Comparison of the Superagonist Complex, ALT-803, to IL15 as Cancer Immunotherapeutics in Animal Models

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Abstract

IL15, a potent stimulant of CD8+ T cells and natural killer (NK) cells, is a promising cancer immunotherapeutic. ALT-803 is a complex of an IL15 superagonist mutant and a dimeric IL15 receptor αSu/Fc fusion protein that was found to exhibit enhanced biologic activity in vivo, with a substantially longer serum half-life than recombinant IL15. A single intravenous dose of ALT-803, but not IL15, eliminated well-established tumors and prolonged survival of mice bearing multiple myeloma. In this study, we extended these findings to demonstrate the superior antitumor activity of ALT-803 over IL15 in mice bearing subcutaneous B16F10 melanoma tumors and CT26 colon carcinoma metastases. Tissue biodistribution studies in mice also showed much greater retention of ALT-803 in the lymphoid organs compared with IL15, consistent with its highly potent immunostimulatory and antitumor activities in vivo. Weekly dosing with 1 mg/kg ALT-803 in C57Bl/6 mice was well tolerated, yet capable of increasing peripheral blood lymphocyte, neutrophil, and monocyte counts by >8-fold. ALT-803 dose-dependent stimulation of immune cell infiltration into the lymphoid organs was also observed. Similarly, cynomolgus monkeys treated weekly with ALT-803 showed dose-dependent increases of peripheral blood lymphocyte counts, including NK, CD4+, and CD8+ memory T-cell subsets. In vitro studies demonstrated ALT-803–mediated stimulation of mouse and human immune cell proliferation and IFNγ production without inducing a broad-based release of other proinflammatory cytokines (i.e., cytokine storm). Based on these results, a weekly dosing regimen of ALT-803 has been implemented in multiple clinical studies to evaluate the dose required for effective immune cell stimulation in humans. Cancer Immunol Res; 4(1): 1–12. ©2015 AACR.

Introduction

Therapeutic approaches using common γ-chain cytokines, such as IL2, for patients with cancer offer the potential for curative antitumor immune responses (1, 2). However, IL2 therapy has limitations due to its significant toxicity and immunosuppressive activity mediated by T regulatory cells (Tregs; refs. 3–6). Alternative approaches employing the other γ-chain cytokines have focused on stimulating effector immune cells against tumors without inducing Tregs or IL2-associated capillary leak syndrome (7, 8).

IL15, like IL2, has the ability to stimulate T-cell and natural killer (NK)–cell responses through the IL2 receptor β-common γ chain (IL2Rβγc) complex (9, 10). However, these cytokines exhibit functionally distinct activities due to differential interactions with their unique receptor subunits. IL2 is produced as a soluble protein that binds to immune cells expressing IL2Rβγc or IL2Rαβγc complexes. In contrast, IL15 and its IL15Rαβ chain are coexpressed by monocytes/macrophages and dendritic cells and subsequently displayed as a cell surface IL15:IL15Rαβ complex, which is trans-presented to neighboring immune cells expressing IL2Rβγc (10, 11). Due to these differences, unlike IL2, IL15 does not support maintenance of Tregs and, rather than inducing apoptosis of activated CD8+ T cells, provides antian apoptotic signals (9, 12). IL15 also has nonredundant roles in the development, proliferation, and activation of NK cells (13–15). IL15 does not induce significant capillary leak syndrome in mice or non-human primates (NHP), suggesting that IL15-based therapies may provide the immunostimulatory benefits of IL2 with fewer adverse effects (16, 17).

Because IL15Rα is considered an integral part of the active cytokine complex, various therapeutic strategies are being explored using soluble preassociated IL15:IL15Rα complexes (18–23). For example, IL15:IL15Rα/Fc (i.e., soluble IL15Rα linked to an Ig Fc domain) was found to exhibit up to 50-fold greater activity than free IL15 in stimulating mouse CD8+ T-cell proliferation (19). In addition, IL15:IL15Rα/Fc increased efficacy...
compared with IL.15 in various mouse tumor models (18, 21, 24, 25). These effects may be partially due to an approximately 150-fold increase in II.15/R9 binding affinity for the IL.15: II.15/R9 complex compared with free IL.15 (26). Moreover, the IL.15:II.15/R9/Fc complex greatly enhanced IL.15 half-life and bioavailability in vivo (18), suggesting advantages of IL.15:II.15/R9/Fc over free IL.15 as an immunotherapeutic (27). Although nonclinical studies demonstrated the pharmacodynamic (PD) and safety profiles of IL.15 in NIH/3T3 (17, 28–30), similar studies have not yet been reported to support clinical development of IL.15:II.15/R9 complexes.

