

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

Peter R. Rhode<sup>1</sup>, Jack O. Egan<sup>1</sup>, Wenxin Xu<sup>1</sup>, Hao Hong<sup>2</sup>, Gabriela M. Webb<sup>3,4</sup>, Xiaoyue Chen<sup>1</sup>, Bai Liu<sup>1</sup>, Xiaoyun Zhu<sup>1</sup>, Jinghai Wen<sup>1</sup>, Lijing You<sup>1</sup>, Lin Kong<sup>1</sup>, Ana C. Edwards<sup>1</sup>, Kaiping Han<sup>1</sup>, Sixiang Shi<sup>2</sup>, Sarah Alter<sup>1</sup>, Jonah B. Sacha<sup>3,4</sup>, Emily K. Jeng<sup>1</sup>, Weibo Cai<sup>2</sup>, and Hing C. Wong<sup>1</sup>

## Abstract

IL15, a potent stimulant of CD8<sup>+</sup> 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  $\alpha$ Su/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<sup>+</sup>, and CD8<sup>+</sup> memory T-cell subsets. *In vitro* studies demonstrated ALT-803-mediated stimulation of mouse and human immune cell proliferation and IFN $\gamma$  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); 49–60. ©2015 AACR.

## Introduction

Therapeutic approaches using common  $\gamma$ -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  $\gamma$ -chain cytokines have focused on stimulating effector immune cells against tumors without inducing Tregs or IL2-associated capillary leak syndrome (7, 8).

<sup>1</sup>Altor BioScience Corporation, Miramar, Florida. <sup>2</sup>Departments of Radiology and Medical Physics, University of Wisconsin, Madison, Wisconsin. <sup>3</sup>Vaccine & Gene Therapy Institute, Oregon Health & Science University, Portland, Oregon. <sup>4</sup>Oregon National Primate Research Center, Oregon Health & Science University, Portland, Oregon.

**Note:** Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

P.R. Rhode and J.O. Egan contributed equally to this article.

**Corresponding Author:** Hing C. Wong, Altor BioScience Corporation, 2810 North Commerce Parkway, Miramar, FL 33185. Phone: 954-443-8600; Fax: 954-443-8610; E-mail: [hingwong@altorbioscience.com](mailto:hingwong@altorbioscience.com)

**doi:** 10.1158/2326-6066.CIR-15-0093-T

©2015 American Association for Cancer Research.

IL15, like IL2, has the ability to stimulate T-cell and natural killer (NK)-cell responses through the IL2 receptor  $\beta$ -common  $\gamma$  chain (IL2R $\beta\gamma_c$ ) complex (9, 10). However, these cytokines exhibit functionally distinct activities due to differential interactions with their unique  $\alpha$  receptor subunits. IL2 is produced as a soluble protein that binds to immune cells expressing IL2R $\beta\gamma_c$  or IL2R $\alpha\beta\gamma_c$  complexes. In contrast, IL15 and its IL15R $\alpha$  chain are coexpressed by monocytes/macrophages and dendritic cells and subsequently displayed as a cell surface IL15:IL15R $\alpha$  complex, which is trans-presented to neighboring immune cells expressing IL2R $\beta\gamma_c$  (10, 11). Due to these differences, unlike IL2, IL15 does not support maintenance of Tregs and, rather than inducing apoptosis of activated CD8<sup>+</sup> T cells, provides antiapoptotic 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 IL15R $\alpha$  is considered an integral part of the active cytokine complex, various therapeutic strategies are being explored using soluble preassociated IL15:IL15R $\alpha$  complexes (18–23). For example, IL15:IL15R $\alpha$ /Fc (i.e., soluble IL15R $\alpha$  linked to an Ig Fc domain) was found to exhibit up to 50-fold greater activity than free IL15 in stimulating mouse CD8<sup>+</sup> T-cell proliferation (19). In addition, IL15:IL15R $\alpha$ /Fc increased efficacy

compared with IL15 in various mouse tumor models (18, 21, 24, 25). These effects may be partially due to an approximately 150-fold increase in IL2R $\beta\gamma_c$  binding affinity for the IL15:IL15R $\alpha$  complex compared with free IL15 (26). Moreover, the IL15:IL15R $\alpha$ /Fc complex greatly enhanced IL15 half-life and bioavailability *in vivo* (18), suggesting advantages of IL15:IL15R $\alpha$ /Fc over free IL15 as an immunotherapeutic (27). Although nonclinical studies demonstrated the pharmacodynamic (PD) and safety profiles of IL15 in NHPs (17, 28–30), similar studies have not yet been reported to support clinical development of IL15:IL15R $\alpha$  complexes.

To advance IL15:IL15R $\alpha$ -based therapies into clinical testing, we have created a complex (referred to as ALT-803) between a novel human IL15 superagonist variant (IL15N72D) and a human IL15R $\alpha$  sushi domain–Fc fusion protein (22, 23). We have previously shown that the IL15N72D mutation increases IL2R $\beta\gamma_c$  binding and IL15 biologic activity by approximately 5-fold (22). This IL15 superagonist fusion protein complex, ALT-803 (IL15N72D:IL15R $\alpha$ Su/Fc), exhibited superior immunostimulatory activity, a prolonged serum half-life, and more potent antimyeloma activity compared with IL15 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 IL15 in mice bearing solid tumors. In addition, ALT-803 was retained in lymphoid organs to a greater extent than IL15, 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 effects 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 IL15 (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 Altor 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 (OneBlood; 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% CO<sub>2</sub>.

### 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; Macrocylics), radiolabeling with <sup>64</sup>CuCl<sub>2</sub> (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 <sup>64</sup>Cu-labeled ALT-803 or IL15 (~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- $\mu$ g/kg i.v. dose, respectively. For proliferation assays, human PBMCs and mouse CD3<sup>+</sup> T cells enriched from splenocytes (CD3<sup>+</sup> 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 IL15 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 ( $5 \times 10^5$ /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 ( $2 \times 10^5$ /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 cynomolgus monkeys

A study was performed under Good Laboratory Practice guidelines to evaluate the effects of multidose administration of ALT-803 in cynomolgus 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 ocular function. Blood was collected for hematology, chemistry, coagulation, and immune cell analysis before dosing and on SD5, 26, and 36. Immunogenicity 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

