteins are readily produced and easily purified from cell culture supernatant at a relatively high level. Since ALT-803 is comprised of an IL-15N72D:IL-15RαSuFc dimer, the results of the studies discussed above suggest that ALT-803 can be used to generate multivalent disease-specific fusion proteins.

Based on previous data demonstrating that ALT-803 can enhance rituximab’s anti-tumor activity, we created 2B8T2M, a novel CD20-specific fusion protein complex. Four single chain domains of the monoclonal antibody, rituximab, were genetically fused to N termini of IL-15N72D and IL-15RαSuFc proteins. This molecule exhibits tri-specific binding activity through its recognition of CD20 on B lymphoma cells, IL-2/15Rβγc on immune cells, and
Fc receptor on NK cells and macrophages (Figure 2). When cultured with Daudi tumor cells, 2B8T2M shows enhanced pro-apoptotic activity that is comparable to >600-fold higher concentration of rituximab alone and is dependent on CD20 binding. Additionally, 2B8T2M is significantly more effective at inducing ADCC against Daudi tumor cells by human immune cells compared to rituximab. A biodistribution study of the fusion protein demonstrated that adding a targeting domain to ALT-803 does not alter the molecule’s ability to home to lymphoid tissues and be retained there for at least 70 hours. In Daudi lymphoma-bearing mice, 2B8T2M treatment results in significantly lower tumor burden and improved survival compared to rituximab treatment alone. Specific B cell depletion by 2B8T2M was also observed in cynomolgus monkeys and importantly, no significant adverse events were observed. These results demonstrate that fusing a single chain antibody domain to ALT-803 is an effective strategy to potentiate tumor targeting immunotherapeutic molecule without compromising its immunostimulatory capabilities. Based on these results, 2B8T2M is currently in late-stage pre-clinical development.

Generating a single agent with multiple tumor targets and anti-tumor activities may be more beneficial compared to a combination of individual molecules for treatment of cancer. Our data show that linking a CD20 targeting antibody to ALT-803 retains its superior IL-15 activity while mediating more specific tumor killing. In addition to improved anti-tumor efficacy, the ALT-803 scaffold may also provide a vehicle for preferential delivery to lymphoid organs to allow proper and prolonged immune activation.

**Future Opportunities**

In order to overcome the various immunological barriers and improve clinical outcomes in cancer, immunotherapeutics must focus on stimulating different cellular immune pathways and overcoming the highly suppressive tumor microenvironment. IL-15-mediated...
immune cell activation by ALT-803 is expected to be attenuated by induction of immune checkpoint such as PD-1 expression by immune effector cells and PD-L1 expression by tumor cells\textsuperscript{23,24}. Co-administration of checkpoint inhibitors, such as anti-PD-1 and anti-PD-L1, with ALT-803 may aid in overcoming the immunosuppressive tumor microenvironment and prolonging ALT-803-stimulated T and NK cell activity and persistence. Potent immune stimulation and blocking suppression can be achieved in a single therapeutic agent using the ALT-803 protein scaffold by combining the highly potent immunostimulatory cytokine, IL-15, with checkpoint inhibitors for preventing immune exhaustion, therapeutic monoclonal antibodies and TCRs for disease-cell targeting, and protein-based molecules for neutralizing immune suppression. Also, ALT-803 could be used to create a single molecule fused with various cytokines for exogenous expansion and stimulation of immune cells for adoptive cell therapy. Adding infectious agent-specific recognition domains to the ALT-803 scaffold has the potential to create novel fusion proteins against virally infected cells for treatment of infectious diseases as well. Thus, ALT-803 is versatile protein scaffold that provides an opportunity for creating unlimited numbers of novel and highly potent immunostimulatory fusion molecules for various clinical applications.

**Conflict of interest statement**

The authors are employees and shareholders of Altor BioScience.

**References**