To advance IL.15:II.15/R9-based therapies into clinical testing, we have created a complex (referred to as ALT-803) between a novel human IL.15 superagonist variant (II.15N72D) and a human II.15/R9 sushi domain–Fc fusion protein (22, 23). We have previously shown that the II.15N72D mutation increases II.15/R9, binding and IL.15 biologic activity by approximately 5-fold (22). This II.15 superagonist fusion protein complex, ALT-803 (II.15N72D:II.15/R9Su/Fc), exhibited superior immunostimulatory activity, a prolonged serum half-life, and more potent antitumor activity compared with IL.15 in mouse models (23, 31). In this article, we further evaluate the antitumor activity, biodistribution, PD, and toxicity of ALT-803 in mice. We found that ALT-803 was significantly more efficacious than IL.15 in mice bearing solid tumors. In addition, ALT-803 was retained in lymphoid organs to a greater extent than IL.15, consistent with its more potent immunostimulatory and antitumor activities in vivo. Evaluation of ALT-803 toxicity in mice compared with its efficacious dose in various tumor models indicated that this complex has a wide therapeutic dose range. Finally, studies of multidose ALT-803 treatment in cynomolgus monkeys and dose-dependent efficacy on human immune cell activity provided results consistent with those from mouse studies and further defined a weekly dosing regimen suitable for initiation of human clinical studies.

Materials and Methods

Reagents and animals

ALT-803 was generated as previously described (23). Recombinant human IL.15 (17) was kindly provided by Dr. Jason Yovandich (NCI, Frederick, MD). Antibodies used to characterize immune cell phenotype and activation markers are described in Supplementary Table S1. C57BL/6 and BALB/c mice were obtained from Harlan Laboratories, and cynomolgus monkeys were supplied by Yunnan Laboratory Primate, Inc. (YLP) and the Oregon National Primate Research Center. All animal studies (mouse biodistribution at University of Wisconsin, other mouse studies at Altior BioScience, and monkey studies at YLP and Oregon Health and Science University) were conducted under Institutional Animal Care and Use Committee–approved protocols.

Cell lines and human peripheral blood mononuclear cells

The murine B16F10 (CRL-6475) and CT26 (CRL-2638) tumor cell lines were obtained from the American Type Culture Collection in 2009 and 2013, respectively. Within 1 week of receipt, the cells were viably cryopreserved and stored in liquid nitrogen. Both tumor cell lines were shown to be free of Mycoplasma contamination. In each experiment, one frozen vial was expanded for use, and the cells were authenticated by confirming cell morphology in vitro and reproducible tumor growth characteristics in mice of the control treatment groups. Human peripheral blood mononuclear cells (PBMC) were isolated using Histopaque (Sigma) from peripheral blood of anonymous donors (Onellood; refs. 17, 23). Human PBMCs, mouse splenocytes, and CT26 cells were cultured in R10 media (32). B16F10 cells were cultured in IMDM (HyClone) supplemented with 10% FBS. Cells were incubated at 37°C with 5% CO2.

PET imaging and tissue biodistribution studies

Protein conjugation methods with 2-S-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA; Macrocyclics), radiolabeling with 64CuCl2 (UW-Madison cyclotron facility), and subsequent purification were conducted as previously described (33). For serial PET imaging, C57BL/6 mice were injected i.v. with 3 to 7 MBq of 64Cu-labeled ALT-803 or IL.15 (~3.7 GBq/mg protein). Static PET scans were performed on anesthetized animals at various times after injection using an Inveon microPET/microCT hybrid scanner (Siemens). Data acquisition, image reconstruction, and region-of-interest analysis to calculate the percentage injected dose per gram of tissue (%ID/g) for major organs were conducted as previously described (33, 34). At various times after injection, mice were euthanized and blood, lymph nodes, thymus, and major organs/tissues were collected and weighed. The radioactivity in each tissue was measured using a gamma-counter (Perkin Elmer) and presented as %ID/g.

In vitro studies

Cytokine-release and proliferation assays were conducted on human and mouse immune cells using ALT-803 as soluble protein or as soluble or air-dried plastic-immobilized proteins prepared according to Stebbing and colleagues (35). ALT-803 was tested at 0.08, 0.8, and 44 nmol/L, which correspond to maximal serum concentrations in humans of a 0.3-, 3.0-, and 170-μg/kg i.v. dose, respectively. For proliferation assays, human PBMCs and mouse CD3+ T cells enriched from splenocytes (CD3+ T Cell Enrichment column; R&D Systems) were labeled with CellTrace Violet (Invitrogen) and cultured in PBS- or ALT-803–containing wells. As a positive control, 27 nmol/L of mAb to CD3 (145-2C11 for mouse splenocytes and OKT3 for human PBMCs; BioLegend) was added to separate wells in the same assay formats. Cells were incubated for 4 days and then analyzed by flow cytometry to determine cell proliferation based on violet dye dilution. In addition, human and mouse immune cells were cultured as described above for 24 and 96 hours. Cytokines released into the media were measured using human and mouse cytometric bead array (CBA) Th1/Th2/Th17 cytokine kits as per the manufacturer’s instructions (BD Biosciences). For assessment of immune cell subset and activation markers, human PBMCs were cultured in various concentrations of ALT-803, stained under appropriate conditions with marker-specific antibodies (Supplementary Table S1), and analyzed on a FACsVerse flow cytometer (BD Biosciences) using FACSuite software.