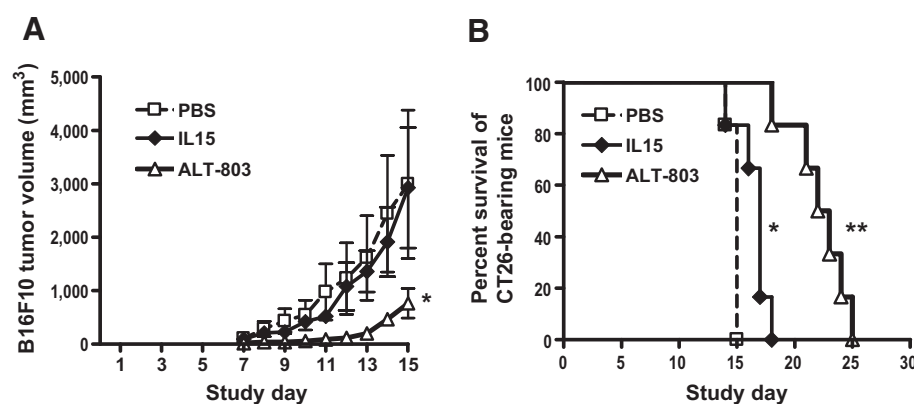
Data are expressed as the mean  $\pm$  SE. Survival data were analyzed using the log-rank test and Kaplan–Meier method. Comparisons of continuous variables were done using Student *t* tests or ANOVA (two-tailed; GraphPad Prism Version 4.03). *P* values  $< 0.05$  were considered statistically significant.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

## Results

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

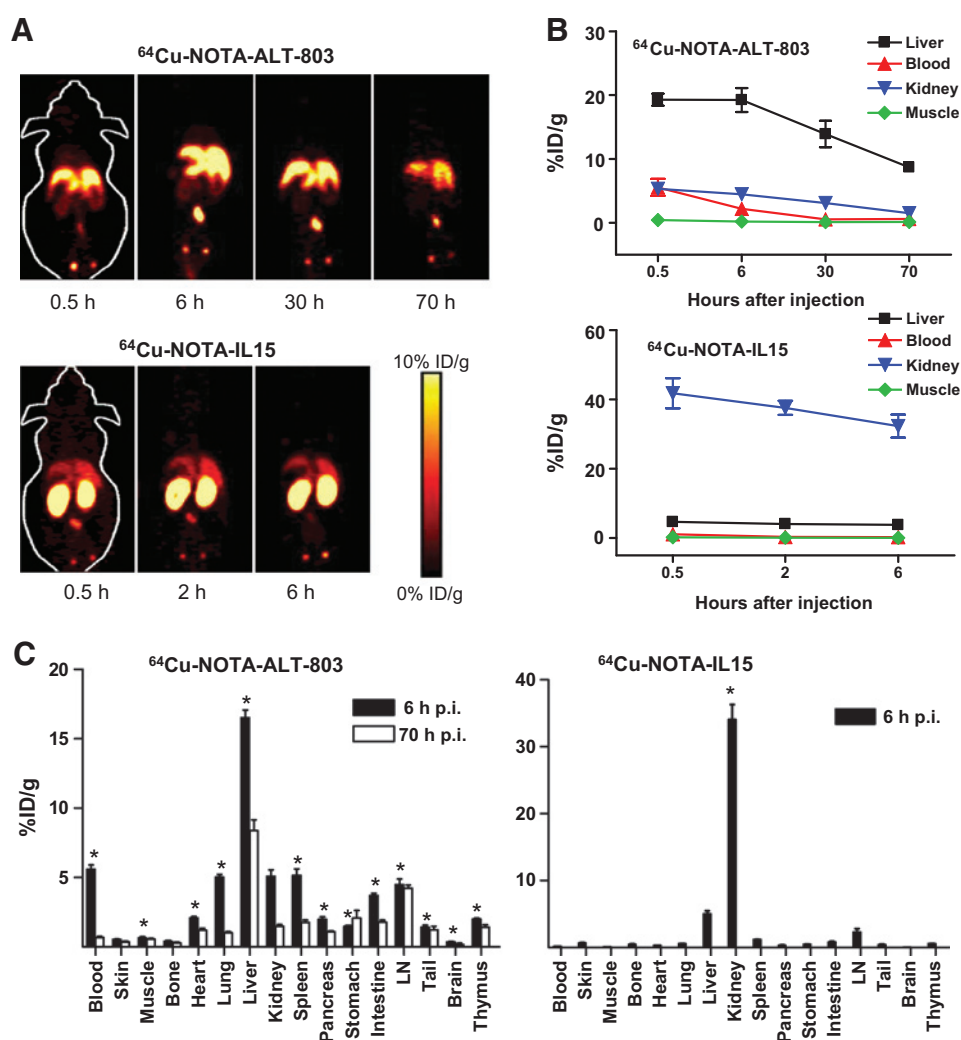
In mice bearing myeloma tumors, single-dose treatment of ALT-803 was found to provide significant reduction of tumor burden compared with an equivalent dose of IL15 (36). To extend these findings to solid tumors, we compared the antitumor activity of ALT-803 and IL15 against s.c. B16F10 melanoma tumors 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:IL15R $\alpha$  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)



**Figure 1.**

Comparative antitumor activity of ALT-803 and IL15 in immunocompetent mice bearing solid tumors. A, changes in tumor volume of subcutaneous B16F10 melanoma tumors in C57BL/6 mice treated with i.v. PBS, 0.2 mg/kg ALT-803, or 0.06 mg/kg IL15 (IL15 molar equivalent dose of 0.2 mg/kg ALT-803) on days 1 and 8 after tumor injection. Data points are expressed as mean  $\pm$  SE ( $n = 5$  mice/group). \*,  $P < 0.05$  comparing ALT-803 vs. IL15. B, survival curves of BALB/c mice ( $n = 6$ /group) injected i.v. with CT26 colon carcinoma cells and subsequently treated with i.v. 0.25 mg/kg IL15 on study days 1 to 5, and 8 to 12, or with 0.2 mg/kg ALT-803, or PBS on study days 1, 4, 8, and 11. \*,  $P < 0.05$  comparing IL15 vs. PBS; \*\*,  $P < 0.01$  comparing ALT-803 vs. IL15 or PBS. The results shown for both tumor models are representative of at least three independent experiments.

**Figure 2.**

Quantitative PET scan imaging and biodistribution of ALT-803 and IL15 in C57BL/6 mice. A, serial coronal PET images at different time points after injection of  $^{64}\text{Cu}$ -ALT-803 (0.5, 6, 30, and 70 hours) or  $^{64}\text{Cu}$ -IL15 (0.5, 2, and 6 hours). B, time-activity curves of quantitative PET imaging of the liver, kidney, and muscle upon i.v. injection of  $^{64}\text{Cu}$ -ALT-803 and  $^{64}\text{Cu}$ -IL15. Data are expressed as mean percentages + SE of %ID/g ( $n = 4$ ). C, tissue biodistribution of  $^{64}\text{Cu}$ -ALT-803 (left) and  $^{64}\text{Cu}$ -IL15 (right) in C57BL/6 mice at 6 hours and 70 hours after injection. Data are expressed as mean percentages + SE of %ID/g ( $n = 4$ /group).