Antitumor efficacy and toxicity of ALT-803 in mice

Comparative efficacy of ALT-803 and IL.15 was assessed in immunocompetent mice bearing s.c. B16F10 melanoma tumors or CT26 colon carcinoma metastases. C57BL/6 mice were injected s.c. with B16F10 cells (5 × 107/mouse) and then treated with i.v.
ALT-803, IL15, or PBS as described in the figure legends. Tumor volume was measured as described (36). In the second model, BALB/c mice were injected i.v. with CT26 cells (2 × 10⁶/mouse) and treated with i.v. ALT-803, IL15, or PBS as described in the figure legends. Mouse survival based on humane endpoint criteria was monitored to generate survival curves.

Toxicity studies were conducted in C57BL/6 mice (~10–11 weeks old) that were injected i.v. with 0.1, 1.0, or 4.0 mg/kg ALT-803 or PBS weekly for 4 weeks [study days (SD) 1, 8, 15, and 22]. Assessments, including physical examination, serum chemistry, hematology, gross necropsy, body and organ weight, and histopathology, were performed on mice sacrificed 4 days (SD26) or 2 weeks (SD36) after treatment. In a second study, C57BL/6 mice were treated with four weekly i.v. injections of 0.1 or 1.0 mg/kg ALT-803 or PBS, and toxicity assessments were performed 4 days (SD26) or 4 weeks (SD50) after treatment.

**Toxicity, pharmacodynamics, and pharmacokinetics of ALT-803 in cynomolagus monkeys**

A study was performed under Good Laboratory Practice guidelines to evaluate the effects of multidose administration of ALT-803 in cynomolagus monkeys. Animals (2–3 years old, 5 animals/sex/group) were treated weekly for 4 weeks (SD1, 8, 15, and 22) with 0.03 or 0.1 mg/kg ALT-803 or PBS administered i.v. over about 3 minutes. Throughout the in-life study phase, animals were assessed for clinical and behavioral changes, food consumption, body weight, cardiac function, and oculur function. Blood was collected for hematology, chemistry, coagulation, and immune cell analysis before dosing and on SD5, 26, and 36. Immunogeticity testing and pharmacokinetics (PK) analyses were conducted using validated ELISA methods. Urine was collected for urinalysis (predosing and SD4, 25, and 35). Clinical pathology assessments, including physical examination, gross necropsy, organ weight measurements, and histopathology, were performed 4 days (SD26) and 2 weeks (SD36) after treatment.

A separate time course study was conducted on 2 animals (5-year-old females) injected i.v. with 0.1 mg/kg ALT-803 on SD1, 8, 15, and 22. Blood and serum were collected as indicated in the legend for Fig. 7C and D. Serum cytokines were measured using NHP CBA Th1/Th2 cytokine kits (BD Biosciences). Immune cell phenotyping was conducted on blood samples after lysis of red blood cells and staining with antibodies specific to immune cell phenotype markers (Supplementary Table S1). For Ki67 assessment, cells were fixed with BD FACS Lysing solution (BD Biosciences) prior to antibody staining.

**Data analysis**

Data are expressed as the mean ± SE. Survival data were analyzed using the log-rank test and Kaplan–Meier method. Comparisons of continuous variables were done using Student t tests or ANOVA (two-tailed; GraphPad Prism Version 4.03). P values < 0.05 were considered statistically significant. PK analysis was conducted as previously described (23).

**Results**

**Comparative efficacy of ALT-803 and IL15 against solid tumors in immunocompetent mice**

In mice bearing myeloma tumors, single-dose treatment of ALT-803 was found to provide significant reduction of tumor burden compared with an equivalent dose of IL15 (36). To extend these findings to solid tumors, we compared the antitumor activity of ALT-803 and IL15 against s.c. B16F10 melanoma tumors or CT26 colon carcinoma metastases, which are sensitive to IL15-based therapies (24, 37). As shown in Fig. 1A, B16F10 cells injected s.c. into the flank of C57BL/6 mice developed into palpable tumors by day 7 and progressed rapidly over the next 8 days. Treatment of tumor-bearing mice with IL15 on days 1 and 8 failed to affect tumor growth. In contrast, ALT-803 administered at a molar cytokine equivalent dose (i.e., 0.06 mg/kg of IL15 equals 0.2 mg/kg of ALT-803) significantly inhibited growth of B16F10 tumors compared with IL15 (P < 0.05) or PBS (control; P < 0.01) treatment. These results are comparable with previous studies demonstrating superior efficacy of preassociated IL15:IL15Rα complexes against s.c. and metastatic B16 tumors (18, 24).