\*, significantly greater distribution of ALT-803 vs. IL15 (left) or IL15 vs. ALT-803 (right) at 6 hours after injection ( $P < 0.05$ ). The results shown are representative of two independent experiments. LN, lymph node; p.i., post injection.

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 compared with IL15 observed *in vivo* and in our previous efficacy studies in hematologic tumor models (23, 31).

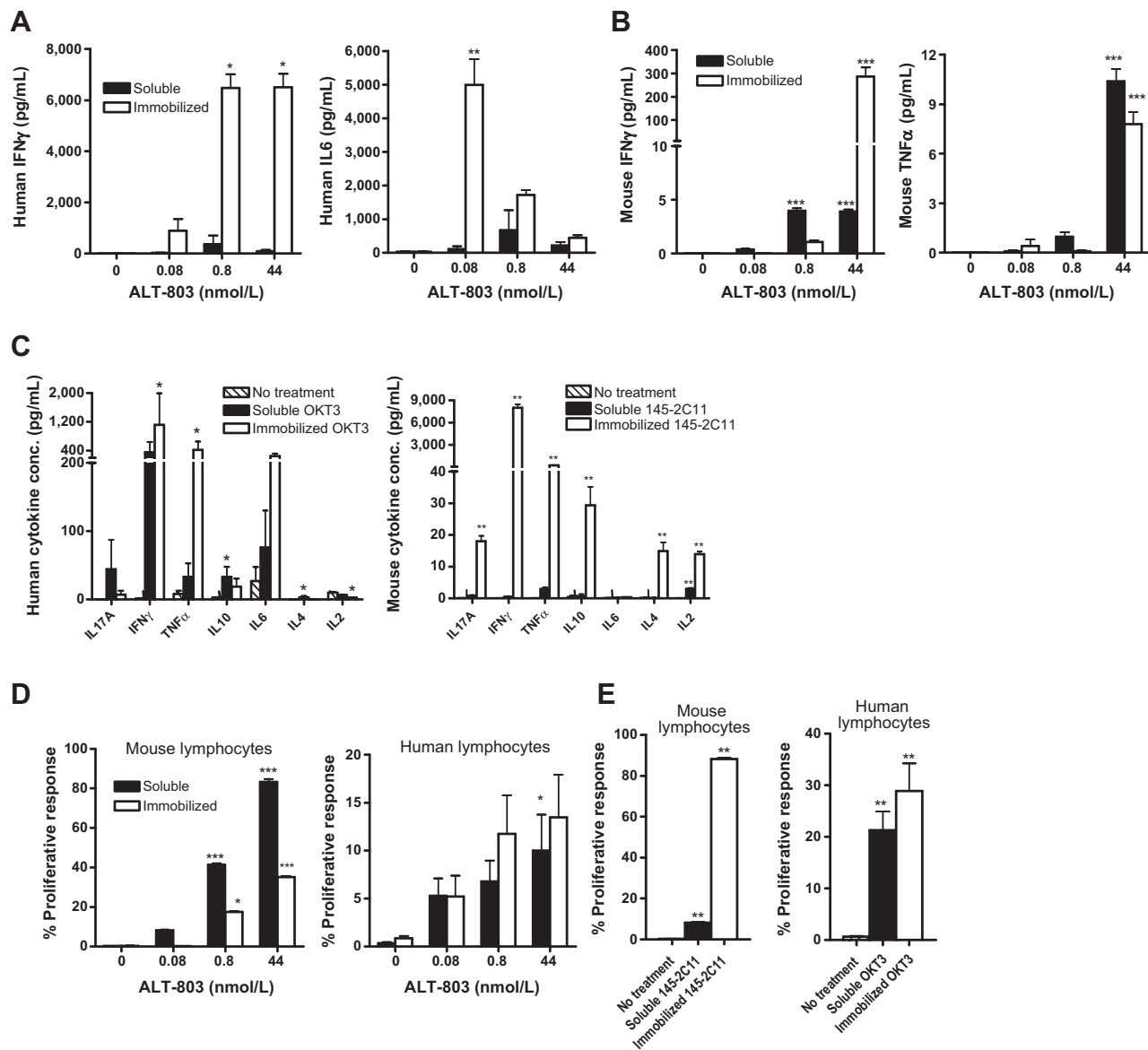
#### Biodistribution of ALT-803 in mice

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  $^{64}\text{Cu}$ -labeled ALT-803 or IL15. Serial noninvasive PET scans of C57BL/6 mice at different times after injection showed that  $^{64}\text{Cu}$ -IL15 was rapidly cleared via the renal pathway, consistent with previous reports (38), whereas ALT-803 was cleared primar-

ily 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%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 IFN $\gamma$  release by human PBMCs. Soluble IL6 was also



**Figure 3.** 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 IFN $\gamma$  and IL6 were observed and are plotted, whereas no significant differences in the levels of human TNF $\alpha$ , IL4, IL10, or IL17A were found among the treatment groups. Bars represent mean cytokine concentration  $\pm$  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 IFN $\gamma$  and TNF $\alpha$  levels were significantly induced by ALT-803, but there were no treatment-mediated changes in the levels of IL6, IL2, IL10, IL4, and IL17A. Bars represent mean cytokine concentration  $\pm$  SE. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$  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  $\pm$  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 TNF $\alpha$ , 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 IFN $\gamma$ , TNF $\alpha$ , IL10, and IL4 (Fig. 3C).

Compared with human immune cells, mouse splenocytes exhibited a similar but less intense response for IFN $\gamma$  release following incubation with ALT-803 (Fig. 3B). ALT-803 also induced TNF $\alpha$  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 IFN $\gamma$  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 IFN $\gamma$  release (Fig. 3A and B), suggesting IL2R $\beta\gamma_c$  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 CD8 $^+$  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 IL15R $\alpha$ /IL2R $\beta\gamma_c$  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 granzyme 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*.