In the CT26 colon carcinoma metastases model (Fig. 1B), IL15 administered as five 0.25-mg/kg doses per week (10 doses total)
for 2 weeks resulted in modest improvement in survival of tumor-bearing mice compared with the control group (median survival time (MST): IL15, 17 days vs. PBS, 15 days; \( P < 0.05 \)), consistent with previously published results (37). However, less frequent dosing with ALT-803 (four 0.2-mg/kg doses over 2 weeks) provided significantly better survival benefit than either IL15 or PBS (MST; ALT-803; 22.5 days; \( P < 0.01 \) vs. IL15 or PBS). Notably, this enhanced efficacy was observed with a cumulative molar cytokine dose of ALT-803 that was 9% of the IL15 dose. Together, these results are consistent with potent immunostimulatory activity of ALT-803 that was 9% of the IL15 observed in vivo and in our previous efficacy studies in hematologic tumor models (23, 31).

**Biodistribution of ALT-803 in mice**

Earlier studies indicated that ALT-803 had a 25-hour serum half-life in mice compared with <40 minutes observed for IL15 (23). To further explore the PK properties of these molecules, biodistribution studies were conducted in mice administered \(^{64}\text{Cu}-\text{labeled ALT-803 or IL15. Serial noninvasive PET scans of C57BL/6 mice at different times after injection showed that}^{64}\text{Cu-IL15} \text{ was rapidly cleared via the renal pathway, consistent with previous reports (38), whereas ALT-803 was cleared primarily via the hepatobiliary pathway (Fig. 2A and B). In accordance with PK analysis,}^{64}\text{Cu-ALT-803 exhibited a longer circulatory half-life than}^{64}\text{Cu-IL15. Tissue distribution of the}^{64}\text{Cu-labeled proteins was determined at 6 hours (IL15 and ALT-803) and 70 hours (ALT-803) after injection (Fig. 2C). These results corroborated the findings from the PET scans by showing elevated uptake of}^{64}\text{Cu-IL15 and}^{64}\text{Cu-ALT-803 in the kidneys and liver, respectively, 6 hours after injection. In addition,}^{64}\text{Cu-ALT-803 levels were elevated in the lungs, spleen, and lymph nodes 6 hours after injection and persisted at >4\% \text{ID/g in the lymph nodes for at least 70 hours after injection, at which time}^{64}\text{Cu-IL15 was not detectable. Thus, ALT-803 not only exhibits a longer serum half-life but also greater distribution and a longer residence time in the lymphoid organs than IL15.}

**Immunostimulatory effects of ALT-803 on murine and human immune cells**

To assess the ALT-803–mediated responses of mouse and human immune cells, studies were conducted with human PBMCs and mouse splenocytes incubated with soluble or plastic-immobilized ALT-803 (Fig. 3). Incubation with immobilized ALT-803 for 1 day (data not shown) or 4 days (Fig. 3A) resulted in elevated IFNγ release by human PBMCs. Soluble IL6 was also
ALT-803 induces cytokine release and proliferation of mouse and human immune cells in vitro. A, human PBMCs (n = 3 from normal donors) were incubated for 4 days in wells containing media and the indicated concentrations of soluble or plastic-immobilized ALT-803. At the end of the incubation period, concentrations of proinflammatory cytokines in the culture media were assessed using a cytometric bead array. ALT-803-mediated changes in human IFNγ and IL6 were observed and are plotted, whereas no significant differences in the levels of human TNFα, IL4, IL10, or IL17A were found among the treatment groups. Bars represent mean cytokine concentration ± SE. *, P < 0.05; **, P < 0.01 comparing ALT-803 vs. media control. B, CD3-enriched mouse splenocytes (n = 6) were incubated for 4 days in media containing soluble or immobilized ALT-803, and concentrations of proinflammatory cytokines in the culture media were assessed as described in A. Mouse IFNγ and TNFα levels were significantly induced by ALT-803, but there were no treatment-mediated changes in the levels of IL6, IL2, IL4, and IL17A. Bars represent mean cytokine concentration ± SE. *, P < 0.05; **, P < 0.01 comparing ALT-803 vs. media control. C, positive control wells for the assays shown in A and B contained 27 nM of soluble and immobilized anti-CD3 Ab (145-2C11 for mouse splenocytes and OKT3 for human PBMCs) and were assessed for immune cell cytokine release as described above. D and E, CD3-enriched mouse splenocytes (n = 6; left) and human PBMCs (n = 3) were labeled with CellTrace violet and cultured for 4 days in media containing soluble or immobilized ALT-803 as described in A. As a positive control, 27 nmol/L of anti-CD3 Ab was added to separate wells (E) in the same assay formats. At the end of the incubation period, the cells were analyzed by flow cytometry to determine cell proliferation based on violet dye dilution. Bars represent mean ± SE of percent total lymphocytes that showed decreased violet labeling (i.e., proliferating cells). *, P < 0.05; **, P < 0.01; ***, P < 0.001 comparing ALT-803 or anti-CD3 Ab vs. media control. The results shown for plastic-immobilized ALT-803 aqueous solution were equivalent to those observed for ALT-803 that was air-dried onto the assay wells. In each case, the results shown are representative of at least two independent experiments.

Increased in 4-day PBMC cultures treated with ALT-803; however, this effect was not dose-dependent. In contrast, ALT-803 had no effect on TNFα, IL4, IL10, or IL17A release in 4-day PBMC cultures (data not shown). When tested in parallel cultures, a positive control anti-CD3 mAb induced release of IFNγ, TNFα, IL10, and IL4 (Fig. 3C).
Compared with human immune cells, mouse splenocytes exhibited a similar but less intense response for IFNγ release following incubation with ALT-803 (Fig. 3B). ALT-803 also induced TNFα production by splenocytes, but showed no significant effect on IL6, IL2, IL10, IL4, and IL17A concentrations. Conversely, murine lymphocytes incubated with immobilized mAb to CD3 showed significantly elevated release of all of the cytokines tested except for IL6 (Fig. 3C). Together, these findings indicate that ALT-803 primarily stimulates IFNγ production by human and mouse immune cells, in contrast with the broad profile of cytokines induced by mAb to CD3.

The ability of ALT-803 to induce in vitro proliferation of CellTrace Violet-labeled human and mouse immune cells was also evaluated. Pronounced proliferation of mouse lymphocytes was evident following incubation with 0.8 to 44 nmol/L soluble or immobilized ALT-803 (Fig. 3D). Up to 83% of the cells in the high-dose soluble ALT-803 group underwent 1 to 6 rounds of cell division during the 4-day incubation period. Little or no proliferation was detected in untreated murine cells or those treated with 0.08 nmol/L soluble ALT-803. As expected, murine lymphocytes incubated with immobilized mAb to CD3 exhibited strong proliferative responses (Fig. 3E). ALT-803 dose-dependent lymphocyte proliferation was also observed in human PBMC cultures, but to a lesser extent than that seen for mouse cells (Fig. 3D). Overall, <20% of all human lymphocytes proliferated in response to high-dose ALT-803, and these responses were lower than those induced by the positive control mAb to CD3 (Fig. 3E). The mechanisms responsible for the differential responsiveness of mouse and human immune cells to ALT-803 (and mAb to CD3) stimulation are not understood.

Both soluble and immobilized forms of ALT-803 were capable of stimulating mouse and human immune cell proliferation. However, immobilized ALT-803 (44 nmol/L) was more potent than soluble ALT-803 at stimulating IFNγ release (Fig. 3A and B), suggesting IL2Rγc crosslinking and stronger signaling provided by immobilized ALT-803 are required for this response. In addition, significant variations in the immunostimulatory activity of both ALT-803 and mAb to CD3 were observed in lymphocytes collected from different human donors, likely due to different genetic and environmental factors shaping immune responses in these individuals.

The immunostimulatory activity of ALT-803 was further assessed in 7-day cultures of human PBMCs. Treatment with 0.5 nmol/L of soluble ALT-803 resulted in 2.1-fold increase (range, 1.4–3.1, n = 7) in lymphocyte counts (Fig. 4A). These effects were due to increased numbers of CD69+ and CD4+ T cells (3.0- and 1.8-fold, respectively) and NK cells (2.8-fold), whereas CD19+ B-cell and Treg counts were not significantly changed by incubation with ALT-803 compared with control. Similar effects were seen with equivalent concentrations of IL15, consistent with previous studies reporting comparable in vitro activity of these proteins on human PBMCs bearing IL15Rα/IL2Rγc complexes (32). Titration studies showed that 0.07 nmol/L ALT-803 significantly increased CD8+ T-cell numbers in 7-day human PBMC cultures (Fig. 4B). In addition, cell surface activation marker expression of CD69 on NK and CD8+ T cells and CD25 on NK and CD4+ T cells was stimulated by ALT-803 in a dose-dependent manner (Fig. 4C). Consistent with increased cytotoxic activity against NK-sensitive cells and tumor cells (32), ALT-803 also induced increased granyme B and perforin expression in both human NK cells and CD8+ T cells (Fig. 4D). Together, these findings indicate that ALT-803 at a concentration as low as 0.01 nmol/L is capable of activating human immune cells in vitro.