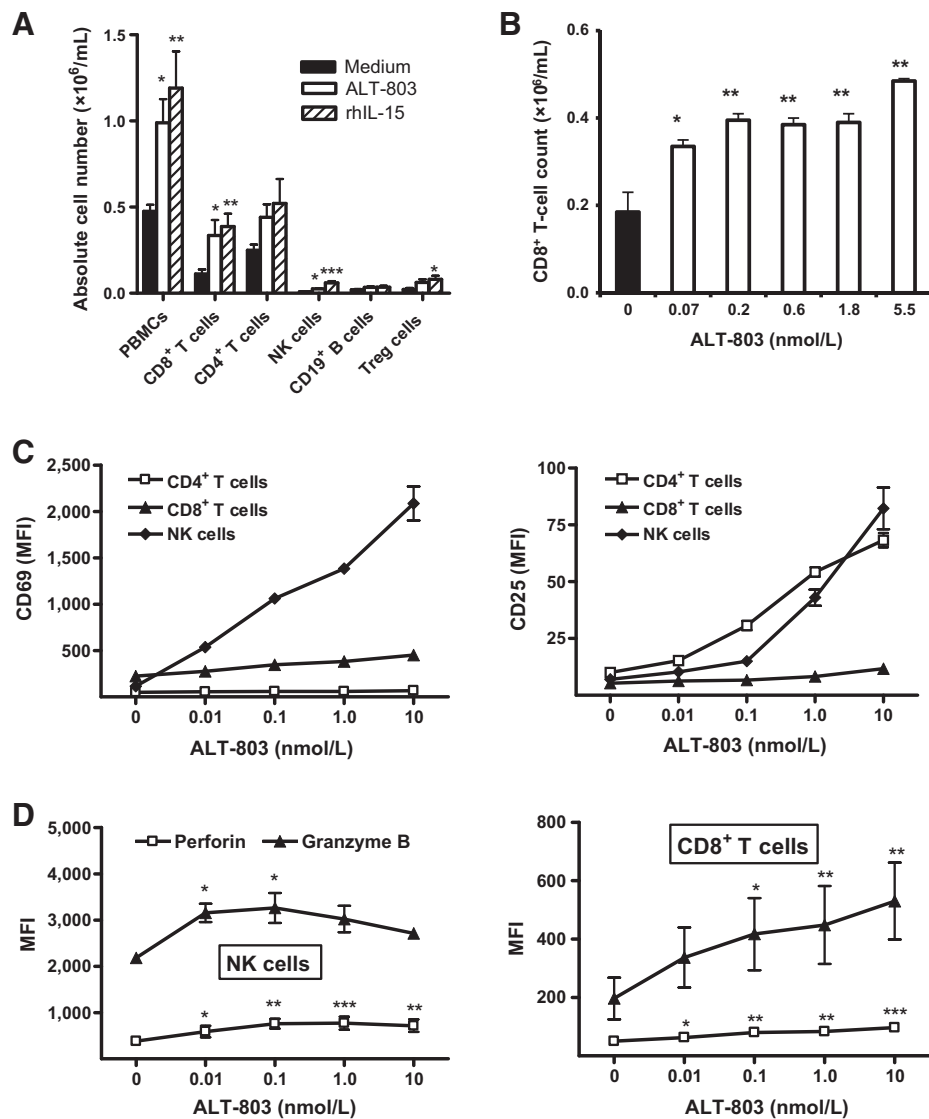
#### Toxicity of ALT-803 in mice

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 IL15R $\alpha$ /IL2R $\beta\gamma_c$  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 multidose 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 ( $C_{max}$ ) of 30 nmol/L for 0.1 mg/kg ALT-803 was consistent with full recovery of the administered dose, whereas  $C_{max}$  and  $AUC_{INF}$  values indicated approximately 30% less recovery at the 0.03-mg/kg dose. However, even at the low dose level, the  $C_{max}$  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).