Toxicity of ALT-803 in mice

The results of comparative binding of ALT-803 to immune cells from mice, cynomolgus monkeys, and healthy human donors were consistent with previously reported species-specific differences in IL15 binding to IL15Rα/IL2Rγc complexes (Supplementary Fig. S3; ref. 39) and verified that mice and cynomolgus monkeys are appropriate species for evaluating the range of ALT-803–mediated effects. Thus, the safety and PD profiles of ALT-803 were assessed in healthy C57Bl/6 mice injected i.v. with 0.1, 1.0, or 4.0 mg/kg ALT-803 or PBS weekly for 4 consecutive weeks (Fig. 5A). Mice receiving 4.0 mg/kg ALT-803 exhibited signs of toxicity (i.e., weight loss) and mortality between 4 and 20 days after treatment initiation. Postmortem necropsy did not determine the cause of death, but observations (i.e., pulmonary edema, and enlarged lymph nodes and spleen) were consistent with cytokine-induced inflammatory responses (40, 41). Treatment-related mortality was not observed in mice treated with 1.0 or 0.1 mg/kg ALT-803 (n = 50/dose level). Dose-dependent increases in spleen weights and white blood cell (WBC) counts were seen 4 days after the last dose of ALT-803 (SD26; Fig. 5B). Of the WBCs, absolute counts for lymphocytes, neutrophils, and monocytes all increased 8-fold in 1.0 mg/kg ALT-803–treated mice compared with controls. By 2 weeks after treatment (SD36), lymphocyte counts returned to control levels, but neutrophil counts remained elevated in 1.0 mg/kg ALT-803–treated mice (Fig. 5B). Histopathologic analysis verified ALT-803 dose-dependent stimulation of immune cell infiltration and hyperplasia in the spleen, liver, thymus, kidneys, lungs, and lymph nodes on SD26, and to a lesser degree on SD36. Similar results were observed in a second study where C57BL/6 mice were treated with four weekly ALT-803 doses and assessed on SD26 and SD50 (i.e., 4 weeks after treatment). The results of these studies define the tolerable dose of midose ALT-803 treatment of up to 1.0 mg/kg in mice in a weekly dosing regimen for 4 weeks.

Toxicity, PK, and PD profiles of ALT-803 in cynomolgus monkeys

Based on allometric scaling to the tolerable murine dose, the activity and toxicity profiles of multidose i.v. treatment of ALT-803 at 0.1 and 0.03 mg/kg were assessed in healthy cynomolgus monkeys. PK analysis after the first dose estimated the terminal half-life of ALT-803 to be 7.2 to 8 hours, which did not appear to differ significantly between dose levels (Fig. 6; Supplementary Table S2). The maximum serum concentration (Cmax) of 30 nmol/L for 0.1 mg/kg ALT-803 was consistent with full recovery of the administered dose, whereas Cmax and AUIC values indicated approximately 30% less recovery at the 0.03-mg/kg dose. However, even at the low dose level, the Cmax of 6 nmol/L in the serum was >50-fold higher than the 0.1 nmol/L concentration found to stimulate immune cell proliferation and activation in vitro.

Monkeys receiving four consecutive weekly injections of ALT-803 showed a dose-dependent reduction in appetite during the first 2 weeks. However, there were no significant differences in mean body weights or any other dose-related clinical or behavioral observations among the groups. In addition, organ weights were not significantly different in ALT-803–treated animals compared with controls (summarized in Supplementary Table S3).
The most biologically relevant changes observed after weekly ALT-803 treatment were dose-dependent increases in peripheral blood WBC and lymphocyte counts (Fig. 7A). After the 4-week dosing period (SD26), animals receiving 0.1 mg/kg ALT-803 showed a 1.5-fold increase in absolute lymphocyte numbers, which returned to control levels following a 2-week recovery period (SD36). Of the lymphocyte subsets, transient dose-dependent increases in NK-cell and CD4<sup>+</sup> and CD8<sup>+</sup> T-cell counts were seen after treatment (Fig. 7B). Increased peripheral blood monocyte counts were observed in 0.1 mg/kg ALT-803–treated monkeys, whereas peripheral blood neutrophil levels were not different among the treatment groups.

In addition, there was a dose-dependent increase in mild multifocal lymphocytic infiltration in the liver, kidneys, and lungs of ALT-803–treated monkeys based on histopathology analysis conducted on SD26 (Supplementary Table S4).
Scattered mild liver necrosis was also observed with increased frequency in ALT-803–treated animals. Clinical chemistry at this time point showed a decrease in serum albumin in the high-dose ALT-803 group compared with controls, which may be a consequence of inflammatory responses in the liver. However, serum liver enzymes were not elevated in ALT-803–treated animals compared with controls. Bone marrow hyperplasia was observed in most animals and increased in severity in the high-dose ALT-803–treated group. Lesions in the majority of affected organs in the ALT-803–treated groups were reduced in incidence and severity by SD36 and were consistent with findings in the control animals.

Eight of 22 animals in the ALT-803 treatment groups developed detectable anti–ALT-803 antibodies after multidose treatment. The pharmacologic consequences of these responses are unclear because there were no postdosing allergic reactions and no effects on ALT-803–mediated responses in animals that developed anti–ALT-803 antibodies.

The effects of ALT-803 on T-cell subpopulations were also assessed in cynomolgus monkeys receiving four consecutive weekly injections at 0.1 mg/kg. Consistent with the results described above, multidose ALT-803 treatment resulted in an increase in blood CD4+ and CD8+ T-cell and CD16+ NK-cell counts over the treatment course (Fig. 7C). Of the CD8+ T cells, effector memory (EM) and to a lesser extent central memory (CM) T-cell counts increased shortly after treatment initiation, whereas naive CD8+ T-cell counts were elevated compared with predose counts as the 4-week treatment course proceeded. Similarly, naive, CM, and EM CD4+ T-cell counts were elevated after the first dose of ALT-803, resulting in an approximately 3-fold increase in blood CD4+ T cells. The changes in blood lymphocyte counts were associated with increased expression of the proliferation marker Ki-67 in CD16+ NK cells and memory CD8+ and CD4+ T cells (Fig. 7D), indicating the ALT-803–mediated effects are due to increased cell proliferation rather than merely redistribution. Assessment of serum
samples collected in this study indicated that there was no significant induction of IFNγ, TNFα, IL6, IL5, IL4, or IL2 levels during the 4-week ALT-803 treatment course. Overall, the observed changes in peripheral blood and tissue lymphocytes after ALT-803 treatment of cynomolgus monkeys were consistent with transient effects reported for NHPs treated with IL15 after ALT-803 treatment of cynomolgus monkeys were consistent with transient effects reported for NHPs treated with IL15 (17, 28, 29).

Discussion

In the present study, we assessed the in vitro activity and in vivo efficacy, toxicity, and PD profiles of an IL15 superagonist fusion protein complex, ALT-803 (IL15N72D:IL15RαSu/Fc), in animal models to provide the rationale for the initial clinical dose regimen. We have previously found that treatment of mice with a single i.v. dose of ALT-803 resulted in significant increases in spleen weight and CD8+ T-cell and NK-cell counts that were not observed after IL15 treatment (23). Moreover, a single dose of ALT-803, but not IL15 alone, effectively eliminated well-established murine myeloma in the bone marrow of tumor-bearing mice (31). Extending these findings to solid tumor models, we found that ALT-803 (even at less than 10% of cumulative cytokine concentrations) was more efficacious than IL15 for antitumor responses in mice bearing s.c. B16 melanoma tumors or CT26 colon carcinoma metastases. These effects were likely due in part to the >20-fold longer in vivo half-life of ALT-803 compared with IL15 (23).

The results of biodistribution experiments reported here confirm the hypothesis that the pharmacologic properties of ALT-803 are highly differentiated from IL15. Consistent with previous reports using 125I-labeled IL15 (38), 64Cu-IL15 was very rapidly cleared from circulation with the kidney as the major site of IL15 accumulation. 64Cu-labeled IL15 also showed low uptake by the liver and lymph nodes and little or no retention by other tissues. The poor bioavailability of i.v.-administered IL15 in lymphoid tissues and its rapid clearance have been important factors in determining the optimal immunostimulatory treatment regimens of IL15, which have focused on daily or continuous i.v. administration (17, 42). In contrast, the biodistribution of 64Cu-ALT-803 showed significantly longer circulation in the blood, lower kidney uptake, and much broader and prolonged accumulation in multiple tissues. Particularly, quantitative PET imaging showed about 5-fold higher amounts of 64Cu-ALT-803 in the liver compared with that of 64Cu-IL15 at 1.5 to 6 hours after injection. In addition, the spleen, lungs, and lymph nodes had elevated uptake of ALT-803 (i.e., >4%ID/g) 6 hours after injection and lymph node localization persisted without diminishing for at least 70 hours after treatment. Thus, ALT-803 is distributed to and retained in the lymphoid organs to a greater extent than IL15, which is consistent with its more potent immunostimulatory activity in vivo.