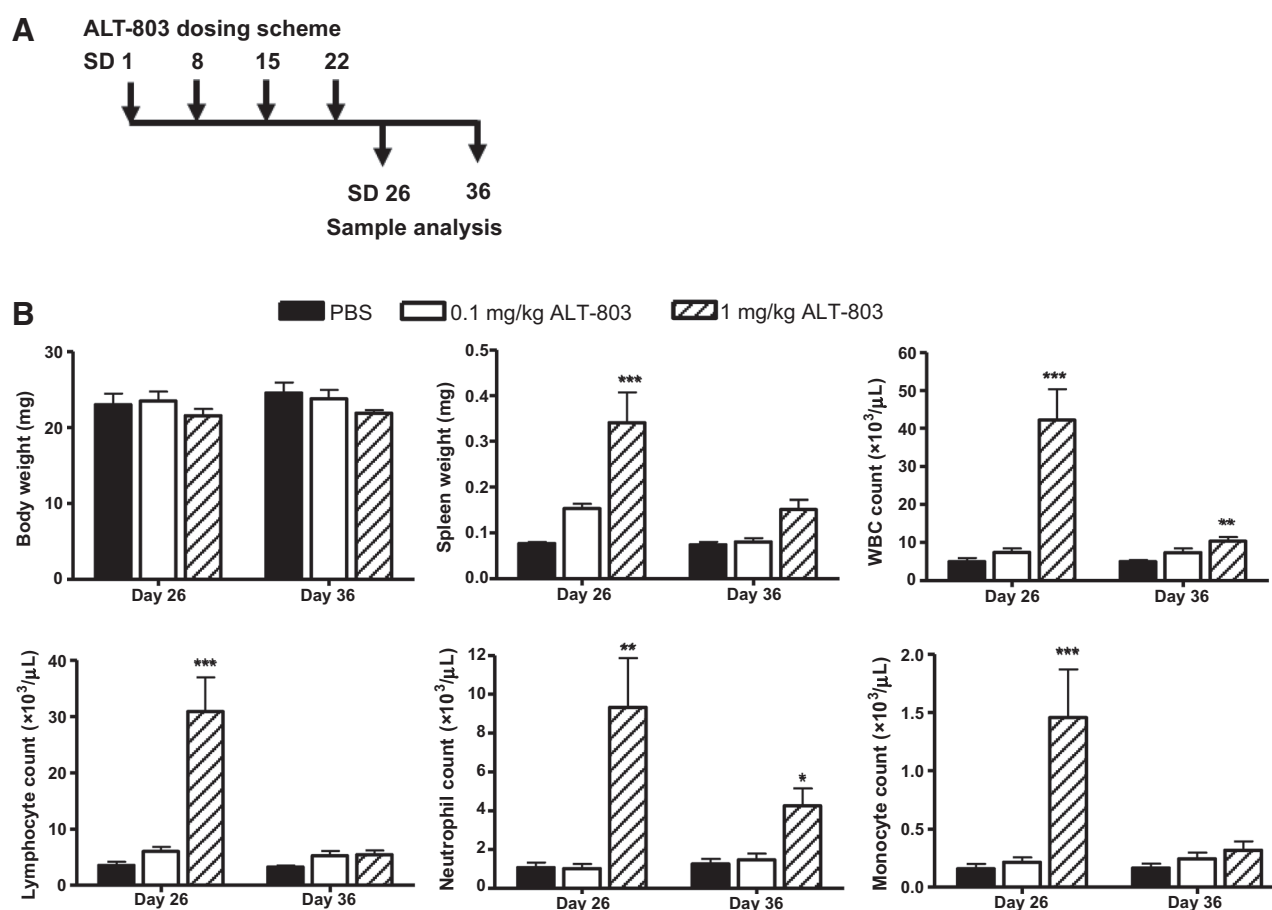
**Figure 4.**

ALT-803 stimulates proliferation and activation of human NK cells and T cells *in vitro*. A, human PBMCs ( $n = 7$  normal donors) were cultured for 7 days in media alone or media containing 0.5 nmol/L ALT-803 or IL15. At the end of the incubation period, changes in absolute cell counts were determined following staining with fluorochrome-conjugated antibodies against CD8a, CD4, CD335, CD19, and CD4/CD25/FOXP3. Bars represent mean  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  comparing ALT-803 vs. media control. B, ALT-803 ranging from 0.07 to 5.5 nmol/L was added to human PBMC cultures ( $n = 2$  donors). After 7 days, changes in CD8<sup>+</sup> T-cell counts were assessed. Bars represent mean  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  comparing ALT-803 (open bars) vs. media control (closed bar). C, representative normal donor PBMCs were cultured for 5 days in the indicated concentrations of ALT-803. Cells were then harvested, and expression of the cell surface activation markers CD25 and CD69 was determined on CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and NK cells by flow cytometry. The symbols represent mean fluorescence intensity (MFI)  $\pm$  SE of triplicates. Similar treatment-related changes were obtained with other human donor PBMCs though control levels of CD25 and CD69 varied between individuals. D, human PBMCs ( $n = 2$  donors) were cultured for 5 days in the indicated concentrations of ALT-803. Following stimulation, cells were harvested and analyzed for intracellular perforin (open squares) and granzyme B (closed triangles) expression in NK cells and CD8<sup>+</sup> T cells. The symbols represent the MFI  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  comparing ALT-803 vs. media control. In each case, the results shown are representative of at least two independent experiments.

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).



**Figure 5.**

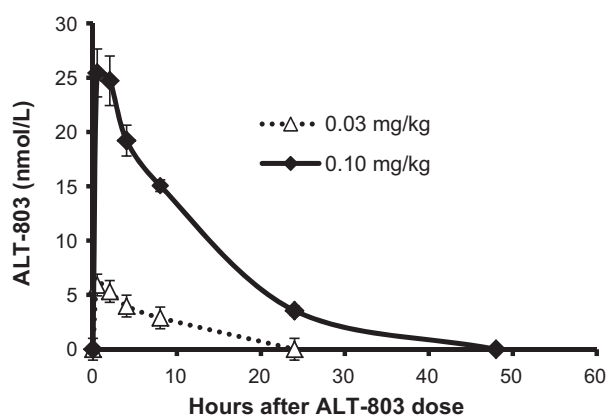
ALT-803 treatment changes spleen weight and peripheral blood leukocyte counts in C57BL/6 mice. A, study design schema. C57BL/6 mice ( $n = 10$  males and 10 females/group) were treated with i.v. PBS, 0.1 mg/kg ALT-803, or 1.0 mg/kg ALT-803 once weekly for 4 weeks. Four days after the last injection (day 26), clinical assessments including physical examination, blood chemistry, hematology, gross necropsy, body and organ weight measurements, and histopathology were performed on 5 females and 5 males per group. Similar assessments were performed on the remaining 5 females and 5 males per group 14 days after the last treatment (day 36). B, there were no changes in body weights with treatment, whereas a dose-dependent increase in spleen weight was observed. Dose-dependent changes in absolute numbers of WBCs, lymphocytes, neutrophils, and monocytes were plotted. Bars represent mean  $\pm$  SE of combined data from male and female mice. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  comparing ALT-803 vs. PBS. Similar results were obtained in a second independent study of C57BL/6 mice treated weekly for 4 weeks with 0.1 or 1.0 mg/kg ALT-803 or PBS and assessed 4 days ( $n = 10$  mice/sex/group) or 4 weeks after the last injection ( $n = 5$  mice/sex/group; data not shown).

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<sup>+</sup> and CD8<sup>+</sup> T-cell and CD16<sup>+</sup> NK-cell counts over the treatment course (Fig. 7C). Of the CD8<sup>+</sup> T cells, effector memory (EM) and to a lesser extent central memory (CM) T-cell counts increased shortly after treatment initiation, whereas naïve CD8<sup>+</sup> T-cell counts were elevated compared with predose counts as the 4-week treatment course proceeded. Similarly, naïve, CM, and EM CD4<sup>+</sup> T-cell counts were elevated after the first dose of ALT-803, resulting in an approximately 3-fold increase in blood CD4<sup>+</sup> T cells. The changes in blood lymphocyte counts were associated with increased expression of the proliferation marker Ki-67 in CD16<sup>+</sup> NK cells and memory CD8<sup>+</sup> and CD4<sup>+</sup> T cells (Fig. 7D), indicating the ALT-803-mediated effects are due to increased cell proliferation rather than merely redistribution. Assessment of serum





**Figure 6.**

PK of ALT-803 in cynomolgus monkeys. Serum concentrations of ALT-803 were evaluated in blood samples obtained from cynomolgus monkeys ( $n = 5/\text{sex}/\text{group}$ ) before dosing and at 0.5, 2, 4, 8, 24, and 48 hours following a single i.v. dose of 0.03 mg/kg or 1.0 mg/kg ALT-803. The symbols represent the mean concentration  $\pm$  SE of ALT-803 in the serum of each animal analyzed independently.

samples collected in this study indicated that there was no significant induction of IFN $\gamma$ , TNF $\alpha$ , 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 twice weekly at up to 0.1 mg/kg or daily at 10 to 50  $\mu\text{g}/\text{kg}$  (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:IL15R $\alpha$ Su/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).