In mice, maximal immune cell stimulation occurs 4 days after ALT-803 dosing, suggesting that once- or twice-weekly administration of ALT-803 may be suitable for initial clinical testing (23, 31). Dose-ranging studies of four weekly ALT-803 i.v. injections in C57BL/6 mice showed progressively increased immunologic effects from 0.1 mg/kg to 4.0 mg/kg. At the intermediate 1.0-mg/kg ALT-803 dose, parallel increases in mouse spleen weight and peripheral blood cell counts for lymphocytes, neutrophils, and monocytes were observed. Consistent with the pattern of ALT-803 tissue biodistribution, immune cell infiltration and hyperplasia were seen in the spleen, liver, thymus, kidneys, lungs, and lymph nodes. Treatment with 4.0 mg/kg ALT-803 resulted in mortality of about half of the mice 4 to 6 days after the initial dose. Similar mortality due to NK-cell–mediated acute hepatocellular injury was recently reported in mice receiving four daily 11-μg/dose (~0.5 mg/kg) injections of preassociated murine IL15:IL15Rα/Fc complex (41). However, such toxicity was not apparent in 1.0 mg/kg ALT-803–treated mice, as serum liver enzyme concentrations were comparable with those in controls. Overall, the 1.0-mg/kg ALT-803 dose showed tolerable but significant immune cell stimulation in mice when given as a weekly treatment. In comparison, a single injection of 0.05 mg/kg ALT-803 had potent antitumor activity in 5T33 myeloma–bearing C57BL/6 mice, suggesting that the therapeutic window of ALT-803 spans a more than 20-fold dose range (31).

Consistent with these observations, multidose ALT-803 administration to cynomolgus monkeys resulted in dose-dependent increases in peripheral blood lymphocytes, primarily NK and CD8+ and CD4+ memory T cells, as well as lymphocytic infiltration in the liver, kidneys, and lungs. Modest or no treatment-dependent effects were seen with other blood cell types. These results contrast with previous studies of IL15 administration to macaques and rhesus monkeys where the major toxicity reported was grade 3/4 transient neutropenia (17, 29). Evaluation of peripheral tissues from IL15–treated monkeys suggests that this event was due to neutrophil migration from the blood to tissues mediated by an IL15-initiated IL18-dependent signaling cascade (17). Consistent with these findings, grade 3/4 neutropenia adverse effects have been reported for 5 of 9 cancer patients receiving daily i.v. IL15 infusions at 0.3 μg/kg (43). The lack of ALT-803 effects on peripheral blood neutrophil counts in cynomolgus monkeys may reflect differential sensitivity of these cells to ALT-803 compared with IL15, a hypothesis that will be further evaluated in in vitro and human clinical studies of ALT-803.

Preclinical studies in animal models have been traditionally used to predict toxicities and determine the initial dose level of...
immunotherapeutics for clinical trials. However, recent evidence showed that these animal models alone may not be sufficient to predict the safety profiles of immunomodulatory molecules in humans (35, 44). An approach using an in vitro assessment of human immune cells was proposed to supplement the animal models and was employed in this study to further evaluate ALT-803 effects on cytokine release. Comparative studies of human and mouse lymphocytes showed that ALT-803 provided dose-dependent stimulation of IFNγ release and immune cell proliferation in vitro. These results were consistent with the ability of ALT-803 to stimulate CD8⁺ T-cell proliferation and IFNγ release in mice bearing myeloma...
tumors (31). Because the antitumor activity of ALT-803 in this mouse tumor model was dependent on both CD8+ T cells and IFNγ (31), these responses may be important indicators of potential clinical activity in cancer patients receiving ALT-803 treatment. We also found that ALT-803 at <0.1 nmol/L could induce activation and cytokotoxicity marker expression on human NK cells and T cells in vitro. However, unlike the mAb to CD3, ALT-803 did not stimulate release of TNFα, IL6, IL5, IL4, or IL2 was observed after multidose 0.1-mg/kg ALT-803 administration to cynomolgus monkeys.

In dose-ranging toxicity studies, no adverse effects were observed in mice treated with 0.1 mg/kg ALT-803 or in cynomolgus monkeys treated with 0.03 mg/kg ALT-803, suggesting that human dosing at approximately 10 μg/kg would be justified based on standard allometric scaling approaches (45). In addition, administration of 5 μg/kg ALT-803 is anticipated to achieve a maximum serum concentration of >1 nmol/L (based on the NHP PK studies), which is sufficient to induce significant human T-cell and NK-cell proliferation and activation in vitro. Based on these results, an initial dose of ALT-803 at 1.0 to 5.0 μg/kg/dose in human clinical trials is expected to provide immune cell stimulation without overt toxicologic effects. This range is consistent with that of the recently published dose-escalation study of IL15 administered daily in patients with metastatic malignancies (43).

In summary, the results of the present study support phase I clinical evaluation of weekly dosing of ALT-803, which has been initiated under FDA-approved clinical protocols for treatment of patients with advanced solid tumors (ClinicalTrials.gov: NCT01946789), multiple myeloma for treatment of patients with advanced solid tumors (NCT01885897), and relapsed hematologic malignancy following allogeneic stem cell transplant (NCT01885897).

Disclosure of Potential Conflicts of Interest

P.R. Rhode, J.O. Egan, W. Xu, X. Chen, S. Liu, X. Zhu, J. Wen, L. You, L. Kong, A.C. Edwards, K. Han, S. Alter, E.K. Jeng, and H.C. Wong have ownership interest (including patents) in Altor BioScience Corp. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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