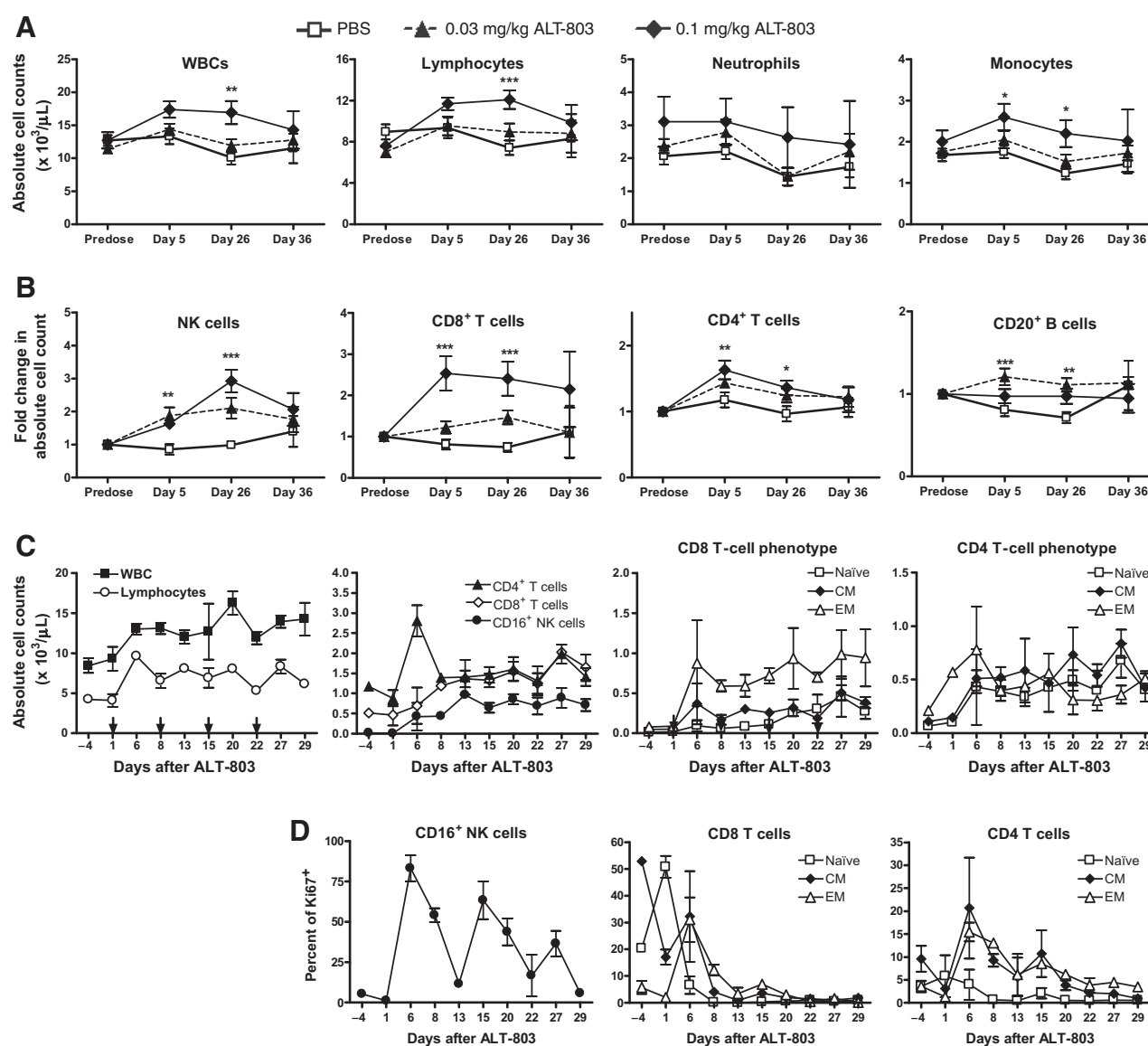
The results of biodistribution experiments reported here confirm the hypothesis that the pharmacologic properties of ALT-803 are highly differentiated from IL15. Consistent with previous reports using  $^{125}\text{I}$ -labeled IL15 (38),  $^{64}\text{Cu}$ -IL15 was very rapidly cleared from circulation with the kidney as the major site of IL15 accumulation.  $^{64}\text{Cu}$ -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  $^{64}\text{Cu}$ -labeled 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  $^{64}\text{Cu}$ -ALT-803 in the liver compared with that of  $^{64}\text{Cu}$ -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- $\mu\text{g}/\text{dose}$  ( $\sim 0.5$  mg/kg) injections of preassociated murine IL15:IL15R $\alpha$ /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  $\mu\text{g}/\text{kg}$  (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



**Figure 7.**

ALT-803 administration increases peripheral blood cell counts of lymphocyte subsets in cynomolgus monkeys. A and B, cynomolgus monkeys ( $n = 5/\text{sex}/\text{group}$ ) were injected i.v. (days 1, 8, 15, and 22) with PBS, 0.03 mg/kg ALT-803, or 0.1 mg/kg ALT-803 once weekly for 4 consecutive weeks. Blood was collected before dosing, 4 days after the first and fourth dose (i.e., days 5 and 26, respectively), and 14 days after completion of treatment (i.e., day 36). Blood samples were evaluated for changes in absolute cell numbers for leukocyte (A) and lymphocyte subsets (B). The symbols represent the mean  $\pm$  SE of combined data from male and female animals. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  comparing ALT-803 vs. PBS. C and D, in a second independent study, cynomolgus monkeys ( $n = 2$ ) were injected i.v. at days 1, 8, 15, and 22 (indicated by arrows) with 0.1 mg/kg ALT-803 once weekly for 4 consecutive weeks. Blood was taken on days -4, 1 (predosing), 6, 8 (predosing), 13, 15 (predosing), 20, 22 (predosing), 27, and 29. Cells were stained with antibodies to CD3, CD8, CD45, CD20, CD14, CD16, and CD56 for NK cells, gating on CD14<sup>-</sup>, CD45<sup>+</sup>, CD3<sup>-</sup>, CD20<sup>-</sup>, CD16<sup>+</sup>, and CD56<sup>-</sup> cells. For CD4<sup>+</sup> and CD8<sup>+</sup> T cells, antibodies to CD3, CD4, and CD8 were used as well as CD28 and CD95 for staining of memory subsets (46). Absolute cell counts were calculated based on the percentage of the particular cell subset and the WBC count. C, absolute blood cell counts during the ALT-803 treatment course were determined for WBCs, lymphocytes, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NK cells, and naïve, CM, and EM phenotypes for each of the CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets. D, cell proliferation was determined as percentage of Ki67<sup>+</sup>-positive cells based on the lymphocyte subsets described in C. The symbols represent the mean  $\pm$  range.

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 IFN $\gamma$  release and immune cell proliferation *in vitro*. These results were consistent with the ability of ALT-803 to stimulate CD8<sup>+</sup> T-cell proliferation and IFN $\gamma$  release in mice bearing myeloma

tumors (31). Because the antitumor activity of ALT-803 in this mouse tumor model was dependent on both CD8<sup>+</sup> T cells and IFN $\gamma$  (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 cytotoxicity marker expression on human NK cells and T cells *in vitro*. However, unlike the mAb to CD3, ALT-803 did not stimulate release of TNF $\alpha$ , IL4, or IL10 by human PBMCs, suggesting that ALT-803 will not trigger a cytokine storm typical of immunostimulatory CD3 or CD28 agonists (35, 44). Supporting this hypothesis, no induction of serum IFN $\gamma$ , TNF $\alpha$ , IL6, IL5, IL4, or IL2 was observed after multidose 0.1-mg/kg ALT-803 administration to cynomolgus monkeys.

In dose-ranging toxicology 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  $\mu$ g/kg would be justified based on standard allometric scaling approaches (45). In addition, administration of 5  $\mu$ g/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  $\mu$ g/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 (NCT02099539), and relapsed hematologic malignancy following allogeneic stem cell transplant (NCT01885897).

## References

- Atkins MB, Lotze MT, Dutcher JP, Fisher RI, Weiss G, Margolin K, et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol* 1999;17:2105–16.
- Atkins MB, Regan M, McDermott D. Update on the role of interleukin 2 and other cytokines in the treatment of patients with stage IV renal carcinoma. *Clin Cancer Res* 2004;10:6342S–6S.
- Tarhini AA, Agarwala SS. Interleukin-2 for the treatment of melanoma. *Curr Opin Investig Drugs* 2005;6:1234–9.
- Ahmadzadeh M, Rosenberg SA. IL-2 administration increases CD4<sup>+</sup> CD25<sup>+</sup> (hi) Foxp3<sup>+</sup> regulatory T cells in cancer patients. *Blood* 2006;107:2409–14.
- Cesana GC, DeRaffele G, Cohen S, Moroziewicz D, Mitcham J, Stoutenburg J, et al. Characterization of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. *J Clin Oncol* 2006;24:1169–77.
- Sim GC, Martin-Orozco N, Jin L, Yang Y, Wu S, Washington E, et al. IL-2 therapy promotes suppressive ICOS<sup>+</sup> Treg expansion in melanoma patients. *J Clin Invest* 2014;124:99–110.
- Cheever MA. Twelve immunotherapy drugs that could cure cancers. *Immunol Rev* 2008;222:357–68.
- Sim GC, Radvanyi L. The IL-2 cytokine family in cancer immunotherapy. *Cytokine Growth Factor Rev* 2014;25:377–90.
- Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* 2006;6:595–601.
- Waldmann T, Tagaya Y, Bamford R. Interleukin-2, interleukin-15, and their receptors. *Int Rev Immunol* 1998;16:205–26.
- Dubois S, Mariner J, Waldmann TA, Tagaya Y. IL-15R $\alpha$  recycles and presents IL-15 *In trans* to neighboring cells. *Immunity* 2002;17:537–47.
- Sanjabi S, Mosaheb MM, Flavell RA. Opposing effects of TGF- $\beta$  and IL-15 cytokines control the number of short-lived effector CD8<sup>+</sup> T cells. *Immunity* 2009;31:131–44.
- Yoon SR, Kim TD, Choi I. Understanding of molecular mechanisms in natural killer cell therapy. *Exp Mol Med* 2015;47:e141.
- Marcais A, Cherfils-Vicini J, Viant C, Degouve S, Viel S, Fenis A, et al. The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells. *Nat Immunol* 2014;15:749–57.
- Yang M, Li D, Chang Z, Yang Z, Tian Z, Dong Z. PDK1 orchestrates early NK cell development through induction of E4BP4 expression and maintenance of IL-15 responsiveness. *J Exp Med* 2015;212:253–65.
- Munger W, DeJoy SQ, Jeyaseelan R Sr., Torley LW, Grabstein KH, Eisenmann J, et al. Studies evaluating the antitumor activity and toxicity of interleukin-15, a new T cell growth factor: comparison with interleukin-2. *Cell Immunol* 1995;165:289–93.
- Waldmann TA, Lugli E, Roederer M, Perera LP, Smedley JV, Macalister RP, et al. Safety (toxicity), pharmacokinetics, immunogenicity, and impact on elements of the normal immune system of recombinant human IL-15 in rhesus macaques. *Blood* 2011;117:4787–95.

## Disclosure of Potential Conflicts of Interest

P.R. Rhode, J.O. Egan, W. Xu, X. Chen, B. 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

**Conception and design:** P.R. Rhode, W. Xu, B. Liu, X. Zhu, J. Wen, L. You, W. Cai, H.C. Wong

**Development of methodology:** J.O. Egan, W. Xu, B. Liu, X. Zhu, J. Wen, L. You, K. Han

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J.O. Egan, W. Xu, H. Hong, G.M. Webb, X. Chen, B. Liu, X. Zhu, J. Wen, L. You, L. Kong, A.C. Edwards, S. Shi, J.B. Sacha, W. Cai

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** P.R. Rhode, J.O. Egan, W. Xu, H. Hong, B. Liu, X. Zhu, J. Wen, L. You, S. Shi, W. Cai, H.C. Wong

**Writing, review, and/or revision of the manuscript:** P.R. Rhode, J.O. Egan, W. Xu, H. Hong, G.M. Webb, B. Liu, X. Zhu, J. Wen, L. You, A.C. Edwards, S. Alter, J.B. Sacha, E.K. Jeng, W. Cai, H.C. Wong

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** W. Xu, S. Alter, W. Cai, E.K. Jeng, H.C. Wong

**Study supervision:** P.R. Rhode, W. Xu, X. Zhu, W. Cai, H.C. Wong

## Acknowledgments

The authors thank Norman H. Altman, V.M.D., and Carolyn Cray, Ph.D., University of Miami, Division of Comparative Pathology, for assistance with the mouse toxicity studies.

## Grant Support

This study was supported by NCI grants 2R44CA156740-02 and 2R44CA167925-02 (to H.C. Wong), NCI grants 1R01CA169365 and P30CA014520, the University of Wisconsin-Madison, and the American Cancer Society 125246-RSG-13-099-01-CCE (to W. Cai).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 8, 2015; revised August 21, 2015; accepted September 3, 2015; published OnlineFirst October 28, 2015.

18. Stoklasek TA, Schluns KS, Lefrancois L. Combined IL-15/IL-15Ralpha immunotherapy maximizes IL-15 activity in vivo. *J Immunol* 2006; 177:6072–80.
19. Rubinstein MP, Kovar M, Purton JF, Cho JH, Boyman O, Surh CD, et al. Converting IL-15 to a superagonist by binding to soluble IL-15Ra. *Proc Natl Acad Sci U S A* 2006;103:9166–71.
20. Mortier E, Quemener A, Vusio P, Lorenzen I, Boublik Y, Grotzinger J, et al. Soluble interleukin-15 receptor alpha (IL-15R alpha)-sushi as a selective and potent agonist of IL-15 action through IL-15R beta/gamma. Hyperagonist IL-15 x IL-15R alpha fusion proteins. *J Biol Chem* 2006;281:1612–9.
21. Dubois S, Patel HJ, Zhang M, Waldmann TA, Muller JR. Preassociation of IL-15 with IL-15R alpha-IgG1-Fc enhances its activity on proliferation of NK and CD8+/CD44high T cells and its antitumor action. *J Immunol* 2008;180:2099–106.
22. Zhu X, Marcus WD, Xu W, Lee HI, Han K, Egan JO, et al. Novel human interleukin-15 agonists. *J Immunol* 2009;183:3598–607.
23. Han KP, Zhu X, Liu B, Jeng E, Kong L, Yovandich JL, et al. IL-15:IL-15 receptor alpha superagonist complex: High-level co-expression in recombinant mammalian cells, purification and characterization. *Cytokine* 2011;56:804–10.
24. Epardaud M, Elpek KG, Rubinstein MP, Yonekura AR, Bellemare-Pelletier A, Bronson R, et al. Interleukin-15/interleukin-15R alpha complexes promote destruction of established tumors by reviving tumor-resident CD8+ T cells. *Cancer Res* 2008;68:2972–83.
25. Bessard A, Sole V, Bouchaud G, Quemener A, Jacques Y. High antitumor activity of RLI, an interleukin-15 (IL-15)-IL-15 receptor alpha fusion protein, in metastatic melanoma and colorectal cancer. *Mol Cancer Ther* 2009;8:2736–45.
26. Ring AM, Lin JX, Feng D, Mitra S, Rickert M, Bowman GR, et al. Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. *Nat Immunol* 2012;13:1187–95.
27. Wu J. IL-15 agonists: the cancer cure cytokine. *J Mol Genet Med* 2013;7:85.
28. Mueller YM, Petrovas C, Bojczuk PM, Dimitriou ID, Beer B, Silvera P, et al. Interleukin-15 increases effector memory CD8+ T cells and NK Cells in simian immunodeficiency virus-infected macaques. *J Virol* 2005;79: 4877–85.
29. Berger C, Berger M, Hackman RC, Gough M, Elliott C, Jensen MC, et al. Safety and immunologic effects of IL-15 administration in nonhuman primates. *Blood* 2009;114:2417–26.
30. Lugli E, Goldman CK, Perera LP, Smedley J, Pung R, Yovandich JL, et al. Transient and persistent effects of IL-15 on lymphocyte homeostasis in nonhuman primates. *Blood* 2010;116:3238–48.
31. Xu W, Jones M, Liu B, Zhu X, Johnson CB, Edwards AC, et al. Efficacy and mechanism-of-action of a novel superagonist interleukin-15: interleukin-15 receptor alpha Su/Fc fusion complex in syngeneic murine models of multiple myeloma. *Cancer Res* 2013;73:3075–86.
32. Rosario M, Liu B, Kong L, Collins L, Schneider SE, Chen X, et al. The IL-15-based ALT-803 complex enhances FcgammaRIIIa-triggered NK cell responses and in vivo clearance of B cell lymphomas. *Clin Cancer Res* 2015 Sep 30 [Epub ahead of print].
33. Zhang Y, Hong H, Engle JW, Bean J, Yang Y, Leigh BR, et al. Positron emission tomography imaging of CD105 expression with a <sup>64</sup>Cu-labeled monoclonal antibody: NOTA is superior to DOTA. *PLoS One* 2011;6: e28005.
34. Hong H, Yang Y, Zhang Y, Engle JW, Barnhart TE, Nickles RJ, et al. Positron emission tomography imaging of CD105 expression during tumor angiogenesis. *Eur J Nucl Med Mol Imaging* 2011;38:1335–43.
35. Stebbings R, Findlay L, Edwards C, Eastwood D, Bird C, North D, et al. "Cytokine storm" in the phase I trial of monoclonal antibody TGN1412: better understanding the causes to improve preclinical testing of immunotherapeutics. *J Immunol* 2007;179:3325–31.
36. Wen J, Zhu X, Liu B, You L, Kong L, Lee HI, et al. Targeting activity of a TCR/IL-2 fusion protein against established tumors. *Cancer Immunol Immunother* 2008;57:1781–94.
37. Yu P, Steel JC, Zhang M, Morris JC, Waldmann TA. Simultaneous blockade of multiple immune system inhibitory checkpoints enhances antitumor activity mediated by interleukin-15 in a murine metastatic colon carcinoma model. *Clin Cancer Res* 2010;16:6019–28.
38. Kobayashi H, Carrasquillo JA, Paik CH, Waldmann TA, Tagaya Y. Differences of biodistribution, pharmacokinetics, and tumor targeting between interleukins 2 and 15. *Cancer Res* 2000;60:3577–83.
39. Eisenman J, Abdieh M, Beers C, Brasel K, Kennedy MK, Le T, et al. Interleukin-15 interactions with interleukin-15 receptor complexes: characterization and species specificity. *Cytokine* 2002;20:121–9.
40. Carson WE, Yu H, Dierksheide J, Pfeffer K, Bouchard P, Clark R, et al. A fatal cytokine-induced systemic inflammatory response reveals a critical role for NK cells. *J Immunol* 1999;162:4943–51.
41. Biber JL, Jabbour S, Parihar R, Dierksheide J, Hu Y, Baumann H, et al. Administration of two macrophage-derived interferon-gamma-inducing factors (IL-12 and IL-15) induces a lethal systemic inflammatory response in mice that is dependent on natural killer cells but does not require interferon-gamma. *Cell Immunol* 2002;216:31–42.
42. Sneller MC, Kopp WC, Engelke KJ, Yovandich JL, Creekmore SP, Waldmann TA, et al. IL-15 administered by continuous infusion to rhesus macaques induces massive expansion of CD8+ T effector memory population in peripheral blood. *Blood* 2011;118:6845–8.
43. Conlon KC, Lugli E, Welles HC, Rosenberg SA, Fojo AT, Morris JC, et al. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J Clin Oncol* 2015;33:74–82.
44. Romer PS, Berr S, Avota E, Na SY, Battaglia M, tenBerge I, et al. Preculture of PBMCs at high cell density increases sensitivity of T-cell responses, revealing cytokine release by CD28 superagonist TGN1412. *Blood* 2011;118: 6772–82.
45. Food and Drug Administration, HHS. Guidance for industry on estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. *Fed Regist* 2005;70:42346.
46. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: human memory T-cell subsets. *Eur J Immunol* 2013;43:2797–809.